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Validation of reproduction tests with molluscs – Establishment of an OECD test guideline for the identification of endocrine and reproductive toxic effects in snails

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Validation of reproduction tests with molluscs – Establishment of an OECD test guideline for the identification of endocrine and reproductive toxic effects in snails

by

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Kurzbeschreibung

Ziel des Projekts war die Entwicklung eines OECD-Prüfrichtlinien-Entwurfs für einen Reproduktionstest mit *Potamopyrgus antipodarum*, einschließlich der dafür notwendigen Validierungs- und Optimierungsarbeiten. Zusätzlich sollten experimentelle Arbeiten zur Entwicklung eines kompletten Lebenszyklustest durchgeführt und eine Standardarbeitsanweisung (SOP) erstellt werden. Weiterhin sollten im Rahmen der von Frankreich koordinierten Validierungsstudien zur Entwicklung eines Reproduktionstests mit *Lymnaea stagnalis* experimentelle Arbeiten durchgeführt werden.
Die erarbeitete SOP des Reproduktionstests mit *P. antipodarum* wurde optimiert und validiert. In dem 28-tägigen Reproduktionstest werden Mortalität und die Zahl der Embryonen in der Bruttasche pro Weibchen als Endpunkte aufgenommen. Zur Optimierung der Zuchtbedingungen wurden verschiedene Testmedien sowie unterschiedliche Besatzdichten der Schnecken untersucht. Weiterhin wurde der Einfluss der Temperatur und der Photoperiode auf die Reproduktion von *P. antipodarum* über den Jahresverlauf ermittelt. Die Ergebnisse zeigen, dass optimale Zuchtbedingungen mit einer Temperatur von 16°C und einem Hell:Dunkel Rhythmus von 16:8 h erreicht werden können. Die Optimierung der Futterdosis im Reproduktionstest erleichtert die Bestimmung der Effekte von reproduktionstoxischen Substanzen und von Chemikalien, die zu einer Reproduktionssteigerung der Schnecken führen. Eine Unterversorgung oder Reduktion der Energiereserven der Schnecken wird dabei vermieden.

Um das vorgeschlagene Testdesign zu validieren und mögliche Probleme bei der Durchführung des Reproduktionstests mit *P. antipodarum* gemäß SOP zu identifizieren, wurden drei Ringtests mit insgesamt 16 Partnern unter Koordination der Goethe-Universität durchgeführt. Für die Validierungsstudien wurden Cadmium, Tributylzinn, Prochloraz, Trenbolon, Triclocarban und Triclosan als Testsubstanzen ausgewählt. In den drei Ringtests erwies sich der Reproduktionstest als robust und reproduzierbar, weil die Mehrzahl der Partnerlabore vergleichbare NOEC-, LOEC-, EC_{10}- und EC_{50}-Werte mit überlappenden 95%-Konfidenzintervallen ermittelten. Zum Projektende wurde eine Entwurfsversion der Prüfrichtlinie des Reproduktionstests mit *P. antipodarum* bei der OECD eingereicht und soll im Juli 2015 zur öffentlichen Kommentierung bereitgestellt werden (siehe Anhang 8.3). Die Ergebnisse des Vorhabens tragen somit gezielt zur Weiterentwicklung von Testmethoden und Bewertungskonzepten für die Regulierung reproduktionstoxischer (z.B. endokrin wirksamer) Chemikalien in REACH, sowie von Pflanzenschutzmitteln, Arzneimitteln und Bioziden bei.

Darüber hinaus wurden zwei mögliche Testdesigns für einen vollständigen Lebenszyklustest mit *P. antipodarum* entwickelt und deren Durchführbarkeit und Eignung evaluiert. Ein Entwurf für eine SOP des Lebenszyklustestes findet sich im Anhang 8.2.

Abstract

The aim of the project was the development of an OECD draft test guideline for the reproduction test with *Potamopyrgus antipodarum* including the required validation and optimisation. Additionally, a full life cycle test with *P. antipodarum* should be developed and a standard operating procedure (SOP) should be compiled. Furthermore, to contribute to the validation studies of the reproduction test with *Lymnaea stagnalis* coordinated by France, experimental assistance should be performed.

The existing SOP of the reproduction test with *P. antipodarum* has been optimised and validated. In the 28 days reproduction test the number of embryos in the brood pouch, reflecting the individual reproduction effort in snails, and adult mortality serve as endpoints. To optimise the culture conditions of the snails, different test media and snail densities were investigated. Also, the influence of temperature and photoperiod on the reproduction of *P. antipodarum* was stud-

ied during the course of the year. The results show that the optimum breeding conditions are at a temperature of 16°C and a light:dark regime of 16:8 h. An optimisation of the food dose during the test facilitates the assessment of both reproductive toxicants and substances enhancing reproduction without causing food limitation or influencing energy reserves of the snails.

To validate the proposed test design and to identify issues in performing the SOP of the *P. antipodarum* reproduction test, three ring tests with 16 partners were performed coordinated by Goethe University. Within these ring trials cadmium, tributyltin, prochloraz, trenbolone, triclocarban and triclosan were chosen as test chemicals. For the three ring tests the robustness and the reproducibility of the reproduction test has been proven as most of the partner laboratories detected comparable NOEC, LOEC, EC_{10} and EC_{50} values with overlapping 95%-confidence intervals for the latter. At the end of the project a draft test guideline for the reproduction test with *P. antipodarum* was submitted to OECD and will be made available for an international commenting round (see Annex 8.3). The outcome of the project contributes to the further development of test methods and evaluation concepts for the regulation of reproductive-toxic (e.g. endocrine active) chemicals in REACH, as well as pesticides, pharmaceuticals and biocides.

Furthermore, to extend the reproduction test, two possible test designs for a full life cycle test with *P. antipodarum* were developed and the suitability of these test designs was evaluated. The draft SOP for full life cycle test with *P. antipodarum* can be found in annex 8.2.

Table of contents

List of figures

List of tables

List of abbreviations

4-NP	4-Nonylphenol
ASV	Air saturation value
BPA	Bisphenol A
Cd	Cadmium
cDNA	Complementary desoxyribonucleic acid
DEFRA	Department of Environment, Food and Rural Affairs
DES	Diethylstilbestrol
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
DRP	Detailed Review Paper
E_2	17β-Estradiol
EC_X	Effect concentration, where x% effect occurs
EDC	Endocrine Disrupting Chemical
EE_2	17α-Ethinylestradiol
ER	Estrogen receptor
FLC	Full life cycle
HPLC	High Performance Liquid Chromatography
i.a.	Inter alia
INRA	French National Institute for Agricultural Research
LOD	Limit of determination/detection
LOEC	Lowest observed effect concentration
LOQ	Limit of quantification/quantitation
mRNA	Messenger ribonucleic acid
MR	Medium renewal
MT	17α-Methyltestosterone
NOEC	No observed effect concentration
OECD	Organisation for Economic Co-operation and Development
OP	4-Tert-octylphenol
PCZ	Prochloraz
PLC	Partial life cycle
qPCR	Quantitative real-time-polymerase chain reaction
RNA	Ribonucleic acid
SD	Standard deviation

SEM	Standard error of the mean
SGR	Specific growth rate
SOP	Standard Operating Procedure
SPE	Solid phase extraction
TBT	Tributyltin
TCC	Triclocarban
TCS	Triclosan
TEG	Triethylene glycol
TR	Trenbolone
TWM	Time-weighted mean
UBA	German Federal Environment Agency
VMGeco	Validation management group on ecotoxicity testing
Vtg	Vitellogenin

Zusammenfassung

Ziel des Projekts „Validierung von Mollusken-Reproduktionstests - Etablierung einer OECD-Testguideline zum Nachweis endokriner und reproduktionstoxischer Effekte bei Schnecken“ (ValMolRepro II) war die Entwicklung eines OECD-Prüfrichtlinien-Entwurfs für einen Reproduktionstest mit *Potamopyrgus antipodarum*, einschließlich der dafür notwendigen Validierungs- und Optimierungsarbeiten. Zusätzlich sollten experimentelle Arbeiten zur Entwicklung eines kompletten Lebenszyklustest durchgeführt und eine Standardarbeitsanweisung (SOP) erstellt werden. Weiterhin sollten im Rahmen der von Frankreich koordinierten Validierungsstudien zur Entwicklung eines Reproduktionstests mit *Lymnaea stagnalis* experimentelle Arbeiten durchgeführt werden.

In dem Vorgängerprojekt ValMolRepro I (FKZ 3708 61 402) wurde ein *Detailed Review Paper* (DRP) *on Molluscs Life-cycle Toxicity Testing* erarbeitet und als OECD-Veröffentlichung publiziert (OECD 2010). Im DRP wurden drei prinzipiell geeignete Molluskenarten sowie mögliche Testdesigns vorgeschlagen. Bei einer der vorgeschlagenen Testspezies handelt es sich um die Neuseeländische Zwergdeckelschnecke *P. antipodarum*, die sich bereits gegenüber endokrin wirksamen Chemikalien (z.B. Organozinnverbindungen) und anderen Substanzen (z.B. Kupfer) sensitiv zeigte. Im Rahmen des Projektes ValMolRepro I wurde das Testdesign für einen 28-tägigen Reproduktionstest mit *P. antipodarum* entwickelt und eine entsprechende SOP erstellt. Bei diesem Test werden als Endpunkte die Mortalität und die Zahl der Embryonen in der Bruttasche pro Weibchen ermittelt. In einem internationalen Ringtest mit der ersten Version des Testdesgins, an dem vier Partnerlabore teilnahmen, erwies sich diese als robust und problemlos anwendbar. Als Testsubstanzen dienten Bisphenol A (BPA) und Cadmium (Cd). Die vier Labore konnten einen konzentrationsabhängigen Rückgang der Embryonenzahl unter Cd-Exposition beobachten. Die Ergebnisse aus drei von vier Laboren bestätigten die erwartete Erhöhung der Embryonenzahl unter BPA-Exposition Die Analyse der Wasserproben ergab, dass sich BPA mit der Versuchsdauer zunehmend abbaut. Möglicherweise wurde das durch den Biofilm in den Testgefäßen verursacht. Das Ergebnis dieses Ringtests war daher, dass in künftigen Validierungsstudien mit *P. antipodarum* die Testgefäße während des Versuchs ausgetauscht werden sollten, um die Entwicklung eines Biofilms an den Glaswänden zu minimieren. Darüber hinaus sollte für die Validierung eine weniger leicht abbaubare Chemikalie, wie beispielsweise 4-tert-Octylphenol (OP), verwendet werden. Weiterhin erwies sich die Unterscheidung von „beschalten“ und „unbeschalten“ Embryonen als zu schwierig, weshalb für künftige Studien auf diese Differenzierung verzichtet und stattdessen ausschließlich die Gesamtzahl der Embryonen erfasst werden sollte. Eine zusätzliche Folgerung aus dem Ringtest mit der ersten Version des Testdesigns war, den Einfluss der Temperatur auf die Reproduktion der Schnecken und damit auf die Effekte der Prüfsubstanz zu untersuchen. Die Effekte von Prüfsubstanzen können, abhängig davon, ob sie eine reproduktionsfördernde oder –hemmende Wirkung zeigen, durch eine besonders hohe oder sehr niedrige Embryonenzahl der Kontrolltiere im Test maskiert werden. Aus diesem Grund sollten experimentell die optimale „Reproduktionsspanne“ bzw. mittlere Embryonenzahl im Brutraum von Kontrolltieren ermittelt werden, bei der sich sowohl reproduktionshemmende wie reproduktionsfördernde Effekte von Testsubstanzen abbilden lassen.

Darüber hinaus sollte, in Anlehnung an das bereits bestehende Testprotokoll für *L. stagnalis* (OECD 2010), ein Testdesign für einen kompletten Lebenszyklustest mit *P. antipodarum* entwickelt werden. Zur Optimierung der Zuchtbedingungen sollte zusätzlich der Einfluss verschiedener Testmedien und Temperaturen auf die Reproduktion der Schnecken untersucht werden.

Im ersten Projektabschnitt wurde eine umfangreiche Literaturrecherche zu den in Expositionsversuchen mit *P. antipodarum* und *L. stagnalis* für unterschiedliche Umweltchemikalien ermittelten Effektkonzentrationen durchgeführt. Der auf den Effektkonzentrationen basierende Sensitivitätsvergleich der beiden Spezies wurde in Zusammenarbeit mit dem französischen Partner vom Institut national de la recherche agronomique (INRA) in Rennes ausgearbeitet. Die Recherche basiert auf bereits veröffentlichten sowie unveröffentlichten Daten, sogenannter „grauer Literatur" (wie Diplom-, Master- oder Doktorarbeiten). Der Fokus der Literaturstudie lag auf Untersuchungen, die chronische Effekte auf Reproduktion, Wachstum, Mobilität und Mortalität erfassen. Für lediglich neun Substanzen (BPA, Cd, Kupfer, 17α-Ethinylöstradiol, Irgarol, 4-tert-Nonylphenol, Tributylzinn, Vinclozolin, Zink) konnten Effektkonzentrationen aus vergleichbaren Expositionsversuchen mit beiden Schneckenarten ermittelt werden. Eine Korrelationsanalyse der LOEC-Werte beider Spezies ergab, dass *P. antipodarum* tendenziell sensitiver auf eine Exposition gegenüber diesen Chemikalien reagiert als *L. stagnalis*.

Optimierung der Zuchtbedingungen

Eine stabile Laborzucht mit *P. antipodarum* ist für die Entwicklung eines OECD-Standardtestsystems unabdingbar, um die ausreichende Versorgung mit Testorganismen zu gewährleisten. Um die Zuchtbedingungen der Schnecken zu optimieren, wurden drei Testmedien untersucht. Dazu wurden die Tiere über eine Dauer von 84 Tagen jeweils in ISO-Medium, Elendt M4-Medium (OECD 2004) oder DRP-Medium (OECD 2010) gehältert. Die Ergebnisse zeigen, dass das Elendt M4-Medium gegenüber den anderen beiden Medien aufwändiger herzustellen ist, eine kürzere Haltbarkeit aufweist und die Bildung eines Biofilms in den Testgläsern begünstigt. Daher eignet sich das Elendt M4-Medium für die Hälterung und Testung von *P. antipodarum* nicht.

Zusätzlich wurde in einem zweiten Versuch der Einfluss des DRP- und ISO-Mediums auf die Entwicklung und das Wachstum von juvenilen *P. antipodarum* untersucht. Hierfür wurden Adulttiere entweder in DRP- oder ISO-Medium gehältert und die entlassenen Neonaten gezählt. Nach vierwöchiger Exposition lag die mittlere Anzahl an entlassenen Jungtieren bei 60. Davon überlebten im DRP-Medium 77,6% während es im ISO-Medium lediglich 63,4% waren. Weiterhin stellte sich heraus, dass bei einer Langzeithaltung der Adulttiere im ISO-Medium die Anzahl an Embryonen in der Bruttasche der Weibchen zurückging. Daher eignet sich ISO-Medium nicht für eine dauerhafte Zucht von *P. antipodarum*. Eine mögliche Erklärung ist die unterschiedliche Zusammensetzung der beiden Medien. Während das ISO-Medium lediglich aus vier, in deionisiertem Wasser gelösten Salzen besteht, wird für das DRP-Medium unter anderem TropicMarin®-Meersalz verwendet, das laut Herstellerangaben über 70 Spurenelemente enthält, wodurch Mangelerscheinungen der adulten und juvenilen Schnecken verhindert werden können.

Über den Jahresverlauf wurde der Einfluss der Temperatur und Photoperiode auf die Reproduktion von *P. antipodarum* untersucht. Neben den bereits im DRP (OECD 2010) vorgeschlagenen Standardbedingungen (16°C, Hell:Dunkel-Rhythmus von 16:8 h), wurden Schnecken an eine Temperatur von 20°C und einer Photoperiode von 16:8 h (Hell:Dunkel) akklimatisiert. Zusätzlich wurden Schnecken bei verschiedenen Photoperioden (Gleichtagbedingungen mit 12:12 h Hell:Dunkel bzw. Kurztagbedingungen mit 8:16 h Hell:Dunkel) und 16°C gehalten. Die mittlere Embryonenzahl der Tiere wurde für die Standardbedingungen über einen Zeitraum von drei Jahren monatlich aufgenommen. Die durchschnittliche Embryonenzahl schwankte dabei in der Laborzucht zwischen 5,48 (April 2014) und 11,7 (Dezember 2014), ohne ein saisonales Muster zu zeigen. Bei 20°C lagen die Embryonenzahlen der Schnecken in einem ähnlichen Bereich wie unter Standardbedingungen. Allerdings trat bei 20°C eine erhöhte Mortalität der Juvenilen auf. Daher ist eine dauerhafte und nachhaltige Kultur von *P. antipodarum* bei dieser Temperatur nicht zu empfehlen. Die Haltung der Schnecken unter Gleichtagbedingungen (12:12 h Hell:Dunkel) ist ebenfalls nicht geeignet, da die mittlere Embryonenzahl in manchen Monaten unter 5 sank und die Mortalität der Adulttiere sehr hoch war. Diese Ergebnisse unterstützen die Standardbedingungen für die Zucht von *P. antipodarum* bei einer Temperatur von 16°C und einem Hell:Dunkel-Rhythmus von 16:8 h.

Optimierung der Testbedingungen

Ein weiteres Projektziel war die Optimierung der Testbedingungen. Es war bereits bekannt, dass die Besatzdichte der Tiere die Reproduktionsleistung beeinflussen kann. Deshalb wurde in einer Versuchsreihe der Einfluss unterschiedlicher Besatzdichten auf die Embryonenzahl von *P. antipodarum* untersucht. In der ersten Phase des Experimentes wurden Gruppen von 5, 10, 20, 40, 80 und 160 Schnecken in jeweils 200 mL DRP-Medium über einen Zeitraum von vier Wochen gehalten. Nach 28 Tagen wurde die Embryonenzahl in der Bruttasche von zehn Schnecken pro Replikat ermittelt. In der zweiten Phase des Versuches wurden die zuvor bei unterschiedlichen Dichten gehaltenen Schnecken in einem vierwöchigen Reproduktionstest unter Standardbedingungen gegenüber Cd exponiert. Die Ergebnisse der ersten Phase zeigten, dass die Reproduktionsleistung von *P. antipodarum* bei mittlerer Individuendichte höher war als bei niedriger bzw. sehr hoher Dichte. Die maximale Embryonenzahl trat in der Dichtegruppe mit 20 Schnecken pro 200 mL Medium auf. Die Ergebnisse des Reproduktionstests mit Cd in der zweiten Phase des Experiments zeigen, dass die Besatzdichte die Sensitivität gegenüber Cd modulieren kann. Je höher die Besatzdichte in Phase 1 war, umso stärker ging die Embryonenzahl während des Reproduktionstests zurück, das heißt umso sensitiver reagierten die Schnecken auf Cd. Diese Ergebnisse belegen die Beeinflussung der Reproduktion von *P. antipodarum* durch die Individuendichte vor dem Testbeginn. Um diesen Dichtestress zu vermeiden, sollte in der Laborzucht von *P. antipodarum* eine maximale Dichte von 100 Schnecken pro Liter nicht überschritten werden.

Zusätzlich wurden Reproduktionstests mit drei unterschiedlichen Testgefäßgrößen (enthalten 200, 400 oder 800 mL DRP-Medium) durchgeführt, um den Einfluss der unterschiedlichen Volumina und Dichten auf die Reproduktion von *P. antipodarum* zu untersuchen. Für eine standardisierte Testdurchführung wäre es vorteilhaft, dass bei Verwendung kleinerer Gefäße der

Verbrauch von Medium und Testchemikalien reduziert werden würde. Nach 28 Tagen wurde die Embryonenzahl der Schnecken ermittelt. Es zeigt sich kein signifikanter Unterschied zwischen den Behandlungsgruppen. Hierbei sollte beachtet werden, dass Substanzen bei kleineren Testgefäßen, aufgrund des größeren Oberflächen-Volumen-Verhältnisses, eher an die Glaswand absorbieren können und somit nicht mehr bioverfügbar sind. Daher hängt die Wahl des geeigneten Testgefäßes von den physiko-chemischen Eigenschaften der Testsubstanz ab. Für den Reproduktionstest mit *P. antipodarum* können Testgefäße mit einem Volumen von 200 bis 800 mL verwendet werden.

Der Einfluss von Lösemitteln auf die Reproduktion von *P. antipodarum* spielt für die Testung ebenso eine wichtige Rolle. In einem 28-tägigen Reproduktionstest wurde die Toxizität von insgesamt sechs Lösemitteln untersucht. Aceton, Methanol, Ethanol, Dimethylsulfoxid (DMSO) und Triethylenglykol (TEG) wurden in einem Konzentrationsbereich zwischen 0,02 und 12,5 mL/L getestet. Zusätzlich wurde der Einfluss von Eisessig auf die Reproduktion der Schnecken bei Konzentrationen von 0,01 bis 2,43 mL/L untersucht. Lediglich unter Exposition gegenüber Eisessig konnte eine signifikant erhöhte Mortalität beobachtet werden. Alle Schnecken starben bei den drei höchsten Testkonzentrationen (0,27; 0,81; 2,43 mL/L) als Folge des starken Abfalls des pH-Werts in diesen Gruppen. In den niedrigeren Konzentrationen trat kein Effekt auf die Reproduktion der Schnecken auf. Bei Exposition gegenüber Aceton und Methanol gingen die Embryonenzahlen bereits ab einer Konzentration von 0,1 mL/L signifikant zurück. Im Gegensatz dazu trat im Versuch mit Ethanol eine signifikante Steigerung der Reproduktion bei Konzentrationen von 0,1 und 0,5 mL/L auf. Ebenso konnte bei einer DMSO-Konzentration von 2,5 mL/L ein signifikanter Anstieg der Embryonenzahlen im Vergleich zur Kontrollgruppe festgestellt werden. Die Exposition von *P. antipodarum* gegenüber TEG hatte keinen Einfluss auf die Reproduktionsleistung bis zu einer Konzentration von 2,5 mL/L. Lediglich bei der höchsten getesteten Konzentration von 12,5 mL/L kam es zu einem signifikanten Rückgang der Embryonenzahl. Aufgrund der bereits im niedrigen Konzentrationsbereich aufgetretenen Effekte, können Aceton, Methanol und Ethanol nicht als Lösemittel für den Reproduktionstest mit *P. antipodarum* empfohlen werden. Dagegen sind DMSO, TEG und Eisessig als Lösemittel für die Testung mit *P. antipodarum* geeignet.

Im Verlauf eines Jahres wurden insgesamt sechs Versuche mit OP in einem nominalen Konzentrationsbereich zwischen 1 und 100 µg/L durchgeführt. Ziel war es, für künftige Validierungsstudien eine chemisch stabilere Substanz als BPA zu finden, welche ebenfalls einen reproduktionssteigernden Effekt auf die Schnecken aufweist. Weiterhin sollte ein möglicher saisonaler Einfluss auf die Reproduktion untersucht werden und der Frage nachgegangen werden, ob ein möglicher saisonaler Einfluss die Effekte endokriner Disruptoren maskieren kann. Die ersten drei Tests wurden zwischen November 2011 und April 2012 durchgeführt. In allen drei Versuchen führte die Exposition von *P. antipodarum* gegenüber OP zu einem signifikanten Anstieg der Embryonenzahl bei Konzentrationen von 3 und 10 µg/L. Bei höheren Konzentrationen lag die Reproduktionsleistung der Schnecken auf dem Niveau der Kontrolle. Dagegen konnte im Februar 2012 eine signifikante Steigerung der Embryonenzahl ab 3 µg/L in allen Konzentrationen beobachtet werden. Die folgenden drei Reproduktionstests wurden von Juni bis Oktober 2012 durchgeführt. In diesen Versuchen stieg die Embryonenzahl im Vergleich zur

Kontrolle in den OP-exponierten Gruppen nicht signifikant an. Eine mögliche Erklärung für die unterschiedlichen Ergebnisse der beiden Testreihen sind die hohen Embryonenzahlen der Kontrollgruppen der letzten drei Versuche. Während der Tests stieg die Embryonenzahl der Schnecken in den Kontrollen signifikant gegenüber dem Teststart an. Dies ist vermutlich auf unterschiedliche Futterdosierungen in der Zucht und während des Reproduktionstests zurückzuführen.

Um den Einfluss der Futterdosis auf die Reproduktionsleistung von *P. antipodarum* zu untersuchen und die Testbedingungen weiter zu optimieren, wurden Versuche mit drei unterschiedlichen Futterdosierungen durchgeführt. Dazu wurden Cd als reproduktionstoxische Substanz in einem nominalen Konzentrationsbereich von 1,56 bis 25 µg/L sowie Triclocarban (TCC) als potentiell reproduktionsfördernde Chemikalie in einem nominalen Konzentrationsbereich von 0,10 bis 10 µg/L in vierwöchigen Reproduktionstests mit *P. antipodarum* eingesetzt, bei denen ein Viertel (62,5 µg/Schnecke x Tag), die volle (250 µg/Schnecke x Tag) sowie die doppelte der im DRP (OECD 2010) empfohlenen Futtermenge (500 µg/Schnecke x Tag) TetraPhyll® gefüttert wurden. Am Ende der Tests wurden die Protein-, Glykogen- und Lipidgehalte der Schnecken ermittelt, um den Einfluss der Futterdosis und der Schadstoffexposition auf den Energiestoffwechsel zu untersuchen. Die Ergebnisse zeigten, dass die Reproduktionsleistung der Kontrolltiere beider Reproduktionstests (mit Cd und TCC) nach 28 Tagen im Vergleich zum Teststart (T_0)signifikant zunahm. Dieser Anstieg ist möglicherweise auf die unterschiedliche Fütterung unter Zucht- und Testbedingungen zurückzuführen, wobei unterschiedliche Besatzdichten der Schnecken und Wasserwechsel-Intervalle ebenfalls eine Rolle spielen können. Bei einer Futterdosis von 62,5 µg/Schnecke x Tag war nach 28 Tagen der Anstieg der Embryonenzahl bei den Kontrolltieren jedoch am geringsten. Weiterhin lag der Energiegehalt der Kontrolltiere bei allen drei Futterdosierungen auf dem gleichen Niveau, sodass die Tiere auch bei der niedrigsten Futterdosis nicht hungerten.

