人為由来環境変化に対する生物の適応戦略と小進化

平成２６年度～平成３０年度私立大学戦略的研究基盤形成支援事業
研　究　成　果　報　告　書

平成31年3月

学校法人名　東洋大学
大　学　名　東洋大学
研究組織名　生命環境科学研究センター
研究代表者　柏田 祥策
（東洋大学 生命科学部 教授）

目次

はしがき

東洋大学生命環境科学研究センターは，私立大学戦略的研究基盤形成支援事業に採択され，平成 26 年度から 30 年度までの 5 年間「人為由来環境変化に対する生物の適応戦略と小進化」と題して研究を実施致しました。本報告書は，その 5 年間の研究を纏めたものです。

生命環境科学研究センターでは，人間活動に起因する様々な環境汚染あるいは環境変動に適応して生き残りを図っている環境生物のドラスティックな生命活動に焦点を当てて，人類の至上命題ともいえる「環境の持続的開発と人類の発展」の実現に貢献する研究を行っています。地球上の生物は様々な環境変化に戦略的に適応しながら進化を重ねてきました。しかし少なくとも数万年単位で進行する自然現象としての環境変化と，数日から数か月単位で劇的に変化する人為的環境変化とは，時空間的な隔たり大き過ぎることから，劇的な人為的環境変化が突発した場合には，将来に亘ってどのような生態影響が引き起こされるのかについては不明な部分が多いのが現状です。

本研究で対象環境の一つとした渡良瀬川流域は，足尾銅山鉱毒事件という劇的かつ長期的な重金属汚染を受けながらも，現在は豊かな生態系を回復していることが知られています。そこで本研究では，重金属あるいは近年の環境汚染が問題となっている抗生物質による化学環境圧に対する微生物叢，藻類，淡水無脊椎動物および魚類の生物応答および防御機構さらには環境適応戦略について，野外調査研究および室内実験研究を行いました。これら人為由来の環境変化に対する生物個体群の生存戦略，適応能に関する知見は，科学技術に支えられた人間社会が抱えている多様性ある生態系保全および持続性社会の構築といった社会問題を解決するための科学的資産となります。

5 年間に亘る本研究活動の結果，顕著な研究成果を上げることが出来るとともに国際研究拠点としての研究基盤形成を成立させることが出来ました。さらに大学院教育においても優秀な人材を輩出することが出来ました。本研究を遂行した 5 年間，国内外から実に多くの方々の御理解，御協力，御支援等を受けることが出来ました。此処に衷心より感謝の意を表します。

今後とも一層の御指導と御鞭撻を賜りますよう深く御願い申し上げます。

平成 31 年 3 月 31 日

東洋大学生命環境科学研究センター長
柏田　祥策

（様式 2）

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プロジェクト番号	S1411016

平成 26 年度～平成 30 年度「私立大学戦略的研究基盤形成支援事業」
研究成果報告書概要

1 学校法人名 東洋大学 2 大学名 東洋大学

3 研究組織名 生命環境科学研究センター

4 プロジェクト所在地 群馬県邑楽郡板倉町泉野1－1－1東洋大学板倉校地

5 研究プロジェクト名 人為由来環境変化に対する生物の適応戦略と小進化

6 研究観点 研究拠点を形成する研究

7 研究代表者

研究代表者名	所属部局名	職名
柏田 祥策	生命科学研究科	教授

8 プロジェクト参加研究者数 8 名

9 該当審査区分 理工・情報 生物・医歯 人文・社会

10 研究プロジェクトに参加する主な研究者

研究者名	所属・職名	プロジェクトでの研究課題	プロジェクトでの役割
柏田 祥策	生命科学研究科・教授	魚類に対する化学汚染影響と生物多様性	生態系進化影響評価の研究統括
長坂 征治	生命科学研究科・教授	藻類に対する化学汚染影響と耐性	生態系進化の影響評価
東端 啓貴	生命科学研究科・准教授	微生物叢に対する化学汚染影響と耐性	生態系進化の影響評価
梅原 三貴久	生命科学研究科・教授	生物組織における代謝成分に関する動態解析	環境応答に関わる低分子化合物の分析支援・分析支援の研究統括
山本 浩文	生命科学研究科・教授	化学汚染における薬物代謝と動態解析	環境応答に関わる低分子化合物の分析支援
宮西 伸光	食環境科学研究科・教授	環境変化と適応における戦略的糖鎖変動解析	環境応答に関わる高分子化合物の分析支援
（共同研究機関等）			
坂本 正樹	富山県立大学・講師	淡水無脊椎生物および食物網に対する化学物質曝露影響	生態系進化の影響評価
立田 晴記	琉球大学・教授	生態系進化に対する環境変化影響研究	生物個体群および生態系の進化解析

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＜研究者の変更状況（研究代表者を含む）＞

旧

ﾌﾟﾛｼﾞｪｸﾄでの研究課題	所属・職名	研究者氏名	プロジェクトでの役割
環境変化と適応における戦略的糖鎖変動解析	生命科学研究科・准教授	宮西　伸光	環境応答に関わる高分子化合物の分析支援

（変更の時期：平成 27 年 4 月 1 日）

新

変更前の所属・職名	変更（就任）後の所属・職名	研究者氏名	プロジェクトでの役割
生命科学研究科・准教授	食環境科学研究科・教授	宮西　伸光	環境応答に関わる高分子化合物の分析支援

旧

ﾌﾟﾛｼﾞｪｸﾄでの研究課題	所属・職名	研究者氏名	プロジェクトでの役割
生物組織における代謝成分に関する動態解析	生命科学研究科・准教授	梅原　三貴久	環境応答に関わる低分子化合物の分析支援

（変更の時期：平成 27 年 4 月 1 日）

新

変更前の所属・職名	変更（就任）後の所属・職名	研究者氏名	プロジェクトでの役割
生命科学研究科・准教授	生命科学研究科・教授	梅原　三貴久	環境応答に関わる高分子化合物の分析支援・分析支援の研究統括

旧

ﾌﾟﾛｼﾞｪｸﾄでの研究課題	所属・職名	研究者氏名	プロジェクトでの役割
化学汚染における薬物代謝と動態解析	生命科学研究科・教授	山本　浩文	分析支援の研究統括

（変更の時期：平成 27 年 4 月 1 日）

新

変更前の所属・職名	変更（就任）後の所属・職名	研究者氏名	プロジェクトでの役割
生命科学研究科・教授	生命科学研究科・教授	山本　浩文	環境応答に関わる高分子化合物の分析支援

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プロジェクト番号	S1411016

旧

ﾌﾟﾛｼﾞｪｸﾄでの研究課題	所属・職名	研究者氏名	プロジェクトでの役割
生態系進化に対する環境変化影響研究	琉球大学・准教授	立田　晴記	生物個体群および生態系の進化解析

（変更の時期：平成 27 年 8 月 1 日）

新

変更前の所属・職名	変更（就任）後の所属・職名	研究者氏名	プロジェクトでの役割
琉球大学・准教授	琉球大学・教授	立田　晴記	生物個体群および生態系の進化解析

11　研究の概要（※　項目全体を10枚以内で作成）

（1）研究プロジェクトの目的・意義及び計画の概要

人間活動に起因する化学物質（重金属，医農薬品類など）による環境汚染は，生態系構成生物を改変するほどの影響力を持つ。慢性的な化学物質による環境汚染に対して，生物は進化の中で獲得した異物代謝機能あるいは抗酸化機能を活用して生き残りを図っている。しかし汚染環境に適応した生物は，遺伝形質の偏りによって種内生物多様性が低下することによる生態系の脆弱化，あるいは新たに獲得した形質が他生物の生存・繁殖に脅威となる可能性が懸念されている。本研究では，化学物質による環境汚染が生物および生態系，さらには生態系進化に与える影響を評価するために，渡良瀬川流域および江戸川水系を中心とした野外調査および生物・化学分析を行い，それぞれの河川に代表的な人為由来環境変化である重金属汚染および抗菌剤汚染に対する生物個体および個体群の戦略的環境適応（小進化）を明らかにする。本研究は，水環境生態系の保全および持続的社会の構築に資する。

本研究は，【水環境生態系研究分野】および【生物機能統合解析分野】の2つに分かれて，互いに連携協働補完しながら密接な関係を保ち，次の年次計画の下，研究を推進している。

平成 26 年度～29 年度

【水環境生態系研究分野】は，渡良瀬川流域および江戸川水系を対象として，慢性的な重金属および医薬品類（ニューキノロン系抗菌剤）汚染に対する微生物叢および生物個体群（藻類，淡水無脊椎動物および魚類）の生存戦略について生物多様性を含む生態調査およびストレス応答研究を行う。

【生物機能統合解析分野】は，化学環境汚染などのストレスに特有のマーカー分子の分析および定量法を確立するとともに，その遺伝子応答が生物個体群の成長に与える影響について集団遺伝学および進化遺伝学的な解析を行う。

平成 29-30 年度

2つの研究分野において得られたデータを基に，人為由来環境変化に対する生物の戦略的適応進化を評価し，生物資源の革新的産業応用を目指す。

（2）研究組織

●研究代表者　柏田祥策（役割：研究全体の統括および責任者）
【水環境生態系研究分野】 4 名　柏田祥策（生態毒性学，環境分析化学，分野長），長坂征治（藻類科学），東端啓貴（微生物学），坂本正樹（水圏生態学）
【生物機能統合解析分野】 4 名　梅原三貴久（植物生理学，分野長），山本浩文（天然物化学），宮西伸光（糖鎖生物学，研究機器管理長），立田晴記（個体群生態学）
●大学院生 4 名および PD 2 名が，野外環境調査研究，室内実験研究などに専従している。
●研究チーム間の連携状況：【水環境生態系研究分野】の研究員が野外から採取した環境

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試料などの化学分析について，【生物機能統合解析分野】の研究員が密接かつ強力に支援しながらデータの蓄積を行い，それを【水環境生態系研究分野】の研究員に還流することでより深度のあるデータ解析を進めている。共同研究機関（琉球大学，富山県立大学および独立行政法人産業技術研究所）とも密接に様々なレベルでの情報共有を行い，常に議論を深め合っている。

（3）研究施設・設備等

●研究施設の面積及び使用者数

施設番号 B1　生命環境科学研究センター（RC 造），使用総面積 80 平方メートル，使用登録者数 52 名（平成 26 年度），58 名（平成 27 年度），73 名（平成 28 年度），34 名(平成 29 年度)，30 名(平成 30 年度)

施設番号 B2　東洋大学板倉キャンパス 5 号館（実験棟 5106，5207，5209，5210，5211 および 5302 実験室），使用総面積 318 平方メートル，使用登録者数 52 名（平成 26 年度），58 名（平成 27 年度），73 名（平成 28 年度），34 名(平成 29 年度)，30 名(平成 30 年度)

●主な研究装置，設備の名称及びその利用時間数

研究装置：　質量分析計　（タンパク質・糖鎖微量迅速解析システム）AXIMA　Resonance，利用時間 301 時間 40 分。LESA　AB SCIEX LC-MS/MS システム TripleTOF 5600，利用時間 1639 時間。

研究設備：　ナノ分光イメージングシステム Cytoviva®-LEICA　DM　2500，利用時間 772 時間。デジタルマイクロスコープ　VHX-5000，利用時間　1031　時間　34　分。ICP-MS　装置 NexION300D，利用時間 1821 時間 30 分。フレキシブルマイクロプレートリーダーインフィニット M1000Pro，利用時間　257　時間。生体微細構造解析凍結試料作製装置クリオスタットⅣCM 3050S，利用時間 1623.5 時間。

（4）研究成果の概要　※下記、13及び14に対応する成果には下線及び＊を付すこと。

各年度に分けて以下の通り記す。

●平成 26 年度（初年度）

7 月採択，11 月生命環境科学研究センター改修工事着工（1 月末竣工），3 月 4 台の大型分析機器搬入。渡良瀬遊水地，渡良瀬川，思川，江戸川および佐潟における野外調査開始。

【水環境生態系研究分野】では，以下の2つの研究を計画した。

1. 重金属および抗菌剤等による汚染の四次元分布解析および比較評価（年 4 回調査）
2. 生物調査および生物体内における汚染化学物質分布調査（年 4 回調査）

1 について，重金属分布の四次元分布解析は 1960 年-2010 年については，平面図+時間経過での解析までは終了したが，3 次元空間分布までには至らなかった。抗菌剤については，環境試料の採取のみを行った。

2 について，土壌および底質に生息する微生物群集のメタゲノム解析を行うための試料採取を行った。安定同位体分析（炭素および窒素）による食物網調査，マイクロサテライトを用いた集団遺伝的解析による生物多様性評価，消化管内容物の環境 DNA 分析による食物網調査などを含む生物調査，重金属汚染の現況把握，毒性実験および関連バイオマーカー調査についても環境試料の採取を行った。

【生物機能統合解析分野】では，以下の4つの研究を計画した。

1. タンパク質・糖鎖微量迅速解析の分析プラットフォーム構築
2. 代謝物のマスイメージングの分析プラットフォーム構築
3. 生体組織における汚染化学物質の定量分析技術の構築
4. 渡良瀬川流域，佐潟，思川，江戸川水系周辺の情報収集

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全ての計画において，環境試料の採取あるいは一部については代替機器などを使用して分析を行ったが，本計画に必要となる ICP-MS，MALDI-TOF-MS および LC-TOF-MS が調整準備中のため分析は次年度以降の持越しとなった。

平成 26 年度は初年度であり，研究環境の整備を行う一方で，既存研究環境を用いて可能な限りの研究を遂行した。次年度以降，より積極的な研究活動を展開することとなった。

●平成 27 年度

初年度における遅滞の回復に留意し，本年度研究計画の完全履行を目標にした。

【水環境生態系研究分野】では，以下の3つの研究を計画した。

1. 重金属および抗菌剤等による汚染の四次元分布解析および比較評価
2. 生物調査および生物体内における汚染化学物質分布調査
3. 化学物質曝露による生物応答が生物多様性に与える影響に関する予備的研究

1 の詳細である，「渡良瀬川流域，佐潟，思川，江戸川水系における底質コア等に含まれる重金属濃度および抗菌剤等の鉛直分布を ICP-MS，LC/MS/MS 等を用いて分析して，当該化学物質の四次元状況を把握する」および「水中の当該化学物質濃度を測定して，河川水中の汚染化学物質の起源，過去における動的平衡等を把握する」については，重金属濃度についてはおおよその目的は達せられた。抗菌剤分析については分析法検討を行った。

2 の詳細である，「採取した土壌コアおよび底質コアに生息する微生物群集のメタゲノム解析を行い，化学汚染が土壌・底質の微生物群集構造に与える影響を地球化学的に評価する」については，初回の解析を終えた。「微生物，藻類，水生植物，動物プランクトンおよび魚類について，安定同位体分析，集団遺伝的解析などによる生物多様性評価」については，安定同位体を用いた食物網解析および魚類個体群の集団遺伝的解析は一部終了した。「重金属汚染の現況把握，毒性実験および関連バイオマーカー調査を開始する」については，ウグイ体内重金属濃度は評価を終え，実験室内における毒性実験および関連バイオマーカー調査を開始した。抗菌剤の影響について研究を開始した。

3 の詳細である，「既報文献値および前年度の野外調査によって得られた化学物質の四次元分布結果から，様々な濃度での化学物質曝露に対する微生物，藻類，水生植物，動物プランクトンおよび魚類の生物応答，成長，個体群成長，群集構造，再生産などに与える影響を評価する」については，モデル物質として銀ナノ粒子または銅を用いたメダカおよび藻類の生物応答および銀ナノ粒子のメダカ個体群への影響について評価した。抗菌剤についてはまだ不十分であるので次年度継続して行うことにした。

【生物機能統合解析分野】では，以下の4つの研究を計画した。

1. タンパク質・糖鎖微量迅速解析の分析プラットフォーム構築および基礎解析
2. 代謝物のマスイメージングの分析プラットフォーム構築およびマスイメージングによる汚染化学物質の動態解析
3. 生体組織における汚染化学物質の定量分析技術の構築および動態解析
4. 渡良瀬川流域，佐潟，思川，江戸川水系周辺の情報収集

1 について，構築ならびに基礎解析が終了して，実サンプルの解析に取り組んでいる。

2 について，親物質（抗菌剤）の分析条件検討は終了した。

3 について，分析条件検討が終了して，実サンプルの分析体制が整った。

4 について，重金属の解析はほぼ完了したが，抗菌剤については継続中である。

●平成 28 年度

【水環境生態系研究分野】では，以下の4つの研究を計画した。

1. 重金属および抗菌剤等による汚染の四次元分布解析および比較評価
2. 生物調査および生物体内における汚染化学物質分布調査
3. メダカ・エコトキシコゲノミクス評価システムの開発
4. 化学物質曝露による生物応答が生物多様性に与える影響に関する研究

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1については，おおよその目的は達せられた。抗菌剤分析については条件が整い開始された(1 の計画の詳細については平成 27 年度参照)。

2 については，そのデータ収集は終了したが影響評価については次年度に行う。安定同位体を用いた食物網解析については，精度に問題は残しているがその全体像を見るに足るデータを得ることができた。集団遺伝的解析はほぼ完了した。総合的な評価は次年度に行う。

環境 DNA 分析を用いた食物網調査については，現時点では不要と判断した。対象生物として，ウグイに加えてタニシおよび底生動物を追加した。タニシについては重金属に対する生物応答を確認することが出来，底生動物（主に水生昆虫）については環境回復にともなう個体群変遷を確認することが出来た。藻類については成長阻害と酸化ストレス量を評価することが出来た。ウグイに関しては，体内重金属濃度は評価を終え，現在，実験室内における毒性実験および関連バイオマーカー調査を行っている。抗菌剤については分析方法が確定したので，次年度も研究を継続して行う(2 の計画の詳細については平成 27 年度参照)。

3 の詳細である，「重金属または抗菌剤の曝露による遺伝子応答をより生態学的に解釈して評価するためのメダカ・エコトキシコゲノミクス評価システムの開発」については，研究を開始した。

4 の詳細である，「既報文献値および前年度の野外調査によって得られた化学物質の四次元分布結果から，様々な濃度での化学物質曝露に対する微生物，藻類，水生植物，動物プランクトンおよび魚類の生物応答，成長，個体群成長，群集構造，再生産などに与える影響を評価する。」については，今年度は，メダカおよびイネの成長に与えるモデル重金属銀ナノ粒子の影響について糖鎖科学的解析を行い，かつ渡良瀬遊水地由来の藻類およびミジンコ類において重金属耐性と見られる新規データを獲得できた。抗菌剤についてはまだ不十分であるので次年度継続して行う。

【生物機能統合解析分野】では，以下の4つの研究を計画した。

1. タンパク質・糖鎖微量迅速解析の分析プラットフォーム構築および基礎解析
2. 代謝物のマスイメージングの分析プラットフォーム構築およびマスイメージングによる汚染化学物質の動態解析
3. 生体組織における汚染化学物質の定量分析技術の構築および動態解析
4. 渡良瀬川流域，佐潟，思川，江戸川水系周辺の情報収集

1 について，構築ならびに基礎解析が終了して，実サンプルの解析を開始した。

2 について，抗菌剤の分析は順調に進行した。マスイメージング分析は次年度行う。

3 について，分析体制が整い，分析を開始した。

4 について，環境分析データ収集とともに順調に進んでいる。

●平成 29 年度

【水環境生態系研究分野】では，以下の4つの研究を計画した。

1. 当該化学物質分布の四次元解析および比較評価（初年度からの継続）
2. 生物調査および生物体内における化学物質分布調査（初年度からの継続）
3. メダカ・エコトキシコジェノミクス評価システムの開発
4. 安定同位体分析，環境 DNA 分析などの結果を用いた栄養段階・食物網の評価検討

1 について，「渡良瀬川流域，佐潟，思川，江戸川水系における底質コア等に含まれる重金属濃度および抗菌剤等の鉛直分布を ICP-MS，LC/MS/MS 等を用いて分析して，当該化学物質の四次元状況を把握する。」および「水中の当該化学物質濃度を測定して，河川水中の汚染化学物質の起源，過去における動的平衡等を把握する。」を行った。重金属濃度についてはおおよその目的は達せられた。抗菌剤の分析およびデータ蓄積は順調に進んだ。

2 について，「採取した土壌コアおよび底質コアに生息する微生物群集のメタゲノム解析を行い，化学汚染が土壌・底質の微生物群集構造に与える影響を地球化学的に評価する。」については，昨年度までにデータ収集は終了している。また，銅耐性菌を発見・分離して，その

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性状について，「新種」の可能性も含めて詳細調査を行っている。「微生物，藻類，水生植物，動物プランクトンおよび魚類について，安定同位体分析（炭素および窒素），マイクロサテライト解析などによる生物多様性評価」については，安定同位体を用いた食物網解析については，昨年度に終了している。マイクロサテライト解析については，追加で調査を始めた渡良瀬川上流の草木ダムに放流以前のウグイの存在が期待されており，追加で分析を行った。「消化管内容物の環境 DNA 分析による食物網調査などを含む生物調査」については，食物網解析データ解析の結果，昨年度報告で不要と判断された。「重金属汚染の現況把握，毒性実験および関連バイオマーカー調査を開始する」については，既に開始しており，昨年度の報告書に，藻類，ミジンコおよびウグイに加えて，タニシおよび底生動物を追加した。ウグイに関しては，体内重金属濃度は評価を終え，実験室内における毒性実験および関連バイオマーカー調査を行った。

3について，「既報文献値および前年度の野外調査によって得られた化学物質の四次元分布結果から，様々な濃度での化学物質曝露に対する微生物，藻類，水生植物，動物プランクトンおよび魚類の生物応答，成長，個体群成長，群集構造，再生産などに与える影響を評価する。」については，銅耐性菌，銅耐性藻類および銅耐性ミジンコの探索を行い，それぞれを発見あるいは分離した。銅耐性菌については新種か否かも含めた，それぞれの銅耐性獲得に関する分子生物学的解析を行った。「重金属または抗菌剤の曝露による遺伝子応答をより生態学的に解釈して評価するためのメダカ・エコトキシコジェノミクス評価システムの開発」については，メダカのゲノム解析は終了しているが遺伝子の機能（アノテーション）について全て判明しておらず，また他の脊椎生物（ヒトあるいはマウス）の遺伝情報を援用しても相同性の観点から無意味であることから，モデル重金属銀ナノ粒子を曝露したメダカの RNAseq 解析データを用いて Whole Transcriptome 解析を行い，可能な限りのメダカ・エコトキシコジェノミクス評価を行った。結果として評価システムの開発には至らなかったが，免疫毒性という新たな知見を得ることができた。

4について，「既報文献値および前年度の野外調査によって得られた化学物質の四次元分布結果から，様々な濃度での化学物質曝露に対する微生物，藻類，水生植物，動物プランクトンおよび魚類の生物応答，成長，個体群成長，群集構造，再生産などに与える影響を評価する。」については，昨年度から引き続きメダカおよびイネの成長に与えるモデル重金属銀ナノ粒子の影響について糖鎖科学的解析を行った。さらに新規データとして，メダカに対するモデル重金属銀ナノ粒子の免疫毒性と絶滅リスクを明らかにした。薬剤耐性菌の分布解析については順調に進んでいる。

【生物機能統合解析分野】では，以下の4つの研究を計画した。

1. タンパク質・糖鎖微量迅速解析による基礎解析
2. マスイメージングによる生理活性代謝物質の動態解析
3. 生体組織における汚染化学物質の定量分析
4. 生物の環境適応および小進化に関する遺伝子群の機能に関する数理統計解析

1について，まずメダカ成魚に銀ナノコロイドを曝露し、臓器の糖鎖構造を解析した結果、特異的な酸性糖鎖が発現していることを明らかにした。一方、イネに銀ナノコロイドを曝露しても顕著な糖鎖構造の変化は認められなかったが、発芽後に特異的に発現が増加する糖鎖の存在を明らかにした。

2 について，抗生物質の分析については順調に進行しており、2年分のデータを取得したのち、渡良瀬川および江戸川における濃度変動に関する傾向をつかむ。マスイメージングについては、まだ明確なデータが得られていない。

3 について，コマツナおよびイネにおける抗生物質の影響を調査し、植物体内に取り込まれた量を分析したところ、植物体内に存在する量は極微量であることが明らかとなった。

4について，環境分析データ収集とともに順調に進んでいる。「土壌および水圏中の汚染化

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学物質の動態解析」については，重金属の解析はほぼ完了し，抗生物質については分析データが揃いつつある。

●平成 30 年度

【水環境生態系研究分野】では，以下の3つの研究を計画した。

1. 当該化学物質分布の四次元解析および比較評価
2. 生物調査および生物体内における化学物質分布調査
3. 渡良瀬川流域，佐潟，思川および江戸川水系における生物多様性評価

【生物機能統合解析分野】では，以下の研究を計画した。

1.【水環境生態系研究分野】のすべての数値データ，バイオイメージデータを統合解析することで，人為的由来環境変化に対する生物の適応戦略と小進化を明らかにする

当該年度は，本研究の最終年度であった。【水環境生態系研究分野】および【生物機能統合解析分野】におけるそれぞれの成果を統合して，以下に示した最終成果を挙げた。

【重金属および抗菌剤等による汚染の四次元分布解析および影響の時空間的評価】

渡良瀬川における生態影響の時空間変化を影響相加モデルを用いて解析した結果から，銅に代表されるような重金属汚染の影響が歴史的に大きかったと予想される通り，1960 年代には銅による顕著な生態影響が予測された。しかし，1970 年にかけて銅濃度が顕著に減少したことに伴い，銅に起因する影響割合は大きく減少し，特に 1970 年代以降の中・下流域では，BOD を指標とする有機物汚濁の影響が顕在化していたことが予測された。渡良瀬川で観測された底生動物指標の時空間的変化とモデルにより推定された影響割合の変化は概ね一致しており，対照河川が渡良瀬川本流の調査地点の比較対象として適切かどうか（流程変化は厳密には考慮できていない）などの課題は残るが，当該比較の結果は影響相加モデルにより予測された影響割合が合理的な値であることを示唆している。抗生物質については，底質中細菌からの抗生物質耐性遺伝子の検出および河川水中濃度の分析を行い，渡良瀬川および江戸川における抗生物質の汚染とその耐性菌の存在を確認できたが，底質からの抗生物質分析における最終精製が確立できず，詳細解析は未完了となった。

【生物調査および生物体内における汚染化学物質分布調査および化学物質曝露による生物応答が生物多様性に与える影響に関する研究】

〇細菌

渡良瀬遊水地の重金属汚染土壌サンプルから単離した銅耐性細菌 AN20SW1 株の表現性状および生化学的性状について，本菌はグラム陰性を示し、本菌の生育温度範囲と生育 pH 範囲は他の種よりも広く、本菌の至適生育温度は他の種よりも高い値を示した。また、本菌は、7.5%（w/v）の塩化ナトリウム存在下でも増殖することができた。これらの性状は、他の *Lysinibacillus* 属標準株と比較して特徴的なものであり、本菌を新種として提案できる可能性が示された。

〇植物プランクトン

渡良瀬川における年次的な銅濃度の減少にともない，草木ダムにおける植物プランクトンの多様性が上昇するという因果関係が浮き彫りになった。ここで用いた因果推論（Granger 因果）は，環境中の銅が直接的にプランクトンの多様性に影響を与えていることを意味するものではないが，環境の改善（銅濃度の減少）が植物プランクトンの多様性を押し上げる（隠された）至近要因の改善に働いたことがデータおよび理論として初めて明らかになった。興味深かったのは，栄養塩であるリンと多様性指数の関連性が不明瞭であったことであり，豊富な栄養塩類の存在が様々な植物プランクトン類の増加を必ずしも助けるわけではないことが示唆された。近年開発された非線形統計理論が，野外の個体群変動を駆動させる要因を特定出来ることを明らかにしたことは生態学研究として大変意義深く，環境変動に対する生物群集の反応性の違い（遺伝分散−共分散構造の変化といった小進化を含む）をとらえるのにも適していると考えられる。

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さらに国立環境研究所の微生物系統保存施設に保存されている渡良瀬川および南極の2箇所から単離された植物プランクトン *Stichococcus bacillaris* Nägeli 株（以下、渡良瀬株および南極株）に明らかな銅耐性の違いが再発見された。とくに培養後の細胞の銅含有量の測定結果からは、渡良瀬株の方が南極株よりも含有量が低く抑えられており、渡良瀬株が銅曝露下で銅の吸収を抑える、あるいは排出を促進することで、細胞内銅濃度を維持していることが示唆された。また次世代シーケンサーを用いたゲノム解析および 18SrDNA の系統解析から渡良瀬株と南極株が他の近縁藻類株と比べて近い株であること、さらに銅代謝関連遺伝子の発現解析結果から2つの株で同様に銅過剰が認識されていることと銅輸送体遺伝子の発現に2株間で違いがあることが示唆された。すなわち、*Stichococcus bacillaris* Nägeli 株は少なくとも細胞への銅の取り込みや金属の代謝を変化させることで，渡良瀬株は高い銅耐性を獲得したものと考えられた。

○動物プランクトン

一般に，重金属耐性が高い遺伝子型の生物個体群は，他の環境ストレスや種内での資源競争に弱いことが多く，環境改善後すぐに感受性の高い個体群に置き換わると考えられている。現在の谷中湖の湖水中銅濃度が毒性影響を及ぼすレベルに無いにもかかわらず，ゾウミジンコの銅耐性は，対照区と同等な感受性を持つ集団（クローン）と明らかな耐性を持つ集団（クローン）という，有為な変動（耐性の多様性）があった。耐性の高いゾウミジンコが生息する理由としては，銅濃度の高い他の水域からの移入の可能性が挙げられる。実際に，足尾銅山跡地から渡良瀬遊水地までを繋いでいる渡良瀬川および遊水地の周辺土壌あるいは河川堆積物においては，土壌法の銅濃度 125 mg/ L を超える地点が多く存在している。すなわちそれらに生息する耐性の高い遺伝子型の個体群が遊水地に恒常的に流入していると考えられた。

また過去に深刻な汚染の歴史を有する生態系の構造的特徴と生物の適応様式を明らかにすることを目的として，谷中湖（渡良瀬貯水池）および佐潟（新潟県）におけるモニタリング調査および食物網構造解析を行った。その結果，谷中湖と佐潟はどちらも富栄養湖に該当するが，両湖の水質や生態系構造は大きく異なることがわかった。これは湖盆形態や魚類の現存量の違いに起因するものと思われる。さらに，谷中湖の食物網構造において重金属汚染の影響を特徴づける傾向は認められなかった。

○魚類

草木ダム，渡良瀬川，谷中湖および思川でそれぞれ採捕された淡水魚ウグイについて，マイクロサテライトマーカーを用いたウグイの集団構造解析の結果，草木ダムおよび谷中湖のウグイは明らかに渡良瀬川および思川のウグイとは異なる集団であった，さらに草木ダムと谷中湖のウグイは互いに異なる集団であることも判明した。理由としては，渡良瀬川および思川のウグイは茨城県那珂川由来のウグイが放流されていること，草木ダムは渡良瀬川の上流に位置しているが放流の履歴が無くかつ下流からのウグイの遡上が出来ないこと，谷中湖のウグイは下流の利根川から遡上してきたウグイが流入している可能性などが考えられた。また草木ダムのウグイ肝臓における重金属濃度の内，とくに亜鉛，砒素および鉛において環境濃度と比例しない逆転現象が見られた。この傾向は異なる集団である渡良瀬川のウグイでも同様であった。そこで重金属曝露に対する種々のバイオマーカーを測定してそのメカニズム解明を試みた。その結果，草木ダムおよび渡良瀬川のウグイの MT，GSTA および GSTP 誘導遺伝子発現量は思川のウグイに比べて有意に高く，少なくとも高い重金属排出能力を持つことが示唆された。さらに草木ダムのウグイは，渡良瀬川および思川に比べ，鉛曝露のマーカーである血中 ALAD 活性の Vmax および肝臓中 GSH 濃度が有意に高く，他集団とは異なる重金属応答性を持つことが考えられた。

人間活動に起因する化学物質による環境汚染は，生態系を破壊して，その構成生物を改変するほどの影響力を持つ。足尾銅山鉱毒事件発生時から 100 年以上に亘り，一時的な高

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濃度そして慢性的な比較的高濃度の重金属に曝露され続けてきた生物集団（細菌，植物プランクトン，底生動物，動物プランクトンおよび魚類）は，本研究で明らかにしてきた通り，何らかの重金属耐性を獲得してきたことが明らかとなった。さらに近年，問題が顕在化している抗生物質についても渡良瀬川および江戸川で検出され，かつその耐性菌の存在も明らかとなった。これらの化学環境圧による生物の変化は，本研究の主眼である生物小進化として説明し得る。しかし，現実の生態学的解釈では，このような生物は，遺伝形質の偏りによって種内の遺伝的多様性が低下して生態系が脆弱化したり，あるいは新たに獲得した形質が他生物の生存に対して脅威となったりする可能性があることが知られている。

本研究成果は，狭義には水圏生態系の保全に資するものであるが，広義には人類に求められている「環境」と「開発」を共存させず，未来世代への責任を果たさず，現在世代の欲求のみを推進した場合に発生した甚大な生態学的影響に関する情報を，事後の 100 年間という生態影響の具体的事例を挙げて提供することで，改めて持続可能な社会の構築の必要性を証明するものである。

＜優れた成果が上がった点＞

- 1960 年代から現在までの渡良瀬川底質における重金属濃度の時空間解析を行い，過去 50 年に亘る重金属濃度推移の可視化に成功した。*1
- 渡良瀬川上流の重金属汚染土壌の菌叢は，重金属（特に銅）濃度依存的に非汚染土壌と異なる菌叢を形成することを明らかにした。*2
- 渡良瀬川および江戸川における抗生物質の汚染とその耐性菌の存在を確認できた。
- 渡良瀬遊水地の重金属汚染土壌から単離されたバクテリア，渡良瀬川から単離された藻類，谷中湖のミジンコが重金属（銅）に対して高い耐性を持つことを明らかにした。*3
- 草木ダムを含む渡良瀬川の河川水中の重金属濃度は，思川よりも高い。しかし，ウグイ肝臓内重金属濃度は，銅を除いて逆の傾向（低い体内濃度）を示した。その理由として，草木ダムおよび渡良瀬川のウグイの MT, GSTA および GSTP 誘導遺伝子発現量が思川のウグイに比べて有意に高いことから，これらを背景とした高い重金属排出能力が示唆された。さらに草木ダムのウグイは，渡良瀬川および思川に比べ，鉛曝露のマーカーである血中 ALAD 活性の Vmax および肝臓中 GSH 濃度が有意に高く，他集団とは異なる重金属応答性を持つことが考えられた。これらのよって，高いバックグラウンド濃度を示す銅以外の重金属の体内濃度の低下を招いたと考えられた。*4
- 渡良瀬川および思川における研究対象魚類ウグイの集団遺伝的解析を行い，両河川におけるウグイ個体群が遺伝的には相同であること，一方で渡良瀬遊水地の谷中湖のウグイおよび草木ダムのウグイも全く異なる個体群であることを初めて明らかにした。*5
- 長期慢性的な化学物質の曝露が，広く環境生物に対して耐性を獲得させることを明らかにすることができた。

＜課題となった点＞

【水環境生態系研究分野】バイオマーカー研究，メダカ・エコトキシコゲノミクス評価システムの開発が途上である。水圏生態系の最上位生物である魚類，本研究ではウグイおよびモデル生物メダカに集中してバイオマーカー／エコトキシコゲノミクス研究を行う。

【生物機能統合解析分野】抗生物質の化学分析において特に底質から抽出方法および代謝物分析が途上である。

＜研究成果の副次的効果（実用化や特許の申請など研究成果の活用の見通しを含む。）＞

本研究の目標である「化学汚染による生態系進化への影響の評価手法」はかなり前衛的な研究であるが，環境変動予測評価の実用化のための萌芽研究として重要である。さらに化学汚染は地球規模の問題である。諸外国研究機関（ノルウェー，チリ，台湾，フィリピン，マレーシア，ケニアなど）との共同研究を検討あるいは開始するなど国際連携を深めつつある。

＜自己評価の実施結果と対応状況＞

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研究プロジェクトに参加する主な研究者（8 名）および東洋大学事務職員（1 名）で構成される毎月1回の定例運営会議（議長：研究代表者　柏田祥策）を開催して各研究員の研究進捗報告会を行い，研究者の自己評価を実施して研究結果に反映する努力対応を行っている。

＜外部（第三者）評価の実施結果と対応状況＞

研究活動を評価するために，以下に示す外部評価委員（平成26年度6名，平成27年度5名，および平成28年度5名）を任用し，これまで平成27年3月9日，平成28年1月25日，平成29年1月15日，平成30年3月2日および平成31年2月5日に研究活動の評価委員会を開催した。

①石川　英律（いであ株式会社主査研究員），②江面　浩（筑波大学教授），③北脇秀敏（東洋大学教授・副学長），④斉藤和季（千葉大学教授），⑤塩月孝博（国立研究開発法人農業・食品産業技術総合研究機構主席研究員），⑥杉浦則夫（筑波大学名誉教授）（平成 26 年度のみ）（五十音順）

評価項目は，センターの運営・研究体制，研究費の使途，研究進捗状況，達成度など 10 項目から成り，評価結果に対して真摯に対応して改善に取り組んでいる。総合評価は 5 年連続で概ね良好である。具体的な修正点および対応結果について以下に記す。

●平成 26 年度

「水域に関する調査が含まれているので環境分野（土木・衛生工学）分野の専門家のノウハウを導入して欲しい（北脇）」→国土交通省利根川上流河川事務所との連絡を密にした。「産業連携により効果を上げて欲しい（北脇）」→東洋大学―パーキンエルマージャパン株式会社「SP-ICP-MS 分析技術検討会」を発足させた。

●平成 27 年度

「各課題間でさらなる情報共有による連携が望まれる（塩月）」→定例運営会議以外でのコミュニケーションが密になるように努力した。「社会に役立つ視点を強化し，他地域への応用できるような視点を入れると有利なのではないか（北脇）」→国際連携を推進して，フィリピン，チリおよびノルウェーの教育研究機関との連携を図っている。「外部機関との連携を強化して行ってほしい（江面）」→国土交通省，水産試験場，漁業協同組合などとの連携を強化した。

●平成 28 年度

「役立つ」という視点で研究目標を明確にする方が良い（北脇）」→他の地域環境への応用を検討中。「次世代研究者の育成も重要（斉藤）」→PD および大学院生の育成をより一層重視することにした。博士課程学生 1 名が日本学術振興会特別研究員（DC2）に採用された。

●平成 29 年度

「センター終了後の構想を作る必要がある(北脇)」→平成 31 年度の新規研究に向けて調整を始めた。

●平成 30 年度

特になし。

＜研究期間終了後の展望＞

本研究で対象とした渡良瀬川流域は局所的な環境問題であるが，類似した環境汚染による生態系への影響は地球上で頻発している。その影響は，環境および資源開発の持続可能性のみならず，人類社会の持続可能性の危機であると警鐘が鳴らされている。現在，国際連合では，持続的開発目標（Sustainable Development Goals; SDGs）を掲げて関連問題の解決に取り組もうとしている。持続可能な社会を構築していくためには，化学物質の毒性を理解するのみならず，生態系に対する影響というリスクを正しく評価することが必要不可欠である。今後は，本研究成果の論文公表に努めとともに，同様な問題を持つ国・地域と共有を進める活動を行い，SDGs の達成に貢献する。

＜研究成果の副次的効果＞

副次的効果として，以下の効果を発揮することが出来た。

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【研究成果の社会還元】
1. ナノテクノロジー標準化国内審議委員会　環境・安全分科会委員（経産省産総研）
2. 金属のリスク評価検討　ワーキンググループ委員（環境省国環研）
3. 化学物質審議会（安全対策部会）委員(経産省)
【地域連携】
3. 群馬県水産試験場・農政部への講師派遣
4. 熊本県水俣市・水俣環境アカデミアとの連携研究
5. 熊本県水俣市・みなまた環境テクノセンターとの連携研究
6. 島根県水産技術センターとの連携研究
【国内共同研究】
7. 桐蔭横浜大学　宮坂研究室　新エネルギー素材安全性評価
8. 東京大学　三谷研究室　放射線毒性研究
9. 環境省　国立水俣病総合研究センター　水銀毒性研究
【国際産学共同研究】
10. パーキンエルマー米国本社・日本支社　金属ナノ粒子測定法開発および国際標準化検討
【国際共同研究】
11. 臺灣中原大学（台湾）環境科学研究
12. 国立臺灣大学（台湾）メダカ毒性研究
13. Fartehr Saturnino Uninos 大学（フィリピン）環境科学研究
14. Universidad de Concepción（チリ）環境化学分析・大学院生受入研究
15. Fundación MERI（チリ） 環境科学研究
16. Akvaplan-Niva（ノルウェー）環境科学研究
17. The University of Tromsø（ノルウェー）植物科学
18. Institut français de recherche pour l'exploitation de la mer (Ifremer)（フランス）生態毒性研究
19. Universidade de Aveiro（ポルトガル）生態毒性研究
20. University of Guam Marine Laboratory（米国） 生態毒性研究
21. Palau International Coral Reef Center （パラオ共和国）生態毒性研究
22. University of Louisiana, Lafayette（米国） 免疫化学研究

12　キーワード（当該研究内容をよく表していると思われるものを8項目以内で記載してください。）

（1）重金属　（2）抗菌剤　（3）時空間解析
（4）渡良瀬川　（5）江戸川　（6）バイオマーカー
（7）生態系進化　（8）生物多様性

13　研究発表の状況（研究論文等公表状況。印刷中も含む。）
上記、11(4)に記載した研究成果に対応するものには＊を付すこと。

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なし

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10. 加藤有美恵, 片岡知里, 有吉理, 多賀須誠樹, 楢崎隆仁, 立田晴記, 柏田祥策 (2017) 銀ナノ粒子のメダカ個体群に対する生態リスクは成長依存的か？, 第 23 回日本環境毒性学会研究発表会, 9 月 1-2 日, 東洋大学白山キャンパス
11. 玉井聡子, 岩崎雄一, 柏田祥策 (2017) 2 値データの解析には一般化線形モデルを使いましょう:割算値の利用からの脱却のススメ, 第 23 回日本環境毒性学会研究発表会, 9 月 1-2 日, 東洋大学白山キャンパス
12. 坂本正樹, 小田悠介, 岩崎雄一, 長坂征治, 柏田祥策:谷中湖の食物網構造と優占種(ゾウミジンコ)の Cu 感受性, 第 23 回日本環境毒性学会研究発表会, 9 月 1-2 日, 東洋大学白山キャンパス
13. 小林夕樹, 柏田祥策, 坂本正樹(2017)重金属汚染の有無が湖沼プランクト群集レベルで耐性に及ぼす影響. 日本陸水学会甲信越支部会第 43 回研究発表会, 山梨県南都留郡(2017 年 11 月 25－26 日)
14. *1, 2, 4, 5 岩崎雄一, 古井知, 北村大樹, 富山英明, 立田晴記, 柏田祥策(2017)渡良瀬川に生息するウグイの重金属蓄積応答:汚染河川で低い組織中金属濃度？, 第64回日本生態学会大会, 早稲田大学(2017 年 3 月 14－18 日)
15. *1, 2, 4, 5 多賀須誠樹, 征矢真広, 岩崎雄一, 柏田祥策(2017)既往調査データから底生動物相の回復過程を追えるか？:渡良瀬川における過去 50 年間の金属濃度変化との関係, 第 64 回日本生態学会大会, 早稲田大学(2017 年 3 月 14－18 日)
16. *3 清水佑一, 川瀬俊吾, 浅香貴啓, 柏田祥策, 長坂征治(2017)渡良瀬遊水地から単離された藻類の銅に対する応答評価, 日本農芸化学会 2017 年度大会、京都女子大学(2017 年 3 月 17—20 日)
17. 堀内里紗, 中島由加里, 柏田祥策, 宮西伸光(2016)銀ナノコロイド曝露を受けたイネ初期生長時における糖鎖の挙動. 第 35 回日本糖質学会年会、高知市文化プラザかるぽーと(2016 年 9 月 1－3 日)
18. *1, 3 岩崎雄一, 多賀須誠樹, 柏田祥策(2016)渡良瀬川における重金属濃度と底生動物相の時空間的変化, 応用生態工学会第20回大会(20周年記念東京大会), 東京大学(2016 年 9 月 2－6 日)
19. Truptimayee Behera, Minakshi M Behera, Shosaku Kashiwada, Sudhansu S Mishra, Saubhaghya M Samantray, Bhagyashree Mohanty, Priyabrat Swain(2016)Toxicological effects of Zinc oxide nanoparticles (nano-ZnO) on three species of freshwater algae, 第 22 回日本環境毒性学会研究発表会, 愛媛大学(2016 年 9 月 6－7 日)
20. *1, 4, 5 岩崎雄一, 古井知, 富山英明, 北村大樹, 立田晴記, 柏田祥策(2016)渡良瀬川

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に生息するウグイの重金属蓄積応答，第 22 回日本環境毒性学会研究発表会，愛媛大学（2016 年 9 月 6－7 日）

21. *1，2，4，5 富山英明，北村大樹，鏡良弘，東端啓貴，長坂征治，岩崎雄一，柏田祥策（2016）渡良瀬川の重金属汚染の時空間的変化：現在の底質菌叢との相関．日本陸水学会第 81 回大会，琉球大学（2016 年 11 月 3－6 日）
22. *3 一野寛登，小田悠介，岩崎雄一，長坂征治，柏田祥策，坂本正樹（2016）過去の重金属汚染がゾウミジンコの Cu 感受性に与える影響．日本陸水学会甲信越支部会第 42 回研究発表会，長野県小諸市（2016 年 11 月 26－27 日）
23. 青山洸貴・真野浩行・坂本正樹（2016）淡水マイクロコズム実験系を用いた Ag の生態影響評価．日本陸水学会甲信越支部会第 42 回研究発表会，長野県小諸市（2016 年 11 月 26－27 日）
24. 森田千暁，河鎭龍，真野浩行，戸田任重，花里孝幸，坂本正樹（2015）個体群・群集レベルでの生態毒性影響評価，日本陸水学会甲信越支部会第 41 回研究発表会，新潟県新発田市（2015 年 11 月 28－29 日）
25. *1，3 小田悠介，河鎭龍，片岡知里，柏田祥策，戸田任重，坂本正樹（2015）過去の重金属汚染の有無による湖沼生態系構成種の感受性と群集構造への影響，日本陸水学会甲信越支部会第 41 回研究発表会，新潟県新発田市（2015 年 11 月 28－29 日）
26. *1，3 坂本正樹，河鎭龍，真野浩行，片岡知里，柏田祥策（2015）有害化学物質による湖沼生物群集への影響：種・個体レベルから個体群・群集レベルへ，日本陸水学会第 80 回大会，北海道大学函館キャンパス（2015 年 9 月 26 日—29 日）
27. 堀内里紗、遠坂翼、廣津直樹、舘野浩章、平林淳、宮西伸光（2015）イネ（*Oryza sativa*）由来レクチンの精製及び性状解析、第 64 回日本応用糖質科学会大会、奈良春日野国際フォーラム　甍～I・RA・KA～（2015 年 9 月 16－18 日）
28. 古田島大輔、脇坂卓実、清水香里、堀内里紗、柏田祥策、宮西伸光（2015）銀ナノコロイド曝露を受けたメダカ胚の糖鎖解析、第 64 回日本応用糖質科学会大会、奈良春日野国際フォーラム　甍～I・RA・KA～（2015 年 9 月 16－18 日）
29. 河鎭龍，加茂将史，坂本正樹（2015）水質（硬度、pH）の違いによる銅の急性毒性への影響カブトミジンコとオオミジンコの比較，第 21 回日本環境毒性学会研究発表会，東洋大学白山キャンパス（2015 年 9 月 2－3 日）
30. 坂本正樹，河鎭龍，真野浩行，片岡知里，柏田祥策（2015）個体群・群集レベルでの生態毒性影響評価へ：種レベル試験と結果を直接比較できることの重要性，第 21 回日本環境毒性学会研究発表会，東洋大学白山キャンパス（2015 年 9 月 2－3 日）
31. 岩崎雄一（2015）Travis S. Schmidt, William H. Clements（2015）野外調査及びマイクロコスム実験における河川底生動物の金属に対する感受性の違い，第 21 回日本環境毒性学会研究発表会，東洋大学白山キャンパス（2015 年 9 月 2 日－3 日）
32. *1，2，4 多賀須誠樹，岩崎雄一，柏田祥策（2015）底生動物相の重金属汚染からの回復：1964～76 年の渡良瀬川における調査結果，第 21 回日本環境毒性学会研究発表会，東洋大学白山キャンパス（2015 年 9 月 2 日－3 日）
33. 加茂将史，岩崎雄一（2015）メダカ個体群モデルの構築: どの個体レベルの形質への影響が集団絶滅に重要か？，第 21 回日本環境毒性学会研究発表会，東洋大学白山キャンパス（2015 年 9 月 2 日－3 日）
34. 岩崎雄一，小谷健輔，益永茂樹，柏田祥策（2015）NOEC から EC10 への代替は 95%の種が保護できる濃度に影響を及ぼすか？，第 21 回日本環境毒性学会研究発表会，東洋大学白山キャンパス（2015 年 9 月 2 日－3 日）
35. 柴田賢一，雨宮隆，伊藤公紀（2015）相対群集代謝による群集レベル代謝活性の解析，日本生態学会第 62 回全国大会，鹿児島大学（2015 年 3 月 18－22 日）

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36. Yuichi Iwasaki, Travis S. Schmidt, William H. Clements（2015）Ranking sensitivities of aquatic insects to metals in the field and stream microcosms, 日本生態学会第 62 回全国大会, 鹿児島大学（2015 年 3 月 18－22 日）
37. *1, 4 多賀須誠樹, 頭士泰之, 征矢真広, 古井知, 太田将平, 片岡知里, 林岳彦, 立田晴記, 柏田祥策（2014） 渡良瀬川における重金属汚染 100 年の四次元解析および生物の環境適応戦略, 第 20 回日本環境毒性学会研究発表会, 冨山国際会議場（2014 年 9 月 10－11 日）
38. 綱取泰広, 松村和也, 間世田英明, 柏田祥策, 清水和哉（2014）抗菌剤曝露が及ぼす硝化反応の阻害, 第 20 回日本環境毒性学会研究発表会, 冨山国際会議場（2014 年 9 月 10－11 日）

＜研究成果の公開状況＞（上記以外）

シンポジウム・学会等の実施状況、インターネットでの公開状況等
ホームページで公開している場合には、URL を記載してください。

●生命環境科学研究センターのホームページ http://www.aqua-env.org/
●平成 26 年 11 月 26 日　東洋大学生命環境科学研究センター開設記念シンポジウム
柏田祥策 「環境汚染に適応する生物進化の可能性」
長坂征治 「化学汚染に対する藻類の適応戦略と生態系への影響」
梅原三貴久 「生物の環境適応能力を評価するための新技術」
立田晴記 「環境毒性研究における生態学の重要性」
●平成 27 年 3 月 9 日　東洋大学生命環境科学研究センター竣工開所式
●平成 27 年 11 月 28 日　東洋大学生命環境科学研究センター　公開シンポジウム
坂本正樹 「ミジンコ類に対する金属毒性と水質, 金属形態の関係」
岩崎雄一 「銅などの重金属濃度が河川大型無脊椎動物に及ぼす影響」
●平成 28 年 12 月 16 日　東洋大学生命環境科学研究センター研究進捗報告シンポジウム
岩崎雄一 「渡良瀬川における重金属汚染の時空間変化と生物応答」
宮西伸光 「重金属影響におけるタンパク質糖鎖マーカー」
坂本正樹 「重金属汚染と水圏生態系影響」
Truptimayee Behera・梅原三貴久 「江戸川流域における抗生物質およびその代謝物の分布」
●平成 29 年 9 月 2 日　東洋大学生命環境科学研究センター・シンポジウム「海洋汚染による生態影響とその対応策」
Lionel Camus 「北極圏における海洋汚染
小山　次朗 「海産生物による生態毒性試験」
楠井　隆史 「海産生物による排水毒性の評価」
岡村　秀雄 「船底防汚剤と海洋汚染」
大嶋　雄治 「マイクロプラスチックの海洋汚染と生態影響：ミニレビュー」
●平成 30 年 11 月 30 日　東洋大学生命環境科学研究センター　総括シンポジウム
Marianne Frantzen　「Pollution status and concerns in the Norwegian Arctic」
Kirsten Krause　「Molecular and functional dissection of Cuscuta plant parasitism」
北村　大樹 「渡良瀬川流域における重金属汚染の今昔」
東端　啓貴 「渡良瀬遊水地から単離された銅耐性細菌 *Lysinibacillus* sp. AN20SW1 株の特徴」

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長坂　征治 「重金属汚染がもたらす微細藻類の環境適応と小進化」
坂本　正樹 「重金属汚染がもたらす動物プランクトンの環境適応と小進化」
竹内　久登 「重金属汚染がもたらす魚類の環境適応と小進化」
岩崎　雄一 「渡良瀬川の水生生物相に何が起こっていたか：水質及び生物調査データから紐解く」
柏田　祥策 「東洋大学における生命環境科学研究のこれから」

14　その他の研究成果等

【企業との連携実績】
*1 東洋大学ーパーキンエルマージャパン株式会社「SP-ICP-MS 分析技術検討会」の発足（平成 28 年 6 月）
【国際共同研究】
*1 チリ国コンセプシオン大学との共同研究「南極圏における重金属の環境挙動研究」（平成 29 年 3 月）
*1 チリ国メリ基礎研究センター：メリモユ生態系研究所との包括的学術交流に関する協定締結（平成 29 年 3 月）
【国際ワークショップ開催】
*1-5 東洋大学-Tromso 大学（ノルウェー）-Akvaplan niva 研究所（ノルウェー）における国際共同研究ワークショップ（2017 年 3 月 6-7 日）

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15 「選定時」及び「中間評価時」に付された留意事項及び対応

＜「選定時」に付された留意事項＞
なし

＜「選定時」に付された留意事項への対応＞
なし

＜「中間評価時」に付された留意事項＞
なし

＜「中間評価時」に付された留意事項への対応＞
なし

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16 施設・装置・設備・研究費の支出状況（実績概要） （千円）

年度・区分		支出額	内訳						
			法人負担	私学助成	共同研究機関負担	受託研究等	寄付金	その他（　　）	備考
平成26年度	施設	25,650	15,884	9,766	0	0	0	0	
	装置	180,084	91,180	88,904	0	0	0	0	
	設備	80,148	30,701	49,447	0	0	0	0	
	研究費	56,817	32,534	24,283	0	0	0	0	
平成27年度	施設	0	0	0	0	0	0	0	
	装置	0	0	0	0	0	0	0	
	設備	0	0	0	0	0	0	0	
	研究費	37,006	23,257	13,749	0	0	0	0	
平成28年度	施設	0	0	0	0	0	0	0	
	装置	0	0	0	0	0	0	0	
	設備	0	0	0	0	0	0	0	
	研究費	38,319	25,886	12,433	0	0	0	0	
平成29年度	施設	0	0	0	0	0	0	0	
	装置	0	0	0	0	0	0	0	
	設備	0	0	0	0	0	0	0	
	研究費	37,767	23,647	14,120	0	0	0	0	
平成30年度	施設	0	0	0	0	0	0	0	
	装置	0	0	0	0	0	0	0	
	設備	0	0	0	0	0	0	0	
	研究費	34,523	19,909	14,614	0	0	0	0	
総額	施設	25,650	15,884	9,766	0	0	0	0	
	装置	180,084	91,180	88,904	0	0	0	0	
	設備	80,148	30,701	49,447	0	0	0	0	
	研究費	204,432	125,233	79,199	0	0	0	0	
総計		490,314	262,998	227,316	0	0	0	0	

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17 施設・装置・設備の整備状況 （私学助成を受けたものはすべて記載してください。）

《施　設》（私学助成を受けていないものも含め、使用している施設をすべて記載してください。）　（千円）

施設の名称	整備年度	研究施設面積	研究室等数	使用者数	事業経費	補助金額	補助主体
生命環境科学研究センター	平成26年	80 ㎡	8	73	25,650	9,766	私学助成
5号館 (5106, 5207, 5209, 5210, 5211, 5302実験室)	平成21年	318 ㎡	6	73			

※ 私学助成による補助事業として行った新増築により、整備前と比較して増加した面積

0 ㎡

《装置・設備》（私学助成を受けていないものは、主なもののみを記載してください。）　（千円）

装置・設備の名称	整備年度	型番	台数	稼働時間数	事業経費	補助金額	補助主体
（研究装置）							
質量分析計（タンパク質・糖鎖微量迅速解析システム）	2014	AXIMA Resonance	1	302 h	54,588	27,294	私学助成
LESA AB SCIEX LC-MS/MS システム	2014	TripleTOF 5600	1	1639 h	125,496	61,610	私学助成
				h			
				h			
				h			
（研究設備）							
ナノ分光イメージングシステム	2014	Cytoviva®-LEICA DM 2500	1	772 h	17,297	10,671	私学助成
デジタルマイクロスコープ	2014	VHX-5000	1	1031 h	13,515	8,338	私学助成
ICP-MS装置	2014	NexION300D	1	1822 h	23,000	14,190	私学助成
フレキシブルマイクロプレートリーダー	2014	インフィニットM1000Pro	1	257 h	17,655	10,893	私学助成
生体微細構造解析凍結試料作製装置	2014	クリオスタットⅣ CM3050S	1	1624 h	8,681	5,355	私学助成
（情報処理関係設備）							
なし				h			
				h			
				h			
				h			
				h			

18 研究費の支出状況　（千円）

年度	平成　26　年度			
小科目	支出額	積算内訳		
		主な使途	金額	主な内容
教育研究経費支出				
消耗品費・準備品	10,592	試薬	5,330	実験試薬等
		その他	5,262	ビペット、電子顕微鏡等
光熱水費	0		0	
通信運搬費	166	メール便	105	書類送付
		その他	61	サンプル送付等
印刷製本費	154	印刷	154	シンポジウム用ポスターおよびニュースレターの印刷
旅費交通費	2,424	海外旅費	1,685	学会参加等
		国内旅費	739	調査等
報酬・委託料	2,433	業務委託	1,276	解析業務等
		その他	1,157	評価委員謝礼等
（　その他　）	2,078	会合費	542	シンポジウム懇親会費等
		その他	1,536	修繕費等
計	17,847			
アルバイト関係支出				
人件費支出（兼務職員）	1,266	研究・実験補助等	1,266	時給　900円,1100円, 年間時間数　1405.5時間 実人数　9人
教育研究経費支出				
計	1,266			
設備関係支出（1個又は1組の価格が500万円未満のもの）				
教育研究用機器備品	34,698	実験機器	4,968	エバネッセント蛍光スキャナー
			29,730	Agilent 2200 Tape Station　等実験機器及び解析用PC
図書				
計	34,698			
研究スタッフ関係支出				
リサーチ・アシスタント	196	研究補助	196	学内1人
ポスト・ドクター	2,810	研究業務等	2,810	学内2人
研究支援推進経費				
計	3,006			学内3人

法人番号	131070

年　　度	平成　　27　　年度			
小 科 目	支 出 額	積　算　内　訳		
		主 な 使 途	金　額	主　な　内　容
教　育　研　究　経　費　支　出				
消耗品費・準備品	10,932	試薬	5,226	実験試薬等
		その他	5,706	実験器具等
光 熱 水 費	6,714	電気料金	6,273	電気代
		その他	441	ガス・水道代
通信運搬費	184	切手	125	書類送付
		その他	59	サンプル送付等
印刷製本費	119	印刷	119	シンポジウム用ポスターおよびニュースレターの印刷
旅費交通費	4,000	海外旅費	2,541	学会参加等
		国内旅費	1,459	調査等
報酬・委託料	2,203	業務委託	1,412	解析業務等
		その他	791	評価委員謝礼等
（　その他　）	3,434	修繕費	1,164	実験機器の修繕費
		その他	2,270	会合費等
計	27,586			
ア　ル　バ　イ　ト　関　係　支　出				
人件費支出（兼務職員）	1,919	研究・実験補助等	1,919	時給　920、1100円，年間時間数　1,756時間 実人数　9人
教育研究経費支出				
計	1,919			
設 備 関 係 支 出（1個又は1組の価格が500万円未満のもの）				
教育研究用機器備品	631	研究・実験用機器	631	顕微鏡観察システム
図　　書				
計	631			
研　究　ス　タ　ッ　フ　関　係　支　出				
リサーチ・アシスタント	243	研究補助	243	学内2人
ポスト・ドクター	6,627	研究業務	6,627	学内2人、外国1名
研究支援推進経費				
計	6,870			学内4人、外国1人

年　　度	平成　　28　　年度			
小 科 目	支 出 額	積　算　内　訳		
		主 な 使 途	金　額	主　な　内　容
教　育　研　究　経　費　支　出				
消耗品費・準備品	10,865	試薬	9,378	実験試薬等
		その他	1,487	実験器具等
光 熱 水 費	4,078	電気料金	3,497	電気代
		その他	581	ガス・水道代
通信運搬費	152	切手	113	書類送付
		その他	39	サンプル送付等
印刷製本費	267	印刷	267	年次報告書等
旅費交通費	6,431	海外旅費	3,333	学会参加等
		国内旅費	3,098	調査等
報酬・委託料	3,397	業務委託	2,390	解析業務等
		その他	1,007	評価委員謝礼等
（　その他　）	1,672	賃借料	404	レンタカー代
		その他	1,268	会合費等
計	26,862			
ア　ル　バ　イ　ト　関　係　支　出				
人件費支出（兼務職員）	2,021	研究・実験補助等	2,021	時給　920、940、1100円，年間時間数　1,690.5時間 実人数　14人
教育研究経費支出				
計	2,021			
設 備 関 係 支 出（1個又は1組の価格が500万円未満のもの）				
教育研究用機器備品	945	情報機器	588	PC等
		実験機器	357	サーマルサイクラー
図　　書				
計	945			
研　究　ス　タ　ッ　フ　関　係　支　出				
リサーチ・アシスタント	0			
ポスト・ドクター	8,491		8,491	学内1人、外国1人
研究支援推進経費				
計	8,491			学内1人、外国1人

法人番号	131070

年　　度	平成　　29　　年度			
小 科 目	支 出 額	積　算　内　訳		
		主 な 使 途	金　額	主　な　内　容
教　育　研　究　経　費　支　出				
消耗品費・準備品	7,389	試薬	6,838	実験試薬等
		その他	551	実験器具等
光　熱　水　費	4,135	電気料金	3,483	電気代
		その他	652	ガス・水道代
通信運搬費	166	切手	117	書類送付
		その他	49	サンプル送付等
印刷製本費	495	印刷	495	年次報告書等
旅費交通費	10,792	海外旅費	6,199	学会参加等
		国内旅費	4,593	調査等
報酬・委託料	1,909	業務委託	1,653	解析業務等
		その他	256	評価委員謝礼等
（　その他　）	1,201	賃借料	14	webサーバー利用料
		その他	1,187	会合費等
計	26,087			
ア　ル　バ　イ　ト　関　係　支　出				
人件費支出（兼務職員）	1,864		1,864	時給　940、960円，年間時間数　1,771時間 実人数　5人
教育研究経費支出				
計	1,864			
設 備 関 係 支 出（1個又は1組の価格が500万円未満のもの）				
教育研究用機器備品	1,494	情報機器	499	PC
		実験機器	995	照明付培養棚、バイオシェーカー
図　　書	102			
計	1,596			
研　究　ス　タ　ッ　フ　関　係　支　出				
リサーチ・アシスタント				
ポスト・ドクター	8,220		8,220	学内1人、外国1人
研究支援推進経費				
計	8,220			学内1人、外国1人

年　　度	平成　　30　　年度			
小 科 目	支 出 額	積　算　内　訳		
		主 な 使 途	金　額	主　な　内　容
教　育　研　究　経　費　支　出				
消耗品費・準備品	7,071	試薬	7,071	実験試薬等
光　熱　水　費	4,824	電気料金	4,511	電気代
		その他	313	ガス・水道代
通信運搬費	189	切手	118	書類送付
		その他	71	サンプル送付等
印刷製本費	230	印刷	230	年次報告書等
旅費交通費	12,974	海外旅費	9,606	学会参加等
		国内旅費	3,368	調査等
報酬・委託料	1,707	業務委託	1,153	解析業務等
		その他	554	評価委員謝礼等
（　その他　）	1,083	賃借料	1	Webサイトのドメイン登録料
		その他	1,082	会合費等
計	28,078			
ア　ル　バ　イ　ト　関　係　支　出				
人件費支出（兼務職員）	1,894		1,894	時給　960、990、1100、1375円，年間時間数　1,734時間 実人数　11人
教育研究経費支出				
計	1,894			
設 備 関 係 支 出（1個又は1組の価格が500万円未満のもの）				
教育研究用機器備品	496	実験機器	496	携帯マルチ水質計
図　　書	50			
計	546			
研　究　ス　タ　ッ　フ　関　係　支　出				
リサーチ・アシスタント	75		75	学内1人
ポスト・ドクター	3,930		3,930	学外2人
研究支援推進経費				
計	4,005			学内1人、学外2人

H26 年度　業績一覧

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Salinity increases the toxicity of silver nanocolloids to Japanese medaka embryos†‡

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To investigate the effects of salinity on the toxicity of silver nanocolloids (SNCs, 28.4 nm in diameter) in aquatic environments (freshwater, brackish water, and seawater), we exposed 15 medaka eggs in triplicate to SNCs at 10 mg L^{-1} in different salinities of embryo-rearing medium (ERM) (1×, 5×, 10×, 15×, 20×, and 30×) until hatching (1× ERM and 30× ERM have osmotic pressures equivalent to freshwater and seawater, respectively). With increasing concentration of ERM, SNCs aggregated to 437.3 nm in diameter in 30× ERM solution. Simultaneously, soluble silver chloro complexes (various combinations of $[AgCl]^0$, $[AgCl_2]^{1-}$, $[AgCl_3]^{2-}$, and $[AgCl_4]^{3-}$) were calculated to have been formed. The patterns of the absorption spectra of SNCs and $AgNO_3$ (a reference compound) differed markedly in ERM at different salinities, indicating that different soluble silver complexes were present in each solution. With increasing salinity, the chorion resistance decreased, and the salinity in the medaka eggs, as indicated by the osmotic pressure, increased. Simultaneously, uptake of SNCs or other silver complexes into the embryos also increased compared with that of $AgNO_3$ in 20× and 30× ERM. In the presence of SNCs in 20× ERM, embryo hatching rate and full body lengths of post-hatch larvae were significantly lower than those with $AgNO_3$. The toxic effects of SNCs on the hatching rate increased significantly in media of high salinity and were greater than those of $AgNO_3$. SNCs and related silver chloro complexes exhibited higher bioavailability and medaka embryo toxicity in saline conditions than did $AgNO_3$. SNCs pose greater ecological risks to fish embryos in high-salinity aquatic environments than in freshwater environments.

Nano impact

Little information is available on the environmental fate of nanomaterials, including silver nanocolloids (SNCs). Environmental factors such as salinity, pH, and temperature could influence the fate of such materials. Salinity—one of the most important aquatic environmental factors—is likely to affect the fate of SNCs, including soluble silvers such as silver chloro complexes. We demonstrated that the bioavailability of SNCs and related soluble silver compounds was greater in seawater than in freshwater, thus increasing the toxicity of SNCs to medaka embryos compared with that of a control $AgNO_3$ solution. Our findings provide novel insights into the aquatic environmental interactions and ecological risks of silver nanomaterials in terms of environmental health.

Introduction

Numerous chemicals are being developed to improve and maintain human quality of life, but the release of such chemicals and chemical wastes into the environment can pose ecological risks. Because nanomaterials and nano-industries are emerging rapidly on international markets,[1] the ecological risks posed by nanomaterials must be considered. Silver nanomaterials have been developed mainly as antibacterial healthcare products and now account for half of the international nanomaterial market.[2] Along with this rise in the use of silver nanomaterials have come studies of their toxicity. For example, the toxicity of silver nanoparticles to zebrafish (*Danio rerio*) embryos is dose dependent in terms of increased mortality, decreased heart rate, reduced hatching rate, abnormal development, and increased catalase activity; mortality is also particle-size dependent[3–5] and capping-material dependent.[3] Silver ions released from silver nanomaterials are considered to be important factors in the

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† *Safety:* The Japanese medaka used were treated humanely in accordance with the institutional guidelines of Toyo University, with due consideration for the alleviation of distress and discomfort.

‡ Electronic supplementary information available. See DOI:10.1039/c4en00175c

toxicity of silver nanomaterials; the release efficiency of silver ions depends on the type or presence of capping material.[6] To minimise the toxic effects of capping materials and to study the toxic effects of nano-sized silver, water-dispersed nanocolloidal silver with uncapped and naked particles has been employed.[7] Previously, using Japanese medaka (*Oryzias latipes*), our research team revealed that the abnormalities in embryo development induced by silver nanocolloids (SNCs) were attributable to the disruption of embryogenesis gene expression.[7] However, there is little information on the mechanism of toxicity of silver nanomaterials or on their ecological effects.

To address the ecological issue of silver nanomaterials, bioassays have been performed in fish, algae, daphnia, sea urchins, shrimp, sea hares, and coral.[3,8–12] Fish eggs and post-hatch larvae are more susceptible than the adult stage to chemical exposure. The chemical susceptibility of these fish stages is advantageous in aquatic toxicological studies, as it helps us to understand and predict the ecological risks of chemicals. Fish eggs have a chorion that protects the embryo from the surrounding environment;[13] the mechanism by which xenobiotics permeate through the chorion remains unclear. Mucus is likely to play a role in the cell membrane permeation and bioavailability of nanoparticles.[14,15] Hayashi *et al.*[16] have found in earthworms that wrapping the nanoparticles in a native protein corona helps in cellular interaction and membrane permeation of the nanoparticles. Fish gills, which are covered in mucous membrane, play the most important role in the uptake of xenobiotics in larvae and adult fish. Fish gills are among the target organs in nanotoxicology,[17] and Yue *et al.*[18] have reported the toxicity of silver nanoparticles to a fish gill cell line. However, in medaka embryogenesis the gills are still not functional at stage 31 (3 days before hatching),[19] which means that the gills are not yet a route of uptake of silver nanoparticles. The chorion has no mucus and has soft tissue on its surface. Sakaizumi[20] studied the toxic interactions between methyl mercury and salinity in Japanese medaka eggs and found that increasing the osmotic pressure of the test solution enhanced the toxicity of the methyl mercury. Sumitani *et al.*[21] used medaka eggs to investigate the toxicity of landfill leachate; they found that osmotic equivalency of leachate to eggs was the key to inducing abnormalities in embryogenesis. Furthermore, in our previous study[22] we found that plastic nanoparticles (39.4 nm in diameter) easily permeated the medaka egg chorion under brackish conditions [500 mOsm in 15× embryo-rearing medium (ERM)].

It was initially considered possible that, in Pleuronectidae fishes, chemicals could permeate through the egg chorion *via* pores distributed on its surface; however, scanning electron microscope (SEM) images have revealed that these pores do not go right through the chorion.[23] We therefore still do not know how nanoparticles penetrate the fish egg chorion. However, the studies mentioned above have shown that chemical bioavailability in the egg is affected by the ambient osmotic pressure.

Japanese medaka is commonly used as a typical freshwater fish model in toxicology[24] and ecotoxicology.[25] Thirteen *Oryzias* species have been identified in East to South Asia, and most of these inhabit marine or brackish waters.[26] Although Japanese medaka is a freshwater fish, it is able to live in not only freshwater but also brackish water or seawater. Because Japanese medaka has evolved in moving northward from south Asia, it has adapted to high-salinity conditions and has highly developed chloride cells.[27] Hence, Japanese medaka eggs are able to hatch normally in seawater (within 8 to 10 days at 25 °C).[28] These characteristics make this species markedly different from other freshwater fish models such as the zebrafish. Our goal here was to use Japanese medaka embryos to evaluate the effects of environmental aquatic salinity on the bioavailability and toxicity of nano-sized silver.

Materials and methods

Silver nanocolloids

Purified SNCs (20 mg L^{-1}, 99.99% purity, 81.1% Ag^+ at pH 7, mean particle diameter *ca.* 28.4 ± 8.5 nm suspended in distilled water; see the SEM image in Fig. S1†) were purchased from Utopia Silver Supplements (Utopia, TX, USA). Diluted SNC solution (a mixture of silver colloids and Ag^+) for exposure tests was prepared with different concentrations of ERM (1×, 5×, 10×, 15×, 20× or 30×) (1× ERM consisted of 1.0 g NaCl, 0.03 g KCl, 0.04 g $CaCl_2{\cdot}2H_2O$ and 0.163 g $MgSO_4{\cdot}7H_2O$ in 1 L of ultrapure water; pH adjusted to 7.2 with 1.25% $NaHCO_3$ in ultrapure water) to simulate conditions ranging from freshwater to seawater. $AgNO_3$ was used as a reference compound for SNCs. See the ESI† for details.

Medaka eggs

Eggs of *O. latipes* orange-red strain at embryonic developmental stage 21 (brain regionalisation and otic vesicle formation stage) were harvested, rinsed with 1× ERM, and then used in the exposure tests. All medaka embryos used were at stage 21, because our previous study had revealed that this stage was more sensitive than other stages to SNCs.[7] See the ESI† for details.

Toxicity testing of SNCs at 1× ERM (freshwater conditions)

To examine the toxic effects of SNCs on medaka embryos, 15 medaka eggs (stage 21) in triplicate were exposed to 5 mL of SNCs (1, 5, 10 or 18 mg L^{-1}) in 1× ERM at pH 7 during incubation at 25 °C in the dark until hatching or for 14 days. The test solutions were renewed once a day. During exposure, every day, exposed eggs were observed under a dissecting microscope (SZ-ET, Olympus Co., Tokyo, Japan). The cumulative hatching rate was counted for 14 days. Medaka eggs in 1× ERM at pH 7 without SNCs were used as controls. To examine the effects of the ERM on SNC toxicity, we replaced the ERM with ultrapure water at pH 7. Full details of the

method used are given in our previous paper.[7] See the ESI† for details.

Wave scanning of silver solutions

For qualitative analysis of SNCs or $AgNO_3$, or of soluble silver chloro complex formation ($[AgCl]^0$, $[AgCl_2]^-$, $[AgCl_3]^{2-}$ and $[AgCl_4]^{3-}$), the absorbances of 10 mg L^{-1} SNC or $AgNO_3$ solution (in 1×, 5×, 10×, 15×, 20×, or 30× ERM, or in ultrapure water) and of 0.625, 1.25, 2.5, 5, and 10 mg L^{-1} SNC or $AgNO_3$ solution in 30× ERM were scanned in triplicate with a UV-vis-NIR spectrophotometer (UV-3600, Shimadzu Co., Kyoto, Japan). SNC or $AgNO_3$ solution was mixed with each ERM or with ultrapure water. The mixture was left at room temperature for 24 h and then subjected to scanning. Portions of the SNC solutions were filtered through a 3-kDa membrane filter to examine the absorbance of soluble silvers without particles. Both filtered and unfiltered solutions were subjected to scanning. See the ESI† for details.

Salinity-dependent production of silver chloro complexes

Formation of silver chloro complexes was calculated by using the free program Visual MINTEQ version 3.0 (http://www.lwr.kth.se/English/OurSoftware/vminteq); we also used stepwise formation constants for Cl^- and Ag^+.[29,30] To calculate silver chloro complex production, we used the Ag concentrations detected as soluble silver in 1×, 5×, 10×, 15×, 20×, or 30× ERM (Fig. 3e).

Toxicity testing of SNCs or $AgNO_3$ at different ERM salinities

SNCs at concentrations as high as 18 mg L^{-1} were not lethal to the medaka eggs in 1× ERM, and all of the exposed eggs hatched (Fig. S2†). Therefore, 15 medaka eggs (stage 21) in triplicate were exposed to 5 mL of SNCs (10 mg L^{-1}) or $AgNO_3$ (15.7 mg L^{-1}, as 10 mg L^{-1} silver) in each concentration of ERM (1×, 5×, 10×, 15×, 20×, or 30×) at pH 7 and 25 °C in the dark until hatching or for 14 days. There were no significant differences in dissolved oxygen concentration (8.30 ± 0.04 mg L^{-1}) among the ERM solutions. The test solutions were renewed once a day. On day 6 of exposure, heart rate per 15 s was counted and eye size (diameter) was measured. The hatch rate was counted for 14 days. Full body lengths of post-hatch larvae were measured on hatching day under a dissecting microscope (SZ-ET, Olympus Co., Tokyo, Japan) with a micrometer. Medaka eggs in 1× to 30× ERM at pH 7 were used as controls.

Measurement of the diameter of aggregated SNCs in ERM solutions

To investigate how ERM solutions of different salinity affected the status of colloidal silver and how silver aggregates were formed, SNCs (20 mg L^{-1}) in triplicate were added to each concentration of ERM (pH 7; 1×, 2.5×, 5×, 10×, 15×, or 30×) to a final concentration of 10 mg L^{-1}; the mixture was then stirred for 24 h at 25 °C in the dark. The mixtures were subjected to particle diameter measurement with a Zetasizer Nano ZS two-angle particle and molecular size analyser (Malvern Instruments Ltd, Malvern, Worcestershire, United Kingdom). Precipitates were observed under a dissecting microscope.

Measurement of osmotic pressure

Two hundred medaka eggs (stage 21) were incubated in each concentration of ERM (1×, 5×, 10×, 15×, 20×, or 30×) at pH 7 and 25 °C in the dark for 24 h, and then osmotic pressures of their body fluids were measured. See the ESI† for details.

Measurement of electrical resistance

To examine salinity-dependent ion permeation, we measured the electrical resistance of the egg chorion in each concentration of ERM (1×, 5×, 10×, 15×, 20×, or 30×) at pH 7 and 25 °C. See the ESI† for details.

Measurement of silver uptake by medaka embryos and soluble silver

To investigate the effects of osmotic pressure on silver uptake through the chorion, 15 medaka eggs (stage 21) in triplicate were exposed to SNCs (10 mg L^{-1}) or $AgNO_3$ (15.7 mg L^{-1}, as 10 mg L^{-1} silver) in each concentration of ERM (1×, 5×, 10×, 15×, 20×, or 30×) at pH 7 and 25 °C in the dark for 5 days (before hatching). The test solutions were renewed once a day. After exposure (on day 6), the eggs were washed with fresh and clean ERM at the respective concentrations (5 mL, 5 times). The embryos were then dechorionated with medaka hatching enzyme. Finally, the dechorionated embryos were washed with fresh and clean ERM at the respective concentrations (5 mL, 5 times). The amount of silver that accumulated in the dechorionated embryos was analysed by using inductively coupled plasma mass spectrometry (ICP-MS). To calculate the silver concentrations in the embryos, the fresh weight of a single embryo was measured with an analytical balance (MS204S, Mettler Toledo International Inc., Griefensee, Switzerland) to be 0.658 ± 0.027 mg. Test solutions (50 μL) were filtered through a 3-kDa membrane filter to obtain soluble silver; the concentration of soluble silver was then analysed by using ICP-MS. See the ESI† for details.

Statistical analyses

Data were analysed by using analysis of variance (ANOVA, $P < 0.05$) and Dunnett's *a posteriori* test to evaluate the impact of silver compared with references.

Results and discussion

Characterization of SNC and $AgNO_3$ solutions at different ERM concentrations

To characterize the SNCs, we measured their aggregated size and investigated the forms of soluble silver present, including silver chloro complexes.

SNCs aggregated with increasing salinity; we measured their diameters in ERMs of different salinities. The diameter of colloidal silver was 28.4 ± 8.5 nm in distilled water; the diameter was 67.8 ± 19.4 nm in 1× ERM (which has an osmotic pressure equivalent to that of freshwater) and 437.3 ± 71.6 nm in 30× ERM (which has an osmotic pressure equivalent to that of seawater). In higher concentrations of ERM (2.5×, 5×, 10×, 15×, or 30×) the measured diameters were larger than those in distilled water or 1× ERM and ranged between 352.8 and 504.5 nm. Between 2.5× ERM and 30× ERM, the average diameter was 435.2 ± 62.5 nm. There were no significant differences among the diameters at these salinities (Fig. S3†).

Generally, when silver is added to saline solutions, soluble silver complexes such as silver chloro complexes ($[AgCl]^0$, $[AgCl_2]^-$, $[AgCl_3]^{2-}$ and $[AgCl_4]^{3-}$) are produced.[29,30] These complexes each have their own absorption spectra.[29] SNC or $AgNO_3$ solutions were scanned with a UV-vis-NIR spectrophotometer (Fig. 1a and b, respectively) and different scan data were obtained in the UV-vis range. In ultrapure water, the maximum absorption wavelengths of the SNC (396 nm) and $AgNO_3$ (198 nm) solutions differed markedly (Fig. 1a and b). The peak absorbance at 396 nm, representing the presence of SNCs dispersed as colloids, was almost the same as the 390 nm reported by Liu and Hurt[31] for silver nanoparticles (diameter, 1.9 nm). In filtered or unfiltered 30× ERM solution the peak at 396 nm disappeared; instead, a peak at *ca.* 220 nm representing soluble silver emerged in both SNC solutions (Fig. 1a). The scan data for these filtered and unfiltered SNC solutions were almost the same. This was because in the unfiltered solution of SNCs in 30× ERM, portions of the SNCs were aggregated and were precipitated out (Fig. S4†). Although a whole, unfiltered solution of SNCs in 30× ERM was subjected to scanning, it consisted largely of supernatant, whereas in the equivalent filtered solution, the aggregated SNCs, including precipitates, were filtered out. Precipitation was not observed in $AgNO_3$ in 30× ERM solution.

In ultrapure water, the maximum absorption wavelength of $AgNO_3$ solution (198 nm) was almost the same as the secondary peak for SNCs in ultrapure water (196 nm) (Fig. 1a and b). The scan data for the filtered and unfiltered $AgNO_3$ solutions were almost the same, because in both cases the $AgNO_3$ was ionized and formed silver chloro complexes in solution.

Thus, with SNCs there was a peak at 396 nm in ultrapure water. In contrast, although the absorption spectra of soluble silver from SNCs and $AgNO_3$ in the 30× ERM solutions differed slightly, soluble silver had a peak at about 210 to 220 nm in all of the 30× ERM solutions (Fig. 1a and b). This meant that the UV-vis-NIR spectrophotometer detected both SNCs and soluble silvers. In this case, aggregated SNCs were precipitated and were thus not detected by the UV-vis-NIR spectrophotometer.

Production of soluble silver chloro complexes ($[AgCl]^0$, $[AgCl_2]^-$, $[AgCl_3]^{2-}$, and $[AgCl_4]^{3-}$) depends on the chloride concentration.[29,30] To determine the differences in complex formation between the SNC and $AgNO_3$ solutions, we obtained scan data at different silver or ERM concentrations.

In solutions that had different silver concentrations but were fixed at 30× ERM, although peaks at 219 nm (SNCs) and 213 nm ($AgNO_3$) emerged, the patterns of the absorption spectra of all SNC solutions were similar; this was also true for all of the $AgNO_3$ solutions (Fig. 1c and d).

In solutions that had different ERM concentrations but were fixed at 10 mg L^{-1} silver concentration, the patterns of the absorption spectra of SNCs differed among different ERM solutions. The peak shifted gradually from 201 nm at 1× ERM to 208 and 219 nm at 30× ERM. The patterns of the absorption spectra of $AgNO_3$ did not differ among the different ERM solutions, although the peak shifted gradually from 207 nm at 1× ERM to 213 nm at 30× ERM (Fig. 1e and f).

To examine the salinity-dependent production of soluble silver chloro complexes from SNCs or $AgNO_3$, we measured the concentrations of soluble silver in filtered SNC or $AgNO_3$ solutions. Although the initial concentrations in the two types of solution were the same at 10 mg L^{-1}, the soluble silver concentrations detected increased with increasing ERM concentration (Fig. 3e). The complex species included in each solution would have differed among salinities, because the patterns of the absorption spectra differed markedly (Fig. 1e and f).

By using the concentrations of Ag detected as soluble silver in 1×, 5×, 10×, 15×, 20×, and 30× ERM (Fig. 3e), we calculated the theoretical concentrations of silver chloro complexes (Fig. 2a and b). Major silver chloro complexes were $[AgCl]^0$, $[AgCl_2]^-$, and $[AgCl_3]^{2-}$ in both the SNC solution and the $AgNO_3$ solution (Fig. 2c and d). These accounted for more than 99% of the soluble silvers. Ag^+ accounted for less than 1% of the soluble silvers (Tables S1 and S2†). This calculation was based on the hypothesis that existing soluble silver would react with anions, including Cl^-, OH^-, HCO_3^-, and SO_4^{2-} (all of which were derived from the ERM), or with NO_3^- (which was derived from the $AgNO_3$), in each solution. Although there were marked differences between the patterns of the absorption spectra of SNCs and $AgNO_3$ (Fig. 1e and f), the two sets of complex-formation data simulated by the software program were almost the same and could not explain the different patterns of the absorption spectra (Fig. 2a to d and Tables S1 and S2†). In theory, $[AgCl_4]^{3-}$ must have been produced;[29,30] however, the software program does not support $[AgCl_4]^{3-}$. Moreover, insoluble AgCl is not supported; however, precipitates were formed in the SNC solution (Fig. S4†) but not in the $AgNO_3$ solution. When stepwise formation constants were used for Cl^- and Ag^+, $[AgCl_4]^{3-}$ was the dominant chloro complex, followed by $[AgCl]^0$, $[AgCl_2]^-$, and $[AgCl_3]^{2-}$ (Fig. S5a and S5b and Table S3†). Regardless, both types of calculations indicate that at least $[AgCl]^0$, $[AgCl_2]^-$, and $[AgCl_3]^{2-}$ must be major complexes and may be involved in the toxic effects of SNCs.

Although the patterns of the absorption spectra of SNCs and $AgNO_3$ differed markedly and precipitates formed only in the SNC solution, the simulation results for soluble silvers

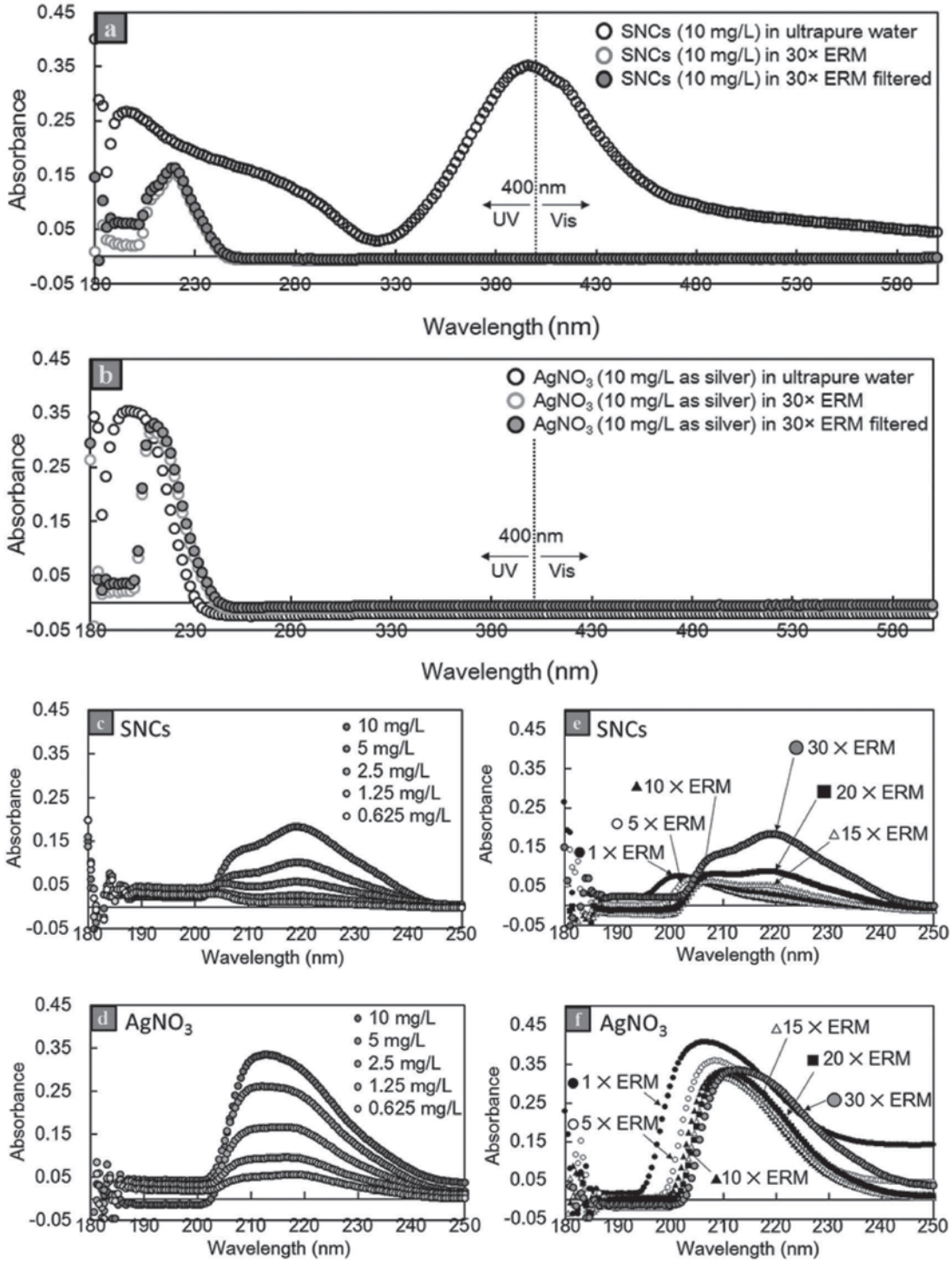

Fig. 1 Wavelength scanning of silver nanocolloids (SNCs) and silver nitrate solutions. (a) SNCs (10 mg L^{-1}) in ultrapure water or 30× ERM (filtered and unfiltered). (b) Silver nitrate (10 mg L^{-1} as silver) in ultrapure water or 30× ERM (filtered and unfiltered). (c) SNCs (0.625 to 10 mg L^{-1}) in 30× ERM. (d) Silver nitrate (0.625 to 10 mg L^{-1}) in 30× ERM. (e) SNCs (10 mg L^{-1}) in 1× to 30× ERM. (f) Silver nitrate (10 mg L^{-1} as silver) in 1× to 30× ERM. Blank data (ultrapure water or each ERM) have been subtracted from all data shown.

could not explain the different patterns of absorption spectra and the precipitation. The difference is probably related to the physicochemical effects of SNCs, but currently we have no clues as to its cause.

Toxic effects of SNCs at 1× ERM (freshwater conditions)

No hatching inhibition, malformation or mortality was observed in any of the medaka eggs exposed to SNCs at 1 to 18 mg L^{-1} in 1× ERM. The rates of hatching (Fig. S2†),

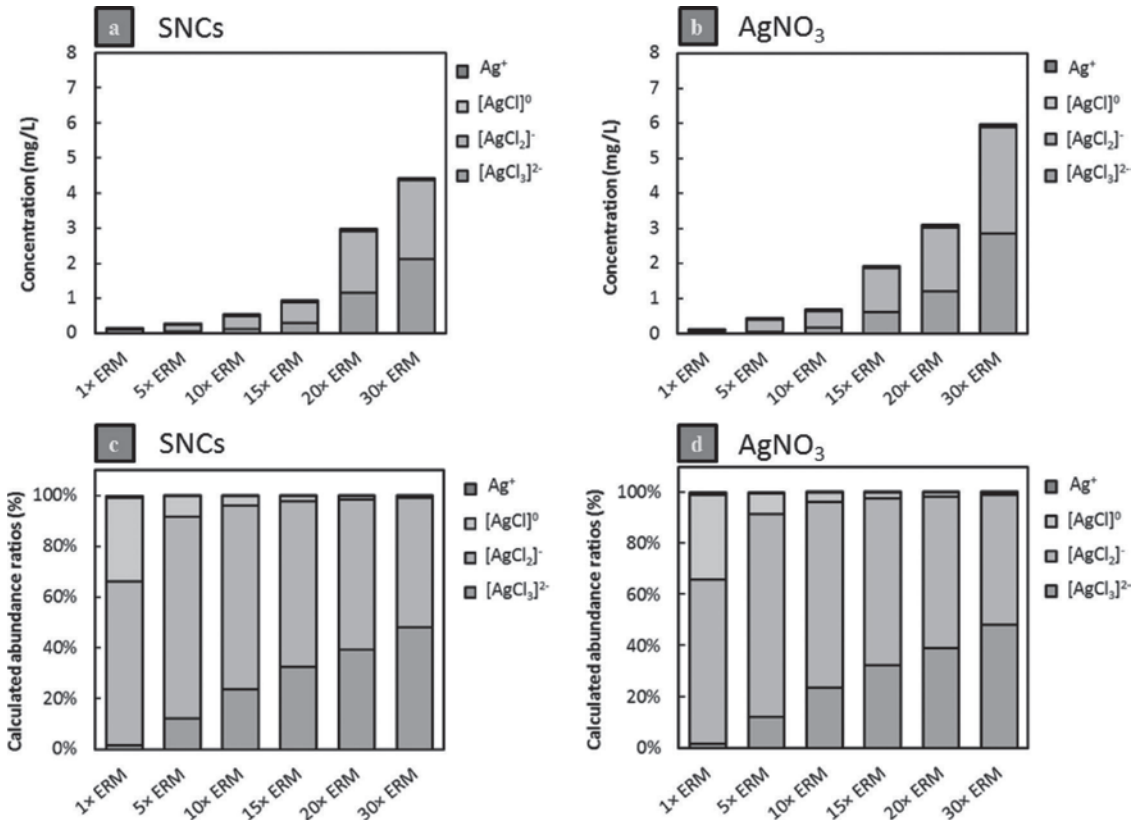

Fig. 2 Calculated concentrations and abundance ratios of silver ions and silver chloro complex species. Concentrations of silver ions and silver chloro complex species in different concentrations of ERM were calculated by using Visual MINTEQ version 3.0. (a) Concentrations of silver ion and silver chloro complex species in SNC solution. (b) Concentrations of silver ion and silver chloro complex species in silver nitrate ($AgNO_3$) solution. (c) Abundance ratios of silver ions and silver chloro complex species in SNC solution. (d) Abundance ratios of silver ions and silver chloro complex species in $AgNO_3$ solution.

malformation, and mortality (data not shown) were almost 100%, 0.0%, and 0.0%, respectively, at all SNC concentrations. However, in ultrapure water, which has very low ionic strength, the presence of SNCs led to higher mortality rates than in 1× ERM; the 50% lethal concentration in 96 h (96 h LC_{50}) was 0.051 (0.0386 to 0.0703, 95% confidence limits) mg L^{-1} (data not shown). Park *et al.*[32] reported a similar toxicity decline: the toxicity of citrate-capped silver nanoparticles was higher in water of low ionic strength than in water of high ionic strength. Groh *et al.*[33] summarised the effects of chloride concentration on the toxicity of silver nanoparticles to fish; they concluded that "high chloride concentrations in the exposure medium pose the risk of underestimating the LC_{50} values for silver nanoparticles." These alterations in silver toxicity are thus associated with changes in the ionic strength of the test solution. In ultrapure water, silver can exist as free Ag^+ and exhibit toxicity; however, in some ionic solutions such as freshwater, free Ag^+ combines with Cl^- to form insoluble AgCl;[29,30] toxicity thus declines.

Salinity-dependent silver toxicity (freshwater to seawater conditions)

Medaka eggs were exposed to SNCs or to $AgNO_3$ at 10 mg L^{-1} (as silver) at different salinities. We then measured phenotypic biomarkers, namely hatching rate, full body length of post-hatch larvae, heart rate per 15 s, and eye size. In the controls, all embryos hatched at salinities ranging from 1× to 30× ERM; however, the hatching rate of SNC-exposed embryos decreased to 71% in 20× ERM (ANOVA, $P < 0.01$) compared with that in SNC-exposed embryos at 1× ERM, and only 2% of embryos hatched in 30× ERM (Fig. 3a). $AgNO_3$ did not significantly inhibit hatching by 20× ERM; it completely inhibited hatching only in 30× ERM compared with $AgNO_3$-exposed embryos at 1× ERM ($P < 0.01$). Full body length in the post-hatch larvae was consistently 4.55 to 4.69 mm at all ERM concentrations in the controls. With SNC exposure, although in 1× to 15× ERM the body length was in a similar range (4.33 to 4.59 mm) as in the controls, the average length decreased significantly to 3.77 in 20× ERM compared with that of SNC-exposed embryos at 1× ERM; moreover, it decreased to 3.75 mm in 30× ERM (statistical comparison was not performed at this concentration because of an insufficiency of samples) (Fig. 3b). $AgNO_3$ exposure caused no significant difference in full body length compared with that in 1× ERM. Heart rate per 15 s in the controls was not constant and ranged from 29.6 to 32.2 throughout the range from 1× to 30× ERM. Although the heart rate was not very stable in the controls, or with SNC or $AgNO_3$ exposure, with SNC or

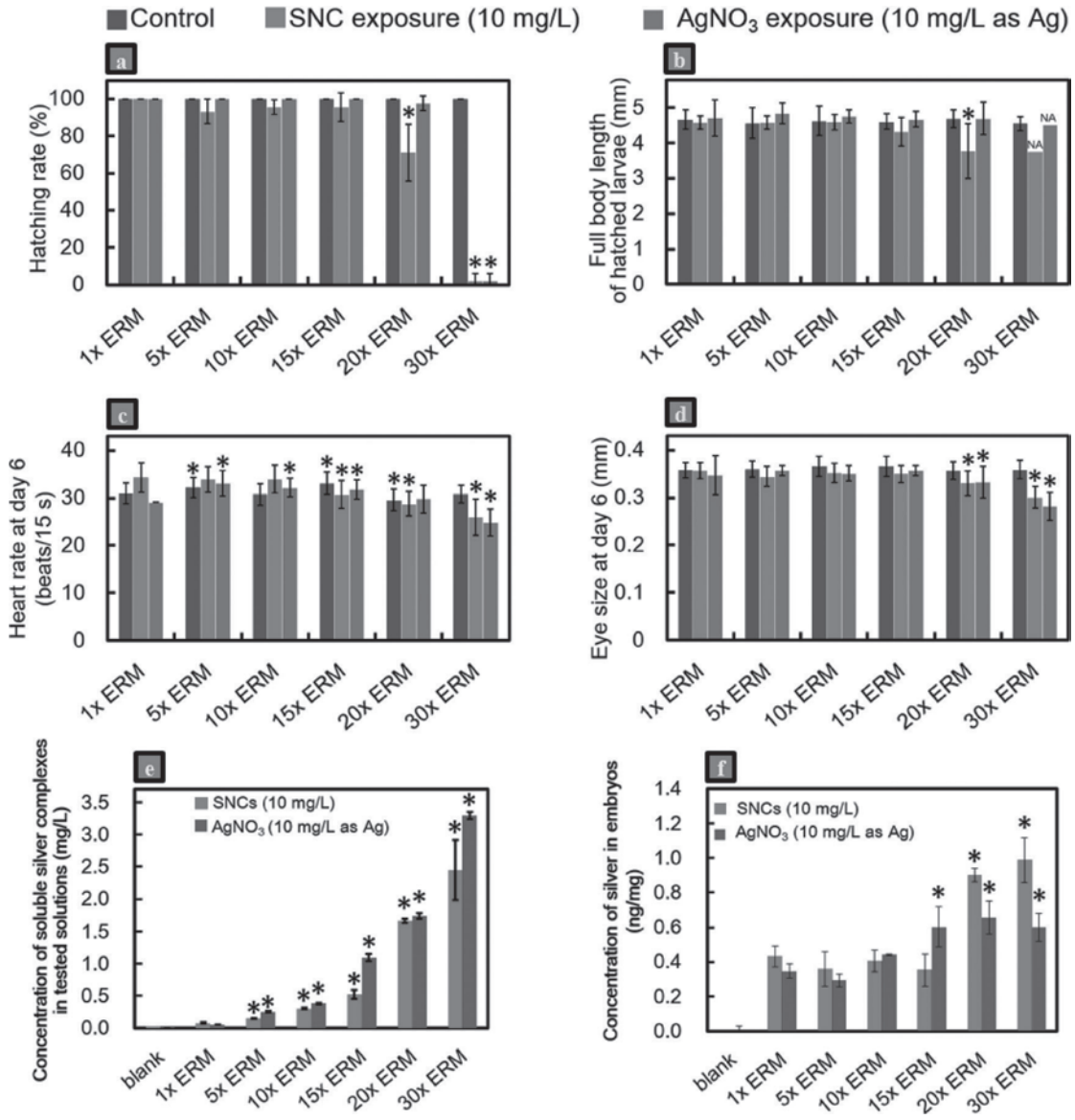

Fig. 3 Measured phenotypic biomarkers and uptake of silver in medaka eggs exposed to silver nanocolloids (SNCs) or silver nitrate. Medaka eggs at stage 21 were exposed to SNCs (10 mg L^{-1}) or silver nitrate (10 mg L^{-1} as silver) in different concentrations of ERM for 6 days. (a) Hatching rate. (b) Full body length. (c) Heart rate per 15 s. (d) Eye size. (e) Concentrations of soluble silver complexes released from SNCs or silver nitrate into test solutions. (f) Silver concentrations in embryos exposed to SNCs or silver nitrate in different concentrations of ERM. *Significant difference compared with the respective 1× ERM solution. NA: not available because only one larva hatched.

$AgNO_3$ exposure the heart rate was significantly ($P < 0.01$) lower in both types of solution at 30× ERM than at 1× ERM (Fig. 3c). Eye size was stable at 0.357 to 0.366 mm at all ERM concentrations in the controls. In 1× to 15× ERM, eye size was in a similar range (0.344 to 0.357 mm) with SNC or $AgNO_3$ exposure as in the controls; however, in 20× ERM and 30× ERM it was significantly lower ($P < 0.01$) with SNC or $AgNO_3$ exposure than in 1× ERM (Fig. 3d).

Groh *et al.*[33] stated that chloride reduces the toxicity of silver nanoparticles. However, the salinities they examined were lower than 1× ERM. In our research, SNC toxicity was higher in ultrapure water than in 1× ERM. Under freshwater conditions, free Ag^+ is no longer dominant; soluble complexes ($[AgCl]^0$ and $[AgCl_2]^-$) are formed and dominate (Fig. 2a to d, Fig. S5a and S5b, and Tables S1 to S3†). These complexes seem to be less toxic than free Ag^+. In 1× to 30× ERM, $[AgCl_3]^{2-}$ or $[AgCl_4]^{3-}$, or both, emerged and dominated along with $[AgCl_2]^-$ (Fig. 2a to d, and S5a and S5b†). Simultaneously, the exposed medaka embryos exhibited toxic effects (Fig. 3a to d). $[AgCl_3]^{2-}$ or $[AgCl_4]^{3-}$—or both—seem to have toxicity.

Thus, although the concentration of SNCs or $AgNO_3$ in all test solutions was 10 mg L^{-1} (as silver), these four biomarkers were affected by an increase in salinity. Notably, the hatching rate was the most sensitive to SNC toxicity, and the toxicity of SNCs to hatching was greater than that of $AgNO_3$.

To measure the concentrations of soluble silver complexes, we analysed filtered solutions of SNCs or $AgNO_3$ at 10 mg L^{-1} (as silver) with ICP-MS (Fig. 3e). The concentration of soluble silver increased from 0.08 mg L^{-1} (SNCs) and 0.06 mg L^{-1} ($AgNO_3$) in 1× ERM to 2.45 mg L^{-1} (SNCs) and 3.30 mg L^{-1} ($AgNO_3$) in 30× ERM. In 20× ERM, although the soluble silver concentrations in the SNC and $AgNO_3$ solutions were the same, the hatching rate was significantly ($P < 0.01$) inhibited in the SNC solution but not in the $AgNO_3$ solution (compare Fig. 3a and e).

The accumulated silver concentrations in the embryos were 0.43 ± 0.060 ng mg^{-1} (SNCs, 1× ERM) and 0.35 ± 0.042 ng mg^{-1} ($AgNO_3$, 1× ERM); these increased to 0.99 ng mg^{-1} (SNCs, 30× ERM) and 0.60 ng mg^{-1} ($AgNO_3$, 30× ERM) with the increase in salinity. In our previous study[7] using other silver nanocolloids (3.6 nm in diameter, with a 96 h LC_{50} of 1.39 ± 0.02 mg L^{-1} in ultrapure water) made by a different company, silver was detected at levels of 0.025 ng mg^{-1} in medaka embryos exposed to 0.5 mg L^{-1} in ultrapure water. In our present study, medaka eggs were exposed to silver at 10 mg L^{-1}—a concentration 20 times the 0.5 mg L^{-1}. Generally higher concentrations in ambient water cause higher bioaccumulations in aquatic organisms. This is why greater accumulation of silver occurred in our present study. Measurement of the concentrations of silver in the embryos revealed that significantly more silver accumulated with SNC exposure than with $AgNO_3$ exposure in 20× and 30× ERM ($P < 0.01$) (Fig. 3f). The greater toxicity with SNCs than with $AgNO_3$ at 20× ERM (Fig. 3a and b) probably occurred because the bioavailability of silver through the egg chorion was significantly higher with SNCs than with $AgNO_3$ in 20× or 30× ERM ($P < 0.01$).

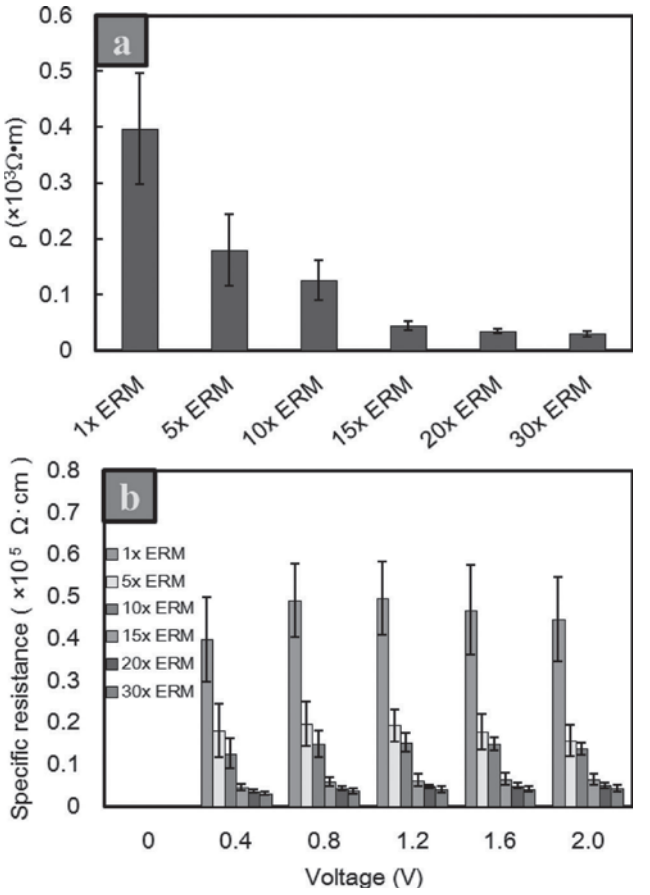

Fig. 4 Electrical resistance of medaka eggs in different concentrations of ERM (a) at a constant voltage of 0.4 V and (b) at voltages of 0.4 to 2.0 V.

Osmotic pressures in medaka egg embryos and bioavailability of SNCs

Salinity-dependent membrane permeation of chemicals has been reported in the cases of mercury chloride,[20] contaminant chemicals at waste-disposal landfill sites,[21] and nanoparticles.[22] To investigate salinity-dependent increases in the bioavailability of silver, we measured the electrical resistance of the chorion membrane in each concentration of ERM (1×, 5×, 10×, 15×, 20×, or 30×) (Fig. S6 and Formula S1†). Although the mean egg diameter was 1.29 ± 0.02 mm at all ERM concentrations and did not change during incubation at any of the concentrations (Table S4†), the electrical resistance of the chorion membrane decreased with increasing salinity (*i.e.* with increasing ERM concentration from 1× to 30×) under a constant voltage (0.4 V) (Fig. 4a). This trend was the same at higher voltages measured up to 2.0 V (Fig. 4b). Thus, there was salinity-dependent ion permeation between the chorion and the ERM. Hence, the increasing accumulation of SNCs or soluble silver chloro complexes, or both, by embryos *via* the chorion membrane can be explained by increasing osmotic pressure of ERM and decreasing resistance.

In addition, incubation of the eggs in ERM at different concentrations for 24 h revealed that the osmotic pressure of embryonic fluids increased with increasing ERM concentration, as Machado and Podrabsky[34] reported (Fig. S7a†). There was a linear relationship ($r = 0.9754$, $P < 0.01$) between the osmotic pressure of the embryonic fluids and the osmotic pressure (and by implication the salinity) of the incubation solution (Fig. S7b†).

In our previous study, water-suspended fluorescent nanoparticles were demonstrated to penetrate through the medaka egg chorion in 1× ERM; particles 474 nm in diameter showed the greatest uptake into the egg. Furthermore, uptake of nanoparticles increased with increasing salinity.[22] In this study, we estimated the aggregated diameter of SNCs in 30× ERM to be 437.3 nm (Fig. S3†)—close to the size that shows the greatest uptake. Moreover, a study of the toxicity of contaminant chemicals at waste-disposal landfill sites found that medaka embryonic malformation was induced most frequently when there was osmotic equivalency of leachate to eggs.[21] These findings support the salinity dependence of the increase in silver bioavailability in the embryos.

Conclusion

We demonstrated here that increasing salinity facilitates the uptake of ion-charged compounds such as SNCs and silver

chloro complexes into medaka eggs *via* the chorion. Salinity affects the electrical resistance of the chorion membrane and the embryonic osmotic pressure; these in turn affect the permeation of ion-charged chemicals through the chorion. Not only are potentially toxic silver chloro complexes released, but also the bioavailability of SNCs is higher than that of $AgNO_3$ in saline solutions, meaning that SNCs have greater medaka embryo toxicity. Although we do not yet know precisely which SNC-related compounds have toxic effects, silver nanotoxicity must be taken into consideration in high-salinity aquatic environments to a greater extent than in freshwater environments.

Acknowledgments and grant information

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Metal Mixture Modeling

APPLICATION OF A GENERALIZED LINEAR MIXED MODEL TO ANALYZE MIXTURE TOXICITY: SURVIVAL OF BROWN TROUT AFFECTED BY COPPER AND ZINC

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Abstract: Increased concerns about the toxicity of chemical mixtures have led to greater emphasis on analyzing the interactions among the mixture components based on observed effects. The authors applied a generalized linear mixed model (GLMM) to analyze survival of brown trout (*Salmo trutta*) acutely exposed to metal mixtures that contained copper and zinc. Compared with dominant conventional approaches based on an assumption of concentration addition and the concentration of a chemical that causes x% effect (ECx), the GLMM approach has 2 major advantages. First, binary response variables such as survival can be modeled without any transformations, and thus sample size can be taken into consideration. Second, the importance of the chemical interaction can be tested in a simple statistical manner. Through this application, the authors investigated whether the estimated concentration of the 2 metals binding to humic acid, which is assumed to be a proxy of nonspecific biotic ligand sites, provided a better prediction of survival effects than dissolved and free-ion concentrations of metals. The results suggest that the estimated concentration of metals binding to humic acid is a better predictor of survival effects, and thus the metal competition at the ligands could be an important mechanism responsible for effects of metal mixtures. Application of the GLMM (and the generalized linear model) presents an alternative or complementary approach to analyzing mixture toxicity. *Environ Toxicol Chem* 2015;9999:1–5. © 2015 SETAC

Keywords: Windermere humic aqueous model (WHAM) humic acid Concentration addition Response addition Binomial distribution Random effect

INTRODUCTION

Predicting and analyzing the toxicity of chemical mixtures is a central but challenging topic in environmental toxicology [1,2]. Understanding critical toxic mechanisms and developing theoretically sound approaches for the prediction of mixture toxicity are ideal goals but are difficult to achieve. To date, 2 classical but sometimes arbitrarily applied methods, concentration addition (i.e., similar joint action) and response addition (i.e., independent joint action), have been adopted to categorize the major types of chemical interactions and to predict mixture toxicity [3]. In addition, based on toxicity tests with single chemicals and their mixtures, the extent of the observed mixture toxicity is typically categorized as additive, more than additive (synergistic), and less than additive (antagonistic) [4–6]. In testing chemical interactions, the concentration addition approach, which generally assumes similar modes of action and often requires parallel concentration–response relationships among the mixture components, is predominantly used [5–7]. A common procedure is to quantify concentration–response relationships for individual chemicals; estimate the concentration of each chemical that causes x% effect (ECx; often 50% is used); calculate the sum of toxic units by adding up the ratios of each chemical concentration in the mixture divided by its corresponding ECx; and, based on the sum of toxic units, compare predicted and observed effects to evaluate chemical interactions (see Laetz et al. [8] and Faust et al. [9] for more detailed examples).

Survival response is binary (i.e., dead or alive) and is commonly investigated in acute toxicity tests with fish or invertebrates (e.g., *Daphnia magna*). The effect on survival rate traditionally has been analyzed using nonlinear regression models that assume the normal (Gaussian) function as the error distribution [10,11]. However, such models have some statistical drawbacks (see Kerr and Meador [12] for more details). For example, the calculation of the proportion (the number of living organisms divided by the total number of organisms tested) loses information on the sample size (the number of organisms added in each chamber); 90 surviving organisms out of 100 organisms and 9 out of 10 are equal (0.9) in terms of proportion. In contrast, the generalized linear model (GLM) with a binomial distribution can analyze survival data while retaining the sample size information (using raw binary data instead of aggregated proportions) and thus can be used in an ecotoxicology context [12].

