Effekte des Schwermetalls Platin auf die frühe Individualentwicklung des Zebrabärblings (*Danio rerio*) und der Paradies-Schnecke (*Marisa cornuarietis*)

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
Raphaela Osterauer
aus Dachau

Tübingen
2010

Tag der mündlichen Prüfung:	07.03.2011
Dekan:	Prof. Dr. W. Rosenstiel
1. Berichterstatter:	Prof. Dr. H.-R. Köhler
2. Berichterstatter:	Prof. Dr. B. Sures
3. Berichterstatter:	Prof. Dr. J. Oehlmann

Slugs are just snails who´ve been
mugged by other snails
~Andy Riley~

Inhaltsverzeichnis

Zusammenfassung

1. Promotionsthema

Effekte des Schwermetalls Platin auf die frühe Individualentwicklung des Zebrabärblings (*Danio rerio*) und der Paradies-Schnecke (*Marisa cornuarietis*)

2. Einleitung

2.1 Grundlagen

Die Umweltbelastung durch Chemikalien nahm mit der wirtschaftlichen Entwicklung stetig zu und hat seit den 1950er Jahren immer wieder zu schwerwiegenden Umweltkatastrophen geführt. Das damit einhergehende wachsende Bewusstsein der Menschen bezüglich dieser Umweltproblematik führte u.a. zur Etablierung der Ökotoxikologie. Diese fächerübergreifende Wissenschaft, welche aus der Verbindung der Toxikologie und Ökologie hervorging, untersucht die Auswirkungen chemischer Substanzen und physikalischer Einwirkungen auf die belebte Umwelt (Fent, 2007). Dabei werden die Wirkungen auf allen biologischen Ebenen untersucht – von Biomolekülen über Zellen, einzelnen Geweben und Organen, Individuen, Populationen und Lebensgemeinschaften bis hin zu Ökosystemfunktionen. So können über eine Risikoabschätzung, welche chemisch-physikalische Eigenschaften von Chemikalien sowie deren Wirkungen in verschiedenen Expositionszenarien berücksichtigt, eventuelle Gefahren für die Umwelt und den Menschen erkannt werden.

Eine Quantifizierung von Umweltbelastungen und der daraus resultierenden Effekte auf Organismen kann anhand so genannter Biomarker erfolgen. Biomarker sind molekularbiologische, biochemisch-physiologische, zelluläre oder histologische Antworten von Organismen, die durch Umweltstress induziert werden (Adams, 2000a; Kaiser, 2001; Triebskorn, 2003). Sie stellen Frühwarnsysteme dar, die wirksam sind, bevor Schädigungen von Populationen oder Ökosystemen erkennbar werden (Adams et al., 1989, 2000b). Dabei kann man den *biomarker of effect*, welcher integrativ die Wirkung der Gesamtheit aller einwirkenden Einflüsse wiederspiegelt, vom *biomarker of exposure* unterscheiden, welcher eine eindeutige Antwort auf eine bestimmte Exposition aufzeigt. Die Auswirkungen der Chemikalien auf verschiedene biologische Ebenen unterscheiden sich nicht nur hinsichtlich ihres zeitlichen Auftretens und ihrer Empfindlichkeit, sondern auch in ihrer

Komplexität (Fent, 2007). Dabei gilt, dass ein untersuchter Endpunkt umso sensitiver ist und umso schneller reagiert, je niedriger die biologische Ebene ist, der er zuzuordnen ist. Seine ökologische Relevanz dagegen ist umso größer, je höher die biologische Ebene ist, die er repräsentiert (Fent, 2007). Zu diesen Endpunkten gehören molekulare Parameter, wie z.B. die Induktion von Stressproteinen oder die Schädigungen des Genmaterials, histologische Veränderungen sowie Disruptionen der Embryonalentwicklung. Um Auswirkungen von Chemikalien von der molekularen bis hin zur Populationsebene erfassen zu können, ist es wichtig Methoden anzuwenden, die unterschiedliche biologische Ebenen adressieren.

Die in der vorliegenden Arbeit untersuchten Stressproteine, auch sogenannten Hitzschockproteine (Hsp), werden im Organismus verstärkt induziert, wenn dieser proteotoxisch wirkenden physikalischen oder chemischen Stressoren ausgesetzt ist (Feder & Hofmann, 1999). Die Expression dieser Hsp dient dem Schutz der Zelle, indem sie den Erhalt der Proteinstruktur unterstützen und Proteine, die durch äußere Einflüsse eine Veränderung in ihrer Faltung erlitten haben, bis zu einem gewissen Grad wiederherstellen. Hitzeschockproteine mit einem Molekulargewicht von 68-74 kD (Hsp70-Familie) sind in der Ökotoxikologie bislang am besten untersucht und dienen daher als etablierter molekularer *biomarker of effect* um proteotoxische Eigenschaften von Umweltgiften zu beurteilen (Hallare et al., 2004; Köhler et al., 1992; Scheil et al., 2008).

Ein weiterer vielfach angewandter und etablierter molekularer Biomarker ist der Comet Assay, auch bekannt als Einzelzell-Gelelektrophorese. Anhand diesem schnell durchzuführenden, einfachen und sensitiven Test wird die Gentoxizität nach einer *in vivo*, *in vitro* oder *in situ* Exposition gegenüber einem Schadstoff untersucht, indem DNA-Strangbrüche und alkali-labile Stellen in der DNA in einzelnen Zellen detektiert werden.

Histopathologische Biomarker, welche in der Abfolge biologischer Organisation weiter oben anzusiedeln sind, stellen Gewebs- und Organveränderungen als Antwort auf toxische Chemikalieneinwirkung dar (Meyers & Hendricks, 1982). Sie sind etablierte Werkzeuge, um Schadeffekte von Chemikalien auf Organismen zu untersuchen (Triebskorn et al., 2000, 2004) und repräsentieren sowohl zeitlich integrierte endogene, als auch exogene Einflüsse auf den Organismus (Stebbing, 1985). Biomarker auf zellulärer Ebene besitzen den Vorteil, dass sie genauere Informationen über den Wirkort liefern (Triebskorn, 2003) und eine geringere Variabilität aufweisen als biochemische Marker. Weiterhin sind sie bei der Interpretation biochemischer Ergebnisse z.B. der Stressproteininduktion hilfreich, da geringe Hsp-Level sowohl im ansteigenden Bereich (geringe Toxizität der

Substanz), als auch im abfallenden Bereich der Hsp-Optimumskurve nach Eckwert et al. (1997) angesiedelt werden können (pathologische Schädigung der molekularen Stressreaktion). Somit zeigen geringe biochemische Effekte in Verbindung mit schwachen zellulären Antworten eine geringe Belastung und geringe Proteinlevel in Kombination mit starken zellulären Schädigungen eine starke Belastung des Organismus an.

Embryonale Entwicklungsprozesse reagieren besonders empfindlich und sensibel auf Einwirkungen von Xenobiotika (Eaton et al., 1978; McKim, 1977). Daher sind Embryotests geeignete Methoden, um schädigende Effekte von Umweltgiften auf Organismen zu untersuchen. Der Embryotest mit dem Zebrabärbling ist ein etablierter Biotest. Er wurde von Nagel (2002) als Alternative zum akuten Fischtest vorgeschlagen und hat Eingang in die standardisierte Toxizitätsprüfung gefunden. Der Embryotest mit *Marisa cornuarietis* wurde von Schirling et al. (2006) entwickelt und bietet einen vielversprechenden Ansatz für die ökotoxikologische Routineuntersuchung. Während des Embryotests wird die Embryonalentwicklung der Organismen zu festgelegten Zeitpunkten beobachtet und letale, subletale und teratogene Endpunkte wie z.B. Mortalität, Entwicklung der Augen und Fühler, Mißbildungen, Herzschlagrate und Schlupfzeitpunkt registriert, welche Reaktionen der Individuen auf die zu untersuchenden Substanzen anzeigen.

Als Schwermetalle werden diejenigen Metalle bezeichnet, die eine Dichte von > 6 g/cm^3 aufweisen. Darunter fallen essentielle Metalle wie Eisen, Kupfer und Zink, aber auch für Lebewesen nicht essentielle und in erhöhten Konzentrationen stark toxisch wirkende Metalle wie Cadmium, Blei und Quecksilber. In der Umwelt liegen Schwermetalle gelöst als freie Ionen, anorganische oder organische Komplexe, an Partikel gebunden, als schwerlösliche Metall-Salze oder elementar vor. Die ökotoxikologische Wirkung von Schwermetallen hängt dabei im Wesentlichen von der Metallspeziierung ab, welche ihrerseits vom pH-Wert, der Alkalinität und der Konzentration anorganischer und organischer Liganden im Medium abhängt (Fent, 2007).

Zu den Schwermetallen gehören auch die Platingruppenelemente (PGE) Platin (Pt), Palladium (Pd) und Rhodium (Rh), welche vielfach in chemischen, pharmazeutischen und elektronischen Industrieprodukten eingesetzt werden. Im besonderen Maße werden diese Metalle seit den 1980er Jahren in Kraftfahrzeugkatalysatoren verwendet, wodurch die Emission von toxischen Verbindungen wie Stickoxiden, Kohlenmonoxiden und aromatischen Kohlenwasserstoffverbindungen signifikant reduziert wurde. Obwohl ein unumstrittener Nutzeffekt des Abgaskatalysators besteht, wurden bald Bedenken

über die Toxizität der PGE und deren Einfluss auf die Umwelt laut, da sie durch die mechanische und thermische Beanspruchung des Katalysators in zunehmendem Maße in die Umwelt eingetragen werden (König et al., 1992; Palacios et al., 2000). Die PGE-haltigen Partikel deponieren überwiegend am Straßenrand, können jedoch mit dem Wind auch großflächig verbreitet werden (Alloway & Ayres, 1997) und gelangen neben dem Eintrag in die terrestrische Umwelt mit dem Regen und dem Regenabflusswasser der Straßen auch in aquatische Systeme (De Vos et al., 2002; Wang & Sun, 2009; Whiteley & Murray, 2005). Pt-Konzentrationen in Oberflächengewässern und im Sediment liegen normalerweise im niedrigen ng/L bzw. ng/g Bereich. Dennoch wurden z.B. in einer Entwässerungsanlage der Bundesautobahn A5 bei Frankfurt am Main Werte von 15-78 ng/L Pt (Zereini et al., 1997) und im Straßenabflussgewässer einer mit 16000 Kfz/Tag befahrenen Straße Spitzenwerte von bis zu 1,1 µg/L Pt gefunden (Laschka et al., 1996). Im Sediment eines Versickerbeckens an der Bundesautobahn A3 wurden sogar Pt-Gehalte von bis zu 208 ng/g Pt nachgewiesen (Golwer & Zereini, 1998). Wichtig für die Einschätzung des Umweltrisikos dieser Edelmetalle ist die Klärung der Bioverfügbarkeit für Tiere und Pflanzen. Von Kfz-Katalysatoren emittierte PGE werden überwiegend in metallischer Form und an Partikel gebunden freigesetzt (Moldovan et al., 2002). Entgegen der früheren Annahme, Pt sei biologisch inert, konnte jedoch gezeigt werden, dass Pt unter bestimmten Bedingungen in der Umwelt gelöst werden kann und somit in biologisch aktiver Form vorliegt (Klaassen, 1996; Lustig et al., 1998). Auch natürlich vorkommende Komplexbildner wie beispielsweise Huminstoffe können die Löslichkeit der PGE erhöhen (Lustig et al., 1998; Sures & Zimmermann, 2007). Tatsächlich konnte die Bioverfügbarkeit der sowohl partikulären als auch in der Wassersäule gelösten PGE inzwischen für einige terrestrische und aquatische Pflanzen (z.B. Djingova et al., 2003; Farago & Parsons, 1994) und Tiere (z.B. Ek et al., 2004; Sures et al., 2001; Zimmermann et al., 2004) nachgewiesen werden. Die Effekte der PGE auf Organismen sind bisher allerdings nur wenig erforscht. Einzelne Studien zeigen Effekte der PGE auf zellulärer Ebene wie z.B. die Induktion von Metallothioneinen und Hsp70 bei *Dreissena polymorpha* (Frank et al., 2008; Singer et al., 2005) oder histologische Veränderungen bei der Sprague-Dawley-Ratte und dem Zebrabärbling (Gagnon et al., 2006; Jouhaud et al., 1999).

Eine weitere wichtige Eintragsquelle von Pt in die Umwelt – mit etwa 3-12% der von Kfz-Katalysatoren in Europa freigesetzten Menge (Kümmerer et al., 1999) – sind Krankenhäuser und Kläranlagen, da Pt-Komplexe in Form von Cisplatin, Carboplatin und Oxaliplatin als Zytostatika vielfach in der Krebsheilkunde

Anwendung finden. Diese Pt-Komplexe hemmen die DNA-Synthese nachhaltig und zu einem gewissen Grad auch die RNA- und Proteinsynthese (Klaassen, 1996).
Bedeutende Bestandteile von aquatischen Ökosystemen sind Fische und wasserlebende Schnecken. Fische sind die einzige Gruppe primär wasserlebender Vertebraten und häufig Endglieder der Nahrungskette und stellen somit eine ökologisch bedeutende Gruppe dar. Der Zebrabärbling *Danio rerio* gehört zur Familie der Cyprinidae und stammt aus Südostasien, wo er in Flussbiotopen mit dichter Randvegetation vorkommt (Börries, 2001). Er gehört zu den Modellorganismen der Entwicklungsbiologie und der molekularen Genetik (Kimmel, 1989; Nüsslein-Volhard, 1994) und dient auch in der Ökotoxikologie und Toxikologie als international etablierter Testorganismus (Nagel & Isberner, 1998). Der Zebrabärbling ist einfach und kostengünstig zu hältern und legt ganzjährig eine große Anzahl an transparenten Eiern. Seine Embryonalentwicklung erfolgt schnell und synchron (Schlupfzeitpunkt temperaturabhängig 72-96 Stunden nach der Befruchtung) und ist somit einfach zu beobachten.
Da der Großteil aller bekannten Arten Invertebraten und diese maßgebend in die meisten Nahrungsnetze integriert sind, besitzen Untersuchungen über Auswirkungen von Chemikalien auf Invertebraten ebenfalls große Bedeutung. Ein Modellorganismus der Invertebraten, welcher vor allem in jüngerer Zeit in der Ökotoxikologie immer mehr an Bedeutung gewonnen hat, ist die Paradies-Schnecke *Marisa cornuarietis*. Diese getrenntgeschlechtliche prosobranche Schnecke gehört zu der Familie der Apfelschnecken (Ampullariidae) und ist in Flüssen und Brackwassergebieten Süd- und Mittelamerikas beheimatet. *M. cornuarietis* besitzt zusätzlich zu ihrer Kieme eine Lunge (eine Einstülpung des Mantelhöhlenepithels) und kann so in sauerstoffarmen Gewässern überleben. Als weitere Besonderheit legt sie ihr Gelege im Wasser ab und nicht an Land, wie es bei anderen Apfelschnecken der Fall ist. Die Embryonen entwickeln sich innerhalb von 10-14 Tagen in transparenten Eiern, die von einer gallertigen Masse zusammengehalten werden (Demian & Yousif, 1973a). In diversen ökotoxikologischen Studien wurde gezeigt, dass *M. cornuarietis* sehr sensitiv auf Schwermetallexposition (z.B. Sawasdee & Köhler, 2009; Schirling et al., 2006) sowie auf endokrine Disruptoren (z.B. Oehlmann et al., 2000, 2006; Schulte-Oehlmann et al., 2000) reagiert.

In Vorversuchen zu dieser Arbeit zeigte sich, dass einige Paradies-Schnecken, die während der Embryonalentwicklung gegenüber einer Pt-Konzentration von 100 µg/L $PtCl_2$ exponiert wurden, keine äußere Schale ausbilden konnten. Im Gegensatz zum archetypischen Bauplan prosobrancher Schnecken wurde dabei

darüber hinaus die Kieme nicht von einer Mantelhöhle eingeschlossen, sondern verblieb unbedeckt am posterioren Teil des Eingeweidesacks.

Die anatomischen Veränderungen, wie sie normalerweise während der Ontogenese von *M. cornuarietis* vonstatten gehen, wurden ausführlich von Demian & Yousif (1973a,b,c,d, 1975) beschrieben und werden im Folgenden in Bezug auf die Schalenentstehung und die Torsion genauer geschildert: Während der Embryonalentwicklung wird die ektodermale Schalendrüse am posterioren Ende des Embryos angelegt, dreht sich später im Laufe der Torsion nach links und bildet den schalensekretierenden Mantelrand. In dieser Entwicklungsphase überwächst der Mantelrand und mit ihm die Schale den Eingeweidesack mitsamt der Organe dorsolaterad nach rechts und bildet die Mantelhöhle, welche sich nach anterior hin öffnet. Die Torsion, das Schlüsselmerkmal der Gastropoden, ist ein Prozess während der Embryonalentwicklung, bei dem sich der Eingeweidesack 180° relativ zur embryonalen Kopf-Fuß Achse dreht. Daraus resultiert, dass der Darm U-förmig ist, der After anterior zu liegen kommt, die Mantelhöhle sich über den Eingeweidesack stülpt und die Kieme vor dem Herzen zu liegen kommt.

Alle Schnecken bilden irgendwann während ihrer Ontogenese eine Schale unterschiedlicher Form, Größe und Lage. Die Schale der Mollusken besteht aus einer organischen Matrix und Calciumcarbonat ($CaCO_3$), welches aus Calcium and Bicarbonat-Ionen gebildet wird (Rousseau et al., 2003). Das Enzym Carboanhydrase (CA) katalysiert die Reaktion $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$ und ist für die reversible Hydration von CO_2 verantwortlich. Dieses Enzym ist weiterhin mit Geweben assoziiert, die bei Kalzifizierungs- und Mineralisierungsprozessen wie z.B. der Bildung der embryonalen Schale bei Mollusken eine Rolle spielen (Takaichi et al., 2003; Wilbur & Saleuddin, 1983). Es stellte sich heraus, dass einige Schwermetalle Carboanhydrase-Hemmer sind (Christensen & Tucker, 1976; Morgan et al., 1997; Vitale et al., 1999) und Veränderungen in der Enzymkinetik hervorrufen, wodurch spezifische metabolische Syteme gestört werden können.

2.2 Beschreibungen der Studien

Die vorliegende Arbeit beschäftigte sich mit den Auswirkungen verschiedener Konzentrationen des Schwermetalls Pt (0, 0,1, 1, 10, 50, 100 und für einige Untersuchungen auch 200 µg/L $PtCl_2$) auf verschiedene Endpunkte während der frühen Individualentwicklung der beiden aquatischen Süßwasserorganismen *D. rerio* und *M. cornuarietis*.

Im ersten Teil der Dissertation wurden ökotoxikologisch relevante Untersuchungen durchgeführt. Hierfür wurde die Bioverfügbarkeit von Pt für beide Organismen

mittels chemischer Analytik bestimmt, sowie anhand von Embryotests die Embryonalentwicklung beider Organismen bei Exposition gegenüber Pt beobachtet (Kapitel 1). Weiterhin wurden die durch Pt induzierte Induktion der Stressproteine sowie histopathologische Gewebe- und Organveränderungen untersucht (Kapitel 2). Um das gentoxische Potential von Pt auf beide Organismen während der Embryonalentwicklug abzuschätzen, wurde der Comet Assay angewandt (Kapitel 3). Im zweiten Teil der Dissertation (Kapitel 4) wurden Studien durchgeführt, welche die durch höhere Pt-Konzentrationen (100 und 200 µg/L $PtCl_2$) induzierte Bauplanveränderung bei *M. cornuarietis* adressierten. Es wurde untersucht, ob der Verlust der äußeren Schale auch bei der pulmonaten Schnecke *Planorbarius corneus* auftritt oder durch andere Metalle (Pd und Lithium (Li)) hervorgerufen werden kann. Die Akkumulation von Pt in exponierten *M. cornuarietis* wurde mittels chemischer Analytik bestimmt. Um das sensitive „Fenster" während der Embryonalentwicklung zu finden, in welchem Pt die Ausbildung einer äußeren Schale verhindert, wurden Pulsexpositionsexperimente durchgeführt. Die Überlebensfähigkeit der Schnecken ohne äußere Schalen wurde bestimmt, indem diese bis zu ihrem natürlichen Ableben aufgezogen wurden. Um eine mögliche Interaktion von Calcium mit der durch Pt induzierten Bauplanveränderung zu untersuchen, wurden die Schneckenembryonen gegenüber äquimolaren Mischungen von Pt^{2+}- und Ca^{2+}-Konzentrationen exponiert. Da Schwermetalle das bei der Schalenbildung mitverantwortliche Enzym Carboanhydrase hemmen können (Christensen & Tucker, 1976; Vitale et al., 1999; Morgan et al., 1997), wurde bei diesen Schnecken die Aktivität dieses Enzyms bestimmt. Mittels des Chromogens Diaminobenzidin konnte der schalensekretierende Mantelrand angefärbt werden und so sein Schicksal während der Ontogenese verfolgt werden. Um die veränderte Morphologie Pt-exponierter Schnecken und deren Fähigkeit zur Biomineralisation näher zu untersuchen, wurden die Methoden Histologie, Rasterelektronenmikroskopie und synchrotrone Röntgenstrahlen-Phasenkontrast-Mikrotomographie angewandt.

Der jeweilige Eigenanteil bei den einzelnen Kapiteln dieser Promotionsarbeit wird separat auf den Seiten 34-35 der vorliegenden Schrift dargelegt.

2.3 Fragestellungen

Im ersten Teil der Dissertation sollte die Frage beantwortet werden, in welchem Maße Pt für die beiden Organismen *D. rerio* und *M. cornuarietis* verfügbar ist und somit akkumuliert werden kann. Es sollte untersucht werden, ob eine

Platinexposition während der Embryonalentwicklung ein schädigendes Potential auf die beiden Organismen ausübt und, wenn ja, ob dieses auch bei umweltrelevanten Konzentrationen auftritt. Es wurde weiterhin untersucht, ob nach einer Platinexposition während der Embryonalentwicklung eine molekulare Stressreaktion auftritt, histopathologische Schädigungen erkennbar sind und gentoxische Veränderungen durch Pt ausgelöst werden können. Es sollte dabei auch beobachtet werden, in welchem Maße sich *D. rerio* und *M. cornuarietis* als Vertreter aquatischer Vertebraten und Invertebraten hinsichtlich ihrer Sensitivität gegenüber der Platineinwirkung unterscheiden. Für diese Untersuchungen wurden chemische Analytikmethoden, Embryotests, Untersuchungen der Hsp70-Induktion, histologische Untersuchungen und der Comet Assay angewandt.

Da sich bei Vorversuchen im Embryotest ab einer Konzentration von 100 µg/L $PtCl_2$ eine Veränderung des prosobranchen Bauplans zeigte, war die Zielsetzung des zweiten Teils der Dissertation die Untersuchung dieses Effekts, um mögliche Ursachen dafür herauszufinden. Dabei galt es, folgende Fragen zu beantworten: In welchem sensitiven „Fenster" während der Embryonalentwicklung interagiert Pt mit der Schalenbildung? Tritt dieser Effekt auch bei anderen Schneckenarten auf? Wie lange sind Schnecken ohne äußere Schale lebensfähig? Übt Pt einen Einfluss auf die Aktivität des mit an der Schalenbildung beteiligten Enzyms Carboanhydrase aus? Beruht dieser ontogenetische Effekt der Schaleninternalisierung möglicherweise auf einer Interaktion mit dem Calcium-Metabolismus? Kommt es auch bei Exposition gegenüber anderen PGE (Pd, Rh und Iridium (Ir)) und dem Leichtmetall Li zu einer Interaktion mit der Schalenbildung bei *M. cornuarietis*? In diesem Zusammenhang war es überdies von Interesse, das Wachstum des schalensekretierenden Gewebes während der durch Pt veränderten Ontogense zu verfolgen.

3. Material und Methoden

3.1 Experimenteller Aufbau

Die Testsubstanz der vorliegenden Arbeit war $PtCl_2$ (Platin-Standard, 1000 µg/mL in 2% HCl, Ultra Scientific, Germany). Die Experimente wurden mit Embryonen und Juvenilen der Paradies-Schnecke *Marisa cornuarietis* (Ampullariidae), Nachkommen eines auf Wildfängen beruhenden Zuchtansatzes der Universität Frankfurt/Main, und des Zebrabärblings *Danio rerio* (Cyprinidae) durchgeführt, welche aus eigener Nachzucht des Wildtypstamm WIK, ZFIN ID: ZDB-GENO-010531-2 stammten. In der vorliegenden Arbeit wurden Pt-Konzentrationen von

0,1, 1, 10, 50 und 100 µg/L $PtCl_2$ auf beide Versuchsorganismen untersucht. In ausgewählten Fällen (Gentoxizität bei beiden Versuchsorganismen und Induktion von Bauplanveränderungen bei *M. cornuarietis*) wurden Versuche mit 200 µg/L $PtCl_2$ durchgeführt.

Die Exposition der Eier beider Versuchsorganismen fand in 50 mL-Polystyrol-Petrischalen statt, um eine Absorption von Pt an Glas zu vermeiden. Um Eier des Zebrabärblings für die verschiedenen Versuche zu gewinnen, wurden am Abend zuvor Laichkästen in die Aquarien eingesetzt, welche mit einem Siebeinsatz zum Schutz vor Laichraub sowie Laichwatte als Laichstimulus versehen waren. Durch das Einschalten des Lichtes am Morgen wurde das Laichen der Weibchen induziert. Eine Stunde später wurden die Eier entnommen und vorläufig gegenüber den verschiedenen Pt-Konzentrationen oder Kunstwasser (Kontrolle) exponiert. Nach einer 90-minütigen Inkubation im Wärmeschrank bei 26 °C konnten befruchtete Eier erkannt und entnommen werden und weiter exponiert werden. Die Eigelege der Paradies-Schnecke, welche nachts gelegt und an Steine oder Aquarienwände angeheftet wurden, wurden am Morgen entnommen und die Eier mittels einer Rasierklinge separiert. Für *D. rerio* diente Kustwasser (OECD Guideline 203, Annex 2) als Kontroll- und Verdünnungsmedium. Da sich *M. cornuarietis* in diesem Kunstwasser nicht entwickeln kann, diente Aquarienwasser als Kontroll- und Verdünnungsmedium. Für die Embryotests wurden die Embryonen und Larven des Zebrabärblings von der Befruchtung an 96 Stunden gegenüber den verschiedenen $PtCl_2$-Konzentrationen exponiert. Da sich die Paradies-Schnecke vergleichsweise langsam entwickelt, wurden ihre Embryonen von der Befruchtung an 14 Tage exponiert, um möglichst vergleichbare Lebensstadien von beiden Organismen (von der Befruchtung bis zum Schlupf) zu untersuchen. Die Stressprotein- und histologischen Untersuchungen, die analytischen Messungen und die Mikrotomographie wurden mit Larven von *D. rerio* durchgeführt, welche von der Befruchtung an 7 Tage gegenüber den verschiedenen $PtCl_2$ Konzentrationen exponiert wurden und mit Embryonen von *M. cornuarietis*, welche von der Befruchtung an 26 Tage gegenüber den verschiedenen $PtCl_2$ Konzentrationen exponiert wurden. Für die gentoxischen Untersuchungen wurden die Embryonen und Larven des Zebrabärblings von der Befruchtung an 96 Stunden und die Embryonen der Paradies-Schnecke 8 Tage gegenüber den verschiedenen $PtCl_2$ Konzentrationen exponiert. Die Untersuchungen zur Internalisierung der Schale und Bauplanveränderung bei platinexponierten *M. cornuarietis* wurden mit Embryonen durchgeführt, welche zu verschiedenen Zeitpunkten und mit

unterschiedlich langen Expositionszeiten während der Embryonalentwicklung gegenüber Pt exponiert wurden (siehe Kapitel 4).

3.2 Embryotests

Für die Embryotests wurden die bei den Schnecken in der Nacht und bei den Fischen am Morgen abgelegten und befruchteten Eier, wie im vorherigen Kapitel beschrieben, gegenüber den verschiedenen Pt-Konzentrationen und der Kontrolle exponiert. Es wurden jeweils 10 Zebrabärblings- und 20 Paradies-Schneckenembryonen pro Petrischalen exponiert. Die Expositionszeit für *D. rerio* betrug 96 Stunden, die für *M. cornuarietis* 14 Tage. Während dieser Zeit wurden die Embryonen und Larven bei 26 °C im Klimaschrank bei einem Hell-Dunkel Rhythmus von 12:12 Stunden kultiviert und nur für die Untersuchungen der Endpunkte während der Embryonalentwicklung zu festgelegten Zeitpunkten entnommen und unter einem Binokular beobachtet. Die Embryotests wurden mit jeweils 9 Replikaten durchgeführt. Das Expositionsmedium der Schnecken wurde täglich, das der Fische alle zwei Tage komplett erneuert.

3.3 Stressproteinanalysen

Nach Ende der Expositionszeit wurden jeweils 10 Replikate von Pools à 8 Zebrabärblingslarven bzw. 2 Paradies-Schnecken in flüssigem Stickstoff schockgefroren und bis zur Weiterverarbeitung bei -20 °C aufbewahrt. Anschließend wurden die Proben mit einer entsprechenden Menge Extraktionspuffer homogenisiert, zentrifugiert und der Gesamtproteingehalt des Überstandes entsprechend des Protokolls von Bradford (1976) bestimmt. Anhand einer SDS-PAGE (modifiziert nach Laemmli, 1970) wurden die Proteine der Größe nach aufgetrennt und anschließend in einer semi-dry Transferkammer aus Polyacrylamidgelen auf Nitrocellulosemembranen überführt. Mittels Antikörperbindung (erster Antikörper: mouse monoclonal anti-human hsp70 IgG, zweiter Antiköper: Peroxidase-gekoppelter goat anti-mouse IgG) und anschließernder Peroxidasefarbreaktion wurden die Proteinbanden detektiert, densitometrisch quantifiziert und mit einem einheitlichen Standard (WIK *D. rerio*) verglichen.

3.4 Histologische Untersuchungen

Nach Beendigung der Expositionszeit wurden bei allen Ansätzen jeweils 10 Individuen einige Tage in Bouin´s Lösung fixiert. Zur Entwässerung wurden die Proben in einer aufsteigenden Alkoholreihe (70%, 80%, 90%, 96%, 100% EtOH)

jeweils 3 × 10 min gewaschen und anschließend in Technovit eingebettet. Mit Hilfe eines automatischen Mikrotoms wurden Serienschnitte von 4 µm Dicke für *D. rerio* and 5 µm Dicke für *M. cornuarietis* angefertigt und auf Objektträger aufgebracht. Die Schnitte wurden mit Hämatoxylin/Eosin gefärbt, mit Deckgläsern versehen und lichtmikroskopisch ausgewertet. Die Auswertung erfolgte sowohl deskriptiv als auch semi-quantitativ über eine Einteilung detektierter Symptome in verschiedene Kategorien nach Köhler & Triebskorn (1998) sowie Scheil et al. (2010), wobei für jedes der 10 Replikate 3 Schnitte pro Organ bewertet wurden und daraus jeweils ein Mittelwert gebildet wurde.

3.5 Analytische Messungen

3.5.1 Bestimmung der Platinakkumulation in den Organismen

Nach Beendigung der jeweiligen Expositionszeiten gegenüber 0,1 – 100 µg/L $PtCl_2$ wurden die Tiere in flüssigem Stickstoff schockgefroren und bis zur Weiterverarbeitung bei -20 °C aufbewahrt. Die Messung der Platinakkumulation in den Organismen erfolgte mittels adsorptiver kathodischer Stripping-Voltammetrie (ACSV), nachdem die Organismen über eine Hochdruckveraschung unter Zugabe von Salzsäure und Salpetersäure nach Zimmermann et al. (2001, 2003) verdaut wurden. Um die für die Voltammetrie störende Salpetersäure aus der Lösung zu entfernen, wurde diese durch Zugabe von Schwefelsäure und Salzsäure sowie anschließendem Erhitzen verdampft. Die voltammentrischen Messungen wurden mit einem VA Prozessor 693, VA-Stand 694 (Metrohm, Germany) durchgeführt, wobei genauere instrumentale Einstellungen in Zimmermann et al. (2001, 2003) und Haus et al. (2009) aufgeführt sind. Nach einer Standardadditionmethode, welche mit verdünnten Metallstandards durchgeführt wurde, konnte über eine lineare Regressions (R^2 der Geraden > 0,99) der Platingehalt in den Proben bestimmt werden.

M. cornuarietis, welche gegenüber einer Konzentration von 200 µg/L $PtCl_2$ exponiert waren, wurden nach einem Mikrowellenverdau nach Sures et al. (1995) mittels elektrothermaler Atomabsorptionsspektrophotometrie (ET-AAS) auf ihre Platinakkumulation hin untersucht.

Die Nachweisgrenze für Pt wurde als dreifache Standardabweichung der Platinmessungen der „blanks“ definiert, welche als Negativkontrolle ebenfalls die analytische Prozedur ohne Zugabe von tierischem Material durchlaufen hatten.

3.5.2 Bestimmung der Platinkonzentration in den Expositionsmedien

Der Platingehalt in den Expositionsmedien mit Nominalkonzentrationen von 1 – 100 µg/L $PtCl_2$ wurde mittels induktiv gekoppelter Plasma Massenspektrometrie (ICP-MS; Elan 5000, Perkin Elmer, Germany) bestimmt, wobei die Metalle Yttrium und Thulium als interne Standards verwendet wurden. Die Pt-Konzentrationen in den Proben wurden mittels einer Regressionsgeraden ermittelt, welche durch Messungen verschiedener Pt-Konzentrationen einer Pt-Standardlösung gebildet wurde. Aufgrund der geringen Pt-Konzentration im Expositionsmedium mit der Nominalkonzentration von 0,1 µg/L $PtCl_2$, wurde dieses Medium mittels ACSV analysiert, wobei die Prozedur dieselbe war wie diejenige für die Untersuchung der Bioakkumulation in den Organismen. Expositionsmedien mit einer Nominalkonzentration von 200 µg/L $PtCl_2$ wurden nach einem Mikrowellenverdau nach Sures et al. (1995) mittels ET-AAS analysiert.

Die Nachweisgrenze für Pt wurde hier ebenfalls als dreifache Standardabweichung der Platinmessungen der „blanks“ definiert, welche als Negativkontrolle die analytische Prozedur durchlaufen hatten.

3.6 Expositionsexperimente

Für die chronischen Expositionen zur Gewinnung von Schnecken ohne äußere Schale wurden 9 Replikate mit jeweils 20 Embryonen gegenüber 100 und 200 µg/L $PtCl_2$ exponiert. Für die Pulsexpositionsversuche wurden Embryonen gegenüber 200 µg/L $PtCl_2$ für jeweils einen Tag (Tag 3, 4, 5, oder 6 nach der Befruchtung) oder für zwei Tage (Tage 3+4, 4+5, 5+6, 6+7 oder 7+8 nach der Befruchtung) exponiert. Davor und danach wurden sie jeweils wieder gegenüber Kontrollwasser exponiert. Negativkontrollen wurden durchgehend gegenüber Kontrollwasser, Positivkontrollen gegenüber 200 µg/L $PtCl_2$ exponiert. Am 9. Tag nach der Befruchtung wurden die Schnecken ohne externe Schale ausgezählt. Das Expositionsmedium wurde in allen Expositionsexperimenten täglich erneuert. Die Pulsexpositionsversuche wurden mit 4 Replikaten (ausgenommen die Positiv- und Negativkontrolle im ersten Durchlauf der Zweitagespulsexposition mit 8 Replikaten) mit jeweils 20 Embryonen durchgeführt. Um die Entwicklung von Schnecken mit interner Schale nach dem Schlupf zu beobachten, wurden diese bis zu ihrem Ableben aufgezogen. Dazu wurden sie nach dem Schlupf in Kontrollwasser gehältert und täglich mit Fisch-Flockenfutter gefüttert.

3.7 Messung der Carboanhydrase-Aktivität

Embryonen von *M. cornuarietis* ohne äußere Schale, welche gegenüber 200 µg/L $PtCl_2$ exponiert waren, wurden 10 Tage nach der Befruchtung in Phosphatpuffer homogenisiert und das Homogenat anschließend zentrifugiert. Die Carboanhydrase-Aktivität wurde mit der pH-Methode nach Henry (1991) und Vitale et al. (1999) gemessen. Dazu wurde eine Mischung aus Reaktionsmedium (Mannitol, Saccharose und Tris-phosphat), Zentrifugationsüberstand und mit CO_2 versetztem Mineralwasser verwendet und der pH-Abfall für 25 Sekunden mit einem pH-Meter gemessen. Für die Kontrolle wurde Phosphatpuffer anstelle des enzymhaltigen Zentrifugationsüberstandes verwendet. Der Proteingehalt der Proben wurde nach dem Protokoll von Bradford (1976) bestimmt. Die Berechnung der spezifischen Carboanhydraseaktivität erfolgte nach Burnett et al. (1981). Dieser Versuch wurde mit 3 Replikaten (Pools à fünf Schnecken) durchgeführt.

3.8 Diaminobenzidin-Färbung

Die Diaminobenzidin (DAB)-Färbung wird gewöhnlich bei Versuchen als Negativkontrolle für eine Antikörperbindung angewendet. Da sich in eigenen Untersuchungen zeigte, dass DAB spezifisch den Mantelrand von Embryonen der Paradies-Schnecke anfärbt, wurden Embryonalstadien von *M. cornuarietis* unterschiedlichen Alters (Tag 3, 3,5, 4, 5 und 6 nach der Befruchtung), welche gegenüber 200 µg/L $PtCl_2$ exponiert waren, diesbezüglich untersucht. Dazu wurden die Embryonen aus der Eihülle präpariert und in Formaldehyd fixiert. Nachdem sie mit PTw (PBS mit 0,01% Tween 20) gewaschen worden waren, wurden sie über Nacht mit PTw+N (PTw mit 5% Goat Serum) inkubiert, daraufhin erneut mit PTw gewaschen und anschließend in einer 0,3 mg/mL DAB Lösung inkubiert. Durch Zugabe von H_2O_2 wurde die braune Färbung durch die endogene Peroxidaseaktivität des schalensekretierenden Mantelrandes sichtbar und die Embryonen konnten unter einem Lichtmikroskop betrachtet werden.

3.9 Synchrotrone Röntgenstrahlen-Phasenkontrast-Mikrotomographie

Eine Schnecke ohne äußere Schale, welche gegenüber 100 µg/L $PtCl_2$ exponiert wurde, sowie ein Kontrollindividuum wurden über Nacht in 100% Ethanol fixiert, „Kritisch-Punkt"-getrocknet und auf für die Elektronenmikroskopie vorgesehene Träger montiert. Die Röntgenstrahlen-Mikrotomographie wurde mit dem Teilchenbeschleuniger ID19 im ESRF in Grenoble, Frankreich durchgeführt. Die Geräteeinstellungen und die Datenverarbeitung erfolgten nach den Beschreibungen von Heethoff & Cloetens (2008).