In den Reproduktionstests mit TCC konnten reproduktionssteigernde Effekte lediglich bei der niedrigsten und der mittleren Futterdosis (250 µg/Schnecke x Tag) beobachtet werden. Diese Effekte blieben bei der höchsten Futterdosis (500 µg/Schnecke x Tag) aus. Die Ergebnisse zeigen, dass eine hohe Futterdosis die Effekte reproduktionsfördernder Substanzen maskieren kann. Während in den TCC-exponierten Gruppen bei keiner der drei Futterdosierungen der Protein- oder Glykogengehalt signifikant gegenüber der Kontrolle verändert war, nahm der Lipidgehalt der Schnecken bei allen Futterdosierungen signifikant und in direkter Abhängigkeit von der TCC-Konzentration zu (LOEC: 0,02 - 0,052 µg/L). Diese Zunahme der Lipidgehalte war unabhängig von der gesteigerten Reproduktion der Tiere, da in der höchsten getesteten TCC-Konzentration ein reproduktionstoxischer Effekt und gleichzeitig die höchsten Lipidgehalte auftraten. Die signifikant erhöhten Lipidgehalte von *P. antipodarum* deuten darauf hin, dass es sich bei TCC um ein Obesogen handeln könnte.

Die Reproduktionstests mit Cd ergaben, dass die Embryonenzahl der gegenüber Cd exponierten Schnecken mit steigernder Konzentration abnahmen. Die NOEC-Werte lagen zwischen 1,05 µg/L (500 µg/Schnecke x Tag) und 4,65 µg/L (62,5 und 250 µg/Schnecke x Tag). Die berechneten Effektkonzentrationen liegen für die drei getesteten Futterdosierungen in einem ähn-

lichen Bereich. Die Analyse der Energiereserven zeigte, dass die Fitness der Schnecken durch eine Exposition gegenüber Cd negativ beeinträchtigt wird, da es zu einem Abfall der Protein-, Glykogen- und Lipidgehalte mit steigernder Cd-Konzentration kam. Dieser Effekt könnte auf die Mobilisierung von Energiereserven zur Detoxifizierung zurückzuführen sein.

Die Experimente zur Futterdosis ergaben, dass die Zwergdeckelschnecken, die ein Viertel der im DRP (OECD 2010) empfohlenen Menge an TetraPhyll® erhielten, die geringste Reproduktionssteigerung im Vergleich zur Zucht aufwiesen und sowohl reproduktionstoxische als auch reproduktionssteigernde Effekte abbilden konnten, ohne dass ihre Energiereserven beeinträchtigt wurden. Im Hinblick auf diese Ergebnisse ist eine Futtermenge von 62,5 µg/Schnecke x Tag die geeignetste Dosis für den Reproduktionstest mit *P. antipodarum* und daher im die Prüfrichtlinienentwurf vorzusehen.

Validierungsstudien

Um das vorgeschlagene Testdesign zu validieren und mögliche Probleme bei der Durchführung des Reproduktionstests mit *P. antipodarum* zu identifizieren, wurden in diesem Projekt drei internationale Ringtests mit insgesamt 16 Partnern aus dem Bereich Universität, Behörde und Industrie aus neun verschiedenen Ländern durchgeführt.

Im ersten Validierungsringtest wurde Cd in fünf Nominalkonzentrationen (1,56; 3,13; 6,25; 12,5; 25 µg/L) inklusive Negativkontrolle, ohne die Verwendung eines Lösemittels, getestet. Neben Cd wurde auch Tributylzinn (TBT) in einem nominalen Konzentrationsbereich von 10 bis 1000 ng Sn/L untersucht. Da TBT in Eisessig gelöst wurde, wurde eine zusätzliche Lösemittelkontrolle mit Eisessig (10 µL/L) getestet. In der Validierung 2 wurden Prochloraz und Trenbolon als Testsubstanzen ausgewählt. Prochloraz wurde in einem nominalen Konzentrationsbereich von 3,2 bis 320 µg/L und Trenbolon in Nominalkonzentrationen zwischen 10 und 1000 ng/L getestet. Hierbei wurde DMSO (10 µL/L) als Lösemittel verwendet. In einem dritten Validierungsringtest wurden TCC und Triclosan (TCS) in fünf Nominalkonzentrationen (0,1; 0,3; 1,00; 3,00; 10,0 µg/L), unter Verwendung von DMSO (10 µL/L) als Lösemittel, getestet. Um den Transport der Schnecken als Stressfaktor auszuschließen, wurden diese für mindestens 13 Tage im jeweiligen Partnerlabor akklimatisiert, bevor der Reproduktionstest gestartet wurde. Die Expositionskonzentrationen der Substanzen wurden analytisch bestimmt. Alle berechneten Effektkonzentrationen der Validierungsstudien beziehen sich auf die zeitlich gewichteten mittleren (TWM) Konzentrationen, da diese meist mehr als 20% von den Nominalkonzentrationen abwichen.

Die Reproduktionstests mit Cd lieferten vergleichbare Ergebnisse. Alle Labore konnten einen konzentrationsabhängigen Rückgang der Embryonenzahlen beobachten. Die Validierungsstudie lieferte ähnliche Ergebnisse wie der Ringtest mit Cd im Vorgängerprojekt (SIERATOWICZ UND OEHLMANN 2011). Die mittleren Effektkonzentrationen (mit Variationskoeffizient) für die EC_{10}, EC_{50}, NOEC und LOEC aus beiden Studien betrugen 6,53 µg/L (35,5%), 14,2 µg/L (21,8%), 6,45 µg/L (50,5%) und 12,6 µg/L (42,2%). Die Differenz zwischen der niedrigsten und höchsten Effektkonzentration machte den Faktor 1,7 (EC_{50}-Werte) bis 3,9 (LOEC-Werte) aus. Die Ergebnisse der Reproduktionstests mit TBT in der Validierung 1 belegen ebenfalls die gute Re-

produzierbarkeit. Die in den Laboren jeweils ermittelten niedrigsten und höchsten Effektkonzentrationen differierten zwischen dem Faktor 4,8 (LOEC-Werte) und 13,5 (EC_{10}-Werte). Die arithmetischen Mittelwerte (mit Variationskoeffizient) für EC_{10}, EC_{50}, NOEC und LOEC aller teilnehmenden Partner betrugen 35,6 ng Sn/L (76,9%), 127 ng Sn/L (39,3%), 39,2 ng Sn/L (68,3%) und 75,7 ng Sn/L (77,0%). Für die Berechnung der TWM-Konzentrationen konnten wegen der hohen Kosten der TBT-Analytik lediglich zwei Medienwechsel berücksichtigt werden.

In der zweiten Validierungsstudie (Validierung 2) konnten alle teilnehmenden Labore einen Rückgang der Reproduktion von *P. antipodarum* mit steigender Prochloraz-Konzentration beobachten. Die NOEC-Werte variierten hierbei zwischen 21,3 µg/L und 40,4 µg/L und die LOEC-Werte zwischen 31,4 µg/L und 194 µg/L. Die gute Reproduzierbarkeit der Ergebnisse zeigte sich ebenfalls in den EC_{10}- und EC_{50}-Werten (basierend auf gemessenen Konzentrationen). Die arithmetischen Mittelwerte (mit Variationskoeffizient) für EC_{10} und EC_{50} betrugen 24,1 µg/L (61,3%) und 336 µg/L (75,7%). Keines der teilnehmenden Labore konnte einen konzentrationsabhängigen Rückgang der Embryonenzahlen von *P. antipodarum* bei Exposition gegenüber Trenbolon beobachten.

An dem dritten Ringtest (Validierung 3), bei dem TCC und Triclosan (TCS) als Testchemikalien ausgewählt wurden, nahmen vier Partnerlabore teil. In einem der teilnehmenden Labore waren die Tests wegen einer erhöhten Mortalität in der Lösemittelkontrolle nicht valide. Zwei der drei verbleibenden Labore beobachteten eine konzentrationsabhängige Abnahme der Embryonenzahl von *P. antipodarum* nach 28-tägiger Exposition gegenüber TCC (NOEC: 0,121 und 0,681 µg/L; LOEC: 0,340 und 1,52 µg/L), während das dritte Labor keine Effekte auf die Reproduktion in dem gemessenen Konzentrationsbereich (0,04 - 1,64 µg/L) feststellen konnte. Bei den Reproduktionstests mit TCS ermittelte einer der Partner eine signifikante Reduktion der Embryonenzahl bei der höchsten getesteten Konzentration (NOEC: 0,480 µg/L; LOEC: 0,964 µg/L), während die anderen beiden Labore keinen Effekt von TCS auf die Embryonenzahl nachweisen konnten. In dieser Validierungsstudie wurde die Futtermenge von 250 µg/Schnecke x Tag (Validierung 1 und 2) auf 62,5 µg/Schnecke x Tag reduziert, um das Risiko eines Pilzwachstums durch verbleibende Futterreste auf dem Gefäßboden und der dadurch bedingten erhöhten Mortalität der Versuchstiere zu minimieren. Die Embryonenzahlen der Schnecken in der Kontrollgruppe waren auf demselben Niveau wie bei den Kontrolltieren in den Validierungen 1 und 2, und unterschieden sich nicht signifikant voneinander.

Insgesamt wurden in den Validierungsstudien (einschließlich des Ringtests mit der ersten Version des Testdesigns aus ValMolRepro I, FKZ 3708 61 402) 43 Reproduktionstests durchgeführt. Ein Labor musste aufgrund sehr niedriger gemessener TBT-Konzentrationen einen Versuch wiederholen. Zusätzlich konnten fünf Partner die Validitätskriterien nicht erfüllen, weil in einem Labor technische Probleme die Einhaltung der vorgegebenen Temperatur zwischen 15°C und 17°C verhinderten und die anderen vier bei einzelnen biologischen Kriterien abwichen (maximale Kontrollmortalität; minimale Embryonenzahl bei den Kontrollschnecken, die aus einer anderen Zucht stammten). Für alle Testsubstanzen konnten in den Partnerlaboren vergleichbare Ergebnisse erzielt und somit die Reproduzierbarkeit des Tests nachgewiesen wer-

den. Die meisten Labore konnten vergleichbare NOEC-, LOEC-, EC_{10}- oder EC_{50}-Werte mit überlappenden 95%-Konfidenzintervallen ermitteln. Das Labor A konnte zudem die Reproduzierbarkeit des Tests innerhalb eines Labors für die Testsubstanzen Cd und TBT erfolgreich nachweisen.

Insgesamt konnte gezeigt werden, dass sich der Reproduktionstest mit *P. antipodarum* gut für die Chemikalientestung eignet. Der Test ist sehr gut handhabbar, und die Ergebnisse sind reproduzierbar. Die „Validation Management Group on Ecotoxicity Testing (VMGeco)" der OECD wertete bei ihrer 10. Sitzung (10. - 12. Dezember 2014) die Validierungsstudien für den Reproduktionstest als erfolgreich und forderte zur Einreichung eines Prüfrichtlinien-Entwurfs für den Reproduktionstest mit *P. antipodarum* auf. Dieser wird 2015 bei der OECD für die offiziellen Kommentierungsabläufe eingereicht und kann nach Bestätigung durch die Mitgliedsstaaten 2016 als OECD-Prüfrichtlinie in Kraft treten. Die Ergebnisse des Vorhabens tragen somit zur Weiterentwicklung von Testmethoden und Bewertungskonzepten für die Regulierung reproduktionsbeeinflussender Chemikalien in REACH sowie von Pflanzenschutzmitteln, Arzneimitteln und Bioziden und zur Beurteilung langfristiger, subletaler Substanzeffekte auf Populationen und Ökosysteme bei. Unter den aquatischen Invertebraten handelt sich hierbei um den ersten Nicht-Arthropoden-Test, der im Rahmen des „Conceptual Framework for Endocrine Disrupters" als Level 4 Assay (OECD 2012a) erfolgreich validiert wurde. Damit werden die Mollusken als sensitive und ökologisch wichtige Invertebraten-Gruppe im verfügbaren Instrumentarium der OECD-Prüfrichtlinien berücksichtigt.

Vollständiger Lebenszyklus-Test

Mit dem 28-tägigen Reproduktionstest können ausschließlich Substanzeffekte auf das adulte Stadium von *P. antipodarum* untersucht werden. Die Effekte von Chemikalien auf frühere Lebensstadien werden dabei nicht betrachtet, obwohl diese möglicherweise sensitiver sind.

Um eine potentielle Erweiterung des Reproduktionstests als Partial-Life-Cycle (PLC) Test zu einem Full-Life-Cycle (FLC) Testdesign zu untersuchen, wurde ein Sensitivitätsvergleich von juvenilen und adulten *P. antipodarum* durchgeführt. Hierfür wurden adulte und frisch entlassene Neonaten in einem 28-tägigen Versuch gegenüber Cd und TBT exponiert. Als Endpunkte dienten Wachstum (im FLC), Reproduktion (im PLC) und Mortalität (im FLC und PLC). Die Versuche zeigten, dass Cd und TBT einen signifikanten Einfluss auf Mortalität, Reproduktion und Wachstum der Schnecken hatten.

Im Reproduktionstest mit Cd und adulten Schnecken lag die Mortalität in der Kontrolle bei 1,25%. Die LC_{10} betrug 14,1 µg/L und die LC_{50} 38,2 µg/L. Die mittlere Embryonenzahl der Kontrollgruppe war 11,5 und bei Cd-Konzentrationen von 12,5 µg/L, 25 µg/L und 50 µg/L war die Reproduktion der Schnecken signifikant reduziert. Der EC_{10}- und EC_{50}-Wert für den Endpunkt Reproduktion betrug 9,73 µg/L bzw. 11,3 µg/L. Die Kontrollmortalität in dem Versuch mit juvenilen *P. antipodarum* lag bei 25%. Ein signifikanter Anstieg der Mortalität wurde bei 25 µg/L und 50 µg/L ermittelt. Hierbei überlebte keiner der eingesetzten Neonaten. Es wurde eine LC_{10} von 12,1 µg/L und eine LC_{50} von 15,0 µg/L ermittelt. Nach 28-tägiger Cd-Exposition zeigte sich

bei Konzentrationen von 6,25 µg/L und 12,5 µg/L ein signifikant reduziertes Wachstum. Für diesen Endpunkt wurden EC-Werte von 1,16 µg/L (EC_{10}) und 3,82 µg/L (EC_{50}) ermittelt.

Im Reproduktionstest mit TBT und adulten Schnecken überlebten 2,5% der eingesetzten Kontrolltiere nicht. Eine signifikant erhöhte Mortalität trat bei der höchsten Testkonzentration (838 ng Sn/L) auf, bei der 87,5% der Schnecken starben. Die berechnete LC_{10} und LC_{50} lagen bei 278 ng Sn/L und 499 ng Sn/L. Die durchschnittliche Embryonenzahl in Negativ- und Lösemittelkontrolle betrug 18,9 bzw. 19,3. Hierbei lag kein signifikanter Unterschied vor. Ab einer TBT-Konzentration von 27,8 ng Sn/L (= LOEC) konnte ein signifikanter Rückgang der Embryonenzahl beobachtet werden. Die EC_{10} und EC_{50} betrugen 12,7 ng Sn/L bzw. 125 ng Sn/L. Bei dem TBT-Versuch mit juvenilen *P. antipodarum* lag die Mortalität in der Kontrolle bei 30% und stieg mit steigender TBT-Konzentration an. Bei der höchsten getesteten Konzentration verstarben 85% der eingesetzten Neonaten. Die LC_{10} war 11,4 ng Sn/L, und die LC_{50} war 194 ng Sn/L. Das Schalenwachstum der juvenilen Schnecken wurde ebenfalls durch TBT negativ beeinträchtigt. Die spezifische Wachstumsrate (SGR) sank mit steigender TBT-Konzentration. Der daraus resultierende EC_{10}- und EC_{50}-Wert betrug 5,88 ng Sn/L bzw. 175 ng Sn/L. Die Ergebnisse zeigen, dass juvenile *P. antipodarum* nur geringfügig sensitiver auf eine Exposition gegenüber Cd oder TBT reagieren als Adulttiere, wobei die EC-Werte sich mit ihren 95%-Konfidenzintervallen überlappen und die Unterschiede daher nicht signifikant sind. Für eine endgültige Bewertung der Sensitivität juveniler Schnecken müssten mehr Tests mit anderen Chemikalien durchgeführt werden.

Im Rahmen des Projektes ValMolRepro II wurden zwei mögliche Testdesigns für einen FLC-Test mit *P. antipodarum* entwickelt und diese getestet.

Das Testdesign des FLC-Tests mit *P. antipodarum* gliedert sich in drei Phasen. In der Phase 1 wird zunächst die F0-Generation analog zum PLC-Test exponiert, indem jeweils 200 Adulttiere in 5 L-Glassaquarien eingebracht werden, die mit vier Litern DRP-Medium befüllt sind. Es sollten vier Replikate pro Expositionsgruppe verwendet werden. Nach 28 Tagen werden die adulten Schnecken aus dem Testsystem entfernt und die Jungtiere, die während der vierwöchigen Exposition entlassen wurden, wachsen in der Phase 2 des FLC-Tests bis zur Geschlechtsreife heran. Das Erreichen der Geschlechtsreife soll anhand einer Stichprobe von zehn Tieren in regelmäßigen Abständen untersucht werden. Pro Replikat sollten wenigstens zehn Tiere der F1-Generation überlebt haben, um in der abschließenden Phase 3 deren Reproduktion erfassen zu können.

Im Oktober 2011 wurde ein erster FLC-Test mit diesem Testdesign durchgeführt. Um das vorgeschlagene Testdesign zu evaluieren, wurde der Versuch lediglich mit einer Kontrollgruppe, bestehend aus vier Replikaten, durchgeführt. Nach 28 Tagen wurden die Adulttiere entnommen und nach weiteren 28 Tagen (Tag 56 des gesamten Experiments) wurde die Anzahl an überlebenden juvenilen Schnecken erfasst. Die Juvenilenzahl lag zwischen 26 und 122 und schwankte somit zwischen den vier Replikaten beträchtlich. Aufgrund der niedrigen Anzahl an überlebenden Jungtieren wurde der FLC-Test vorzeitig beendet. Eine inadäquate Futterquelle und die dadurch bedingte hohe Mortalität der Tiere ist eine mögliche Erklärung für die geringe Anzahl an juvenilen Schnecken.

Um dieser Hypothese nachzugehen, wurde mit frisch entlassenen Neonaten ein Futterversuch durchgeführt. Der Test verlief über 56 Tage. Als Endpunkt diente die Mortalität der Schnecken. Es wurden drei verschiedene Futterqualitäten in drei Replikaten mit jeweils 15 Tieren getestet. Neben fein gemahlenem TetraPhyll® und fein gemahlenen Sera® Spirulina-Tabs wurde die Grünalge *Scenedesmus acutus* var. *acutus* als Futterquelle *ad libitum* angeboten. Nach 56 Tagen betrug die Mortalität bei Fütterung mit der Grünalge lediglich 11,1%. Im Gegensatz dazu war die Mortalität in den anderen beiden Expositionsgruppen deutlich höher. Bei Fütterung mit Sera® Spirulina starben 93,3% der eingesetzten juvenilen Schnecken, in der TetraPhyll®-Gruppe 88,9%. Als Schlussfolgerung für das erste Testdesign lässt sich festhalten, dass Glasaquarien für einen FLC-Test mit *P. antipodarum* nicht geeignet sind. Da die frisch entlassenen Neonaten eine Größe von lediglich 450 bis 500 µm aufweisen, sind kleine Testgefäße sinnvoller, um die Juvenilen unter einem Stereomikroskop zu beobachten. Weiterhin zeigte der Futterversuch, dass juvenile und adulte *P. antipodarum* unterschiedliche Futterpräferenzen aufweisen. Daher sollte in einem FLC-Test mit Grünalgen gefüttert werden, um das Überleben und das Wachstum der Neonaten zu sichern. Außerdem scheint das erste Testdesign weniger geeignet, da in der Phase 1 viele Adulttiere benötigt werden. Zur Optimierung des Testdesigns sollte der FLC-Test mit frisch entlassenen Neonanten beginnen.

Im zweiten Testdesign beginnt der Versuch mit frisch aus der Bruttasche entlassenen Juvenilen von *P. antipodarum*. Zunächst werden vor Versuchsbeginn Adulttiere in Gefäße mit frischem DRP-Medium eingesetzt. Eine Woche später beginnt der Versuch. Es werden die entlassenen Juvenilen abgesammelt und in Kristallisierschalen (vier Replikate pro Expositionsgruppe) mit einer Dichte von 1000 Neonaten/L eingebracht. In dieser Phase werden die Jungtiere mit *S. acutus* var. *acutus ad libitum* gefüttert. Haben die Schnecken eine mittlere Schalenlänge von 1,5 mm erreicht, werden sie in 500 mL-Bechergläser (gefüllt mit 400 mL DRP-Medium) transferiert. Die Besatzdichte wird auf 75 Juvenilen/L angepasst. Zusätzlich zur Fütterung mit Grünalgen werden die Schnecken mit TetraPhyll® (0,1 mg/Schnecke x Tag) gefüttert. Der Versuch endet, wenn die Schnecken in der Kontrollgruppe eine mittlere Schalenlänge von 3,5 mm erreicht haben. Endpunkte sind hierbei Mortalität, Wachstum und die Embryonenzahl der Schnecken am Ende des Versuchs. Im Jahr 2014 wurde ein FLC-Test mit diesem Testdesign durchgeführt. Als Testsubstanz diente Cd bei drei Nominalkonzentrationen (2, 4 und 8 µg/L). Die Dauer des FLC-Tests betrug 159 Tage.

In den ersten beiden Wochen des Versuchs starben in allen Expositionsgruppen, einschließlich der Kontrolle, mehr als 50% der Jungtiere. Bis zum Versuchstag 70 überlebten in der Kontrolle lediglich 27,3%. Die Mortalität nahm mit der Cd-Konzentration zu, so dass bei der höchsten Konzentration (8 µg/L) mit nur 6,41% signifikant weniger Tiere als in der Kontrolle überlebten. Am Tag 70 wurde die Dichte der Schnecken auf 75 Neonanten/L bzw. auf 25 Neonaten/L in der 8 µg/L-Gruppe angepasst. Nach dieser Dichteanpassung nahm ab dem Versuchstag 98 bei einer Konzentration von 2 µg/L die Mortalität signifikant zu. Als mögliche Ursache konnte ein Pilzwachstum in den Testgefäßen identifiziert werden.

Zum Beginn des Tests lag die mittlere Schalenlänge bei 0,5 mm. Nach 159 Tagen betrug die mittlere Schalenlänge in der Kontrolle 3,8 mm. Die Schnecken, die gegenüber 8 µg Cd/L expo-

niert wurden, waren mit 3,3 mm im Vergleich zur Kontrolle kleiner, diese Differenz war jedoch nicht signifikant. Es traten keine signifikanten Effekte von Cd auf das Wachstum der Schnecken im FLC-Test auf. Am Ende des Tests wurde die Embryonenzahl der Schnecken bestimmt. Die mittlere Embryonenzahl der Kontrollgruppe lag bei 18,2 und nahm mit steigender Cd-Konzentration ab. Bei einer Konzentration von 2 µg/L betrug die mittlere Embryonenzahl 17,5, bei 4 µg/L und 8 µg/L nur noch 8,15 bzw. 6,38.

Die Experimente zum FLC-Test zeigten, dass bei der Arbeit mit juvenilen *P. antipodarum* besondere Sorgfalt notwendig ist. Aufgrund der langen Testdauer und der hohen Mortalität in den ersten Testwochen ist es eine Herausforderung, einen FLC-Test erfolgreich abzuschließen. Die Neonanten im FLC-Test zeigten sich im Vergleich zu Adulttieren im Reproduktionstest leicht sensitiver gegenüber einer Exposition mit Cd. Das zweite entwickelte Testdesign ist weiter zu optimieren. Außerdem müssten juvenile *P. antipodarum* gegenüber weiteren Chemikalien exponiert werden, um die Frage zu beantworten, inwieweit juvenile Schnecken im Vergleich zu adulten sensitiver auf Schadstoffe reagieren. Nur wenn eine signifikant höhere Sensitivität der Neonaten für eine Reihe von Testsubstanzen gezeigt werden kann, erscheint der zusätzliche Aufwand eines FLC-Tests mit *P. antipodarum* gegenüber dem Reproduktionstest gerechtfertigt. Ein SOP-Entwurf für den FLC-Tests mit *P. antipodarum* ist als Anhang 8.2 beigefügt.

Summary

The aim of the project "Validation of a mollusc reproduction test – Establishment of an OECD test guideline for the assessment of endocrine and reproduction toxic effects in snails" (ValMolRepro II) was the development of an OECD draft test guideline for the reproduction test with *Potamopyrgus antipodarum* including the required validation and optimisation. Additionally, a full life cycle test with *P. antipodarum* should be developed and a standard operating procedure (SOP) should be compiled. Furthermore, to contribute to the validation studies of the reproduction test with *Lymnaea stagnalis* coordinated by France, experimental assistance should be performed.

In the predecessor project (project code 3708 61 402, ValMolRepro I) a *Detailed Review Paper* (DRP) *on Molluscs Life-cycle Toxicity Testing* (OECD 2010) was compiled and is available at the OECD webpage. Here, three appropriate molluscan species are proposed and adequate test designs were envisaged. One of the proposed test species is the New Zealand mud snail *P. antipodarum*, which has been proven to be sensitive to endocrine disrupting chemicals (e.g. organotins) and other substances (e.g. copper). In ValMolRepro I a test design of the reproduction test with *P. antipodarum* over 28 days, including a SOP, was developed. In this test the number of embryos in the brood pouch, reflecting the individual reproduction effort in snails, and adult mortality serve as main endpoints. To analyse the robustness of the test and to validate the test conditions a first ring test was conducted with four participating laboratories. Bisphenol A (BPA) and cadmium (Cd) were chosen as test chemical. All four laboratories found a concentration-dependent decrease of embryo numbers under Cd exposure. The expected BPA-dependent increase of reproduction was only affirmed by three out of four laboratories. Analytical data indicated a faster BPA degradation with increasing test duration. A possible cause was the increasing biofilm development in test beakers. The outcome of this project for future validation work with *P. antipodarum* was that test beakers should be changed during the test to reduce biofilm development. Also a more stable reproduction enhancing compound like 4-tert-octylphenol (OP) should be used for validation. Furthermore, the number of total embryos should serve as a sole endpoint for reproduction and the discrimination of shelled and unshelled embryos should be omitted. Additionally, the temperature dependency of reproduction and hence of substances-related effects on reproduction should be further investigated. Also, an optimal reproduction range for the number of embryos in the brood pouch of control snails, to facilitate the assessment of both reproductive toxicants and substances enhancing reproduction, should be defined and set as a validity criterion. As an extension of the reproduction test with *P. antipodarum*, a full life cycle test should be developed comparable to proposed protocol for *L. stagnalis* in OECD (2010). To optimise culture conditions, an alternative culture media and temperatures had to be analysed to increase the reproductive output of the snails.