Moreover, the GLM has an appealing feature in its application to analysis of the mixture toxicity. Suppose we have toxicity data on survival of a species after exposure to each of 2 chemicals (M_1, M_2) and their mixtures. The expected survival rate (S) can be modeled using the binomial GLM with a logit link

$$S = \frac{1}{1 + exp(-(\beta_0 + \beta_1 M_1 + \beta_2 M_2 + \beta_3 M_1 M_2))} \quad (1)$$

and this equation can be expressed as

$$\begin{aligned} \text{logit}\ (S) &= \log\left(\frac{S}{1-S}\right) \\ &= \beta_0 + \beta_1 M_1 + \beta_2 M_2 + \beta_3 M_1 M_2 \end{aligned} \quad (2)$$

where β_0, β_1, β_2, and β_3 are regression coefficients to be estimated. The term $\beta_3 M_1 M_2$ corresponds to the influence of the chemical interaction, and the importance can be evaluated by

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simply determining the statistical significance of β_3 or by performing model selection based on information criteria such as Akaike's information criterion (AIC; [13]).

In addition to within-test variability, nonsimultaneous toxicity testing of the individual chemicals and their mixtures can increase variability (e.g., changes in initial conditions of test organisms) and result in invalid data interpretations [14] if not addressed properly. In the framework of GLM, the additional variability that affects the intercept of the concentration–response curve can conventionally be handled by including dummy variables in the model. For example, if toxicity tests were performed in n different time periods, n–1 dummy variables would be needed. However, because such additional variability is not usually the main research focus but instead is considered a nuisance factor, it is preferable to use a smaller number of variables to simplify the data handling and the resulting model. In this regard, a simple extension of the GLM called the generalized linear mixed model (GLMM) would be effective, because the GLMM requires only an additional parameter (called a random effect; see *Methods* for further explanation). The GLMMs rarely have been used in ecotoxicological studies [15], but are frequently used in ecology [16]. Simple introductions to GLMMs are available elsewhere [17].

The present study demonstrates an application of the GLMM to analyze survival data from toxicity tests using metal mixtures. We used available data on survival responses of brown trout (*Salmo trutta*) to copper and zinc [18]. Through this application, we test whether the concentration of metals binding to humic acid is a better predictor of metal effects on survival of brown trout than 2 other measures: dissolved and free-ion concentrations of metals. The concentration of metals binding to humic acid, estimated using a chemical speciation model (Windermere humic aqueous model [WHAM]), has been recently proposed as a new predictor of metal exposure [19]. This approach (referred to in the present study as the WHAM-HA approach, a term also used by Iwasaki et al. [20]) uses humic acid as a proxy of organism binding and has been applied to responses of river macroinvertebrates to metals and protons in field and microcosm settings [19–21] as well as to copper toxicity to duckweed (*Lemna minor* [22]). Evidence is accumulating for the use of the WHAM-HA approach to model the effects of metals on aquatic organisms [20,22–24], and the present study provides additional support for this approach.

METHODS

Toxicity data

The data used in the present study were from acute toxicity tests conducted with brown trout exposed to zinc, copper, and their mixtures [18]. For those tests, brown trout eggs were obtained from the Colorado Division of Wildlife's Fish Research Hatchery in Bellevue, Colorado (USA), and swimup-stage fry hatched from those eggs were used for the tests (6–9 wk old: average length and weight were 35.4 mm and 0.408 g, respectively). A total of 8 flow-through toxicity tests were conducted at 5 different times (January to March 2002), including 4 single-metal tests and 4 mixture tests in which the target mass ratios of zinc to copper were 4:1, 8:1, 16:1, and 32:1 (Supplemental Data, Figure S1). These target ratios were selected to bracket the expected zinc:copper individual-metal median lethal concentration (LC50) ratio of 12:1. The concentration ranges of dissolved zinc and copper were <10 μg/L to 2340 and <0.5 μg/L to 116 μg/L, respectively, and the measured concentrations are reported in Davies et al. [18]. Each toxicity test had 6 exposure concentrations (including a control) and 4 replicate chambers per concentration. Ten fish were randomly distributed to each chamber, and mortality was frequently monitored and recorded. Although the original toxicity tests lasted for 7 d, we used the initial 96-h survival data because mortality did not increase considerably thereafter.

Dissolved concentrations of copper and zinc (0.45-μm-filtered) were measured daily from a water sample taken from 1 of 4 replicates in each exposure concentration. Other water quality characteristics were measured daily in 2 randomly chosen exposure chambers from each of the 3 serial diluters used [25]. For the present analysis, the water quality characteristics were pH (range of mean values across 8 tests: 7.00–7.35), water temperature (11.6–12.5°C), hardness (44.7–55.1 mg/L $CaCO_3$), and alkalinity (32.4–41.8 mg/L $CaCO_3$). Mean values obtained from each test were used as the inputs for the metal speciation.

Predictors of metal exposure

In addition to measured concentrations of dissolved metals, 2 predictors of metal exposure were used in the present study: free-ion concentrations of metals (mol/L) and concentrations of metals binding to particulate humic acid, ν (mol/g). These 2 predictors were estimated using WHAM 7 (version 7.0.2; [26]) according to the procedure described in Iwasaki et al. [20].

The inputs used for the estimation, approximately half of which were obtained from Davies et al. [18], were concentrations of magnesium, calcium, sodium, potassium, chloride, sulfate, zinc, and copper; pH; water temperature; alkalinity; and colloidal humic acid. The value of colloidal humic acid was calculated from that of dissolved organic carbon (DOC); that is, colloidal humic acid = DOC × 2 × 0.65 [27]. Because DOC and concentrations of sodium, potassium, chloride, and sulfate were not measured in the original study, we used average values of 1.7 mg/L, 3.1 mg/L, 0.6 mg/L, 3.4 mg/L, and 11.0 mg/L, respectively, from recent samples [28]. Also, concentrations of magnesium and calcium were unavailable at the time of the tests and were estimated from measured hardness values by assuming the mass ratio of 11.8 (Ca/Mg [28]). Concentrations of metals less than detection limits (10 μg/L for zinc, and 0.5 μg/L for copper) were assigned to be one-half of the detection limits. In addition to the estimated concentrations of colloidal humic acid in the exposure waters, a small concentration (1.0×10^{-15} g/L) of particulate humic acid was specified in the WHAM-input chemistry to allow prediction of the concentration of metals bound to particulate humic acid (ν). Although several parameters were not measured in the original study, the laboratory has a long history of conducting toxicity tests with the same water source (dechlorinated municipal tap water), and measured values have been stable and changed little over time. Based on this fact and some additional analyses, it is unlikely that our conclusions are affected by the use of concentrations estimated from recent measurements.

Statistical analysis

To test which of the 3 predictors of metal exposure (dissolved concentration, free-ion concentration, or ν) provided a better estimate of metal mixture effects, we developed GLMMs with a binomial distribution and logit link function for each of these predictors. The expected survival rate S_{ij} at concentration i in test period j is modeled as

$$\text{logit}\ (S_{ij}) = \beta_0 + \beta_1 Zn_{ij} + \beta_2 Cu_{ij} + \beta_3 Zn_{ij} Cu_{ij} + r_j \qquad (3)$$

where Zn_{ij} and Cu_{ij} are concentrations of the metals in each predictor category, and r_j is a random effect used to consider the

variability attributed to nonsimultaneous testing. The random effect r_j is assumed to be normally distributed with a mean of 0 and a standard deviation of *sigma*, which is to be estimated. This assumption for the random effect is commonly employed [17] and is the default in the R package used (see below). In addition, to accommodate a case that inclusion of the chemical interaction provides limited improvement of the model fitting, we also included a simpler model ($\beta_0 + \beta_1 \mathrm{Zn}_{ij} + \beta_2 \mathrm{Cu}_{ij} + r_j$). We then fit a total of 6 GLMMs (the 2 models in each of 3 predictor categories) to the whole dataset and ranked models based on calculated AIC values. The AIC is an estimator of Kullback–Leibler information and provides a parsimonious property to selected models [29,30]. The form of the estimator has 2 terms: first, -2 log(L) is a measure of lack of fit, and second, $+2$K is a correction for asymptotic bias. A lower value of AIC indicates a more parsimonious model that makes a better prediction. All statistical analyses were performed using R version 2.15. 3 [31] with the R package glmmML, in which Gauss–Hermite quadrature is used to approximate the likelihood for estimating model parameters [16]. An example R code for running the GLMM and data used in the present analysis can be found in the Supplemental Data.

RESULTS AND DISCUSSION

Predictors of metal exposure

Among the 6 GLMMs tested, each pair of which (models with and without the interaction term) employed 1 of the 3 predictors of toxicity (dissolved metal concentration, free-ion concentration, or ν), the models that used ν had the smallest AIC values among them by AIC differences (ΔAIC) of approximately 6 to 8 compared with the higher AIC models (Table 1 and Fig. 1). Although use of arbitrary cutoffs requires caution [29], if the AIC value for one model (say, model A) exceeds the AIC value for another model (model B) by 4 to 7, then there is some support for the claim that model B is better [30]. If the AIC difference is >10, there is strong support for that claim. Thus, these results provide moderate support for the use of the WHAM-HA approach to modeling toxicity of metal mixtures.

This finding is consistent with that of Iwasaki et al. [20], who analyzed microcosm data on responses of macroinvertebrate communities to metal mixtures (cadmium, copper, zinc), but the resulting ranking of dissolved-metal and free-ion concentrations as predictors of toxicity differed between the 2 studies. The results from the present study and from Iwasaki et al. [20] thus suggest that the competition of metals for binding to organisms could be an important mechanism for predicting effects of metal mixtures. Based on relationships between observed and predicted survival rates of brown trout (Fig. 1), however, predictions of the 3 predictor categories did not vary considerably, and even the best model with ν had some prediction error. Therefore, although the WHAM-predicted concentration of metals binding to particulate humic acid (ν) is clearly worthy of consideration as a predictor of metal mixture toxicity, further research is needed to reach more convincing conclusions. Moreover, although we focused on 2 metals (copper and zinc) in the present study, application of the WHAM-HA approach to soft metals (e.g., inorganic mercury), which exhibit different coordination preferences [32,33], might require some adjustments.

Metal interaction

The regression coefficients of the GLMM interaction term were positive regardless of the predictors used, implying that the interaction of copper and zinc caused less-than-additive toxicity in the brown trout dataset. The models with the interaction term generally had smaller AIC values in all predictor categories (Table 1), but the differences in AIC values were <2 and not considered to be substantial [30]. Thus, the evidence for a nonadditive underlying interaction is not conclusive based on the current dataset. In fact, when dissolved concentrations of the metals were used as the predictor of mixture toxicity, the model assuming additivity had a lower AIC value than the model with the interaction term. Although the difference in AIC values (0.4) is not substantial, this result indicates that the interpretation for the interactions in mixture toxicity tests can change depending on predictors used; another example can be found in Meyer et al. [34].

Application of GLMMs to mixture toxicity

The present study has demonstrated the application of an extension of the GLM (GLMM) to analyze mixture ecotoxicity data and statistically test the extent of the interactions (i.e., less than additive, additive, more than additive). Such approaches rarely have been employed, although a few applications that used a linear mixed model (i.e., using a normal error distribution) to investigate the metal interactions are available [35,36]. This is rather surprising because the GLM and GLMM have been introduced into ecotoxicology [12,15], and more sophisticated applications such as the use of Bayesian inference [37] have been available. Compared with conventional approaches such as those based on the concentration addition approach, the major advantages are 2-fold: first, binary response variables such as survival can be modeled without any transformations, and thus the approaches can take sample size

Table 1. Results of statistical analysis using generalized linear mixed models

		Model coefficients					
Predictor	Model[a]	β_0	β_1	β_2	β_3	*sigma*	AIC[b]
Dissolved	1	5.31	−1.47E-01	−8.01E-03	4.67E-05	0.87	180.68
	2	5.25	−1.42E-01	−7.50E-03	NA	0.96	180.28
Free-ion	1	4.39	−3.37E+08	−7.72E+05	1.74E+13	0.65	178.79
	2	4.35	−3.22E+08	−7.16E+05	NA	0.77	179.90
ν	1	7.83	−4.47E+04	−1.57E+04	2.34E+07	0.89	172.44
	2	7.60	−4.24E+04	−1.44E+04	NA	1.06	173.89

[a]Model 1 is the model with the interaction (i.e., full model; see Equation 3), and mode1 2 is the model without the interaction.
[b]A lower Akaike's information criterion (AIC) value indicates a more parsimonious model that makes a better prediction.
ν = concentrations of metals binding to particulate humic acid; NA = not available.

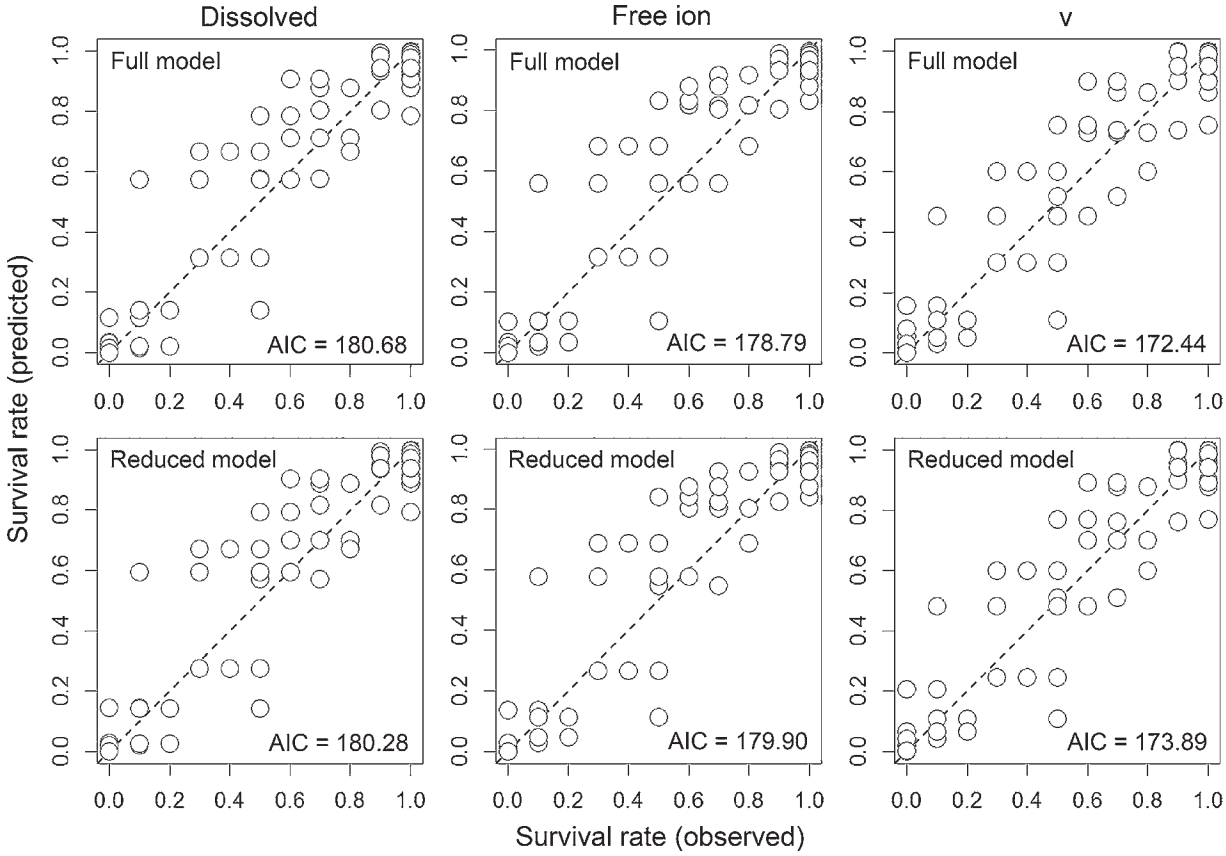

Figure 1. Observed versus predicted survival rates obtained from full and reduced models with 3 predictors of metal exposure ($n = 192$). Full model is the model with the interaction (see Equation 3), and reduced model is the model without the interaction. A lower Akaike's information criterion (AIC) value indicates a more parsimonious model that makes a better prediction. v = concentrations of metals binding to particulate humic acid.

into consideration (i.e., using raw binary data instead of aggregated proportions); and second, the importance of the interaction can be statistically tested in an intuitive manner.

In contrast, an appealing advantage of the conventional concentration addition approach is that ECx, particularly EC50 (or LC50), can be obtained from the literature and used for evaluation of the chemical interactions. Although concentration addition itself does not necessarily assume that the concentration–response curves are parallel [38,39], concentration addition approaches such as toxic unit summation and toxic equivalency factor require such assumption [7] to be able to validly predict the amount of toxicological impairment at a specified number of toxic units or toxic equivalents other than 1.0 (see Laetz et al. [8] as a practical example). Also, researchers using the concentration addition and response addition approaches often ignore the uncertainty associated with the point estimate of ECx, which may lead to unreliable conclusions on chemical interactions (see also Meyer et al. [4] in the present issue). By applying the GLM or GLMM to all the toxicity data (i.e., single and mixture toxicity data), such uncertainties associated with data are necessarily taken into account.

In addition to the GLMM approach demonstrated in the present study and conventional concentration addition and response addition approaches, other methods are used to analyze mixture toxicity [40–42], such as an isobole-based approach [41] and a more comprehensive analysis tool [42]. An interesting research question is to investigate how the choice of these methods affects the subsequent interpretation of the chemical interactions. However, probably more importantly, use of those approaches without a solid understanding of the toxic mechanisms occurring in a mixture is solely arbitrary. Mechanistic understanding and modeling are needed for a comprehensive evaluation of the effects of chemical mixtures [43].

In the present study, we used the random effect to statistically consider the variation because of nonsimultaneous toxicity tests. The use of random effects (called the mixed model) is an advantageous way to take into account circumstances in which the data within a single group (e.g., test period, chamber) have common features of correlation. The mixed model thus helps to handle pseudoreplication [44], particularly when data that are analyzed are not statistically independent. If all the experiments are conducted at the same time and the data are statistically independent, the inclusion of a random effect is unnecessary, and the GLM (the model without random effect) can be used. Moreover, 2 or more random effects can be modeled if needed (e.g., the influence of different diluters). Overall, the use of the GLMM and GLM is an alternative or complementary approach to analyze mixture toxicity to binary biological responses such as survival.

SUPPLEMENTAL DATA

Tables S1–S2. (31 KB XLS).
Figure S1. (168 KB PDF).

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Data availability—Data used in the present study may be found in the online Supplemental Data.

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Free Silver Ion as the Main Cause of Acute and Chronic Toxicity of Silver Nanoparticles to Cladocerans

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Abstract We investigated the interspecific variation of silver nanoparticle (SNP) sensitivity in common cladocerans (*Daphnia magna*, *D. galeata*, and *Bosmina longirostris*) and the exact cause of both acute and chronic toxicity focusing on the form of silver (NPs and ions). Materials tested were non-surface-coated silver nanocolloids (SNCs) and $AgNO_3$. The results of the acute toxicity tests support the theory that the effects of SNPs on aquatic organisms is mainly due to Ag^+ released from SNPs. Among the three cladocerans, *D. galeata* was more sensitive to silver (as Ag^+) than both *D. magna* and *B. longirostris*. Moreover, the chronic toxicity of SNCs was also derived from dissolved silver (especially Ag^+). SNCs (as total silver concentration) showed far lower chronic compared with acute toxicity to daphnids because the amount of dissolved silver decreased in the presence of prey algae. The chronic endpoint values (EC_{10} values for net reproductive rate and the probability of survival to maturation) did not differ largely from acute ones (48-h EC_{50} obtained from acute toxicity tests and 48-h LC_{50} estimated by the biotic ligand model) when the values were calculated based on Ag^+ concentration. The α value (concentration at which intrinsic population growth rate is decreased to zero) estimated by a power function model was a reliable parameter for assessing the chronic toxicity of silver.

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Despite rapid nanotechnology development, biochemical mechanisms on acute and chronic toxicity of nanomaterials are still uncertain. Nano-silver is one of the most frequently used nanomaterials because of its antimicrobial activity. Fabrega et al. (2011) reviewed the behavior and effects of silver nanoparticles (SNPs) in aquatic environments and pointed out the necessity to develop analytical and metrological methods to detect, quantify, and characterize SNPs under ambient conditions. Despite the lack of experimental validation, the predicted environmental concentrations of SNPs (*e.g.*, surface water, sludge treatment plant effluents, and sludge-treated soil) are estimated in the range of ng L^{-1} and mg kg^{-1} (Gottschalk et al. 2009).

In aquatic environments, SNPs can affect prokaryotes, invertebrates, and fish at a few ng L^{-1} (see Fabrega et al. 2011 for review). However, the potential toxicity of SNPs differs depending on their own properties (*e.g.*, particle size, presence/absence and type of capping agent, purity, and dissolution rate) and environmental conditions (*e.g.*, water chemistry and temperature). For instance, small particles tend to show high toxicity to aquatic organisms (Kennedy et al. 2012). SNPs penetrate into the algal cell if the particle size is smaller than the cell wall pore (5–20 nm) (Navarro et al. 2008a). The depositions of SNPs have also been observed in fish eggs and embryos (Asharani et al. 2008; Kashiwada et al. 2012). Starch-capped and bovine serum albumin-capped SNPs and released free ions

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(Ag^+) exert oxidative stress, DNA damage, and cellular proliferation delay (Asharani et al. 2008). The expression of six embryogenesis- and morphogenesis-related genes (*ctsL*, *tpm1*, *rbp*, *mt*, *atp2a1*, and *hox6b6*) in fish were significantly affected by SNP (non-surface coating, particle size = 3.8 ± 1.0 nm) exposure (Kashiwada et al. 2012). SNP-specific toxicity has also been observed in an invertebrate (*Daphnia magna*), in which mitochondrial function (proton efflux) of the embryo was more severely affected by SNP exposure than that of $AgNO_3$ (Stensberg et al. 2014). Poynton et al. (2012) reported that polyvinylpyrrolidone (PVP)-coated SNPs (particle size = 35 nm) and $AgNO_3$ affected the different gene expressions of *D. magna*: the former disrupted protein metabolism- and signal transduction-related gene expression profiles, and the latter caused a downregulation of developmental processes, particularly in sensory development.

In contrast, the results of laboratory bioassays have indicated that toxicity to algae and cladocerans is attributed mainly to dissolved silvers released from SNPs, especially Ag^+ (Navarro et al. 2008b; Burchardt et al. 2012; Kennedy et al. 2012; Völker et al. 2013; Newton et al. 2013; Ribeiro et al. 2014). The most likely mechanism causing acute toxicity of Ag^+ to aquatic animals is the inhibition of Na^+ uptake by the gills as a result of the impact on Na^+, K^+-ATPase activity (Hogstrand and Wood 1998; Bianchini and Wood 2003). Dissolution of Ag^+ from SNPs should be dependent on the properties of the test media (chemical composition, pH, and temperature) and SNPs (size, shape, and capping agent) (Fabrega et al. 2011). In fact, the acute toxicity results (48-h LC_{50}) for *D. magna* as dissolved silver concentration are comparable for gum arabic-coated, polyethylene glycol-coated and PVP-coated SNPs and $AgNO_3$ solutions (Newton et al. 2013). Thus, the 48-h LC_{50} of SNPs for *Ceriodaphnia dubia* were remarkably consistent with the value predicted by the biotic ligand model (BLM; Niyogi and Wood 2004) when expressed on the basis of dissolved silver (Kennedy et al. 2012). Although the BLM has not yet been calibrated to predict SNP toxicity, existing metal speciation and toxicity models might be effective tools for this purpose.

For ecological risk assessment, extrapolation from toxicological data obtained at the individual level into effects at the population level is inevitable (Tanaka and Nakanishi 2001). Population level effects can be evaluated by life-history data obtained from chronic toxicity tests; however, only a few studies addressing chronic SNP toxicity have been performed on aquatic animals (*Daphnia*: Zhao and Wang 2011; Blinova et al. 2013; Völker et al. 2013). According to Zhao and Wang (2011), the lowest-observable effect concentration of carbonate-coated SNPs for reproduction in *D. magna* was <1/10 of the 48-h EC_{50}. As a result, they concluded that the mechanisms of chronic effects were caused by the low food quality of algae associated with SNPs and the low depuration of ingested SNPs. Völker et al. (2013) showed that PVP-coated SNPs (particle size < 20 nm) affected reproduction in three *Daphnia* species at far lower concentrations than the 48-h EC_{50}. The investigators also showed that SNP toxicity on *Daphnia* increased with multigeneration exposures. These empirical data suggest that ecological risk assessment based on acute toxicity data (*e.g.*, 48-h EC_{50} values) is not yet sufficient to explain the impacts of SNPs on aquatic ecosystems. Contrary to those reports, Blinova et al. (2013) recorded lower chronic than acute toxicity of PVP-coated and protein-coated SNPs (particle size 6–12.5 nm) to *D. magna*. Note, however, that the information about chronic SNP toxicity is limited. Moreover, interspecific variation of sensitivity in cladocerans has seldom been investigated, except for standard test organisms (*D. magna*, *D. pulex*, and *C. dubia*), even for dissolved silver.

The aims of the present study were to determine (1) the interspecific variation of SNP sensitivity in common cladocerans and (2) the exact cause of both acute and chronic toxicity focusing on the form of silver (nanoparticles and ions). We evaluated the sensitivity of three cladocerans (*D. magna*, *D. galeata*, and *Bosmina longirostris*) to SNCs (a mixture of colloidal and ionic silver) compared with $AgNO_3$ by acute toxicity tests. The selected organisms (*D. galeata* and *B. longirostris*) are common lake species distributed in Eurasia and North America and often dominate the zooplankton community (Alonso 1991). Furthermore, we obtained the whole life-table data sets of two daphnids (*D. magna* and *D. galeata*) by chronic toxicity tests to evaluate the net reproductive rate (R_0) and intrinsic population growth rate (r) under different silver concentrations. With the above-mentioned data sets, we estimated the EC_{10} for R_0 and the probability of survival to maturation. In addition, we evaluated the silver concentration (α) at which *r* reduces to 0 by fitting the concentration-*r* data to a power function model (Tanaka and Nakanishi 2001). The response of the *r* value to pollutant concentration approximates was in many cases nearly quadratic (Tanaka and Nakanishi 2001). By comparing the silver concentrations (total silver and Ag^+) that affect immobilization (48-h EC_{50}) and reproduction (EC_{10} values and α) in daphnids, we re-examined the hypothesis that toxicity is mainly attributed to dissolved silvers released from SNPs.

Materials and Methods

Test Animals

Three cladoceran species (*D. magna*, *D. galeata*, and *B. longirostris*) were used in the present study. Single

clones of *D. magna* and *D. galeata* were obtained from the National Institute for Environmental Studies, Japan. These stock cultures have been maintained for 30 years at the institute. The *B. longirostris* clonal culture was established from animals collected in Lake Suwa (36°2′N, 138°5′E), Japan. We cultured these cladocerans in 1-L glass beakers. COMBO medium (Kilham et al. 1998) containing *Chlorella vulgaris* (Chlorella Industry Co. Ltd, Fukuoka, Japan) was used as the culture medium. The medium was changed once a week. The food concentrations were 5×10^5 cells mL^{-1} in the *D. magna* and *D. galeata* cultures and 1×10^5 cells mL^{-1} in the *B. longirostris* culture. We added *C. vulgaris* to the culture medium every second or third day. Stock cultures were kept under constant laboratory conditions (20 ± 1 °C; 16 h of light to 8 h of darkness).

SNCs and $AgNO_3$

Purified SNCs (99.99 % purity, non-surface coating, suspended in deionized water) were purchased from Utopia Silver Supplements (Utopia, Texas, USA). Diluted SNC solutions (a mixture of colloidal and ionic silver) for exposure tests were prepared with ultrapure water. The silver purity and concentration were validated by inductively coupled plasma mass spectrometry (ICP-MS) analysis using a Thermo Scientific X Series 2 (Thermo Scientific, Pittsburgh, Pennsylvania, USA): actual concentration and average diameter were 24.46 mg/L and 79.9 nm, respectively. The toxicity of SNCs was compared with dissolved Ag from reagent-grade $AgNO_3$ (Wako Pure Chemical Industries Ltd., Osaka, Japan). A stock solution of $AgNO_3$ (20 mg L^{-1}) was prepared by dissolving the compounds in distilled water.

Bioassays

The laboratory conditions (20 ± 1 °C, 16 h of light to 8 h of darkness) were the same as those for the stock cultures. Procedure for the acute toxicity tests for daphnids (*D. magna* and *D. galeata*) were performed according to Organisation for Economic Co-operation and Development (OECD) guideline no. 202 (OECD 2004) and with slight modifications of the guideline for *B. longirostris*. Female neonates (<24 h old) from the third or later broods were used in all tests. For the tests on *D. magna* and *D. galeata*, the nominal concentrations of SNCs and $AgNO_3$ were 5.92–28.56 μg L^{-1} (common rate = 1.3; no. of treatments = 7) and 1.01–3.73 μg L^{-1} (common rate = 1.3; no. of treatments = 6), respectively. For *B. longirostris*, SNCs and $AgNO_3$ were 3.85–8.45 μg L^{-1} (common rate = 1.3; no. of treatments = 4) and 0.78–3.73 μg L^{-1} (common rate = 1.3; no. of treatments = 7), respectively. A control (0 μg L^{-1}) was also prepared for each assay. For all of the test media, pH was adjusted to 7.0 with minimal drop additions of 10 % (v v^{-1}) HCl as required. Tests on daphnids were performed in four 50-mL glass beakers containing 50 mL of each test solution, and five neonates were introduced into a beaker. The procedure for *B. longirostris* was the same as that in our previous assay (Sakamoto et al. 2005). Ten glass bottles (12 mL) were filled with each test solution. One bosminid individual was placed into each bottle, and the exposure test was started. The mouth of each bottle was covered with a cover glass to exclude air from the test water so as to avoid trapping the animals at the water surface. Test individuals were not fed during the assays. At 48 h after the exposure began, the numbers of immobilized individuals were counted. Dissolved oxygen (DO) and pH were measured at the beginning and end of the tests in the controls and the highest test substance concentrations. The physicochemical conditions met the criteria: Values at the start and end were 8.70 ± 0.54 mg L^{-1} (mean ± SD) and 9.04 ± 0.69 mg L^{-1} for DO and 7.04 ± 0.03 and 7.35 ± 0.14 for pH.

Chronic toxicity tests were performed for *D. magna* and *D. galeata* according to OECD guideline no. 211 (OECD 2012). Nominal concentrations of SNC and $AgNO_3$ were 11.6–93.52 μg L^{-1} (common rate = 2.0; no. of treatments = 4) and 2.18–17.44 μg L^{-1} (common rate = 2.0; no. of treatments = 4) for *D. magna*, and 7.55–60.40 μg L^{-1} (common rate = 2.0; no. of treatments = 4) and 1.55–12.40 μg L^{-1} (common rate = 2.0; no. of treatments = 4) for *D. galeata*, respectively. The lowest dose in each assay was equal to the 48-h EC_{50} calculated using nominal silver concentrations. A control (0 μg L^{-1}) was also prepared for each assay. A female neonate was put into a 50-mL glass beaker containing 50 mL of the each test solution with food (*C. vulgaris*, 5×10^5 cells mL^{-1}). The replicate number of individuals at the start of the experiment was 10/treatment. Each animal was transferred to a clean beaker containing new test solution with food every second day, at which point neonates born to the experimental animal were counted and removed. Parturition, mortality, and number of newborns were checked daily until all of the individuals had died.

Water-Chemistry Analysis

To measure the absolute concentrations of cation (Na^+, Mg^{2+}, K^+ and Ca^{2+}), anion (Cl^-, NO_2^-, NO_3^-, PO_4^{3-} and SO_4^{2-}), dissolved organic carbon (DOC), and dissolved inorganic carbon (DIC), 100 mL water was collected from each test solution at the start of both the acute and the chronic toxicity tests. Each water sample was filtered through a Whatman GF/C filter. The concentrations of cations and anions were measured using ion chromatography (ICS-

Table 1 Concentrations of cations, anions, DOC, and DIC (mg L^{-1}) in test media and 48-h LC_{50} (Ag μg L^{-1}) for *D. magna* estimated by BLM

Test type	Cation				Anion					Carbon		BLM
	Na^{+} [a,b]	Mg^{2+} [a]	K^{+} [a,b]	Ca^{2+} [a,b]	Cl^{-} [a,b]	NO_2^{-} [a]	NO_3^{-} [a,b]	PO_4^{a}	SO_4^{2-} [a,b]	DOC[a,b]	DIC[a,b]	48-h LC_{50} (Ag^{+})
Acute (all species)	32.50	3.69	3.13	9.49	18.82	0	64.03	0.33	14.99	00[c]	3.86	0.56
Chronic (*D. magna*)	30.79	3.89	3.25	8.77	24.52	0.62	56.15	1.44	14.49	0.39[c]	3.86	0.51
Chronic (*D. galeata*)	32.25	3.78	3.60	9.11	28.27	0.45	55.86	1.27	12.04	0.83[c]	3.86	0.49

[a] Parameters used for metal speciation

[b] Parameters used for BLM

[c] Subtracted 1.4 mg C/L (in EDTA) from total DOC

1600; Thermo Fisher Scientific, Waltham, Massachusetts, USA), and DOC and DIC were evaluated with a TOC analyzer (multi N/C 3100, Analytik Jena AG, Jena, Germany).

Silver concentration was quantified from a 1.5-mL sample collected at the start of the bioassays. The water samples were centrifuged at 3,500×*g* for 10 min at 4 °C to remove the large solids (algal cells) before instrumental analysis. Total silver concentration was quantified from the supernatant. Dissolved silver (size < 1.5 nm) was separated from the particles through a 3-kDa membrane filter tube (Amicon Ultra-0.5, EMD Millipore Corporation, Billerica, Massachusetts, USA) where 0.5 mL supernatant was centrifuged at 14,000×*g* at 4 °C for 10 min. Two milliliters of HNO_3 (Ultrapur-100, specific gravity 1.42, Kanto Chemical, Tokyo, Japan) was added to the samples (0.4 mL supernatant for the total Ag or 0.1 mL filtrate for the dissolved Ag) in a 50-mL Teflon beaker (Sanplatec, Osaka, Japan). The mixture was heated at 110 °C until just before it dried out. Then 2.0 mL of ultrapure nitric acid and 0.5 mL of hydrogen peroxide (for atomic absorption spectrometry, Kanto Chemical) were added to the beaker and heated until just before the mixture dried out. The residue was dissolved with 1.0 % ultrapure nitric acid solution to a volume of 12.0 mL and then subjected to ICP-MS analysis (determination limit 0.03–1 μg/L).

Free-ion (Ag^{+}) concentrations in the test media were estimated using the freeware program Visual MINTEQ version 3.0 (http://www.lwr.kth.se/English/OurSoftware/vminteq) and then used to calculate the silver toxicities (EC_{50}). Input data sets are listed in Table 1. Binding of metal ions to dissolved organic matter was modeled using the NICA-Donnan formulation (Milne et al. 2001; Milne et al. 2003).

Data Analysis

Values of 48-h EC_{50} with 95 % confidence intervals (CIs) were estimated by fitting acute toxicity data to a two-parameter log-logistic model using the drc package (Ritz and Streibig 2005) in R version 2.15.2 [R Development Core Team, Vienna, Austria (http://www.R-project.org/)]:

$$f(x) = \frac{1}{1 + \exp\{b(\log(x) - \log(e))\}}$$

where $f(x)$ is the probability of immobilization, x is the measured silver concentration (total Ag or Ag^{+}), b is the relative slope at the inflection point, and e is the inflection point of the fitted line (equivalent to the dose required to cause a 50 % response).

Life table data obtained from the chronic toxicity tests were used to estimate the parameters related to population growth. Net reproductive rate (R_0) was calculated as

$$R_0 = \sum l_x m_x$$

where l_x and m_x are the probability of surviving from birth to age class x and number of offspring for a female in age class x, respectively (Stearns 1992). EC_{10} values for R_0 with 95 % CIs were estimated by a three-parameter log-logistic model using the drc package in *R* version 2.15.2:

$$f(x) = \frac{d}{1 + \exp\{b(\log(\mathrm{x}) - \log(e))\}}$$

where $f(x)$ is the R_0 value, x is the silver concentration (total Ag or Ag^{+}), b is the relative slope at the inflection point, d is the maximum value of R_0, and e is the inflection point of the fitted line. The EC_{10} values for the probability of survival to maturation (first parturition) were estimated by the above-mentioned two-parameter log-logistic model. Intrinsic population growth rate (r) was estimated using the dominant eigenvalue (λ) of the Leslie matrix for each treatment (Case 2000).

$$\lambda = \exp(r)$$

The silver concentration (α) at which r reduces to 0 was estimated by a power function model (Tanaka and Nakanishi 2001),

$$r = r_{\max}\left\{1 - \left(\frac{x}{\alpha}\right)^{\beta}\right\}$$

Table 2 Effective concentrations of SNCs and $AgNO_3$ for the different end point parameters of tested cladocerans

End point	Species	SNCs		$AgNO_3$
		Total silver	Ag^+	Ag^+
Behavior (48-h EC_{50})	*D. magna*	2.43	0.90	0.25
		[2.18, 2.68][a]	[0.79, 1.01]	[0.23, 0.26]
	D. galeata	2.16	.23	0.16
		[1.90, 2.41]	[0.16, 0.31]	[0.13, 0.19]
	B. longirostris	2.90	0.60	0.22
		[2.16, 3.65]	[0.57, 0.63]	[0.19, 0.25]
R_0 (EC_{10})	*D. magna*	20.88	1.20	0.09
		[−13.53, 55.29]	[0.43, 1.97]	[−0.15, 0.34]
	D. galeata	14.22	1.00	0.02
		[7.45, 20.99]	[0.63, 1.37]	[−0.11, 0.15]
Survival to maturation (EC_{10})	*D. magna*	3.77	0.35	0.34
		[−0.43, 7.98]	[0.01, 0.69]	[−0.16, 0.84]
	D. galeata	9.76	0.75	0.28
		[4.52, 15.00]	[0.44, 1.07]	[−0.09, 0.65]

[a] 95 % CIs (Ag μg L^{-1})

where x is the exposure concentration, r_{max} is the r in control, and α and β are parameters. Respectively, α and β are associated, with the magnitude of toxicity and the curvature of responses in r to exposure concentration, x.

As a reference value, we estimated the 48-h LC_{50} value of Ag^+ for *D. magna* by the BLM for each test condition using the freeware program BLM version 2.2.3 [HydroQual (http://www.hydroqual.com/wr_blm.html)].

Results

Water-Chemistry Analysis

Hardness of the test media was approximately 39 mg (as $CaCO_3$). Because there was no marked variation of the cation and anion concentrations, the BLM estimated similar 48-h LC_{50} values (Ag^+ for *D. magna*) in the three media (Table 1). Measured silver concentrations in the SNC and $AgNO_3$ solutions were far lower than the nominal ones. The percentage recoveries were 6–66 % for SNCs and 5–38 % for $AgNO_3$. By employing the metal speciation results, approximately 48 % of the dissolved silver was free ion (Ag^+), and the rest was $AgCl_{(aq)}$ (49 %) and $AgCl_2^-$ (2 %) in the acute toxicity tests. In the chronic toxicity tests, Ag^+, $AgCl_{(aq)}$, and $AgCl_2^-$ presented in the media were approximately 38, 58, and 4 %, respectively. Other silver species (*e.g.*, $AgSO_4^-$, $AgNO_{2(aq)}$, and $AgNO_{3(aq)}$) were <0.5 % of the total dissolved silver. Although the COMBO medium contained ethylene diamine tetraacetic acid (EDTA) (3.4 mg L^{-1}, 1.4 mg C L^{-1}) as a chelating agent, the concentrations of chelated silver ($AgEDTA^{3-}$ and $AgHEDTA^{2-}$) were negligibly low (<0.01 %).

Bioassays

The 48-h EC_{50} value of SNCs based on the measured total silver concentrations did not differ largely among the three cladocerans (Table 2). However, the sensitivity of *D. galeata* to SNCs was greater than that of the other two species when the values were calculated with the Ag^+ concentrations. A similar trend was observed for $AgNO_3$. The 48-h EC_{50} value of SNCs (0.90 μg L^{-1}, for Ag^+) and $AgNO_3$ (0.25 μg L^{-1}) for *D. magna* were comparable with the 48-h LC_{50} value (0.56 μg L^{-1}) estimated by the BLM (Table 1).

In each chronic toxicity test, daily observation was continued until all of the test individuals had died, and thus the experimental period differed between treatments. In the controls, *D. magna* reached greater net reproductive rates ($R_0 = 71.9$ in SNCs experiment and 68.8 in $AgNO_3$ exp.) than *D. galeata* ($R_0 = 44.5$ in SNCs exp. and 53.0 in $AgNO_3$ exp.) (open circles in Fig. 1). SNCs did not affect the daphnids' R_0 when the total silver concentrations were <14.5 μg L^{-1} (Fig. 1a, c). The EC_{10} values of SNCs (as total silver) for R_0 were far greater than the 48-h EC_{50} value, although the broad range of 95 % CIs indicate that the estimation is highly uncertain (Table 2). In contrast, $AgNO_3$ was highly toxic to daphnids: All of the *D. magna* neonates died within 1 day at 1.93 μg L^{-1}. Although SNC toxicity was lower than that of $AgNO_3$, the dose ranges did not differ between the chemicals in Ag^+ concentration (Fig. 2a, b). The SNC EC_{10} values based on Ag^+ concentrations (1.20 μg L^{-1} for *D. magna* and 1.00 μg L^{-1} for *D. galeata*) for R_0 did not differ largely from the 48-h EC_{50} values. The $AgNO_3$ EC_{10} values for R_0 (0.09 μg L^{-1} for *D. magna* and 0.02 μg L^{-1} for *D. galeata*) were 1 to 50 times lower than those of SNCs (as Ag^+), but the 95 % CIs were very wide. A similar trend was observed in the probability of survival to maturation (Table 2; Fig. 2c, d). Calculated SNC EC_{10} values for maturation tended to be lower than those for R_0 and were comparable with the 48-h EC_{50} values.

The predicted SNC concentrations (α) at which the intrinsic growth rate (r) reaches 0 was approximately 26 μg L^{-1} for *D. magna* and 20 μg L^{-1} for *D. galeata* (Table 3; Fig. 3). The estimated α value of $AgNO_3$ for *D.*

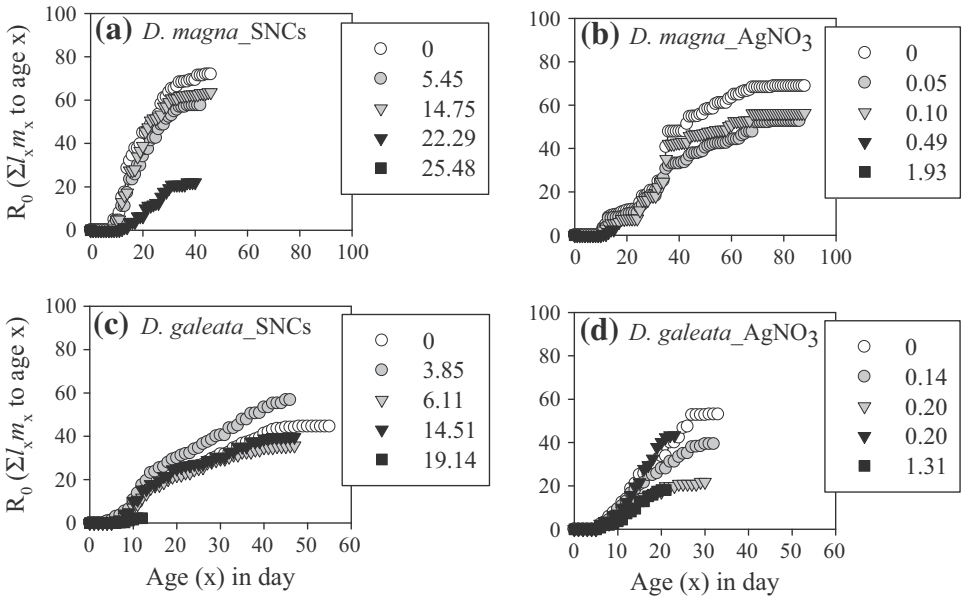

Fig. 1 Effects of SNCs and $AgNO_3$ on the net reproductive rates (R_0) of **a**, **b** *D. magna* and **c**, **d** *D. galeata* in the chronic toxicity tests. Numerals in each label indicate measured silver concentrations (μg L^{-1})

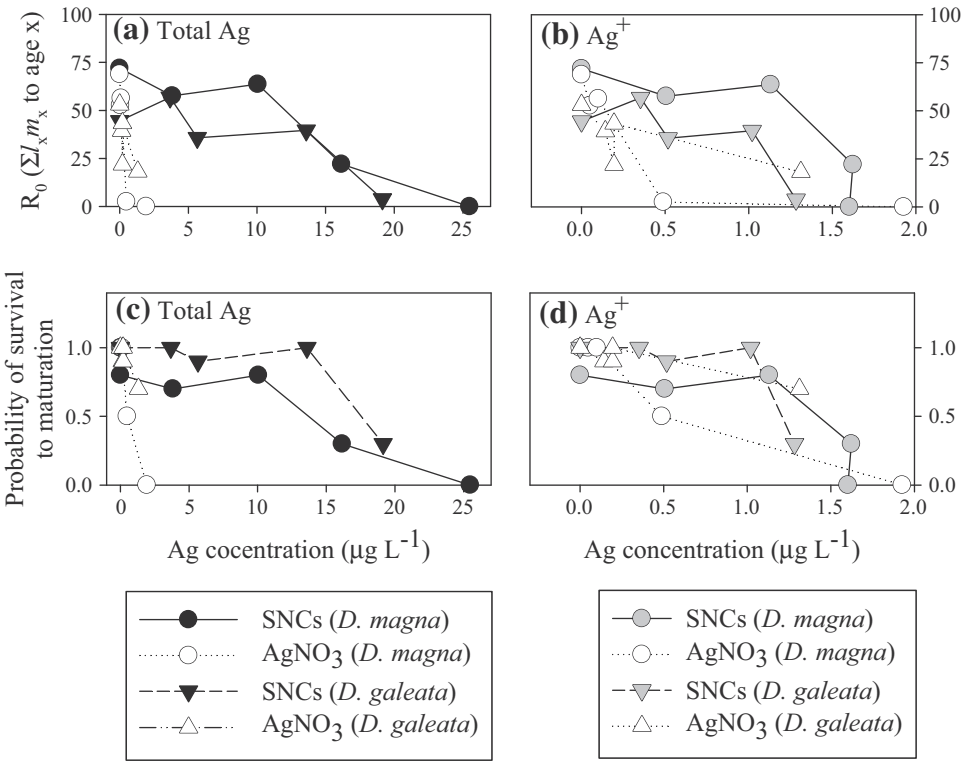

Fig. 2 Effects of SNCs and $AgNO_3$ on the R_0 (**a**, **b**) and the probability of survival to maturation (**c**, **d**) of *D. magna* and *D. galeata*. Left (**a**, **c**) and right (**b**, **d**) *panels* show the results based on the total silver and Ag^+ concentrations, respectively

magna was 0.8 μg L^{-1}. We could not estimate the $AgNO_3$ value for *D. galeata* because r (0.22 day^{-1}) at the highest dose was very high [only 25 % lower than the control (Fig. 3b)]. Despite the fact that SNCs were less toxic to daphnids than $AgNO_3$, the α Ag^+ values in SNCs were very low (1.9 μg L^{-1} for *D. magna* and 1.3 μg L^{-1} for *D. galeata*).

Discussion

The present results do not support the view that SNCs (as total silver concentration) are more toxic to cladocerans than $AgNO_3$ (as Ag^+) when survival rate, swimming behaviour, and reproductivity are used as end points. Likewise, laboratory studies suggest that acute toxicity of

Table 3 Estimated α and β values in chronic toxicity tests

Species	Exp.	Conc.[a]	r_{max}	Parameter in model	
				$\alpha \pm$ SE[b]	$\beta \pm$ SE
D. magna	SNCs	Total	0.27	25.99 ± 2.28	4.76 ± 2.44
		Ag^+		1.86 ± 0.14	5.39 ± 2.73
	$AgNO_3$	Ag^+	0.18	0.75 ± 0.01	1.04 ± 0.01
D. galeata	SNCs	Total	0.28	19.60 ± 0.52	13.33 ± 14.14
		Ag^+		1.31 ± 0.03	16.10 ± 17.08
	$AgNO_3$	Ag^+	0.30	Not estimated	Not estimated

[a] Concentration used for calculation (total Ag or Ag^+)

[b] Estimated dose level (Ag $\mu g\ L^{-1}$) for $r = 0$

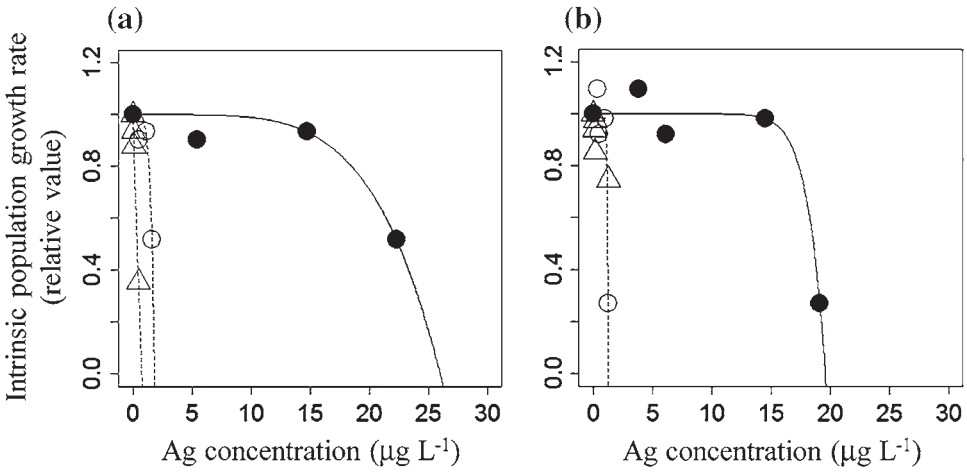

Fig. 3 Relationships between silver concentration and the intrinsic population growth rate of *D. magna* (**a**) and *D. galeata* (**b**). *Closed circle* SNCs as total silver; *open circle* SNCs as Ag^+; *triangle* $AgNO_3$ as Ag^+

SNPs to zooplankton and phytoplankton is attributed mainly to dissolved silvers released from SNPs (Burchardt et al. 2012; Kennedy et al. 2012; Völker et al. 2013; Newton et al. 2013; Ribeiro et al. 2014).

All three species (*D. magna*, *D. galeata*, and *B. longirostris*) exhibited a 48-h EC_{50} values ten times greater for SNCs than $AgNO_3$ (Table 2). The SNC 48-h EC_{50} values based on Ag^+ concentrations were comparable with those of $AgNO_3$ and the estimated 48-h LC_{50} value by BLM (Table 1), although the SNC values tended to be greater. In our acute toxicity tests, sensitivity to Ag^+ was *D. galeata* > *B. longirostris* ≥ *D. magna*. Völker et al. (2013) also reported that the 48-h EC_{50} value of PVP-coated SNPs (particle size < 20 nm) for *D. galeata* (13.9 $\mu g\ L^{-1}$) was approximately ten times lower than that of *D. magna* (121 $\mu g\ L^{-1}$). They also showed that *D. magna* was more sensitive to $AgNO_3$ than *D. galeata* (48-h EC_{50} = 1.1 $\mu g\ L^{-1}$ for *D. magna* and 2.1 $\mu g\ L^{-1}$ for *D. galeata*). However, our results contradict this: *D. galeata* was slightly more sensitive to $AgNO_3$ than *D. magna*. Although we have found no other report addressing silver toxicity to *D. galeata*, this species may generally show greater sensitivity to heavy metals [*e.g.*, copper (Bossuyt and Janssen 2005) and zinc (Vesela and Vijverberg 2007)]. In addition, this is the first report on the toxicity of silver to *B. longirostris*. *B. longirostris* is more sensitive to copper than either *D. galeata* or *D. magna* (Koivisto et al. 1992). Vesela and Vijverberg (2007) reported that the sensitivity of neonates of four *Daphnia* spp. to zinc was positively correlated with body size (the sensitivity was *D. galeata* > *D. pulex* > *D. pulicaria* > *D. magna*). The metabolic rate and the amount of accumulated metal ions per unit of body volume are greater in small-sized than large-sized species (Grosell et al. 2002; Bianchini et al. 2002). In the present study, approximate neonate sizes were *B. longirostris* (250 μm) < *D. galeata* (450 μm) < *D. magna* (1,100 μm), meaning that size-dependent sensitivity is inapplicable to all cladoceran species.

Copper and silver ions are thought to render their effect through a similar physiological mechanism whereby they inhibit Na^+ uptake by the gills of aquatic animals (Niyogi and Wood 2004). Therefore, the mechanism causing the contradiction observed in bosminid sensitivity might be elucidated by the investigation of whole-body sodium uptake and Na^+, K^+-ATPase activity (Bianchini and Wood 2003). In addition, because downsizing vessels and volumes enlarges the surface/volume ratio, which in turn affects the toxicity of potentially adsorbing substances such as SNPs (Baumann et al. 2013), the smaller test vessels

used for the *Bosmina* (12-mL bottles) tests compared with those used for the *Daphnia* (50-L beaker) tests could have caused decreased toxicity of the silver.

Population-level effects of chemicals are generally evaluated based on both the survival and reproductive rate of the individuals. However, some researchers have found that mortality during chronic toxicity tests is a more critical end point for silver than per-capita fecundity (Nebeker et al. 1983; Blinova et al. 2013). Here, we show the effects of SNCs on the net reproductive rate (R_0), the probability of survival of tested individuals to maturation, and the intrinsic population growth rate (r) (Figs. 2 and 3). The 95 % CIs of EC_{10} values for R_0 and maturation tended to have a broad range, thus implying high uncertainty of the estimation (Table 2). However, we found that the EC_{50} (acute toxicity) and EC_{10} (chronic toxicity) values did not vary largely when they were calculated using Ag^+ concentrations. The α values (silver concentrations at $r = 0$) were greater than the EC_{10} for R_0 and maturation (Table 3). Mortality, especially during juvenile stages, exerted a strong influence on the total offspring number. Among the three chronic toxicity end points compared, probability of survival to maturation was the most sensitive to SNCs, which supports the conclusion by Blinova et al. (2013) that mortality is a convenient end point to assess the chronic toxicity of SNPs.

There are two contrasting laboratory findings on the relationship between acute and chronic toxicity of SNPs. The first is that SNPs influence daphnid reproduction at concentrations lower than the 48-h EC_{50} (Zhao and Wang 2011; Völker et al. 2013). A chronic effect (low offspring number) results from altered energy-reserve fractions (*e.g.*, lipids) (Muyssen and Janssen 2001). Thus, Völker et al. (2013) concluded that the lower 21-day EC_{10} compared with the 48-h EC_{50} (and EC_{10}) recorded in their experiments was due to the inhibition of algal food ingestion by accumulated PVP-coated SNPs in the daphnids' gut. In the present study, however, SNCs did not affect daphnid reproduction even at concentrations five to six times greater (as total silver) than the 48-h EC_{50} value (Fig. 1a, c). The contradictory results of those reports may be explained by the size-dependent influx rates of the SNPs (Zhao and Wang 2012). The influx rate (ingestion is the dominant uptake pathway) of SNPs into daphnids is size dependent with smaller particles exhibiting high values. The average particle sizes of silver used by Völker et al. (2013) and the present study were < 20 nm (58 nm in the test medium) and 80 nm, respectively. Unfortunately, we did not measure the particle size or hydrodynamic diameter in the COMBO medium; however, we assumed that particles formed large aggregates resulting in the low influx rates.

The second study, by Blinova et al. (2013), showed a lower chronic than acute toxicity to *D. magna*. This is consistent with our results. The toxicity of SNPs to *D. magna* decreases with the addition of algae (Allen et al. 2010), in which organic materials may either alter the availability of ionic Ag or stimulate active sodium uptake by Na^+, K^+-ATPase (Bianchini and Wood 2003; Glover and Wood 2004). Moreover, Stevenson et al. (2013) elucidated that extracellular DOC compounds produced by algal cells mitigate the citrate-coated SNP toxicity to *Chlamydomonas reinhardtii*. In the present study, chronic SNC end point values as total silver concentration for *D. magna* (EC_{10} for $R_0 = 20.88$ μg L^{-1}, $\alpha = 25.99$ μg L^{-1}) were approximately ten times greater than that of acute values (48-h $EC_{50} = 2.43$ μg L^{-1}) (Tables 2 and 3). Similar acute/chronic ratios were also observed in *D. galeata*. Such low chronic toxicity of SNPs might be explained by the low dissolved silver/total silver concentrations in the chronic toxicity tests. The metal speciation results showed that the relative abundance of Ag^+ in the dissolved silver did not differ largely among the toxicity tests (40–50 %) despite slightly greater DOC concentrations in the chronic ones. For instance, Ag^+/total silver concentration in SNC test media in the acute and chronic toxicity tests for *D. magna* were 35–68 % and 6–9 %, respectively. This resulted in the high acute/chronic toxicity SNC ratios. In fact, the dose ranges in the chronic toxicity tests did not differ between SNCs and $AgNO_3$ in Ag^+ concentrations (Figs. 2 and 3). Moreover, similar EC_{10} values and 48-h SNCs and $AgNO_3$ EC_{50} were obtained when they were calculated using Ag^+ concentrations (Table 2). The estimated α value was only two to five times greater than the 48-h EC_{50} value based on the Ag^+ concentrations (Table 3). These results indicate that Ag^+ was the main toxic substance for the cladocerans. However, as is also shown in the 48-h EC_{50} value, the EC_{10} value, and the α of $AgNO_3$ as Ag^+ for daphnids tended to be lower than those of SNCs, suggesting that the exact cause of SNP toxicity is still uncertain. Nevertheless, similar BLM-estimated 48-h LC_{50} values for *D. magna* [Table 1 (0.49–0.56 μg L^{-1})] suggest that the chemical composition of the media affecting the interaction between daphnid biotic ligands and bioavailable silver did not differ among the assays.

The low dissolved:total silver ratio in the chronic toxicity tests implied that a large amount of dissolved silver went missing from the SNC test media. The instrumental analysis was performed after two centrifugal separation processes. First, the water samples were centrifuged to remove the algal cells. Afterward, dissolved silver (<1.5 nm) in the supernatant was separated from the particles with a 3-kDa membrane filter tube. Therefore, it is conceivable that the dissolved silver was absorbed by the algal cells and/or other organic matter (>1.5 nm), which decreased the toxicity of SNCs to daphnids (Allen et al. 2010). Chronic toxicity tests

can be expressed as bitrophic level experiments in another way. In lake ecosystems, cladocerans always exist with their prey algae, and thus our findings might be useful for SNP ecological risk assessment.

The impact of anthropogenic stress on ecosystems often depends not only on the level of pollution but also on the community structure of the particular ecosystem (Sakamoto and Tanaka 2013). However, we still have insufficient knowledge on how SNPs affect aquatic ecosystems through the modification of food web structure with species-specific sensitivities. Our results forecast that zooplankton communities dominated by *D. galeata* are relatively vulnerable to SNP pollution. Moreover, we found that acute and chronic SNC toxicity (composed of non-surface-coated silver nanocolloids and ionic silver) to cladocerans was mainly derived from dissolved silver (especially Ag^+) in the test medium. Chronic SNC toxicity was lower than acute toxicity because of low dissolved/total silver in the former. The chronic end point values (SNC EC_{10} values for R_0 and probability of survival to maturation) did not differ largely from acute ones (48-h EC_{50} and BLM-estimated 48-h LC_{50} value) when the values were calculated based on Ag^+ concentration. However, very wide 95 % CIs of EC_{10} values indicate that the estimation is highly uncertain. The α values estimated by fitting r to a power function model were reliable parameters for assessing the chronic toxicity of silver. In the present study, all of the end point values tended to be greater for SNCs than for $AgNO_3$ even if they had been calculated using Ag^+ concentrations. Further study is needed to clarify this contradiction.

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Effects of silver nanocolloids on early life stages of the scleractinian coral *Acropora japonica*

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ABSTRACT

In this study, the effects of silver nanocolloids (SNCs) on the early life stages of the reef-building coral *Acropora japonica* were investigated. The tolerance of this species to SNC contamination was estimated by exposing gametes, larvae, and primary polyps to a range of SNC concentrations (0, 0.5, 5, 50, and 500 μg l^{-1}). Pure SNCs were immediately ionized to Ag^{+} in seawater and concentrations of ≥50 μg l^{-1} SNC had a significant detrimental effect on fertilization, larval metamorphosis, and primary polyp growth. Exposure to 50 μg l^{-1} SNC did not significantly affect larval survival; however, the larvae were deformed and lost their ability to metamorphose. At the highest concentration (500 μg l^{-1} SNC), all gametes, larvae, and primary polyps died. These experiments provide the first data on the effects of silver-nanomaterial-contaminated seawater on cnidarians, and suggest that silver nanomaterials can influence the early development of corals through anthropogenic wastewater inputs.

1. Introduction

Nanotechnology is rapidly developing in a variety of industries. In recent years, silver nanomaterials, including nanocolloids and nanoparticles, have been widely used in hygiene products and industry for their antibacterial activity. Such uses of silver nanomaterials carry a high risk of impacting aquatic environments through anthropogenic wastewater inputs (Wijnhoven et al., 2009). Several reports describe the impacts of silver nanoparticles on marine organisms, such as shellfish (e.g. Ringwood et al., 2010; Gomes et al., 2013) and sea urchins (e.g. Gambardella et al., 2013; Šiller et al., 2013). However, the effects of seawater contaminated with silver nanomaterials on cnidarians remain unexplored. Scleractinian corals (Cnidaria: Anthozoa) play important roles as primary producers and providers of structural habitat for other marine organisms in ecosystems. Because coral live in shallow areas that permit the penetration of light for photosynthesis, they may be influenced by nanomaterials from anthropogenic wastewater inputs. There are few studies about the effects of nanomaterials on cnidarians. The behavior of freshwater hydra *Hydra vulgaris* is reportedly disrupted by rod-shaped semiconductor nanoparticles (Malvindi et al., 2008), and the scleractinian coral *Montastraea faveolata* expelled algal symbionts when exposed to titanium dioxide (TiO_2) nanoparticles (Jovanović and Guzmán, 2014). Corals have been used as the test animal for investigating the effects of environmental perturbations such as high and low temperature (Suwa et al., 2008), hypo-osmosis (Kerswell and Jones, 2003), ocean acidification (e.g. Suwa et al., 2010), biocides (e.g. Watanabe et al., 2007), herbicides (e.g. Jones et al., 2003), cyanide (Jones and Hoegh-Guldberg, 1999), oils (e.g. Negri and Heyward, 2000), and metals (e.g. Harland and Brown, 1989). The genus *Acropora* is one of the most widespread, abundant, and species-rich (113–180 species) coral genera in Pacific coral reefs (Veron, 2000; Wallace, 1999). The early life stages of these corals have frequently been used in eco-toxicological studies (e.g. Watanabe et al., 2007; Negri et al., 2007; Morita et al., 2009) because it is easy to obtain *Acropora* gametes. In this study, it is hypothesized that silver nanocolloids (SNCs) may have an impact on the early life stages of *Acropora japonica*. To test this hypothesis, the tolerance of this species to SNC contamination

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was estimated by exposing gametes, larvae, and primary polyps to a range of initial SNC concentrations (0, 0.5, 5, 50, and 500 μg l^{-1}).

2. Materials and methods

2.1. Coral sampling

Gravid colonies of *A. japonica* were collected from Okinoshima, Tanabe Bay, Wakayama, Japan (33°71′N, 135°3′E) 6 d before spawning. The colonies were maintained in a running seawater tank under natural light conditions at the Seto Marine Biological Laboratory, Field Science Education and Research Center, Kyoto University, Wakayama, Japan. Coral spawning took place at night after the full moon in July 2012. Gametes were collected after spawning in accordance with Morita et al. (2006).

2.2. Silver nanocolloids

Silver nanocolloidal solution (25.7 mg l^{-1}) as measured by inductively coupled plasma mass spectrometry (ICP-MS; Thermo Scientific X Series 2, Thermo Scientific, PA, USA) was purchased from Utopia (TX, USA). The diameter of the silver nanocolloids (SNC) was determined using an ultra-high resolution scanning electron microscope SU8000 series (HITACHI, Tokyo, Japan) operated at 120 kV. Particle size was confirmed to be 57.2 ± 3.6 nm ($n = 3$, mean ± SD) in ultrapure water using a Delsa Nano Zeta Potential and Submicrometer Particle Size Analyzer (Beckman Coulter, Inc., Fullerton, CA). The Zeta potential of the SNC was −45.1 ± 1.9 mV in ultrapure water and but could not be measured in seawater due to the presence of salt. The SNC solution was diluted to nominal concentrations of 0.5, 5, 50, and 500 μg l^{-1} with 0.22-μm membrane-filtered seawater (MFSW). MFSW served as a control. The volume of purified water was adjusted between the four SNC solutions and the control condition because the amount of purified water added as part of the SNC stock solution ranged from 0 v/v% in the control to 1.95 v/v% in the 500 μg l^{-1} SNC condition. A 1-ml sample of all experimental seawater was collected immediately before and after each experiment and was preserved in a freezer at −30 °C for Ag analysis. The total amount of Ag from SNC and Ag^+ in each water sample was measured by ICP-MS. To isolate Ag^+ from the SNC solution (a mixture of silver colloids and Ag^+), 0.5 ml of test solution was filtered through a 3-kDa membrane filter (0.5-ml centrifugal-type filter, EMD Millipore Corporation, Billerica, MA, USA) at 14,000 × *g* and 4 °C for 10 min; this filter size was chosen because the mean diameters of the SNCs and Ag^+ were 57.2 nm and 0.162 nm (Shannon, 1976), respectively, and the 3-kDa membrane excludes particles ≥2 nm. The Ag^+ concentration in the filtered solution was measured using ICP-MS. Two milliliters of ultrapure nitric acid (Ultrapur-100, specific gravity 1.42, Kanto Chemical Co., Tokyo, Japan) was added to 100-μl water samples in a 50-ml Teflon beaker (Sanplatec Co., Osaka, Japan). The mixture was heated to 110 °C until almost all of the liquid had evaporated. Two milliliters of ultrapure nitric acid and 0.5 ml of hydrogen peroxide (for atomic absorption spectrometry, Kanto Chemical Co., Tokyo, Japan) were then added to the beaker and heated until the mixture was nearly dry. The residue was dissolved with 1.0% ultrapure nitric acid solution to a volume of 12.0 ml and then subjected to ICP-MS analysis. Measurements were conducted in triplicate and the data were averaged. All exposure experiments were conducted in a thermostatic room maintained at 27.0 ± 0.5 °C for the fertilization experiment and 27.0 ± 0.3 °C for other experiments. The water temperature was logged every 15 min throughout the experiments using data loggers (Thermochron iButtons DS1922; Maxim Integrated Products, Sunnyvale, CA, USA).

2.3. Fertilization experiment

Four crosses using gametes from four spawned colonies of *A. japonica* were performed. Each sperm–egg combination was considered to be a separate cross. All crosses were performed in a plastic cup filled with 200 ml of SNC solution and crosses were replicated three times at each SNC concentration. Approximately 200 eggs were combined with sperm at a final concentration of 10^5 sperm ml^{-1}. Fertilized eggs were fixed with 5% formalin 2 h after the addition of sperm, and the number of unfertilized eggs and developing embryos were counted under a dissecting microscope to calculate the rate of fertilization.

2.4. Larval experiment

Planula larvae of *A. japonica* were prepared by mixing gametes from all of the spawned colonies. Planula larvae were maintained in a container with 0.10-μm cartridge filtered seawater until the experiment started. Water was exchanged twice per day. Individual 5-day-old larvae were added to the wells of 24-well plastic culture plates (Iwaki Glass, Tokyo, Japan). Each well contained 2 ml experimental SNC seawater. Four plates containing 20 larvae (20 larvae per plate × 4 plates) were prepared for each SNC treatment. Surviving larvae were counted every 2 d during the 10-day culture experiments. SNC-contaminated MFSW was exchanged once per day during the experiment.

2.5. Larval metamorphosis experiment

The ability of the coral larvae to metamorphose after 24 h of exposure to SNC was examined using the coral metamorphosis-inducer peptide Hym-248 (Iwao et al., 2002). We added 4 ml peptide solution (1×10^{-6} M, dissolved in MFSW) to each well of a 24-well plastic culture plate. One larva that had been pre-exposed to SNC for 24 h was added to the peptide solution in each well. Four plates containing 20 larvae (20 larvae per plate × 4 plates) were prepared for each SNC treatment. Thus, metamorphosis of 80 larvae was observed for each SNC condition. The number of metamorphosed larvae was counted after 12 h of exposure to the peptide. Larvae were considered to have metamorphosed normally when they had developed septa (Iwao et al., 2002) and had become bilaterally symmetric in appearance.

2.6. Polyp experiment

Primary polyps were prepared according to Suwa et al. (2010). Primary polyps were prepared by inducing the settlement of 7-day-old *A. japonica* larvae using Hym-248. A 20-μl aliquot of 2×10^{-4} M Hym-248 in MFSW was added to each well of a 6-well plastic culture plate (Iwaki Glass, Tokyo, Japan). A peptide solution was created by mixing individual drops containing four larvae in 20 μl MFSW with individual 20-μl drops of peptide. Seven drops of this peptide solution was added to each well, for a total of 28 larvae and 280 μl of peptide. After the induction of metamorphosis, 10 ml of MFSW was added to each well of the plate. Larvae that settled on the seawater surface and on the sides of the plastic culture plates were removed, whereas those that settled at the bottom of the wells were used for the experiment. In each treatment, five 6-well culture plates, each containing approximately 25 settled polyps were prepared and maintained with a daily change of experimental seawater for 10 d. After 2 and 10 d, polyp size was evaluated by measuring the projected areas occupied using a digital camera (E-330; Olympus, Tokyo, Japan) connected to a dissecting microscope (SMZ 645; Nikon, Tokyo, Japan) and the ImageJ 1.38 program (National Institutes of Health, Bethesda, MD, USA).

2.7. Statistical analysis

The rates of fertilization, larval survivorship, and metamorphosis did not conform to parametric assumptions, and thus differences between treatments were assessed using Kruskal–Wallis ANOVA followed by Steel's *post hoc* pairwise comparison with the control. Differences in the growth of polyps were analyzed using nested ANOVA followed by Dunnett's pairwise comparison with the control. All statistical analyses were performed using JMP 10.0.2 software (SAS Institute, Cary, NC, USA).

3. Results

Almost all SNC in all treatments was ionized to Ag^+ regardless of the amount SNC added (Table 1). The concentrations of total Ag (SNC and Ag^+) and Ag^+ in the control condition were below the quantification limit.

The fertilization rate of *A. japonica* was significantly lower for gametes exposed to 50 and 500 μg l^{-1} SNC than for the controls (Fig. 1, Kruskal–Wallis $\chi^2 = 15.9$, df = 4, $p < 0.05$; paired comparisons using Steel's test, both $p < 0.05$). No fertilization success was observed for gametes exposed to 500 μg l^{-1} SNC. Larval survivorship was significantly decreased relative to the controls after 2 d of exposure (Fig. 2, Kruskal–Wallis $\chi^2 = 14.6$, df = 4, $p < 0.05$; paired comparisons using Steel's test, each $p < 0.05$), and all larvae died after 4 d of exposure to 500 μg l^{-1} SNC. The survival rates of larvae exposed to 0.5, 5 and 50 μg l^{-1} SNC were not significantly different from that of the controls (Fig. 2, Steel's test, each $p > 0.05$). However, all larvae exposed to 50 μg l^{-1} SNC stopped swimming and were malformed after 2 d of exposure (Fig. 3B), whereas control larvae were rod-shaped and continued swimming (Fig. 3A). Larvae

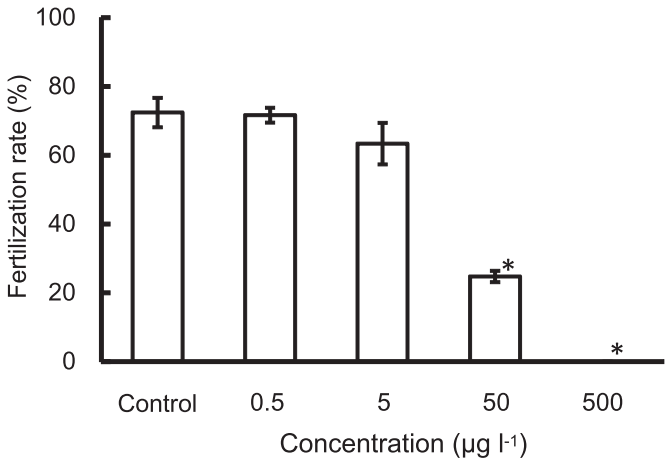

Fig. 1. Fertilization rate 2 h after mixing sperm with the eggs of *Acropora japonica* subjected to various concentrations of silver nanocolloids (SNCs). In each repetition, the fertilization success of 200 eggs was recorded. Asterisks indicate the statistical significance compared with the control condition ($p < 0.05$, Kruskal–Wallis ANOVA/Steel's pair-wise comparison). Error bars = SD ($n = 4$).

Table 1
Conditions of Ag during experiments. Summary of chemical Ag conditions in each experiment. Seawater sampling was conducted before and after each experiment, except for the fertilization experiment, for which sampling was conducted only before starting the experiment.

Life stage	Nominal Ag (μg l^{-1})	Timing of sampling	SNC and Ag^+ (μg l^{-1})	Ag^+ (μg l^{-1})	Quantitation limit (μg l^{-1})	Temperature (°C)
Fertilization	0	Before experiment	nd	nd	0.92	27.1 ± 0.6
Fertilization	0.5	Before experiment	1.83 ± 2.09	2.61 ± 1.25	0.92	27.1 ± 0.6
Fertilization	5	Before experiment	7.50 ± 2.80	8.28 ± 1.59	0.92	27.1 ± 0.6
Fertilization	50	Before experiment	61.4 ± 2.57	68.4 ± 2.16	0.92	27.1 ± 0.6
Fertilization	500	Before experiment	548 ± 10.2	545 ± 16.3	0.92	27.1 ± 0.6
Metamorphosis	0	Before experiment	nd	nd	2.75	26.6 ± 0.3
Metamorphosis	0.5	Before experiment	4.16 ± 1.96	6.11 ± 3.40	2.75	26.6 ± 0.3
Metamorphosis	5	Before experiment	10.2 ± 3.62	9.46 ± 1.24	2.75	26.6 ± 0.3
Metamorphosis	50	Before experiment	75.7 ± 3.42	76.8 ± 1.90	2.75	26.6 ± 0.3
Metamorphosis	500	Before experiment	621 ± 5.57	638 ± 7.07	2.75	26.6 ± 0.3
Metamorphosis	0	After experiment	nd	nd	0.92	26.6 ± 0.3
Metamorphosis	0.5	After experiment	1.40 ± 0.77	2.19 ± 0.74	0.92	26.6 ± 0.3
Metamorphosis	5	After experiment	7.84 ± 0.83	7.99 ± 1.11	0.92	26.6 ± 0.3
Metamorphosis	50	After experiment	63.6 ± 3.19	62.5 ± 0.62	0.92	26.6 ± 0.3
Metamorphosis	500	After experiment	656 ± 12.0	591 ± 8.99	0.92	26.6 ± 0.3
Larvae	0	Before experiment	nd	nd	1.24	26.6 ± 0.3
Larvae	0.5	Before experiment	5.40 ± 2.77	4.60 ± 1.44	1.24	26.6 ± 0.3
Larvae	5	Before experiment	17.6 ± 10.5	14.2 ± 2.87	1.24	26.6 ± 0.3
Larvae	50	Before experiment	37.7 ± 9.00	46.2 ± 1.32	1.24	26.6 ± 0.3
Larvae	500	Before experiment	346 ± 44.5	385 ± 32.0	1.24	26.6 ± 0.3
Larvae	0	After experiment	nd	nd	1.36	26.6 ± 0.3
Larvae	0.5	After experiment	2.26 ± 0.93	1.82 ± 0.54	1.36	26.6 ± 0.3
Larvae	5	After experiment	7.77 ± 1.72	11.7 ± 4.94	1.36	26.6 ± 0.3
Larvae	50	After experiment	67.5 ± 4.54	69.8 ± 3.72	1.36	26.6 ± 0.3
Larvae	500	After experiment	303 ± 68.6	348 ± 15.5	1.36	26.6 ± 0.3
Primary polyp	0	Before experiment	nd	nd	1.24	26.6 ± 0.3
Primary polyp	0.5	Before experiment	1.82 ± 0.87	2.80 ± 2.28	1.24	26.6 ± 0.3
Primary polyp	5	Before experiment	15.0 ± 6.82	10.6 ± 1.00	1.24	26.6 ± 0.3
Primary polyp	50	Before experiment	93.3 ± 6.84	99.2 ± 1.83	1.24	26.6 ± 0.3
Primary polyp	500	Before experiment	757 ± 18.2	785 ± 2.25	1.24	26.6 ± 0.3
Primary polyp	0	After experiment	nd	nd	1.36	26.6 ± 0.3
Primary polyp	0.5	After experiment	0.63 ± 0.57	3.53 ± 1.38	1.36	26.6 ± 0.3
Primary polyp	5	After experiment	0.72 ± 0.60	1.58 ± 0.79	1.36	26.6 ± 0.3
Primary polyp	50	After experiment	39.0 ± 2.04	39.5 ± 1.11	1.36	26.6 ± 0.3
Primary polyp	500	After experiment	360 ± 13.9	438 ± 6.13	1.36	26.6 ± 0.3

The limit of quantitation is 3.3 times the limit of detection.
Background values of Ag^+ in seawater were measured and subtracted from the data of samples.
nd: Not detected, means ± SD, $n = 3$.

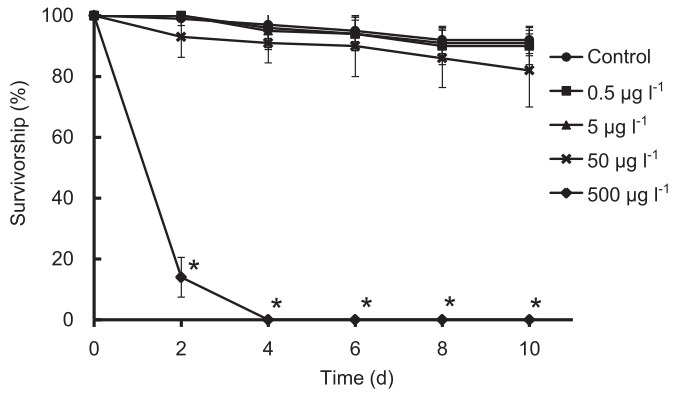

Fig. 2. Survivorship of *Acropora japonica* larva after a 10-d exposure to various concentrations of silver nanocolloids (SNCs). In each repetition, the survivorship of 20 larvae was recorded. Asterisks indicate the statistical significance compared with the control condition ($p < 0.05$, Kruskal–Wallis ANOVA/Steel's pair-wise comparison). Error bars = SD ($n = 5$).

exposed to 0.5 and 5 μg l^{-1} SNC showed same morphology and behavior to those in the control condition. The metamorphosis rate of larvae exposed to 50 and 500 μg l^{-1} SNC for 24 h was significantly lower than that of the controls (Fig. 4, Kruskal–Wallis $\chi^2 = 23.8$, df = 4, $p < 0.05$; paired comparisons using Steel's test, each $p < 0.05$). All larvae metamorphosed normally under control conditions, whereas 14.0% and 0% of larvae successfully metamorphosed in 50 and 500 μg l^{-1} SNC, respectively.

Polyps were significantly smaller in 50 μg l^{-1} SNC (0.49 ± 0.02 mm^2, means ± SD) than in the control condition (Figs. 3C and 5, 0.94 ± 0.02 mm^2, means ± SD, nested-ANOVA, $F_{3,666} = 728$, $p < 0.05$; paired comparisons by Dunnett's test, $p < 0.05$) after 2 d of exposure. All polyps exposed to 50 μg l^{-1} SNC were malformed (Fig. 3D) and all polyps exposed to 500 μg l^{-1} SNC died after 2 d of exposure. Polyps exposed to 50 μg l^{-1} SNC remained malformed and the projected area of polyps exposed to 5 μg l^{-1} SNC was not significantly different from that of controls even after 10 d of exposure (Fig. 5B, Dunnett's test, $p > 0.05$).

4. Discussion

Silver nanomaterials are widely used in hygiene products and industry for their antibacterial activity and have a potentially high risk of negative impacts on aquatic environments through anthropogenic wastewater inputs (Wijnhoven et al., 2009). Marine animals in nearshore and marine areas around estuaries are at particular risk of harm from silver nanomaterials. However, the effects of silver nanomaterials on cnidarians, including corals, remain unexplored. In this study, the effects of seawater contaminated with SNC on the early life stages of the coral *A. japonica* were investigated.

This is the first study of the effects of silver nanomaterials in corals and cnidarians. Exposure of the coral *A. japonica* to SNC-contaminated seawater had negative impacts on fertilization, larval survival, larval metamorphosis, and primary polyp growth at concentrations of ≥50 μg l^{-1}. SNC at concentrations of 0.1–1000 μg l^{-1} does not affect the fertilization of sea urchins, although developmental delay and anomalies were induced by 72 h of exposure to 0.1 μg l^{-1} SNC (Gambardella et al., 2013). In the present study, exposure to 50 μg l^{-1} SNC did not significantly decrease larval survival, but the larvae were deformed and lost their ability to metamorphose. This deformation of larvae has also been reported for oysters after exposure to 0.16 μg l^{-1} SNC (Ringwood et al., 2010) and sea urchins after exposure to 300 μg l^{-1} SNC (Šiller et al., 2013). The difference in the effective concentrations found in these studies may be due to the species under investigation or the experimental conditions. The degree of ionization and size of particles, in addition to the concentration of the particles, influence the toxicity of silver nanomaterials (Kennedy

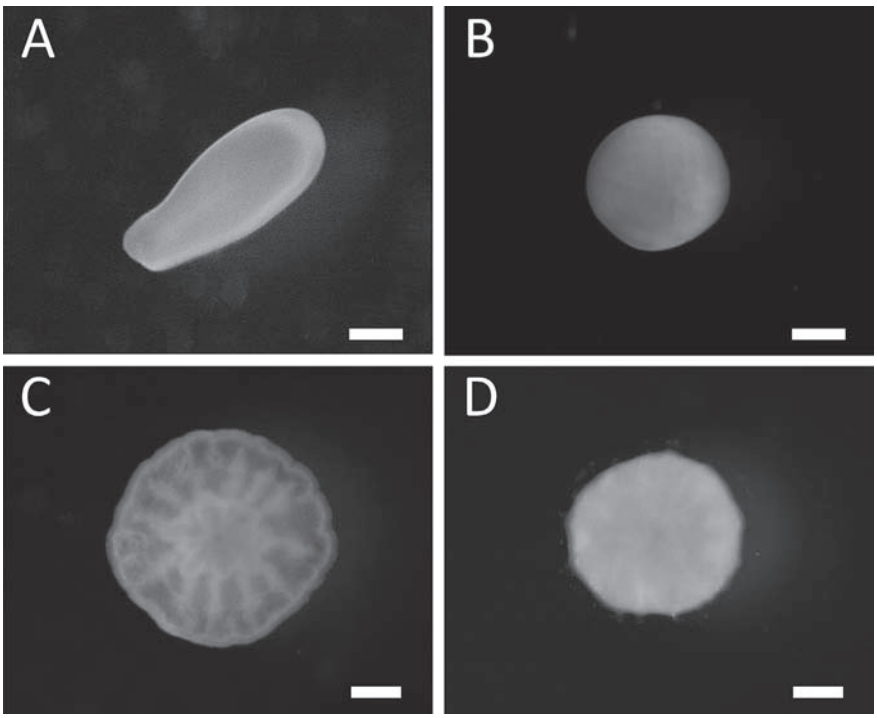

Fig. 3. Representative images of *Acropora japonica* larvae (A, B) and primary polyps (C, D) under different conditions of silver nanocolloid exposure. A larva and primary polyp in the control condition (A, C) and exposed to 50 μg l^{-1} silver nanocolloid (SNC)-contaminated seawater for 2 d (B, D). Scale bar = 200 μm.

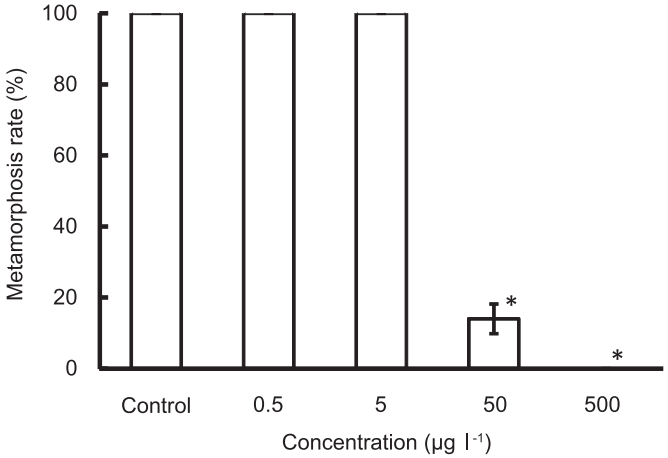

Fig. 4. Metamorphosis rate of *Acropora japonica* larvae that were pre-exposed to different silver nanocolloid (SNC) concentrations for 24 h. In each repetition, the metamorphosis of 20 larvae was recorded. Asterisks indicate the statistical significance compared with the control condition ($p < 0.05$, Kruskal–Wallis ANOVA/Steel's pair-wise comparison). Error bars = SD ($n = 5$).

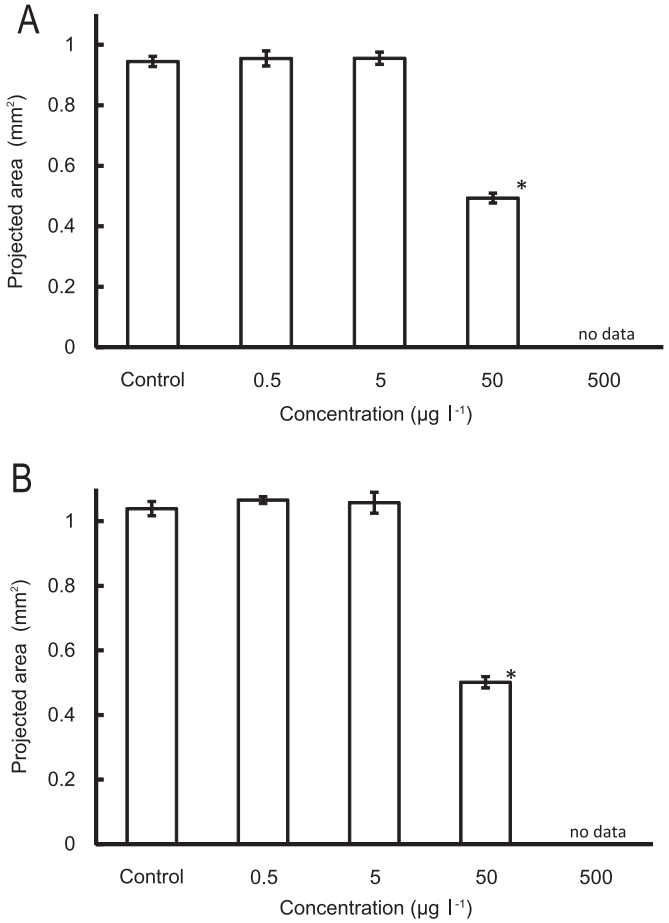

Fig. 5. Areas of occupation by primary polyps of *Acropora japonica* after 2 d (A) and 10 d (B) of incubation with different concentrations of silver nanocolloids (SNCs). In each repetition, the occupied areas of approximately 40 primary polyps were recorded. Asterisks indicate the statistical significance compared with the control condition ($p < 0.05$, nested ANOVA/Dunnett's pair-wise comparison). Error bars = SD ($n = 5$).

et al., 2010). For example, Šiller et al. reported that Ag^+ ions are more toxic to sea urchin larvae than citrate-capped SNC, of which less than 1% is ionized (Šiller et al., 2013). Almost all of the SNC used in the present study was ionized to Ag^+ ions. There have been no reports detailing the toxicity of Ag^+ ions to corals. In a study of the effects of metal ions on coral fertilization, copper ions were reported to have the highest level of toxicity among lead, zinc, cadmium and nickel ions and the lowest effective concentrations of copper on the fertilization success of *Acropora tenuis* and *Acropora longicyathus*, were 66.6 and 23.6 μg l^{-1} (Reichelt-Brushett and Harrison, 2005). Larval settlement success of *A. tenuis* is also significantly decreased by 42.0 μg l^{-1} of ionic copper (Reichelt-Brushett and Harrison, 2000). These values for the lowest effective copper dose are similar to that of the lowest effective ionic silver concentrations of 46.2–68.4 μg l^{-1} found in the present study. This suggests high toxicity of silver ions to coral in the early stages of development.

In addition to the degree of ionization, internal bioaccumulation of SNC should also be considered. Bioaccumulation of SNC has been reported in some marine molluscs (Zuykov et al., 2011; Al-Sid-Cheikh et al., 2013; Li et al., 2013). In the scallop *Chlamys islandica*, larger silver nanoparticles accumulated in the digestive system over a longer period, and had a different distribution, than smaller particles (Al-Sid-Cheikh et al., 2013). In adult corals, metal ion bioaccumulation was investigated both in the field (Reichelt-Brushett and McOrist, 2003) and in indoor exposure experiments (Bastidas and García, 2004; Bielmyer et al., 2010). These studies show that symbiotic algae, *Symbiodinium* spp. (zooxanthellae), accumulate more metal ions than their coral host. This suggests that the expulsion of algae is a detoxifying mechanism for corals. Although there is still no evidence for bioaccumulation of SNC or other nanomaterials in corals, increased expulsion of zooxanthellae from coral after exposure to TiO_2 nanoparticles has been reported (Jovanović and Guzmán, 2014). Nonetheless, internally accumulated particulate contaminants may damage corals chronically, even after the contaminants have been removed from the surrounding water column.

The physiological mechanism underlying the effects of SNC on marine organisms is still not well understood. In sea urchin embryos, cholinesterase activity is inhibited by metal nanomaterials, including SNC (Gambardella et al., 2013). In adult coral colonies of *Montastraea franksi*, DNA is damaged and the expression pattern of oxidative stress genes is altered by copper ions (Schwarz et al., 2013). The expression of oxidative stress gene HSP 70 is increased by TiO_2 nanoparticles in the adult colonies of *M. faveolata* (Jovanović and Guzmán, 2014). It is hypothesized that SNC induces DNA damage and alterations of gene expression patterns in corals.

In conclusion, pure SNC is immediately ionized to Ag^+ and this may influence multiple early life stages of corals. However, knowledge concerning the effects of SNC on coral and other marine organisms is still poor. Studies investigating the relationship between toxicity and level of SNC ionization, the effects of internal SNC bioaccumulation, the physiological mechanism underlying the effects of SNC, the effects of SNC on multiple life stages, synergistic effects of SNC and other environmental factors, and effects of long-term exposure to low levels of SNC are necessary to understand the toxicity of SNC to marine organisms.

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Video Article

Salinity-dependent Toxicity Assay of Silver Nanocolloids Using Medaka Eggs

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Abstract

Salinity is an important characteristic of the aquatic environment. For aquatic organisms it defines the habitats of freshwater, brackish water, and seawater. Tests of the toxicity of chemicals and assessments of their ecological risks to aquatic organisms are frequently performed in freshwater, but the toxicity of chemicals to aquatic organisms depends on pH, temperature, and salinity. There is no method, however, for testing the salinity dependence of toxicity to aquatic organisms. Here, we used medaka (*Oryzias latipes*) because they can adapt to freshwater, brackish water, and seawater. Different concentrations of embryo-rearing medium (ERM) (1×, 5×, 10×, 15×, 20×, and 30×) were employed to test the toxicity of silver nanocolloidal particles (SNCs) to medaka eggs (1× ERM and 30× ERM have osmotic pressures equivalent to freshwater and seawater, respectively). In six-well plastic plates, 15 medaka eggs in triplicate were exposed to SNCs at 10 mg $L^{\#1}$ in different concentrations of ERM at pH 7 and 25 °C in the dark.

We used a dissecting microscope and a micrometer to measure heart rate per 15 sec and eye diameter on day 6 and full body length of the larvae on hatching day (section 4). The embryos were observed until hatching or day 14; we then counted the hatching rate every day for 14 days (section 4). To see silver accumulation in embryos, we used inductively coupled plasma mass spectrometry to measure the silver concentration of test solutions (section 5) and dechorionated embryos (section 6).The toxicity of the SNCs to medaka embryos obviously increased with increasing salinity. This new method allows us to test the toxicity of chemicals in different salinities.

Video Link

The video component of this article can be found at http://www.jove.com/video/53550/

Introduction

Since the establishment of the Organisation for Economic Co-operation and Development (OECD) test guidelines for testing chemicals in 1979, 38 test guidelines have been published in Section 2 of the guidelines, Effects on Biotic Systems[1]. All of the aquatic organisms tested have been from freshwater habitats, namely freshwater plants; algae; invertebrates such as daphnia and chironomids; and fishes such as medaka, zebrafish, and rainbow trout. Compared to saltwater environments, freshwater environments are more directly affected by human economic and industrial activities. Therefore, freshwater environments have been prioritized for testing because they are at higher risk from pollution.

In coastal areas, including estuaries, salinities vary between brackish water and seawater conditions, and these areas are often polluted by industrial activity[2]. Coastal areas and their associated wetlands are characterized by high ecological biodiversity and productivity. Coastal ecosystems must therefore be protected from chemical pollution. However, there has been limited ecotoxicological research in brackish water and seawater habitats.

Sakaizumi[3] studied the toxic interactions between methyl mercury and salinity in Japanese medaka eggs and found that increasing the osmotic pressure of the test solution enhanced the toxicity of the methyl mercury. Sumitani *et al*.[4] used medaka eggs to investigate the toxicity of landfill leachate; they found that the osmotic equivalency of leachate to the eggs was the key to inducing abnormalities during embryogenesis. In addition, Kashiwada[5] reported that plastic nanoparticles (39.4 nm in diameter) easily permeated through the medaka egg chorion under brackish conditions (15× embryo rearing medium (ERM)).

A typical small fish model, the Japanese medaka (*Oryzias latipes*) has been used in basic biology and ecotoxicology[6]. Japanese medaka can live in conditions ranging from freshwater to seawater because of their highly developed chloride cells[7]. They are therefore likely to be useful for testing in conditions with a wide range of salinities.

Protocol

The Japanese medaka used in this study were treated humanely in accordance with the institutional guidelines of Toyo University, with due consideration for the alleviation of distress and discomfort.

1. Silver Nanocolloids (SNCs)

1. Purchase purified SNCs (20 mg $L^{#1}$, 99.99% purity, particle mean diameter about 28.4 ± 8.5 nm suspended in distilled water).
2. Validate the purity and concentration of the silver by inductively coupled plasma mass spectrometry (ICP-MS) analyses according to operating manual[8]. The pretreatment method for ICP-MS analyses is described in section 7.

2. Preparation of SNC Solutions (Mixtures of Silver Colloids and Ag^+) with Different Salinities

1. Prepare 60× ERM consisting of 60 g NaCl, 1.8 g KCl, 2.4 g $CaCl_2 \cdot 2H_2O$, and 9.78 g $MgSO_4 \cdot 7H_2O$ in 1 L of ultrapure water; adjust the pH to 7.0 with 1.25% $NaHCO_3$ in ultrapure water.
2. Stir the ERM solution at 25 °C overnight.
3. Mix SNCs with diluted ERM. Prepare 40 ml of each SNC-ERM mixed solution. The final concentration is 10 mg $L^{#1}$ of SNCs in different concentrations of ERM (1×, 5×, 10×, 15×, 20×, or 30×).
4. Adjust pH of the SNC-ERM mixed solution to 7.0 with 0.625% $NaHCO_3$ in ultrapure water. pH adjustment is very important in preparing the SNC solution, because Ag^+ release is facilitated by acidic conditions[9].
5. Use $AgNO_3$ as a reference compound for SNCs.
    1. Mix $AgNO_3$ with diluted ERM. Prepare 40 ml of $AgNO_3$-ERM mixed solution at an $AgNO_3$ concentration of 15.7 mg L^{-1} (10 mg $L^{#1}$ silver) in different concentrations of ERM (1×, 5×, 10×, 15×, 20×, or 30×).
    Note: To examine silver colloid toxicity, $AgNO_3$ solution, which is a source of soluble silver, is used as a reference compound for SNCs, which are a mixture of silver colloids and soluble silver.

3. Medaka Culture and Egg Harvesting

1. Obtain the medaka (*O. latipes*) (orange-red strain) (60 males and 60 females).
2. Culture medaka as groups (20 males and 20 females as one group) in 1x ERM in 3-L tanks by using a medaka flow-through culturing system.
    1. Culture at the following conditions:
    pH range of the culture medium: 6.2 to 6.5
    light:dark cycle: 16:8 hr
    temperature of the culture medium: 24 ± 0.5 °C
    osmotic pressure of the culture medium: 257 mOsm
3. Feed medaka on *Artemia salina* nauplii at 10:00 (once a day) and feed an artificial dry fish diet at 09:00, 11:00, 13:00, 15:00, and 17:00 (five times a day).
    1. Obtain *A. salina* nauplii.
    2. Prepare 5 L of a 3.0% salt solution in a plastic beaker.
    3. Add 30 g of brine shrimp eggs to the salt solution in the beaker.
    4. Incubate the eggs at 25 °C for 48 hr with bubbling (4 L $min^{#1}$) using an aeration pump.
    5. After 48 hr, stop the bubbling.
    6. Allow the solution to stand for 5 to 10 min to separate the hatched *A. salina* nauplii (lower part of the solution) from the unhatched eggs and eggshells (upper part of the solution).
    7. Remove the upper layer of the solution by decantation.
    8. Filter the lower portion of the solution through a sieve with openings of 283 μm, and collect the nauplii that pass through on a net with openings of 198 μm.
    9. Feed the nauplii to the medaka within 6 hr.
4. After the female medaka have spawned, remove the external egg clusters gently from the females' bodies or collect the eggs from the bottom of the fish tank by using a small net (net size 5 cm × 5 cm, hole size 0.2 mm × 0.2 mm).
5. Rinse the egg cluster with flowing tap water for 5 sec.
6. Add all of the rinsed egg clusters to 30× ERM solution.
7. Remove the clusters from the solution after 1 min and place the egg clusters between dry paper towels and roll gently.
8. Put the eggs back into the 30× ERM.
9. Select fertilized eggs under a dissecting microscope.
10. Place selected 810 eggs in 1× ERM in six-well plastic plates by using forceps.
11. Incubate the eggs at 25 ± 0.1 °C in an incubator until developmental stage 21. (Developmental stages of the medaka embryos were defined from the work of Iwamatsu[10].)
12. Pick out incubated eggs at developmental stage 21 under a dissecting microscope.
13. Rinse selected eggs with 1× ERM.
14. Subject the rinsed eggs to exposure experiments (section 4).

4. Toxicity Testing of SNCs or $AgNO_3$ at Different ERM Salinities

1. Rinse medaka eggs (stage 21) three times with test solution [SNCs (10 mg $L^{#1}$) or $AgNO_3$ (15.7 mg $L^{#1}$ as 10 mg $L^{#1}$ silver) at each concentration of ERM (1×, 5×, 10×, 15×, 20×, or 30×) at pH 7]. As controls, use eggs in 1× to 30× ERM at pH 7.
2. Add 15 rinsed eggs to 5 ml of each test solution in six-well plastic plates. (Perform the exposure experiments three times for SNC or $AgNO_3$ toxicity testing using each test solution.)
3. Wrap the plates in aluminum foil.
4. Incubate the wrapped plates at 25 °C in the dark until hatching or for 14 days.
5. Observe the exposed eggs every 24 hr for biological changes and dead eggs (**Figures 1** and **2**).

6. Exchange the test solutions every 24 hr.
7. Perform observations as follows.
    1. On day 6 of exposure, count the heart rate (per 15 sec) of medaka embryos under a dissecting microscope by using a stopwatch (**Figure 3a**).
    2. On day 6 of exposure, measure the eye size (diameter) of medaka embryos under a dissecting microscope by using a micrometer (**Figure 3b**).
    3. On hatching day, measure the full body lengths of larvae under a dissecting microscope by using a micrometer (**Figure 3c**).
    4. Count the total number of exposed eggs that hatch over the 14 days (**Figure 3d**).

5. Isolation of Soluble Silver from SNC Solution, and Silver Analysis

1. Isolate soluble silver from each SNC solution (a mixture of silver colloids and soluble silver) by filtering through a 3-kDa membrane filter at 14,000 x g and 4 °C for 10 min. Use a 3-kDa membrane filter to isolate soluble silver from the SNCs, because the reported mean diameter of aggregated SNCs in 1× ERM is 67.8 nm[11] and that of Ag^+ is 0.162 nm[12]; the 3-kDa membrane excludes particles with diameters of 2 nm or more[13].
2. Measure the silver concentration in 50 µl of filtered solution (= the soluble silver concentration) by ICP-MS analysis (**Figure 3e**) according to the ICP-MS operating manual[8]. The pretreatment method for the ICP-MS analyses is described in section 7.

6. Measurement of Silver Bioaccumulation in Medaka Embryos

1. Expose medaka eggs (stage 21) to SNCs or $AgNO_3$ as described in section 4.
2. On day 6 of exposure, remove chorion from the egg (*i.e.*, dechorion) by using medaka hatching enzyme according to the protocol described in the *Medaka Book*[14].
3. Measure the silver concentration of the dechorioned eggs by ICP-MS analysis according to the ICP-MS operating manual[8] (**Figure 3f**). The pretreatment method for the ICP-MS analyses is described in section 7.

7. Measurement of Silver Concentration by ICP-MS Analysis

1. Add samples [50 µl of silver solution (for validation of the silver concentration; section 1); three dechorionated embryos (section 5); or 50 µl of filtered solution (section 5)] to a 50-ml Teflon beaker.
2. Add 2.0 ml of ultrapure nitric acid to the 50-ml beaker.
3. Heat the mixture on a hot plate at 110 °C until just before it dries out (about 3 hr).
4. To dissolve the organic matter completely, add 2.0 ml of ultrapure nitric acid and 0.5 ml of hydrogen peroxide to the beaker.
5. Heat the mixture again on the hot plate until just before it dries out (about 3 hr).
6. Dissolve the residue in 4 ml of 1.0% ultrapure nitric acid solution.
7. Transfer 4 ml of solution to a centrifuge tube.
8. Repeat 7.6 to 7.7 twice (a total of three times). The final volume is 12.0 ml.
9. Measure the silver concentration of the sample (dissolved in 1.0% ultrapure nitric acid) by using ICP-MS analysis according to the operating manual[8].
    1. Use an internal and an external standard solution (See Materials List) to quantify the silver concentration. The internal and external standard solution is accredited by American Association for Laboratory Accreditation (A2LA). Detection limits of silver were 0.0018 ng ml^{-1} (solution) and 0.016 ng mg-$weight^{-1}$ (embryo body).

Representative Results

The effect of salinity on SNC toxicity was very obvious: the induction of deformity or death was salinity dependent (**Figures 1** and **2**). We measured phenotypic biomarkers (heart rate, eye size, full body length, and hatching rate) in SNC (10 mg L^{-1})-exposed embryos. These phenotypic biomarkers revealed salinity-dependent SNC toxicity.

Heart rates ranged from 29.6 to 32.2 beats/15 sec throughout 1× to 30× ERM in the controls. However, they decreased significantly ($P < 0.01$) with SNC or $AgNO_3$ exposure in 30× ERM (**Figure 3a**). Decreasing heart rate indicates deteriorating health. There were no significant differences in full body length of the larvae under control or $AgNO_3$ exposure at salinities ranging from 5× to 30× ERM compared with the respective 1× ERM solutions. Body length was consistently 4.55 to 4.69 mm. However, body length decreased significantly ($P < 0.01$) to 4.33 and 3.77 mm, as a result of SNC exposure in 15× and 20× ERM compared with the respective 1× ERM solutions; moreover, it decreased to 3.75 mm in 30× ERM (statistical analysis was not available at 30× ERM because only one hatched) (**Figure 3c**). Decreasing full body length indicates growth inhibition. There were no significant differences in eye diameter in the controls at salinities ranging from 1× to 30× ERM compared with 1× ERM; eye diameter was consistently 0.357 to 0.366 mm. However, it decreased significantly upon SNC or $AgNO_3$ exposure in 20× or 30× ERM compared with in the respective 1× ERM solutions (**Figure 3b**). Decreasing eye diameter indicates developmental inhibition of the nervous system. All control eggs hatched within 14 days. However, upon SNC exposure in 20× and 30× ERM the hatching rate decreased significantly to 71% and 2%, respectively, of the rate in 1× ERM ($P < 0.01$) (**Figure 3d**). Also, upon $AgNO_3$ exposure it decreased significantly in 30× ERM ($P < 0.01$). Decreasing hatching rate indicates the toxic effect of the presence of SNCs or $AgNO_3$. These four phenotypic biomarkers therefore show salinity dependent SNC toxicity.

Salinity increases water-soluble metal complex formation, and these complexes might have toxic effects[3,8]. In our study, ICP-MS analyses of silver revealed that the soluble silver concentrations in the test solutions increased as the salinity increased; the silver concentration in the embryos also increased (**Figures 3e** and **f**).

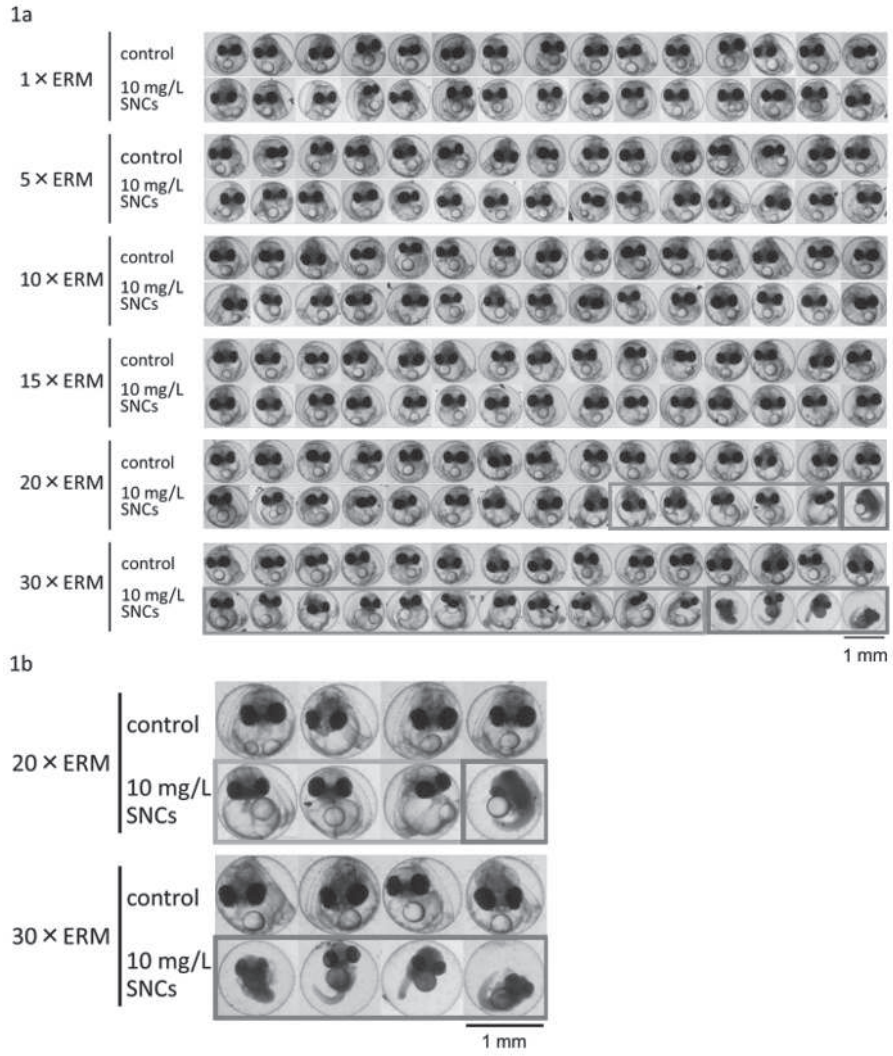

Figure 1. Increasing salinity increases SNC toxicity. Mortality and number of abnormally developed embryos increased with increasing salinity under SNC exposure. **(a)** Image array of medaka eggs exposed to 10 mg $L^{\#1}$ SNC solution at different ERM concentrations. Images are typical of medaka eggs exposed to SNCs and observed under a dissecting microscope. Control medaka eggs were well developed, and all of them hatched in 1× to 30× ERM. At 10 mg $L^{\#1}$ SNC exposure, although all of the medaka eggs hatched in 1× to 15× ERM, developmental deformities (red outlined rectangles, unhatched) and embryos unhatched within 14 days (green outlined rectangles, unhatched) were observed at 20× and 30× ERM. **(b)** Magnified images of the lower right of (a). Please click here to view a larger version of this figure.

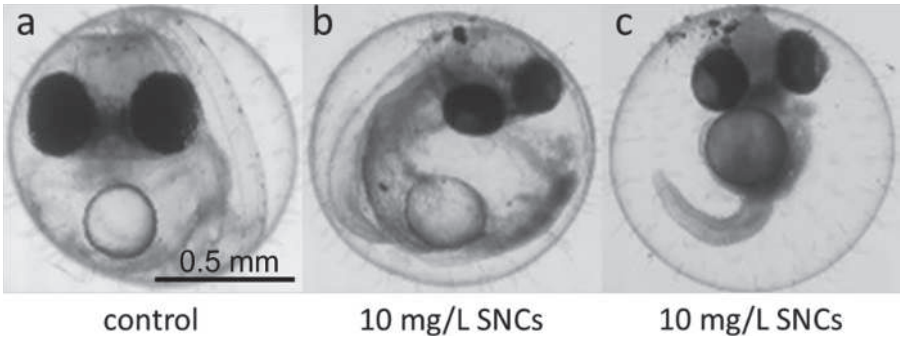

Figure 2. Typical phenotypic biomarkers of medaka eggs exposed to SNCs. Medaka eggs at developmental stage 21 were exposed to SNCs (10 mg $L^{\#1}$) in different concentrations of ERM for 6 days. **(a)** Control medaka embryo with normal development. **(b)** Developmental deformity (light degree of damage). This embryo displayed pericardiovascular edema; tubular heart; blood clots; inadequate development of the blood vessels (and thus ischemia), spinal cord, tail, eyes, and brain; and a short tail. **(c)** Developmental deformity (heavy degree of damage). This embryo showed destruction of the yolk sack; inadequate development of the blood vessels (and thus ischemia), spinal cord, tail, eyes, and brain; and a short tail. The signs in b) and c) were observed upon SNC exposure in 20× and 30× ERM. Please click here to view a larger version of this figure.

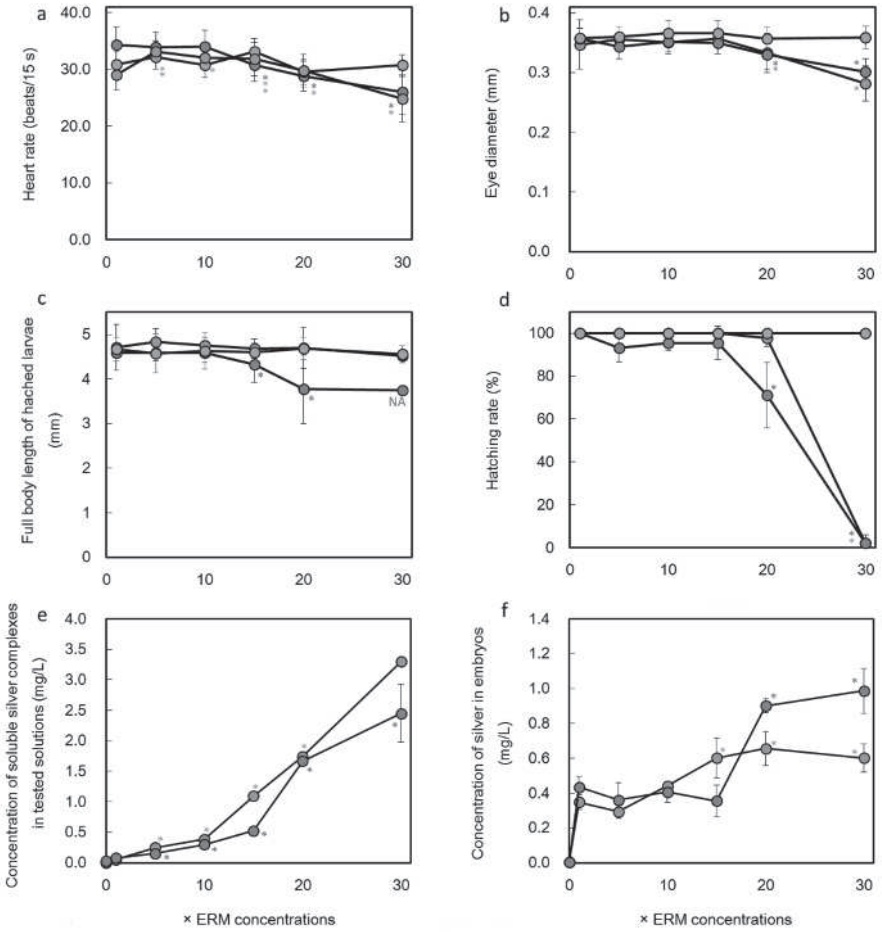

Figure 3. Effects of exposure to SNCs or silver nitrate on toxicological biomarkers during medaka egg development. Developmental stage 21 medaka eggs exposed to SNCs (10 mg $L^{\#1}$) or silver nitrate (10 mg $L^{\#1}$ as silver) in a series of ERMs were observed for 6 days. [blue] Control (ERM); [red] SNCs at 10 mg $L^{\#1}$ in ERM; [green] $AgNO_3$ at 10 mg $L^{\#1}$ as silver in ERM. **(a)** Heart rate per 15 sec. Decreasing heart rate indicates deteriorating health. **(b)** Eye diameter. Decreasing eye diameter indicates developmental inhibition of the nervous system. **(c)** Full body length. Decreasing full body length indicates growth inhibition. **(d)** Hatching rate. Decreasing hatching rate indicates the toxic effect of the presence of SNCs. **(e)** Concentrations of soluble silver complexes from SNCs or silver nitrate in test solutions (mg $L^{\#1}$). **(f)** Silver concentrations in embryos exposed to SNCs or silver nitrate in a series of ERMs. *Significant difference (analysis of variance, $P < 0.05$) compared with the respective 1× ERM solution. NA: not available because only one hatched. Error bars indicate standard deviation. Please click here to view a larger version of this figure.