3.10 Rasterelektronenmikroskopische Untersuchungen

Schneckenembryonen unterschiedlichen Alters, welche gegenüber 100 oder 200 µg/L $PtCl_2$ exponiert waren, wurden in Glutaraldehyd fixiert, in Cacodylatpuffer gewaschen und in Osmium-tetroxyd inkubiert. Anschließend wurden sie in einer aufsteigenden Alkoholreihe (70%, 80%, 90%, 96%, 100% EtOH) entwässert. Nach einer „Kritisch-Punkt"-Trocknung wurden die Tiere auf Träger montiert, mit einer Goldschicht bedampft und unter einem Rasterelektronenmikroskop des Modells Cambridge Stereoscan 250 Mk2 untersucht.

3.11 Einzelzell-Gelelektrophorese (Comet Assay)

Die Durchführung des Comet Assays erfolgte entsprchend des Protokolls von Kosmehl et al. (2006) für Embryonen des Zebrabärblings. Nach Ende der Expositionszeit gegenüber den verschiedenen Pt-Konzentrationen sowie der Kontrolle wurden jeweils 20 Zebrabärblingslarven und 10 Paradies-Schneckenembryonen in einer Benzocainlösung betäubt. Mittels eines Glas/Glas-Gewebehomogenisators wurden die Zellen der Embryonen unter Zugabe von phosphatgepufferter Salzlösung (PBS) mechanisch isoliert. Die so gewonnene Zellsuspension wurde durch Gaze in ein Eppendorfgefäß pipettiert, zentrifugiert und mit Agarose vermischt.

Als Positivkontrollen dienten Embryonen, welche gegenüber bekannten gentoxischen Agenzien (H_2O_2 bzw. UV-Licht) exponiert wurden. Dazu wurden unter Kontrollbedingungen kultivierte Embryonen direkt vor Beginn des Tests für eine Stunde gegenüber einer 1%-igen H_2O_2-Lösung exponiert. Für die UV-Licht-Bestrahlung wurden die Zellen der bei Kontrollbedingungen aufgezogenen Embryonen direkt nach der mechanischen Isolierung in einem Eppendorfgefäß für 5 min bei einer Wellenlänge von 254 nm und einer Leistung von 8 Watt bestrahlt.

Um zu gewährleisten, dass während der Zellisolation keine zu hohe Zellschädigung auftrat, wurden 20 µL der filtrierten Zellsuspension mit einer Trypanblaulösung vermischt, auf einen Objektträger aufgetragen, mit einem Deckglas versehen und unter einem Lichtmikroskop ausgewert. Der Comet Assay wurde nur mit Zellsuspensionen durchgeführt, welche ≤ 20% geschädigte Zellen aufwiesen.

Der Comet Assay wurde nach dem Protokol von Singh et al. (1988) mit einigen Änderungen von Schnurstein & Braunbeck (2001) durchgeführt. Dazu wurde die Zellsuspension-Agarosemischung wie bei einem Sandwich zwischen zwei Schichten von Agarose auf aufgerauhte Objektträger aufgebracht. Zur Zerstörung der Zellmembranen wurden die Objektträger in einer stark alkalischen Lyselösung abgedunkelt im Kühlschrank inkubiert. Die Denaturierung der DNA erfolgte durch

Inkubation der Objektträger in einem Elektrophoresebehälter in alkalischem Elektrophoresepuffer. Die DNA wurde anschließend mittels Elektrophorese aufgetrennt und nach Beendigung der Elektrophorese in Trispuffer neutralisiert. Vor der Auswertung mittels eines Fluoreszenzmikroskops (Aristoplan; Leica, Germany) wurde die DNA mit Ethidiumbromid angefärbt. Für jede Behandlung wurde das *Tail Moment* (Intensität des DNA-Schweifes × Länge des Schweifes) von 100 zufällig ausgewählten Zellkernen auf 2 Objektträgern (50 Kerne pro Objektträger) ausgewertet.

4. Ergebnisse und Diskussion

1. Ökotoxikologische Untersuchungen zur Toxizität verschiedener Platinkonzentrationen auf die Embryonalentwicklung des Zebrabärblings (*Danio rerio*) und der Paradies-Schnecke (*Marisa cornuarietis*)

Kapitel 1: Osterauer, R., Haus, N., Sures, B., Köhler, H.-R. 2009. Uptake of platinum by zebrafish (*Danio rerio*) and ramshorn snail (*Marisa cornuarietis*) and resulting effects on early embryogenesis. Chemosphere 77: 975–982.

In der ersten Studie dieser Dissertation wurde die Bioakkumulation von Pt in den Versuchsorganismen *D. rerio* und *M. cornuarietis*, welche gegenüber verschiedenen Konzentrationen (0, 0,1, 1, 10, 50 und 100 µg/L $PtCl_2$) exponiert worden waren, sowie die Auswirkungen dieser Expositionen auf die Embryonalentwicklung untersucht.
Es zeigte sich, dass beide Organismen Pt stark akkumulierten. Abhängig von den verschiedenen Pt-Konzentrationen in den Expositionsmedien, welche ebenfalls analytisch bestimmt wurden, ergaben sich bei *D. rerio* Bioakkumulationsfaktoren (BAF) von 5 bis 55. Bei *M. cornuarietis* wurden sogar BAF von 218 bis 724 erreicht. In den Embryotests waren die beiden Endpunkte Herzschlagrate und Schlupfzeitpunkt bei beiden Organismen betroffen: Die Herzfrequenz sank mit zunehmender Pt-Konzentration bei beiden Organismen signifikant ab. Bei *D. rerio* war der Schlupfzeitpunkt bei den Konzentrationen 50 und 100 µg/L $PtCl_2$ signifikant gegenüber der Kontrolle verzögert, bei *M. cornuarietis* bei den Konzentrationen 10, 50 und 100 µg/L $PtCl_2$. Bei *M. cornuarietis* waren im Embryotest weiterhin die Endpunkte Gewicht und Augenentwicklung betroffen. Das Gewicht der geschlüpften Schnecken nahm mit zunehmender Pt-Konzentration im Expositionsmedium, mit Ausnahme des Gewichts der Schnecken welche gegenüber

0,1 und 50 µg/L $PtCl_2$ exponiert waren, signifikant ab. Die Entwicklung der Augen war bei Schnecken, welche gegenüber den Konzentrationen 1, 10, 50 und 100 µg/L $PtCl_2$ exponiert waren signifikant verzögert.
Diese Studie zeigt, dass *M. cornuarietis* in der Lage dazu ist, Pt in der dargereichten Form außergewöhnlich stark zu akkumulieren. Vergleichsdaten von adulten *Dreissena polymorpha* (Sures & Zimmermann, 2007) zeigen, dass juvenile *M. cornuarietis* nach einer vergleichbaren Expositionszeit etwa 40-mal mehr Pt akkumulieren. Vergleichsdaten zu *D. rerio* mit anderen Fischen schwanken stark. Für *Anguilla anguilla* lagen die BAFs in der gleichen Größenordnung (Zimmermann et al., 2004), wohingegen der BAF für *D. rerio* in einer anderen Studie wesentlich höher lag (Jouhaud et al., 1999). Dieses Phänomen liegt möglicherweise an den unterschiedlichen Expositionszeiten, den Altersklassen der Versuchstiere, oder der unterschiedlichen Speziierung des jeweils untersuchten Platinsalzes. Die vorliegende Studie zeigt weiterhin, dass Pt auch schon bei einer umweltrelevanten Konzentration von 0,1 µg/L $PtCl_2$ einen Effekt auf die Embryonalentwicklung der beiden untersuchten Organismen ausübte. Wie es auch schon für andere Metalle beschrieben wurde (Hallare et al., 2005; Schirling et al., 2006; Sawasdee & Köhler, 2009), reagierte *M. cornuarietis* dabei sehr sensitiv auf die Platinexposition und zeigte neben den beschriebenen Effekten auf die Herzschlagrate und Schlupfrate auch eine Entwicklungsverzögerung, welche bei *D. rerio* nicht festgestellt wurde.

Kapitel 2: Osterauer, R., Köhler, H.-R., Triebskorn, R. 2010. Histopathological alterations and induction of hsp70 in ramshorn snail (*Marisa cornuarietis*) and zebrafish (*Danio rerio*) embryos after exposure to $PtCl_2$. Aquat. Toxicol. 99: 100–107.

In dieser Studie wurden die Hsp70-Induktion und histopathologische Veränderungen bei den beiden Versuchstieren *D. rerio* und *M. cornuarietis* nach Exposition gegenüber verschiedenen Pt-Konzentrationen (0, 0,1, 1, 10, 50 und 100 µg/L $PtCl_2$) während der frühen Individualentwicklung untersucht. Bei Larven des Zebrabärblings zeigten die beiden untersuchten Organe Leber und Darm Veränderungen durch die Platinexposition. Der Darm befand sich bei allen untersuchten Pt-Konzentrationen in einem Zustand leichter Reaktion auf den Schadstoff. Die Symptome bestanden in einer Ablösung der Darmzellen von der Basallamina, in basaler Vakuolisierung des Zytoplasmas, in unregelmäßiger Kompartimentierung der Darmzellen und in einer erhöhten Anzahl an Schleimzellen und traten bei den Konzentrationen von 1, 50 und 100 µg/L $PtCl_2$ signifikant verstärkt gegenüber der Kontrolle auf.

Die Leber des Zebrabärblings befand sich vor allem bei den höheren Pt-Konzentrationen von 50 und 100 µg/L $PtCl_2$ in einem Zustand starker Reaktion auf den Schadstoff. Ab einer Konzentration von 1 µg/L $PtCl_2$ zeigte sich ein signifikanter Anstieg der beobachteten Effekte gegenüber der Kontrolle. Diese Effekte umfassten Entzündungsreaktionen mit erhöhter Makrophagenanzahl, Erweiterung der Kapillarräume, unterschiedliche Dichte des Zytoplasmas und der Zellkerne, und – bei den Konzentrationen 50 und 100 µg/L $PtCl_2$ – Vakuolisierung des Zytoplasmas, eine leichte Anschwellung der Leberzellen und eine Verdichtung des Chromatins (Karyopyknose).

Bei der Paradies-Schnecke zeigten sich histopathologische Effekte des Platins auf die Epidermis, die Mitteldarmdrüse und die Kieme. Bei allen untersuchten Konzentrationen befand sich die Epidermis der Schnecken lediglich in einem Zustand leichter Reaktion. Die beobachteten Symptome von Zell- und Zellkernvergrößerung, unregelmäßigen apikalen Zelloberflächen und seltener einer Abschuppung der Epidermis traten bei den Konzentrationen 10, 50 und 100 µg/L $PtCl_2$ signifikant verstärkt im Vergleich zur Kontrolle auf. Die Kieme zeigte bei 50 und 100 µg/L $PtCl_2$ eine Reaktion auf die Schadstoffexposition. Dieses zeigte sich in signifikant häufiger auftretenden Unregelmäßigkeiten der Morphologie der Kiemenlamellen und -zellen, in Vergrößerungen der Lamellenzwischenräume und der Kiemenzellkerne, in einer erhöhten Anzahl an Schleimzellen und in Chromatinverdichtungen. Die Mitteldarmdrüse der Paradies-Schnecke wurde am stärksten durch Pt geschädigt und zeigte schon ab einer Konzentration von 0,1 µg/L $PtCl_2$ einen signifikanten Anstieg der beobachteten Effekte gegenüber der Kontrolle. Die Effekte bestanden in erweiterten Hämolymphräumen und Tubulilumina, einer Abflachung des Epithels, unregelmäßig geformten Zellen, in apikalen zytoplasmatischen Ausstülpungen („surface blebbing“), in einer erhöhten Vakuolenanzahl bei Verdauungszellen sowie im verstärkten Auftreten nekrotischer Zellen und nahmen bei ansteigender Konzentration zu, so dass sich vor allem bei den Konzentrationen 50 und 100 µg/L $PtCl_2$ die Mitteldarmdrüse im Zustand einer starken Reaktion auf den Schadstoff befand.

Bei *D. rerio* wurde eine erhöhte Hsp70-Induktion nur bei der höchsten getesteten Konzentration von 100 µg/L $PtCl_2$ gefunden. *M. cornuarietis* zeigte hingegen keine erhöhten Hsp70 Werte bei allen untersuchten Pt-Konzentrationen.

In dieser Studie zeigte sich ein organ- und schadstoffspezifisches Reaktionsbild. Die Leber bei Fischen und die Mitteldarmdrüse bei Schnecken sind die Hauptorgane der Schwermetallentgiftung und -akkumulation (Sastry & Gupta, 1978, 1979; Tanhan et al., 2005) und wurden bei dieser Untersuchung am stärksten durch Pt

geschädigt. Bei *M. cornuarietis* entspricht die niedrigste untersuchte Konzentration von 0,1 µg/L $PtCl_2$, welche einen signifikanten Unterschied hervorrief (LOEC – lowest observed effect concentration), einer Konzentration, wie sie in Straßenabflussgewässern gefunden wurde (Laschka et al., 1996). Pt zeigte für die untersuchten Konzentrationen eine geringe proteinschädigende Wirkung auf *D. rerio* und erwies sich als nicht proteotoxisch für *M. cornuarietis*. Da Singer et al. (2005) bei *Dreissena polymorpha* eine erhöhte Hsp70-Induktion beobachtete, war in der vorliegenden Studie möglicherweise die Expositionsdauer für eine erhöhte Stressproteininduktion nicht ausreichend. Die Ergebnisse dieser Studie unterstreichen die Wichtigkeit der Kombination von Biomarkern auf verschiedenen biologischen Ebenen, denn, obwohl die Hsp70-Induktion oft als Biomarker zur Risikoabschätzung eines potentiellen Schadstoffes herangezogen wird, hätte die Stressproteinantwort alleine den Schadeffekt der untersuchten Pt-Konzentrationen auf *D. rerio* und *M. cornuarietis* unterschätzt.

Kapitel 3: Osterauer, R., Faßbender, C., Braunbeck, T., Köhler, H.-R. 2011. Genotoxicity of platinum in embryos of zebrafish (*Danio rerio*) and ramshorn snail (*Marisa cornuarietis*). Sci. Total Environ. 409: 2114–2119.

In dieser Studie wurde anhand der Einzelzell-Gelelektrophorese (Comet Assay) das gentoxische Potential verschiedener Konzentrationen des Platinsalzes $PtCl_2$ (0, 0,1, 1, 10, 50, 100 und 200 µg/L) auf embryonale Stadien des Zebrabärblings (*Danio rerio*) und der Paradies-Schnecke (*Marisa cornuarietis*) untersucht. Beide in diesem Versuch eingesetzten Positivkontrollen (Wasserstoffperoxid (H_2O_2) und UV-Licht) bewirkten DNA-Schäden bei beiden Versuchstieren, wobei die 1%-ige H_2O_2-Lösung, in welcher die Embryonen für eine Stunde exponiert wurden, die DNA beider Organismen stärker schädigte als die fünfminütige Bestrahlung von bereits isolierten Zellen mit UV-Licht.
Für *D. rerio* waren die in dieser Studie getesteten Pt-Konzentrationen nicht gentoxisch, wohingegen schon ab 1 µg/L $PtCl_2$ signifikant erhöhte DNA-Schäden bei *M. cornuarietis* auftraten. Andere Untersuchungen dieser Dissertation (Kapitel 1) zeigten, dass *M. cornuarietis* bei einer etwa 3,5 mal längeren Expositionszeit 50 mal mehr Pt akkumulierte als *D. rerio*, was auf eine außergewöhnlich starke Akkumulationsfähigkeit der Paradies-Schnecke für dieses Metall hinweist. Obwohl *M. cornuarietis* in dieser Studie nur doppelt so lange gegenüber Pt exponiert war (8 Tage), ist anzunehmen, dass auch nach dieser Zeit vielfach mehr Pt akkumuliert wurde als in *D. rerio*, welcher aufgrund der schnelleren Embryonalentwicklung nur

4 Tage exponiert wurde. Somit könnte die längere Expositionszeit und eine höhere Akkumulation die Gentoxizität von Pt für *M. cornuarietis* erklären. Möglicherweise spielen aber auch speziesabhängige Unterschiede in den Reparaturmechanismen oder in der Kapazität metallbindender Proteine (Metallothioneine), deren Induktion bei Hühnerembryonen nach einer Pt(VI)-Exposition (Gagnon and Patel, 2007) und bei *Dreissena polymorpha* nach einer Pd(II)-Exposition (Frank et al., 2008) beobachtet wurde, für die unterschiedlichen Ergebnisse bei beiden Versuchsorganismen eine Rolle.

Mit zunehmender Pt-Konzentration nahm die Stärke der DNA-Schädigung bei *M. cornuarietis* zu, mit Ausnahme der höchsten getesteten Konzentration von 200 µg/L $PtCl_2$, welche signifikant geringer ausfiel als die DNA-Schädigung bei 100 µg/L. Dieser Effekt ist möglicherweise auf eine cytotoxische Wirkung der höchsten getesteten Konzentration zurückzuführen, die sich hemmend auf die Exzisionsreparatur und damit auf die Anzahl von Strangbrüchen auswirken könnte. Möglicherweise ist auch die veränderte Morphologie der Schnecken auschlaggebend, welche bei einer Exposition gegenüber 200 µg/L $PtCl_2$ während der Embryonalentwicklung u.a. keine äußeren Schalen entwickeln konnten. Diese grundlegende Bauplanveränderung, die im zweiten Teil meiner Dissertation eingehender untersucht wurde, könnte auch zu einer veränderten Antwort des Organismus auf den Schadstoff geführt haben.

Untersuchungen zur Gentoxizität von Pt wurden bisher überwiegend mit Platinverbindungen durchgeführt, welche als Zytostatika in der Krebsheilkunde verwendet werden (Blasiak et al., 1999; Unger et al., 2009). Einzelne Studien untersuchten die Gentoxizität der PGE Pt, Pd und Rh an *in vitro* Zellkulturen und fanden ein mutagenes Potential von Pt (Bünger et al., 1996; Pelka et al., 2009). Gagnon et al. (2006) untersuchten den Effekt des Platinsalzes H_2PtCl_6 (Pt(IV)) auf das Moos *Sphagnum* und die Sprague-Dawley Ratte und fanden ebenfalls ein gentoxisches Potential. In der vorliegenden Studie wurde erstmals das gentoxische Potential von Pt auf embryonale Stadien der Süßwasserorganismen *D. rerio* und *M. cornuarietis* untersucht. Es konnte gezeigt werden, dass, speziesabhängig und abhängig von verschiedenen Faktoren, sehr unterschiedliche Mutagenitäten bei den untersuchten Organismen auftraten. Da in der Umwelt Pt-Konzentrationen von bis zu 1,1 µg/L (Laschka et al., 1996) auftreten, legen diese Ergebnisse nahe, dass derartige Pt-Kontaminationen ökotoxikologisch nicht unbedenklich sind.

2. Untersuchungen zur platininduzierten Bauplanveränderung bei *Marisa cornuarietis* nach Platinexposition.

Kapitel 4: Osterauer, R., Marschner, L., Betz, O., Gerberding, M., Sawasdee, B., Cloetens, P., Haus, N., Sures, B., Triebskorn, R., Köhler, H.-R. 2010. Turning snails into slugs: Induced body plan changes and formation of an internal shell. Evol. Dev. 12: 474–483.

Nachdem sich in Vorversuchen zeigte, dass es ab einer bestimmten Pt-Konzentration während der Embryonalentwicklung der Paradies-Schnecke zu Veränderungen des Bauplans „prosobrancher Gastropoden" kommt, wurde dieses Phänomen in der vorliegenden Studie mittels unterschiedlichster Methoden untersucht. Dreißig Prozent der Schnecken, welche gegenüber 100 µg/L $PtCl_2$ exponiert wurden, und 100% der gegenüber 200 µg/L $PtCl_2$ exponierten Schnecken entwickelten während der Embryonalentwicklung keine Mantelhöhle und damit auch keine äußere Schale. Die Kieme verblieb somit unbedeckt hinter dem Herzen und der Viszeralsack bildete Hämocoel-Blasen. Die Torsion jedoch fand, zumindest teilweise statt, wie an der Position des Anus und der leichten Verschiebung der Kiemenposition erkennbar war. Pulsexpositionsversuche zeigten, dass das sensitive „Fenster", in welchem eine Platinexposition zu diesem Effekt führte, bei einer Temperatur von 26 ± 1°C an den Tagen 4 und 5 nach der Befruchtung lag.
Einzig äquimolare Konzentrationen von Ca^{2+}, welche der Pt^{2+}-enthaltenden Expositionslösung beigefügt wurden, vermochten diesen Effekt großteils zu verhindern. In diesem Fall trat der Effekt bei nur 16% der Schnecken auf. Von den gehäusetragenden Schnecken waren jedoch einige Schalen nicht groß genug, um den gesamten Eingeweidesack zu überdecken. Analyseverfahren ergaben allerdings, dass nicht, wie erwartet, durch die Calciumzugabe weniger Pt akkumuliert wurde, so dass es eher nahe liegt, dass Ca^{2+} platinsensitive Signaltransduktionswege stabilisiert.
Die ebenfalls getesteten PGE Rh^{2+}, Ir^{2+} (unpublizierte Daten) und Pd^{2+} führten nicht zu dem oben beschriebenen Effekt. Nur hohe Konzentrationen von Li^{+} (2,5 und 3 mg/L LiCl), einem Metall welches bei *Loligo pealeii* und *Xenopus laevis* zu Interaktionen mit dem positionalen System vorwiegend ekdodermaler Gewebe führte (Crawford, 2003; Kao et al., 1986) induzierten in einzelnen Fällen Schnecken ohne äußere Gehäuse.
Durch Färbung des schalensekretierenden Mantelrandes mit Diaminobenzidin bei unterschiedlichen Stadien während der Embryonalentwicklung und mittels

Rasterelektronenmikroskopie konnte beobachtet werden, dass sich der Mantelrand nicht, wie bei sich normal entwickelnden Schnecken, dreht und über den Eingeweidesack wächst, sondern an der ursprünglich embryonalen Position verbleibt und später vom Eingeweidesack überwachsen wird. Mit Hilfe der synchrotronen Röntgenstrahlen-Phasenkontrast-Mikrotomographie konnte gezeigt werden, dass daraufhin die Schale gedrungen und tütenförmig ins Körperinnere der Schnecke wächst. Die durch Pt induzierten Bauplanveränderungen traten nicht nur bei der prosobranchen Paradies-Schnecke, sondern auch bei den pulmonaten Posthorn-Schnecken *Biomphalaria glabrata* (N. Spiegel, unpublizierte Daten) und *P. corneus* auf. Da bei Pulmonaten die Mantelhöhle als Lunge dient, war bei diesen Schnecken, welche ebenfalls aufgrund der Platinexposition keine Mantelhöhle ausbildeten, die Lebensdauer auf wenige Wochen beschränkt.
In dieser Studie wurde erstmals eine durch einen Schadstoff induzierte Schaleninternalisierung nachgewiesen, welche im Laufe der Evolution mehrfach und unabhängig voneinander bei verschiedenen Molluskentaxa aufgetreten ist. Weiterhin wurde gezeigt, dass mindestens zwei Prozesse, welche mit dem Prozess der Torsion assoziiert sind, von diesem entkoppelt wurden. Dies waren die Bildung einer Mantelhöhle und die Position der Kieme, welche in diesem Fall hinter dem Herzen verblieb. Es konnte gezeigt werden, dass minimale Einflüsse auf das Entwicklungsprogramm während der Embryogenese zu nachhaltigen Veränderungen des Bauplans führen können.

5. Synopsis

Im ersten Teil der vorliegenden Dissertation wurden ökotoxikologische Untersuchungen zur Toxizität verschiedener Pt-Konzentrationen während der Embryonalentwicklung des Zebrabärblings und der Paradies-Schnecke durchgeführt. Analytische Messungen ergaben, dass Pt in den untersuchten Organismen in hohem Maße angereichert wurde. Bis auf die Ergebnisse zur Induktion des Stressproteins Hsp70 zeigte sich bei allen Biomarkern, dass *M. cornuarietis* sehr sensitiv gegenüber einer Pt-Exposition reagierte. Dies kann u.a. auf den außergewöhnlich hohen akkumulierten Pt-Gehalt zurückgeführt werden. Die Studien zeigen, dass Pt auch bereits bei umweltrelevanter Konzentration schädliche Einflüsse auf die Embryonalentwicklung der beiden untersuchten Organismen ausübt. So wurde bei einer Exposition gegenüber 0,1 µg/L $PtCl_2$ und allen höheren untersuchten Konzentrationen die Herzschlagrate beider Organismen signifikant verzögert und bei *M. cornuarietis* histopathologische Effekte der

Mitteldarmdrüse beobachtet. Ab einer Konzentration von 1 µg/L $PtCl_2$ waren bei *D. rerio* histopathologische Effekte auf Leber und Darm sichtbar und bei *M. cornuarietis* zeigte sich eine Verzögerung der Embryonalentwicklung und eine Schädigung der DNA.
Pt ist bis heute das am häufigsten vorkommende PGE in der Umwelt (Pan et al., 2009; Tsogas et al., 2009). Und da der Einsatz der PGE als Kfz-Katalysator in Zukunft vermutlich noch ansteigen wird und Konzentrationen, wie sie in den vorliegenden Studien untersucht wurden, in Straßenabflussgewässern gefunden wurden (Laschka et al., 1996), sollten sich dringend weitere Studien anschließen, welche die Auswirkungen der PGE auf die belebte Umwelt untersuchen.

Der zweite Teil der Dissertation befasste sich mit Untersuchungen zur platininduzierten Bauplanveränderung bei *Marisa cornuarietis*, welche nach einer Exposition gegenüber 100 und 200 µg/L $PtCl_2$ auftraten. Es zeigte sich, dass Individuen von *M. cornuarietis*, welche innerhalb eines kleinen „Zeitfensters" während der Embryonalentwicklung (Tag 4 und 5 nach der Befruchtung) gegenüber oben erwähnten Pt-Konzentrationen exponiert wurden, eine Umstrukturierung des prosobranchen Bauplans erfuhren: die Kieme verblieb am Hinterende der Schnecke und der schalenbildende Mantelrand verblieb an der ursprünglich embryonalen Stelle, so dass keine externe, sondern eine in den Körper wachsende kegelförmige interne Schale gebildet wurde. Dieser Effekt beschränkt sich dabei nicht nur auf *M. cornuarietis*, sondern konnte in modifizierter Form inzwischen auch bei den pulmonaten Schnecken *B. glabrata* (N. Spiegel, unpublizierte Daten) und *P. corneus* beobachtet werden. Allerdings sind diese Schnecken nicht, wie *M. cornuarietis*, über viele Monate hinweg lebensfähig, sondern sterben nach wenigen Wochen, da sich keine Mantelhöhle und somit auch keine Lunge, wie gewöhnlich bei den Pulmonaten, ausbildet. Der oben beschriebene Effekt ist bisher nur bei Pt, nicht jedoch bei den anderen untersuchten PGE Rh, Ir (unpublizierte Daten) und Pd aufgetreten.
Die hier vorgestellten Befunde sprechen dafür, dass sich während der Evolution die Körpergestalt von Organismen durch vergleichsweise geringfügige Modifikationen von Signaltransduktionswegen sprunghaft verändert haben könnte und bestärkt die Ansicht, dass die Individualentwicklung modular verläuft, dass heißt, drastische Veränderungen während der Entwicklung einer Variablen geschehen können, ohne die restlichen Variablen des Gesamtsystems zu beeinflussen. Da während der Evolution mehrfach und unabhängig voneinenander eine Internalisierung und/oder Reduktion der Schale stattfand (z.B. bei Tintenfischen

und Nacktschnecken) könnten die in diesen Untersuchungen erzeugten bauplanveränderten Schecken als entwicklungsbiologische Modelle für die Erklärung der Evolution innerer Schalen bei Weichtieren dienen. Da davon auszugehen ist, dass dieser Effekt von Pt auf eine veränderte Regulation von Entwicklungsgenen zurückzuführen ist, sollten sich zukünftige Untersuchungen zu Modifikationen dieser Genaktivitäten anschließen.

6. Literatur

Adams, S.M., Shepard, K.L., Greeley, M.S., Jr. Ryon, M.G., Jimenez, B.D., Shugart, L.R., McCarthy, J.F., Hinton, D.E., 1989. The use of bioindicators for assessing the effects of pollutant stress in fish. Mar. Environ. Res. 28: 459–464.

Adams, S.M., 2000a. Assessing sources of stress to aquatic ecosystems using integrated biomarkers. Biological resource managment connecting science and policy: 17-29.

Adams, S.M., Greenley, M.S., Ryon, M.G., 2000b. Evaluating effects of contaminants on fish health at multiple levels of biological organisation: Extrapolating from lower to higher levels. Hum. Ecol. Risk Assess. 6: 15–27.

Alloway, B.J., Ayres, D.C., 1997. Chemical principles of environmental pollution p. 147. London: Blackie Academic and Professional.

Blasiak, J., Kowalik, J., Trzeciak, A., Wojewodzka, M., 1999. Cytotoxicity and DNA damage and repair in human lymphocytes exposed to three anticancer platinum drugs. Neoplasma 46: 61–63.

Börries, A., 2001. Das Zierfischverzeichnis: Zebrabärbling/Zebrafisch *Danio rerio*. http://www.zierfischverzeichnis.de/klassen/pisces/cypriniformes/cyprinidae/danio_rerio.htm (Letzter Zugriff: 14.10.2010).

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72: 248–254.

Burnett, L.E., Woodson, P.B.J., Rietow, M.G. and Vilicich, V.C., 1981. Crab gill intra-epithelial carbonic anhydrase plays a major role in haemolymph CO_2 and chloride ion regulation. J. Exp. Biol. 92: 243–254.

Bünger, J., Stork, J., Stalder, K., 1996. Cyto- and genotoxic effects of coordination complexes of platinum, palladium and rhodium in vitro. Int. Arch. Occup. Environ. Health 69: 33–38.

Christensen, G.M, Tucker, J.H., 1976. Effects of selected water toxicants on the in vitro activity of fish carbonic anhydrase. Chem.-Biol. Interact. 13: 181–92.

Crawford, K., 2003. Lithium chloride inhibits development along the animal vegetal axis and anterior midline of the squid embryo. Biol. Bull. 205: 181–182.

Demian, E.S., Yousif, F., 1973a. Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda: Ampullariidae). I. General outlines of development. Malacologia 12: 123–150.

Demian, E.S., Yousif, F., 1973b. Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda: ampullariidae). II. Development of the alimentary system. Malacologia 12: 151–174.

Demian, E.S., Yousif, F., 1973c. Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda: Ampullariidae). III. Development of the circulatory and renal systems. Malacologia 12: 175–194.

Demian, E.S., Yousif, F., 1973d. Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda: Ampullariidae). IV. Development of the shell gland, mantle and respiratory organs. Malacologia 12: 195–211.

Demian, E.S., Yousif, F., 1975. Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda: Ampullariidae). V. Development of the nervous system. Malacologia 15: 29–42.

De Vos, E., Edwards, S.J., Mc Donald, I., Wray, D.S., Carey, P.J., 2002. A baseline survey of the distribution and origin of platinum group elements in contemporary fluvial sediments of the Kentish Stour, England. Appl. Geochem. 17: 1115 –1121.

Djingova, R., Kovacheva, P., Wagner, G., Markert, B., 2003. Distribution of platinum group elements and other traffic related elements among different plants along some highways in Germany. Sci. Total Environ. 308: 235–246.

Eaton, J.G., McKim, J.M., Holcombre, G.W., 1978. Metal toxicity to embryos and larvae of seven freshwater fish species. I. Cadmium. Bull. Environ. Contam. Toxicol. 19: 95–103.

Eckwert, H., Alberti, G., Köhler, H.-R., 1997. The induction of stress proteins (hsp) in *Oniscus asellus* (Isopoda) as a molecular marker of multiple heavy metal exposure: I. Principles and toxicological assessment. Ecotoxicology 6: 249–262.

Ek, K.H., Rauch, S., Morrison, G.M., Lindberg, P., 2004. Platinum group elements in raptor eggs, faeces, blood, liver and kidney. Sci. Total Environ. 334–335: 149–159.

Farago, M.E., Parsons, P.J., 1994. The effects of various platinum metal species on the water plant *Eichhornia Crassipes (Mart.)* Solms. Chem. Spec. Bioavail. 6: 1–12.

Feder, M.E., Hofmann, G.E., 1999. Heat-shock proteins, molecular chaperones and the stress response: Evolutionary and ecological physiology. Ann. Rev. Physiol. 61: 243–282.

Fent, K., 2007. Ökotoxikologie: Umwelchemie-Toxikologie-Ökologie, 3., überarb. und erw. Aufl., Georg Thieme Verlag, Stuttgart, New York.

Frank, S., Singer, C., Sures, B., 2008. Metallothionein (MT) response after chronic palladium exposure in the zebra mussel, *Dreissena polymorpha*. Environ. Res. 108: 309–314.

Gagnon, Z.E., Newkirk, C., Hicks, S., 2006. Impact of Platinum Group Metals on the Environment: A Toxicological, Genotoxic and Analytical Chemistry Study. J. Environ. Sci. Health A, 41: 397–414.

Gagnon, Z.E., Patel, A., 2007. Induction of metallothionein in chick embryos as a mechanism of tolerance to platinum group metal exposure. J. Environ. Sci. Health A 42: 381–87.

Golwer, A., Zereini, F., 1998. Anreicherung anorganischer Stoffe in Sedimenten eines Versickerbeckens der Bundesautobahn A3 bei Frankfurt am Main. Straße und Autobahn 4: 189–199.

Hallare, A.V., Köhler, H.-R., Triebskorn, R., 2004. Developmental toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and ist solvent, DMSO. Chemosphere 56: 659–666.

Hallare, A.V., Schirling, M., Luckenbach, T., Köhler, H.-R., Triebskorn, R., 2005. Combined effects of temperature and cadmium on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos. J. Therm. Biol. 30: 7–17.

Haus, N., Eybe, T., Zimmermann, S., Sures, B., 2009. Is microwave digestion using TFM vessels a suitable preparation method for Pt determination in biological samples by adsorptive cathodic stripping voltammetry? Anal. Chim. Acta 635: 53–57.

Heethoff, M. and Cloetens, P.A., 2008. Comparison of synchrotron X-ray phase contrast tomography and holotomography for non-invasive investigations of the internal anatomy of mites. Soil Organisms 80: 205–215.

Henry, R.P., 1991. Techniques for measuring carbonic anhydrase activity *in vitro*: the electrometric delta pH and pH stat methods. In: Dodgson, S.J., Tashien, R.E., Gros, G. & Carter, N.D. (eds). *The Carbonic Anhydrases*: *Cell. Physiol. Mol. Gen.* New York: Plenum Press. pp. 119–125.

Jouhaud, R., Biagianti-Risbourg, S., Vernet, G., 1999. Effets du platine chez *Brachydanio rerio* (Téléostéen, Cyprinidé). I. Toxicité aiguë; bioaccumulation et histopathologie intestinales. J. Appl. Ichthyol. 15: 41–48.

Kaiser, J., 2001. Bioindicators and Biomarkers of Environmental Pollution and Risk Assessment. Science Publishers, Inc. Enfield (NH), USA, Plymouth, UK.

Kao, K.R., Masui, Y., Elinson, R.P., 1986. Lithium-induced respecification of pattern in *Xenopus laevis* embryos. Nature (London) 322: 371–373.

Kimmel, C.B., 1989. Genetics and early development of zebrafish. Trends Gen. 5: 283–288.

Klaassen, C.D., 1996. Casarett & Doull's Toxicology—The Basic Science of Poisons, Fifth Edition; McGraw-Hill: New York, 725–726.

Kosmehl, T., Hallare, A.V., Reifferscheid, G., Manz, W., Braunbeck, T., Hollert, H., 2006. A novel contact assay for testing genotoxicity of chemicals and whole sediments in zebrafish embryos. Environ. Toxicol. Chem. 25: 2097–106.

Kümmerer, K., Helmers, E., Hubner, P., Mascart, G., Milandri, M., Reinthaler, F., Zwakenberg, M., 1999. European hospitals as a source for platinum in the environment in comparison with other sources. Sci. Total Environ. 225: 155–165.

Köhler, H.-R., Triebskorn, R., 1998. Assessment of the cytotoxic impact of heavy metals on soil invertebrates using a protocol integrating qualitative and quantitative components. Biomarkers 3: 109–127.

Köhler, H.-R., Triebskorn, R., Stöcker, W., Kloetzel, P.M., Alberti, G., 1992. The 70 kD Heat Shock Protein (hsp70) in Soil Invertebrates: A Possible Tool for Monitoring Environmental Toxicants. Arch. Environ. Contam. Toxicol. 22: 334-338.

König, H.P., Hertel, R.F., Koch, W., Rosner, G., 1992. Determination of platinum emissions from a three-way catalyst-equipped gasoline engine. Atmos. Environ. 26A: 741–745.

Laemmli, U.K., 1970. Cleavage of structural proteins durino assembly of the head of bacteriophage T4. Nature 227: 680–684.

Laschka, D., Striebel, T., Daub, J., Nachtwey, M., 1996. Platinum in rainwater discharges from roads. UWSF – Z Umweltchem Ökotox 8: 124–129.

Lustig, S., Zang, S., Beck, W., Schramel, P., 1998. Dissolution of metallic platinum as water soluble species by naturally occurring complexing agents. Mikrochim. Acta 129: 189–194.

McKim, J.M., 1977. Evaluation of tests with early life stages of fish for predicting long-term toxicity. J. Fish Res. Bd. Can. 34: 1148–54.

Meyers, T.R., Hendricks, J.D., 1982. A summary of tissue lesions in aquatic animals induced by controlled exposures to environmental contaminants, chemotherapeutic agents, and potential carcinogens. Mar. Fish Rev. 44: 1–17.

Moldovan, M., Palacios, M.A., Gomez, M.M., Morrison, G., Rauch, S., McLeod, C., Ma, R., Caroli, S., Alimonti, A., Petrucci, F., Bocca, B., Schramel, P., Zischka, M., Pettersson, C., Wass, U., Luna, M., Saenz, J.C., Santamaria, J., 2002. Environmental risk of particulate and soluble platinum group elements released from gasoline and diesel engine catalytic converters. Sci. Total Environ. 296: 199–208.

Morgan, I.J., Henry, R.P., Wood, C.M., 1997. The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) is inhibition of Na- and Cl- transport. Aquat. Toxicol. 39: 145–63.

Nagel, R., Isberner, K., 1998. Testing of chemicals with fish – a critical evaluation of tests with special regard to zebrafish. In: Braunbeck, T., Hinton, D.E. & Streit, B. (1998): Fish Exotox, Birkhäuser, Basel.

Nagel, R., 2002. DarT: The Embryo Test with the Zebrafish *Danio rerio* – a General Model in Ecotoxicology and Toxicology. ALTEX 19, Suppl 1.

Nüsslein-Volhard, C., 1994. Of flies and fishes. Science 266: 572–576.