In the first project part an extensive literature review has been performed to compare the sensitivity of *P. antipodarum* and *L. stagnalis* towards chemical exposure. The evaluation of literature refers to selected documents and intends to provide a critical synthesis of previous research. The sensitivity comparison across species should help to provide a background to further OECD validation studies with both gastropod species. The literature study was conducted in cooperation with the French National Institute for Agri-cultural Research (INRA). The generation of this report bases on combining

available data of effect concentrations from "peer-reviewed literature" and "grey literature" of unpublished dissertations and in-house studies. Evaluations focus exclusively on primary literature (original research results) dealing with chronic effects on reproduction, growth, mobility and mortality. The synopsis of data reveals that only nine substances (BPA, Cd, copper, 17α-ethinylestradiol, irgarol, 4-tert-nonylphenol, tributyltin, vinclozolin, and zinc) have been tested in both species and provide data for a comparison of effect concentrations. The LOEC values for both species were used for a correlation analysis. This correlation demonstrates that *P. antipodarum* tends to respond slightly more sensitive towards exposure of identical substances compared with *L. stagnalis*. However, a sound analysis would require more data to allow for a final appraisal.

Optimisation of culturing conditions

A long-term maintenance of a stable laboratory culture of *P. antipodarum* is an important prerequisite for the development of an OECD standard test system. To optimise the culture conditions of the snails, three different test media were investigated. Snails were kept for 84 days in either ISO medium, Elendt M4 medium (OECD 2004) or in DRP medium (OECD 2010), which has routinely been used for the culturing of snails at Goethe University. The results indicate that the Elendt M4 medium was more labour-intensive to prepare, exhibited a lower durability and test vessels filled with Elendt M4 showed a more intensive biofilm development. Consequently, this medium is less suitable for culturing and testing of *P. antipodarum* compared to DRP and ISO medium. Additionally, a second test was performed to assess the influence of DRP and ISO medium on the development and growth of juvenile snails. Therefore adult snails were maintained in either DRP or ISO medium and released and survived juvenile snails were counted. It turned out that after four weeks exposure the mean number of released juvenile snails in both media was 60. Here, 77.6% and 63.4% survived in DRP and ISO medium, respectively. Also, if adult snails are longer cultured in ISO medium, the embryo numbers in the brood pouch of the snails were declining. The experiments show that ISO medium is less suitable for culturing and testing of *P. antipodarum*. A possible reason could be its rather simple composition with only four salts solved in deionised water. In contrast to this, DRP medium contains Tropic Marin® sea salt, which provides over 70 trace elements according to the manufacture's specification. This circumstance may prevent deficiency symptoms in adult and juvenile snails.

Studies on the influence of temperature and photoperiod on the reproduction of *P. antipodarum* were conducted during the course of the year to optimise culturing conditions. Besides to the standard conditions with 16°C and a light:dark regime of 16:8 h described in the DRP (OECD 2010), snails were acclimatized to a temperature of 20°C with a light:dark regime of 16:8 h. Furthermore, snails were acclimatized to an equinoctial photoperiod (12:12 h) and to a short-day exposure with a light:dark regime of 8:16 h, both at 16°C to investigate the influence of the photoperiod on reproduction. The mean embryo numbers of *P. antipodarum* cultured under standard conditions (16°C, 16:8 h light:dark) were monthly recorded over more than 3 years. The average embryo numbers of the laboratory culture ranges between a minimum of 5.48 in April 2014 and a maximum of 11.7 in December 2014. A seasonal variation in reproduction of the snails was not observed if compared to the embryo numbers of field collected snails over the course of a year. The reproductive output over the course of the year of snails maintained at 20°C and a photoperiod of 16:8 h (light:dark) was in a similar range like in snails cultured un-

der standard conditions. Besides that, a higher mortality of juvenile snails was observed at 20°C compared to the culture of 16°C. Therefore, a permanent breeding of *P. antipodarum* is not feasible at a temperature of 20°C. The culturing of *P. antipodarum* under equinoctial conditions seems to be inappropriate, as embryo numbers in some cases were below 5 as one of the validity criteria of the reproduction test, indicating a poor health status of the snails. Also the culturing of *P. antipodarum* under short-day conditions (8:16 h light:dark) seems to be not suitable, as mortality of snails was high and within replicates the variability increases with exposure duration. Therefore, the results support the final choice of the culturing conditions at 16°C and a photoperiod of 16:8 h light:dark.

Optimisation of test conditions

Another project part dealt with the optimisation of test conditions. As it is known that animal density may impact reproduction, embryo numbers were assessed for different snail densities in a series of experiments with *P. antipodarum*. In a first phase of the experiment groups of 5, 10, 20, 40, 80 and 160 snails per 200 mL DRP medium were maintained over four weeks and the number of embryos was counted in 10 snails per replicate on day 28. In the second phase of the experiment the previous density stress was replaced by Cd exposure within a 28 days reproduction test. The results of phase 1 showed that the reproductive output of *P. antipodarum* was higher at higher densities compared to low densities. These positive effects are probably caused by a waterborne substance produced by *P. antipodarum*. The highest embryo numbers were found in the density group with 20 snails per 200 mL DRP medium. The reproduction test with Cd in phase 2 showed that snail densities modulated the sensitivity for Cd, with higher densities in the pre-exposure phase 1 resulting in a higher sensitivity for Cd in phase 2. These findings demonstrate that the parameter density is able to alter the reproduction of *P. antipodarum* and that a prior high density of snails could enhance the sensitivity to a toxicant. To avoid density stress during culturing a maximum density of 100 snails/L should not be exceeded.

Additionally, a reproduction test with three different sizes of test vessels (containing 200, 400 or 800 mL DRP medium) was conducted to investigate the influence of different volumes and densities on the reproduction of *P. antipodarum*. The use of smaller test vessels would reduce the consumption of medium, chemicals and space for testing. After 28 days the embryo numbers of the snails were assessed. The mean embryo numbers did not differ between treatments. However, smaller test vessels may adsorb a higher share of the test substance due to a bigger surface-volume relationship. So the choice of the right test vessels depends on the physico-chemical properties of the substance. As a modification for the test conduct the volume of the test media may range between 200 mL and 800 mL depending on the physico-chemical properties of the test compounds.

The influence of solvents on reproduction of *P. antipodarum* was assessed in a series of 28-days reproduction test. The OECD-recommended solvents acetone, methanol, ethanol, dimethyl sulfoxide (DMSO) and triethylene glycol (TEG) were tested at concentrations ranging from 0.02 to 12.5 mL/L. Additionally, glacial acetic acid was tested at a concentration range from 0.01 to 2.43 mL/L. None of the tested solvents affected mortality significantly, except for glacial acetic

acid. Here, all exposed snails died at the three highest test concentrations (0.27, 0.81, 2.43 mL/L) due to a strong decrease of the pH in these exposure groups. The embryo numbers in snails exposed to lower concentrations of glacial acetic acid were on the level of control snails. Acetone and methanol caused a significant reduction in embryo numbers already at a concentration of 0.1 mL/L. In contrast, ethanol caused a reproductive-increasing effect at 0.1 and 0.5 mL/L. DMSO caused a significant increase of the embryo numbers only at 2.5 mL/L compared to the control group. The exposure of *P. antipodarum* towards TEG had no effect on the reproductive output up to a concentration of 2.5 mL/L. Only at the highest test concentration of 12.5 mL/L a significant reduction of the embryos was observed. Because of their effects on reproduction of *P. antipodarum* at low concentrations, acetone, methanol and ethanol cannot be recommended as solvents in reproduction tests with this snail. However, DMSO, TEG and glacial acetic acid are well suited solvents for tests with *P. antipodarum*.

Due to the chemical instability of BPA, which has been tested in the first validation studies, reproduction tests with OP were performed to find an alternative, more stable reproduction increasing substance for further validation studies. During the course of a year six reproduction tests with OP at a nominal concentration range between 1 and 100 µg/L were performed. The aim of this study was to analyse if a possible seasonal reproductive pattern could mask reproduction increasing effects of endocrine disruptors in ecotoxicological tests. The first three tests were conducted between November 2011 and April 2012. In all three test series OP caused an increase in embryo numbers at 3 and 10 µg/L. In April 2012 peak embryo numbers occurred at 10 µg/L. At higher concentrations the reproductive output decreased to embryo numbers comparable to the control groups. In February 2012 a significant increased embryo number was detected at all concentrations above 3 µg/L. The remaining three reproduction tests were performed between June and October 2012. Here no significant increase of embryo numbers in OP-exposed groups could be detected compared to control. A likely reason for the different results compared to the first three tests was the high embryo numbers in control groups in the last three tests. It was observed that the embryo numbers in the last three tests resulted in control reproduction rates considerably higher than at the beginning of the test. This issue is probably caused by differences between the food amounts under culture and test conditions.

To find out if the amount of food influences the increase of embryo numbers in a reproduction test after 28 days and in order to optimise the test design of the reproduction test with *P. antipodarum*, reproduction tests with three different food doses were performed. Therefore, Cd was used as toxic substance at nominal concentrations ranging from 1.56 to 25.0 µg/L and triclocarban (TCC) was used as potentially reproduction stimulating chemical at nominal concentrations between 0.10 and 10.0 µg/L. Reproduction tests were carried out with *P. antipodarum* in which a quarter (62.5 µg/snail x day), the full dose (250 µg/snail and day) and twice the dose (500 µg/snail x day) of TetraPhyll® proposed in the DRP (OECD 2010) were tested. Furthermore, body protein, glycogen and lipid contents were determined to investigate the influences of food dosage and pollutant exposure on the energy reserves of the snails.
The reproduction in control groups of both reproduction tests (with Cd and TCC) compared to test start (T_0) showed a significant increase of the embryo numbers. This increase may be a result of differences in the supply of food between culturing and test conditions, but may also

reflect the influence of different snail densities and frequencies of medium renewal between snail culture and the reproduction test. Under the food dose of 62.5 µg/snail x day, the increase of embryo numbers in control snails was lowest in both tests over the course of the experiment. Additionally, the measured energy content in control snails under all three food doses was on the same level, showing that snails at the lowest food dose did not starve.
The reproduction test with TCC showed that reproduction increasing effects could be observed only for the lowest and the median food dose (250 µg/snail x day) but not for the highest food dose (500 µg/snail x day). These results demonstrate that a higher food dose is able to mask reproduction-increasing effects of substances. Interestingly, the results of the energy content showed no significant effect on the protein or the glycogen content. In contrast, a significant increase in the lipid content was determined with increasing TCC concentrations for all food doses (LOEC: 0.02 - 0.052 µg/L). This enhancement cannot be linked with the increased reproduction of the snails, as a toxic effect on the embryo numbers was observed at the highest test concentration of TCC. The result of significantly increased lipid content in TCC exposed snails is a first indication that TCC may represent an obesogen.
Furthermore, the reproduction tests with Cd showed that the embryo numbers in Cd exposed snails decreased with increasing Cd concentrations, independent from food dose. NOEC values ranged between 1.05 µg/L (500 µg/snail x day) and 4.65 µ/L (62.5 and 250 µg/snail x day). The calculated effect concentrations are in similar ranges for all tested food doses. The results of the energy content also show that the fitness of the snails is negatively affected by an exposure towards Cd with a declining trend of protein, glycogen and lipid in all feeding scenarios. This effect might be due to a mobilization of the energy reserves for detoxification. In conclusion, snails of the lowest food dose (62.5 µg/snail x day) showed the lowest reproductive-increase compared to the brood stock over the course of the 28-days reproduction tests. Additionally, reproduction stimulating as well as toxic effects could be mapped. Therefore, a food dose of 62.5 µg/snail x day turned out to be the most appropriate food dose for the snails and should be recommended in the draft test guideline for the reproduction test with *P. antipodarum*.

Validation studies

To validate the proposed test conditions and identify issues in performing the SOP of the reproduction test with *P. antipodarum*, 16 partners participated in three validation exercises for the reproduction tests coming from academia, government or industry in 10 countries.

In the first validation, Cd was tested at five nominal concentrations (1.56, 3.13, 6.25, 12.5, 25 µg/L) including a negative control and without the use of solvents. Tributyltin (TBT) was also tested at a nominal concentration range between 10 and 1000 ng Sn/L. Additionally a solvent control with glacial acetic acid (10 µL/L) was tested. In validation 2 prochloraz and trenbolone were chosen as test chemicals at a nominal concentration range from 3.2 to 320 µg/L and from 10 to 1000 ng/L, respectively. DMSO was used as a solvent (10 µL/L). In a third validation run both TCC and triclosan (TCS) were tested at five nominal concentrations (0.1, 0.3, 1.00, 3.00, 10 µg/L) and DMSO (10 µL/L) was used as a solvent. After shipping to the participating laboratories snails were acclimated for at least 13 days prior to the reproduction test to avoid stress

from shipping. Exposure concentrations in validation exercises were analytically verified. All effect concentrations provided in these validation studies refer to time weighted mean (TWM) concentrations, because TWM concentrations deviated by more than 20% from nominal concentrations.

The reproduction tests with Cd showed a good agreement among participating laboratories. All partners observed a concentration dependent decrease of the number of embryos. The results of the Cd tests are in accordance with the outcome of the former validation run with Cd (SIERATOWICZ AND OEHLMANN 2011). By calculation of the mean effect concentrations (with coefficient of variation) of both validation studies, the EC_{10}, EC_{50}, NOEC and LOEC from all laboratories are 6.53 µg/L (35.5%), 14.2 µg/L (21.8%), 6.45 µg/L (50.5%) and 12.6 µg/L (42.2%), respectively with a minimum of a 1.7-fold difference (EC_{50} values) and a maximum of a 3.9-fold difference (LOEC values). The results of the reproduction tests with TBT in validation II also showed a good accordance among partners. Effect concentrations exhibit a minimum of a 4.8-fold difference (LOECs) and a maximum of a 13.5-fold difference (EC_{10} values). The mean values (with coefficient of variation) for EC_{10}, EC_{50}, NOEC and LOEC values from these laboratories are 35.6 ng Sn/L (76.9%), 127 ng Sn/L (39.3%), 39.2 ng Sn/L (68.3%) and 75.7 ng Sn/L (77.0%), respectively. Some uncertainties concerning the measured concentrations might have influenced the results, because TWM concentrations have been calculated based on only two measuring intervals, due to the high costs of chemical analyses for TBT.

In the second validation all laboratories found a decrease of the embryo numbers with increasing concentrations of prochloraz. The NOEC varies between 21.3 µg/L and 40.4 µg/L. The LOEC is in the range between 31.4 µg/L and 194 µg/L. The good match of results is also reflected by the EC_{10} and EC_{50} values (based on measured concentrations). The mean values (with coefficient of variation) are 24.1 µg/L (61.3%) and 336 µg/L (75.7%), respectively. The EC_{10} values from all laboratories overlap with their 95%-confidence intervals. None of the participating laboratories found a concentration dependent decrease of the embryo numbers in the brood pouch of *P. antipodarum* under exposure to trenbolone.

In validation 3 four laboratories participated in this round robin and TCC and triclosan (TCS) were chosen as test chemicals. In one of the participating laboratories the test was not valid (increased mortality in the solvent control). Two of the other three laboratories observed a concentration-depended decrease of embryo numbers in *P. antipodarum* after 28 days exposure to TCC (NOEC 0.121 and 0.681 µg/L; LOEC 0.340 and 1.52 µg/L) while the third laboratory did not found any effects in the measured concentration range (0.02 – 1.64 µg/L). For TCS one laboratory reported significantly reduced embryo numbers at the highest test concentration (0.964 µg/L as LOEC; NOEC = 0.480 µg/L) while the other two laboratories found no effects in the measured concentration range (<0.004 – 1.36 µg/L and 0.057 – 3.17 µg/L, respectively). In this validation study the food level was reduced to 62.5 µg/snail x day compared to 250 µg/snail x day in validations 1 and 2 to reduce the amount of unconsumed food and the resulting risk of fungus growth which may contribute to an increased mortality of the test organisms. The embryo numbers in the control snails were on the same level like in validations 1 and 2

without any statistically significant deviations in the negative controls among validation studies.

In total, three validation studies and a first ring test in ValMolRepro I (SIERATOWICZ AND OEHLMANN 2011) were conducted and within these 43 reproduction tests have been performed. Thereof one laboratory had to repeat the reproduction test with TBT due to very low concentrations of the test substance and five laboratories did not achieve the given validity criteria for the following reasons. One laboratory had technical issues to satisfy the temperature between 15°C and 17°C and the other four laboratories did not meet the biological criteria (maximum control mortality; or minimum embryo numbers in control groups in snails coming from a different culture). For all tested chemicals the inter-laboratory reproducibility of the test has been shown as most of the laboratories detected comparable NOEC, LOEC, EC_{10} and EC_{50} values with overlapping 95%-confidence intervals for the latter, even if difficult to handle substances were chosen as test compounds (e.g. TBT or trenbolone). In validation 3 the actually measured test concentrations of TCC and TCS were probably too low to induce significant effects on reproduction in all participating laboratories. Furthermore, in the validation run with former test design and in validation 1 the repeatability/intra-laboratory reproducibility could be demonstrated as laboratory A repeated the reproduction test with TBT and Cd.

All in all, the reproduction test turned out to be a practical tool for the risk assessment of chemicals. The test is good manageable and the results are reproducible. At the 10th meeting (10. - 12. December 2014) of the Validation Management Group on Ecotoxicity Testing (VMGeco) of the OECD the group acknowledged the validation exercises and requested the submission of a draft test guideline for the reproduction test with *P. antipodarum* which will be submitted in June 2015. After international commenting rounds and the acceptance by the member states, it is planned to release the test guideline in 2016. Therefore, the outcome of the project contributes to the further development of test methods and evaluation concepts for the regulation of reproductive-influencing chemicals in REACH, as well as pesticides, pharmaceuticals and biocides and moreover for the assessment of long-term and/or sublethal substance effects on populations and ecosystems. It is the first aquatic non-arthropod-test, which was successfully validated within the *Conceptual Framework for Endocrine Disrupters* as a level 4 assay (OECD 2012a). Thereby, molluscs are getting considered as a sensitive and ecological important group of invertebrates in the guideline program for the risk assessment of chemicals of the OECD.

Full life cycle test

Within the reproduction test with *P. antipodarum*, only the effects of substances on adult snails can be investigated. The effects of chemicals on earlier life stages are not considered although these early stages may represent the most sensitive stage of life.

To investigate a potential extension of the existing 28-days reproduction test as partial life cycle (PLC) test protocol to a full life cycle (FLC) test design, which incorporates both juvenile and adult life stages, a comparative sensitivity analysis with juvenile and adult *P. antipodarum* was performed. Therefore, adult snails and newly hatched neonates were exposed to Cd and TBT for 28 days and apical endpoints like growth (in the FLC), reproduction (in the PLC) and mortality

(in FLC and PLC) were compared. These experiments with adult and juvenile *P. antipodarum* showed that Cd and TBT have significant impacts on survival, reproduction and growth of the snails. In the Cd reproduction test with adults mortality in control was 1.25%. Calculated LC_{10} and LC_{50} were 14.1 µg/L and 38.2 µg/L, respectively. The mean embryo number in control was 11.5 and a significant decrease of the mean embryo numbers were observed at concentrations of 12.5, 25 and 50 µg/L. Here, embryo numbers varied between 0.85 (50.0 µg/L) and 2.40 (12.5 µg/L). Calculated EC_{10} and EC_{50} values for the endpoint reproduction were 9.73 and 11.3 µg/L, respectively. Control mortality of juvenile snails in the growth test was 25.0%. Juvenile survival was significantly affected at 25 and 50 µg Cd/L. All snails exposed to these concentrations died during the test. Calculated LC_{10} and LC_{50} were 12.1 and 15.0 µg/L, respectively. After 28 days of exposure to Cd, growth was significantly reduced at concentrations of 6.25 and 12.5 µg/L. The growth of juvenile snails could not be assessed for the two highest test concentrations due to 100% mortality. Calculated EC_{10} and EC_{50} for the endpoint growth were 1.16 and 3.82 µg/L, respectively. In the TBT reproduction test with adult snails mortality in controls was 2.50%. A significantly increased mortality occurred at the highest test concentration of 838 ng Sn/L, where 87.5% of the snails died during the test. The calculated LC_{10} and LC_{50} value were 278 and 499 ng Sn/L, respectively. The mean number of embryos per female in the control and solvent control was 18.9 and 19.3, respectively and did not differ significantly. A significant decrease of embryos was observed at 27.8 ng Sn/L (= LOEC) and higher concentrations. The calculated EC_{10} and EC_{50} were 12.7 and 125 ng Sn/L, respectively. Mortality in the control group of the juvenile growth test was 30% and increased with increasing TBT concentrations up to 85% mortality at the highest test concentration. Calculated LC_{10} and LC_{50} were 11.4 and 194 ng Sn/L, respectively. Also shell length of juvenile *P. antipodarum* was negatively affected by the exposure to TBT. The specific growth rate (SGR) decreased with increasing TBT concentrations and resulted in an EC_{10} and EC_{50} of 5.88 and 175 ng Sn/L, respectively. The results indicate that juvenile snails react slightly more sensitive towards the exposure of Cd and TBT although generally 95% confidence intervals overlap so that differences are not statistically significant. For a final evaluation of juvenile's sensitivity, more tests should be performed to answer this question.

Within the project, two possible test designs of a FLC test with *P. antipodarum* were developed and have been tested.

Test design 1 of a FLC test with *P. antipodarum* comprises three phases. Firstly, the F0-generation is exposed in a PLC test over 28 days (phase 1). Therefore, 200 adult snails are introduced into five litres glass aquaria filled with four litres of DRP medium. Four replicates per exposure group should be used for the test. After 28 days of exposure, adult snails are removed from the test system and in a second phase (phase 2), released neonates are allowed to grow up until they reach sexual maturity. The sexual maturity status is regularly checked by a sample of ten individuals per replicate. A minimum of 10 snails per replicate from F1-generation should survive to assess reproduction in phase 3 of the test.

A first approach of a FLC test with *P. antipodarum* according to test design 1 was conducted in October 2011. Here, a control group with four replicates was used to evaluate the test design.

After 28 days, adult snails were removed from the test system and after 28 additional days (day 56 of the entire experiment) surviving juveniles per replicate were counted. The number of surviving neonates between the four control replicates differed considerably between 26 and 122. Due to the low number of snails, the FLC test had to be ended. A possible explanation for the insufficient number of living juveniles could be a high mortality caused by the absence of a suitable food source.

Therefore, a feeding experiment with neonates and different food types was conducted. The test lasted for 56 days and the endpoint was mortality. A maximum of 24 h old juvenile snails were used for testing. Three different food types with three replicates and 15 neonates each were tested. Here, fine grounded Tetraphyll®, fine grounded Sera® Spirulina and green algae of *Scenedesmus acutus* var. *acutus* were offered as food sources and fed *ad libitum*. The test showed that after 56 days feeding with green algae only 11.1% of the juvenile snails died, whereas high mortalities of 93.3% and 88.9% were observed in the groups fed with Sera® Spirulina and TetraPhyll®, respectively. As a conclusion from test design 1, aquaria are no suitable vessels for a FLC test with *P. antipodarum*. As freshly hatched neonates are only between 450 and 500 µm in size, small test vessels are sufficient and allow the observation of juvenile snails under a stereo microscope. Also, the feeding experiment demonstrated that there are differences in food preferences between adult and juvenile snails of *P. antipodarum*. Therefore, the feeding of green algae should be included when conducting a FLC test to ensure survival and growth of the neonates. Furthermore, test design 1 is not ideal as many adult snails are needed in phase I. To optimise the test design, a FLC test should start with the F0-generation and a defined number of neonates should be introduced into the test system.

The developed test design 2 differs from test design 1 as it starts with the freshly hatched juveniles of *P. antipodarum* to avoid the consumption of high numbers of adult snails. First, adult snails from a laboratory culture are transferred into fresh medium and allowed to reproduce for one week. Afterwards, released juvenile snails are introduced into the test system. Crystallizing dishes with four replicates per exposure group are used as test vessels and snails are introduced at a density of 1000 neonates/L. The snails are fed *ad libitum* with *S. acutus* var. *acutus*. After reaching a mean shell length of 1.5 mm, juveniles are transferred into 500 mL glass beakers (filled with 400 mL medium) and density is adjusted to 75 juvenile snails/L. From then on, snails are additionally fed with fine grounded TetraPhyll® (0.1 mg/snail & day). The test ends when animals in control groups reach a mean size of 3.5 mm. Endpoints of the test are mortality, growth and embryo numbers of the snails. A FLC test according to test design 2 was conducted in 2014. Cd was chosen as test chemical at three nominal concentrations (2, 4 and 8 µg/L). The test lasted 159 days.

Within the first two weeks of the experiment high mortalities were observed in all exposure groups. In controls and Cd exposed groups more than 50% of juveniles died. Up to day 70 survival rate in control was 27.3% and decreased with increasing Cd concentrations. At the highest test concentration (8 µg/L) survival rate was significantly reduced to 6.41%. Thereafter, density was adjusted to 75 neonates/L or to 25 neonates/L for 8 µg/L. After the adjustment of densities on day 70, mortality at 2 µg/L increased significantly starting at day 98. A probable

cause for the high mortalities throughout the test is the development of fungus in the test vessels. At test start, mean shell length was 0.5 mm. Juveniles grew continuously during the experiment. After 159 days mean shell length in controls was 3.8 mm. Snails in the 8 µg/L exposure group were smaller compared to controls. This was not statistically significant. After 159 days of exposure to 8 µg Cd/L snails exhibited a mean size of 3.3 mm. No significant effects of Cd on growth were found in the FLC test. At the end of the test, the mean number of embryos per female was determined. The mean embryo number in controls was 18.2 and decreased with increasing Cd concentrations. 17.5 embryos were found in the brood pouch of the snails at 2 µg/L. At 4 µg/L and 8 µg Cd/L the number of embryos was reduced to 8.15 and 6.38, respectively.