Discussion

Medaka is a freshwater fish that is highly tolerant to seawater; it is not well known that the original natural habitat of this fish was saltwater off the Japanese coast[6]. Hence, medaka fish have well-developed chloride cells[7]. This unique property provides scientists with a new way to test the toxicity of chemicals in the environment as a function of salinity (freshwater to seawater) by using only a single species of fish.

To obtain medaka eggs at stage 21, eggs must be harvested every morning and selected at stage 20. Usually, medaka pairs start mating in the early morning (just before sunrise) and produce eggs by sunrise. Eggs harvested in the morning must be at about stage 10 or 11. If there is a need to control egg development before the start of the experiment, egg development can be slowed by using temperatures of 15 to 20 °C before stage 21 is reached. Measuring the silver concentration (soluble silver) in the test solutions and in dechorionated embryos was important to our investigation of the salinity dependence of SNC toxicity. Hatching enzyme is the best biologically suitable enzyme for removing the chorion, because its high specificity means that it has no harmful proteinase. Other proteinases are not recommended. So far, the only hatching enzyme available is that for medaka; this is one limitation of this method.

The obvious effect of salinity on the outcome of the chemical toxicity tests demonstrated that simulating such natural aquatic properties as realistically as possible, as in our experiments, was useful for investigating the toxicity of chemicals in the environment. The discovery that SNC toxicity due to high silver concentrations was increased by salinity is highly applicable to the ecotoxicology of pollutant chemicals in all aquatic areas. In the case of general chemical toxicity testing in seawater, there is as yet no fish model nominated by authorized international organizations (*e.g.*, the OECD and International Organization for Standardization). Among the freshwater fishes (*e.g.*, medaka, zebrafish, carp, rainbow trout, and fathead minnow) that have been used for chemical toxicity testing, only the medaka has all of the advantages of salinity adaptation, hatching enzyme availability, high fecundity, and a size sufficiently small for easy use in laboratory experiments. Furthermore, medaka can be adapted to a wide temperature range (2 to 38 °C)[6]. In aquatic environments, salinity and temperature are the most important environmental influences on the fate of chemicals; our method should therefore be modifiable for a range of aquatic environmental research.

Disclosures

The authors declare that they have no competing financial interests.

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特集 1 生態系における汚染の動態と影響

生物群集の応答から金属の“安全”濃度を推定する：野外調査でできること

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Estimating “safe” concentrations of metals from responses of biological communities: What field survey can provide for ecological risk assessments

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要旨：化学物質の生態リスク評価（特に生態影響評価）は、主に室内毒性試験結果に基づき行われる。単一の種を用いた室内毒性試験は、比較的簡便で手法が標準化されているという実務的なメリットがある一方で、その結果のみから実環境における生物個体群や群集の応答を予測することには限界がある。そこで本稿では、実際の野外での生物群集の応答を直接観察できる野外調査に着目し、まず、その利点及び欠点、生態影響評価手法（室内試験、模擬生態系試験、野外実験、野外調査）における位置づけを整理した。さらに、底生動物調査結果に基づき金属の“安全”濃度を推定した事例から、①交絡要因の影響をできるだけ排除した調査デザインや計画を丁寧に選択すること、②分位点回帰を用いて複数の調査データをメタ解析することで、野外調査が室内試験結果を基にする生態リスク評価結果の信頼性を評価・補完する情報を提供できることを示した。最後に、生態系保全を目的とした今後の化学物質の管理施策を見据え、生物モニタリングや野外調査の導入が提供しうるさらなるメリットについて考察した。

キーワード：生物モニタリング、河川管理、底生無脊椎動物、水環境、生態毒性学

はじめに：生態学と生態リスク評価

化学物質の生物・生態系への影響を評価する生態毒性学は、私の知る限り、現在の生態学会において非常にマイナーな研究分野である。しかしながら、過去から現在にかけてこの分野が生態学会と関連が薄いかといえば、そうでもない。例えば、日本生態学会誌として初めて刊行された 1954 年（第 4 号）には農薬散布後の水田昆虫相の変化が報告されている（吉目木 1954）。また、本稿のテーマでいえば、『河川の生態学（1972 年出版）』の著者の 1 人である御勢が 1960 ～ 61 年に、鉱山廃水の影響を受けた日本各地の河川における底生動物相の調査結果を立て続けに報告しており（御勢 1960a, 1960b, 1961）、化学物質による汚染がより顕著であった比較的過去からその生態影響は“生態学”の中で扱われてきている。

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化学物質の生態毒性を評価した研究は古くから存在するが（例えば、Powers 1917；Hynes 1960）、生態毒性学（Ecotoxicology）という用語自体は 1970 年代に Truhaut により、自然または人為的汚染物質による、生態系の構成要素などへの毒性影響を研究する毒性学の一分野と定義された（Truhaut 1977）。毒性学が伝統的に個体レベルに重点を置いているのに対し、当初から生態毒性学は集団（個体群レベル）、即ちより高次の生物学的階層を意識していることが Truhaut（1977）から伺える。

化学物質の生態リスク評価の標準的な枠組みは、National Research Council（1983）のヒト健康リスク評価の枠組みをおおよそ踏襲した形で行われ、「問題設定・曝露評価・影響（有害性）評価・リスク同定」という手順で通常算定される（林ほか 2010）。曝露評価では、主に環境中での濃度を測定・予測することで行われ、「生物一般がどれくらい対象物質にさらされるか」の指標となる。影響評価では、室内毒性試験から得られる生存、繁殖、

表 1. 生態影響評価における手法の比較。

手法	生物種	コスト	操作性	現実性	時間スケール	空間スケール
室内実験	主に 1 種	小	高	低	短	小
模擬生態系実験	数～多種	中程度～大	低～高	中程度	短～中程度	中程度
野外実験	多種	中程度～大	低～高	中程度	短～中程度	中程度～大
野外調査	多種	中程度～大	低	高	長	大

模擬生態系試験はメソコスムやマイクロコスム実験を含む（早坂ほか（2013）に詳しい）。野外実験は、野外環境（例えば河川）での操作実験を指す。野外調査は、汚染の程度の異なる複数の地点で（空間的に）調査するものと、特定の地点で時間的に繰り返して（あるいは定期的に）調査するもの（生物モニタリング）を含む。あくまで相対的な比較をもとにした目安であり、個々の実験･調査計画によって異なりうることに留意。なお、数理モデルを用いたモデリングは除いている。

成長等への無影響濃度を収集し、「生物一般に顕著な影響を及ぼさない濃度」が推定される（室内毒性実験の簡単な例は、林ほか（2010）を参照）。利用する毒性試験の選択基準や「生物一般に顕著な影響を及ぼさない濃度」の推定方法などの細かい点は異なるにしても、後者の影響評価と同様の手順が我が国の水質環境基準の策定でも踏まれている。室内毒性試験の利用は、手法が標準化されており再現性が高いこと（多国間の規制において重要）、また比較的簡便でコストが低いという利点がある一方で、単一の生物種を用いた試験は非現実的であり、室内試験から野外応答を予測するのは困難であるといった批判は古くから行われている（Cairns 1983；Levin et al. 1984；Cairns 1988）。Cairns（1988） の「putting the eco in ecotoxicology」というタイトルが、ごく近年の環境毒性化学会（Society of Environmental Toxicology and Chemistry; SETAC）の北米年次大会の中で行われるセッション名などに使用されていることからも、依然根強い研究・議論が続いていることが分かる（例えば、2009 年に行われた SETAC North America 30th Annual Meeting）。

そこで、本稿では室内毒性試験結果に基づく生態リスク評価（特に、影響評価）を補足・補完する手法として野外調査に着目し、河川底生動物調査結果から亜鉛など金属の“安全”濃度を推定した最近の研究成果を中心に紹介し、化学物質の生態リスク評価における野外調査の有用性や今後の展望について考察する。野外での応答から推定される安全濃度（顕著な影響を及ぼさない濃度）は、上述した生態リスク評価における「生物一般に顕著な影響を及ぼさない濃度」と比較可能なものであり、影響評価や基準値の設定の適切性を評価する上で重要な参考情報となりうるものである（Iwasaki et al. 2011；Iwasaki and Ormerod 2012；Iwasaki et al. 2013a）。しかしながら、例えば、環境省中央環境審議会水環境部会水生生物保全小委員会では「フィールドで観察されるものは（中略）一つの物質による生態系への影響の程度を定量的に分離・特定することは困難であると考え、環境基準の導出に当たって採用しない（環境省 2004）」という意見もあがっており、行政機関では、生態リスク評価実施時における野外調査に対する評価が必ずしも高いとはいえない。

生態リスクの影響評価手法：野外調査の位置づけ

野外調査の最も大きな強みは、実環境における生物群集の応答を直接観測できることである。化学物質が及ぼす生態影響の評価手法を、(a) 単一種を用いた室内毒性試験、(b) 模擬生態系試験（マイクロコスムやメソコスムなど室内や屋外で構築した模擬生態系を用いた実験）、(c) 野外実験、(d) 野外調査の 4 つに分けた場合（表 1）、前者ほど操作性が高く、必要となる人的金銭的コストは少ないが、後者ほど対象とする時空間的スケールが大きくなり、実際の環境中における生物応答をより直接的に観察可能である（表 1 では「現実性」と表現した）。これら手法の相対的な比較から、野外調査に関するその他の欠点としては、注目する要因以外の影響の制御が難しくデータのばらつきが大きくなるため結果の解釈や因果の推論が難しくなること、および調査地点数（サンプル数）が限られてくるため結果の一般化が難しくなることが挙げられる。

しかしながら、これら 2 つの欠点はそれぞれ、①交絡要因の影響を可能な限り排除できる調査デザインや解析方法を丁寧に選択すること、②既往の野外調査結果やモニタリングデータを分位点回帰などの適切な統計解析を用いてメタ解析することで一定程度解決することが可能である。①についていえば、完全にランダムに調査地点を選択するよりも（例：Clements et al. 2000）、例えば、

汚染地点と似通った環境（例えば、標高、地質、植生、河川サイズなどに加えて、流速や底質などの河川内環境）を持つ非汚染地点を対照地点に設定することで、これらの影響をできるだけ排除した比較が可能になる。河川では、上流から下流にかけての河川環境（水温や河川地形、エネルギー基盤など）の変化に伴って、底生動物群集の種組成が変化することは一般的に受けいれられている（例えば、河川連続体仮説（Vannote et al. 1980）など）。他方、特定の汚染源から流入してきた化学物質の濃度は、下流にいくにしたがって流量増加などに伴い減少することが多い。そのため、一つの河川においてこの流程変位に伴う濃度減少と底生動物相の変化を関連付ける研究が過去多く行われていたが、それらの研究では、そもそも河川環境の流程変化が交絡要因として働いており、適切な野外調査デザインとはいえない（Clements 1994；岩崎 2011a）。

次節では、河川底生動物の種数や個体数に顕著な影響を及ぼさない金属濃度（安全濃度）を推定した研究を例として取り上げ、①休廃止鉱山周辺の河川において丁寧な地点選択を行うことでデータのばらつきを減らし、着目する要因（ここでは亜鉛）の影響を推定すること、及び②既往の野外調査結果をメタ解析することで、より普遍性のある結果を導き出すことに取り組んだ事例を紹介する。

野外調査結果から金属の安全濃度を推定する

背景

御勢による一連の論文からも分かるように（御勢 1960a, 1960b, 1961）、金属汚染は古くて新しい世界的にも重要な問題の一つである（Newman and McIntosh 1991；Luoma and Rainbow 2008）。金属種によっても異なるが、日本では工場や事業所、下水処理場からの排水や休廃止鉱山からの抗廃水などが負荷源として挙げられ（中西ほか 2006；中西・恒見 2008；中西ほか 2008a, 2008b）、例えば、亜鉛が高濃度で検出される地点は上記の 3 つが主要な負荷源として推定されている（中西ほか 2008b）。日本において水生生物の保全を目的とする水質環境基準の必要性については 1993 年の中央環境審議会答申で指摘され、2003 年に初めて亜鉛について設定された（淡水域：30 μg/L：より詳細な背景は岩崎・及川（2009）参照）。この基準値は、複数の毒性試験結果を比較した結果、水生昆虫の一種であるヒラタカゲロウ類の成長阻害に影響を及ぼさない濃度（すなわち、無影響濃度）をもとに設定されている。この基準値の全国的な維持・達成を図るため、日本では、亜鉛の一律排水基準が 5 mg/L から 2 mg/L に強化されている。同様に、欧州など各国においても、金属の水質環境基準に相当するものが設定されており、いずれの場合も、室内毒性試験の結果をもとに導出されている。したがって、これらの基準値前後における金属濃度が野外群集に及ぼす影響を明らかにすること、特に自然条件下において水生生物の種数や個体数に顕著な影響を及ぼさない濃度を推定することは、室内毒性試験結果から導出された基準値の適切性を評価する上で有用な情報を提供するだろう。

本稿で着目する河川大型無脊椎動物（以下、底生動物）は、河川生態系において中間的な栄養段階に位置し（Rosenberg and Resh 1993；中村 2013）、汚染物質に様々な感受性を示す種によって構成されている。底生動物は比較的定着性であり、古くから河川生態系の状態を表す指標生物として広く用いられており（津田 1964；野崎・山崎 1995；Barbour et al. 1999；Birk et al. 2012）、野外調査や模擬生態系実験によって金属に対する応答を調べた研究も少なくない（岩崎 2011b）。魚類に比べて経済的な（あるいはレクリエーション的な）価値は一般的に劣るが、簡易な現地調査法によって（室内でのソーティングと同定に比較的労力がかかるが）、多様な分類群が少なくない個体数（生物量）で収集できるのが底生動物を調査対象とする利点である。

休廃止鉱山周辺の河川での底生動物調査

まず、著者らが休廃止鉱山周辺の河川において交絡因子による影響をできるだけ排除できるように調査地点を選択し、亜鉛濃度の影響を評価した研究について紹介する。当該調査では、河川底生動物群集の種数（分類群数）や個体数に顕著な影響を及ぼさない亜鉛濃度を推定するために、休廃止鉱山周辺の 3 水系の上流域に設定した計 25 地点の早瀬において、底生動物を採取し（礫単位採取法）、水質項目（溶存態金属濃度（亜鉛（Zn）、銅（Cu）、カドミウム（Cd）、鉛（Pb））、BOD、DO など）、物理環境項目（川幅､流速､水深､礫サイズ）を測定した（Iwasaki et al. 2009, 2011, 2012）。これらの 3 水系での調査結果をまとめて解析した結果、解析対象としたすべての底生動物種数で、亜鉛濃度の影響についてはある一定濃度までは影響がないとするモデルが最良モデルとして選ばれ、基準値の 2 ～ 3 倍程度の亜鉛濃度でも底生動物の種数はほとんど減少しないことが示唆された（図 1 に全底生動物とカゲロウ目の種数の例を示す）。この際、丁寧な地点選

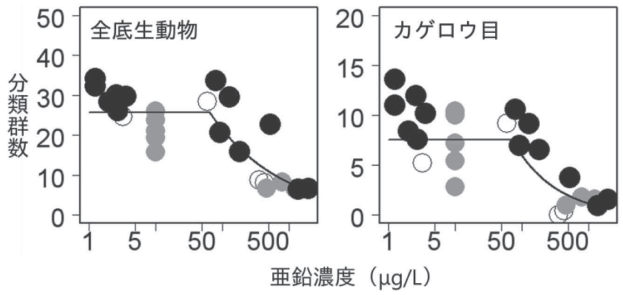

図 1. 亜鉛濃度と河川底生動物の種数との関係（Iwasaki et al. 2011 より改変）。異なる色のプロットは異なる河川を示す。黒線が AIC 最小の最良モデル（一定の濃度までは影響がないと仮定する閾値モデル）を示す。

択に加えて、亜鉛等金属濃度以外の影響をできるだけ排除するために、測定したその他の物理化学的項目を用いた主成分分析から得られた 11 個の主成分を重回帰分析に用いてモデルを構築し、赤池情報量規準によるモデル選択を行った。ここでは、種数に着目して紹介したが、群集レベルの個体数（例えば、総個体数やカゲロウ目の個体数）についても同様の結果が得られている（岩崎ほか未発表）。また、単一水系（兵庫県市川水系）での調査で優占した分類群の個体数を対象に亜鉛濃度との関係を評価した場合でも、上記の結果を支持する結果が報告されている（Iwasaki et al. 2012）。以上の結果は底生動物の種数の保全という観点で、亜鉛の基準値は安全側に設定されていることを示唆していると言えるだろう。

同様の研究例として、Schmidt et al.（2010）では米国コロラド州周辺の 153 の河川地点での底生動物調査に基づき、金属濃度と底生動物群集の種数や個体数との関係を定量化し、複数の金属濃度が水質クライテリア（water quality criteria）未満であった地点における影響について考察している。これらの研究結果は、対象生物の生態を慎重に考慮し、できるだけ物理化学的な特徴が似通った地点で調査することによって不必要なばらつきを抑え、野外調査結果データからも濃度反応関係を推定できることを示している。

英国・米国・日本の底生動物調査結果のメタ解析

一方で、以上の研究はあくまで特定の対象地域（あるいは流域）で実施されたものであり、より広域における一般性が担保されているわけではない。したがって、この課題を乗り越えるためには、異なる地域（例えば、生物地理区や大陸）での生物調査結果をプールして（まとめて）解析する（広義の）メタ解析が有用である（Glass 1976；Koricheva et al. 2013）。その場合、前述した野外調査研究と比較して、調査方法や調査場所の違いからデータのばらつきが無視できなくなり、平均値を回帰する通常の回帰分析では、考慮していない変数の影響によって注目する説明変数と応答変数同士の関係を正確に推定することが極端に難しくなる（図 2）。しかしながら、多くのデータを収集し分位点回帰を用いることでこの問題をカバーすることができる（詳細は、図 2 とその説明文を参照）。分位点回帰の紹介は Cade and Noon（2003）および Schmidt et al.（2012）に詳しいが、金属汚染のように生物の生息を制限するような要因は上位の分位点（例えば、90％や 95％）を回帰することによって、着目する説明変数の影響を推定する方法である。

著者らは、英国、米国、日本の金属汚染河川や酸性河川で主に実施された底生動物調査データ（合計 400 地点超）にこの分位点回帰を適用し、銅（Cu）、亜鉛（Zn）、カドミウム（Cd）、マンガン（Mn）の 4 つの金属について底生動物の種数に顕著な影響を及ぼさない閾値濃度（“安全”濃度）を推定した（図 3A；Iwasaki and Ormerod 2012）。この解析では各金属濃度を説明変数に用いて、ある一定の濃度まで影響がないとする閾値モデル（図 3A）に加え、切片のみのモデル（$y = a$：a は切片）、線形モデル（$y = a + b*x$：x は対数変換した金属濃度、b は回帰係数）、指数モデル（$y = a + b*\exp(x)$）の 3 つの分位点回帰モデルを当てはめたが、いずれの金属データでも閾値モデルが最良であった。分位点回帰から得られた安全濃度は、信頼区間は大きいものの、大変興味深いことに室内毒性試験結果から導出された各国の基準値等と概ね重複しており（図 3B）、野外調査データからそれら基準値の信頼性を補完する結果を提供することができたといえるだろう。Crane et al.（2007）も同様のアプローチで、英国イングランドとウェールズでの野外調査結果から科レベルの個体数と分類群数を用いて金属の安全濃度の推定を試みている。

野外調査ができることと課題

ばらつきが大きいなどの理由で通常敬遠されがちな野外調査ではあるが、以上の研究結果が示すように、丁寧な調査デザインやメタ解析（及び適切な統計手法）を用いることで、濃度反応関係や安全濃度の推定に利用することができ、室内試験結果を基にする生態リスク評価結果の信頼性を評価・補完できる情報を提供できるといえるだろう。室内毒性試験結果から自然条件下における生

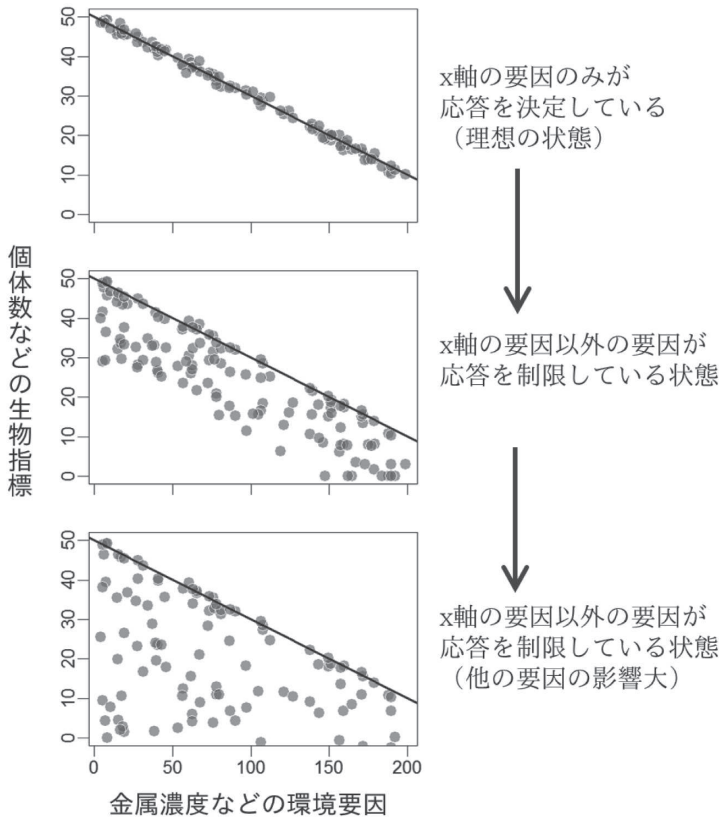

図 2. 高分位点を回帰する場合の分位点回帰の概念図（Cade and Noon 2003 より改変）。最上部のパネルは、主に x 軸の要因のみによって y 軸の応答が制限されていることを示す（それ以外の環境条件は y 軸の値が最大となる「理想の状態」と仮定）。下部のパネルにいくにしたがい、x 軸以外の（考慮していない）要因が y 軸の応答を制限することでばらつきが大きくなっている。すなわち、y 軸の変数を制限するような説明変数であれば、高分位点を回帰することで説明変数のみの影響を抽出することが理論的には可能になる。なお、着目する説明変数と高い相関にある変数が別に存在する場合、分位点回帰を用いても得られた濃度反応関係の解釈には注意が必要になるが、多くのデータを収集することでそのような高い相関を持つ変数は少なくなることも予想される。実際に、限られた負荷源から金属が排出される単一水系内では高い相関（例えば、r>0.9）を示す金属濃度も、図 3A に示した解析データでは亜鉛とカドミウムを除いて（r=0.89）、金属濃度間の相関は 0.05-0.53 と低くなっている。

物・生態系への影響を（事前に）正確に予測することが困難であることを考えれば、野外の生物個体群や群集の応答を調査・モニタリングしていくことは効果的な管理を実施していく上で実質的に不可欠といえるだろう。

とはいえ、もちろん野外調査も万能ではない。例えば、著者らの上記の研究では説明変数として単一の金属濃度を用いたが、実際、野外は複数の金属で汚染されており、休廃止鉱山周辺の河川ではそれらの濃度が相関していることも多い（Iwasaki et al. 2011）。複数の金属存在下で、上述の研究のように単一の金属（例えば亜鉛）の影響のみを考えることは、その金属の影響を過大評価する（すなわち、安全濃度は過小評価されている）可能性が高いと考えられるが、この仮定は十分に検証されているわけではない。例えば､オオミジンコを用いた短期間（48 時間）の毒性試験ではあるが、カドミウム一定濃度下において銅濃度を増加させると、一度生存率が上昇するといった現象も最近観測されている（Meyer et al. 2015）。金属の複合存在下によって、どのような曝露指標を用いるべきか、まだまだ議論が始まったばかりである（Iwasaki et al. 2013b；Balistrieri and Mebane 2014；Farley et al. 2015）。

したがって、野外での生物応答を理解するためには、室内毒性試験はもちろん、メソコスムやマイクロコスムといった模擬生態系試験や野外実験とも組み合わせて、研究を進めていく必要があるだろう。注意すべき点は、

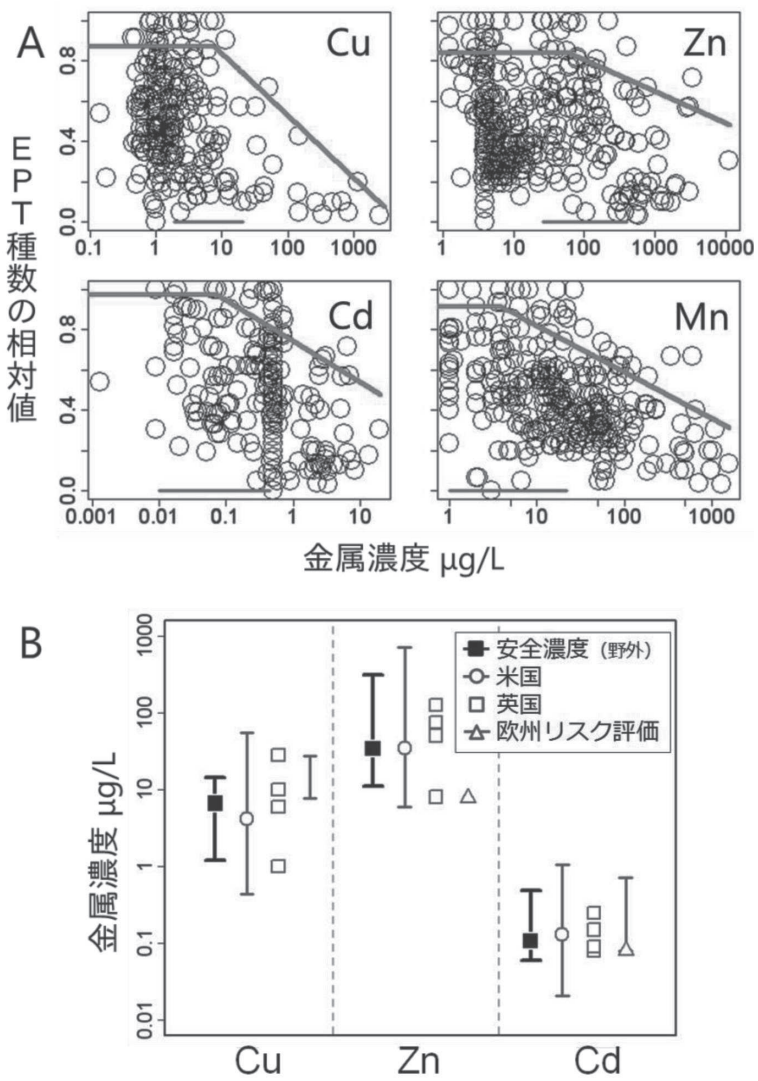

図 3.（A）各金属濃度と EPT 種数（カゲロウ目、カワゲラ目、トビケラ目の分類群数の和）の関係。EPT 種数は各調査ごとの最大値を用いて標準化して解析に用いている。折れ線は推定された分位点回帰モデル（分位点＝ 0.95）、各パネル下部にある直線は分岐点（安全濃度）の 95％信頼区間を示す。なお､回帰線より下側にあるプロットは、対象とした金属以外の影響を受けていると仮定される（図 2 も参照）。（B）野外データより推定した安全濃度（エラーバーは 95％信頼区間）と室内毒性試験を基にした水質環境基準（米国、英国）及び予測無影響濃度（欧州リスク評価）との比較。水質環境基準及び予測無影響濃度は硬度によって連続的に変化するため、野外調査データで観測された硬度の最小及び最大値に基づき計算した値をエラーバーで表示している（英国の水質環境基準は硬度により離散的に変化する）。いずれの図も、Iwasaki and Ormerod（2012）より改変。

表 1 に示したように各手法の利点と欠点である。特に、水生昆虫は野外調査結果から推定された底生動物の金属感受性と室内毒性試験やマイクロコスム実験から推定されたそれが顕著に異なることが示されている（Brix et al. 2011；Clements et al. 2013；Iwasaki et al. unpublished）。既往の試験では若齢な個体が毒性試験に用いられていないこと、時空間的なスケールの制限から複雑な生活史がカバーできていないことなどがその理由として議論されている。また、ごく最近になって、カゲロウ類の一種を用いた室内試験で幼虫時期よりも羽化時に金属感受性が高くなることも報告されている（Wesner et al. 2014）。他方、特定の地点における生物相に及ぼす要因を考えた場合、野外ではその地点からの移出と他の地点からの移入が重要な役割を担っている。例えば、非汚染地点からの底生

動物の流下が、汚染地点における生物相の変化を見かけ上緩和していることも考えられる（Beltman et al. 1999）。以上まとめると、当然ではあるが、時空間的スケールに制限がある室内実験などは現象の背後にあるメカニズムの理解、野外調査は実際の環境下で起きている現象の記述・観測に比較的適していることになるだろう。なお、本稿では触れなかったが、室内毒性試験結果などと組み合わせた数理モデル（個体群モデルや生態系モデル）の利用も、生態リスク評価において重要な役割を担う。

今後の展望：野外調査・生物モニタリングのすすめ

ここまでは、野外調査として、主にある特定の時期に複数の地点でスナップショット的に行われる生物調査を想定し、その結果から室内毒性試験から導かれる水質環境基準などの適切性や信頼性を評価することを中心に紹介してきた。これに加えて、特定の地点で時間的に繰り返す生物調査（以下、生物モニタリングと呼ぶ）は、化学物質の生態リスク評価結果を評価・補完することができるだけでなく、同時に後述する新たなメリットが得られると予想されるが、現時点ではほとんど利用されていない。以降では、実際の管理・施策にどのように組み込むかという具体的な議論を抜きに生物モニタリングのメリットについて論じるが、理想論としても、今後の管理を考える上でそのメリットを認識・共有しておくことは重要だと考える。

化学物質濃度の管理から、生態系保全中心の管理へ

亜鉛の水質環境基準のように、今日までに複数の化学物質について水生生物を保全するための基準値が設定されている。しかしながら、環境基準の目標が水生生物の保全にもかかわらず、その管理では行政や事業所が環境中や排水中の化学物質の濃度を一定未満に下げることに努力と関心が注がれる（加茂ほか 2009）。実際に、法的拘束力があるのは、環境基準の設定を受けて通常設定・強化される排水基準である。

しかしながら、施策の効果や達成度を評価するために、野外での生物調査や生物モニタリングをこの規制の仕組みの中に組み込むことは環境基準の本来の目的からして必然とも思われる（例えば、加茂ほか 2009；岩崎 2011b）。一つの例として、当該物質の影響が懸念される（生態リスクが高い）河川を優先的に抽出し、排水が流入する直上（対照地点）および直下の地点において、生物モニタリングを継続的に実施することで、排水規制の効果を測ることができるだろう。このような取り組みは現状の化学物質の管理に以下の 3 つの現実的に重要な視点を提供すると考えられる。まず、化学物質の濃度のみの管理から、生態系保全の視点が加わることである。“われわれが守りたいのは、化学物質の濃度ではなく生態系であることを忘れてはならない”のである（加茂ほか 2009）。コストをかけて排水処理を行っている事業者のなかには、実質的な生物保全効果を知りたいと思う事業者も少なくないだろう。

化学物質の個別管理から統合的な管理へ

2 つ目として、化学物質の管理に生態系保全の視点が加わると、化学物質の影響だけでなくより広い視点で保全方策を考える必要性が必然的に出てくることである。例えば、いくら水質が改善しても、河床がコンクリート張りで生息場が単調であれば、生物相の回復はあまり見込めないだろう。また、図 3 に示したように、金属濃度が低い地点において河川底生動物の種数が制限されている地点が多く存在することからも金属濃度以外の要因を考慮する必要性は明らかである（図 2 も合わせて参照のこと）。一方で、金属などの水質汚染は特定の負荷源またはノンポイント負荷源から流域の比較的広い範囲（特定の負荷源であれば、その下流域）に影響を及ぼすため、生物相の回復に重要となる種プールに影響を及ぼす可能性があり（Sundermann et al. 2011）、通常小規模で行われる物理環境の改善の効果を直接・間接的に制限してしまうかもしれない（Kail et al. 2012；Palmer et al. 2014）。すなわち、河川の生物相は複数の非生物学的および生物学的要因から影響を受けている。

したがって、各々の化学物質に新たな排水規制を設けて（あるいは既存の排水規制を強化して）個別に管理するよりも、化学物質以外の要因の影響も総合的に勘定して、優先順位付けを行った上で効果的な対策をとることが理想的である。複合的な影響を評価・管理することは決して簡単なことではないが、効果が小さい（あるいはほとんどない）と予想される対策を回避できるというインセンティブは小さくないだろう。また、行政だけではなく、市民レベルの活動（例えば、近年、活発になってきている小さな自然再生：三橋 2012）や企業の CSR（企業の社会的責任）活動とも連携して、流域内での問題点を抽出・整理していくことも非常に重要であろう。

何を守るべきか。何を守りたいか。という問いとの対峙

さらに、そのような生物モニタリングを通じて、「我々

は一体なにをどこまで守りたいのか。守るべきなのか。」という課題を直面することになるだろう。この問いは、化学物質の生態リスク評価において重要な出発点であるが、現在までむしろ曖昧に扱われてきている（Iwasaki and Clements 2015）。生態リスク評価における無影響濃度の利用がその典型的な例である。無影響濃度（NOEC: no observed effect concentration）は室内毒性試験結果から得られる統計学的に有意な影響が観測されなかった最大濃度である。したがって、無影響濃度は影響がまったくない濃度に対応するわけではなく、単に設定した実験デザインや統計処理方法に依存して決定される（岩崎ほか 2013）。実際に、無影響濃度において、生存率や産卵数などの測定された指標に数％～ 20％程度の減少が観測されている（Crane and Newman 2000）。このように、生態リスク評価は、本質的な問いに対して具体的で明確な解を準備せずに、個体群や生物多様性の保全といった抽象的な目的のもとに、統計学的な有意性に依拠して今日まで進んできたともいえるだろう。

中長期的に生物モニタリングを行い、実施された施策や対策の効果を評価し、さらなる対策を検討・実施することによって、この「なにをどこまで守りたいか。守るべきか。」という問いに、管理者や利害関係者が市民とともに具体的な答えを準備する必要が出てくるだろう。そして、そのような個別具体的事例の集積を、より一般的な生態リスク評価における保全目標の設定や評価方法自体にフィードバックすることによって、現状の統計学的有意性への依拠から脱却することができるのではないかとひそかに期待している。

長期モニタリングすることのメリット：時空間的広がり

JaLTER（Japan Long-Term Ecological Research Network；http://www.jalter.org/、2014 年 10 月 9 日確認）の設立にあるように、生態系の理解や保全、将来予測を促進する上で、長期モニタリングデータは欠かせない。特に気候変動に伴う水温等の変化が今後どのように生物相に影響する（している）のか調査する上で、そのような時系列データは不可欠である（Vaughan and Ormerod 2014）。Vaughan and Ormerod（2014）では、イングランド及びウェールズにおける 1991 ～ 2011 年の長期のモニタリング調査結果から、底生動物の分布の北上は気候変動によるものではなく、水質の改善によるものであることを示している。河川の水生生物（特に底生動物や魚類）についていえば、日本においても実は、長期間のモニタリングデータがすでに存在する地域も存在する。例えば、神奈川県川崎市では 1979 年から 2011 年までの間に川崎市内の 110 地点で魚類及び底生動物調査が実施されている（小林・岩渕 2013）。このようなモニタリングデータを着実に蓄積し、時空間的に分析することで（データの蓄積だけでなく活用も重要）、長期的な生物相の変化やその要因を特定することも可能になるだろう。まずは、上述の川崎市のデータや 1990 年度から実施されている日本全国の一級水系（河川）を主な対象とした河川水辺の国勢調査などの生物データと国及び地方公共団体が実施している公共用水域の水質測定結果とリンクさせて解析することによって、モニタリングから得られるメリットをより広く認知してもらうことも最初のステップとして重要となるだろう。

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Does the Choice of NOEC or EC10 Affect the Hazardous Concentration for 5% of the Species?

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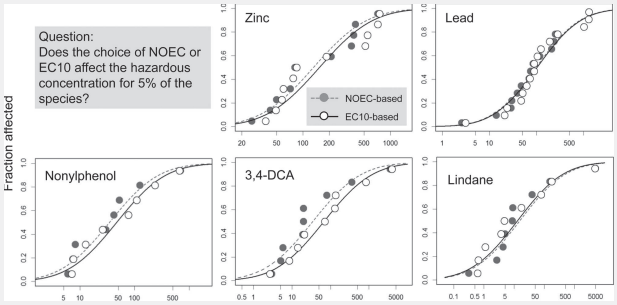
S *Supporting Information*

ABSTRACT: We evaluated if the choice of no observed effect concentration (NOEC) or a 10% effect concentration (EC10) affects the hazardous concentrations for 5% of the species (HC5s) estimated from species sensitivity distributions (SSDs). By reviewing available literature reporting NOECs and reanalyzing original toxicity data to estimate EC10s, we developed two SSDs for five chemicals (zinc, lead, nonylphenol, 3,4-dichlorobenzenamine, and lindane) based separately on 9−19 EC10s and NOECs. On average, point estimates of HC5s based on EC10s were 1.2 (range of 0.6−1.9) times higher than those based on NOECs. However, both EC10-based and NOEC-based HC5s estimated for five substances were on the same order of magnitude, and their 95% confidence intervals overlapped considerably. Thus, although EC10 was chosen as a representative of EC*x* in this study, our results suggest that the choice of EC*x* (e.g., EC5, EC10, or EC20) or NOEC does not largely affect the resulting HC5s. Therefore, use of NOECs would be acceptable particularly in regulatory contexts, although the NOEC has important shortcomings and should be used with caution.



INTRODUCTION

No observed effect concentration or level (NOEC or NOEL, respectively), which is the highest concentration that does not cause a statistically significant adverse effect in a toxicity test, is one of the commonly used toxic measurements in ecological risk assessments (ERAs). However, because of substantial shortcomings, the NOEC and a related toxicity measurement [i.e., lowest observed effect concentration (LOEC)] have been heavily criticized for more than 30 years,[1−3] the reasons being that (1) statistical significance (i.e., estimation of NOEC and LOEC) depends on experimental design, data variability, sample size, effect size, and statistical analysis used (including the significance level chosen), (2) statistical insignificance does not guarantee ecological, biological, or ecotoxicological insignificance, and (3) the magnitude of the effect at the NOEC or LOEC is not explicitly defined. Indeed, several studies demonstrated that effect sizes at NOECs are mostly between 0 and 20%.[4,5]

Recently, Landis and Chapman[1] called for the ban of using NOECs and LOECs and for more emphasis on concentration−response approaches. This editorial stimulated a line of discussion that both agrees and disagrees with the original call.[6−10] The most frequently applied alternative is to estimate the *x*% effect concentration (EC*x*) based on the concentration−response relationship. Values of 5−20% have been commonly suggested or used as the *x*% (e.g., refs 11−13). Because the replacement of the NOEC with an EC*x* (e.g., EC10) will likely be conducted at least gradually, it is important to determine if and how it affects the outcome of ERAs.

Species sensitivity distributions (SSD) have been frequently applied to estimate the hazardous concentration for 5% of the species (HC5), which is used as a "safe" concentration (e.g., for environmental water quality criteria) and a predicted no effect concentration (PNEC) in ERAs,[14−16] mostly by applying a safe (or assessment) factor. The SSD is typically estimated by fitting a statistical distribution (e.g., a log-normal distribution) to multiple NOECs. Although the relationship between NOEC and EC*x* has been evaluated in a few studies,[4,5] it is uncertain how the use of EC*x* instead of NOEC changes the resulting HC5.

In this study, we evaluated how the choice of EC10 or NOEC affects the resulting HC5s. Although EC10 was operationally selected as a representative EC*x*, discussion about using other values such as EC5 and EC20 can be found below. To this end, we first reviewed published literature that

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employed the SSD approach using NOECs (rarely ECx) and reanalyzed the original toxicity data to quantify concentration−response relationships that allow us to estimate EC10. Then, we estimated two SSDs for five chemicals [zinc, lead, nonylphenol, 3,4-dichlorobenzenamine (3,4-DCA), and γ-hexachlorocyclohexane (lindane)] based separately on EC10s and NOECs and compared the resulting HC5s. Using this procedure, we also estimated the magnitude of the effect (in this study, predicted percent reduction compared to controls) at NOECs and LOECs and the EC10/NOEC ratios to investigate relationships between NOECs an EC10s. Determining whether and how the use of EC10 changes the resulting HC5 provides essential knowledge for performing ERAs and setting environmental quality criteria. Even if there is little impact, such information can help optimize risk assessment processes.

METHODS

Data. We referred to 15 risk assessment documents that include ecological risk assessments performed by the Research Center for Chemical Risk Management, National Institute of Advanced Industrial Science and Technology, Japan (https://unit.aist.go.jp/riss/crm/mainmenu/e_1.html), and Versteeg et al.[17] that estimated SSDs for 11 substances. From these sources, we chose five substances based on two criteria: (1) the original SSD was estimated based mostly on NOECs, and (2) EC10s can be determined for more than four to eight species, which is the minimum database size that is generally accepted for regulatory contexts.[18] Furthermore, only ecotoxicity tests for which the data could be obtained from original literature and tests that included more than four concentration levels, including controls, were used to estimate EC10s. The numbers of toxicity tests analyzed for the five chemicals were zinc[19] (17), lead[20] (19), nonylphenol[21] (9), 3,4-DCA[17] (16), and lindane[17] (13).

From the original literature used for the estimation of NOECs, measured concentrations (if unavailable, nominal concentrations) and response variables (e.g., survival) were collected to estimate the corresponding EC10s. If requisite data were available in only figures, we obtained numerical data using GSYS2.2 [Japan Nuclear Reaction Data Centre (JCPRG) Digitizing software (http://www.jcprg.org/gsys/gsys-j.html)]. Note that if the raw data were unavailable and the EC10 was estimated and used in the original study, we used the reported EC10 for this study. Although bioavailability affects a metal's toxicity,[22] we did not consider bioavailability in this study because our aim was to compare HC5s based on EC10s and NOECs.

Data Analysis. Concentration−response relationships were modeled with the "drc" package (version 2.3-96) in R version 3.0.2.[23,24] Individual toxicity data sets were fitted by a total of nine models (log-logistic, Weibull-1, and Weibull-2 functions with two to four parameters), and the best model with the smallest value of Akaike's information criterion (AIC) was selected. A smaller AIC value indicates a more parsimonious model that makes better prediction (see, e.g., ref 25).

The best models selected for most of the data sets were log-logistic, Weibull-1, and Weibull-2 models with two and three parameters. The three-parameter log-logistic model (LL.3) is

$$y = \frac{d}{1 + \exp\{b[\log(x) - \log(e)]\}} \quad (1)$$

where x is the substance concentration, b is the slope at concentration e, and d is the maximal response value. In the two-parameter log-logistic model (LL.2), the value of d is set to 1. Similarly, the three-parameter Weibull-1 model (W1.3) and three-parameter Weibull-2 model (W2.3) are

$$y = d\exp(-\exp\{b[\log(x) - \log(e)]\}) \quad (2)$$

$$y = d[1 - \exp(-\exp\{b[\log(x) - \log(e)]\})] \quad (3)$$

respectively, and the value of d is 1 in the two-parameter Weibull-1 model (W1.2) and two-parameter Weibull-2 model (W2.2). Further details of other models can be found in refs 23 and 24. By using the best models selected for individual toxicity data sets, we estimated the EC5, EC10, and EC20 and the percent reductions at the NOEC and LOEC compared to the control responses.

We then fit the NOECs and EC10s separately to a log-normal distribution (i.e., estimated SSD) and estimated HC5s and their 95% confidence limits.[26] If there were multiple NOECs or EC10s for a species, the geometric mean was calculated and used to derive the SSD.

RESULTS AND DISCUSSION

Details about the species, end points, test durations, response concentrations, and best-fit concentration−response models in the 74 toxicity data sets for the five chemicals are provided in Tables S1−S5 of the Supporting Information. For each chemical, 9−19 NOECs were available, although one and three EC10s were used as surrogate NOECs for zinc and lead, respectively, because the NOEC and LOEC were not reported for those studies (Tables S1 and S2 of the Supporting Information). Major end points were survival (34%), reproduction (23%), growth (18%), and population growth (14%) (Tables S1−S5 of the Supporting Information). Other end points were fish deformity, algal biomass, insect emergence, time to hatch, and survival time. The numbers of species ranged from 8 to 16, and fish (47%), crustaceans (28%), and algae (9%), which are common taxonomic groups in general toxicity tests, dominated the data sets.

Percent Reduction Predicted at NOEC and LOEC. Among the five chemicals, no significant difference in the estimated magnitude of effect (i.e., percent reduction) at the NOECs or LOECs was observed [Kruskal−Wallis test; $p = 0.31$ and 0.72, respectively (Figure S1 of the Supporting Information)]. After all the data had been pooled, the medians (and ranges) of percent reductions estimated at the NOECs and LOECs were 5.3% (0.0−67.6%) and 34.0% (2.7−99.8%), respectively [$n = 69$ (Figure 1)]. This result indicates that, on average, the NOEC approximately equals the EC5. The distribution of percent reductions at the NOECs was not uniform, with 50% of reductions at the NOECs being ≤5%, 70% of reductions being ≤10%, and 85% of reductions being ≤20%. It is rather surprising that, in approximately 15% of cases, the actual effects at NOECs were larger than 20%. In contrast, the percent reductions at the LOECs were distributed almost uniformly from 0 to 100%. Given the reductions at the NOECs, this is simply because any percent reductions can be observed at the LOECs depending on the shape of concentration−response curves. These results are generally consistent with previous studies.[4,5] For example, the mean percent reductions that were observed and predicted at NOECs (algae, cladocerans, and zebrafish; $n = 22$) were 4.7 and 3.0%,

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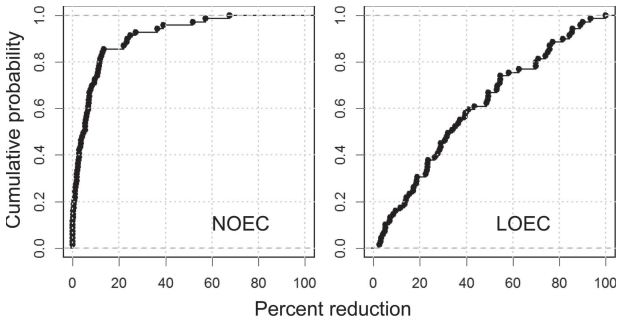

Figure 1. Cumulative probability distribution of percent reductions (i.e., effect magnitudes) observed at the no observed effect concentration (NOEC) and lowest-observed effect concentration (LOEC). Data were pooled across the five chemicals evaluated in this study (n = 69). Tests in which only EC10s were available or raw data were unavailable were excluded.

respectively,[5] which is close to our estimate despite different toxicity data sets used.

Relationship between NOEC and EC10. The median (and range) of the distribution of EC10/NOEC ratios was 1.3 [0.1−6.5 (Figure S2 of the Supporting Information)], indicating that EC10s were usually higher than NOECs, as with obtained results described above. Among all the toxicity data, the EC10 was lower than the NOEC in approximately 30% of the cases (Figure 2). These results are similar to those of Isnard et al.,[5] who reported the median EC10/NOEC ratio was 1.32.

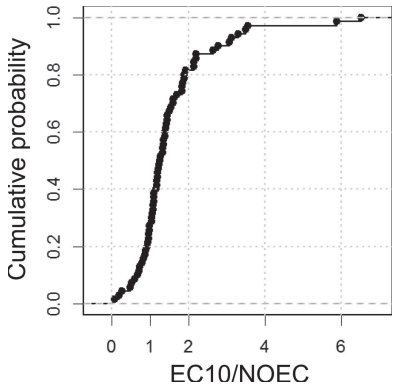

Figure 2. Cumulative probability distribution of ratios of the 10% effect concentration (EC10) to the no observed effect concentration (NOEC). Data were pooled across the five chemicals evaluated in this study (n = 70). Tests in which only EC10s were available were excluded.

Influences of Using EC10 on Species Sensitivity Distributions. On average, HC5s estimated on the basis of EC10s (hereafter termed the EC10-based HC5) were 1.2 times higher than those estimated on the basis of NOECs [range of ratios of (EC10-based HC5)/(NOEC-based HC5) of 0.6−1.9 (Table 1 and Figure 3)]; only for lindane was the EC10-based HC5 (0.17 μg/L) lower than the NOEC-based HC5 (0.27 μg/L). However, both EC10-based and NOEC-based HC5s estimated for five substances were on the same order of magnitude (Table 1). The smaller range of (EC10-based HC5)/(NOEC-based HC5) ratios is somewhat surprising given that the range of the EC10/NOEC ratios was relatively

Table 1. Estimated Hazardous Concentration for 5% of Species (HC5) Based on No Observed Effect Concentrations (NOECs) and 10% Effect Concentrations (EC10s)

	NOEC-based		EC10-based	
substance	HC5	95% confidence interval	HC5	95% confidence interval
zinc	21.5	4.2−46.3	22.3	3.8−50.3
lead	6.61	0.99−16.9	7.99	1.34−19.61
nonylphenol	2.75	0.10−8.78	3.48	0.11−11.38
3,4-DCA[a]	1.12	0.01−4.59	2.12	0.03−8.74
lindane	0.27	0.00−1.31	0.17	0.00−0.90

[a]3,4-Dichlorobenzenamine.

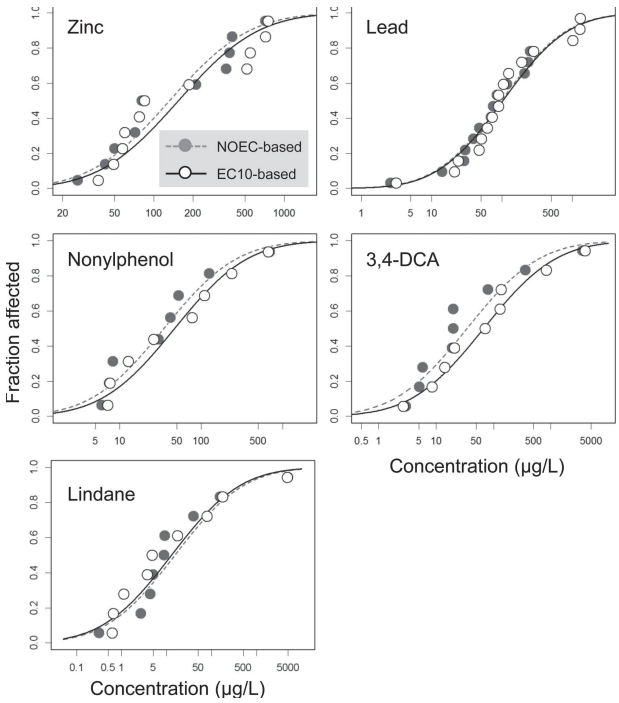

Figure 3. NOEC- and EC10-based species sensitivity distributions for zinc, lead, nonylphenol, 3,4-dichlorobenzenamine (3,4-DCA), and lindane. NOEC represents no observed effect concentration and EC10 the 10% effect concentration.

large (0.1−6.5). This is because HC5s are less affected by individual toxic values because SSDs are derived from multiple NOECs or EC10s, and we fitted the log-normal distribution to the data (in other words, log-transformed values of NOECs and EC10s were used to derive SSDs). Overall, the close overlap of the 95% confidence intervals for EC10-based HC5s with those for NOEC-based HC5s suggests that the choice of EC10 or NOEC has little influence on the resulting HC5s.

Implications and Limitations. This study provides empirical evidence that the choice of EC10 or NOEC does not largely affect the resulting HC5s estimated from SSDs. If EC5s or EC20s were used instead of NOECs to calculate HC5s, the resulting point estimates of HC5 would have decreased or increased marginally, but the confidence intervals of HC5s derived from EC5s and EC20s would have still overlapped those derived from NOECs. Indeed, because NOECs generally corresponded to EC5s in the current data

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sets, the EC5-based HC5 was closer to the NOEC-based HC5 (data not shown).

Other factors affect SSDs and thus HC5s: (1) how to estimate the SSD (selection of statistical distributions and use of bootstrap-based and regression-based estimations),[14,27,28] (2) how to deal with multiple toxicity data for a species,[29] and (3) how to determine proportions of multiple taxonomic groups included in an SSD (e.g., how to adjust the proportions of algae, invertebrates, and vertebrates).[29,30] By deriving chronic SSDs for 15 substances, Duboudin et al.[29] demonstrated that the latter two factors had more influence on the estimation of HC5s than the first factor. Also, the selection of toxicity data used and acute versus chronic definitions can be very important.[31] Furthermore, there remains considerable uncertainty about the extrapolation from results of simplified laboratory toxicity tests to field effects.[32] Therefore, we suggest that the overall influence of the choice of NOEC or EC10 on the estimation of HC5 is trivial.

We conclude that the use of NOECs would be practically acceptable for calculation of HC5s, particularly in regulatory contexts, because our results indicate that HC5s derived from EC10s or NOECs do not differ much. However, we emphasize that NOECs have important shortcomings as discussed elsewhere (see the Introduction). We suggest that actual magnitudes of effect (e.g., percent reduction of survival, reproduction, etc.) be provided for NOECs in cases of using or reporting them. This is essential for transparent ERAs, as the NOEC should not be considered as the concentration at which there are no effects. In addition, sensitive traits in terms of NOECs might not correspond to traits critical for population-level consequences[33] that are determined through nonlinear interaction among effects on individual-level traits such as survival and reproduction. Thus, information about actual effect size at the NOEC is unquestionably important in interpretation of the test results. Note that the use of EC*x* has a similar issue and the *x* value must be carefully chosen.[7] Likewise, reporting uncertainty in estimates of EC*x* is also important (see, e.g., Tables S1−S5 of the Supporting Information) for assessing the reliability, although such information for NOECs cannot be generally estimated. Finally, we recommend that concentration−response relationships be quantitatively modeled to the extent possible[1] and that model parameters as well as raw data be reported so readers can generate EC*x* values such as the EC10.

■ ASSOCIATED CONTENT

Supporting Information

Summary table for zinc (Table S1), summary table for lead (Table S2), summary table for nonylphenol (Table S3), summary table for 3,4-dichlorobenzenamine (Table S4), summary table for lindane (Table S5), percent reduction of the end point response estimated at the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) for the five chemicals evaluated in this study (Figure S1), and relationships between the no observed effect concentration (NOEC) and the 10% effect concentration (EC10) for the five chemicals evaluated in this study (Figure S2). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b02069.

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A Continuous Need To Determine What We Should Protect In Ecological Risk Assessments

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WHAT TO PROTECT

What aspects of ecosystems should we protect from the adverse impacts of chemicals? How much of an effect on these end points would be acceptable? While a commonly advocated protection goal is viability of species,[1] which is developed typically at the population (e.g., density) or community level (e.g., species richness), these are long-standing and fundamental questions that have been rather ambiguously dealt with in general ecological risk assessments and in the establishment of water quality criteria. Use of no observed effect concentration (NOEC) is a typical example. Using laboratory toxicity testing on individual- (or organismal-) level end points such as survival and reproduction, NOEC is determined solely by statistical significance which depends on experimental design, sample size, effect size, and data variability. Who knows what real ecological consequences have resulted from this reliance on statistical significance.

BE AWARE OF IDEALS AND REALITY WHEN DETERMINING WHAT TO PROTECT

Pursuing ideals is important. However, we should also recognize that we cannot protect all aspects of all ecosystems exposed to chemicals, particularly as the number of registered chemicals continues to increase. Here, we introduce two hypothetical questions that one of the authors often asks undergraduate students in the classroom. Suppose each of us has a small pond with a fish population of value in her/his home. Then, students are asked "Let us assume contamination from an industrial plant is occurring in the pond. Which level do you want to protect? Note that in this case the industrial plant pays for the cost of protection." Students are asked to pick a single protection level from four choices (suborganismal, individual, and population levels, and no protection; Figure 1). The second similar question

Increase ↑ Cost ↓ Zero

Protection level	Example: a fish population
A. Sub-organismal	Damage on DNA or cells is unacceptable
B. Individual	Damage on DNA or cells is acceptable, but any mortality is unacceptable.
C. Population	Some mortality is acceptable but the population extinction is unacceptable
D. No protection	You are not interested at all.

Figure 1. Hypothetical protection levels and corresponding examples. In this example, it is assumed that end points at lower levels of biological organization are more sensitive to pollution and their protection is more costly.

is "Assume that domestic wastewater from your house was discharged into your pond. What do you want to protect? Note that in this case the homeowner (i.e., you) should pay for the cost of protection." Interestingly, in this situation the choice often changes to an assessment of higher-level end points (say, a student who chose "suborganismal level" in case of industrial payment picked "population level" in case of her/his payment).

A likely reason for the switch is that if an individual is not required to pay then they pursue an ideal. In contrast, if an individual is required to pay they select a practically feasible answer. The circumstances supposed in ecological risk assessment and management are usually closer to the latter situation because we usually have limited resources that can be allocated. Thus, in order to better address the fundamental questions raised above, one of our suggestions here is that stakeholders and risk assessors/managers explicitly recognize the gap between what we personally (ideally) want to protect and what we can/should practically protect. At one extreme, do you pay for protecting each individual within a population of mayflies or *Daphnia*? Furthermore, by explicitly recognizing the gap between our personal preferences and what we can practically achieve, we can identify the critical studies necessary to answer our two fundamental questions: (1) What aspects of ecosystems should we protect?; and (2) How much of an effect on this ecosystem would be acceptable?

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It should be stressed that we are not claiming that what we should (or can) protect is more important than what we personally (or ideally) want to protect, or that providing practical answers is more valuable than, say, seeking any effect of chemicals. However, given an environmental standard for a chemical, it is rather easy to find an effect below the standard, and is difficult to find a more convincing (and feasible) way to establish a more defensible standard. Therefore, balancing these efforts would be a key for improving ecological risk assessments, especially given that the number of potential assessment end points developed at suborganismal- (e.g., genes[2]) and ecosystem-levels (e.g., ecosystem services[3]) has been increasing owing to recent technological and scientific developments.

■ CALL FOR DISCUSSION AND STUDIES ADDRESSING WHAT TO PROTECT

Although the fundamental questions will likely remain unsolved, we believe these suggestions would aid in conducting more convincing ecological risk assessment and management. There will never be a single answer (or end point) that can be applied to all management decisions. In addition, following accumulation of scientific knowledge and development of technologies as well as changes in societal values, what we should protect would likely change. Therefore, we hope discussion and research directly addressing the fundamental questions will be intensively performed.

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我々は何を守るべきか？：生態リスク評価における根深い問題を問い続ける必要性

A need to keep asking what we should protect in ecological risk assessments

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ABSTRACT

化学物質の影響から生態系の“何を”守るべきなのか？分子生物学的手法を筆頭とする最近の技術発展と知見の増加によりエンドポイントとなりうる候補が増えてきている一方で，この重要な問いは生態リスク評価やそれに関わる研究において曖昧に扱われてきている。本稿では，守る対象を決める際の理想と現実について一例を紹介し，“何を理想的に守りたいのか（あるいは，何を研究したいか）”と“何を守るべきなのか（あるいは守ることができるのか）”のギャップを利害関係者やリスク管理・評価者（すなわち，行政や研究者など）が明示的に認識しておく必要があること，さらにはそれらに対する努力配分のバランスをうまくとる必要があることを指摘した。加えて，エンドポイントの設定にあたっては時空間的なスケールを考慮することの重要性についても議論した。「何を守るべきか」という問題に対する決定的な解は依然準備されておらず，これからも重要な問いとして根強く残ることが予想されるが，具体的なエンドポイント（あるいは保全目標）を設定し共有することは，化学物質管理以外の環境対策を統合的かつ合理的に推し進める上でも肝要となる。今後この“重要な問い”により直接的に切り込む研究や活発な議論が行われることを期待したい。

Key words: 評価エンドポイント，保全目標，生態リスク管理，合意形成，リスク認知

1. 何を守るべきか？

我々は，化学物質の影響から生態系の“何を”守るべきなのか（あるいは守りたいのか）？どの程度の影響であれば許容可能なのか？これらは根強く残る重要な問いであり（以下では，“重要な問い”と呼ぶ），生態リスク評価（水質環境基準の設定なども含む）においてむしろ曖昧に扱われてきた。典型的な一例としては，生態リスク評価における無影響濃度（no observed effect concentration: NOEC）の利用が挙げられる。NOEC は室内毒性試験を用いて単に統計学的な有意性に基づいて決定され，その統計的な有意性はサンプルサイズ，効果量（影響の大きさ），データのばらつきに依存する[1)]。すなわち，NOEC は影響がまっ

たくない濃度を意味せず，実際に，NOECと等しい濃度レベルにおいて，数％～20%の影響が観測されている[2]。このように統計学的有意性に単純に依拠してきたことが，実際の生態系においてどのような影響を引き起こしてきたかについては，誰も知り得ないだろう。NOECの代替として挙げられているx%影響濃度（x% effect concentration：ECx）の利用についての議論もどのようにこのx%を決めるかという重要な課題を抱えている[3, 4]。

2. エンドポイント候補の増加

一般的に用いられる保全目標は個体群の存続（多種では種多様性の保全）であり，個体群（または群集）レベルで定められる[5]。しかしながら，最近の技術発展と知見の増加により，エンドポイントとなりうるエンドポイント候補は増えてきているといえるだろう。例えば，分子生物学的手法の発展により，個体内の遺伝子やタンパク質の構造・機能に対する様々な影響を測定することが可能になっている。また，生態系の構造だけでなく，水循環や水質の調整や食糧生産などといった生態系から人が受ける恩恵（生態系サービスと呼ばれる）の保全も注目されてきている。少し変わった例としては，最近，羽化途中の水生昆虫が一般に毒性試験に用いられる生活史段階（幼虫）よりも感受性が高いことがウスバコカゲロウ属の一種を用いた実験により示された[6]。河川からの羽化昆虫はクモ類や鳥類のきわめて重要な餌となるため[7, 8]，この結果は，河川生態系と陸域生態系のつながり（さらにいえば陸上の生物）を保護するためには，水生昆虫の異なる生活史段階において生態影響を評価することが必要であることを示唆している。このように感受性の高いエンドポイントを探索･特定することは奨励されるべきであるし，それによって自然環境中の汚染物質の影響に関する私たちの知見は修正・更新される。一方で，最初に提示した重要な問いは未解決のままであり，エンドポイント候補の増加は同時に悩ましい課題ともいえるだろう。例えば，林（2013）[9]は，特に分子生物学的手法の発展を背景に「“護るべきもの”と“見ることができるもの”の間のギャップは近年むしろ広がり続けている」と考察している。なお，現状，日本で現在実施されている生態リスク評価において採用されている生態毒性試験は概ね経済協力開発機構（OECD）の試験ガイドラインに調和したものであり，*in vivo*で生存，繁殖，成長等の個体レベルのエンドポイントを評価したものが主流であることに留意する必要がある。

3. 守る対象を決める際の理想と現実

理想を追求することが重要である。しかしながら，それと同時に，Chemical Abstracts Service（CAS）に登録されている化学物質の数は増加しており，すべての生態系のすべての要素を保全することは不可能であることも常に認識しておかなければならない。

ここで，大学生向けの講義で私がよくする2つの仮想的な質問を紹介したい。まず，（各人の）自宅の池に貴重な魚類種（1種）が生息していると仮定する。最初に，「その池に近くの工場の排水が流れ込むことになってしまった状況を想定します。そのときにあなたは，どの保護レベルを求めますか？なお，その排水処理は工場が行うものであり，あなたはお金を払う必要はありません。」と質問し，Fig.1の4つの保護レベル（遺伝子または細胞（個体内の）レベル，個体レベル，個体群レベル，なにもしない）のうちから，1つを選択してもらう。次に，同じ仮定で，「その池に自分の家の排水が流れ込むことになってしまった状況を想定します。そのときにあなたは，どの保護レベルを求めますか？なお，この排水処理にはあなたはお金を払う必要があります。」と質問する。すると興味深いことに，1つ目の質問で個体内あるいは個体レベルを選択した学生が，自分で支払う必要がある2つ目の質問では，個体群レベルまたはなにもしないを際だって選ぶようになる。

この変化の主要因としては，自分で支払う必要がない場合には（ただ乗りできるため）自分の理想を追求し，自分で支払う場合にはより現実的（実現可能な）解を選んでいることが考えられる。話を元に戻すと，生態リスク評価や管理で想定している状況は多くの場合，前者よりも後者の想定に近く，この例から実務的に実現可能な解を探すことを肝に銘じておくことの重要性が示唆できる。すなわち，最初に示した重要な問いによりうまく対処するためには，“何を理想的に（個人的に）守りたいのか（あるいは，何を研究したいか)”と“何を守るべきなのか（あるいは守ることができるのか)”のギャップを利害関係者やリスク管理・評価者（すなわち，行政や研究者など）が明示的に認識しておく必要があるだろう。例えば，あるミジンコの集団の一個体を保護するために，我々はお金を払うことができるだろうか。果たしてどの程度の研究者がこのギャップを意識して研究を進めているかは計り知れないが，それによって研究の目的や到達点をより明確に持つことができ，重要な問いに回答を準備する上で有用な研究や事例をより蓄積することができると思われる。

このようなギャップを認識・理解する上で，候補も含めた利用可能なすべてのエンドポイントをそれが実際のリスク評価に使われたことがあるかどうかも含めて，整理することは有用かもしれない。例えば，一般的な評価エンドポイントについては米国環境保護庁がすでに収集し，リスト化されている[10]。ただし，“何を理想的に（個人的に）守りたいのか”よりも“何を守るべきなのか（あるいは守ることができるのか)”，あるいは“影響を見つけること”よりも“実務的に実現可能な解を見つけること”がより重要であるというように優劣をつけることはできないし，本稿にもそのような意図はない。重要なのはそれらに対する努力配分のバランスをうまくとることであろう。

4. おわりに

最後に，エンドポイントの設定にあたって，適切な時空間的なスケールを考慮し，選択することの重要性についても述べておきたい。例えば，野外調査は，比較的限られた地域（例：複数の河川地点）においてサンプルを採取することで行われる。では，ある調査で，ある汚染源から 1km 下流までにある地点ではその種が不在であり，上流や 1km 以上下流の地点には生息が確認されたとしよう。その際，「どのスケールを基に我々は許容される影響を決めるべきか」という１つの単純

費用大 ↑ ↓ 費用なし

保護レベル	例（貴重種に対して･･･）
A. 遺伝子または細胞レベル	DNAや細胞への影響が許容できない
B. 個体レベル	個体内へのダメージは許容するが，一匹でも死亡することは許容できない
C. 個体群レベル	多少の死亡は許容するが，この種がいなくなる（絶滅する）ことは許容できない
D. なにもしない	興味がない

Fig.1. 保護レベルとその例
この例では，より低次の生物学的階層のエンドポイントの感受性が高く，保護する上でよりコストがかかることを仮定している。

な疑問が出てくる。ここでは簡単のために，エンドポイントはこの種の個体群の存続性とし，在不在により判断するとする。もし，地点ごとこの評価を行うのであれば，この想定されている状況は当該種が確認されない地点が存在するため許容できない，と判断される。反対に，一本の河川でみると，生息は確認されているため，許容可能となる（ただし，時間的な経過には注意が必要である）。このような時空間的な拡張は生態リスク評価自体をより複雑にしてしまうものではあるが，前述したように潜在的に利用可能な評価エンドポイントが増加・拡張している現状を踏まえると，保全対象とする時空間的なスケールを明確に定義しておく（あるいは各々が少なくとも意識しておく）意義は小さくないだろう。

依然“何を守るべきか”問題に対する決定的な解は準備されておらず，これからも重要な問いとして根強く残るだろう。すべての管理・対策において絶対的に適用可能な１つの回答を準備することは不可能であろうし，科学的知見の蓄積や技術の発展，さらには価値観の変化にともない，“何を守るべきか”も変化するものであろう。しかしながら，具体的なエンドポイント（あるいは保全目標）を設定し共有することは，化学物質管理以外の環境対策を統合的かつ合理的に推し進める上でも肝要となる。今後この“重要な問い”により直接的に切り込む研究や活発な議論が行われることを期待したい。

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10. 清水佑一，川瀬俊吾，浅香貴啓，柏田祥策，長坂征治，渡良瀬遊水地から単離された藻類の銅に対する応答評価，日本農芸化学会 2017 年度大会，京都女子大学（発表予定：2017 年 3 月 17―20 日）

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Three-dimensional visualization of green fluorescence protein-labelled *Edwardsiella tarda* in whole Medaka larvae

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Abstract

The invasive fish pathogen *Edwardsiella tarda* is common in aquatic environments and causes the environmentally and economically destructive emphysematous putrefactive disease called edwardsiellosis. In order to understand the organism's infection pathway, medaka larvae (*Oryzias latipes*) were immersion-infected with *E. tarda* labelled with green fluorescence protein (GFP) and then visualized in three dimensions under confocal laser microscopy and light-sheet fluorescence microscopy. Confocal microscopy revealed GFP-labelled *E. tarda* in the mouth, head, gill bridges, gill cover, skin, membrane fin, gastrointestinal tract and air bladder, and in the caudal vein, somite veins, caudal artery and caudal capillaries. Light-sheet microscopy additionally showed GFP-labelled *E. tarda* in the pharyngeal cavity, muscle of the pectoral fin and cardiac atrium and ventricle. These findings suggest that during its infection of fish, *E. tarda* initially adheres to, and invades, the epithelial cells of the skin, gills and gastrointestinal tract (through the pharyngeal cavity); *E. tarda* then enters the blood vessels to access organs, including the air bladder and heart.

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Safety: *The Japanese medaka used were treated humanely according to the Institutional Animal Care and Use Committee guidelines of Toyo University, with due consideration for the alleviation of distress and discomfort.*

Keywords: Edwardsiella tarda, green fluorescence protein, Medaka, path of infection, three-dimensional imaging.

Introduction

Edwardsiella tarda, which was named after the American biologist P. R. Edwards (1901–1966) (Ewing *et al.* 1965; Meyer & Bullock 1973), is a common pathogen that causes putrefactive disease leading to mass mortality in several fish species, including eel (Gutierrez *et al.* 1993), flounder (Han *et al.* 2006), carp (Sae-Oui, Muroga & Nakai 1984) and chinook salmon (Amandi *et al.* 1982). Epizootics of *E. tarda* in the United States, Japan, Europe and Asian countries have led to enormous biological and economic losses (Mohanty & Sahoo 2007). Furthermore, *E. tarda* can infect reptiles, birds, humans and other mammals (Thune, Stanley & Cooper 1993), making infection with this bacterium not only a threat to fish industries but also an environmental and public health concern. *Edwardsiella tarda* is an opportunistic pathogen of fish. Exposure of fish to environmental stressors such as high temperature, high carbon dioxide or ammonia concentration or low dissolved oxygen makes them more susceptible to *E. tarda*, hastening the onset, and increasing the severity, of infection (Thune *et al.* 1993).

Little information is available regarding how *E. tarda* migrates through the fish it infects. Use of GFP-labelled *E. tarda* revealed that the organism adhered to and invaded the epithelioma

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papillosum cells of carp (Ling *et al.* 2000); a subsequent study found that the gastrointestinal tract, gills and body surface of fish were the sites of entry of *E. tarda* (Ling *et al.* 2001). In addition, GFP-labelled *Vibrio anguillarum* – the leading cause of haemorrhagic septicaemia in fish – was detected in both the gastrointestinal tract and skin of infected transparent zebra fish (O'toole *et al.* 2004). In the current study, we investigated distribution of GFP-labelled *E. tarda* in infected medaka larva using confocal microscopy and provided 3D data using light-sheet fluorescence microscopy of whole mounts.

Material and methods

Medaka larvae

The orange-red strain of medaka (*Oryzias latipes*) was obtained from the National Institute for Environmental Studies, Japan. Breeding groups of medaka were fed *Artemia salina* nauplii twice daily and Otohime β-2 (Marubeni Nissin Feed Co. Ltd.) three times daily. The fish were maintained under a 16:8-h light:dark cycle at 25 ± 0.5 °C in the Environmental Health Sciences Laboratory at Toyo University. After the female medaka had spawned, the external egg clusters were removed. Fertilized eggs were selected and rinsed with embryo-rearing medium (ERM; 1.0 g NaCl, 0.03 g KCl, 0.04 g $CaCl_2{\cdot}2H_2O$, 0.163 g $MgSO_4{\cdot}7H_2O$ in 1 L of ultrapure water, adjusted to pH 7.2 with 1.25% $NaHCO_3$ in water; filter-sterilized). To avoid any biological contamination, following work were done in clean bench. Egg were placed in sterilized ERM by filtered 0.22-μm membrane and incubated at 25 ± 0.1 °C until hatching. Hatched larvae were harvested, rinsed with ERM and placed in 6-well plates (15 larvae and 5 mL of ERM per well) for further study. All reagents were purchased from Nacalai Tesque.

GFP-labelled *E. tarda*

Wild-type *E. tarda* (strain FK1051) was a kind gift of Professor Yasushi Okinaka (Hiroshima University, Japan). The strain was isolated from diseased Japanese flounder, *Paralichthys olivaceus,* which was captured at Hiroshima, Japan. Suezawa *et al.* (2016) have reported that *E. tarda* FK1051 strain is virulent for goldfish. Cultures were grown in 802 medium (10 g hipolypepton, 2 g yeast extract, 1 g $MgSO_4{\cdot}7H_2O$ in 1 L of ultrapure water, adjusted to pH 7.0; the composition of 802 medium is defined by the National Institute of Technology and Evaluation, Tokyo, Japan) at 30 °C in the dark. Stock cultures were maintained at −80 °C in a suspension of 802 medium containing 20% glycerol. To generate GFP-labelled organisms, *E. tarda* was transformed with the plasmid pGFPuv (Clontech Laboratories) using electroporation (ELEPO21 In Vitro High Energy Electroporator; Nepa Gene Co., Ltd.) as described previously (Ling *et al.* 2000). Because pGFPuv has an ampicillin resistance marker, transformed *E. tarda* were plated on 802 medium containing 1.5% agar and ampicillin. Colonies that were resistant to ampicillin and fluoresced bright green (pGFPuv) under UV light were selected. Plasmid stability for 7 days was confirmed using a previously described stability assay (Ling *et al.* 2000). GFP-labelled *E. tarda* were cultured in 802 medium containing ampicillin for 24 h at 30 °C in the dark and then used in infection assays of medaka larvae.

Infection of medaka larvae with GFP-labelled *E. tarda*

A 24-h culture of GFP-labelled *E. tarda* (10 mL) was centrifuged at 3900***g*** for 10 min at 4 °C. The supernatant was removed, and the pelleted GFP-labelled *E. tarda* were rinsed twice with ice-cold ERM (5 mL each rinse) and resuspended in ERM (5 mL, 25 °C, 10^9 cfu mL^{-1}). To infect medaka larvae, ERM solution (5 mL) was removed from each well (containing 15 medaka larvae) of a 6-well plate and replaced with 5 mL of ERM containing GFP-labelled *E. tarda* (immersion infection); the infections were performed in triplicate. The 6-well plates were covered with foil to prevent light exposure and incubated at 25 °C for 48 h. After 48 h, medaka larvae infected with *E. tarda* were anesthetized in ice-cold ERM, rinsed twice with ice-cold ERM (5 mL each rinse), fixed in 4% paraformaldehyde (w/w) in PBS for 24 h and then stained with DAPI (VectaShield Mounting Medium with DAPI; Vector Laboratories) or BODIPY FL phalloidin (ThermoFisher Scientific) for 12 h. Medaka larvae in ERM in the absence of GFP-labelled *E. tarda* were used as control (uninfected condition). The stained medaka larvae were observed

under a fluorescence dissecting microscope with a GFP filter (M165 FC; Leica Microsystems), a confocal microscope (TCS-SP5 II, Leica Microsystems) and a light-sheet fluorescence microscope (Lightsheet Z.1, Carl Zeiss Microscopy Co., Ltd.) to detect GFP-labelled *E. tarda*. Wavelengths for observation were 360-nm emission and 460-nm excitation for DAPI, 505-nm emission and 512-nm excitation for BODIPY and 395-nm emission and 509-nm excitation for GFP. Autofluorescence was detected from liver during GFP observations. Light field images were taken using a digital microscope, VHX-5000 (Keyence).

Imaging analyses

Imaging analyses were performed using the software LAS AF Lite (Leica Microsystems) for confocal microscopy and ZEISS ZEN for Lightsheet Z.1 (Carl Zeiss Microscopy Co., Ltd.) and IMARIS V 6.1.0 (BITPLANE) for light-sheet microscopy.

Results

Fluorescence microscopy

Green fluorescence protein-associated fluorescence was detected in all larvae, which then underwent further observation as described below.

Confocal laser microscopy

Confocal laser microscopic image of control (uninfected) larvae is shown in Fig. 1a–d, and the image of GFP-labelled *E. tarda*-infected larvae is shown in Fig. 1a′, a″, b′, c′, c″ and d′. No GFP signals were detected from control larvae. GFP signals were detected on the mouth, some gill bridges, gill cover and body surface (Fig. 1a′ and a″) and in the membrane fin, gastrointestinal tract and air bladder (Fig. 1b′). GPF fluorescence also was noted in the caudal vein and somite veins (Fig. 1c′ and c″), as well as in the caudal artery and caudal capillaries (Fig. 1d′).

Light-sheet microscopy

Using the ZEISS ZEN software, GFP fluorescence was noted around the mouth (Fig. 2a), head (Fig. 2b), gill cover (Fig. 2c), muscle of pectoral fin (Fig. 2d), membrane fin and areas of the anus and gastrointestinal tract (Fig. 2e). In addition, a single 2- to 3-μm (the same length as *E. tarda* (Mohanty & Sahoo 2007)), bacterium-shaped spot of GFP colour was detected within the region supplied by the caudal artery (Fig. 2f). Although the ZEISS ZEN software was used to provide high-resolution imaging analyses, it did not yield sufficient information here for three-dimensional (3D) imaging. To visualize the interaction between GFP-labelled *E. tarda* and medaka larvae in greater detail, we used IMARIS software to obtain 3D cross-sectional images from the medulla oblongata to the pharyngeal cavity (Fig. 3a), the spinal cord to the heart (Fig. 3b), the spinal cord to the liver (Fig. 3c) and the spinal cord to the anus through the gastrointestinal tract (Fig. 3d). GFP signals were detected in the gills (Fig. 3a), the atrium and ventricle of the heart and pharyngeal cavity (Fig. 3b), the muscle of the pectoral fin and liver (Fig. 3c) and the gastrointestinal tract and anus (Fig. 3d). Regarding GFP signal of liver, autofluorescence was observed, because green fluoresce was detected from liver of control (uninfected) larvae.

Discussion

Previous studies using GFP-labelled *E. tarda* indicated that the organism adhered to and invaded fish through cells of the epithelioma papillosum and infected fish intramuscularly (Ling *et al.* 2001) and that the gastrointestinal tract, gills and body surface were the pathogen's sites of entry (Ling *et al.* 2001). In addition, another fish pathogen, *V. anguillarum*, was found to infect through the gastrointestinal tract and skin (O'toole *et al.* 2004). Here, confocal and light-sheet microscopy revealed GFP-labelled *E. tarda* in the mouth, head, gill bridges, gill cover, skin, membrane fin, gastrointestinal tract, air bladder, caudal vein, somite veins, caudal artery and caudal capillaries of medaka larvae. Furthermore, 3D image analysis showed GFP-labelled *E. tarda* in the pharyngeal cavity, muscle of the pectoral fin and atrium and ventricle of the heart. In another study, oral or intraperitoneal administration of a protein-bound polysaccharide enhanced resistance to *E. tarda* and eliminated the pathogen from the blood (Park & Jeong 1996); these results imply that *E. tarda* is distributed in the pharyngeal cavity, gastrointestinal tract and vasculature, thus supporting our current findings.

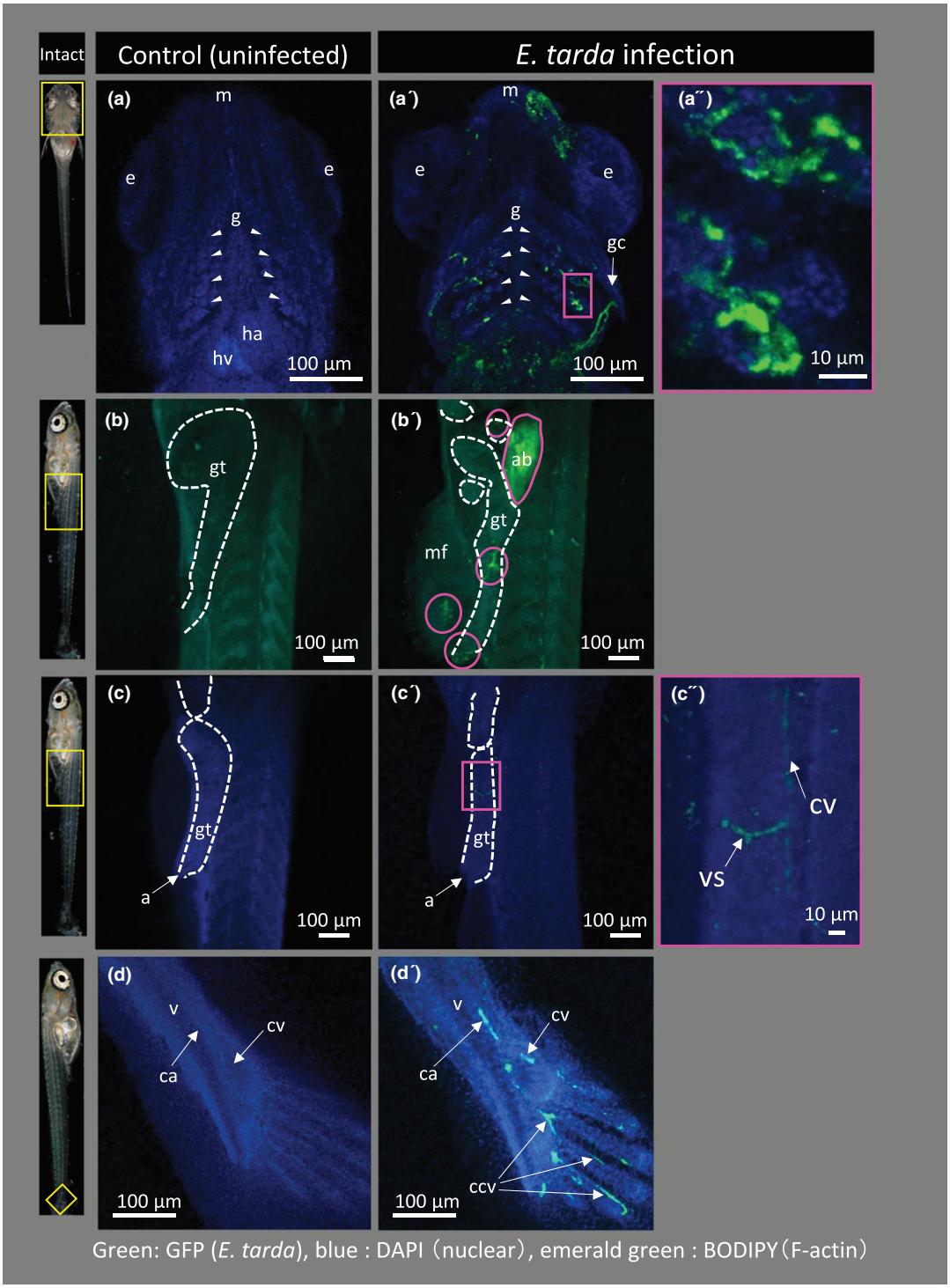

Figure 1 Confocal laser microscopy of medaka larva infected with green fluorescence protein (GFP)-labelled *Edwardsiella tarda*. Confocal laser microscopic image of control (uninfected) larvae is shown in a, b, c and d, and the image of GFP-labelled *E. tarda*-infected larvae is shown in a′, a″, b′, c′, c″ and d′. Images a, a′ and a″ were taken from the ventral view. Image b, b′, c, c′, c″, d and d′ were taken from lateral view. Images a′, a″, c′, c″ and d′ were taken from the same larva. a, anus, ab, air bladder; ca, caudal artery; ccv, caudal capillary vessel; cv, caudal vein; e, eye; g, gill; gc, gill cover; gt, gastrointestinal tract; ha, atrium of heart; hv, ventricle of heart; m, mouth; mf, membrane fin; v, vertebra; vs, vein of somite.

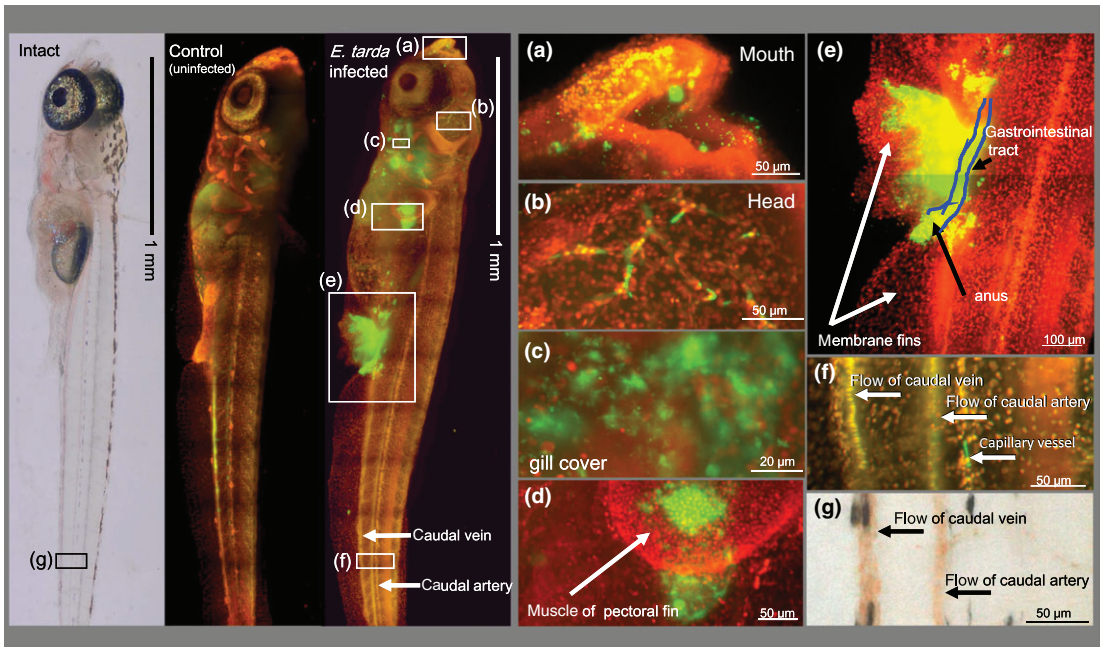

Figure 2 Light-sheet microscopy of medaka larva infected with green fluorescence protein (GFP)-labelled *Edwardsiella tarda*. Imaging analyses were performed using ZEISS ZEN software.

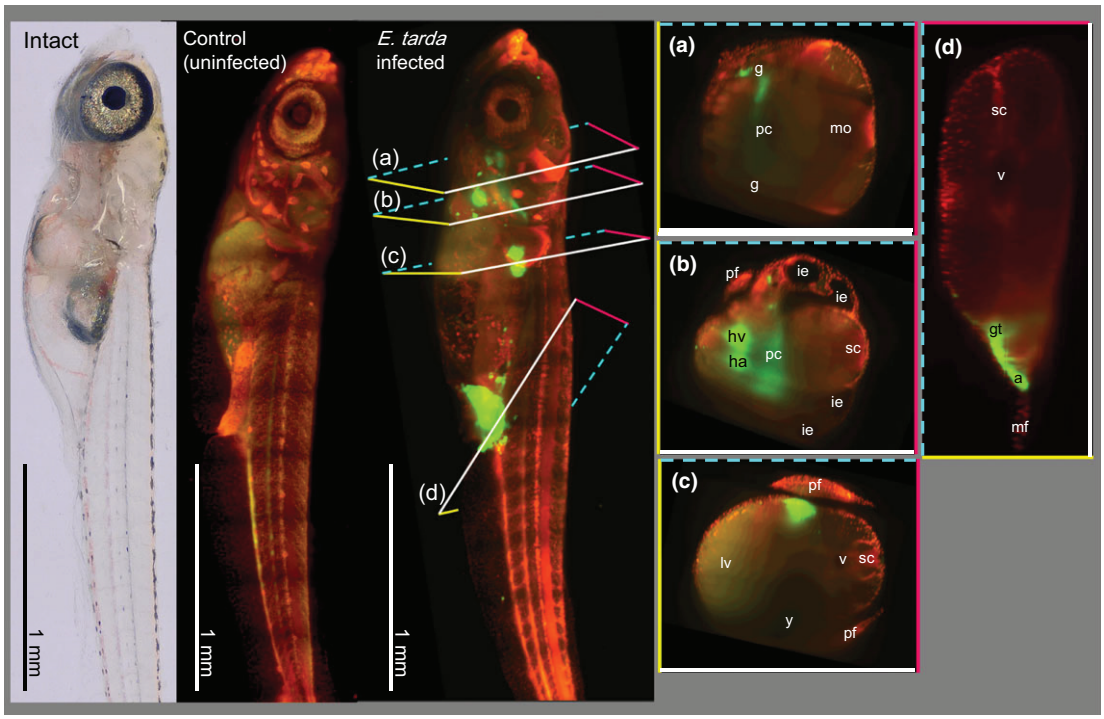

Figure 3 Light-sheet microscopy of medaka larva infected with green fluorescence protein (GFP)-labelled *Edwardsiella tarda*. Imaging analyses were performed using IMARIS software. a, anus; g, gill; gt, gastrointestinal tract; ha, atrium of heart; hv, ventricle of heart; ie, inner ear; lv, liver; mf, membrane fin; mo, medulla oblongata; pc, pharyngeal cavity; pf, pectoral fin; sc, spinal cord; v, vertebra; y, yolk.

According to this study at 48 h post-infection and a chronological study of Ling *et al.*, we assumed the following pathway of infection of medaka larvae with *E. tarda*. The pathogen may adhere to and invade epithelial cells of the skin, gills and gastrointestinal tract (*via* the pharyngeal cavity). From these sites, *E. tarda* gains access into blood vessels to reach various organs, including the air bladder and heart. The improved understanding of pathogenicity that we obtained here will facilitate efforts to prevent and combat infections with *E. tarda* and other fish pathogens. In addition, combining confocal microscopy with light-sheet microscopy enabled us to obtain detailed, 3D information from mounts of whole fish; this combined technique is very promising for imaging-based research.

Conclusions

Using GFP-labelled *E. tarda* to infect medaka larvae showed that the pathogen distributed into head, pharyngeal cavity, gill cover, gill, atrium and ventricle of the heart, muscle of pectoral fin, membrane fin and areas of the anus and gastrointestinal tract.

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Salinity-dependent toxicity of water-dispersible, single-walled carbon nanotubes to Japanese medaka embryos

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ABSTRACT: To investigate the effects of salinity on the behavior and toxicity of functionalized single-walled carbon nanotubes (SWCNTs), which are chemical modified nanotube to increase dispersibility, medaka embryos were exposed to non-functionalized single-walled carbon nanotubes (N-SWCNTs), water-dispersible, cationic, plastic-polymer-coated, single-walled carbon nanotubes (W-SWCNTs), or hydrophobic polyethylene glycol-functionalized, single-walled carbon nanotubes (PEG-SWCNTs) at different salinities, from freshwater to seawater. As reference nanomaterials, we tested dispersible chitin nanofiber (CNF), chitosan-chitin nanofiber (CCNF) and chitin nanocrystal (CNC, i.e. shortened CNF). Under freshwater conditions, with exposure to 10 mg l^{-1} W-SWCNTs, the yolk sacks of 57.8% of embryos shrank, and the remaining embryos had a reduced heart rate, eye diameter and hatching rate. Larvae had severe defects of the spinal cord, membranous fin and tail formation. These toxic effects increased with increasing salinity. Survival rates declined with increasing salinity and reached 0.0% in seawater. In scanning electron microscope images, W-SWCNTs, CNF, CCNF and CNC were adsorbed densely over the egg chorion surface; however, because of chitin's biologically harmless properties, only W-SWCNTs had toxic effects on the medaka eggs. No toxicity was observed from N-SWCNT and PEG-SWCNT exposure. We demonstrated that water dispersibility, surface chemistry, biomedical properties and salinity were important factors in assessing the aquatic toxicity of nanomaterials. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: carbon nanotube; chitin nanomaterial; medaka; embryo; salinity; toxicity

Introduction

Since concern has arisen about the environmental hazards and risks posed by nanomaterials, there have been many toxicological investigations of carbon and metal nanomaterials. Carbon nanotubes (CNTs) are among the most industrially important nanomaterials and are, therefore, generating environmental concern. Zhu *et al.* (2006) used *Stylonychia mytilus* to investigate the biological interactions and toxicity of raw, multi-walled CNTs (MWCNTs) and showed that the MWCNTs induced dose-dependent growth inhibition to the cell, and exclusively localized to the mitochondria of the cell by electron microscopic analysis. Smith *et al.* (2007) reported effects of raw, single-walled CNTs (SWCNTs) on the gills of rainbow trout (*Oncorhynchus mykiss*); which were a dose-dependent rise in the ventilation rate, gill pathologies and mucus secretion with SWCNT's precipitation on the gill mucus. Fish gills are important organs for gas exchange; they are also the first point of xenobiotic contact and uptake into the fish's body. CNTs, which attached to gills, could penetrate epithelial cells of gills, and enter blood circulation. In a previous study, using fluorescence polystyrene latex nanoparticles resulted in nanomaterials passing through the see-through medaka gill membrane or other biological membrane barriers, and then they entered the blood and were distributed to other organs (Kashiwada, 2006). Cheng *et al.* (2007) exposed zebrafish eggs to raw SWCNTs at 120 mg l^{-1} and observed a significant hatching delay.

Since Klaine *et al.* (2008) reviewed the behavior, fate, bioavailability and effects of nanomaterials, there have been only a few ecotoxicological reviews of CNTs (Zhao and Liu, 2012; Du *et al.*, 2013; Jackson *et al.*, 2013). Despite the relatively large number of toxicological studies of nanomaterials, only very limited research information on the fate of nanomaterials in aquatic environments is available (Ferry *et al.*, 2009; Burns *et al.*, 2013). Nanomaterials readily aggregate in solution, and even more so in saltwater. Salinity is an important aquatic environmental determinant of the behavior and fate of chemicals, as are pH and temperature. Most aggregated nanomaterials are no longer nano-sized; instead, they are micron-sized in all dimensions. Generally, it is more difficult for these bigger materials to pass through biological membranes. Salinity also has other interesting effects on the behavior and bioavailability of nanomaterials.

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Kashiwada (2006) reported that fluorescence cationic polystyrene latex nanoparticles (39.4 nm in diameter) passed through the see-through medaka chorion in a salinity-dependent manner; the greatest penetration by nanoparticles occurred in a salinity equivalent to half-strength seawater (brackish water). Recently, our group (Kataoka *et al.,* 2015) found that salinity (from freshwater to seawater) increased the toxicity of silver nanocolloids (SNC) to medaka embryos, and salinity equivalent to seawater simultaneously increased the bioavailability of silver in SNC than salinity equivalent to freshwater, which was more effective as reference compounds in analyzes than was $AgNO_3$. SWCNTs have a greater aquatic toxicity than do MWCNTs (Jackson *et al.,* 2013); however, raw CNTs are considered less toxic than other nanomaterials (e.g. SNC), because raw CNTs are nearly insoluble in any solvent owing to their bundle-shaped configurations (Zhao and Liu, 2012). Raw CNT can get dispersibility by 'functionalization' which is chemical modification such as amidation and esterification of the nanotube-bound carboxylic acids (Sun *et al.,* 2002). Hence, increasing dispersibility using functionalized CNTs is critical in both their practical use and their toxicological evaluation. Functionalized CNTs have been developed and are commercially available, and some of them are water dispersible (Hersam, 2008). Although raw CNTs do not readily cross biological barriers, functionalized CNTs that are water dispersible may cross biological barriers and have unforeseen effects on aquatic organisms in various aquatic environments (freshwater to seawater). Our goal here was to evaluate the effects of salinity on the toxicity of three types of functionalized SWCNTs in the embryos of Japanese medaka (*Oryzias latipes*).

Material and methods

Functionalized carbon nanotubes and reference chitin nanomaterials

A water-dispersible, single-walled carbon nanotube (W-SWCNT) suspension (10 mg ml^{-1}) prepared from SWCNTs coated with cationic plastic polymer was purchased from Nanocs Inc. (New York, NY, USA). Polyethylene glycol–functionalized (PEG)-SWCNTs were purchased from Nanocs Inc. PEG-SWCNTs are neutrally charged and hydrophobic. PEG-SWCNTs were added to ultrapure water to a concentration of 100 mg ml^{-1}. Non-functionalized-SWCNTs (N-SWCNTs) were purchased from Nanocs Inc. and added to ultrapure water to a concentration of 100 mg ml^{-1}. Transmission electron microscopy (TEM; JEOL-2100, JEOL Ltd., Tokyo, Japan) images of the three kinds of carbon nanotubes in ultrapure water are shown in Figure 1. The three kinds of carbon nanotubes had a long, narrow strip shape, with diameters less than 50 nm. Dynamic light scattering (DLS) revealed the diameters of W-SWCNTs, PEG-SWCNTs and N-SWCNTs in ultrapure water to be much larger, at 203.7 ± 65.1, 127.7 ± 0.8 and 4340.5 ± 1259.5 nm, respectively. (Details are given below.) Three kinds of chitin nanomaterial, a highly crystalline fiber, namely chitin nanofiber (CNF), chitosan-chitin nanofiber (CCNF) and chitin nanocrystal (CNC, i.e. shortened CNF), were employed as reference materials for W-SWCNTs, PEG-SWCNTs and N-SWCNTs, because they also have a long, narrow strip shape, with diameters less than 50 nm (Fig. 1). CNF, CCNF and CNC solutions (1.0%, pH 4) were synthesized at the Shinsuke Ifuku Laboratory of Tottori University. The

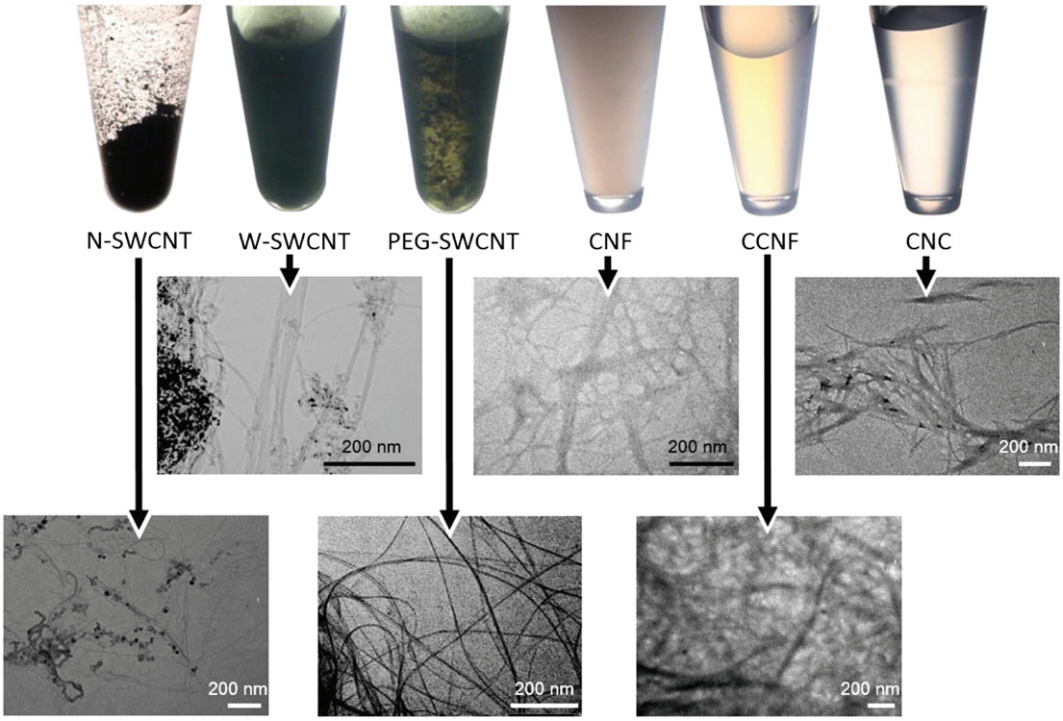

Figure 1. Features of N-SWCNTs, W-SWCNTs, PEG-SWCNTs, CNF, CCNF, and CNC in ultrapure water (10 mg ml^{-1}), and the respective transmission electron microscope images. CNC, chitin nanocrystal; CCNF, chitosan-chitin nanofiber; CNF, chitin nanofiber; ERM, embryo-rearing medium; N-SWCNT, non-functionalized, single-walled carbon nanotube; PEG-SWCNT, hydrophobic polyethylene glycol–functionalized, single-walled carbon nanotube; W-SWCNT, water-dispersible, cationic, plastic-polymer-coated, single-walled carbon nanotube.

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diameters of CNF, CCNF and CNC, as measured by DLS analysis, were 7909.3 ± 337.0, 2387.0 ± 174.8 and 241.5 ± 2.2 nm, respectively. (Details are given below.) Chitin nanofibers are highly crystalline and are not water soluble; however, they are water dispersible because of their high surface electric charge (Fan *et al.,* 2008, 2010). CNF, CCNF and CNC are, therefore, hydrophilic and water dispersible compounds. For detailed information on the preparation of chitin nanomaterials, please see previous papers (Gopalan Nair and Dufresne, 2003; Ifuku *et al.,* 2009, 2014). In comparison with the TEM data, the DLS measurements gave much larger sizes for the six nanomaterials. It is well known that DLS measurements are biased towards larger sizes because DLS measures the hydrodynamic size of particles, which includes both the core and the coating, whereas TEM measures only the electron-dense core of dried nanomaterials (van der Zande *et al.,* 2012).

Physicochemical characterization of SWCNTs and chitin-based nanomaterials

DLS and a laser Doppler method were used to evaluate the diameter and zeta potential of the three kinds of SWCNTs and three kinds of chitin-based nanomaterials using an ELSZ-2000ZS Zeta-potential & Particle size Analyzer (Otsuka Electronics Co., Ltd, Osaka, Japan). This apparatus can be used to evaluate the zeta potential of suspended nanomaterials even if the solution is saline. The three kinds of SWCNTs or three kinds of chitin-based nanomaterials were suspended in 10 mg l^{-1} ultrapure water or in each concentration of ERM (medaka embryo-rearing medium; Yamamoto, 1939; 1×, 5×, 10×, 15×, 20×, or 30×) at pH 7 and 25 °C (1× ERM and 30× ERM have osmotic pressures equivalent to freshwater and seawater, respectively). Twenty-four hours later, the suspensions were vortexed for 20 s and then subjected to analysis for measurement the diameter and zeta potential. Each analysis was performed five times. Nanomaterial diameters were calculated by cumulant analysis. Data are shown in Figure 2.

Medaka eggs

Adult Japanese killifish medaka (*O. latipes*, drR strain) were reared in dechlorinated tap water at 25 °C under a 16:8-h light:dark cycle and were fed either *Artemia salina* nauplii hatched within 24 h or artificial fish food (Otohime β1; Marubeni Nisshin Feed Co., Tokyo, Japan) five times a day. Embryos of *O. latipes* at developmental stage 21 (brain regionalization and otic vesicle formation stage) were harvested, rinsed with 1× ERM, and then used in the exposure tests. Developmental stages of the medaka embryos were defined from the work of Iwamatsu (2004). For detailed information on the medaka culturing and egg collection, please see previous papers (Kataoka *et al.,* 2015 and Kataoka and Kashiwada, 2016). All medaka embryos used were at stage 21 because our previous study had revealed that this stage was more sensitive than other stages to SNC (Kashiwada *et al.,* 2012). Japanese medaka is commonly used as a typical freshwater fish model in toxicology (Van Wettere *et al.,* 2013) and ecotoxicology (Kashiwada *et al.,* 2008). Although Japanese medaka is a freshwater fish, it can live in not only freshwater but also brackish water or seawater. Because Japanese medaka has evolved in moving northward from south Asia, it has adapted to highly saline conditions and has highly developed chloride cells (Miyamoto *et al.,* 1986). Hence, Japanese medaka eggs can hatch normally in seawater (within 8 to 10 days at 25 °C) (Inoue and Takei, 2002). These characteristics make this species markedly different from other freshwater fish models such as the zebrafish.

Toxicity testing of CNTs at 1× ERM (freshwater conditions)

To examine the toxic effects of CNTs on medaka embryos, using a six-well plastic plate (Greiner CELLSTAR®; Sigma-Aldrich Japan Inc., Tokyo, Japan), 15 medaka embryos (stage 21) in triplicate were exposed to 5 ml of N-SWCNTs, W-SWCNTs, or PEG-SWCNTs (1 or 10 mg l^{-1}) or to CNF, CCNF, or CNC (10 mg l^{-1}, as references) in 1× ERM at pH 7 during incubation at 25 °C in the dark until hatching or for 14 days, whichever came first. Test solutions were renewed once a day. During exposure, exposed embryos were observed every day under a dissecting microscope (Leica M165 FC; Leica Microsystems Co., Tokyo, Japan). Heart rate and eye diameter were measured on day 6. Cumulative hatching rate was counted for 14 days. Medaka eggs in 1× ERM at pH 7 in the absence of nanomaterials were used as controls. For detailed information on the measurement, please see previous papers (Kataoka and Kashiwada, 2016).

Toxicity testing of CNTs at different ERM salinities

Only W-SWCNTs at 10 mg l^{-1} inhibited the hatching of medaka eggs in 1× ERM (Fig. 3C). Therefore, 15 medaka eggs (stage 21) in triplicate were next exposed to 5 ml of N-WCNTs (10 mg l^{-1}), W-SWCNTs (1 or 10 mg l^{-1}) or PEG-SWCNTs (10 mg l^{-1}) or to CNF, CCNF or CNC (10 mg l^{-1}, as references) in each concentration of ERM (1×, 5×, 10×, 15×, 20×, or 30×) at pH 7 and 25 °C in the dark for 7 days. The 1× ERM and 30× ERM have osmotic pressures equivalent to freshwater and seawater, respectively. There were no significant differences in dissolved oxygen concentration (8.30 ± 0.04 mg l^{-1}) among the ERM solutions. Test solutions were renewed once a day. On day 6 of exposure, heart rate per 15 s was counted, and eye size (diameter) was measured. Survival rates were counted for 5 days. Medaka eggs in 1× to 30× ERM at pH 7 in the absence of nanomaterials were used as controls. For detailed information on the measurement, please see previous papers (Kataoka and Kashiwada, 2016).

Analysis using electron microscopy

For scanning electron microscopic (SEM) observations, medaka embryos were exposed to each nanomaterial at 10 mg l^{-1} in each concentration of ERM (1×, 5×, 10×, 15×, 20×, or 30×) under the same conditions as mentioned above and then harvested on day 6. Eggs were washed with fresh concentrations of ERM and fixed in 2.5% glutaraldehyde and 0.1 M phosphate buffer (pH 7.4) on ice for 2 h. The eggs were then dehydrated serially with 50%, 70%, 80%, 90% and 100% ethanol. The dehydrated eggs were soaked in a solution of *t*-butyl alcohol: ethanol (1:1) for 30 min. This was followed by soaking in *t*-butyl alcohol twice, for 30 min each time. The eggs were vacuum freeze-dried and then subjected to SEM analysis (VE-8800; Keyence, Osaka, Japan) at 10 to 20 kV. Magnifications were ×5000 and ×10000.

For transmission electron microscopic (TEM) observations, the specimens were prepared by placing a 10-μl drop of the nanomaterials suspension onto an SiO-coated Cu grid. After 30 s, the grid was allowed to dry. Samples assessed by TEM were not stained.

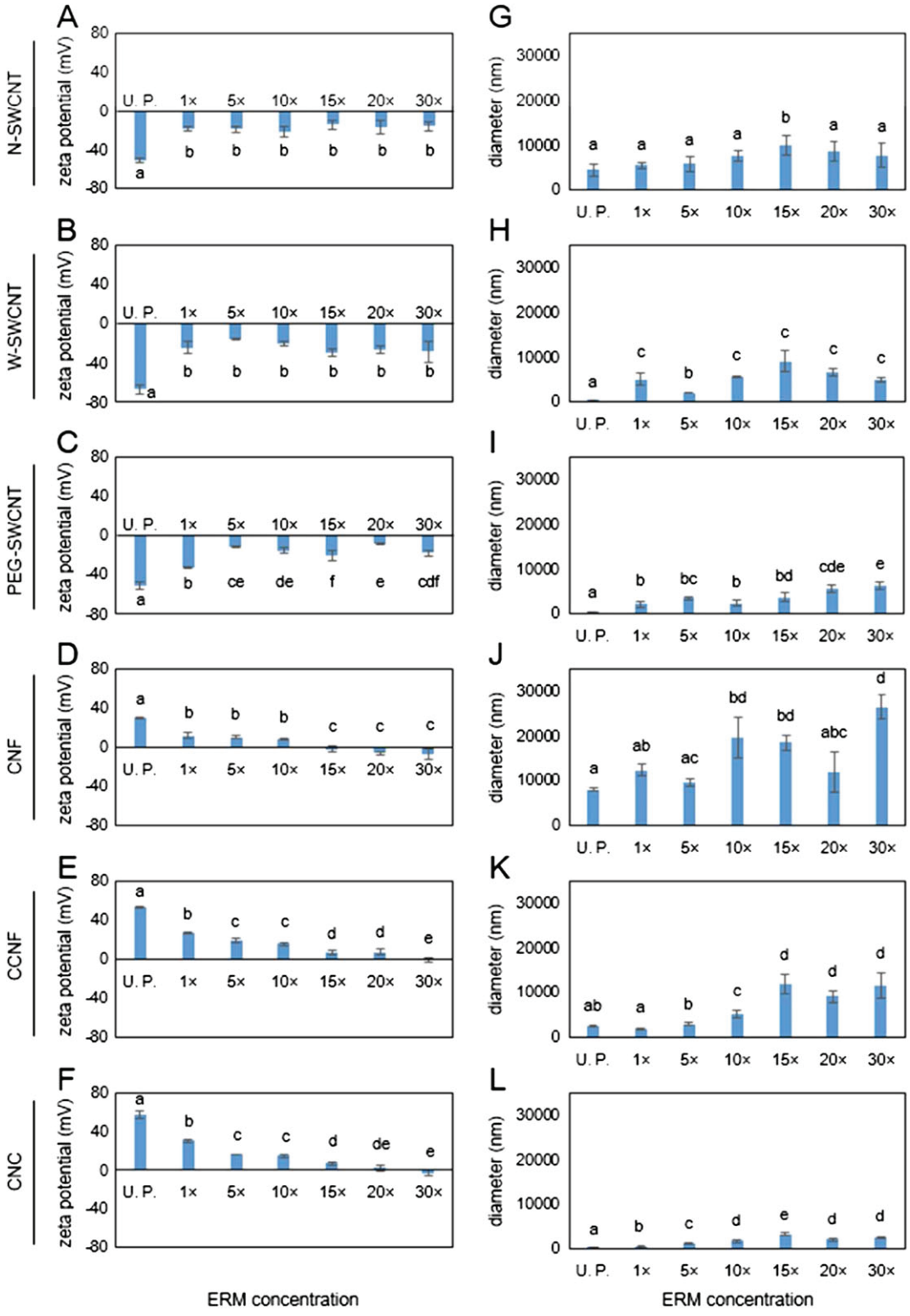

Figure 2. Physicochemical characterization of SWCNTs and chitin-based nanomaterials. Zeta potential and diameter of N-SWCNTs (A and G), W-SWCNTs (B and H), PEG-SWCNTs (C and I), CNF (D and J), CCNF (E and K), or CNC (F and L) in ultrapure water or different concentrations of embryo-rearing medium (ERM) were measured with an ELSZ-2000ZS Zeta-potential & Particle size Analyzer. U.P., ultrapure water. Different letters indicate significant difference at $P < 0.05$ based on one-way ANOVA and Tukey's multiple comparison test. Other abbreviations as for Fig. 1.

Statistical analyzes

We performed one-way analysis of variance (one-way ANOVA) and Dunnett's test for comparing each treatment group to a control group, if not specified (see e.g. Fig. 2). All statistical analyzes were done using Excel 2013 (Microsoft Office 2013®; Microsoft Japan, Tokyo, Japan) and R version 3.3.0 (http://www.R-project.org/). We chose a significance level (α) of 0.05 for all analyzes.