OECD Guideline for Testing of Chemicals., 1992. Test Guideline 203. Fish, Acute Toxicity Test. OECD Guideline for Testing of Chemicals.

Oehlmann, J., Schulte-Oehlmann, U., Tillmann, M., Markert, B., 2000. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part I: Bisphenol A and octylphenol as xeno-estrogens. Ecotoxicology 9: 383–397.

Oehlmann, J., Schulte-Oehlmann, U., Bachmann, J., Oetken, M., Lutz, I., Kloas, W., Ternes, T.A., 2006. Bisphenol A induces superfeminization in the ramshorn

snail *Marisa cornuarietis* (Gastropoda: Prosobranchia) at environmentally relevant concentrations. Environ. Health Perspect 114: 127–133.

Palacios, M.A., Gómez, M.M., Moldovan, M., Morrison, G.M., Rauch, S., McLeod, C., Ma, R., Laserna, J., Lucena, P., Caroli, S., Alimonti, A., Petrucci, F., Bocca, B., Schramel, P., Lustig, S., Zischka, M., Wass, U., Stenbom, B., Luna, M., Saenz, J.C., Santamaría, J., Torrens, J.M., 2000. Platinum-group elements: quantification in exhaust fumes and surface modifications of the catalytic surface. Sci. Total Environ. 257: 1–15.

Pan, S., Zhang, G., Sun, Y., Chakraborty, P., 2009. Accumulating characteristics of platinum group elements (PGE) in urban environments, China. Sci. Total Environ. 407: 4248–4252.

Pelka, J., Gehrke, H., Esselen, M., Turk, M., Crone, M., Brase, S., Muller, T., Blank, H., Send, W., Zibat, V., Brenner, P., Schneider, R., Gerthsen, D., Marko, D., 2009. Cellular uptake of platinum nanoparticles in human colon carcinoma cells and their impact on cellular redox systems and DNA integrity. Chem. Res. Toxicol. 22: 649–659.

Rousseau, M., Plouguerne, E., Wan, G., Wan, R., Lopez, E., and Fouchereau-Peron, M., 2003. Biomineralization markers during a phase of active growth in *Pinctada margaritifera*. Comp. Biochem. Physiol. 135: 271–278.

Sastry, K.V., Gupta, P.K., 1978. Effect of mercuric chloride on the digestive system of *Channa punctatus*. A histopathological study. Environ. Res. 16: 270–278.

Sastry, K.V., Gupta, P.K., 1979. The effect of cadmium on the digestive system of the teleost fish, *Heteropneustes fossilis*. Environ. Res. 19: 221–230.

Sawasdee, B., Köhler, H.-R., 2009. Embryo toxicity of pesticides and heavy metals to the ramshorn snail, *Marisa cornuarietis* (Prosobranchia). Chemosphere 75: 1539–1547.

Scheil, V., Triebskorn, R., Köhler, H.-R., 2008. Cellular and stress protein responses to the UV-filter 3-benzylidene camphor in the amphipod crustacean *Gammarus fossarum* (Koch 1835). Arch. Environ. Contam. Toxicol. 54: 684–689.

Scheil, V., Zürn, A., Triebskorn, R., Köhler, H.-R., 2010. Embryo development, stress protein (Hsp70) responses and histopathology in zebrafish (*Danio rerio*) following exposure to nickel chloride, chlorpyrifos and binary mixtures of them. Environ. Toxicol. 25: 83–93.

Schirling, M., Bohlen, A., Triebskorn, R., Köhler, H.-R., 2006. An invertebrate embryo test with the apple snail *Marisa cornuarietis* to assess effects of potential developmental and endocrine disruptors. Chemosphere 64: 1730–1738.

Schnurstein, A., Braunbeck, T., 2001. Tail moment versus tail length—Application of an in vitro version of the comet assay in biomonitoring for genotoxicity in native surface waters using primary hepatocytes and gill cells from zebrafish (*Danio rerio*). Ecotoxicol. Environ. Saf. 49: 187–196.

Schulte-Oehlmann, U., Tillmann, M., Market, B., Oehlmann, J., 2000. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in laboratory. Part II. Triphenyltin as a xeno-androgen.. Ecotoxicology 9, 399–412.

Singer, C., Zimmermann, S., Sures, B., 2005. Induction of heat shock proteins (hsp70) in the zebra mussel (*Dreissena polymorpha*) following exposure to platinum group metals (platinum, palladium and rhodium): comparison with lead and cadmium exposures. Aquat. Toxicol. 75: 65–75.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175: 184–191.

Spiegel, N., 2011. Auswirkungen von Platin auf die Embryonalentwicklung von *Biomphalaria glabrata* (Mollusca, Planorbidae). Staatsexamensarbeit, Eberhard-Karls-Universität Tübingen

Stebbing, A.R.D., 1985. A possible synthesis. In: B.L. Bayne (Editors), The Effects of Stress and Pollution on Marine Animals. Praeger, New York.

Sures, B., Taraschewski, H. and Haug, C., 1995. Determination of trace metals (Cd, Pb) in fish by electrothermal atomic absorption spectrometry after microwave digestion. Anal. Chim. Acta 311: 135–139.

Sures, B., Zimmermann, S., Messerschmidt, J., von Bohlen, A., Alt, F., 2001. First report on the uptake of automobile catalyst emitted palladium by European eels (*Anguilla anguilla*) following experimental exposure to road dust. Environ. Pollut. 113: 341–345.

Sures, B., Zimmermann, S., 2007. Impact of humic substances on the aqueous solubility, uptake and bioaccumulation of platinum, palladium and rhodium in exposure studies with *Dreissena polymorpha*. Environ. Pollut. 146: 444–451.

Takaichi, S., Mizuhira, V., Hasegawa, H., Suzaki, T., Notoya, M., Ejiri, S., Ozawa, H. and Van Wyk, J.H., 2003. Ultrastructure and early embryonic shell formation in the terrestrial pulmonate snail, *Euhadra hickonis*. J. Moll. Stud. 69: 229–244.

Tanhan, P., Sretarugsa, P., Pokethitiyook, P., Kruatrachue, M., Upatham, E.S., 2005. Histopathological alterations in the edible snail, *Babylonia areolata* (spotted babylon), in acute and subchronic cadmium poisoning. Environ. Toxicol. 20: 142–149.

Triebskorn, R., 2003. Biomarkeruntersuchungen an einheimischen Fischen: Moderne Instrumente zur Erfassung von komplexen Gewässerbelastungen. Schriftenreihe des Landesfischereiverbandes Bayern 9: 37–42.

Triebskorn, R., Adam, S., Casper, H., Honnen, W., Müller, E. F., Pawert, M., Schramm, M., Schwaiger J., Köhler, H.-R., 2000. Biomarkers as diagnostic tools for evaluating toxicological effects of past water quality conditions on stream organisms. Ecotoxicology 11: 451–465.

Triebskorn, R., Casper, H., Heyd, A., Eikemper, R., Köhler, H.-R., Schwaiger, J., 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac. Part II: Cytological effects in liver, kidney, gills and intestine of rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 68: 151–166.

Tsogas, G.Z., Giokas, D.L., Vlessidis, A.G., Aloupi, M., Angelidis, M.O., 2009. Survey of the Distribution and Time-Dependent Increase of Platinum-Group Element Accumulation Along Urban Roads in Ioannina (NW Greece). Water Air Soil Pollut. 201: 265–281.

Unger, F.T., Klasen, H.A., Tchartchian, G., de Wilde, R.L., Witte, I., 2009. DNA damage induced by cis- and carboplatin as indicator for in vitro sensitivity of ovarian carcinoma cells. BMC CANCER 9, Article Number: 359.

Vitale, A.M., Monserrat, J.M, Castilho, P., Rodriguez, E.M., 1999. Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulata* (Decapoda, Grapsidae). Comp. Biochem. Physiol. C 122: 121–129.

Wang, X.S., Sun, C., 2009. Pt and Pd concentrations and source in urban roadside soils from Xuzhou, China. Environ. Geol. 56: 1129–1133.

Whiteley, J.D., Murray, F., 2005. Autocatalyst-derived platinum, palladium and rhodium (PGE) in infiltration basin and wetland sediments receiving urban runoff. Sci. Total Environ. 341: 199–209.

Wilbur, K.M., Saleuddin, A.S.M., 1983. Shell formation. In: Saleuddin, A.S.M., Wilbur, K.M. (Eds.), The Mollusca, vol. 4. Academic Press, New York, London, pp. 235–287.

Zereini F, Alt F, Rankenburg K, Beyer JM, Artelt S., 1997. The distribution of platinum group elements (PGE) in the environmental compartments of soil, mud, roadside dust, road sweeping and water: emission of the platinum group elements (PGE) from motor vehicle catalytic converters. UWSF–Z Umweltchem. Ökotox. 9: 193 –200.

Zimmermann, S., Menzel, C.M., Berner, Z., Eckhardt, J.D., Stuben, D., Alt, F., Messerschmidt, J., Taraschewski, H., 2001. Trace analysis of platinum in biological samples: a comparison between sector field ICP-MS and adsorptive cathodic stripping voltammetry following different digestion procedures. Anal. Chim. Acta 439: 203–209.

Zimmermann, S., Messerschmidt, J., von Bohlen, A., Sures, B., 2003. Determination of Pt, Pd and Rh in biological samples by electrothermal atomic absorption spectromety as compared with adsorptive cathodic stripping voltammetry and total-reflexion X-ray fluorescence analysis. Anal. Chim. Acta 498: 93–104.

Zimmermann, S., Baumann, U., Taraschewski, H., Sures, B., 2004. Accumulation and distribution of platinum and rhodium in the european eel *Anguilla anguilla* following aqueous exposure to metal salts. Environ. Pollut. 127: 195–202.

Eigenanteil an den durchgeführten Arbeiten bei den in der vorliegenden Dissertation integrierten Publikationen

Kapitel 1:

Osterauer, R., Haus, N., Sures, B. and Köhler, H.-R., 2009. Uptake of platinum by zebrafish (*Danio rerio*) and ramshorn snail (*Marisa cornuarietis*) and resulting effects on early embryogenesis. Chemosphere 77: 975–982.

Vollständiger Eigenanteil an der Versuchsplanung, Durchführung und Auswertung. Die analytischen Messungen wurden unter Mithilfe von Nadine Haus (Universität Duisburg-Essen) durchgeführt. Die fachliche Betreuung erfolgte durch Prof. Dr. Bernd Sures (Universität Duisburg-Essen) und Prof. Dr. Heinz-R. Köhler (Universität Tübingen).

Kapitel 2:

Osterauer, R., Köhler, H.-R. and Triebskorn, R., 2010. Histopathological alterations and induction of stress proteins in ramshorn snail (*Marisa cornuarietis*) and zebrafish (*Danio rerio*) after exposure to $PtCl_2$ during embryogenesis. Aquat. Toxicol. 99: 100–107.

Vollständiger Eigenanteil an der Versuchsplanung, Durchführung und Auswertung. Die fachliche Betreuung erfolgte durch Prof. Dr. Rita Triebskorn (Universität Tübingen) und Prof. Dr. Heinz-R. Köhler (Universität Tübingen).

Kapitel 3:

Osterauer, R., Faßbender, C., Braunbeck, T., Köhler, H.-R., 2011. Genotoxicity of platinum in embryos of zebrafish (*Danio rerio*) and ramshorn snail (*Marisa cornuarietis*). Sci. Total Environ. 409: 2114–2119.

Vollständiger Eigenanteil an der Versuchsplanung, Durchführung und Auswertung. Die Testerlernung und Auswertung fand an der Universität Heidelberg statt. Die fachliche Betreuung erfolgte durch Christopher Fassbender (Universität Heidelberg) und Prof. Dr. Heinz-R. Köhler (Universität Tübingen), sowie durch Prof. Dr. Thomas Braunbeck (Universität Heidelberg).

Kapitel 4:

Osterauer, R., Marschner, L., Betz, O., Gerberding, M., Sawasdee, B., Cloetens, P., Haus, N., Sures, B., Triebskorn, R., Köhler, H.-R., 2010. Turning snails into slugs: Induced body plan changes and formation of an internal shell. Evol. Dev. 12: 474–483.

Vollständiger Eigenanteil an der Versuchsplanung, Durchführung und Auswertung bei den Expositionsversuchen mit Platin, der Histologie, der Carboanhydrase-Messung und der Diaminobenzidin-Färbung. Die analytischen Messungen wurden unter Mithilfe von Nadine Haus (Universität Duisburg-Essen) durchgeführt. Die rasterelektronischen Bilder wurden von mir und Leonie Marschner (Universität Tübingen) in der Arbeitsgruppe von Prof. Dr. Oliver Betz (Universität Tübingen) erstellt. Der Großteil der Pulsexpositionsversuche wurde von mir (Universität Tübingen) durchgeführt. Ein Versuch zur zweitägigen Pulsexposition mit Platin wurde von Leonie Marschner (Universität Tübingen) durchgeführt. Die Arbeiten zu den Pulsexpositionsversuchen mit Platin und Calcium, und die Versuche mit *Planorbarius corneus*, sowie die Optimierung des Kunstwassers für *M. cornuarietis* wurden von Leonie Marscher (Universität Tübingen) durchgeführt. Die Versuche mit Lithium und Palladium wurden von Banthita Sawasdee (Universität Tübingen) durchgeführt. Die Untersuchung der internen Schalen mittels synchrotroner Röntgenstrahlen-Phasenkontrast-Mikrotomographie wurde von Dr. Peter Cloetens (European Synchrotron Radiation Facility, Grenoble, France) in Zusammenarbeit mit Prof. Dr. Oliver Betz (Universität Tübingen) durchgeführt.
Die fachliche Betreuung erfolgte durch Prof. Dr. Heinz-R. Köhler (Universität Tübingen; experimentelles Design), Prof. Dr. Rita Triebskorn (Universität Tübingen; Histologie), Prof. Dr. Bernd Sures (Universität Duisburg-Essen; Analytik) und Dr. Matthias Gerberding (Universität Hohenheim bzw. MPI für Entwicklungsbiologie, Tübingen; Entwicklungsbiologie).

Kapitel 1: Uptake of platinum by zebrafish (*Danio rerio*) and ramshorn snail (*Marisa cornuarietis*) and resulting effects on early embryogenesis

Raphaela Osterauer [a], Nadine Haus [b], Bernd Sures [b], Heinz-R. Köhler [a]

[a] Animal Physiological Ecology Department, University of Tübingen, Konrad-Adenauer-Str. 20, 72072 Tübingen, Germany

[b] Department of Applied Zoology/Hydrobiology, University of Duisburg-Essen, Universitätsstr. 5, 45141 Essen, Germany

Abstract

Platinum group elements (PGEs), platinum, palladium and rhodium are widely used in automobile catalytic converters. PGEs are emitted into the environment and enter the aquatic ecosystem *via* runoff rainwater. The present study investigated the bioavailability of platinum chloride for the zebrafish (*Danio rerio*) and the ramshorn snail (*Marisa cornuarietis*) and determined the bioaccumulation rate of platinum. Applying the fish early life stage assay for *D. rerio* (DarT) and the *Marisa* embryo toxicity test ("Mariett") for *M. cornuarietis*, effects of platinum chloride on the embryonic development were investigated. Platinum concentrations tested in this study ranged from environmentally relevant concentrations of 38 ng L^{-1} up to a concentration of 74.2 µg L^{-1} for *D. rerio* and of 200 ng L^{-1} up to 98.7 µg L^{-1} for *M. cornuarietis*. Platinum was found to be accumulated in both organisms. Bioaccumulation factors (*BAFs*) were in the range of 5–55 for *D. rerio* and of 218.4–723.9 for *M. cornuarietis*, depending on the tested Pt concentrations. During the embryonic development, platinum was shown to alter the heart rate of both organisms already at the lowest tested concentration. At higher concentrations, platinum decelerated the hatching rate of the embryos of both species. Additionally, a retardation of the general development and a loss of weight due to platinum exposure was observed in *M. cornuarietis*. Results of this study contribute important data on the ecotoxicity of a rarely studied element.

Keywords: Platinum group elements (PGEs), Bioavailability, Embryonic development, Ecotoxicology

Chemosphere, 2009, 77: 975–982

Introduction

Since the introduction of the platinum group elements (PGEs) platinum, palladium and rhodium in automobile catalytic converters, the emission rate of platinum into the environment increased substantially (Barbante et al., 2001). As a consequence of chemical and physical processes and mechanical abrasion during vehicle operation, exhaust containing PGEs is released (Barefoot, 1997). Dust containing PGEs mainly deposits along roadsides and reaches aquatic ecosystems *via* road runoff or atmospheric transportation. PGEs used as catalytic converters are mainly emitted in metallic form and are bound to particles (Moldovan et al., 2002). It has been shown, however, that these metals can be transformed through environmental processes, e.g. through oxidation (Lustig et al., 1996). Speciation of metals is a very important factor as it determines the degree of bioavailability and toxicity. In fact, up to 10% of the total platinum emissions are soluble (Hill and Mayer, 1977; König et al., 1992) and, contrary to the assumption that emitted noble metals behave inert, platinum is ionizable in the environment and thus potentially biologically active (Klaassen, 1996). Furthermore, the presence of humic substances and further natural complexing agents may increase the solubility and the biological availability of these noble metals (Lustig et al., 1998; Zimmermann et al., 2002, 2003b). Accordingly, bioaccumulation of particulate as well as dissolved platinum has been shown for terrestric and aquatic plants and animals (Lustig et al., 1997; Jouhaud et al., 1999; Rauch and Morrison, 1999; Moldovan et al., 2001; Sures et al., 2001; Zimmermann et al., 2002; Sures and Zimmermann, 2004). Despite their broad distribution in the environment, however, little is known about adverse effects of PGEs.

Fish and aquatic gastropods are important elements of aquatic ecosystems and are therefore widely used as model organisms for ecotoxicological studies (Nagel and Isberner, 1998; Oehlmann et al., 2006). The zebrafish (*Danio rerio*) is a tropical fish belonging to the family Cyprinidae. This test organism is easy and cost-effective to maintain, breeds almost all year round and has a short generation time. Through a transparent chorion the embryonic development is easy to observe. Because of the broad knowledge about its morphometrical, biochemical and physiological features (Hisoaka and Battle, 1958; Kimmel et al., 1995) the zebrafish is a widely accepted model organism in ecotoxicology used to assess the risk of potentially noxious pollutants (Hill et al., 2005). The embryo test with *D. rerio* (DarT) using zebrafish embryos was suggested by Nagel (2002) to replace the acute fish test with juvenile or adult fish. The test is based on the fact that embryonic developmental stages are

usually regarded to be most sensitive to chemicals during fish development (McKim et al., 1975; Eaton et al., 1978; Suter et al., 1987; Luckenbach et al., 2001). Furthermore, the test provides advantages in respect to animal protection laws and for ethical reasons. During the test, which normally lasts for 48 h, observation of defined sublethal endpoints provides information about the effects of tested substances. The test can be prolonged to 96 h or even 120 h to detect effects on additional sublethal endpoints.

Because 95% of all known species are invertebrates, a toxicity assessment of a given chemical should also consider invertebrates. In this context, a species which has gained more and more attention during the last years is the ramshorn snail *Marisa cornuarietis* (Ampullariidae), a freshwater snail originating from Central and South America. This species has been used in a number of ecotoxicological studies revealing a high sensitivity to endocrine disruptive substances (Oehlmann et al., 2000; Schulte-Oehlmann et al., 2000; Oehlmann et al., 2006) and to heavy metals (Schirling et al., 2006; Sawasdee and Köhler, 2009). The largely transparent chorion also allows an unhampered observation of the embryonic development in this species. Recently, Schirling et al. (2006) have developed the *Marisa* embryo toxicity test ("Mariett") which turned out to be a promising tool in invertebrate embryonic toxicology. The test takes approximately 12 d, from fertilisation up to the hatching of the embryos.

In the present study, effects of different concentrations of the bivalent platinum ion – as a representative of PGEs – are investigated on the early development of the ramshorn snail and the zebrafish. To determine the accumulative potential of platinum in both species, platinum accumulation in the tissue of the organisms was determined. Particular emphasis was laid on the comparison of the severity of platinum effects on embryos of both test species.

Material and methods

Source and maintenance of the test animals

Test animals used in this study were *D. rerio* and *M. cornuarietis*. A zebrafish breeding stock (*D. rerio*, strain: WIK, ZFIN ID: ZDB-GENO-010531-2) was originally obtained from the Max-Planck-Institute for Developmental Biology, Tübingen, Germany (C. Nüsslein-Volhard group) and a breeding stock of ramshorn snail (*M. cornuarietis*) derived from Frankfurt/Main University, Germany (J. Oehlmann group).

Both sexes of *D. rerio* and both sexes of *M. cornuarietis*, respectively, were kept together in 150 and 230 L aquaria under the following conditions: temperature: 26 ± 1 °C, pH: 7.5–8, conductivity for fish: 400 µS cm^{-1}, for snails: 1000 µS cm^{-1}, light/dark regime: 12 h/12 h. Water for fish was exchanged every 10–14 d, for snails every week. The fish were fed twice a day, in the morning with commercially available artificial diet (Nutrafin Max, Hagen, Germany) and in the evening with frozen food from unpolluted sources (mosquito larvae, Mysis, Moina, Artemia). The snails were fed a commercially available artificial diet (Nutrafin Max, Hagen, Germany) once a day.

Table 1 Aqueous concentrations of platinum as determined by inductively coupled plasma mass spectrometry and adsorptive cathodic stripping voltammetry in the exposure media used for tests with *Danio rerio* and *Marisa cornuarietis*. Data show means ± standard deviation of 3 aliquots.

Nominal concentration of $PtCl_2$ [µg L^{-1}]	Aqueous concentration of platinum used for *Danio rerio* [µg L^{-1}]	Aqueous concentration of platinum used for *Marisa cornuarietis* [µg L^{-1}]
0	below the detection limit	below the detection limit
0.1	0.202 ± 0.015	0.038 ± 0.002
1	1.230 ± 0.207	0.872 ± 0.304
10	8.084 ± 0.142	3.662 ± 0.186
50	44.238 ± 1.100	33.732 ± 0.211
100	98.659 ± 0.962	74.203 ± 5.273

Experimental design

Whenever eggs of *M. cornuarietis* were required, snails were fed fresh carrots 2 d before. Egg masses, laid at night were collected from the aquaria in the respective mornings. To receive the eggs of *D. rerio*, spawning traps covered with stainless steel mesh were placed on the bottom of the aquaria in the evening. The next morning spawning and fertilisation was initiated by sudden illumination of the aquaria, and terminated one hour later by removal of the spawning boxes. Eggs could be gathered through sieving the eggs which had been deposited in the spawning boxes.

Eggs of fish or snails were exposed to platinum chloride (platinum standard solution 1000 µg mL^{-1}, Ultra Scientific, Germany). The following nominal concentrations were applied: 0.1, 1, 10, 50 and 100 µg L^{-1} $PtCl_2$. Due to the known precipitation of Pt during exposure experiments (Sures and Zimmermann, 2007) aqueous Pt concentrations were determined in the exposure media (see Table 1).

For fish, reconstituted water (OECD Guideline 203, Annex 2) served as the overall control. Since reconstituted water was found to be inappropriate for *M. cornuarietis* maintenance, control tap water was taken from the snail aquaria.

After fertilisation, eggs of *D. rerio* (n = 10) or *M. cornuarietis* (n = 20) were distributed to plastic Petri dishes containing the different concentrations of $PtCl_2$ or the control medium. The exposure period was 96 h for *D. rerio*. As embryonic development in *M. cornuarietis* is slower, snail eggs were exposed for 14 d. This time was chosen relatively to the hatching date of *M. cornuarietis* which was compared to that of *D. rerio*. Both assays were conducted with nine replicates. Throughout the exposure period, embryos were kept at 26 °C in a climate chamber and were only removed for monitoring their development from blastula to early life stages at defined time points using a stereomicroscope (detailed description of the endpoints in Table 2). At any time of observation, coagulated embryos were removed to avoid contamination of the medium. During the embryo tests, animals were not fed.

To compare similar developmental stages in both *D. rerio* and *M. cornuarietis*, the much slower development of *M. cornuarietis* necessitated different lengths of the exposure period for the two species. Since exposure time was a maximum of 7 d for *D. rerio* and 26 d for *M. cornuarietis* in biomarker battery studies which have been conducted in parallel to the reported investigations but are not subject to this article, accumulation of Pt was measured after 7 d of exposure to $PtCl_2$ for *D. rerio* and after 26 d for *M. cornuarietis*. In this context also the weight of *M. cornuarietis* was measured after 26 d of exposure. Since *D. rerio* nourish on yolk up to the 12th day after fertilisation (Rombough, 2002), fish were not fed during the exposure time of 7 d. *M. cornuarietis*, however needed to be supplied with equal portions of commercially available artificial diet (Nutrafin Max, Hagen, Germany) once a day after the eclosion.

Table 2 Details of the test procedures for the embryo test with *Danio rerio* and *Marisa cornuarietis*

	Danio rerio	*Marisa cornuarietis*
Specified time points of observation	8, 12, 24, 48, 60, 72, 84 and 96 h after fertilisation	every 24 hours at 9.00h a.m.
Replacement of the medium	every 48 hours	every 24 hours
Test procedure arranged according to	OECD guideline for the *Early Life Stage* toxicity-Test (OECD 210, 1992b) and Schulte & Nagel (1994).	Schirling et al. (2006)
Endpoints	% of mortality, individuals showing gastrulation, somite formation, blood circulation, pigmentation, tail detachment, defects of the eyes and the brain, edemas, heart rate (heart beats per minute), and hatching success	% of mortality, individuals developing tentacles and eyes, malformations, heart rate and hatching success
A test was considered valid, if	90% of the control animals did not show any pathological effects	80% of the control animals did not show any pathological effects

Analytical procedure

Analysis of accumulated platinum in the organisms

After the respective exposure times, animals were frozen in liquid nitrogen and stored at -20 °C. Platinum accumulated in the organisms was detected with adsorptive cathodic stripping voltammetry (ACSV) after digestion *via* high pressure ashing according to Zimmermann et al. (2001, 2003a). Fresh tissue samples (*D. rerio* about 8 mg each and *M. cornuarietis* about 100 mg each) were digested by adding 4 mL HNO_3 (65 vol.%, sub-boiled) and 0.5 mL HCl (30 vol.%, suprapure, Merck, Germany) into 70 mL quartz vessels. Using a high pressure-asher (HPA-S, Anton Paar, Austria) samples were completely mineralized at a temperature of 320 °C and pressure conditions of 12 MPa. To determine contamination during the preparation process and the analytical limit of detection (LOD) procedural blanks were prepared without the insertion of sample material in a regular manner. As

nitric acid interferes with the voltammetric detection, it has to be removed. Therefore 0.2 mL H_2SO_4 (95 vol.% suprapure quality, Merck, Germany) and 0.5 mL HCl (30 vol.%, suprapure quality, Merck, Germany) were added to the digestion solution and evaporated to 0.4 mL. Then 0.5 mL of HCl was added for another evaporation. This step was repeated until no further nitric acids evaporated out of the digestion solution (visually assessed by the absence of brownish gases). The resulting solution was filled up to 5 mL with double deionized water. Voltammetric measurements were conducted with a VA prozessor 693, VA-Stand 694 (Metrohm, Germany). Detailed instrumental setups are listed in Zimmermann et al. (2001, 2003a) and Haus et al. (2009). Platinum concentrations of the electrolyte solution were checked for contamination prior to each sample measurement. Pt content in the samples was calculated from the resulting regression line ($R^2 > 0.99$) after a standard addition method, which was performed with diluted metal standard solutions (Certi Pur, 1000 mg L^{-1} H_2PtCl_6 in HCl 2 M, Merck, Germany). The LOD for platinum in the samples was defined to be threefold the standard deviation of the measurements of blanks. For the average sample weight of 8 mg for *D. rerio* the LOD was found to be 1.62 ng g^{-1} and for the average sample weight of 100 mg for *M. cornuarietis* it was found to be 0.12 ng g^{-1}.

Analysis of platinum in the exposure medium

The content of platinum in the exposure medium was measured with inductively coupled plasma mass spectrometry (ICP-MS, Elan 5000, Perkin Elmer, Germany) for nominal concentrations ranging from 1 to 100 µg L^{-1} $PtCl_2$. Yttrium and thulium were used as internal standards. Platinum concentrations in the water samples were calculated from the corresponding regression lines by using different concentrations of a platinum standard solution (1000 µg mL^{-1}, Ultra Scientific, Germany). The LOD for platinum was defined to be threefold the standard deviation of the measurements of blanks andwas found to be 0.21 µg L^{-1}. Because of the low concentration of platinum in the exposure medium with the nominal concentration of 0.1 µg L^{-1}, it was measured with adsorptive cathodic stripping voltammetry (ACSV). Therefore samples of 1 mL were digested using the HPA-S. The detection procedure was the same as for the tissue material. Each sample was analyzed in triplicate.

Data analyses

For statistical analyses means and standard deviations were calculated using Microsoft Excel. Graphs were generated using Microsoft Excel or SigmaPlot 2000 (SPSS Science, USA). For data which corresponded with normality (Shapiro–Wilk test, JMP 4.0, SAS Systems, USA) the parametric multiple comparison Tukey–Kramer test (JMP 4.0, SAS Systems, USA) was applied to compare means of all treatment groups versus the control. For non-parametric data the Wilcoxon test (JMP 4.0, SAS Systems, USA) was used to detect significant differences between the treatment groups versus the control.

Levels of significant differences were defined to be slightly significant for $0.01 < p \leq 0.05$ (*), significant for $0.001 < p \leq 0.01$ (**), and highly significant for $p \leq 0.001$ (***).

For graphs showing the accumulated Pt in the organisms, "best fit" functions were selected on the basis of the R^2 level (R^2 for *D. rerio* = 0.99, R^2 for *M. cornuarietis* = 0.95). Data for the hatching rate for both organisms and the development of the eyes for *M. cornuarietis* were subjected to linear regression analyses (SigmaPlot 2000, SPSS Science, USA).

Bioaccumulation factors (*BAFs*) were calculated according to the equation

$BAF = c_o / c_m$

in which *BAF* is the quotient between the Pt concentration in the organism (c_o) and the Pt concentration in the exposure medium (c_m). *BAFs* were meant to refer to the platinum uptake from the water. However, since *M. cornuarietis* exposured for bioaccumulation measurements was fed post hatch with uncontaminated fish flakes, a possible uptake of Pt portions absorbed to the food cannot be excluded.

Results

Bioaccumulation of platinum in D. rerio

To facilitate the reading of this article, nominal concentrations are used throughout. Analyses of platinum in *D. rerio* showed a clear uptake and accumulation of this metal in fish embryos (Table 3, Fig. 1a). In control animals platinum concentrations were below the LOD. For exposed embryos *BAFs* were found to range between 5 and 55 (Table 3). Platinum uptake of *D. rerio* was found to be concentration depending and rose constantly with increasing concentrations of platinum in the exposure media ($R^2 = 0.99$, see Fig. 1a).

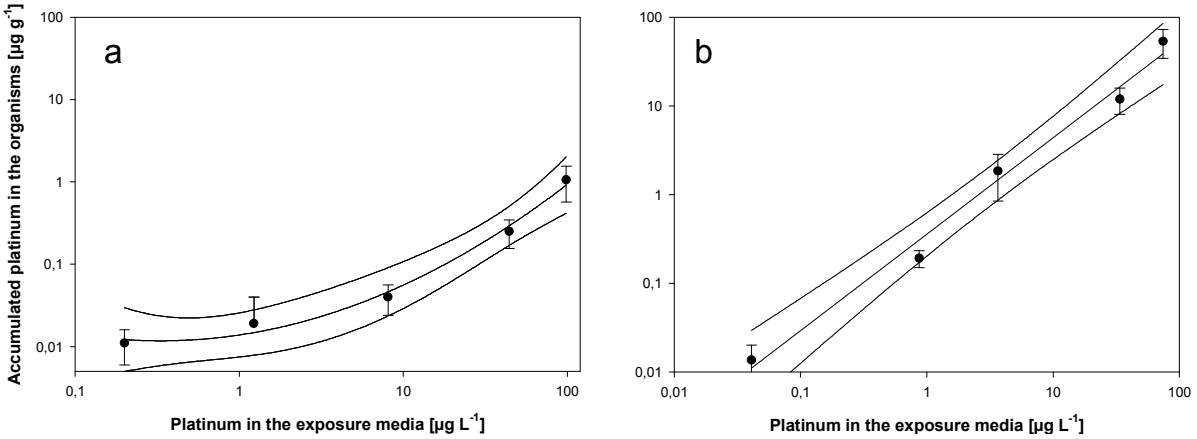


Fig. 1. Pt concentrations in (a) *Danio rerio* after 7 d of exposure, for control n = 7, for 0.1 µg L^{-1} Pt n = 4, for 1 and 50 µg L^{-1} Pt n = 6, for 10 and 100 µg L^{-1} Pt n = 5 of 20 individuals each, R^2 = 0.99, y = 0.00096x^2 + 0.0011x + 0.017 and in (b) *Marisa cornuarietis* after 26 d of exposure in relation to Pt concentrations in the exposure medium, n = 8 pools of 10 individuals each, R^2 = 0.95, y = 0.687x + 1.598.

Bioaccumulation of platinum in M. cornuarietis

Compared with the fish exposure aqueous Pt concentrations are considerably lower than nominal concentrations (Table 1). In controls of *M. cornuarietis*, the platinum concentrations were below the LOD. The *BAFs* for *M. cornuarietis* ranged from 218.4–723.9 and are displayed in Table 3. Platinum uptake of *M. cornuarietis* is linearly correlated with Pt concentrations in the exposure medium (R^2 = 0.95, see Fig. 1b).

Table 3 Nominal concentrations (= groups), concentrations of platinum in whole body of either *Danio rerio* or *Marisa cornuarietis* embryos and calculated bioaccumulation factors.
[1]: For control n = 7; for 0.1 µg L^{-1} Pt n = 4; for 1 and 50 µg L^{-1} Pt n = 6; for 10 and 100 µg L^{-1} Pt n = 5 of 20 individuals each.
[2]: Bioaccumulation factors considering aqueous Pt concentrations
[3]: n=8 pools of 10 individuals each.

Groups	Platinum accumulation in *D. rerio* [µg g^{-1}][1]	Bioaccumulation factor[2] for *D. rerio*	Platinum accumulation in *M. cornuarietis* [µg g^{-1}][3]	Bioaccumulation factor[2] for *M. cornuarietis*
0	below the detection limit		below the detection limit	
0.1	0.011± 0.005	55	0.014 ± 0.007	368.4
1	0.019 ± 0.021	15.4	0.192 ± 0.041	218.4
10	0.040 ± 0.016	5	1.849 ± 1.998	505.5
50	0.255 ± 0.094	5.7	11.967 ± 3.903	354.9
100	1.056 ± 0.493	10.7	53.709 ± 19.238	723.9

Embryo test with D. rerio

Endpoints significantly affected by platinum exposure in zebrafish embryos were heart rate and hatching rate.

At all Pt concentrations tested the average heart rate of *D. rerio* was significantly diminished in comparison to the control. Furthermore, the decrease of the average heart rate was enhanced with higher Pt concentrations in the exposure medium (Fig. 2a). The LOEC of platinum affecting the heart rate was found to be 0.1 µg L^{-1} $PtCl_2$.

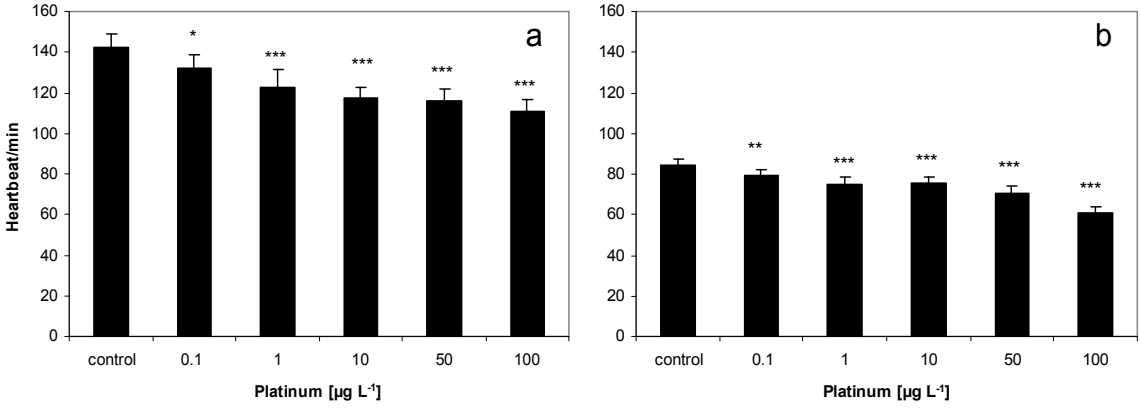


Fig. 2. Average heart rate of (a) *Danio rerio* exposed to platinum, measured 48 h after ertilisation at 26 °C, n = 9 pools of 10 individuals each and (b) *Marisa cornuarietis* exposed to platinum, measured at day 9 after fertilisation at 26 °C, n = 9 pools of 20 individuals each, levels of significant differences are 0.01 < p ≤ 0.05 (*), 0.001 < p ≤ 0.01 (**) and p ≤ 0.001 (***).

Fig. 3a shows the hatching rate of embryos during the test. The hatching rate of animals exposed to the two highest concentrations of 50 and 100 µg L^{-1} $PtCl_2$ was affected by the metal exposure. At the last two observation points at 84 h and 96 h after fertilisation the hatching success of fish exposed to 50 and 100 µg L^{-1} $PtCl_2$ was significantly reduced in comparison to the control treatment. For all other observed endpoints (mortality, percentage of individuals showing gastrulation, somite formation, blood circulation, pigmentation, tail detachment, defects of eyes and brain, edemas) no influence of Pt could be determined.