The FLC experiments demonstrate that particular care is required when working with *P. antipodarum* early life stages. Due to the long test duration and high mortality during the first weeks, it is difficult to complete such a test successfully. Neonates in a FLC test were slightly more sensitive towards an exposure to Cd compared to adult snails in the reproduction test. However, test design 2 has to be further optimised (e.g. reducing mortality at the beginning of the FLC test) and juvenile *P. antipodarum* should be exposed towards different chemicals to answer the question if neonates are more sensitive compared to adults. Only if a significantly higher sensitivity of snails can be demonstrated for a number of test compounds in the FLC test compared to the reproduction test, the additional time and effort of the FLC test is justified. A first draft SOP for a full life cycle test with *P. antipodarum* can be found in annex 8.2.

1 Research and development project: Definition and aims

The aim of the project "Validation of reproduction test with molluscs – Establishment of an OECD test guideline for the assessment of endocrine and reproduction toxic effects in snails" was to develop a draft OECD test guideline for reproduction tests with molluscs. The project focused on the reproduction test with the New Zealand mud snail *Potamopyrgus antipodarum*. Several international ring trials of a working group from British, French, Danish and German institutions have been performed, planned and coordinated. To extend the reproduction test, a full life cycle test, including a standard operating procedure (SOP), has been developed. To contribute to the validation studies of the reproduction test with *Lymnaea stagnalis* coordinated by France, Goethe University has participated in ring trials with *L. stagnalis*.

1.1 State of knowledge

Aquatic ecosystems are highly susceptible to anthropogenic impacts. Many man-made chemicals are known to affect growth, development and reproduction of aquatic organisms. Especially Endocrine Disrupting Chemicals (EDCs), pharmaceuticals and pesticides have become a major problem for the aquatic environment due to their effectiveness at comparatively low concentrations. Although molluscs respond particularly sensitive to many pollutants, including EDCs, no standard tests with representatives of this species-rich phylum are established for routine chemical testing in Europe so far.

GOURMELON AND AHTIAINEN (2007) suggested the development of a 28 days-mollusc reproduction test for OECD purposes with regard to a publication by DUFT ET AL. (2007). The suitability of three test designs for the development of a standard method was discussed. The presented test species were the parthenogenetic freshwater mudsnail *P. antipodarum* and two gonochoristic species, the freshwater ramshorn snail *Marisa cornuarietis* and the marine netted whelk *Nassarius reticulatus* because these species have been shown to be remarkably susceptible to xenohormones like 17α-methyltestosterone (MT), 17α-ethinylestradiol (EE_2) and industry chemicals acting as EDCs (e.g. 4-tert-octylphenol (OP), Bisphenol A (BPA)). DUFT ET AL. (2007) finally recommend *P. antipodarum* for the development of a mollusc reproduction test.

In 2008, the German Federal Environment Agency (UBA) started the predecessor project "Validation of a mollusc reproduction test" (internal project name: ValMolRepro I, project code 3708 61 402). The project aimed (a) to prepare a Detailed Review Paper (DRP) which summarises the state-of-the-art of science and technology regarding mollusc testing and (b) to develop a standard test procedure with a selected mollusc species. The DRP on molluscs life-cycle toxicity testing (OECD 2010) had been submitted and made available to the public on the OECD webpage in 2010. Furthermore, experimental work has been performed with the aim to establish a reproduction test with the snail *P. antipodarum*, which had been proposed in the DRP as one of the most promising candidate species for a standardised test guideline next to the pond snail *Lymnaea stagnalis* and the Pacific oyster *Crassostrea gigas*. In the *P. antipodarum* reproduction test the number of embryos in the brood pouch, reflecting the individual reproduction effort in snails, and adult mortality serve as main endpoints. The aim of the investigations was to demonstrate that apical effects of EDCs can be assessed by an increased or de-

creased embryo production.

Investigations regarding the test design showed that a test duration of 28 days is sufficient to detect significant substance effects. Concerning reproduction, counting of embryos in the brood pouch at the end of the exposure period turned out to be a more reliable parameter than estimating the number of released juveniles during the test. In consultation with the funding organisations, the UBA and UK Department of Environment, Food, and Rural Affairs (DEFRA), BPA and cadmium (Cd) were selected for a first international method validation.

A nominal concentration range from 5 to 40 µg BPA/L was considered suitable to assess a concentration dependent and significant increase of embryo numbers in snails. In pre-tests this effect could be detected at a concentration of 40 µg/L (lowest observed effect concentration, LOEC) at a temperature of 16°C. Snails, which have been acclimatised to a temperature of 7°C and 25°C, respectively, showed a higher sensitivity towards BPA (LOEC: 10 µg/L). However, the mean embryo number in the 7°C and 25°C control groups were significantly lower than in control snails tested at 16°C. Additionally measured BPA concentrations declined during the test at 25°C, indicating a more accelerated degradation (SIERATOWICZ ET AL. 2011).

A pre-test with Cd and *P. antipodarum* resulted in the expected reproduction toxic effects at 5 and 25 µg Cd/L (concentration range between 0.2 and 125 µg Cd/L). 100% mortality occurred at 125 µg Cd/L.

Four laboratories (Antwerp University in Belgium, Cefas Laboratories in the United Kingdom, ECT Oekotoxikologie GmbH in Germany and Goethe University in Germany) participated in the following validation exercise for the reproduction test. Five nominal concentrations of each substance (BPA: 2.5 – 40 µg/L; Cd: 1.56 – 25 µg/L) were tested without the use of solvents.

All four laboratories found a concentration-dependent decrease of embryo numbers under Cd exposure. EC_{10} values in three out of four tests ranged between 3.46 and 4.52 µg Cd/L (parameter: total embryo number in the brood pouch). EC_{50} values were in a narrow range, too (11.3 to 13.2 µg Cd/L). One laboratory reported considerably lower effect concentrations (EC_{10}: 0.689 µg Cd/L, EC_{50}: 4.59 µg Cd/L). Also BPA caused a significant decrease of total embryos at the lowest test concentration (2.5 µg/L) in this laboratory. The response resembled an U-shaped concentration effect curve and for the highest exposure group the reproduction level of the control was reached again. Two other laboratories recorded an increasing reproduction with an inverted U-shaped concentration effect curve but with no significant increase at the highest test concentration. The fourth laboratory reported also an increasing embryo number in BPA exposed snails but this effect followed a monotonic concentration response relationship (EC_{10}: 1.19 µg BPA/L; EC_{50}: 10.5 µg BPA/L).

All in all, the reproduction test turned out to be a practical tool. Cd results were reproducible. The expected BPA-dependent increase of reproduction was only affirmed by three out of four laboratories. Analytical data indicate a faster BPA degradation with increasing test duration. A possible cause is the increasing biofilm development in test beakers. Following recommendations made for future validation work with *P. antipodarum* were considered for the current project:

- Change of test beakers in the middle of the test period to reduce biofilm development.
- Selection of a more stable reproduction-enhancing compound like OP.
- Only total embryo numbers should be counted because most of the participating laboratories found it difficult to distinct between shelled and unshelled embryos.
- Temperature dependency of reproduction and hence of substance effects should be further investigated. An optimal temperature interval and range of embryo numbers in the brood pouch should be defined so that effects of test compounds on reproduction are not masked.
- The development of a life cycle test for *Potamopyrgus*, comparable to the *L. stagnalis* protocol (OECD, 2010), requires further investigations and data, for example on mean developmental time until sexual maturity under laboratory conditions and the mean percentage of juveniles reaching this stage.
- Breeding conditions, e.g. regarding breeding medium and temperature, should be optimised to increase the reproductive output of the breeding culture and to support the supply of snails for the validation studies.

Additionally, Goethe University participated in ring tests with *L. stagnalis*, coordinated by the French National Institute for Agricultural Research (INRA, leading scientists: Virginie Ducrot and Laurent Lagadic).

The focus of the present project is on the optimisation of the test and breeding conditions for *P. antipodarum* to finally draw up a draft OECD test guideline on a reproduction test with *P. antipodarum* and additionally enlarge the developed reproduction test into a full life cycle test. The outcome of the project contributes to further development of test methods and evaluation concepts for the regulation of reproductive-influencing chemicals in REACH, as well as pesticides, pharmaceuticals and biocides and moreover for the assessment of long-term and/or sub-lethal substance effects on populations and ecosystems. It is the first aquatic non-arthropod-test, which was successfully validated within the *Conceptual Framework for Endocrine Disrupters* as a level 4 assay (OECD 2012a). Thereby, molluscs are getting considered as a sensitive and ecological important group of invertebrates in the guideline program for the risk assessment of chemicals of the OECD.

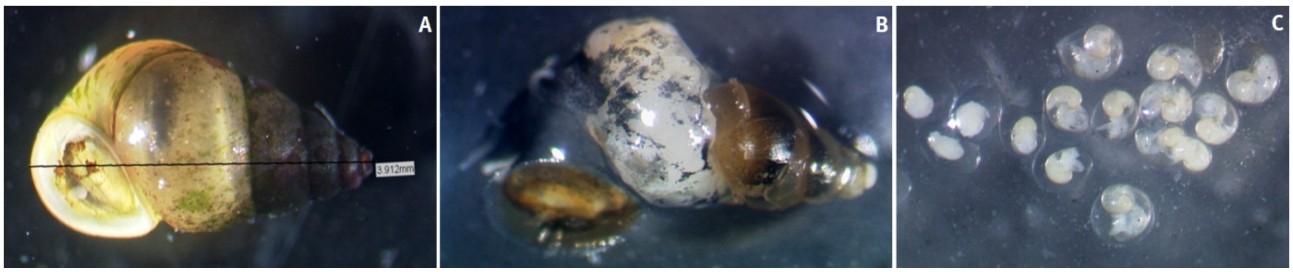
1.2 Test species *Potamopyrgus antipodarum*

The freshwater mud snail *P. antipodarum* originates from New Zealand and has been introduced to other parts of the world, mainly with ballast water of ships. They are found in running waters from small creeks to streams, lakes and estuaries as it tolerates salinities up to 15‰ (COPE AND WINTERBOURN 2004, KINZELBACH 1995, JACOBSEN AND FORBES 1997). The shell height of adult snails averages about 4.3 mm and can reach up to 6 mm. They feed on detritus, algae and bacteria (DUFT ET AL. 2007). European populations consist almost completely of parthenogenetically reproducing females whereas populations in their ancestral distribution area exhibit an almost balanced male to female ratio. Three genotypes of *P. antipodarum* are found in Europe (clones A, B, C). Clone A is dominating in freshwater ecosystems in continental Europe,

clone B in estuaries in the Baltic Sea and clone C is spread in the United Kingdom (HAUSER ET AL. 1992).

The snails are ovoviviparous, i.e. developing eggs are retained in the female brood pouch until the juveniles hatch. After removing the shell from the snail and opening the brood pouch, embryos can easily be removed for analysing the reproductive output of each female by counting the number of developing embryos (Fig. 1). It is also possible to distinguish between unshelled and shelled embryos as it is assumed that the number of unshelled represents the more sensitive parameter when investigating substance effects on the reproduction of the snails (DUFT ET AL. 2003a, b).

Figure 1: *Potamopyrgus antipodarum* with shell (A), without shell and clearly visible brood pouch (central, white, B) and embryos removed from the brood pouch (left: unshelled, right shelled, C).



1.3 Test design description (status 2011) and evaluation method

In the course of the project the experimental conditions have been optimised due to the results obtained in the different studies. At the beginning of this project experimental conditions were as follows:

Using animals originating from all female laboratory cultures, adult *P. antipodarum* of a defined size class (3.7-4.2 mm) were exposed at 16°C in a 28 days reproduction test to a concentration range of the test substance. The test substance was spiked into the water and adult snails are subsequently introduced into 1 L test beakers containing 800 mL aerated medium (see table 1). Dissolved oxygen content should be kept above 60% of the air saturation value (ASV). However the test vessels should be aerated as little as possible to avoid stripping of test chemicals. Water quality parameters (pH, dissolved oxygen content, conductivity, temperature and total nitrite content) should be measured before the water renewal in all test vessels. Additional measurements of ammonium and nitrate should be made if necessary. Constant photoperiods of 16 hours light to 8 hours darkness should be maintained at an intensity that does not exceed 500 lx at the water surface. A semi-static test design was applied with medium renewals at least three times a week for all exposure groups and controls. Each test concentration and the control were tested in four replicates with ten snails each.

Survival was regularly determined and dead snails were removed from test vessels. During the test snails are fed three times per week with finely grounded TetraPhyll® (0.25 mg per snail x day, Tetra GmbH, Melle, Germany) after renewal of the exposure media. At the end of the test snails were anaesthetized with $MgCl_2$ but may also be quick-frozen in liquid nitrogen

and stored at -20°C for later analysis. Adult's shell length and the number of embryos in the brood pouch are determined. The individual per female number of embryos is assessed separately after carefully cracking the shell of the parent specimen and opening of the brood pouch. The number of embryos with and without shell and the total embryo number per female were recorded. Table 1 summarizes the test conditions (status 2011) given for the experimental conditions.

Table 1: Summary of main experimental conditions according to the standard operating procedure from 2011.

Test duration	28 days
Test water	Reconstituted water (with 0.3 g Tropic Marin® salt and 0.18 g $NaHCO_3$ per 1L de-ionised water) water quality requirements: pH 7.5 – 8.5, conductivity 770 ± 100 µS/cm, oxygen concentration ›60% ASV
Test vessels	1 L glass beakers with lids; 800 mL test solution
Water renewal	At least 3 times per week
Temperature	16 ± 1°C
Light intensity	300 – 500 lx
Photoperiod	16:8 h L:D
Food source	Finely ground TetraPhyll®
Feeding	0.25 mg/animal and day
Snails origin	Laboratory culture
Test snails size	3.7 – 4.2 mm
Snails density	10 snails per 800 mL (4 replicates per tested concentration)
Core test endpoints	Mortality, reproduction

1.4 Statistical evaluation

At the beginning of the project the statistical analysis of the results based on the weighted mean method with standard error (SEM) according to TAYLOR (1997).Therefore some of the results obtained in this project are analysed based on this method. In the course of the project this evaluation approach was changed in consultation with the OECD mollusc expert group and the experienced statistician John Green (DuPont) with expert knowledge in bio-statistics. The calculation of weighted treatment means gives more weight to replicate means with lower variance. However, a lower variability in a replicate does not mean that this replicate provides a better capture of the mean response. The final statistical evaluation of embryo numbers as proposed in the draft test guideline is now based on arithmetic means per replicate. To increase the statistical power of this method the experimental design has been adapted: The number of replicates has been increased to six (instead of four) and the number of snails per replicate has been reduced to six (instead of ten). This experimental design has already been applied during

the validation exercises 2 and 3. The statistical analysis of data is described in the draft test guideline (see annex 8.3) and recommends the following procedure:

Statistical analysis of the embryo numbers per female can be done by using one-way ANOVA with Dunnett's test, Williams' test or stepdown Jonckheere-Terpstra test or additionally by the student t-test. It is recommended to consider the transformation of data if needed for meeting the requirements of the particular statistical test (OECD 2006). As non-parametric alternatives one can consider Dunn's or Mann-Whitney's test. EC_X-values, including their associated lower and upper 95% confidence limits, are calculated using appropriate statistical methods (e.g. logistic or Weibull function, trimmed Spearman-Karber method, or simple interpolation). To compute the EC_{10}, EC_{50} or any other EC_X, the complete data set should be subjected to regression analysis.

2 First project part: Comparison of the test organisms *Lymnaea stagnalis* and *Potamopyrgus antipodarum*

2.1 Aim of the literature study

In physiology, "sensitivity" means the capacity of an organism to respond to stimulation taking into account that the degree of susceptibility to stimulation can be rather different between species (species-specificity). Ideally a sensitivity study across species would apply identical test designs and chemicals and evaluate the same endpoints (e.g. mortality, growth, reproduction) including comparable parameters (e.g. NOEC, LOEC, EC_X). Since VON DER OHE AND LIESS (2004) evaluated the relative sensitivity of a selected choice of aquatic invertebrates towards organic and metal contaminants, freshwater gastropods are regarded as being a comparably insensitive group of test organisms. Due to a limited number of toxicants and endpoints available for all taxa, VON DER OHE AND LIESS (2004) considered exclusively acute toxicity data. The standard test organism *Daphnia magna* served as reference species. The relative sensitivity of other aquatic invertebrate taxa was expressed as the logarithmic proportion relative to the sensitivity of *D. magna*. Except for stoneflies, the sensitivity to organic compounds of all other taxa turned out to be less compared to *D. magna* and the order Cladocera. However, the authors acknowledged these findings may not be transferred to chemicals with receptor-mediated (e.g. EDCs) or taxon-specific modes of action. Indeed effect studies on the impact of EDCs on Cladocera, namely *D. magna*, indicated that water flees respond comparably insensitive towards the exposure to xenohormones. A NOEC of 3.16 mg BPA/L was reported by CASPERS (1998) for effects in a 21 days reproduction test with daphnids. The author observed no statistically significant effects on the moulting frequency between the control and BPA exposed groups. Therefore it was concluded that chronic endpoints (reproduction, moulting behaviour) in *D. magna* are of similar sensitivity like the acute endpoint investigated in the 48 hours immobilization test according to OECD guideline 202 (OECD 2004). These findings were confirmed by the study of BRENNAN ET AL. (2006) who determined ecotoxicological effects of four compounds with known estrogenic activity in vertebrates (0.2-1.0 mg 17ß-estradiol/L (E_2), 0.1-0.5 mg diethylstilbestrol/L (DES), 0.2-1.0 mg BPA/L and 0.02-0.1 mg 4-nonylphenol/L (4-NP)) in a two generation test with *D. magna*. EC_{50} acute values both, for immobilization and impacts on the moulting frequency, were in the mg/L range for all compounds tested except for BPA, which had no impact on moulting at all.

In contrast freshwater gastropods have been proven to be very sensitive towards EDCs and vertebrate-type steroids. An enhanced reproductive performance and/or feminisation was observed for the caenogastropod species *Potamopyrgus antipodarum* (table 2, 4) and *Marisa cornuarietis* and the pulmonate *Lymnaea stagnalis* (table 3, 5, 6) during exposure to EE_2, BPA or alkylphenols in the low nano and microgram/L range (DUFT ET AL. 2003a, b, JOBLING ET AL. 2004, OEHLMANN ET AL. 2006, SEGNER ET AL. 2003, SIERATOWICZ ET AL. 2011). Reproductive impairment and/or masculinisation have been reported for more than hundred gastropod species exposed to androgenic compounds like methyltestosterone, tributyltin or triphenyltin (DUFT ET AL. 2003a, DUCROT ET AL. 2010b, LEUNG ET AL 2007, SCHULTE-OEHLMANN ET AL. 2004, WOIN AND

BRÖNMARK 1992). In summary, it can be concluded that acute tests with crustaceans are not representative for chronic tests with mollusc species and should not be used as reference for an interspecies sensitivity comparison.

However, the evaluation of peer-reviewed effect studies of even closer related species like *P. antipodarum* and *L. stagnalis* may demonstrate that the comparability of investigations - even on the same species - is extremely limited. Nevertheless an overview of the findings may provide hints whether the two species can be considered as comparable or complementary in terms of their ecology and degree of sensitivity towards a chemical. It might be possible that effects observed in both test species cover different concentration ranges. The latter would influence the choice of species, e.g. for field monitoring purposes indicating that one species could be a better choice at highly polluted sites since this species can survive where populations of the other cannot. In this context MINCHIN ET AL. (1996) examined the varying TBT contamination within a shipping harbour to evaluate the use of two prosobranch species. The distribution of the dogwhelk *Nucella lapillus* was limited by reduced salinity and the effects of TBT, while the periwinkle *Littorina littorea* exhibited a more extensive range of habitats and turned out to be less sensitive towards TBT exposure. Although indices of both species did not overlap to a significant extent it appeared that first masculinisation effects were observed in female *L. littorea* when *N. lapillus* females became almost sterile and *N. lapillus* population were in danger of extinction. However, test species complemented each other and allowed a monitoring of a broader range of habitats.

In conclusion the current study aims to compare the sensitivity of the mud snail *P. antipodarum* and of the pond snail *L. stagnalis* towards chemical exposure, based on the results of an extensive literature review. The evaluation of literature refers to selected documents and intends to provide a critical synthesis of previous research. The sensitivity comparison across species shall help to provide a background to further OECD validation studies with both gastropod species. Key points for further research concern similarities and differences in test design and sampling, husbandry conditions, data analysis and interpretation of findings. A synopsis of available data may help to identify lacks of knowledge in research, variables in species sensitivity, promising ecotoxicological endpoints and to avoid unnecessary duplication of research.

2.2 Methods

The literature study was conducted in 2011 in corporation with the French National Institute for Agricultural Research (INRA). The generation of this report bases on combining available data of effect concentrations from “peer-reviewed literature” and “grey literature” of unpublished dissertations and in-house studies. Evaluations focus exclusively on primary literature (original research results) dealing with chronic effects on reproduction, growth, mobility and mortality.

2.2.1 Data banks consulted

Data and information for this study were gathered through databank search using the key words "*Potamopyrgus antipodarum* + toxicity + reproduction" and "*Lymnaea stagnalis* + toxicity + reproduction". It however should be mentioned that keyword search did not inevitably result in studies of interest (primary data) as cross-references of closely related studies (e.g. on other species or endpoints) were sampled out by the search engine as well.

The following databanks have been consulted:

- Federal Environment Agency Germany (UBA) data banks:
  - UBA data bank ETOX (Information System Ecotoxicology and Environmental Quality Values): no entries for *P. antipodarum*; 6 entries for *L. stagnalis* dealing with reproduction
  - UBA data bank OPAC including ULIDAT (Environmental Literature and Information): 2 entries for *P. antipodarum* dealing with reproduction; none for *L. stagnalis*
  - UBA data bank UFORDAT (FEA R&D-Project Reports): no entries
- Web of Science: 55 entries for *P. antipodarum,* thereof 27 dealing with reproduction; 159 for *L. stagnalis*, thereof 11 dealing with reproduction
- Biological Abstracts: 20 entries for *P. antipodarum*, thereof 9 dealing with reproduction; 98 for *L. stagnalis*, thereof 14 dealing with reproduction
- Zoological Records: 3 entries for *P. antipodarum*, thereof 2 dealing with reproduction; 21 for *L. stagnalis*, thereof 5 dealing with reproduction
- USEPA Ecotox database (including "grey literature" and internal reports): 64 entries for *P. antipodarum*, thereof 41 dealing with reproduction; 164 for *L. stagnalis*, thereof 118 dealing with reproduction
- PubMed – NCBI: 30 entries for *P. antipodarum*, thereof 18 dealing with reproduction; 105 for *L. stagnalis*, thereof 12 dealing with reproduction

2.2.2 Evaluation criteria

To assure comparability of independent experiments, only those toxicity studies providing calculated EC_x, NOEC and/or LOEC values were considered.

For many studies it was not possible to calculate these data either because of non-monotonic concentration-effect relationships, too small number of exposure groups and replicates or non-significant data within a concentration-response. It has however to be noted that several endocrine-mediated effect studies result in non-monotonic concentration-effect relationships.

Acute toxicity data were not considered as the OECD tests to be developed with molluscs are chronic tests. Field and wastewater studies represent typical mixture studies and were conducted for *P. antipodarum* exclusively and did not provide comparable parameters for comparison so that these studies were also not taken into account. Furthermore mono-substance studies on chemicals of minor relevance for reproduction and development (e.g. microcystin, calcium, and nitrate) were not considered.

2.3 Results of the literature study

2.3.1 Peer-reviewed studies (original data)

Table 2: *Potamopyrgus antipodarum*. Effect concentrations and NOEC/LOEC values of selected exposure studies. Abbreviations: n.a. (not applicable), n.d. (not determined).