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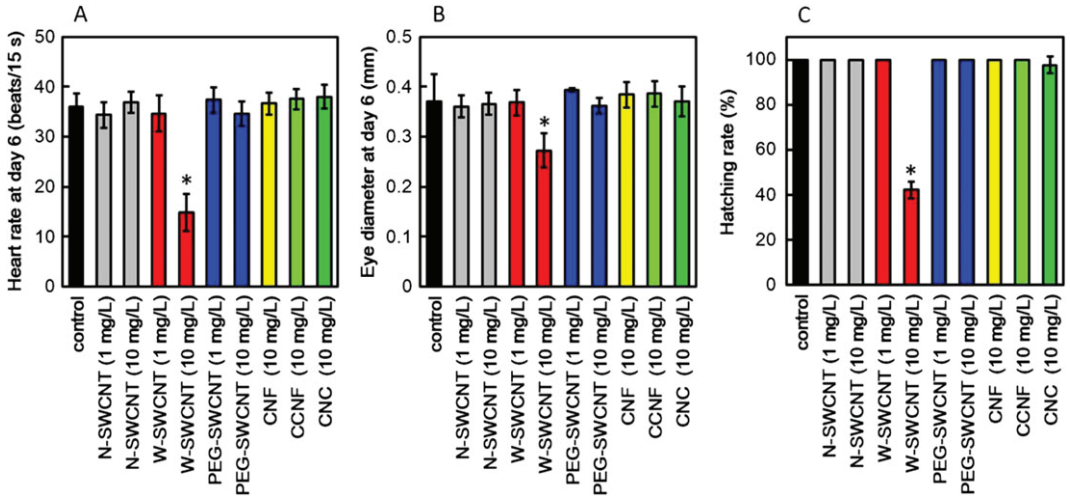

Figure 3. Effects of different kinds of nanomaterials on medaka embryo development under freshwater conditions (1× embryo-rearing medium). Unlike W-SWCNTs, the N-SWCNTs, PEG-SWCNTs, CNF, CCNF and CNC did not affect the heart rate (A), eye diameter (B) or hatching rate (C). *Dunnett's test compared with control (1× ERM which is the absence of nanomaterials), $P < 0.05$. Abbreviations as for Fig. 1.

Results

Physicochemical characterization of SWCNTs and chitin-based nanomaterials

We measured the zeta potential and diameter of the SWCNTs and chitin-based nanomaterials in ultrapure water or different concentrations of ERM (1×, 5×, 10×, 15×, 20× and 30× ERM) (Fig. 2). The absolute zeta potentials value of SWCNTs were significantly smaller in different concentrations of ERM than in ultrapure water (Fig. 2A–C). At different ERMs, the zeta potentials of SWCNTs were stable. In contrast, the absolute zeta potentials value of the three chitin-based nanomaterials were significantly lower than those in ultrapure water and decreased with increasing ERM concentration (Fig. 2D–F). A low absolute zeta potential value indicates aggregation. These results showed that the salinity decreased the dispersibility of nanomaterials. The diameters of the N-SWCNTs were in a similar range (5394.0 to 10001.6 nm) regardless of increasing salinity (ultrapure water to 30× ERM) (Fig. 2G). In contrast, W-SWCNTs and PEG-SWCNTs were significantly bigger in ERM than in ultrapure water (Fig. 2H–I). The sizes of W-SWCNTs at all ERM concentrations were in a similar range (1939.6 to 8921.9 nm). The sizes of PEG-SWCNTs were stable at 2042.1 nm to 3594.0 nm in 1× to 15× ERM but were significantly bigger in 20 × ERM and 30× ERM at 5558.1 and 6142.0 nm, respectively. With some exceptions, the diameters of the three kinds of chitin-based nanomaterials increased with increasing ERM concentration (Fig. 2J–L). The diameters of CNF were greatest (9523.4 to 26 448.0 nm), followed in order by CCNF (1740.1 to 11 771.8 nm) and CNC (445.4 nm to 3108.0 nm) in different concentrations of ERM.

Toxicity of CNTs at different salinities (freshwater to seawater conditions)

After exposure of medaka embryos to 1 or 10 mg l^{-1} N-WCNTs, W-SWCNTs or PEG-SWCNTs, only 10 mg l^{-1} W-SWCNTs resulted in a significantly decreased heart rate, eye diameter and hatching rate (Fig. 3). Exposure to N-SWCNTs or PEG-SWCNTs, or to the reference CNF, CCNF or CNC, at 10 mg l^{-1} in 1× ERM had no vital effects. Light microscopic observations of exposed eggs compared with control eggs (Fig. 4A) revealed that N-SWCNTs were absorbed ontothe chorionic villi of the chorion (Fig. 4B). In normal developments, yolk sack lost volume, and instead growing embryo body increased volume in the egg (Fig. 4A). In embryos exposed to W-SWCNTs, the yolk sack and embryo body shrank simultaneously (Fig. 4C). W-SWCNTs were adsorbed on to the egg chorion; the yolk sacks of 57.8% of embryos shrank and then, yellowish yolk leaked from the yolk sack; furthermore, embryos were dead and no longer available heart rate in this condition (within 24 h of exposure, Fig. 4C); however, 42.2% of eggs exposed to W-SWCNTs were able to develop and hatch (Fig. 4D). 4.4% of larvae exposed to W-SWCNTs had severe defects in the spinal cord, membranous fin and tail formation (Fig. 4J–K), unlike the control larvae (Fig. 4I). They were no longer swimming like larvae in the control.

Exposure to N-SWCNTs, PEG-SWCNTs, or the three reference chitin nanomaterials resulted in no malformations (Fig. 4B, E, F, G and H). Upon CNF exposure, the CNF became wrapped around the eggs and had a soft, absorbent-cotton-like appearance, although this had no biological effects (Fig. 4F).

We also tested the toxicity of the nanomaterials at different salinities. Only W-SWCNTs at 10 mg ml^{-1} exhibited a significant ($P < 0.05$) salinity-dependent increase in toxicity, in the form of a reduced heart rate, eye diameter and survival rate (Fig. 5A–C).

Adsorption and distribution of nanomaterials on the egg chorion surface

Eggs exposed to each nanomaterial at 10 mg l^{-1} in each concentration of ERM (1×, 5×, 10×, 15×, 20× and 30× ERM) were subjected to SEM analyses to observe their adsorption. The W-SWCNTs were adsorbed densely all over the surface of the egg chorion which was exposed in 1× ERM (Fig. 6C); the surface was obviously different from that in the unexposed eggs incubated in 1× ERM (control, Fig. 6A). The surface of the unexposed egg chorion had a characteristic pattern of numerous craters (Fig. 6A). Only a few aggregated N-SWCNTs and PEG-SWCNTs were found on the surface of the egg which was exposed in 1× ERM (Fig. 6B and D); otherwise, the egg-surface patterns were similar to those in the controls (Fig. 6A). As with W-SWCNTs, the

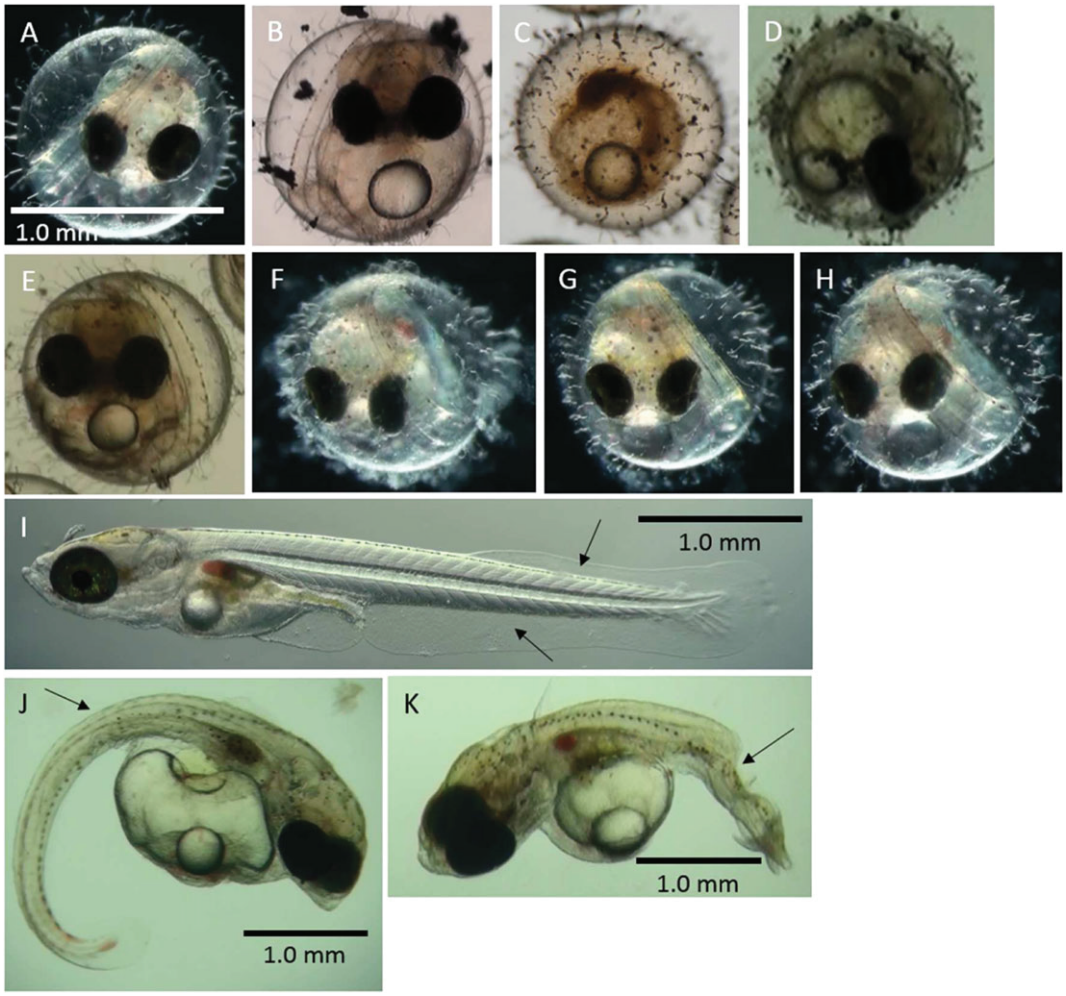

Figure 4. Light microscopic images of medaka embryos exposed to N-SWCNTs, W-SWCNTs, PEG-SWCNTs, CNF, CCNF, or CNC at 10 mg l^{-1} in 1× ERM. Shown are embryos in control (A, day 6) or with exposure to N-SWCNTs (B, day 6), W-SWCNTs (shrank yolk sack) (C, day 1), W-SWCNTs (D, day 6), PEG-SWCNTs (E, day 6), CNF (F, day 6), CCNF (G, day 6), or CNC (H, day 6). In (F) the egg chorion is wrapped in adsorbent-cotton-like CNF. Also shown are a hatched control medaka larva (I, day 7, black arrows indicate membranous fin) and medaka embryos exposed to W-SWCNTs (J and K, day 7). Defects in the membranous fin and tail are visible (J and K). Abbreviations as for Fig. 1.

reference CNF, CCNF and CNC were crowded all over the egg chorion surface (Fig. 6E, F and G), but unlike with the W-SWCNTs, they caused no toxicity. We exposed SWCNTs or chitin nanomaterials in each concentration of ERM (1×, 5×, 10×, 15×, 20×, and 30× ERM), the adsorbed nanomaterials on the surface of the egg chorion which were exposed in 5× to 30× ERM were similar to that of 1× ERM (data not shown).

Discussion

There are two basic ways of increasing dispersibility. The first is by physically adsorbing small molecules or polymers onto the surfaces of CNTs (producing, for example, plastic-polymer-coated W-SWCNTs) through hydrophobic interaction, π–π interactions or supermolecular inclusions; the other is to modify functional groups on the surface covalently by chemically decorating their side-walls and tips (producing, for example, PEG-SWCNTs) (Hersam, 2008). Here, we tested the toxicity of non-functionalized-SWCNTs, plastic-polymer-coated W-SWCNTs and PEG-SWCNTs to medaka embryos. Only W-SWCNTs exhibited toxicity (decreased heart rate, eye diameter, and hatching rate, as well as teratogenicity, including severe defects of the spinal cord, membranous fin and tail formation) at 10 mg l^{-1}. Regarding the effects of salinity on the zeta potential and diameter, there was no significant change in N-SWCNT and W-SWCNT, and there were significant increases in PEG-SWCNT among 1× to 30× ERM. Only PEG-SWCNT exhibited increases in the zeta potential and diameter with an increase in salinity. However, there was no obvious relationships among physicochemical characterization, salinity and toxicity in this study. Using zebrafish eggs, Asharani *et al.* (2008) tested the embryotoxicity of water-dispersible MWCNTs. The water-dispersible MWCNTs were prepared by acid treatment of raw MWCNTs using a concentrated H_2SO_4 and HNO_3 mixture (Hu *et al.,* 2003; Zhang *et al.,* 2003). These water-dispersible MWCNTs caused hatching delay and mortality in a dose-dependent manner from 60 to 300 mg l^{-1}. The teratogenic effects such as a bent notochord, mucus production and apoptosis were found only in embryos injected with 10 nl of 500 mg l^{-1} (5 ng) water-dispersible MWCNTs (Asharani *et al.,* 2008). Using Chinese rare minnow (*Gobiocypris rarus*) eggs, Zhu *et al.* (2014) tested the embryo toxicity of water-dispersible SWCNTs prepared by acid treatment of raw SWCNTs using concentrated HNO_3. They observed malformation

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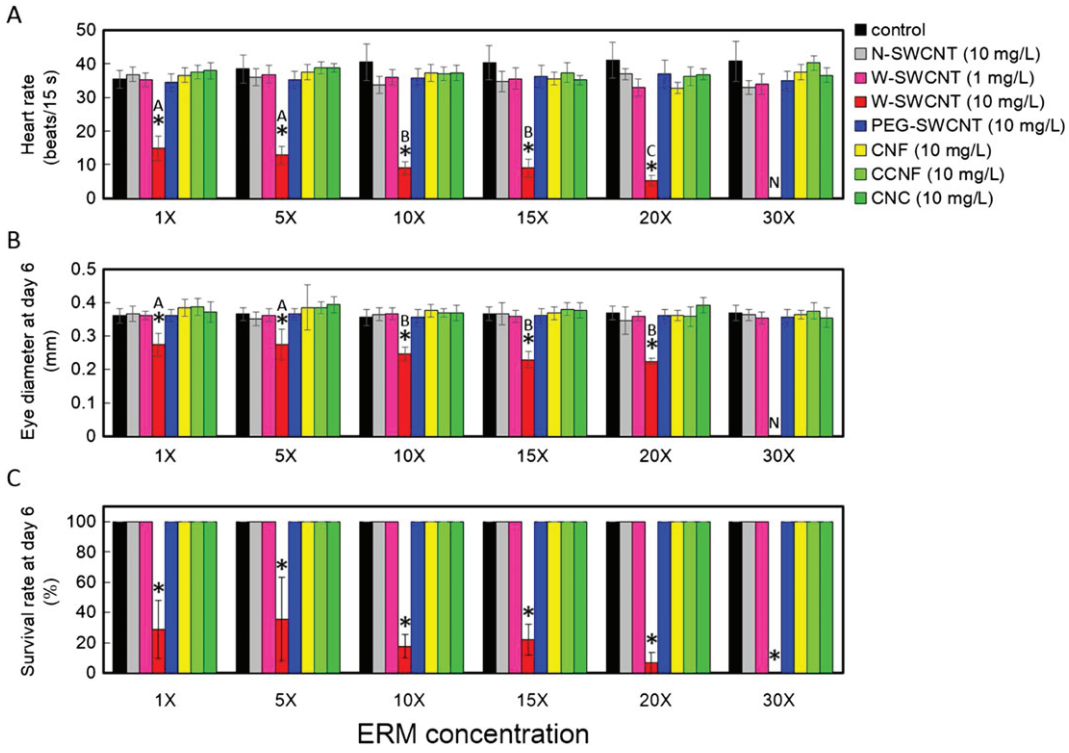

Figure 5. Salinity-dependent vital effects of W-SWCNTs on medaka embryo development in 1× to 30× embryo-rearing medium (ERM). Heart rate (A), eye diameter (B), and survival rate (C) at day 6. * Dunnett's test compared with controls at each concentrations of ERM, $P < 0.05$. Different letters indicate significant difference among the W-SWCNT (10 m l^{-1}) based on *t*-test multiple comparison test, $P < 0.05$. N, not available. Abbreviations as for Figure 1.

and mortality in a dose-dependent manner from 80 to 320 mg l^{-1}, but only at and after 72 h post-fertilization. Our results (showing the toxicity of water-dispersible nanotubes) and those of these studies suggest that dispersibility is an important factor in aquatic nanotoxicity.

Zhu *et al.* (2014) observed SWCNT adsorption by fish (*Gobiocypris rarus*) eggs and found that the chorion protected the embryos from exposure. Our medaka egg chorions were covered by W-SWCNTs, CNF, CCNF or CNC, but, interestingly, only the W-SWCNTs exhibited toxicity. In previous studies, we have demonstrated that fluorescence polystyrene latex nanoparticles and SNC (aggregated Ag^{+}) penetrate through the chorion and cause toxicity (Kashiwada, 2006; Kataoka *et al.,* 2015). However, the issue remains as to how chemicals penetrate biological membranes and whether the pores of such membranes are large enough to allow the passage of synthetic nanomaterials. Verma *et al.* (2008) found that 'While some biomacromolecules can penetrate or fuse with cell membranes without overt membrane disruption, no synthetic material of comparable size has shown this property yet'. Furthermore, they stated that cationic nano-objects pass through cell membranes by generating transient holes and that this process results in cytotoxicity. Furthermore, Zhu *et al.* (2006) reported that the MWCNTs induced dose-dependent growth inhibition to *Stylonychia mytilus*, and exclusively localized to the mitochondria. It suggests that growth inhibition of the cells might be a result of the damage to mitochondria. W-SWCNTs such as cationic polystyrene latex nanomaterials, when coated with cationic plastic polymers, may penetrate the medaka egg chorion readily and cause toxicity. To date, we could not observe any evidence that materials penetrated causing toxicities to the embryos. Zhu *et al.* (2014) reported that although water-dispersible SWCNTs (which are functionalized by HNO_3 treatment) at 320 mg l^{-1} covered the eggs completely, the chorion was an effective protective barrier, and acute toxicity was not observed. The surface chemistry of nanomaterials is thus important for their behaviors and interactions with the egg chorion.

We found here that the toxicity of W-SWCNTs at a constant concentration (10 mg l^{-1}) increased with increasing salinity. Salinity-dependent increases in the toxicity and bioavailability of nanomaterials to medaka embryos have been reported previously in studies using polystyrene latex nanoparticles and SNC (Kashiwada *et al.,* 2012; Kataoka *et al.,* 2015). We (Kataoka *et al.,* 2015) have found that the electrical resistance of the chorion membrane decreases with increasing salinity. This suggests that there is salinity-dependent ion permeation between the chorion and the ERM.

Although W-SWCNTs, CNF, CCNF and CNC are all hydrophilic and dispersible, CNF, CCNF and CNC were not toxic. Chitin is a natural polysaccharide. Chitin, chitosan, and their derivatives are widely used in engineering, wound healing and functional foods, and they are now considered to have promising biomedical applications, including drug delivery, because of their antitumor and anti-inflammatory effects (Azuma *et al.,* 2014). As with asbestos, inflammation is a toxic effect of some nanomaterials (Yamawaki and Iwai, 2006). CNF, CCNF and CNC probably penetrate the medaka egg chorion because of their water-dispersibility and surface cationic charged properties, but their lack of toxicity may be explainable by their anti-inflammatory effects. Biomedical properties are thus important in assessing the toxicity of nanomaterials.

The salinities of aquatic environments vary from freshwater to seawater. Salinity is an important factor in both the bioavailability and the toxicity of charged nanomaterials. A previous study of the

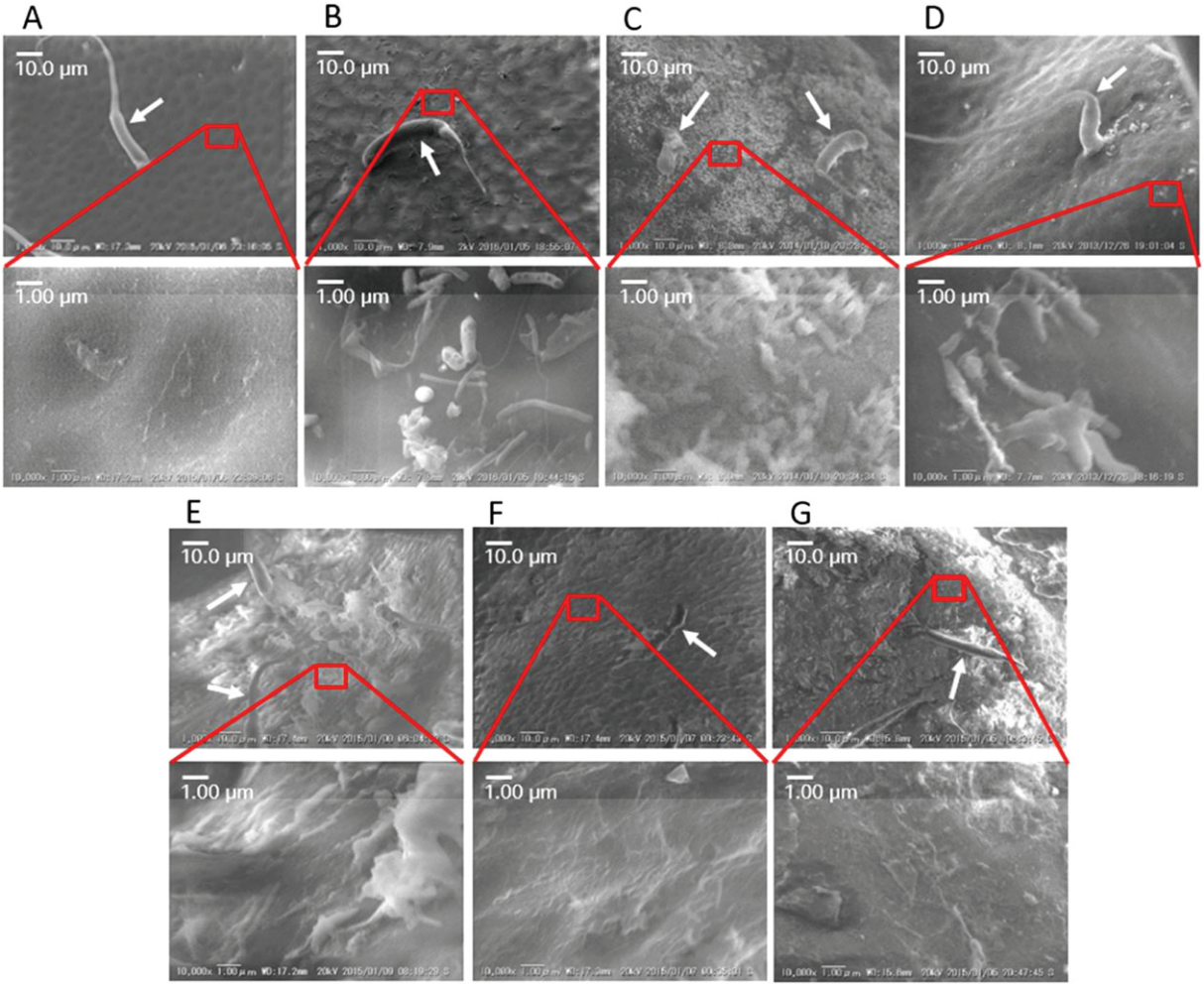

Figure 6. Scanning electron microscope images of the chorion surfaces of medaka eggs. Control egg (A), and eggs exposed to N-SWCNTs (B), W-SWCNTs (C), PEG-SWCNTs (D), CNF (E), CCNF (F), or CNC (G) at 10 mg l^{-1} in 1× embryo-rearing medium (day 6). White arrows indicate villi. A number of short villi are distributed over the whole surface of the chorion. Red boxes and lines delineate areas magnified in each set of the lower panels. Abbreviations as in Fig. 1.

fate of gold nanorods in saline estuaries revealed that surface charge controlled the initial behavior of the nanorods in the aquatic environment: positively charged gold particles entered the food chain via feeding strategies associated with the filtering of organic planktonic solids such as floc, whereas negatively charged gold particles entered the food chain via strategies associated with the searching of sediment surfaces for food (Burns *et al.,* 2013). Cationic W-SWCNTs may, therefore, enter the food chain via the feeding of organisms on organic floc.

Conclusions

W-SWCNTs were adsorbed densely over the surface of the egg chorion and then exhibited acute toxicity to medaka embryos, probably because of penetration through the egg chorion. Also, the embryotoxicity of W-SWCNTs increased with increasing salinity from freshwater to seawater. N-SWCNTs and PEG-SWCNTs exhibited no toxicity, and they were not adsorbed onto the egg chorion. Like W-SWCNTs, the CNF, CCNF and CNC used here (for the first time to our knowledge) as reference materials were adsorbed thickly onto the egg chorion; however, they were not toxic, probably because of their biochemically harmless properties. We demonstrated here that not only water dispersibility, surface chemistry (in the case of N-SWCNTs, W-SWCNTs and PEG-SWCNTs) and biomedical properties (in the case of CNF, CCNF, and CNC), but also salinity, were important factors in assessing the aquatic toxicity of nanomaterials.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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アセスメント係数を用いる方法と種の感受性分布方法から導出される予測無影響濃度（PNEC）の比較

Comparison of Predicted No Effect Concentrations (PNECs) Derived by Using Assessment Factor and Species Sensitivity Distribution methods

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SUMMARY

Running laboratory toxicity tests is costly and time consuming, and therefore extrapolations based on the limited toxicological information are necessary in performing the ecological risk assessment/management of chemical substances. Two widely-used extrapolation methods are the use of assessment factors (AF) as adopted in, for example, the Japanese chemical substances control law, and the other is the application of species sensitivity distribution (SSD). In this study, we theoretically investigated the performance of two extrapolation methods by comparing predicted no effect concentrations (PNECs) derived by the methods. For the comparison, two criteria were adopted. One criterion considers that an extrapolation method which derives a PNEC smaller than the hazardous concentration for 5% of species (HC5) with higher possibility is better. Another criterion considers that an extrapolation method which sets a PNEC closer to the HC5 is better. The AF method adopted by the chemical substances control law was superior under the first criterion but was inferior under the second criterion. Our results show that there is no unique assessment method that always defeat the others, and thereby suggests that a better strategy may be required to compile the merits of existing extrapolation methods depending on data and goal of protection in risk assessments. (201 words / approx. 200 words)

Key words: ecological risk assessment, chemical substances control law, species sensitivity distribution, HC5

1. はじめに

化学物質の有害性情報を収集することは多大なコストが必要となるため、一部の社会的関心が高い物質を除けば、有害性情報は限られている。生物は多種多様であり、例えある生物１種に対する有害性情報が得られたとしても、より高い感受性を持つ生物種が存在するのではないかという危惧が常につきまとう[1]。そのため生態リスク評価では、より感受性の高い種の存在を考慮して、既知の情報を何らかの形で外挿し予測無影響濃度（predicted no effect concentration, 以下 PNEC）を導出している[2]。

PNEC をどのように導出するかは、理想的には生態系におけるどのようなリスクを評価したいか、例えば保全の対象が個体か個体群か、群集レベルか等、評価の目的に応じて変わりうるものであるが、実際には個体レベルの毒性影響をもとに PNEC の導出が行われることが多い。主な導出方法として、不確実係数を用いて導出する方法（以下 assessment factor: AF 方法と述べる）[3, 4] と種の感受性分布（SSD）を用いた方法（以下 SSD 方法と述べる）[5] があげられる。AF 方法における AF の大きさは、対応する法律や国によっても様々である。本研究で例として用いる化学物質の審査及び製造等の規制に関する法律（以下、化審法）のスクリーニング評価における AF の設定方法を表１に示す。

SSD 方法では、化学物質の有害性は生物種ごとに異なるが非常に多くの生物種で有害性（例えば、無影響濃度 : no observed effect concentrati

Table 1. Uncertainty factors used to derive predicted no effect concentration（PNEC）for risk assessments for aquatic organisms under chemical substances control law（Available from http://www.meti.go.jp/policy/chemical_management/kasinhou/files/information/ra/screening.pdf）. ACR stands for acute chronic ratio.

採用する毒性値			種間外挿の UF	急性から慢性への UF(ACR)	室内試験から野外への UF	不確実係数積 UFs
3 つの栄養段階の慢性毒性試験結果がある場合の最小の無影響濃度(NOEC)			-	-	10	10
2 つの栄養段階の慢性毒性試験結果がある場合の最小の NOEC			5	-	10	50
1 つの栄養段階の慢性毒性試験結果がある場合の NOEC			10	-	10	100
3 つの栄養段階の急性毒性試験の半数致死(影響)濃度 [L(E)C50]がある場合の最小の L(E)C50			-	ACR	10	10×ACR
慢性毒性試験結果が欠けている栄養段階の急性毒性値がそろわない場合の小さい方の L(E)C50			10	ACR	10	100×ACR
ACR	藻類		-	20	-	-
	ミジンコ	アミン類	-	100	-	-
		アミン類以外	-	10	-	-
	魚類		-	100	-	-

on, NOEC）を調査すれば、それら有害性値はある統計分布で表現できると仮定し、その統計分布からほとんどの生物種に影響がないと考えられる濃度を推定する。どの統計分布が適切であるかについては議論があるものの[6]、対数正規分布が仮定されることが多い。

SSD 方法は、European Commission が作成しているガイダンス[4]による方法が比較的受け入れられているが、評価の目的に応じて様々である。評価の大まかな流れは、まず NOEC が従う SSD を推定し、その分布から 95% の種で影響がない濃度（hazardous concentrations for 5% of the species: HC5）を推定する。SSD 方法においても AF が用いられ、推定された HC5 を AF で除して PNEC とする。AF は 5 がデフォルトであるが、データの質や量に応じて 5 から 1 の間に調整される[4]。SSD から推定される HC5 がそのまま PNEC として扱われることもある。

本研究の目的は、これら二つの方法（AF 方法と SSD 方法）で導出された PNEC がどのように異なるかを調べ、それぞれどのような利点を持っているかを考察することである。AF の設定方法については、化審法のスクリーニング評価（表 1）を採用する。急性毒性値も評価の対象に含めた解析も可能ではあるが解析やシナリオの設定が複雑となるため、本研究では慢性毒性のみが利用可能であるという状況を考えた。以下では、化審法の AF 適用ルールを用いた PNEC の推定方法を略して化審法 AF 方法と呼ぶこととする。なお、どちらの方法を使うべきかという「べき論」を問うことが本研究の目的ではない。そもそもどちらの評価方法がより良いか、より適切かというのは単純な二項対立的問題でなく、データの質と量に依存して議論されるべき問題であり、どのようなリスクを評価するか（つまり評価の目的は何か、評価軸を何にするか）で変わりうる。すなわち、ある条件がそろえば SSD 方法の適用がより適当であり、その他の条件では AF 方法がより適当といった議論が適切であろう。幾つかの評価軸が同時に存在する場合は、両者ともに優れた方法と判断される可能性すらある。しかしながら、その条件とは何かという議論は不足しているし、そもそも両者の利点や欠点を比較するにはどのような評価軸を設けるのかという点についても議論は不足している。後述する条件で、慢性毒性値（NOEC）を仮想的に生成し、AF 方法および SSD 方法を用いて PNEC を導出することで両者の利点や差異を比較することが本研究の目的である。

2. 方法

2.1 定義と仮定

本研究で用いた定義は以下である

1. 一つの生物種は一つの化学物質に対し一つの NOEC を持つ
2. NOEC の母集団は対数正規分布に従う
3. この分布を真の SSD と呼ぶ
4. 真の SSD から導出される 95% 保護濃度（HC5）を真の HC5 と呼ぶ
5. 真の HC5 をリスクの許容レベルとする。つまり、真の HC5 が管理目標である

定義 2 にある対数正規分布であるが、以下全ての NOEC は対数（底は 10）を取った値で解析を行うため、実際には正規分布に従う NOEC の対数変換値で考えることになる。以下、平均、標準偏差と述べた場合、対数変換された NOEC から求められる平均と標準偏差を意味する。

本研究で用いた仮定は以下である

1. 任意の生物種についてあらかじめ NOEC を知る方法はない
2. 生物種は、藻類、甲殻類、魚類のどれかの分類群に属する
3. ある生物の毒性試験を実施する際、その生物種の分類群をあらかじめ知る方法はない
4. 毒性試験は全て慢性毒性試験であり、急性毒性試験は考えない

仮定 1 は NOEC は母集団からランダム抽出されることを意味している。仮定 3 は、3 つの分類群（仮定 2）からどの生物種を選ぶかもランダム

であることを意味している。なお、この枠組みでは、確率としては低いが、生物種 100 種を試験したところ、全て藻類であったという状況も生成される。仮定 4 は解析を単純化するために設けた仮定である。化審法 AF 方法では急性毒性に対しても AF が定められているため（表 1）特段必要としない仮定であるが、SSD 方法では急性毒性と慢性毒性が混在する場合、目的に応じて急性毒性だけで分布を推定する場合や、急性慢性比を用いて急性毒性を慢性毒性に外挿して、慢性毒性として分布を推定する等、幾つか方法が存在する。様々な仮定や方法が付加的に存在すると解析が煩雑になるため、ここでは慢性毒性のみを考慮することとした。SSD 方法においてどの方法を採用するかはシナリオに依存する。シナリオが適切に設定されれば、本研究の方法論は応用可能である。

2.2 PNEC の導出

まず、真の SSD の標準偏差に任意の値を設定する。次いで、その分布から NOEC（実際には log（NOEC））を n 個抽出する。

化審法 AF 方法では、抽出した NOEC に対し、等確率（=1/3）で 3 つの分類群のどれかを割り当てる。抽出された NOEC の中から最も低い値を選び、選ばれた分類群数に応じた AF（表 1）で除して PNEC とする。なお、この方法では、NOEC が最低一つあれば評価できるため $n \geq 1$ である。

SSD 方法では、抽出した NOEC から SSD を推定し、HC5 を推定する。HC5 を更に AF で除した値が PNEC と定められるが、AF の大きさについては幾つか任意に設定し、結果を示す際に AF の大きさについても言及する。なお、標準偏差を推定する必要があるため、この方法では $n \geq 2$ である。

2.3 二つの方法の評価基準

本研究では，以下の二つの基準でどちらの評価が優れているかを判定した。

- [基準 1] 真の HC5 より低い PNEC が導出できるか
- [基準 2] 真の HC5 により近い PNEC を導出できるか

基準 1 では、管理目標である真の HC5 より低い PNEC を導出できなかった場合に「失敗」と考える。そして、その失敗が起きる確率（失敗率）を計算し、その確率がより低い手法を良い手法と考える。この方法では、導出された PNEC が真の HC5 より少しだけ低くても、非常に低くても同様に「成功」と判断される。定義上、本研究では真の HC5 がリスクの許容レベルであるため、真の HC5 よりも低すぎる PNEC でリスク評価を行えば、リスクを過剰に推定することにも繋がる。そのため，そのような過度に安全側の PNEC の導出にペナルティーを考えるのが基準 2 である。こちらの基準では、各方法で導出された PNEC と真の HC5 の距離（の平均値）が近い方を良い方法と考える。

基準 1 では、化審法 AF 方法では失敗率を解析的に導出できたため（付録 1）、解析解を用いる。SSD 方法ではランダム抽出を多数回繰り返すというモンテカルロシミュレーションで失敗確率を推定した。基準 2 の評価については、両者ともモンテカルロシミュレーションで解析を行った。繰り返し数はいずれの基準でも 10000 回とした。いずれの場合も、真の SSD の標準偏差とサンプルサイズである n の値によって結果がどう変わるかを調べた。

なお、以上の解析において、仮定される真の SSD の平均値は本研究の結果に全く影響を与えない。平均値は分布全体を右または左に移動させる効果しか持たないためである（抽出される NOEC 値全体が高くなるか低くなるかだけの問題であるため）。そのため本研究では平均値を 0 に固定した。平均値は 1μg/L とも言っても良いが、本解析では単位も重要ではない。

3. 結果

3.1 基準 1 での結果

化審法 AF 方法で真の HC5 より高い PNEC を導出してしまう確率（失敗率）を求めた結果を図 1 に示す。概して、真の SSD の標準偏差が大きい時、つまり種ごとの感受性のばらつきが大きい時、失敗率は高い。また、真の SSD の標準偏差が大きければ、多くの種を調べることで失敗率を単調に減少させることができるが、小さい時はより多くの種数を調べることで失敗率が上がることもある。例えば、標準偏差が 0.6 であれば、2 種調べると 1 種だけで PNEC を設定するよりも、失敗率を下げられるが、3 種調べると失敗率は逆に増加する。種を沢山調べるとより異なる分類群から選ばれる確率も上昇し、そのため適用する AF が減少することによって生じる逆転現象である。より多くの NOEC を調べればより正しい評価に近づくと考えられがちだが、化審法 AF 方法では調べる種数の増加によって適用される AF は減少するため PNEC 導出の失敗率を増やす可能性があることには注意が必要だろう。

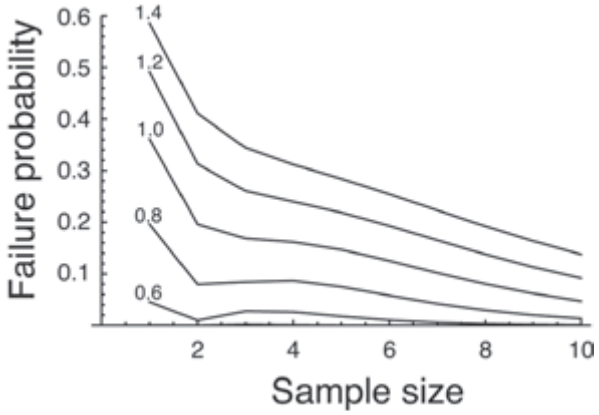

Fig. 1 Probability of failure for deriving a PNEC lower than the true HC5 based on AF-method. Numbers (0.2- 1.4) in the panel indicate standard deviations (SD) of true SSDs assumed. Any further larger sample sizes yield qualitatively similar results, and hence the sample size of 10 was set to be the maximum value in the panel. Lines for smallest and the second smallest SDs. (i.e., SDs of 0.2 and 0.4) are invisible due to very low probabilities. The minimum sample size is 1 for the AF-method. Readers who are interested in actual values in the figure and other values with different SDs and sample sizes should be refer to the analytical result appearing in the Appendix.

次に SSD 方法において、AF を 5 とした場合に安全側の PNEC 導出に失敗する確率を図 2 a に示す。真の SSD の標準偏差が大きいと失敗率は高くなるという結果については、スクリーニング評価と同様である。ただし、標本サイズ（すなわち，NOEC が得られた生物種の数）を増やすことで、どの標準偏差においても失敗率を単調に減少させることができる（図 2a）。次に、SSD 方法でも AF を 1 として失敗率を計算したのが図 2b である。この場合、どの標準偏差でも失敗率はほぼ同じになる。標本サイズが増えると失敗率は減るという点については図 2a 同様だが、8 種以上調べたところで失敗率は 0.5 を少し上回った値で下げ止まりになる。これは当然の結果で、次の理由による。SSD 方法では各標本は真の SSD から抽出されたものであるため、標本サイズを上げれば上げるほど、推定される HC5 は真の HC5 へと近づく。ただし、推定誤差が存在するため HC5 の推定値は真の HC5 の周りでばらつくことになる。仮にそのばらつきが正規分布で表されるとすると、半分が真の HC5 よりも高い値になり、その半分が失敗と判断される。図 2b で失敗率が 0.5 より若干高いのは、実際には、推定された HC5 のばらつきは正規分布ではなく、右に（つまり値が高い方に）偏った非心 t 分布に従うためである（詳しくは後述）[7]。

化審法 AF 方法と AF を 5 とした SSD 方法での失敗率の差（Δp）を調べたのが図 3 である。縦軸の値が高いほど、SSD 評価の失敗率が小さいことを意味している。この AF の下では、化審法 AF 方法に比べて SSD 方法の失敗率は、ほとんど同じ（標準偏差が 0.2 の場合。この場合、両者ともに失敗率はほぼゼロ）かまたは高い（$\Delta p<0$）のどちらかであることがわかる。また、標本サイズが小さいとその差は大きく（すなわち、化審法 AF 方法の法が安全側の PNEC を導出できる確率が高く）、標本サイズを大きくするとその差は小さくなるものの図 3 における最大標本サイズである 10 でも依然 Δp は負のままである。

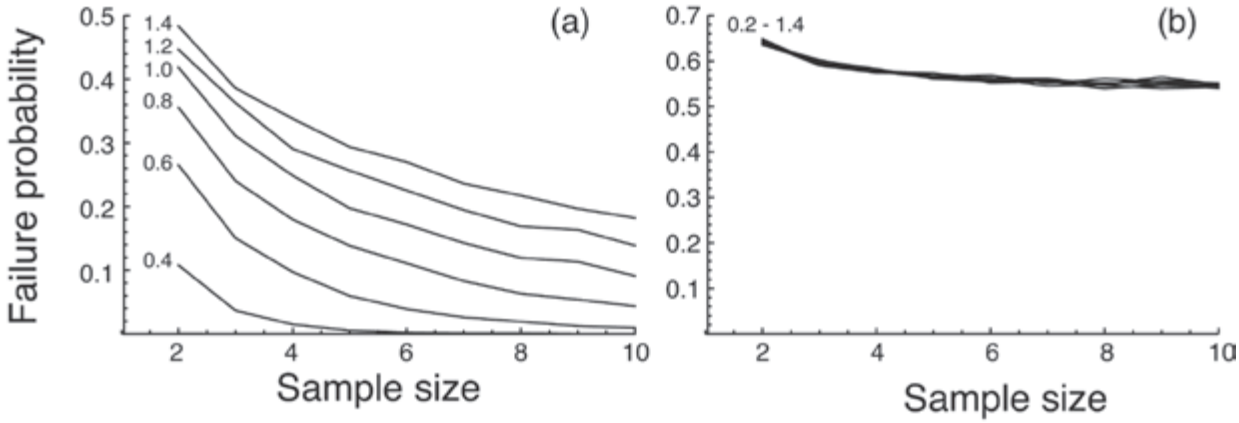

Fig. 2 Probability of failure for setting a PNEC lower than the true HC5 based on SSD method. Numbers (0.2- 1.4) in the panel indicate standard deviations (SD) of true SSDs assumed. Left and right panels show the probabilities when the assessment factors (AF) of 5 and 1 are used, respectively. The minimum required sample size is 2 for performing the SSD method. The maximum sample size of 10 was set to be 10 (see the caption of Figure 1 for the reason).

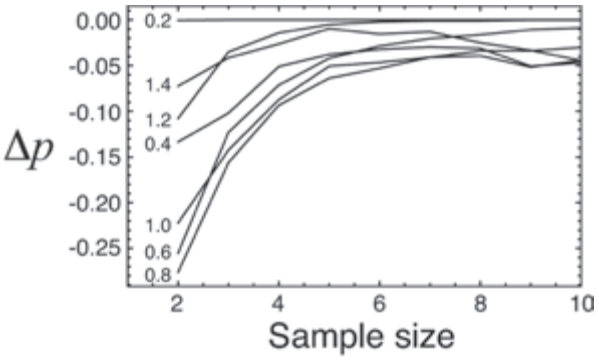

Fig. 3 Difference of failure probabilities between the AF- and and SSD methods. Δp on vertical axis shows the differences, and $\Delta p < 0$ represents that the failure probability in AF method is lower than that in SSD method. Assessment factor (AF) of 5 was used for the SSD method.

失敗率の差は標準偏差が高くなるほど高くなる、といった明確な傾向が見られないという特徴があるが、この理由については定かではない。いずれにしろ、真の HC5 よりも低い PNEC が設定できれば管理は成功と考える基準 1 において、AF を 5 とした SSD 方法に比べて、化審法 AF 方法がより失敗率が低いと言える。

3.2 基準 2 の結果

評価基準 2 では、PNEC が真の HC5 にどれだけ近いかで評価を行う。距離の基準は真の HC5 と PNEC の差の二乗和の平均値（averaged sum of squared errors: ASE）

$$\mathrm{ASE}=\sum_i^{10000}(\log(\mathrm{PNEC}_i)-\log(\text{true HC5}))^2/10000$$

で与えた（図 4）。図 4a が SSD 評価での AF を 5 にした時の結果である。AF が 5 の場合、標本サイズが少なく標準偏差が大きい場合は、SSD 方法による PNEC が真の HC5 よりもかけ離れているが、標本数が 5 より大きければいずれの標準偏差においても SSD 方法がより HC5 に近い PNEC を導出することがわかる。標本数が 10 あれば、いずれの標準偏差においても ASE の差は 0.6 程度であり、化審法 AF 方法は SSD 方法に較べ、平均で 4 倍程度かけ離れた PNEC を導出することを意味している（$10^{0.6}$ は約 4）。

図 4b は SSD 方法での AF を 1 にした時の結果である。標準偏差が大きく標本サイズが小さければ化審法 AF 方法の方がより真の HC5 に近い PNEC を導出するという点については図 4a と同じであるが、図 4b では全体的に ASE の値が上側に寄っている。これは SSD 方法が図 4a の結果よりもより真の HC5 に近い PNEC を導出していることを意味する。この結果は当然で、各 NOEC は真の SSD からランダムに標本抽出されたものであるため、抽出すればするほど真の SSD に近い SSD が推定され、HC5 も真の値に近づく。こ

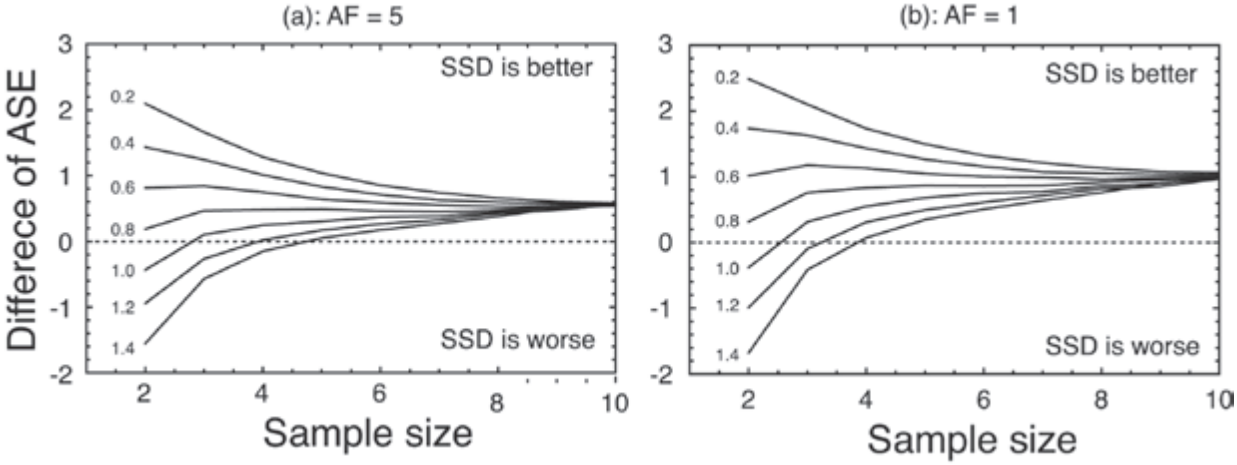

Fig. 4 Comparisons of averaged sum of squared errors（ASEs）based on the AF and SSD methods. A horizontal dashed line shows zero difference（ASEs（difference between the true HC5 and the PNEC）of AF and SSD methods are equal). Positive values on vertical axis show that PNECs by SSD method are closer to true HC5 than that by AF method（i.e., SSD method is better）.（a）and（b） shows the results when AFs of 5 and 1 were used for the SSD method.

の基準では、1 より大きい AF は真の HC5 からかけ離れた PNEC を導出する効果しかない。基準 1 では AF が大きいほど基準を達成しやすかったが、基準 2 では AF が小さいほど達成しやすくなり、評価精度における AF の役割は逆転する。

4. 考察

本研究では，化審法の AF 適用ルールと例として参照した AF 方法（化審法 AF 方法）と SSD 方法で、それぞれから導出された PNEC がどのような特徴を持つかを調べた。真の HC5 より低い PNEC が導出できれば良いと考える基準 1 では、化審法 AF 方法が基準を満たす確率が高かった（図 3：すなわち失敗率が低かった)。ただし、この基準では真の HC5 より非常に低い PNEC と少しだけ低い PNEC を等価に扱うことになる。真の HC5 よりも低くなりすぎることを問題視し、真の HC5 に近い PNEC を導出できる評価方法が良いとするのが､基準 2 である。この基準 2 では、真の SSD の標準偏差が大きくかつ標本サイズが小さい場合を除けば、SSD 評価がより良いとの結果が得られた（図 4)。これらの結果から、どちらの評価手法が優れているのかというのは、そう単純には決められない問題であるということがわかる。これらの手法の平均的な優劣はどのような基準で評価するかによっても変化するし、その他の条件、例えば真の SSD の標準偏差や標本サイズ、SSD 方法に用いる AF の大きさによっても変わる。

生物種の化学物質に対する感受性のばらつきの程度は、評価結果に比較的大きな影響をもたらすようである。この点を指定している研究[8]はごく僅かである。例えば､EU のガイダンスではデータの質や HC5 の推定精度などに応じて AF は 1-5 の間で設定されるとあり、考慮すべき条件が幾つか列挙されているが、感受性のばらつきの程度で AF を変えるべきという考え方は見受けられない。図 1,2 で示したように、いずれの基準でも感受性のばらつきが大きいほど管理は難しくなる（PNEC 設定の失敗確率や真の HC5 と PNEC の差異が増加する)。その理由は明らかで、化学物質に対する感受性のばらつきが小さければ、何種で試験しても同程度の NOEC しか得られないためである。このような場合、感受性のばらつきが大きい場合と比較して個々の NOEC の情報量は相対的に低く、新たに NOEC を収集する価値がほとんどない（あるいは低い）ということを意味している。一方、標準偏差が大きければ NOEC

は生物種ごとにより大きく異なるため、新たにNOEC を取得した場合 、その情報の価値は高い。これは、取得すべき NOEC の数は感受性のばらつきによってある程度最適化できるかもしれないということを意味している。毒性試験の実施には少なくないコストがかかるため、有害性情報を増やすことは容易ではない。感受性のばらつきが事前に推定できれば、それによって定められるNOEC の情報量を考慮することで、何種の生物で有害性情報を収集すべきかという議論もできるだろう。その結果に基づいて、化学物質管理の効率化が行えるはずである。

図 2 及び図 4 に示したように、評価基準によって SSD 方法で使用すべき適切な AF が変わる。安全側の（真の HC5 より低い）PNEC の設定には AF は大きいほど好ましいし（図 2)、真の HC5 との誤差を小さくするのであれば AF は小さいほど良い（図 4)。本研究では二つの基準しか考えていないが、他の基準ではまた、AF の役割が変わってしまうかも知れない。どの基準が一番良いかということを決めるのは難しい問題であり、それぞれの良さを柔軟に取り込んだ上で、適切な AF を定めるという考え方も必要かも知れない。例えば、次のような決め方もあり得るのではないだろうか。

対数正規分布から標本抽出を行って HC5 を推定するには、標本サイズを無限にしない限り推定値には幅が生じ、その幅は非心 t 分布に従う[7]。この幅を利用することで、例えば失敗率を 5% 以下に押さえたい場合に必要な AF の大きさを理論的に求めることができる。図 5 にある f(HC5) が HC5 のばらつきを示す非心 t 分布であり、その形状は推定された SSD の平均、標準偏差および標本サイズで決まる。ここでは SSD の平均は 0、標準偏差は 0.5、標本サイズは 5 の例を示した。図中の CL は f(HC5) の上側信頼区間である。この例では、CL が -0.41、HC5 が -0.82 である。CL が HC5 を下回るように AF を決めれば、95% の確率で推定された HC5 を真の HC5 より小さくできるので、その差（CL-HC5）が採るべき AF の大きさである。底を 10 とした対数で考えているので、この場合

$$UF=10^{(-0.41+0.82)}=2.59$$

となる。つまり、AF を 2.6 程度にすれば、真の HC5 よりも高い PNEC を設定してしまう過ちを 5% 以下に抑えられる。

真の SSD の標準偏差と標本サイズ（取得した NOEC の数）を変化させ 、管理の失敗率を 5% 以下に押さえたい場合に必要な AF の大きさを調べたのが図 6 である。まず、真の SSD の標準偏差が大きいほど，また標本サイズが小さいほど，大きな AF が必要となることがわかる（図 6)。標本サイズが 2 で標準偏差が 1.4 であった場合、失敗率を 5% 以下にするのに必要な AF は 40 以上と非常に大きい。標本サイズが増えるに従い、失敗率を 5% 以下にするのに必要な AF の大きさは急速に小さくなり、標本サイズが 10 あれば真の SSD の標準偏差が 1.4 でも必要な AF は 7.6 程度である。AF の定め方には客観性が必要であるが、同じデータセットを基にしても評価機関によって

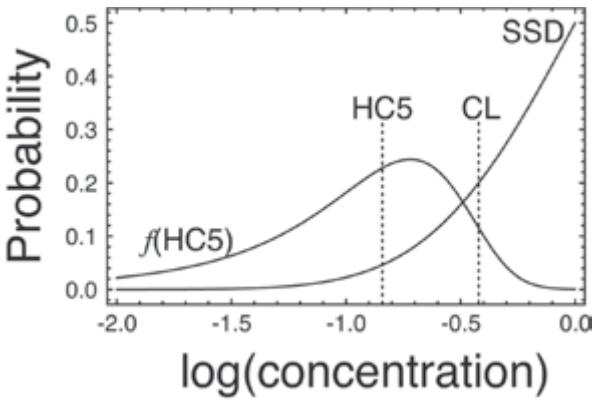

Fig. 5 A true SSD and the distribution of the estimated HC5 (noted by f(HC5) in the panel). HC5 in the figure is the true HC5 determined by the true SSD. The estimated HC5 follows the non-central t distribution of which parameters are the mean and standard deviation of the SSD and the sample size. The upper bound of the 95% confidence interval (noted by CL) is determined by the non-central t distribution. The mean and standard deviation of the SSD in this example are 0 and 0.5, respectively. The sample size in this example is 5.

PNEC が最大 3 桁も異なってしまう[9] という現状を踏まえると、PNEC の導出方法には議論の余地が多分に残されていると言え、その客観性は高いとは言いがたい。AF 方法と SSD 方法のどちらがより適切な PNEC を導出できるかという本研究の本来の目的からは少し異なる指摘となるが、必要な AF の大きさを、分布の形状や標本サイズに応じて柔軟にかつ客観的に定めることができるというのは SSD を用いた評価方法の優れた特性と言えるだろう。

本研究では、真の SSD の平均値や標準偏差は既知という前提で計算を進めたが、現実にはそのような情報を知ることは難しい。幾つかの先行研究からで報告されている SSD の標準偏差を付表 1 にまとめた。全てを網羅したわけではないが、調べた範囲では標準偏差は 0.45-1.17 の間にあり、本研究の結果で示した範囲にある。この情報だけで何かの指針を出すことは難しいが、化学物質の物性値や使用用途等の情報と SSD の標準偏差の関係や急性毒性値に基づく SSD の標準偏差と慢性毒性値に基づく SSD の標準偏差の関係などを調査することで、SSD の標準偏差を事前に推定する方法を検討することもできるだろう。例えば農薬を用いたリスク評価の研究からは、SSD の標準偏差は作用機作により特異的であることが示されている[8,10]。同様のアプローチはその他の化学物質でも可能かもしれない。

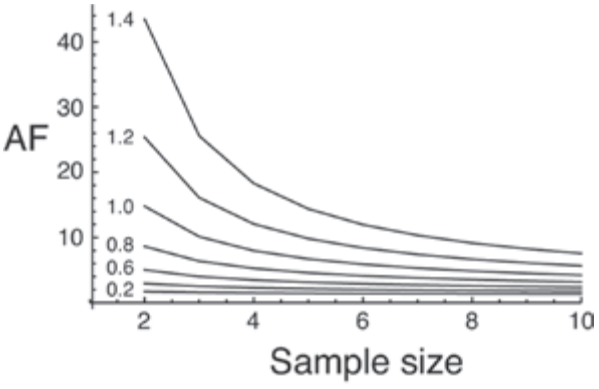

Fig. 6 Estimated assessment factors required to determine a PNEC lower than the true HC5 with the failure probability of less than 5%.

本稿で設けた仮定は、現実では満たされないことも少なくないと考えられる。例えば、本研究では、試験生物種はランダムに抽出されるという仮定をおいたが、その仮定が満たされない場合には HC5 を過大または過小評価する場合があることが示されている[11]。また、本研究では、各生物種の NOEC は 1 つの真の SSD に従うとしたが、特定の作用機序をもった農薬などでは、異なる分類群で異なる SSD に従うことも示されている（実例として，例えば Nagai[8]）。本稿で設けた仮定は、実務レベルで行われている SSD 評価において一般的な仮定だと考えられるが、異なる仮定を用いた場合や仮定が満たされなかった場合において、本研究の結論がどの程度普遍性を持つかについては、上記の点も含めて仮定や定義のより詳細な精査が必要となってくるだろう。

AF を用いた評価方法では、調べた範囲で一番低い値を PNEC の導出に採用する。得られた情報が限られている場合、このアプローチを取らざるを得ないだろう。しかしながら、「AF を用いて最も低い毒性値未満に目標を定めるのだから安全である」と、もしそのように考えるのであるならば、それは安易に過ぎる。調べている生物種数が少なくまた種間の感受性が大きくばらつく場合は、真の HC5 よりも高い PNEC が設定されてしまう確率は決して低くないし（図 1：例えば真の SSD の標準偏差が 1.4 の場合，失敗率は最大約 60%）、実際に農薬登録保留基準を例にそのような現象が報告されている[8]。HC5 を基準とした管理では、5% の種を守らなくても良いのか、という批判が常にあるが、AF 方法でも一定の種が保護の対象から除外されてしまうという点に付いては大同小異である（図 4b）。本研究では除外したが、急性毒性を考慮しても生物種の感受性のばらつきが大きくなれば守られない種が増えるという傾向はおそらく同じだろう。したがって、種の感受性のばらつきに応じて AF を柔軟にかつ客観的に変化させる評価方法を構築するというアプロー

チも今後の戦略の１つとして検討する価値はあるかもしれない。

謝辞

Table A1 におけるニッケルの無影響濃度一覧は、詳細リスク評価書ニッケルの著者の一人である恒見清孝氏が提供くださいました。感謝します。本研究の一部は平成 26-30 年度文部科学省私立大学戦略的研究基盤形成支援事業（S1411016）による支援を受けています。

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Table A1. Examples of standard deviation (SDs) of estimated species sensitivity distribution (SSD) based on chronic toxicity data in previous studies[1)].

substances	number of samples	estimated SD of SSD	source
Ammonia	11	0.97	A1)
Atrazine	13	0.83	A1)
3, 4- DCA	11	1.17	A1)
Linden	18	1.02	A1)
Phenol	9	0.62	A1)
Nonylphenol	8	0.70	A2)
C_{12}TMAC	8	0.45	A1)
LAS[2)]	16	0.60	A1)
Copper[3)]	23	0.89	A1)
Zinc	15	0.46	A3)
Lead	20	0.58	A4)
Nickel	22	0.60	A5)
Cadmium	39	1.04	A6)
Hexavalent Chromium	26	0.70	A7)

1) Geometric means were used to calculate the SD of the SSD when multiple toxicity data were available for the same species.

2) Toxicities of LAS vary depending on the alkyl chain length. No toxicity correction was made in the present study.

3) Toxicities of metals vary depending on water quality. No toxicity correction was made in the present study.

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付録

化審法スクリーニング評価での PNEC 設定失敗率

種の感受性分布は対数正規分布を仮定する。濃度を 10 を底とした対数で変換した値を考えれば、対数正規分布は、正規分布となる。以降、平均と標準偏差と述べた場合、濃度を対数変換した値の平均と標準偏差を意味している。平均 μ, 標準偏差を s として、対数変換済み濃度（x）での累積確率を $f(\mu, s; x)$ とする。種の感受性分布（SSD）は累積確率の分布であるので、$f(\mu, s; x)$ は SSD そのものである。なお、以下の解析では簡単のため慢性影響しか考えない。

NOEC は SSD からランダムに抽出する。表 1 で要求される不確実係数の累積を AF と書く。1 種の NOEC だけで PNEC を導出する場合、種間外挿と野外の外挿それぞれ 10 の累積となり AF=100 である。観測された値を AF で割ると PNEC が得られ、その値は

log（PNEC）= log（NOEC）-log（AF）

と表される。この PNEC が真の HC5 を下回っていれば適切な PNEC の導出は成功で、上回っていれば失敗である。適切な PNEC の導出失敗の

条件は

PNEC > HC5

log（NOEC）–log（AF）> log（HC5）

log（NOEC）> log（HC5）+ log（AF）

log（NOEC）> log（HC5×AF）

と書かれ、この条件を満たす確率、P(log（NOEC）> log[HC5×AF])、を求める。SSD は累積確率分布なので、選んだ NOEC がこの値より低くなる確率（管理が成功する確率）は SSD から直接求められ

P (log（NOEC）< log[HC5×AF]) =

f (μ, s; log[HC5×AF])　…成功確率

P (log（NOEC）> log[HC5×AF]) =

1- f (μ, s; log[HC5×AF])　…失敗確率

である。

NOEC が一種しかなければこの計算で良いが、2 種を考えた場合、2 種とも同じ分類群から選ばれる場合、違う分類群から選ばれる場合を考えないといけない。藻類、ミジンコ、魚類をそれぞれ a, b, c と書くと 2 種を選んだ時の組み合わせは（a, a），（a, b），（a,c），（b, a），（b, b），（b, c），（c, a），（c, b），（c, c）の 9 組ある。2 種を同じ分類群から選ぶ組みは（a, a），（b, b），（c, c）の 3 組みで確率としては 1/3 であり、この場合 AF は 100 となる。残りの 6 組は 2 分類群から選んでいるため AF は 50 となり、その確率は 2/3 である。2 回続けて失敗する確率は各確率の 2 乗で表されるので、HC5 よりも高い PNEC が設定される確率は

$$\frac{1}{3}P\left(\mathrm{PNEC}>\log\left[\mathrm{HC5}\times 100\right]\right)^2+\frac{2}{3}P\left(\mathrm{PNEC}>\log\left[\mathrm{HC5}\times 50\right]\right)^2$$

となる。3 種以上でも同様で、一般に N 種を抽出した時に、1 分類群だけから選ばれる確率は

$$p_1={}_3\mathrm{C}_1\times\left(\frac{1}{3}\right)^N$$

で、2 分類群から選ばれる確率は

$$p_2={}_3\mathrm{C}_1\times\left(\sum_{i=1}^{N-1}{}_N\mathrm{C}_i\right)\left(\frac{1}{3}\right)^N$$

である（全ての分類群から選ばれる確率 p_3 は 1-p_1-p_2）。それぞれの確率で重みづけて和を取ればよいので、失敗の確率は

$$\sum_{i=1}^{3}p_iP\left(\mathrm{PNEC}>\log\left[\mathrm{HC5}\times\mathrm{UF}_i\right]\right)^N$$

となる。ただし、UF_i = {100, 50, 10} である。

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ミニレビュー

河川底生動物を対象とした野外調査結果から金属の“安全”濃度を推定する#

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A journey to estimating “safe” concentrations of trace metals based on macroinvertebrate field surveys in rivers

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は じ め に

農薬を含めた化学物質の生態リスク評価において，我々の保護対象は野外環境中の生物個体群や群集であり，厳密には実験室内で人工的（あるいは半人工的）構築されるそれらではない．最近では，実際の環境を模したメソコスム試験の活用もヨーロッパを中心に進んできているが[1]，過去（特に1990年よりも以前）には，時空間的にも大きなスケールでの生態系操作実験[2,3]や実環境中への化学物質（硫酸[4]や銅[5]，農薬[6]）の意図的な散布などが生態影響の理解や評価の一貫として行われていた．より最近の例では，カナダのオンタリオ州北西部の実験湖エリアにおいて，合成エストロゲンがファットヘッドミノー（個体群）に及ぼす影響を2つの湖を使って7年間かけて実施されたKiddら[7]の大規模な野外操作実験研究がある（著名な学術誌である米国科学アカデミー紀要に掲載）．とはいえ，倫理的問題や設備やコストの問題も考慮すると，野外環境において時空間的スケールの大きい操作実験を行う機会はかなり限られているといえるだろう．

このような時空間スケールの大きい野外操作実験に比べると，OECD（経済協力開発機構）等のガイドラインに従って実施される室内毒性試験は比較的簡便であり，方法が標準化されているため実施場所や実験者による差異も生まれにくく，再現性も高く，人的および経済的コストも低い．したがって，化学物質の生態リスク評価における環境中での予測無影響濃度（Predicted no effect concentration, PNEC）の推定に，室内毒性試験から得られる毒性値（LC_{50}（median lethal concentration；半数致死濃度）やNOEC（No observed effect concentration；統計学的に有意な影響が観測されなかった最大濃度[8]），EC_x（Effect concentration of *x*: *x*% の個体が影響を受けると推定される濃度）など）の利用が世界的に進むのは当然と言えるだろう．最近では，そのような毒性値を複数の種について収集し，対数正規分布などの統計学的分布に当てはめ，群集レベルの影響を予測する方法（種の感受性分布方法）が，データが利用可能な状況では常套手段となり

第41回大会シンポジウムを取りまとめた解説.

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つつある[9–12]．

しかし，室内試験結果から野外における生態影響を予測することが困難であることは古くから指摘がなされている[13,14]．特に，室内試験の結果から導出されるPNECや水質環境基準が「実環境においてどのような意味を持っているのか」という点は，リスク評価者や管理者，利害関係者にとって無視できない重要な問いと言えるが，この問いについて踏み込んだ研究ほとんど行われていない．例えば，化学物質の審査および製造等の規制に関する法律（化審法）のスクリーニング評価では，PNECを導出する際に，室内試験から野外への外挿の不確実係数として10が採用されているが，明確な科学的根拠を提示することは難しい．

このような問いに対し，私がこれまでに着目してきた野外調査研究は，実際の環境における生物への影響を直接観察することができ，「実際に環境中濃度がPNECと同等レベルであった場合に野外で予想される影響の大きさはどの程度か（例えば，生物種数の減少は予想されるか）」といった問いに解答を提示することができる．本稿では，この野外調査に着目して，生態影響評価手法における野外調査の位置づけを確認し，異なる野外調査の方法論について概観した後に，私がこれまでに実施してきた野外調査データを用いた金属の安全濃度推定に関する研究結果を紹介する．最後に，特に広域レベルで実施される生物モニタリングを用いた野外生態影響評価の可能性についても言及した．なお，野外調査に着目した化学物質の生態影響評価という文脈では，既出の拙著[15]も参考にされたい．内容の重複はあるが，管理ではなく評価により重点を置き，本稿ではより踏み込んだ記述を加えるようにした．

1.　化学物質の生態影響評価手法

化学物質の生態影響評価手法は，(1) 室内毒性試験，(2) 模擬生態系試験（マイクロコスムやメソコスムなど），(3) 野外実験，(4) 野外調査の4つに区分できる（表1）．ここでは，野外調査と比較することで，各手法の得手不得手を考察する．日本では，農薬登録の際に，藻類（緑藻：*Pseudokirchneriella subcapitata*），ミジンコ（オオミジンコ），魚類（メダカまたはコイ）の急性の室内毒性試験結果が求められるが，先にも述べたとおり，これらの室内毒性試験は，野外調査に比べて操作性（および再現性）が高く，同条件で行われた試験間での比較が容易で，必要となる人的金銭的コストは少ないと言えるだろう（ただ，外部委託した場合の費用という意味では状況によって明確に優劣はつけにくいかもしれない）．一方で，単一種を用いて室内の比較的安定した条件および限られた時間内で行われる室内毒性試験の結果が，変動が大きく同時に多種が存在する野外環境での影響をどの程度反映しているか（あるいは予測できるか）は難しい問題である．

複数の生物種を用いるマイクロコスムやメソコスムなどの模擬生態系実験は，単純な系で行われる室内毒性試験に比べ実環境をより再現している．また，濃度操作が可能なため一般的に野外調査よりも操作性は高いといえるだろう．ただし，通常数週間から数か月で行われるため，時空間スケールには依然として限界がある[16]．例えば，一年間の限られた時期に使用され，環境中での分解も比較的速い農薬のような物質であれば，このような試験結果から得られるものは少なくないだろう．一方で，休廃止鉱山由来の廃水が流入する重金属汚染河川では，曝露が長期間続いており，野外での生態影響を理解するうえで，時間的なスケールが制限されていることの重要性はより大きくなると考えられる[17]．空間スケールの限界も含めて模擬生態系実験の結果から野外での応答を理解することに対する批判は存在するが[18,19]，少なくとも化学物質濃度と野外生物群集の応答との因果関係や種間相互作用が存在する多種の応答を理解するうえで，模擬生態系実験が果たす役割は大きいだろう．なお，模擬生態系実験については，早坂ら（2013）[16]に詳述されている．

野外実験の例については「はじめに」で言及した通りではあるが，例えば，汚染河川と非汚染河川の底質（礫）を入れ換えて生物の定着応答を比較する実験[20]や，実河川にケージを設置する実験（例えば，ケージ内に一定期間放流された魚類の金属蓄積と生体内応答を評価する研究[21]）など，より時空間スケールの小さい実験も含まれる．化学物質の生態影響評価ではあまり使われていない印象を受けるが，野外調

表1.　化学物質の生態影響評価手法の比較

手法	生物種	費用	操作性	時空間スケール	現実性
室内毒性試験	主に1種	低	高	小	低
模擬生態系実験	数～多種	中程度～高	低～高	中程度	中程度
野外実験	多種	中程度	低～高	中程度～大	中程度
野外調査	多種	中程度～高	低	大	高

模擬生態系実験は，規模によってメソコスム実験やマイクロコスム実験等と呼ばれる．野外実験は，野外環境での操作実験を指す（詳細は本文も参照のこと）．野外調査の種類については，図1を参照のこと．なお，各手法に対する評価は，あくまで相対的な比較による目安であり，個別の実験・調査計画によって異なることが考えられる．

査に比べて実験設定を操作可能であり，検証する仮説に適した野外実験環境があれば，“お手軽な”影響評価方法にもなりうる．しかしながら，大小の出水が頻繁に起こる日本の河川のように，攪乱の多い環境では，長期間の実験をすることは現実的に困難であり，時間スケールの課題は依然残るかもしれない．

2.　異なるデザインの野外調査

では，野外調査はどうだろうか．他の手法内でも具体的な方法論には多様性はあるが，河川を例にとれば，野外調査も図1のように大まかに4つに分けられるだろう．まず，点源の汚染源がある場合に，その直上と直下に調査地点を設定し，生物調査をする方法である（方法1）．この方法1では，両地点を近傍に設定でき，流量等の河川環境の変化の影響は最小限に抑えられると考えられるため，当該廃水流入の影響を効果的かつ比較的容易に推定できるだろう．一方で，事例研究の要素は強く，例えば廃水に含まれる単一の化学物質の影響を抽出・推定することは容易ではないだろう．方法2は，汚染地点と非汚染地点で時間に継続した生物モニタリングを行うことで，水質等の変化がない状況における生物相の時間的変化を非汚染地点で捉えつつ，汚染地点において化学物質の負荷低減（あるいは負荷増加）による生物相の変化を評価するものである（BACIデザインと呼ばれる[22]）．

一方で，野外調査から特定の化学物質の生態影響を評価する場合は，方法3または方法4を利用することが多くなるだろう（図1）．方法3は，汚染の程度の異なる複数の河川（例えば，重金属汚染河川および非汚染河川[23,24]）において，地点の違いによる影響を排除するために複数の地点を設定し，生物調査を実施することで，当該汚染が野外の生物群集に及ぼす影響を評価する方法である．この際，交絡因子の影響を可能な限り排除できる調査デザインや解析方法を丁寧に選択することが肝となる[15]．例えば，汚染地点と似通った環境（例：標高や河川サイズなどに加えて，流速や底質などの河川内環境）の非汚染地点を選出することで，これらの着目する要因以外の影響をできるだけ排除した比較が可能になる．ただし，例えば，休廃止鉱山の廃水に複数の金属，下水処理場廃水に複数の化学物質が含まれているように，この方法でも特定の化学物質のみの影響を抽出することは容易ではないことに留意されたい．

調査地域が限られる方法3では，得られた結果の他河川への適用可能性に課題が残る．この課題を解決するために，方法4では，さらに多くの河川および地点で調査を実施する．ただし，対象調査地点を増やすと，調査地点の選定は困難を極めるため，着目する化学物質以外の要因を制御することがさらに難しくなり，データのばらつきも大きくなるという別の課題が生まれる．しかし，これらの課題は分位点回帰などの適切な統計解析を用いることで一定程度解決することが可能である．

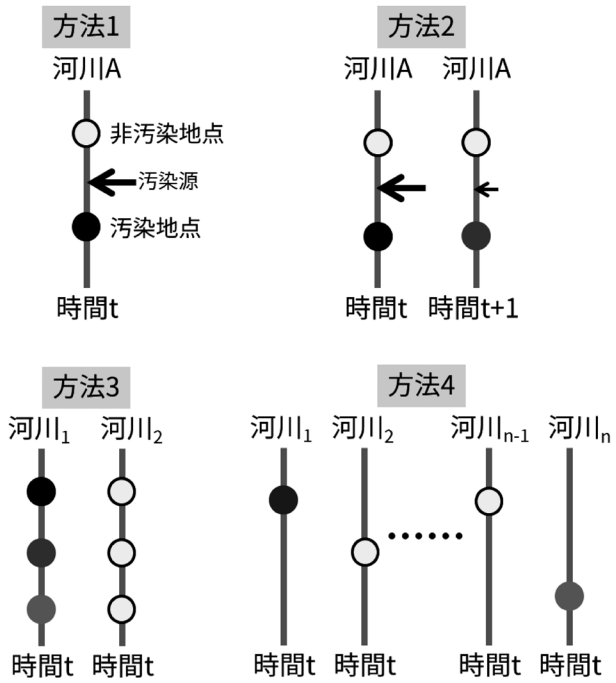

図1.　化学物質の生態影響評価を目的とした野外調査．方法1：汚染流入源（例：排水口）の直上直下で生物調査を行う；方法2：汚染負荷の除去・低減（あるいは増加）の前後で，対照地点とともに生物相の変化を調査する；方法3：汚染河川と非汚染河川で複数の地点で調査を行う（この調査デザインでは，河川の違いによる影響を考慮できないため，厳密には汚染・非汚染ともに複数河川を設定して調査することが好ましい）；方法4：多くの河川で調査地点を設定し，生物調査を実施する．

3.　野外調査結果から安全濃度を推定する

本章では，これらの方法3および4を用いて，重金属汚染河川で実施した底生動物調査結果から，亜鉛等の金属が顕著な影響を及ぼさない濃度（安全濃度）を推定した結果を紹介したい．対象とした大型無脊椎動物（以下，底生動物）は河川生態系において中間的な栄養段階に位置し[25]，汚染物質に様々な感受性を示す種によって構成されているため，野外調査や模擬生態系実験によって金属に対する応答を調べた研究も少なくない[26,27]．また，簡易な現地調査法によって，多様な分類群が採集できるのが底生動物を対象とする大きな利点の1つである．

まず，日本の休廃止鉱山周辺の河川において重金属汚染以外の影響をできるだけ排除できるように調査地点を設定し（方法3），亜鉛濃度と底生動物の種数（分類群数）の関係を推定した研究について紹介する．この調査では，宮城県迫川，山形県寒河江川，兵庫県市川の3水系の上流域に設定した計25地点（早瀬）において，最大怪15〜25 cmの礫から底生動物を採集し，水質（亜鉛等の金属濃度，生物化学的酸素要求量，溶存酸素濃度など），物理環境（川幅，流速まど）

を調査した[23,24,28]．これらの調査結果を用いた重回帰分析の結果，カゲロウ目の種数などすべての底生動物種数で，亜鉛濃度の影響についてはある一定濃度までは影響がないとするモデルが赤池情報量規準（AIC）によって最良モデルとして選ばれ，基準値の2〜3倍程度の亜鉛濃度でも底生動物の種数はほとんど減少しないことが示唆された（図2：カゲロウ目の種数の例）．これらの調査結果では，亜鉛とその他の金属（銅，鉛，カドミウム）の濃度は高く相関しており，亜鉛濃度の影響のみを抽出できていないため，安全濃度は過小推定されていることが考えられる．以上の結果は，底生動物の種数の保全という観点でみれば，亜鉛の基準値は安全側の値に設定されていることを示唆している[23,28]．

次に，より広域で行われた底生動物調査結果を用いて，金属の安全濃度を推定した研究を紹介したい（方法4に該当）．このような研究では，前述した野外調査研究と比較して，解析対象とする調査データ内での方法や調査地域・地点の環境の多様性から，データのばらつきが大きくなるが，分位点回帰を用いることでこの問題に対処することができる（図3Aの脚注参照）．このようなデータに対する分位点回帰の適用についてはCade and Noon（2003）[29]およびSchmidt *et al.*（2012）[30]に詳しいが，生物の生息個体数や種数を制限するような要因を解析対象とする場合は，高分位点（例えば，95%）を回帰することによって着目する説明変数の影響を推定することが一般的である（図3A）．

著者らは，この分位点回帰を利用して，英国，米国，日本の金属汚染河川や酸性河川で主に実施された底生動物調査結果（合計400地点超）から，銅，亜鉛，カドミウム，マンガンの4つの金属について底生動物の種数に顕著な影響を及ぼさない閾値濃度（安全濃度）を推定した[31]（図3Bに銅とカドミウムの例を示した）．この解析では，直線モデルや指数モデルなど計4つの分位点回帰モデルをデータに当てはめたが，いずれの金属データでも閾値のあるモデルが最良であっ

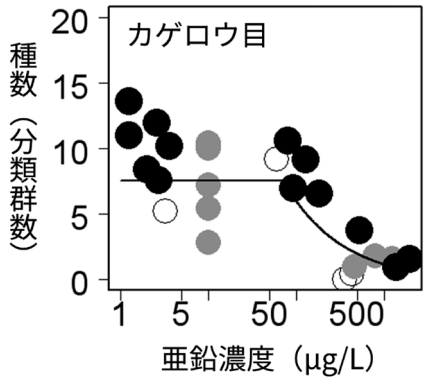

図2.　亜鉛濃度とカゲロウ目種数との関係（Iwasaki *et al.*[28]より改変）．異なるプロットは異なる河川を意味する，黒線は赤池情報量規準最小の閾値モデルを示す．

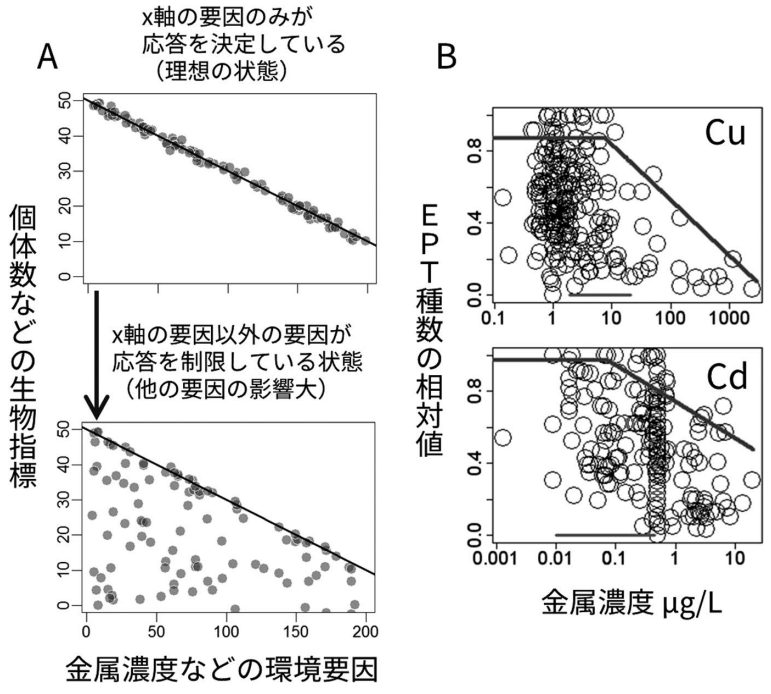

図3.　（A）高分位点を回帰する場合の分位点回帰の概念図（Cade and Noon[29]より改変）．上図はx軸の説明変数のみによってy軸の応答が制限されていることを示す（それ以外の環境条件はy軸の値が最大となる「理想の状態」と仮定する），下図では，x軸以外の（考慮されていない）要因がy軸の応答を制限することでばらつきが大きくなっている，しかしながら，高分位点を回帰することで説明変数のみの影響を抽出できる．（B）分位点回帰の適用例．銅およびカドミウム濃度とEPT種数（カゲロウ目，カワゲラ目，トビケラ目の分類群数の和）の相対値の関係．EPT種数は各調査の最大値を用いて標準化した値を解析に用いている，折れ線は分位点回帰モデル（95%タイル），図下部にある直線は閾値（安全濃度）の95%信頼区間を示す．

た（AICによるモデル選択）．推定された安全濃度の95%信頼区間は大きく（図3B），その解釈には注意が必要であるが，興味深いことに，銅，カドミウム，亜鉛の推定安全濃度は，室内毒性試験結果から導出された各国の基準値等と概ね重複しており[31]，室内試験ベースの基準値の信頼性を補完する結果を野外データから提供することができたといえる．他にも，調査範囲は英国イングランドおよびウェールズと限定されているが，Crane *et al.*（2007）[32] も底生動物調査結果から，分位点回帰を用いて金属の安全濃度を推定している．

4. おわりに

本稿では，化学物質の生態影響評価において，利用可能な手法を概観したうえで，野外調査による影響評価方法の利点と欠点を説明した．野外調査結果に基づく化学物質の生態影響評価は，その他の手法に比べてあまり実施されていないが[15]，適切な調査デザイン（例：対象生物の生態を理解したうえでの丁寧な調査地点の選定）や，メタ解析および適切な統計手法（本稿では，分位点回帰）を用いることで，濃度反応関係や安全濃度の推定に利用することができる．このような調査研究から得られる結果は，室内試験ベースの生態リスク評価結果の信頼性を評価・補完できる有用な情報を提供できるため，今後積極的に活用されるべきだろう．

最後に，図3Bで紹介したような膨大な野外データは，実は日本でも収集されてはいるが，有効活用されないままで眠っていることを強調しておきたい．例えば，地域レベルでは，神奈川県川崎市では1979年から2011年までの間に川崎市内の110地点で魚類および底生動物調査が実施されている[33]．また，国土交通省の河川水辺の国勢調査（水国調査）では，日本全国の一級水系109水系等を対象に魚介類調査，底生動物調査，植物調査，鳥類調査，両生類・爬虫類・哺乳類調査，陸上昆虫類等調査が1990年度から実施されている．この水国調査では，本稿で着目した底生動物では，合計約1000地点において5年で1度は調査が行われるように実施されており，調査結果自体は河川環境データベース[34]で公開されている．ただし，csv形式で落手できる生データを解析できるように整理するには少なくない労力が必要である．一方で，河川等における化学物質のモニタリングデータも，公共用水域の環境基準地点等の全国約9000箇所で行われる水質測定結果（環境数値データベース[35]）や国土交通省が所管する観測所における水質測定結果（水文水質データベース[36]）がある．したがって，これらの生物および化学データと組み合わせることができれば，野外での生態影響評価を実施することが可能であるが，残念ながら，データベース間で調査地点の照合が進んでないことなどが障壁となり，そのような解析はほとんど行われていない．今後は，このようなモニタリングデータの解析事例を増やしていくことも，化学物質の生態影響評価において，野外での生物調査データの活用を増やして行くうえで重要なステップになるだろう．

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Comment

More practical and gentler guides are required for non-mathematicians in ecotoxicology and beyond Comment on "Physics of metabolic organization" by Marko Jusup et al.

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Use of dynamic energy budget (DEB) model [1], and/or other bioenergetic models would be definitely a key in ecotoxicological applications and ecological risk assessments. One of the critical reasons to anticipate so is that we are required to reduce animal use in ecotoxicity testing that usually measures effects of chemicals on survival or reproduction of organisms [2]. Consequently, the prediction of population-level consequences based on ecotoxicological modeling and suborganismal-level effects evaluated by *in vitro* testing would have more significant value. In this regard, the modeling that can link the sub-organismal responses to organismal- (e.g., survival and reproduction) and then population-levels consequences would be really valuable although challenging [3,4]. Particularly, DEB models have the potential for providing a mechanistic link between sub-organismal and organismal levels once the effects of chemicals on biogenetics (i.e., growth, increased maintenance cost, etc.) are assessed [3]. It should be noted that, even though a considerable amount of work is required to develop such mechanistic models for local populations of a given species [3], a time-consuming model development may not be necessary for "general" ecological risk assessments as with the case that use of "standard/surrogate" test species such as *Daphnia* is accepted in many regulatory contexts. What will be required is probably the agreement on which models/scenarios are used for the assessments.

To that end, Jusup et al. [1] provide a detailed but relatively beginner-friendly guide to DEB model with an emphasis on the theoretical background. For example, the section 2 quite nicely depicts the reasons why they think keeping the DEB model simple is critical and justified. However, the latter sections contain many mathematical equations, which is often not a very effective way to communicate theory to biologists [5]. Indeed, I believe that, even though a considerable number of DEB applications are available in ecotoxicology [6], still a limited number of people understand well enough and can handle DEB models because of that difficulty (a hurdle for non-mathematicians in ecotoxicology and beyond), I would thus strongly argue that, to increase use of DEB models in ecotoxicological and other applications, more *practical* and *hopefully gentler* guides including a series of open courses would definitely be required for non-mathematicians in ecotoxicology and beyond. Taking an example from personal experience, I had never thought that I would be doing the matrix population modeling [7,8] before I had read a good introductory book

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written by Akçakaya et al. [9]. What I would like to emphasize here is that DEB researchers need to put themselves in the place of non-mathematically oriented potential users in order to achieve wider acceptance.

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Short communication

Aeromonas veronii biovar sobria Associated with Mortalities of Riverine Ayu *Plecoglossus altivelis* in the Tama River

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ABSTRACT—In July 2016, there were mortalities of riverine ayu *Plecoglossus altivelis* in a tributary of the Tama River, Japan. A Gram-negative, motile and short rod-shaped bacterium was dominantly isolated from all examined dead fish, and identified as *Aeromonas veronii* biovar sobria. Biochemical characteristics and *gyrB* sequence of the present strains differed from those of *A. veronii* strains from ayu in previous years. The present strains also caused higher mortalities to ayu than *A. veronii* strains previously isolated. These results indicate that the present mortalities of riverine ayu in the Tama River were caused by high pathogenic *A. veronii* biovar sobria.

Key words: *Aeromonas veronii* biovar sobria, *Plecoglossus altivelis*, riverine fish, pathogenicity, the Tama River

The ayu *Plecoglossus altivelis*, a representative freshwater fish species in Japan, has long held an important position among riverine fishes as a target for recreational fisheries and as food for human consumption. Therefore, hatchery-produced or wild (captured from lakes, rivers or sea coasts) ayu are released annually into many rivers to enhance riverine stocks, despite indications that many released fish are at risk of several bacterial infections. In particular, bacterial infection by *Flavobacterium psychrophilum* (bacterial cold-water disease) (Iida and Mizokami, 1996) and *Edwardsiella ictaluri* (Sakai *et al.*, 2008) has become one of the most serious problems for riverine ayu management in recent years. Consequently, the infection status of both pathogens in hatchery-produced and wild ayu has been investigated throughout Japan (Kumagai, 2016; Iida *et al.*, 2016).

In July 2016, bacteria that differed from *F. psychrophilum* and *E. ictaluri* were isolated from dead ayu collected following mass mortalities of riverine ayu in the tributary of the Tama River, Japan. The bacteria were identified as *Aeromonas veronii*, which has been frequently isolated from aquatic environments (Albert *et al.*, 2000) and fish intestines (Namba *et al.*, 2007). Although some studies report that *A. veronii* causes disease in farmed and ornamental fishes (Rahman *et al.*, 2002; Sreedharan *et al.*, 2011; Smyrli *et al.*, 2017), the majority of aeromonads causing damage in Japanese aquaculture have been identified as *A. hydrophila* and *A. salmonicida* (Jo and Onishi, 1980; Kitao *et al.*, 1985; Rahman *et al.*, 2001; Yamamoto, 2017). In the present study, we investigated the characteristics and pathogenicity of strains from diseased ayu and concluded that the mortalities of riverine ayu found in the tributary were caused by *A. veronii*.

Materials and Methods

Bacterial examination

There were two mass mortalities of riverine ayu in the tributary of the Tama River in July 2016, when the daily average water temperature rapidly increased above 23°C. Since we could not sample freshly dead ayu in the first mortality event, we obtained 16 dead and 14 living fish (captured by angling) in the second event. Bacterial isolation from the kidney were performed using trypto-soya agar (TSA, Nissui) and the plates with inoculum were incubated for 48 h at 25°C. Cell morphology and motility of the bacterial strains were examined microscopically by Gram staining and the wet-mount method, respectively, and strains were molecularly identified to species using a partial (500-bp) 16S rRNA sequence from the 5′ region (Namba *et al.*, 2007). Additionally, we tested for the presence of *F. psychrophilum* and *E. ictaluri* in sampled ayu according to the methods of our previous study (Takeuchi *et al.*, 2016).

Biochemical and phylogenetic characterization

Of the strains identified as *A. veronii* in the present study by partial 16S rRNA sequencing, 15 strains (“present strains”) were biochemically characterized using API 20E (BioMerieux) according to the manufacturer’s instructions. The derived API profiles were compared with those of the eight *A. veronii* strains from the kidney of ayu and pale chub *Opsariichthys platypus* captured in the Tama River Basin in 2012 and 2014 (“previous strains” from asymptomatic or *E. ictaluri* infected fish), and reference strains from the intestine of common carp

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Cyprinus carpio (HPI4 and CWP11; Namba *et al.*, 2008) and human patients (JCM7375; Hickman-Brenner *et al.*, 1987). Additionally, for phylogenetic characterization, we performed *gyrB* gene (1,100 bp) amplification and direct sequencing using extracted DNA from present strains and the primers developed by Yáñez *et al.* (2003). After genome assembly, the sequences were compared to sequence data in GenBank using BLAST (blastn) algorithms (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and aligned with sequences of previous strains and reference strains using Clustal X (Thompson *et al.*, 1997). A maximum likelihood phylogenetic tree of aligned sequences was constructed with Kimura's 3-parameter model using MEGA 6 software (http://www.megasoftware.net/), and the robustness of the phylogenetic results were tested by bootstrap analysis with 1,000 iterations.

Experimental infection

To assess the pathogenicity of *A. veronii* strains obtained in the present study, we performed an experimental infection of ayu using the three present strains from dead ayu (AAr1608, AAr1614, and AAr1615), three previous strains (AAr1412, AAr1216, and AAr1218), and one reference strain from the intestine of common carp (CWP11). Stock cultures of all strains in tryptic soy broth (TSB, Difco) containing 10% glycerol at –80°C, were transferred and grown on TSA at 25°C for 24 h and then cultured in 300 mL TSB with shaking at 25°C for 9 h. Hatchery-produced ayu (body weight: 12.0 ± 1.9 g), obtained from the Freshwater Experimental Station, Kanagawa Prefectural Fisheries Technology Center, were acclimated to experimental conditions at 20°C–25°C for 5 days prior to the experiment.

Following a 10-fold dilution of bacterial suspensions of each strain with dechlorinated tap water (final bacterial density: 2.1–3.4 × 10^7 CFU/mL), experimental fish were immersed in the suspensions at 25°C for 30 min, while control fish were exposed to 10-fold diluted TSB. The fish exposed to each strain (n = 10 per strain) were then reared at 25°C in a 50-L glass aquarium with filtration and aeration equipment, and monitored for 10 days. Identification of isolates from the kidneys of dead and moribund fish was performed by direct sequencing of the *gyrB* gene as described above.

Results and Discussion

Most dead ayu and some living fish obtained during the mass mortality of riverine ayu in the river tributary showed external and internal clinical signs such as hemorrhaging of the lower jaw or body surface, reddening at the base of the ventral fin or anus, and ascites (Fig. 1). Of these signs, reddening of the anus and ascites are known as typical clinical signs of *E. ictaluri* infection (Sakai *et al.*, 2008; Takeuchi *et al.*, 2016), so at first, we assumed that the mortality was caused by *E. ictaluri*. However, *E. ictaluri* was detected in only 31.3% (five of 16 fish) of dead ayu and 28.6% (4/14) of the living; *F. psychrophilum* was not detected in any sampled fish. On the other hand, unknown bacteria, which were Gram negative, motile, and short rod-shaped, were isolated from 100% (16/16) of dead ayu and 57.1% (8/14) of living fish. The partial 16S rRNA sequences derived from these bacteria (accession nos., LC311422- LC311447) most closely matched that of *A. veronii* (KT998815).

A. veronii was originally described as a novel species in the genus *Aeromonas* in 1987 (Hickman-Brenner *et al.*, 1987). It is divided into two biovars ("sobria" and "veronii") on the basis of biochemical characteristics such as the activity of arginine dihydrolase (ADH) and ornithine decarboxylase (ODC) (Janda and Abbott,

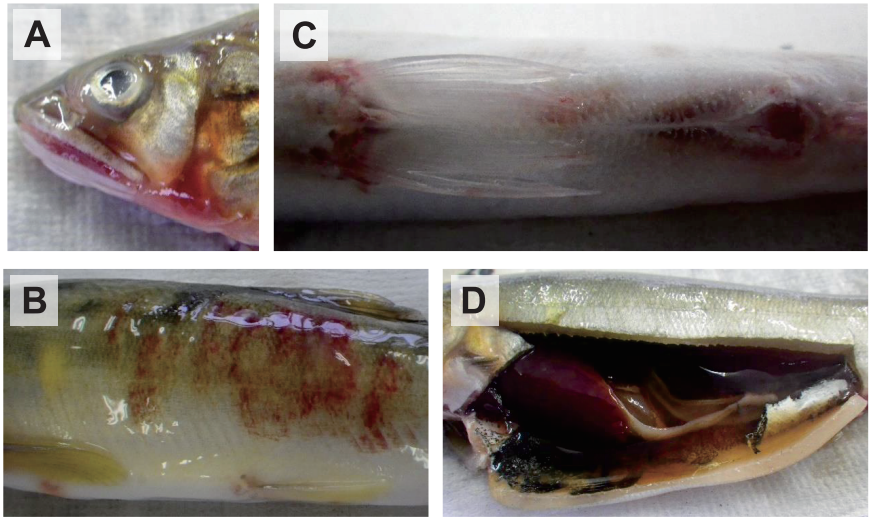

Fig. 1. Typical clinical signs in dead ayu found in the tributary of the Tama River in July 2016: hemorrhaging of lower jaw (A) or body surface (B), reddening at the base of the ventral fin or anus (C), and ascites (D).

1998), and there have been several reports of fish disease caused by *A. veronii* biovar sobria in Asia and Europe (Rahman *et al.*, 2002; Sreedharan *et al.*, 2011; Smyrli *et al.*, 2017). All present and previous strains in the present study were ADH-positive and ODC-negative in the API 20E test, and were assumed to be *A. veronii* biovar sobria. However, the API profiles of the present strains differed from those of previous strains, and *gyrB* gene sequences from the present strains (LC311630–LC311644) formed a cluster different from other sequences except for the sequence of *A. veronii* isolated from diseased European seabass *Dicentrarchus labrax* in Greece (AERO NS; Smyrli *et al.*, 2017; KF636138) (Fig. 2). Smyrli *et al.* (2017) reported that mortality of European seabass caused by *A. veronii* was observed in Agean Sea and the Black Sea. These results and information suggest that the *A. veronii* biovar sobria isolated in the present study may have been introduced from other aquatic environments, inside and outside the country.

In our experimental infection by bath exposure, the dead ayu showed clinical signs similar to those observed in naturally infected fish, and *A. veronii* with *gyrB* sequences matching those of the strains used for exposure were isolated from all dead or moribund fish. The cumulative mortalities of ayu exposed to previous strains were 20%–40%, whereas over 80% of fish exposed to the present strains were dead by 3 days post exposure (Fig. 3). These results indicate that the *A. veronii* found

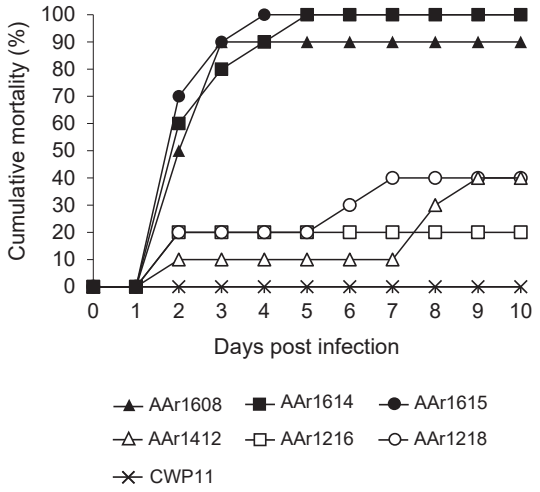

Fig. 3. Cumulative mortality of ayu challenged by exposure to *Aeromonas veronii* isolated from riverine fish collected in the Tama River Basin compared with that of ayu exposed to a reference strain from the intestine of common carp (CWP11). No dead fish were observed in the control group (exposed with TSB). Solid symbols: "present strains"; hollow symbols: "previous strains"; ×: "reference strain".

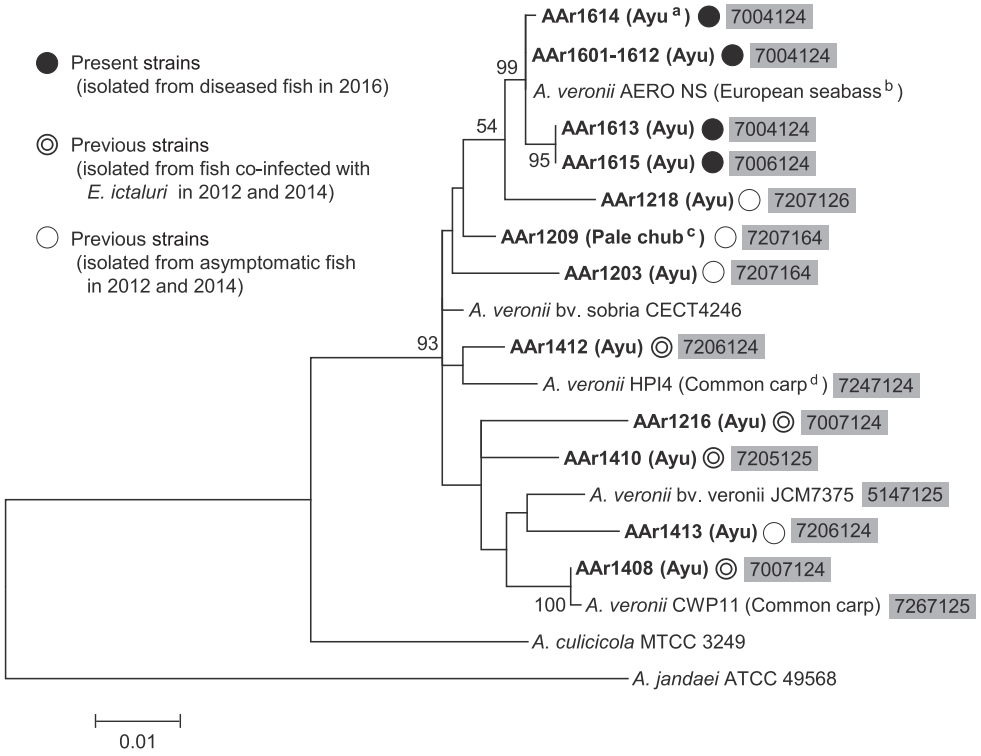

Fig. 2. Phylogenetic tree showing the relationships among *Aeromonas veronii* strains from riverine fish collected in the Tama River Basin. The tree was inferred from *gyrB* gene sequences by the maximum likelihood method. The scale bar represents a 1% sequence difference. Numbers at nodes are bootstrap values (> 50%) after 1,000 iterations. The name of the fish species in parentheses and the shaded boxes show the origins and API 20E profiles of each strain, respectively. [a]*Plecoglossus altivelis*, [b]*Dicentrarchus labrax*, [c]*Opsariichthys platypus*, [d]*Cyprinus carpio*.

in the present study has high pathogenicity to ayu compared to other strains. We conclude that the mass mortalities of riverine ayu found in the tributary of the Tama River in July 2016 were caused by high pathogenic *A. veronii* biovar sobria with different properties than previous strains.

Acknowledgements

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Effects of silver nanocolloids on plant complex type *N*-glycans in *Oryza sativa* roots

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Silver nanomaterials have been mainly developed as antibacterial healthcare products worldwide, because of their antibacterial activity. However, there is little data regarding the potential risks and effects of large amounts of silver nanomaterials on plants. In contrast, *N*-glycans play important roles in various biological phenomena, and their structures and expressions are sensitive to ambient environmental changes. Therefore, to assesse the effects of silver nanomaterials, we focused on the correlation between *N*-glycans and the effects of silver nanomaterials in plants and analyzed *N*-glycan structures in *Oryza sativa* seedlings exposed to silver nanocolloids (SNCs). The phenotype analysis showed that the shoot was not affected by any SNC concentrations, whereas the high SNC exposed root was seriously damaged. Therefore, we performed comparative *N*-glycan analysis of roots. As a result, five of total *N*-glycans were significantly increased in SNC exposed roots, of which one was a free-*N*-glycan with one beta-*N*-acetylglucosamine residue at the reducing end. Our results suggest that the transition of plant complex type *N*-glycans, including free-*N*-glycans, was caused by abnormalities in *O. sativa* development, and free-*N*-glycan itself has an important role in plant development. This study originally adapted glycome transition analysis to environmental toxicology and proposed a new category called "Environmental glycobiology".

Nanomaterial is a general term for small substances that are 1–100 nm in diameter. Nanomaterials have many unique electrical, chemical, and physical properties and are used in electronics, medicine, and healthcare fields. Silver nanomaterials have been developed and mainly used for their antibacterial activities in clothes, appliances, cosmetics, and plastics. However, there have been concerns that silver nanomaterials are likely released into the aquatic environment through factory and household wastewater on a large scale, and there are also concerns regarding the effects of silver nanomaterials on aquatic organisms and ecological systems. The toxicity of silver nanomaterials in the embryos of aquatic organisms such as medaka and zebrafish has been reported[1,2]. The toxicity affected the expression of morphogenesis- and cell proliferation-related genes and induced the increase of severe development abnormalities and mortality. The toxicity of silver nanomaterials was dependent on the particle size, shape, and capping materials[3,4]. Toxicity of silver nanomaterials has also been reported in plants, with the toxicity affecting germination, development, and photosynthetic efficiency because of the induction of oxidative stress, cytotoxicity, and genotoxicity[5,6]. For example, silver nanoparticle exposure significantly reduced root elongation, shoot and root fresh weights, and total chlorophyll and carotenoid contents[7]. Colman *et al.* showed that low silver nanoparticle concentrations caused a decrease in biomass[8]. Furthermore, the toxicity of silver nanoparticles affects the expression of several proteins that are mainly involved in primary metabolism and cell defense in wheat seedlings[9]. At a gene level, silver nanoparticles activate gene expression involved in plant cellular events, including cell proliferation, metabolism, and hormone signaling pathways[10]. The above-mentioned studies showed that silver nanomaterials have high toxicity in plants. Therefore, the risk assessment of silver nanomaterials in plants is important.

Asparagine (N)-linked glycans (*N*-glycans) are comprised several types of monosaccharides, forming complex compositions and linkage types. *N*-Glycan has a trimannosyl core structure [Man alpha1–6(Man alpha1-3) Man beta1-4GlcNAc beta1-4GlcNAc-Asn], which is a common feature in eukaryotes. Many *N*-glycan structures are linked to proteins or peptides and are closely involved in all life phenomena, such as development, signaling,

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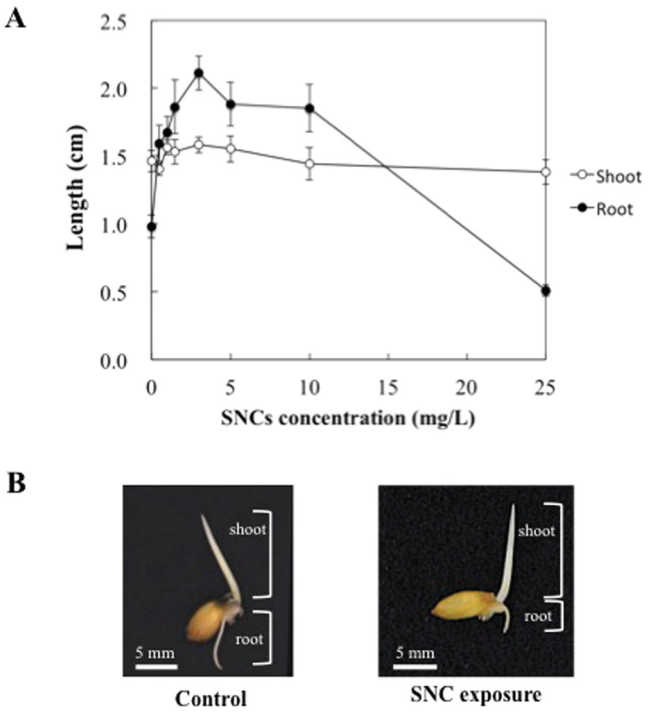

Figure 1. Phenotype analysis of *O. sativa* seedling exposed by SNCs. (**A**) Length of shoots and roots, opened circles indicated shoot and closed circles indicated root. Error bars represent ± one standard deviation from the mean of 20 replicates. (**B**) Overall phenotypes of *O. sativa* seedling.

and cell-to-cell recognition. In plants, *N*-glycan structures are categorized into three main types: high-mannose, complex, and paucimannose types; except for hybrid types. A characteristic of plant-specific *N*-glycans is the addition of beta1,2-xylose and alpha1,3-fucose to the trimannosyl core structure. High-mannose type *N*-glycans are synthesized in endoplasmic reticulum (ER), and other type *N*-glycans are synthesized in the Golgi apparatus. Paucimannose type *N*-glycans are linked to vacuole proteins and complex type *N*-glycans are linked to secretory proteins. In addition, cell alterations are reflected in gene expressions through cell signaling, whereas *N*-glycan is synthesized as a result of the integral expression of glycosyltransferase genes, and *N*-glycan structure is sensitive to slight environmental changes[11]. Therefore, *N*-glycan structural analysis is valuable for the risk assessment of silver nanomaterial toxicity in plants. However, there is little data regarding the toxicity of silver nanomaterials in glycobiology. In this study, to assess the effects of silver nanomaterials, we focused on the correlation of *N*-glycan structures and the effect of silver nanomaterials in *Oryza sativa* and analyzed the *N*-glycan structures in SNC exposed *O. sativa* seedlings.

Results and Discussion

Phenotype analysis of *O. sativa* seedling exposed to silver nanocolloids (SNCs). To observe the effect of SNCs on *O. sativa* seedlings, *O. sativa* seeds were grown with and without SNC exposure. Germination rate was 95% (control), 100% (SNCs 0.5 mg/L), 100% (SNCs 1.0 mg/L), 95% (SNCs 1.5 mg/L), 100% (SNCs 3.0 mg/L), 95% (SNCs 5.0 mg/L), 90% (SNCs 10 mg/L), 100% (SNCs 25 mg/L) after 48 h incubation. The result shows that there is no effect on germination rate at any concentration of SNC for 48 h exposure in *O. sativa*. Figure 1A shows the results of root and shoot elongation in *O. sativa* exposed to SNCs at 0 (control), 0.5, 1.0, 1.5, 3.0, 5.0, 10, and 25 mg/L for 96 h. Shoot and root length of control was 1.46 ± 0.08 cm and 0.98 ± 0.08 cm, respectively. The shoot elongation was not affected at any SNC concentration, whereas the root length increased from 0.5 to 10 mg/L based on Fig. 1A; however, in roots exposed to 25 mg/L SNCs, the lengths were two times lower than those of the control. Representative images of control and 25 mg/L SNC exposed *O. sativa* seedlings are shown in Fig. 1B. From the phenotype analysis, root length was seriously affected by 25 mg/L of SNC exposure.

The effect of SNCs was also observed in other plants. SNCs also have a significant effect on *Arabidopsis thaliana* and poplar development[12]. SNCs are mainly present in two forms: silver nanoparticles and free ions (Ag^+), which are derived from silver nanoparticles. Free Ag^+ is more poisonous than SNCs because of its oxidative potency. Wang *et al.*[12] demonstrated that free Ag^+ tends to accumulate in *A. thaliana* roots. Previous studies also showed that silver nanoparticles or free Ag^+ inhibited the growth of *O. sativa* roots[13], and these materials affect cell metabolism-related proteins[14]. In addition, the effects of SNCs or free Ag^+ occur in ER- and vacuole-localized proteins of *Eruca sativa*[15]. Nair *et al.* reported that total sugar levels are decreased in SNC exposed *O. sativa* seedlings[7]. From these reports and our observation, SNCs and SNC derived molecules may affect *N*-glycans that are synthesized in the ER and Golgi apparatus of *O. sativa* roots. Therefore, we focused on the correlation of *N*-glycan structures and silver nanomaterials in *O. sativa* and analyzed the *N*-glycan structures in SNC exposed *O. sativa* roots.

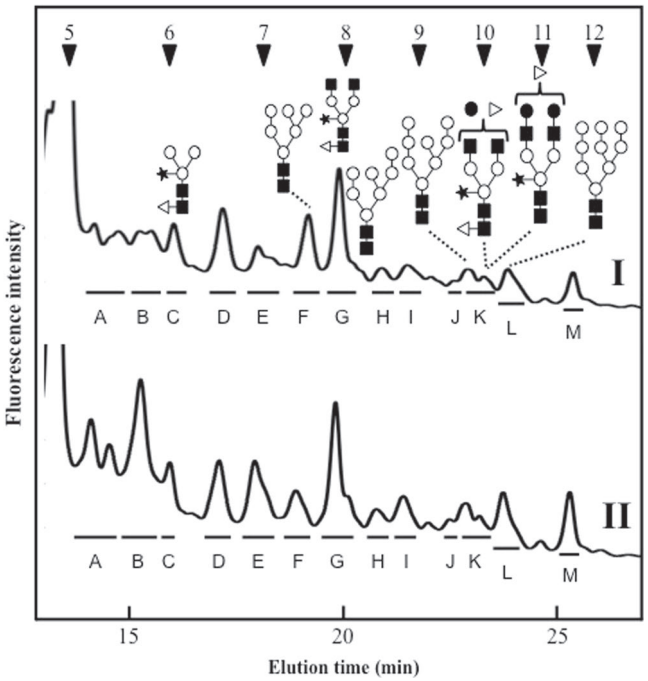

Figure 2. Results of size-fractionation HPLC analysis of PA-*N*-glycans derived from *O. sativa* seedlings. (I) Control, (II) SNCs exposure, PA-*N*-glycans were applied to a Cosmosil $5NH_2$-MS column (4.6 ID × 150 mm). Arrowheads 5–12 indicate the degree of polymerization of PA-isomaltooligomer. The opened circle, closed square, opened triangle, closed star, closed circle represent mannose, *N*-acetylglucosamine, fucose, xylose, and galactose residues, respectively.

***N*-Glycan analysis of SNC exposed *O. sativa* roots.** *N*-Glycans were prepared by hydrazinolysis, *N*-acetylation, and pyridylamination (PA). The resulting PA-*N*-glycans were separated according to their degree of saccharide polymerization by size-fractionation HPLC. Then, *N*-glycans of control and 25 mg/L of SNC exposed roots were compared (Fig. 2I, control and II, SNC treatments). Thirteen peaks were detected (indicated by bars). The areas of peaks B, E, and M increased in SNC exposed roots, and in particular, peak B was extremely increased in SNC exposed roots. Therefore, to identify each *N*-glycan structure in detail, reversed phase HPLC was performed, and branched *N*-glycan isomers were separated. Reversed phase HPLC analysis revealed three major peaks (peaks E1, E2, and E3) of peak E (Fig. 3). Comparing the HPLC elution times with known-position *N*-glycans, peaks E1, E2, and E3 coincided with those of ^{GN}M3FX, $_{GN}$M3FX, and GN2M3X, respectively. Similarly, one major peak, M1, was detected in reversed phase HPLC analysis, and peak M1 coincided with the elution position of Gal2F2GN2M3FX.

Table 1 shows the identified *N*-glycan structures and ratios derived from peaks E1, E2, E3, and M1, and each *N*-glycan is shown in terms of percentage proportion relative to GN2M3FX (peak G). The largest *N*-glycan Gal2F2GN2M3FX was increased three fold after SNC exposure. For other plant complex type *N*-glycans, ^{GN}M3FX, $_{GN}$M3FX, and GN2M3X, SNC exposure caused up to five- or six-fold higher accumulations than the control. In general, complex type *N*-glycans are formed in from the *cis*-Golgi to *medial* Golgi apparatus, and higher complex modifications occur downstream in the synthetic pathway. Recent reports showed that protein-linked complex type *N*-glycans are related to proper targeting and functioning of linking proteins[16,17] and also showed that complex type *N*-glycans play an important role in resistance to external stresses such as salt stress[11,18,19]. These reports showed that the Golgi-localized *N*-glycan synthetic enzymes are related to plant growth and development, and their defect inhibited growth and caused abnormalities. Therefore, the transition of complex type *N*-glycans may be related to the disorder of Golgi-localized *N*-glycan synthetic enzymes and genes in SNC exposed roots. The relatively small *N*-glycans ^{GN}M3FX, $_{GN}$M3FX, and GN2M3X were affected by SNCs more significantly. These results imply that the upstream part of the *N*-glycan complex pathway was affected by SNCs; therefore, the downstream part of the synthetic pathway was less affected for more complicated *N*-glycans such as Gal2F2GN2M3FX. These results may provide evidence that the intermediate complex type *N*-glycans play an important role in plants under excessive stress conditions.