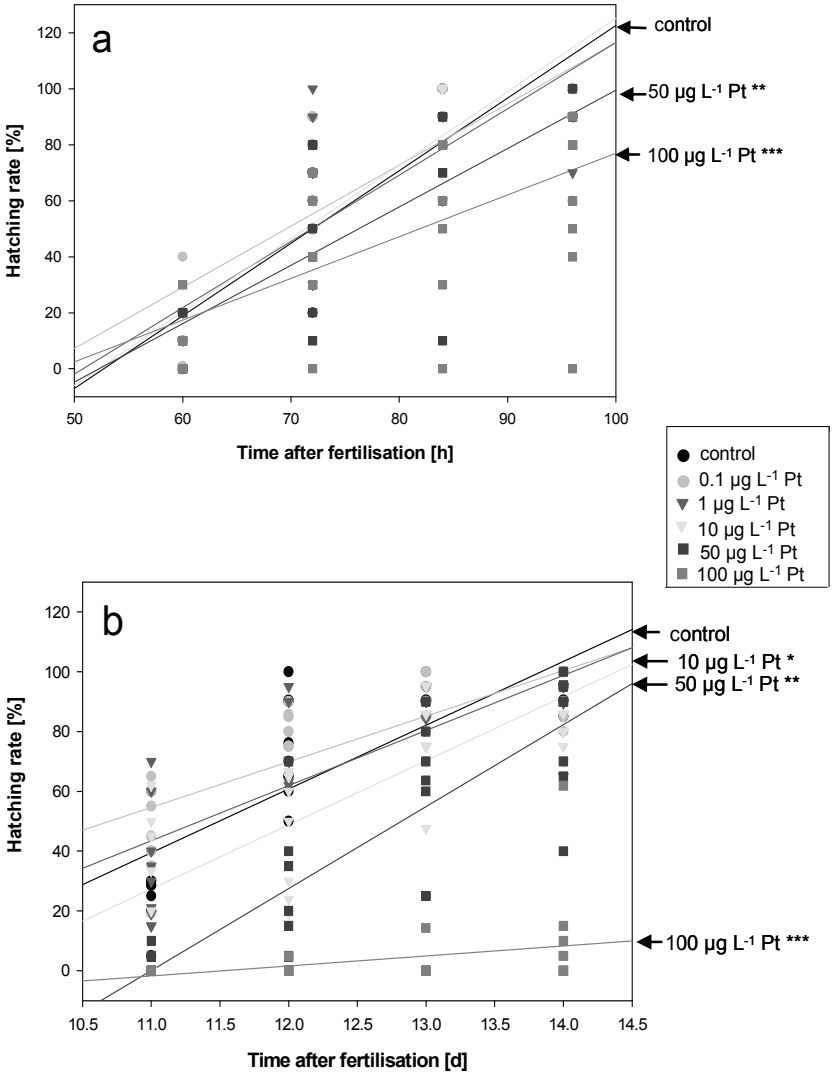


Fig. 3. Hatching success of a) *Danio rerio*, n=9 pools of 10 individuals each and b) *Marisa cornuarietis*, n=9 pools of 20 individuals each, in % of the eggs initially introduced during the test, linear regression. Levels of significant differences are $0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**) and $p \leq 0.001$ (***).

Embryo test with M. cornuarietis

During the embryonic development of *M. cornuarietis* LOEC of $PtCl_2$ concerning the heart rate was found to be the same as for *D. rerio*. Besides the hatching rate, which was also affected by platinum, further endpoints of *M. cornuarietis* were impaired in comparison to *D. rerio*. These endpoints included weight and eye development.

At all Pt concentrations tested the average heart rate of *M. cornuarietis* was significantly reduced versus the control (Fig. 2b). Hatching success of embryos in response to platinum exposure is shown in Fig. 3b. The hatching rate of animals exposed to 10, 50 and 100 µg L^{-1} $PtCl_2$ was affected by the pollutant. Embryos exposed to 100 µg L^{-1} $PtCl_2$ hatched only sporadically. Thus the hatching rate of this treatment was significantly reduced during the entire test period. The hatching rate of embryos exposed to 50 µg L^{-1} $PtCl_2$ was also significantly postponed during the test except for the last observation point at day 14. The hatching rate ofembryos exposed to 10 µg L^{-1} $PtCl_2$ was significantly retarded at day 12 after fertilisation in comparison to the control treatment.

Weight of *M. cornuarietis* measured after 26 d of exposure was gradually reduced with increasing concentrations of platinum (0.1 and 50 µg L^{-1} $PtCl_2$ treatments lacked significance, see Fig. 4a).

Contact to $PtCl_2$ lead to a retardation of the formation of the eyes in *M. cornuarietis*. At 100 µg L^{-1} $PtCl_2$ formation of the eyes was significantly reduced at day 6 and 7, whereas in the treatment groups of 1, 10 and 50 µg L^{-1} $PtCl_2$, this parameter was only found to be significantly reduced at day 6 (Fig. 4b).

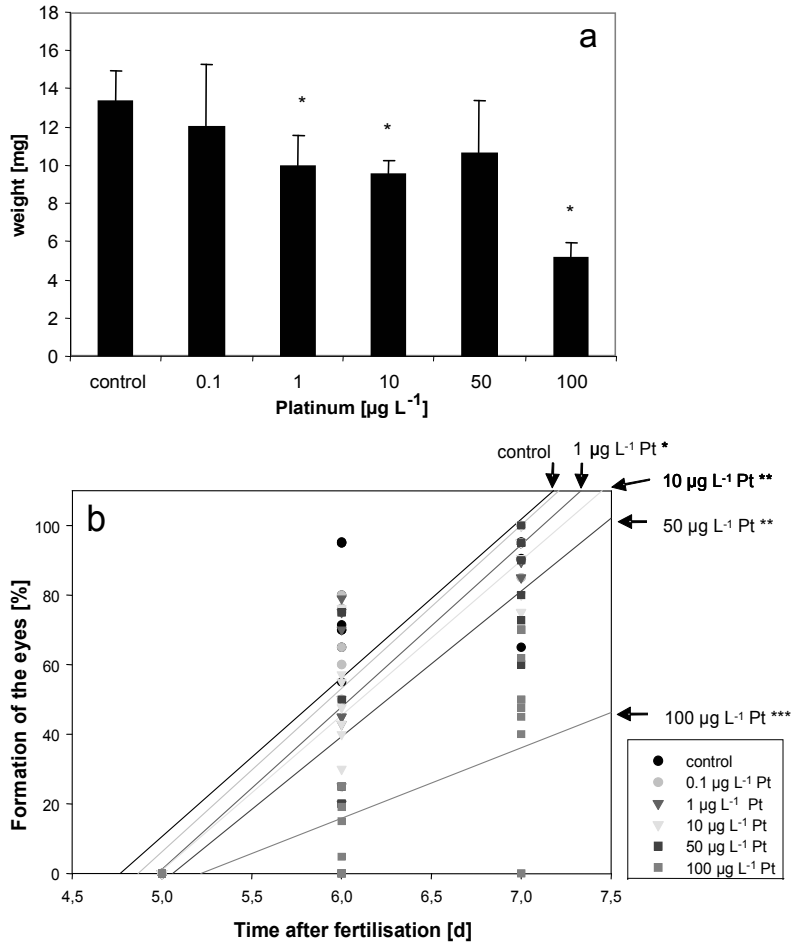


Fig. 4. Effects of platinum on a) weight of *Marisa cornuarietis*, exposed to platinum, measured 26 days after fertilisation, n= 3 pools of 40 individuals each and b) formation of the eyes of *Marisa cornuarietis*, exposed to platinum during the embryo test, n=9 pools of 20 individuals each, linear regression. Levels of significant differences are $0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**) and $p \leq 0.001$ (***).

Discussion

Uptake of platinum by the organisms

Uptake of PGEs was shown for many aquatic organisms, such as the freshwater isopod *Asellus aquaticus* (Moldovan et al., 2001), the amphipods *Gammarus pulex* and *Dikerogammarus villosus* (Haus et al., 2007), the European eel *Anguilla anguilla* (Zimmermann et al., 2004), the barbel *Barbus barbus* (Sures et al., 2005) or the oligochaete *Lumbriculus variegatus* (Veltz et al., 1996). In this study, the bioaccumulation rate of platinum in *M. cornuarietis* and *D. rerio* was investigated. Even though the exposure period of *M. cornuarietis* was only 3.5 times longer than that of *D. rerio*, the snails accumulated about 50 times more platinum than *D. rerio*.

On the one hand this higher *BAF* in *M. cornuarietis* might be due to the shorter exposure period of the zebrafish, which was chosen to compare biological effects of Pt on similar embryonic stages in both species. Another aspect regards the metal uptake. For both organisms there are three possible ways for the metal to enter the body: the body surface, the gills, and the alimentary tract. Since fish were not fed and the gills were not developed fully at the investigated stage, the main Pt uptake likely occured *via* the body surface. In contrast to *D. rerio*, *M. cornuarietis* were fed post hatch because of the length of the exposure time and gills were further developed than those of *D. rerio*. Therefore, Pt uptake occured also *via* the gills and the food to which at least part of Pt probably adsorbed to. A good portion of the snail is their hepatopancreas. Since the hepatopancreas is known to be one main organ for the accumulation of heavy metals (Dallinger, 1992; Tanhan et al., 2005) and together with above mentioned factors, it might be plausible, that much more Pt is accumulated in *M. cornuarietis* than in *D. rerio*, which have small organs bioaccumulating Pt like liver and kidney at the investigated point of development. Furthermore, during the exposure time *D. rerio* have been longer protected by the chorion, than *M. cornuarietis*.

The difference in Pt bioaccumulation between the two test species likely is even larger than calculated, since measured Pt concentrations in the exposure media used for *M. cornuarietis* were constantly lower than those *D. rerio* was subjected to. The preparation of the exposure media for both species was identical except for the water. For *D. rerio* reconstituted water (OECD Guideline 203, Annex 2) was used. Since reconstituted water was found to be inappropriate for *M. cornuarietis* maintenance, water was taken from the snail aquaria, which originated from tap water. An important factor for the solubility of metals is the pH. However, since pH values of both water types were the same, this factor can be excluded from an explanation. One possible reason for the lower Pt concentration in the exposure media for the snails could be that platinum, which was present in the water in an ionic form could have been adsorbed to suspended organic particles present in the water. Such natural organic particles were not present in the artificial water used for fish. Furthermore, the conductivity for reconstituted water which depends on the concentration of free ions was 600 µS cm^{-1}, whereas the conductivity for the used aquaria water was about 1000 µS cm^{-1}. In the latter case, some anions could possibly have precipitated a small portion of Pt as insoluble salts.

Since this study presents the first results of platinum effects on aquatic gastropods, we can best compare our results with those obtained from bivalves. A few studies have been conducted with the zebra mussel (*Dreissena polymorpha*), in which the

uptake rates of PGEs were investigated (Zimmermann et al., 2002, 2005; Sures and Zimmermann, 2007). In comparison to adults of *D. polymorpha*, which were exposed to approximately 70 µg L^{-1} Pt (Sures and Zimmermann, 2007), embryonic stages of *M. cornuarietis* accumulated about 40 times more platinum after a similar exposure time. This might be indicative for a high capability of *M. cornuarietis* to take up dissolved platinum compared to *D. polymorpha*, the latter of which is not known to be very sensitive to pollution. Alternatively, this effect might be a consequence of higher metabolic rates of larval stages or might be due to the activation of defence systems which may have been better developed in adults than in larvae or which may have been actually engaged or affected by embryonic development.

In comparison to Zimmermann et al. (2004) who reported BAFs of 2.6–8.7 for Pt in the European eel exposed to $PtCl_4$, the *BAFs* of 5–55 found for *D. rerio* in the present study were in a similar order of magnitude. In contrast, Jouhaud et al. (1999) calculated a *BAF* of 225 when analysing the accumulation rate of Pt in the intestine of adult *D. rerio* after a similar exposure time of 7 d to H_2PtCl_6. This investigation raises the question whether adult fish may be able to uptake much more platinum or if the speciation of dissolved platinum results in such a varying extent of bioaccumulation in organisms as supported by Rauch and Morrison (1999) who described a higher uptake rate of platinum by the freshwater isopod *A. aquaticus* for Pt IV than for Pt II, whereas Zimmermann (2002) found a higher uptake rate of Pt II by *D. polymorpha* than for Pt IV.

Effects of platinum on early embryogenesis

In addition to data on Pt accumulation in animals our results are the first which show effects of platinum on the embryonic development, a sensitive phase for the impact of pollutants.

For both organisms, platinum had an impact on the average heart rate and the hatching success during embryonic development, being more distinct for *M. cornuarietis* (Figs. 2 and 3). It was also found to have a stronger impact on the embryonic development of *M. cornuarietis* since the development of the eyes was retarded and the weight of the animals reduced (Fig. 4).

As described earlier for other metals, *M. cornuarietis* appears to be more sensitive to platinum exposure than *D. rerio* (Hallare et al., 2005; Schirling et al., 2006; Sawasdee and Köhler, 2009). Also the different *BAFs* and bioaccumulation

dynamics discussed above may account for the fact that more endpoints are affected in *M. cornuarietis*.

The decreased heart rate probably could have resulted from down-regulation of the entire metabolism as a consequence of greater energy demands for metal elimination/detoxification. It could, however, also be interpreted as an adaptive response to limit the ion exchange with a medium containing toxic substances or as a pathologic effect of platinum on the heart. Adverse effects of chlorides (platinum chloride solution was used for the exposure) can be excluded, since other metal chlorides were tested earlier and had no effects on heart rates of *D. rerio* and *M. cornuarietis* although they were dosed manyfold higher than concentrations used in the present study (Hallare et al., 2005; Sawasdee and Köhler, 2009).

Hatching success is supposed to be a sensitive endpoint of the *Danio* embryo toxicity assay. Dave and Xiu (1991) found a delayed hatching for several heavy metals, notably for copper and nickel. The retarded hatching observed in the present study could be a protective response to postpone the time of more intense contact to the pollutant, since the chorion is known to act as a barrier which reduces permeation of pollutants (Fent, 1992; Fent and Meier, 1994). Furthermore, this effect could also result from a trade-off in energy allocation towards pollutant elimination/detoxification, and thus less energy may be left for the hatching process. Alternatively, the delayed hatching might also be a result of a generally retarded development caused by the pollutant (Rosenthal and Alderdice, 1976).

Platinum lead to a retarded development of *M. cornuarietis*, as observed in the development of the eyes. A retardation of embryonic processes has also been shown for other heavy metals before (Waterman, 1937; Thompson and Bannigan, 2008; Sawasdee and Köhler, 2009). The loss of weight in *M. cornuarietis* due to Pt exposure has also been described for other species exposed to heavy metals (Gilani and Alibhai, 1990).

In the environment, aqueous platinum concentrations are generally in the lower ng L^{-1} range, but in run-off water they were found to be in a µg L^{-1} range (Helmers et al., 1994; Laschka et al., 1996). LOEC data of 0.1 µg L^{-1} $PtCl_2$ found in the present study together with observations on a ~10% solubility of platinum suggests an ecological relevance for organisms being exposed to platinum in their environment. Only limited information on adverse effects of platinum on aquatic organisms has been published so far. Veltz et al. (1996) determined the 96 h LC_{50} for the oligochaete *L. variegatus*. They found a varying toxicity of tetravalent platinum depending on water hardness and temperature. Jouhaud et al. (1999) found ultrastructural changes in the anterior intestine of adult *D. rerio* induced by

16 µg L^{-1} of platinum applied as H_2PtCl_6. Studies of Singer et al. (2005) with *D. polymorpha* revealed that platinum induces the expression of heat shock proteins (hsp70) after 25–32 d of exposure to soluble Pt II. The threshold Pt concentration for the induction of heat shock proteins was from 43 to 58 µg g^{-1} Pt in the mussel tissue, which was considerably lower than that of cadmium. Based on the available studies, it appears that PGEs, although being noble metals, might be of environmental concern. Recently it was shown, that the exposure of *D. polymorpha* to low concentrations of palladium results in increased metallothionein concentrations (Frank et al., 2008), showing that there is a need for detoxification of Pd in the mussel. An estimated level of 250 ng L^{-1} Pd was sufficient to increase MT levels after 5 weeks of exposure. Although this Pd level is still above environmental Pd concentrations, an induction of MT due to chronic Pd exposure at contaminated sites such as sites which receive road runoff cannot be excluded. These investigations are just a starting point for a comparative risk assessment of PGEs which seems to be necessary in order to select the ecotoxicologically least problematic compounds for future catalytic converter technology.

Acknowledgements

Studienstiftung des deutschen Volkes and Landesgraduiertenförderung Baden-Württemberg are highly acknowledged for financial support.

References

Barbante, C., Veysseyre, A., Ferrari, C., Van de Velde, K., Morel, C., Capodaglio, G., Cescon, P., Scarponi, G., Boutron, C., 2001. Greenland snow evidence of large scale atmospheric contamination for platinum, palladium, and rhodium. Environ. Sci. Technol. 35: 835–839.

Barefoot, R.R., 1997. Determination of platinum at trace levels in environmental and biological materials. Environ. Sci. Technol. 31: 309–314.

Dallinger, R., 1992. Strategies of metal detoxification in terrestrial invertebrates. In: Dallinger, R., Rainbow, P.S. (Eds.), Ecoxoticology of Metals in Invertebrates. Lewis Publ, Boca Raton, FL, pp. 245–289.

Dave, G., Xiu, R., 1991. Toxicity of mercury, copper, nickel, lead, and cobalt to embryos and larvae of zebrafish, *Brachydanio rerio*. Arch. Environ. Contam. Toxicol. 21: 126–134.

Eaton, J.G., McKim, J.M., Holocombre, G.W., 1978. Metal toxicity to embryos and larvae of seven freshwater fish species. I. Cadmium. Bull. Environ. Contam. Toxicol. 19: 95–103.

Fent, K., 1992. Embryotoxic effects of tributyltin on the minnow *Phoxinus phoxinus*. Environ. Pollut. 76: 187–194.

Fent, K., Meier, W., 1994. Effects of triphenyltin on fish early life stages. Arch. Environ. Contam. Toxicol. 27: 224–231.

Frank, S., Singer, C., Sures, B., 2008. Metallothionein (MT) response after chronic palladium exposure in the zebra mussel, *Dreissena polymorpha*. Environ. Res. 108: 309–314.

Gilani, S.H., Alibhai, Y., 1990. Teratogenicity of metals to chick embryos. J. Toxicol. Environ. Health 30: 23–32.

Hallare, A.V., Schirling, M., Luckenbach, T., Köhler, H.-R., Triebskorn, R., 2005. Combined effects of temperature and cadmium on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos. J. Therm. Biol. 30: 7–17.

Haus, N., Zimmermann, S., Wiegand, J., Sures, B.: 2007. Occurrence of platinum and additional traffic related heavy metals in sediments and biota. Chemosphere 66: 619–629.

Haus, N., Eybe, T., Zimmermann, S., Sures, B., 2009. Is microwave digestion using TFM vessels a suitable preparation method for Pt determination in biological samples by adsorptive cathodic stripping voltammetry? Anal. Chim. Acta 635: 53–57.

Helmers, E., Mergel, N., Barchet, R., 1994. Platinum in ash from sewage sludge incinerators and in grass. UWSF – Z Umweltchem. Ökotox. 6: 130–134.

Hill, R.F., Mayer, W.J., 1977. Radiometric determination of platinum and palladium attrition from automotive catalysts. IEEE. Trans. Nucl. Sci. 24: 2549–2554.

Hill, A.J., Teraoka, H., Heidemann, W., Peterson, R.E., 2005. Zebrafish as a model vertebrate for investigating chemical toxicity. Rev. Toxicol. Sci. 86: 6–19.

Hisoaka, K.K., Battle, H.I., 1958. The normal developmental stages of the zebrafish *Brachydanio rerio* (Hamilton–Buchanan). J. Morphol. 102: 311–321.

Jouhaud, R., Biagianti-Risbourg, S., Vernet, G., 1999. Effets du platine chez *Brachydanio rerio* (Téléostéen, Cyprinidé). I. Toxicité aiguë; bioaccumulation et histopathologie intestinales. J. Appl. Ichthyol. 15: 41–48.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203: 253–310.

Klaassen, C.D., 1996. Casarett & Doull's Toxicology—The Basic Science of Poisons, fifth ed. McGraw-Hill, New York. pp. 725–726.

König, H.P., Hertel, R.F., Koch, W., Rosner, G., 1992. Determination of platinum emissions from a three-way catalyst-equipped gasoline engine. Atmos. Environ. 26: 741–745.

Laschka, D., Striebel, T., Daub, J., Nachtwey, M., 1996. Platinum in rainwater discharges from roads. UWSF–Z Umweltchem. Ökotox. 8: 124–129.

Luckenbach, T., Triebskorn, R., Müller, E., Oberemm, A., 2001. Toxicity of waters from two streams to early life stages of brown trout (*Salmo trutta f. Fario L.*), tested under semi-field conditions.. Chemosphere 45: 571–579.

Lustig, S., Zang, S., Michalke, B., Schramel, P., Beck, W., 1996. Transformation behaviour of different platinum compounds in a clay-like humic soil: speciation investigations. Sci. Total Environ. 188: 195–204.

Lustig, S., Zang, S., Michalke, B., Schramel, P., Beck, W., 1997. Platinum determination in nutrient plants by inductively coupled plasma mass spectrometry

with special respect to the hafnium oxide interference. Fresen. J. Anal. Chem. 357: 1157–1163.

Lustig, S., Zang, S., Beck, W., Schramel, P., 1998. Dissolution of metallic platinum as water soluble species by naturally occurring complexing agents. Mikrochim. Acta 129: 189–194.

McKim, J.M., Arthur, J.W., Thorslund, T.W., 1975. Toxicity of linear alkylate sulfonate detergent to larvae of four species of freshwater fish. Bull. Environ. Contam. Toxicol. 14: 1–7.

Moldovan, M., Rauch, S., Gomez, M., Palacios, M.A., Morrison, G., 2001. Bioaccumulation of palladium, platinum and rhodium from urban particulates and sediments by the freshwater isopod *Asellus aquaticus*. Water Res. 35: 4175– 4183.

Moldovan, M., Palacios, M.A., Gomez, M.M., Morrison, G., Rauch, S., McLeod, C., Ma, R., Caroli, S., Alimonti, A., Petrucci, F., Bocca, B., Schramel, P., Zischka, M., Pettersson, C., Wass, U., Luna, M., Saenz, J.C., Santamaria, J., 2002. Environmental risk of particulate and soluble platinum group elements released from gasoline and diesel engine catalytic converters. Sci. Total Environ. 296: 199–208.

Nagel, R., Isberner, K., 1998. Testing of chemicals with fish–a critical evaluation of tests with special regard to zebrafish. In: Braunbeck, T., Hinton, D.E., Streit, B. (Eds.), 1998 Fish Exotoxicology. Birkhäuser, Switzerland, pp. 337–351.

Nagel, R., 2002. DarT: the embryo test with the zebrafish *Danio rerio* – a general model in ecotoxicology and toxicology. Altex 19: 38–48.

OECD Guideline for Testing of Chemicals, 1992a. Test Guideline 203. Fish, Acute Toxicity Test. OECD Guideline for Testing of Chemicals.

OECD Guideline for Testing of Chemicals, 1992b. Test Guideline 210. Fish, Early-life Stage Toxicity Test. OECD Guideline for Testing of Chemicals.

Oehlmann, J., Schulte-Oehlmann, U., Tilmann, M., Markert, B., 2000. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in laboratory. Part I: Bisphenol A and octylphenol as xeno-estrogens. Ecotoxicology 9: 383–397.

Oehlmann, J., Schulte-Oehlmann, U., Bachmann, J., Oetken, M., Lutz, I., Kloas, W., Ternes, T.A., 2006. Bisphenol A induces superfeminization in the ramshorn snail *Marisa cornuarietis* (Gastropoda: Prosobranchia) at environmentally relevant concentrations. Environ. Health. Perspect. 114: 127–133.

Rauch, S., Morrison, G.M., 1999. Platinum uptake by the freshwater isopod *Asellus aquaticus* in urban rivers. Sci. Total Environ. 235: 261–268.

Rombough, P., 2002. Gills are needed for ionoregulation before they are needed for O_2 uptake in developing zebrafish, *Danio rerio*. J. Exp. Biol. 205: 1787–1794.

Rosenthal, H., Alderdice, D.F., 1976. Sublethal effects of environmental stressors, natural and pollutional, on marine fish eggs and larvae. J. Fish Res. Board Can. 33: 2047–2065.

Sawasdee, B., Köhler, H.-R., 2009. Embryo toxicity of pesticides and heavy metals to the ramshorn snail, *Marisa cornuarietis* (Prosobranchia). Chemosphere 75: 1539–1547.

Schirling, M., Bohlen, A., Triebskorn, R., Köhler, H.-R., 2006. An invertebrate embryo test with the apple snail *Marisa cornuarietis* to assess effects of potential developmental and endocrine disruptors. Chemosphere 64: 1730–1738.

Schulte, C., Nagel, R., 1994. Testing acute toxicity in the embryo of zebrafish, *Brachydanio rerio*, as an alternative to the acute fish test: Preliminary results. Atla 22: 12–19.

Schulte-Oehlmann, U., Tillmann, M., Market, B., Oehlmann, J., 2000. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in laboratory. Part II. Triphenyltin as a xeno-androgen.. Ecotoxicology 9: 399– 412.

Singer, C., Zimmermann, S., Sures, B., 2005. Induction of heat shock proteins (hsp70) in the zebra mussel (*Dreissena polymorpha*) following exposure to platinum

group metals (platinum, palladium and rhodium): comparison with lead and cadmium exposures. Aquat. Toxicol. 75: 65–75.

Sures, B., Zimmermann, S., Messerschmidt, J., von Bohlen, A., Alt, F., 2001. First report on the uptake of automobile catalyst emitted palladium by European eels (*Anguilla anguilla*) following experimental exposure to road dust. Environ. Pollut. 113: 341–345.

Sures, B., Zimmermann, S., 2004. Untersuchungen zur Toxizität der Platingruppenelemente Pt, Pd und Rh. Zwischenbericht anlässlich des Statusseminars von BWPLUS am 02. und 03. März 2004 im Forschungszentrum Karlsruhe, Germany

Kapitel 2: Histopathological alterations and induction of hsp70 in ramshorn snail (*Marisa cornuarietis*) and zebrafish (*Danio rerio*) embryos after exposure to $PtCl_2$

Raphaela Osterauer [a], Heinz-R. Köhler [a], Rita Triebskorn [a,b]

[a] Animal Physiological Ecology Department, University of Tübingen, Konrad-Adenauer-Str. 20, 72072 Tübingen, Germany

[b] Transfer Center for Ecotoxicology and Ecophysiology, Blumenstrasse 13, 72108 Rottenburg, Germany

Abstract

The platinum group metals (PGMs) platinum (Pt), palladium (Pd), and rhodium (Rh) are used in automobile catalytic converters, from which they have been emitted into the environment to an increasing degree during the last 20 years. Despite the bioavailability of these metals to plants and animals, studies determining the effects of PGMs on organisms are extremely rare. In the present study, effects of various concentrations of $PtCl_2$ (0.1, 1, 10, 50 and 100 µg/L) were investigated with respect to the induction of hsp70 and histopathological alterations in the zebrafish, *Danio rerio* and the ramshorn snail, *Marisa cornuarietis*. Histopathological investigations revealed effects of Pt on both species, which varied between slight and strong cellular reactions, depending on the $PtCl_2$ concentration. The hsp70 level in *M. cornuarietis* did not show an increase following Pt exposure whereas it was significantly elevated at 100 µg/L $PtCl_2$ in *D. rerio*.

Keywords: platinum, *Marisa cornuarietis*, *Danio rerio*, Hsp70, histopathology

Aquatic Toxicology, 2010, 99: 100–107

Introduction

The intake of the platinum group metals (PGMs) platinum (Pt), palladium (Pd), and rhodium (Rh) into the environment has significantly increased during recent decades, due to their use in automobile catalytic converters (reviewed in Hoppstock and Sures, 2004; Zereini and Alt, 2000). Until recently, metallic forms of PGMs were generally considered as inert, with respect to their reactivity. However, it has been shown that these metals are ionizable and thus potentially biologically active (Klaassen, 1996). Accordingly, many studies examined their bioaccumulation in a variety of freshwater organisms (Haus et al., 2007; Moldovan et al., 2001; Sures and Zimmermann, 2007; Zimmermann et al., 2004). Despite the constant increase of these metals in the environment and their bioaccumulative potential, studies on the toxic effects of PGMs are still extremely rare.

An established vertebrate model organism used to assess the risk of potentially toxic substances is the zebrafish, *Danio rerio* (Hill et al., 2005). Also, with regard to invertebrates, several molluscan species have been shown to serve as good experimental models to detect and evaluate potentially harmful substances (Duft et al., 2007; Melo et al., 2000; Oehlmann et al., 2007). In this context, *Marisa cornuarietis*, an ampullariid freshwater snail, which is indigenous in Central and South America, has been shown to react in a highly sensitive way to metal exposure (Sawasdee and Köhler, 2009; Schirling et al., 2006). Since investigations revealed developing fish embryos and larvae to be more sensitive to chemicals than adult fish (Bresch, 1991; Eaton et al., 1978), investigations in the present study were conducted with fish and snails exposed to $PtCl_2$ during their embryonic development.

The so-called 'heat shock proteins' (hsp) are chaperones, which play a vital role in normal cellular function and contribute to correct protein folding (Gething and Sambrook, 1992). They are, e.g., induced when new and active proteins are needed. The hsps with a molecular weight of 68–74 kD (the hsp70 family) are best known among all hsps. They are also inducible under adverse conditions, such as exposure to chemicals, physiological stress, or virus infection (Bächtle, 2000; Welch, 1993), which all act on the integrity of intracellular proteins. They are able to restore partly denaturated protein structures and, therefore, serve as components of the cellular protection system. Based on known correlations of the hsp70 level of an organism or organ with the intensity of proteotoxic stress, these proteins are used as measures to assess the proteotoxic effect of pollutants (De Pomerai, 1996; Köhler et al., 1992; Lewis et al., 1999). However, the kinetic reaction

of the hsp70 activation at increasing exposure to stressors, describes an optimum curve (homeostasis, compensation, non-compensation): both low and very high stress loads result in a low hsp70 level, because high stress intensities overcharge the protective hsp70-system (Köhler and Eckwert, 1997).
Histopathological responses include changes in cell and tissue structure in response to toxic pollutants (Meyers and Hendricks, 1982) and serve as basic indicators for assessing the conditions in a particular water ecosystem (Lajtner et al., 1996). Histopathological characteristics of the investigated organs mirror the health status of an organism and reflect endogenous and exogenous impacts on organisms integrated over time. Strong histopathological effects have been accompanied by a collapse of the hsp70-system (Scheil et al., 2008; Triebskorn et al., 2005). Therefore, histopathological investigations are helpful tools to detect an overcharge of the hsp70-system in case of low hsp70 levels (non-compensative part of the optimum curve). Since the investigation of responses on different biological levels leads to a better understanding of the health condition of animals exposed to chemicals, the present study investigates effects of various concentrations of $PtCl_2$ on the hsp70 level, as well as histopathological alterations in the developing organs in embryos of the zebrafish *D. rerio* and the ramshorn snail *M. cornuarietis*.

Materials and Methods

Test animals and maintenance of cultures

Embryos of a zebrafish breeding stock (*D. rerio*, strain: WIK (a commonly used wild type line of zebrafish), ZFIN ID: ZDB-GENO-010531-2) originally obtained from the Max-Planck-Institute for Developmental Biology, Tübingen, Germany (C. Nüsslein-Volhard group), and embryos of a ramshorn snail breeding stock (*M. cornuarietis*) originally obtained from Frankfurt/Main University, Germany (J. Oehlmann group), served as test animals in this study.
D. rerio and *M. cornuarietis* cultures were both kept in aerated and filtered 150 and 230 L aquaria under the following conditions: temperature: 26 ± 1 °C, pH: 7.5–8, conductivity for fish: 400 µS/cm, for snails: 1000 µS/cm, light/dark regime: 12 h/12 h. The medium for fish was exchanged every 10–14 days, and for snails every week. The fish were fed a commercially available artificial diet (Nutrafin Max flakes, Hagen, Germany) in the morning and frozen food from unpolluted sources (mosquito larvae, *Mysis*, *Moina*, *Artemia*) in the evening. The snails were fed a

commercially available artificial diet (Nutrafin Max flakes, Hagen, Germany) once a day.

Exposure of eggs and test substance

The substance investigated in the present study was $PtCl_2$ (platinum standard solution 1000 µg/mL, Ultra Scientific, Wesel, Germany). Nominal concentrations tested were 0.1, 1, 10, 50 and 100 µg/L $PtCl_2$.
When eggs of *M. cornuarietis* were required, egg masses, laid during the night, were collected from the aquaria the following morning. To gather eggs of *D. rerio*, spawning traps covered with stainless steel mesh were placed on the bottom of the aquaria in the evening. After 1 h of spawning and fertilisation, initiated by sudden illumination of the aquaria the following morning, deposited eggs were collected by sieving the content of the spawning boxes.
After fertilisation, eggs of fish (n = 4 replicates of 40 eggs each) or snails (n = 4 replicates of 20 eggs each) were distributed to plastic Petri dishes containing 40 mL of the different concentrations of $PtCl_2$ each, or the control medium. For fish, reconstituted water (OECD, 1992) served as the overall control. Because reconstituted water was found to be inappropriate for *M. cornuarietis* maintenance, tap water taken from the snail aquaria served as control medium.
Since Sures and Zimmermann (2007) described precipitation of Pt during exposure experiments, real concentrations of Pt were determined in the exposure media. These data have been published and discussed by Osterauer et al. (2009) and are as follows: 0.20, 1.23, 8.08, 44.24, and 98.66 µg/L Pt for *D. rerio* and 0.04, 0.87, 3.66, 33.73, and 74.20 µg/L Pt for *M. cornuarietis* corresponding to nominal concentrations of 0.1, 1, 10, 50, and 100 µg/L $PtCl_2$, respectively. To facilitate the reading of this article, the applied nominal concentrations for both organisms are used throughout.
The exposure period was 7 days for *D. rerio*. As embryonic development of *M. cornuarietis* is much slower than that of *D. rerio*, snail eggs were exposed for 26 days. *D. rerio* nourishes on yolk up to the 12th day after fertilisation (Rombough, 2002). Therefore, the fish were not fed during the exposure time of 7 days. *M. cornuarietis*, however, needed to be fed with equal portions of commercially available artificial diet (Nutrafin Max, Hagen, Germany) once a day after hatching. Throughout the exposure period, embryos were kept at 26 °C in a climate cabinet with a light/dark regime of 12 h each. Exchange of the test medium was accomplished every day for *M. cornuarietis* and every second day for *D. rerio*.

Sample processing for hsp70 determination

Embryos were pooled (8 embryos per sample for *D. rerio* and 2 embryos per sample for *M. cornuarietis*), frozen in liquid nitrogen and stored at -20 °C. For subsequent analysis of hsp70, 10 samples were used. Prior to homogenisation (by sonification for 5 s at 28 W for *D. rerio* and by mechanical homogenisation for *M. cornuarietis*), extraction buffer (80 mM potassium acetate, 4 mM magnesium acetate, 20 mM HEPES pH 7.5 and 2% protease inhibitor) was added to the frozen samples (20 µL for *D. rerio*, 140 µL for *M. cornuarietis*). Subsequently, the homogenised samples were centrifuged (10 min, 20,000 g at 4 ◦C). The total protein concentration of each supernatant was determined according to Bradford (1976). A constant total protein mass (20 µg per lane) was loaded on a 12% polyacrylamide gel using the buffer system described by Laemmli (1970). The gels were run at 80 V for 15 min and at 120 V for another 60 min. The separated proteins were electrically transferred to nitrocellulose membranes by semi-dry blotting (2 mA/cm^2 of one gel, 10 V, 2 h). After saturation for 90 min in a solution of 50% horse serum in TBS ((Tris-) buffered saline: 50 mM Tris pH 5.7, 150 mM NaCl), the membranes were washed in TBS for 5 min. A mouse monoclonal antibody raised against human hsp70 was diluted 1:5000 in 10% horse serum/TBS and incubated with membranes overnight at room temperature. The used antibody has been proven to detect hsp70 very specifically in a wide variety of species, also in fish and gastropods (Köhler et al., 2009; Hallare et al., 2005). After washing in TBS for 5 min, the membranes were incubated for 2 h with the peroxidase coupled secondary antibody (goat anti-mouse IgG, dilution 1:1000 in 10% horse serum/TBS) at room temperature. After a final washing of the membranes, 4-chloro(1)naphtol and 0.015% H_2O_2 in 30 mM Tris, pH 8.5, containing 6% methanol was used to visualize the antibody complex. Finally, the filters were dried for 1–2 h and each membrane was scanned. The mean grey value intensities were quantified by densitometric image analysis (Herolab E.A.S.Y. Win 32, Germany) and related to a consistent standard from WIK *D. rerio*.

Sample processing for histopathological investigations

Embryos were fixed in Bouin's solution for several days. Subsequently, they were washed for 3 × 10 min with 70% EtOH containing one drop of 32% NH_3 for a better elimination of the picric acid. For dehydration, embryos were washed in a graded series of EtOH (70%, 80%, 80%, 96% 100%) for 3 × 10 min each and finally embedded in Techno-Vit (Heraeus Kulzer, Germany). Using an automatic microtome

(2050 Supercut, Reichert-Jung, Germany), serial sections of 4 µm thickness for *D. rerio* and 5 µm for *M. cornuarietis*, were cut and mounted on microscope slides. The slices were stained with hematoxylin/eosin, covered with Eukitt (Roth, Germany) and evaluated microscopically using a light microscope (Axioskop 2, Zeiss, Germany).

For *M. cornuarietis*, histopathological changes in the mantle, gill, and digestive gland were investigated, for *D. rerio*, alterations of liver and gut were checked.

For histopathological investigation, organs of 10 animals per treatment were qualitatively described and semi-quantitatively evaluated. Three different sections per organ and animal were qualitatively described and a mean assessment value (MAV) of these 3 different sections per organ was calculated according to Triebskorn et al. (1997).

For semi-quantification of effects, histopathological symptoms were classified according to the state of cellular pathology: control status, status of reaction and status of destruction (Tables 1 and 2). According to a protocol of Köhler and Triebskorn (1998) and some modifications published by Scheil et al. (2010), the different categories were chosen on the basis of the quantitative abundance of histopathological effects (which are summarized in Tables 1 and 2). Categories were defined as follows:

- Category 1: 90–100% of cells in control status, 0–10% in reaction status.
- Category 2: 50–90% of cells in control status, 10–50% in reaction status.
- Category 3: 50–100% of cells in reaction status, 0–50% in the control status.
- Category 4: 25–75% of cells in status of destruction.
- Category 5: 75–100% of cells in status of destruction.

Table 1 Histopathological symptoms in tissues of *Danio rerio* used for classification into categories 1 to 5. Modified according to Triebskorn (2007).

	Control status	Status of reaction	Status of destruction
Liver	▪ Regularly shaped hepatocytes with round nuclei and homogeneous cytoplasm ▪ Slightly extended capillary spaces and lumina of the hepatic tubules ▪ Only single macrophages	▪ Increased amount of macrophages ▪ Atrophy or hypertrophy of hepatocytes and/or nuclei ▪ Variances in density of cytoplasm and/or nuclei ▪ Dilation of capillaries and/or intercellular spaces ▪ Onset of vacuolisation	▪ Intense inflammatory reaction with high amount of macrophages ▪ Necrosis (caryolysis, caryopycnosis) ▪ Intense vacuolization of cytoplasm
Gut	▪ Highly prismatic cells with basally located nuclei ▪ Mucocytes containing mucus vacuoles ▪ Smooth apical and basal surfaces ▪ Homogenous cytoplasm with apical microvilli	▪ Increased amount of macrophages ▪ Irregular apical and/or basal surfaces ▪ Atrophy or hypertrophy of cells and/or nuclei ▪ Basal vacuolization ▪ Partly ablation from the basal lamina ▪ Variances in density of cytoplasm and/or nuclei ▪ Alteration of compartmentation ▪ Increased amount of mucus cells	▪ Intense inflammatory reaction with high amount of macrophages ▪ Necrosis (caryolysis, caryopycnosis) ▪ Extensive ablation of basal surface from basal lamina

Table 2 Histopathological symptoms in tissues of *Marisa cornuarietis* used for classification into categories 1 to 5.