Tests with *Potamopyrgus antipodarum*			
Substance and reference	**Test duration**	**Endpoint**	**Effect concentration**
Ammonia ALONSO & CARMAGO (2009)	40 days	Activity/Immobility	NOEC/LOEC = 20/70 µg/L (activity), NOEC/LOEC = 70/130 µg/L (immobility)
Atrazine GERARD & POULLAIN (2005)	35 days	Mobility	n.d. (only two concentrations tested)
Bisphenol A, DUFT ET AL. (2003a)	56 days	Reproduction (enhanced embryo production)	EC_{10} = 0.22 µg/kg, EC_{50} =24.5 µg/kg (sediment) (after 2 weeks) EC_{10} = 0.19 µg/kg, EC_{50} = 5.67 µg/kg (sediment) (after 4 weeks)
Bisphenol A JOBLING ET AL. (2004)	63 days	Reproduction (enhanced embryo production)	NOEC = 1 µg/L; LOEC = 5 µg/L (non-monotonic)
Bisphenol A GAGNAIRE ET AL. (2009)	14 -28 days	Reproduction (Vg-like protein levels enhanced)	LOEC (increase) = 100 µg/L
Bisphenol A SIERATOWICZ ET AL. (2011)	28 days	Reproduction (enhanced embryo production)	NOEC = 20 µg/L, LOEC = 40 µg/L (at 16°C) NOEC = 5 µg/L, LOEC = 10 µg/L (at 7°C & 25°C)
Cadmium DORGELO ET AL. (1995)	Up to 20 weeks	Reproduction, growth	EC_{50} = 16 µg/L (growth), LOEC = 25 µg/L (reproduction)
Cadmium FORBES ET AL. (1995)	21 days	Growth (inhibition)	n.a. (only one concentration tested)
Cadmium MØLLER ET AL. (1996)	48 hours or 30 days (+ pre exposure)	Growth rate (inhibition)	n.d.
Cadmium JENSEN ET AL. (2001)	Up to 56 days (depending on recorded parameter)	Growth rate, reproduction (clone B)	7.29 µg Cd/g dw (sediment)
Cadmium SIERATOWICZ ET AL. (2011)	28 days	Reproduction (reduced embryo production)	NOEC= 1 µg/L, LOEC = 5 µg/L, EC_{10} = 1.3 µg/L, EC_{50} = 11.5 µg/L
Carbamazepine OETKEN ET AL. (2005)	28 days	Growth, reproduction, mortality	n.d. effects for single concentrations; do not follow a monotonic or concentration response
Chloroxurane KONSAKE ET AL. (1988)	20 days	Embryo development (reduced hatching rate)	ED_{50} = $6x10^{-5}$ M (17.5 mg/L)

Tests with *Potamopyrgus antipodarum*			
Substance and reference	**Test duration**	**Endpoint**	**Effect concentration**
Copper DORGELO ET AL. (1995)	Up to 20 weeks	Reproduction, growth	EC_{50} = 13 µg/L (growth), LOEC = 30 µg/L (reproduction)
EE2 JOBLING ET AL. (2004)	63 days	Reproduction (reduced embryo production)	NOEC = 5 ng/L; LOEC = 25 ng/L (non-monotonic)
EE2 SIERATOWICZ ET AL. (2011)	28 days	Reproduction (enhanced embryo production)	NOEC = 25 ng/L; LOEC = 50 ng/L
Fadrozole GUST ET AL. (2010b)	42 days	Reproduction (reduced embryo production and cumulative number of neonates released)	LOEC (embryo production) = 6.2 µg/L LOEC (cumulative number of neonates) = n.d. (effects for single concentrations; no monotonic or nonmonotonic concentration response)
Fluoride ion ALONSO & CAMARGO (2011)	28 days	Reproduction (cumulative embryo number/female)	NOEC = 4.6 mg/L LOEC = 9.5 mg/L
Fluoride ion ALONSO & CAMARGO (2011)	7-14 days	Velocity decrease	LOEC = 17.5 mg/L NOEC = 5.2 mg/L
Fluoxetine NENTWIG (2007)	56 days	Reproduction (reduced embryo production)	EC_{10} = 0.81 µg/L
Fluoxetine PÉRY ET AL. (2008)	42 days	Reproduction (reduced number of newborns per female)	NOEC =13 µg/L LOEC = 69 µg/L
Fluoxetine GUST ET AL. (2009)	42 days (adults), 14 weeks (juveniles)	Reproduction (cumulative number of neonates)	LOEC = 3.7 µg/L (non-monotonic)
Fluoxetine GUST ET AL. (2011b)	56 days	Reproduction, biomarker for metabolism	n.d. (only one concentration tested)
Leachates from plastic and glass bottles WAGNER & OEHLMANN (2009)	56 days	Growth, reproduction, mortality	n.a.
3-(4´-Methylbenzyli-dene)-campher, 3-benzylidene-campher SCHMITT ET AL. (2008)	56 days (sediment exposure)	Reproduction (increased embryo production)	EC_{50} = 1.17 mg 4-MBC/kg dw (sediment) LOEC = 0.28 mg 3-BC/kg dw (sediment)
Methyltestosterone DUFT ET AL. (2007)	56 days	Reproduction (decreased embryo production)	EC_{10} = 12.0 ng/L EC_{50} = 160 ng/L
4-tert-Nonylphenol DUFT ET AL. (2003b)	56 days	Reproduction (increased embryo production)	LOEC = 10 µg/kg (sediment)
Octylphenol DUFT ET AL. (2003b)	28 days	Reproduction (increased embryo production)	EC_{10} = 4 ng/kg, EC_{50} = 70 ng/kg (sediment) (after 4 weeks)
Octylphenol JOBLING ET AL. (2004)	63 days	Reproduction	NOEC = 1 µg/L; LOEC = 5 µg/L (non-monotonic)
Octylphenol GAGNAIRE ET AL. (2009)	14-28 days	Reproduction (Vg-like protein levels)	LOEC (increase) = 1 µg/L LOEC (decrease) = 100 µg/L
Polycyclic musk HHCB PEDERSEN ET AL. (2009)	84 days (adults) or from birth to first re-	Growth, vitality, reproduction, mortality, develop-	LOEC (decrease reproduction) = 10 µg/g dw (sedi-

Tests with *Potamopyrgus antipodarum*			
Substance and reference	**Test duration**	**Endpoint**	**Effect concentration**
	production (juveniles)	ment	ment) NOEC (decrease reproduction) = 1 µg/g dw (sediment)
Sediments (River) DUFT ET AL. (2002)	28 days	Reproduction	n.a.
Sediments (River) STACHEL ET AL. (2002)	28 days	Reproduction	n.a.
Sediments (River) OETKEN ET AL. (2005)	28 days	Reproduction (embryo production)	n.a.
Sediments (Lake) MAZUROVÁ ET AL. (2008)	56 days	Reproduction (embryo production), mortality	n.a.
Sediments (River) SCHMITT ET AL. (2010)	28 days	Growth, reproduction (embryo production), mortality	n.a.
Sediment extracts/fractions of river basins SCHMITT ET AL. (2010, 2011)	28 days	Growth, reproduction (embryo production), mortality	n.a.
Sediments (River) TUIKKA ET AL. (2011)	56 days	Growth, reproduction (embryo production), mortality	n.a.
Stream mesocosm with metal mixture (copper, zinc) HICKEY & GOLDING (2002)	34 days	Relative (%) change in abundance	EC_{20} = 1.4 CCU (Cumulative criterion Unit) EC_{50} = 4.4 CCU
River water GUST ET AL. (2011a)	28 days	Mortality, growth, reproduction, biomarker for metabolism	n.a.
Tributyltin DUFT ET AL. (2003a,b)	28 days	Growth, reproduction (embryo production), mortality	Reproduction: EC_{10} (unshelled) = 0.98 µg/kg (sediment) EC_{50} (unshelled) = 45.8 µg/kg (sediment) EC_{10} (shelled) = 10.6 µg/kg (sediment) EC_{50} (shelled) = 173 µg/kg (sediment)
Tributyltin GAGNAIRE ET AL. (2009)	14 - 28 days	Reproduction (Vg-like protein levels)	LOEC (decrease) = 5 ng TBT/L
Triclocarban GUIDICE & YOUNG (2010)	28 days	Reproduction (increase in embryo production)	EC_{10} (unshelled) = 0.5 µg/L EC_{50} (unshelled) = 2.5 µg/L
Triphenyltin DUFT ET AL. (2003a,b)	56 days	Growth, reproduction (decrease in embryo production), mortality	Reproduction EC_{10} (unshelled) = 0.03 µg/kg (sediment) EC_{50} (unshelled) = 0.74 µg/kg (sediment) EC_{10} (shelled) = 0.05 µg/kg (sediment) EC_{50} (shelled) = 23.6 µg/kg (sediment)

Tests with *Potamopyrgus antipodarum*			
Substance and reference	**Test duration**	**Endpoint**	**Effect concentration**
Wastewater JOBLING ET AL. (2004)	42 days	Growth, reproduction (embryo production), mortality	n.a.
Wastewater GUST ET AL. (2010a,c)	21-28 days	Reproduction (embryo production), mortality	n.a.
Wastewater STALTER ET AL. (2010)	28 days	Reproduction (embryo production), mortality	n.a.
Wastewater WATTON & HAWKES (1982)	21 weeks	Abundance and biomass reduction, reproduction (decreased embryo production)	n.a.
Zinc DORGELO ET AL. (1995)	Up to 20 weeks	Growth inhibition	EC_{50} = 103 µg/L

Table 3: *Lymnaea stagnalis*. Effect concentrations and NOEC/LOEC values of selected exposure studies. Abbreviations: n.a. (not applicable), n.d. (not determined).

Tests with *Lymnaea stagnalis*			
Substance and reference	**Test duration**	**Endpoint**	**Effect concentration**
1,1,2-Trichloroethane ADEMA & VINK (1981)	16 days	Development/hatching	EC_{50} = 36 mg/L
2,2-Dibromobiphenyl WILBRINK ET AL. (1987)	28 and 42 days	Reproduction (inhibited egg mass production)	n.a. only one concentration tested
2,2-Dichlorobiphenyl WILBRINK ET AL.(1992)	7 days (food exposure)	Reproduction (start oviposition & oviposition latency after clean water stimulus)	LOEC = 7.5 g/kg (300 µg / 40 mg) for start oviposition and oviposition latency
2,4,5-Trichlorophenoxyacetic BLUZART & SEUGÉ (1983)	68-330 days	Reproduction (reduced cumulative egg number and number of eggs/egg mass), growth, survival	LOEC/NOEC = 40/20 mg/L (survival) LOEC/NOEC = 40/20 mg/L (growth) LOEC/NOEC (Reproduction) = n.d. (effects for single concentrations; do not follow a monotonic or nonmonotonic concentration response)
2,4-Dichloroaniline SLOOFF & CANTON (1983)	40 days (reproduction) 7 days (hatching)	Reproduction, hatching	NOEC = 1 mg/L (reproduction) NOEC = 3.2 mg/L (hatching)
3,4-Dichloroaniline ADEMA & VINK (1981)	16 days	Development/hatching	EC_{50} = 1 mg/L
4,4-Dibromobiphenyl WILBRINK ET AL. (1987)	28 and 42 days	Reproduction (inhibited egg mass production)	n.a. only one concentration tested
Acetone BLUZART ET AL. (1979)	287 days	Growth inhibition, reproduction	LOEC = 0.1% (1mL/L, growth), NOEC /LOEC = 0.1/0.2% (1mL/2mL/L fecundity)
Alpha-HCH CANTON & SLOOFF (1977)	70 days/	Reproduction (inhibition of egg production), hatching (reduced), heart-beat rate (reduced)	EC_{50} = 250 µg/L (reproduction) EC_{50} = 230 µg/L (hatching) LOEC = 20 µg/L (heart-beat rate)
Aluminium DOBRANSKYTE ET AL. (2006)	30 days	Feeding behavior suppression	LOEC = 250 µg/L
Atrazine RUSSO & LAGADIC (2004)	21 days	Physiology (increased hemocyte density)	LOEC = 50 µg/L (504 hours)
Bisphenol A SEGNER ET AL. (2003)	21 days	Hatching, genital malformation	LOEC = 0.32 µg/L (hatching) LOEC = 3.2 µg/L (malformation) LC50/7 d = 5.94 µg/L LC50/21 d = 0.71 µg/L
Cadmium COEURDASSIER ET AL. (2007)	28 days	Growth (inhibition)	EC_{50} = 142 µg/L
Cadmium GOMOT (1998)	49 days	Reproduction (reduced mean egg number/egg mass, egg masses/week),	LOEC = 200 µg/L (egg number/egg mass) LOEC = 200 µg/L (hatching)

Tests with *Lymnaea stagnalis*			
Substance and reference	**Test duration**	**Endpoint**	**Effect concentration**
		Development (reduced number of hatchlings)	LOEC = 400 µg/L (egg masses/egg week)
Carbaryl SEUGÉ & BLUZART (1983)	28-420 days	Survival, growth, reproduction (reduced number of egg-masses/snail, reduced cumulative egg number and number of eggs/egg mass)	LOEC = 1 mg/L (survival) LOEC/NOEC (growth) = n.d. (effects for single concentration; no monotonic or non-monotonic concentration response) LOEC/NOEC (reproduction) = n.d.
Chlorpropham KOSANKE ET AL. (1988)	20 days	Impairment of embryo development	ED_{50} = 7x10^{-5} M (15 mg/L)
Chloroxurone KOSANKE ET AL. (1988)	20 days	Embryo survival	ED_{50} =6x10^{-5} M
Cobalt DE SCHAMPHELAERE ET AL. (2008)	28 days	Growth (inhibition)	NOEC = 26.0 µg/L (growth) LOEC = 79.0 µg/L (growth)
Copper BRIX ET AL. (2011)	30 days	Growth, mortality	EC_{20} = 1.8 µg/L (growth) NOEC = 2.3 µg/L (growth) LOEC = 4.9 µg/L (growth) EC_{20} = 5.6 µg/L (survival) NOEC = 4.0 µg/L (survival) LOEC = 7.0 µg/L (survival)
Copper NG ET AL. (2011)	28 days	Mortality	n.a. EC_{20}/EC_{50} = higher than the highest Cu conc. tested (survival)
Cycloate KOSANKE ET AL. (1988)	20 days	Embryo survival	ED_{50} =7x10^{-6} M (1.505 mg/L)
DDT WOIN & BRÖNMARK (1992)	45 days	Reproduction (cumulative egg number decreased)	LOEC = 50.0 µg/L
Dieldrin ADEMA & VINK (1981)	16 days	Development/hatching	EC_{50} = 18 µg/L
Dimethoate SLOOFF & CANTON (1983)	40 days (reproduction) 7 days (hatching)	Reproduction, hatching	NOEC = 10 mg/L (reproduction) NOEC = 32 mg/L (hatching)
Dinitro-*o*-cresol SLOOFF & CANTON (1983)	40 days (reproduction) 7 days (hatching)	Reproduction, hatching	NOEC = 32 µg/L (reproduction) NOEC = 1 mg/L (hatching)
Diquat COUTELLEC ET AL. (2008)	10 days	Growth (inhibition), development (delay)	NOEC / LOEC = 22.2 / 44.4 µg/L (growth, adults) LOEC = 222.2 µg/L (development juv.) LOEC = 222.2 µg/L (reduced hatching)
EE_2 SEGNER ET AL. (2003)	21 days	Hatching, growth of hatchlings, reproduction	LOEC = 1 µg/L (hatching) LOEC = 0.5 µg/L (growth) LOEC = 0.5 µg/L (reproduction)

Tests with *Lymnaea stagnalis*			
Substance and reference	**Test duration**	**Endpoint**	**Effect concentration**
Fenthion Seugé & Bluzart (1983)	28-420 days	Survival, growth, reproduction (reduced number of egg-masses/snail, reduced cumulative egg number and number of eggs/egg mass)	LOEC = 2 mg/L (survival) LOEC = 2 mg/L (growth) LOEC/NOEC (reproduction) = n.d.
Fomesafen Jumel et al. (2002)	84 days (mesocosm & lab study)	Growth, reproduction	n.a. only one concentration tested
Fomesafen Russo et al. (2007)	21 days	lysosome membrane fragility (increase, NRRT), oxidative burst of hemocytes (ROS)	LOEC = 10 µg/L (membrane fragility) LOEC = 10 µg/L (ROS)
Lead Grosell et al. (2006)	30 days	Growth	EC_{20} = 4.0 µg/L NOEC = 12.0 µg/L LOEC = 16.0 µg/L
Lindane Seugé & Bluzart (1983)	28-420 days	Survival, growth, reproduction (reduced number of egg-masses/snail, reduced cumulative egg number and number of eggs/egg mass)	LOEC = 1 mg/L (survival) LOEC = 2 mg/L (growth) LOEC/NOEC (reproduction) = n.d.
MCPA Woin & Brönmark (1992)	45 days	Reproduction (cumulative egg number decreased)	LOEC = 10.0 mg/L
Molybdate De Schamphelaere et al. (2010)	28 days	Growth rate (length, biomass)	NOEC = 200 mg/L (length) LOEC = 388 mg/L (length) EC_{10} = 211.3 mg/L (length) NOEC = 200 mg/L (biomass) LOEC = 388 mg/L (biomass) EC_{10} = 221.8 mg/L (biomass)
4-tert-Nonylphenol Czech et al. (2001)	84 days	Reproduction	LOEC = 100 µg/L
Nonylphenol metabolite (4(3',6'-dimethyl-3'-heptyl)-phenol) Lalah et al. (2006)	20 days	Development (delay), reduced hatching success, increased mortality	n.a. only one concentration (105 µg/L) tested
p-Nitrotoluene Slooff & Canton (1983)	40 days (reproduction) 7 days (hatching)	Reproduction, hatching	NOEC = 320 µg/L (reproduction) NOEC = 10 mg/L (hatching success)
Pentachlorphenol Adema & Vink (1981)	16 days	Development/hatching	EC_{50} = 130 µg/L LC_{50} = 180 µg/L
Pentachlorphenol Crossland & Wolff (1985)	16 days	Mortality	LC_{50} = 180 µg/L
Pentachlorphenol Slooff & Canton (1983)	40 days (reproduction) 7 days (hatching)	Reproduction, hatching	NOEC = 10 µg/L (reproduction) NOEC = 3.2 µg/L (hatching)
Potassium-Dichromate Slooff & Canton (1983)	40 days (reproduction) 7 days (hatching)	Reproduction, hatching	NOEC = 320 µg/L (reproduction) NOEC = 1 mg/L (hatching)

Tests with *Lymnaea stagnalis*			
Substance and reference	**Test duration**	**Endpoint**	**Effect concentration**
Reglone 2® (diquat dibromide) DUCROT ET AL. (2010a)	Embryos, 8 weeks, water exposure, 20 °C	Hatching rate Development duration	LOEC = 6.7 µg/L LOEC = 3.2 µg/L
Reglone 2® (diquat dibromide) DUCROT ET AL. (2010a)	FLC, 184 days of water exposure, 20°C	Survival, Growth, Age at first spawning, Cumulated fecundity	All LOEC = 3.2 µg/L
Simazine KOSANKE ET AL. (1988)	20 days	Embryo mortality	ED_{50} = <10^{-7} M (<20.1 µg/L)
Sodium-Bromide SLOOFF & CANTON (1983)	40 days (reproduction) 7 days (hatching)	Reproduction, hatching	NOEC = 10 mg/L (reproduction) NOEC = 3200 mg/L (hatch)
Terbutryne KOSANKE ET AL. (1988)	20 days	Embryo mortality	ED_{50} = <10^{-7} M
Tetrapropylene-Benzene-Sulphonate SLOOFF & CANTON (1983)	40 days (reproduction) 7 days (hatching)	Reproduction, hatching	NOEC = 320 µg/L (reproduction) NOEC = 3.2 mg/L (hatching)
Tributyltin CZECH ET AL. (2001)	49 days	Reproduction	LOEC = 100 µg/L
Tributyltin LEUNG ET AL. (2007)	40/150 days	Development & growth (40 days), survival hatchlings (150 days), population growth rate	Development NOEC = 0.01 µg TBT/L; LOEC = 1 µg TBT/L Growth NOEC = 0.01 µg TBT/L; LOEC = 1 µg TBT/L Survival NOEC = 0.01 µg TBT/L; LOEC = 1 µg TBT/L NOEC = 2.75 µg TBT/L (population growth rate)
Tributyltin-Oxide MATHIJSSEN ET AL. (1989)	33 days	Reproduction	EC_{50} = 0.38 µg/L
Triphenyltin-Hydroxide VAN DER MAAS AT AL. (1972)	35 days	Reproduction	TC = 2.0 µg/L
Vinclozolin DUCROT ET AL (2010b)	Adults, 21 days, water exposure	Survival Feeding behaviour	LOEC = 2500 µg/L LOEC = 250 µg/L
Vinclozolin DUCROT ET AL (2010b)	Juveniles, 21 days, water exposure	Growth Feeding behaviour	n.s. (>2500 µg/L) LOEC = 250 µg/L
Vinclozolin DUCROT ET AL (2010b)	Adults (young), 21 days, water exposure	Cumulated fecundity	LOEC = 0.025 µg/L
Vinclozolin DUCROT ET AL (2010b)	Adults (old), 21 days, water exposure	Cumulated fecundity	LOEC = 250 µg/L
Zinc DE SCHAMPHELAERE & JANSSEN (2010)	28 days	Growth rate reduction	EC_{10} = 672 µg/L (mean value of 6 EC_{10} values) EC_{50} = 964 µg/L (mean value of 6 EC_{10} values)

2.3.2 In-house studies (unpublished original data)

Table 4: In-house tests with *Potamopyrgus antipodarum* performed at Goethe University Frankfurt am Main (Germany). Reproduction was assessed as the number of embryos in the snails' brood pouch. n. s. (not significant).

In-house studies with *Potamopyrgus antipodarum* performed at Goethe University			
Substance and reference	Test duration	Endpoint	Effect concentration
Benzophenone 3 THESIS KAISER (2007)	56 days, sediment exposure	Reproduction	n. s. (>50 mg/kg)
Bisphenol A DISSERTATION CASEY (2000)	63 days, water exposure	Reproduction	LOEC = 5 µg/L
Bisphenol A ValMolRepro (2011)	28 days, water exposure	Reproduction	EC_{10} = 1.19 µg/L EC_{50} = 10.5 µg/L
Cadmium DISSERTATION OETKEN (1999)	42 days, water exposure	Reproduction	LOEC = 0.1 µg/L (unbounded)
Cadmium VALMOLREPRO (2011)	28 days, water exposure	Reproduction	EC_{10} = 5.65 µg/L EC_{50} = 12 µg/L
Cadmium THESIS RUDZKI (1998)	42 days, water exposure	Creep rate	LOEC = 0.1 µg/L
Carbamazepine OETKEN ET AL (2005)	28 days, water exposure	Reproduction	LOEC = 10 µg/L
Chrome DISSERTATION OETKEN (1999)	42 days, water exposure	Reproduction	LOEC = 5 µg/L
Chrome THESIS RUDZKI (1998)	42 days, water exposure	Creep rate	LOEC = 2 µg/L
Ciprofloxacin DISSERTATION NENTWIG (2006)	56 days, water exposure	Reproduction	LOEC = 0.8 µg/L
Clofibric acid DISSERTATION NENTWIG (2006)	56 days, water exposure	Reproduction	LOEC = 0.04 µg/L (unbounded)
EE2 DISSERTATION CASEY (2000)	63 days, water exposure	Reproduction	LOEC = 25 ng/L
Fenarimol THESIS SCHOLZ (2003)	84 days, water exposure	Reproduction	n. s. (>331 ng/L)
Fenarimol THESIS HASENBANK (2004)	56 days, water exposure	Reproduction	n. s. (>3000 ng/L)
Galaxolide (HHCB) THESIS ANNUSSEK (2006)	56 days, sediment exposure	Reproduction	LOEC = 0.02 mg/kg
Irgarol In-house-study FFM (unpublished)	28 days	Reproduction	NOEC = <0.05 µg/L LOEC = 0.05 µg/L
Nano silver THESIS GRÄF (2012)	28 days, water exposure	Reproduction	n. s. (>50 µg/L)
Nano silver THESIS GRÄF (2012)	28 days, water exposure	Reproduction	LOEC = 50 µg/L
Nickel THESIS RUDZKI (1998)	42 days, water exposure	Creep rate	LOEC = 0.5 µg/L (unbounded)
Nonylphenol	56 days, sediment	Reproduction	n. s. (>300 µg/kg)

In-house studies with *Potamopyrgus antipodarum* performed at Goethe University			
Substance and reference	**Test duration**	**Endpoint**	**Effect concentration**
Dissertation Di Benedetto (2009)	exposure		
Octyl-methoxycinnamate Dissertation Kaiser (2012)	56 days, sediment exposure	Reproduction	LOEC = 0.4 mg/kg
4 *tert*-Octylphenol Dissertation Casey (2000)	63 days, water exposure	Reproduction	LOEC = 25 µg/L
Platinum salt Dissertation Galluba (2012)	56 days, water exposure	Reproduction	LOEC = 0.33 µg/L
Tonalide (AHTN) Dissertation Di Benedetto (2009)	56 days, sediment exposure	Reproduction	EC_{10} = 1.73 mg/kg
Primidone Neptune (2009)	28 days, water exposure	Reproduction	n. s. (› 20 mg/L)
Terbutryne Dissertation Di Benedetto (2009)	56 days, sediment exposure	Reproduction	n. s. (› 31.25 µg /kg)
Tributyl phosphate Dissertation Di Benedetto (2009)	56 days, sediment exposure	Reproduction	LOEC = 0.05 mg/kg (unbounded)
Tris(1,3-dichloriso-propyl) phosphate Dissertation Di Benedetto (2009)	56 days, sediment exposure	Reproduction	LOEC = 31.3 mg/kg
Tributyltin Dissertation Schulte-Oehlmann (1997)	180 days, water exposure	Reproduction	TC = 5 ng/L (calculated threshold concentration)
Tributyltin Thesis Hasenbank (2004)	56 days, water exposure	Reproduction	EC_{10} = 53.4 ng Sn/L EC_{50} = 163 ng Sn/L
Titanium dioxide Thesis Giebner (2011)	28 days, water exposure	Reproduction	LOEC = 100 µg/L
Tramadol Neptune (2009)	28 days, water exposure	Reproduction	LOEC = 0.032 mg/L (unbounded)
Vinclozolin Thesis Scholz (2003)	84 days, water exposure	Reproduction	LOEC = 114 ng/L
Zinc Thesis Rudzki (1998)	42 days, water exposure	Creep rate	LOEC = 1 µg/L

Table 5: In-house tests with *Lymnaea stagnalis* performed at Goethe University (Frankfurt am Main, Germany). LC_{50} (substance concentration that gives half-maximal response), NOEC (no observed effect concentration), LOEC (lowest observed effect concentration).

In-house studies with *Lymnaea stagnalis* performed at Goethe University			
Substance and reference	**Test duration**	**Endpoint**	**Effect concentration**
Bisphenol A THESIS GATZKE (2010)	Embryos, until hatching, water exposure, 15°C, 20°C, 25°C	Hatching success, development, transition into next developmental stage	$LOECs_{15°C}$ = 2 mg/L (transition into next stage); 8 mg/L (mortality); 4 mg/L (hatching decline) $LOECs_{20°C}$ = 2 mg/L (transition into next stage); 4 mg/L (mortality); 4 mg/L (hatching decline) $LOECs_{25°C}$ = 2 mg/L (transition into next stage); 4 mg/L (mortality); 4 mg/L (hatching decline)
Bisphenol A THESIS KIPOUROU (2010)	56 days, water exposure	Mortality, growth, biomass, reproduction	LOEC >300 µg/L (unbounded, >30% mortality in the control) (mortality, growth, biomass, reproduction)
Cadmium THESIS KIPOUROU (2010)	56 days, water exposure	Mortality, growth biomass, reproduction	LOEC = 120 µg/L (mortality) LOEC = only 15 µg/L (increased biomass) LOEC = 240 µg/L (decreased number of egg masses per snail)
Copper USEPA Ecotox database	28 days	Hatching success, reproduction	NOEC = 104 µg/L, LC_{50} = 146 µg/L (juveniles, hatching) NOEC = 200 µg/L (adults, 21 days) LC_{50} = 475 µg/L (adults, 48 h)
Dimethylformamid USEPA Ecotox database	21 days	Hatching success	NOEC = 3 µg/L LC_{50} = 1.59 g/L
Irgarol In-house-study FFM (unpublished)	28 days	Reproduction	NOEC = 0.32 µg/L LOEC = 0.8 µg/L
Lindane USEPA Ecotox database	21 days	Hatching success	NOEC = 3 mg/L
Tributyltin THESIS BANDOW (2009)	21 days	Hatching success, reproduction	NOEC = 0.1 µg/L, LC_{50} = 0.36 µg/L (juveniles, hatching) NOEC = 0.4 µg/L (adults, reproduction)

Table 6: In-house tests with *Lymnaea stagnalis* performed at INRA (France). n.s. = not significant, NOEC (no observed effect concentration), LOEC (lowest observed effect concentration).