Free-*N*-glycan analysis of SNC exposed *O. sativa* roots. Reversed phase HPLC analysis revealed that peak B1 were eluted at around 3 min; therefore, to purify peak B1, twice reversed phase HPLC was performed (Fig. 4A). Peak B1 was further analyzed using MALDI-TOF mass spectrometry and sequential enzyme digestion. Mass spectrometry analysis of peak B1 showed that the *m/z* ratio was 1143.90 (Na^+), which corresponded to $(Hex)_3(HexNAc)_2(Pent)_1$-PA. The *m/z* ratio and elution position on reversed phase HPLC revealed that the *N*-glycan structure of peak B1 was predicted to be a free-GNM3X structure [$GlcNAc_1Man_3Xyl_1GlcNAc_1$-PA] with one GlcNAc residue at the reducing end. To ascertain the *N*-glycan structure of peak B1, peak B1 was enzymatically digested with two exoglycosidases. Peak B1 [$GlcNAc_1Man_3Xyl_1GlcNAc_1$-PA] was converted

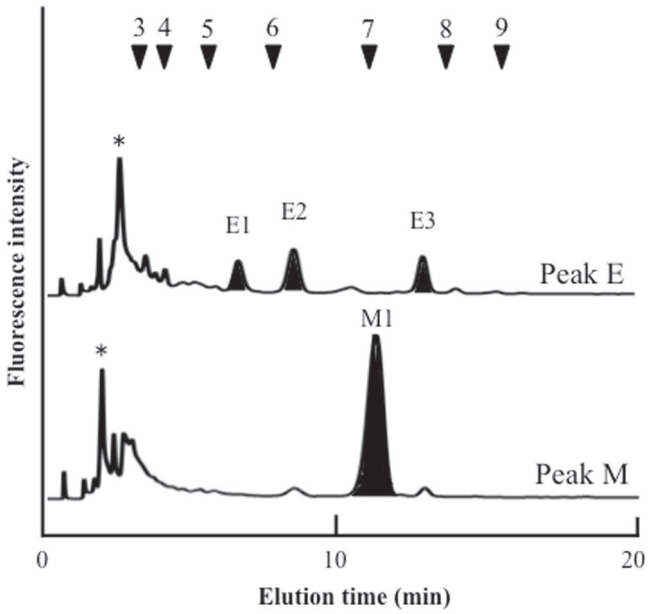

Figure 3. Result of reversed phase HPLC analysis of peaks E and M. The closed areas indicated elution positions of known-*N*-glycan. The peaks marked by the asterisks indicated non-specific peaks.

Peak	Structure	Abbreviation	Ratio	
			Control	SNCs exposure
E1	GlcNAcβ1-2Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA; Xylβ1 on 2; Fucα1 on 3	^{GN}M3FX	3 (0.07)	15 (0.02)
E2	Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA; Xylβ1 on 2; Fucα1 on 3	$_{GN}$M3FX	5 (0.06)	24 (0.01)
E3	GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA; Xylβ1 on 2	GN2M3X	3 (0.04)	18 (0.01)
M1	Fucα1 on 4; Galβ1-3GlcNAcβ1-2Manα1-6(Galβ1-3GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA; Fucα1 on 4; Xylβ1 on 2; Fucα1 on 3	Gal2F2GN2M3FX	18 (0.02)	60 (0.00)

Table 1. Estimated *N*-glycan structures obtained from peaks E1, E2, E3, and M1. Standard errors are in parentheses. Each *N*-glycan was also expressed in terms of percentage proportion relative to the GN2M3FX structure.

to $Man_3Xyl_1GlcNAc_1$-PA, releasing one GlcNAc residue by beta-*N*-acetylhexosaminidase (Fig. 4B-II), and $Man_3Xyl_1GlcNAc_1$-PA was further converted to $Man_1Xyl_1GlcNAc_1$-PA, releasing two mannose residues by alpha-mannosidase (Fig. 4B-III). Thus, peak B1 was assigned as free-GNM3X structure with one GlcNAc residue at the reducing end (Fig. 4B-I).

Peak G is a major component of *O. sativa* roots (Fig. 2). As a result of reversed phase HPLC, two major peaks were detected; as a result of *N*-glycan two-dimensional mapping, the retention time of peak G2 corresponded to that of known position *N*-glycan, GN2M3FX[20], and the other one was eluted at around 3 min (Fig. 5A, peak G1). In a similar way to peak B1, peak G1 was analyzed using MALDI-TOF mass spectrometry and exoglycosidase digestion. Mass spectrometry analysis showed that the *m/z* ratio was 1456.67 (Na^+), which corresponded to $(Hex)_7$ $(HexNAc)_1$-PA. The mass value and elution position of reversed phase HPLC revealed that peak G1 was predicted to be a free-M7 structure [$Man_7GlcNAc_1$-PA]. To confirm the *N*-glycan structure, peak G1 was enzymatically digested with alpha-mannosidase. As a result, peak G1 [$Man_7GlcNAc_1$-PA] was converted to $Man_4GlcNAc_1$-PA, $Man_3GlcNAc_1$-PA, $Man_2GlcNAc_1$-PA, and $Man_1GlcNAc_1$-PA, releasing from three to six mannose residues by alpha-mannosidase (Fig. 5B-II); therefore, peak G1 was assigned as free-M7 structure.

As shown in Table 2, the proportion of free-GNM3X (peak B1) increased six fold after SNC exposure. In contrast to free-GNM3X, free-M7 increased three fold after SNC exposure (Table 2). To date, high-mannose type free-*N*-glycans have been detected in plant during development[21,22], and other complex type free-*N*-glycans have been detected in the culture broth of rice cultured cells[23] and *Egeria densa*[24], and most of them have Lewis a structure [Gal beta1-3(Fuc alpha1-4)GlcNAc beta1-] that is characteristic of the *N*-glycan of

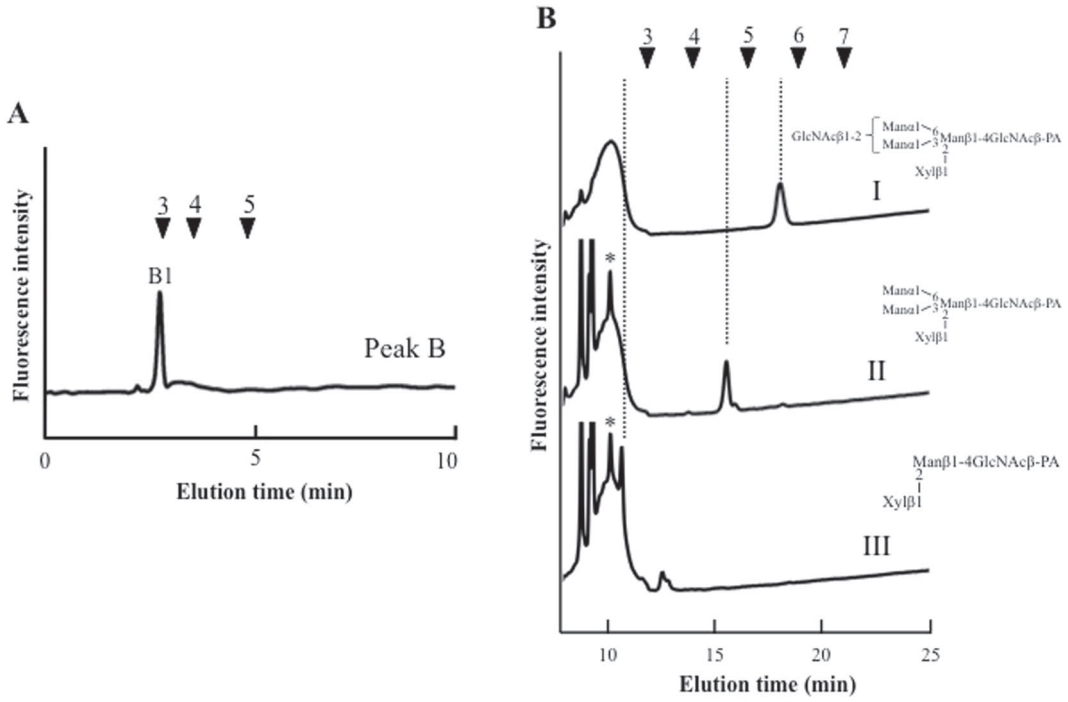

Figure 4. Structural analysis of peak B1. (**A**) Result of second reversed phase HPLC analysis of peak B1. (**B**) Sequential enzyme digestions of peak B1, I: peak B1, II: beta-*N*-acetylhexosaminidase digestion of I, III: alpha-mannosidase digestion of II. The peaks marked by the asterisks indicated non-specific peaks.

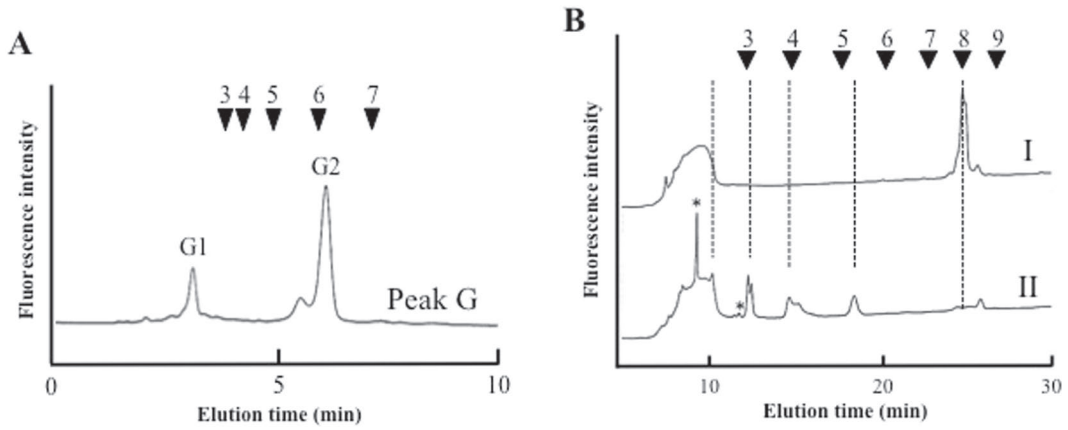

Figure 5. Structural analysis of peak G1. (**A**) Result of reversed phase HPLC analysis of peak G. (**B**) Exoglycosidase digestion of peak G1, I: peak G1, II: alpha-mannosidase digestion of I. The peaks marked by the asterisks indicated non-specific peaks.

extracellular glycoproteins. Maeda *et al.* discussed that a mechanism responsible for the production of complex type free-*N*-glycans is present under special or artificial conditions and native plant tissues[24]. In animal cells, the accumulation of sialyl free-*N*-glycans is caused by a decline in free-*N*-glycan metabolism by basal autophagy[25]. Mkhikian *et al.* demonstrated that alternative *N*-glycan structures were generated under unusual growth conditions[26]. These observations suggest that the occurrence of specific *N*-glycan structures is involved in cell conditions under excessive stress conditions.

The elongation of *O. sativa* shoots was unaffected by SNC exposure (Fig. 1A), and *N*-glycan structures were also unaffected. We suggested that a root defense mechanism serves to protect shoot development from SNC toxicity, and *O. sativa* roots may have a defense mechanism against soil environmental changes. Although the generation of high-mannose type free-*N*-glycans is generally caused by the deglycosylation from misfolded glycoproteins in ER-associated degradation system[27], there is almost no information about the biological significance of complex type free-*N*-glycan. Our results showed that the increase of complex type free-*N*-glycans was caused by the effect of SNCs, suggesting that complex type free-*N*-glycan itself in *O. sativa* roots has an important role for resistance mechanism against excessive environmental changes. *N*-Glycan is one of the post-translational

Peak	Structure	Abbreviation	Ratio	
			Control	SNCs exposure
B1	GlcNAcβ1-2 (Manα1-6 / Manα1-3) Manβ1-4GlcNAcβ-PA; Xylβ1-2	Free-GNM3X	9 (0.00)	58 (0.00)
G1	Manα1-2 Manα1-2 (Manα1-6 / Manα1-3) Manα1-6 / Manα1-3 Manβ1-4GlcNAc-PA	Free-M7	16 (0.02)	45 (0.06)

Table 2. Proposed free-*N*-glycan structures. Standard errors are in parentheses.

modifications, and the structure and quantity are sensitive to ambient environment. In addition, *N*-glycans are generated by the result of the integrated multiple gene expression, and the biosynthesis is strictly controlled by many glycosyltransferases and glycosidases in each specific organelle. Though, it is difficult to identify which genes are specifically affected under stress condition, *N*-glycan analysis can derive the related genes and synthetic regions. Therefore, *N*-glycan analysis can predict to the initial response to environmental changes at comprehensively, and the transition is valuable for studying the relationship between environmental changes and biological response. As the case for monitoring the signaling pathway of plant under environmental changes, novel plant nanobionics approach has been reported[28]. Our results revealed a correlation between free-*N*-glycans and plant development under excessive stress conditions and also demonstrated that free-*N*-glycan transitions are valuable as stress markers for assessing trace environmental changes. This is the first report of the relationship between "environmental changes and glycome transition", and the present study originally adapted glycome transition to environmental toxicology and proposed a new category called "Environmental glycobiology".

Materials and Methods

Chemicals. Purified SNCs (28.4 ± 8.5 nm, release 81.1% Ag^+ suspended in distilled water[29]) were purchased from Utopia Silver Supplements (Utopia, TX, USA). Cosmosil packed columns, $5NH_2$-MS (4.6 ID × 150 mm) and $5C_{18}$-P (4.6 ID × 150 mm), were purchased from Nacalai Tesque (Kyoto, Japan). Known-position PA-sugar chains were purchased from Takara Bio Inc. (Shiga, Japan). alpha-Mannosidase (from jack bean) was purchased from Sigma (MO, USA). beta-*N*-acetylhexosaminidase (from jack bean) was purchased from Prozyme (CA, USA).

Plant materials and sample preparation. Seeds of *O. sativa* (Koshihikari) were supplied by Itakura Agricultural Cooperative Society, Japan. Before each experiment, seeds were washed five times with water and then washed twice with deionized water. The washed twelve seeds were then placed in a plate and immersed in 7 mm depth of water (control) or each concentration of SNCs suspension (0.5, 1.0, 1.5, 3.0, 5.0, 10.0, and 25.0 mg/L of SNCs). The plates were placed in a controlled environmental chamber, and kept at 37 °C for 96 h in the dark. The resulting seedlings were washed and then dried.

Preparation of pyridylaminated *N*-glycans from *O. sativa* seedlings. *N*-Glycan preparation was performed according to the method of Natsuka *et al.*[30]. Dried shoots and roots were ground in a mortar at room temperature, and a ten milligram sample was used. *N*-Glycans were prepared by hydrazinolysis, *N*-acetylation. The reducing ends of the liberated *N*-glycans were then tagged with a fluorophore, 2-aminopyridyne (pyridylaminated *N*-glycans; PA-*N*-glycans), as described in previous paper[20]. These preparations were performed following details in Hase *et al.*[31] with minor modifications.

Separation of PA-*N*-glycans. Size-fractionation HPLC was performed in a Cosmosil $5NH_2$-MS column (4.6 ID × 150 mm) at a flow rate of 0.8 mL/min at 40 °C. PA-*N*-glycans were detected with a fluorescence spectrophotometer at 310 nm excitation and 380 nm emission. Reversed phase HPLC was performed on a Cosmosil $5C_{18}$-P column (4.6 ID × 150 mm) at a flow rate of 1.5 mL/min at 40 °C. The detection of PA-*N*-glycans performed by use of fluorescence spectrophotometer at 315 nm excitation and 400 nm emission. Each HPLC conditions were described in previous paper[20].

Mass spectrometry analysis of PA-glycans. MALDI-TOF mass spectrometry analysis was then performed using an AXIMA resonance instrument (Shimadzu) in reflector mode. Sample preparation was described in previous paper[20].

Glycosidase digestion of PA-*N*-glycans. A two picomoles of PA-*N*-glycans was prepared in 1 microL of accessory reaction buffer (5 mM $CaCl_2$, 10 mM ammonium acetate buffer, pH 4.5) and 2 microL of D. D. W, 1 microL of beta-*N*-acetylhexosaminidase (0.05 units/microL) was added, and the mixture was incubated at 37 °C for 4 h, and then 1 microL of jack bean alpha-mannosidase (19 units/mg) and 5 microL of 10 mM ammonium acetate buffer (pH 4.5) were added, and the mixture was incubated at 37 °C for 1 h. alpha-Mannosidase digestion was described in previous paper[20]. To stop all reactions, the mixtures were boiled for 5 min at 95 °C, and the mixture was analyzed by size-fractionation HPLC.

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Author Contributions

R.H., Y.N., S.K. and N.M. conceived and designed the experiments, R.H. and Y.N. performed sample preparation and *N*-glycan analysis. R.H. wrote the manuscript.

Additional Information

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Sensitivity of medaka (*Oryzias latipes*) to 4-nonylphenol subacute exposure; erythrocyte alterations and apoptosis

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ABSTRACT

The present study was undertaken to assess the effects of the endocrine-disrupting compound; 4-nonylphenol (4-NP) in medaka (*Oryzias latipes*). The frequencies of erythrocyte alterations, apoptosis, and micronuclei were used as biological indicators of damage. Medaka were exposed 15 days to 4-NP at three sublethal concentrations (50, 80, and 100 µg/l 4-NP) and results compared with those of a previous study using catfish as an animal model. Exposure of medaka resulted in a dose-dependent increase in the frequency of erythrocyte alterations, apoptosis and micronucleus (MN). Many morphological alterations and nuclear abnormalities were observed, including acanthocytes, lobed nucleus, eccentric nucleus, fragmented nucleus, blebbed nucleus, binuclei, deformed nucleus, notched nucleus, hemolysed cells, crenated cells, teardrop-like cells, and schistocytes. Mortality was recorded after treatment with 80 and 100 µg/l 4-NP, indicating that medaka are more sensitive than catfish to 4-NP exposure. We concluded that, 4-NP causes several malformations in the shape and number of erythrocytes in medaka, indicating its genotoxicity.

1. Introduction

Increasing environmental pollution, especially chemical pollution, as a result of increasing industrialization is a major problem worldwide. One group of chemicals that causes pollution are the nonylphenol ethoxylates (NPEs), which have been found in aquatic environments, particularly in river water (Clark et al., 1992; Rivero et al., 2008; Tsuda et al., 2000) and have been reported to contaminate aquatic animals including fish and amphibians during their adult life and at sensitive stages of development (Cox, 1996; Giger et al., 1984; Marcomini et al., 1990; Radhaiah et al., 1987; Soto et al., 1991). The breakdown product of these chemicals in aquatic systems is 4-nonylphenol (NP), which is more stable and persistent than the NPEs (Kim et al., 2002; Rivero et al., 2008; Sakai, 2001; Sone et al., 2004; Uguz et al., 2003). It has been shown to be toxic in animals including bees, siders, fish, molluscs, and crustaceans, with hemotoxic, oxidative stressor, estrogenic, histopathological, genotoxic, lethal, and antifertility effects (Cox, 1996; Flouriot et al., 1995; Mekkawy et al., 2011; Rivero et al., 2008; Salanitro et al., 1988; Sayed and Ismail, 2017; Sayed et al., 2012; Sayed et al., 2011; Sayed et al., 2016b; Servos, 1999; Staples et al., 2004; Vazquez-Duhalt et al., 2005).

The induction by various chemicals of micronuclei (MN) and abnormal erythrocyte morphology in fish has been studied to evaluate water quality, the health of species and potential risks (Al-Sabti and Metcalfe, 1995; Ferraro et al., 2004; Grisolia and Starling, 2001; Talapatra and Banerjee, 2007). Fish erythrocytes are favored because the frequency of alterations in both cytoplasm and nuclei can be a marker of cytotoxicity, genotoxicity, hemotoxicity, and oxidative stress (Bushra et al., 2002; Gomes et al., 2015; Joshi et al., 2002; Mekkawy et al., 2011; Sayed et al., 2017; Sayed et al., 2016c).

The frequency of alterations in fish erythrocytes, the induction of MN, and their apoptosis have been studied after exposure UVA, pesticides, hydrocarbons, and toxins (Bahari et al., 1994; Bombail et al., 2001; Bushra et al., 2002; Mekkawy et al., 2011; Sayed et al., 2016c; Talapatra and Banerjee, 2007). Apoptosis is associated with signs of abnormal cell morphology including cell shrinkage, membrane blebbing and chromatin condensation (Cavas et al., 2005; Murakawa et al., 2001; Sayed and Hamed, 2017; Talapatra and Banerjee, 2007).

Medaka (*Oryzias latipes*) are frequently used as an animal model for aquatic toxicology because they are prolific daily egg-layers under ideal breeding conditions, mature quickly and are a small size that allows for large sample sizes in treatment groups (Sayed et al., 2017; Sayed et al.,

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2014). Medaka erythrocytes have been used for genotoxicity studies after UVA- and gamma-IR (Sayed et al., 2014; Sayed et al., 2016a; Sayed et al., 2016b; Sayed et al., 2017). The present study is the first to examine the ability of 4-NP to induce apoptosis, MN, and morphological alterations in blood erythrocytes of medaka compared with its reported effects in the African catfish, *Clarias gariepinus*.

2. Materials and methods

2.1. Medaka

Sexually mature WT (Hd-rR) adult female medaka (*O. latipes*) were used. The fish were kept at 26–28 °C under a 14 h light/10 h dark cycle. They were fed a powdered diet (Tetra-min, Tetra Werke Co., Mells, Germany) and brine shrimp (*Artemia franciscana*) three times a day.

2.2. Nonylphenol

4-NP was obtained from Dr. Ehrenstorfer GmbH, Augsburg, Germany (purity, 99.9%).

2.3. Experimental setup

The adapted adult fish were subdivided into four groups (six fish per group): a control group and three 4-NP-treated groups (50, 80, and 100 µg/l daily for 15 days). The 4-NP doses were selected based on those used by Mekkawy et al. (2011). The experimental conditions during the experiment were the same as during acclimatization with half the volume of water and 4-NP changed every day. Physicochemical parameters and the mortality rate were assessed (Table 1).

2.4. Blood sample collection

Blood samples were collected from the caudal veins of the control and 4-NP treated fish. Each sample was immediately placed on ice to prevent endogenous DNA damage during sample preparation and to inhibit DNA repair in the unfixed cells (Collins, 2004). Blood smears were prepared on clean glass slides (triplicate slides from each fish).

2.5. Apoptosis detection

Apoptotic erythrocytes were detected using acridine orange (AO) staining (Cat. No. A1031, Life Technologies, Carlsbad, CA, USA). A modified protocol (Sayed et al., 2016a) was used to detect apoptosis in red blood cells (RBCs). In brief, after preparation of blood smears on clean glass slides, the slides were washed in 1 × phosphate-buffered saline (PBS) (pH = 7.2). AO buffer (17 µg/l AO in 1 × PBS) was added to the slides for 30 min in the dark. Decolorization was achieved by washing the slides every 30 min for 2 h with 1 × PBS. Fixation was in 4% paraformaldehyde for 5 min. Cells were observed under a fluorescence microscope (BX50; Olympus) equipped with a digital color video camera (DP-70; Olympus).

2.6. Micronucleus, nuclear abnormalities, and morphological alterations

The smears were fixed by dipping the slides in absolute methanol, allowing them to air-dry, and then staining with May-Grünwald solution for 15 min, followed by 6% Giemsa stain for 30 min as reported previously (Tavares-Dias and Moraes, 2003). Slides were selected on the basis of staining quality, then coded, randomized, and scored by person blinded to treatment. In each group, 10,000 cells (minimum of 1000 per slide) were examined using a previously published method (Al-Sabti and Metcalfe, 1995) under a 40 × objective with a 10 × eyepiece to identify MN and morphologically-altered erythrocytes in separate studies. Established criteria for identifying MN (Schmidt, 1975) were followed strictly to ensure accurate scoring.

2.7. Statistical analysis

The mean, standard division, and range were calculated for all values. Differences between groups were evaluated by one-way analysis of variance using SPSS software (SPSS, 1998) at the 0.05 significance level and Dunnett's posttests treating one group as the control and comparing it with all the other groups.

2.8. Ethics statement

All experiments were performed in accordance with the Japanese laws and guidelines for the care of experimental animals and The University of Tokyo Animal Experiment Enforcement Rules. The protocols were approved by the Committee on the Ethics of Animal Experiments of The University of Tokyo (Permit Number: C-14-02).

3. Results

3.1. Detection of morphological alterations, nuclear abnormalities and apoptosis in erythrocytes

Fig. 1a shows a blood smear from normal fish and demonstrates the normal structure of medaka's erythrocytes, which are oval with a centrally located nucleus.

Exposure of medaka to sublethal concentrations of 4-NP (50, 80, and 100 µg/l) resulted in morphological changes in the erythrocytes and the appearance of some abnormal types of cells. The major alterations in the erythrocytes of the fish treated with 50 µg/l 4-NP (Fig. 1b) were the appearance of acanthocytes (Ac); erythrocytes with fewer projections from their surface. Fig. 1c and d shows other morphological changes in the erythrocytes of fish treated with 80 µg/l 4-NP, including echinocytes or crenated cells (Cr), where the red blood cells develop an irregular cell surface with numerous projections, Teardrop-like cells (Tr), whose shape resembles a tear with pointed apices, schistocytes (Sh), notched nuclei (Non), and hemolysed cells (HC), where lysis the cell membrane had occured.

The fish exposed to 100 µg/l 4-NP showed many alterations in their blood (Fig. 2a–d) including acanthocytes. In addition, many nuclear abnormalities were apparent including fragmented nuclei (Fn), deformed nuclei (Dn), eccentric nuclei (Ecn), lobed nuclei (Ln), and Mn, one or more Mn were present per cell in most observations, notched

Table 1
Physiochemical parameters and mortality rate (mean ± SE) after exposure of adult female medaka *Oryzias latipes* to different doses of 4-nonylphenol (4-NP).

100 µg/l 4-NP	80 µg/l 4-NP	50 µg/l 4-NP	Control	Treatments Parameters
4.5 ± 0.28 (5.6 – 3.2)[a]	5.35 ± 0.63 (8 – 2.5)[a]	5.85 ± 0.64 (8.8 – 3.2)[a]	5.51 ± 0.40 (7.9 – 4.1)[a]	Dissolved Oxygen (mg/l)
6.95 ± 0.12 (7.6 – 6.66)[b]	7.24 ± 0.13 (7.7 – 6.81)[ab]	7.3875 ± 0.12 (7.9 – 7)[ab]	7.74 ± 0.20 (8.6 – 7)[a]	pH
24.35 ± 0.12 (24.8 – 23.8)[a]	24.44 ± 0.13 (25 – 24)[a]	24.15 ± 0.14 (24.8 – 23.6)[a]	24.54 ± 0.69 (29.3 – 23.4)[a]	Temperature °C
0.13 ± 0.13 (1 – 0.00)[a]	0.13 ± 0.13 (1 – 0.0)[a]	0.00 ± 0.00 (0.00 – 0.00)[a]	0.00 ± 0.00 (0.0 – 0.0)[a]	Mortality rate%

* Different letters indicate there is a significant difference at ($p \leq 0.05$).

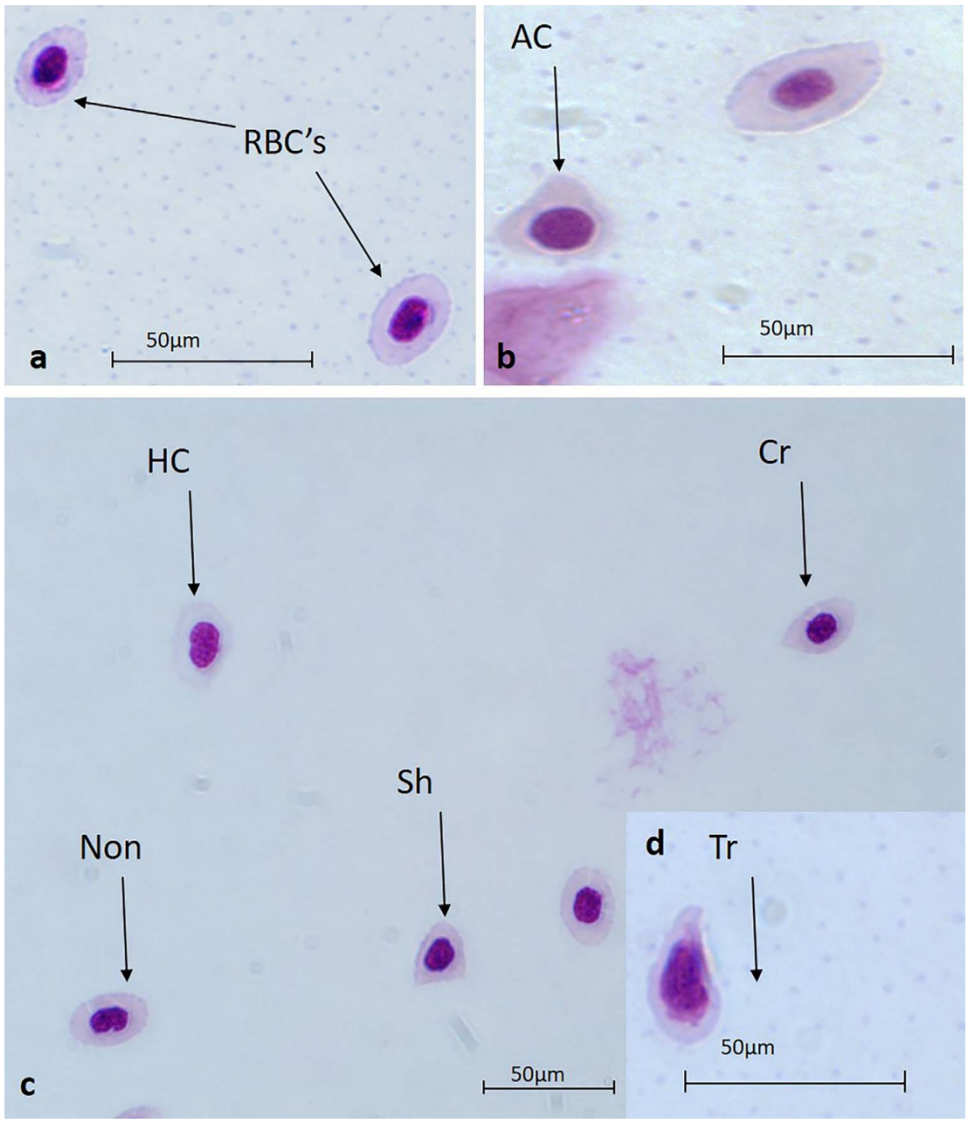

Fig. 1. Giemsa staining of blood film from WT (Hd-rR) adult female medaka (*Oryzias latipes*) showing morphological alterations, (a) WT control; (b) WT after 50 µg/l 4-NP exposure for 15 days; (c and d) WT after 80 µg/l 4-NP exposure; RBC's red blood cells; Ac, acanthocytes; Hc, hemolysed cell; Cr, crenated cells; Tr, teardrop-like cells; Non, notched nucleus; Sh, schistocyte. (Scale bar = 50 µm).

nuclei (Non), binuclei (Bin), and blebbed nuclei (Bn).

Fig. 3 shows typical acridine-orange stained apoptotic erythrocytes in which appear light green under the microscope, from the different NP-treated groups and controls.

3.2. Variation in altered, apoptotic and MN erythrocytes

At all 4-NP doses tested the frequency of erythrocyte cytoplasmic abnormalities including nuclear abnormalities (Fn, Bin, Ln, Ln, ecn, and Non) was elevated compared with controls, with the highest frequency at 100 µg/l 4-NP exposure (Table 2).

Compared with controls, a significant increase in the frequency of apoptotic erythrocytes was observed in fish exposed to 4-NP with the highest frequency observed in fish exposed to 100 µg/l 4-NP (24.3 ± 3 0.36). The frequencies of apoptosis as a function of 4-NP dose are presented in Table 2.

As shown in Table 2, MN increased in the groups treated with 4-NP compared with the control one and the percentage of MN increased significantly with increasing 4-NP dose ($p \leq 0.05$). The percentage of MN was 0.3 ± 0.15% in controls, 1.5 ± 0.27% in fish treated with 50 µg/l 4-NP, 2.9 ± 0.53% in those treated with 80 µg/l 4-NP, and 6.5 ± 0.95% in those treated with 100 µg/l 4-NP.

4. Discussion

The toxic effects of 4-NP have been studied using different animal models, which indicated that, these effects differ between species. The aim of this study was to assess and compare the adverse effects of different doses of 4-NP in *O. latipes* with those reported for *C. gariepinus* (Mekkawy et al., 2011) using selected biomarkers.

Such toxicological research in fish requires biomarkers that can provide an early warning of effects in different organs. Although because of the high impact of using fish erythrocytes as individual cells for environmental pollution studies, some recent studies have used piscine cell lines for environmental toxicity testing (Bols et al., 2005), in this study we used erythrocytes as the target organ for detection of cytotoxicity and genotoxicity to allow the use of simple, reliable, rapid, and sensitive techniques such as measuring the frequency of erythrocyte alterations, nuclear abnormalities including MN, and apoptosis (Fig. 4).

Using different test methods, 4-NP has been shown to be toxic in different types of fish at concentrations from 17 to 3000 µg/l (Servos, 1999). The LC50 pf NP for *O. latipes* was reported to be from 1 to 10,000 µg/l (Yoshimura, 1986). We selected the doses used in this study based on these previous data and the comparison study (Mekkawy et al., 2011), even though they are high and are very rarely seen in aquatic systems.

Many studies have scored morphological and nuclear abnormalities

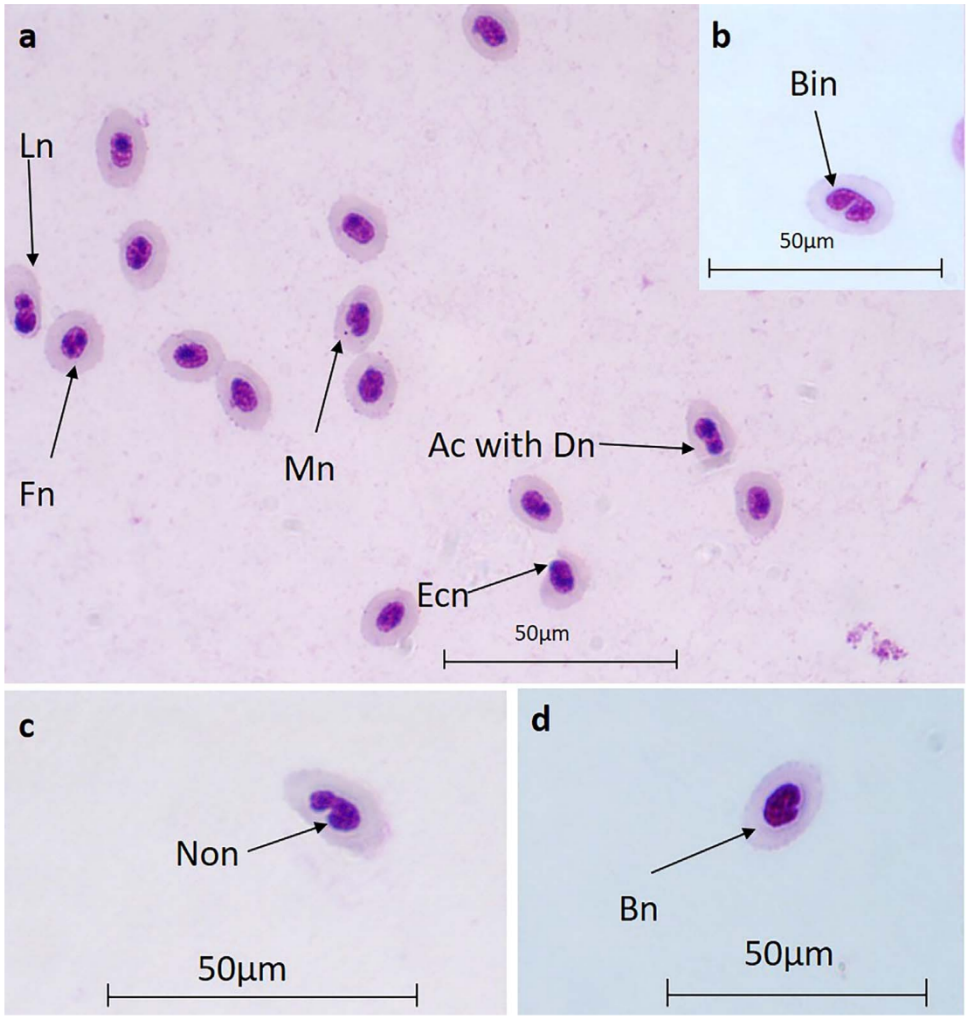

Fig. 2. Giemsa staining of blood film from WT (Hd-rR) adult female medaka (*Oryzias latipes*) showing morphological alterations, (b) WT after 100 µg/l 4-NP exposure for 15 days; Ac, acanthocytes; Ln, lobed nucleus; Ecn, eccentric nucleus; Fn, fragmented nucleus; Mn, micronucleus; Bin, binuclei; Dn, deformed nucleus; Non, notched nucleus; Bn, blebbed nucleus. (Scale bar = 50 µm).

in erythrocytes together with MN as biomarkers for genotoxicity after exposure to radiation and chemical pollution (Ayllon and Garcia-Vazquez, 2000; Ergene et al., 2007; Gomes et al., 2015; Mekkawy et al., 2011; Sayed et al., 2013; Sayed et al., 2017; Sayed et al., 2016c; Sayed et al., 2015b; Sharma et al., 2014). Because of the close relationship between DNA damage and the frequencies of these biomarkers, and their simplicity, reliability, and sensitivity, erythrocyte alterations including nuclear abnormalities are considered to be as a powerful tool for the study of genotoxic and cytotoxic damage in eukaryotes (Al-Sabti and Metcalfe, 1995; Gomes et al., 2015; Mekkawy et al., 2011; Sayed et al., 2017; Sayed et al., 2016c).

Apoptosis induction after 4-NP treatment has been reported in tissue and blood cells of many fish species (Mekkawy et al., 2011; Miura et al., 2005; Murakawa et al., 2001; Sayed and Hamed, 2017; Weber et al., 2003; Yi et al., 2009). One of the causes of apoptosis is DNA damage; we will confirm the existence of such damage in medaka erythrocytes in the second part of this study using γ-H2AX (data under preparation). It has been suggested that the high level of erythrocyte damage and apoptosis seen in fish erythrocytes is because of their short lifespan (1–3 months) (Udroiu, 2006). However, it has also been suggested that the genetic instability caused by toxins could act directly to cause the increased erythrocyte and nuclear abnormalities (ENAs) (Gomes et al., 2015). Our results have demonstrated that many types of ENAs were observed after 4-NP exposure. The study by Gomes et al. (2015) shed light on a possible mechanism underlying the occurrence of different ENAs in genotoxicity studies. In the present study, the frequency of MN increased after 4-NP exposure in a linear dose-dependent manner. These results are consistent with others indicating a dose-dependent increase in damage after exposure to 4-NP (Al-Sharif, 2012; Mekkawy et al., 2011; Sayed and Ismail, 2017; Sharma and Chadha, 2017), carbosulphan (Nwani et al., 2011), 2,4-dicholorophenoxy acetic acid (Ateeq et al., 2005), UVA (Sayed et al., 2013; Sayed et al., 2016c) and arsenic (Sayed et al., 2015a).

Sharma et al. (2014) reported the cytotoxic effects of NP as a decrease in the ratio of polychromatic/normochromatic erythrocytes. They attributed this decrease to either direct cytotoxicity or DNA damage leading to apoptosis. Our results support this concept because exposure to 4-NP induced erythrocyte alterations, nuclear abnormalities and apoptosis in all exposed groups.

Lysis of erythrocytes leading to a reduction in their hemoglobin content causing a type of macrocytic anemia was recorded after 4-NP exposure in *C. gariepinus* (Satyanarayanan et al., 2011; Sayed et al., 2011). The direct damage to erythrocyte membranes presented in that study was detectable by an increase in MCV and MCH after 4-NP exposure (Satyanarayanan et al., 2011; Sayed et al., 2011). In contrast, some authors have attributed cellular damage after toxin exposure to an increase in several enzymes (ALT, AST, and ALP) in fish (Mekkawy et al., 2010; Sayed et al., 2011).

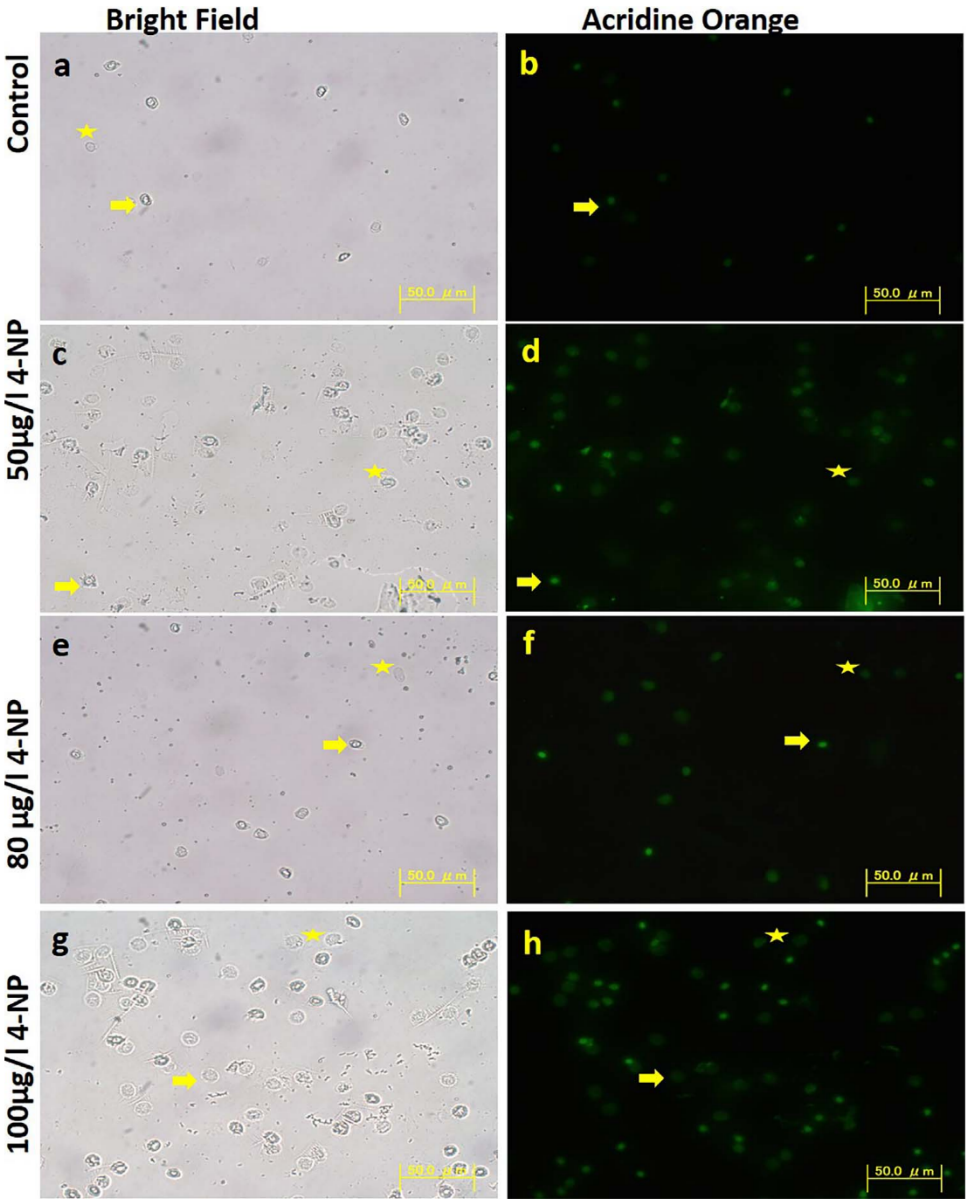

Fig. 3. Apoptosis detection in sexually mature WT (Hd-rR) adult female medaka (*Oryzias latipes*). (a and b) control; (c and d) WT after 50 µg/l 4-NP exposure for 15 days (e and f) WT after 80 µg/l 4-NP exposure for 15 days; (g and h) WT after 100 µg/l 4-NP exposure for 15 days. Cells fluorescing light green after acridine orange staining were considered apoptotic. Arrowheads indicate apoptotic cells, asterisks indicate nonapoptotic cells. (Scale bar = 50 µm).

Because of the importance of DNA for life, clarification of the risk associated with the DNA-damaging effects of 4-NP requires more scientific reports research (Sharma and Chadha, 2017). Recently, it has been reported that the nuclear abnormalities observed after gamma-irradiation of medaka were associated with DNA double-strand breaks, with a strong relationship between these alterations and phosphorylation after γ-H2AX staining (Sayed et al., 2017). Gomes et al. (2015) in a study of the effects of exposure to cadmium of *Oreochromis niloticus*, suggested a possible explanation for the high level of apoptosis in the present study: it may be the result of the cell's response to DNA damage by arresting the cell cycle to assist in DNA repair or by initiating apoptosis to remove damaged cells.

5. Conclusion

The present study showed that, 4-NP is cytotoxic to medaka because it induced a dose-dependent significant increase in erythrocyte alterations, nuclear abnormalities, and apoptosis. These effects may suppress

Table 2
Percentage of altered, apoptotic and micronucleated erythrocytes (mean ± SE) % after exposure to different doses of 4-nonylphenol per 100 cells of WT (Hd-rR) adult female medaka *Oryzias latipes*.

100 µg/l 4-NP (n = 5)	80 µg/l 4-NP (n = 5)	50 µg/l 4-NP (n = 6)	Control (n = 6)	Treatments Parameters
49.9 ± 6.06 (67 – 11)^{a}	19.1 ± 1.91 (22 – 3)^{b}	3.1 ± 0.64 (6 – 0)^{b}	1.1 ± 0.28 (2 – 0)^{b}	Altered cells
24.3 ± 3.36 (36 – 12)^{a}	5.3 ± 0.73 (9 – 2)^{b}	4.2 ± 3.95 (41 – 0)^{b}	2.2 ± 0.63 (7 – 1)^{b}	Apoptotic cells
6.5 ± 0.95 (10 – 1)^{a}	2.9 ± 0.53 (5 – 0)^{b}	1.5 ± 0.27 (3 – 0)^{bc}	0.3 ± 0.15 (1 – 0)^{c}	MN

*Different letters indicate there is a significant difference at ($p \leq 0.05$)

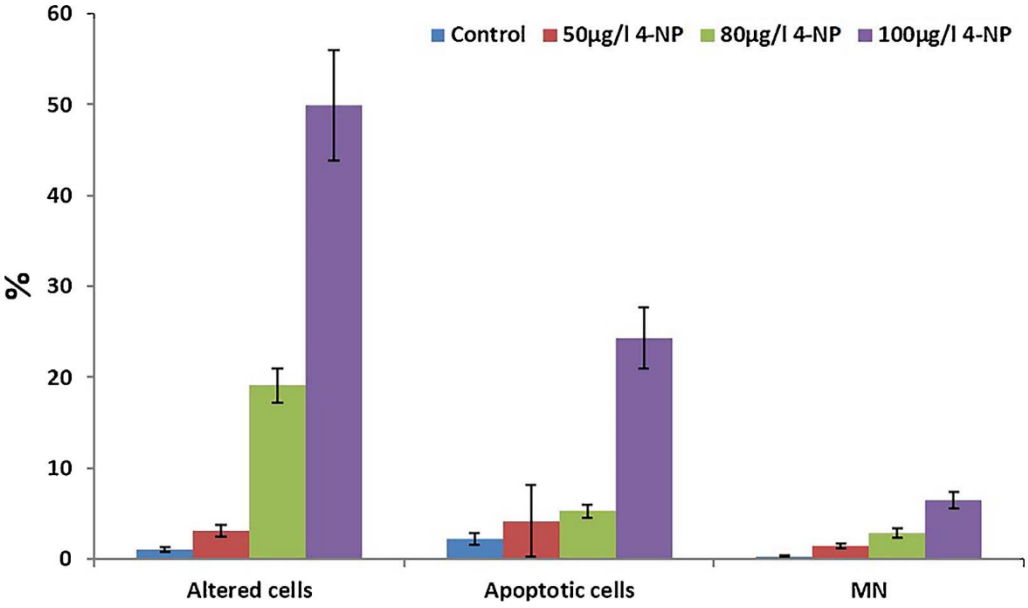

Fig. 4. Percentage of altered, apoptotic and micronucleated erythrocytes (mean ± SE) after exposure to different doses of 4-NP per 100 cells of WT (Hd-rR) adult female medaka *Oryzias latipes*.

normal growth, biological activities, and immunity in both natural and culture environments. Medaka; WT (Hd-rR) is more sensitive to 4-NP than *Clarias gariepinus* and *channa punctaus*, indicating that it is an excellent animal model for toxicological studies.

Declaration of interest statement

We declare no conflicts of interest.

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Comparative toxicities of silver nitrate, silver nanocolloids, and silver chloro-complexes to Japanese medaka embryos, and later effects on population growth rate☆

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ABSTRACT

Fish embryo toxicology is important because embryos are more susceptible than adults to toxicants. In addition, the aquatic toxicity of chemicals depends on water quality. We examined the toxicities to medaka embryos of three types of silver—$AgNO_3$, silver nanocolloids (SNCs), and silver ions from silver nanoparticle plates (SNPPs)—under three pH conditions (4.0, 7.0, and 9.0) in embryo-rearing medium (ERM) or ultrapure water. Furthermore, we tested the later-life-stage effects of SNCs on medaka and their population growth. "Later-life-stage effects" were defined here as delayed toxic effects that occurred during the adult stage of organisms that had been exposed to toxicant during their early life stage only. $AgNO_3$, SNCs, and silver ions were less toxic in ERM than in ultrapure water. Release of silver ions from the SNPPs was pH dependent: in ERM, silver toxicity was decreased owing to the formation of silver chloro-complexes. SNC toxicity was higher at pH 4.0 than at 7.0 or 9.0. $AgNO_3$ was more toxic than SNCs. To observe later-life effects of SNCs, larvae hatched from embryos exposed to 0.01 mg/L SNCs in ultrapure water were incubated to maturity under clean conditions. The mature medaka were then allowed to reproduce for 21 days. Calculations using survival ratios and reproduction data indicated that the intrinsic population growth rate decreased after exposure of embryos to SNC. SNC exposure reduced the extinction time as a function of the medaka population-carrying capacity.

1. Introduction

Because of the emergence of silver nanotechnology and the global growth of related industries, the effects of silver on aquatic environments are being studied, and silver nanotoxicology is emerging as a research area. Silver is comparatively rare in the Earth's crust. Although silver concentrations tend naturally to be elevated in crude oil and in water from hot springs and steam wells, anthropogenic sources can also be associated with elevated concentrations of silver (Howe and Dobson, 2002b). The maximum concentrations of total silver that have been reported in aquatic systems are 6.0 μg/L (groundwater), 260 μg/L (river water), and 300 μg/L (treated photoprocessing wastewater) (Howe and Dobson, 2002a). Silver concentrations are relatively high in aquatic organisms near sewage outfalls, electroplating plants, and mine waste dumps (Eisler, 1996). In addition to conventional anthropogenic silver sources, emerging new silver nanomaterial products likely are contributing to predicted increases in silver concentrations in aquatic environments. According to maximum scenario modeling, silver concentrations are expected to reach 18 μg/L in sewage treatment plants and 320 ng/L in river water (Blaser et al., 2008). Furthermore, the release of silver ion, which is very toxic to organisms, from silver is pH dependent (Kashiwada et al., 2012), thus prompting concern because of the wide range of pH of environmental waters (~4–~12) (Schwedt, 2001). The fate of silver and

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silver ion in aquatic environments is currently poorly understood.

Various organisms have been used to investigate the biological effects of silver nanomaterials in aquatic environments. These species include algae (Navarro et al., 2015), water fleas (Kim et al., 2016; Sakamoto et al., 2014), trout (Salari Joo et al., 2013), carp (Oprsal et al., 2015), sea urchins (Magesky and Pelletier, 2015), and coral (Suwa et al., 2014). In addition, medaka and zebrafish are frequently used as small-fish models for nanotoxicology. The eggs of these two species have similar advantages for embryogenesis research (i.e., a transparent chorion and rapid embryo development), and sufficient genomic information is available on both. Previously, we demonstrated that silver nanocolloids (SNCs) interfere with medaka embryogenesis by disrupting vital gene expression (Kashiwada et al., 2012). Moreover, we have found that Ag^+ released from SNCs and from the silver chloro-complexes that form from Ag^+ and Cl^- ions induces silver toxicity in medaka under environmentally relevant conditions (Kataoka et al., 2015). In addition, the toxicity of Ag^+ is higher than that of silver chloro-complexes; the LC_{50} value of SNCs in ultrapure water (0.050 mg/L; 95% confidence limit, 0.039 to 0.070 for 96 h at pH 7.0) is lower than that in embryo-rearing medium (ERM), which contains chloride ions (>10 mg/L for 96 h at pH 7.0) (Kataoka et al., 2015).

In nanotoxicology, $AgNO_3$ has been used as a reference silver-ion source in toxicological studies of nanosized silver (Newton et al., 2013; Scown et al., 2010; van der Zande et al., 2012), but $AgNO_3$ also includes NO_3^-, and a silver-ion source devoid of nitrate ions is needed. In this regard, all three silver nanomaterials coated with different materials are three to 10 times less toxic to medaka eggs than $AgNO_3$ on a mass-concentration basis (Kwok et al., 2012); thus, the toxicity of silver nanomaterials depends on how efficiently silver ions are released from the nanomaterials. Both $AgNO_3$ and silver nanomaterials show similar toxicity to adult medaka at silver ion concentrations of 10–100 μg/L (Kim et al., 2011). Indeed, silver nanomaterials are sources of dissolved silver (silver ions or silver chloro-complexes or both), and the silver nanomaterials themselves contribute to toxicity in fish (Kwok et al., 2012). Not only the resulting concentration of dissolved silver but also the size of the nanomaterial may be important for toxicity. For example, the growth, reproduction, and behavior of *Caenorhabditis elegans* are adversely affected by ZnO nanoparticles in a particle-size-dependent manner (Khare et al., 2015). In another study (Neubauer et al., 2015), the production of reactive oxygen species depended on the size of palladium and nickel nanoparticles.

The biological effects associated with metal nanomaterials are known to be due to the presence of dissolved metals (ions and complexes) as well as reactive oxygen species; irritation can also occur through contact with the nanomaterials themselves. However, determining the ecological risks posed by chemicals simply by using individual toxicity data is difficult. The probability of population extinction is determined partly by the reduction in the population's intrinsic growth rate as a result of acute and chronic toxicity (Stark et al., 2004). Ecological parameters such as population growth are well known to be important in estimating the ecotoxicological effects on an organism's growth and reproduction (Gentile et al., 1982). To achieve more realistic ecological risk assessments of chemicals, we need to use indices of population dynamics, such as population carrying capacity relative to time to extinction (Lande, 1993). Previously, we successfully assessed the ecological risk of the neurotoxic insecticide carbaryl on medaka populations by using later-life viability and population growth rate (Kashiwada et al., 2008).

Here, to investigate and compare the biological effects of different types of silver on medaka eggs at different pH, we developed a silver nanoparticle plate (SNPP), in which silver nanoparticles are fixed to the bottoms of the wells in a six-well plastic plate. Only silver ions (without nitrate ion) are released from the adhered nanoparticles and into solution for exposure of medaka embryos. We conducted a comparative toxicity study of $AgNO_3$, SNCs, and dissolved silver ions from SNPPs in medaka embryos under different pH conditions. Furthermore, we determined the ecological risk of SNCs to medaka embryos. To examine the later-life effects of SNC exposure on medaka embryos, we used later-life survival ratios and reproduction data to calculate the per-capita growth rate; we then simulated the time to extinction as a function of the environment's medaka population carrying capacity as an indicator of the ecological risk posed by SNCs to medaka populations.

2. Materials and methods

2.1. Medaka eggs (embryos)

Japanese medaka (*Oryzias latipes*, orange-red strain, inbred line) were obtained from the medaka broodstock of the National Institute for Environmental Studies (Tsukuba, Japan). Breeding groups of medaka were incubated at Toyo University and were fed *Artemia salina* nauplii and Otohime β-2 (Marubeni Nissin Feed, Tokyo, Japan). The fish were maintained in a medaka culture system (Small Fish Culture System Type Meito-Hikosaka, Meitosuien, Nagoya, Japan). Tap water, which was dechlorinated by using an activated carbon filter and temperature controlled (25 ± 0.5 °C), was supplied (pH 7.8) to the medaka culture system. Room conditions included a 16:8-h light:dark photocycle and a temperature of 25 ± 0.5 °C.

Spawned eggs were harvested from female medaka, and fertilized eggs were collected under a dissecting microscope (model M165-FC, Leica Microsystems, Tokyo, Japan). The fertilized eggs were rinsed with ERM, which was composed of 1.0 g NaCl, 0.03 g KCl, 0.04 g $CaCl_2 \cdot 2H_2O$, and 0.163 g $MgSO_4 \cdot 7H_2O$ in 1 L of ultrapure water; the pH was adjusted to 7.2 by using 1.25% $NaHCO_3$ in water solution, and the medium was then filter-sterilized. Egg embryos were placed in the ERM and incubated at 25 ± 0.1 °C.

Stage 21 medaka embryos (at the brain regionalization and otic-vesicle formation stage (Iwamatsu, 1994)) were used in these experiments. The medaka embryo goes through 39 developmental stages before hatching; we chose stage 21 because it is the one most susceptible to the effects of SNCs (Kashiwada et al., 2012). Stage 21 embryos were harvested, rinsed with ultrapure water or ERM, and used immediately. All reagents were purchased from Nacalai Tesque (Kyoto, Japan).

2.2. SNCs

Purified SNC solution (20 mg/L; 99.99% purity; particle diameter in distilled water, 28.4 ± 8.5 nm) was purchased (Utopia Silver Supplements, Utopia, TX, USA). The purity and concentration of the silver were validated by inductively coupled plasma–mass spectroscopy (ICP-MS) analyses (X series 2, Thermo Scientific, Pittsburgh, PA, USA). Transmission electron microscopy (TEM) of SNCs was performed (model 2100, JEOL, Tokyo, Japan) (Fig. 1a). SNC solutions (mixtures of SNCs and silver ions) for exposure tests were prepared in ultrapure water or ERM at three different pH values (4.0, 7.0, or 9.0). The pH of the test solution was adjusted by using minimal-drop additions of 0.1 mol/L HNO_3 solution (for pH 4), 1.25% $NaHCO_3$ in water (for pH 7), or 0.1 mol/L NaOH solution (for pH 9). Distributions of particle size (diameter) and the zeta potential of 1 mg/L SNCs at pH 4.0, 7.0, or 9.0 were measured (Zetasizer Nano ZS analyzer, Malvern Instruments, Malvern, Worcestershire, United Kingdom). Before the pH adjustment, the zeta potential of the SNC solution was −28.68 mV. In the pH-adjusted ultrapure water (pH 4.0, 7.0, or 9.0), the three peaks of the zeta potential of the SNCs

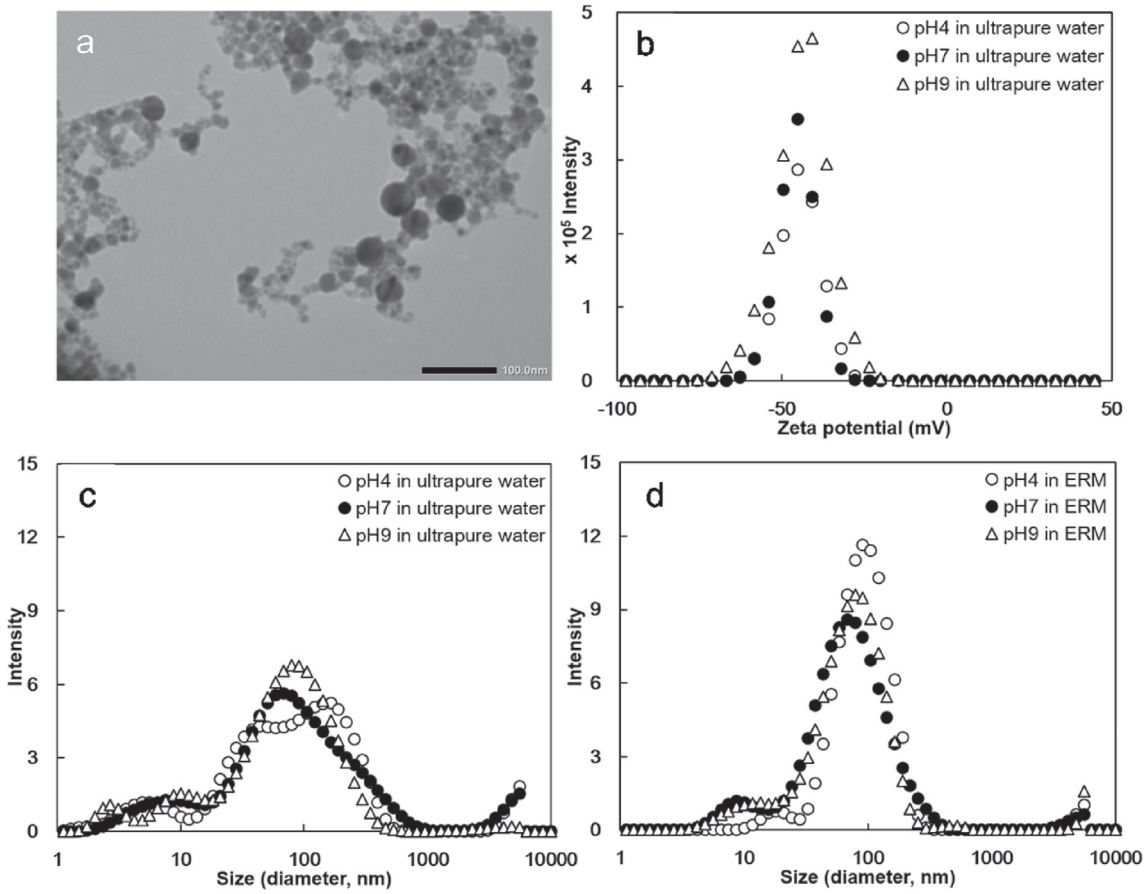

Fig. 1. Transmission electron microscopy (TEM) image of silver nanocolloids (SNCs), and graphs of their size distribution and zeta potentials in ultrapure water and embryo-rearing medium (ERM) at different pH values. TEM image of SNCs (a). Zeta potential (mV) in ultrapure water (b). Size distribution of SNCs in ultrapure water (c). Size distribution of SNCs in ERM (d). All parameters were measured at pH 4.0, 7.0, and 9.0.

overlapped at about −48 mV (Fig. 1b). The particle size distributions had peaks at 164.2 nm (with a shoulder peak at 43.8 nm; pH 4.0), 70.8 nm (pH 7.0), and 85.1 nm (pH 9.0) (Fig. 1c). In the pH-adjusted ERM, the particle size distributions had peaks at 96.1 nm (pH 4.0), 67.8 nm (pH 7.0), and 82.4 nm (pH 9.0) (Fig. 1d). Generally, particles with a zeta potential greater than about ±30 mV tend to be dispersed, rather than aggregated, in solution. Although we were unable to measure the zeta potential in the ERM because of the presence of electrolytes, the SNCs in the pH-adjusted ultrapure water or ERM were estimated to be well dispersed, despite the presence of electrolytes (HNO_3, $NaHCO_3$, NaOH, and other components) in ERM.

2.3. SNPPs for toxicological studies of free silver ion

We developed SNPPs for testing the toxicological effects of silver ions in the absence of nitrate ion and related compounds formed in charge-balanced solutions (Fig. S1).

2.4. Exposure of medaka eggs to silver at different pH

We exposed a group of 15 stage-21 medaka embryos in triplicate to 5 mL of 0.05 mg/L SNCs (that is, the LC_{50} value) in ERM or ultrapure water at pH 4.0, 7.0, or 9.0 in six-well plastic plates; the embryos were incubated at 25 ± 0.1 °C with protection from light until hatching or for 14 days, whichever came first. To expose medaka embryos to silver ions, SNPPs with a 50-nm-thick layer of silver were prepared as described in the Supplemental Information and Fig. S1; medaka embryos were exposed in triplicate as for SNCs. In addition, $AgNO_3$ (0.05 mg/L as Ag) was used as a silver reference; and ERM or ultrapure water at pH 4.0, 7.0, or 9.0 was used as an untreated control in each case. Test solutions were renewed every 24 h. Silver concentrations in the test solutions during exposure were measured by ICP-MS. Every 24 h, exposed embryos were observed for morphological abnormalities under a dissection microscope equipped with a digital camera (model M165 FC, Leica Microsystems). Other reagents were purchased from Nacalai Tesque.

2.5. Calculation of abundance ratios of silver ion species

Formation of silver chloro-complexes and other compounds in ERM and ultrapure water was calculated by using the free program Visual MINTEQ version 3.0 (https://vminteq.lwr.kth.se/).

2.6. ICP-MS analyses of silver

Details of the ICP-MS analysis have been given previously (Kataoka et al., 2015). To separate SNCs and dissolved silver from each SNC solution, a 1-mL sample was filtered by using a centrifuge cassette with a 3-kDa cut-off membrane (Amicon Ultra 3K device, Millipore, Billerica, MA, USA) at 14,000 × *g* for 10 min at 4 °C. The eluate then underwent ICP-MS analysis as dissolved silver.

2.7. Exposure to SNCs and post-exposure incubation to determine the impact on medaka population growth

Five replicate groups of stage-21 medaka embryos ($n = 15$ per group) were exposed to 0.01 mg/L (close to 1/5 LC_{50}) SNCs at pH 7.0 in ultrapure water until hatching. After hatching, all larvae were confirmed to be alive and were rinsed in clean ERM; each group was then moved into 200 mL of fresh ERM in a glass dish and fed with artificial feed (Hikari Lab 130, Kyorin, Hyogo, Japan) once daily for 3 days and thereafter with *A. salina* nauplii. At 7 days posthatch, the larvae were moved to 500-mL acrylic resin aquariums (90 mm × 150 mm × 90 mm) in another medaka culture system and fed *Artemia salina* nauplii and artificial food Otohime β-2 (Marubeni Nissin Feed, Tokyo, Japan) until the medaka reached sexual maturity. During incubation, the numbers of surviving medaka were recorded daily.

2.8. Assessment of effect of SNCs on growth of medaka populations

To evaluate the influence of SNC exposure on medaka populations, we first estimated the per-capita growth rate (r), a summary index that represents the ability of each population to proliferate. The index r was estimated by fitting the life table data for each exposure treatment to the Euler–Lotka equation (Bernstein, 2003). The equation is

$$\sum_t l_t m_t e^{-rt} = 1,$$

where t is age in days, l_t is survivorship until age t, and m_t is per capita fecundity. Because females began to spawn at about 60 days posthatch, we started to record fecundity (the number of spawned eggs/day/female), fertility (the number of fertilized eggs/day/female), and hatchability (the number of hatched larvae/day/female) at 60 days so as to estimate r. The number of newborn females is usually taken as m_t. We therefore identified the sex of all hatched larvae (941 control larvae and 614 exposure-group larvae) by using PCR analysis of a medaka sex-determination gene (*DMY*/*DMRT1*) (Matsuda et al., 2007; Otake et al., 2006). Briefly, for DNA extraction, the larvae were heated at 95 °C in 25 μL of Tris-EDTA buffer for 10 min; the supernatant was used as the template for PCR amplification using primers for *DMY* and *DMRT1* (Shinomiya et al., 2004). After estimating r, we then calculated the finite rate of increase (λ), defined as the number of times the population multiplies in a unit of time, which is expressed as the natural antilogarithm of r (Birch, 1948).

We randomly selected five mating pairs from each treatment and incubated them in the medaka culture system for 21 days. We then measured the numbers of eggs spawned and fertilized to obtain the fertilization ratio. We identified the sex of all larvae to obtain the sex ratio and calculated the arithmetic mean fecundity across the pairs to estimate m_t.

To compare population vulnerability between the control and exposure treatments, we estimated the average time to extinction, $T(K)$, as a function of the population carrying capacity K according to equation 5a of Lande (1993), that is,

$$T(K) = \frac{1}{\bar{r}} \int_1^K \frac{e^{2\bar{r}(N-1)/V}}{N} dN - \frac{\log K}{\bar{r}},$$

where $\bar{r}$ is the mean population growth rate derived from averaging r over replicates, N is population size, and V is variance in individual fitness per unit time. We assumed $V = 1$, according to Lande (1993).

All calculations were performed by using a program code developed for the statistical program R (http://www.R-project.org).

2.9. Statistical analyses

Data were analyzed by using normality testing and the t-test to evaluate the effect of silver compared with the untreated control (Table S1). In addition, we performed one-way analysis of variance and Dunnett's test (Tables 1 and 2). All statistical analyses were done by using Excel 2013 (Microsoft Japan, Tokyo, Japan) or the statistical program R. We chose a significance level (α) of 0.05 for all analyses.

3. Results

3.1. Dissolved silver ions from SNPPs

The concentrations of silver ions dissolved from SNPPs in ultrapure water were measured by using ICP-MS. In ultrapure water, the silver concentration was significantly higher at pH 4.0 (0.57 ± 0.22 mg/L) than at pH 7.0 and pH 9.0 (both 0.08 ± 0.02 mg/L) (t-test, $P < 0.05$) (Table 2). In ERM, the silver concentration was the same (0.04 ± 0.01 mg/L) regardless of the pH (Table 1). In addition, we found that the bottom surfaces of the SNPPs tested with ERM turned from the original purple to white after use, whereas the surfaces of the SNPPs in ultrapure water remained purple (Figs. S1e and S1g). The SEM images revealed that the whitening was due to the presence of white crystals (probably silver chloride) that had grown from the particles on the bottoms of the SNPPs used with ERM but not with ultrapure water (Figs. S1f and S1h).

3.2. Speciation of silver ions

Usually, soluble silver complexes such as silver chlorocomplexes ($[AgCl]^0$, $[AgCl_2]^-$, and $[AgCl_3]^{2-}$) are produced when silver is dissolved in saline solutions (Eisler, 1996; Spiro, 1963). Here, $[AgCl]^0$ and $[AgCl_2]^-$ were the two major silver chlorocomplexes, accounting for 33.0% and 64.5%, respectively, of all silver ion species formed when ERM was used (Figure S2a through S2c). Ag^+ accounted for almost 100% of the silver ion species in the ultrapure water at all three pH levels (Figure S2d through S2f).

3.3. Morphological deformities induced by silver exposure

Medaka embryos exposed to $AgNO_3$, SNCs, or silver ions from SNPPs had various morphological deformities. Specifically, exposure of embryos to $AgNO_3$, SNCs, or silver ions from SNPP in ultrapure water or ERM induced various malformations, including underdevelopment of the eyes and brain, short spinal cord, ischemia, blood clots, pericardiovascular edema, tubular heart, and vascular defects. Although we noted the same malformations as after exposure of embryos to SNCs in our previous study (Kashiwada et al., 2012), the incidences of these abnormalities varied depending on the pH and salinity of the test solution (Fig. 2, Tables 1 and 2).

3.4. Toxicity of silver in ERM and effects of pH

For the ERM controls (ERM without silver), hatchability was 91.1%, 100%, and 100% at pH 4.0, 7.0, and 9.0, respectively (Table 1). In addition, hatchability of ERM control larvae was significantly lower at pH 4.0 compared with pH 7.0. In addition, eye size, heart rate, and full body length of posthatch larvae were lower at pH 4.0 than pH 7.0 ($P < 0.05$), and time to hatch and rates of ischemia and pericardiovascular edema were greater ($P < 0.05$). Although eye size was decreased at pH 9.0 compared with pH 7.0, it appeared

Table 1
Toxic effects of dissolved silver from $AgNO_3$ (as a silver reference), silver nanocolloids (SNCs), or silver ions from silver nanoparticle plates (SNPPs) in medaka embryos in embryo-rearing medium (ERM).

	ERM only (control)			$AgNO_3$			SNCs			SNPPs		
	pH 4.0	pH 7.0	pH 9.0	pH 4.0	pH 7.0	pH 9.0	pH 4.0	pH 7.0	pH 9.0	pH 4.0	pH 7.0	pH 9.0
Ag concentration in complete solution (mg/L)	–	–	–	0.06 (0.00)	0.06 (0.00)	0.07 (0.00)	0.10 (0.00)	0.11 (0.00)	0.10 (0.00)	0.04 (0.01)	0.04 (0.01)	0.04 (0.01)
filtrate from 3-kDa membrane (mg/L)	–	–	–	–	–	–	0.04 (0.00)	0.04 (0.00)	0.04 (0.00)	–	–	–
Underdevelopment of eyes or brain (%)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	2.2 (3.8)
Short spinal cord (%)	2.2 (3.8)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Ischemia or pericardiovascular edema (%)	8.9 (3.8)$^{*\$}$	0.0 (0.0)$^{\#}$	0.0 (0.0)$^{\#}$	0.0 (0.0)$^{\#}$	0.0 (0.0)$^{\#}$	0.0 (0.0)$^{\#}$	20.0 (6.7)$^{*\#\$}$	0.0 (0.0)$^{\#}$	0.0 (0.0)$^{\#}$	2.2 (3.8)	6.7 (6.7)	0.0 (0.0)$^{\#}$
Blood clots (%)	4.4 (3.8)	2.2 (3.8)	0.0 (0.0)	4.4 (7.7)	0.0 (0.0)	0.0 (0.0)	11.1 (13.9)	2.2 (3.8)	0.0 (0.0)	6.7 (0.0)	2.2 (3.8)	2.2 (3.8)
Tubular heart and vascular defects (%)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	2.2 (3.8)	0.0 (0.0)	0.0 (0.0)	2.2 (3.8)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	2.2 (3.8)
Eye size (mm in diameter)	0.35 (0.02)$^{*\$}$	0.38 (0.01)$^{\#}$	0.37 (0.01)$^{\#}$	0.34 (0.03)$^{*\$}$	0.37 (0.02)$^{\#}$	0.37 (0.01)$^{\#}$	0.35 (0.03)$^{*\$}$	0.38 (0.01)$^{\#}$	0.38 (0.02)$^{\#}$	0.36 (0.01)$^{*\$}$	0.35 (0.02)$^{*\$}$	0.37 (0.02)$^{*\#}$
Heart rate of embryo at day 5 (no. of beats/15 s)	31.0 (4.6)$^{*\$}$	37.7 (3.0)$^{\#}$	38.0 (2.9)$^{\#}$	30.3 (3.3)$^{*\$}$	37.0 (3.2)$^{\#}$	40.7 (2.5)$^{*\#\$}$	34.4 (5.2)$^{*\#\$}$	38.0 (2.6)$^{\#}$	38.7 (1.8)$^{\#}$	40.0 (3.4)$^{*\#\$}$	38.1 (4.0)$^{\#}$	39.5 (3.0)$^{\#}$
Time to hatch (d)	7.6 (0.8)$^{*\$}$	6.1 (0.3)$^{\#}$	6.0 (0.0)$^{\#}$	8.8 (2.2)$^{*\#\$}$	7.0 (0.2)$^{*\#\$}$	7.0 (0.2)$^{*\#\$}$	7.2 (0.8)$^{*\$}$	6.0 (0.4)$^{\#}$	6.0 (0.0)$^{\#}$	6.6 (0.9)$^{\#}$	7.0 (1.2)$^{\#}$	6.1 (0.8)$^{\#}$
Hatchability in 14 d (%)	91.1 (3.8)$^{*\$}$	100.0 (0.0)$^{\#}$	100.0 (0.0)$^{\#}$	18.0 (15.0)$^{*\#\$}$	100.0 (0.0)$^{\#}$	100.0 (0.0)$^{\#}$	60.0 (11.5)$^{*\#\$}$	100.0 (0.0)$^{\#}$	100.0 (0.0)$^{\#}$	77.8 (7.7)$^{*\$}$	100.0 (0.0)$^{\#}$	100.0 (0.0)$^{\#}$
Full body length of posthatch larvae (mm)	4.1 (0.2)*	4.2 (0.2)	4.2 (0.2)	4.2 (0.2)	4.3 (0.2)$^{\#}$	4.3 (0.1)$^{\#}$	4.1 (0.2)$^{*\$}$	4.2 (0.2)	4.2 (0.2)$^{\#}$	3.9 (0.2)$^{*\#\$}$	4.1 (0.2)	4.1 (0.3)

Standard errors are in parentheses.
$^{*}P < 0.05$ (Dunnett test) compared with data from control at pH 7.0.
$^{\#}P < 0.05$ (Dunnett test) compared with data from control at pH 4.0.
$^{\$}P < 0.05$ (Dunnett test) compared with data from control at pH 9.0.

Table 2
Toxic effects of dissolved silver from $AgNO_3$ (as a silver reference), silver nanocolloids (SNCs), or silver ions from silver nanoparticle plates (SNPPs) on medaka embryos in ultrapure water.

	Ultrapure water (control)			$AgNO_3$			SNCs			SNPP		
	pH 4.0	pH 7.0	pH 9.0	pH 4.0	pH 7.0	pH 9.0	pH 4.0	pH 7.0	pH 9.0	pH 4.0	pH 7.0	pH 9.0
Ag concentration in complete solution (mg/L)	–	–	–	0.03 (0.01)	0.03 (0.01)	0.03 (0.00)	0.04 (0.00)	0.05 (0.01)	0.05 (0.01)	0.57 (0.22)	0.08 (0.02)	0.08 (0.02)
filtrate from 3-kDa membrane (mg/L)	–	–	–	–	–	–	0.04 (0.00)	0.02 (0.00)	0.02 (0.00)	–	–	–
Underdevelopment of eyes or brain (%)	NA	0.0 (0.0)	0.0 (0.0)	NA	NA	0.0 (0.0)	NA	13.3 (0.1)$^{*\$}$	2.2 (3.8)$^{*\$}$	NA	NA	NA
Short spinal cord (%)	NA	0.0 (0.0)	0.0 (0.0)	NA	NA	11.1 (10.2)$^{*\$}$	NA	13.3 (0.1)$^{*\$}$	2.2 (3.8)	NA	NA	NA
Ischemia or pericardiovascular edema (%)	NA	0.0 (0.0)	2.2 (3.8)	NA	NA	0.0 (0.0)	NA	4.4 (7.7)*	2.2 (3.8)*	NA	NA	NA
Blood clots (%)	NA	0.0 (0.0)	0.0 (0.0)	NA	NA	0.0 (0.0)	NA	6.7 (0.1)$^{*\$}$	2.2 (3.8)$^{*\$}$	NA	NA	NA
Tubular heart and vascular defects (%)	NA	0.0 (0.0)	0.0 (0.0)	NA	NA	0.0 (0.0)	NA	8.9 (0.0)$^{*\$}$	0.0 (0.0)	NA	NA	NA
Eye size (mm in diameter)	NA	0.37 (0.01)	0.38 (0.01)*	NA	NA	0.35 (0.02)$^{\$}$	NA	0.32 (0.03)$^{*\$}$	0.31 (0.11)$^{*\$}$	NA	NA	NA
Heart rate of embryo at day 5 (no. of beats/15 s)	NA	34.6 (4.1)	35.7 (4.0)	NA	NA	36.9 (3.1)	NA	31.3 (4.4)$^{*\$}$	31.3 (6.8)$^{*\$}$	NA	NA	NA
Time to hatch (d)	NA	7.8 (0.7)	7.9 (1.1)	NA	NA	7.9 (0.4)	NA	9.4 (1.3) $^{*\$}$	8.2 (1.3)$^{*\$}$	NA	NA	NA
Hatchability in 14 days (%)	0.0 (0.0)$^{*\$}$	91.1 (7.7)$^{\#}$	93.3 (6.7)$^{\#}$	0.0$^{*\$}$	0.0$^{*\$}$	17.1 (14.8)$^{*\$}$	0.0 (0.0)$^{*\$}$	55.6 (40.7)$^{\#}$	60.0 (43.7)$^{\#}$	0.0 (0.0)$^{*\$}$	0.0 (0.0)$^{*\$}$	0.0 (0.0)$^{*\$}$
Full body length of post-hatched larvae (mm)	NA	4.2 (0.2)$^{\$}$	4.0 (0.2)*	NA	NA	3.9 (0.5)*	NA	3.7 (0.3)$^{*\$}$	3.5 (0.7)$^{*\$}$	NA	NA	NA

NA, not available owing to acute lethality within 24 h.
Standard errors are in parentheses.
$^{*}P < 0.05$ (Dunnett test) compared with data from control at pH 7.0.
$\#P < 0.05$ (Dunnett test) compared with data from control at pH 4.0.
$\$P < 0.05$ (Dunnett test) compared with data from control at pH 9.0.

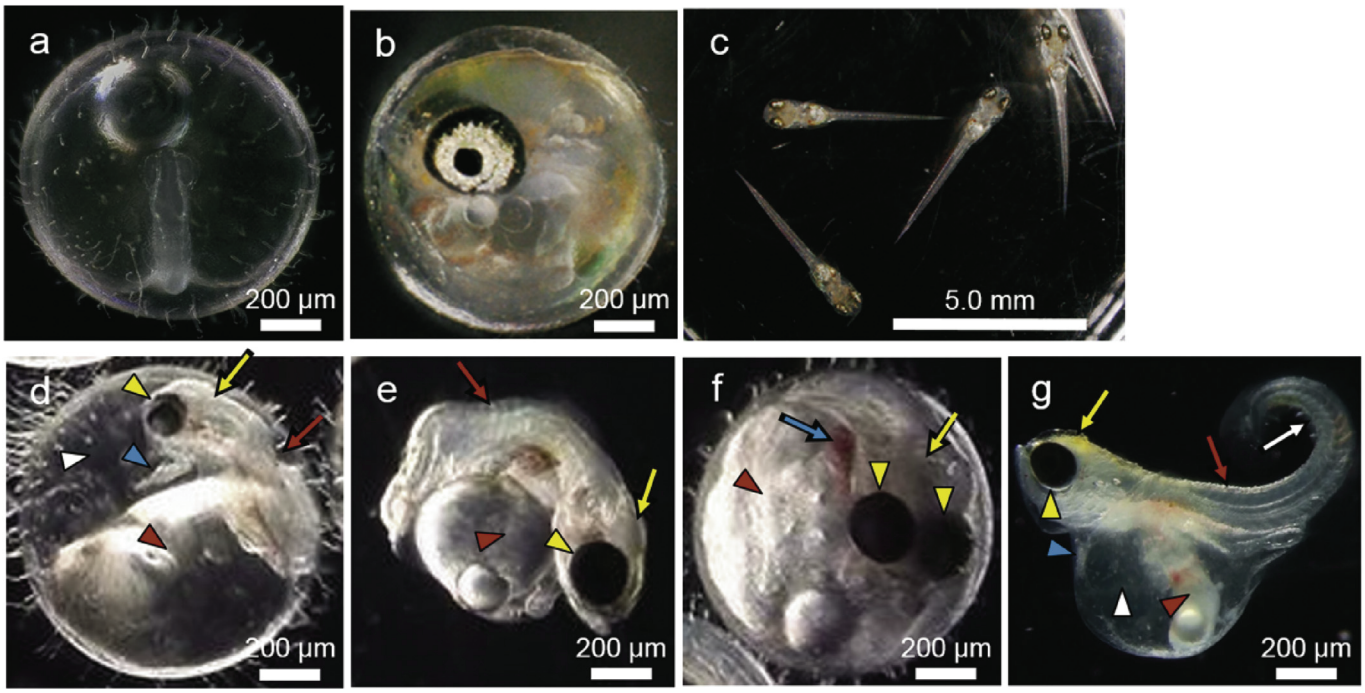

Fig. 2. Morphological toxic effects of silver on medaka embryos. Medaka embryos were exposed to silver from stage 21 through stage 38 (before hatching). Controls: stage 21 (a), stage 38 (b), and posthatch larvae (c). Typical malformations of medaka exposed to silver (same number of posthatch days as stage 38): underdevelopment of eyes (d, e, f, and g: yellow arrowheads) and brain (d, e, f, and g: yellow arrows), short spinal cord (d, e, and g: red arrows), ischemia and vascular defects in yolk sac (d, e, f, and g: red arrowheads), blood clots (f: blue arrow), tubular heart (d and g, blue arrowheads), pericardiovascular edema (d and g: white arrowheads), and kyphosis (g: white arrow). Egg chorion has been removed in e and g.

that, overall, pH 4.0 was more detrimental to medaka embryogenesis than was pH 9.0.

When the embryos were exposed to $AgNO_3$ (0.06–0.07 mg/L as dissolved silver) in ERM, hatchability was 18.0%, 100%, and 100% at pH 4.0, 7.0, and 9.0, respectively (Table 1). Hatchability and eye size at pH 4.0 were significantly reduced in 0.06 mg/L $AgNO_3$ compared with ERM ($P < 0.05$); there were no biological effects at pH 7.0 or 9.0. Therefore, acidic conditions enhanced the toxicity of silver to medaka embryogenesis.

After the exposure of medaka embryos to SNCs (0.10–0.11 mg/L as total silver, 0.04 mg/L as dissolved silver) in ERM, hatchability was 60.0%, 100%, and 100% at pH 4.0, 7.0, and 9.0, respectively (Table 1). Notably, hatchability at pH 4.0 was lower in the SNC-exposed larvae than the ERM controls ($P < 0.05$). In addition, eye size, heart rate, and full body length of posthatch larvae were decreased, and time to hatch and rates of ischemia and pericardiovascular edema at pH 7.0 were increased, in silver-exposed medaka compared with controls ($P < 0.05$); there were no biological effects at pH 7.0 or 9.0. Acidic conditions therefore again enhanced the toxicity of silver to medaka embryogenesis.

When medaka embryos were exposed to dissolved silver (0.04 mg/L as total silver) from SNPPs in ERM, hatchability was 77.8%, 100%, and 100% at pH 4.0, 7.0, and 9.0 respectively (Table 1). Compared with the values for pH 7.0 ERM controls, hatchability was decreased after pH 4.0 exposure; eye size was smaller after exposure at pH 4.0, 7.0, and 9.0; and full body length of posthatch larvae was decreased after exposure at pH 4.0 ($P < 0.05$ for all comparisons).

3.5. Toxicity of silver in ultrapure water and effects of pH

For the controls (ultrapure water without silver), hatchability was 0.0%, 91.1%, and 93.3% at pH 4.0, 7.0, and 9.0, respectively (Table 2). Ultrapure water exerted 24-h acute lethality at pH 4.0. Moreover, full body length in posthatch larvae was decreased and eye size was increased at pH 9.0 compared with that in controls. However, no severe biological defects were noted in ultrapure water at pH 7.0 or 9.0, and the hatched medaka were healthy, despite the extended times to hatch compared with those in ERM ($P < 0.05$) (Table 1).

After exposure to $AgNO_3$ (0.03 mg/L as dissolved silver) in ultrapure water, hatchability was 0.0%, 0.0%, and 17.1% at pH 4.0, 7.0, and 9.0, respectively (Table 2). The toxicity of $AgNO_3$ at pH 4.0, 7.0, and 9.0 was clearly more severe in ultrapure water than in ERM. Eye size and full body length of posthatch larvae were significantly lower at pH 9.0 than in the water controls at pH 7.

Exposure of embryos to SNCs (0.04–0.05 mg/L as total silver; 0.02–0.04 mg/L as dissolved silver) in ultrapure water led to hatchabilities of 0.0%, 55.6%, and 60.0% at pH 4.0, 7.0, and 9.0, respectively (Table 2).

At pH 7.0 and 9.0 in the ultrapure water, the silver concentrations from SNCs were in the same range as those for $AgNO_3$; however, SNCs were comparatively less toxic than $AgNO_3$. Nevertheless, rates of underdevelopment of the eyes and brain, short spinal cord, ischemia and pericardiovascular edema, and blood clots were higher after SNC exposure at pH 7.0 and 9.0 than in the ultrapure water controls at pH 7.0; furthermore, SNC exposure at pH 7.0 and 9.0 reduced the heart rate and full body length of posthatch larvae compared with those at pH 7.0 in the ultrapure water controls. In addition, tubular heart and vascular defects and extended time to hatch occurred more often from exposure to SNCs at pH 7.0 than in the ultrapure water controls at the same pH (Table 2). Finally, the dissolved silver concentration from SNPPs in ultrapure water was pH dependent: it was 0.57, 0.08, and 0.08 mg/L at pH 4.0, 7.0, and 9.0, respectively (Table 2). All pH conditions were associated with 100% 24-h acute lethality.

3.6. Effects of SNCs on population growth rate

Medaka embryos were exposed to SNCs at 0.01 mg/L in ultrapure water until hatching. As mentioned earlier, exposure to 0.01 mg/L SNCs in ultrapure water had no significant effect on hatchability, time to hatch, or survival rate until mating (Table S1). However, mating tests of adult pairs over a 21-day period revealed that fecundity, fertility, and hatchability were reduced in adults that had been exposed to SNCs as embryos (Fig. S3 and Table S2). The calculated intrinsic growth rates (r) (mean [SE]) were 0.273 (0.008) and 0.235 (0.009) in the control and SNC exposure groups, respectively, yielding finite rates of increase (λ) of 1.314 (0.010) and 1.265 (0.011) in the control and SNC exposure groups, respectively. Both r and λ differed significantly between the control and SNC exposure groups (t-test, $P < 0.001$). By using the r values, we estimated the average time to extinction as a function of the population-carrying capacity of the environment. Time to extinction was shorter after SNC exposure than in the controls; SNC exposure of medaka embryos thus induced later-life effects in the medaka population (Fig. 3). For example, time to extinction at $T(30)$ differed by approximately six-fold between the control and exposure treatments (1.80×10^6 in controls; 2.66×10^5 in SNC exposure group).

4. Discussion

We examined the comparative toxicities of $AgNO_3$, SNCs, and silver ions from SNPPs to medaka embryos and the effects of embryonic SNC exposure on the later-life growth of medaka populations.

The wide range of pH values in natural aquatic environments can affect the fate of SNCs. Here, our tests of the effects of pH on the zeta potential and aggregation of SNCs revealed that changing the pH had no observable effect on the zeta potential of SNCs. We estimated that the SNCs were well dispersed and formed aggregates with a broad peak at around 100 nm in size in either ultrapure water or ERM, although we were unable to measure the zeta potential of SNCs in ERM (Fig. 1b). These data suggest that medaka embryos were exposed to relatively the same size of SNCs regardless of whether embryos were in ultrapure water or ERM at pH 4.0, 7.0, or 9.0. Subsequent tests revealed that pH markedly influenced medaka embryogenesis, and acidic conditions (pH 4.0) had an overall greater biological effect on medaka embryogenesis than did alkaline conditions (pH 9.0) (Tables 1 and 2). These effects were more severe in ultrapure water than in ERM. Hatchability and time to hatch are well known and important ecological and ecotoxicological biomarkers. In the controls at pH 4.0, hatchability was reduced to 91.1% in ERM and to 0.0% in ultrapure water. In the controls at pH 7.0, although hatchability did not differ significantly between ERM (100%) and ultrapure water (91.1%) ($P = 0.12$), time to hatch was significantly extended from 6.1 days in ERM to 8.7 days in ultrapure water ($P < 0.05$). Acidic conditions thus had a direct biological effect on medaka embryogenesis, but the buffering action of ERM alleviated the effects of the acidic conditions.

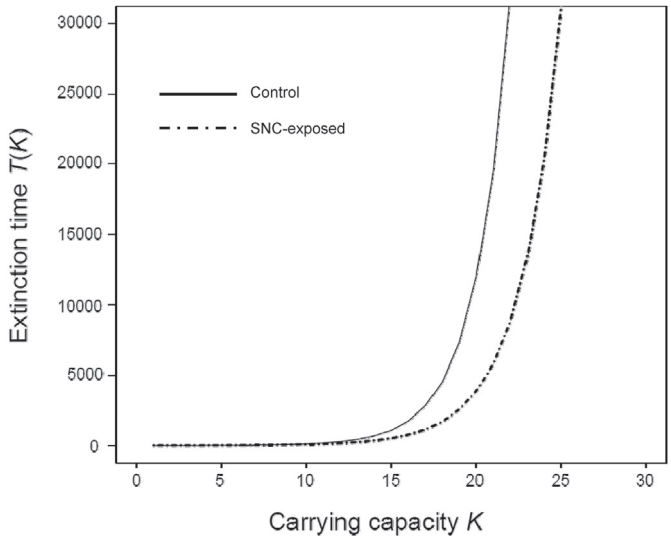

Fig. 3. Ecological risks posed by silver nanocolloids (SNCs), as assessed by time to extinction ($T(K)$) in relation to the medaka carrying capacity (K) of the environment. Solid line indicates control medaka. Dashed line indicates SNC-exposed medaka.

We then tested the effects of pH on the toxicity of silver to medaka embryos. In our previous study, SNCs were more lethal to medaka embryos in ultrapure water than in ERM. That is, the 96-h LC_{50} value was 0.050 (0.039–0.070, 95% efficiency limit) mg/L at pH 7.0 in ultrapure water; in contrast, the lethal toxicity of SNCs was decreased in ERM (LC_{50} value > 10.0 mg/L) (Kataoka et al., 2015). ERM reduced the toxicity of SNCs likely because of the formation of silver chloride complexes (lower toxic form) from Ag^+ (higher toxic form) (Kataoka et al., 2015). Silver ion toxicity reportedly decreases when silver complexes with humic acid (Kim et al., 2013). In our present study, exposure to either $AgNO_3$ or SNCs was markedly more toxic in ultrapure water than in ERM (Tables 1 and 2). ERM alleviated the toxicity of both $AgNO_3$ and SNCs.

Exposure of medaka embryos to silver ions from SNPPs in ultrapure water induced 100% acute toxicity at all three pH values tested. In addition, the dissolved concentration of silver was pH dependent. In this case, a high silver concentration and acidic conditions were directly lethal factors. In contrast, in the case of SNPPs with ERM, acute toxicity was abolished in ERM. Our analyses of silver ion speciation revealed that, in ultrapure water, Ag^+ accounted for almost 100% of species from the three silver compounds. In ERM, two major silver chloro-complexes ($[AgCl]^0$ and $[AgCl_2]^-$) accounted for all of the species (Figure S2d through S2f). Therefore, this different silver speciation likely underlies the different toxicities of the silver ion solutions.

Comparison of the toxic effects of $AgNO_3$ and SNCs in ultrapure water at pH 7.0 and 9.0 revealed that $AgNO_3$ was overall more toxic than SNCs. In the SNC exposure experiment in ultrapure water, the concentrations of silver were 0.05 mg/L (total silver) and 0.02 mg/L (dissolved silver) at both pH 7.0 and 9.0; these concentrations were similar to those of $AgNO_3$ (0.03 mg/L). Nevertheless, at these two pH values, SNCs exhibited lower toxicity than $AgNO_3$ (Tables 1 and 2). In terms of differentiation of the toxicities of silver nanoparticles and silver ions, a study using adult medaka found that toxicity was due to silver ions and that silver nanoparticles had no effect on acute toxicity (Kim et al., 2011). Moreover, solutions containing high ratios of silver ions to silver nanoparticles in suspension were more toxic to medaka embryos than those with lower ratios, suggesting that silver ions had greater adverse effects (Lee et al., 2014).

When we used SNPPs, the concentrations of dissolved silver were higher in ultrapure water than in ERM and were highest (0.57 mg/L) in ultrapure water at pH 4.0. SEM analyses suggested that the white crystals were silver chloride; ERM is an artificial freshwater containing 0.018 M chloride (0.64 g/L as Cl). Silver chloride is insoluble in water. In addition, because the white crystals covered the entire surfaces of the SNPP, the concentrations of dissolved silver were all low (0.04 mg/L) in ERM (Table 1).

Even in the absence of any marked toxicity to medaka embryos or reduction in later-life survival rates (Table S1), later-life reproductive measures, including fecundity, fertility, and hatchability of offspring, were affected (Table S2). Subsequently, both the intrinsic

growth rate and the finite rate of increase were reduced, and we demonstrated a shortened time to extinction relative to the environment's carrying capacity for the medaka population (Fig. 3). Previously, we reported similar research regarding the neurotoxic pesticide carbaryl: exposure at the larval stage, although not necessarily at the embryo stage, had significant toxic effects on population growth during later-life stages (Kashiwada et al., 2008). Therefore, the toxic cascade likely differs between heavy metals and neurotoxicants. In the current study, although the concentration of silver to which medaka eggs was exposed was much lower than the LC_{50} value, we found that 1) exposure during early life can lead to effects during the adult stage, and 2) population-level effects must be considered during ecological risk assessment of chemicals. Therefore, ecological risk assessments of hazardous chemicals must incorporate the concept of population dynamics.

5. Conclusions

In the current study, the malformations and other toxic effects induced in medaka embryos were similar among the three types of silver tested. In ultrapure water, toxicity due to SNCs was lower than that from $AgNO_3$ and silver ions from SNPPs, and using ERM further reduced silver toxicity. In addition, silver toxicity was higher under acidic conditions than under neutral or alkaline conditions. Exposure of medaka eggs to 0.01 mg/L SNCs until hatching had no significant toxic effects on embryo development or hatching, or on later-life survival after rearing under clean conditions. However, population growth was decreased significantly, even when fish were transferred to clean conditions after hatching.

Ethical use of animals

The Japanese medaka used were treated humanely according to the Institutional Animal Care and Use Committee guidelines of Toyo University, with due consideration for the alleviation of distress and discomfort.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2017.10.028.

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RAPID COMMUNICATION

Note on important and novel findings

Lower sensitivity of cyprinid fishes to three acetylcholinesterase inhibitor pesticides: an evaluation based on no-effect concentrations

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Abstract Researchers have suggested that cyprinid fishes are less sensitive to chemical stress than comparable fish families, yet few empirically based evaluations of this hypothesis have been conducted. In this study, we developed a generalized linear mixed model in which the no-effect concentrations (NECs; threshold concentration below which no effect on survival is predicted during prolonged exposure) of 29 fish species from 13 families exposed to an acetylcholinesterase inhibitor pesticide (carbaryl, chlorpyrifos, or malathion) were used as the response variable. The corresponding specific somatic maintenance (SSM) rates, as a size-independent proxy for fish metabolism and a categorical variable regarding whether the species is a cyprinid, were used as the predictor variables. We included SSM rates in the analysis because previous work demonstrated that they are negatively correlated with NECs. Our results indicate that the NECs for cyprinid fishes were significantly higher than those for other fishes, suggesting that cyprinids are indeed less sensitive to the three studied pesticides. Although the SSM rates were negatively related with the NECs, the actual relationship between the two was not clear, implying that the importance of SSM rates may depend on the taxonomic group tested.

Keywords Species sensitivity · Trait · Tolerance · Resistance · Freshwater fish

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Introduction

Determining which species are sensitive to chemical stress and understanding the underlying reasons are critical for cost-effective ecological risk assessments (Ippolito et al. 2012). Cyprinid fishes play a crucial rule in ecosystems by affecting other organisms, including algae, zooplankton, invertebrates, and vertebrates (Winfield and Townsend 1991). Members of Cyprinidae are generally reported to be less sensitive (i.e., more resistant) to chemical compounds (Teather and Parrott 2006) than other families (such as Salmonidae) and have been found inhabiting contaminated rivers and lakes (Heath 1995; Kaneko et al. 1999). However, given the numerous toxicity data available in the literature (e.g., Brix et al. 2001), it is surprising that only a few empirical studies have tested the lower sensitivity of cyprinids to chemicals versus that of other fish families. To our knowledge, Teather and Parrott (2006) is the only study that addressed this issue; they compiled a large acute toxicity dataset (i.e., median lethal concentration; LC50) on 9–195 chemicals for eight freshwater fish species, including three cyprinids.

Sensitivities to chemical stress, even if water quality is carefully controlled, still depend on (i) biological factors (e.g., measured endpoints such as survival and growth, and

life stages considered) and (ii) test conditions (e.g., exposure duration) that may have distinct effects on different species. Stark et al. (2004) demonstrated that the same level of effect on survival and/or fecundity (i.e., a 50% reduction) leads to different population-level impacts on different invertebrate species, suggesting the importance of life-history traits in ranking the population susceptibility. Nevertheless, that the rankings of sensitivities to chemicals are usually evaluated with time-dependent measures (e.g., Teather and Parrott 2006; Woltering 1984), such as the no-observed-effect concentration (NOEC) and the LC50 derived from results of toxicity tests for limited periods of time, causes the results to be potentially misleading. Developing validated population models for long-lived species such as fish is difficult. Thus, a promising way to avoid potential problems and prevent exposure duration from affecting estimated sensitivity rankings is the use of time-independent toxicity measures such as the no-effect concentration (NEC; Baas and Kooijman 2015). The NEC is the threshold concentration below which no effect on survival is predicted during a prolonged exposure (i.e., 0% lethal concentration at an infinite time of exposure).

To test the hypothesis that members of Cyprinidae are less sensitive to chemicals than other fish families, we compared the NEC values for three acetylcholinesterase inhibitor pesticides (carbaryl, chlorpyrifos, and malathion). The dataset made available by Baas and Kooijman (2015), which was used as is in the present study, included estimates of NEC for 29 fish species from 13 families. Because that study reported a negative correlation between specific somatic maintenance (SSM) rate (i.e., metabolic rate) as a size-independent proxy for fish metabolism and NEC as a quantifier of toxicity, we took the former variable into account when testing the hypothesis on the lower sensitivity of cyprinid fishes.

Methods

Data

The complete dataset used in this study is available as the electronic supplementary material of Baas and Kooijman (2015). Briefly, the number of NECs reported for carbaryl, chlorpyrifos, and malathion were 16, 9, and 6, respectively. The 13 families (and the total number of NECs) included in the dataset were Acipenseridae (1), Anguillidae (1), Atherinopsidae (1), Centrarchidae (3), Channidae (2), Cichlidae (2), Cyprinidae (12), Ictaluridae (1), Melanotaeniidae (1), Osphronemidae (1), Percidae (2), Poeciliidae (1), and Salmonidae (3). Among the 12 available Cyprinidae NECs for the three studied pesticides, only those for common carp (*Cyprinus carpio* L.) and white carp (*Cirrhinus mrigala* F. Hamilton) were given for two different pesticides. NECs for carbofuran, although available in the dataset of Baas and Kooijman (2015), did not cover Cyprinidae and hence were excluded from our analyses.

The NEC (μmol l^{-1}) plays a key role in the simplest mechanistic model for the survival of organisms exposed to toxicants and can be estimated from the related time-series toxicity data (for further details about NEC, see Baas and Kooijman 2015; Jager et al. 2011; Kooijman and Bedaux 1996). Furthermore, SSM rates (kJ d^{-1} cm^{-3}; a measure of the daily volume-specific maintenance costs for living tissue at 20 °C) were included in the statistical analysis, because Baas and Kooijman (2015) concluded that a higher SSM rate increases a species' sensitivity to pesticides based on NECs for a wide range of species, including non-fish species. The SSM rate is a key parameter for a class of energy budget models based on principles of physiological energetics and used to describe the full life cycle of individual organisms (Baas and Kooijman 2015; Jusup et al. 2017; Nisbet et al. 2000). The SSM rate assigned to each species was (i) a direct estimate for the species in question whenever available, or (ii) a substitute estimate for a related species generally from the same taxon (where the quality of the data available for the estimation of the SSM rate was also considered). Because substitute SSM rates were used for nearly three-quarters of species in this study, we took particular care to check whether our conclusion regarding the sensitivity of cyprinid fishes to the three studied pesticides was affected by the substitutions (see section "Results and discussion").

Statistical analysis

To test whether the NECs of three pesticides for cyprinids were higher than those for other fish families, we developed a linear mixed model with a Gaussian distribution. The NEC was used as the response variable. As predictor variables, we used the SSM rate of each fish species and a categorical variable (i.e., taking a value of 0 or 1) to represent whether the species belonged to Cyprinidae or not (Cyprinidae in the formula below). The NECs and SSM rates were $\log_{10}$-transformed for this analysis. The linear mixed model tested was:

$$\log\left(\mathrm{NEC}_{ij}\right) = \beta_0 + \beta_1 \mathrm{Cyprinidae}_{ij} + \beta_2 \log\left(\mathrm{SSM}_{ij}\right) + r_i + \varepsilon_{ij},$$

where β_0, β_1, and β_2 are regression coefficients to be estimated, and r_i is a random effect used to account for variability attributed to different pesticides (for an introduction to random effects, see Bolker et al. 2009; Faraway 2006). The random effect r_i and residual term ε_{ij} are assumed to be normally distributed with a mean of 0 and a standard deviation estimated from the data. Subscripts i and j refer to individual pesticide and fish species, respectively.

Statistical analyses were performed using R version 3.0.3 (R Development Core Team 2014; http://CRAN.R-project.org/) with the R package "nlme". The significance level was set to 0.05 for the analysis.

Results and discussion

Application of the linear mixed model indicated that the estimated coefficient ± standard error for the model intercept, categorical Cyprinidae variable, and SSM rate were 3.62 ± 1.60 ($p = 0.032$), 1.36 ± 0.49 ($p = 0.010$), and −0.65 ± 0.31 ($p = 0.047$), respectively. The coefficients of random effects (i.e., r_i) for carbaryl, chlorpyrifos, and malathion were 1.87, −1.79, and −0.08, respectively. Accordingly, cyprinids were found to have higher NECs for the three acetylcholinesterase inhibitor pesticides than other examined fish families (Fig. 1, upper panels), with a few exceptions. Our results suggest that cyprinids are generally less sensitive to the studied pesticides than other families. Furthermore, the observed lower sensitivities (i.e., higher NECs) of cyprinid fishes are unlikely to be explained by their higher SSM rates than those of other fishes (Fig. 2) because the higher SSM rate as a size-in-

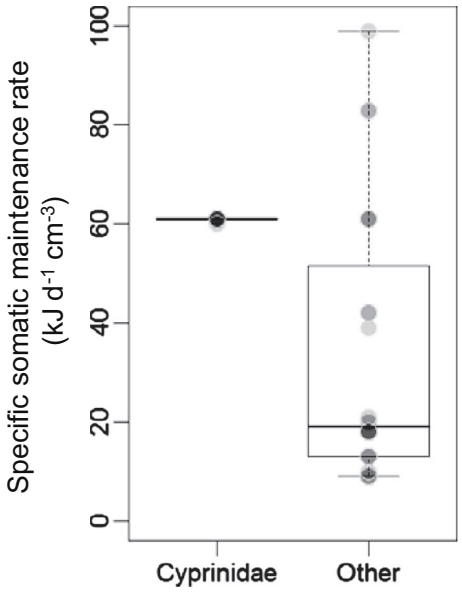

Fig. 2 Specific somatic maintenance (SSM) rates of cyprinid and other fishes. All the SSM rates shown were model-estimated (Baas and Kooijman 2015). *The bold horizontal line*, *box*, *error bars*, and *gray dots* indicate the median, interquartile range, 1.5 ×interquartile range, and raw data, respectively. For cyprinid fishes, only the direct (non-substituted) estimates of SSM rates are included in this figure

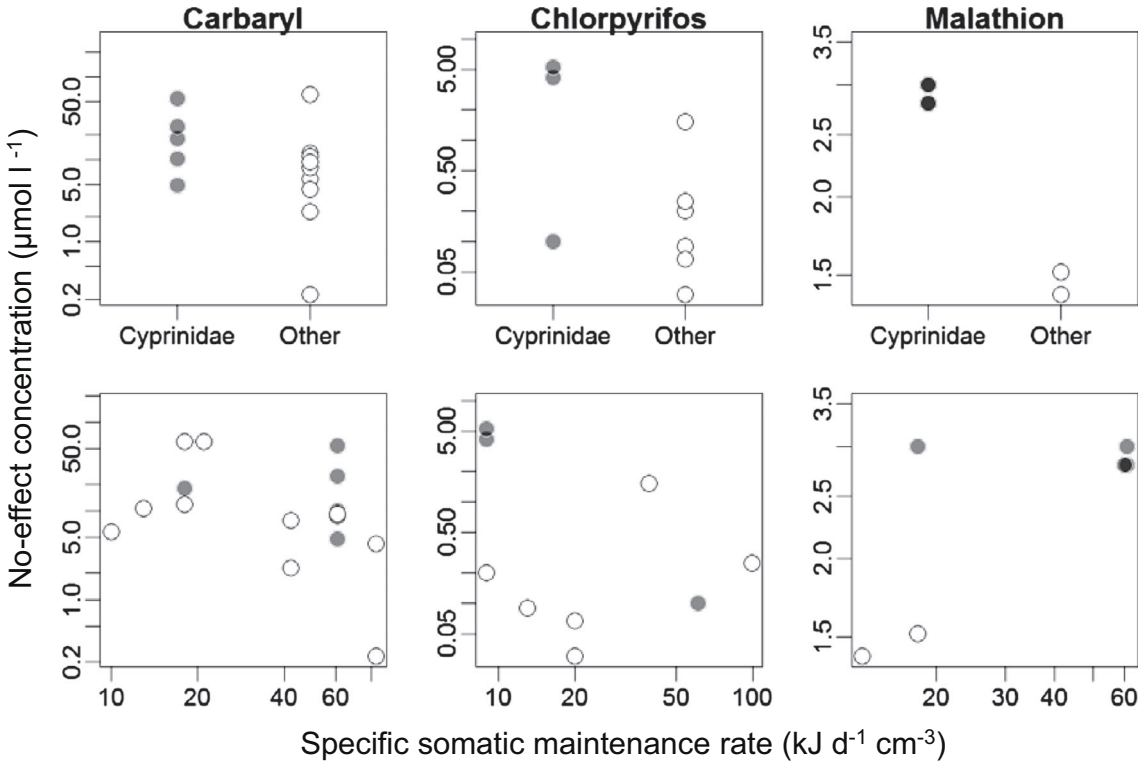

Fig. 1 Relationships between no-effect concentration and whether the fish species belongs to Cyprinidae or not (*upper panels*) and specific somatic maintenance rate (*lower panels*). *Gray circles* represent cyprinid fishes

dependent proxy for faster metabolism is expected to lead to a lower NEC (Baas and Kooijman 2015).

The uncertainty (i.e., under- or overestimation of SSM rates) introduced by the substitution of the SSM rates for many species might have affected our results. To ensure the robustness of our main conclusion regarding the lower sensitivity of cyprinid fishes, we excluded the SSM rate parameter from the regression analysis and confirmed that the categorical Cyprinidae variable was still a significant predictor ($p = 0.032$). Therefore, it is unlikely that the uncertainty in the estimated SSM rates affected our main finding regarding the lower sensitivity of cyprinids to the studied pesticides.

In the full linear mixed model with both predictor variables, although relatively large variations were observed, NECs were negatively correlated with the SSM rates (Fig. 3). This result supports the general conclusion of Baas and Kooijman (2015), who included taxa other than fish (e.g., invertebrates) in their analysis. That this negative correlation, however, was not readily apparent from the actual relationships between the NECs and the SSM rates (Fig. 1, lower panels) suggests that the importance of SSM rates may depend on the taxonomic group being tested.

The sensitivities to pesticides discussed here are based only on the effect on survival because the NEC is a threshold concentration below which no effect on survival is predicted during prolonged exposure. However, population-level consequences (such as the probability of extinction) are determined not only by survival but also by other individual-level traits, including reproduction and growth (Nisbet et al. 2000). Thus, it would be ideal to estimate and compare the sensitivity (or vulnerability) of organisms to chemicals by using population models that can incorporate such traits into the calculation of population growth rates and/or equilibrium population size (Hanson and Stark 2012; Hayashi et al. 2009; Iwasaki et al. 2010, 2013).

Overall, our findings based on a time-independent toxicity measure (i.e., NEC) provide additional evidence for the hypothesis that cyprinid fishes are less sensitive to chemicals than other fish families. Because the present study was limited to only three pesticides with a specific mode of action, however, further research comparing the sensitivities to other kinds of chemicals (e.g., metals) is required to reach a more compelling conclusion. Furthermore, cyprinids are agastric fishes whose lower sensitivity to chemicals is matched by other agastric fishes such as mosquitofish (*Gambusia affinis* S. F. Baird and Girard) and guppies (*Poecilia reticulata* W. K. H. Peters), which were found to be less sensitive to chemicals compared to coho salmon, bass, and bluegill (Teather and Parrott 2006). Although the mechanistic link is uncertain, this or related traits might be important for understanding the lower sensitivity. A more comprehensive analysis for identifying the underlying mechanisms would be valuable for generalizing and predicting the sensitivity of species to chemicals. In doing so, the use of NECs or measures based on population-level effects should help elicit more ecologically and ecotoxicologically relevant evidence.