	Control status	Status of reaction	Status of destruction
Epidermis	▪ Prismatic cells with basally located nuclei ▪ Filled mucocytes	▪ Irregular apical surfaces ▪ Dilatation of intercellular spaces ▪ Atrophy or hypertrophy of cells and/or nuclei ▪ Empty and/or destroyed mucocytes ▪ Higher or lower amount of mucocytes	▪ Desquammation ▪ Very large intercellular spaces ▪ Destroyed epidermis
Hepato-pancreas	▪ Small hemolymph spaces between the tubules ▪ Small tubule lumen ▪ Digestive cells with smoth apex and regularly shaped microvilli ▪ Regular compartmentation of digestive cells (small apical and larger basal vacuoles) ▪ Basophilic cells with dense cytoplasm and large roundish nucleus with high amounts of heterochromatin	▪ Large hemolymph spaces between the tubules ▪ Enlarged tubule lumen ▪ Dilatation of intercellular spaces ▪ Flattened epithelium ▪ Irregular shape of cells ▪ Apical surface blebs of digestive cells ▪ Increased amount and/or enlarged vacuoles in digestive cells ▪ Irregular compartmentation of digestive cells ▪ Varying amount and shape of basophilic cells ▪ Altered densitiy of the cytoplasm	▪ Destroyed tubules ▪ Necrosis of digestive and basophilic cells
Gill	▪ Columnar cells with long cilia ▪ Round to oval nuclei with dense heterochromatin ▪ Single mucus-secreting goblet cells	▪ Enlargement of nuclei ▪ Increase of mucus cells ▪ Varying amount of mucus-secreting cells ▪ Irregular shape of cells ▪ Enlarged interlamellar space	▪ Necrosis of cells ▪ Very large interlamellar spaces ▪ Pycnotic nuclei

Statistical analysis

For normally distributed data (Shapiro–Wilk test, JMP 4.0, SAS Systems, USA), the parametric multiple comparison Tukey–Kramer test (JMP 4.0, SAS Systems, USA) was used. Data not corresponding to normal distribution were tested using the nonparametric distribution-independent Wilcoxon test (JMP 4.0, SAS Systems, USA) to detect significant differences between the respective treatment groups and the control group. Differences were considered not to be significant for $p > 0.05$ and were significant for $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***).

Results

Histopathological effects of platinum on D. rerio

Qualitative analysis

The only organs of *D. rerio*, which could reliably be evaluated histologically at the investigated state of development, were the gut and the liver. Both organs were affected by Pt, particularly the liver at high Pt concentrations.

Gut: Most dominant effects of Pt on the gut of *D. rerio* were ablation of gut cells from the basal lamina, basal vacuolisation of the cytoplasm, irregular compartmentation, and an increase in the number of mucus cells (Fig. 1b and c). These effects were prominent from 1 µg/L $PtCl_2$ onwards, getting more severe at higher concentrations.

Liver: At 1 µg/L and higher concentrations of $PtCl_2$, inflammatory reactions with increasing numbers of macrophages, dilation of capillary spaces and high variability in the density of cytoplasm and/or nuclei, occurred in the liver of *D. rerio*. Further prominent effects on the liver, especially at the highest concentrations of 50 and 100 µg/L $PtCl_2$ were vacuolisation of the cytoplasm, beginning of cloudy swelling of the hepatocytes, and caryopycnosis (Fig. 1e and f).

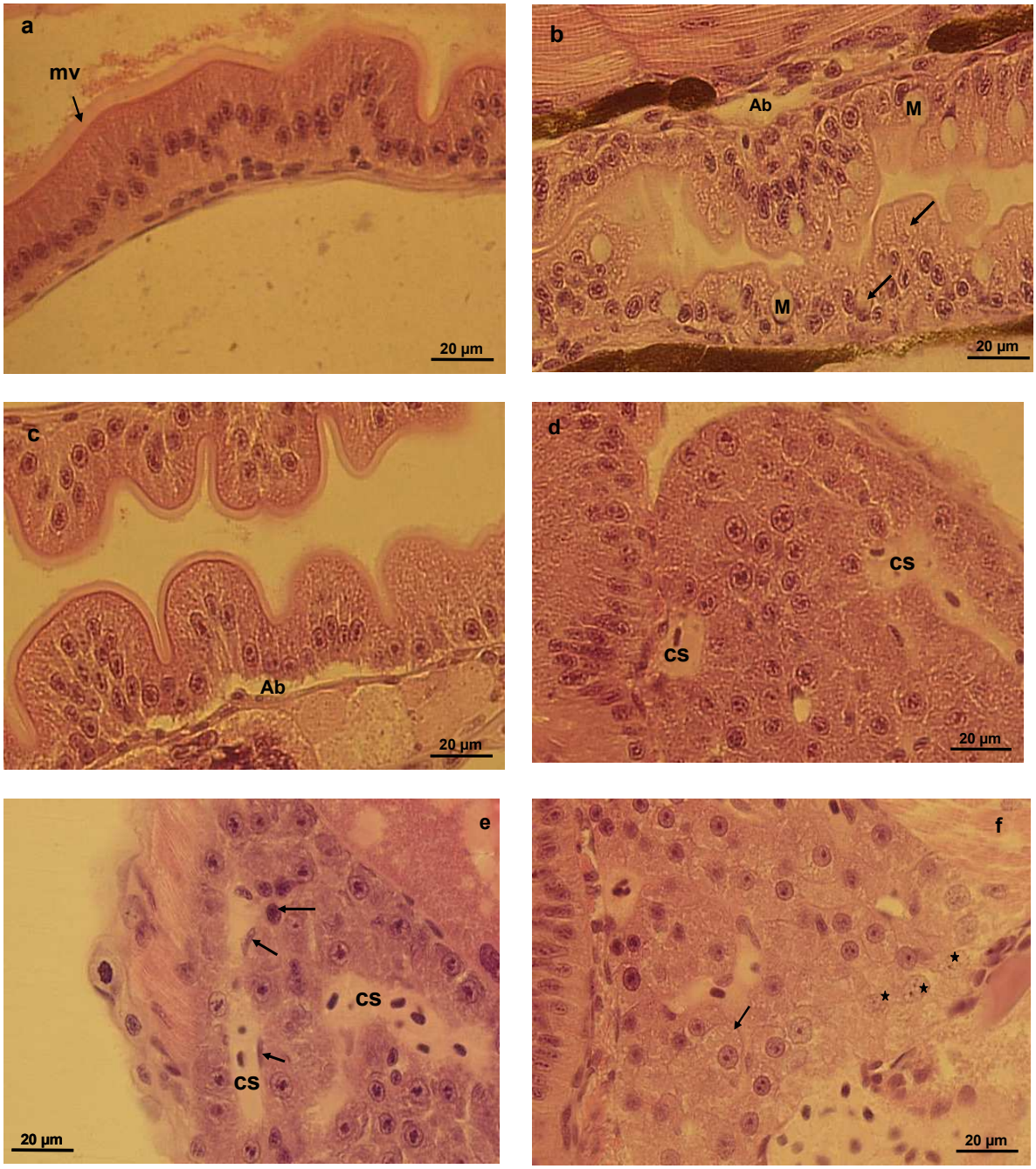


Fig. 1. (a) Gut of a control fish with highly prismatic cells, homogenous cytoplasm and apical microvilli (mv); (b) and (c) gut of fish exposed to 50 µg/L $PtCl_2$ showing an increased number of mucocytes (M), vacuolisation (arrows) and ablation from the basal lamina (Ab); (d) liver of a control fish with regularly shaped hepatocytes, round nuclei, eosin-positive cytoplasm and capillary spaces (cs); (e) liver of fish exposed to 50 µg/L $PtCl_2$ indicating slightly extended capillary spaces, macrophages and variances in density of nuclei (arrows); (f) liver of fish exposed to 100 µg/L $PtCl_2$ showing beginning of cloudy swelling of hepatocytes (arrow) unregularly shaped cells and nuclei and variances in density of nuclei and cytoplasm (asterisks).

Semi-quantitative evaluation

Although effects of Pt on the gut of *D. rerio* were significantly stronger at concentrations of 1, 50 and 100 µg/L $PtCl_2$, compared to the control impairment of the gut was generally low, being in a status of slight reaction (Fig. 2). A significant

increase in the above-described effects of Pt on the liver of *D. rerio* was observed for 1 and 10 µg/L $PtCl_2$ compared to the livers of the control animals. Controls of the liver have been classified into the status of slight reaction. Livers of animals, exposed to 50 and 100 µg/L $PtCl_2$ showed strong reactions and were assessed between categories 4 and 5, being highly significantly impaired compared to the livers of the control animals (Fig. 2).

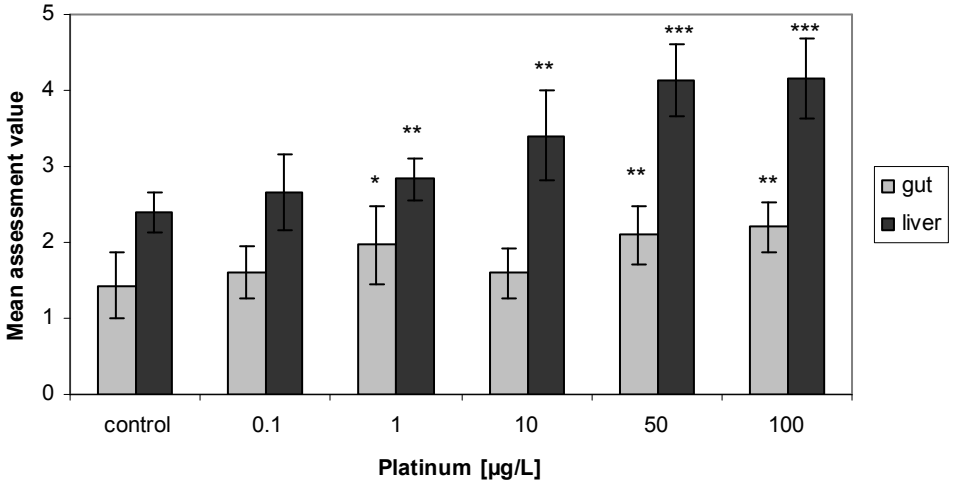


Fig. 2. Mean assessment values of the qualitative evaluations of 3 different sections per organ for the conditions of gut and liver of *Danio rerio* 7 days after fertilisation, exposed to different concentrations of $PtCl_2$ (means ± SD), n = 10. Levels of significant differences are $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***).

Histopathological effects of platinum on M. cornuarietis

Qualitative analysis

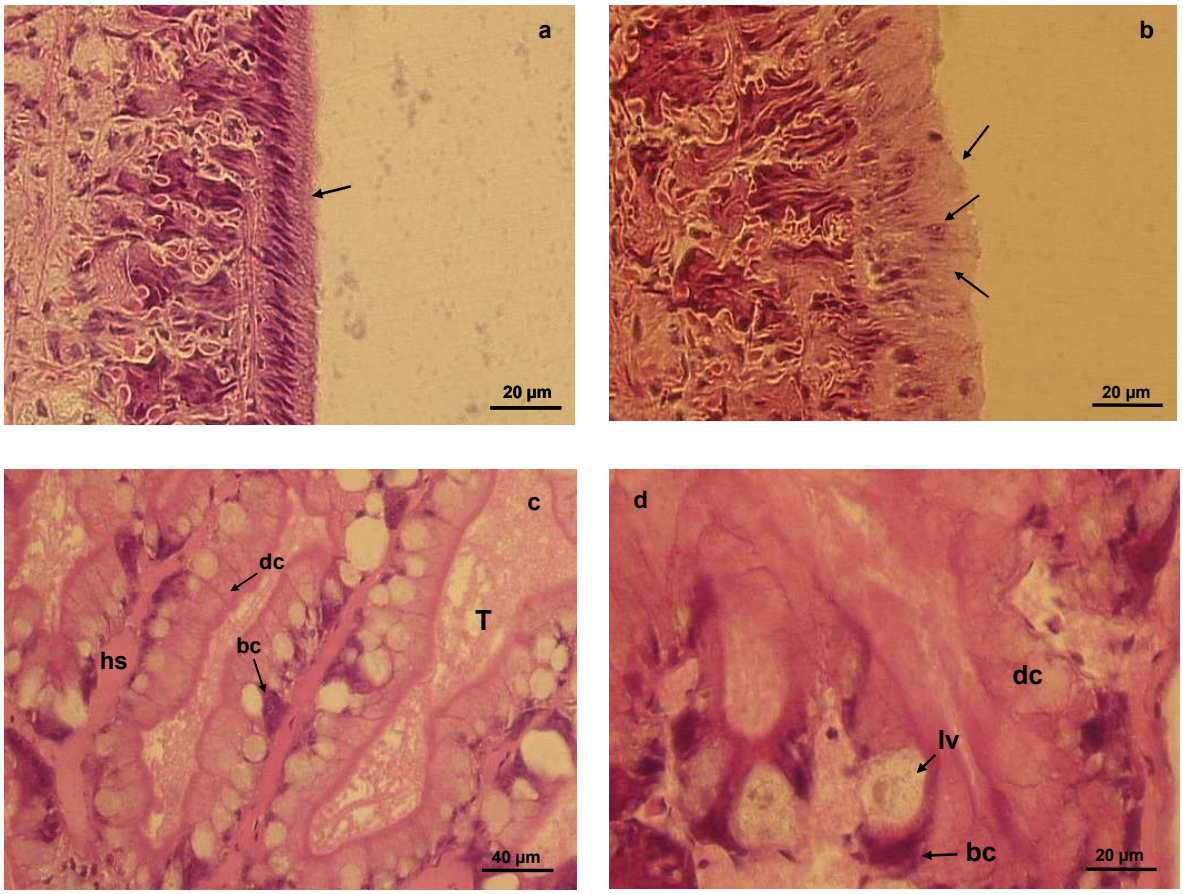
Histopathological investigations revealed effects of Pt on epidermis, hepatopancreas, and gills of *M. cornuarietis* (Fig. 3). Only little modification of the epidermae structure occurred due to Pt exposure. The hepatopancreas as an organ for storage and detoxification of heavy metals and the gills, which have been permanently in direct contact with the metal, were modified and showed strong reactions due to Pt exposure.

Epidermis: Alterations of the epidermis of *M. cornuarietis* due to Pt exposure were significantly increased at 10, 50 and 100 µg/L $PtCl_2$, and were characterized mainly by hypertrophy of cells and nuclei, irregular apical surfaces, and rarely, desquammation of parts of the epidermis (Fig. 3b).

Hepatopancreas: With rising concentrations of Pt, effects on the hepatopancreas of *M. cornuarietis* became more severe, resulting in strong reactions of the

hepatopancreas following exposure to high concentrations of 50 and 100 µg/L $PtCl_2$. Changes were characterized by large hemolymphe spaces between the tubules, enlarged tubule lumen, flattened epithelia, irregular shape of cells, cytoplasmatic protuberances of digestive cells, increased amount of vacuoles in digestive cells, and rarely, necrosis of digestive and basophilic cells at 50 and 100 µg/L $PtCl_2$ (Fig. 3d and e).

Gills: Due to Pt exposure, lamellae of gills and the shape of their cells appeared to be very irregular. Interlamellar spaces were extensively enlarged, hypertrophy of nuclei, pycnotic nuclei, and an increase in mucocyte number appeared (Fig. 3g and h). These alterations occurred most dominantly at the high concentrations of 50 and 100 µg/L $PtCl_2$.



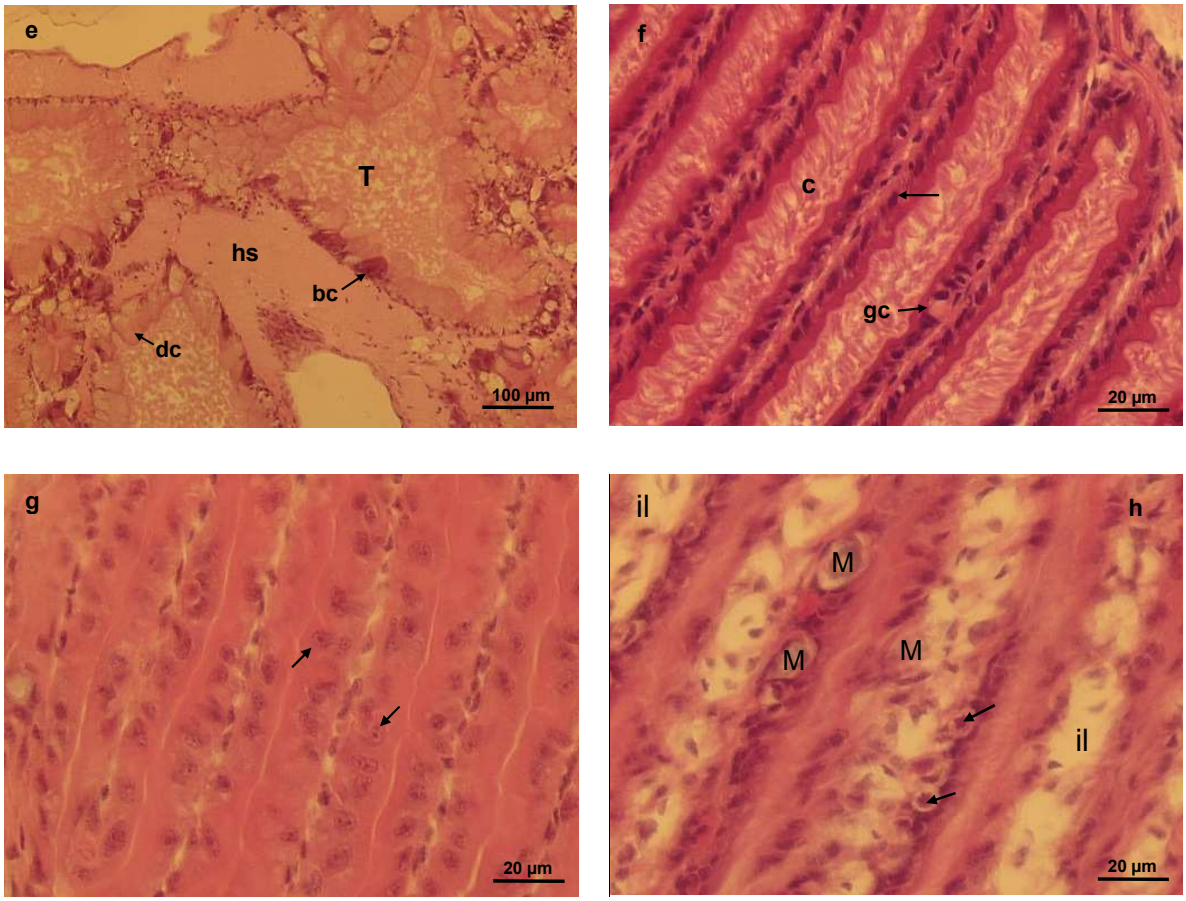


Fig. 3. (a) Epidermis of a control snail. The arrow indicates the regular apical surface of the prismatic cells; (b) epidermis of a snail exposed to 0.1 µg/L $PtCl_2$ showing hypertrophy of cells and nuclei and irregular apical surface of the prismatic cells (arrows); (c) hepatopancreas of a control snail with basophilic cells (bc), digestive cells (dc), tubuli lumen (T), hemolymphe spaces (hs); (d) and (e) hepatopancreas of snails exposed to 100 µg/L $PtCl_2$ indicating dilated hemolymphe spaces, irregular shape of digestive cells, enlarged tubules, enlarged lysosomal vacuoles (lv) and flattened epithelium; (f) gill of a control snail with nuclei with dense heterochromatin (arrow), cilia (c) and goblet cells (gc); (g) gill of a snail exposed to 100 µg/L $PtCl_2$ displaying hypertrophy of nuclei (arrows); (h) gill of a snail exposed to 50 µg/L $PtCl_2$ showing an increased amount of mucocytes (M), caryopycnosis (arrows) and dilated interlamellar spaces (il).

Semi-quantitative evaluation

Effects of Pt on the epidermis of *M. cornuarietis* were generally weak and were classified into a status of slight reaction, being significantly elevated at concentrations of 10, 50 and 100 µg/L $PtCl_2$, versus the control (Fig. 4). Gills of controls, on the average, had already been classified into a status of slight reaction. At concentrations of 50 and 100 µg/L $PtCl_2$, the conditions of the gills were significantly impaired showing reactions as described above (Fig. 4). A significant increase of the severity of effects of Pt on the hepatopancreas of *M. cornuarietis* was observed already at 0.1 µg/L $PtCl_2$ and higher concentrations, compared to the hepatopancreas' of control animals. The hepatopancreas' of animals exposed to 50

and 100 µg/L $PtCl_2$, which were assessed to category 3, showed highly significantly enhanced effects compared to hepatopancreas' of control animals (Fig. 4).

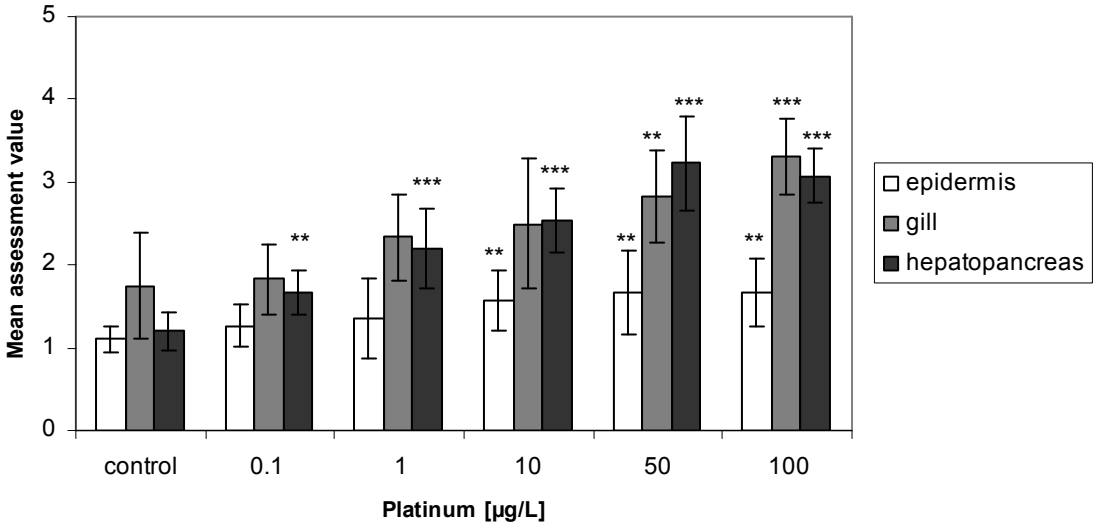


Fig. 4. Mean assessment values of the qualitative evaluations of 3 different sections per organ for the conditions of epidermis, gills and hepatopancreas of *Marisa cornuarietis* 26 days after fertilisation, exposed to different concentrations of $PtCl_2$ (means ± SD), n = 10. Levels of significant differences are $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

Proteotoxicity of platinum

Heat shock proteins (hsp70) were detected in all embryo samples of control groups and Pt treatment groups. For *D. rerio* the hsp70 response was increased significantly only for animals exposed to 100 µg/L $PtCl_2$ (Fig. 5). Hsp70 levels of *M. cornuarietis* did not differ significantly from the control in all Pt treatment groups. Therefore hsp70 expression in *M. cornuarietis* is not shown.

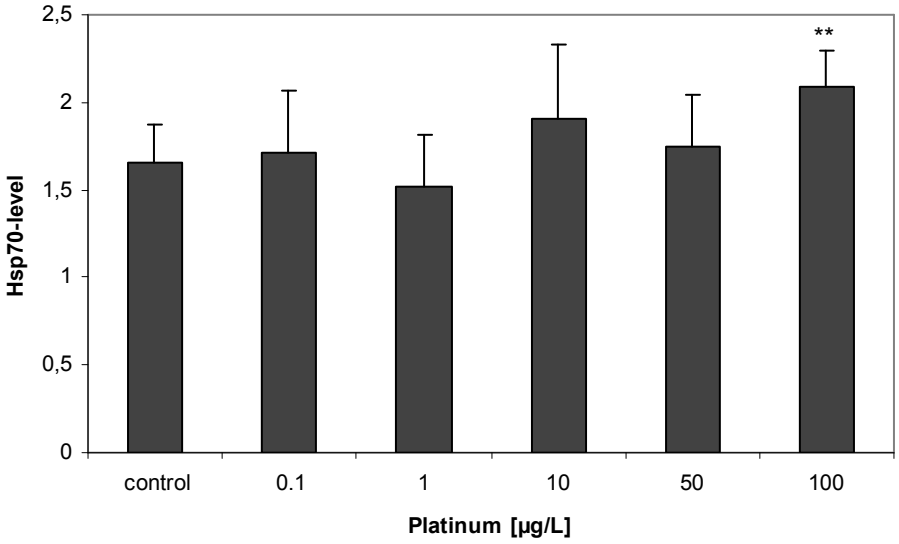


Fig. 5. Average hsp70 level in *Danio rerio* after exposure to various concentrations of $PtCl_2$, 7 days after fertilisation, n = 10 of 8 individuals each (except for 50 µg/L $PtCl_2$ n = 9 of 8 individuals each). Level of significant difference is $0.001 < p \leq 0.01$ (**).

Discussion

Uptake of metals by aquatic animals can result in biochemical, physiological, and histopathological alterations of their tissues and organs (Köhler et al., 1998; Triebskorn et al., 2008). The combined application of histopathology and hsp70 induction as a cellular and a biochemical response is often used (Hallare et al., 2005; Triebskorn et al., 2002, 2005) and leads to a more comprehensive assessment of potentially harmful substances. Therefore, the present study investigated histopathological effects and induction of hsp70 due to known concentrations of Pt (as analytically determined before by Osterauer et al., 2009) on *D. rerio* and *M. cornuarietis*.

Effects of platinum on D. rerio

Histopathologically investigated organs of *D. rerio* were gut and liver. In the present study, the liver, as the main organ for detoxification of harmful substances, showed the most severe symptoms with strong reactions at high concentrations of Pt (50 and 100 µg/L $PtCl_2$). Also, other investigations have revealed the liver to be the organ most affected by metal impact (Sastry and Gupta, 1978, 1979). The above-described Pt effects in the liver of *D. rerio*, like increased invasion of macrophages and dilated capillary spaces, are probably unspecific reactions. Sastry and Gupta (1978) investigated effects of $CdCl_2$ on *Heteropneustes fossilis* and found many

similar effects on the liver like pycnotic nuclei, vacuolisation of the cytoplasm and increased number of phagocytes. Hypertrophy of nuclei and caryopycnosis have also been described in the gills of *M. cornuarietis* and indicate that a main intracellular target of Pt is the nucleus. Most dominant effects of Pt on the gut of *D. rerio* were ablation of gut cells from the basal lamina, basal vacuolisation of the cytoplasm, irregular compartmentation and an increase of mucus cells. Vacuolisation of submucosal structures were also found in the fish *Channa punctatus* and *Puntius goionotus* following exposure to the metals mercury and cadmium (Banerjee and Bhattacharya, 1995; Kruatrachue et al., 2003). Several studies revealed metals to increase the mucus cell activity in the intestine of fish (e.g. Crespo et al., 1986; Sastry and Gupta, 1978). Mucus may dilute toxic substances and is possibly able to detoxify them (Andreozzi et al., 1994). Jouhaud et al. (1999) investigated effects of Pt^{4+} (H_2PtCl_6) on the gut of *D. rerio* and found Pt to be bioaccumulated in the gut. Their histopathological investigations revealed ultrastructural changes in the anterior intestine following exposure to 16 µg/L Pt. In their study, adaptive responses like fusion between villi and degenerative effects like lysis and necrosis of mucus cells and alterations in submucosa structures, were found. A significantly elevated hsp70 induction was found for *D. rerio* only at the highest test concentration (100 µg/L $PtCl_2$), indicating a low proteotoxicity of Pt for *D. rerio*.

Effects of platinum on M. cornuarietis

Gastropods are long known to be able to accumulate metals exceedingly (Dallinger and Wieser, 1984; Kurihara and Suzuki, 1987). Therefore, they have often been used as indicator organisms to assess metal pollution in the field (Coughtrey and Martin, 1977; Meincke and Schaller, 1974). In the present study, histopathological investigations of *M. cornuarietis* revealed the epidermis to be only slightly affected by Pt exposure, whereas hepatopancreas and gills showed strong reactions. The hepatopancreas is the main organ for detoxification, nutrient absorption and metabolism, and is known to be the main target organ for the accumulation of heavy metals (Dallinger and Wieser, 1984; Tanhan et al., 2005). In the present study, effects of Pt on the hepatopancreas were observed at ≥ 0.1 µg/L, including symptoms which probably derive from unspecific reactions. For example, enlarged hemolymphe spaces between the tubules have also been described in *Lymnea stagnalis* after exposure to Thiodan® (Ünlü et al., 2005) and in *Planorbarius corneus* due to exposure to endosulfan (Otludil et al., 2004). Also, vacuolisation in digestive

cells in *L. stagnalis*, due to Thiodan® exposure, has been observed before (Ünlü et al., 2005). In a previous study, Osterauer et al. (2009) found the weight of newly hatched *M. cornuarietis* to be reduced after exposure to 1, 10 or 100 µg/L $PtCl_2$. Damages in the hepatopancreas, like those described in the present study, supposedly result in a lower ability of the animals to digest food.

The main symptoms in the gills of *M. cornuarietis*, observed at ≥ 10 µg/L $PtCl_2$, were enlarged interlamellar spaces, irregular lamellae and irregular shape of the cells. These symptoms also seem to be nonspecific reactions. Further symptoms were hypertrophy of nuclei, pycnotic nuclei and an increase of the number of mucocytes. These symptoms have also been observed in the gill of the snail *Babylonia aerolata* after exposure to cadmium (Tanhan et al., 2005). Since the gill is a vital organ in aquatic organisms concerning oxygen uptake, an enhanced production of mucus might be a first reaction to mechanically protect the epithelia. However, an enhanced mucus production trades off against the capacity of oxygen transportation and therefore, may decrease the vitality of an organism.

Despite the fact that histopathological effects of Pt exposure in *M. cornuarietis* were observed, Pt concentrations used in this study did not induce elevated hsp70 responses in *M. cornuarietis*. Singer et al. (2005) investigated the induction of hsp70 in *Dreissena polymorpha* exposed to Pt^{4+} and found the induction threshold for Pt to be 43–58 µg/g. This bioaccumulated concentration is in the same order of magnitude as in our study, since previous studies of Osterauer et al. (2009) found bioaccumulated Pt in *M. cornuarietis* to be 53.7 µg/g after exposure to 100 µg/L $PtCl_2$ for 26 days. In the study of Singer et al. (2005) an increased level of hsp70 was induced after 32–39 days of exposure, whereas in the present study, *M. cornuarietis* was exposed to a maximum of 100 µg/L $PtCl_2$ for only 26 days. These results show that in different species or life stages the hsp70 response to a factor can be different and, compared to their study, the length of the exposure period might have been too short to reach the threshold level of the tissue metal concentration to induce hsp70.

For detoxification of non-essential metals, some metals, e.g. Cd, Zn and Co, are bound to metallothioneins (Kägi and Schäffer, 1988). Since Frank et al. (2008) found Pd to induce metallothioneins, the results of this study could infer that Pt, as chemically akin with Pd, may also be bound to metallothioneins. This could also be an explanation for the low hsp70 induction due to Pt exposure. Although hsp70 is known to be expressed in a variety of taxa and is a widely used response to assess the risk of a potentially toxic substance, hsp70 studies should be accompanied by other studies investigating effects on different biological levels as seen in the

present study, where the sole application of hsp70 measurements without histopathological investigations would have underestimated the toxic effects of Pt on *M. cornuarietis*.

Concentrations generally found in the water ecosystem are in the lower ng/L range (Ravindra et al., 2004). LOEC data found in the present study were 1 µg/L $PtCl_2$ for *D. rerio* and 0.1 µg/L $PtCl_2$ for *M. cornuarietis*. Although these concentrations are above environmental concentrations, effects of Pt on organisms due to chronic Pt exposure and at contaminated sites, such as run-off water sites, where Pt concentrations were found to be in a µg/L range (Laschka et al., 1996), cannot be excluded.

Conclusions

The present study investigated histopathological alterations and induction of hsp70 due to exposure of 0.1, 1, 10, 50 and 100 µg/L $PtCl_2$ on the ramshorn snail *M. cornuarietis* and the zebrafish *D. rerio*. The results of the present study indicate Pt to be capable of inducing histopathological alterations in the tissues of the freshwater organisms *D. rerio* and *M. cornuarietis*. Furthermore, Pt was shown to be slightly proteotoxic for *D. rerio* at the highest test concentration of 100 µg/L $PtCl_2$. Although there are a great number of publications on PGMs (for review see Kalavrouziotis and Koukoulakis, 2009; Ravindra et al., 2004; Zimmermann and Sures, 2004), most of these manuscripts deal exclusively with their distribution in the environment and their bioavailability for plants and organisms, but do not address the effects. Therefore, the present study basically broadens the knowledge of the effects of Pt in aquatic organisms. Furthermore, these results are necessary to comparatively assess the risk of elements used for catalytic converter technology and to help find the least harmful solution for controlling automobile exhaust fumes.

Acknowledgements

Studienstiftung des deutschen Volkes and Landesgraduiertenförderung Baden-Württemberg are highly acknowledged for financial support.

References

Andreozzi, G., Antonucci, R., Affatato, C., Gargiulo, G., Battaglini, P., 1994. The effect of cadmium on the intestine of *Carassius auratus*. Anat. Histol. Embryol. 23: 102–111.

Banerjee, S., Bhattacharya, S., 1995. Histopathological changes induced by chronic nonlethal levels of elsan, mercury and ammonia in the snail intestine of *Channa punctatus* (Bloch). Ecotoxicol. Environ. Saf. 31: 62–68.

Bächtle, C., 2000. Im stress. NEWSletter 1: 22–25.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72: 248–254.

Bresch, H., 1991. Early life-stage test in zebrafish versus a growth test in rainbow trout to evaluate toxic effects. Bull. Environ. Contam. Toxicol. 46: 641–648.

Coughtrey, P.J., Martin, M.H., 1977. The uptake of lead, zinc, cadmium and copper by the Pulmonate mollusc, *Helix aspersa* Müller, and its relevance to monitoring of heavy metal contamination of the environment. Oecologia 73: 91–98.

Crespo, S., Nonnotte, G., Colin, A., Leray, C., Nonnotte, L., Aubree, A., 1986. Morphological and functional alterations induced in trout intestine by dietary cadmium and lead. J. Fish Biol. 28: 69–80.

Dallinger, R., Wieser, W., 1984. Patterns of accumulation, distribution and liberation of Zn, Cu, Cd and Pb in different organs of the land Snail *Helix pomatia* L. Comp. Biochem. Physiol. 79C: 117–124.

De Pomerai, D.I., 1996. Heat-shock proteins as biomarkers of pollution. Hum. Exp. Toxicol. 15: 258–279.

Duft, M., Schmitt, C., Bachmann, J., Brandelik, C., Schulte-Oehlmann, U., Oehlmann, J., 2007. Prosobranch snails as test organisms for the assessment of endocrine active chemicals—an overview and a guideline proposal for a

reproduction test with the freshwater mudsnail *Potamopyrgus antipodarum*. Ecotoxicology 16: 169–182.

Eaton, J.G., McKim, J.M., Holcombre, G.W., 1978. Metal toxicity to embryos and larvae of seven freshwater fish species. I. Cadmium. Bull. Environ. Contam. Toxicol. 19: 95–103.

Frank, S., Singer, C., Sures, B., 2008. Metallothionein (MT) response after chronic palladium exposure in the Zebra mussel, *Dreissena polymorpha*. Environ. Res. 108: 309–314.

Gething, M.-J., Sambrook, J., 1992. Protein folding in the cell. Nature (Lond.) 355: 33–45.

Hallare, A.V., Schirling, M., Luckenbach, T., Köhler, H.-R., Triebskorn, R., 2005. Combined effects of temperature and cadmium on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos. J. Therm. Biol. 30: 7–17.

Haus, N., Zimmermann, S., Wiegand, J., Sures, B., 2007. Occurrence of platinum and additional traffic related heavy metals in sediments and biota. Chemosphere 66: 619–629.

Hill, A.J., Teraoka, H., Heidemann, W., Peterson, R.E., 2005. Zebrafish as a model vertebrate for investigating chemical toxicity. Rev. Toxicol. Sci. 86: 6–19.

Hoppstock, K., Sures, B., 2004. Platinum-group Metals. In: Merian, E., Anke, M., Ihnat, M., Stoeppler, M. (Eds.), Elements and their Compounds in the Environment. Wiley, VCH, Weinheim, Germany, pp. 1047–1086.

Jouhaud, R., Biagianti-Risbourg, S., Vernet, G., 1999. Effets du platine chez *Brachydanio rerio* (Téléostéen, Cyprinidé). I. Toxicité aiguë; bioaccumulation et histopathologie intestinales. J. Appl. Ichthyol. 15: 41–48.

Kalavrouziotis, I.K., Koukoulakis, P.H., 2009. The environmental impact of the platinum group elements (Pt, Pd, Rh) emitted by the automobile catalyst converters. Water Air Soil Pollut. 196: 393–402.

Kägi, J.H.R., Schäffer, A., 1988. Biochemistry 27: 8509–8515.

Klaassen, C.D., 1996. Casarett & Doull's Toxicology—The Basic Science of Poisons, Fifth ed. McGraw-Hill, New York, pp. 725–726.

Köhler, H.-R., Triebskorn, R., Stöcker, W., Kloetzel, P.-M., Alberti, G., 1992. The 70 kD heat shock protein (hsp70) in soil invertebrates: a possible tool for monitoring environmental toxicants. Arch. Environ. Contam. Toxicol. 22: 334–338.

Köhler, H.-R., Eckwert, H., 1997. The induction of stress proteins (hsp) in *Oniscus asellus* (Isopoda) as a molecular marker of multiple heavy metal exposure. II: Joint toxicity and transfer to field situations. Ecotoxicology 6: 263–274.

Köhler, H.-R., Triebskorn, R., 1998. Assessment of the cytotoxic impact of heavy metals on soil invertebrates using a protocol integrating qualitative and quantitative components. Biomarkers 3: 109–127.

Köhler, H.-R., Belitz, B., Eckwert, H., Adam, R., Rahman, B., Trontelj, P., 1998. Validation of hsp70 stress gene expression as a marker of metal effects in *Deroceras reticulatum* (Pulmonata): correlation with demographic parameters. Environ. Toxicol. Chem. 17: 2246–2253.