In-house studies with *Lymnaea stagnalis* performed at INRA (F)			
Substance and reference	**Test duration**	**Endpoint**	**Effect concentration**
Azoxystrobine DUCROT ET AL. (unpublished)	Embryos, 5 weeks, water exposure, 20°C	Hatching rate	LOEC = 2500 µg/L
Boscalid DUCROT ET AL. (unpublished)	Embryos, 5 weeks, water exposure, 20°C	Hatching rate	n.s. (›156 mg/L)
Bromoxinyl DUCROT ET AL. (unpublished)	Embryos, 5 weeks, water exposure, 20°C	Hatching rate	LOEC = 3000 µg/L
Cadmium DUCROT ET AL. (unpublished)	Adults, 8 weeks water exposure, 20°C	Survival Growth Reproduction	LOEC = 180 µg/L LOEC = 180 µg/ LOEC ‹80 µg/L
Clomazone DUCROT ET AL. (unpublished)	Embryos, 5 weeks, water exposure, 20°C	Hatching rate	LOEC = 30 mg/L
Chlordecone GUISTI ET AL. (unpublished)	Adults, 21 days water exposure, 20 °C	Survival, Growth Fecundity Reproduction: egg-abnormality	n.s. (›110 µg/L) LOEC = 10 µg/L LOEC = 4.5 µg/L
Cyprodinil DUCROT ET AL. (unpublished)	Embryos, 5 weeks, water exposure, 20°C	Hatching rate	LOEC = 160 µg/L
Cyproterone acetate GUISTI ET AL. (unpublished)	Adults, 21 days water exposure, 20 °C	Survival, Growth, Fecundity Reproduction: egg-abnormality	n.s. (›50 ng/L) LOEC = 4.5 ng/L
Deltametrhin DUCROT ET AL. (unpublished)	Embryos, 5 weeks, water exposure, 20°C	Hatching rate	LOEC = 0.05 µg/L
Diflufenican DUCROT ET AL. (unpublished)	Embryos, 5 weeks, water exposure, 20°C	Hatching rate	n.s. (›5 mg/L)
Dimetachlor DUCROT ET AL. (unpublished)	Embryos, 5 weeks, water exposure, 20°C	Hatching rate	LOEC = 6400 µg/L
Epoxiconazole DUCROT ET AL. (unpublished)	Embryos, 5 weeks, water exposure, 20°C	Hatching rate	LOEC = 1250 µg/L
Fenitrothion GUISTI ET AL. (unpublished)	Adults, 21 days water exposure, 20 °C	Survival, Growth, Fecundity Reproduction: egg-abnormality	n.s. (›240 µg/L) LOEC = 22 µg/L
Isoproturon DUCROT ET AL. (unpublished)	Embryos, 5 weeks, water exposure, 20°C	Hatching rate	LOEC = 100 µg/L
Mesosulfuron DUCROT ET AL. (unpublished)	Embryos, 5 weeks, water exposure, 20°C	Hatching rate	LOEC = 20 mg/L
Napropamide	Embryos, 5 weeks,	Hatching rate	LOEC = 6400 µg/L

In-house studies with *Lymnaea stagnalis* performed at INRA (F)			
Substance and reference	**Test duration**	**Endpoint**	**Effect concentration**
DUCROT ET AL. (unpublished)	water exposure, 20°C		
Prochloraze DUCROT ET AL. (unpublished)	Embryos, 5 weeks, water exposure, 20°C	Hatching rate	LOEC = 640 µg/L
Prosulfocarb DUCROT ET AL. (unpublished)	Embryos, 5 weeks, water exposure, 20°C	Hatching rate	n.s. (>15 mg/L)
Testosterone GUISTI ET AL. (unpublished)	Adults, 21 days water exposure, 20 °C	Survival, Growth, Fecundity Reproduction: egg-abnormality	n.s. (>110 ng/L) LOEC <2 ng/L
Tributyltin GUISTI ET AL. (unpublished)	Adults, 21 days water exposure, 20 °C	Survival Growth Feeding behavior Fecundity Reproduction: egg-abnormality	LOEC = 10 µg TBT/L LOEC = 1180 ng TBT/L LOEC = 1180 ng TBT/L LOEC = 540 ng TBT/L LOEC = 110 ng TBT/L
Vinclozolin GUISTI ET AL. (unpublished)	Adults, 21 days water exposure, 20 °C	Reproduction (egg-abnormality)	LOEC = 10 ng/L

2.4 Comparison and conclusion of the literature study

Tables 2 - 6 summarize EC_x, NOEC, and LOEC values of substances from peer-reviewed and in-house studies on *P. antipodarum* and *L. stagnalis*. The synopsis of these data reveals that only nine substances (see table 7) have been tested in both species and provide data for a comparison of effect concentrations. For the other chemicals listed under tables 2 - 6 endpoints and parameters investigated were not comparable.

Table 7: Effect concentrations of compounds tested in both *Potamopyrgus antipodarum* and *Lymnaea stagnalis*.

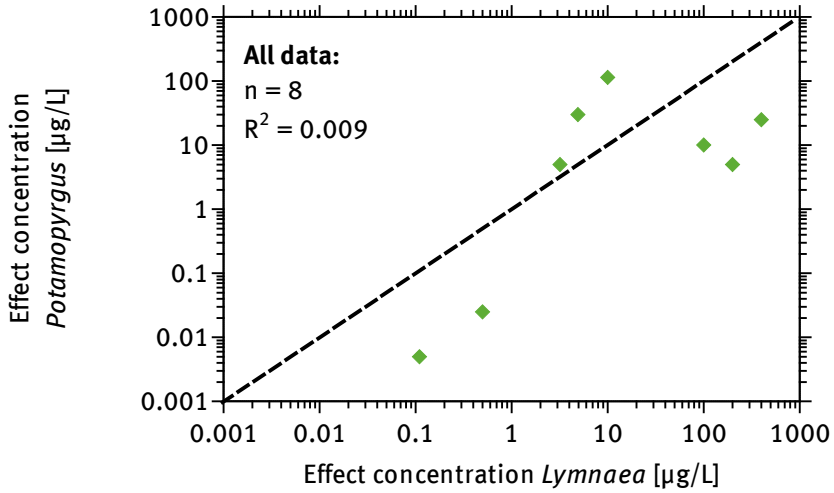
Compound	EDC?	Endpoint	Parameter	*P. antipodarum*	*L. stagnalis*
Bisphenol A	X	Reproduction	NOEC	1 µg/L	
			NOEC	5 µg/L	
			NOEC	20 µg/L	
			NOEC	20 µg/L	
			LOEC	5 µg/L	3.2 µg/L
			LOEC	10 µg/L	
			LOEC	40 µg/L	
			LOEC	100 µg/L	
			LC_{50}		5.94 µg/L
		Development	LC_{50}		0.71 µg/L
			LOEC		0.32 µg/L
Cadmium		Growth	EC_{50}	16 µg/L	142 µg/L
			LOEC		180 µg/L
		Survival	LOEC		180 µg/L
		Reproduction	LOEC	5 µg/L	200 µg/L

Compound	EDC?	Endpoint	Parameter	*P. antipodarum*	*L. stagnalis*
			LOEC	25 µg/L	400 µg/L
			LOEC		‹80 µg/L
			NOEC	1 µg/L	
			EC_{10}	1.3 µg/L	
			EC_{50}	11.5 µg/L	
		Development	LOEC		200 µg/L
Copper		Growth	EC_{50}	13 µg/L	
		Reproduction	LOEC	30 µg/L	4.9 µg/L
			NOEC		2.3 µg/L
			EC_{20}		1.8 µg/L
		Mortality	EC_{20}		5.6 µg/L
			LOEC		7.0 µg/L
			NOEC		4.0 µg/L
EE_2	X	Reproduction	LOEC	25 ng/L	0.5 µg/L
			LOEC	50 ng/L	
			NOEC	5 ng/L	
			NOEC	25 ng/L	
		Growth	LOEC		0.5 µg/L
		Development	LOEC		1.0 µg/L
Irgarol		Reproduction	NOEC	‹ 0.05 µg/L	0.32 µg/L
			LOEC	0.05 µg/L	0.8 µg/L
4-tert-Nonylphenol	X	Reproduction	LOEC	10 µg/kg	100 µg/L
Tributyltin	X	Reproduction	EC_{10}	0.98 µg/kg	
			EC_{10}	10.6 µg/kg	
			EC_{50}	45.8 µg/kg	0.38 µg/L
			EC_{50}	173 µg/kg	
			LOEC		100 µg/L
			LOEC		540 ng TBT/L
			LOEC		110 ng/L
			TC/LOEC	5 ng/L	2.0 µg/L
		Development	NOEC		0.01 µg/L
			LOEC		1.0 µg/L
		Growth	NOEC		0.01 µg/L
			LOEC		1.0 µg/L
			LOEC		1.18 µg/L
		Mortality	NOEC		0.01 µg/L
			LOEC		1.0 µg/L
			LOEC		10 µg/L
		Population Growth Rate	NOEC		2.75 µg/L
		Feeding behav-	LOEC		540 ng/L

Compound	EDC?	Endpoint	Parameter	*P. antipodarum*	*L. stagnalis*
		ior			
Vinclozolin	X	Reproduction:	LOEC	114 ng/L	10 ng/L
			LOEC		0.025 µg/L
			LOEC		250 µg/L
		Survival	LOEC		2500 µg/L
		Feeding behaviour	LOEC		250 µg/L
			LOEC		250 µg/L
Zinc		Growth	EC_{10}		672 µg/L
			EC_{50}	103 µg/L	964 µg/L

Based on the data in table 7, LOECs of BPA, Cd (2x), copper, EE_2, irgarol, 4-NP, TBT and vinclozolin for *P. antipodarum* and *L. stagnalis* were correlated using the statistical computer program GraphPad Prism® (version 5.0, GraphPad Software, San Diego, CA, USA) and are visualised in figure 2.

Figure 2: *Potamopyrgus antipodarum* and *Lymnaea stagnalis*. Correlation of effect data (LOECs) for bisphenol A, cadmium (2x), copper, 17α-ethinylestradiol, irgarol, 4-tert-nonylphenol, tributyltin and vinclozolin.



The correlation (Fig. 2) demonstrates that *P. antipodarum* tends to respond slightly more sensitive towards exposure of identical substances compared with *L. stagnalis*. However, a sound analysis would require more data to allow for a final opinion.

3 Second project part: Optimisation of culture conditions

3.1 Effects of different test media

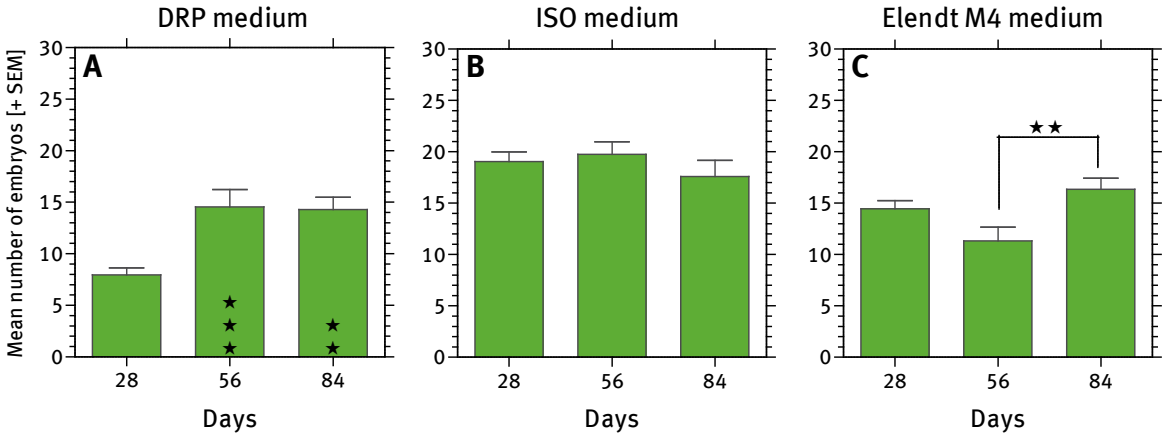
A long-term maintenance of a stable laboratory culture of *Potamopyrgus antipodarum* is an important prerequisite for the development of an OECD standard test system. To optimise the culture conditions of the snails, investigations on three different test media were performed in 2011. Results are published by SIERATOWICZ ET AL. (2013).

In a first test, snails were kept for 84 days in either ISO medium, Elendt M4 medium (OECD 2004) or in DRP medium (OECD 2010), which is routinely used for the culturing of snails at Goethe University. For each medium three replicates with 30 snails each were used. Animals were not acclimatized before being introduced into the ISO and Elendt M4 media. Medium was renewed twice per week and snails were fed three times per week with TetraPhyll®. Test vessels were changed after 4 and 8 weeks of exposure. In time intervals of 4 weeks, 10 snails per replicate were removed and embryo numbers were counted.

Figure 3 shows the mean embryo numbers per female after exposure to DRP, ISO and Elendt M4 medium. The mean number of embryos at the beginning of the test was 13.8 (n = 20). After 28 days, snails maintained in DRP medium (Fig. 3A) exhibited a mean embryo number of 7.97, which significantly increased to 14.6 and 14.3 after 56 and 84 days, respectively. Snails in ISO medium (Fig. 3B) exhibited mean embryo numbers between 17.6 and 19.8, which did not vary significantly over the duration of the test. Snails maintained in Elendt M4 medium (Fig. 3C) had a mean embryo number of 14.5 on day 28 which decreased to 11.3 on day 56. On day 84 mean embryo numbers increased to 16.4, which is significantly higher compared to the embryo numbers on day 56. After 4 and 8 weeks, embryo numbers between media differed significantly. On day 28, snails kept in ISO medium and Elendt M4 medium exhibited significantly more embryos compared to snails maintained in DRP medium. After 8 weeks of exposure, snails of the ISO medium group had significantly more embryos in the brood pouch compared to snails kept in Elendt M4 or DRP medium. On day 84, embryo numbers of snails cultured in the different media were not significantly different from each other.

Overall, it turned out that the Elendt M4 medium was more labour-intensive to prepare, exhibited a lower durability and test vessels filled with Elendt M4 developed more biofilm. Consequently, this medium is less suitable for culturing and testing of *P. antipodarum* compared to DRP and ISO medium.

Figure 3: Total embryo numbers (mean with standard error) of *Potamopyrgus antipodarum* after 28, 56 and 84 days in DRP medium (A), ISO medium (B) and M4 medium (C) (3 replicates with 30 snails each). Asterisks indicate significant differences (Bonferroni´s test), ★★ = p ‹ 0.01 ★★★ = p ‹ 0.001.

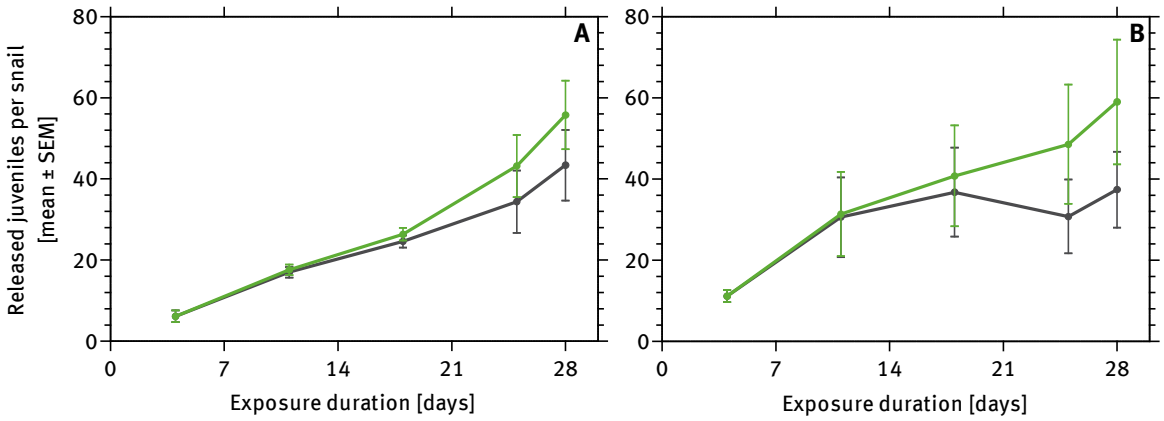


Modified according to SIERATOWICZ ET AL. (2013)

A second test was performed to assess the influence of DRP and ISO medium on the development and growth of juvenile snails. Adult snails with a shell length between 3.7 - 4.1 mm were acclimatized to ISO medium. For testing 5 replicates with 10 snails each were used. Snails were maintained in glass cuvettes with 300 mL of DRP or ISO medium. Twice a week 50% of the medium was exchanged by new medium and snails were fed with TetraPhyll®. By means of a stereo microscope neonates were counted and mortality was recorded once a week.

Figure 4 shows the number of released (green line) and survived (grey line) juvenile snails. Within the first three weeks, the number of offspring in ISO medium (Fig. 4B) was higher compared to DRP medium (Fig. 4A). After 4 weeks exposure the mean number of released juvenile snails in DRP medium and ISO medium was 55.8 and 59.0, respectively. Here, 77.8% and 63.4% survived in DRP and ISO medium, respectively.

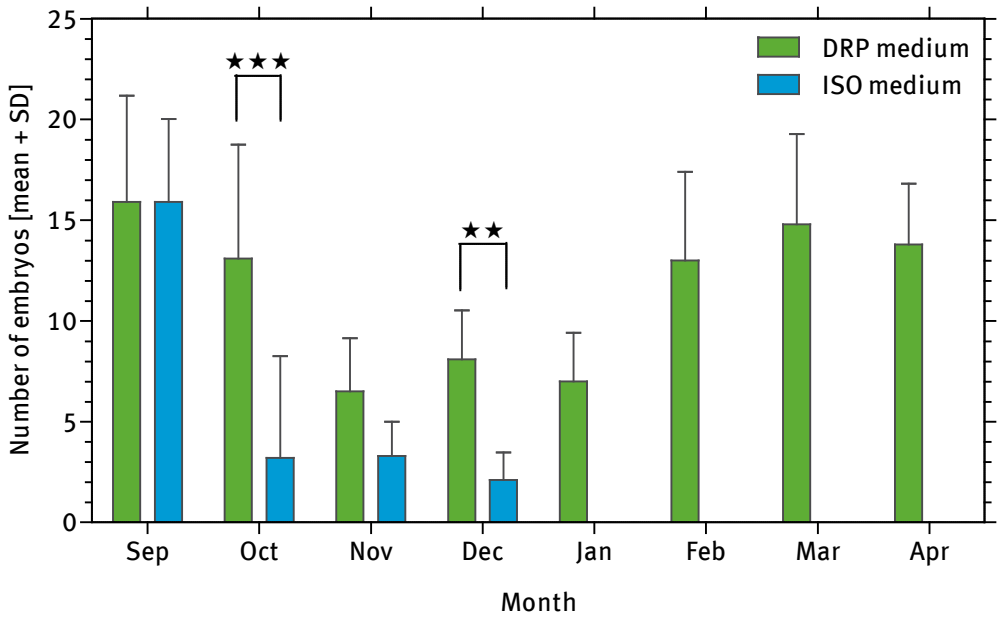
Figure 4: Released (green line, mean with standard error) and surviving (grey line) juveniles of *Potamopyrgus antipodarum* over 28 days exposure to DRP medium (A) and ISO medium (B). n = 5 replicates.



In August 2011 specimens of *P. antipodarum* were collected in the river Lumda, near Rabenau, and were introduced into the laboratory. 600 to 700 adult snails (shell length between 3.0 and 4.2 mm) were transferred in glass aquaria either filled with DRP or ISO medium. They were maintained under culture conditions. A partial renewal of the water was done three times per week and the snails were fed with TetraPhyll® *ad libitum*. In a monthly time interval, 10 snails each were removed and the mean embryo number per female was assessed.

Figure 5 shows the mean embryo numbers in DRP medium (September 2011 - April 2012) and in ISO medium (September 2011 - December 2011). After 4 weeks of exposure (September 2011) the mean number of embryos was 15.9 in both media. Over the course of the year, a seasonal variation of embryo numbers in DRP medium occurred with a typical decrease in autumn and winter months and an increase in spring. Already SIERATOWICZ ET AL. (2011) observed a seasonal reproduction cycle in a laboratory culture of *P. antipodarum*. Compared to DRP medium, snails maintained in ISO medium show a significant reduction in reproduction. The mean embryo number in December was 2.1 whereas 8.1 embryos were found in the brood pouch of snails in DRP medium. As this value is already under the validity criteria of 5 embryos per female, the culturing in ISO medium was stopped.

Figure 5: Total embryo numbers (mean + standard deviation) of *Potamopyrgus antipodarum* in culture per month, maintained in DRP medium (green) or ISO medium (blue). Asterisks indicate significant differences (unpaired t-test), ★★ = p ‹ 0.01 ★★★ = p ‹ 0.001. n = 10 snails.



The results indicate that ISO medium seems to be not suitable for culturing and testing of *P. antipodarum*. A possible reason could be the composition of 4 salts solved in deionised water. In contrast to this, DRP medium contains Tropic Marin® sea salt, which provides over 70 trace elements according to the manufacture's specification. This circumstance may prevent deficiency symptoms of the adult and juvenile snails.
Therefore, the use of ISO medium for culturing and testing of snails is not advisable. Instead, the DRP medium should be applied.

3.2 Reproduction of *Potamopyrgus antipodarum* during the course of the year in laboratory culture

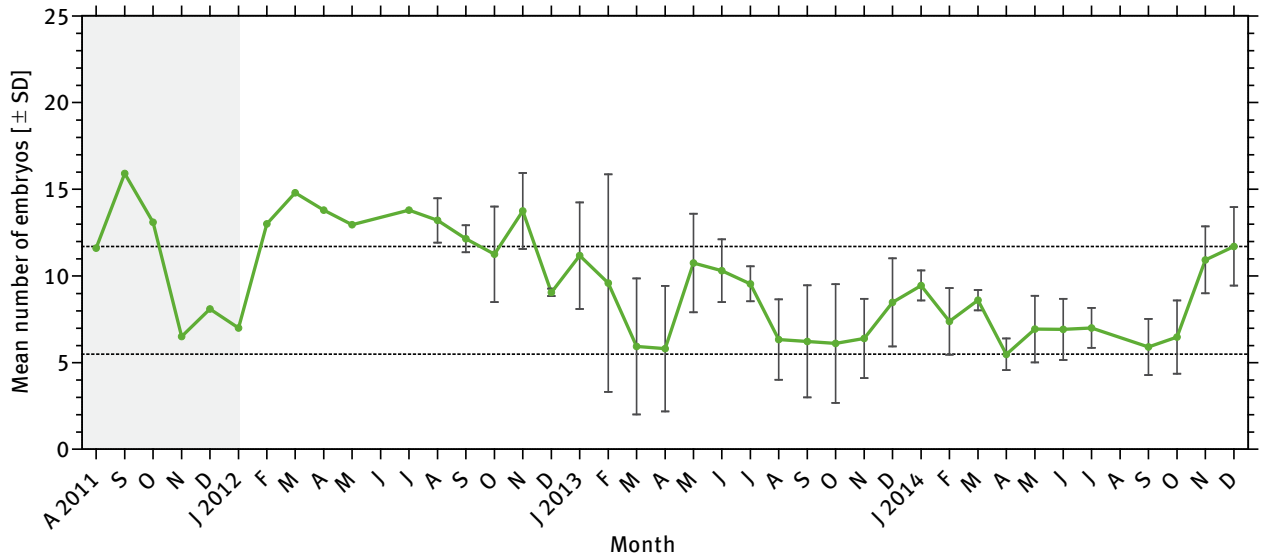
Studies on the influence of temperature and photoperiod on the reproduction of *P. antipodarum* were conducted during the course of the year to optimise culturing conditions. Besides to the standard conditions with 16°C and a light:dark regime of 16:8 h described in the DRP (OECD 2010), snails were acclimatized to a temperature of 20°C with a light:dark regime of 16:8 h. Furthermore, snails were acclimatized to a equinoctial photoperiod (12:12 h) and to a short-day exposure with a light:dark regime of 8:16 h, both at 16°C to investigate the influence of the photoperiod on the reproduction.

The mean embryo numbers of *P. antipodarum* cultured under standard conditions (16°C, 16:8 h light:dark) were monthly recorded over more than 3 years and are shown in figure 6. The snail culture was transferred into a new animal building of Goethe University Frankfurt in January 2012, characterized by different background colours in figure 6 (until January 2012 grey, white after January 2012). From August to November 2011 a season related variation of

embryo numbers is visible. In September 2011 a maximum of 15.9 embryos was observed. Afterwards the embryo numbers decreased in winter months to a minimum of 6.5 embryos per female (November 2011). This circadian reproductive pattern was already observed and described by SIERATOWICZ ET AL. (2011).

After moving the snail culture to the new location, an increase of the embryo numbers was observed until March 2012. The seasonal variation in reproduction until February 2012 may be caused by the climate chamber in the old animal house. This room was provided with a window, so that the snails were not only exposed to the constant and artificial lighting regime (16:8 h light:dark) but also to the natural irradiation. As a consequence, snails showed a seasonal variation in reproduction. This fluctuation of embryo numbers disappeared gradually after the snails were moved to the new building. The new climate room had no windows, so that the snails were not influenced by the natural light:dark rhythm. Hereby, an external influence on the snails could be prevented. Since December 2012, the average embryo numbers ranges between a minimum of 5.48 in April 2014 and a maximum of 11.7 in December 2014. Consequently, a seasonal variation in reproduction of the snails was not observed any longer.

Figure 6: Total embryo numbers (mean values; standard deviation for the period between August 2012 and December 2014) of *Potamopyrgus antipodarum* at a temperature of 16°C and a light:dark regime of 16:8 h from August 2011 to December 2014 (n = 1 - 4 replicates). Dashed lines mark the range of the minimum and maximum achieved embryo numbers between the years 2013 and 2014. Light grey background marks the laboratory culture at the old location and the white background shows the culture in the climate chamber at the new location of Goethe University Frankfurt.