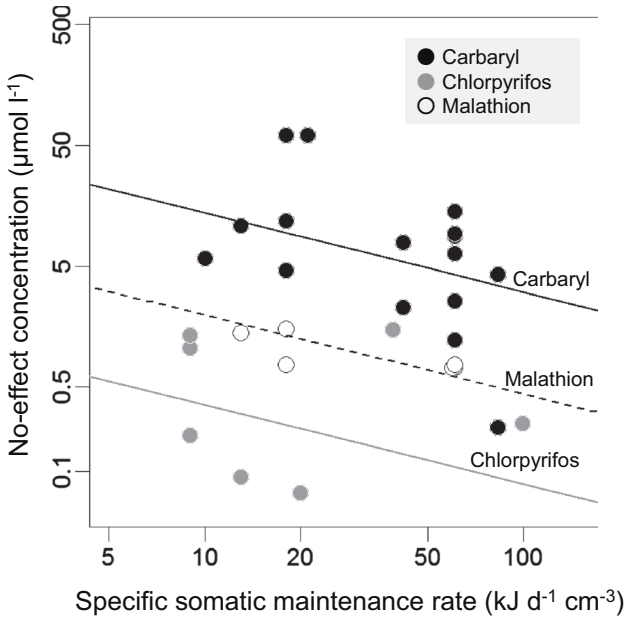

Fig. 3 Relationships between specific somatic maintenance rates and estimates of no-effect concentrations (NECs) for three pesticides. Estimates of NECs were calculated by removing the "Cyprinidae" effect, and regression lines were estimated by the linear mixed model (see the caption of Fig. 1 for more details)

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ASIA/OCEANIA REPORT

Spatiotemporal changes in water quality along a historically metal-contaminated river: a retrospective analysis of 50 years of monthly monitoring data

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Abstract The Watarase River, running through Japan's northern Kanto region, has a long history of trace-metal contamination originating from the Ashio Copper Mine. Given the historical importance of incidents at this mine, understanding spatiotemporal environmental changes in the river, including changes in water quality, is important. By using long-term water-quality monitoring data (1960–2010), we aimed to reconstruct the spatiotemporal changes in six water-quality variables—the concentrations of three metals (copper, zinc, arsenic), biochemical oxygen demand (BOD), chemical oxygen demand (COD), and concentration of nitrate-nitrogen—along the Watarase River using generalized additive mixed models. The modeling results clearly demonstrate that during the 1960 and 1970s, metal pollution levels (as represented by copper and zinc) greatly decreased (from 450–2300 to 8–39 μg Cu L^{-1} and from 490–1500 to 17–52 μg Zn L^{-1}), whereas organic pollution levels, as represented by the BOD and COD increased. Unique changes were observed in the cases of arsenic and nitrate-nitrogen (e.g., marked increases in the 1960s). From the 1980s until 2010, gradual decreases in the levels of metal and organic pollution were generally observed. Only in the 2000s were annual mean concentrations of copper in the lower reaches of the Watarase River lower than the US Environmental Protection Agency (EPA) water-quality criterion.

Keywords Copper mining · Generalized additive mixed model · Heavy metal · Longitudinal trend · Water quality monitoring

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Introduction

Environmental pollution with chemicals such as trace metals is a major issue worldwide in the management of river ecosystems (Luoma and Rainbow 2008). Long-term monitoring programs and spatially extensive field surveys of aquatic organisms in contaminated environments offer valuable opportunities to understand the field impacts of chemicals and to test ecological risk predictions based on laboratory toxicity testing (Iwasaki and Ormerod 2012; Iwasaki et al. 2013). In particular, long-term monitoring of chemical (e.g., water quality) and biological (e.g., species diversity) parameters after the removal or mitigation of pollution in aquatic environments can be used to investigate ecological responses and recovery trajectories. However, the abundance of such studies is limited (Clements et al. 2010; Mebane et al. 2015; Stockdale et al. 2014). One

possible reason is a lack of data availability: there are limited opportunities to perform both chemical analyses and biological surveys in long-term monitoring programs (Merrington et al. 2014).

In Japan, the Watarase River, which runs through the northern Kanto region, has a long history of heavy-metal contamination (Fig. 1). Heavy-metal contamination originating from the Ashio Copper Mine (1610–1973) became evident in the late nineteenth century and is well known as the first-recognized serious environmental pollution problem in Japan (Ui 1992). In addition to causing water pollution, mining activities have had severe impacts on agriculture (e.g., soil pollution), forestry (forest destruction), and fisheries (mass fish deaths) in the Watarase River basin (e.g., Ui 1992). Therefore, revealing spatiotemporal environmental changes in the Watarase River, including changes in water quality, would be valuable given the historical importance of incidents at the Ashio Copper Mine.

Water quality along the Watarase River has been monitored monthly since 1960 at the earliest by the Watarasegawa River Office (2013). However, the temporal availability of measurement data varies among the individual monitoring sites along the river (Fig. 1). Because available motoring data are scattered in space and time, statistical modeling (in this study, interpolation) is needed to achieve a more spatiotemporally complete understanding of changes in water quality along the river. We therefore aimed to reconstitute the spatiotemporal changes in water quality measured in the long-term monitoring program along the Watarase River by applying a generalized additive mixed model

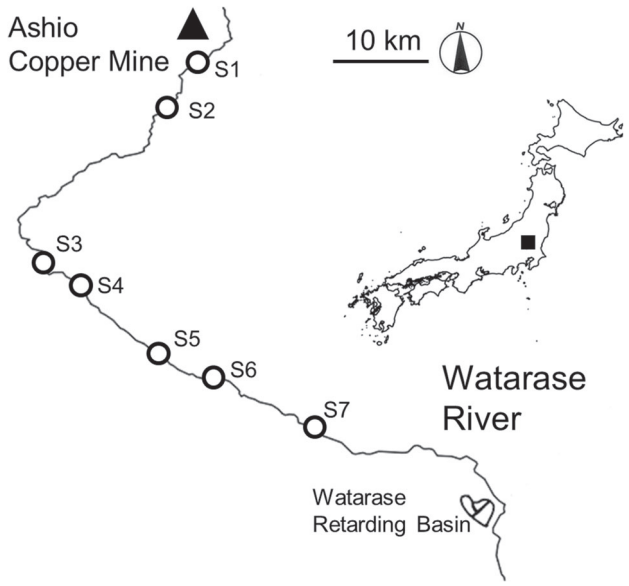

Fig. 1 Water-quality monitoring sites (S1 to S6) on the Watarase River, Japan

(GAMM). The GAMM is an extension of the generalized additive model and enables the modeling of nonlinear patterns for which little is known regarding an appropriate model form (Wood 2003, 2006; see "Materials and methods" for more details). For the modeling, we used the concentrations of copper, zinc, and arsenic as indicators of the heavy-metal contamination caused by copper mining activities. Additionally, because 12% by area of the land use within the river basin is urbanized and 16% is agricultural (Watarasegawa River Office 2012), we included biochemical oxygen demand (BOD), chemical oxygen demand (COD), and concentration of nitrate-nitrogen in our modeling to gain an understanding of the spatiotemporal trends of organic and nutrient pollution from these urban or agricultural areas. Biological surveys such as those using benthic macroinvertebrates, along with limited water-quality measurements have occasionally been performed to evaluate the ecological impacts of pollution in the Watarase River (e.g., Gose 1960; Ide and Arai 1978; Suzuki et al. 2004). Coupling the modeling outputs from our study with results of these biological surveys should create opportunities to assess the river's long-term biological recovery from pollution.

Materials and methods

Water-quality monitoring data

All measurement data on water quality (total concentrations of zinc, copper, and arsenic; BOD; COD; nitrate-nitrogen) at monitoring sites along the Watarase River (Fig. 1 and Table S1 in the electronic supplementary material: ESM) were acquired from the Watarasegawa River Office (2013), Kanto Regional Development Bureau, Ministry of Land, Infrastructure, Transport, and Tourism (MLIT), Japan (MLIT 2009). We digitized measurement values recorded in PDF (Portable Document Format) files; digitized raw data are available upon request. In the data set, BOD represents the 5-day biochemical oxygen demand (i.e., BOD_5); COD was measured using the potassium permanganate method (MLIT 2009). Lead was also measured, but 90% of lead measurements (mostly later than 1965) were below the limits of quantification (LOQs) (decreasing from 80 to 1 $\mu g\ L^{-1}$ in recent years) at the most downstream site (S7, Fig. 1) and the circumstances were similar at other sites. Thus, we did not include lead in this study.

Monthly measurements of water-quality variables at the seven sites from 1960 to 2010 were used for statistical modeling. In 1960, monitoring started at four sites (S2, S3, S6, S7), but monitoring periods varied among sites.

Detailed information (e.g., location, monitoring period) is available in Table S1. Measurements below LOQs were found only for metals (zinc, copper, arsenic) and were assigned values of half of the LOQs. Note that more complex modeling approaches are potentially applicable (e.g., Bayesian approach; Hayashi and Kashiwagi 2011), but no method that can readily be incorporated into the GAMM approach for analysis of censored data is currently available (see Helsel 2005, 2012). A complementary analysis we performed by excluding the below-LOQ data did not materially affect results given that our primary focus was to capture spatiotemporal trends by estimating annual averages of water-quality variables. In addition, because the concentrations of copper and zinc measured during 1988–1991 were markedly lower than those in more recent years (approximately one tenth) and the stated value of their LOQ (0.000 mg L^{-1}) was unclear, those data were not included in our study.

Statistical analysis

Visual investigation of data suggested that temporal changes in some water-quality variables were not linear (e.g., see Fig. 2 for copper). GAMMs were thus fitted with year and distance from S1 as predictor variables using a smooth function (thin-plate regression spline) to capture nonlinear spatiotemporal changes in the annual averages of water-quality variables (Wood 2003, 2006). The smooth

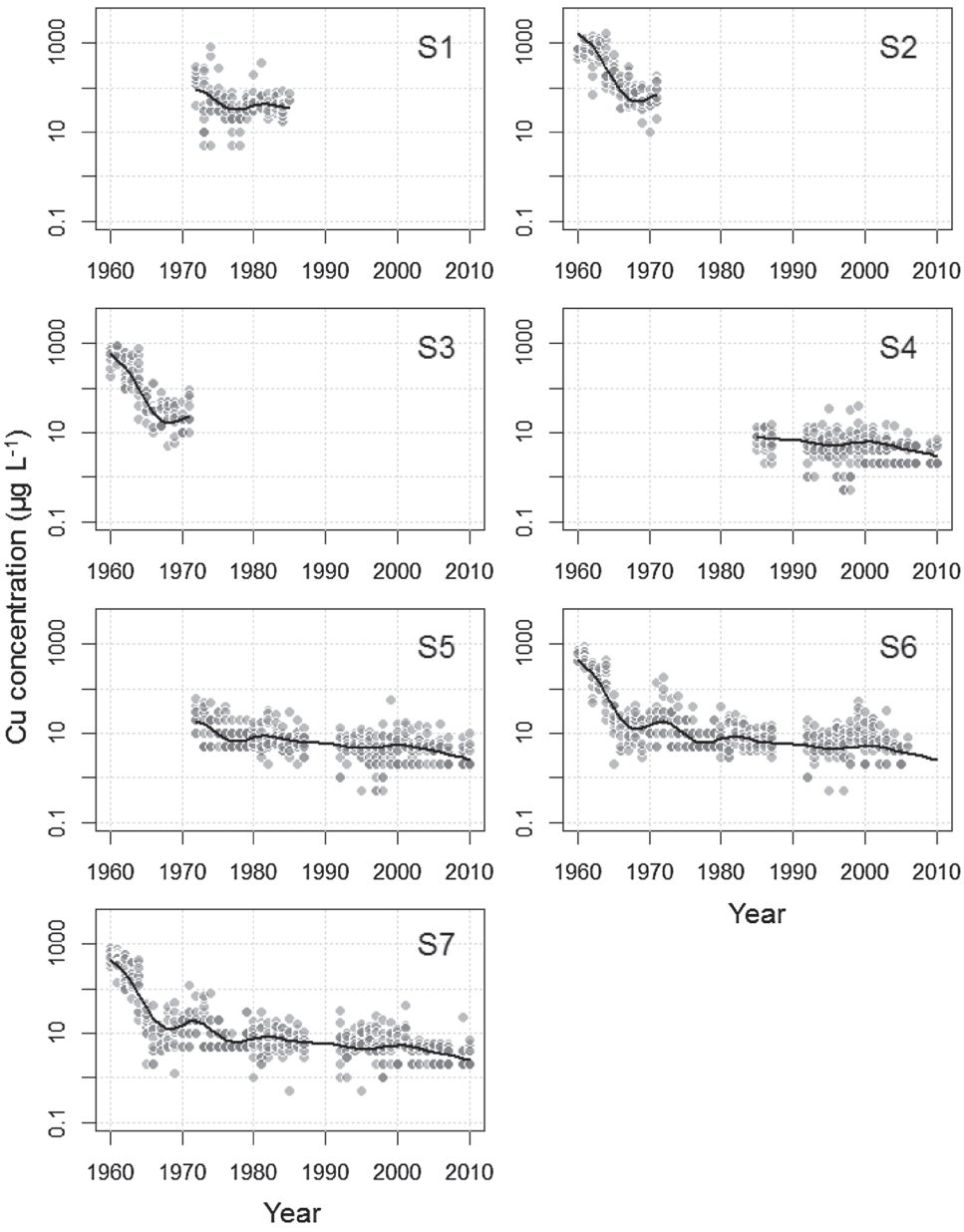

Fig. 2 Temporal changes in concentrations of total copper (Cu) at seven monitoring sites along the Watarase River (see Fig. 1 for locations). Individual data points are monthly monitoring measurements. *Solid lines* are modeled trends using the generalized additive mixed model

function does not assume a strict form and thereby provides more flexible modeling of changes in the response variable. Note that this modeling assumes the temporal trend is similar at all monitoring sites and the spatial (upstream–downstream) trend is similar during the monitoring period (i.e., 1960–2010; see below for more discussion). Measured values of water-quality variables were log transformed for this analysis. A random effect and an autoregressive correlation structure of order 1 (AR1; Hastie and Tibshirani 1990) were included in the models to take into account the autocorrelation among within-year observations at individual sites and the interyear autocorrelation, respectively. All statistical analyses were performed in R v. 3.2.2 software (R Core Team 2015) with the R package "mgcv" (v. 1.8-9; Wood 2003).

Results and discussion

Spatiotemporal changes in water quality

GAMMs were developed for six water-quality variables (zinc, copper, arsenic, BOD, COD, and nitrate-nitrogen). The coefficients of correlation between annual averages estimated by using the GAMMs and those calculated from monthly measurements ranged from 0.88 to 0.98, and the differences between the predicted and observed values of all variables were mostly within a factor of 2 (Fig. 3). All variables showed nonlinear temporal changes but comparatively linear spatial trends (Fig. 4).

Temporal changes in the concentrations of copper and zinc were similar but were different from that of arsenic (see Fig. 4 for the general spatiotemporal trends). Concentrations of copper and zinc decreased dramatically from 1960 (approximate range: copper, 450–2300 μg L^{-1}; zinc, 490–1500 μg L^{-1}) to the late 1970s (copper, 8–39 μg L^{-1}; zinc, 17–52 μg L^{-1}). This decrease was probably attributable to the mine closure in 1973 and the establishment of the Water Pollution Control Law in 1971. Concentrations then decreased gradually and were still decreasing in 2010 (copper, 3–13 μg L^{-1}; zinc, 6–17 μg L^{-1}; Fig. 2; Fig. S1). In contrast, arsenic concentrations showed a marked increase in 1968 (maximum concentration 180 μg L^{-1}) at all monitoring sites, likely because of an increase in the concentration in treated mine wastewater (Kiryu City Waterworks Bureau 1982). After this increase, arsenic concentrations generally

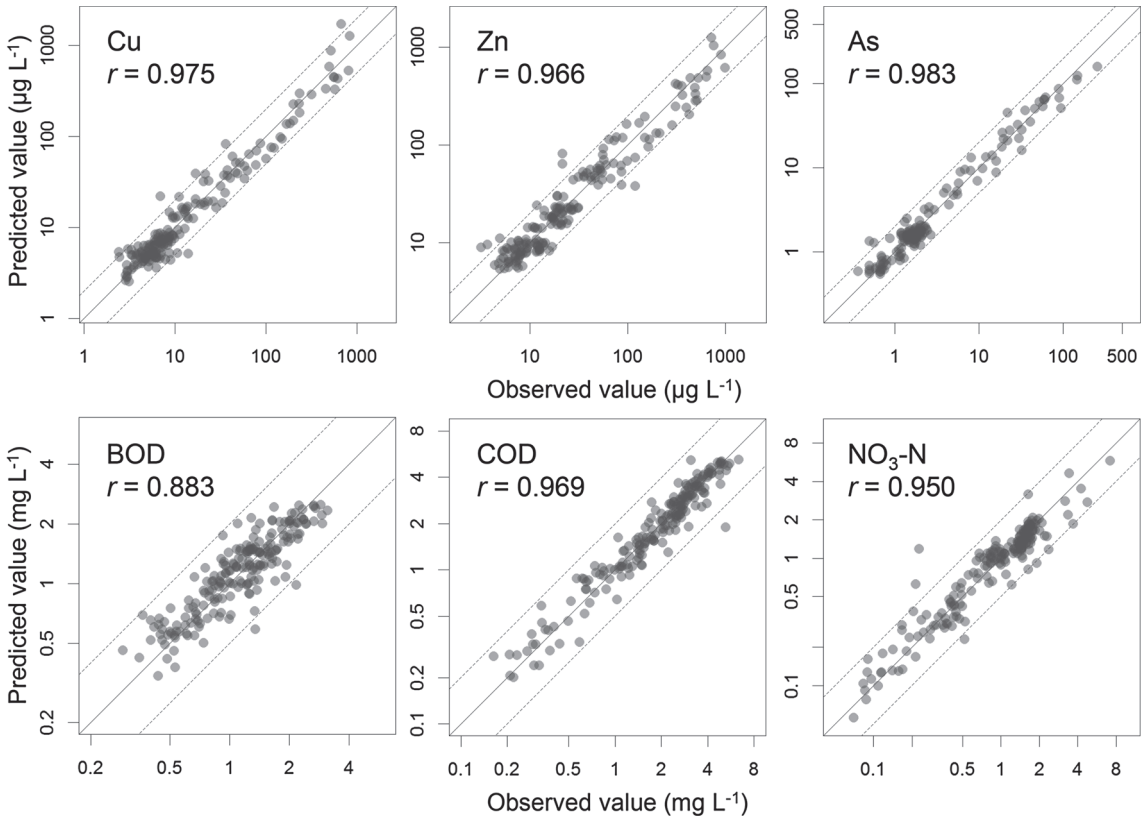

Fig. 3 Relationships between annual averages predicted by generalized additive mixed models and those calculated from observations of copper (Cu), zinc (Zn), and arsenic (As) concentrations, biochemical oxygen demand (BOD), chemical oxygen demand (COD), and nitrate-nitrogen (NO_3–N) concentration. *Solid line* corresponds to the line of perfect agreement; *dashed lines* correspond to a factor of ±2

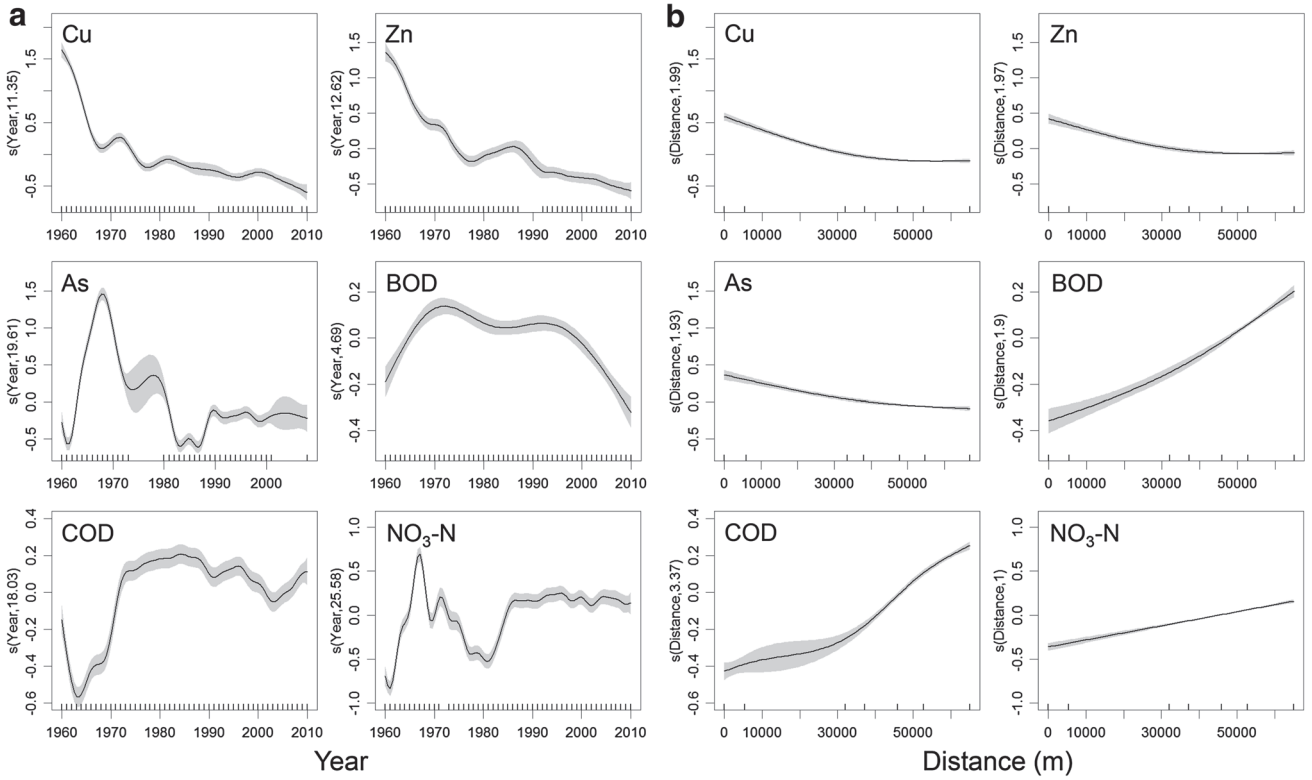

Fig. 4 Estimated spatiotemporal changes (**a** year; **b** distance from S1) in water-quality variables [copper (Cu), zinc (Zn), arsenic (As), biochemical oxygen demand (BOD), chemical oxygen demand (COD), and nitrate-nitrogen (NO_3–N)]. *Vertical axis* is a standardized measure of the corresponding water-quality variable; the number in *parentheses* is the estimated degrees of freedom for the smoothing function (i.e., a more "complex" curve has a larger number of degrees of freedom, and if the number is 1, then the estimated regression line is straight). The *solid line* is the estimated smoothing curve, and the *shaded area* is the 95% confidence band

decreased and had reached 1–3 μg L^{-1} by 2010 (Fig. 4; Fig. S2). Concentrations of all of these metals were higher upstream, close to the Ashio Copper Mine, and gradually decreased downstream (Fig. 4).

Only in the 2000s were the annual mean concentrations of copper in the lower reaches of the Watarase River lower than the US Environmental Protection Agency (EPA) water-quality criterion (continuous concentrations of 3–5 μg L^{-1} at a water hardness of 30–50 mg L^{-1}; US EPA 2002). Note that a Japanese water-quality standard for copper has not yet been established. Annual mean concentrations of zinc in the lower reaches were below the Japanese water-quality standard (30 μg L^{-1} for freshwater; Ministry of Environment 2016) after 1974, and those concentrations at the most upstream site (S1) were below the standard after 1996 (Fig. S1). Annual mean concentrations of arsenic were usually lower than the US EPA criterion of 150 μg L^{-1} (Fig. S2), although "safe" concentrations for arsenic vary depending on the jurisdiction: for example, the value is 5 μg L^{-1} in the Canadian water quality guideline (http://ceqg-rcqe.ccme.ca/en/index.html) but 50 μg L^{-1} in the UK water quality standard (http://evidence.environment-agency.gov.uk/ChemicalStandards/).

In contrast to the declines in copper and zinc concentrations, BOD, COD, and nitrate-nitrogen increased from 1960 to the 1970s (see Fig. 4 for the general spatiotemporal trend and Figs. S3 to S5 for individual sites). Trends differed after the 1970s, although slight decreases were generally observed (Fig. 4). However, temporal changes in actual values were not as distinct as those of metals: for example, spatial variation in BOD was 0.3–1.0 L^{-1} in 1960 and 0.7–2.1 mg L^{-1} in 1970 (Fig. S3). The range of BOD then gradually decreased to 0.3–0.8 mg L^{-1} by 2010. Identifying drivers of these temporal changes is challenging. However, in general, they are likely associated with industrialization and urbanization, as well as population increases, in the river basin during periods of rapid economic growth. They are also likely related to the establishment of environmental regulations (e.g., the Water Pollution Control Law in 1971) and to the improvement of sewerage systems (Japan Society on Water Environment 1999). Spatial changes in BOD, COD, and nitrate-nitrogen were similar (i.e., they increased downstream; Fig. 4), and higher rates of increase in BOD and COD were detected in the lower reaches than in the upstream reaches (e.g., downstream of S3; Fig. 4). This was likely the result of the

contribution of activities in urbanized and agricultural areas located downstream.

Limitations on estimates obtained from GAMMs

By using the GAMMs that we developed, we could predict the six water-quality variables at almost any location along the Watarase River (see the ESM for model estimates provided for the river during 1960–2010). This capacity should be useful for evaluating biological responses to water quality on the basis of time-series biomonitoring data. However, some caution should be paid to the use of the model outputs, even though they are essentially statistical interpolations.

First, water-quality measurement data for the upstream reaches (i.e., at sites S1 and S2) after 1985 were unavailable in the data set used, so it would be advisable to validate the model estimates by comparing them with other measured data before use. Water-quality monitoring from 2011 to 2015 ($n = 20$; Kiryu City Waterworks Bureau 2016) indicated that average concentrations of total copper, zinc, and arsenic at S1 were 12, 21, and 3 μg L^{-1}, respectively. These monitoring results were closely consistent with our model estimates of 12, 16, and 3 μg L^{-1} in 2010. In addition, 2004 measurements ($n = 2$ or 3) of copper, zinc, arsenic, BOD, COD, and nitrate-nitrogen at S2 are available from another survey (Suzuki et al. 2004). Their averages (13, 14, and 2 μg L^{-1}; 1.2, 0.8, and 0.7 mg L^{-1}, respectively) were comparable with our estimates (16, 19, and 4 μg L^{-1}; 0.4, 0.7, and 0.6 mg L^{-1}), except in the case of BOD. Results of these comparisons indicate that, particularly after the 2000s, our models provide reasonable values in the upper reaches in cases where original measurement data are lacking.

Second, the multipurpose Kusaki Dam (total reservoir capacity 60,500,000 m^3) was built just downstream of S2 in 1977. Because our GAMMs do not explicitly take into account the influence of dam operation on water quality, model estimates for areas just downstream of the dam should be used with caution.

Third, as noted in "Materials and methods", GAMMs used here assume that temporal trends in water quality are consistent at all locations along the river. This assumption is likely reasonable for metals, because their major source is the mine wastewater (a point source located upstream). However, the sources of BOD, COD, and nitrate-nitrogen are generally diffuse (Campbell et al. 2005), and this diffuse nature could have added uncertainty to our model estimates. Therefore, we recommend that some validation be performed before our model estimates are used in further studies, particularly if the original measurements are unavailable in the corresponding locations or years.

Last, but not least, during the monitoring period (i.e., 1960–2010), water-quality-analysis techniques markedly changed (e.g., the introduction of inductively coupled plasma–mass spectrometry; ICP-MS), and knowledge of quality control and assurance improved. Unfortunately, detailed information on when and how such changes were applied to the monitoring data sets used was unavailable. However, for example, original data show that quantification levels for copper and zinc indeed declined in the 1980s. Because higher concentrations of metals were observed earlier in the monitoring period, the higher LOQs in this period are unlikely to be a major issue in interpreting our modeling results. If we were to take into account the above-mentioned technical changes (e.g., when ICP-MS was introduced for metal analysis) in the modeling, more accurate and statistically defensible estimates could be obtained.

Concluding remarks

We reconstructed the spatiotemporal changes in six water-quality variables along the Watarase River using GAMMs. The results clearly demonstrated that metal pollution levels, as represented by copper and zinc, greatly decreased during the 1960s and 1970s, whereas organic pollution levels, as represented by BOD and COD, increased, possibly because of other factors such as urban and agricultural activities. Relatively different temporal patterns of change were observed during this period in the cases of arsenic and nitrate-nitrogen. Thereafter, the levels of metal and organic pollution generally decreased and were still decreasing in 2010, except COD. Further research using geographic information systems and hydrological and water-quality modeling (e.g., Arnold et al. 1998; Ryo et al. 2015) is required to more specifically identify the reasons behind spatiotemporal changes in these water-quality variables.

Accumulation of knowledge and experience from long-term monitoring studies is vital for developing future ecological risk management strategies (Iwasaki 2016). However, a limited number of long-term biomonitoring studies have been done in metal-contaminated rivers and streams worldwide (Clements et al. 2010; Mebane et al. 2015). To our knowledge, virtually no such Japanese studies are available (but see Watanabe et al. 2000, who investigated the long-term recovery of macroinvertebrates in a metal-contaminated river), despite the fact that many rivers in this country have been affected by discharge from abandoned mines (Naito et al. 2010). Our modeling outputs for water-quality variables, coupled with a series of biological surveys conducted along the Watarase River (e.g., for macroinvertebrates: Ide and Arai 1978; Suzuki et al. 2004), allow us to investigate relationships between these

variables and ecosystem recovery trajectories. This is our ongoing work.

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Introducing a new standardized nanomaterial environmental toxicity screening testing procedure, ISO/TS 20787: aquatic toxicity assessment of manufactured nanomaterials in saltwater Lakes using *Artemia sp*. nauplii

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RESEARCH ARTICLE

Introducing a new standardized nanomaterial environmental toxicity screening testing procedure, ISO/TS 20787: aquatic toxicity assessment of manufactured nanomaterials in saltwater Lakes using *Artemia sp*. nauplii

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ABSTRACT

This paper introduces a new standardized testing procedure for nanomaterial environmental toxicity (International Organization for Standardization/Technical Specification (ISO/TS) 20787): 'aquatic toxicity assessment of manufactured nanomaterials in saltwater lakes using *Artemia sp.* Nauplii' intended to generate more reliable and repeatable aquatic toxicity data testing manufactured nanomaterials, using *Artemia sp*., to evaluate their possible ecotoxicity in saltwater lake ecosystems. The principles behind testing with *Artemia sp*. are reviewed and the paper gives an overview of research published between 2009 and 2018 in which manufactured nanomaterials were tested using *Artemia sp*.

Abbreviations: AChE: acetylcholinesterase; AAS: atomic absorption spectrometry; AgNPs: silver nanoparticles; AFM: atomic force microscope; AP: acid phosphatase; ASTM: American Society for Testing and Materials; AuNPs: gold nanoparticles; CAT: catalase; CHN: carbon hydrogen nitrogen; CNTs: carbon nanotubes; DLS: dynamic light scattering; EC50: effective concentration 50%; EDX: energy dispersive X-ray analyzer; FESEM: field emission scanning electron microscope; FTIR: Fourier transform infrared spectroscopy; GPx: glutathione peroxidase; GST: glutathione S-transferase; ICP-MS: inductively coupled plasma mass spectrometry; ISO: International Organization for Standardization; LC50: lethal concentration for 50% individuals; MDA: malondialdehyde; MNMs: manufactured nanomaterials; MWCNTs: multi-walled carbon nanotubes; NPs: nanoparticles; OECD: Organization for Economic Co-operation and Development; ROS: reactive oxygen species; SSA: swimming speed alteration; sp: species; SWCNTs: single wall carbon nanotubes; SOD: superoxide dismutase; TAOC: total antioxidant capacity; TC: Technical Committee; TEM: transmission electron microscope; TG: test guideline; TP: total protein; TR: Technical Report; TS: Technical Specification; UV-vis: ultraviolet and visible light; XRD: X-ray diffraction

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Introduction

The rapid development of nanotechnology is potentially affecting every aspect of industry and society, leading to a need for establishing international standards for nanotechnologies. The International Organization for Standardization/Technical Committee 229 (ISO/TC 229) addresses standardization for nanotechnologies. *Standardization for nanotechnologies* will promote commercialization of nanoproducts and eventually global economic development, and also contribute to protection of humans and the environment from adverse effects, which are derived from the possible hazards and the exposure to manufactured nanomaterials (MNMs) during their life cycle.

When performing tests for aquatic toxicity, it is important to ensure actual exposure to the material tested, which can be challenging for nanomaterials. One issue when testing MNMs is that they may undergo agglomeration, sedimentation, dissolution, and transformation by reacting with the dispersion or exposure media (Tantra et al. 2011). However, test organisms are exposed to colloids or particle-sediment mixtures rather than chemicals in solution and can also be exposed to a mixture of particles and reaction products, including ions, reactive oxygen species, etc. (OECD 2012). An OECD (Organization for Economic Co-operation and Development) publication, Guidance Document on Aquatic Toxicity Testing of Difficult Test Chemicals and Mixtures, included considerations for testing sparingly soluble chemicals (OECD 2000). Furthermore, the OECD collected the lessons learnt when preparing nanomaterials for testing, resulting in the 'Guidance on Sample Preparation and Dosimetry for the Safety testing of Manufactured Nanomaterials' (OECD 2012), but further guidance may be needed for testing insoluble, sparingly soluble or partially reactive MNMs. The fate of MNMs depends both on the

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intrinsic and extrinsic physicochemical properties of nanomaterials (Rasmussen et al. 2018), and this increases the challenges of testing MNMs in an aquatic environment. Therefore, the availability of internationally standardized or harmonized methods for testing would facilitate the assessment of aquatic toxicity of MNMs.

In the case of the aquatic environment, a variety of test organisms can be used to assess the possible adverse effects of chemicals, including MNMs. For regulatory testing purposes, the OECD test guidelines (TGs) are applied globally, and they focus mainly on the fresh water environment using fish, *Daphnia*, and algae as the representative test organisms for the three trophic levels relevant for testing aquatic toxicity (http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm). The OECD TGs were originally developed for testing chemicals, not for insoluble or poorly soluble particles such as MNMs. A preliminary review of the OECD TGs indicated the need to adapt and modify them to ensure their applicability to MNMs (OECD 2009). This adaptation is currently ongoing within the OECD test guidelines program, and for environmental testing a new TG318, Dispersion Stability of Nanomaterials in Simulated Environmental Media, has been adopted (OECD 2017), and several more are underway.

Brine shrimps (*Artemia sp.*) are ubiquitous in saline lakes worldwide, (Lavens and Sorgeloos 1996), making them one of the most widely occurring euryhaline organisms. In fact, the salinity of many saltwater lakes is so high that *Artemia* is one of the few organisms that can survive along with some specific species of bacteria and microalgae. Thus, *Artemia* is the representative species for saline lakes, and it may be used in ecotoxicity testing.

There are several advantages of using *Artemia sp*. as a biological model for assessing saltwater aquatic toxicity of MNMs, and they are listed in the following: (i) less concern about animal welfare than for vertebrate species; (ii) extensive knowledge of *Artemia sp*. biology for culturing it in laboratories (Nunes et al. 2006); (iii) wide geographic distribution of *Artemia sp*. in saline lakes; (iv) testing using *Artemia sp*. nauplii (newborn brine shrimp) is simple and cost-effective; v) the small body-size of *Artemia sp*. nauplii (0.44 millimeters) allows testing in small containers; (vi) *Artemia sp*. adapt to a wide range of water salinity (5 g/L–300 g/L) and temperatures (6–40 °C); vii) *Artemia sp*. are simple to maintain in the laboratory; viii) the short life cycle of *Artemia sp*. makes them suitable for growth, reproduction, and short-term toxicity tests; ix) *Artemia sp*. cysts are commercially and readily available, and the cysts remain viable after being stored for years under cool and dry conditions, and free-swimming nauplii hatch within ca. 24 h on immersion of the cysts in saline water; and x) hatching from cysts yields organisms of similar age, genotype, and physiological condition. However, based on the adaptability of *Artemia sp*. to such a wide range of water salinity and temperatures, *Artemia sp*. is not a very sensitive organism, and may lead to false negatives. Thus, it should, for example, not be used as a general substitute for *Daphnia* testing (Persoone and Wells 1987). *Artemia sp*. may be useful as a test organism in saline water where *Daphnia* cannot be used as test organism.

In the past, several international attempts have been made by various organizations to standardize aquatic toxicity testing with *Artemia sp*., however without resulting in an internationally standardized method. In the 1980s, a simple acute toxicity test using *Artemia sp.* nauplii was developed by the *Artemia* Reference Center. The test is a static test, which is based on then existing methods. Its duration is 24 h, the life-stage is nauplii, and the response criterion is mortality expressed as the LC_{50} (Lethal Concentration for 50% of individuals) (Persoone and Wells 1987). A Round Robin test to examine intra- and inter-laboratory variabilities was conducted in the early 1989 with 129 responses received in total from 81 laboratories from Europe and 48 laboratories from Canada and the United States of America (USA) (Persoone et al. 1993). The outcome of the Round Robin test was satisfactory, but the overall precision was not impressive, as the coefficient of variation was 50% in Europe and USA, and 33% in Canada. Among the reasons for this variability were that some participants were unfamiliar with the new methodology for ecotoxicological testing (Van Steertegem and Personne 1993). After these Round Robin test activities, only little progress has been made to internationally standardize method(s) for *Artemia* toxicity testing.

Currently, regulatory use is essentially limited to the United States Environmental Protection Agency's (USEPA) use of *Artemia sp*. for testing oil-spill dispersants. However, the Italian National Institute for Environmental Protection and Research, the Italian Water Research Institute, and the Italian Agency for Standardization in the Chemical sector proposed a protocol for testing immobilization/mortality of *Artemia* (APAT IRSA-CNR 2003). Several researchers have used *Artemia sp*. as a test organism for salt water aquatic nanotoxicology (Table 1). However, due to lack of an agreed and standardized protocol for testing aquatic toxicity using *Artemia sp*., data from these studies are effectively not reproducible. Moreover, important gaps in the description of the testing performed were identified (Libralato 2014), and Libralato proposed that, when reporting test results in future, parameters such as water oxygen saturation, pH, and conductibility should be stated. As that information could not be extracted from the literature reviewed it is not recorded in Table 1, but otherwise Table 1 reports on the degree to which Libralto's proposed parameters are reported in the reviewed literature.

This paper describes the considerations behind the recent ISO/Technical Specification (TS) 20787 (2017) and outlines its content. ISO/TS 20787 proposes a procedure for acute toxicity testing in saline lakes with *Artemia sp*., using the acute endpoints of immobilization and hatching rate as the main endpoints. As the hatching of *Artemia* cysts occurs within about 24 h, testing using *Artemia* is acute testing.

Other endpoints, including chronic endpoints, such as mortality (both short-term and long-term) and biomass productivity or reproductive ability, can also be considered through long-term exposure to sub-acute concentrations and measurement of several biomarkers as indicated in Table 1, last column. Only two papers that consider chronic exposure

Table 1. Summary of aquatic nanotoxicity experiments using *Artemia sp.* published between 2009 and 2018.

No. and Reference/year	MNMs/range of concentration tested	Particle size / morphology / measurement method(s)	Tested species	Life stage	Media used for exposure/salinity	Exposure vessel (type/volume/ density); No of replicates	Lighting/ temperature/feeding (see note)	Exposure time	Additional control/ Reference chemical	Toxicity endpoint(s)
1. Jeong et al. 2009	Nine kinds of MNMs; 0.05–20 mg/L	Less than 100 nm (measurement method not determined)	*Artemia sp.*	Cysts	Filtered and auto-claved sea water	24-well plate; 1 ml	12 h-12 h light-dark; 25 °C	24 h	Not used	Hatching rate
2. Cornejo-Garrido et al. 2011	Pb NPs; 1, 10, 100, and 1000 μM	<50 nm (TEM)	*A. salina*	Instar I–II (Stage not declared clearly)	Artificial seawater (Instant Ocean®); 37 g/L	96-well plates; 0.1 ml; 10 nauplii per well	25 ± 1 °C	24 h	$K_2Cr_2O_7$: (29.42 mg/L) caused 48.3% mortality	Mortality
3. Fatouros et al. 2011	SWCNTs; 100 mg/L	Diameter: 0.88–1 nm (estimated from Raman); Also, TEM and FTIR done.	*Artemia sp.*	Nauplii (Stage not declared clearly)	Artificial sea water (Tropic Marin®)	48 well-plate; 1 ml; 20–25 larvae per well; triplicate	27 °C	24 h	Not used	Visual accumulation/ Elimination
4. Kowalska-Góralska et al. 2011	Ag NPs; 5–100 mg/L	Not determined	*A. salina*	Fully developed (unknown stage)	ISO stand-ard freshwater	Erlenmeyer flasks; 10 animal per flask	18 °C	6 h	$AgNO_3$ (for comparison)	Mortality
5. Radhika et al. 2011	Fullerene (0.04–0.88 mg/L); TiO_2 NPs (0.5–500 mg/L)	TEM: Filtered C60 (10–20 nm), Sonicated C60 (50–100 nm), Sonicated TiO_2 (200 nm), Filtered TiO_2 (30–50 nm).	*A. salina*	Instar I; Instar II; Metanauplius; Zoea; Aadult	Filtered sea water; 35 g/L	Closed Petri dish; 10 ml; 10 per vessel; 5 replicates	Darkness; 27 ± 1 °C; No feeding	48 h	Not used	Mortality; LC_{50}; Studying the effects of MNMs sonication and filtration on toxicity;
6. Ashtari et al. 2012	Silica-encapsulated magnetic NPs; 10, 100, and 1000 mg/L	70 nm (TEM); EDS also done	*A. salina*	Nauplii (unknown stage)	Artificial seawater (unknown source); 38 g/L	Vial (unknown material and volume); 10 per vial; Shaking.	Room temperature.	24 h	Gallic acid ($C_7H_6O_5$): LD_{50}=20 mg/L	Mortality; LD_{50}
7. Falugi et al. 2012	Ag NPs; 0.1–100 mg/L	1–10 nm	*A. salina*	Instar I	Not determined	Not determined	Not determined	48 h	Not used	Mortality; LC_{50}
8. Arulvasu et al. 2014	Ag NPs; 2–12 nm	30–40 nm (FESEM); Also, EDX done	*Artemia sp.*	Decapsulated cysts; Instar I nauplii	33 g/L	12-well plates; 2 ml; 10 nauplii or 20 cysts per well; Intermittent flow-through condition; Triplicate	Not described very well.	24 h (cysts); 48 h (nauplii)	Not used	Hatching rate; Mortality; LC_{50}; DNA damage (comet assay); apoptosis
9. Ates et al. 2013a	NPs of Zn and ZnO (10, 50, and 100 mg/L)	Zn: 30-80, 40–135 nm (TEM); 2110, 1952 nm (DLS); ZnO: 10–55, 90–230 nm (TEM); 2103, 1820 nm (DLS)	*A. salina*	Instar I	Seawater; 29–30 g/L	500 ml conical plastic containers	16 h–8 h light-dark; 24 ± 2 °C; No feeding	Up to 96 h	Not used	Mortality; Accumulation/ Elimination; Oxidative stress (MDA)
10. Ates et al. 2013b	TiO_2 NPs; 10–100 mg/L	99.5 % rutile polymorph; 8–40 nm (TEM); 210–1833 nm (DLS)	*A. salina*	Nauplii (stage not determined), and adults	Filtered artificial seawater (Instant Ocean®); 29–30 g/L	Conical plastic containers: 500 and 1500 ml for nauplii and adults respectively; 3 replicates; Mixing exposure media through gentle aeration	16 h–8 h light-dark; 24 ± 2 °C; No feeding	Up to 96 h	Not used	Mortality; Accumulation/ Elimination; Lipid peroxidation (MDA)
11. Karthik et al. 2013	Au NPs	35 ± 5 nm (TEM); Also, UV-vis and XRD analysis done	*Artemia sp.*	Not determined	Not determined	Not determined	Not determined	Not determined	Not used	Mortality
12. Clemente et al. 2014	TiO_2 NPs; 6.25-1000 mg/L	25 nm (Manufacturers' specifications); 700–1000 nm (DLS); UV–Vis done to determine suspension stability in exposure media (at 0, 3, 6, and 24 h)	*A. salina*	**Acute:** Instar II; **Chronic:** Nauplii for growth test and adults for biochemical assay	Red Sea Salt®; 30 g/L; (For the growth tests, 3% saccharose was added)	**Acute:** Glass Petri dishes (5 cm diameter); 10 ml; 5 nauplii per dish; quadruplicate; Test solution renewed every day; **Chronic:** For growth test: 24–well polystyrene plates; 2 ml; 10 nauplii per well; for biochemical assay: Petri dishes	**Acute:** 20 ± 1 °C; No feeding; **Chronic:** Feeding on microalgae	48 h (acute); 96 h (chronic)	Not used	**Acute:** Mortality; EC_{50}; (Interestingly *A. salina* was more sensitive than *Daphnia similis*); **Chronic:** Growth rate; Biochemical parameters (GPx, GST, CAT; SOD; AP)

(*continued*)

Table 1. Continued.

No. and Reference/year	MNMs/range of concentration tested	Particle size / morphology / measurement method(s)	Tested species	Life stage	Media used for exposure/salinity	Exposure vessel (type/volume/ density); No of replicates	Lighting/ temperature/feeding (see note)	Exposure time	Additional control/ Reference chemical	Toxicity endpoint(s)
						(10 cm diameter); 50 ml; 100 adult per dish; triplicate; Test solution renewed every day				
13. Gambardella et al., 2014	NPs of SnO_2, CeO_2, Fe_3O_4; 0.01, 0.1, 1 mg/L	Manufacturer information: SnO_2 (61 nm), CeO_2 (50–105 nm), Fe_3O_4 (20–30)	*A. salina*	Instar I	Filtered natural seawater; 34 ± 1 g/L	24-well polystyrene plates; 1 ml; 10–15 nauplii per ml; 3 replicates	16 h–8 h light-dark; 20 °C	48 h	Not used	Mortality; Visual accumulation; Swimming Speed Alteration (SSA); Biochemical parameters (Cholinesterase, GST, CAT activity)
14. Pretti et al. 2014	Graphene (two kinds); 0.1–10 mg/L	Lateral size 5–25 μm and ≈ 550 nm; Thickness: 5–30 and 0.35 nm (Company information); DLS done	*A. salina*	Not determined	Filtered natural sea water; 35 g/L	100 ml glass flasks; 2000 per flask; 3 replicates	16 h–8 h light-dark; 20 ± 1 °C	24 h (for acute mortality test); 48 h (for oxidative stress)	Not used	Mortality; Visual accumulation; Oxidative stress: Lipid peroxidation, GPx, CAT
15. Rodd et al. 2014	Carbon black NPs; 50–1000 mg/L	SEM and DLS techniques were used but results not appear in the paper.	*A. franciscana*	Instar I	Simulated seawater (Instant Ocean®)	Glass scintillation vial; 15 ml; 250 larvae per vial	19 °C	24 h	Not used	Mortality; Visual accumulation; Measuring hsp70 protein levels
16. Tavana et al. 2014	Ag NPs and TiO_2 NPs; 0.1-100 mg/L	12.65 nm AgNPs and 17.5 nm TiO_2 NPs (TEM); UV-vis done to estimate particle dispersibility over time	*A. franciscana*	Instar I	Natural seawater (Urmia lake); 35 g/L	Glass beaker; 1000 ml	22 °C	12 h (Ag NPs); 48 h (TiO_2 NPs)	Not used	Accumulation/ Elimination
17. Vijayan et al, 2014	NPs of Au and Ag; Conc. not declared clearly	2–17 nm Ag NPs and 2–19 nm Au NPs (TEM); Also UV-vis, FTIR, FESEM, EDX, and XRD analysis done	*A. salina*	Nauplii (Stage not declared clearly)	Sterilized sea water	24-well plates; 1 ml; 10 nauplii per well	25 °C	24 h	Not used	Mortality; LC_{50}
18. Ates et al. 2015	Alpha and gamma Al_2O_3 NPs; 5–100 mg/L	a-Al_2O_3 (50 nm and 3.5 μm) and g-Al_2O_3 (5 nm and 0.4 μm); Also, TEM, DLS, FTIR, XRD analysis done	*A. salina*	Nauplii (stage not determined)	Filtered artificial seawater (Instant Ocean®); 29–30 g/L	Conical plastic containers: 500 nauplii; 3 replicates; Mixing exposure media via gentle aeration	16 h–8 h light-dark; 24 ± 2 °C; No feeding;	Up to 96 h	Not used	Mortality; Accumulation/ Elimination; Lipid peroxidation (MDA)
19. Becaro et al. 2015	Ag NPs; 0.00015–1.5 mg/L	2–18 nm (TEM); 8.12 nm (DLS); UV-vis	*A. salina*	Nauplii (Stage not declared clearly)	Artificial seawater (Sera Premium ®); 30 g/L	Beakers; 30 ml; 10 nauplii per beaker; duplicate.	20 ± 2 °C	48 h	Borohydride (for comparison)	Mobility; EC_{50}
20. Callegaro et al. 2015	TiO_2 NPs; 0.01–90 mg/L	80% Aanatase and 20% Rutile; TEM, CHN, DLS, Stability analyzer (LUMifuge®), and fluorescence spectrophotometry were done	*A. franciscana*	Instar II	Filtered artificial seawater; 34 ± 1 g/L	24-well plates; 2 ml; 10 nauplii per well; 3 replicates	12 h–12 h light-dark; 25 ± 1 °C	48 h	$CuSO_4.5H_2O$: 48 h EC_{50}<6.5 mg/L; Alginates (as dispersant control)	Mortality
21. Gambardella et al. 2015	Ag NPs; 1, 5, 10, and 50 mg/L	1–10 nm (unknown); 990 nm (DLS)	*A. salina*	Instar I	Filtered natural seawater; 36.9 g/L	Multi-well plates; 1 ml; 10–15 nauplii per ml; 3 replicates	Darkness; 20 ± 0.5 °C	48 h	$K_2Cr_2O_7$	Mortality and Swimming Speed Alteration (SSA): The second one had a higher sensitivity
22. Mesarič et al. 2015	Carbon black (CB), Graphene oxide (GO),	TEM (CB: 13nm, GO: 0.5–5 μm, MWCNTs: 5.7-15nm); Also, Zeta	*A. salina*	Instar I	Filtered natural sea water; Unknown source and salinity	24-well polystyrene plates; 1 ml; 10–15	16 h–8 h light-dark; 20 °C; No feeding	48 h	Not used	Mortality; LC_{50}; Visual accumulation; Swimming Speed

(*continued*)

Table 1. Continued.

No. and Reference/year	MNMs/range of concentration tested	Particle size / morphology / measurement method(s)	Tested species	Life stage	Media used for exposure/salinity	Exposure vessel (type/volume/ density); No of replicates	Lighting/ temperature/feeding (see note)	Exposure time	Additional control/ Reference chemical	Toxicity endpoint(s)
	and MWCNTs; 10–1000 mg/L	potential, SEM, EDS analysis done				nauplii per well; 3 replicates				Alteration (SSA); Biochemical parameters (Cholinesterase, GST, CAT)
23. Nogueira et al. 2015	NPs of TiO_2, NiO, Fe_2O_3: 8.2–20 mg/L	TiO_2 (spherical anatase, <25 nm); NiO (nearly spherical, 100, and 10–20 nm); Fe_2O_3 (nanorods, d = 40–130 nm, l = 250–600 nm)	*A. salina*	Instar II	Unknown source of salinity; 35 g/L	Multiwell plate; 3 replicates	Darkness; 25 °C	24 h	Not used	Immobilization; No toxicity was observed
24. Rajabi et al. 2015	Various organic and inorganic NPs; 1.56–400 mg/L	From 6 to up to 464.6 nm	*A. salina*	Nauplii (unknown stage)	Unknown source of artificial sea water; 35 g/L	96 well plates; 200 µL; 10 nauplii per well; 3 replicates	Unknown	24 h	Not used	Mortality; LC_{50}
25. Ates et al. 2016	NiO and CoO NPs	TEM: NiO (20–90 nm) and CoO (40–120 nm); Ions release measured (ultrafiltration + ICP-MS); Also DLS, analysis done	*A. salina*	Nauplii (stage not determined), and adults	Filtered artificial seawater (Instant Ocean®); 29–30 g/L	Conical plastic containers: 500 and 1500 ml for nauplii and adults respectively; 3 replicates	16 h–8 h light-dark; 24 ± 2 °C; No feeding;	Up to 96 h	Not used	Accumulation; Lipid peroxidation (MDA)
26. Bergami et al. 2016	Polystyrene (PS) NPs; 5–100 mg/L	TEM: PS-COOH (40 nm), PS-NH_2 (50 nm); Also Zeta potential and DLS analysis done.	*A. franciscana*	Instar I	Filtered natural sea water; 38 g/L	24-well plates; 2 ml; 10 nauplii per well; 3 replicates; Static condition; Mixing exposure media via gentle aeration	Darkness; 25 ± 1 °C; No feeding.	48 h	$K_2Cr_2O_7$	Mortality; Amount of molts; Visual accumulation/Elimination
27. Daglioglu et al. 2016	Pd NPs (0.01, 0.1, 1 mg/L)	Unknown	*A. salina*	Not determined clearly	Filtered artificial sea water	Covered conical plastic vessel; 100 ml; 50 per vessel; 3 replicates; Mixing exposure media via gentle aeration	16 h–8 h light-dark; 24 ± 2 °C; No feeding	Up to 96 h	K_2PdCl_4 (for comparison)	Mortality
28. Jemec et al. 2016	Ag NPs	20.4 ± 6.8 nm (TEM); 123.8 ± 12.2 nm (DLS); 46–68% Ag^+ dissolution	*A. franciscana*	Hydrated Dechorionated eggs (cysts)	Rock salt; 25 g/L	Unknown	Continuous illumination; 28 °C	24 h	$AgNO_3$ (for comparison)	Hatching rate; 24 h EC_{50}: 1.3 mg/L
29. Johari et al. 2016	ZnO NPs; 1, 10, 100 mg/L	25–30 nm (TEM)	*A. salina*	Instar II	Natural seawater (Urmia lake); 30 g/L	Glass beaker; 500 ml	29 °C	24 h (Accumulation); 28 d (Trophic transfer)	Not used	Accumulation/ Elimination; Trophic transfer to fish (*Danio rerio*)
30. Kos et al. 2016	Ag NPs; 1–125 mg/L	19 ± 21 nm (TEM); 144–169 nm (DLS); 46% Ag^+ species in the stock suspension (ultracentrifugation-+ AAS)	*A. franciscana*	Instar I	Synthetic saltwater (SSW); 31.83 g/L	24 well polypropylene plates; 2 ml; 10 nauplii per well; 10 replicates	16 h–8 h light-dark ; 25 °C; No feeding	48 h	$K_2Cr_2O_7$: 48 h EC_{50}= 21.2 ± 3.7 mg/ L $K_2Cr_2O_7$	Immobilization; 48 h EC_{50}: 36.48 mg/L
31. Ozkan et al. 2016	NPs of TiO_2 and $AgTiO_2$ (0.03–100 mg/L)	SEM: TiO_2: 9.014–267 nm, $AgTiO_2$: 1.049–4620 nm; XRD done; Also DLS measurements done after 0, 24, and 96 h exposure.	*A. salina*	Not determined clearly	Filtered artificial sea water (Instant Ocean salt); 30 g/L	Covered conical Polyethylene vessel; 100 ml; 50 per vessel; 3 replicates; Mixing exposure media via gentle aeration	16 h–8 h light-dark; 25 ± 1.5 °C; No feeding	Up to 96 h	Not used	Mortality; LC_{50}; Morphological Changes; Accumulation/ Elimination
32. Rahmani et al. 2016	Ag NPs; 0.5, 1, 2 mg/L	12.65 nm (TEM)	*A. salina*	Adult	Natural seawater (Urmia lake); 35 g/L		28 °C		Not used	

(*continued*)

Table 1. Continued.

No. and Reference/year	MNMs/range of concentration tested	Particle size / morphology / measurement method(s)	Tested species	Life stage	Media used for exposure/salinity	Exposure vessel (type/volume/ density); No of replicates	Lighting/ temperature/feeding (see note)	Exposure time	Additional control/ Reference chemical	Toxicity endpoint(s)
33. Balalakshmi et al. 2017	Au NPs	25 nm (TEM); Also UV-vis, SEM, FTIR, and XRD analysis done	*Artemia sp.*	Instar I	Sterile seawater (unknown source); 30 g/L	Glass beaker; 500 ml; 300 adults per beaker 24-well plates; 3 ml; 10 nauplii per well; 3 replicates	Not determined	24 h (Accumulation); 14 d (Trophic transfer) 48 h	Not used	Accumulation; Trophic transfer to fish (*Danio rerio*) Mortality
34. Bergami et al. 2017	Polystyrene (PS) NPs; 0.1–5 mg/L	TEM: PS-COOH (40 nm), PS-NH_2 (50 nm); Also Zeta potential and DLS analysis done.	*A. franciscana*	Instar I	Filtered natural sea water; 38 g/L	**Short term:** Glass beakers; 100 ml; 200 nauplii per beaker; Static condition. **Long term:** Flasks; 30 ml; 10 nauplii per flask; 3 replicates; Test solution renewed every 2–3 days	**Short term:** Darkness; 25 ± 1 °C; No feeding. **Long term:** 16 h–8 h light-dark; 25 ± 1 °C; Feeding on microalgae	48 h (short term); 14 d (long term)	Not used	**Short term:** Gene expression (RT q-PCR) analysis on molting genes (clap and cstb). **Long term:** Mortality; LC_{50}; Visual disposition of fluorescent NPs.
35. Bhuvaneshwari et al. 2017	NPs of ZnO and Anatase TiO_2 (10, -160 mg/L)	ZnO: rods and spherical, length: 45.3 ± 17, width: 25.1 ± 5.1 nm (TEM); TiO_2: 18.3 ± 3.2 nm (TEM); Zn^{2+} release from NPs were measured	*A. salina*	Untreated eggs; Instar II	Filtered and sterilized natural sea water; 37 ± 0.5 g/L	Not determined clearly	No feeding	24 h (for cysts); 48 h (for nauplii)	Not used	Hatching rate of cysts; Mortality of nauplii; Accumulation/ Elimination; ROS generation; Oxidative stress (CAT)
36. Lacave et al. 2017	PVP/PEI-coated Ag NPs; 1–10 mg/L	5.08 ± 2.03 nm (TEM); 90–100 nm (DLS); 20–29.6% Ag^{+} dissolution	Unknown (*Artemia sp.*)	Instar I or instar II	Unknown salt source; 33 g/L	Covered 24-well polystyrene microplates; 2 ml; 5–7 nauplii per well	Continuous illumination; 18.5 °C	48 h	Not used	Immobilization; Dietary transfer of NPs to fish
37. Madhav et al. 2017	CuO NPs; 1–90 mg/L (Cysts and nauplii), 50–800 mg/L (Adults)	114 ± 36 nm (SEM); DLS done after 0, 24, and 48 h (339–347, 518–574, and 825–855 nm, respectively); Also EDX, XRD, and FTIR, done.	*A. salina*	Cyst, nauplii (1 d, 2 d, and 7 d old), and adult	Filtered and sterilized seawater; 32 g/L	**Cysts and nauplii:** 12-well microplates; 2 ml; 10 cyst/nauplii per well. Shaking (60 RPM); Triplicate. **Adults:** Glass beaker; 25 ml; 10 nauplii per beaker; Triplicate.	Room temperature	24 h (cysts); 48 h (nauplii and adults)	Not used	Hatching rate; Mortality; LC_{50}; Accumulation; Histopathology; Biochemical parameters (RG, GST, CAT)
38. Muthukrishnan et al. 2017	Ag NPs; 25–200 mg/L	41.84 nm (TEM); Spherical shape	*Artemia sp.*	Nauplii (unknown stage) or adults	Seawater; unknown salinity	Unknown; 100 ml	16 h–8 h light-dark; 26 ± 2 °C; No feeding	Up to 108 h	Not used	Mortality; LC_{50}; Accumulation/ Elimination
39. Sugantharaj David et al. 2017	CeO_2 NPs; 10-320 mg/L	15 ± 3.5 nm (TEM); 130 ± 20 nm (SEM); 510 ± 80 nm (DLS); UV-vis done to estimate particle dispersibility over time; Ions dissolution measured (centrifugation + passing through ultra filteration disc + ICP-OES)	*A. salina*	Cysts; Nauplii (instar I)	Filtered and sterilized natural seawater; 37 g/L	**For cysts:** 24-well plates **For Nauplii:** Not determined.	Not determined clearly	48 h	Bulk CeO_2 (for comparison)	Hatching rate of cysts; Mortality; LC_{50}; Accumulation; Biochemical parameters (TP, ROS, CAT, SOD, AChE, GST)
40. Wang et al. 2017	α-Fe2O3 NPs; 25–600 mg/L	TEM, SEM, FTIR, XRD, and DLS done. Also ion release measured (centrifugation + ICP-MS)	*A. salina*	Cysts (capsulated and decapsulated), Nauplii (instar I, II, and III)	Filtered natural seawater; 30 g/L	**For cysts:** 24-well plates; 1 ml; 10 cyst per well; Shaking. **For Nauplii:** 24-well plates; 1 ml;	**For cysts:** Continuous illumination; 28 °C. **For Nauplii:** 16 h–8 h light-dark; 28 °C; No feeding.	**For cysts:** Up to 36 h **For Nauplii:** 24 h	Not used	Hatching rate of cysts; Mortality and swimming inhibition of Nauplii; Morphology (Visual, SEM); Uptake of NPs

(*continued*)

Table 1. Continued.

No. and Reference/year	MNMs/range of concentration tested	Particle size / morphology / measurement method(s)	Tested species	Life stage	Media used for exposure/salinity	Exposure vessel (type/volume/ density); No of replicates	Lighting/ temperature/feeding (see note)	Exposure time	Additional control/ Reference chemical	Toxicity endpoint(s)
						10 nauplii per well; Shaking; Octuplicate.				(Visual, TEM); Accumulation/ Elimination; Biochemical parameters (ROS, MDA, TAOC)
41. Zhu et al. 2017a	Fe_3O_4 NPs (25–600 mg/L)	186 nm to 19.9 μm (DLS); 170 nm (TEM)	*A. salina*	Cysts (capsulated and decapsulated), Nauplii (instar I, II, and III)	Filtered natural seawater; 30 g/L	**For cysts:** 24-well plates; 1 ml; 10 cyst per well; Shaking. **For Nauplii:** 100 ml beakers; 1000 nauplii per vessel	**For cysts:** Continuous illumination; 28 °C. **For Nauplii:** Not determined clearly	**For cysts:** Up to 36 h **For Nauplii:** 24 h	Not used	Hatching rate of cysts; Mortality and swimming inhibition of Nauplii; Morphology; Accumulation; Biochemical parameters (ROS, MDA, TAOC, SOD, CAT, GPx)
42. Zhu et al. 2017b	Oxidized-MWCNTs (25–600 mg/L)	Length ranged from 25 to 575 nm (TEM); Hydrodynamic size ranged from 117 nm to 121 μm (DLS)	*A. salina*	Cysts (capsulated and decapsulated), Nauplii (instar I, II, and III)	Filtered natural seawater; 30 g/L	**For cysts:** 24-well plates; 1 ml; 10 cyst per well; Shaking. **For Nauplii:** 24-well plates; 1 ml; 10 nauplii per well; Shaking; Octuplicate.	**For cysts:** Continuous illumination; 28 °C. **For Nauplii:** 16 h–8 h light-dark; 28 °C; No feeding.	**For cysts:** Up to 36 h **For Nauplii:** 24 h	$FeCl_3$ (for comparison)	Hatching rate of cysts; Mortality and swimming inhibition of Nauplii; Morphology; Accumulation/ Elimination; Biochemical parameters (ROS, MDA, TAOC, SOD, CAT, GPx)
43. Zhu et al. 2017c	Graphene oxide (GO); 25–600 mg/L	TEM, SEM, FTIR, and DLS done.	*A. salina*	Cysts (capsulated and decapsulated), Nauplii (instar I, II, and III)	Filtered natural seawater; 30 g/L	**For cysts:** 24-well plates; 1 ml; 10 cyst per well; Shaking. **For Nauplii:** 24-well plates; 1 ml; 10 nauplii per well; Shaking; Octuplicate.	**For cysts:** Continuous illumination; 28 °C. **For Nauplii:** 16 h–8 h light-dark; 28 °C; No feeding.	**For cysts:** Up to 36 h **For Nauplii:** 24 h	Not used	Hatching rate of cysts; Mortality and swimming inhibition of Nauplii; Morphology (Visual, SEM); Uptake of NPs (TEM); Accumulation/ Elimination; Biochemical parameters (ROS, MDA, TAOC, SOD, CAT, GST, GPx)
44. Bhuvaneshwari et al. 2018	TiO_2 NPs; 0.1, 1, 10 mg/L	24.6 ± 4.5 nm (TEM); DLS done after 0, 24, and 48 h	*A. salina*	Instar II	Sterilized natural seawater; Unknown salinity	Not determined clearly	No feeding	48 h	Not used	Mortality; LC_{50}; (*A. salina* was more sensitive than marine microalgae, *Dunaliella salina*); Accumulation/ Elimination; Histopathological effects; Biochemical parameters (ROS, SOD, CAT); Trophic transfer of NPs from algae to Artemia
45. Darwesh et al. 2018	Nano chitosan; 5000–20,000 ppm	3–13 nm (TEM); FTIR done	*A. salina*	Nauplii (unknown stage)	Sea water (Unknown source and salinity)	Vial; 5 ml; 10 per vial	Room temperature	24 h	Chitosan (for comparison)	Mortality
46. Kim et al. 2018	Carbon nanodots	1-4 nm (AFM); Up to 20 nm (DLS); Also TEM, Raman, FTIR, UV–vis, and XPS done	*A. franciscana*	Instar I	Artificial sea water	48-well plates; 0.4 ml; 50 nauplii per well; triplicate	Continuous illumination; 25 °C	48 h	$K_2Cr_2O_7$	Mortality (immobilization); Bioimaging (fluorescent microscope)

(*continued*)

Table 1. Continued.

No. and Reference/year	MNMs/range of concentration tested	Particle size / morphology / measurement method(s)	Tested species	Life stage	Media used for exposure/salinity	Exposure vessel (type/volume/ density); No of replicates	Lighting/ temperature/feeding (see note)	Exposure time	Additional control/ Reference chemical	Toxicity endpoint(s)
47. Leng et al. 2018	Nanocapsules of Iginate/EUDRAGIT® S 100-enclosed chitosan–calcium phosphate-loaded Fe-bLf	DLS, SEM	*A. salina*	Instar I	Artificial Seawater, 38 g/L	Bijoux bottle; 2 ml; 10–15 nauplii per bottle	Continuous illumination; 28 °C	24 h	$K_2Cr_2O_7$: 24 h LC_{50}= 64.15 mg/L	Mortality; LC_{50}
48. Lu et al. 2018	TiO_2 NPs; 5–400 mg/L	<500 nm (TEM); 562-22,700 nm (DLS)	*A. salina*	Instar I	Artificial sea water; 30 ± 2 g/L	**Static system:** Glass beaker; 20 ml; 10 nauplii per beaker **Dynamic system:** Conical flask; 20 ml; 10 nauplii per beaker; Rotation =100 r/min	16 h–8 h light-dark; 25 °C; No feeding	48 h	$K_2Cr_2O_7$: 24 h EC_{50}= 30.12 mg/L (static system) and 26.47 mg/L (dynamic system).	Mortality; LC_{50}; Visual accumulation/ Elimination; (Effect of TiO_2 NPs on the toxicity of phenanthrene and Cd^{2+} was evaluated)
49. Murugesan et al. 2018	Graphene oxide quantum dots; 10–160 mg/L	2–8 nm (TEM); Also EDX, FTIR, XRD, Raman, and XPS done	*A. salina*	Mature nauplii (unknown stage)	Sterilized artificial sea water; 35 g/L	24-well plates; 1 ml; 10 nauplii per well	Not determined clearly	48 h	H_2O_2 (as positive control)	Mortality; LC_{50}; Bioimaging (fluorescent microscope)
50. Rotini et al. 2018	CuO NPs; 10, 20, 40, and 80 mg/L	SEM; DLS done at 0 and 24 h; Metal dissolution (centrifugation + ICP MS)	*A. franciscana*	Instar II or III	ASTM Artificial Seawater (ASTM 2004); 34 g/L	24-well plates; 1 ml; 10 nauplii per well	Not determined	48 h	$CuSO_4{\cdot}5H_2O$ (for comparison)	Mortality; LC_{50}
51. Sarkheil et al. 2018	ZnO NPs; 1–30 mg/L	32.28 ± 13.30 nm (TEM); 79.31 nm (DLS); 12.43% Zn^{++} dissolution (ultracentrifugation filter + AAS)	*A. franciscana*	Instar I for acute tests and Instar II for bioaccumulation	Artificial seawater (13045 Process®; Aqua Craft®), 35 g/L	Glass vessels; 100 ml; 10 nauplii per vessel; 10 replicates	12 h–12 h light-dark ; 30 °C; No feeding	96 h	Not used	Immobilization; 96 h EC_{50}: 4.86 mg/L; Accumulation/ Elimination
52. Schiavo et al. 2018	ZnO NPs; **Acute:** 10, 50, and 100 mg/L; **Chronic:** 0.03–0.5 mg/L	Referred to previous publication	*A. salina*	Instar II	ASTM Artificial Seawater (ASTM 1998)	**Acute:** Glassware containers, 50 ml; 5 nauplii per well; triplicate. **Chronic:** Beaker; 50 ml; 10 nauplii per beaker; 4 replicates; Organism transfer to new beaker 3 times a week	**Acute:** Darkness; 25 °C; No feeding **Chronic:** 14 h–10 h light-dark; 25 ± 2 °C; Feeding on microalgae	96 h (acute); 14 d (chronic)	$ZnSO_4$ (for comparison)	Mortality; EC_{50}; Growth (body length)
53. Yang et al. 2018	TiO_2 NPs; 10 mg/L	Anatase; TEM; EDX; DLS done to determine NPs sedimentation over time (0, 4, 8, 12, 16, 20, and 24 h).	*A. salina*	Instar II	Filtered artificial seawater (Instant Ocean®)	0.5 ml test solution per nauplius	14 h–10 h light-dark; 25 °C	Up to 96 h	Not used	Effect of TiO_2 NPs on the trophic transfer of arsenic from *Nannochloropsis maritima* to *A. salina* nauplii was evaluated. Biochemical parameters (CAT, SOD, AChE)

AFM: Atomic Force Microscopy; AP: Alkaline phosphatase; CAT: Catalase; CHN: Carbon, Hydrogen, and Nitrogen analyzer; DLS: Dynamic Light Scattering; DNA: Deoxyribonucleic Acid; EC_{50}: Half Maximal Effective Concentration; EDS(EDX): Energy-dispersive X-ray Spectroscopy; FESEM: Field Emission Scanning Electron Microscope; FTIR: Fourrier Transformed Infrared Spectroscopy; GPx: Glutathione peroxidase; GST: Glutathione S-transferase; LC_{50}: The Lethal Concentration required to kill 50% of the population; LD_{50}: The lethal dose at which 50% of the population are killed in a given period of time; MDA: Malondialdehyde; MNM: Manufactured Nanomaterial; NP: Nanoparticle; ROS: Reactive oxygen species; SEM: Scanning Electron Microscope; SOD: Superoxide dismutase; TAOC: Total Antioxidant Capacity; TEM: Transmission Electron Microscope; UV-vis: Ultra Violet Visible; XPS: X-ray Photoelectron Spectroscopy; XRD: X-ray Diffraction.

Although EC_{50} is widely used term for *Artemia* toxicity testing, LD_{50} and LC_{50} have been used in the publications. For the **Lighting/temperature/feeding** absence of information means that the information was not stated in the article, whereas "No feeding" means that the article stated this.

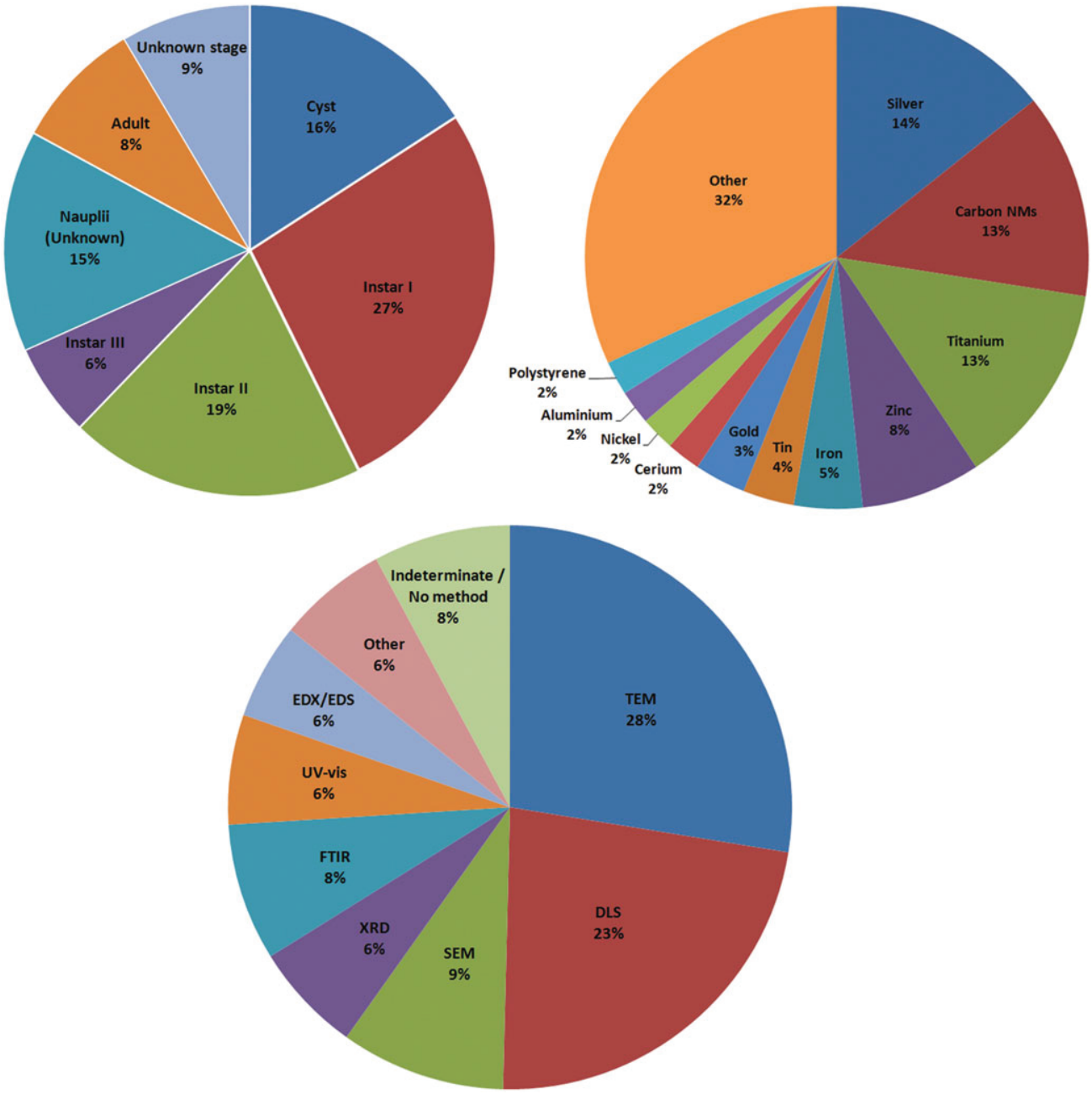

Figure 1. *Artemia sp.* life stages (top left), frequency of chemistry of tested MNMs (top right), and frequency of methods for MNMs characterization (bottom) in aquatic nanotoxicity studies using *Artemia sp.*, based on 53 studies of 91 different nanomaterials.

were identified. However, the chronic tests are possible, for example, effects of MNM on the life cycle and growth of *Artemia*. ISO/TS 20787 (2017) does not address details of such tests.

Information on aquatic nanomaterial toxicology studies using *artemia sp*

We reviewed 53 papers published between 2009 and 2018 on aquatic nanomaterial toxicology studies and the review results are summarized in Table 1. The columns present the following information: (1) reference and year of publication, (2) MNM tested and the concentration range tested, (3) particle size and morphology, and measurement method(s), (4) tested species (5) life stage of *Artemia*, (6) media used for exposure and salinity, (7) exposure vessel (type/volume/density) and number of replicates, (8) Lighting/temperature/feeding (9) exposure time, (10) additional control and reference chemical, and (11) toxicity endpoints.

The 53 studies using *Artemia* provide data on toxicity of 91 different types of nanomaterials. Silver, titanium dioxide, and various carbon-based MNMs (including CNTs, fullerene, carbon black, carbon nanodots, and graphene) are the MNMs most frequently tested for aquatic toxicity using *Artemia sp.* (Figure 1).

In the studies, various methods have been used for characterizing the size (distribution) of the MNMs (Figure 1) with

TEM and DLS being the most frequently used methods. In 10 studies, either no characterization was performed, or the characterization method(s) used is not specified, or only the specifications provided by the manufacturer are stated. In five studies, only one method was used for MNMs characterization, while in 38 studies, two or more methods were used for characterization. In eight studies, ion release from particles was measured by various techniques.

In 11 studies, the dispersion state of MNMs was tracked over time using DLS (eight cases) or UV-vis (three cases).

Among the 53 studies, *Artemia salina* and *Artemia franciscana* were used in 36 and 10 studies, respectively, and the species used was not specified in seven studies (*Artemia sp.*). In *Artemia*'s life stage, 13 studies *Artemia* cysts were used for toxicity test (hatchability was considered as the endpoint) and capsulated (five studies) and decapsulated cysts (six studies) were used, while in two cases, there was no information on whether the cysts were dechorionated or not. The naupliar stage was used 55 times to evaluate the nanomaterial toxicity, among which the naupliar stage was not clearly specified in 12 cases, and in other cases instar I, II, and III were used 22, 16, and 5 times, respectively. The adult stage was used in seven cases. In five studies, the *Artemia* life stage was not identified (Figure 1).

In exposure media sources and salinity, interestingly Kowalska-Góralska et al (2011) used ISO standard freshwater (ISO 6107–3 1993) for toxicity testing with *Artemia*. Otherwise, the salinity range stated in the studies was 25 g/L–38 g/L with the following sub-ranges: 25 g/L–30 g/L (14 studies), 31 g/L–35 g/L (15 studies), and 36 g/L–38 g/L (eight studies), whereas in 15 studies, the salinity of the exposure media was not stated. Natural and artificial seawater were each used in 22 cases, while in eight cases the source of salinity was not stated. Saltwater was filtered in 21 cases and sterilized in six cases. ASTM artificial seawater was used in two studies.

The temperature range used in the studies was 18–30 °C with the following sub-ranges: 18–20 °C (nine studies), 21–25 °C (20 studies), and 26–30 °C (16 studies), whereas in 13 studies, the temperature used during the tests is not mentioned. Regarding the light conditions during the various studies the following was observed: 16–8 h light-dark photoperiod (16 cases), 14–10 h light-dark photoperiod (two cases), 12–12 h light-dark photoperiod (three cases), complete darkness (six cases) and continuous illumination (seven cases) and in 20 studies, the light conditions used during the tests were not stated. Regarding feeding in 20 studies, it was clearly stated that *Artemia* was not fed during the experiment (reported as no feeding in Table 1); however, in three studies, nutrition on microalgae is mentioned.

To maintain MNMs in suspension, the exposure media was mixed either through gentle aeration (five cases) or shaking (six cases) in two studies the exposure media was renewed during the experiment, while in one long-term study, the exposed organisms were transferred to new beaker three times a week. In three cases, tests were done under static conditions, whereas the remaining studies did not declare whether they applied static conditions or renewed exposure media. Furthermore, a variety of containers were used for the testing, of which 24-well plates are most used. In many cases, the size of the containers used is not clearly indicated, and the container size-range varied from 400 µl–1.5 L.

In test duration, most toxicity tests were short-term tests with a duration from six (one study) to 108 h (one study), and the 24-hour (19 studies) and 48-hour (22 studies) tests were the most frequent ones, but also 96 h tests (six studies) and 36 h test (four studies) were performed. Only two long-term experiments, lasting 14 d, were identified (Bergami et al. 2017; Schiavo et al. 2018).

The toxicity endpoint(s) listed in column 11 in Table 1, gives an overview of the many biomarkers that may indicate a toxic effect. ISO/TC 20787 focuses on hatching rate and immobilization, but in addition also changes in levels of Alkaline phosphatase, Catalase, Glutathione peroxidase, Glutathione S-transferase, Malondialdehyde, Reactive oxygen species, Superoxide dismutase, and Total Antioxidant Capacity are relevant indicators of possible toxic effects.

In developing a standardized method for aquatic toxicity testing of nanomaterials using *Artemia sp.*, all pros and cons of the reviewed literature were considered. This led to the agreement within ISO of a new standardized procedure on 'aquatic toxicity assessment of manufactured nanomaterials in saline lakes using *Artemia sp*. nauplii' (ISO/TS 20787 2017) as a step towards generating more reliable and repeatable aquatic toxicity data for saline lake ecosystems using *Artemia sp*. nauplii. It is recognized that *Artemia sp.* is not the most sensitive test organism, but a representative organism for saline lakes and ISO/TS 20787 should overcome at least some of the previous problems in using *Artemia sp*. data.

Scope and content of the proposed ISO document

The scope of ISO/TS 20787 (2017) states that it defines a test method, aiming to maximize repeatability and reliability of testing, to determine whether MNMs are toxic to aquatic organisms, specifically *Artemia sp.* nauplius, and that the method uses *Artemia sp.* nauplii in a simulated environment, artificial seawater, to assess effects of nanomaterials. It is intended for use by ecotoxicological laboratories capable of hatching and culturing *Artemia sp*. nauplii in a simulated environment of artificial seawater.

The ISO TS uses the hatching rate of the cysts and immobilization of the nauplii (or exhibiting adverse effects, including abnormal behavior) as a measure of the toxicity of the nanomaterial.

The ISO TS suggests materials and instruments for the proposed procedure, as well as test organisms and chemicals to be used for the testing. While different species of *Artemia sp.* can be used, *Artemia salina* and *Artemia franciscana* are the preferred test species, given their widespread availability compared to other species of *Artemia*. Brine shrimp cysts are widely available from many commercial sources. The *Artemia sp*. nauplii should be produced by hatching high-quality cysts (hatching percentage more than 80%), and experiments should be performed with nauplii at the same stage of

Table 2. Preparation and characterization of nanomaterial dispersion for aquatic nanotoxicity using *Artemia sp.* with ISO/TS 20787.

Dispersion step	Procedure
Preparation	• Suspension: use stirring, sonication, functionalization, or biocompatible reagents • Vehicle: use less than 100 mg/ml of organic solvents, emulsifiers or dispersants
Characterization	• DLS or ultrasonic attenuation spectroscopy
Stability of stock suspension	• Characterize size distribution stability over time • Determine concentration and metal ions in case of metal MNMs
Stability of nanomaterial suspension in artificial seawater	• Stability and actual concentration of MNMs • Degree of aggregation/agglomeration and amount of metal ion in media
Preparation of exposure media	• Minimize contamination with algae or bacterial growth by sterilization or filtration

development. It is demonstrated that the sensitivity to chemicals varies from one geographical *Artemia* strain to another (Sorgeloos et al. 1978) and thus the geographical origin of cysts should be known and recorded.

Required chemicals include artificial seawater, potassium dichromate, Lugol's solution (Lugol's iodine), sodium hypochlorite (5.25%wt. NaOCl), and a sodium hydroxide solution (400 g/L NaOH). In addition, routine aquatic toxicity testing equipment for measuring the salinity and total organic carbon are needed.

Preparation and characterization of dispersed nanomaterials

For the procedure outlined in ISO/TS 20878, the characterization of the MNM being tested, and preparation and characterization of the dispersion are key steps, see Table 2. The preparation of the MNM dispersion should be well documented based on written standard operating procedures (e.g. OECD 2012; Hartmann et al. 2015), as the dispersion step impacts the overall test results. The test dispersions can be prepared in a two-step procedure by first preparing a stock suspension and then dilute aliquots of this prior to the testing. Dispersion of the nanomaterial in the stock suspension can be achieved by stirring, sonication, or via functionalizing groups or biocompatible dispersant reagents. As sonication will transfer energy into the dispersion, it should be conducted in a manner that avoids, for example, the generation of new materials or loss of material from the sonicator tip to the suspension. Additionally, dispersing the test item in the stock solution may be facilitated by using organic solvents, emulsifiers, or dispersants, the concentration of which should not exceed 100 mg/L. Chemicals that have a lethal effect on *Artemia sp.* should also be avoided. When using vehicles or cosolvents, an additional control should be conducted by exposing the test organisms to a concentration of the vehicle or cosolvent that corresponds to the most concentrated suspension of the test nanomaterial.

Most nanomaterials tend to strongly agglomerate/aggregate in water, a behavior that can be exacerbated in saltwater. Thus, before toxicity testing, it should be ensured that the nanomaterials are well dispersed in the artificial seawater. It is also recommended to further characterize the test nanomaterial, including the degree of aggregation/agglomeration (or change of particle size distribution). The dispersion state of the nanomaterials should be characterized using DLS (dynamic light scattering), and the procedure described in ISO 22412 (2017), may be used, as may other suitable methods, such as ultrasonic attenuation spectroscopy (ISO 20998–1, 2006). The size distribution of the dispersed MNM and its stability over time need to be characterized at specified intervals (e.g. 6 h, 24 h, and 48 h) both for the stock solution and during the experiment. In addition, the concentration of MNM in the stock suspension needs to be evaluated using an appropriate method. In the case of metal-based nanomaterials, which may partly convert to metal ions, the proportion of metal ions and nanoparticles need to be determined both for the stock solution and the exposure media.

All samples (or suspensions) used for exposure of *Artemia sp*. nauplii to the MNM should be freshly prepared from stock suspension of the MNM (e.g. at a concentration of 1000 mg/L in the stock suspension). Aliquots of the stock suspension should be added directly to sterilized artificial seawater to achieve the relevant MNM concentration in the exposure media. Although *Artemia sp*. does not need sterile conditions for hatching and growth, an effort should be made to minimize the development of unicellular algae and bacterial contamination. Sterile filtration using 0.1 μm filters is recommended as the best method for sterilizing artificial seawater without altering its chemistry.

Testing of nanomaterial toxicity using *Artemia* nauplii

Table 3 shows the steps for hatching *Artemia* cysts. Stored cysts are hatched in sterilized artificial seawater, harvested, and the hatching percentage is calculated.

The test container volume should be at least 5 ml per 5 animals per concentration group and control. The test can be conducted using semi-static (renewal) test medium when the test nanomaterial concentration is not stable. As already noted, an additional control containing the dispersant should be prepared when using dispersant reagents for the dispersion. At least five nauplii should be exposed to each test concentration for 48 h. At least five test concentrations should be used, arranged in a geometric series with a separation factor not exceeding '2.2', and if less than five concentrations are used a justification should be provided. The highest concentration tested should result in 100% immobilization, while the lowest concentration tested should cause no observable effect. The water temperature should range

Table 3. Steps for hatching *Artemia* cysts for aquatic nanotoxicity experiments.

Hatching steps	Procedure
Preparation of artificial seawater	• Salinity of 35 ± 1 g/L • Aeration and stabilization for 24 h • pH of 8.0 ± 0.5
Storage of cysts	• Once opened, use within two months • For long-term storage, freeze until use
Disinfection of cysts	• Soak cysts for 30 min at 50 g cysts/liter of 200 ppm NaOCl • Wash cysts 3 times with distilled water on 125 μm screen.
Hatching	• Aerate into transparent hatching vessels • Incubate disinfected cysts for 24 h in 1000 ml artificial seawater at density of 2 g/liter
Harvesting nauplii	• Remove floating cysts from water surface • Collect nauplii from bottom of conical vessel by siphoning onto fine mesh screen (<150 μm) • Rinse collected nauplii with artificial seawater
Calculation of hatching percentage	• Take 6250 μl sub-samples out of each incubator and fix with Lugol's solution • Count hatched nauplii (N), umbrella-stage cysts (U), and unhatched embryos (E) • Hatching percentage (H) = (N × 100) ÷ (N + U+E).

between 25 °C and 28 °C and be constant for each test within ±1 °C. A 16 h light and 8 h dark cycle is recommended. The nauplii should not be fed during the test period (48 h). In addition, the number of immobilized nauplii should be counted at 24 h and 48 h from the start of the test.

Parameters including the oxygen concentration, pH, total concentration of test nanomaterial, the stability of the dispersion, and the size distribution of the nanomaterials should also be measured at the start and at the end of the test. The concentration of test nanomaterials should be maintained within ±20% of the nominal or measured initial concentration throughout the test.

Data should be summarized and presented in a tabular form which includes the control and treatment groups, number of nauplii used, and percentage immobilized nauplii at 24 h and 48 h. The EC_{50} with a 95% confidence limit should also be determined based on an appropriate statistical analysis. Where standard methods of calculating the EC_{50} (i.e. Probit) are not applicable to data obtained, the geometric mean of the highest concentration causing no immobility and the lowest concentration producing 100% immobility should be used to approximate the EC_{50}.

The test report should contain information on the test nanomaterial, for example, in accordance with ISO/Technical Report (TR) 13014 (2012), including the nanomaterial physicochemical properties, particle morphology by TEM or SEM, source of the test nanomaterial (manufacturer's code, catalog or formulation number, batch number or date of manufacture, and trade name), equipment used, and dispersion characterization and stability of the test nanomaterial in the stock suspension and in artificial seawater. For reporting test results, test conditions such as water temperature and hours of light/darkness, and bioassay results should also be described, including, but not limited to, the following:-number and percentage of nauplii immobilized or exhibiting adverse effects (including abnormal behavior) in controls and each treatment group at each observation time; calculated 48 h EC_{50} with 95% confidence limit; anddata confirming validity of results.

- EC_{50} of potassium dichromate,
- Mortality percentages for controls.

The test is considered valid if the following three conditions are fulfilled: (i) in the controls, including a control containing the maximum concentration of the dispersant reagent, no more than 10% of the nauplii have been immobilized; (ii) the 48 h EC_{50} of potassium dichromate (positive control) is within the range of the reported concentrations for each *Artemia sp*. used; and (iii) the dissolved oxygen concentration at the end of the test is higher than 3 mg/L in the control and test vessels.

Conclusions

Artemia sp. appears to be useful as a test organism in saline water where Daphnia cannot be used as test organism, but it should not be used in general as a substitute for Daphnia testing, as *Artemia sp.* is not a very sensitive organism due to its ability to adapt to a wide range of water salinity and temperatures. However, *Artemia sp*. represents a unique ecosystem, saline lakes, and it is associated with less concern about animal welfare than vertebrate species. Furthermore, extensive knowledge has been gained on *Artemia sp.* biology for culturing it in laboratories (Nunes et al. 2006). Moreover, testing with *Artemia sp.* nauplii is simple and cost-effective as the smallness of *Artemia sp.* nauplii allows testing in small containers, and *Artemia sp.* are simple to maintain in the laboratory. *Artemia sp.* has a short life cycle that makes them suitable for short-term toxicity tests. *Artemia sp.* cysts are commercially available and remain viable for years, when stored under dry and cool conditions, hatching free-swimming nauplii of similar age, genotype, and physiological condition within about 24 h of immersion in saline water. Furthermore, *Artemia sp.* inhabits saline lakes that are widely distributed geographically.

This paper outlines early efforts to develop procedures for using *Artemia sp.* as a test organism and gives an overview of the more recent research into testing MNMs using *Artemia sp.* as described in 53 research papers published between 2009 and 2018, which describe the toxicity test outcomes for 91 different MNMs using *Artemia sp.* as the test organism.

The overview includes the test conditions, as far as reported, and the *Artemia sp.* life stage tested.

Based on these efforts, ISO developed a new technical specification, presented in this paper, for the nanomaterial-specific aquatic toxicity assessment of manufactured nanomaterials in saline lakes using *Artemia sp.* nauplii as the test organism. Among the several endpoints that can be considered for *Artemia sp.*, the ISO TS uses the hatching rate of the cysts and immobilization (or exhibiting adverse effects, including abnormal behavior) of the nauplii as measures of the toxicity of the nanomaterial. ISO/TS 20787 presents a test that may fill a gap for obtaining ecotoxicology information for saline lakes, identified by Libralato (2014). The ISO TS requires, for example, for the hatching phase that the origin of cysts and their storage/maintenance conditions is reported, and it suggests using artificial seawater for which the brand or composition should be stated. In addition, parameters, such as water oxygen saturation and pH value, the temperature and timing and the photo-period duration should be recorded as well.

The advantage of the new ISO/TS is that, it provides a standardized procedure for the testing with *Artemia sp.*, which is an important step toward generating more reliable and repeatable aquatic toxicity data using *Artemia sp.* nauplii. Furthermore, the new ISO/TS includes a positive control (potassium dichromate), thus addressing some of the concerns expressed previously about tests performed with *Artemia sp.*

The ISO/TS 20787 thus provides a procedure for short-term toxicity testing for saline lakes. In the future *Artemia sp.,* testing may also be standardized, for example, for growth and reproduction tests.

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Short communication

Aeromonas veronii biovar sobria Associated with Mortalities of Riverine Ayu *Plecoglossus altivelis* in the Tama River

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ABSTRACT—In July 2016, there were mortalities of riverine ayu *Plecoglossus altivelis* in a tributary of the Tama River, Japan. A Gram-negative, motile and short rod-shaped bacterium was dominantly isolated from all examined dead fish, and identified as *Aeromonas veronii* biovar sobria. Biochemical characteristics and *gyrB* sequence of the present strains differed from those of *A. veronii* strains from ayu in previous years. The present strains also caused higher mortalities to ayu than *A. veronii* strains previously isolated. These results indicate that the present mortalities of riverine ayu in the Tama River were caused by high pathogenic *A. veronii* biovar sobria.

Key words: *Aeromonas veronii* biovar sobria, *Plecoglossus altivelis*, riverine fish, pathogenicity, the Tama River

The ayu *Plecoglossus altivelis*, a representative freshwater fish species in Japan, has long held an important position among riverine fishes as a target for recreational fisheries and as food for human consumption. Therefore, hatchery-produced or wild (captured from lakes, rivers or sea coasts) ayu are released annually into many rivers to enhance riverine stocks, despite indications that many released fish are at risk of several bacterial infections. In particular, bacterial infection by *Flavobacterium psychrophilum* (bacterial cold-water disease) (Iida and Mizokami, 1996) and *Edwardsiella ictaluri* (Sakai *et al.*, 2008) has become one of the most serious problems for riverine ayu management in recent years. Consequently, the infection status of both pathogens in hatchery-produced and wild ayu has been investigated throughout Japan (Kumagai, 2016; Iida *et al.*, 2016).

In July 2016, bacteria that differed from *F. psychrophilum* and *E. ictaluri* were isolated from dead ayu collected following mass mortalities of riverine ayu in the tributary of the Tama River, Japan. The bacteria were identified as *Aeromonas veronii*, which has been frequently isolated from aquatic environments (Albert *et al.*, 2000) and fish intestines (Namba *et al.*, 2007). Although some studies report that *A. veronii* causes disease in farmed and ornamental fishes (Rahman *et al.*, 2002; Sreedharan *et al.*, 2011; Smyrli *et al.*, 2017), the majority of aeromonads causing damage in Japanese aquaculture have been identified as *A. hydrophila* and *A. salmonicida* (Jo and Onishi, 1980; Kitao *et al.*, 1985; Rahman *et al.*, 2001; Yamamoto, 2017). In the present study, we investigated the characteristics and pathogenicity of strains from diseased ayu and concluded that the mortalities of riverine ayu found in the tributary were caused by *A. veronii*.

Materials and Methods

Bacterial examination

There were two mass mortalities of riverine ayu in the tributary of the Tama River in July 2016, when the daily average water temperature rapidly increased above 23°C. Since we could not sample freshly dead ayu in the first mortality event, we obtained 16 dead and 14 living fish (captured by angling) in the second event. Bacterial isolation from the kidney were performed using trypto-soya agar (TSA, Nissui) and the plates with inoculum were incubated for 48 h at 25°C. Cell morphology and motility of the bacterial strains were examined microscopically by Gram staining and the wet-mount method, respectively, and strains were molecularly identified to species using a partial (500-bp) 16S rRNA sequence from the 5′ region (Namba *et al.*, 2007). Additionally, we tested for the presence of *F. psychrophilum* and *E. ictaluri* in sampled ayu according to the methods of our previous study (Takeuchi *et al.*, 2016).

Biochemical and phylogenetic characterization

Of the strains identified as *A. veronii* in the present study by partial 16S rRNA sequencing, 15 strains (“present strains”) were biochemically characterized using API 20E (BioMerieux) according to the manufacturer’s instructions. The derived API profiles were compared with those of the eight *A. veronii* strains from the kidney of ayu and pale chub *Opsariichthys platypus* captured in the Tama River Basin in 2012 and 2014 (“previous strains” from asymptomatic or *E. ictaluri* infected fish), and reference strains from the intestine of common carp

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Cyprinus carpio (HPI4 and CWP11; Namba *et al.*, 2008) and human patients (JCM7375; Hickman-Brenner *et al.*, 1987). Additionally, for phylogenetic characterization, we performed *gyrB* gene (1,100 bp) amplification and direct sequencing using extracted DNA from present strains and the primers developed by Yáñez *et al.* (2003). After genome assembly, the sequences were compared to sequence data in GenBank using BLAST (blastn) algorithms (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and aligned with sequences of previous strains and reference strains using Clustal X (Thompson *et al.*, 1997). A maximum likelihood phylogenetic tree of aligned sequences was constructed with Kimura's 3-parameter model using MEGA 6 software (http://www.megasoftware.net/), and the robustness of the phylogenetic results were tested by bootstrap analysis with 1,000 iterations.

Experimental infection

To assess the pathogenicity of *A. veronii* strains obtained in the present study, we performed an experimental infection of ayu using the three present strains from dead ayu (AAr1608, AAr1614, and AAr1615), three previous strains (AAr1412, AAr1216, and AAr1218), and one reference strain from the intestine of common carp (CWP11). Stock cultures of all strains in tryptic soy broth (TSB, Difco) containing 10% glycerol at –80°C, were transferred and grown on TSA at 25°C for 24 h and then cultured in 300 mL TSB with shaking at 25°C for 9 h. Hatchery-produced ayu (body weight: 12.0 ± 1.9 g), obtained from the Freshwater Experimental Station, Kanagawa Prefectural Fisheries Technology Center, were acclimated to experimental conditions at 20°C–25°C for 5 days prior to the experiment.

Following a 10-fold dilution of bacterial suspensions of each strain with dechlorinated tap water (final bacterial density: 2.1–3.4 × 10^7 CFU/mL), experimental fish were immersed in the suspensions at 25°C for 30 min, while control fish were exposed to 10-fold diluted TSB. The fish exposed to each strain (n = 10 per strain) were then reared at 25°C in a 50-L glass aquarium with filtration and aeration equipment, and monitored for 10 days. Identification of isolates from the kidneys of dead and moribund fish was performed by direct sequencing of the *gyrB* gene as described above.

Results and Discussion

Most dead ayu and some living fish obtained during the mass mortality of riverine ayu in the river tributary showed external and internal clinical signs such as hemorrhaging of the lower jaw or body surface, reddening at the base of the ventral fin or anus, and ascites (Fig. 1). Of these signs, reddening of the anus and ascites are known as typical clinical signs of *E. ictaluri* infection (Sakai *et al.*, 2008; Takeuchi *et al.*, 2016), so at first, we assumed that the mortality was caused by *E. ictaluri*. However, *E. ictaluri* was detected in only 31.3% (five of 16 fish) of dead ayu and 28.6% (4/14) of the living; *F. psychrophilum* was not detected in any sampled fish. On the other hand, unknown bacteria, which were Gram negative, motile, and short rod-shaped, were isolated from 100% (16/16) of dead ayu and 57.1% (8/14) of living fish. The partial 16S rRNA sequences derived from these bacteria (accession nos., LC311422- LC311447) most closely matched that of *A. veronii* (KT998815).

A. veronii was originally described as a novel species in the genus *Aeromonas* in 1987 (Hickman-Brenner *et al.*, 1987). It is divided into two biovars ("sobria" and "veronii") on the basis of biochemical characteristics such as the activity of arginine dihydrolase (ADH) and ornithine decarboxylase (ODC) (Janda and Abbott,

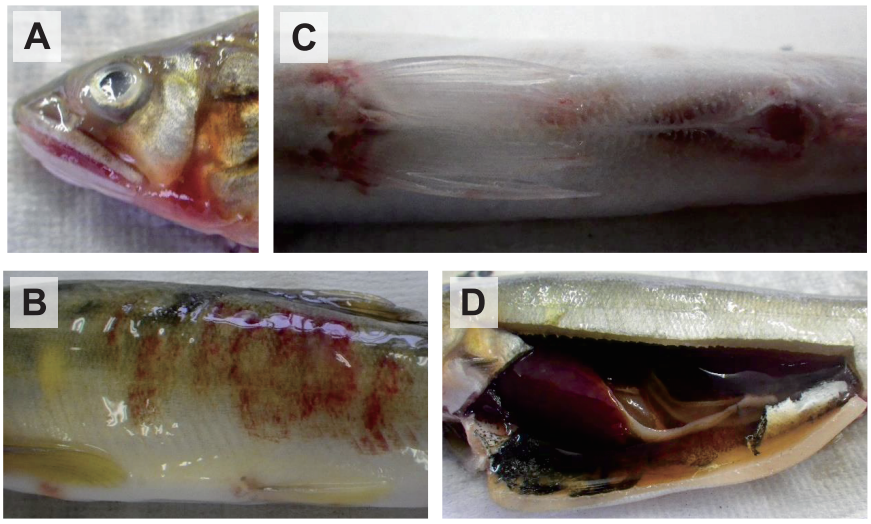

Fig. 1. Typical clinical signs in dead ayu found in the tributary of the Tama River in July 2016: hemorrhaging of lower jaw (A) or body surface (B), reddening at the base of the ventral fin or anus (C), and ascites (D).

1998), and there have been several reports of fish disease caused by *A. veronii* biovar sobria in Asia and Europe (Rahman *et al.*, 2002; Sreedharan *et al.*, 2011; Smyrli *et al.*, 2017). All present and previous strains in the present study were ADH-positive and ODC-negative in the API 20E test, and were assumed to be *A. veronii* biovar sobria. However, the API profiles of the present strains differed from those of previous strains, and *gyrB* gene sequences from the present strains (LC311630–LC311644) formed a cluster different from other sequences except for the sequence of *A. veronii* isolated from diseased European seabass *Dicentrarchus labrax* in Greece (AERO NS; Smyrli *et al.*, 2017; KF636138) (Fig. 2). Smyrli *et al.* (2017) reported that mortality of European seabass caused by *A. veronii* was observed in Agean Sea and the Black Sea. These results and information suggest that the *A. veronii* biovar sobria isolated in the present study may have been introduced from other aquatic environments, inside and outside the country.

In our experimental infection by bath exposure, the dead ayu showed clinical signs similar to those observed in naturally infected fish, and *A. veronii* with *gyrB* sequences matching those of the strains used for exposure were isolated from all dead or moribund fish. The cumulative mortalities of ayu exposed to previous strains were 20%–40%, whereas over 80% of fish exposed to the present strains were dead by 3 days post exposure (Fig. 3). These results indicate that the *A. veronii* found

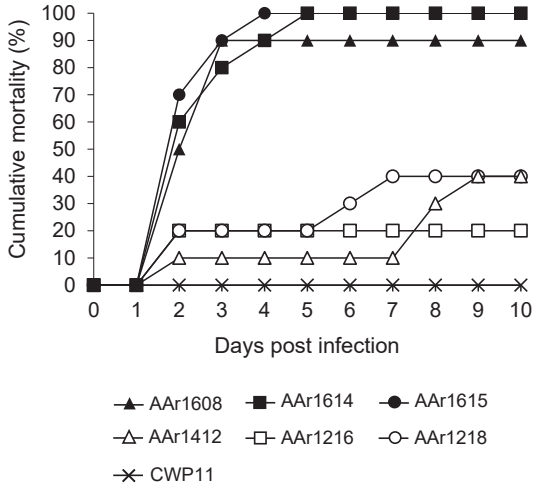

Fig. 3. Cumulative mortality of ayu challenged by exposure to *Aeromonas veronii* isolated from riverine fish collected in the Tama River Basin compared with that of ayu exposed to a reference strain from the intestine of common carp (CWP11). No dead fish were observed in the control group (exposed with TSB). Solid symbols: "present strains"; hollow symbols: "previous strains"; ×: "reference strain".

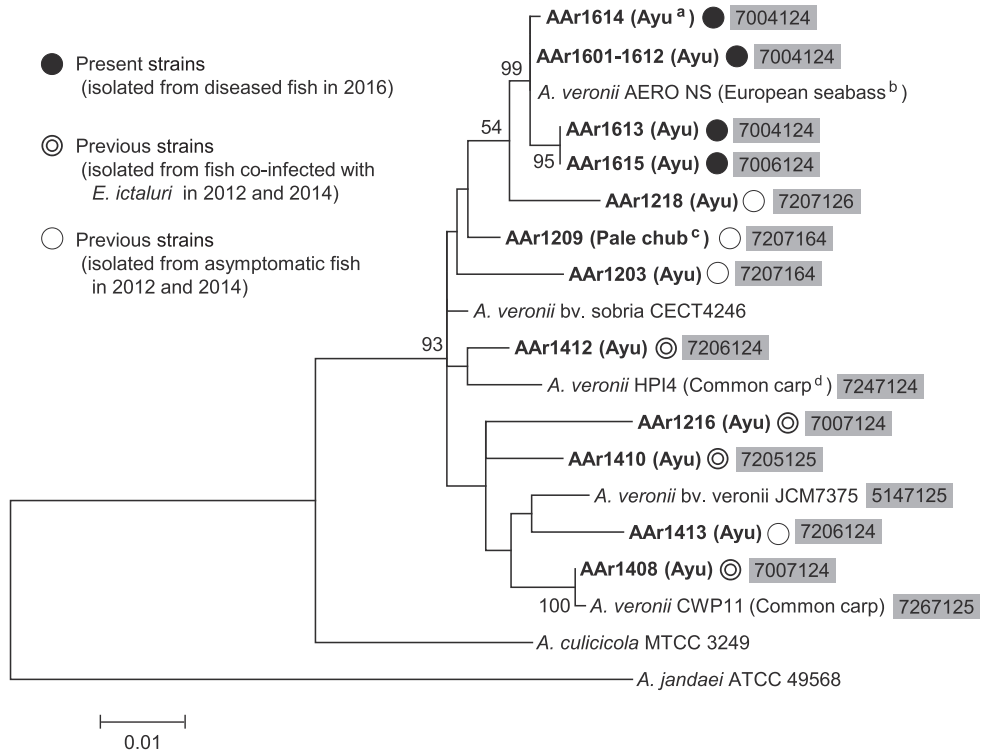

Fig. 2. Phylogenetic tree showing the relationships among *Aeromonas veronii* strains from riverine fish collected in the Tama River Basin. The tree was inferred from *gyrB* gene sequences by the maximum likelihood method. The scale bar represents a 1% sequence difference. Numbers at nodes are bootstrap values (> 50%) after 1,000 iterations. The name of the fish species in parentheses and the shaded boxes show the origins and API 20E profiles of each strain, respectively. [a]*Plecoglossus altivelis*, [b]*Dicentrarchus labrax*, [c]*Opsariichthys platypus*, [d]*Cyprinus carpio*.

in the present study has high pathogenicity to ayu compared to other strains. We conclude that the mass mortalities of riverine ayu found in the tributary of the Tama River in July 2016 were caused by high pathogenic *A. veronii* biovar sobria with different properties than previous strains.

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Radiocesium dynamics in the aquatic ecosystem of Lake Onuma on Mt. Akagi following the Fukushima Dai-ichi Nuclear Power Plant accident

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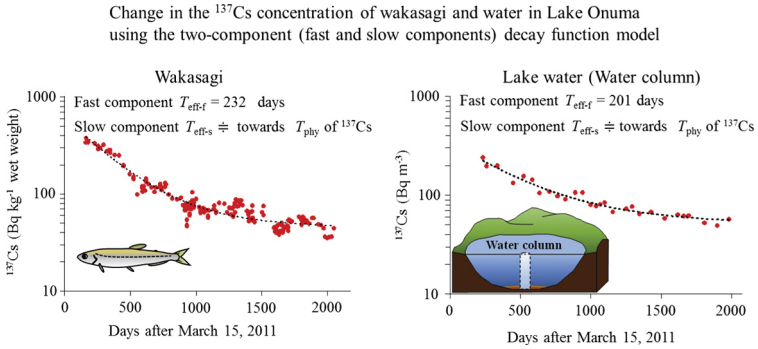
HIGHLIGHTS

- Radiocesium dynamics of ecosystem in Lake Onuma after FDNPP accident was assessed.
- ^{137}Cs decay was estimated using samples collected from 2011 to 2016.
- ^{137}Cs levels in wakasagi almost reached a state of dynamic equilibrium.
- Parts of aquatic ecosystems exhibited different decay processes with wakasagi.
- ^{137}Cs contamination in lake water affected aquatic organisms.

GRAPHICAL ABSTRACT



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ABSTRACT

Understanding ecosystem dynamics of radionuclides is necessary to ensure effective management for food safety. The Fukushima Dai-ichi Nuclear Power Plant (FDNPP) accident on March 11, 2011 released large amounts of radiocesium (^{134}Cs and ^{137}Cs) and contaminated the environment across eastern Japan. In this study, we aimed to elucidate the temporal dynamics of ^{137}Cs in the aquatic ecosystem of Lake Onuma on Mt. Akagi. The effective ecological half-life (T_{eff}) of ^{137}Cs in fishes, western waterweed (*Elodea nuttallii*), seston (phytoplankton and zooplankton), and lake water was estimated using survey data of ^{137}Cs concentration collected from 2011 to 2016, and single- and two-component decay function models (SDM and TDM, respectively). The decay processes of ^{137}Cs concentrations in wakasagi (*Hypomesus nipponensis*), pale chub (*Zacco platypus*), phytoplankton, and total ^{137}Cs concentrations of the water column (WC) in the lake were well suited by the TDMs. The T_{eff} in the fast component of the TDMs in these samples ranged from 0.49 to 0.74 years. The T_{eff} in the slow component of the TDMs could converge towards the physical half-life of ^{137}Cs. Nearly five and a half years after the FDNPP accident, we concluded that ^{137}Cs concentrations approached a state of dynamic equilibrium between some aquatic

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Effective ecological half-life
Decay function model

organisms (wakasagi, pale chub, and phytoplankton) and the environment (lake water). However, the decay processes of ^{137}Cs concentrations in Japanese dace (*Tribolodon hakonensis*), western waterweed, zooplankton, and particulate- and dissolved-forms in the WC were better predicted for the SDM. The total ^{137}Cs concentrations in inflowing river and spring waters were one to two orders of magnitude lower than lake water under normal flow conditions. However, particulate ^{137}Cs contamination level in the river water was high after heavy rains. Overall, ^{137}Cs contamination levels have significantly decreased in Lake Onuma, but monitoring surveys should be continued for further understanding of the reduction processes.

1. Introduction

The accident at the Fukushima Dai-ichi Nuclear Power Plant (FDNPP) of the Tokyo Electric Power Company that occurred following the Great East Japan Earthquake on March 11, 2011 released large amounts of radioactive materials including radiocesium (^{134}Cs and ^{137}Cs) into the environment (Hirose, 2016; Steinhauser et al., 2014). In particular, the FDNPP accident-derived radiocesium contaminated most parts of eastern Japan through atmospheric transport, as confirmed by simulation (Morino et al., 2011, 2013) and airborne monitoring surveys (NRA, 2017). Radiocesium has long physical half-life (T_{phy}) (T_{phy} of ^{134}Cs = 2.06 years, T_{phy} of ^{137}Cs = 30.2 years) and accumulates in the muscle of vertebrates including fishes (Fukuda et al., 2013; Malek et al., 2004; McCreedy et al., 1997; Yamamoto et al., 2014). FDNPP accident-derived radiocesium deposited on inland waters has accumulated to high concentrations in freshwater fishes through bioconcentration in the ecosystem (Arai, 2014a, 2014b; Matsuda et al., 2015; Mizuno and Kubo, 2013; Tsuboi et al., 2015; Wada et al., 2016; Yoshimura and Yokoduka, 2014). A strict standard for allowable ^{134}Cs plus ^{137}Cs level of 100 Bq kg^{-1} wet weight in general food was enforced from April 1, 2012 under the Food Sanitation Law in Japan, although a provisional standard on ^{134}Cs plus ^{137}Cs contamination level of 500 Bq kg^{-1} wet weight had been applied following the FDNPP accident (Gilmour et al., 2016). Hence, it is important to understand the distribution and dynamics of radiocesium contamination across regions to prevent internal and external exposure of humans to radiocesium.

Following the Chernobyl Nuclear Power Plant (CNPP) accident in 1986, the biological impact and dynamics of ^{137}Cs in aquatic ecosystems were extensively investigated and discussed (IAEA, 2006). Numerous studies on ^{137}Cs contamination in freshwater fishes have been carried out in European countries (Elliott et al., 1993; Håkanson et al., 1989; Jonsson et al., 1999; Saxén et al., 2010; Smith et al., 2000; Ugedal et al., 1995). Despite low ^{137}Cs concentration in water, freshwater fishes exhibit high ^{137}Cs concentrations due to bioconcentration. In particular, ^{137}Cs concentrations in freshwater fishes inhabiting closed lakes have declined at slower rates in comparison to fishes in rivers or open lakes (Bulgakov et al., 2002; Rask et al., 2012; Sarkka et al., 1995; Saxén et al., 2010; Saxén and Ilus, 2008).