Köhler, H.-R., Lazzara, R., Dittbrenner, N., Capowiez, Y., Mazzia, C., Triebskorn, R., 2009. Snail phenotypic variation and stress proteins: do different heat response strategies contribute to Waddington's widget in field populations? J. Exp. Zool. (Mol. Dev. Evol.) 312B: 136–147.

Kruatrachue, M., Rangsayatorn, N., Pokethitiyook, P., Upatham, E.S., Singhakaew, S., 2003. Histopathological changes in the gastrointestinal tract of fish, *Puntius gonionotus*, fed on dietary cadmium. Bull. Environ. Contam. Toxicol. 71: 561–569.

Kurihara, Y., Suzuki, T., 1987. Removal of heavy metals and sewage sludge using the mud snail, *Cipangopaludina-chinensis-malleata reeve* in paddy fields as artificial wetlands. Water Sci. Technol. 19: 281–286.

Laemmli, U.K., 1970. Cleavage of structural proteins durino assembly of the head of bacteriophage T4. Nature 227: 680–684.

Lajtner, J., Erben, R., Klobucar, G.I.V., 1996. Histopathological effects of phenol on the digestive gland of *Amphimelania holandri* Fér. (Gastropoda, Prosobranchia). Bull. Environ. Contam. Toxicol. 57: 458–464.

Laschka, D., Striebel, T., Daub, J., Nachtwey, M., 1996. Platinum in rainwater discharges from roads. UWSF–Z Umweltchem. Ökotox. 8: 124–129.

Lewis, S., Handy, R.D., Cordi, B., Billinghurst, Z., Depledge, M.H., 1999. Stress proteins (HSP's): methods of detection and their use as an environmental biomarker. Ecotoxicology 8: 351–368.

Meincke, K.F., Schaller, K.H., 1974. Use of the snail *Helix pomatia* L. on cultivated land as an indicator of environmental pollution with Zn, Pb and Fe. Oecologia 15: 393–398.

Melo, L.E.L., Coler, R.A., Watanabe, T., Batalla, J., 2000. Developing the gastropod *Pomacea lineata* (Spix) as a toxicity test organism. Hydrobiologia 429: 73–78.

Meyers, T.R., Hendricks, J.D., 1982. A summary of tissue lesions in aquatic animals induced by controlled exposures to environmental contaminants, chemotherapeutic agents, and potential carcinogens. Mar. Fish. Rev. 44: 1–17.

Moldovan, M., Rauch, S., Gomez, M., Palacios, M.A., Morrison, G., 2001. Bioaccumulation of palladium, platinum and rhodium from urban particulates and sediments by the freshwater isopod *Asellus aquaticus*.Water Res. 35: 4175–4183.

Oehlmann, J., Di Benedetto, P., Tillmann, M., Duft, M., Oetken, M., Schulte-Oehlmann, U., 2007. Endocrine disruption in prosobranch molluscs: evidence and ecological relevance. Ecotoxicology 16: 29–43.

OECD Guideline for Testing of Chemicals, 1992. Test Guideline 203. Fish, Acute Toxicity Test. OECD Guideline for Testing of Chemicals.

Osterauer, R., Haus, N., Sures, B., Köhler, H.-R., 2009. Uptake of platinum by zebrafish (*Danio rerio*) and ramshorn snail (*Marisa cornuarietis*) and resulting effects on early embryogenesis. Chemosphere 77: 975–982.

Otludil, B., Cengiz, E.I., Yildirim,M.Z., Ünver, Ö., Ünlü, E., 2004. The effects of endosulfan on the great ramshorn snail *Planorbarius corneus* (Gastropoda, Pulmonata): a histopathological study. Chemosphere 56: 707–716.

Ravindra, K., Bencs, L., Van Grieken, R., 2004. Platinum group elements in the environment and their health risk. Sci. Total Environ. 318: 1–43.

Rombough, P., 2002. Gills are needed for ionoregulation before they are needed for O_2 uptake in developing zebrafish, *Danio rerio*. J. Exp. Biol. 205: 1787– 1794.

Sastry, K.V., Gupta, P.K., 1978. Effect of mercuric chloride on the digestive system of *Channa punctatus*. A histopathological study. Environ. Res. 16: 270–278.

Sastry, K.V., Gupta, P.K., 1979. The effect of cadmium on the digestive system of the teleost fish, *Heteropneustes fossilis*. Environ. Res. 19: 221–230.

Sawasdee, B., Köhler, H.-R., 2009. Embryo toxicity of pesticides and heavy metals to the ramshorn snail, *Marisa cornuarietis* (Prosobranchia). Chemosphere 75: 1539–1547.

Scheil, V., Triebskorn, R., Köhler, H.-R., 2008. Cellular and stress protein responses to the UV filter 3-benzylidene camphor in the amphipod crustacean *Gammarus fossarum* (Koch 1835). Arch. Environ. Contam. Toxicol. 54: 684–689.

Scheil, V., Zürn, A., Triebskorn, R., Köhler, H.-R., 2010. Embryo development, stress protein (Hsp70) responses and histopathology in zebrafish (*Danio rerio*) following exposure to nickel chloride, chlorpyrifos and binary mixtures of them. Environ. Toxicol. 25: 83–93.

Schirling, M., Bohlen, A., Triebskorn, R., Köhler, H.-R., 2006. An invertebrate embryo test with the apple snail *Marisa cornuarietis* to assess effects of potential developmental and endocrine disruptors. Chemosphere 64: 1730–1738.

Singer, C., Zimmermann, S., Sures, B., 2005. Induction of heat shock proteins (hsp70) in the zebra mussel (*Dreissena polymorpha*) following exposure to platinum group metals (platinum, palladium and rhodium): comparison with lead and cadmium exposures. Aquat. Toxicol. 75: 65–75.

Sures, B., Zimmermann, S., 2007. Impact of humic substances on the aqueous solubility, uptake and bioaccumulation of platinum, palladium and rhodium in exposure studies with *Dreissena polymorpha*. Environ. Pollut. 146: 444– 451.

Tanhan, P., Sretarugsa, P., Pokethitiyook, P., Kruatrachue, M., Upatham, E.S., 2005. Histopathological alterations in the edible snail, *Babylonia areolata* (spotted babylon), in acute and subchronic cadmium poisoning. Environ. Toxicol. 20: 142–149.

Triebskorn, R., Köhler, H.-R., Honnen, W., Schramm, M., Adams, S.M., Müller, E.F., 1997. Induction of heat shock proteins, changes in liver ultrastructure, and alterations of fish behavior: are these biomarkers related and are they useful to reflect the state of pollution in the field? J. Aquat. Ecosyst. Stress Recov. 6: 57–73.

Triebskorn, R., Adam, S., Casper, H., Honnen, W., Pawert, M., Schramm, M., Schwaiger, J., Köhler, H.-R., 2002. Biomarkers as diagnostic tools for evaluating effects of unknown past water quality conditions on stream organisms. Ecotoxicology 11: 451–465.

Triebskorn, R., Bader, K., Linder, B., Köhler, H.-R., 2005. Snails and slugs as non-targets for environmental chemicals. IOBC–WPRS Bull. 28: 1–7.

Triebskorn, R., 2007. Mixture toxicity of environmental chemicals in zebrafish (*Danio rerio*) larvae: Histopathological effects of imidacloprid and thiacloprid. Interim Report, NoMiracle (NOvel Methods for Integrated Risk Assessment of CumuLative stressors in Europe).

Triebskorn, R., Telcean, I., Casper, H., Farkas, A., Sandu, C., Stan, G., Colărescu, O., Dori, T., Köhler, H.-R., 2008. Monitoring pollution in River Mureş, Romania, part II: metal accumulation and histopathology in fish. Environ. Monit. Assess. 141: 177–188.

Ünlü, E., Cengiz, E.I., Yildirim, M.Z., Otludil, B., Ünver, Ö., 2005. Histopathological effects in tissues of snail *Lymnaea stagnalis* (Gastropoda, Pulmonata) exposed to sublethal concentration of Thiodan® and recovery after exposure. J. Appl. Toxicol. 25: 459–463.

Welch, W., 1993. Stress-proteine. Spektrum der Wissenschaft, 40–47.

Zereini, F., Alt, F. (Eds.), 2000. Anthropogenic Platinum-group Element Emission: Their Impact on Man and Environment. Springer, Heidelberg, Germany.

Zimmermann, S., Baumann, U., Taraschewski, H., Sures, B., 2004. Accumulation and distribution of platinum and rhodium in the European eel *Anguilla anguilla* following aqueous exposure to metal salts. Environ. Pollut. 127: 195–202.

Zimmermann, S., Sures, B., 2004. Significance of platinum group metals emitted from automobile exhaust gas converters for the biosphere. Environ. Sci. Pollut. Res. 11: 194–199.

Kapitel 3: Genotoxicity of platinum in embryos of zebrafish (*Danio rerio*) and ramshorn snail (*Marisa cornuarietis*)

Raphaela Osterauer [a], Christopher Faßbender [b], Thomas Braunbeck [b], Heinz-R. Köhler [a]

[a] Animal Physiological Ecology Department, University of Tübingen, Konrad-Adenauer-Str. 20, 72072 Tübingen, Germany

[b] Aquatic Ecology and Toxicology Section, Department of Zoology, University of Heidelberg, Im Neuenheimer Feld 230, 69120 Heidelberg, Germany

Abstract

The metal platinum is *inter alia* used for industrial and medical purposes. Due to its application in automobile catalytic converters and as an anti-cancer drug, Pt enters the aquatic environment *via* road runoff and hospital sewage and raises concerns about its environmental impact and toxicity to organisms. Therefore, the genotoxicity of Pt at 0, 0.1, 1, 10, 50, 100 and 200 µg/l $PtCl_2$ was tested on two freshwater organisms, zebrafish (*Danio rerio*) and ramshorn snail (*Marisa cornuarietis*) using the single cell gel electrophoresis, also called comet assay. $PtCl_2$ did not show any genotoxicity for *D. rerio* at the tested concentrations, whereas significantly elevated DNA damage was observed in *M. cornuarietis* at 1 µg/l $PtCl_2$ and beyond. The results of the study suggest a high sensitivity of *M. cornuarietis* concerning the genotoxic impact of $PtCl_2$.

Keywords Aquatic organisms, $PtCl_2$, DNA damage, Comet assay

Science of the Total Environment, 2011, 409: 2114–2119

Introduction

In the last decades, an increased release of the platinum group elements (PGE) platinum (Pt), palladium (Pd), and rhodium (Rh) into various environmental compartments has taken place. These metals are predominantly used in automobile catalytic converters to reduce toxic exhaust fumes (for overview, see Hoppstock and Sures, 2004; Wiseman and Zereini, 2009). It is well-known that toxicological effects of metals decisively depend on their speciation (Sabbioni et al., 1985). PGE are emitted from catalytic converters in particulate form, mainly in elemental form or as oxides (Moldovan et al., 2002), but, due to transformation processes, and also dependant from particle size (Artelt et al., 1999), a considerable amount becomes soluble in water and is, therefore, bioavailable and mobile in the environment (Hill and Mayer, 1977; König et al., 1992; Lustig et al., 1996, 1998).

Besides its application in the automobile industry, Pt is used as catalyst in various chemical production processes, in textile industry, electrical engineering, jewelry production and dentistry. Metalloorganyls like cisplatin (cis-diaminodichloroplatinum [II]) and carboplatin (diamino(1.1-cyclobutandicarboxylatoplatinum [II]) are commonly used as anti-cancer drugs in medical applications. In comparison to traffic-borne emissions, the contribution of hospital-related Pt to environmental exposure levels is rather low (Kümmerer et al., 1999). However, considering the occurrence of soluble and therefore potentially bioreactive Pt metabolites in hospital effluents, properties regarding mobility, sorption, and toxicity for aquatic organisms might be of great concern already at very small quantities. Since anti-cancer drugs often act by inhibiting cell growth or directly killing cells, many cytostatic drugs are categorized as CMR-drugs (carcinogenic, mutagenic, reprotoxic). Furthermore, some cytostatics have been shown to excert ecotoxicity, reproduction toxicity, and genotoxicity to non-target organisms (Lenz et al., 2007; Zounkova et al., 2010).

Aquatic ecosystems are the first sink, since relatively high concentrations of Pt are discharged *via* road runoff water (Laschka et al., 1996) and hospital sewage (Kümmerer et al., 1999). Therefore, fish and aquatic gastropods represent suitable model organisms for the testing of Pt pollutants because they might be exposed to in the environment. The zebrafish (*Danio rerio*) is a freshwater cyprinid and a model organism for toxicological, genetic, and ecotoxicological investigations (Hill et al., 2005; Nüsslein-Volhard, 1994). Gastropods have been known for long to naturally accumulate metals and have consequently been used as bioindicators for the assessment of aquatic metal pollution (Liang et al., 2004). The ramshorn snail

(*Marisa cornuarietis*), an ampullariid freshwater snail originating from Central and South America, has gained attention through studies investigating endocrine disruptors (Oehlmann et al., 2007; Schulte-Oehlmann et al., 2000) and has also been shown to react sensitively to heavy metal exposure during its embryonic development (Osterauer et al., 2009, 2010a,b; Sawasdee and Köhler, 2009; Schirling et al., 2006). Both model organisms are *inter alia* characterized by a transparent chorion allowing direct observation of the development of embryos, which seems to be a very sensitive stage for environmental pollutants (Luckenbach et al., 2001; Pickering and Gast, 1972). Beside developmental parameters such as heart rate, time of hatching, and developmental abnormalities which are endpoints observed in the embryo tests with *D. rerio* (Nagel, 2002), and with *M. cornuarietis* (MariETT; Schirling et al., 2006), other biomarkers are accessible in the developing embryos. Genotoxicity is a crucial field of study, since induction of mutations may results in population-level effects (Anderson and Wild, 1994; White et al., 1999). A widely used method to test the genotoxic hazard of a pollutant is the comet assay, also known as single cell gel electrophoresis. This test detects single and double DNA strand breaks and alkali-labile sites in single cells (Fairbairn et al., 1995; Lee and Steinert, 2003; Singh et al., 1988) and has been adapted to test genotoxic effects in embryos of *D. rerio* some years ago (Kosmehl et al., 2006).
The present study aims at evaluating the genotoxic potential of environmentally relevant and higher concentrations of $PtCl_2$ in embryos of *D. rerio* using the comet assay according to the protocol of Kosmehl et al. (2006) and at adapting this protocol for testing the genotoxicity of identical $PtCl_2$ concentrations in embryonic stages of the snail *M. cornuarietis*.

Materials and methods

Maintenance of the test animals, egg production and exposure scenario

Adult *D. rerio* and *M. cornuarietis* were kept in aerated and filtered 120–230 l aquaria with a minimum of 1 l of water per fish and 0.5 l of water per snail on average under the following conditions: temperature: 25±1 °C, pH: 7.5–8, water conductivity for fish: 400 µS/cm, for snails: 820 µS/cm, light/dark regime: 12 h/12 h. 33% of the aquarium water volume for fish was exchanged every 10–14 days and 50% of the water volume for snails every week. The fish were fed fish flake food (Nutrafin Max flakes, Hagen, Germany) and frozen food (mosquito larvae,

Mysis, Moina, Artemia), twice a day. The snails were solely fed fish flake food (*Nutrafin Max flakes*, Hagen, Germany) once a day.

Egg masses of *M. cornuarietis*, laid at night, were collected from the aquaria in the respective mornings. To obtain eggs of *D. rerio*, spawn traps covered with stainless steel mesh and spawning substrate were placed in the aquaria in the evening. The next morning, spawning and fertilization was triggered by the onset of illumination of the aquaria. One hour later, eggs were collected which had been deposited in the spawning boxes.

Eggs of fish or snails were transferred to 50 ml plastic Petri dishes containing different solutions of $PtCl_2$ (platinum standard solution of 1000 µg/ml in 2% HCl, Ultra Scientific, Wesel, Germany) with the following nominal concentrations: 0.1, 1, 10, 50, 100 and 200 µg/l $PtCl_2$ and the control medium, respectively.

After 90 min incubation at 26 °C in a climate chamber, fertilized eggs of *D. rerio* could be selected and were transferred to the respective final Petri dishes containing the above-mentioned solutions or the control medium. For *D. rerio*, reconstituted water (OECD Guideline for Testing of Chemicals, 1992) was used as a control. Since *M. cornuarietis* cannot develop in the reconstituted water used for *D. rerio* (Osterauer et al., 2009), tap water from the snail aquaria served as the control.

Following the procedure by Kosmehl et al. (2006), the exposure period was 96 h for *D. rerio*. However, *M. cornuarietis* has a much longer embryonic development. Therefore, the exposure period was 8 d for *M. cornuarietis*. Throughout the exposure period, embryos were kept at 26±1 °C in a climate chamber with a light/dark regime of 12 h/12 h. The medium was completely exchanged every second day for *D. rerio* and daily for *M. cornurietis*.

Due to known precipitation of Pt during exposure (Sures and Zimmermann, 2007), real concentrations of Pt in the exposure media of identical exposure scenario as in the present study were determined as published by Osterauer et al. (2009, 2010b) and are as follows: 0.20, 1.23, 8.08, 44.24, and 98.66 µg/l Pt for *D. rerio* and 0.04, 0.87, 3.66, 33.73, 74.20, and 163.4 µg/l Pt for *M. cornuarietis* corresponding to nominal concentrations of 0.1, 1, 10, 50, and 100 µg/l $PtCl_2$ for *D. rerio* and 0.1, 1, 10, 50, 100, and 200 µg/l $PtCl_2$ for *M. cornuarietis*. For the nominal concentration of 200 µg/l $PtCl_2$ used for *D. rerio*, no data on the real concentration are available. To simplify the reading of this article, the applied nominal concentrations are used throughout.

Comet assay

For each tested Pt concentration, after the exposure period, 20 hatched embryos of *D. rerio* or 10 embryos of *M. cornuarietis*, which had been dissected from the chorion prior to the assay, were anaesthetized in a saturated benzocain (Sigma, Deisenhofen, Germany) solution. For mechanical cell isolation, embryos of either species were homogenized using a 2-ml glass/glass tissue grinder (Dounce-type; Witeg, Wertheim, Germany) with a defined pestle/wall distance of 30–80 µm adding 2 ml of phosphate buffered saline (PBS). 1 ml of the resulting cell suspension was filtered through a 70 µm gauze (Verseidag, Krefeld, Germany) into a 1.5 ml reaction tube and centrifuged for 10 min at 4 °C and 200×*g*. The pellet was resuspended in 1 ml PBS and again centrifuged for 7 min at 4 °C and 180×*g*. The pellet was resuspended in 100 µl PBS and mixed with 90 µl low melting agarose (LMA; Peqlab, Erlangen, Germany).
Known genotoxicants (H_2O_2 and UV light) served as positive controls. For this end, prior to the assay, animals were incubated for 1 h to 1% hydrogen peroxide (H_2O_2) dissolved in the respective control medium. For UV light treatment, isolated cells were exposed to UV light (254 nm) in a 1.5 ml reaction tube for 5 min (Vilber Lourmat, Eberhardzell, Germany).
For cytotoxicity testing, 20 µl of the filtered cell suspension was mixed with 10 µl of 0.4% trypan blue solution (Sigma, Deisenhofen, Germany). The cells were spread on a microscope slide and overcasted with a cover slip. Subsequently, 100 randomly selected cells were evaluated using a light microscope (×400 magnification; Axiostar plus, Zeiss, Jena, Germany). Genotoxicity testing was carried out only with cell suspension preparations showing a cell viability of ≥80%.

The alkaline comet assay was performed as described by Kosmehl et al. (2006). Fully frosted microscope slides (Langenbrink, Emmendingen, Germany) were degreased in 100% ethanol and coated with 1% normal melting agarose (NMA; Peqlab, Erlangen, Germany) in PBS. Subsequently, this agarose layer was scraped off using a cover slip. After drying the slides at 37 °C for 5 min, these were coated with 200 µl 0.5% NMA. The slides were cooled for 3 min on ice and dried for 10 min at 37 °C. 90 µl of the above mentioned cell suspension-LMA-mix was spread on top of this first permanent agarose layer. The slides were cooled for 3 min on ice and dried for 5 min at 37 °C. Finally, a third layer of 90 µl 0.5% NMA coated the cell-containing agarose layer. After another cooling (3 min) and drying (5 min), the slides were incubated in a lysis solution (100 mM EDTA, 2.5 M NaCl, 1% (v/v) Triton X-

100, and 10% (v/v) DMSO, pH 13) in the dark at 4 °C for 90 min. For DNA unwinding, slides were immersed in the dark for 20 min in a horizontal electrophoresis tank containing a cooled alkaline buffer (12 g/l NaOH and 0.37 g/l EDTA). The electrophoresis was run at 28 V and 250 mA for 20 min in the same buffer. Subsequently, the slides were neutralized for 2 min in Tris buffer (400 mM Tris-HCl, pH 7.4). Prior to image analysis, DNA was stained by adding 60 µl of a 20 µg/ml ethidium bromide (Sigma, Deisenhofen, Germany) solution.

Image analysis and statistics

For image analysis, the slides were evaluated at ×340 magnification using a fluorescent microscope (Aristoplan; Leica, Wetzlar, Germany) equipped with an image analysis system (Optilas, Munich, Germany) with a greyscale CCD camera (JAI Pulnix TM-765E Kinetic, Glostrup, Denmark) and the Komet 5.5 software (Kinetic Images, Liverpool, UK).

In cells with DNA damage, migrating DNA causes the image of a comet tail. For each treatment, the tail moment (intensity of the DNA tail × length of the tail) of 100 randomly chosen single nucleoids were evaluated on two replicate slides (50 cells on each slide). Image examples of typical comets are shown in Fig. 1.

Normal distribution of data was checked with the Shapiro-Wilk test. Since not all data were normally distributed, they were analyzed for significance (ANOVA-on-ranks) using SigmaStat 3.1 software (Systat, Erkath, Germany), followed by a Dunnett *post*-hoc test (SigmaStat 3.1, Systat, Errath, Germany) to identify significant differences between the groups. Differences were considered to be significant for $p \leq 0.05$ (*).

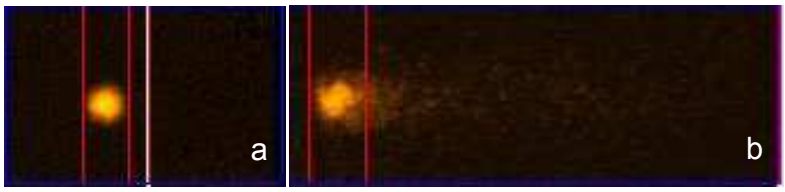


Fig. 1. Images illustrating the induction of DNA damage described as tail moment in cells of *M. cornuarietis* exposed to $PtCl_2$ in the comet assay. a) control, and b) 100 µg/l $PtCl_2$.

Results

Genotoxicity of UV light and H_2O_2 in D. rerio and M. cornuarietis

To control for the correct operation of the comet assay, two known genotoxic agents with different modes of action and different application times during the assay were chosen as positive controls: UV light was applied on isolated cells which were irradiated for 5 min directly after the cell isolation, and H_2O_2 exposure was applied on whole embryos which were exposed to 1% H_2O_2 for 1 h, just before the start of the assay.

In both organisms, exposure to H_2O_2 induced stronger DNA damage than irradiation with UV light (Figs. 2 and 3).

Means of tail moments of negative controls did not differ significantly between the both test species. Likewise, in H_2O_2-treated embryos, medians of tail moments of both test species were similar. However, UV light caused significantly more damage in the DNA from cells of *M. cornuarietis* than in the DNA of *D. rerio*-derived cells.

Genotoxicity of $PtCl_2$ in D. rerio

Comet assay data revealed no genotoxic hazard of the tested $PtCl_2$ concentrations in cells derived from *D. rerio* (Fig. 2).

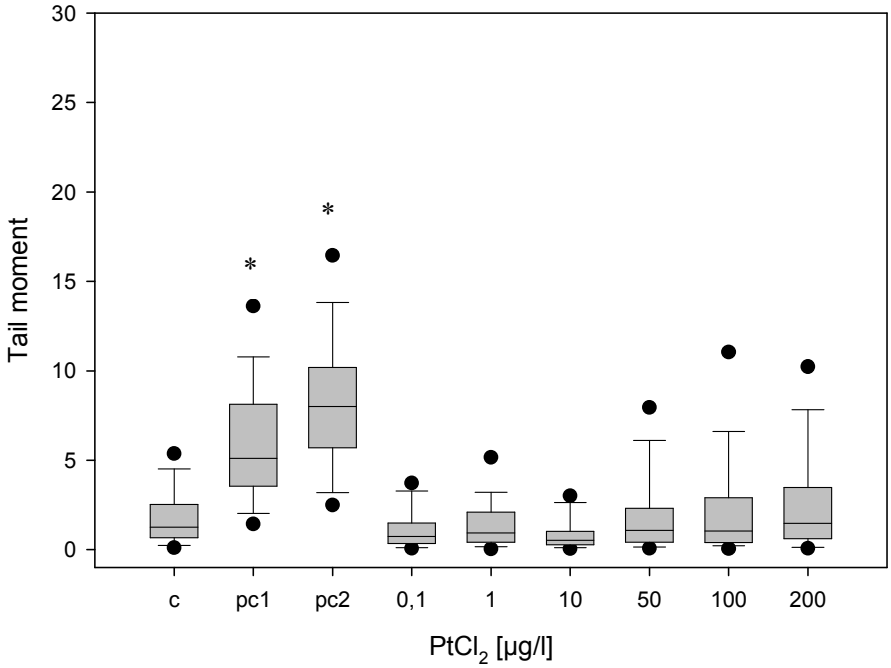


Fig. 2. DNA fragmentation as tail moment (intensity of the DNA tail × length of the tail) in cells isolated from *Danio rerio* 4 days post-fertilization and exposed to different concentrations of $PtCl_2$ or known genotoxicants as positive controls (pc1: ultraviolet light, pc2: hydrogen peroxide). Data are given as box plot of 100 nucleoids. Shaded boxes display the following percentiles: 25 (lower edge), 50 = median (horizontal bar) and 75 (upper edge); whiskers above and below the box represent the 90th and 10th percentile; black dots refer to 5th (lower dot) and 95th (upper dot) percentiles. Level of significant difference *versus* the control is $p \leq 0.05$ (*).

Genotoxicity of $PtCl_2$ in M. cornuarietis

In contrast to the results obtained for *D. rerio*, $PtCl_2$ revealed a considerable genotoxic hazard potential for *M. cornuarietis*. Except for the lowest tested concentration of 0.1 µg/l $PtCl_2$, tail moments of Pt-exposed snail cells were significantly higher than controls (Fig. 3). With rising concentration of $PtCl_2$ up to 100 µg/l, an increasing tail moment was observed in cells of *M. cornuarietis*. Medians of the tail moments of cells of *M. cornuarietis* exposed to 50 and 100 µg/l $PtCl_2$ were in the same order of magnitude or even slightly higher than the tail moments of the positive controls. Only the tail moments recorded for snails exposed to the highest tested concentration of 200 µg/l $PtCl_2$ were, though elevated above negative control levels, significantly lower than after exposure to 100 µg/l $PtCl_2$ (Fig. 3).

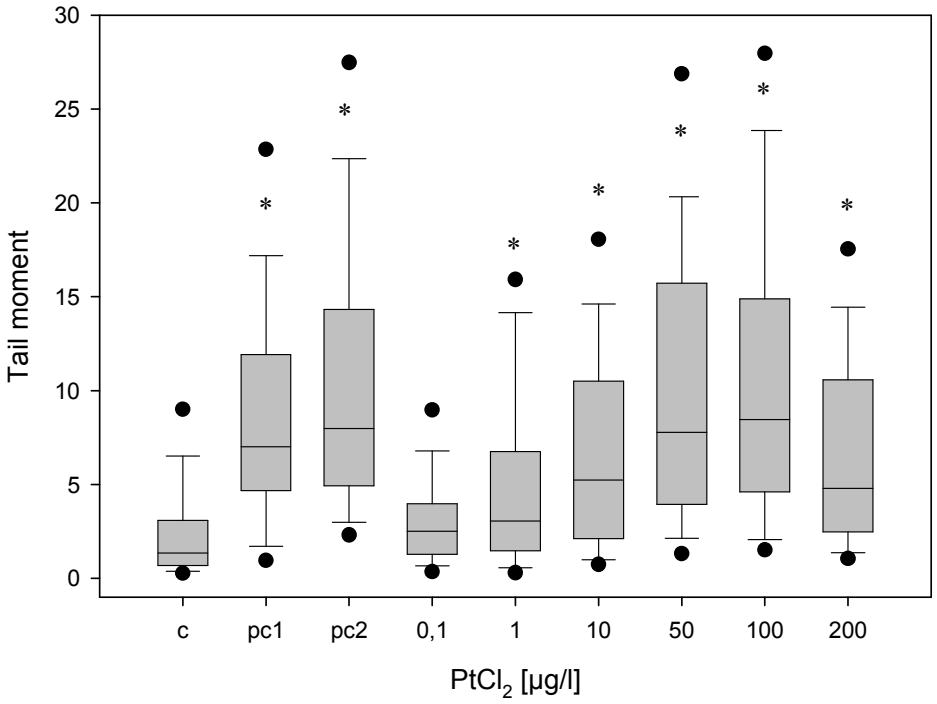


Fig. 3. DNA fragmentation as tail moment (intensity of the DNA tail × lenght of the tail) in cells isolated from *Marisa cornuarietis* 8 days post-fertilization and exposed to different concentrations of $PtCl_2$ or known genotoxicants as positive controls (pc1: ultraviolet light, pc2: hydrogen peroxide). Data are given as box plot of 100 nucleoids. Box plot percentiles are described in Fig. 2. Level of significant difference *versus* the control is $p \leq 0.05$ (*).

Discussion

Many heavy metals are known to be genotoxic to cells *in vitro* (Bünger et al., 1996; Hartwig, 1995). In most cases, genotoxicity of metal ions to cell cultures or animal models seems to be mainly mediated by oxidative stress leading to lipid peroxidation and DNA damage, by interference with DNA repair and/or DNA replication processes or by production of free radicals (Bertin and Averbeck, 2006; Flora et al., 2008; García-Lestón et al., 2010; Hartwig, 1995). Knowledge about the genotoxicity of Pt is mostly related to Pt complexes used as anti-tumor agents (Blasiak et al., 1999; IARC, 1987; Unger et al., 2009). However, also studies investigating the genotoxicity of Pt nanoparticles or Pt salts have been conducted: Barefoot (1997) reports that Pt is emitted from catalysts in the form of nanocrystals attached to alumina particles. Subsequently, a small portion of Pt can be solubilized and might thus become mobile in the environment (Whiteley and Murray, 2003). Pelka et al. (2009) investigated the genotoxic potential of Pt nanoparticles on human colon carcinoma cells and found them to significantly

induce DNA strand breaks after 3 h of exposure. Gagnon et al. (2006) investigated genotoxic effects of 0.1, 1, and 10 mg/l H_2PtCl_6 (PtIV) on *Sphagnum* moss and Sprague-Dawley rats. After an exposure period of 3 weeks for *Sphagnum* and 4 weeks for the rat (daily gavage), respectively, genotoxicity of Pt on capitula cells of *Sphagnum* moss and rat liver cells was shown by single cell electrophoresis at all investigated concentrations. Even though concentrations used by Gagnon et al. (2006) exceeded those of the present study by far, these authors also found a dose-dependent increase in DNA damage in both *Sphagnum* and rat. Genotoxicity of different Pt salts ($PtCl_2$, $PtCl_4$, and $(NH_4)_2PtCl_4$) at concentrations between 25 and 1000 µM, as well as Pd ($PdCl_2$ and $(NH_4)_2PdCl_4$) and Rh ($RhCl_3$) salts to human lymphocytes were investigated by Migliore et al. (2002) in the micronucleus assay. They found a significant induction of micronuclei for each Rh and Pt compound tested, whereas different Pt compounds exerted differential genotoxic hazards: $PtCl_4$ was found to be more genotoxic than $PtCl_2$ and $(NH_4)_2PtCl_4$. In addition, using a modified comet assay, the authors tested the oxidative DNA damage, because metal genotoxicity might act *via* oxidative mechanisms as described above. They found oxidative DNA damage only for $PtCl_4$ and $RhCl_3$, but not for $PtCl_2$ and $(NH_4)_2PtCl_4$, the two latter of which induce DNA damage probably *via* direct action only. As a consequence, of course, genotoxicity of Pt compounds strongly depends on the oxidation state of Pt, structure, and conformation (Gebel et al., 1997). Bünger et al. (1996) tested the mutagenicity of Pt salts (K_2PtCl_6, $(NH_4)_2PtCl_4$, and $(NH_4)_2PtCl_6$), Pd salts (K_2PdCl_6, $(NH_4)_2PdCl_4$, and $(NH_4)_2PdCl_6$), and Rh salts (K_2RhCl_5 and $(NH_4)_3RhCl_6$) on two established mouse and human cell lines using the Ames test. They found a mutagenic potential of all investigated Pt salts which appears to be based on a variety of mechanisms damaging the DNA. In their study, Rh salts were less genotoxic than Pt salts, and for Pd salts a mutagenic potential could be found. These results later were corroborated by Migliore et al. (2002).

As represented by the cited papers, investigations on the genotoxicity of platinum group elements have mostly been conducted using either human or other mammalian cell lines, or cells derived from plants. Studies investigating genotoxic effects of Pt on embryonic stages of fish or gastropods species have been lacking so far. Therefore, the present study basically broadens the knowledge about the genotoxic hazard of environmentally relevant Pt concentrations on taxa which might come into contact with Pt released into aquatic habitats from hospitals and/or roads.

As Pt^{2+} was tested in the form of $PtCl_2$, it should be mentioned that chloride itself is not known to be genotoxic, whereas Pt in different application forms (platinum nanoparticles, K_2PtCl_4, $PtCl_4$, H_2PtCl_6, $(NH_4)_2PtCl_4$) has been shown to be genotoxic (Lantzsch and Gebel, 1997; Migliore et al., 2002; Pelka et al., 2009). Therefore, it can be assumed that genotoxicity observed in the present study is mediated by Pt^{2+} and not by chloride. Moreover, the control tap water used for *M. cornuarietis* contained far more chloride (24.55 mg/l; Stadtwerke Tübingen, 2009) than it was provided by the very small amounts of $PtCl_2$ (a maximum of 53.3 µg/l chloride). Another possible side effect which should be discussed is the influence of pH values of the test media. However, since the amount of added $PtCl_2$ diluted in 2% HCl in the present study was so small (0.1–200 µl for 1 l of water each), pH-values of the test solutions did not differ from the control media and therefore can be excluded from exerting any genotoxic action.

H_2O_2 and UV light are both known and widely used DNA-damaging agents (Kosmehl et al., 2006; Lee and Steinert, 2003) and are, therefore, suitable agents as positive controls for DNA damage. In the present study, isolated cells of both species were irradiated with UV-light according to the protocol of Kosmehl et al. (2006). Even though illumination intensity may have varied between the different experiments (due to slight variations in lamp operation and/or transparency of the cell suspension), the results showed irradiation with UV-light to be a well suited positive control to compare the response of both test species. In contrast, H_2O_2 was applied to whole embryos. Being relevant for the comparison of snail and fish it needs to be considered that most of the body surface of *M. cornuarietis* was protected by the shell and even the shell aperture was probably closed by the operculum, whereas hatched embryos of *D. rerio* were exposed to this substance without any mechanism of physical protection.

In the present study, genotoxic effects of $PtCl_2$ were only visible in cells of *M. cornuarietis* exposed to concentrations ranging from 1 to 200 µg/l, but not in cells of *D. rerio*. Embryos of *D. rerio*, kept at 26 °C, normally hatch around 72–96 h post fertilization (hpf; Nagel, 2002). $PtCl_2$ is known to postpone hatching in *D. rerio* (Osterauer et al., 2009) and, also in the present study, higher test concentrations of $PtCl_2$ caused a hatching delay of *D. rerio*: Following exposure to 50 µg/l $PtCl_2$ only 50% of the embryos hatched at 96 hpf; in the 100 µg/l $PtCl_2$ treatment, 20% hatched; and in the 200 µg/l $PtCl_2$ treatment, only 10% hatched after 96 hpf. In those treatments, most of the embryos were protected by the chorion throughout

exposure, which could be a reason for the absent genotoxicity of $PtCl_2$ even at higher tested concentrations, since the chorion of fish is known to act as a barrier which reduces permeation of pollutants (Fent, 1992; Fent and Meier, 1994). Also embryos of *M. cornuarietis* were protected by their chorion throughout exposure time – they normally hatch 11-14 dpf at 26 °C (Osterauer et al., 2009) – but, however, genotoxicity of $PtCl_2$ could be recorded here. For this reason we conclude that Pt^{2+} is able to pass the chorion to some extent, probably *via* ionic transport mechanisms, as other bivalent metals like zink or nickel also were shown to cause adverse effects on the chorion-encased *M. cornuarietis* embryo (Sawasdee and Köhler, 2009). Obviously, the most crucial parameters influencing Pt genotoxicity in the two investigated test species seem to be exposure time and Pt accumulation rate, since *M. cornuarietis* was exposed for 8 days, whereas *D. rerio* was exposed for only 4 days. The elongated exposure time for *M. cornuarietis* was chosen because of a much slower development compared to *D. rerio*. However, the longer exposure period might have contributed to the genotoxicity of $PtCl_2$ for *M. cornuarietis*. Osterauer et al. (2009, 2010b) determined the accumulation rate of $PtCl_2$ concentrations, as they were tested in the present study, in both species (except for *D. rerio* exposed to 200 µg/l $PtCl_2$). They found *M. cornuarietis* after exposure for 26 days post-fertilization (dpf) to accumulate 50 times more Pt than *D. rerio* exposed for 7 dpf. Although exposure time ratio in the present study was different (*M. cornuarietis* was only two times, not 3.5 times longer exposed than *D. rerio*), *M. cornuarietis* most likely accumulated more Pt than *D. rerio*, which probably also resulted in stronger DNA damage. Also species-dependent differences in mechanisms of DNA repair cannot be excluded as an explanation for the strikingly different effects of $PtCl_2$ in the two test organisms. Metal-binding proteins, so-called metallothioneins (MT) have been shown to play a protective role against Pt(IV) toxicity as it was shown by an increased MT expression in chick embryos (Gagnon and Patel, 2007). However, MT induction in fish and gastropods exposed to Pt(II) have not been described in the literature so far. For other heavy metals, an increased MT induction has been observed in fish (Roeva et al., 1999) and gastropods, even prosobranch gastropods (Amiard et al., 2006; Leung et al., 2000). And for Pd(II), another platinum group element, an increase in MT was found in *Dreissena polymorpha* (Frank et al., 2008). In this context, possible differences in MT induction and effectivity in Pt(II) scavenging between fish and snail could have contributed to the diverging effects of $PtCl_2$ in the both investigated test species.