Additionally, the reproduction during the course of a year of field collected animals was investigated and is presented in figure 7. Animals were collected in the small streams Lumda (near Rabenau, Hesse) and Kalbach (near Frankfurt am Main, Hesse). The seasonal variation in reproduction is obviously stronger compared to the results from the laboratory cultured snails

(Fig. 6), with high embryo numbers in spring and summer months and a decline in winter. Here, the mean embryo number ranges between 4.70 (November 2014) and 17.1 (August 2014).

Figure 7: Total embryo numbers (mean with standard deviation) of *Potamopyrgus antipodarum* collected in the field. From November 2013 to August 2014 animals were obtained from Lumda (near Rabenau, Hesse) and from September to December 2014 animals were taken from Kalbach (near Frankfurt am Main, Hesse) (n = 4 replicates). Dashed lines mark the range of the minimum and maximum achieved embryo numbers between the years 2013 and 2014 in the laboratory culture under standard conditions (16°C, 16:8 h light:dark).

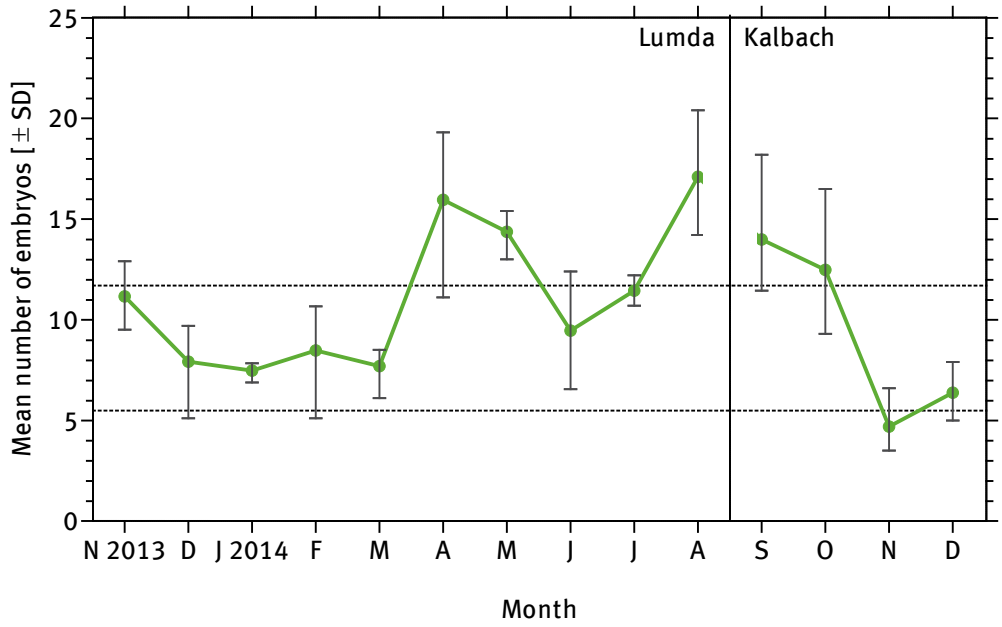


Figure 8 shows the results of the monthly recording of embryo numbers per snail at a temperature of 20°C and a photoperiod of 16:8 h (light:dark). By comparison with snails cultured at 16°C, reproduction is not increased at a higher temperature. During the investigated period the mean number of embryos varies between 5.2 (November 2014) and 11.6 (May 2014). Thus, values lie within the reproduction range of snails maintained at standard conditions (dashed lines in Fig. 8). Here again no seasonal variation in reproduction is perceptible.
Besides that, a higher mortality of juvenile snails was observed at 20°C compared to the culture of 16°C. Therefore, a permanent breeding of *P. antipodarum* is not feasible at a temperature of 20°C. Furthermore in several studies it has been demonstrated, that temperature impacts the sensitivity of snails towards different toxicants. MØLLER ET AL. (1994) performed 48 h acute toxicity tests with *P. antipodarum* and Cd at 3 different temperatures (5°C, 15°C, 20°C) and found that mortality increased with increasing temperatures. In addition, it has been also found that temperature is a reproduction modulation factor with the highest number of embryos at 16°C compared to other tested temperatures (GUST ET AL. 2011a, SIERATOWICZ ET AL. 2011).

Figure 8: Total embryo numbers (mean with standard deviation) of *Potamopyrgus antipodarum* at a temperature of 20°C and a light:dark regime of 16:8 h from September 2013 to December 2014 (n = 4 replicates). Dashed lines mark the range of the minimum and maximum achieved embryo numbers between the years 2013 and 2014 in the laboratory culture under standard conditions (16°C, 16:8 h light:dark).

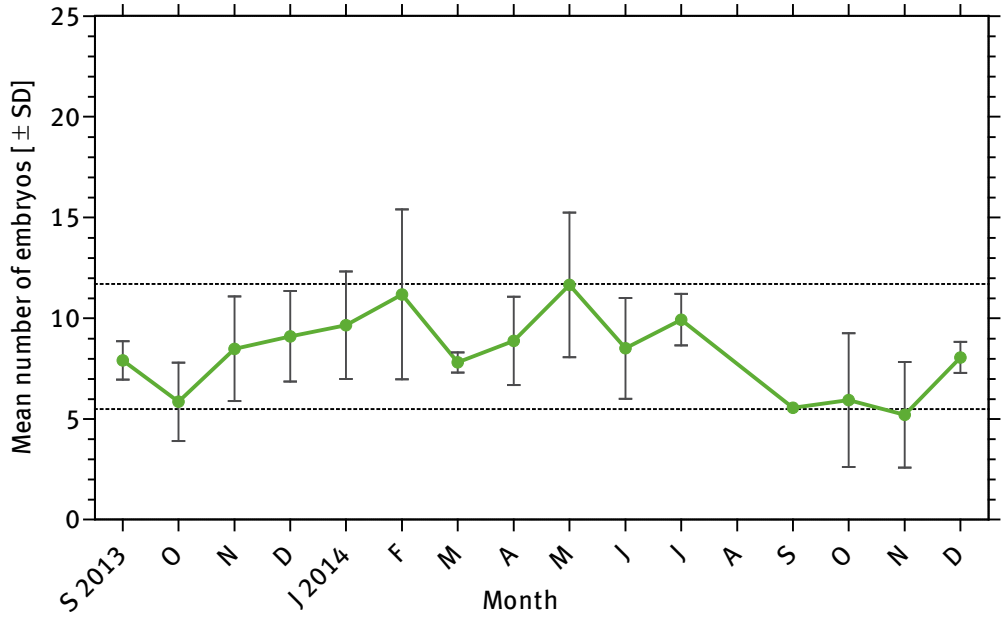
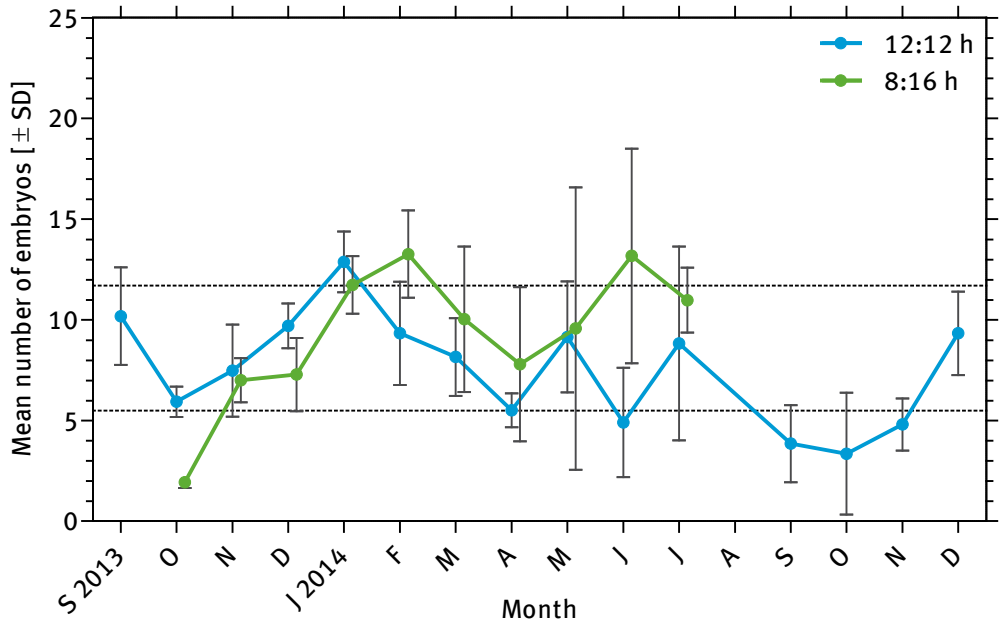


Figure 9 shows the results of the monthly analyses of embryo numbers from snails maintained at 16°C and different photoperiods. The blue line represents the reproduction of snails at equinoctial conditions (12:12 h light:dark). Here, embryo numbers vary between 3.35 (October 2014) and 12.9 (January 2014). In comparison to standard conditions, the reproduction range is greater, but also no seasonal variation, as observed by SIERATOWICZ ET AL. (2011), is noticeable. The culturing of *P. antipodarum* under equinoctial conditions seems to be inappropriate, as embryo numbers in some cases were below 5 as one of the validity criteria of the reproduction test, indicating a poor health status of the snails.

The green line in figure 9 represents the monthly recording of embryo numbers of snails maintained under short-day conditions. The low value in October 2013 is most probably an outlier, because snails were introduced into new glass aquaria without an existing biofilm on the glass walls. Thus, the low reproduction may be due to the lack of this biofilm which provides additional nourishment for the snails. Also under short-day conditions no seasonal variation of reproduction could be observed over the course of the year. Additionally, within replicates the variability increases with exposure duration and also a high mortality of the snails was observed. Therefore, the keeping of snails at short-day conditions is not recommended. It seems that short-day conditions have a direct or indirect influence on the fitness of the snails, so that culturing of *P. antipodarum* under these conditions is not feasible.

Figure 9: Total embryo numbers (mean with standard deviation) of *Potamopyrgus antipodarum* at a temperature of 16°C and a light:dark regime of 12:12 h (blue line from September 2013 to December 2014) and 8:16 h (green line from October 2013 to July 2014) (n = 4 replicates). Dashed lines mark the range of the minimum and maximum achieved embryo numbers between the years 2013 and 2014 in the laboratory culture under standard conditions (16°C, 16:8 h light:dark).



The results support the culturing conditions already described in the DRP (OECD 2010). While testing in dark is an option, for photo-labile substances, a long-term culturing under short-day or equinoctial conditions is not recommendable. Also the temperature of 20°C does not seem to be suitable, as a high juvenile mortality was observed under these conditions.

3.3 Summary: Optimisation of culturing conditions

To investigate the suitability of different test media, snails were kept for 84 days in either ISO medium, Elendt M4 medium (OECD 2004) or in DRP medium (OECD 2010). In time intervals of 4 weeks, the reproductive output of the snails was examined. On day 28, snails kept in ISO medium and Elendt M4 medium exhibited significantly more embryos compared to DRP medium. After 8 weeks, snails maintained in ISO medium had significantly more embryos than those kept in DRP or Elendt M4 medium. After 84 days, the embryo numbers of snails cultured in the different media were not significantly different from each other. However, Elendt M4 medium was more labour-intensive to prepare and vessels filled with this medium developed more biofilm compared to the other media. Therefore, Elendt M4 medium is less suitable for culturing and testing with *P. antipodarum*. In a second test, the influence of DRP medium and ISO medium on the development and growth of juvenile snails was investigated. Over 28 days neonates were counted and mortality was recorded once a week. The number of released juvenile snails

was comparable in both media, but the mortality of neonates maintained in ISO medium was higher than in DRP medium.

Besides these tests field collected snails were acclimatized either to DRP medium or ISO medium for a longer duration. In a monthly time interval, 10 snails each were removed and the mean embryo number per female was assessed. After 4 weeks of exposure the mean number of embryos was similar in both media. However, after four months of exposure the embryo numbers in ISO medium showed up to a 3.9-fold decrease compared to the DRP medium. These results indicate that both ISO medium and Elendt M4 medium are less suitable for the culturing of *P. antipodarum* and consequently DRP medium is proposed as the sole medium in the draft test guideline for the reproduction test.

Studies on the influence of temperature and photoperiod on the reproduction of field collected *P. antipodarum* were conducted during the course of the year to optimise culturing conditions. Besides to the standard conditions with 16°C and a light:dark regime of 16:8 h described in the DRP (OECD 2010), snails were acclimatized to a temperature of 20°C with a light:dark regime of 16:8 h. Furthermore, snails were acclimatized to a equinoctial photoperiod (12:12 h) and to a short-day exposure with a light:dark regime of 8:16 h, both at 16°C. While in the field a seasonal variation of the embryo numbers is known, reproductive fluctuations disappeared gradually over the time at standard conditions in the laboratory. The average embryo numbers ranges between a minimum of 5.48 in April 2014 and a maximum of 11.7 in December 2014. Consequently, a seasonal variation in reproduction of the snails under culture conditions was not observed any longer.

The culturing of snails at a temperature of 20°C and a photoperiod of 16:8 h light:dark resulted in a comparable pattern like for snails maintained under standard conditions. Besides that, a higher mortality of juvenile snails was observed at 20°C compared to 16°C. Therefore, a permanent breeding of *P. antipodarum* at 20°C is not feasible and should not be recommended in the draft test guideline. The culturing of *P. antipodarum* under equinoctial conditions seems to be inappropriate, as embryo numbers in some cases were below 5 as one of the validity criteria of the reproduction test, indicating a poor health status of the snails. Under short-day conditions (8:16 h light:dark) no seasonal variation of reproduction was observed over the course of the year. Additionally, within replicates the variability increases with exposure duration and also a high mortality of the snails was observed. Therefore, the keeping of snails at short-day conditions is not recommended. Short-day conditions seem to compromise the fitness of the snails, so that culturing of *P. antipodarum* under these conditions is not feasible.

The results support the culturing conditions described in the DRP (OECD 2010). Culturing and testing of the snails should be done in DRP medium at a temperature of 16°C and under long-day conditions (16:8 h light:dark). These conditions are recommended in the draft test guideline.

4 Third project part: Pre-validation

4.1 Optimisation of test conditions

4.1.1 Density effects on the reproduction of *Potamopyrgus antipodarum*

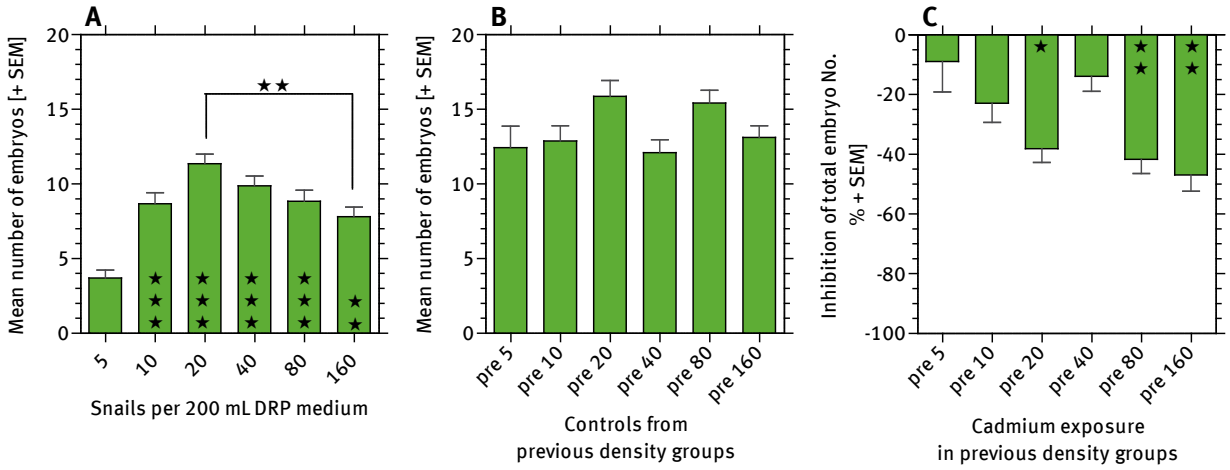
Test conditions such as temperature, day length, and food quality or population density are sensitive parameters, which can significantly alter the endpoint of interest, e.g. reproduction. It has been shown that the embryo production of *Potamopyrgus antipodarum* is affected by the number of coexisting parthenogenetic females (NEIMAN 2006, NEIMAN ET AL. 2013).

Tests on the effects of different snail densities on the reproduction of *P. antipodarum* have been performed within the project in 2011 and are published by SIERATOWICZ ET AL. (2013). For the first part of the experiment, groups of 5, 10, 20, 40, 80 and 160 snails per 200 mL DRP medium (4 replicates each, 8 replicates for the lowest density group) were maintained over 4 weeks. On termination of the first phase of the experiment, embryos were counted in 10 snails per replicate (5 in the lowest density group for 4 of the 8 replicates). Remaining snails were immediately exposed towards Cd (12 µg/L). Density stress was replaced by Cd exposure and a reproduction test was performed as described above in the second phase of the experiment (section 1.3). After 28 days of exposure the embryo numbers per snail were assessed. In a second test, the optimal snail density for testing was investigated. For this purpose 4, 10, 40 or 80 snails per 800 mL medium were maintained over 12 weeks. Embryo numbers per female were assessed after 4, 8 and 12 weeks.

Figure 10 shows the results of snails kept in the density stress experiment with a following Cd exposure. Figure 10A provides the mean embryo numbers in the different density groups. Only 3.70 embryos were found in the brood pouch of snails maintained at the lowest density. In all other tested groups the embryo numbers were significantly higher compared to 5 snails/200 mL medium. The highest embryo numbers (11.4) were observed at the group of 20 snails/200 mL medium. These findings are in line with results from NEIMAN ET AL. (2013). The authors found a higher reproductive output at higher densities compared to low density conditions. The authors suggested that these positive effects are probably caused by a waterborne substance produced by *P. antipodarum*.

Figures 10B and 10C show the mean number of embryos after a subsequent maintenance in control medium or in medium with 12 µg Cd/L. Control embryo numbers of snails taken from different density populations did not differ significantly. In contrast, snail densities affected the sensitivity for Cd in the subsequent reproduction test. The results indicate that the sensitivity increases with snail density in the pre-exposure experiment. A significant higher reduction of embryo numbers was observed in snails taken from the 20, 80 and 160 snails/200 mL medium.

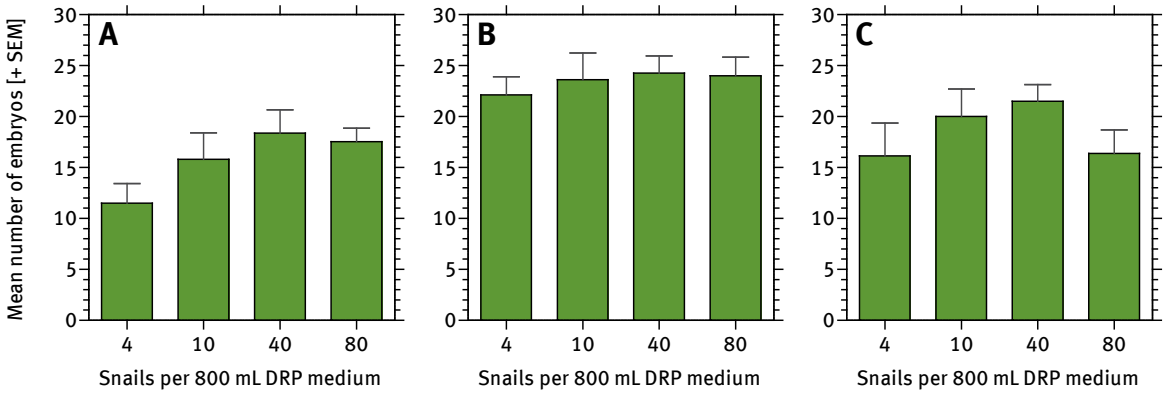
Figure 10: Total embryo numbers (mean with standard error) of *P. antipodarum* after 4 weeks in DRP medium at varying snail densities (A), subsequent 4 weeks exposure of 10 control snails from respective previous density groups (B) and inhibition of total embryo numbers (% with standard error) relative to the respective control shown in (B) after 4 weeks exposure to 12 µg Cd/L (C). Asterisks indicate significant differences to the lowest density group (Bonferroni's test). ★ = p ‹ 0.05, ★★ = p ‹ 0.01 ★★★ = p ‹ 0.001, n = 4 replicates.



Modified according to SIERATOWICZ ET AL. (2013)

Figure 11 illustrates the results of the density experiment over 12 weeks. Here, no significant differences between the varying density groups could be observed after 4 (Fig. 11A), 8 (Fig. 11B) or 12 weeks (Fig. 11C) exposure.

Figure 11: Total embryo numbers (mean with standard error) of *P. antipodarum* kept in DRP medium for 4 (A), 8 (B) and 12 (C) weeks at different densities. n = 4 replicates.



Modified according to SIERATOWICZ ET AL. (2013)

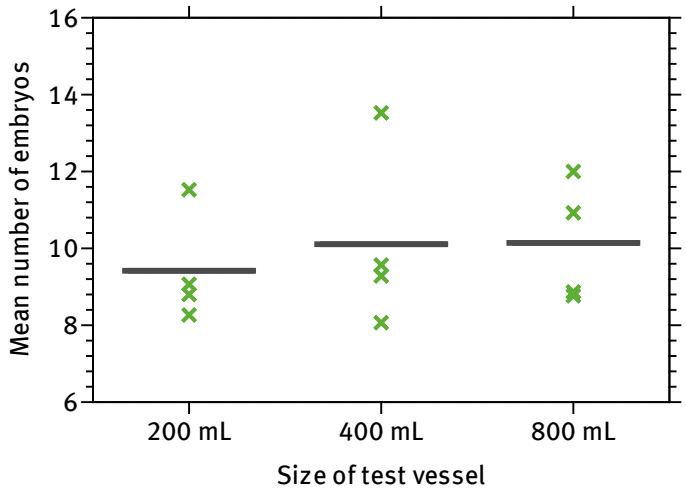
These findings demonstrate that the parameter density is able to alter the reproduction of *P. antipodarum* and that a prior high density of snails could enhance the sensitivity to a toxicant. To avoid density stress, during culturing a maximum density of 100 snails/L should not be exceeded.

4.1.2 Effects of test vessel size on the reproduction of *Potamopyrgus antipodarum*

A reproduction test with three different types of test vessels was conducted to investigate the influence of different volumes and densities on the reproduction of *P. antipodarum*. The use of smaller test vessels would reduce the consumption of medium, chemicals and space for testing.

In addition to the recommended glass vessels containing 800 mL medium smaller vessels filled with 400 mL and 200 mL were tested according to the DRP (OECD 2010). After 28 days the embryo numbers of the snails were assessed. The results are presented in figure 12. The mean embryo numbers did not differ significantly between treatments. These results indicate that the chosen test vessels and the resulting densities do not influence the reproduction in the test. These results confirm the outcome of the study conducted by SIERATOWICZ ET AL. (2013) suggesting to reduce the medium volume in the reproduction test with *P. antipodarum* to 400 mL. However, smaller test vessels may adsorb a higher fraction of the test substance due to a bigger surface-volume relationship. So the choice of the appropriate test vessels depends on the physicochemical properties of the substance. As a modification for the test conduct the volume of the test media may range between 200 mL and 800 mL depending on the physico-chemical properties of the test compounds. This has been proposed in the validation report submitted to OECD (OECD 2015a).

Figure 12: Total embryo numbers of *Potamopyrgus antipodarum* after 4 weeks in 200 mL, 400 mL and 800 mL media (crosses: replicate mean; line: treatment mean). n = 4 replicates with 10 - 15 snails each.



4.1.3 Study of reproductive effects of several solvents

To assess the influence of solvents on the reproduction on *P. antipodarum*, tests have been performed for the following solvents recommended by OECD (2000): acetone, methanol, ethanol, and triethylene glycol (TEG). Additionally, dimethyl sulfoxide (DMSO) was also tested. Snails were exposed to concentrations of 0.02, 0.1, 0.5, 2.5 and 12.5 mL/L in a 28-days reproduction test. The reproductive effect of glacial acetic acid was also investigated, because this solvent was planned to be used for the reproduction tests with tributyltin in validation 1 (see section 5.1). Glacial acetic acid was tested in a concentration range from 0.01 to 2.43 mL/L. After 28 days exposure the embryos in the brood pouch of the snails were counted and weighted means were calculated. Reproduction tests with acetone, methanol, ethanol, DMSO and glacial acetic acid were performed from 08.04.2013 - 03.05.2013 and for TEG test were conducted from 02.09.2013 to 30.09.2013 (for description of test design see section 1.3). The results of the reproduction tests are shown in figures 13 and 14.

None of the tested solvents affected mortality significantly, except for glacial acetic acid. Here, all exposed snails died at the three highest test concentrations (0.27, 0.81, 2.43 mL/L) due to a strong decrease of the pH in these exposure groups.

Figure 13A shows the influence of acetone on the number of total embryos after 28 days of exposure. Already at a concentration of 0.1 mL/L a significant reduction of the embryo numbers compared to control was observed. Only the lowest concentration (0.02 mL/L) did not show a significant effect on the reproduction. Also methanol had negative effects on the reproductive output of *P. antipodarum* (Fig. 13B), which was significantly reduced at concentrations between 0.1 and 2.5 mL/L. At the highest test concentration of 12.5 mL/L the toxic effect of methanol could only be observed by trend. In contrast, ethanol caused a reproductive-increasing effect at 0.1 and 0.5 mL/L (Fig. 13C). This phenomenon was also observed for *D. magna* by ZHANG & BAER (2000).

Figure 13: Total embryo numbers (weighted mean + standard deviation) of *P. antipodarum* after four weeks of exposure to acetone (A), methanol (B) and ethanol (C) (4 replicates with 10 snails each). Asterisks indicate significant differences compared to control (Dunnett´s test), ★ = p ‹ 0.05, ★★ = p ‹ 0.01 ★★★ = p ‹ 0.001.