In Gunma Prefecture, relatively high concentrations of ^{134}Cs plus ^{137}Cs (30–100 kBq m^{-3}) was deposited after the FDNPP accident (Hirose, 2016; NRA, 2017). The Gunma prefectural government initiated measurement of radiocesium concentrations in agricultural, forestry, livestock, and fishery products for food safety control immediately after the accident. In August 2011, 640 Bq kg^{-1} wet weight of ^{134}Cs plus ^{137}Cs was detected in wakasagi (*Hypomesus nipponensis*) from Lake Onuma on Mt. Akagi in the Gunma Prefecture (Suzuki et al., 2016; Suzuki and Tsunoda, 2013; Mori et al., 2017). This was higher than levels in wakasagi from other lakes in Gunma, Tochigi, or Fukushima Prefectures where similar radiocesium contamination levels were confirmed by airborne monitoring surveys (MAFF, 2017; Mori et al., 2017; Suzuki and Tsunoda, 2013; Wada et al., 2016). Mt. Akagi area and Lake Onuma are popular tourist destinations, and wakasagi fishing is an important tourist attraction from autumn to winter. The radiocesium contamination of wakasagi has significantly damaged tourism in the area. Therefore, it is particularly important for local residents around Lake Onuma to elucidate the mechanism and forecast the future of radiocesium contamination in the area. Furthermore, Mt. Akagi area has relatively heavy rainfall from June to September because the climate of Japan is largely under the influence of the East Asian monsoon (Kondo and Hamada, 2011; Kono, 1993). Thus, the measurement of radiocesium in this area under the East Asian monsoon climate may be useful in understanding the dynamics and contamination of radioactive materials in the environment.

When ^{137}Cs contamination occurs in natural ecosystems, radiological risks can be evaluated from the duration of ^{137}Cs persistence in populations of certain species in the biota. For such cases, the effective ecological half-life (T_{eff}) or ecological half-life (T_{eco}) is used to assess ^{137}Cs dynamics in the environment (Iwata et al., 2013; Jonsson et al., 1999; Pröhl et al., 2006; Smith and Beresford, 2005; Smith et al., 2000). In addition, the concentration ratio (CR) value, which is the ratio of the ^{137}Cs concentration in aquatic organisms to that in the lake water, is a useful environmental parameter (IAEA, 2010; IAEA, 2004; Kaeriyama et al., 2015).

In the previous report of our group (Mori et al., 2017), ^{137}Cs was measured for soil and lake sediment on Mt. Akagi surrounding Lake Onuma by sequential extraction, and abundance ratios of soluble and insoluble species were estimated by determining the radiocesium concentrations of each sample. In this study, we measured radiocesium levels in aquatic organisms (fishes and aquatic plant) in Lake Onuma since August 2011 to evaluate ^{137}Cs contamination levels and temporal changes in the aquatic resources. In addition to aquatic species, radiocesium concentrations were determined in the environment (lake water) and potential food resources (seston), all of which can affect the radiocesium concentration of aquatic organisms. Though many studies on the dynamics of radiocesium concentrations in fishes have been reported (e.g. Iwata et al., 2013; Ugedal et al., 1995; Wada et al., 2016), studies on the dynamics in lake water and seston have been limited. To predict the future of ^{137}Cs contamination levels in aquatic organisms and lake water, we derived the T_{eff} for ^{137}Cs by constructing decay function models and the CR value of ^{137}Cs based on samplings performed from 2011 to 2016 in the lake. Additionally, this paper reports our monitoring results of radiocesium concentrations in inflowing spring and river waters, including impact of the heavy rainfall in September 2013.

2. Materials and methods

2.1. Study area

Samples were obtained from Lake Onuma, a crater lake, situated at an altitude of 1345 m in Mt. Akagi, approximately 190 km southwest of the FDNPP (Fig. 1). The lake has a surface area of 0.87 km^2, watershed area of 4.82 km^2, volume of 0.0078 km^3, average water depth of 9.1 m, and maximum depth of 17.5 m (Kondo and Hamada, 2011). The lake is semi-closed with limited amount of inflow and runoff water and an average water residence time of 2.3 years. The only inflowing river is the Kakuman River, which has its source in the Kakumanbuchi marshland. The lake is stratified during summer (June–September) and winter (January–March) seasons, and mixed during spring (April–May) and autumn (October–December) seasons. The lake surface water is frozen during winter seasons (Kondo and Hamada, 2011; Kono, 1993).

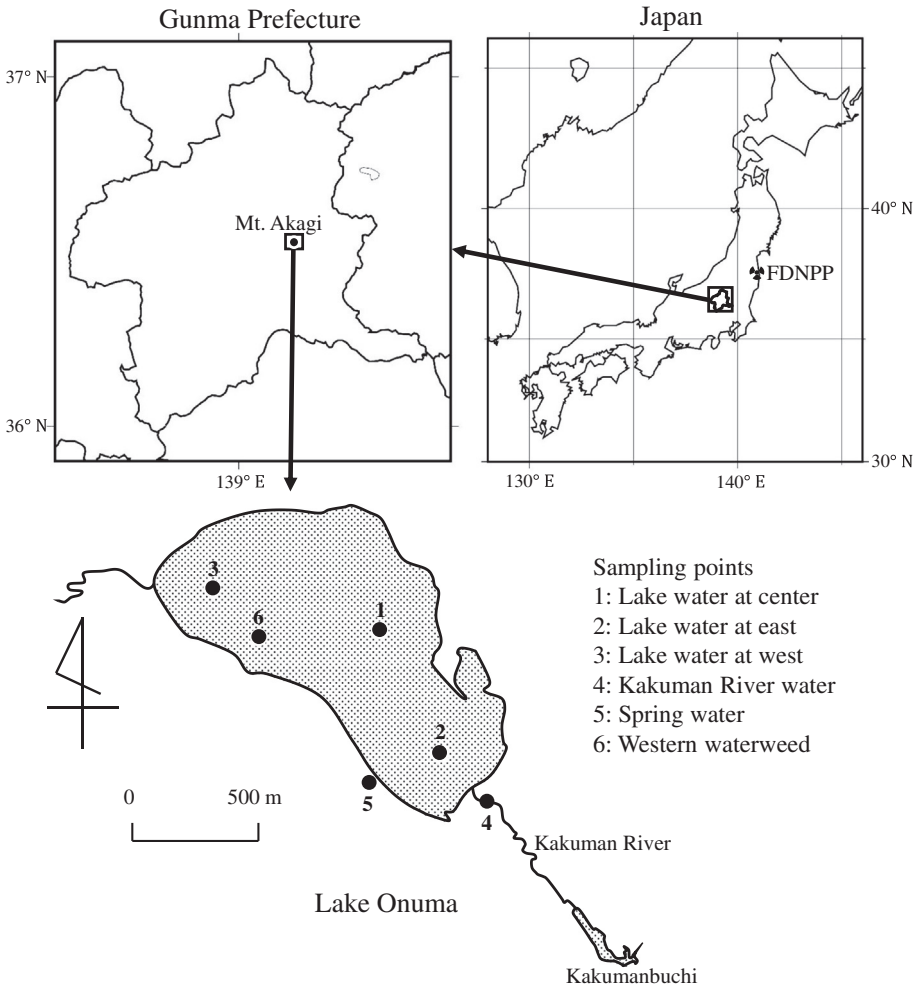

Fig. 1. Map showing sampling locations in Lake Onuma on Mt. Akagi.

2.2. Sampling

Radiocesium measurements at Lake Onuma were initiated in August 2011. Fish species including wakasagi, pale chub (*Zacco platypus*), and Japanese dace (*Tribolodon hakonensis*), aquatic plant western waterweed (*Elodea nuttallii*), seston (phytoplankton and zooplankton), lake water, inflowing river water, and spring water were collected periodically from the lake (n = 194, 24, 37, 40, 48 (32 and 16), 167, 23, and 10, respectively). Cryo-preserved wakasagi samples, which were collected before the FDNPP accident on August 28, 2007, August 24, 2010, and January 24, 2011, were used. Radiocesium concentrations were measured using high-purity germanium semiconductor detector as described in Section 2.2.3.

2.2.1. Fish and aquatic plant sampling and pre-treatment

Fish samples were collected by fishing, cast netting, or electrofishing (Fish Shocker IIIS, Frontier Electric Co., Shizuoka, Japan). In Lake Onuma, wakasagi, pale chub, and Japanese dace are the dominant fishes (Suzuki and Tsunoda, 2013). The average life span of wakasagi, pale chub, and Japanese dace in Lake Onuma are 1–2 years, 3 years, and over 3 years, respectively (Natsumeda et al., 2010; Suzuki et al., 2016). Western waterweed samples were collected by towing a small anchor at the depth of 2–4 m (Fig. 1). Fish and western waterweed samples were washed using tap water to remove dirt. Subsequently, all samples were minced using food processors (MM22, Yamamoto Electric Corp., Fukushima, Japan) or kitchen knives. Fish samples were pooled and the entire whole body including head, organs, and contents of the digestive gland were minced. The minced samples were packed in one of the follow sample cups: 100 mL U8 (Umano Kagaku Youki Co. Ltd., Osaka, Japan), 490 mL Hi-PACK S-60 (Entec Co. Ltd., Niigata, Japan), 880 mL Lustroware B-313 (Iwasaki Industry Inc., Nara, Japan), or 2 L Marineri containers (Sugiyama-Gen Co. Ltd., Tokyo, Japan). The radiocesium concentration in fish and western waterweed samples are expressed as the amount of radioactivity (Bq) per unit wet weight (kg).

2.2.2. Seston sampling and pre-treatment

Seston samples were collected by towing Kitahara's surface plankton nets (NXX13; mesh size 100 μm, NXX7; mesh size 200 μm, Rigo Co. Ltd., Tokyo, Japan) at 1 m depth. The phytoplankton bloom in Lake Onuma has been validated during vernal and autumnal circulation periods (Kondo and Hamada, 2011; Kono, 1993). Kitahara's NXX13 surface plankton net was used for collecting phytoplankton during these seasons. In contrast, zooplanktons were collected during the summer using Kitahara's NXX7 surface plankton net. As nourishment supply from the bottom sediment is suppressed by lake stratification during the summer season, zooplanktons become dominant as phytoplankton biomass decrease (Kondo and Hamada, 2011; Kono, 1993). Material other than seston, such as fallen leaves, was removed from seston samples manually. Thereafter, the collected samples were centrifuged at 2000 $\times g$ for 15 min and supernatant was discarded. This procedure was executed twice. The resultant precipitate was used for radiocesium concentration measurements. Species compositions of plankton were confirmed using light microscope (BH-2, Olympus Corporation, Tokyo,

Japan). Centrifuged seston samples were packed in unchanged form in U8 or S-60 containers. Water content of the centrifuged samples was measured after heating at 105 °C for 120 min. In addition, after July 2015 samples were dried using a freeze dryer (FDU-1200, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The radiocesium concentration of seston samples are expressed as the amount of radioactivity (Bq) per unit wet and dry weight (kg). Seston samples were fractionated using centrifuge separation method under the same rotation speeds, dry weight data was used to calculate the T_{eff} due to difference in moisture content.

2.2.3. Water sampling and pre-treatment

Approximately 40 L lake water samples were collected from the surface (0 m), middle (8 m), and bottom layers (15 m) at the center of the lake (Fig. 1 and Table S7). Surface layer water samples were collected using a bucket. The middle and bottom layer samples were collected using a Von Dorn water sampler (6 L volume, Rigo Co. Ltd., Tokyo, Japan). Nearly 40 L of the inflowing river and spring water samples were collected using a bucket (Fig. 1 and Table S7). Following collection, 20 L of the non-filtered raw water sample was used to measure total radiocesium concentration that was the sum of concentrations in the particulate- and dissolved-forms. Dissolved radiocesium was obtained by filtration of the raw water sample using 0.45 μm diameter cartridge filter within 8 h of collection. Total and dissolved radiocesium concentrations were measured using a high-purity germanium semiconductor detector. The particulate radiocesium concentration was obtained by subtracting dissolved radiocesium concentration from the total radiocesium concentration. In addition, water samples (3–20 L) collected from the center, east, and west of the lake, inflowing river water, and spring water were used to measure total radiocesium concentration (Fig. 1 and Table S7). Water samples were co-precipitated using ammonium-dodeca-molybdo-phosphate (AMP) and cesium chloride (CsCl), as described by Aoyama and Hirose (2008). The water samples were adjusted to pH 1.0–1.6 using nitric acid (HNO_3). The samples were agitated for 30 min after adding 0.26 g CsCl as a carrier, and for 60 min after adding 4.00 g AMP as an adsorbent for Cs^+. Subsequently, the specimens were left at room temperature overnight. The AMP/Cs compounds were recovered by decantation and suction filtration, and the weight yield was determined after drying at room temperature. The dried AMP/Cs compounds were packed in polyethylene bags (3.5 cm × 7.0 cm) for radiocesium concentration measurements. The concentrations in water samples were expressed as the amount of radioactivity (Bq) per cubic meter (m^3).

2.3. Measurement of radiocesium concentration

Radiocesium concentrations in fish, western waterweed, and seston samples were measured with gamma-ray spectrometry using high-purity germanium semiconductor detector (GC2020-7500SL, Canberra, Meriden, USA), coupled to a multichannel analyzer (DSA1000, Canberra Meriden, USA), and Spectrum Explorer software (Canberra, Meriden, USA) at Gunma Agricultural Technology Center (GATC). The germanium semiconductor detector at GATC was calibrated using a standard appropriate for the sample volume (MX033U8PP for the U-8 container, MX033SPS for the S-60 container, MX033SPL for the B-313 container and MX033MR for the 2-L Marinelli container, Japan Radioisotope Association, Tokyo, Japan). The counting times of ^{137}Cs in fish, western waterweed, and seston samples were set to maintain the counting error to <5%, 10%, and 10%, respectively. Radiocesium concentrations in water samples were measured at the GATC, Low Level Radioactivity Laboratory (LLRL) and Ogoya Underground Laboratory (OUL) of Kanazawa University, or the Atomic Energy Research Laboratory (AERL) of Tokyo City University employing gamma-ray spectrometry using high-purity germanium semiconductor detectors (LLRL: GC4019, Canberra, Meriden, USA; GEM30195 and other similar models, Seiko EG&G Ortec Co. Ltd., Chiba, Japan; OUL: EGPC-90-220-R and other similar models, Canberra, Meriden, USA; AERL: GEM20–70, Seiko EG&G Ortec Co. Ltd., Chiba, Japan) and a multichannel analyzer (LLRL and OUL: Multiport II, Canberra, Meriden, USA; AERL: MCA-7, Seiko EG&G Co. Ltd., Chiba, Japan) for 1–3 days. The Gamma semiconductor detectors at LLRL, OUL, and AERL were calibrated using an International Atomic Energy Agency (IAEA) Irish Sea water reference standard (IAEA-443) (Pham et al., 2011). Gamma-ray peaks used in the measurements were 604.7 keV for ^{134}Cs and 661.7 keV for ^{137}Cs. Decay correction of radiocesium concentrations were performed for each sampling date. The detection limit of radiocesium was defined as the concentration that was three times the standard deviation of counting error. The coincidence-sum effect of gamma-rays from ^{134}Cs was corrected.

2.4. Calculation of ^{137}Cs concentration in the water column

The concentration distribution of ^{137}Cs in the water column (WC) at the center of the lake was approximated using quadratic polynomial equations in Microsoft Excel. The coefficients (a_1 and a_2) and constant term (a_3) were calculated from three measurement values at 0 m, 8 m, and 15 m of the sampling depth (see 2.2.3) in the WC (Table S7), respectively, and the average concentration of ^{137}Cs in the WC was calculated using Eq. (1):

$$\left[^{137}\mathrm{Cs}\right](\mathrm{Bq\,m^{-3}}) = \frac{\int_0^d (a_1x^2 + a_2x + a_3)dx}{d} \times 1000 \quad (1)$$

where, d is the water depth at the center of lake during each investigation. ^{137}Cs concentration in the WC at the center of the lake was expressed as the amount of ^{137}Cs (Bq) per cubic meter (m^3).

3. Theory

3.1. Model selection and calculation of T_{eff} for ^{137}Cs

The decline in ^{137}Cs concentration in a natural ecosystem can be modeled using the single-component decay function model (SDM) (Eq. (2)) or the two-component decay function model (TDM) constructed from the fast (first) and slow (second) components (Eq. (3); Jonsson et al., 1999; Smith et al., 2000):

$$Q_t = Qe^{-kt} \quad (2)$$

$$Q_t = Q_1e^{-k_1t} + Q_2e^{-k_2t} \quad (3)$$

where, t is the number of days elapsed since March 15, 2011, when the main deposition of ^{137}Cs occurred in Gunma Prefecture (Morino et al., 2011); Q, Q_1, and Q_2 are the initial ^{137}Cs concentrations of SDM, the fast component of TDM, and the slow component of TDM at time t = 0 (initial value), respectively; Q_t is the ^{137}Cs concentration after t; k, k_1, and k_2 (day^{-1}) are the effective decay rate constant of SDM, effective decay rate constant in the fast component of TDM, and effective decay rate constant in the slow component of TDM, respectively.

All the parameters of decay models were estimated using the "nls" function R version 3.2.2 (R Development Core Team, 2015). The model selection (SDM and TDM) were compared using Akaike's information criterion (AIC: Akaike, 1987). The model with a smaller AIC value was selected as a "better" model (Burnham and Anderson, 2002); if the difference in AIC values between two models is <2, there is still substantial support for the model with a larger AIC value; if the difference is between 4 and 7, there is considerably less support; if the difference is >10, there is essentially no support. We chose a significance level (α) of 0.05 for our analysis.

The T_{eff} in the SDM, T_{eff} in the fast component in the TDM ($T_{eff\text{-}f}$), and T_{eff} in the slow component in the TDM ($T_{eff\text{-}s}$) were calculated using k, k_1, or k_2, respectively, as shown in Eq. (4) (Smith and Beresford, 2005):

$$T_{eff}, T_{eff-f}, \text{or } T_{eff-s} = \frac{\ln 2}{\text{decay rate constant } (k, k_1, \text{or } k_2)} \quad (4)$$

3.2. Definitions of T_{eff}, T_{eco}, and T_{phy}

The T_{eff} corresponds to the time required for 50% decline in the radiocesium levels in a given population in its natural environment, and therefore combines both physical decay and ecological decay (Smith and Beresford, 2005). The T_{eco} is the change in radiocesium concentration attributable solely to the natural ecosystem. The inverse of T_{eff} is the sum of the inverses of T_{phy} and T_{eco}, as shown in Eq. (5) (Smith and Beresford, 2005):

$$\frac{1}{T_{eff}} = \frac{1}{T_{phy}} + \frac{1}{T_{eco}} \quad (5)$$

We used only ^{137}Cs concentrations for the T_{eff} and T_{eco} estimations because ^{134}Cs data in samples from later sampling dates were below detection limits as a result of shorter T_{phy}. Furthermore, the T_{eff} in the FDNPP accident-derived ^{137}Cs was calculated without correction because concentrations of atmospheric nuclear testing-derived ^{137}Cs or the CNPP accident-derived ^{137}Cs in wakasagi were very low compared to the FDNPP accident-derived ^{137}Cs concentrations.

3.3. Definition of the CR value

The level of ^{137}Cs contamination in aquatic organisms is commonly defined in terms of a CR value as shown in Eq. (6) (IAEA, 2010):

$$\text{CR value}\left(\text{L kg}^{-1}\right) = \frac{^{137}\text{Cs concentration of aquatic organisms}\left(\text{Bq kg}^{-1}\text{ wet weight}\right)}{\text{Total}\,^{137}\text{Cs concentration of lake water}\left(\text{Bq L}^{-1}\right)} \quad (6)$$

The CR value was calculated using the monthly mean value of ^{137}Cs concentration in aquatic organisms and total ^{137}Cs concentration of lake water (Eq. (1)).

4. Results and discussion

4.1. ^{137}Cs concentration in fish

Wakasagi samples collected from Lake Onuma before the FDNPP accident were low ^{137}Cs concentrations (1.0–1.4 Bq kg^{-1} wet weight) (Table S1, Nos. 1–3). However, higher ^{137}Cs levels (340–377 Bq kg^{-1} wet weight) (Table S1, Nos. 4–5) were detected in the wakasagi in August 2011, a few months after the FDNPP accident.

Fig. 2(a) shows the ^{137}Cs concentrations in wakasagi from August 2011 to October 2016 and the decay in ^{137}Cs in the wakasagi samples was rapid from August 2011 to September 2012. Detailed information on radiocesium concentration in wakasagi is listed in Table S1. The difference in AIC values of the SDM and TDM was 185.5 (Table 1), providing reasonable support that the TDM was better for predicting the decay process of ^{137}Cs concentrations in wakasagi. The $T_{eff\text{-}f}$ and $T_{eff\text{-}s}$ in the TDM were estimated at 232 days (0.64 years) and 51,268 days (140.4 years), respectively (Table 1), although the coefficient k_2 was not statistically significant ($P > 0.05$) (Table 1). For aquatic organisms such as fishes, the SDM and fast component of the TDM correspond to the biological elimination of ^{137}Cs, which is rapidly excreted due to metabolism. In contrast, the slow component of the TDM represents the state of dynamic equilibrium between the fishes and lake environments due biological, physical, and chemical reactions, and is suggested to correspond to the physical decay rate of ^{137}Cs (Jonsson et al., 1999; Rowan et al., 1998; Smith et al., 2000; Ugedal et al., 1997). Indeed, the theoretical value of k_2 in the TDM (i.e., the physical decay rate of ^{137}Cs $= 6.29 \times 10^{-5}$) was within the estimated 95% confidence interval of k_2 (-3.33×10^{-4}–3.60×10^{-4}) (Table 1). The $T_{eff\text{-}s}$ of ^{137}Cs concentrations in wakasagi has become long-term and could converge towards the T_{phy} of ^{137}Cs.

Fig. 2(b) shows ^{137}Cs concentrations in pale chub from November 2011 to July 2016 (Table S2). The difference in AIC values of the SDM

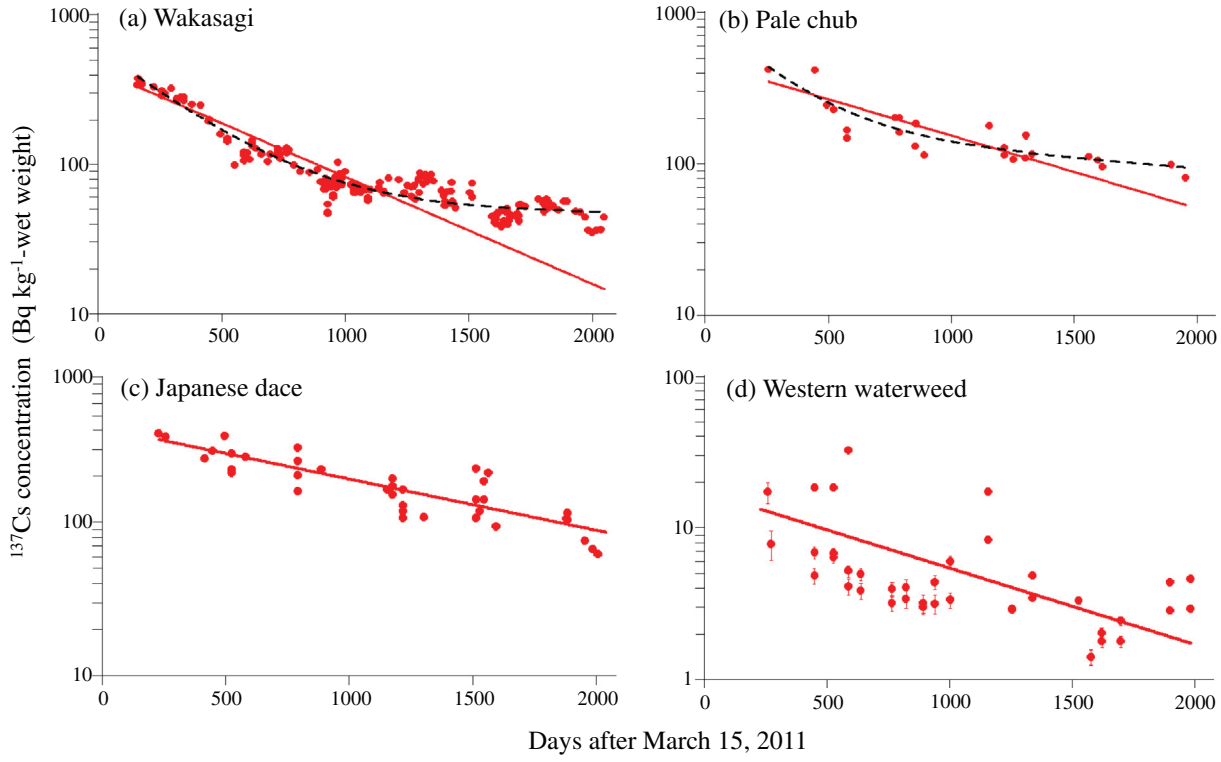

Fig. 2. Change in the ^{137}Cs concentration (Bq kg^{-1} wet weight) of three fish species ((a) wakasagi, (b) pale chub, and (c) Japanese dace) and (d) western waterweed in Lake Onuma collected in 2011–2016. The solid red lines indicate the fit of the single-component decay function model (SDM). The dashed black line indicates the fit of the two-component decay function model (TDM). The error bars indicate counting errors. Results of the fitted SDM and TDM are presented in Tables 1.

Table 1
Detailed information on results of parameters for fitted the single-component decay function model (SDM) and the two-component decay function model (TDM) of ^{137}Cs concentrations in aquatic organisms and water column (WC) in Lake Onuma. The unit of initial value of fishes, western waterweed, seston (phytoplankton and zooplankton), and WC (lake water) are Bq kg^{-1} wet weight, Bq kg^{-1} wet weight, Bq kg^{-1} dry weight, and Bq m^{-3}, respectively.

Species	Decoy function model	Initial value	Decay rate (day^{-1})	Standard error		*P*-value		T_{eff} or $T_{eff\text{-}f}$ (days)	T_{eco} or $T_{eff\text{-}f}$ (days)	Initial value	Decay rate (day^{-1})	Standard error		*P*-value		$T_{eff\text{-}s}$ (days)	$T_{eco\text{-}s}$ (days)	AIC
		Q or Q_1	k or k_1	Q or Q_1	k or k_1	Q or Q_1	k or k_1			Q_2	k_2	Q_2	k_2	Q_2	k_2			
Wakasagi	SDM	4.32×10^2	1.66×10^{-3}	1.27×10^1	4.52×10^{-5}	<0.001	<0.001	419	435									1756.9
	TDM	5.48×10^2	2.99×10^{-3}	1.56×10^1	2.21×10^{-4}	<0.001	<0.001	232	237	4.83×10^1	1.35×10^{-5}	1.50×10^1	1.76×10^{-4}	0.002	0.939	51,268	−14,027	1571.4
Pale chub	SDM	4.66×10^2	1.11×10^{-3}	5.89×10^1	1.66×10^{-4}	<0.001	<0.001	626	663									260.1
	TDM	7.69×10^2	3.89×10^{-3}	2.77×10^2	2.21×10^{-3}	0.012	0.094	178	181	1.68×10^2	2.98×10^{-4}	1.45×10^2	5.47×10^{-4}	0.258	0.544	2328	2953	254.3
Japanese dace	SDM	4.17×10^2	7.71×10^{-4}	2.86×10^1	7.70×10^{-5}	<0.001	<0.001	899	979									386.5
Western waterweed	SDM	1.73×10^1	1.16×10^{-3}	4.90×10^0	3.99×10^{-4}	0.001	0.006	598	632									253.7
Phytoplankton	SDM	2.01×10^3	1.23×10^{-3}	2.25×10^2	1.73×10^{-4}	<0.001	<0.001	565	596									450.5
	TDM	2.77×10^3	2.58×10^{-3}	5.28×10^2	1.11×10^{-3}	<0.001	0.028	269	276	2.00×10^2	-3.73×10^{-4}	3.39×10^2	9.12×10^{-4}	0.560	0.686	−1859	−1591	444.8
Zooplankton	SDM	5.61×10^2	8.23×10^{-4}	1.00×10^2	1.58×10^{-4}	<0.001	<0.001	842	912									173.5
Total in the WC (raw lake water)	SDM	2.54×10^2	1.02×10^{-3}	1.38×10^1	7.19×10^{-5}	<0.001	<0.001	682	727									238.6
	TDM	2.71×10^2	3.45×10^{-3}	3.94×10^1	1.35×10^{-3}	<0.001	0.017	201	205	1.16×10^2	4.13×10^{-4}	4.82×10^1	4.13×10^{-4}	0.024	0.108	1677	1978	218.7
Dissolved in the WC	SDM	7.14×10^1	8.14×10^{-4}	1.26×10^1	1.82×10^{-4}	<0.001	<0.001	851	923									191.8
	TDM	1.48×10^2	4.47×10^{-3}	4.71×10^2	9.41×10^{-3}	0.756	0.640	155	157	4.53×10^1	4.89×10^{-4}	5.15×10^1	6.97×10^{-4}	0.389	0.491	1418	1628	194.5
Particulate in the WC	SDM	1.20×10^2	7.11×10^{-4}	1.09×10^1	9.07×10^{-5}	<0.001	<0.001	975	1070									189.5
	TDM	1.38×10^2	9.23×10^{-4}	2.63×10^1	3.82×10^{-4}	<0.001	0.025	751	806	2.00×10^{-1}	-2.22×10^{-3}	1.99×10^0	4.79×10^{-3}	0.921	0.648	−312	−304	190.7

and TDM was 5.8 (Table 1), providing modest support that the TDM was better for predicting the decay process of ^{137}Cs in pale chub. The $T_{eff\text{-}f}$ and $T_{eff\text{-}s}$ in the TDM were estimated at 178 days (0.49 years) and 2328 days (6.37 years), respectively (Table 1), although the model coefficients, k_1 and k_2, were not statistically significant ($P > 0.05$). The theoretical value of k_2 in the TDM was within the estimated 95% confidence interval of k_2 (-8.38×10^{-4}–1.43×10^{-3}) (Table 1).

Fig. 2(c) shows ^{137}Cs concentrations in Japanese dace from October 2011 to September 2016 (Table S3). The parameter estimation of the TDM for Japanese dace did not converge, suggesting that the TDM is not likely to be an appropriate model for this species. The T_{eff} in the SDM was estimated at 899 days (2.46 years), which was longer than wakasagi (419 days) and pale chub (626 days) (Table 1). The T_{eff} was reported to be fundamentally longer in carnivorous and omnivorous fishes with higher longevity compared to herbivorous and planktivorous small fishes with shorter longevity (Mizuno and Kubo, 2013; Ugedal et al., 1995; Wada et al., 2016). Japanese dace are large omnivorous fish with higher longevity, while wakasagi and pale chub are small planktivorous and small herbivorous fish, respectively, with a shorter lifespan (Natsumeda et al., 2010). It is therefore likely that Japanese dace exhibited longer T_{eff} compared to wakasagi and pale chub. The fact that the TDM was better for modeling ^{137}Cs concentration of wakasagi in Lake Onuma agreed with the survey results of ^{137}Cs concentration of wakasagi in Ura-bandai Lakes (LUB) of Fukushima prefecture where similar radiocesium contamination levels were confirmed by airborne monitoring surveys (NRA, 2017; Wada et al., 2016). However, model selections in LUB for calculating T_{eff} were performed using the ^{137}Cs concentration of fishes during the period 2011–2014 (Wada et al., 2016). In order to compare with the results for Lake Onuma, we updated the ^{137}Cs concentrations of fishes in LUB during 2011–2016 using the results of the monitoring on radioactivity level in fisheries products (MAFF, 2017), and performed model selection of SDM or TDM. Wakasagi and Japanese dace in LUB were best suited for TDM and SDM, respectively, similar to Lake Onuma (Table S4). The $T_{eff\text{-}f}$ of wakasagi in the TDM in LUB was estimated at 151 days (0.41 years), and was shorter than Lake Onuma (i.e., 232 days). Initial values (Q_2) and contribution rates (Q_2 / (Q_1 + Q_2)) in the slow component of the TDM were 5.2 Bq kg^{-1} wet weight and 1.1% in the LUB, and 48 Bq kg^{-1} wet weight and 8.1% in Lake Onuma, respectively (Table S4) (Jonsson et al., 1999). Moreover, the decay of ^{137}Cs in the Japanese dace in LUB was better explained by the SDM, and the T_{eff} of the Japanese dace in LUB was 1.45 year and was shorter than Lake Onuma (2.46 years) (Table S4). These results indicated that the ^{137}Cs contamination of wakasagi and Japanese dace in Lake Onuma extended for longer periods of time compared to LUB. In addition, detection of the atmospheric nuclear testing-derived ^{137}Cs or the CNPP accident-derived ^{137}Cs in wakasagi of Lake Onuma suggests that FDNPP accident-derived ^{137}Cs contamination will remain in fishes for longer duration.

Following the CNPP accident, the ^{137}Cs concentrations in freshwater fishes at the lakes in the Bryansk region of the Russian Federation, approximately 200 km from the CNPP, were 0.2–1.6 kBq kg^{-1} wet weight during the period 1990–1992 (Fleishman et al., 1994). In addition, the ^{137}Cs concentrations in adult non-predatory freshwater fish at the Kiev reservoir were 0.2–1.6 kBq kg^{-1} wet weight in 1987 and 0.2–0.8 kBq kg^{-1} wet weight during the period 1990–1995 (IAEA, 2006). Therefore, ^{137}Cscontamination levels of freshwater fishes in Lake Onuma after the FDNPP accident were one-to-two orders of magnitude lower than freshwater fishes after the CNPP accident, and were 2- to-3 times higher than freshwater fishes in the LUB after the FDNPP accident (Wada et al., 2016).

4.2. ^{137}Cs concentration in western waterweed

Fig. 2(d) provides the ^{137}Cs concentration in western waterweed from November 2011 to August 2016. (Table S5). The parameter

estimation of the TDM for western waterweed did not converge, as in the case of Japanese dace. The T_{eff} in the SDM was estimated at 598 days (1.64 years) (Table 1). The differences in the ^{137}Cs concentration of western waterweed were observed between samples on the same collection day. This may be due to the potentially nonuniform distribution of ^{137}Cs concentration of lake sediment in the horizontal direction (Mori et al., 2017; Tsunoda et al., 2014). The ^{137}Cs concentrations in western waterweed were one order of magnitude lower than fishes. Similar results were reported with pondweed (*Potamogeton perfoliatus*) and hair bur-reed (*Sparganium gramineum*) in Finnish lakes after the CNPP accident (Saxén and Ilus, 2008).

4.3. ^{137}Cs concentration in seston

Fig. 3 shows ^{137}Cs concentrations in seston (Table S6). During lake water circulation period (April to May and October to December), the diatom *Aulacoseira* sp. was the dominant species. In the lake, during summer season (June to September), zooplanktons *Bosmina longirostris* and *Holopedium gibberum* became dominant with decrease in phytoplankton biomass. Consequently, the T_{eff} of phytoplankton and zooplankton was calculated separately. The ^{137}Cs concentration of phytoplankton was measured from November 2011 to October 2016. The difference in AIC values of the SDM and TDM was 5.7 (Table 1), providing modest support that the TDM was better for predicting the decay process of ^{137}Cs in phytoplankton. The $T_{eff\text{-}f}$ and $T_{eff\text{-}s}$ in the TDM were estimated at 269 days (0.74 years) and −1859 days (−5.09 years), respectively (Table 1), although the coefficient k_2 was a negative value and not statistically significant ($P > 0.05$) (Table 1). As with other species, the theoretical value of k_2 in the TDM was within the estimated 95% confidence interval of k_2 (-2.33×10^{-3}–1.49×10^{-3}) (Table 1). In contrast, the parameter estimation for the ^{137}Cs concentration of zooplankton from September 2012 to August 2016 by the TDM did not converge. The T_{eff} in the SDM obtained was estimated to be 842 days (2.31 year) (Table 1).

In this study, we observed that the amount of ^{137}Cs per unit wet and dry weight (kg) of phytoplankton (primary producer) was higher than zooplankton (primary consumer) (Fig. 3 and Table S6). This result is inconsistent with previous reports that indicated that higher ^{137}Cs concentrations were detected in species at higher trophic levels (Honda et al., 2012; IAEA, 2004; Rowan et al., 1998). The weakly bound fraction (easy-elution form) of ^{137}Cs in wakasagi, zooplankton, phytoplankton, and lake sediment in Lake Onuma were estimated to be approximately 100%, 80%, 40%, and <10%, respectively (Mori et al., 2017). The

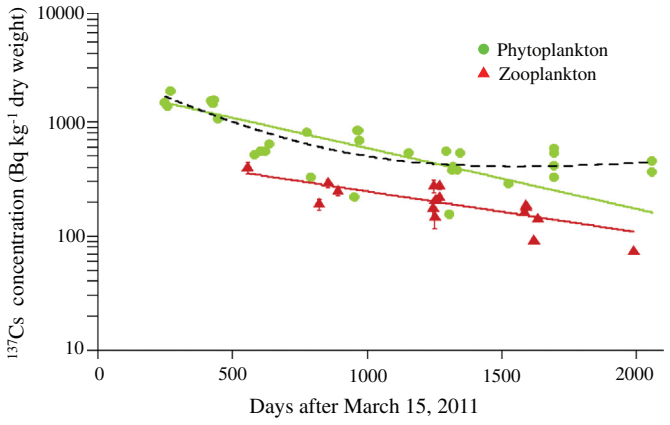

Fig. 3. Change in the ^{137}Cs concentration (Bq kg^{-1} dry weight) of seston (phytoplankton and zooplankton) in Lake Onuma collected between 2011 and 2016. The solid lines (green and red) of seston indicate the fit of the single-component decay function model (SDM). The dashed black line of phytoplankton indicates the fit of the two-component decay function model (TDM). The error bars indicate counting errors. Results of the fitted SDM and TDM are presented in Tables 1.

concentrations of aluminum and titanium in the lake, which are considered to originate from silicate minerals and clay, were highest in phytoplankton followed by zooplankton and wakasagi (Mori et al., 2017). These results suggest that phytoplankton samples were contaminated by the lake sediment or surrounding soil. To evaluate suspended particulate matter contamination derived from lake sediments in phytoplankton samples, the following experiments would be valuable: 1) quantitative determination by measuring chlorophyll *a* level in phytoplankton, 2) fractionation in phytoplankton using membrane filters, and 3) visualization of ^{137}Cs contamination and distribution using imaging plate autoradiography and the scanning electron microscope to explore ^{137}Cs in radioactive particles and to identify the actual ^{137}Cs-adsorbing materials. However, the contamination of zooplankton samples by the lake sediment or surrounding soil was less likely because the lake was stratified during collection (Kono, 1993).

4.4. ^{137}Cs concentration in the lake water

Fig. 4 presents the ^{137}Cs concentration in lake water samples at different depths at the center of Lake Onuma, collected from June 2012 to April 2013. In addition, detailed information on radiocesium concentrations in lake water from November 2011 to August 2016 is listed in Table S7. Radiocesium concentrations varied depending on water depth. The T_{eff} estimation based on the radiocesium concentration of the surface layer alone would be inaccurate because radiocesium concentrations in the bottom layer were particularly high from August to October (Fig. 4 and Table S7). Hence, the ^{137}Cs concentration in the WC was calculated using Eq. (1). Fig. 5 shows the ^{137}Cs concentration in the lake water using WC data (Table S8). Total ^{137}Cs concentrations in the WC (raw lake water) were measured from November 2011 to August 2016. The difference in AIC values of the SDM and TDM was 19.9 (Table 1), providing reasonable support that the TDM was better. The $T_{eff\text{-}f}$ and $T_{eff\text{-}s}$ in the TDM were estimated to be 201 days (0.55 years) and 1677 days (4.59 years), respectively (Table 1), although the coefficient k_2 was not statistically significant ($P > 0.05$) (Table 1). The theoretical value of k_2 in the TDM was within the estimated 95% confidence interval of k_2 (-9.42×10^{-5}–9.21×10^{-4}) (Table 1). The $T_{eff\text{-}s}$ of the total ^{137}Cs concentration in the WC has become long-term, and could converge towards the T_{phy} of ^{137}Cs. A large amount of ^{137}Cs was released in the long-term (1966–1990) from the Sellafield nuclear fuel reprocessing plant in United Kingdom into the Irish Sea. ^{137}Cs concentration in the Irish Sea was >15 Bq L^{-1} in the late 1970s, but it decreased rapidly and became <1 Bq L^{-1} in the mid 1980s because ^{137}Cs was diluted with seawater (Du Bois et al., 2012; Kobayashi et al., 2007). The same phenomenon was observed in the FDNPP accident-derived ^{137}Cs in the North Pacific, (Du Bois et al., 2012; Yoshida et al., 2015). The decay rate of total ^{137}Cs concentrations in Lake Onuma of semi-closed lake water is considered to be slower than that observed in open seawater. In addition, following the CNPP accident, a survey of closed lakes and ponds also revealed that ^{137}Cs concentrations in fishes declined slower in comparison to fishes in rivers and open lakes, due to the slower decline in ^{137}Cs concentrations in the lake water. The average residence time of lake water is suggested to be an important factor determining the ^{137}Cs contamination level of aquatic ecosystems (Bulgakov et al., 2002; IAEA, 2006; Rask et al., 2012; Sarkka et al., 1995). The average residence time of the lake water in Lake Onuma (2.3 years) was longer in comparison to LUB (0.046–0.83 years) of Fukushima prefecture (Kondo and Hamada, 2011; Wada et al., 2016). The ^{137}Cs contamination levels of freshwater fishes in Lake Onuma were higher than those in the LUB. Thus, this study suggests a positive correlation between the average residence time of the lake water and ^{137}Cs concentration of fishes. The longer residence time of lake water is a likely factor contributing to the longer-term contamination of ^{137}Cs in aquatic ecosystems.

Particulate and dissolved ^{137}Cs concentrations in the WC were measured from June 2012 to October 2016. Differences in AIC values in the SDM and TDM of particulate and dissolved ^{137}Cs concentrations in the

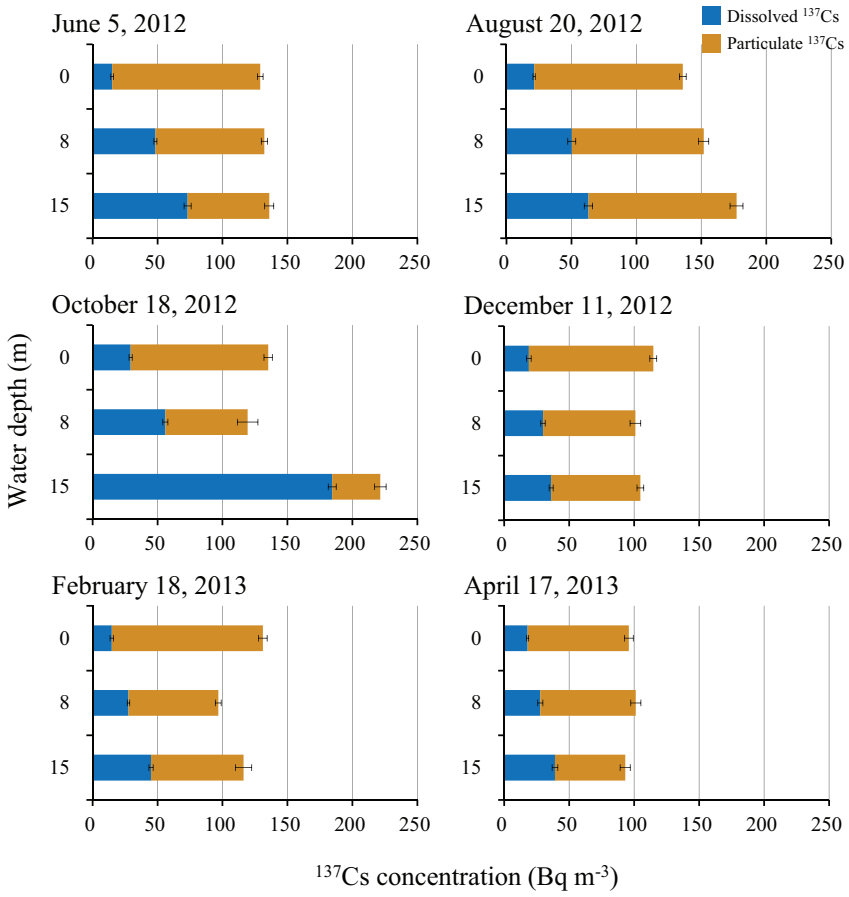

Fig. 4. The particulate and dissolved ^{137}Cs concentrations in the lake water at three water depths (0, 8, and 15 m) in Lake Onuma collected from June 2012 to April 2013.

WC were small (1.2 and 2.7, respectively; Tables 1) and thereby we chose the SDMs for further discussion. The T_{eff} in the SDMs of particulate and dissolved ^{137}Cs concentrations in the WC were estimated at 975 days (2.67 year) and 851 days (2.33 year), respectively (Table 1). Furthermore, the T_{eff} in the SDM of total ^{137}Cs concentrations in the WC was estimated at 929 days (2.54 years) using the data set for the same sampling periods as particulate and dissolved ^{137}Cs concentrations in the WC (June 2012 to October 2016). Decreasing trends of the total, particulate, and dissolved ^{137}Cs concentrations in the WC were similar during the period 2012–2016.

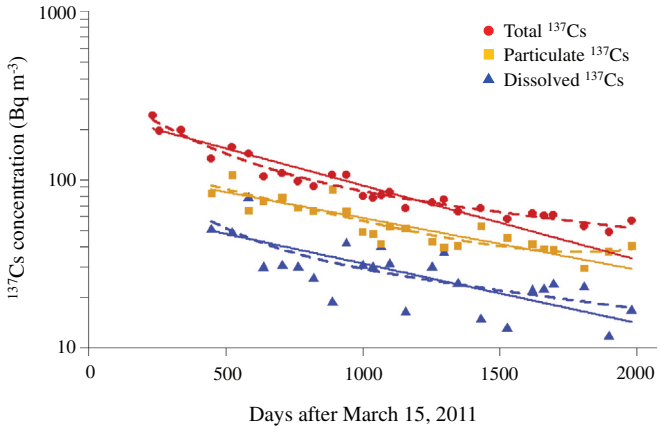

Fig. 5. Change in the ^{137}Cs concentration (Bq m^{-3}) of the water column in Lake Onuma collected in 2011–2016. The solid lines (red, orange, and blue) of lake water indicate the fit of the single-component decay function model (SDM). The dashed lines (red, orange, and blue) of lake water indicate the fit of the two-component decay function model (TDM). Results of the fitted SDM and TDM are presented in Tables 1.

The total radiocesium concentration in the vertical profile of the lake water during the entire circulation period and immediately afterwards (April to June and November to December), and inverse stratification period (January to March) did not register significant differences (Fig. 4 and Table S7). However, an increase in the dissolved radiocesium concentration towards the bottom layer was observed during all seasons (Fig. 4 and Table S7). Similar phenomenon was observed in the Par pond after the Savannah River Plant reactor accident and Lake Maggiore after the CNPP accident (Alberts et al., 1979; Putyrskaya et al., 2009). These concentration gradients suggest that ^{137}Cs is released to the lake water from the lake sediment. In addition, the dissolved radiocesium concentration was very high at the bottom layer in October (Fig. 6 and Table S7). Lake circulation in Lake Onuma starts in September and/or October (Kondo and Hamada, 2011; Kono, 1993), causing an increase in primary production due to phytoplankton bloom and fresh sediment increase. Mori et al. (2017) reported that approximately 40% of the ^{137}Cs in phytoplankton collected in October was in easy-elution form (dissolved-form). Therefore, the easy-elution form of radiocesium was considered to be eluted in the fresh sediment, which was mainly phytoplankton. Moreover, thermocline formed between 9 and 13 m water depths during the survey in October. During this period, the dissolved radiocesium exhibited higher concentration in the bottom layer, as radiocesium eluted from the fresh sediment remained in the bottom layer. However, in 2013, radiocesium concentration in the surface water along the center, east, and west parts of the lake were not different and horizontal change in radiocesium concentration along the surface was not observed (Table S7). Thus, the radiocesium concentration in the lake water was considered to have high correlation with the flow mechanism in the vertical direction and material circulation

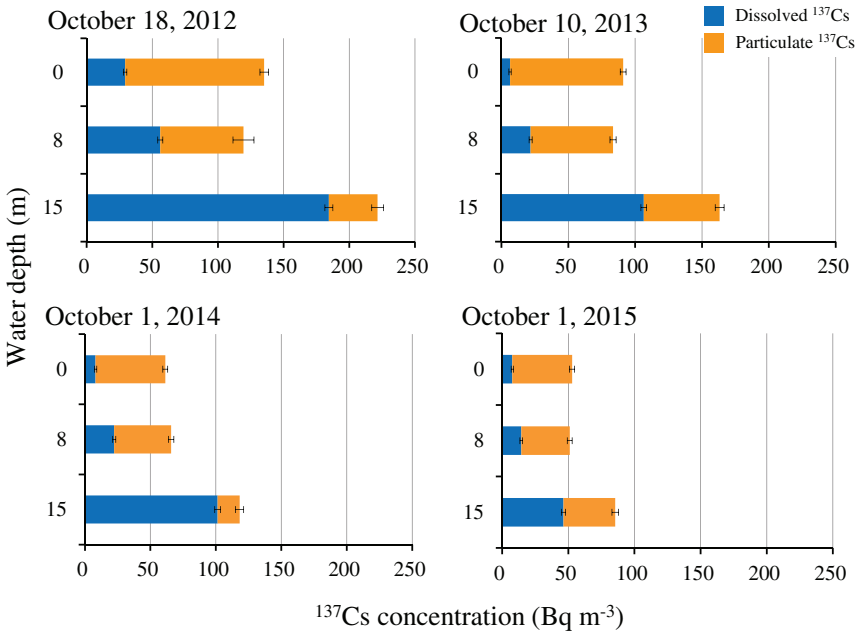

Fig. 6. The particulate and dissolved ^{137}Cs concentrations in the lake water at three water depths (0, 8, and 15 m) in Lake Onuma collected in October from 2012 to 2015.

of the lake water. Further long-term dynamic analysis of particulate and dissolved ^{137}Cs concentrations in the lake will be needed to determine material circulation of ^{137}Cs in the lake.

The total ^{137}Cs concentration in the WC in February 18, 2013 was 109 Bq m^{-3} (Table S8). Based on the lake water volume, 0.85 GBq was present in the lake. Amount of ^{137}Cs in the bottom sediment of the lake in February 2013 was estimated at 14.5 GBq (Tsunoda et al., 2014). Thus, the amount of ^{137}Cs in the bottom sediment was 17.1 times greater than the lake water. The ^{137}Cs deposited on the lake may have been transferred to the bottom sediment, as indicated following the CNPP accident (Kryshew, 1995).

To evaluate differences in the behavior of ^{137}Cs between lake sediment and water, the partition coefficient (K_d) value was calculated. The K_d value of ^{137}Cs is defined as the ratio of the contaminant concentration bound on the solid phase to the contaminant concentration remaining in the liquid phase (IAEA, 2010). The dissolved ^{137}Cs concentration in the water located immediately above lake bottom were 72 Bq m^{-3} (0.072 Bq L^{-1}) on August 19, 2014, and 49 Bq m^{-3} (0.049 Bq L^{-1}) on August 20, 2015 (Table S7). The ^{137}Cs concentrations of lake sediment at depths of 0–3 cm were 2290 Bq kg^{-1} dry weight on August 12, 2014, and 1830 Bq kg^{-1} dry weight on August 28, 2015 (Mori et al., 2017). The K_d values between lake sediment and dissolved lake water (K_d value = lake sediment (Bq kg^{-1} dry weight) / dissolved lake water (Bq L^{-1})) were 3.2–3.8 × 10^4 L kg^{-1}, which are consistent with K_d values of IAEA compilations (IAEA, 2010).

4.5. CR value of ^{137}Cs in aquatic organisms

The CR value of ^{137}Cs is a useful parameter for estimating the dynamics of ^{137}Cs in the environment under equilibrium conditions (Kaeriyama et al., 2015). Therefore, we calculated CR values using data since May 2015 when the ^{137}Cs concentration in the lake water became almost constant and Eq. (6). The mean CR value of ^{137}Cs in wakasagi, pale chub, Japanese dace, western waterweed, phytoplankton, and zooplankton were 8.9 × 10^2, 1.8 × 10^3, 2.0 × 10^3, 5.2 × 10^1, 7.2 × 10^2, and 4.0 × 10^1 L kg^{-1}, respectively (Table 2). The CR values of fishes, western waterweed, and zooplankton were consistent with previous reports (IAEA, 2010; Yankovich et al., 2013). However, the CR values of phytoplankton were slightly higher than the maximum value (6.6 × 10^2 L kg^{-1}) previously reported (Yankovich et al., 2013). Although this result could also indicate that phytoplankton were contaminated with the lake sediment or surrounding soil, the contamination level may be low. Future studies are needed to determine the contamination level of phytoplankton using the above-mentioned methodologies (Section 4.3), as well as to gain a more precise CR value.

4.6. ^{137}Cs concentrations in the spring and river waters

Table 3 summarizes the ^{137}Cs concentrations in the spring water from December 2011 to September 2016. As the sample taken on October 10, 2013 (Table 3, No. 4) was measured using an ultralow background germanium semiconductor detector of OUL in Kanazawa University, radiocesium in spring water was detected (Hamajima and Komura, 2004). The dissolved ^{137}Cs concentration in this sample (0.23 Bq m^{-3}) was similar to the Tone River water in Japan before the CNPP accident (Hirose et al., 1990). Moreover, ^{137}Cs was detected in the river water in Denmark after the CNPP accident, but was not detected in the underground water (Hansen and Aarkrog, 1990). These results agree with the results of our study demonstrating lower contamination levels in the spring water.

The total radiocesium concentrations in river water samples at Kakuman River from November 2011 to August 2016 under normal conditions were lower than the lake water in Lake Onuma (Fig. 7 and Table S9). The radiocesium concentration of the lake water in Lake Onuma was considerable and could not be reduced drastically by the inflowing river and spring water. In contrast, during Typhoon No. 18 (Man-yi) on September 16, 2013, 164 mm of rainfall was recorded within 24 h near Lake Onuma (MLIT, 2017). The total ^{137}Cs

Table 2
Concentration ratio (CR) values (L kg^{-1}) of aquatic organisms.

Species	Number	CR (L kg^{-1}) mean ± SD
Wakasagi	7	8.9 × 10^2 ± 2.0 × 10^2
Pale chub	2	1.8 × 10^3 ± 2.5 × 10^2
Japanese dace	3	2.0 × 10^3 ± 5.8 × 10^2
Western waterweed	5	5.2 × 10^1 ± 1.7 × 10^1
Phytoplankton	2	7.2 × 10^2 ± 1.1 × 10^2
Zooplankton	2	4.0 × 10^1 ± 1.4 × 10^1

Table 3
Radiocesium concentrations (Bq m^{-3} ± counting error, <detection limit) of spring water samples. NA denotes not analyzed. NC denotes not calculated. LLAL and OUL denote Low Level Radioactivity Laboratory and Ogoya Underground Laboratory of Kanazawa University, respectively. AERL denotes Atomic Energy Research Laboratory of Tokyo City University.

No.	Sampling date	Sampling volume (L)	Measuring institution	^{137}Cs (Bq m^{-3})			^{134}Cs (Bq m^{-3})		
				Total	Dissolved	Particulate	Total	Dissolved	Particulate
1	Dec. 26, 2011	20	LLRL	<0.80	NA	NA	<0.83	NA	NA
2	Oct. 18, 2012	40	AERL	<1.8	<0.88	NC	<3.6	<0.92	NC
3	Dec. 11, 2012	40	AERL	<2.8	<1.7	NC	<5.3	<3.8	NC
4	Oct. 10, 2013	40	OUL	1.5 ± 0.13	0.23 ± 0.04	1.3 ± 0.14	0.49 ± 0.08	0.12 ± 0.03	0.36 ± 0.09
5	Nov. 10, 2014	40	AERL	<2.7	<1.4	NC	<3.3	<1.5	NC
6	May 18, 2015	40	AERL	<2.5	<2.5	NC	<2.8	<2.6	NC
7	Aug. 21, 2015	40	AERL	<2.3	<2.3	NC	<2.9	<2.7	NC
8	Nov. 06, 2015	40	AERL	<2.3	<2.2	NC	<2.5	<2.2	NC
9	Aug. 16, 2016	40	AERL	<2.5	<2.5	NC	<3.0	<2.5	NC
10	Sep. 14, 2016	40	AERL	<2.4	<2.6	NC	<2.5	<2.4	NC

concentration in Kakuman River (230 Bq m^{-3}) significantly increased immediately afterwards (Fig. 7, No. 1 arrow and Table S9). In particular, the particulate ^{137}Cs concentration (220 Bq m^{-3}) increased. When several soil particles were observed upon microscopic examination, the origin of particulate radiocesium after the disturbance was assumed to be from soil particles. Rapid increase in the particulate radiocesium concentration after disturbance due to heavy rainfall has also been observed in other rivers in Fukushima prefecture (Kakehi et al., 2016; Nagao et al., 2013, 2015; Sakaguchi et al., 2015; Ueda et al., 2013). This appears to be a universal phenomenon. However, the radiocesium concentration in the Kakuman river water rapidly decreased one day after the passage of Typhoon No. 18 (Fig. 7, No. 2 arrow and Table S9). Moreover, when the radiocesium concentration in the surface layer of the lake water at the center of Lake Onuma was measured two days after the passage of Typhoon No. 18, no changes were observed (Table S7, No. 40). Additionally, no significant change was observed in the radiocesium concentration of wakasagi before and after the passage of Typhoon No. 18 (Table S1, Nos. 51–56). From these results, soil particles containing radiocesium, which flowed into Lake Onuma, are predicted to have rapidly settled to the bottom of the lake. In addition, since the rate of ^{137}Cs re-dissolution from the soil particles is low (Mori et al., 2017; Saito et al., 2014; Tsukada et al., 2008), the soil particles in the river water flowing into Lake Onuma during the disturbance probably had limited impact on the aquatic ecosystem. Although higher ^{137}Cs flux in the lake water and increase in inflowing river water in large-scale lakes with large watershed areas are correlated (Putyrskaya et al., 2009), in Lake Onuma, where the watershed area is relatively small, ^{137}Cs concentrations in wakasagi and the lake water did not change; the influence of heavy rain was considered to be consequently small.

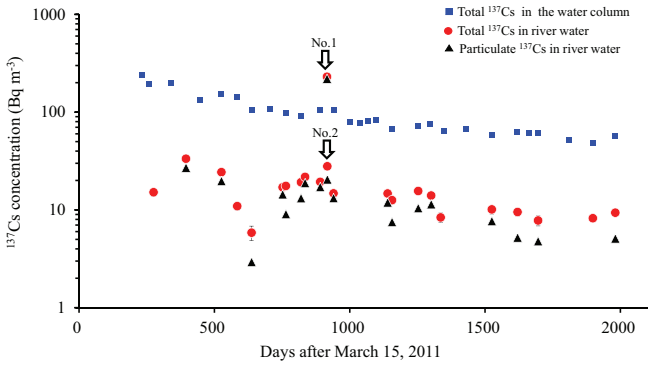

Fig. 7. Change in the ^{137}Cs concentration (Bq m^{-3}) of river water in Kakuman River and total ^{137}Cs concentration in the water column (lake water) in Lake Onuma. No.1 arrow shows Typhoon No.18 (Man-yi) in September 16, 2013. No.2 arrow shows one day after the passage of Typhoon No.18.

4.7. Decay process of ^{137}Cs concentrations in aquatic organisms and ^{137}Cs concentrations in the WC

The decay processes of ^{137}Cs concentrations in wakasagi, pale chub, and phytoplankton, and the total ^{137}Cs concentrations in the WC were best suited for the TDM. The $T_{eff\text{-}f}$ of the TDMs in these samples ranged from 0.49 to 0.74 years. The $T_{eff\text{-}s}$ in the TDMs for these samples could converge towards the T_{phy} of ^{137}Cs, though longer-term monitoring is required for the *P*-value of k_2 to be significant. In the second half of 2016, nearly eight- to eleven-fold times longer than $T_{eff\text{-}f}$ of the TDMs in these samples had elapsed because five and a half years had passed since the FDNPP accident. Therefore, the ^{137}Cs concentrations of Q_1 in these samples decreased from 2^{-8} to 2^{-11} and were <0.5% in the second half of 2016. The decay rates in these samples were quite slow in the second half of 2016 and may have been near the T_{phy} of ^{137}Cs. These results suggest that ^{137}Cs had tended towards a state of dynamic equilibrium between the aquatic organisms (wakasagi, pale chub, and phytoplankton) and lake water in 2016, consistent with previous studies related to the CNPP accident (Jonsson et al., 1999; Pröhl et al., 2006; Smith et al., 2000). Moreover, similar decay rates between ^{137}Cs concentrations in aquatic organisms (wakasagi, pale chub, and phytoplankton) and the total ^{137}Cs concentrations in the WC suggests that they may be controlled by the same processes (Smith et al., 2000). Thus, ^{137}Cs in the lake water has a direct or indirect effect on the accumulation of ^{137}Cs in aquatic organisms, suggesting that measurement of ^{137}Cs concentration in the aquatic ecosystem is essential for grasping the contamination level in lakes. Given the natural variation in data, further data collection is required to achieve a more precise estimation of the *P*-value of k_2 and predict long-term future trends of ^{137}Cs contamination in aquatic organisms.

The decay processes of ^{137}Cs concentrations in Japanese dace, western waterweed, and zooplankton, and the particulate and dissolved ^{137}Cs concentrations in the WC were best suited for the SDM. The decay process in Japanese dace could be due to the possibility that ^{137}Cs did not reach a state of dynamic equilibrium with the lake water, unlike wakasagi and pale chub. The different decay processes of ^{137}Cs between wakasagi and pale chub, which are planktivorous small fishes, and Japanese dace, which is an omnivorous large fish, are probably due to their different ecological niches (habitat and diet) and longevity (Jonsson et al., 1999; Natsumeda et al., 2010). The ^{137}Cs measurements of the particulate- and dissolved-forms in the WC and zooplankton were initiated in June and September 2012, respectively. Sampling started approximately 10–13 months after wakasagi examination and may have contributed to lower ^{137}Cs reading, suggesting that SDM was the better model. However, because ^{137}Cs concentrations in the WC were relatively unchanged or slightly decreased since May 2014 (Table S8) and differences in the AIC values of the SDM and TDM in particulate and dissolved ^{137}Cs concentrations in the WC were <3, the TDMs may become more suitable than the SDMs through future data collection. Furthermore, ^{137}Cs concentrations in Japanese dace and zooplankton slightly decreased since July 2015 (Tables S3 and S6). Because ^{137}Cs concentrations in Japanese dace and zooplankton could reach a

state of dynamic equilibrium in the near future, the TDMs may be then selected. In either case, the ^{137}Cs concentrations in Japanese dace, zooplankton, and particulate and dissolved ^{137}Csconcentrations in the WC must be collected for several years as establishment of the TDM is useful to predict future trends in ^{137}Cs contamination. In western waterweed, the SDM was selected, because ^{137}Cs concentrations were low and the variation in concentrations were large. The ^{137}Cs concentrations in western waterweed will most likely be below detection limits in the near future. Overall, further monitoring is necessary to understand and predict the future changes in ^{137}Cs contamination in Lake Onuma.

5. Conclusions

The present investigation was conducted nearly five and a half years after the FDNPP accident to elucidate the decay process of ^{137}Cs in Lake Onuma aquatic ecosystem in the Gunma Prefecture. The results are summarized as follows:

1) The decay processes of ^{137}Cs concentrations in wakasagi, pale chub, and phytoplankton, and total ^{137}Cs concentrations in the lake water were well suited for TDMs. The $T_{\text{eff-f}}$ of the TDMs in these samples ranged from 0.49 to 0.74 years. The $T_{\text{eff-s}}$ of the TDMs could converge towards the T_{phy} of ^{137}Cs. Contribution of the fast component of the TDM to the decay of ^{137}Cs concentrations in the second half of 2016 was insignificant. We concluded that ^{137}Cs concentrations were likely to be in a state of dynamic equilibrium between the aquatic organisms (wakasagi, pale chub, and phytoplankton) and lake water.
2) The decay process of ^{137}Cs concentration in Japanese dace was well suited for the SDM. The ^{137}Cs did not reach a state of dynamic equilibrium between the Japanese dace and lake water.
3) The decay processes of ^{137}Cs concentrations in western waterweed, zooplankton, and particulate- and dissolved-forms in the lake water were suited for the SDM. This is probably due to large variations in the measurement results or/and delay in initiation of investigation by approximately 10–13 months compared to wakasagi.
4) The dissolved radiocesium concentrations in the lake water were higher at the bottom layer in October, when the lake experiences partial circulation every year.
5) The CR values of ^{137}Cs in fishes, western waterweed, and zooplankton were consistent with previous reports, but the CR value in phytoplankton was slightly higher than that previously reported.
6) The contamination levels in the spring water and inflowing river water samples were low during their normal flow conditions. In contrast, high particulate radiocesium concentration was detected in the lake water following heavy rains in the river water.

From the viewpoint of long-term dynamic analysis of ^{137}Cs in aquatic ecosystems and evaluation of the precise radiological impact of the FDNPP accident, ^{137}Cs concentrations in freshwater fishes in Lake Onuma should be measured regularly over a longer period. Such continued monitoring will improve predictions of future ^{137}Cs contamination in aquatic ecosystems.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.12.017.

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Biomagnification of Tantalum through Diverse Aquatic Food Webs

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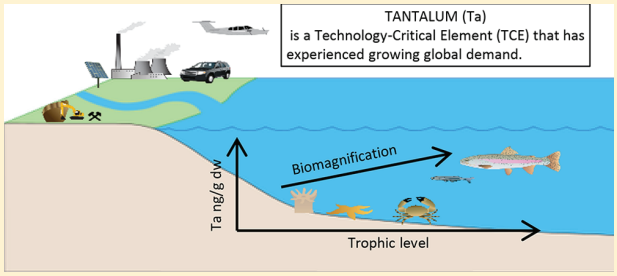
Supporting Information

ABSTRACT: Tantalum (Ta) is a technology-critical element (TCE) that is growing in global demand because of its use in electronic and medical devices, capacitors, aircraft, and hybrid cars. Despite its economic relevance, little is known about its environmental concentrations and the trophodynamics of Ta in aquatic food webs have not been studied. Invertebrates and fishes from coastal marine food webs representing different climatic zones in northwestern Chile, western Chilean Patagonia, and the Antarctic Peninsula were sampled and analyzed for Ta. The trophic level (TL) of species was assessed with nitrogen stable isotopes (δ^{15}N), and carbon stable isotopes (δ^{13}C) were used to trace energy flow in the food webs. Levels of Ta varied among taxa and sites, with the highest values found in fishes (0.53−44.48 ng g^{-1}dry weight) and the lowest values found in invertebrates (0.11−7.80 n ng g^{-1}dry weight). The values of δ^{13}C ranged from −11.79 to −25.66 ‰. Ta biomagnified in all four aquatic food webs, with slopes of log Ta versus TL ranging from 0.16 to 0.60. This has important implications as little is known about its potential toxicity and there may be increased demand for TCEs such as Ta in the future.



INTRODUCTION

Tantalum (Ta) is a rare transition element that is highly corrosion-resistant and stable at high temperatures,[1,2] and it is increasingly used in technology related to renewable energies, electronics, the automotive and aerospace industries, and biomedicine.[3,4] World production of Ta has increased over the last 2 decades, although its extraction remains low (ca. 1000 t per year) when compared to other elements.[5] Although Australia, Brazil, Canada, Ethiopia and Nigeria have produced Ta, countries such as Burundi, Congo and Rwanda (65% of global production since 2014) have used it to finance illegal military operations during civil wars, dubbing it a "conflict mineral".[5,6] Nonetheless, it is estimated that new uses for Ta will increase global demand and production[4] but its environmental concentrations and fate are poorly characterized.[7]

Published data on Ta levels in the environment are scarce, focusing mainly on mineralogical analysis and then abiotic matrices,[7] with only a few reports on Ta in aquatic animals. Ascidians (*Styela plicata*) from Japanese waters had 100−410 μg g^{-1}dw Ta[8] (dw: dry weight) whereas marine organisms from coastal areas of southern England ranged from 0.009 in mollusks to 2.3 μg g^{-1}dw in crustaceans.[9] Chebotina et al.[10] reported the bioconcentration of Ta from water to phytoplankton (>10^{1}) and zooplankton (>10^{7}). Despite evidence of Ta bioaccumulation in aquatic organisms, the factors affecting its concentrations in different species have not been examined.

Metals such as mercury, persistent organic pollutants and organotin compounds are known to biomagnify in diverse aquatic food webs to levels in upper-trophic-level fish that may pose a risk to fish consumers and the fish themselves.[11−13] The trophic level (TL) of species is estimated from δ^{15}N and frequently used to provide a measure of the relative trophic position of organisms within food webs.[11] Levels of contaminants are regressed against TL to understand whether they biomagnify and these relationships can be compared

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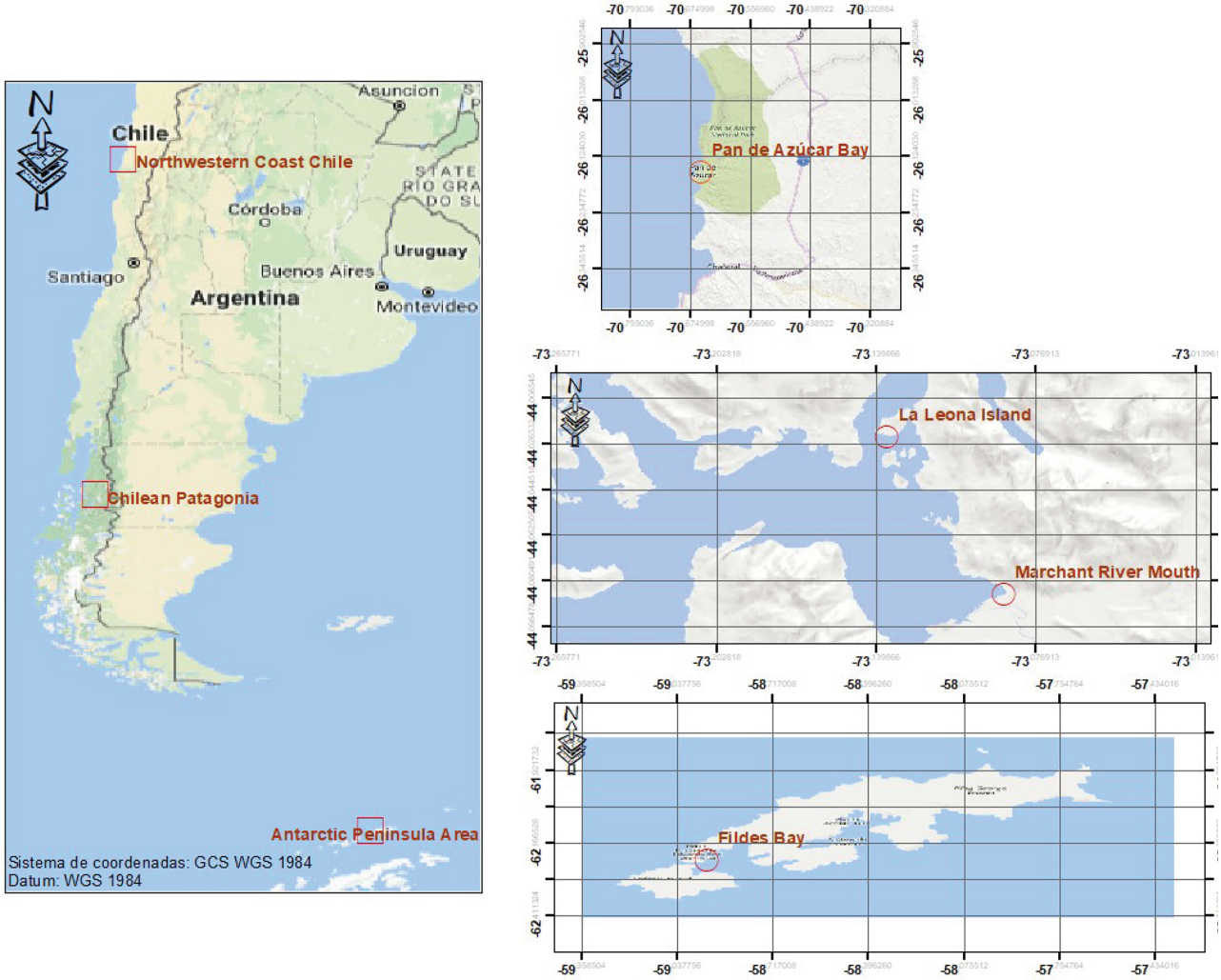

Figure 1. Map of the locations of the marine coastal food webs sampled in 2015 in northwestern Chile, Chilean Patagonia and Antarctica.

among ecosystems differing in species composition, physical and chemical characteristics, and climatic zones.[11,12]

There is a lack of knowledge on the concentrations of Ta in biota and whether this element biomagnifies through aquatic food webs.[7] This is important to address because of the likely increased use of Ta and the potential risk it may pose from dietary exposures.[13] The objectives of the present study were to determine the concentrations of Ta and the relative trophic level of aquatic organisms from marine coastal food webs across three climatic zones in Antarctica and Chile. The results show for the first time that there is an increase in Ta concentrations with increasing trophic level, and that its biomagnification occurred at sites differing in their physical and biological characteristics.

MATERIAL AND METHODS

Field Collections. During the austral summer of 2015, four marine ecosystems with different climatic conditions were sampled in the following regions of the southern hemisphere (Figure 1): northwestern coast of Chile (Sector A), with a tropical hyper-desertic climate;[14] western Chilean Patagonia (Sector B) with a climate classified as template hyper-oceanic;[14] and the Antarctic Peninsula area (Sector C), which is classified as a cold desert.[15] In northwestern Chile, samples were obtained from Pan de Azúcar Bay (26°09′ S, 70°40′ W). In Chilean Patagonia, samples were obtained from two sites: the first was off of La Leona Island (44°1′58″ W, 73°7′56″ W) and the second was at the mouth of the Marchant River (44°5′15″ S, 73°5′6″ W). In Antarctica, samples were obtained from Fildes Bay (62°12′ S, 58°58′ W).

Fishes and invertebrates were collected from each of the locations by SCUBA to ensure the collection of the selected species, as well as to minimize any impacts of sampling. At Pan de Azúcar Bay in northwestern Chile, 8 species of macroinvertebrates and 6 species of fishes were collected (N = 61; Table S1). In Chilean Patagonia, 4 species of macroinvertebrates and 3 species of fishes were collected at the mouth of the Marchant River (N = 31), and 4 species of macroinvertebrates and 3 species of fishes were sampled at La Leona Island (N = 28; Table S2). At Fildes Bay in Antarctica, 9 of both macroinvertebrate and fish species were sampled (N = 55; Table S3). Fish were anaesthetized with BZ-20 (Veterquimica), sacrificed by severing the spinal cord, and sampled for muscle tissues. Soft tissues of mollusks were collected and whole bodies of other macroinvertebrates were retained. All

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specimens were stored at −20 °C until processed in the laboratory.

Laboratory Analyses. Individual fish muscle and soft invertebrate tissues were freeze-dried until dry masses were constant and then were homogenized into a fine powder using a glass mortar and pestle precleaned with 2% Conrad solution (Merck) for 24 h, washed with deionized water and HCl 1 M and rinsed with distilled water.[16] Subsamples (0.2 g) were placed into 50 mL Teflon beaker with 5 mL of ultrapure nitric acid and heated (at 110 °C) until almost dry (about 3 h). Then 5 mL of ultrapure nitric acid and 1 mL of hydrogen peroxide were added, and the mixture was heated again to near dryness (about 3 h). The residue was dissolved in 5 mL of 1% ultrapure nitric acid, filtered with glass fiber filter (<0.45 μm), and then transferred to a centrifuge tube. This digestion and filtration were repeated four times so as to obtain a final volume of 25 mL. Total Ta was determined by mass spectrometry coupled with a plasma inductor (ICP-MS, NexION-350D, PerkinElmer) at the Environmental Health Science Laboratory, Toyo University (Japan).

To ensure the quality of the Ta measurements, a seven-point calibration curve was made and a median response factor used to calculate sample concentrations. The detection limits and quantification limits were 0.0019 and 0.036 ng g^{-1}dw respectively for each batch of samples calculated as 3× and 5× the standard deviation of the blanks (n = 12). A certified reference material (CRM) for Ta in biological materials is not available. Instead, a Custom Claritas PPT grade Tantalum for ICP-MS (CLTA9-1BY) by SPEXertificate (n = 12) and Multielement Calibration Standard 5 by PerkinElmer (n = 12) were used. The internal standard was In (stable isotope of indium, standard atomic weight 115). Triplicates of every 10th sample were analyzed and the accuracy was 0.28 ± 0.29 ng g^{-1} for Ta (n = 54). All Ta values are expressed on a dw basis.

Quantification of Stable Isotopes. Tissues (1 mg) were analyzed for carbon and nitrogen (δ^{13}C and δ^{15}N) isotopes using an elemental mass spectrometer Costech 4010 interfaced with Delta XP at the Stable Isotopes in Nature Laboratory (SINLAB) at the University of New Brunswick (Canada). The stable isotope measurements were reported in delta notation (δ) and in parts per thousand (‰). Two reference materials, N-2 (n = 6) and CH-7 (n = 6), both certified by the International Atomic Energy Agency (IAEA) for isotope values[15,16] were used as well as certified standards of commercially available elements, acetanilide (n = 18) and nicotinamide (n = 18). In addition, three laboratory standards, bovine liver (n = 18), muskellunge muscle (n = 42) and protein (n = 18), were used and they had an average deviation of 0.03‰ from the long-term values. Replicates were performed of every 10th sample and the accuracy was 0.2 ± 0.17‰ for δ^{13}C and 0.14 ± 0.14‰ for δ^{15}N (n = 24).

The raw δ^{15}N values were adjusted by subtracting the average δ^{15}N of primary consumers from each site, thus obtaining $\delta^{15}N_{adj}$ values.[17,18] Raw and lipid-adjusted δ^{13}C data were used to ensure organisms were energetically linked, and details are given in Tables S7−S12 and Figure S1. Consumer δ^{15}N values were also converted to trophic levels (TL) according to the following equation:

$$TL_{consumer} = (\delta^{15}N_{consumer} - \delta^{15}N_{baseline})/\Delta^{15}N + \lambda \quad (1)$$

where λ is the trophic level of the baseline organism, herein 2 for primary consumers. $TL_{consumer}$ is the trophic level of a given consumer, and $\delta^{15}N_{consumer}$ and $\delta^{15}N_{baseline}$ are raw δ^{15}N values of a given consumer and the baseline organism for each site (see Tables S7−S9). A trophic discrimination factor for δ^{15}N ($\triangle^{15}$N) of 3.4‰ was used as in Lavoie et al.[11]

Data Analysis. Levels of Ta were $\log_{10}$-transformed to meet the assumptions of normality and biomagnification was examined with linear regressions as in Lavoie et al.[11] and Yoshinaga et al.[19] using the following equations:

$$\log_{10}[Ta] = b\delta^{15}N_{adj} + a \quad (2)$$

$$\log_{10}[Ta] = b\ TL + a \quad (3)$$

where b in eq 2 is known as the trophic magnification slope (TMS) and the antilog of the slope in eq 3 as the trophic magnification factor (TMF). Analysis of Covariance (ANCOVA) was used to determine whether Ta biomagnification was significantly different in the four food webs. Statistical analyses were performed using JMP from SAS.

RESULTS AND DISCUSSION

The trophic levels of species sampled in northwestern Chile showed TL ranged between 2.21 ± 0.16 and 4.81 ± 0.11; *Crucibulum scutellatum* had the lowest TL and *Hemilutjanus macrophthalmus* had the highest TL. In contrast, in Chilean Patagonia at the Marchant River Mouth *Fissurella* spp. (2.48 ± 0.69) had the lowest trophic level and *Graus nigra* (4.69 ± 0.06) had the highest trophic level. At Leona Island, *Aulacomya ater* (2.21 ± 0.13) and *Pinguipes chilensis* (4.23 ± 0.27) were the species with the lowest and highest TL, respectively. Finally from the Antarctic Peninsula, the TL values ranged from 2 (*Cnemidocarpa verrucosa*) to 4.13 (*Pagothenia borchgrevinki*).

Across all three climatic regions, Ta levels in macroinvertebrates were consistently lower than those in fishes (Tables S4−S6). In macroinvertebrates from the northwestern coast of Chile, the lowest mean Ta level was in sea snails (*Crucibulum scutellatum*, 0.17 ng g^{-1}), a benthic grazer, whereas the highest was in sea stars (*Forcipulatida* spp., 0.83 ng g^{-1}), a benthic predator. In fishes, the lowest and highest mean Ta levels were found in *Pinguipes chilensis* (a benthic-pelagic predator, 2.09 ng g^{-1}) and *Trachurus symmetricus murphyi* (a pelagic predator, 2.86 ng g^{-1}), respectively, and they were 12.3 to 17.6 times higher than the lowest levels found in macroinvertebrates from this location. In western Chilean Patagonia at Marchant River, mean Ta levels ranged from 1.05 ng g^{-1} in the filter-feeding mollusk (*Aulacomya ater*) to 1.51 ng g^{-1} in crabs (*Cancer coronatus*), benthic predators. In fishes, mean Ta was 2.08 ng g^{-1} in *Genypterus chilensis* (a benthic predator) and 2.48 ng g^{-1} in *Eleginops maclovinus* (a benthic-pelagic predator), over 2 times higher than those in mollusks from the same location. Similarly for La Leona Island, the lowest Ta levels were found in macroinvertebrates, and ranged from 0.23 ng g^{-1} in mollusks (*Aulacomya ater*), to 0.37 ng g^{-1} in mollusk (*Concholepas concholepas*), a benthic predator. In fishes from this location, the lowest and highest Ta levels were in *Panguipes chilensis* (0.61 ng g^{-1}) and *Sebastes capensis* (1.84 ng g^{-1}, a benthic predator), respectively. Finally, from the Antarctic Peninsula, the lowest and highest mean Ta levels of all macroinvertebrates were in sea urchin (*Abatus agassizii*, 0.43 ng g^{-1}), which is a benthic forager, and starfish (*Odontaster validus*, 7.8 ng g^{-1}), a predator. Fishes from Antarctica had the highest Ta of all sites examined herein, with mean levels ranging from 2.23 ng g^{-1} in *Pagothenia hansoni* to 14.0 ng g^{-1} in *Notothenia kempi*, both are benthic predators.

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Table 1. Regressions of $\log_{10}$ Ta versus TL for Fishes and Invertebrates Collected from Coastal Sites in Northwestern Chile, Chilean Patagonia and Antarctica[a]

Sector	Location	Slope	Intercept	R^2	p-value	N
A[b]	Pan de Azúcar Bay	0.36 ± 0.04	−1.50 ± 0.17	0.52	<0.0001	61
B[b]	La Leona Island	0.30 ± 0.07	−1.34 ± 0.25	0.39	0.0004	28
	Marchant River Mouth	0.16 ± 0.07	−0.42 ± 0.23	0.15	0.02	34
C[b]	Fildes Bay	0.60 ± 0.10	−1.72 ± 0.35	0.39	<0.0001	55

[a]See Figure 2. Letters indicate significant differences among trophic magnification slopes (TMS). [b]A = northwestern coast of Chile; B = western Chilean Patagonia; C = south Shetland Island (Antarctic Peninsula area).

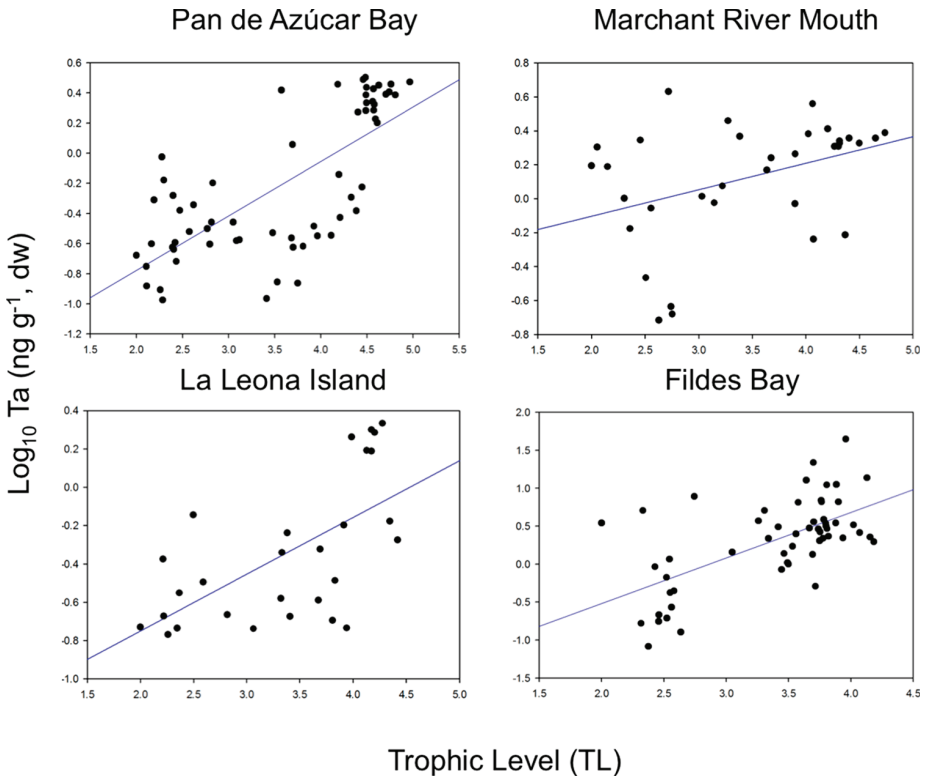

Figure 2. Regressions of log Ta versus trophic level (TL) for fishes and macroinvertebrates collected from coastal, marine food webs in northwestern Chile (Pan de Azucar), Chilean Patagonia (Marchant River Mouth and La Leona Island) and Antarctica (Fildes Bay).

For those taxa collected at several sites, the levels of Ta varied but not consistently across the climatic gradient. More specifically, *Aulacomya ater* had Ta levels at Marchant River Mouth that were 4.5 and 2 times lower than the levels found at nearby La Leona Island and the most northerly site Pan de Azúcar Bay, respectively. In contrast, *Fissurella* spp. had lower Ta levels at Pan de Azúcar Bay (0.31 ng g^{-1}) than at Marchant River Mouth (1.4 ng g^{-1}). Finally, *Concholepas concholepas* showed similar Ta levels of 0.26 ng g^{-1} at Pan de Azúcar Bay and 0.38 ng g^{-1} at La Leona Island; similarly, *Genypterus chilensis* had Ta levels of 1.83 and 2.08 ng g^{-1} from La Leona Island and Marchant River Mouth, respectively. Although nothing is known about the dynamics of Ta in organisms and the factors that affect its uptake and storage, these data suggest that site specific factors may be relevant in determining its environmental fate.

It is possible to make only limited comparisons of the Ta levels in marine species from Chile and Antarctica to data from other regions. Ta values in the current study are much lower than those reported in macroinvertebrates from southern England (ranging from 0.1 to 2 ppm dw),[9] and those in the ascidian *Styela plicata* collected off the coast of Japan (between 100 and 410 ppm dw).[8] It was not possible to find other studies on Ta in fishes.

Trophic Transfer of Ta. In general, in all the food webs studied here (Tables S7−S9 and Figure S1), fishes had $\delta^{13}C$ values that were between those of the macroinvertebrates, indicating reliance on both pelagic and benthic energy sources, as observed in temperate lake food webs.[20] Ta levels increased with the TL of the organisms, showing biomagnification of this element (Table S13 and Figure S2). The TMS ranged from 0.05 at Marchant River Mouth in Chilean Patagonia to 0.18 at Fildes Bay in Antarctica (Table 1 and Figure 2). The slopes of log Ta versus TL were significantly different across sites (site * TL, $p < 0.001$) and translated into TMF values of 2.29 in northwestern Chile, 2.00 and 1.45 at the sites in Chilean Patagonia and 3.98 in Antarctica, indicating that Ta does not consistently biomagnify across sites and that the highest trophic transfer of Ta occurred at the coldest latitude. Indeed, the slope for Antarctica was significantly higher than for all other sites and this may be because these marine food webs are simple and clearly defined on the basis of benthic and pelagic populations,

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which are strongly coupled with each other.[21] The Ta biomagnification slopes for the food webs of Pan de Azúcar Bay and La Leona Island fell between those of the Marchant River and Antarctica sites and were not statistically different from one another (p = 0.46). In contrast, Marchant River Mouth had the lowest TMF and a slope that was significantly lower than those at all other sites (p < 0.012). The lower biomagnification of Ta may be the result of the large inputs of nutrients and other elements from the river to the coast[22,23] that in turn affect the bioaccumulation and trophic transfer of this element, as has been observed for Hg.[11] It was not possible to find either laboratory or field studies on Ta trophodynamics for comparison. The TMF values observed for Ta were mostly lower than those reported for TBT (from 3.88 to 4.62).[13] Also, our log Ta versus $\delta^{15}N_{adj}$ slopes were lower than those reported in a global review of total Hg (0.21 for polar, 0.22 for temperate, 0.16 for tropical systems) and MeHg (0.21 for polar, 0.26 for temperate and 0.14 for tropical systems) in marine environments,[11] suggesting that Ta biomagnifies to a lesser extent than for Hg.[11] The exception was for Ta in the Fildes Bay food web, which had a TMS in the same range of polar areas and slightly higher than tropical. Overall, Ta biomagnified regardless of the latitude and composition of the marine food web, which could be a characteristic of this particular element, as with Hg.[11] We recommend Ta biomagnification be examined in other diverse food webs to develop a broader understanding of how ecosystem characteristics affect the fate of this element.

There is no published information on Ta toxicity in aquatic animals. In mammals, Ta_2O_5 inhalation can cause bronchitis and interstitial pneumonitis.[24] So far, there is a general consensus that Ta does not play a biological role[25] but it is unclear whether the biomagnification of Ta observed herein poses a risk to upper-trophic-level consumers. This becomes important considering that the production and use of Ta will likely increase with the growing demand for new technologies and, as such, is an issue that needs more investigation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.8b00051.

Additional text to the material and methods, tables with the concentrations of stable isotopes of C and N, Ta concentrations in the different trophic levels and different regression analysis (PDF)

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Quantifying Differences in Responses of Aquatic Insects to Trace Metal Exposure in Field Studies and Short-Term Stream Mesocosm Experiments

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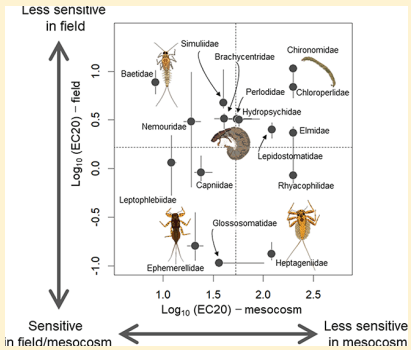
Supporting Information

ABSTRACT: Characterizing macroinvertebrate taxa as either sensitive or tolerant is of critical importance for investigating impacts of anthropogenic stressors in aquatic ecosystems and for inferring causality. However, our understanding of relative sensitivity of aquatic insects to metals in the field and under controlled conditions in the laboratory or mesocosm experiments is limited. In this study, we compared the response of 16 lotic macroinvertebrate families to metals in short-term (10-day) stream mesocosm experiments and in a spatially extensive field study of 154 Colorado streams. Comparisons of field and mesocosm-derived EC_{20} (effect concentration of 20%) values showed that aquatic insects were generally more sensitive to metals in the field. Although the ranked sensitivity to metals was similar for many families, we observed large differences between field and mesocosm responses for some groups (e.g., Baetidae and Heptageniidae). These differences most likely resulted from the inability of short-term experiments to account for factors such as dietary exposure to metals, rapid recolonization in the field, and effects of metals on sensitive life stages. Understanding mechanisms responsible for differences among field, mesocosm, and laboratory approaches would improve our ability to predict contaminant effects and establish ecologically meaningful water-quality criteria.



INTRODUCTION

Characterizing macroinvertebrate taxa as either sensitive or tolerant is of critical importance for investigating impacts of anthropogenic stressors in aquatic ecosystems and for inferring causality.[1−3] Information on the relative sensitivity of species to contaminants and other stressors will improve our ability to conduct biological assessments. Indeed, among approximately 300 biological assessment methods used in the Water Framework Directive monitoring programs,[1] metrics based on relative sensitivity are second only to abundance metrics across different water categories and are most common in stream field studies. More importantly, many ecological risk assessments, such as those that utilize species sensitivity distribution models,[4] assume that species sensitivities observed in the laboratory are consistent with those observed in the field.

Despite the considerable knowledge that has been obtained from field studies,[5−8] our understanding of factors that determine relative sensitivity of aquatic insects to metals in the field and laboratory is relatively poor.[9,10] Results of acute laboratory toxicity tests have shown that aquatic insects are relatively insensitive to metals compared to other freshwater organisms, such as cladocerans and fathead minnows.[11−14] In contrast, field and mesocosm experiments show that some aquatic insects are highly sensitive to metals.[6,9,15]

There are inherent differences between field and laboratory experiments that could account for the differences in responses reported in the literature. For example, differences in the spatial and temporal scale of observations affect exposure. Most laboratory studies are conducted for too short a time interval to account for dietary uptake or unique life history characteristics of aquatic insects, such as the sensitivity of early instars and emerging insects.[9,10,16−19] In contrast, field studies capture natural responses to contaminants, but confounding factors can obscure biological responses, bias results, and limit our ability to identify responses of individual taxa to unique chemical stressors.[20] Additionally, in contrast to laboratory tests and some micro- and mesocosm experiments, field responses reflect properties of open systems (e.g., immigration and migration). For example, a high rate of immigration or recolonization of organisms from uncontaminated sites to contaminated sites may mitigate population and community responses to contaminants.[21]

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The primary goal of this study was to quantitatively assess the response of 16 lotic macroinvertebrate families to metals (Cd, Cu, and Zn) in short-term (10-day) stream mesocosm experiments and a spatially extensive survey of 154 Colorado streams. Sensitivity rankings estimated from 19 individual mesocosm experiments conducted over an 18-year study period were compared to results derived from field data to determine concordance among taxa responses to metals. To our knowledge, this is a first attempt to quantify metal sensitivities of macroinvertebrates using spatially extensive field data and mesocosm experiments.

METHODS

Field Data. A complete description of the study design, benthic macroinvertebrate data, water quality, and quality control/quality assurance procedures were previously published and only briefly described here.[6,15,22] Quantitative benthic macroinvertebrate samples were collected from 154 sites in Colorado during summer base-flow conditions (July to September) from 2003 through 2007. At each location, five replicate benthic samples were collected using a 0.1-m^2 Hess sampler (350-μm mesh net) from shallow riffle areas (<0.5 m). Overlying substrate was scrubbed and disturbed to a depth of approximately 10 cm and the remaining material was washed through a 350-μm mesh sieve. All organisms retained were preserved in 80% ethanol in the field and enumerated in the laboratory. Samples were processed in the laboratory to remove debris and subsampled until 300 organisms (±10%) were removed following methods described by Moulton et al.[23] Invertebrates were identified to the lowest practical taxonomic level (genus or species for most taxa; subfamily for chironomids).[24,25] Means of the five replicate benthic samples were used to calculate the density (number of individuals/0.1 m^2) of taxa at each site. Water quality at field sites was characterized by a range of water hardness (5−163 mg/L $CaCO_3$; i.e., soft to hard water) and dissolved organic carbon (0.3−8.1 mg/L), pH (3.5−8.5; 69% were between 6.5−8.0), and cool temperature (2.8−17.8 °C).[6,15,22] The concentration ranges of dissolved Cd, Cu, and Zn at the field sites were 0.01−7.92, 0.15−934, and 0.25−1940 μg/L, respectively.[6,15,22]

Mesocosm Data. Mesocosm data were obtained primarily (approximately 75%) from published papers[9,26−31] and from Clements and colleagues (unpublished data). All experiments focused on quantifying responses of aquatic insects to individual metals or metal mixtures (Cd, Cu, and Zn). A total of 19 mesocosm experiments were conducted in spring, summer, or fall between 1994 and 2012 at the Colorado State University Stream Research Laboratory (SRL), Fort Collins, CO. The SRL consists of 18 stream mesocosms in a greenhouse that receives natural water directly from a deep, mesotrophic reservoir. Water quality in the mesocosms is typical of mountain streams and was characterized by low water hardness (21−42 mg/L $CaCO_3$) and dissolved organic carbon (2.5−3.0 mg/L), cool temperature (12−16.6 °C), circumneutral pH (6.7−7.8), and low conductivity (57−89 μmhos). The concentration ranges of Cd, Cu, and Zn in mesocosms were <0.1−13.5, 0.5−520, and 3.0−5685 μg/L, respectively. Current in the 20-L mesocosms was provided by paddlewheels that maintain a constant current velocity of 0.45 m/s. Each flow-through stream receives water from a headbox at 1.0 L/min, resulting in a turnover time of approximately 20 min. Natural communities of benthic macroinvertebrates were collected from small to midorder streams using a technique that has been employed for over 25 years to assess ecological responses to a variety of anthropogenic stressors.[9,28,32] Briefly, benthic communities were established on 10 × 10 × 6 cm trays filled with pebble and small cobble substrate placed in the field. Our previous studies have shown that benthic communities colonizing these trays are very similar to those collected from the natural substrate.[31] After 30−40 d of colonization, the trays with their associated communities were removed from the stream, transferred to the SRL, and randomly assigned to metal treatments in stream mesocosms. Treated mesocosms were dosed using peristaltic pumps that dripped in metals from 20-L carboys. At the end of a 10-d exposure period, remaining organisms were removed and preserved in 80% ethanol. All organisms were enumerated in the laboratory and identified to the same level of taxonomic resolution as in the field study.

Concentrations of metals, water hardness, and concentration−response relationships for 16 dominant families of aquatic insects common to both data sets were used in this study. Although the level of taxonomic resolution in mesocosm and field studies was generally to genus or species, data were pooled at the family level to facilitate comparisons between data sets. Although variation in metal sensitivity within aquatic insect families may be important, most of the groups in our study were represented by relatively few genera. Therefore, we believe that comparing ranked sensitivities among families is a valid approach for assessing differences between field and mesocosm responses. The 16 families included representatives from each of the major aquatic insect orders common in Rocky Mountain streams: Ephemeroptera (Baetidae, Ephemerellidae, Heptageniidae, and Leptophlebiidae), Plecoptera (Capniidae/Leuctridae, Chloroperlidae, Nemouridae, and Perlodidae), Trichoptera (Brachycentridae, Glossosomatidae, Hydropsychidae, Lepidostomatidae, Rhyacophilidae), Diptera (Chironomidae and Simuliidae), and Coleoptera (Elmidae).

Statistical Analysis—Sensitivity Ranking Based on EC_{20}. For each field and mesocosm data set, we determined sensitivity rankings for 16 families of aquatic insects based on an effect concentration of 20% (EC_{20}) estimated from concentration−response relationships. Because many of the mesocosm experiments used metal mixtures and because field sites were often contaminated by several metals, we used the cumulative criterion unit (CCU) as an indicator of metal exposure. The CCU was calculated as

$$\mathrm{CCU} = \sum \frac{M_i}{C_i}$$

where M_i and C_i are total or dissolved concentration of metal i and the hardness-adjusted criterion[33] for metal i, respectively. Although CCU has been applied in many studies,[5,9,34] it does not account for several factors (major cations, dissolved organic carbon, and pH) known to influence metal bioavailability.[6,35−37] Because these modifying factors were not routinely measured in the mesocosm experiments, we were unable to include them in the analyses. However, the use of the chronic criterion accumulation ratio (CCAR[6]), which accounts for factors that modify metal bioavailability, did not largely change the sensitivity rankings based on EC_{20} values estimated from field data ($r = 0.92$, see Figures S1 and S2 and the Discussion section for the detailed results). Because routine water-quality parameters in stream mesocosms showed little variation among experiments or treatments, it is unlikely that differences in metal bioavailability among experiments would affect the relative sensitivity rankings for the mesocosm results. The ranges of CCUs were 0.05−130 and 0.56−186 for field and mesocosm data sets, respectively.

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Quantile regression models were used to evaluate concentration−response relationships between CCU and family level abundance at higher quantiles.[38] Estimating changes in higher quantiles allow us to infer how metals limit the ecological potential of streams.[20,38] In the present analysis, we chose to model the 90th quantile of the data (i.e., $\tau = 0.90$), but the sensitivity ranking did not substantially change when the 80th quantile was modeled (see Figure S3). For each regression analysis, four quantile regression models were applied to the data: null or intercept only model ($y = a$), linear ($y = a + b \times \mathrm{CCU}$), and two piecewise models. These models included a traditional piecewise model:

$$y = a + b_1 \times \mathrm{CCU}\ (\text{if CCU} \le c),\ (a - b_2 \times c) + (b_1 + b_2) \times \mathrm{CCU}\ (\text{if CCU} > c)$$

where the slopes before and after the knot (i.e., breakpoint; c in the equation) were freely estimated (but b_1 is assumed to be negative; see the reason below), and a piecewise model that assumed the slope of the first segment (b_1) to be zero (hereafter called as threshold model):

$$y = a\ (\text{if CCU} \le c),\ a + b \times \mathrm{CCU}(\text{if CCU} > c)$$

The first segment was assumed to be negative or zero in these models because the inherent variability in the data made it difficult to estimate more complicated responses (e.g., a hump-shaped relationship) and because our focus was to obtain EC_{20} values relative to maximum abundances at reference conditions. We then selected the best model with the smallest value of Akaike's information criterion among the four models. Akaike's information criterion (AIC) measures the relative quality of a model among a group of models for a given data set.[39] Thus, AIC is a measure of which model best describes the data over other models, and thus can be used for model selection.

Log-transformed values of CCU were used for the analysis of field data,[6] but not for the analysis of mesocosm data. When log-transformed values of CCU were used for the mesocosm data analysis, null models were selected for most insect families (changes observed at higher CCUs were not apparent on a log scale). Thus, we chose to use different scales for modeling responses of insect families in field and mesocosm experiments. This is a reasonable approach for comparing relative sensitivities within the data sets, but requires caution when comparing absolute estimates (i.e., EC_{20}) between data sets.

For each insect family, an EC_{20} (i.e., a CCU value where a 20% reduction is predicted) was calculated using the best model determined by AIC model selection. EC_{20} values were determined by calculating a 20% change in the 90th quantile of abundance from a reference value. Reference values were abundances predicted at the minimum CCU values observed in the field study (CCU = 0.05) or in the mesocosm experiments (0.56). If any best model (null or linear model) indicated no change or an increase in abundance with increasing CCU, we operationally assigned a high CCU value of 200 for the corresponding EC_{20}. To evaluate the uncertainty in our EC_{20} estimate, we used a nonparametric bootstrap method.[40] We generated a *xy*-pairs bootstrap data set with replacement, reparameterized the best model selected in the original data set, estimated the corresponding EC_{20}, and repeated this procedure 1000 times to obtain the median and confidence interval of the EC_{20}. We also estimated EC_{10} and EC_{50} values and verified that these estimates were highly correlated with the EC_{20} values in field and mesocosm data ($r = 0.93$−99; data not shown), indicating that the choice of EC_x had no substantial influence on taxa sensitivity rankings.

All statistical analyses were performed using *R*, version 3.2.3.[41] The parametrization of null and linear quantile regression models was performed using the function "rq" in the R package "quantreg" (version 5.19). For the two piecewise models, the R code available in Iwasaki and Ormerod[42] was used because of the difficulty in the parametrization.

RESULTS

Despite considerable variation in field and mesocosm data, abundances of most dominant families significantly decreased with increasing metal concentrations (Figure 1). On the basis of the quantile regression analysis of field data, the threshold model was selected as the best model for most insect families (Figure 1a and Table S1). In contrast, the best models for the mesocosm experiments were more diverse (Figure 1b and Table S2). The null (intercept only) model was chosen for Chloroperlidae, Rhyacophilidae, Chironomidae, and Elmidae.

The mean of $\log_{10}$-transformed EC_{20} values calculated across all the families were about 1.5 times greater in mesocosm experiments than in the field study (Figure 2 and Table S3). The ratios of mesocosm to field-based EC_{20} values for each family ranged from 1.09 (Baetidae) to 927 (Heptageniidae), and 81% of these ratios were >10. However, as noted above, direct comparison of absolute EC_{20} values estimated from field and mesocosm data should be made with caution. The Pearson's correlation coefficient between field and mesocosm-derived EC_{20} values was low ($r = 0.112$), primarily because some families showed very different relative sensitivities between field and mesocosm experiments. For example, Baetidae was the most sensitive to metals in mesocosm experiments but less sensitive in the field study. In contrast, Heptageniidae was one of the most sensitive families in field study, but less sensitive in mesocosm experiments. Despite these differences, ranked sensitivities were consistent for many families showing relatively higher (e.g., Ephemerellidae), intermediate (e.g., Capniidae, Lepidostomatidae, and Hydropsychidae), and lower sensitivities (e.g., Chironomidae and Chloroperlidae) in the field study and mesocosm experiments. Indeed, if EC_{20} values for Baetidae and Hepetageniidae were excluded, the correlation coefficient increases to 0.44.

DISCUSSION

Comparison of EC_{20} values estimated field and mesocosm data. As expected, comparisons of field and mesocosm-derived EC_{20} values showed that aquatic insects were generally more sensitive to metals in the field. This finding is consistent with previous studies that compared responses from field studies and laboratory toxicity tests.[9,14] Although our study cannot identify the specific mechanisms that were responsible for these differences, they are most likely associated with (1) the relatively short duration (10 d) of mesocosm experiments; (2) difficulty accounting for dietary exposure to metals;[10] (3) failure to consider sensitive life history stages such as early instars and emerging adults;[9,16,17] and (4) underestimation of CCUs in field studies where metals other than Cd, Cu, and Zn might have contributed to the overall impacts. Regarding the short exposure duration, an encouraging finding from a recent study is that responses of aggregated community metrics, such as taxon richness and mayfly abundance, responded similarly in 30-d mesocosm experiments and natural streams.[43]

Comparing Relative Sensitivity in Field and Mesocosm Data. The most significant finding of this study was the difference in relative sensitivity to metals among some invertebrate

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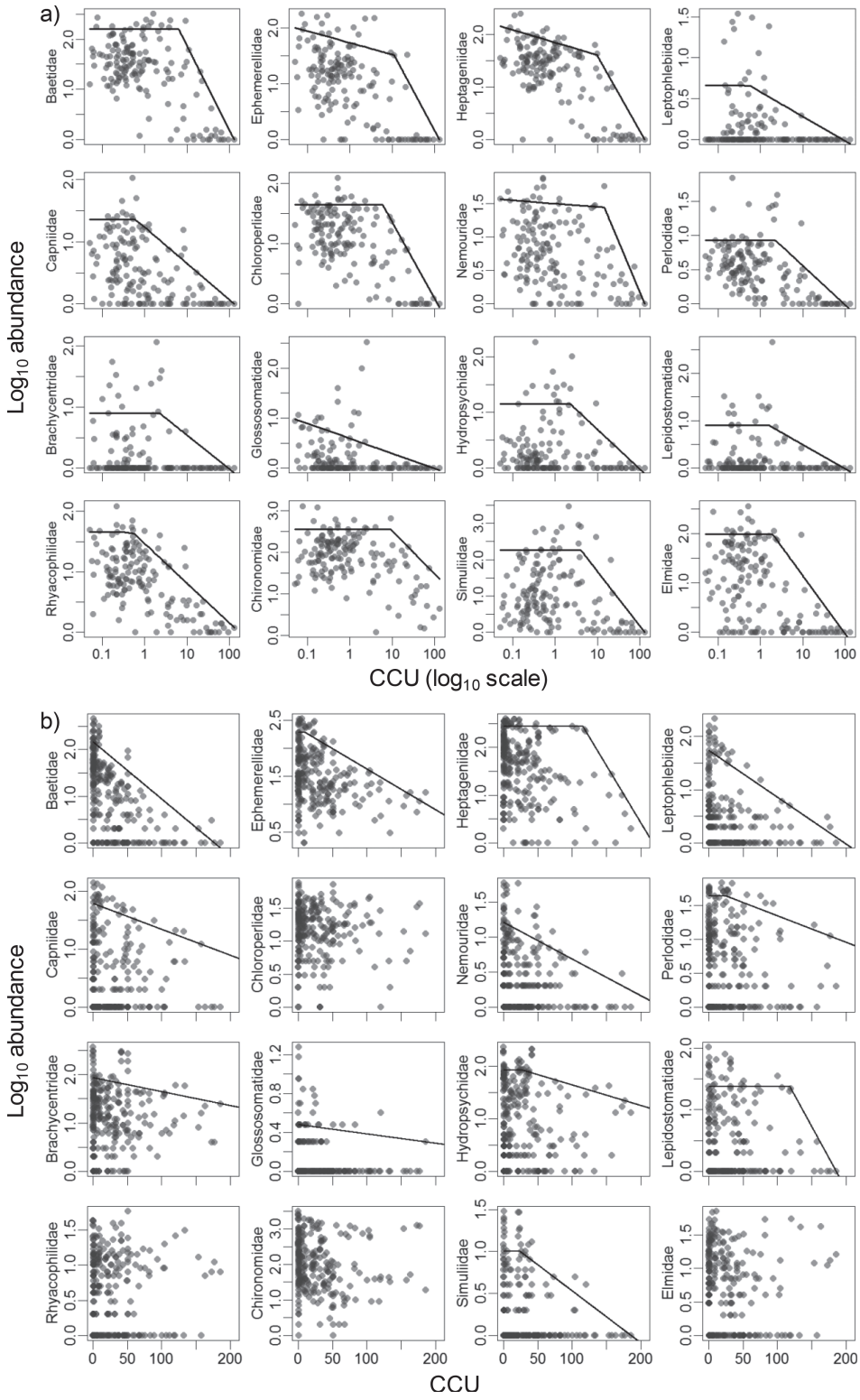

Figure 1. Responses of 16 aquatic insect families to metals in (a) a field study and (b) mesocosm experiments. The solid lines indicate the best model selected for a quantile of 0.90 (no solid line indicates that the null model was selected as best). Metals were expressed as the cumulative criterion unit (CCU), defined as the sum of the measured concentration for each metal divided by the U.S. EPA hardness-adjusted criterion value.[33] Darker points indicate multiple data points at the same position.

families estimated from field and mesocosm data. Previous field studies have shown that among mayflies, baetids are relatively tolerant to metals, whereas heptageniids are considered highly sensitive.[5,7,44−46] Although this trend was observed in the field study, results of mesocosm experiments showed that Baetidae was highly sensitive to metals, whereas Heptageniidae was less

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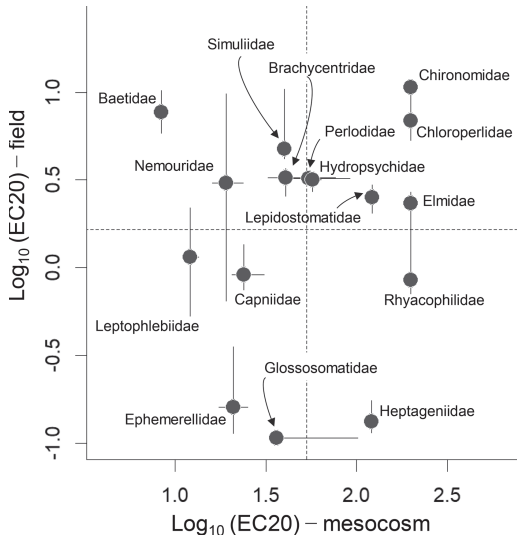

Figure 2. Relationship between effect concentrations of 20% (EC_{20} values) for 16 insect families estimated from field and mesocosm data. The Pearson's correlation coefficient between those log_{10}-transformed EC_{20} values was 0.11, but it was increased to 0.44 if the EC_{20} values for Baetidae and Heptageniidae were excluded (see the text for the details). Error bars indicate 50% confidence intervals estimated by bootstrapping. The dotted lines indicate mean values of log_{10}-tranformed EC_{20} values from field and mesocosm data.

sensitive than expected from field results. Given that mesocosm experiments and field studies measure responses under very different conditions, we should not be surprised to see differences in relative sensitivity among macroinvertebrate groups. In contrast to field studies, mesocosm and laboratory experiments isolate effects of contaminants and provide a direct measure of toxicity primarily associated with aqueous exposure. Mesocosm experiments conducted with natural communities are therefore especially useful for quantifying differences in sensitivity among taxa. In contrast, the distribution and abundance of these same taxa in the field are determined by numerous factors in addition to contaminant concentrations, including rates of immigration, emigration, and emergence; species interactions; and habitat requirements. Defining tolerance or sensitivity of species to contaminants based exclusively on presence or absence in the field without some experimental evidence to support these conclusions may be inappropriate. In fact, quantifying differences in relative sensitivity and developing a better understanding of mechanisms responsible for this variation may provide important insights into the more general question of why we often see such vast differences in responses between laboratory and field studies reported in the literature.[9,11,43]

Multiple factors are likely responsible for the differences we observed in relative sensitivity among aquatic insect families to metals between the mesocosm experiments and the field study. For example, baetid mayflies are widely recognized for their high propensity to drift[47,48] and, despite their sensitivity to dissolved metals, are capable of rapid recolonization in metal-contaminated streams.[49,50] We hypothesize that the apparent tolerance of Baetidae to metals reported in many field studies may be partially explained by their high rate of drift and rapid colonization ability. While invertebrate drift plays an important role in determining the distribution of stream invertebrates,[51] it has received less attention in ecotoxicology (but see[52]). Conversely, the greater tolerance of heptageniid mayflies observed in the mesocosm experiments compared to the field may have resulted from the short duration of these experiments and the limited effects of metal exposure on biofilms. As grazing mayflies, heptageniids in contaminated streams are exposed to very high concentrations of metals associated with periphyton.[34,53] This dietary exposure may be less important in short-term mesocosm experiments as compared to field situations for a number of reasons. First, the short duration of exposure might limit accumulation of metals in biofilm (but see Bradac et al.[54]) and thus reduce dietary exposure, considered a major source of metal accumulation for grazing aquatic insects.[10,55] Second, it is likely that indirect effects of metals exposure on grazing aquatic insects via alterations in the quality of periphyton would be greater in the field than in short-term mesocosm experiments.[56] Thus, the greater sensitivity of heptageniids in the field may result from these effects and other stressors associated with metal-polluted streams. There is evidence from field experiments that heptageniids are more sensitive to metal-contaminated substrate compared to other taxa.[49]

Differences in sensitivity among life stages of aquatic insects may account for some of the variation between field and mesocosm experiments. Because aquatic insects in nature are exposed to metals across all size classes and life stages, we would expect greater sensitivity in the field. Although mesocosm experiments included a range of size classes and provided more realistic exposure conditions compared to laboratory experiments, they did not duplicate all aspects of field conditions. For example, failure to account for effects of metals on emergence, a critical life stage for many aquatic insects, may explain the greater tolerance observed in the mesocosm experiments. Recent studies have suggested that emerging mayflies are more sensitive to metals than larvae.[17] If the effects of metals on adult emergence differed among taxonomic groups, this could explain some of the variation between mesocosm and field results. Finally, the use of CCU as a measure of metal exposure might have led to differences in responses of aquatic insects to different mixtures of metals. Clements et al.[9] demonstrated that Cu alone had significantly greater effects on abundance and richness of aquatic insects than a mixture of Cu and Zn, even though the ranges of CCU were similar.