In *M. cornuarietis*, a dose-related increase of DNA damage was evident, except for the 200 µg/l $PtCl_2$ treatment, which showed a significant decrease of tail moments

in comparison to the 100 µg/l $PtCl_2$ treatment (Fig. 3). The high Pt concentration of 200 µg/l may have inhibited the excision repair mechanism consequently leading to less DNA strand breaks through incomplete excision repair. Another explanation for the observed decrease of tail moments could be that images of highly damaged nuclei show only minimally visible remnants of the original nuclei with DNA so dispersed that it is impossible to measure the full length of the comet tail. In this case, measurements of extensively broken and dispersed DNA comet tails may give false, shorter tail length values than expected.
However, another important aspect is the morphology of snails exposed to 200 µg/l $PtCl_2$: as described recently, *M. cornuarietis* exposed to this Pt concentration during their embryonic development cannot develop an external shell, but change their prosobranchian body plan (the gill remains at the posterior part of the body) and develop a cone-shaped internal shell (Osterauer et al., 2010b). It cannot be excluded that such fundamental body plan modifications may also result in different sensitivity to artificially applied chemicals.

In the present study, $PtCl_2$ was shown to exert genotoxicity to the test species *M. cornuarietis*, a representative for aquatic freshwater gastropods, at concentrations ranging from 1 to 200 µg/l. However, no genotoxic hazard of the tested $PtCl_2$ concentrations was shown for *D. rerio*. Despite the current tendency to substitute Pt by Pd in catalytic converter technology for financial reasons, Pt is, up to now, the predominant platinum group element in the environment (Pan et al., 2009; Tsogas et al., 2009). Although concentrations of Pt found in the aquatic ecosystem are usually in a lower ng/l range, concentrations up to 78 ng/l (Zereini et al., 1997) or even 1.1 µg/l (Laschka et al., 1996) have been measured in road run-off. Since LOEC data of 1 µg/l $PtCl_2$ found in the present study are not far from concentrations measured in the environment, the results of the present study strongly support the strategy to primarily build on Pt-free automobile catalytic converters in the future.

Acknowledgements

The authors would like to thank Frank R. Hoffmann who helped with the comet assay. Studienstiftung des deutschen Volkes and Landesgraduiertenförderung Baden-Württemberg are highly acknowledged for financial support to R.O.

References

Allwood, M., Wright, P., 1993. The Cytotoxic Handbook. Radcliffe Medical Press Oxford.

Amiard, J.C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006. Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers. Aquat. Toxicol. 76: 160–202.

Anderson, S.L., Wild, G.C., 1994. Linking genotoxic responses to reproductive success in ecotoxicology. Environ. Health Perspect. 102: 9–12.

Artelt, S., Creutzenberg, O., Kock, H., Levsen, K., Nachtigall, D., Heinrich, U., Rühle, T., Schlögl, R., 1999. Bioavailability of fine dispersed platinum as emitted from automotive catalytic converters: A model study. Sci. Total Environ. 228: 219–242.

Barefoot, R., 1997. Determination of platinum at trace levels in environmental and biological materials. Environ. Sci. Technol. 31: 309–313.

Bertin, G., Averbeck, D., 2006. Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). Biochimie 88: 1549–1559.

Blasiak, J., Kowalik, J., Trzeciak, A., Wojewodzka, M., 1999. Cytotoxicity and DNA damage and repair in human lymphocytes exposed to three anticancer platinum drugs. Neoplasma 46: 61–63.

Bünger, J., Stork, J., Stalder, K., 1996. Cyto- and genotoxic effects of coordination complexes of platinum, palladium and rhodium in vitro. Int. Arch. Occup. Environ. Health 69: 33–38.

Fairbairn, D.W., Olive, P.L., O'Neill, K.L., 1995. The comet assay: A comprehensive review. Mutat. Res. 339: 37–59.

Flora, S.J., Mittal, M., Mehta, A., 2008. Heavy metal induced oxidative stress & its possible reversal by chelation therapy. Indian J. Med. Res. 128: 501–523.

Gagnon, Z.E., Newkirk, C., Hicks, S., 2006. Impact of Platinum Group Metals on the environment: A Toxicological, Genotoxic and Analytical Chemistry Study. J. Environ. Sci. Health A 41: 397–414.

Gagnon, Z.E., Patel, A., 2007. Induction of metallothionein in chick embryos as a mechanism of tolerance to platinum group metal exposure. J. Environ. Sci. Health A 42: 381–387.

García-Lestón, J., Méndez, J., Pásaro, E., Laffon, B., 2010. Genotoxic effects of lead: an updated review. Environ. Int. 36: 623–636.

Gebel, T., Lantzsch, H., Pleßow, K., Dunkelberg, H., 1997. Genotoxicity of platinum and palladium compounds in human and bacterial cells. Mutat. Res. 389: 183–190.

Hartwig, A., 1995. Current aspects in metal genotoxicity. Biometals 8: 3–11.

Hill, R.F., Mayer, W.J., 1977. Radiometric determination of platinum and palladium attrition from automotive catalysts. IEEE Trans. Nucl. Sci. 24: 2549–2554.

Hill, A.J., Teraoka, H., Heidemann, W., Peterson, R.E., 2005. Zebrafish as a model vertebrate for investigating chemical toxicity. Rev. Toxicol. Sci. 86: 6–19.

Hoppstock, K., Sures, B., 2004. Platinum-Group Metals. In: Merian, E., Anke, M., Ihnat, M., Stoeppler, M., editors. Elements and their compounds in the environment. Wiley-VCH, Weinheim, Germany. p. 1047–1086.

IARC, 1987. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Genetic and Related Effects: An Updating of Selected IARC Monographs from Vols. 1-42, Cisplatin*. IARC, Lyon, Suppl. 6. p. 178–181.

Kosmehl, T., Hallare, A.V., Reifferscheid, G., Manz, W., Braunbeck, T., Hollert, H., 2006. A novel contact assay for testing genotoxicity of chemicals and whole sediments in zebrafish embryos. Environ. Toxicol. Chem. 25: 2097–2106.

König, H.P., Hertel, R.F., Koch, W., Rosner, G., 1992. Determination of platinum emissions from a three-way catalyst-equipped gasoline engine. Atmos. Environ. 26: 741–745.

Kümmerer, K., Helmers, E., Hubner, P., Mascart, G., Milandri, M., Reinthaler, F., Zwakenberg, M., 1999. European hospitals as a source for platinum in the environment in comparison with other sources. Sci. Total Environ. 225: 155-165.

Lantzsch, H., Gebel, T., 1997. Genotoxicity of selected metal compounds in the SOS chromotest. Mutat. Res. 389: 191–197.

Laschka, D., Striebel, T., Daub, J., Nachtwey, M., 1996. Platinum in rainwater discharges from roads. UWSF–Z Umweltchem. Ökotox, 8: 124–129.

Lee, R.F., Steinert, S., 2003. Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Mutat. Res. 544: 43–64.

Leung, K.M.Y., Taylor, A.C., Furness, R.W., 2000. Temperature-dependent physiological responses of the dogwhelk *Nucella lapillus* to cadmium. J. Mar. Biol. Assoc. UK 80: 647–660.

Liang, L.N., He, B., Jiang, G.B., Chen, D.Y., Yao, Z.W., 2004. Evaluation of mollusks as biomonitors to investigate heavy metal contaminations along the Chinese Bohai Sea. Sci. Total Environ. 324: 105–113.

Lustig, S., Zang, S., Michalke, B., Schramel, P., Beck, W., 1996. Transformation behaviour of different platinum compounds in a clay-like humic soil: speciation investigations. Sci. Total Environ. 188: 195–204.

Lustig, S., Zang, S., Beck, W., Schramel, P., 1998. Dissolution of metallic platinum as water soluble species by naturally occurring complexing agents. Mikrochim. Acta 129: 189–194.

McKim, J.M., 1977. Evaluation of tests with early life stages of fish for predicting long-term toxicity. J. Fish Res. Bd. Can. 34: 1148–1154.

Migliore, L., Frenzilli, G., Nesti, C., Fortaner, S., Sabbioni, E., 2002. Cytogenetic and oxidative damage induced in human lymphocytes by platinum, rhodium and palladium compounds. Mutagenesis 17: 411–417.

Moldovan, M., Palacios, M.A., Gomez, M.M., Morrison, G., Rauch, S., McLeod, C., Ma, R., Caroli, S., Alimonti, A., Petrucci, F., Bocca, B., Schramel, P., Zischka, M., Pettersson, C., Wass, U., Luna, M., Saenz, J.C., Santamaria, J., 2002. Environmental risk of particulate and soluble platinum group elements released from gasoline and diesel engine catalytic converters. Sci. Total Environ. 296: 199–208.

Nagel, R., 2002. DarT: the embryo test with the zebrafish *Danio rerio* – a general model in ecotoxicology and toxicology. Altex 19: 38–48.

Nüsslein-Volhard, C., 1994. Of flies and fishes. Science 266: 572–576.

OECD Guideline for Testing of Chemicals, 1992. Test Guideline 203. Fish, Acute Toxicity Test. OECD Guideline for Testing of Chemicals.

Oehlmann, J., Di Benedetto, P., Tillmann, M., Duft, M., Oetken, M., Schulte-Oehlmann, U., 2007. Endocrine disruption in prosobranch molluscs: evidence and ecological relevance. Ecotoxicology 16: 29–43.

Osterauer, R., Haus, N., Sures, B., Köhler, H.-R., 2009. Uptake of platinum by zebrafish (*Danio rerio*) and ramshorn snail (*Marisa cornuarietis*) and resulting effects on early embryogenesis. Chemosphere 77: 975–982.

Osterauer, R., Köhler, H.-R., Triebskorn, R., 2010a. Histopathological alterations and induction of stress proteins in ramshorn snail (*Marisa cornuarietis*) and zebrafish (*Danio rerio*) after exposure to PtCl2 during embryogenesis. Aquat. Toxicol. 99: 100–107.

Osterauer, R., Marschner, L., Betz, O., Gerberding, M., Sawasdee, B., Cloetens, P., Haus, N., Sures, B., Triebskorn, R., Köhler, H.-R., 2010b. Turning snails into slugs: induced body plan changes and formation of an internal shell. Evol. Dev. 12: 474–483.

Pan, S., Zhang, G., Sun, Y., Chakraborty, P., 2009. Accumulating characteristics of platinum group elements (PGE) in urban environments, China. Sci. Total Environ. 407: 4248–4252.

Pelka, J., Gehrke, H., Esselen, M., Türk, M., Crone, M., Bräse, S., Muller, T., Blank, H., Send, W., Zibat, V., Brenner, P., Schneider, R., Gerthsen, D., Marko, D., 2009. Cellular uptake of platinum nanoparticles in human colon carcinoma cells and their impact on cellular redox systems and DNA integrity. Chem. Res. Toxicol. 22: 649–659.

Roeva, N.N., Sidorov, A.V., Yurovitskii, Y.G., 1999. Metallothioneins, proteins binding heavy metals in fish. Biol. Bull. 26: 617–622.

Sabbioni, E., Edel, J., Goetz, L., 1985. Trace metal speciation in environmental toxicology research. Nutr. Res. Suppl. 1: 32–43.

Sawasdee, B., Köhler, H.-R., 2009. Embryo toxicity of pesticides and heavy metals to the ramshorn snail, *Marisa cornuarietis* (Prosobranchia). Chemosphere 75: 1539–1547.

Schirling, M., Bohlen, A., Triebskorn, R., Köhler, H.-R., 2006. An invertebrate embryo test with the apple snail *Marisa cornuarietis* to assess effects of potential developmental and endocrine disruptors. Chemosphere 64: 1730–1738.

Schulte-Oehlmann, U., Tillmann, M., Market, B., Oehlmann, J., 2000. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in laboratory. Part II. Triphenyltin as a xeno-androgen.. Ecotoxicology 9: 399–412.

Schnurstein, A., Braunbeck, T., 2001. Tail moment versus tail length—Application of an in vitro version of the comet assay in biomonitoring for genotoxicity in native surface waters using primary hepatocytes and gill cells from zebrafish (*Danio rerio*). Ecotoxicol. Environ. Saf. 49: 187–196.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell. Res. 175: 184–191.

Sures, B., Zimmermann, S., 2007. Impact of humic substances on the aqueous solubility, uptake and bioaccumulation of platinum, palladium and rhodium in exposure studies with *Dreissena polymorpha*. Environ. Pollut. 146: 444–451.

Tsogas, G.Z., Giokas, D.L., Vlessidis, A.G., Aloupi, M., Angelidis, M.O., 2009. Survey of the Distribution and Time-Dependent Increase of Platinum-Group Element Accumulation Along Urban Roads in Ioannina (NW Greece). Water Air Soil Pollut. 201: 265–281.

Unger, F.T., Klasen, H.A., Tchartchian, G., de Wilde, R.L., Witte, I., 2009. DNA damage induced by cis- and carboplatin as indicator for in vitro sensitivity of ovarian carcinoma cells. BMC Cancer 9: 359.

Wiseman, C.L.S., Zereini, F., 2009. Airborne particulate matter, platinum group elements and human health: A review of recent evidence. Sci. Total Environ. 407: 2493–2500.

White, P.A., Robitaille, S., Rasmussen, J.B., 1999. Heritable reproductive effects of benzo[a]pyrene on the fathead minnow *(Pimephales promelas)*, Environ. Toxicol. Chem. 18: 1843–1847.

Whiteley, J.D., Murray, F., 2003. Anthropogenic platinum group element (Pt, Pd and Rh) concentrations in road dust and roadside soils from Perth, Western Australia. Sci. Total Environ. 317: 121–135.

Kapitel 4: Turning snails into slugs: induced body plan changes and formation of an internal shell

Raphaela Osterauer[a], Leonie Marschner[a], Oliver Betz[b], Matthias Gerberding[c], Banthita Sawasdee[a], Peter Cloetens[d], Nadine Haus[e], Bernd Sures[e], Rita Triebskorn[a,f], Heinz-R. Köhler[a]

[a] Animal Physiological Ecology Department, Institute of Evolution and Ecology, University of Tübingen, Konrad-Adenauer-Str. 20, D-72072 Tübingen, Germany

[b] Department of Evolutionary Biology of Invertebrates, Institute of Evolution and Ecology, University of Tübingen, Auf der Morgenstelle 28E, 72076 Tübingen, Germany

[c] Institute of Zoology, University of Hohenheim, Garbenstr. 30, 70599 Stuttgart, Germany

[d] European Synchrotron Radiation Facility, B.P. 220, RF-38043 Grenoble, France

[e] Department of Applied Zoology/Hydrobiology, Institute of Biology, University of Duisburg-Essen, Universitätsstr. 5, 45141 Essen, Germany

[f] Transfer Center for Ecotoxicology and Ecophysiology, Blumenstrasse 13, 72108 Rottenburg, Germany

Abstact

The archetypal body plan of conchiferan molluscs is characterized by an external calcareous shell, though internalization of shells has evolved independently in a number of molluscan clades, including gastropod families. In gastropods, the developmental process of torsion is regarded as a hallmark that is associated with a new anatomical configuration. This configuration is present in extant prosobranch gastropod species, which predominantly bear external shells. Here, we show that short-term exposure to platinum during development uncouples at least two of the processes associated with torsion of the freshwater snail *Marisa cornuarietis*. That is, the anus of the treated snails is located anteriorly, but the gill and the designated mantle tissue remains in a posterior location, thus preventing the formation of an external shell. In contrast to the prosobranchian archetype, platinum treatment results in the formation of a posterior gill and a cone-shaped internal shell, which persists across the lifetime. This first finding of artificially induced snail-slug conversion was also seen in the pulmonate snail *Planorbarius corneus* and demonstrates that selective alteration of embryonic key processes can result in fundamental changes of an existing body plan and – if altered regulation is inherited – may give rise to a new one.

Evolution and Development, 2010, 12: 474–483

Introduction

It is commonly assumed that the first mollusc-like animals in the early Cambrian were shell-less and only protected by a cuticle with aragonitic spicules or scales (Scheltema and Schander, 2006). Later a calcified shell evolved in the conchiferan clade, which includes the extant Tryblidiida, Gastropoda, Bivalvia, Scaphopoda, and Cephalopoda. Secondary reduction and internalization of the shell has evolved repeatedly and independently in several gastropod taxa (Vitrinidae, Arionidae, Limacidae, Nudibranchia, Velutinidae, Titiscaniidae, Fissurellidae), notably in the cephalopods (except for *Nautilus*), and most likely for greater motility (Furbish and Furbish, 1984). The ontogeny of internal shells has been described in detail for a selection of gastropod species (Furbish and Furbish, 1984; Page, 2000). Even though mechanisms of shell internalization vary between different evolutionary lines, always the interactions between mantle and shell growth have been shown to be modified in the early individual ontogeny.

Extant species of the paraphyletic and globally distributed group of prosobranch gastropods (nonheterobranch gastropods) mostly develop an outer shell (except for, e.g., a few species of Velutinidae, Titiscaniidae, Fissurellidae). Their ontogeny also includes the process of torsion, which is crucial for the anatomical configuration of these snails. Torsion is defined as a process in gastropod ontogenesis that rotates the visceral body 180° relative to the larval headfoot region. As a consequence the digestive tract is U-shaped and the anus is located anteriorly, the mantle cavity is located anteriorly over the back of the head, and the gills are located anteriorly in front of the heart. This process is the key character defining the gastropod class.

It is generally thought that torsion involves a counterclockwise simultaneous movement of the outgrowing mantle, the shell, and the visceral sac, and – following Ernst Haeckel's theory of ontogeny recapitulating phylogeny – that this developmental process recapitulates evolutionary events at the rise of prosobranch gastropods.

In growing snails, the mineralization of the teleoconch takes place at the peripheral edge of the mantle fold and over the entire inner surface of the shell in order to increase shell thickness. Usually, invagination of the dorsal epithelium first occurs to form the shell gland, followed by shell field evagination and the migration of shell material-secreting cells toward the mantle edge (Kniprath, 1981; Waller, 1981). The shell gland originates at the aboral end of the embryo, later shifts to the left and forms the mantle (Demian and Yousif, 1973a). The helical growth of the shell occurs in parallel to the onset of the counterclockwise movement of the outgrowing mantle

due to the process of torsion. The mantle overgrows the visceral sac in the right dorso-lateral direction and opens to the anterior, forming the mantle cavity sheltering the organs such as the gill(s).

The mollusc shell consists of an organic matrix and calcium carbonate ($CaCO_3$), which is formed from calcium and bicarbonate ions present in the extrapallidal fluid (Rousseau et al., 2003). Catalyzing the reaction $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$ and being responsible for reversible hydration of CO_2, the enzyme carbonic anhydrase (CA) is associated with tissues involved in calcification and mineralization processes such as the formation of the embryonic shell (Costlow, 1959; Maren, 1967; Wilbur and Saleuddin, 1983; Takaichi et al., 2003).

The course of anatomical modifications during embryonic development of the prosobranch freshwater snail, *Marisa cornuarietis* (Ampullariidae), is well documented (Demian and Yousif, 1973a–d, 1975). Earlier studies revealed its embryos to be particularly sensitive to metals (Schirling et al., 2006; Sawasdee and Köhler, 2009). Because of increasing importance of platinum group elements in ecotoxicology resulting from their use in automobile catalytic converters, we tested the effect of platinum (Pt) on the embryogenesis of this gonochoric species. Because we found that exposure to Pt separates the process of torsion from mantle cavity formation and therefore prevents the formation of an external shell in *M. cornuarietis* we used different methological approaches to study this effect. To investigate the variability of this effect we also tested other metals like the physicochemical similar element palladium (Pd) and the alkaline metal lithium (Li) on embryos of *M. cornuarietis*. Furthermore, we also tested the effect of Pt on the pulmonate snail *Planorbarius corneus* to compare effects on different snail species.

Materials and methods

Test animals

Test animals used in the present study were the freshwater snails *M. cornuarietis* (Ampullariidae, prosobranch gastropod) and *P. corneus* (Planorbidae, pulmonate). Origin and maintenance of the lab stock culture of the gonochoric species *M. cornuarietis* were described in Osterauer et al. (2009). The breeding stock of the hermaphroditic snail *P. corneus* was gathered in a pond near Tübingen. *P. corneus* were kept in 30 L aquaria containing oxygenized tap water in the following conditions: temperature: 20 ± 1 °C, pH: 8, conductivity: 800 µS/cm, and 12 h/12 h light/dark regime. To ensure optimal water quality, the water of the aquaria was

exchanged every week. The snails were fed once a day with commercially available artificial diet (Nutrafin Max flakes, Hagen, Germany) and casually with fresh carrots or lettuce.

Exposure experiments

Test substances used in the present study were $PtCl_2$ (Ultra Scientific, Wesel, Germany), $PdCl_2$ (Sigma-Aldrich, München, Germany), LiCl (≥ 99%, Fluka, Buchs, Switzerland), and, in combination with $PtCl_2$, $CaCl_2$ (Merck, Darmstadt, Germany). For both, exposure and pulse experiments, single eggs were separated from the egg masses laid during the night and distributed to Petri dishes containing the respective substance or the control medium which was tap water taken from the snail aquaria. The described effects occurred independently of using either tap/aquaria water or reconstituted water after the OECD Test Guideline 203 (1992), modified for *M. cornuarietis*, as a solvent. Concentrations used for chronic exposure (from the day of fertilization until hatch) were 100 and 200 µg/L $PtCl_2$, 50, 100, and 500 µg/L $PdCl_2$, 2.5 and 3 mg/L LiCl for *M. cornuarietis*, and 300, 400, and 500 µg/L $PtCl_2$ for *P. corneus*. For the pulse exposure experiments, *M. cornuarietis* eggs were exposed to 200 µg/L $PtCl_2$ for either 1 (at days 3, 4, 5, or 6 postfertilization) or 2 days (at days 3+4, 4+5, 5+6, 6+7, or 7+8 postfertilization) and subsequently returned to tap/aquaria water again. Furthermore, binary combinations of $PtCl_2$ (200 µg/L) and $CaCl_2 \times 2H_2O$ (0.54 g/L) in which Pt^{2+} and Ca^{2+} concentrations were equimolar, were tested on *M. cornuarietis* (exposure from the day of fertilization until hatch). Negative controls were exposed to tap/aquaria water, positive controls were exposed to 200 µg/L $PtCl_2$ during the whole study.
For all experiments, exposure media were exchanged daily. Throughout the exposure period, embryos were kept at 26 °C in a climate chamber and were only removed for monitoring their development using a stereomicroscope. The numbers of snails without external shell were counted at day 9 postfertilization (dpf) in chronic exposure experiments and at day 11 postfertilization in pulse experiments. To study the postembryonic development of *M. cornuarietis*, hatched snails either were continuously exposed to 100 or 200 µg/L $PtCl_2$ or 200 µg/L $PtCl_2$ plus 0.54 g/L $CaCl_2 \times 2H_2O$ or returned to tap/aquaria water but, in any case, hatched snails were fed with equal portions of commercially available artificial diet (Nutrafin Max, Hagen, Germany) *ad libitum* once a day. The chronic exposure experiments with *M. cornuarietis* embryos and Pt, Pd, and Li were conducted with nine replicate groups (à 20 individuals), the pulse experiments and the exposure experiments with

Pt plus Ca with four replicate groups (except for control and Pt-control in the first run of the 2-day pulses with eight replicate groups) (à 20 individuals) and the exposure experiments with *P. corneus* with two replicate groups at 300 µg/L $PtCl_2$ (à 13 individuals) and at 400 µg/L $PtCl_2$ (à 6 and 11 individuals) and, due to limitations in egg production, 1 group of four individuals at 500 µg/L $PtCl_2$. The Pt-induced internalization of the shell in the investigated gastropod species was found to be highly reproducible and allowed us to induce the described body plan modifications *ad libitum* ever since.

Reconstituted water composition for M. cornuarietis

Reconstituted water consisted of double-distilled water supplemented with KCl (17.94 mg/L), $MgSO_4 \times 7H_20$ (0.19 g/L), $NaHCO_3$ (98.42 mg/L), $CaCl_2 \times 2H_2O$ (0.45 g/L), and NaCl (0.43 mg/L).

Histology

Whole embryos of *M. cornuarietis* at the age of 26 dpf that had been exposed to 200 µg/L $PtCl_2$ throughout were fixed in Bouin´s solution for 1 week. Detailed information on the histological techniques is given in Osterauer et al. (2010). Histological methodology was based on Triebskorn et al. (2005).

Analysis of platinum in M. cornuarietis and in the exposure media

After 26 dpf of exposure to 100 or 200 µg/L $PtCl_2$ or 200 µg/L $PtCl_2$ + 0.54 g/L $CaCl_2 \times 2H_2O$ (equimolar Pt^{2+} and Ca^{2+} concentrations), *M. cornuarietis* were frozen in liquid nitrogen and stored at -20 °C. Depending on the estimated Pt concentrations, quantity of bioaccumulated Pt in the organisms was measured with adsorptive cathodic stripping voltammetry (ACSV) after digestion via high-pressure ashing according to Zimmermann et al. (2001, 2003) or with electrothermal atomic spectrometry (ET-AAS) after microwave-assisted digestion according to Sures et al. (1995). $PtCl_2$ concentrations above 100 µg/L were analyzed by ET-AAS, while lower concentrations were analyzed by ACSV. For controls and *M. cornuarietis* exposed to 100 µg/L $PtCl_2$ the replicate number n was 8, for *M. cornuarietis* exposed to 200 µg/L $PtCl_2$ n=9, for *M. cornuarietis* exposed to 200 µg/L $PtCl_2$ plus equimolar Ca n=5. Depending on the estimated Pt concentrations in the exposure medium for *M. cornuarietis*, concentrations of Pt were measured with inductive coupled plasma

mass spectrometry (ICP-MS, Perkin Elmer model Elan 5000, PerkinElmer Inc., Wellesley, MA, USA) or with ET-AAS (Perkin Elmer model 4100ZL). $PtCl_2$ concentrations above 100 µg/L were analyzed by ET-AAS, while lower concentrations were analyzed with ICP-MS. Each sample was analyzed in triplicate. Detailed descriptions of analytical procedures for ACSV and ICP-MS have been published by Osterauer et al. (2009).

Electrothermal atomic absorption spectrometry (ET-AAS)

Tissue samples of about 20 mg fresh weight each were digested by adding 1.8 mL HNO_3 (65 vol.%, subboiled) into 100 mL perfluoralkoxy vessels. Using a microwave digestion oven (CEM Model MDS-2000, 650 ± 50 W; Spectralab Scientific Inc., Toronto, ON, Canada) samples were digested according to the description of Sures et al. (1995). The resulting solution was filled up to 2 mL with bidistilled water. Analytical measurements were conducted with an atomic absorption spectrophotometer (Perkin Elmer Model 4100ZL). Therefore, 20 µL of each sample, priorily diluted with bidistilled water, were injected into a pyrolytic graphite furnace tube with L`vov platform by an autosampler AS 70. Operation parameters and further procedural descriptions have been published by Zimmermann et al. (2003). The detection limit for Pt in the tissue samples was defined to be threefold the standard deviation of the measurements of procedural blanks. For the average sample weight of 25 mg for *M. cornuarietis* it was found to be 62 ng/g.
For Pt analysis in the exposure medium 0.5 mL of the medium were topped up to 1 mL with bidistilled water and analysed as described above. The detection limit for Pt was defined threefold the standard deviation of the measurements of blanks and was found to be 1.6 µg/L.

Carbonic anhydrase activity

M. cornuarietis at the age of 10 dpf (control animals and animals treated with 200 µg/L $PtCl_2$) were homogenized in 60 µL phosphate buffer (25 mM, pH 7.4). Homogenates were centrifuged for 5 min at 2000 × *g* and 4 °C according to the protocol of Giraud (1981). The supernatant served as enzyme source. The Δ pH method of measuring CA activity was conducted according to the description of Henry (1991) and Vitale et al. (1999). For activity measurement 7.5 mL reaction medium (mannitol, 225 mM; saccharose, 75 mM and tris-phosphate, 10 mM at pH 7.4), 50 µL supernatant and 1 mL of CO_2 containing sparkling mineral water

(Selters, Löhnberg, Germany) were mixed and the pH-drop was measured for 25 s with a high-precision pH meter (WTW ph 391, WTW, Weilheim, Germany). A linear regression of pH data against time was calculated and the estimated slope was adopted as the catalyzed reaction ($b_{catalyzed}$). For control measurements, phosphate buffer was used instead of enzyme containing supernatant and the same procedure was performed. Total protein content was determined according to Bradford (1976). To determine the specific CA activity the formula according to Burnett et al. (1981) was used: SCA = ($b_{catalyzed}$ / $b_{noncatalyzed}$ −1)/mg of total protein. Five snails per sample and 3 replicates were used. All preparations were conducted at low temperature (vessels were kept on ice).

Diamino-benzidine (DAB) staining of mantle edge tissue

DAB staining usually is used as negative control for antibody labelling procedures. In other experiments we found DAB to specifically stain the mantle edge of developing *M. cornuarietis* embryos. Different stages of control embryos and embryos exposed to 200 µg/L $PtCl_2$ (day 3, 3.5, 4, 5 and 6 post fertilization) were mechanically removed from the chorion and fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 30 min. After washing for 4 × 5 min with PTw (PBS with 0.01% Tween 20, Roth, Karlsruhe, Germany) embryos were incubated with PTw+N (PTw with 5% Goat Serum, Jackson ImmunoResearch, West Grove, PA, USA) overnight at 4 °C.
For horseradish peroxidase (HRP) reaction embryos were washed with PTw for 4 × 5 min and 4 × 15 min and afterwards incubated in 0.3 mg/mL DAB solution for 20 min. The reaction was started by adding H_2O_2 to a final concentration of 0.03%. When the brown signal became visible, as could be visually detected under a stereomicroscope, reaction was stopped by washing 2 × 5 min with PTw. Finally, embryos were mounted in a 50% glycerol/DAPI (4´,6-diamidino-2-phenylindole) mix. For visual analysis and photography were used a light microscope and a stereomicroscope (both Zeiss, Jena, Germany).

Synchrotron X-ray phase contrast tomography and holotomography

Whole intact embryos of *M. cornuarietis* exposed to 100 µg/L $PtCl_2$ for 26 dpf were fixed in 100% ethanol overnight, critical point dried (CPD 020, Balzers, Wiesbaden, Germany) and mounted on specimen holder stubs. X-ray tomography was conducted at beamline ID19 (ESRF, Grenoble, France) at an energy of 20.0 keV.

Measurements were performed at three different sample-detector distances, i.e. 16 mm, 100 mm, and 841 mm. The effective pixel resolution amounted to 5.05 µm. Otherwise, instrument settings and further data treatments were done according to the description of Heethoff and Cloetens (2008). Due to limitations in beam time, measurements were conducted with a single animal each of the Pt treated group and of the control group.

Scanning electron microscopy

Embryos of *M. cornuarietis* of different age and exposure (100 or 200 µg/L $PtCl_2$, and controls) were fixed overnight in 2% glutaraldehyde in 0.01 M cacodylate buffer at pH 7.4. Subsequently, the organisms were washed three times with 0.01 M cacodylate buffer and stored in 1% osmium tetroxide overnight. The next day, the specimens were dehydrated in an ascending series of ethanol dilutions. After critical point drying they were fixed on specimen holder stubs, sputter-coated with gold, and viewed with a scanning electron microscop (SEM) (Cambridge Stereoscan 250 Mk2, Cambridge Scientific, Cambridge, UK).

Statistical analyses

Normally distributed data (Shapiro-Wilk test, JMP 4.0, SAS Systems, USA) were tested with the parametric one-way t-test (JMP 4.0, SAS Systems, USA) to detect significant differences between the treatment group and the control. Data not corresponding to normal distribution were tested using the nonparametric distribution-independent Wilcoxon´s test (JMP 4.0, SAS Systems, USA) to detect significant differences between the respective treatment groups and the control group. The alpha level was set at 0.05. Differences were considered to be significant for $p \leq 0.05$ (*) and highly significant for $p \leq 0.01$ (**), and $p \leq 0.001$ (***).

Results

Platinum accumulation and induced body plan changes in M. cornuarietis

To quantify Pt accumulation snails were exposed to 100 or 200 µg/L $PtCl_2$ for the first 26 dpf. Chemical analytical data already have been partly published by Osterauer et al. (2009), and showed exposure of *M. cornuarietis* embryos (within the egg) and juveniles to result in exceptionally high Pt concentrations in the animals:

74.2 ± 5.3 µg/L Pt in the medium (nominal concentration of 100 µg/L $PtCl_2$ corresponding to 73.4 µg/L Pt) led to 53.7 ± 19.2 µg/g Pt wet weight in the animals (bioaccumulation factor 724) and 163.4 ± 2.7 µg/L Pt (nominal concentration of 200 µg/L $PtCl_2$ corresponding to 146.8 µg/L Pt) resulted in a tissue concentration of 90.3 ± 9.9 µg/g Pt wet weight (bioaccumulation factor 553), both indicating a high potential of Pt to interact with developmental processes in this species.
In response to Pt treatment 29.9 ± 32.78% of surviving *M. cornuarietis* juveniles continuously exposed to 74.2 µg/L Pt (85.1 ± 9.41% survival) did not form an external shell, whereas at exposure to 163.4 µg/L Pt (84.4 ± 6.23% survival), all surviving animals were 'shell-less' (100 ± 0%).

To detect the most sensitive stage in embryogenesis in which Pt interferes with the formation of the shell, we conducted two separate runs of a pulse-exposure experiment. These experiments included pulses of 200 µg/L $PtCl_2$ with a duration of either 1 day or 2 days. Data varied between the two experimental runs but indicated the embryonic stages at days 4 and 5 postfertilization to be most susceptible to Pt action on shell formation (Fig. 1).

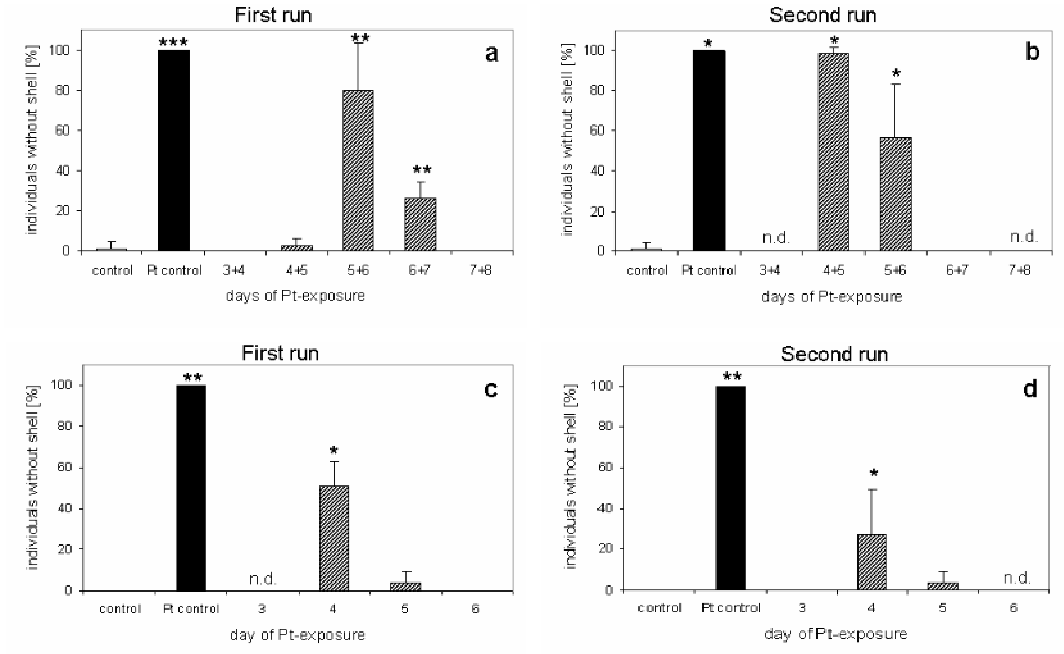


Fig. 1. Pulsed Pt-exposure of *Marisa cornuarietis* to 200 µg/L $PtCl_2$ at different days of embryonic development. Percentages of surviving individuals without external shell at day 11 post fertilization (means ± SD). (a) and (b) Two-days-pulses. (c) and (d) One-day-pulses. For each experiment and run, n = 4 replicates with 20 individuals per replicate (except for control and Pt-control in the first run of two-days-pulses n = 8). Control: water exposure; Pt control: continuous exposure to $PtCl_2$ during the entire embryonic phase; n.d.: not determined. Significance vs. control : *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

We repeatedly raised large numbers of *M. cornuarietis* embryos without external shell by pulse exposure to nominal concentrations of 200 µg/L $PtCl_2$ at days 4 and 5 postfertilization. Subsequently, the embryos in their egg capsules were transferred to uncontaminated water and cultivated at 26 °C. We never obtained reversal of the Pt pulse-induced body plan changes under these conditions and, thus, none of these individuals ever developed an external shell. Without exception, all juveniles shared the following alterations relative to the nontreated snails (Fig. 2a): no mantle cavity was formed at all and hence the gill, which remained posterior to the heart at the hind part of the visceral sac, protruded from the visceral sac into the surrounding water (Fig. 2b). Nevertheless, as indicated by the deflected intestine, the position of the anus at the lower right side (Fig. 2b), and by a slight movement of the gill from hind left to hind right, part of developmental processes associated with torsion was accomplished in all individuals. The hepatopancreas, as usual, surrounds the mid part of the intestine. Dorsal of the hepatopancreas, the hindgut bends to the front. In addition, the visceral sac surface of these snails bloats and blisters to varying degree, forming hemocoel cavities of different sizes (Fig. 2, b, d, and f). An operculum was present (Fig. 2c) and the morphology of the head and ventral part of the body, including the operculum, did not differ from the archetypical body plan (Fig. 2, a, b, and e).