Despite the lack of agreement between field and mesocosm data for some groups, many families exhibited similar relative sensitivities. Chironomids were estimated to be least sensitive in both the field study and mesocosm experiments, and this result is generally consistent with previous empirical studies.[57] In contrast, Ephemerellidae and Glossosomatidae were found to be relatively sensitive in the field study and mesocosm experiments. The vulnerability of ephemerellid mayflies has been reported previously in field[5,50,58] and mesocosm studies,[43] but there is limited information for Glossosomatidae.[50]

Implications for Ecological Risk Assessments. Ecological risk assessments and the establishment of water-quality criteria are based largely on results of laboratory toxicity tests. Although we recognize that laboratory, mesocosm, and field studies are designed to characterize different aspects of concentration–response relationships, there are implicit assumptions in each approach. Specifically, we assume that responses observed in the laboratory are representative of those in the field and that variation in sensitivity among taxa is similar in the field and in the laboratory. This assumption forms the basis of the species sensitivity distribution approach[4,12,13] which uses results from laboratory toxicity tests (e.g., no observed effect concentration) to infer likely field impacts. Our findings suggest that there may not be a direct 1:1 relationship between responses to

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contaminants observed in the field and in the laboratory for some aquatic insects.

We believe that discrepancies among field studies, mesocosm studies, and laboratory experiments represent an opportunity to identify mechanisms driving these differences that could improve our ability to predict the effects of metals on ecosystems. Field studies of contaminated sites provide the greatest degree of ecological realism, but results are often confounded by factors other than the specific chemical(s) of concern. In contrast, laboratory and mesocosm experiments provide the greatest control over potential confounding variables, but are generally conducted at insufficient spatiotemporal scales to characterize longer-term exposure and include critical life stages of aquatic insects (but see ref 43). A more systematic approach that integrates results of field, mesocosm, and laboratory studies and helps identify mechanisms that are responsible for the discrepancies among these approaches will improve our ability to predict contaminant effects on aquatic communities and is a critical research need.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b06628.

Detailed results on model selection, estimated EC_{20} values, and comparison of ranked sensitivities (PDF)

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Notes

Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

The authors declare no competing financial interest.

Field data with U.S. Geological Survey funding are available here https://pubs.usgs.gov/of/2007/1044/. While much of the mesocosm results were previously published,[9,26−31] interested parties should contact William H. Clements for the mesocosm data. Unpublished mesocosm data are not otherwise available at time of publication because they are planned to be published to address the different goals of other projects.

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Effects of silver nanocolloids on plant complex type *N*-glycans in *Oryza sativa* roots

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Silver nanomaterials have been mainly developed as antibacterial healthcare products worldwide, because of their antibacterial activity. However, there is little data regarding the potential risks and effects of large amounts of silver nanomaterials on plants. In contrast, *N*-glycans play important roles in various biological phenomena, and their structures and expressions are sensitive to ambient environmental changes. Therefore, to assesse the effects of silver nanomaterials, we focused on the correlation between *N*-glycans and the effects of silver nanomaterials in plants and analyzed *N*-glycan structures in *Oryza sativa* seedlings exposed to silver nanocolloids (SNCs). The phenotype analysis showed that the shoot was not affected by any SNC concentrations, whereas the high SNC exposed root was seriously damaged. Therefore, we performed comparative *N*-glycan analysis of roots. As a result, five of total *N*-glycans were significantly increased in SNC exposed roots, of which one was a free-*N*-glycan with one beta-*N*-acetylglucosamine residue at the reducing end. Our results suggest that the transition of plant complex type *N*-glycans, including free-*N*-glycans, was caused by abnormalities in *O. sativa* development, and free-*N*-glycan itself has an important role in plant development. This study originally adapted glycome transition analysis to environmental toxicology and proposed a new category called "Environmental glycobiology".

Nanomaterial is a general term for small substances that are 1–100 nm in diameter. Nanomaterials have many unique electrical, chemical, and physical properties and are used in electronics, medicine, and healthcare fields. Silver nanomaterials have been developed and mainly used for their antibacterial activities in clothes, appliances, cosmetics, and plastics. However, there have been concerns that silver nanomaterials are likely released into the aquatic environment through factory and household wastewater on a large scale, and there are also concerns regarding the effects of silver nanomaterials on aquatic organisms and ecological systems. The toxicity of silver nanomaterials in the embryos of aquatic organisms such as medaka and zebrafish has been reported[1,2]. The toxicity affected the expression of morphogenesis- and cell proliferation-related genes and induced the increase of severe development abnormalities and mortality. The toxicity of silver nanomaterials was dependent on the particle size, shape, and capping materials[3,4]. Toxicity of silver nanomaterials has also been reported in plants, with the toxicity affecting germination, development, and photosynthetic efficiency because of the induction of oxidative stress, cytotoxicity, and genotoxicity[5,6]. For example, silver nanoparticle exposure significantly reduced root elongation, shoot and root fresh weights, and total chlorophyll and carotenoid contents[7]. Colman *et al.* showed that low silver nanoparticle concentrations caused a decrease in biomass[8]. Furthermore, the toxicity of silver nanoparticles affects the expression of several proteins that are mainly involved in primary metabolism and cell defense in wheat seedlings[9]. At a gene level, silver nanoparticles activate gene expression involved in plant cellular events, including cell proliferation, metabolism, and hormone signaling pathways[10]. The above-mentioned studies showed that silver nanomaterials have high toxicity in plants. Therefore, the risk assessment of silver nanomaterials in plants is important.

Asparagine (N)-linked glycans (*N*-glycans) are comprised several types of monosaccharides, forming complex compositions and linkage types. *N*-Glycan has a trimannosyl core structure [Man alpha1–6(Man alpha1-3) Man beta1-4GlcNAc beta1-4GlcNAc-Asn], which is a common feature in eukaryotes. Many *N*-glycan structures are linked to proteins or peptides and are closely involved in all life phenomena, such as development, signaling,

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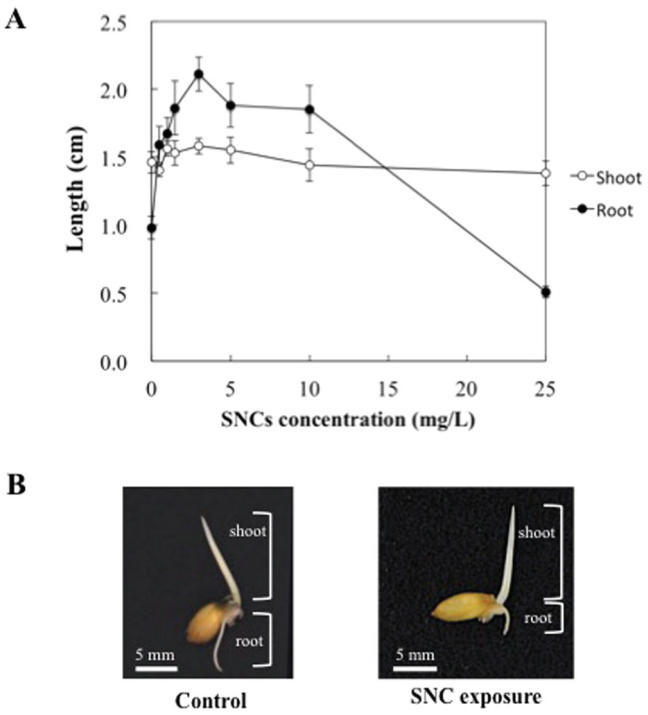

Figure 1. Phenotype analysis of *O. sativa* seedling exposed by SNCs. (**A**) Length of shoots and roots, opened circles indicated shoot and closed circles indicated root. Error bars represent ± one standard deviation from the mean of 20 replicates. (**B**) Overall phenotypes of *O. sativa* seedling.

and cell-to-cell recognition. In plants, *N*-glycan structures are categorized into three main types: high-mannose, complex, and paucimannose types; except for hybrid types. A characteristic of plant-specific *N*-glycans is the addition of beta1,2-xylose and alpha1,3-fucose to the trimannosyl core structure. High-mannose type *N*-glycans are synthesized in endoplasmic reticulum (ER), and other type *N*-glycans are synthesized in the Golgi apparatus. Paucimannose type *N*-glycans are linked to vacuole proteins and complex type *N*-glycans are linked to secretory proteins. In addition, cell alterations are reflected in gene expressions through cell signaling, whereas *N*-glycan is synthesized as a result of the integral expression of glycosyltransferase genes, and *N*-glycan structure is sensitive to slight environmental changes[11]. Therefore, *N*-glycan structural analysis is valuable for the risk assessment of silver nanomaterial toxicity in plants. However, there is little data regarding the toxicity of silver nanomaterials in glycobiology. In this study, to assess the effects of silver nanomaterials, we focused on the correlation of *N*-glycan structures and the effect of silver nanomaterials in *Oryza sativa* and analyzed the *N*-glycan structures in SNC exposed *O. sativa* seedlings.

Results and Discussion

Phenotype analysis of *O. sativa* seedling exposed to silver nanocolloids (SNCs). To observe the effect of SNCs on *O. sativa* seedlings, *O. sativa* seeds were grown with and without SNC exposure. Germination rate was 95% (control), 100% (SNCs 0.5 mg/L), 100% (SNCs 1.0 mg/L), 95% (SNCs 1.5 mg/L), 100% (SNCs 3.0 mg/L), 95% (SNCs 5.0 mg/L), 90% (SNCs 10 mg/L), 100% (SNCs 25 mg/L) after 48 h incubation. The result shows that there is no effect on germination rate at any concentration of SNC for 48 h exposure in *O. sativa*. Figure 1A shows the results of root and shoot elongation in *O. sativa* exposed to SNCs at 0 (control), 0.5, 1.0, 1.5, 3.0, 5.0, 10, and 25 mg/L for 96 h. Shoot and root length of control was 1.46 ± 0.08 cm and 0.98 ± 0.08 cm, respectively. The shoot elongation was not affected at any SNC concentration, whereas the root length increased from 0.5 to 10 mg/L based on Fig. 1A; however, in roots exposed to 25 mg/L SNCs, the lengths were two times lower than those of the control. Representative images of control and 25 mg/L SNC exposed *O. sativa* seedlings are shown in Fig. 1B. From the phenotype analysis, root length was seriously affected by 25 mg/L of SNC exposure.

The effect of SNCs was also observed in other plants. SNCs also have a significant effect on *Arabidopsis thaliana* and poplar development[12]. SNCs are mainly present in two forms: silver nanoparticles and free ions (Ag^+), which are derived from silver nanoparticles. Free Ag^+ is more poisonous than SNCs because of its oxidative potency. Wang *et al.*[12] demonstrated that free Ag^+ tends to accumulate in *A. thaliana* roots. Previous studies also showed that silver nanoparticles or free Ag^+ inhibited the growth of *O. sativa* roots[13], and these materials affect cell metabolism-related proteins[14]. In addition, the effects of SNCs or free Ag^+ occur in ER- and vacuole-localized proteins of *Eruca sativa*[15]. Nair *et al.* reported that total sugar levels are decreased in SNC exposed *O. sativa* seedlings[7]. From these reports and our observation, SNCs and SNC derived molecules may affect *N*-glycans that are synthesized in the ER and Golgi apparatus of *O. sativa* roots. Therefore, we focused on the correlation of *N*-glycan structures and silver nanomaterials in *O. sativa* and analyzed the *N*-glycan structures in SNC exposed *O. sativa* roots.

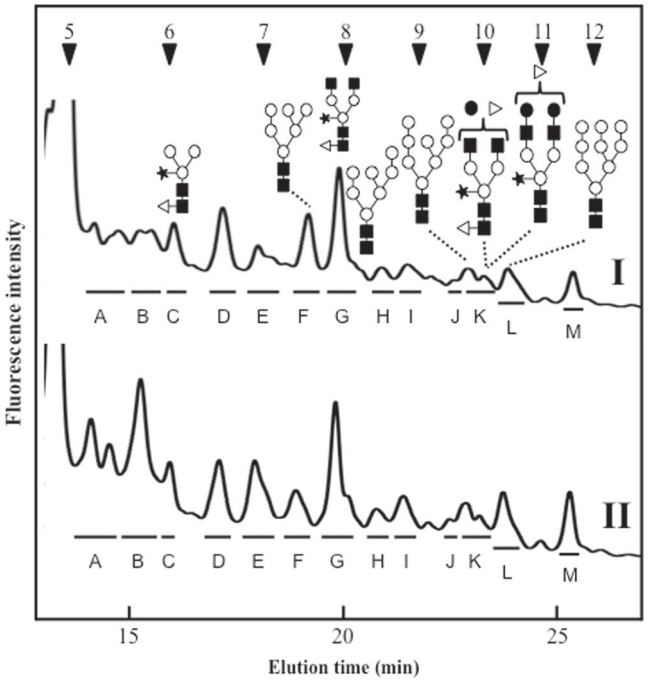

Figure 2. Results of size-fractionation HPLC analysis of PA-*N*-glycans derived from *O. sativa* seedlings. (I) Control, (II) SNCs exposure, PA-*N*-glycans were applied to a Cosmosil $5NH_2$-MS column (4.6 ID × 150 mm). Arrowheads 5–12 indicate the degree of polymerization of PA-isomaltooligomer. The opened circle, closed square, opened triangle, closed star, closed circle represent mannose, *N*-acetylglucosamine, fucose, xylose, and galactose residues, respectively.

***N*-Glycan analysis of SNC exposed *O. sativa* roots.** *N*-Glycans were prepared by hydrazinolysis, *N*-acetylation, and pyridylamination (PA). The resulting PA-*N*-glycans were separated according to their degree of saccharide polymerization by size-fractionation HPLC. Then, *N*-glycans of control and 25 mg/L of SNC exposed roots were compared (Fig. 2I, control and II, SNC treatments). Thirteen peaks were detected (indicated by bars). The areas of peaks B, E, and M increased in SNC exposed roots, and in particular, peak B was extremely increased in SNC exposed roots. Therefore, to identify each *N*-glycan structure in detail, reversed phase HPLC was performed, and branched *N*-glycan isomers were separated. Reversed phase HPLC analysis revealed three major peaks (peaks E1, E2, and E3) of peak E (Fig. 3). Comparing the HPLC elution times with known-position *N*-glycans, peaks E1, E2, and E3 coincided with those of ^{GN}M3FX, $_{GN}$M3FX, and GN2M3X, respectively. Similarly, one major peak, M1, was detected in reversed phase HPLC analysis, and peak M1 coincided with the elution position of Gal2F2GN2M3FX.

Table 1 shows the identified *N*-glycan structures and ratios derived from peaks E1, E2, E3, and M1, and each *N*-glycan is shown in terms of percentage proportion relative to GN2M3FX (peak G). The largest *N*-glycan Gal2F2GN2M3FX was increased three fold after SNC exposure. For other plant complex type *N*-glycans, ^{GN}M3FX, $_{GN}$M3FX, and GN2M3X, SNC exposure caused up to five- or six-fold higher accumulations than the control. In general, complex type *N*-glycans are formed in from the *cis*-Golgi to *medial* Golgi apparatus, and higher complex modifications occur downstream in the synthetic pathway. Recent reports showed that protein-linked complex type *N*-glycans are related to proper targeting and functioning of linking proteins[16,17] and also showed that complex type *N*-glycans play an important role in resistance to external stresses such as salt stress[11,18,19]. These reports showed that the Golgi-localized *N*-glycan synthetic enzymes are related to plant growth and development, and their defect inhibited growth and caused abnormalities. Therefore, the transition of complex type *N*-glycans may be related to the disorder of Golgi-localized *N*-glycan synthetic enzymes and genes in SNC exposed roots. The relatively small *N*-glycans ^{GN}M3FX, $_{GN}$M3FX, and GN2M3X were affected by SNCs more significantly. These results imply that the upstream part of the *N*-glycan complex pathway was affected by SNCs; therefore, the downstream part of the synthetic pathway was less affected for more complicated *N*-glycans such as Gal2F2GN2M3FX. These results may provide evidence that the intermediate complex type *N*-glycans play an important role in plants under excessive stress conditions.

Free-*N*-glycan analysis of SNC exposed *O. sativa* roots. Reversed phase HPLC analysis revealed that peak B1 were eluted at around 3 min; therefore, to purify peak B1, twice reversed phase HPLC was performed (Fig. 4A). Peak B1 was further analyzed using MALDI-TOF mass spectrometry and sequential enzyme digestion. Mass spectrometry analysis of peak B1 showed that the *m/z* ratio was 1143.90 (Na^+), which corresponded to $(Hex)_3(HexNAc)_2(Pent)_1$-PA. The *m/z* ratio and elution position on reversed phase HPLC revealed that the *N*-glycan structure of peak B1 was predicted to be a free-GNM3X structure [$GlcNAc_1Man_3Xyl_1GlcNAc_1$-PA] with one GlcNAc residue at the reducing end. To ascertain the *N*-glycan structure of peak B1, peak B1 was enzymatically digested with two exoglycosidases. Peak B1 [$GlcNAc_1Man_3Xyl_1GlcNAc_1$-PA] was converted

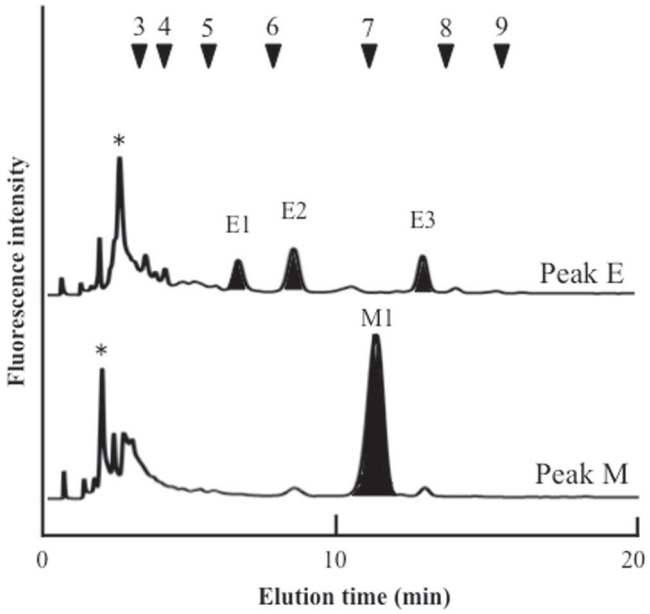

Figure 3. Result of reversed phase HPLC analysis of peaks E and M. The closed areas indicated elution positions of known-*N*-glycan. The peaks marked by the asterisks indicated non-specific peaks.

Peak	Structure	Abbreviation	Ratio	
			Control	SNCs exposure
E1	GlcNAcβ1-2Manα1→6(Manα1→3)Manβ1-4GlcNAcβ1-4GlcNAc-PA; Xylβ1 on 2; Fucα1 on 3	^{GN}M3FX	3 (0.07)	15 (0.02)
E2	Manα1→6(GlcNAcβ1-2Manα1→3)Manβ1-4GlcNAcβ1-4GlcNAc-PA; Xylβ1 on 2; Fucα1 on 3	$_{GN}$M3FX	5 (0.06)	24 (0.01)
E3	GlcNAcβ1-2Manα1→6(GlcNAcβ1-2Manα1→3)Manβ1-4GlcNAcβ1-4GlcNAc-PA; Xylβ1 on 2	GN2M3X	3 (0.04)	18 (0.01)
M1	Fucα1 on 4; Galβ1-3GlcNAcβ1-2Manα1→6(Galβ1-3GlcNAcβ1-2Manα1→3)Manβ1-4GlcNAcβ1-4GlcNAc-PA; Fucα1 on 4; Xylβ1 on 2; Fucα1 on 3	Gal2F2GN2M3FX	18 (0.02)	60 (0.00)

Table 1. Estimated *N*-glycan structures obtained from peaks E1, E2, E3, and M1. Standard errors are in parentheses. Each *N*-glycan was also expressed in terms of percentage proportion relative to the GN2M3FX structure.

to $Man_3Xyl_1GlcNAc_1$-PA, releasing one GlcNAc residue by beta-*N*-acetylhexosaminidase (Fig. 4B-II), and $Man_3Xyl_1GlcNAc_1$-PA was further converted to $Man_1Xyl_1GlcNAc_1$-PA, releasing two mannose residues by alpha-mannosidase (Fig. 4B-III). Thus, peak B1 was assigned as free-GNM3X structure with one GlcNAc residue at the reducing end (Fig. 4B-I).

Peak G is a major component of *O. sativa* roots (Fig. 2). As a result of reversed phase HPLC, two major peaks were detected; as a result of *N*-glycan two-dimensional mapping, the retention time of peak G2 corresponded to that of known position *N*-glycan, GN2M3FX[20], and the other one was eluted at around 3 min (Fig. 5A, peak G1). In a similar way to peak B1, peak G1 was analyzed using MALDI-TOF mass spectrometry and exoglycosidase digestion. Mass spectrometry analysis showed that the *m/z* ratio was 1456.67 (Na^+), which corresponded to $(Hex)_7$ $(HexNAc)_1$-PA. The mass value and elution position of reversed phase HPLC revealed that peak G1 was predicted to be a free-M7 structure [$Man_7GlcNAc_1$-PA]. To confirm the *N*-glycan structure, peak G1 was enzymatically digested with alpha-mannosidase. As a result, peak G1 [$Man_7GlcNAc_1$-PA] was converted to $Man_4GlcNAc_1$-PA, $Man_3GlcNAc_1$-PA, $Man_2GlcNAc_1$-PA, and $Man_1GlcNAc_1$-PA, releasing from three to six mannose residues by alpha-mannosidase (Fig. 5B-II); therefore, peak G1 was assigned as free-M7 structure.

As shown in Table 2, the proportion of free-GNM3X (peak B1) increased six fold after SNC exposure. In contrast to free-GNM3X, free-M7 increased three fold after SNC exposure (Table 2). To date, high-mannose type free-*N*-glycans have been detected in plant during development[21,22], and other complex type free-*N*-glycans have been detected in the culture broth of rice cultured cells[23] and *Egeria densa*[24], and most of them have Lewis a structure [Gal beta1-3(Fuc alpha1-4)GlcNAc beta1-] that is characteristic of the *N*-glycan of

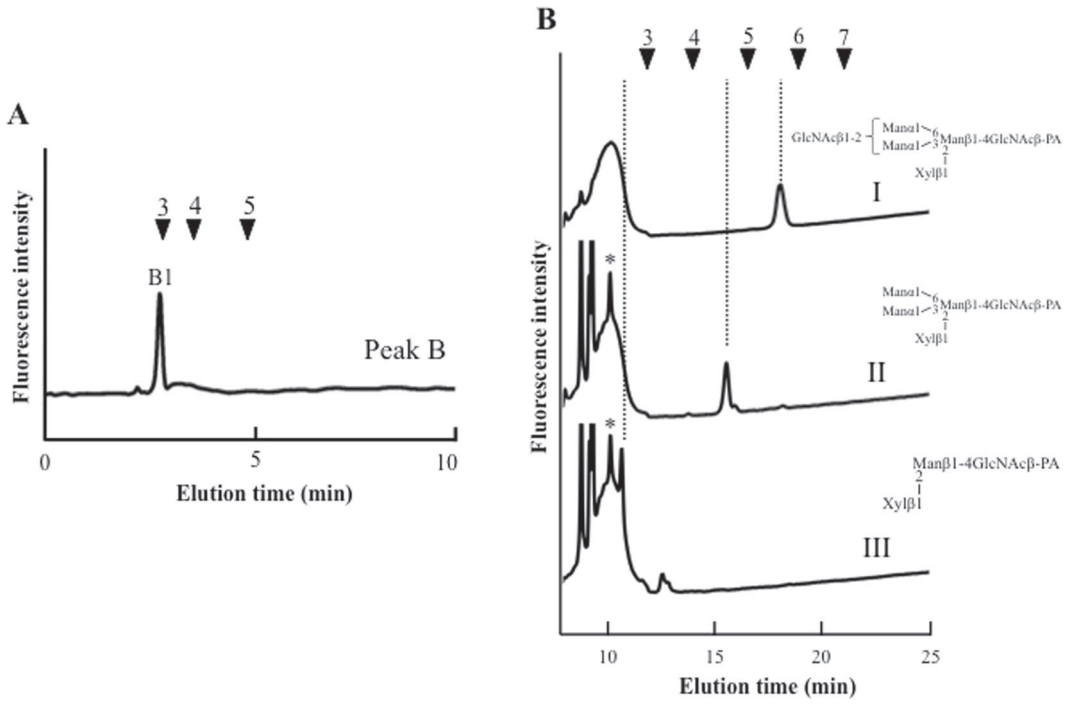

Figure 4. Structural analysis of peak B1. (**A**) Result of second reversed phase HPLC analysis of peak B1. (**B**) Sequential enzyme digestions of peak B1, I: peak B1, II: beta-*N*-acetylhexosaminidase digestion of I, III: alpha-mannosidase digestion of II. The peaks marked by the asterisks indicated non-specific peaks.

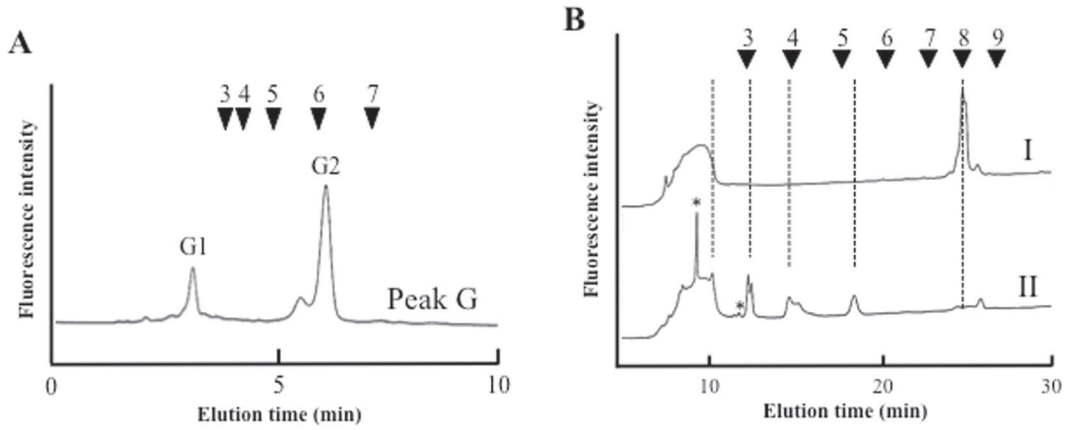

Figure 5. Structural analysis of peak G1. (**A**) Result of reversed phase HPLC analysis of peak G. (**B**) Exoglycosidase digestion of peak G1, I: peak G1, II: alpha-mannosidase digestion of I. The peaks marked by the asterisks indicated non-specific peaks.

extracellular glycoproteins. Maeda *et al.* discussed that a mechanism responsible for the production of complex type free-*N*-glycans is present under special or artificial conditions and native plant tissues[24]. In animal cells, the accumulation of sialyl free-*N*-glycans is caused by a decline in free-*N*-glycan metabolism by basal autophagy[25]. Mkhikian *et al.* demonstrated that alternative *N*-glycan structures were generated under unusual growth conditions[26]. These observations suggest that the occurrence of specific *N*-glycan structures is involved in cell conditions under excessive stress conditions.

The elongation of *O. sativa* shoots was unaffected by SNC exposure (Fig. 1A), and *N*-glycan structures were also unaffected. We suggested that a root defense mechanism serves to protect shoot development from SNC toxicity, and *O. sativa* roots may have a defense mechanism against soil environmental changes. Although the generation of high-mannose type free-*N*-glycans is generally caused by the deglycosylation from misfolded glycoproteins in ER-associated degradation system[27], there is almost no information about the biological significance of complex type free-*N*-glycan. Our results showed that the increase of complex type free-*N*-glycans was caused by the effect of SNCs, suggesting that complex type free-*N*-glycan itself in *O. sativa* roots has an important role for resistance mechanism against excessive environmental changes. *N*-Glycan is one of the post-translational

Peak	Structure	Abbreviation	Ratio	
			Control	SNCs exposure
B1	GlcNAcβ1-2 (Manα1-6 / Manα1-3) Manβ1-4GlcNAcβ-PA; Xylβ1-2	Free-GNM3X	9 (0.00)	58 (0.00)
G1	Manα1-2 Manα1-2 (Manα1-6 / Manα1-3) Manα1-6 / Manα1-3 Manβ1-4GlcNAc-PA	Free-M7	16 (0.02)	45 (0.06)

Table 2. Proposed free-*N*-glycan structures. Standard errors are in parentheses.

modifications, and the structure and quantity are sensitive to ambient environment. In addition, *N*-glycans are generated by the result of the integrated multiple gene expression, and the biosynthesis is strictly controlled by many glycosyltransferases and glycosidases in each specific organelle. Though, it is difficult to identify which genes are specifically affected under stress condition, *N*-glycan analysis can derive the related genes and synthetic regions. Therefore, *N*-glycan analysis can predict to the initial response to environmental changes at comprehensively, and the transition is valuable for studying the relationship between environmental changes and biological response. As the case for monitoring the signaling pathway of plant under environmental changes, novel plant nanobionics approach has been reported[28]. Our results revealed a correlation between free-*N*-glycans and plant development under excessive stress conditions and also demonstrated that free-*N*-glycan transitions are valuable as stress markers for assessing trace environmental changes. This is the first report of the relationship between "environmental changes and glycome transition", and the present study originally adapted glycome transition to environmental toxicology and proposed a new category called "Environmental glycobiology".

Materials and Methods

Chemicals. Purified SNCs (28.4 ± 8.5 nm, release 81.1% Ag^+ suspended in distilled water[29]) were purchased from Utopia Silver Supplements (Utopia, TX, USA). Cosmosil packed columns, $5NH_2$-MS (4.6 ID × 150 mm) and $5C_{18}$-P (4.6 ID × 150 mm), were purchased from Nacalai Tesque (Kyoto, Japan). Known-position PA-sugar chains were purchased from Takara Bio Inc. (Shiga, Japan). alpha-Mannosidase (from jack bean) was purchased from Sigma (MO, USA). beta-*N*-acetylhexosaminidase (from jack bean) was purchased from Prozyme (CA, USA).

Plant materials and sample preparation. Seeds of *O. sativa* (Koshihikari) were supplied by Itakura Agricultural Cooperative Society, Japan. Before each experiment, seeds were washed five times with water and then washed twice with deionized water. The washed twelve seeds were then placed in a plate and immersed in 7 mm depth of water (control) or each concentration of SNCs suspension (0.5, 1.0, 1.5, 3.0, 5.0, 10.0, and 25.0 mg/L of SNCs). The plates were placed in a controlled environmental chamber, and kept at 37 °C for 96 h in the dark. The resulting seedlings were washed and then dried.

Preparation of pyridylaminated *N*-glycans from *O. sativa* seedlings. *N*-Glycan preparation was performed according to the method of Natsuka *et al.*[30]. Dried shoots and roots were ground in a mortar at room temperature, and a ten milligram sample was used. *N*-Glycans were prepared by hydrazinolysis, *N*-acetylation. The reducing ends of the liberated *N*-glycans were then tagged with a fluorophore, 2-aminopyridyne (pyridylaminated *N*-glycans; PA-*N*-glycans), as described in previous paper[20]. These preparations were performed following details in Hase *et al.*[31] with minor modifications.

Separation of PA-*N*-glycans. Size-fractionation HPLC was performed in a Cosmosil $5NH_2$-MS column (4.6 ID × 150 mm) at a flow rate of 0.8 mL/min at 40 °C. PA-*N*-glycans were detected with a fluorescence spectrophotometer at 310 nm excitation and 380 nm emission. Reversed phase HPLC was performed on a Cosmosil $5C_{18}$-P column (4.6 ID × 150 mm) at a flow rate of 1.5 mL/min at 40 °C. The detection of PA-*N*-glycans performed by use of fluorescence spectrophotometer at 315 nm excitation and 400 nm emission. Each HPLC conditions were described in previous paper[20].

Mass spectrometry analysis of PA-glycans. MALDI-TOF mass spectrometry analysis was then performed using an AXIMA resonance instrument (Shimadzu) in reflector mode. Sample preparation was described in previous paper[20].

Glycosidase digestion of PA-*N*-glycans. A two picomoles of PA-*N*-glycans was prepared in 1 microL of accessory reaction buffer (5 mM $CaCl_2$, 10 mM ammonium acetate buffer, pH 4.5) and 2 microL of D. D. W, 1 microL of beta-*N*-acetylhexosaminidase (0.05 units/microL) was added, and the mixture was incubated at 37 °C for 4 h, and then 1 microL of jack bean alpha-mannosidase (19 units/mg) and 5 microL of 10 mM ammonium acetate buffer (pH 4.5) were added, and the mixture was incubated at 37 °C for 1 h. alpha-Mannosidase digestion was described in previous paper[20]. To stop all reactions, the mixtures were boiled for 5 min at 95 °C, and the mixture was analyzed by size-fractionation HPLC.

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Author Contributions

R.H., Y.N., S.K. and N.M. conceived and designed the experiments, R.H. and Y.N. performed sample preparation and *N*-glycan analysis. R.H. wrote the manuscript.

Additional Information

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Sensitivity of medaka (*Oryzias latipes*) to 4-nonylphenol subacute exposure; erythrocyte alterations and apoptosis

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ABSTRACT

The present study was undertaken to assess the effects of the endocrine-disrupting compound; 4-nonylphenol (4-NP) in medaka (*Oryzias latipes*). The frequencies of erythrocyte alterations, apoptosis, and micronuclei were used as biological indicators of damage. Medaka were exposed 15 days to 4-NP at three sublethal concentrations (50, 80, and 100 µg/l 4-NP) and results compared with those of a previous study using catfish as an animal model. Exposure of medaka resulted in a dose-dependent increase in the frequency of erythrocyte alterations, apoptosis and micronucleus (MN). Many morphological alterations and nuclear abnormalities were observed, including acanthocytes, lobed nucleus, eccentric nucleus, fragmented nucleus, blebbed nucleus, binuclei, deformed nucleus, notched nucleus, hemolysed cells, crenated cells, teardrop-like cells, and schistocytes. Mortality was recorded after treatment with 80 and 100 µg/l 4-NP, indicating that medaka are more sensitive than catfish to 4-NP exposure. We concluded that, 4-NP causes several malformations in the shape and number of erythrocytes in medaka, indicating its genotoxicity.

1. Introduction

Increasing environmental pollution, especially chemical pollution, as a result of increasing industrialization is a major problem worldwide. One group of chemicals that causes pollution are the nonylphenol ethoxylates (NPEs), which have been found in aquatic environments, particularly in river water (Clark et al., 1992; Rivero et al., 2008; Tsuda et al., 2000) and have been reported to contaminate aquatic animals including fish and amphibians during their adult life and at sensitive stages of development (Cox, 1996; Giger et al., 1984; Marcomini et al., 1990; Radhaiah et al., 1987; Soto et al., 1991). The breakdown product of these chemicals in aquatic systems is 4-nonylphenol (NP), which is more stable and persistent than the NPEs (Kim et al., 2002; Rivero et al., 2008; Sakai, 2001; Sone et al., 2004; Uguz et al., 2003). It has been shown to be toxic in animals including bees, siders, fish, molluscs, and crustaceans, with hemotoxic, oxidative stressor, estrogenic, histopathological, genotoxic, lethal, and antifertility effects (Cox, 1996; Flouriot et al., 1995; Mekkawy et al., 2011; Rivero et al., 2008; Salanitro et al., 1988; Sayed and Ismail, 2017; Sayed et al., 2012; Sayed et al., 2011; Sayed et al., 2016b; Servos, 1999; Staples et al., 2004; Vazquez-Duhalt et al., 2005).

The induction by various chemicals of micronuclei (MN) and abnormal erythrocyte morphology in fish has been studied to evaluate water quality, the health of species and potential risks (Al-Sabti and Metcalfe, 1995; Ferraro et al., 2004; Grisolia and Starling, 2001; Talapatra and Banerjee, 2007). Fish erythrocytes are favored because the frequency of alterations in both cytoplasm and nuclei can be a marker of cytotoxicity, genotoxicity, hemotoxicity, and oxidative stress (Bushra et al., 2002; Gomes et al., 2015; Joshi et al., 2002; Mekkawy et al., 2011; Sayed et al., 2017; Sayed et al., 2016c).

The frequency of alterations in fish erythrocytes, the induction of MN, and their apoptosis have been studied after exposure UVA, pesticides, hydrocarbons, and toxins (Bahari et al., 1994; Bombail et al., 2001; Bushra et al., 2002; Mekkawy et al., 2011; Sayed et al., 2016c; Talapatra and Banerjee, 2007). Apoptosis is associated with signs of abnormal cell morphology including cell shrinkage, membrane blebbing and chromatin condensation (Cavas et al., 2005; Murakawa et al., 2001; Sayed and Hamed, 2017; Talapatra and Banerjee, 2007).

Medaka (*Oryzias latipes*) are frequently used as an animal model for aquatic toxicology because they are prolific daily egg-layers under ideal breeding conditions, mature quickly and are a small size that allows for large sample sizes in treatment groups (Sayed et al., 2017; Sayed et al.,

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2014). Medaka erythrocytes have been used for genotoxicity studies after UVA- and gamma-IR (Sayed et al., 2014; Sayed et al., 2016a; Sayed et al., 2016b; Sayed et al., 2017). The present study is the first to examine the ability of 4-NP to induce apoptosis, MN, and morphological alterations in blood erythrocytes of medaka compared with its reported effects in the African catfish, *Clarias gariepinus*.

2. Materials and methods

2.1. Medaka

Sexually mature WT (Hd-rR) adult female medaka (*O. latipes*) were used. The fish were kept at 26–28 °C under a 14 h light/10 h dark cycle. They were fed a powdered diet (Tetra-min, Tetra Werke Co., Mells, Germany) and brine shrimp (*Artemia franciscana*) three times a day.

2.2. Nonylphenol

4-NP was obtained from Dr. Ehrenstorfer GmbH, Augsburg, Germany (purity, 99.9%).

2.3. Experimental setup

The adapted adult fish were subdivided into four groups (six fish per group): a control group and three 4-NP-treated groups (50, 80, and 100 µg/l daily for 15 days). The 4-NP doses were selected based on those used by Mekkawy et al. (2011). The experimental conditions during the experiment were the same as during acclimatization with half the volume of water and 4-NP changed every day. Physicochemical parameters and the mortality rate were assessed (Table 1).

2.4. Blood sample collection

Blood samples were collected from the caudal veins of the control and 4-NP treated fish. Each sample was immediately placed on ice to prevent endogenous DNA damage during sample preparation and to inhibit DNA repair in the unfixed cells (Collins, 2004). Blood smears were prepared on clean glass slides (triplicate slides from each fish).

2.5. Apoptosis detection

Apoptotic erythrocytes were detected using acridine orange (AO) staining (Cat. No. A1031, Life Technologies, Carlsbad, CA, USA). A modified protocol (Sayed et al., 2016a) was used to detect apoptosis in red blood cells (RBCs). In brief, after preparation of blood smears on clean glass slides, the slides were washed in 1 × phosphate-buffered saline (PBS) (pH = 7.2). AO buffer (17 µg/l AO in 1 × PBS) was added to the slides for 30 min in the dark. Decolorization was achieved by washing the slides every 30 min for 2 h with 1 × PBS. Fixation was in 4% paraformaldehyde for 5 min. Cells were observed under a fluorescence microscope (BX50; Olympus) equipped with a digital color video camera (DP-70; Olympus).

2.6. Micronucleus, nuclear abnormalities, and morphological alterations

The smears were fixed by dipping the slides in absolute methanol, allowing them to air-dry, and then staining with May-Grünwald solution for 15 min, followed by 6% Giemsa stain for 30 min as reported previously (Tavares-Dias and Moraes, 2003). Slides were selected on the basis of staining quality, then coded, randomized, and scored by person blinded to treatment. In each group, 10,000 cells (minimum of 1000 per slide) were examined using a previously published method (Al-Sabti and Metcalfe, 1995) under a 40 × objective with a 10 × eyepiece to identify MN and morphologically-altered erythrocytes in separate studies. Established criteria for identifying MN (Schmidt, 1975) were followed strictly to ensure accurate scoring.

2.7. Statistical analysis

The mean, standard division, and range were calculated for all values. Differences between groups were evaluated by one-way analysis of variance using SPSS software (SPSS, 1998) at the 0.05 significance level and Dunnett's posttests treating one group as the control and comparing it with all the other groups.

2.8. Ethics statement

All experiments were performed in accordance with the Japanese laws and guidelines for the care of experimental animals and The University of Tokyo Animal Experiment Enforcement Rules. The protocols were approved by the Committee on the Ethics of Animal Experiments of The University of Tokyo (Permit Number: C-14-02).

3. Results

3.1. Detection of morphological alterations, nuclear abnormalities and apoptosis in erythrocytes

Fig. 1a shows a blood smear from normal fish and demonstrates the normal structure of medaka's erythrocytes, which are oval with a centrally located nucleus.

Exposure of medaka to sublethal concentrations of 4-NP (50, 80, and 100 µg/l) resulted in morphological changes in the erythrocytes and the appearance of some abnormal types of cells. The major alterations in the erythrocytes of the fish treated with 50 µg/l 4-NP (Fig. 1b) were the appearance of acanthocytes (Ac); erythrocytes with fewer projections from their surface. Fig. 1c and d shows other morphological changes in the erythrocytes of fish treated with 80 µg/l 4-NP, including echinocytes or crenated cells (Cr), where the red blood cells develop an irregular cell surface with numerous projections, Teardrop-like cells (Tr), whose shape resembles a tear with pointed apices, schistocytes (Sh), notched nuclei (Non), and hemolysed cells (HC), where lysis the cell membrane had occured.

The fish exposed to 100 µg/l 4-NP showed many alterations in their blood (Fig. 2a–d) including acanthocytes. In addition, many nuclear abnormalities were apparent including fragmented nuclei (Fn), deformed nuclei (Dn), eccentric nuclei (Ecn), lobed nuclei (Ln), and Mn, one or more Mn were present per cell in most observations, notched

Table 1
Physiochemical parameters and mortality rate (mean ± SE) after exposure of adult female medaka *Oryzias latipes* to different doses of 4-nonylphenol (4-NP).

100 µg/l 4-NP	80 µg/l 4-NP	50 µg/l 4-NP	Control	Treatments Parameters
4.5 ± 0.28 (5.6 − 3.2)[a]	5.35 ± 0.63 (8 − 2.5)[a]	5.85 ± 0.64 (8.8 − 3.2)[a]	5.51 ± 0.40 (7.9 − 4.1)[a]	Dissolved Oxygen (mg/l)
6.95 ± 0.12 (7.6 − 6.66)[b]	7.24 ± 0.13 (7.7 − 6.81)[ab]	7.3875 ± 0.12 (7.9 − 7)[ab]	7.74 ± 0.20 (8.6 − 7)[a]	pH
24.35 ± 0.12 (24.8 − 23.8)[a]	24.44 ± 0.13 (25 − 24)[a]	24.15 ± 0.14 (24.8 − 23.6)[a]	24.54 ± 0.69 (29.3 − 23.4)[a]	Temperature °C
0.13 ± 0.13 (1 − 0.00)[a]	0.13 ± 0.13 (1 − 0.0)[a]	0.00 ± 0.00 (0.00 − 0.00)[a]	0.00 ± 0.00 (0.0 − 0.0)[a]	Mortality rate%

* Different letters indicate there is a significant difference at ($p \leq 0.05$).

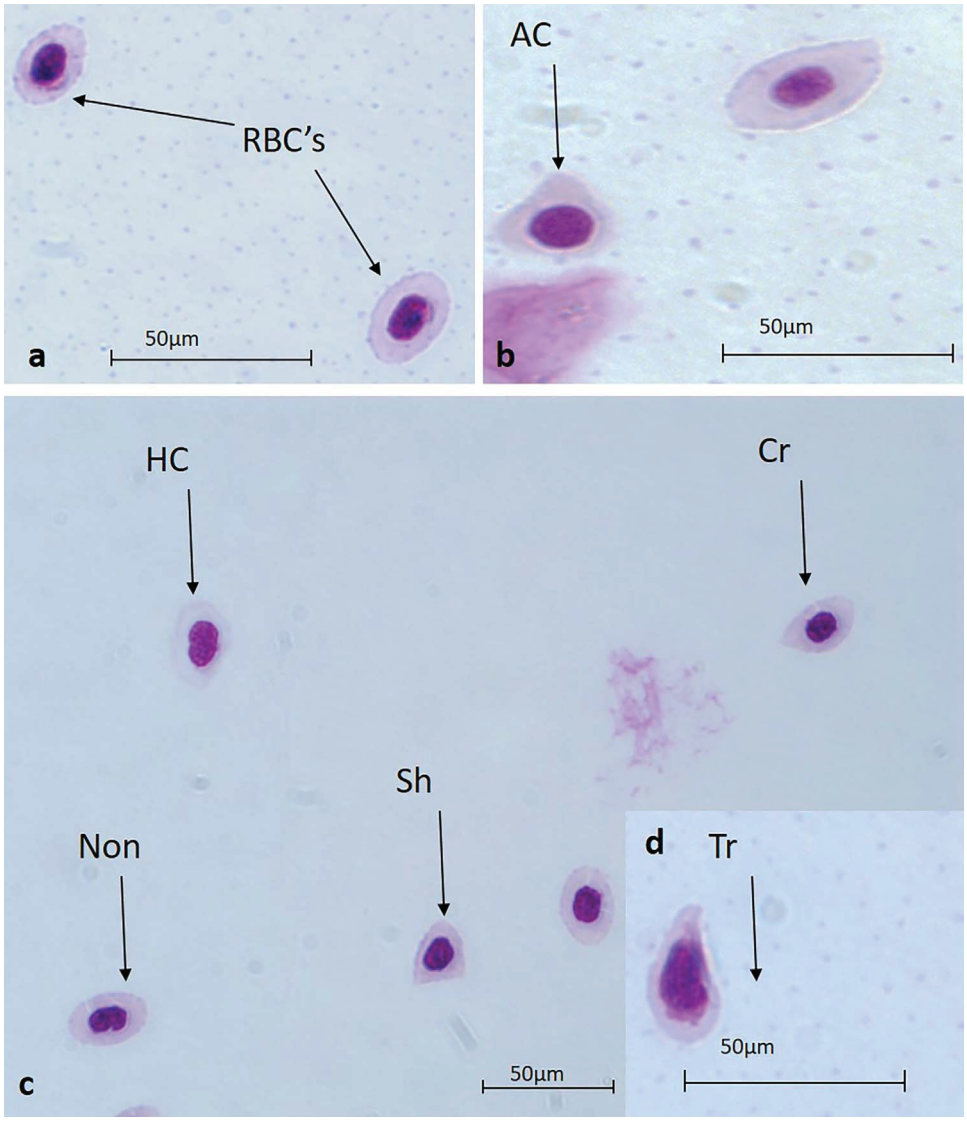

Fig. 1. Giemsa staining of blood film from WT (Hd-rR) adult female medaka (*Oryzias latipes*) showing morphological alterations, (a) WT control; (b) WT after 50 µg/l 4-NP exposure for 15 days; (c and d) WT after 80 µg/l 4-NP exposure; RBC's red blood cells; Ac, acanthocytes; Hc, hemolysed cell; Cr, crenated cells; Tr, teardrop-like cells; Non, notched nucleus; Sh, schistocyte. (Scale bar = 50 µm).

nuclei (Non), binuclei (Bin), and blebbed nuclei (Bn).

Fig. 3 shows typical acridine-orange stained apoptotic erythrocytes in which appear light green under the microscope, from the different NP-treated groups and controls.

3.2. Variation in altered, apoptotic and MN erythrocytes

At all 4-NP doses tested the frequency of erythrocyte cytoplasmic abnormalities including nuclear abnormalities (Fn, Bin, Ln, Ln, ecn, and Non) was elevated compared with controls, with the highest frequency at 100 µg/l 4-NP exposure (Table 2).

Compared with controls, a significant increase in the frequency of apoptotic erythrocytes was observed in fish exposed to 4-NP with the highest frequency observed in fish exposed to 100 µg/l 4-NP (24.3 ± 3 0.36). The frequencies of apoptosis as a function of 4-NP dose are presented in Table 2.

As shown in Table 2, MN increased in the groups treated with 4-NP compared with the control one and the percentage of MN increased significantly with increasing 4-NP dose ($p \leq 0.05$). The percentage of MN was 0.3 ± 0.15% in controls, 1.5 ± 0.27% in fish treated with 50 µg/l 4-NP, 2.9 ± 0.53% in those treated with 80 µg/l 4-NP, and 6.5 ± 0.95% in those treated with 100 µg/l 4-NP.

4. Discussion

The toxic effects of 4-NP have been studied using different animal models, which indicated that, these effects differ between species. The aim of this study was to assess and compare the adverse effects of different doses of 4-NP in *O. latipes* with those reported for *C. gariepinus* (Mekkawy et al., 2011) using selected biomarkers.

Such toxicological research in fish requires biomarkers that can provide an early warning of effects in different organs. Although because of the high impact of using fish erythrocytes as individual cells for environmental pollution studies, some recent studies have used piscine cell lines for environmental toxicity testing (Bols et al., 2005), in this study we used erythrocytes as the target organ for detection of cytotoxicity and genotoxicity to allow the use of simple, reliable, rapid, and sensitive techniques such as measuring the frequency of erythrocyte alterations, nuclear abnormalities including MN, and apoptosis (Fig. 4).

Using different test methods, 4-NP has been shown to be toxic in different types of fish at concentrations from 17 to 3000 µg/l (Servos, 1999). The LC50 pf NP for *O. latipes* was reported to be from 1 to 10,000 µg/l (Yoshimura, 1986). We selected the doses used in this study based on these previous data and the comparison study (Mekkawy et al., 2011), even though they are high and are very rarely seen in aquatic systems.

Many studies have scored morphological and nuclear abnormalities

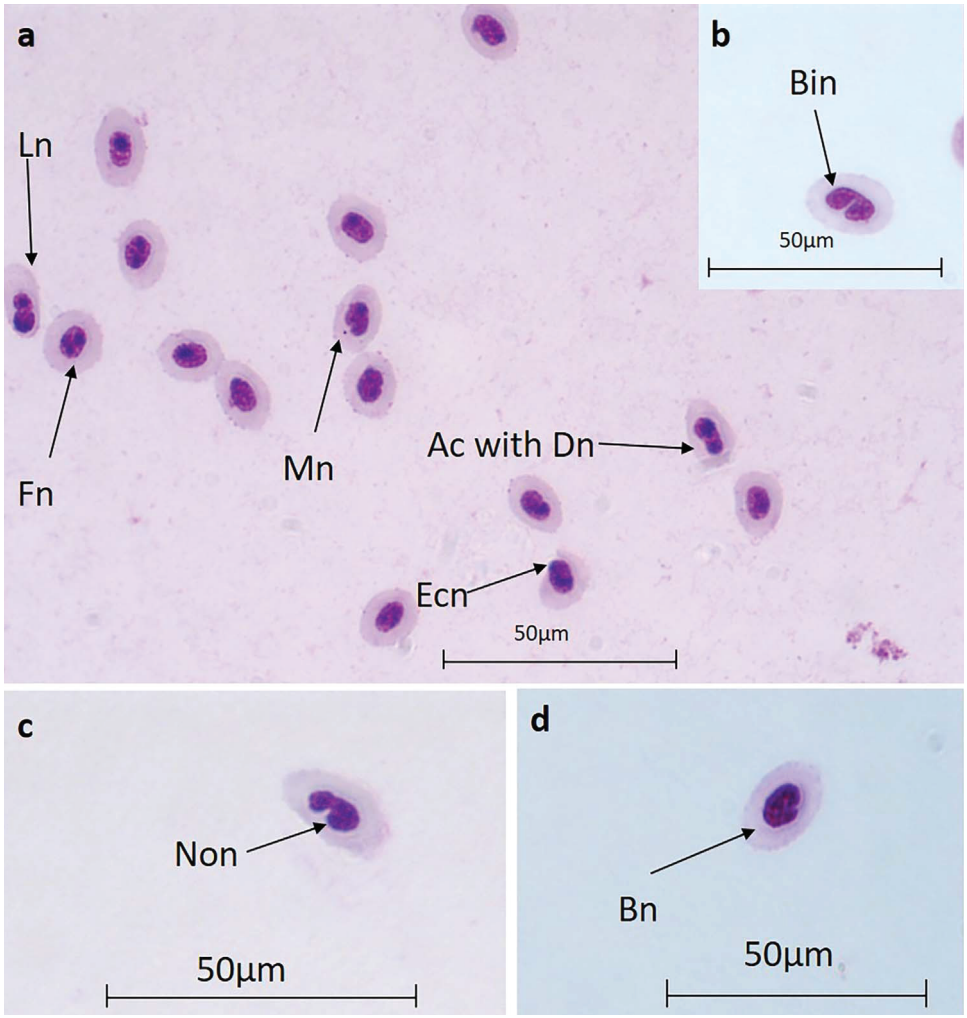

Fig. 2. Giemsa staining of blood film from WT (Hd-rR) adult female medaka (*Oryzias latipes*) showing morphological alterations, (b) WT after 100 µg/l 4-NP exposure for 15 days; Ac, acanthocytes; Ln, lobed nucleus; Ecn, eccentric nucleus; Fn, fragmented nucleus; Mn, micronucleus; Bin, binuclei; Dn, deformed nucleus; Non, notched nucleus; Bn, blebbed nucleus. (Scale bar = 50 µm).

in erythrocytes together with MN as biomarkers for genotoxicity after exposure to radiation and chemical pollution (Ayllon and Garcia-Vazquez, 2000; Ergene et al., 2007; Gomes et al., 2015; Mekkawy et al., 2011; Sayed et al., 2013; Sayed et al., 2017; Sayed et al., 2016c; Sayed et al., 2015b; Sharma et al., 2014). Because of the close relationship between DNA damage and the frequencies of these biomarkers, and their simplicity, reliability, and sensitivity, erythrocyte alterations including nuclear abnormalities are considered to be as a powerful tool for the study of genotoxic and cytotoxic damage in eukaryotes (Al-Sabti and Metcalfe, 1995; Gomes et al., 2015; Mekkawy et al., 2011; Sayed et al., 2017; Sayed et al., 2016c).

Apoptosis induction after 4-NP treatment has been reported in tissue and blood cells of many fish species (Mekkawy et al., 2011; Miura et al., 2005; Murakawa et al., 2001; Sayed and Hamed, 2017; Weber et al., 2003; Yi et al., 2009). One of the causes of apoptosis is DNA damage; we will confirm the existence of such damage in medaka erythrocytes in the second part of this study using γ-H2AX (data under preparation). It has been suggested that the high level of erythrocyte damage and apoptosis seen in fish erythrocytes is because of their short lifespan (1–3 months) (Udroiu, 2006). However, it has also been suggested that the genetic instability caused by toxins could act directly to cause the increased erythrocyte and nuclear abnormalities (ENAs) (Gomes et al., 2015). Our results have demonstrated that many types of ENAs were observed after 4-NP exposure. The study by Gomes et al. (2015) shed light on a possible mechanism underlying the occurrence of different ENAs in genotoxicity studies. In the present study, the frequency of MN increased after 4-NP exposure in a linear dose-dependent manner. These results are consistent with others indicating a dose-dependent increase in damage after exposure to 4-NP (Al-Sharif, 2012; Mekkawy et al., 2011; Sayed and Ismail, 2017; Sharma and Chadha, 2017), carbosulphan (Nwani et al., 2011), 2,4-dicholorophenoxy acetic acid (Ateeq et al., 2005), UVA (Sayed et al., 2013; Sayed et al., 2016c) and arsenic (Sayed et al., 2015a).

Sharma et al. (2014) reported the cytotoxic effects of NP as a decrease in the ratio of polychromatic/normochromatic erythrocytes. They attributed this decrease to either direct cytotoxicity or DNA damage leading to apoptosis. Our results support this concept because exposure to 4-NP induced erythrocyte alterations, nuclear abnormalities and apoptosis in all exposed groups.

Lysis of erythrocytes leading to a reduction in their hemoglobin content causing a type of macrocytic anemia was recorded after 4-NP exposure in *C. gariepinus* (Satyanarayanan et al., 2011; Sayed et al., 2011). The direct damage to erythrocyte membranes presented in that study was detectable by an increase in MCV and MCH after 4-NP exposure (Satyanarayanan et al., 2011; Sayed et al., 2011). In contrast, some authors have attributed cellular damage after toxin exposure to an increase in several enzymes (ALT, AST, and ALP) in fish (Mekkawy et al., 2010; Sayed et al., 2011).

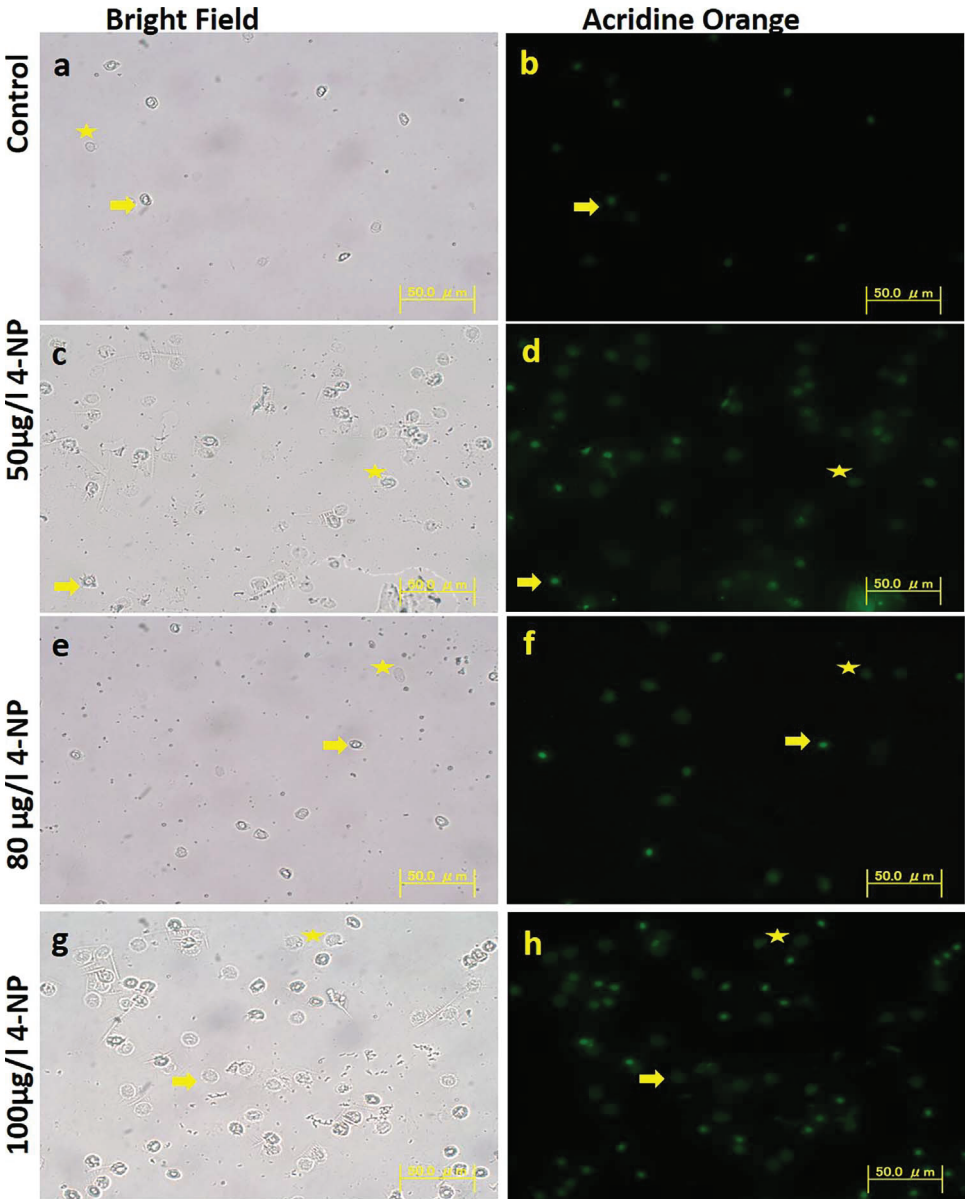

Fig. 3. Apoptosis detection in sexually mature WT (Hd-rR) adult female medaka (*Oryzias latipes*). (a and b) control; (c and d) WT after 50 µg/l 4-NP exposure for 15 days (e and f) WT after 80 µg/l 4-NP exposure for 15 days; (g and h) WT after 100 µg/l 4-NP exposure for 15 days. Cells fluorescing light green after acridine orange staining were considered apoptotic. Arrowheads indicate apoptotic cells, asterisks indicate nonapoptotic cells. (Scale bar = 50 µm).

Because of the importance of DNA for life, clarification of the risk associated with the DNA-damaging effects of 4-NP requires more scientific reports research (Sharma and Chadha, 2017). Recently, it has been reported that the nuclear abnormalities observed after gamma-irradiation of medaka were associated with DNA double-strand breaks, with a strong relationship between these alterations and phosphorylation after γ-H2AX staining (Sayed et al., 2017). Gomes et al. (2015) in a study of the effects of exposure to cadmium of *Oreochromis niloticus*, suggested a possible explanation for the high level of apoptosis in the present study: it may be the result of the cell's response to DNA damage by arresting the cell cycle to assist in DNA repair or by initiating apoptosis to remove damaged cells.

5. Conclusion

The present study showed that, 4-NP is cytotoxic to medaka because it induced a dose-dependent significant increase in erythrocyte alterations, nuclear abnormalities, and apoptosis. These effects may suppress

Table 2
Percentage of altered, apoptotic and micronucleated erythrocytes (mean ± SE) % after exposure to different doses of 4-nonylphenol per 100 cells of WT (Hd-rR) adult female medaka *Oryzias latipes*.

100 µg/l 4-NP (n = 5)	80 µg/l 4-NP (n = 5)	50 µg/l 4-NP (n = 6)	Control (n = 6)	Treatments Parameters
49.9 ± 6.06 (67 − 11)^{a}	19.1 ± 1.91 (22 − 3)^{b}	3.1 ± 0.64 (6 − 0)^{b}	1.1 ± 0.28 (2 − 0)^{b}	Altered cells
24.3 ± 3.36 (36 − 12)^{a}	5.3 ± 0.73 (9 − 2)^{b}	4.2 ± 3.95 (41 − 0)^{b}	2.2 ± 0.63 (7 − 1)^{b}	Apoptotic cells
6.5 ± 0.95 (10 − 1)^{a}	2.9 ± 0.53 (5 − 0)^{b}	1.5 ± 0.27 (3 − 0)^{bc}	0.3 ± 0.15 (1 − 0)^{c}	MN

*Different letters indicate there is a significant difference at ($p \leq 0.05$)

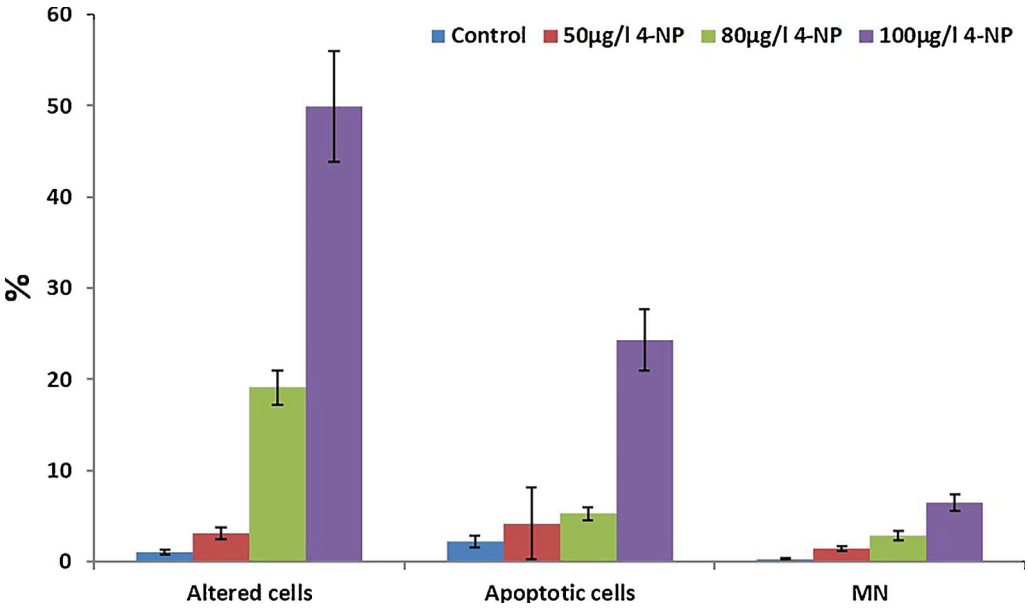

Fig. 4. Percentage of altered, apoptotic and micronucleated erythrocytes (mean ± SE) after exposure to different doses of 4-NP per 100 cells of WT (Hd-rR) adult female medaka *Oryzias latipes*.

normal growth, biological activities, and immunity in both natural and culture environments. Medaka; WT (Hd-rR) is more sensitive to 4-NP than *Clarias gariepinus* and *channa punctaus*, indicating that it is an excellent animal model for toxicological studies.

Declaration of interest statement

We declare no conflicts of interest.

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Comparative toxicities of silver nitrate, silver nanocolloids, and silver chloro-complexes to Japanese medaka embryos, and later effects on population growth rate☆

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ABSTRACT

Fish embryo toxicology is important because embryos are more susceptible than adults to toxicants. In addition, the aquatic toxicity of chemicals depends on water quality. We examined the toxicities to medaka embryos of three types of silver—$AgNO_3$, silver nanocolloids (SNCs), and silver ions from silver nanoparticle plates (SNPPs)—under three pH conditions (4.0, 7.0, and 9.0) in embryo-rearing medium (ERM) or ultrapure water. Furthermore, we tested the later-life-stage effects of SNCs on medaka and their population growth. "Later-life-stage effects" were defined here as delayed toxic effects that occurred during the adult stage of organisms that had been exposed to toxicant during their early life stage only. $AgNO_3$, SNCs, and silver ions were less toxic in ERM than in ultrapure water. Release of silver ions from the SNPPs was pH dependent: in ERM, silver toxicity was decreased owing to the formation of silver chloro-complexes. SNC toxicity was higher at pH 4.0 than at 7.0 or 9.0. $AgNO_3$ was more toxic than SNCs. To observe later-life effects of SNCs, larvae hatched from embryos exposed to 0.01 mg/L SNCs in ultrapure water were incubated to maturity under clean conditions. The mature medaka were then allowed to reproduce for 21 days. Calculations using survival ratios and reproduction data indicated that the intrinsic population growth rate decreased after exposure of embryos to SNC. SNC exposure reduced the extinction time as a function of the medaka population-carrying capacity.

1. Introduction

Because of the emergence of silver nanotechnology and the global growth of related industries, the effects of silver on aquatic environments are being studied, and silver nanotoxicology is emerging as a research area. Silver is comparatively rare in the Earth's crust. Although silver concentrations tend naturally to be elevated in crude oil and in water from hot springs and steam wells, anthropogenic sources can also be associated with elevated concentrations of silver (Howe and Dobson, 2002b). The maximum concentrations of total silver that have been reported in aquatic systems are 6.0 μg/L (groundwater), 260 μg/L (river water), and 300 μg/L (treated photoprocessing wastewater) (Howe and Dobson, 2002a). Silver concentrations are relatively high in aquatic organisms near sewage outfalls, electroplating plants, and mine waste dumps (Eisler, 1996). In addition to conventional anthropogenic silver sources, emerging new silver nanomaterial products likely are contributing to predicted increases in silver concentrations in aquatic environments. According to maximum scenario modeling, silver concentrations are expected to reach 18 μg/L in sewage treatment plants and 320 ng/L in river water (Blaser et al., 2008). Furthermore, the release of silver ion, which is very toxic to organisms, from silver is pH dependent (Kashiwada et al., 2012), thus prompting concern because of the wide range of pH of environmental waters (~4–~12) (Schwedt, 2001). The fate of silver and

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silver ion in aquatic environments is currently poorly understood.

Various organisms have been used to investigate the biological effects of silver nanomaterials in aquatic environments. These species include algae (Navarro et al., 2015), water fleas (Kim et al., 2016; Sakamoto et al., 2014), trout (Salari Joo et al., 2013), carp (Oprsal et al., 2015), sea urchins (Magesky and Pelletier, 2015), and coral (Suwa et al., 2014). In addition, medaka and zebrafish are frequently used as small-fish models for nanotoxicology. The eggs of these two species have similar advantages for embryogenesis research (i.e., a transparent chorion and rapid embryo development), and sufficient genomic information is available on both. Previously, we demonstrated that silver nanocolloids (SNCs) interfere with medaka embryogenesis by disrupting vital gene expression (Kashiwada et al., 2012). Moreover, we have found that Ag^+ released from SNCs and from the silver chloro-complexes that form from Ag^+ and Cl^- ions induces silver toxicity in medaka under environmentally relevant conditions (Kataoka et al., 2015). In addition, the toxicity of Ag^+ is higher than that of silver chloro-complexes; the LC_{50} value of SNCs in ultrapure water (0.050 mg/L; 95% confidence limit, 0.039 to 0.070 for 96 h at pH 7.0) is lower than that in embryo-rearing medium (ERM), which contains chloride ions (>10 mg/L for 96 h at pH 7.0) (Kataoka et al., 2015).

In nanotoxicology, $AgNO_3$ has been used as a reference silver-ion source in toxicological studies of nanosized silver (Newton et al., 2013; Scown et al., 2010; van der Zande et al., 2012), but $AgNO_3$ also includes NO_3^-, and a silver-ion source devoid of nitrate ions is needed. In this regard, all three silver nanomaterials coated with different materials are three to 10 times less toxic to medaka eggs than $AgNO_3$ on a mass-concentration basis (Kwok et al., 2012); thus, the toxicity of silver nanomaterials depends on how efficiently silver ions are released from the nanomaterials. Both $AgNO_3$ and silver nanomaterials show similar toxicity to adult medaka at silver ion concentrations of 10–100 μg/L (Kim et al., 2011). Indeed, silver nanomaterials are sources of dissolved silver (silver ions or silver chloro-complexes or both), and the silver nanomaterials themselves contribute to toxicity in fish (Kwok et al., 2012). Not only the resulting concentration of dissolved silver but also the size of the nanomaterial may be important for toxicity. For example, the growth, reproduction, and behavior of *Caenorhabditis elegans* are adversely affected by ZnO nanoparticles in a particle-size-dependent manner (Khare et al., 2015). In another study (Neubauer et al., 2015), the production of reactive oxygen species depended on the size of palladium and nickel nanoparticles.

The biological effects associated with metal nanomaterials are known to be due to the presence of dissolved metals (ions and complexes) as well as reactive oxygen species; irritation can also occur through contact with the nanomaterials themselves. However, determining the ecological risks posed by chemicals simply by using individual toxicity data is difficult. The probability of population extinction is determined partly by the reduction in the population's intrinsic growth rate as a result of acute and chronic toxicity (Stark et al., 2004). Ecological parameters such as population growth are well known to be important in estimating the ecotoxicological effects on an organism's growth and reproduction (Gentile et al., 1982). To achieve more realistic ecological risk assessments of chemicals, we need to use indices of population dynamics, such as population carrying capacity relative to time to extinction (Lande, 1993). Previously, we successfully assessed the ecological risk of the neurotoxic insecticide carbaryl on medaka populations by using later-life viability and population growth rate (Kashiwada et al., 2008).

Here, to investigate and compare the biological effects of different types of silver on medaka eggs at different pH, we developed a silver nanoparticle plate (SNPP), in which silver nanoparticles are fixed to the bottoms of the wells in a six-well plastic plate. Only silver ions (without nitrate ion) are released from the adhered nanoparticles and into solution for exposure of medaka embryos. We conducted a comparative toxicity study of $AgNO_3$, SNCs, and dissolved silver ions from SNPPs in medaka embryos under different pH conditions. Furthermore, we determined the ecological risk of SNCs to medaka embryos. To examine the later-life effects of SNC exposure on medaka embryos, we used later-life survival ratios and reproduction data to calculate the per-capita growth rate; we then simulated the time to extinction as a function of the environment's medaka population carrying capacity as an indicator of the ecological risk posed by SNCs to medaka populations.

2. Materials and methods

2.1. Medaka eggs (embryos)

Japanese medaka (*Oryzias latipes*, orange-red strain, inbred line) were obtained from the medaka broodstock of the National Institute for Environmental Studies (Tsukuba, Japan). Breeding groups of medaka were incubated at Toyo University and were fed *Artemia salina* nauplii and Otohime β-2 (Marubeni Nissin Feed, Tokyo, Japan). The fish were maintained in a medaka culture system (Small Fish Culture System Type Meito-Hikosaka, Meitosuien, Nagoya, Japan). Tap water, which was dechlorinated by using an activated carbon filter and temperature controlled (25 ± 0.5 °C), was supplied (pH 7.8) to the medaka culture system. Room conditions included a 16:8-h light:dark photocycle and a temperature of 25 ± 0.5 °C.

Spawned eggs were harvested from female medaka, and fertilized eggs were collected under a dissecting microscope (model M165-FC, Leica Microsystems, Tokyo, Japan). The fertilized eggs were rinsed with ERM, which was composed of 1.0 g NaCl, 0.03 g KCl, 0.04 g $CaCl_2 \cdot 2H_2O$, and 0.163 g $MgSO_4 \cdot 7H_2O$ in 1 L of ultrapure water; the pH was adjusted to 7.2 by using 1.25% $NaHCO_3$ in water solution, and the medium was then filter-sterilized. Egg embryos were placed in the ERM and incubated at 25 ± 0.1 °C.

Stage 21 medaka embryos (at the brain regionalization and otic-vesicle formation stage (Iwamatsu, 1994)) were used in these experiments. The medaka embryo goes through 39 developmental stages before hatching; we chose stage 21 because it is the one most susceptible to the effects of SNCs (Kashiwada et al., 2012). Stage 21 embryos were harvested, rinsed with ultrapure water or ERM, and used immediately. All reagents were purchased from Nacalai Tesque (Kyoto, Japan).

2.2. SNCs

Purified SNC solution (20 mg/L; 99.99% purity; particle diameter in distilled water, 28.4 ± 8.5 nm) was purchased (Utopia Silver Supplements, Utopia, TX, USA). The purity and concentration of the silver were validated by inductively coupled plasma–mass spectroscopy (ICP-MS) analyses (X series 2, Thermo Scientific, Pittsburgh, PA, USA). Transmission electron microscopy (TEM) of SNCs was performed (model 2100, JEOL, Tokyo, Japan) (Fig. 1a). SNC solutions (mixtures of SNCs and silver ions) for exposure tests were prepared in ultrapure water or ERM at three different pH values (4.0, 7.0, or 9.0). The pH of the test solution was adjusted by using minimal-drop additions of 0.1 mol/L HNO_3 solution (for pH 4), 1.25% $NaHCO_3$ in water (for pH 7), or 0.1 mol/L NaOH solution (for pH 9). Distributions of particle size (diameter) and the zeta potential of 1 mg/L SNCs at pH 4.0, 7.0, or 9.0 were measured (Zetasizer Nano ZS analyzer, Malvern Instruments, Malvern, Worcestershire, United Kingdom). Before the pH adjustment, the zeta potential of the SNC solution was −28.68 mV. In the pH-adjusted ultrapure water (pH 4.0, 7.0, or 9.0), the three peaks of the zeta potential of the SNCs

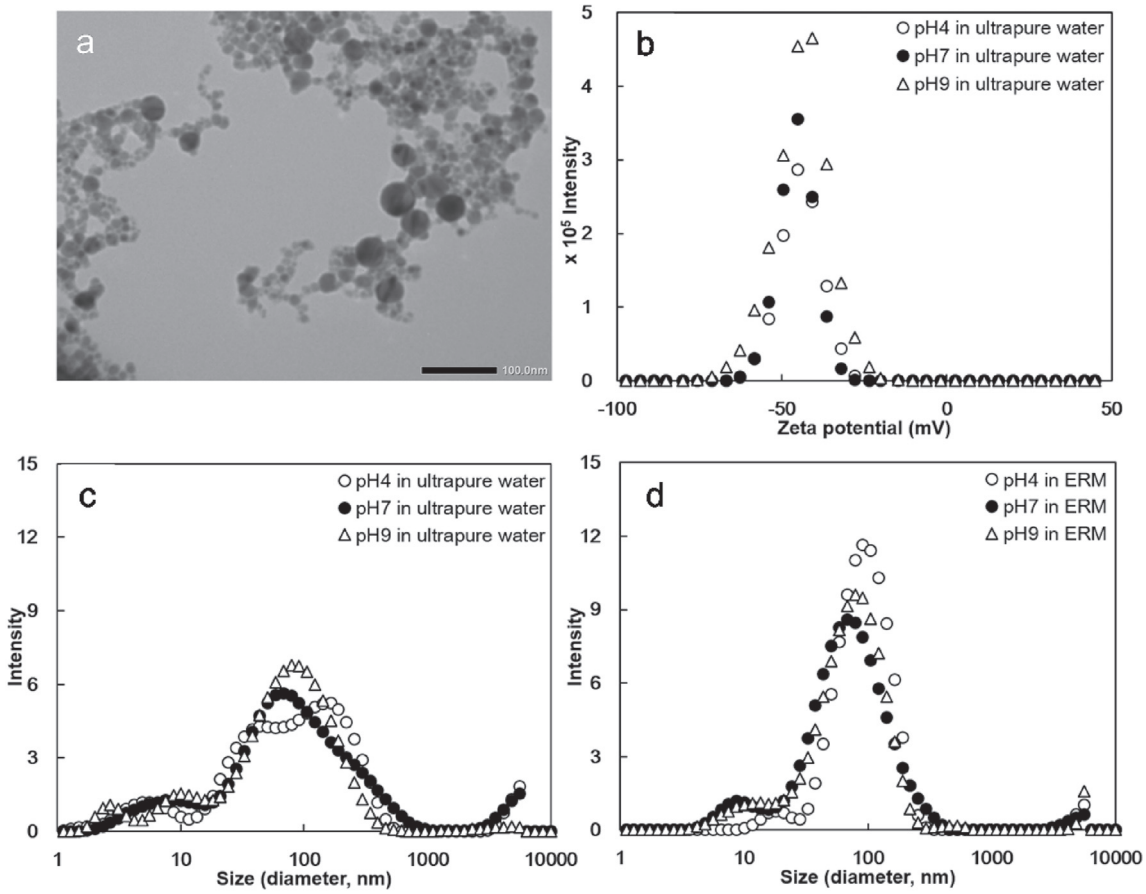

Fig. 1. Transmission electron microscopy (TEM) image of silver nanocolloids (SNCs), and graphs of their size distribution and zeta potentials in ultrapure water and embryo-rearing medium (ERM) at different pH values. TEM image of SNCs (a). Zeta potential (mV) in ultrapure water (b). Size distribution of SNCs in ultrapure water (c). Size distribution of SNCs in ERM (d). All parameters were measured at pH 4.0, 7.0, and 9.0.

overlapped at about −48 mV (Fig. 1b). The particle size distributions had peaks at 164.2 nm (with a shoulder peak at 43.8 nm; pH 4.0), 70.8 nm (pH 7.0), and 85.1 nm (pH 9.0) (Fig. 1c). In the pH-adjusted ERM, the particle size distributions had peaks at 96.1 nm (pH 4.0), 67.8 nm (pH 7.0), and 82.4 nm (pH 9.0) (Fig. 1d). Generally, particles with a zeta potential greater than about ±30 mV tend to be dispersed, rather than aggregated, in solution. Although we were unable to measure the zeta potential in the ERM because of the presence of electrolytes, the SNCs in the pH-adjusted ultrapure water or ERM were estimated to be well dispersed, despite the presence of electrolytes (HNO_3, $NaHCO_3$, NaOH, and other components) in ERM.

2.3. SNPPs for toxicological studies of free silver ion

We developed SNPPs for testing the toxicological effects of silver ions in the absence of nitrate ion and related compounds formed in charge-balanced solutions (Fig. S1).

2.4. Exposure of medaka eggs to silver at different pH

We exposed a group of 15 stage-21 medaka embryos in triplicate to 5 mL of 0.05 mg/L SNCs (that is, the LC_{50} value) in ERM or ultrapure water at pH 4.0, 7.0, or 9.0 in six-well plastic plates; the embryos were incubated at 25 ± 0.1 °C with protection from light until hatching or for 14 days, whichever came first. To expose medaka embryos to silver ions, SNPPs with a 50-nm-thick layer of silver were prepared as described in the Supplemental Information and Fig. S1; medaka embryos were exposed in triplicate as for SNCs. In addition, $AgNO_3$ (0.05 mg/L as Ag) was used as a silver reference; and ERM or ultrapure water at pH 4.0, 7.0, or 9.0 was used as an untreated control in each case. Test solutions were renewed every 24 h. Silver concentrations in the test solutions during exposure were measured by ICP-MS. Every 24 h, exposed embryos were observed for morphological abnormalities under a dissection microscope equipped with a digital camera (model M165 FC, Leica Microsystems). Other reagents were purchased from Nacalai Tesque.

2.5. Calculation of abundance ratios of silver ion species

Formation of silver chloro-complexes and other compounds in ERM and ultrapure water was calculated by using the free program Visual MINTEQ version 3.0 (https://vminteq.lwr.kth.se/).

2.6. ICP-MS analyses of silver

Details of the ICP-MS analysis have been given previously (Kataoka et al., 2015). To separate SNCs and dissolved silver from each SNC solution, a 1-mL sample was filtered by using a centrifuge cassette with a 3-kDa cut-off membrane (Amicon Ultra 3K device, Millipore, Billerica, MA, USA) at 14,000 × *g* for 10 min at 4 °C. The eluate then underwent ICP-MS analysis as dissolved silver.

2.7. Exposure to SNCs and post-exposure incubation to determine the impact on medaka population growth

Five replicate groups of stage-21 medaka embryos ($n = 15$ per group) were exposed to 0.01 mg/L (close to 1/5 LC_{50}) SNCs at pH 7.0 in ultrapure water until hatching. After hatching, all larvae were confirmed to be alive and were rinsed in clean ERM; each group was then moved into 200 mL of fresh ERM in a glass dish and fed with artificial feed (Hikari Lab 130, Kyorin, Hyogo, Japan) once daily for 3 days and thereafter with *A. salina* nauplii. At 7 days posthatch, the larvae were moved to 500-mL acrylic resin aquariums (90 mm × 150 mm × 90 mm) in another medaka culture system and fed *Artemia salina* nauplii and artificial food Otohime β-2 (Marubeni Nissin Feed, Tokyo, Japan) until the medaka reached sexual maturity. During incubation, the numbers of surviving medaka were recorded daily.

2.8. Assessment of effect of SNCs on growth of medaka populations

To evaluate the influence of SNC exposure on medaka populations, we first estimated the per-capita growth rate (r), a summary index that represents the ability of each population to proliferate. The index r was estimated by fitting the life table data for each exposure treatment to the Euler–Lotka equation (Bernstein, 2003). The equation is

$$\sum_t l_t m_t e^{-rt} = 1,$$

where t is age in days, l_t is survivorship until age t, and m_t is per capita fecundity. Because females began to spawn at about 60 days posthatch, we started to record fecundity (the number of spawned eggs/day/female), fertility (the number of fertilized eggs/day/female), and hatchability (the number of hatched larvae/day/female) at 60 days so as to estimate r. The number of newborn females is usually taken as m_t. We therefore identified the sex of all hatched larvae (941 control larvae and 614 exposure-group larvae) by using PCR analysis of a medaka sex-determination gene (*DMY*/*DMRT1*) (Matsuda et al., 2007; Otake et al., 2006). Briefly, for DNA extraction, the larvae were heated at 95 °C in 25 μL of Tris-EDTA buffer for 10 min; the supernatant was used as the template for PCR amplification using primers for *DMY* and *DMRT1* (Shinomiya et al., 2004). After estimating r, we then calculated the finite rate of increase (λ), defined as the number of times the population multiplies in a unit of time, which is expressed as the natural antilogarithm of r (Birch, 1948).

We randomly selected five mating pairs from each treatment and incubated them in the medaka culture system for 21 days. We then measured the numbers of eggs spawned and fertilized to obtain the fertilization ratio. We identified the sex of all larvae to obtain the sex ratio and calculated the arithmetic mean fecundity across the pairs to estimate m_t.

To compare population vulnerability between the control and exposure treatments, we estimated the average time to extinction, $T(K)$, as a function of the population carrying capacity K according to equation 5a of Lande (1993), that is,

$$T(K) = \frac{1}{\bar{r}} \int_1^K \frac{e^{2\bar{r}(N-1)/V}}{N} dN - \frac{\log K}{\bar{r}},$$

where $\bar{r}$ is the mean population growth rate derived from averaging r over replicates, N is population size, and V is variance in individual fitness per unit time. We assumed $V = 1$, according to Lande (1993).

All calculations were performed by using a program code developed for the statistical program R (http://www.R-project.org).

2.9. Statistical analyses

Data were analyzed by using normality testing and the t-test to evaluate the effect of silver compared with the untreated control (Table S1). In addition, we performed one-way analysis of variance and Dunnett's test (Tables 1 and 2). All statistical analyses were done by using Excel 2013 (Microsoft Japan, Tokyo, Japan) or the statistical program R. We chose a significance level (α) of 0.05 for all analyses.

3. Results

3.1. Dissolved silver ions from SNPPs

The concentrations of silver ions dissolved from SNPPs in ultrapure water were measured by using ICP-MS. In ultrapure water, the silver concentration was significantly higher at pH 4.0 (0.57 ± 0.22 mg/L) than at pH 7.0 and pH 9.0 (both 0.08 ± 0.02 mg/L) (t-test, $P < 0.05$) (Table 2). In ERM, the silver concentration was the same (0.04 ± 0.01 mg/L) regardless of the pH (Table 1). In addition, we found that the bottom surfaces of the SNPPs tested with ERM turned from the original purple to white after use, whereas the surfaces of the SNPPs in ultrapure water remained purple (Figs. S1e and S1g). The SEM images revealed that the whitening was due to the presence of white crystals (probably silver chloride) that had grown from the particles on the bottoms of the SNPPs used with ERM but not with ultrapure water (Figs. S1f and S1h).

3.2. Speciation of silver ions

Usually, soluble silver complexes such as silver chlorocomplexes ($[AgCl]^0$, $[AgCl_2]^-$, and $[AgCl_3]^{2-}$) are produced when silver is dissolved in saline solutions (Eisler, 1996; Spiro, 1963). Here, $[AgCl]^0$ and $[AgCl_2]^-$ were the two major silver chlorocomplexes, accounting for 33.0% and 64.5%, respectively, of all silver ion species formed when ERM was used (Figure S2a through S2c). Ag^+ accounted for almost 100% of the silver ion species in the ultrapure water at all three pH levels (Figure S2d through S2f).

3.3. Morphological deformities induced by silver exposure

Medaka embryos exposed to $AgNO_3$, SNCs, or silver ions from SNPPs had various morphological deformities. Specifically, exposure of embryos to $AgNO_3$, SNCs, or silver ions from SNPP in ultrapure water or ERM induced various malformations, including underdevelopment of the eyes and brain, short spinal cord, ischemia, blood clots, pericardiovascular edema, tubular heart, and vascular defects. Although we noted the same malformations as after exposure of embryos to SNCs in our previous study (Kashiwada et al., 2012), the incidences of these abnormalities varied depending on the pH and salinity of the test solution (Fig. 2, Tables 1 and 2).

3.4. Toxicity of silver in ERM and effects of pH

For the ERM controls (ERM without silver), hatchability was 91.1%, 100%, and 100% at pH 4.0, 7.0, and 9.0, respectively (Table 1). In addition, hatchability of ERM control larvae was significantly lower at pH 4.0 compared with pH 7.0. In addition, eye size, heart rate, and full body length of posthatch larvae were lower at pH 4.0 than pH 7.0 ($P < 0.05$), and time to hatch and rates of ischemia and pericardiovascular edema were greater ($P < 0.05$). Although eye size was decreased at pH 9.0 compared with pH 7.0, it appeared

Table 1
Toxic effects of dissolved silver from $AgNO_3$ (as a silver reference), silver nanocolloids (SNCs), or silver ions from silver nanoparticle plates (SNPPs) in medaka embryos in embryo-rearing medium (ERM).

	ERM only (control)			$AgNO_3$			SNCs			SNPPs		
	pH 4.0	pH 7.0	pH 9.0	pH 4.0	pH 7.0	pH 9.0	pH 4.0	pH 7.0	pH 9.0	pH 4.0	pH 7.0	pH 9.0
Ag concentration in complete solution (mg/L)	–	–	–	0.06 (0.00)	0.06 (0.00)	0.07 (0.00)	0.10 (0.00)	0.11 (0.00)	0.10 (0.00)	0.04 (0.01)	0.04 (0.01)	0.04 (0.01)
filtrate from 3-kDa membrane (mg/L)	–	–	–	–	–	–	0.04 (0.00)	0.04 (0.00)	0.04 (0.00)	–	–	–
Underdevelopment of eyes or brain (%)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	2.2 (3.8)
Short spinal cord (%)	2.2 (3.8)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Ischemia or pericardiovascular edema (%)	8.9 (3.8)*$	0.0 (0.0)#	0.0 (0.0)#	0.0 (0.0)#	0.0 (0.0)#	0.0 (0.0)#	20.0 (6.7)*#$	0.0 (0.0)#	0.0 (0.0)#	2.2 (3.8)	6.7 (6.7)	0.0 (0.0)#
Blood clots (%)	4.4 (3.8)	2.2 (3.8)	0.0 (0.0)	4.4 (7.7)	0.0 (0.0)	0.0 (0.0)	11.1 (13.9)	2.2 (3.8)	0.0 (0.0)	6.7 (0.0)	2.2 (3.8)	2.2 (3.8)
Tubular heart and vascular defects (%)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	2.2 (3.8)	0.0 (0.0)	0.0 (0.0)	2.2 (3.8)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	2.2 (3.8)
Eye size (mm in diameter)	0.35 (0.02)*$	0.38 (0.01)#	0.37 (0.01)#	0.34 (0.03)*$	0.37 (0.02)#	0.37 (0.01)#	0.35 (0.03)*$	0.38 (0.01)#	0.38 (0.02)#	0.36 (0.01)*$	0.35 (0.02)*$	0.37 (0.02)*#
Heart rate of embryo at day 5 (no. of beats/15 s)	31.0 (4.6)*$	37.7 (3.0)#	38.0 (2.9)#	30.3 (3.3)*$	37.0 (3.2)#	40.7 (2.5)*#$	34.4 (5.2)*#$	38.0 (2.6)#	38.7 (1.8)#	40.0 (3.4)*#$	38.1 (4.0)#	39.5 (3.0)#
Time to hatch (d)	7.6 (0.8)*$	6.1 (0.3)#	6.0 (0.0)#	8.8 (2.2)*#$	7.0 (0.2)*#$	7.0 (0.2)*#$	7.2 (0.8)*$	6.0 (0.4)#	6.0 (0.0)#	6.6 (0.9)#	7.0 (1.2)#	6.1 (0.8)#
Hatchability in 14 d (%)	91.1 (3.8)*$	100.0 (0.0)#	100.0 (0.0)#	18.0 (15.0)*#$	100.0 (0.0)#	100.0 (0.0)#	60.0 (11.5)*#$	100.0 (0.0)#	100.0 (0.0)#	77.8 (7.7)*$	100.0 (0.0)#	100.0 (0.0)#
Full body length of posthatch larvae (mm)	4.1 (0.2)*	4.2 (0.2)	4.2 (0.2)	4.2 (0.2)	4.3 (0.2)#	4.3 (0.1)#	4.1 (0.2)*$	4.2 (0.2)	4.2 (0.2)#	3.9 (0.2)*#$	4.1 (0.2)	4.1 (0.3)

Standard errors are in parentheses.
*$P < 0.05$ (Dunnett test) compared with data from control at pH 7.0.
#$P < 0.05$ (Dunnett test) compared with data from control at pH 4.0.
$$P < 0.05$ (Dunnett test) compared with data from control at pH 9.0.

Table 2
Toxic effects of dissolved silver from $AgNO_3$ (as a silver reference), silver nanocolloids (SNCs), or silver ions from silver nanoparticle plates (SNPPs) on medaka embryos in ultrapure water.

	Ultrapure water (control)			$AgNO_3$			SNCs			SNPP		
	pH 4.0	pH 7.0	pH 9.0	pH 4.0	pH 7.0	pH 9.0	pH 4.0	pH 7.0	pH 9.0	pH 4.0	pH 7.0	pH 9.0
Ag concentration in complete solution (mg/L)	–	–	–	0.03 (0.01)	0.03 (0.01)	0.03 (0.00)	0.04 (0.00)	0.05 (0.01)	0.05 (0.01)	0.57 (0.22)	0.08 (0.02)	0.08 (0.02)
filtrate from 3-kDa membrane (mg/L)	–	–	–	–	–	–	0.04 (0.00)	0.02 (0.00)	0.02 (0.00)	–	–	–
Underdevelopment of eyes or brain (%)	NA	0.0 (0.0)	0.0 (0.0)	NA	NA	0.0 (0.0)	NA	13.3 (0.1)*$	2.2 (3.8)*$	NA	NA	NA
Short spinal cord (%)	NA	0.0 (0.0)	0.0 (0.0)	NA	NA	11.1 (10.2)*$	NA	13.3 (0.1)*$	2.2 (3.8)	NA	NA	NA
Ischemia or pericardiovascular edema (%)	NA	0.0 (0.0)	2.2 (3.8)	NA	NA	0.0 (0.0)	NA	4.4 (7.7)*	2.2 (3.8)*	NA	NA	NA
Blood clots (%)	NA	0.0 (0.0)	0.0 (0.0)	NA	NA	0.0 (0.0)	NA	6.7 (0.1)*$	2.2 (3.8)*$	NA	NA	NA
Tubular heart and vascular defects (%)	NA	0.0 (0.0)	0.0 (0.0)	NA	NA	0.0 (0.0)	NA	8.9 (0.0)*$	0.0 (0.0)	NA	NA	NA
Eye size (mm in diameter)	NA	0.37 (0.01)	0.38 (0.01)*	NA	NA	0.35 (0.02)$	NA	0.32 (0.03)*$	0.31 (0.11)*$	NA	NA	NA
Heart rate of embryo at day 5 (no. of beats/15 s)	NA	34.6 (4.1)	35.7 (4.0)	NA	NA	36.9 (3.1)	NA	31.3 (4.4)*$	31.3 (6.8)*$	NA	NA	NA
Time to hatch (d)	NA	7.8 (0.7)	7.9 (1.1)	NA	NA	7.9 (0.4)	NA	9.4 (1.3) *$	8.2 (1.3)*$	NA	NA	NA
Hatchability in 14 days (%)	0.0 (0.0)*$	91.1 (7.7)#	93.3 (6.7)#	0.0*$	0.0*$	17.1 (14.8)*$	0.0 (0.0)*$	55.6 (40.7)#	60.0 (43.7)#	0.0 (0.0)*$	0.0 (0.0)*$	0.0 (0.0)*$
Full body length of post-hatched larvae (mm)	NA	4.2 (0.2)$	4.0 (0.2)*	NA	NA	3.9 (0.5)*	NA	3.7 (0.3)*$	3.5 (0.7)*$	NA	NA	NA

NA, not available owing to acute lethality within 24 h.
Standard errors are in parentheses.
*$P < 0.05$ (Dunnett test) compared with data from control at pH 7.0.
#$P < 0.05$ (Dunnett test) compared with data from control at pH 4.0.
$$P < 0.05$ (Dunnett test) compared with data from control at pH 9.0.

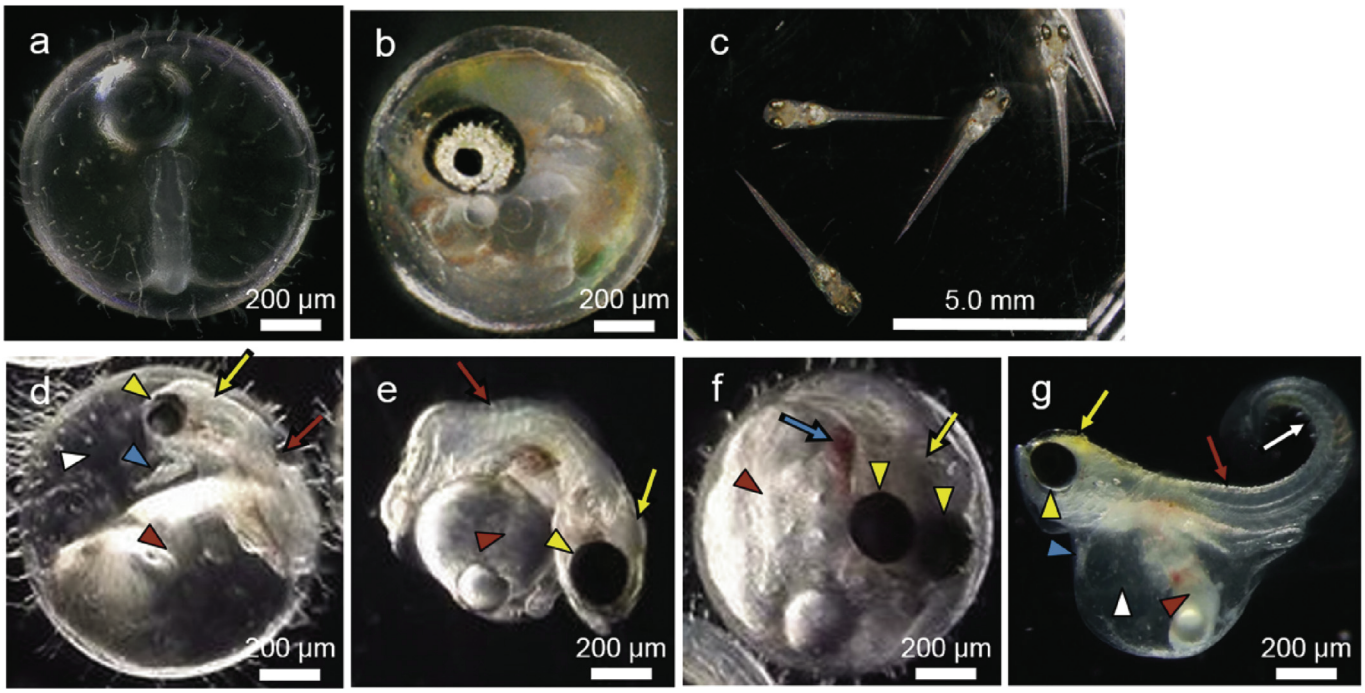

Fig. 2. Morphological toxic effects of silver on medaka embryos. Medaka embryos were exposed to silver from stage 21 through stage 38 (before hatching). Controls: stage 21 (a), stage 38 (b), and posthatch larvae (c). Typical malformations of medaka exposed to silver (same number of posthatch days as stage 38): underdevelopment of eyes (d, e, f, and g: yellow arrowheads) and brain (d, e, f, and g: yellow arrows), short spinal cord (d, e, and g: red arrows), ischemia and vascular defects in yolk sac (d, e, f, and g: red arrowheads), blood clots (f: blue arrow), tubular heart (d and g, blue arrowheads), pericardiovascular edema (d and g: white arrowheads), and kyphosis (g: white arrow). Egg chorion has been removed in e and g.

that, overall, pH 4.0 was more detrimental to medaka embryogenesis than was pH 9.0.

When the embryos were exposed to $AgNO_3$ (0.06–0.07 mg/L as dissolved silver) in ERM, hatchability was 18.0%, 100%, and 100% at pH 4.0, 7.0, and 9.0, respectively (Table 1). Hatchability and eye size at pH 4.0 were significantly reduced in 0.06 mg/L $AgNO_3$ compared with ERM ($P < 0.05$); there were no biological effects at pH 7.0 or 9.0. Therefore, acidic conditions enhanced the toxicity of silver to medaka embryogenesis.

After the exposure of medaka embryos to SNCs (0.10–0.11 mg/L as total silver, 0.04 mg/L as dissolved silver) in ERM, hatchability was 60.0%, 100%, and 100% at pH 4.0, 7.0, and 9.0, respectively (Table 1). Notably, hatchability at pH 4.0 was lower in the SNC-exposed larvae than the ERM controls ($P < 0.05$). In addition, eye size, heart rate, and full body length of posthatch larvae were decreased, and time to hatch and rates of ischemia and pericardiovascular edema at pH 7.0 were increased, in silver-exposed medaka compared with controls ($P < 0.05$); there were no biological effects at pH 7.0 or 9.0. Acidic conditions therefore again enhanced the toxicity of silver to medaka embryogenesis.

When medaka embryos were exposed to dissolved silver (0.04 mg/L as total silver) from SNPPs in ERM, hatchability was 77.8%, 100%, and 100% at pH 4.0, 7.0, and 9.0 respectively (Table 1). Compared with the values for pH 7.0 ERM controls, hatchability was decreased after pH 4.0 exposure; eye size was smaller after exposure at pH 4.0, 7.0, and 9.0; and full body length of posthatch larvae was decreased after exposure at pH 4.0 ($P < 0.05$ for all comparisons).

3.5. Toxicity of silver in ultrapure water and effects of pH

For the controls (ultrapure water without silver), hatchability was 0.0%, 91.1%, and 93.3% at pH 4.0, 7.0, and 9.0, respectively (Table 2). Ultrapure water exerted 24-h acute lethality at pH 4.0. Moreover, full body length in posthatch larvae was decreased and eye size was increased at pH 9.0 compared with that in controls. However, no severe biological defects were noted in ultrapure water at pH 7.0 or 9.0, and the hatched medaka were healthy, despite the extended times to hatch compared with those in ERM ($P < 0.05$) (Table 1).

After exposure to $AgNO_3$ (0.03 mg/L as dissolved silver) in ultrapure water, hatchability was 0.0%, 0.0%, and 17.1% at pH 4.0, 7.0, and 9.0, respectively (Table 2). The toxicity of $AgNO_3$ at pH 4.0, 7.0, and 9.0 was clearly more severe in ultrapure water than in ERM. Eye size and full body length of posthatch larvae were significantly lower at pH 9.0 than in the water controls at pH 7.

Exposure of embryos to SNCs (0.04–0.05 mg/L as total silver; 0.02–0.04 mg/L as dissolved silver) in ultrapure water led to hatchabilities of 0.0%, 55.6%, and 60.0% at pH 4.0, 7.0, and 9.0, respectively (Table 2).

At pH 7.0 and 9.0 in the ultrapure water, the silver concentrations from SNCs were in the same range as those for $AgNO_3$; however, SNCs were comparatively less toxic than $AgNO_3$. Nevertheless, rates of underdevelopment of the eyes and brain, short spinal cord, ischemia and pericardiovascular edema, and blood clots were higher after SNC exposure at pH 7.0 and 9.0 than in the ultrapure water controls at pH 7.0; furthermore, SNC exposure at pH 7.0 and 9.0 reduced the heart rate and full body length of posthatch larvae compared with those at pH 7.0 in the ultrapure water controls. In addition, tubular heart and vascular defects and extended time to hatch occurred more often from exposure to SNCs at pH 7.0 than in the ultrapure water controls at the same pH (Table 2). Finally, the dissolved silver concentration from SNPPs in ultrapure water was pH dependent: it was 0.57, 0.08, and 0.08 mg/L at pH 4.0, 7.0, and 9.0, respectively (Table 2). All pH conditions were associated with 100% 24-h acute lethality.

3.6. Effects of SNCs on population growth rate

Medaka embryos were exposed to SNCs at 0.01 mg/L in ultrapure water until hatching. As mentioned earlier, exposure to 0.01 mg/L SNCs in ultrapure water had no significant effect on hatchability, time to hatch, or survival rate until mating (Table S1). However, mating tests of adult pairs over a 21-day period revealed that fecundity, fertility, and hatchability were reduced in adults that had been exposed to SNCs as embryos (Fig. S3 and Table S2). The calculated intrinsic growth rates (r) (mean [SE]) were 0.273 (0.008) and 0.235 (0.009) in the control and SNC exposure groups, respectively, yielding finite rates of increase (λ) of 1.314 (0.010) and 1.265 (0.011) in the control and SNC exposure groups, respectively. Both r and λ differed significantly between the control and SNC exposure groups (t-test, $P < 0.001$). By using the r values, we estimated the average time to extinction as a function of the population-carrying capacity of the environment. Time to extinction was shorter after SNC exposure than in the controls; SNC exposure of medaka embryos thus induced later-life effects in the medaka population (Fig. 3). For example, time to extinction at $T(30)$ differed by approximately six-fold between the control and exposure treatments (1.80×10^6 in controls; 2.66×10^5 in SNC exposure group).

4. Discussion

We examined the comparative toxicities of $AgNO_3$, SNCs, and silver ions from SNPPs to medaka embryos and the effects of embryonic SNC exposure on the later-life growth of medaka populations.

The wide range of pH values in natural aquatic environments can affect the fate of SNCs. Here, our tests of the effects of pH on the zeta potential and aggregation of SNCs revealed that changing the pH had no observable effect on the zeta potential of SNCs. We estimated that the SNCs were well dispersed and formed aggregates with a broad peak at around 100 nm in size in either ultrapure water or ERM, although we were unable to measure the zeta potential of SNCs in ERM (Fig. 1b). These data suggest that medaka embryos were exposed to relatively the same size of SNCs regardless of whether embryos were in ultrapure water or ERM at pH 4.0, 7.0, or 9.0. Subsequent tests revealed that pH markedly influenced medaka embryogenesis, and acidic conditions (pH 4.0) had an overall greater biological effect on medaka embryogenesis than did alkaline conditions (pH 9.0) (Tables 1 and 2). These effects were more severe in ultrapure water than in ERM. Hatchability and time to hatch are well known and important ecological and ecotoxicological biomarkers. In the controls at pH 4.0, hatchability was reduced to 91.1% in ERM and to 0.0% in ultrapure water. In the controls at pH 7.0, although hatchability did not differ significantly between ERM (100%) and ultrapure water (91.1%) ($P = 0.12$), time to hatch was significantly extended from 6.1 days in ERM to 8.7 days in ultrapure water ($P < 0.05$). Acidic conditions thus had a direct biological effect on medaka embryogenesis, but the buffering action of ERM alleviated the effects of the acidic conditions.

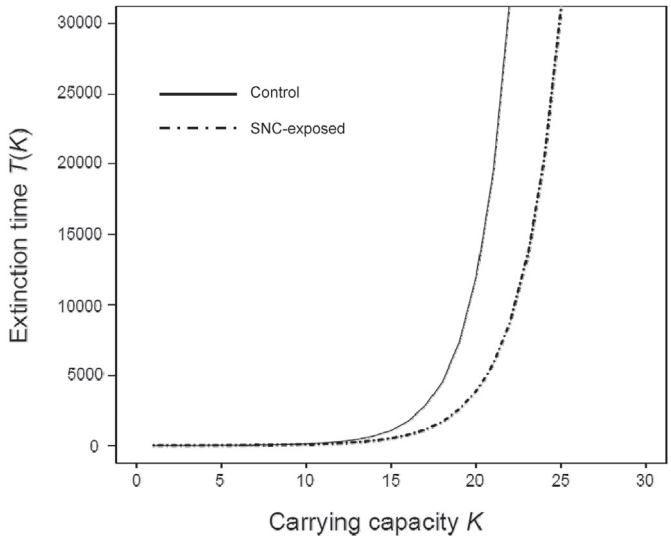

Fig. 3. Ecological risks posed by silver nanocolloids (SNCs), as assessed by time to extinction ($T(K)$) in relation to the medaka carrying capacity (K) of the environment. Solid line indicates control medaka. Dashed line indicates SNC-exposed medaka.

We then tested the effects of pH on the toxicity of silver to medaka embryos. In our previous study, SNCs were more lethal to medaka embryos in ultrapure water than in ERM. That is, the 96-h LC_{50} value was 0.050 (0.039–0.070, 95% efficiency limit) mg/L at pH 7.0 in ultrapure water; in contrast, the lethal toxicity of SNCs was decreased in ERM (LC_{50} value > 10.0 mg/L) (Kataoka et al., 2015). ERM reduced the toxicity of SNCs likely because of the formation of silver chloride complexes (lower toxic form) from Ag^+ (higher toxic form) (Kataoka et al., 2015). Silver ion toxicity reportedly decreases when silver complexes with humic acid (Kim et al., 2013). In our present study, exposure to either $AgNO_3$ or SNCs was markedly more toxic in ultrapure water than in ERM (Tables 1 and 2). ERM alleviated the toxicity of both $AgNO_3$ and SNCs.

Exposure of medaka embryos to silver ions from SNPPs in ultrapure water induced 100% acute toxicity at all three pH values tested. In addition, the dissolved concentration of silver was pH dependent. In this case, a high silver concentration and acidic conditions were directly lethal factors. In contrast, in the case of SNPPs with ERM, acute toxicity was abolished in ERM. Our analyses of silver ion speciation revealed that, in ultrapure water, Ag^+ accounted for almost 100% of species from the three silver compounds. In ERM, two major silver chloro-complexes ($[AgCl]^0$ and $[AgCl_2]^-$) accounted for all of the species (Figure S2d through S2f). Therefore, this different silver speciation likely underlies the different toxicities of the silver ion solutions.

Comparison of the toxic effects of $AgNO_3$ and SNCs in ultrapure water at pH 7.0 and 9.0 revealed that $AgNO_3$ was overall more toxic than SNCs. In the SNC exposure experiment in ultrapure water, the concentrations of silver were 0.05 mg/L (total silver) and 0.02 mg/L (dissolved silver) at both pH 7.0 and 9.0; these concentrations were similar to those of $AgNO_3$ (0.03 mg/L). Nevertheless, at these two pH values, SNCs exhibited lower toxicity than $AgNO_3$ (Tables 1 and 2). In terms of differentiation of the toxicities of silver nanoparticles and silver ions, a study using adult medaka found that toxicity was due to silver ions and that silver nanoparticles had no effect on acute toxicity (Kim et al., 2011). Moreover, solutions containing high ratios of silver ions to silver nanoparticles in suspension were more toxic to medaka embryos than those with lower ratios, suggesting that silver ions had greater adverse effects (Lee et al., 2014).

When we used SNPPs, the concentrations of dissolved silver were higher in ultrapure water than in ERM and were highest (0.57 mg/L) in ultrapure water at pH 4.0. SEM analyses suggested that the white crystals were silver chloride; ERM is an artificial freshwater containing 0.018 M chloride (0.64 g/L as Cl). Silver chloride is insoluble in water. In addition, because the white crystals covered the entire surfaces of the SNPP, the concentrations of dissolved silver were all low (0.04 mg/L) in ERM (Table 1).

Even in the absence of any marked toxicity to medaka embryos or reduction in later-life survival rates (Table S1), later-life reproductive measures, including fecundity, fertility, and hatchability of offspring, were affected (Table S2). Subsequently, both the intrinsic

growth rate and the finite rate of increase were reduced, and we demonstrated a shortened time to extinction relative to the environment's carrying capacity for the medaka population (Fig. 3). Previously, we reported similar research regarding the neurotoxic pesticide carbaryl: exposure at the larval stage, although not necessarily at the embryo stage, had significant toxic effects on population growth during later-life stages (Kashiwada et al., 2008). Therefore, the toxic cascade likely differs between heavy metals and neurotoxicants. In the current study, although the concentration of silver to which medaka eggs was exposed was much lower than the LC_{50} value, we found that 1) exposure during early life can lead to effects during the adult stage, and 2) population-level effects must be considered during ecological risk assessment of chemicals. Therefore, ecological risk assessments of hazardous chemicals must incorporate the concept of population dynamics.

5. Conclusions

In the current study, the malformations and other toxic effects induced in medaka embryos were similar among the three types of silver tested. In ultrapure water, toxicity due to SNCs was lower than that from $AgNO_3$ and silver ions from SNPPs, and using ERM further reduced silver toxicity. In addition, silver toxicity was higher under acidic conditions than under neutral or alkaline conditions. Exposure of medaka eggs to 0.01 mg/L SNCs until hatching had no significant toxic effects on embryo development or hatching, or on later-life survival after rearing under clean conditions. However, population growth was decreased significantly, even when fish were transferred to clean conditions after hatching.

Ethical use of animals

The Japanese medaka used were treated humanely according to the Institutional Animal Care and Use Committee guidelines of Toyo University, with due consideration for the alleviation of distress and discomfort.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2017.10.028.

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