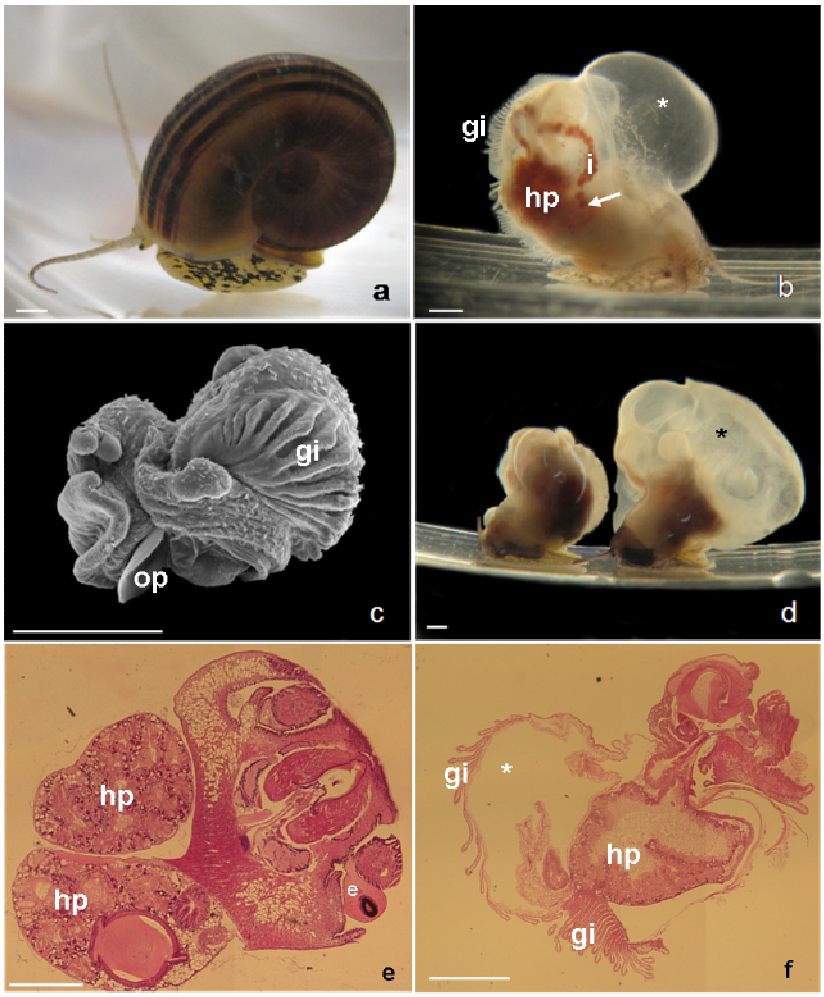


Fig. 2. Images of *Marisa cornuarietis* exposed to 200 µg/L $PtCl_2$ without external shell and control. (a) Control, 3 months old. (b) and (d) Pt-exposed snails without external shell, 2 months old. Arrow indicates position of the anus. (c) Scanning electron micrograph of an embryo without external shell, 14 days post fertilization. (e) Horizontal section of a control animal, 26 days post fertilization, ventral part of the body. (f) Sagittal section of a Pt-exposed animal, 26 days post fertilization, median part of the body. gi: gill, i: intestine, hp: hepatopancreas, op: operculum, asterisks: bloats and blisters of the hemocoel. Scale bars are 500 µm.

Up to now, *M. cornuarietis* individuals with induced body plan changes due to Pt exposure reached a maximum age of 7 months and a maximum length of about 1.5 cm. During their lifetime, the animals steadily grew and gained mass but did not change their outer appearance. So far, the only known way to partly protect embryos from the action of Pt is to supplement the Pt solution with equimolar concentrations of bivalent calcium ions. In experiments with *M. cornuarietis* embryos exposed to 200 µg/L $PtCl_2$ plus 0.54 g/L $CaCl_2 \times 2H_2O$ (equimolar Pt^{2+} and Ca^{2+} concentrations) for 9 days postfertilization, 84 ± 12% of the individuals formed an external shell and 16 ± 12% did not. However, a considerable number of the shelled animals in this experiment only developed a small, cap-like external shell, which was not sufficiently large to cover completely the gill (Fig. 3a).

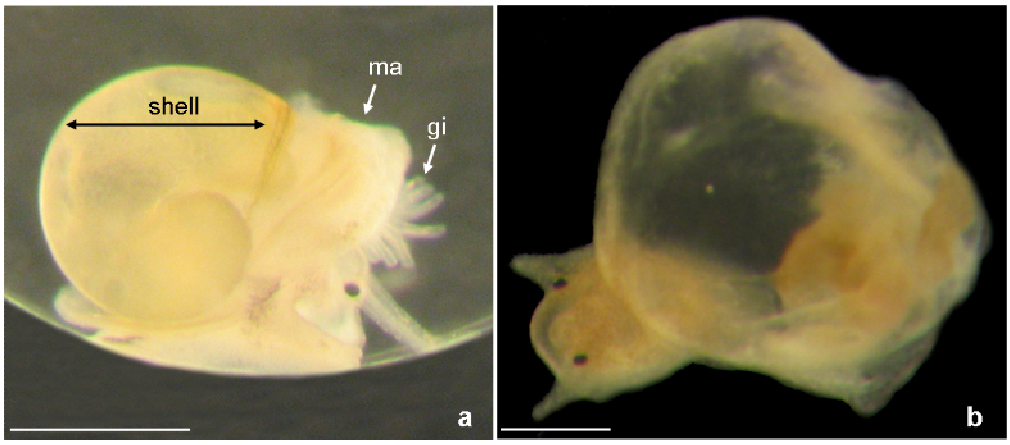


Fig. 3. Pt effect in Ca-supplemented *Marisa cornuarietis* and in *Planorbarius corneus*. (a) *M. cornuarietis* with a small, external shell, 11 days post fertilization, exposed to 200 µg/L $PtCl_2$ and equimolar concentrations of Ca^{2+}, ma: mantle, gi: gills. (b) *P. corneus* without external shell, 23 days post fertilization, exposed to 300 µg/L $PtCl_2$. Scale bars are 500 µm.

Ca supplements did not diminish Pt uptake: snails without an external shell exposed to 200 µg/L $PtCl_2$ accumulated 90.3 ± 9.9 mg/g Pt, snails without an external shell exposed to Pt and equimolar concentrations of Ca accumulated 115 ± 28.1 µg/g Pt, and snails exposed to Pt and equimolar concentrations of Ca, which developed an external shell accumulated 82.5 ± 26.4 µg/g Pt.
In contrast to Pt^{2+}, the physicochemical similar ion Pd^{2+} did not affect shell formation during embryogenesis. Only occasionally were we able to induce shell-less *M. cornuarietis* embryos with high concentrations of Li^{+}: 2.5 mg/L LiCl caused 10.0 ± 6.0% shell-less snails and 3 mg/L LiCl caused 20.0 ± 9.5% shell-less snails.

The fate of the shell secreting edge of the mantle fold and the formation of an internal shell

Since we found DAB to specifically stain the shell-secreting peripheral edge of the mantle fold, we were able to follow the fate of the tissue, which usually forms the mantle fold edge during embryogenesis and to visualize the presumably shell-secreting region of Pt-exposed *M. cornuarietis*. In contrast to the controls, the tissue archetypically designated to form the mantle edge did neither evaginate nor overgrow the visceral sac but remained at the posterior end of the embryo and invaginated into the body (Fig. 4, c–f), thus closing the aperture of the shell gland.

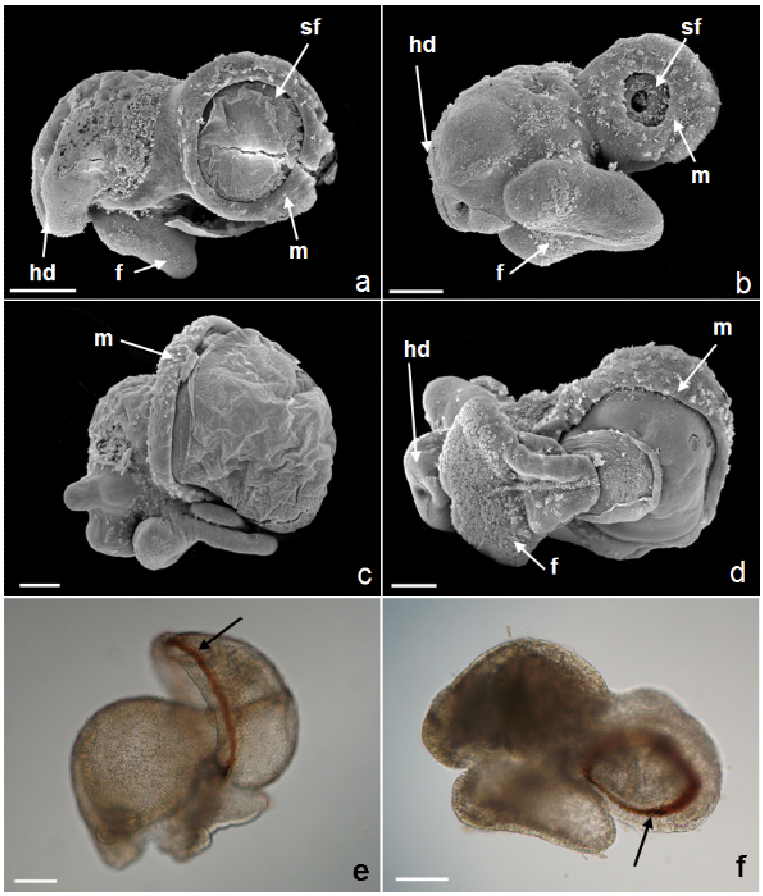


Fig. 4. Location of the shell-secreting peripheral edge of the mantle fold in *Marisa cornuarietis* exposed to 200 µg/L $PtCl_2$ and in controls. Scanning electron micrographs of Pt-exposed (200 µg/L $PtCl_2$) *M. cornuarietis* and controls. (a) Control, 3 days post fertilization. (b) Pt-exposed *M. cornuarietis*, 4 days post fertilization. (c) Control, 5 days post fertilization. (d) Pt-exposed *M. cornuarietis*, 6 days post fertilization. DAB staining (arrows) of the shell-secreting mantle edge of a control individual (e) and a Pt-exposed individual (f), both 5 days post fertilization. Scale bars are 100 µm.

Using SEM, the morphology of the shell gland and the initial protoconch of continuously Pt-exposed embryos and water controls did not differ substantially (Fig. 4, a and b). However, while the shell grows rapidly during the subsequent days of embryogenesis in controls, the opening of the shell gland of Pt-exposed individuals was reduced in size. To test whether this effect and the subsequent lack of an external shell was possibly based on a lack or a drastic diminution of biomineralization activity during the days subsequent to the Pt-sensitive stages, CA activity was measured in embryos continuously exposed to 200 µg/L $PtCl_2$ at day 10 postfertilization. Even though Pt exposure decreased CA activity from 13.8 ± 2.6/mg in controls to 8.5 ± 3.6/mg in shell-less *M. cornuarietis* embryos, this difference was not significant ($p = 0.107$).

Synchrotron X-ray phase-contrast microtomography applied on a 26 dpf old Pt-exposed juvenile revealed the formation of an internal solid circular structure in the

ventral part of the visceral sac, right at the position of the invaginated ectodermal tissue that has been formed the shell gland (Fig. 5a). Starting from this solid structure, with increasing age the snails developed an internal calcareous shell in the shape of a hollow, nonsegmented cone, which surrounds the hepatopancreas in its ventralmost part (Fig. 5).

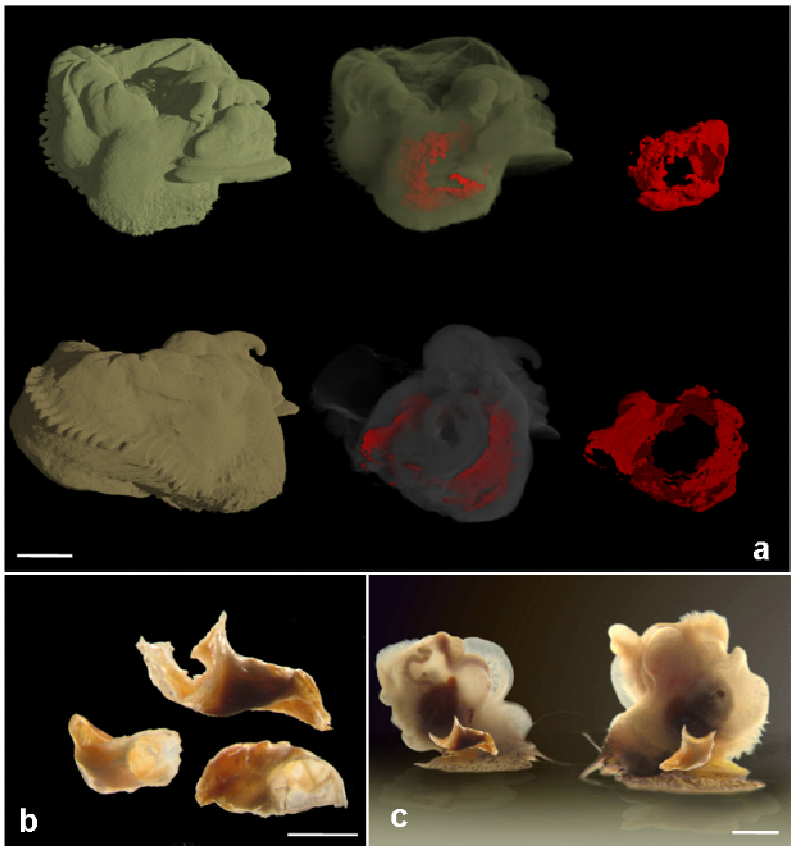


Fig. 5. Internal shells of Pt-exposed *Marisa cornuarietis*. (a) Synchrotron X-ray phase-contrast microtomographs of snails exposed to 100 µg/L $PtCl_2$, 26 days post fertilization. Upper row: view from the right, ahead; lower row: view from the left, rear. Respective left pictures: whole body; pictures in the middle: semi-transparent view of the body; respective right pictures: internal shell fragment. Scale bar is 500 µm. (b) Three examples of internal shells of snails exposed to 200 µg/L $PtCl_2$, about 2 months old. Scale bar is 500 µm. (c) Approximate position of the internal shell in *M. cornuarietis* exposed to 200 µg/L $PtCl_2$, about 2 months old, photo editing. Scale bar is 1 mm.

The phenomenon of Pt-induced body plan changes is not restricted to *M. cornuarietis* but could also be observed in embryos of the pulmonate snail *P. corneus* exposed to nominal concentrations of ≥ 300 µg/L $PtCl_2$ from fertilization until hatch. Also shell-less *P. corneus* did not form a mantle cavity (Fig. 3b). In contrast to *M. cornuarietis*, the longevity of unshelled *P. corneus* was restricted to a maximum of about 2 weeks postfertilization.

Discussion

The present study describes and investigates artificially induced shell internalization in *M. cornuarietis* due to Pt exposure, which corresponds with fundamental body plan changes.

In contrast to Pt^{2+}, the physicochemical similar ion Pd^{2+} and other bivalent metals (Zn^{2+}, Ni^{2+}, Cd^{2+}; Schirling et al., 2006; Sawasdee and Köhler, 2009) did not interfere with shell formation during embryogenesis. Only occasionally high concentrations of Li^{+} (2.5 and 3 mg/L $LiCl_2$), a metal which has been shown to interact with the positional system of predominantly ectodermal tissue in *Xenopus* and *Loligo* (Kao et al., 1986; Crawford, 2003), prevented the formation of an external shell in *M. cornuarietis*. So far, solely the heavy metal Pt seems to specifically interact with key processes during early embryonic development, which inhibits the mantle to evaginate and to overgrow the visceral sac, hence, leading to the growth of an internal shell as it could be traced by the staining of the mantle edge.

Pulse experiments revealed days 4 and 5 postfertilization to be most susceptible to Pt action on shell formation. Data probably varied due to slight variation in culture temperature (26 ± 1 °C) and depending on the exact time of fertilization during the night. During these days of embryonic development the initial stages of the embryonic shell are formed (Demian and Yousif, 1973a). One hypothetical approach to explain the Pt-induced internalization of the shell relies on a possible interaction of Pt with the Ca metabolism and its uptake via Ca trans-membrane transport. Because Ca supplements did not diminish Pt uptake, we conclude that Pt likely interacts with Ca signalling pathways involved in the positional system, which may be stabilized by increasing intracellular levels of Ca. Some heavy metals have been shown to act as inhibitors of the ezyme CA (Christensen and Tucker, 1976; Morgan et al., 1997; Vitale et al., 1999) or to reduce shell mass in snails (Beeby et al., 2002). We therefore tested the CA activity in Pt exposed *M. cornuarietis*. The results indicated a diminuation but no significant difference of the activity in Pt exposed snails. Hence, the mechanisms of the internal shell production seem to be almost as effective as those involved in the growth of the external shell, and the reduction in shell size could probably only be attributed to a compressed shape due to the limited space inside the gastropod's body.

The effect of body plan changes due to Pt exposure could also be observed in embryos of the pulmonate snail *P. corneus*. Also shell-less *P. corneus* did not form a mantle cavity which might indicate that Pt action on the re-direction of presumptive mantle tissue is likely universal in gastropods. In contrast to prosobranch

gastropods, however, pulmonates lack a gill but use their mantle cavity to form a lung. A re-direction of mantle tissue here consequently leads to a lack of any respiratory organ and, therefore, to a short life span in *P. corneus* embryos.

Descriptions of various authors about the time span of torsion (from about 2 min until about 200 h) and its main cause(s) (muscular activity, differential growth, hydraulic activity) in different groups of gastropods vary to a high degree (for detailed informations see Wanninger et al., 2000). Therefore, it can be assumed that ontogenetic torsion of gastropods has been highly modified within different groups subsequent to its rise in early phylogeny.
Because no external shell is formed after exposure to Pt, torsion occurs independently from retractor muscle action on the larval shell in *M. cornuarietis* as it was shown for other gastropod species in earlier studies of Hickman and Hadfield (2001) and Page (2002) who provided evidence for this view. As shown for other organisms (for an overview see Wanninger et al., 2000), our results provide additional independent evidence that several processes are involved in the ontogenetic process of torsion, in contrast to Garstang (1929) and Crofts (1937, 1955) who proclaimed contraction of asymetric larval retractor mussels to be the cause of developmental rotation. We could show that at least two of the processes associated with torsion can be uncoupled during the development of *M. cornuarietis*. That is, the anus of the treated snails is located anteriorly, but the mantle tissue and gill remains in a posterior location. Hence, the process of torsion is neither inevitably connected to mantle cavity formation nor to the translocation of its aperture together with the gill into a frontal position but rather developmentally separated from the distal outgrowth of the mantle epithelium, which is also the prerequisite for an external shell. Both freshwater model species, *M. cornuarietis* and *P. corneus*, go through a 'direct development' lacking a trochophora or veliger larva. Therefore, differential growth may play a crucial role in torsion because muscles are differentiated after the torsion process only.
The fact that only the position of the mantle tissue and the gills but not the anus in Pt-treated *M. cornuarietis* can be uncoupled from torsion processes compared to nontreated animals, might be due to the observations made by Demian and Yousif (1973b) who described that the intestine of this species is entirely endodermal and opens into the mantle cavity at a relatively late stage.

This is the first report on snail–slug conversion and experimentally induced shell internalization in gastropods. Even though the morphological similarity of these

artificial internal shells with internal shell derivatives in extant or fossil molluscan taxa is striking, we do not claim to be able physiologically to trigger exactly what has evolved in cephalopods, nudibranchs, and pulmonate slugs. The mechanisms of shell–mantle interactions in the formation of internal shell derivatives in extant molluscs are manifold and do not follow exactly the same developmental pattern, even though, in all cases and also in our experiments, a shell precursor is overgrown by ectodermal (mantle) tissue (Kniprath, 1981; Page, 2000; Gibson, 2003). Particularly in extant taxa of opisthobranchs, there is evidence for a stepwise reduction of the shell (Wägele and Klussmann-Kolb, 2005) which is contradicting a hypothesis of 'macromutation'- based radical developmental shifts underlying body plan modifications such as detorsion in these heterobranch gastropods. Nevertheless, it is evident from our study that minimal changes in the blueprint for molluscan ontogeny (or the corresponding signal transduction machinery of the positional system) may lead to sudden body plan shifts. This observation is consistent with the notion of modularity, the idea that a subset of variables in a system may be changed independent of the remaining variables in the system (Lipson et al., 2002) – in this case Pt leads to a drastic change during early development to one part of the body without lethal consequences to the whole organism.

We cannot exclude that similar, mutation-based body plan alterations have contributed to the evolution of shell internalizations in several molluscan taxa as we know them today.

Acknowledgements

The authors thank K.-H. Hellmer for technical assistance with the SEM, J. Oehlmann for initially providing a *M. cornuarietis* stock, A. Dieterich for help with tomography imaging, and R. Paxton and two anonymous reviewers for critical comments on the manuscript. Studienstiftung des deutschen Volkes and Landesgraduiertenförderung Baden-Württemberg are highly acknowledged for financial support.

References

Beeby, A., Richmond, L. and Herpé, F., 2002. Lead reduces shell mass in juvenile garden snails (*Helix aspersa*). Environ. Pollut. 120: 283–288.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Burnett, L.E., Woodson, P.B.J., Rietow, M.G. and Vilicich, V.C., 1981. Crab gill intra-epithelial carbonic anhydrase plays a major role in haemolymph CO_2 and chloride ion regulation. J. Exp. Biol. 92: 243–254.

Christensen, G.M. and Tucker, J.H., 1976. Effects of selected water toxicants on the in vitro activity of fish carbonic anhydrase. Chem.Biol. Interact. 13: 181–192.

Costlow, J.D., 1959. Effects of carbonic anhydrase inhibitors on shell development and growth of *Balanus improvisus* Darwin. Physiol. Zool. 32: 177–189.

Crawford, K., 2003. Lithium chloride inhibits development along the animal vegetal axis and anterior midline of the squid embryo. Biol. Bull. 205: 181–182.

Crofts, D.R., 1937. The development of *Haliotis tuberculata* with special reference to organogenesis during torsion. Philos. Trans. R. Soc. Lond. B 228: 219–268.

Crofts, D.R., 1955. Muscle morphogenesis in primitive gastropods and its relation to torsion. Proc. Zool. Soc. Lond. 125: 711–750.

Demian, E.S. and Yousif, F., 1973a. Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda: Ampullariidae). I. General outlines of development. Malacologia 12: 123–150.

Demian, E.S. and Yousif, F., 1973b. Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda: ampullariidae). II. Development of the alimentary system. Malacologia 12: 151–174.

Demian, E.S. and Yousif, F., 1973c. Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda: Ampullariidae). III. Development of the circulatory and renal systems. Malacologia 12: 175–194.

Demian, E.S. and Yousif, F., 1973d. Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda: Ampullariidae). IV. Development of the shell gland, mantle and respiratory organs. Malacologia 12: 195–211.

Demian, E.S. and Yousif, F., 1975. Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda: Ampullariidae). V. Development of the nervous system. Malacologia 15: 29–42.

Furbish, D.R. and Furbish, W.J., 1984. Structure, crystallography, and morphogenesis of the cryptic shell of the terrestrial slug *Limax maximus* (Mollusca, Gastropoda). J. Morphol. 180: 195–212.

Garstang, W., 1929. The origin and evolution of larval forms. Rep. Brit. Assoc. Adv. Sci. Section D: 77–98.

Gibson GD., 2003. Larval development and metamorphosis in *Pleurobranchaea maculata*, with a review of development in the notaspidea (Opisthobranchia). Biol. Bull. 205: 121–132.

Giraud, M.M., 1981. Carbonic anhydrase activity in the integument of the crab *Carcinus maenas* during the intermold cycle. Comp. Biochem. Physiol. 69A: 381–387.

Heethoff, M. and Cloetens, P.A., 2008. Comparison of synchrotron X-ray phase contrast tomography and holotomography for non-invasive investigations of the internal anatomy of mites. Soil Organisms 80: 205–215.

Henry, R.P., 1991. Techniques for measuring carbonic anhydrase activity in vitro: the electrometric delta pH and pH stat methods. In: Dodgson, S.J., Tashien, R.E., Gros, G., Carter, N.D. (eds). The Carbonic Anhydrases: Cell. Physiol. Mol. Gen. New York: Plenum Press. pp. 119–125.

Hickman, C.S., and Hadfield, M.G., 2001. Larval Muscle Contraction Fails to Produce Torsion in a Trochoidean Gastropod: Biol Bull 200: 257-260.

Kao, K.R., Masui, Y. and Elinson, R.P., 1986. Lithium-induced respecification of pattern in *Xenopus laevis* embryos. Nature (Lond.) 322: 371–373.

Kniprath, E. 1981. Ontogeny of the molluscan shell field: a review: Zool. Scr. 10: 61–79.

Lipson, H., Pollack, J.B., Suh, N.P., 2002. On the origin of modular variation. Evolution 56: 1549–1556.

Maren, T.H., 1967. Carbonic anhydrase: chemistry, physiology, and inhibition. Physiol. Rev. 47: 595–781.

Morgan, I.J., Henry, R.P. and Wood, C.M., 1997. The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) is inhibition of Na- and Cl- transport. Aquat. Toxicol. 39: 145–163.

OECD Guideline for Testing of Chemicals, 1992. Test Guideline 203: Fish, Acute Toxicity Test.

Osterauer, R., Haus, N., Sures, B. and Köhler, H.-R., 2009. Uptake of platinum by zebrafish (*Danio rerio*) and ramshorn snail (*Marisa cornuarietis*) and resulting effects on early embryogenesis. Chemosphere 77: 975–982.

Osterauer, R., Köhler, H.-R. and Triebskorn, R., 2010. Histopathological alterations and induction of stress proteins in ramshorn snail (*Marisa cornuarietis*) and zebrafish (*Danio rerio*) after exposure to PtCl2 during embryogenesis. Aquat. Toxicol. 99: 100–107.

Page, L.R., 2000. Inflated protoconchs and internally dissolved, coiled protoconchs of nudibranch larvae: different developmental trajectories achieve the same morphological result. Invertebrate Biol. 119: 278–286.

Page, L.R., 2002. Ontogenetic torsion in two basal gastropods occurs without shell attachments for larval retractor muscles. Evol. Dev. 4: 212–222.

Rousseau, M., Plouguerne, E., Wan, G., Wan, R., Lopez, E. and Fouchereau-Peron, M., 2003. Biomineralization markers during a phase of active growth in *Pinctada margaritifera*. Comp. Biochem. Physiol. 135: 271–278.

Sawasdee, B. and Köhler, H.-R., 2009. Embryo toxicity of pesticides and heavy metals to the ramshorn snail, *Marisa cornuarietis* (Prosobranchia). Chemosphere 75: 1539–1547.

Scheltema, A. H. and Schander, C., 2006. Exoskeletons: Tracing molluscan evolution. Venus 65: 19–26.

Schirling, M., Bohlen, A., Triebskorn, R. and Köhler, H.-R., 2006. An invertebrate embryo test with the apple snail *Marisa cornuarietis* to assess effects of potential developmental and endocrine disruptors. Chemosphere 64: 1730–1738.

Sures, B., Taraschewski, H. and Haug, C., 1995. Determination of trace metals (Cd, Pb) in fish by electrothermal atomic absorption spectrometry after microwave digestion. Anal. Chim. Acta 311: 135–139.

Takaichi, S., Mizuhira, V., Hasegawa, H., Suzaki, T., Notoya, M., Ejiri, S., Ozawa, H. and Van Wyk, J.H., 2003. Ultrastructure and early embryonic shell formation in the terrestrial pulmonate snail, *Euhadra hickonis*. J. Moll. Stud. 69: 229–244.

Triebskorn, R., Bader, K., Linder, B. and Köhler, H.-R., 2005. Snails and slugs as non-targets for environmental chemicals. IOBC Bull. 28: 1–9.

Vitale, A.M., Monserrat, J.M., Castilho, P. and Rodriguez, E.M., 1999. Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulata* (Decapoda, Grapsidae). Comp. Biochem. Physiol. C 122: 121–129.

Wägele, H. and Klussmann-Kolb A., 2005. Opisthobranchia (Mollusca, Gastropoda) – more than just slimy slugs. Shell reduction and its implications on defence and foraging. Frontiers in Zoology 2: 1-18.

Waller, T.R., 1981. Functional morphology and development of veliger larvae of the European Oyster, *Ostrea edulis* Linné. Smithsonian Contrib. Zool. 328: 1–70.

Wanninger, A., Ruthensteiner, B. and Haszprunar, G., 2000. Torsion in *Patella caerulea* (Mollusca, Patellogastropoda): ontogenetic process, timing, and mechanisms. Invert. Biol. 119: 177–187.

Wilbur, K.M. and Saleuddin, A.S.M., 1983. Shell formation. In: Saleuddin, A.S.M., Wilbur, K.M. (Eds.), The Mollusca 4. Academic Press, New York. Pp. 235–287.

Zimmermann, S., Menzel, C.M., Berner, Z., Eckhardt, J.D., Stuben, D., Alt, F., Messerschmidt, J., Taraschewski, H. and Sures, B., 2001. Trace analysis of platinum in biological samples: a comparison between sector field ICP-MS and adsorptive cathodic stripping voltammetry following different digestion procedures. Anal. Chim. Acta 439: 203–209.

Zimmermann, S., Messerschmidt, J., von Bohlen, A. and Sures, B., 2003. Determination of Pt, Pd and Rh in biological samples by electrothermal atomic absorption spectromety as compared with adsorptive cathodic stripping voltammetry and total-reflexion X-ray fluorescence analysis. Anal. Chim. Acta 498: 93–104.

Danksagung

Der überwiegende Teil der vorliegenden Arbeit wurde im Institut für Evolution und Ökologie, Abteilung Physiologische Ökologie der Tiere der Eberhard Karls Universität Tübingen durchgeführt.

Besonders bedanken möchte ich mich bei meinem Doktorvater Prof. Dr. Heinz-R. Köhler, welcher mir stets bei Fragen und Ungewissheiten hilfreich zur Seite stand, sich für mein Thema begeisterte und stets dafür offen und diskussionsbereit war. Prof. Dr. Bernd Sures, Leiter der Abteilung Angewandte Zoologie/Hydrobiologie an der Universität Duisburg-Essen, danke ich für die Hilfe bei der Ausarbeitung meines Themas, für die Betreuung des analytischen Teils meiner Doktorarbeit und für die Erstellung des Zweitgutachtens. Bei seinen DoktorandInnen und MitarbeiterInnen bedanke ich mich für weiterführende fachnahe Diskussionen, die Einlernung in die analytischen Methoden und für die sehr schöne Zeit an der Universität Duisburg-Essen. Ein besonderer Dank gilt dabei Nadine Ruchter, Sabrina Frank, Kerstin Geiss, Milen Nachev und Sonja Zimmermann.

Prof. Dr. Jörg Oehlmann danke ich für die Erstellung des Drittgutachtens.

Bei Prof. Dr. Rita Triebskorn bedanke ich mich für die Betreuung des histologischen Teils meiner Dissertation, sowie für die wichtige Unterstützung und Beratung bei den elektronenmikroskopischen Untersuchungen.

Ein Dank geht auch an Prof. Dr. Thomas Braunbeck, Leiter der Arbeitsgruppe Aquatische Ökologie und Toxikologie an der Universität Heidelberg, in dessen Abteilung ich den Comet Assay erlernen und auswerten durfte. Für die Einarbeitung und Betreuung des Comet Assays danke ich Frank Hoffmann und Christopher Faßbender.

Ebenfalls bedanken möchte ich mich bei Dr. Matthias Gerberding, unter dessen Betreuung ich am Max Planck Institut für Entwicklungsbiologie in Tübingen die Färbung des schalensekretierenden Mantelgewebes durchführen konnte. Und bei Prof. Dr. Oliver Betz bedanke ich mich dafür, dass in dessen Abteilung für Evolutionsbiologie der Invertebraten an der Universität Tübingen rasterelektronische Untersuchungen durchgeführt werden konnten.

Besonders möchte ich mich auch bei den vielen Technischen AssistentInnen bedanken, die mir bei praktischen Belangen und Probenpräparationen hilfreich zur Seite standen: Heidi Casper, Stefanie Krais, Monika Meinert, Julia Straube, Karl-Heinz Hellmer und Manocheer Bahmani.

Bei der Landesgraduiertenförderung Baden-Württemberg und der Studienstiftung des deutschen Volkes bedanke ich mich für die finanzielle Unterstützung in Form von Promotionsstipendien. Und für finanzielle Beihilfen für Kongressbesuche und Aufenthalte an anderen Universitäten zum Erlernen von neuen Methoden bedanke ich mich bei der Reinhold-und-Maria-Teufel Stiftung.

Ein ganz herzlicher Dank geht an alle MitarbeiterInnen, Diplomanden, Doktoranden und Praktikanten unserer Abteilung Physiologische Ökologie der Tiere, die dazu beigetragen haben, dass die Jahre der Doktorarbeit eine Zeit des Lernens, der Zusammenarbeit, der Freude und Bereicherung waren. Besonderer Dank gebührt Irene Gust, Heidi Casper, Stefanie Krais, Timo Haap, Dr. Cornelia Kienle, Nils Dittbrenner, Miriam Langer-Jaesrich, Katja Wallmeier, Katja Bader, Manuela Schneider, Martina Čížcová, Volker Pott, Anja Henneberg, Leonie Marschner, Dr. Volker Scheil und Banthita Sawasdee.

Und natürlich ein Dank an diejenigen, die mich auf meinem Weg begleiten, bereichern und bestärken: Meine lieben Eltern und Freunde.

Publikationsliste

Originalpublikationen in wissenschaftlichen Zeitschriften

Osterauer, R., Köhler, H.-R. 2008. Temperature-dependent effects of the pesticides thiacloprid and diazinon on the embryonic development of zebrafish (*Danio rerio*). Aquat. Toxicol. 86: 485-494.

Scheil, V., Kienle, C., Osterauer, R., Gerhardt, A., Köhler, H.-R. 2009. Effects of 3,4-dichloroaniline and diazinon on different biological organisation levels of zebrafish (*Danio rerio*) embryos and larvae. Ecotoxicology 18: 355–363.

Osterauer, R., Haus, N., Sures, B., Köhler, H.-R. 2009. Uptake of platinum by zebrafish (*Danio rerio*) and ramshorn snail (*Marisa cornuarietis*) and resulting effects on early embryogenesis. Chemosphere 77: 975–982.

Osterauer, R., Köhler, H.-R., Triebskorn, R. 2010. Histopathological alterations and induction of hsp70 in ramshorn snail (*Marisa cornuarietis*) and zebrafish (*Danio rerio*) embryos after exposure to $PtCl_2$. Aquat. Toxicol. 99: 100–107.

Osterauer, R., Marschner, L., Betz, O., Gerberding, M., Sawasdee, B., Cloetens, P., Haus, N., Sures, B., Triebskorn, R., Köhler, H.-R. 2010. Turning snails into slugs: Induced body plan changes and formation of an internal shell. Evol. Dev. 12: 474-483.

Osterauer, R., Faßbender, C., Braunbeck, T., Köhler, H.-R. 2011. Genotoxicity of platinum in embryos of zebrafish (*Danio rerio*) and ramshorn snail (*Marisa cornuarietis*). Sci. Total Environ. 409: 2114–2119.

Köhler, H.-R., Osterauer, R., Marschner, L., McKee, M., Betz, O., Gerberding, M., Sawasdee, B., Cloetens, P., Haus, N., Sures, B., Triebskorn, R. 2010. *Marisa* topless: What platinum may bring about. Comp. Biochem. Physiol. A. 157 (Suppl. 1): S37 (Congress abstract).

Tagungsbeiträge

Osterauer, R., Scheil, V., Köhler, H.-R. 2007. Effects of the insecticides thiacloprid, imidacloprid, diazinon and 3,4-dichloroaniline, one binary mixture and temperature influence on embryonic development of zebrafish (*Danio rerio*). SETAC (Society of Environmental Toxicology and Chemistry) Europe 17th Annual Meeting; The multiple stressors for the environment – present and future challenges and perspectives, Porto, Portugal, 20.-24.05.2007. Poster.

Scheil, V., Osterauer, R., Triebskorn, R., Köhler, H.-R. 2007. Influence of three pesticides, a decomposition product of a pesticide and twobinary mixtures of them on the stress protein level of *Danio rerio* larvae. SETAC (Society of Environmental Toxicology and Chemistry) Europe 17th Annual Meeting; The multiple stressors for the environment – present and future challenges and perspectives, Porto, Portugal, 20.-24.05.2007. Poster.

Langer, M.E., Kienle, C., Osterauer, R., Scheil, V., Triebskorn, R., Gerhardt, A., Köhler, H.-R. 2007. Auswirkungen von Umweltschadstoffen auf verschiedene biologische Organisationsebenen des Zebrabärblings *Danio rerio*. GdCh-Annual Meeting, Osnabrück, Germany, 26.-28.09.2007. Vortrag.

Kienle, C., Langer, M.E., Osterauer, R., Scheil, V., Triebskorn, R., Gerhardt, A., Köhler, H.-R. 2007. Auswirkungen der Pestizide Imidacloprid und Thiacloprid auf verschiedene biologische Organisationsebenen des Zebrabärblings *Danio rerio*. GdCh-Annual Meeting, Osnabrück, Deutschland, 26.-28.09.2007. Poster.

Osterauer, R., Sawasdee, B., Sures, B., Köhler, H.-R. 2007. Environmental toxicity assessment of platinum group elements. Meeting StEvE; Annual meeting of Students in Evolution and Ecology, Tübingen, Germany, 19.-20.10.2007. Vortrag.

Osterauer, R., Sures, B., Köhler, H.-R. 2008. Effects of platinum on the early development of ramshorn snail (*Marisa cornuarietis*) and zebrafish (*Danio rerio*). SETAC (Society of Environmental Toxicology and Chemistry) Europe 18th Annual Meeting; World under stress: scientific and applied issues, Warsaw, Polen, 25.-29.05.2008. Poster.

Osterauer, R., Sures, B., Köhler, H.-R. 2008. Effekte des Schwermetalls Platin auf die frühe Individualentwicklung des Zebrabärblings (*Danio rerio*) und der Paradies-Schnecke (*Marisa cornuarietis*). 11. Edelmetallforum, Emden, Germany, 23.-24.6.2008. Vortrag.

Osterauer, R., Sures, B., Köhler, H.-R. 2008. Effekte des Schwermetalls Platin auf die Embryonalentwicklung der Paradies-Schnecke (*Marisa cornuarietis*) und des Zebrabärblings (*Danio rerio*). Doktorandenforum der Studienstiftung des deutschen Volkes, Köln-Deutz, Germany, 07.-10.12.2008. Vortrag.

Marschner, L., Osterauer, R., Betz, O., Gerberding, M., Cloetens, P., Sures, B., Triebskorn, R., Köhler, H.-R. 2009. Platinum-induced internalization of shell formation in snails. 26th Congress of the new European Society of Comparative Biochemistry and Physiology - Environmental stimuli and their impact on cellular homeostasis and gene regulation, Innsbruck, Austria, 06.-09.09.2009. Poster.

Osterauer, R., Marschner, L., Betz, O., Gerberding, M., Sawasdee, B., Cloetens, P., Haus, N., Sures, B., Triebskorn, R., Köhler, H.-R. 2010. Effects of platinum group elements (PGE) on the embryonic development of the ramshorn snail *Marisa cornuarietis* with special attention to shell formation. SETAC (Society of Environmental Toxicology and Chemistry) Europe 20th Annual Meeting; Science and Technology for Environmental Protection, Sevilla, Spain, 23.-27.05.2010. Poster.

Osterauer, R., Marschner, L., Betz, O., Gerberding, M., Sawasdee, B., Cloetens, P., Haus, N., Sures, B., Triebskorn, R., Köhler, H.-R. 2010. Durch Platin verursachte Bauplanänderungen bei der Paradies-Schnecke *Marisa cornuarietis*. 12. Edelmetallforum, Essen, Germany, 16.-17.06.2010. Vortrag.

Köhler, H.-R., Osterauer, R., Marschner, L., McKee, M., Betz, O., Gerberding, M., Sawasdee, B., Cloetens, P., Haus, N., Sures, B., Triebskorn, R. 2010. *Marisa* topless: What platinum may bring about. 27th Congress of the newEuropean Society of Comparative Biochemistry and Physiology, Alessandria, Italy, 05.-09.09.2010. Vortrag.