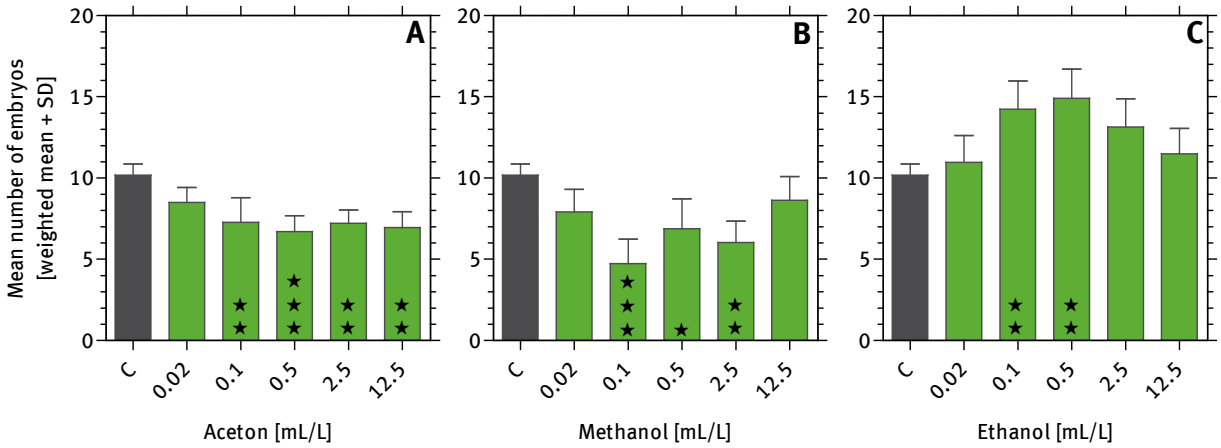


Figure 14 shows the results of the four weeks exposure of *P. antipodarum* towards DMSO, TEG and glacial acetic acid. DMSO caused a significant increase of the embryo numbers at 2.5 mL/L compared to the control group. The remaining concentrations of DMSO had no significant influence on the reproduction of the snails. Besides that, STANGE ET AL. (2012) demonstrated an influence of DMSO on the expression of the estrogen receptor (ER) orthologue in *P. antipodarum*. Because of this, DMSO should only be used in low concentrations with a maximum of 20 µL/L, as proposed by HUTCHINSON ET AL. (2006). Figure 14B shows the results of the test with TEG. Only at the highest test concentration of 12.5 mL/L a significant reduction of the embryos was observed. Therefore, the use of TEG in the reproduction test with *P. antipodarum* is recommended at lower concentrations. The exposure of *P. antipodarum* towards glacial acetic acid (Fig. 14C) up to a concentration of 0.09 mL/L had no effect on the reproductive output of the snails. Therefore, glacial acetic acid seems to be a suitable solvent in a low concentration range for the reproduction test with *P. antipodarum*.

Figure 14: Total embryo numbers (weighted mean + standard deviation) of *P. antipodarum* after four weeks of exposure to DMSO (A), TEG (B) and glacial acetic acid (C) (4 replicates with 10 snails each). Asterisks indicate significant differences compared to control (Dunnett´s test), ★ = p ‹ 0.05, ★★★ = p ‹ 0.001.

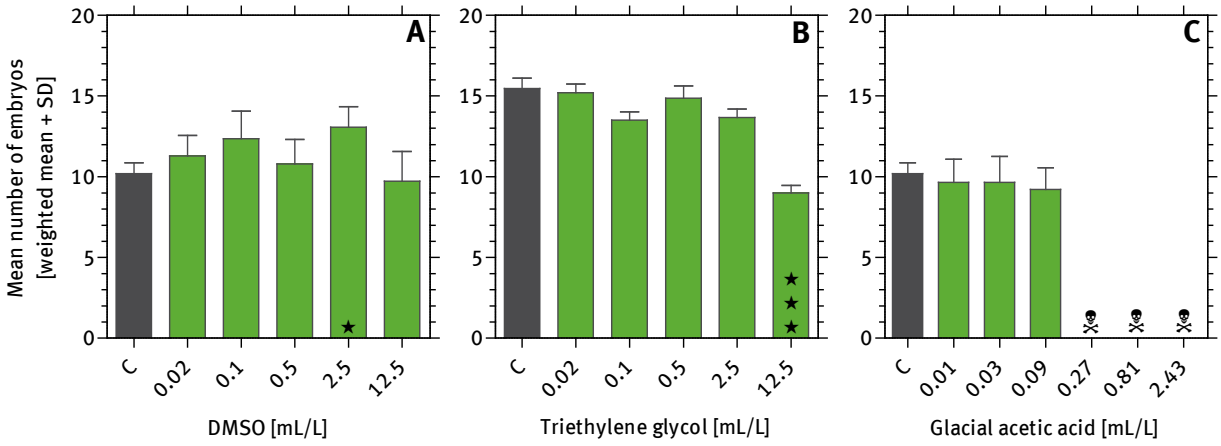


Table 8 summarizes the NOEC and LOEC values for the tested solvents. Because of their effects on reproduction of *P. antipodarum* at low concentrations, acetone, methanol and ethanol cannot be recommended as solvents for reproduction tests with this snail. However, DMSO, TEG and glacial acetic acid are well suited solvents for tests with *P. antipodarum*.

Table 8: NOEC and LOEC values for total embryo numbers of *Potamopyrgus antipodarum* after exposure to solvents in mL/L.

In µL/L	Acetone	Methanol	Ethanol	DMSO	TEG	Glacial acetic acid
NOEC	0.02	0.02	0.02	0.5	2.5	0.09
LOEC	0.1	0.1	0.1	2.5	12.5	-

4.1.4 Summary: Optimisation of test conditions

Temperature, day length, food quality and population density are sensitive parameters, which may significantly alter reproduction of test organisms in toxicity tests. In order to optimise test conditions, the influence of snail density, test vessel size and the effects of solvents on the reproductive output of *P. antipodarum* were investigated.

Density was able to alter the reproduction of the snails. The lowest embryo numbers were found under low density conditions. The highest reproductive output was observed at a density of 20 snails/200 mL. Replacing density stress by Cd exposure in a following reproduction test phase, control embryo numbers of snails taken from culture vessels with different snail densities did not differ significantly after 28 days. In contrast, snail densities affected the sensitivity towards Cd in the subsequent reproduction test. The results indicate that the sensitivity of

snails increases with snail density in the pre-exposure experiment. To avoid density stress, maximum culturing density should not exceed 100 snails/L.

According to the DRP (OECD 2010) three different sizes of test vessels containing 200 mL, 400 mL or 800 mL medium were tested. After 28 days the mean embryo numbers did not differ significantly between treatments. The choice of the appropriate test vessels should depend on the physicochemical properties of the substance when testing.

To find the most appropriate solvent for testing with *P. antipodarum*, reproduction tests with the solvents acetone, methanol, ethanol, DMSO, TEG and glacial acetic acid were performed. Acetone, methanol and ethanol caused significant effects on the reproductive output of the snails and can therefore not be recommended.

Based on these results the draft test guideline should comprise a recommendation to culture the snails with a maximum density of 100 snails/L. Furthermore, as a modification for the test conduct the volume of the test media may range between 200 mL and 800 mL depending on the physico-chemical properties of the test compounds. This has been proposed in the validation report submitted to OECD (OECD 2015a). The use of a solvent should be avoided whenever possible. DMSO, TEG and glacial acetic acid did not show significant effects on reproduction of *P. antipodarum* and should be recommended in the draft test guideline. The maximum solvent concentration should be 20 µL/L.

4.2 Necessity to distinct between shelled and unshelled embryos

According to DUFT ET AL. (2007) the number of unshelled embryos is the most sensitive endpoint in reproduction tests with *P. antipodarum*. However, several other studies did not confirm this hypothesis, including the results of the reproduction tests in validation I. Here the effect concentrations based on total embryo numbers were similar or even more sensitive compared to effect concentrations for the endpoint of unshelled embryos (SIERATOWICZ AND OEHLMANN 2011). An analysis of 58 reproduction tests conducted in the last 15 years at Goethe University revealed that in 36% of the reproduction tests there were no differences between the sensitivity of the total embryo number and the number of shelled or unshelled embryos, respectively. Only in 21% of the reproduction tests the number of unshelled embryos was more sensitive than the total number of embryos. In 19% of the reproduction tests the endpoint number of unshelled embryos was less sensitive compared to the total number of embryos. The analysis of data also showed that in 19% of the reproduction tests the number of unshelled embryos did not differ from the results for the total number of embryos but the number of shelled embryos differed in sensitivity from the two other endpoints. The evaluation of this historical data did not provide a clear indication that the number of unshelled embryos increases the relative sensitivity of the reproduction test. Also participants in validation I found it difficult to distinguish between shelled and unshelled embryos. To avoid mistakes in interpreting the results and to simplify the conduct of the test it was recommended by the OECD mollusc expert group to assess the total embryo number as the sole reproductive endpoint within the test without distinguishing between shelled and unshelled embryos.

4.3 Identification of an ideal reproduction range

Effects of substances altering the reproductive output may be masked if the reproduction of control animals in test is particularly high or low (SIERATOWICZ ET AL. 2011). For this reason, an ideal reproduction range, where both reproduction-toxic and reproduction-increasing effects of chemicals can be assessed, should be defined within this project.

Several tests and studies have been performed to find an ideal reproduction range and will be presented in this section.

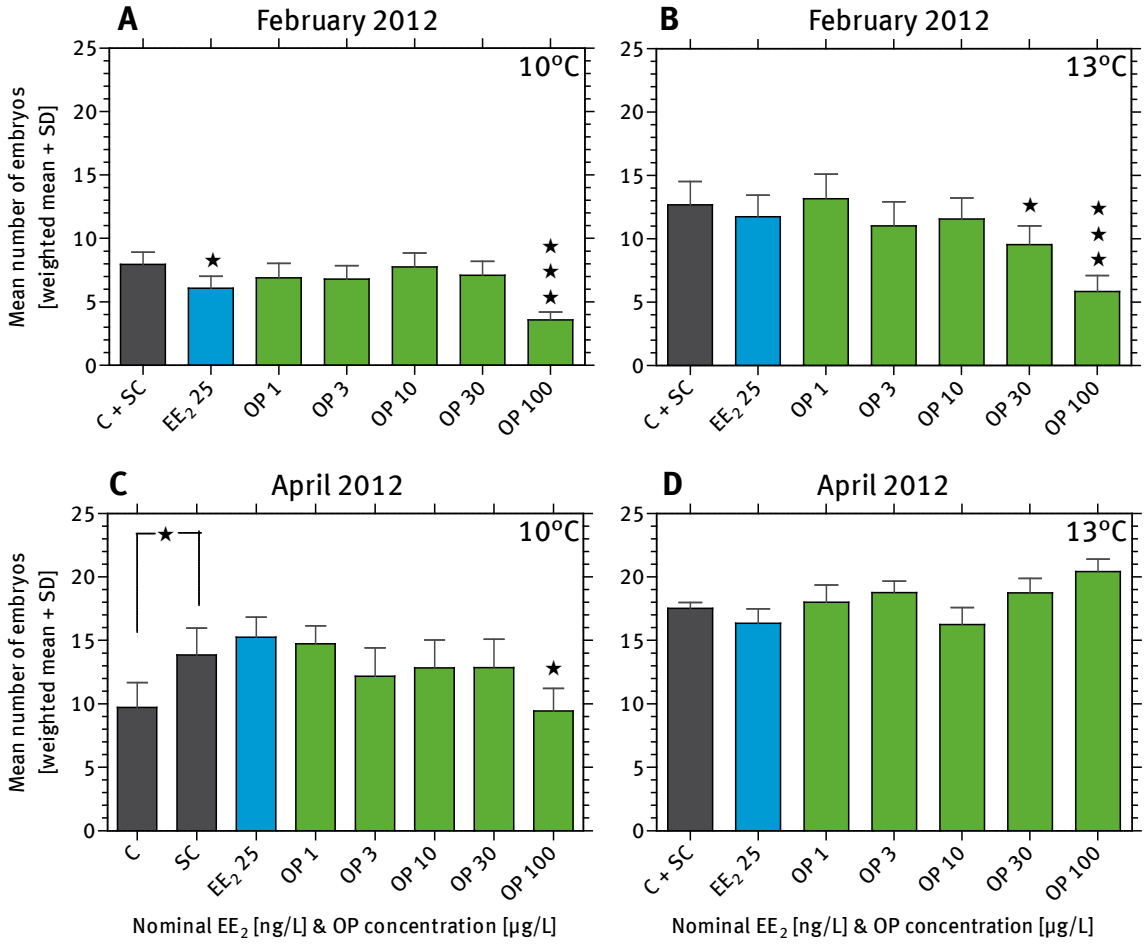
4.3.1 Reproduction of *Potamopyrgus antipodarum* at different temperatures

In January 2012, specimens of *P. antipodarum* were acclimatized to a temperature of 10°C and 13°C, respectively. The sensitivity of snails towards OP at these temperatures was determined during the course of a year and compared with the results of reproduction tests with OP under the standard temperature of 16°C (section 4.4.1).

Reproduction tests with *P. antipodarum* and OP were conducted in February and April 2012 at 10°C and 13°C. OP was tested at nominal concentrations of 1, 3, 10, 30 and 100 µg/L. 17α-ethinylestradiol (EE_2) was used as a positive control at a concentration of 25 ng/L (nominal). As DMSO was used as solvent, a solvent control (10 µL/L) was tested. The results of the reproduction tests are shown in figure 15.

In contrast to the results at 16°C in February 2012 (section 4.4.1, fig. 23B), no significant increase of embryo numbers in OP or EE_2 exposed snails could be detected at temperatures of 10°C (Fig. 15A) and 13°C (Fig. 15B), respectively. At 10°C a significant reduction at 25 ng EE_2/L and 100 µg OP/L was observed. Also at 13°C, the embryo numbers at the two highest test concentrations of OP were significant lower than in the control group.

Figure 15: Total embryo numbers of *P. antipodarum* after exposure to five concentrations of 4-tert-octylphenol (OP, green), 17α-ethinylestradiol (EE_2, blue) and merged solvent control with negative control (C + SC, grey) in February 2012 after 4 weeks at 10°C (A) and 13°C (B) and in April 2012 after 8 weeks at 10°C (C) and 13°C (D). Asterisks indicate significance compared to control and to solvent control (C) (Dunnett´s test), ★ = p ‹ 0.05, ★★★ = p ‹ 0.001. n = 4 replicates with 10 snails each.



At lower temperatures the metabolic rate of poikilothermic animals, like snails, is reduced and embryos develop slower in the brood pouch. To analyse if reproduction-increasing effects only occur at low temperatures after a longer exposure, the duration of the test conducted in April 2012 was prolonged to 56 days. Results of both tests are shown in figure 15C and 15D. At 10°C a significant difference between negative and solvent control occurred, which might be due to an accidental introduction of smaller snails into the negative control. Therefore, the embryo numbers in the exposure groups were referred to the solvent control for statistical analyses. As

already observed in February, no reproduction increasing effects occurred at 10°C or 13°C, respectively. A significant reduction of embryo numbers at 100 µg OP/L occurred at 10°C. Also these findings deviate from the results of the reproduction test with *P. antipodarum* conducted in April 2012 at 16°C (section 4.4.1, fig. 23C). Here, a significant increase was observed at OP concentrations of 3 and 10 µg/L. However, the lack of effects on reproduction should be treated critically, because a fungus growth occurred during the 13°C test, which may have affected reproduction of the snails.

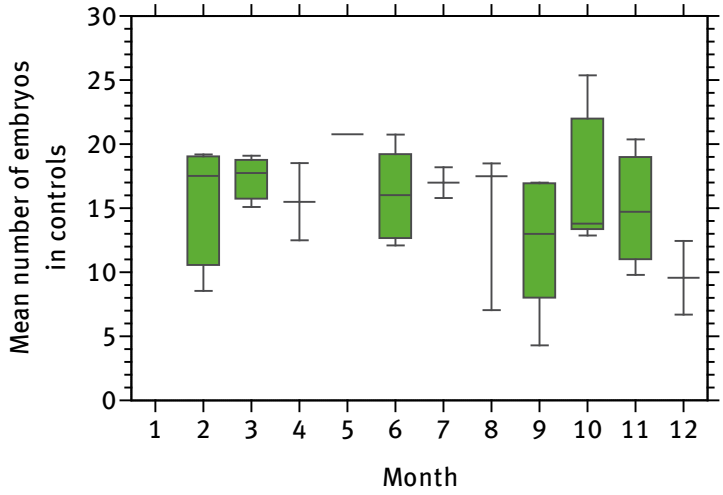
Because of difficulties with the maintenance of a stable culture at 10° and the fungus development at 13°C, it was not possible to conduct more reproduction tests with *P. antipodarum*. The results also underline that the culturing and testing at low temperatures is not feasible and the snails should be kept at 16°C. This temperature is close to the optimal temperature for this species (MØLLER ET AL. 1994, SIERATOWICZ ET AL. 2011).

4.3.2 Former in-house data of reproduction tests with *Potamopyrgus antipodarum*

Historical in-house data from Goethe University Frankfurt were analysed to determine an ideal reproduction range for testing. This data included grey literature (unpublished bachelor, master, diploma and PhD theses).

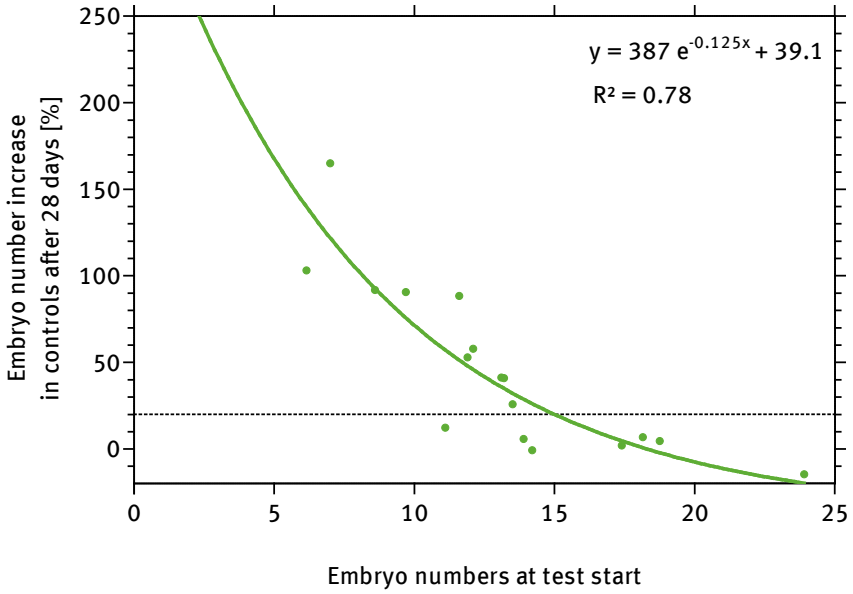
For the evaluation embryo numbers in control groups after a test duration of 28 days were considered. It was examined *inter alia* if a seasonal pattern is displayed over the course of a year under test conditions. The results of this analysis are shown in figure 16. Overall no clear seasonal pattern is discernible although a slight tendency of higher embryo numbers in late spring and early summer with a decrease in late autumn and winter is visible. In most of the tests control embryo numbers were between 10 and 20. However, it should be noted that different snail populations were used in the tests after different acclimation periods and with differing snail densities in the exposure vessels. Taken together, these results indicate that the season of a year does not have a major influence on the results of a reproduction test with *P. antipodarum* even if embryo numbers in the culture reared in the former animal house had exhibited seasonal variations (cf. chapter 3.2).

Figure 16: Mean number of embryos (boxplot with mean (horizontal line within box), 1st and 3rd quartiles (lower and upper box margins), minimum and maximum (whiskers) of *Potamopyrgus antipodarum* in the control groups of reproduction tests conducted at different time of the year. The numbers on the x-axis refer to the month of analysis (January to December).



A further result of the analysis was that the control embryo numbers were usually lower at test start (T_0) than 28 days later (Fig. 17). This observation was examined in detail by comparison of T_0 embryo numbers with the percentage increase of embryo numbers in control snails after four weeks. The correlation is highly significant with a coefficient of determination of 0.78 ($p < 0.001$). If embryo numbers were already high at test start, a further increase of embryo numbers during the course of the test was unlikely. The lower the embryo numbers were at T_0, the higher the embryo numbers increase after 28 days with a maximum increase of 160% in control snails. Various factors may have contributed to this effect. Different snail densities in the culture and during the test are probably one key factor (NEIMAN ET AL. 2013). Also time intervals of medium renewal and feeding rates for snails during the reproduction test and in the snail culture differed. To investigate the influence of the food, reproduction tests have been performed dealing with this issue (section 4.3.3).

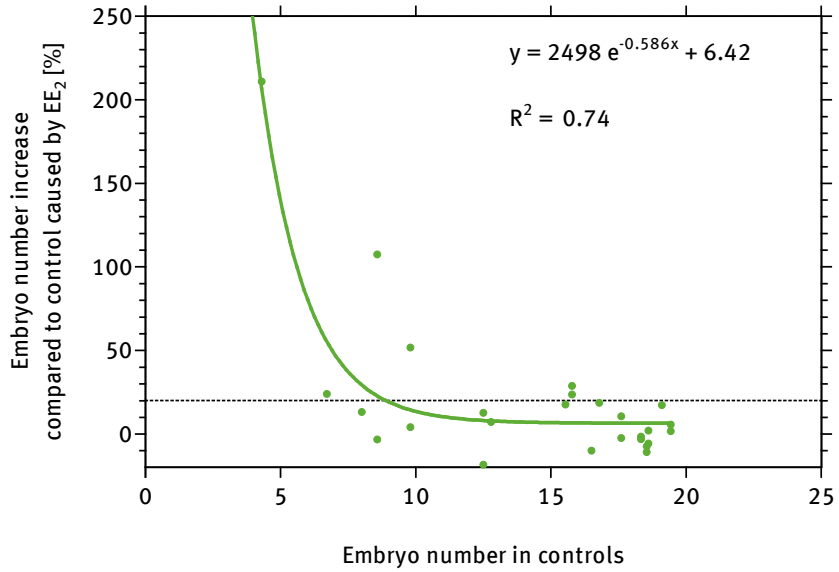
Figure 17: Increase in embryo numbers [%] of *Potamopyrgus antipodarum* in negative controls after 4 weeks in the reproduction test correlated to test start (T_0).



Besides the embryo numbers in negative controls, the positive control (EE_2) also showed considerable variations. Especially, if embryo numbers in the negative control are already high, a further and statistically significant increase under exposure to EE_2 is unlikely. The statistical power of the test (with 4 replicates and 10 snails per replicate) is 0.80, which means the minimum difference of embryo numbers which can be detected as statistically significant is 20% (dashed line in fig. 17 and 18). Therefore, embryo numbers of control snails and the relative increase of embryo numbers under exposure to 25 ng EE_2/L after 28 days was analysed by correlation (Fig. 18). The coefficient of determination was 0.74 and demonstrates a highly significant correlation ($p < 0.001$). This analysis indicates that it becomes increasingly unlikely to detect a significant increase of embryo numbers in snails under exposure to EE_2 if embryo numbers in the negative control exceed a mean value of 10.

The reproduction test with *P. antipodarum* is suitable to assess reproduction-toxic and reproduction-increasing effects of chemicals. However, the reproduction test is not suitable for proving an endocrine mediated mode of action solely on the basis of a decreased or increased embryo number. Further mechanistic information should also be considered to decide about e.g. adverse outcome pathways.

Figure 18: Correlation of the embryo numbers in negative controls of *Potamopyrgus antipodarum* and the reproductive increase caused by 17α-ethinylestradiol (EE_2). d = 28.



4.3.3 Influence of the food dose on the reproduction of *Potamopyrgus antipodarum* under chronic pollutant exposure

In order to optimise the test design of the reproduction test with *P. antipodarum*, a possible influence of different amounts of food on the reproductive output of the snails under chronic substance exposure was investigated. In several feeding experiments with gastropods, a correlation between food and reproduction could be ascertained (AUGUSTO ET AL. 2012, KEAS & ESCH 1997, TER MAAT ET AL. 2007). The aim of these studies was to identify the amount of food, which allows the determination of toxic as well as stimulating effects on the embryo numbers. Therefore, Cd (applied as Cd chloride) was used as substance to reduce reproduction output at nominal concentrations ranging from 1.56 to 25.0 µg/L and triclocarban (TCC) was used as potentially reproduction stimulating chemical at nominal concentrations between 0.10 and 10.0 µg/L. Reproduction tests were carried out with *P. antipodarum* in which a quarter (62.5 µg/snail x day), the full dose (250 µg/snail and day) and the double food dose (500 µg/snail x day) of TetraPhyll® proposed in the DRP (OECD 2010) were tested. Furthermore, body protein, glycogen and lipid contents were determined to investigate the influences of food dosage and pollutant exposure on the energy reserves of the snails (BRADFORD 1976, VAN HANDEL 1965, 1985). Also analytical measurements of water samples have been performed by chemlab GmbH, Bensheim. 73.2% and 26.4% of nominal concentrations for Cd and TCC were achieved (see table 72 and 73 in annex 8.1), respectively. Therefore, calculated effect concentrations are based on measured concentrations. The analytical measurements of TCC at the lowest test concentration (0.10 µg/L) were below the LOD (< 0.04 µg/L). Therefore, according to the recommendation of OECD (2000), the LOD (0.04 µg/L) was assumed for this exposure group to calculate effect concentrations.

The percentage increase of the reproduction in control groups of both reproduction tests compared to test start (T_0) are summarized in table 9. In all three feeding groups a significant increase of the embryo numbers was observed. This increase may be a result of differences in the supply of food between culturing and test conditions, but the influence of snail density and the frequency of medium renewal should also be taken into account. With these tests, an influence of the amount of food on the reproduction of *P. antipodarum* has been demonstrated. With a food dose of 62.5 µg/snail x day, in both tests the lowest increase of the embryo numbers in control snails was detected.

Table 9: Increase of the embryo numbers [%] of *Potamopyrgus antipodarum* in negative controls after 28 days in the reproduction tests with cadmium (test No. 1) and triclocarban (test No. 2). n = 6 replicates.

	Increase of embryo numbers in controls after 28 days [%]		
	62.5 µg/snail x day	250 µg/snail x day	500 µg/snail x day
Test No. 1	94	138	155
Test No. 2	68	174	153

The reproduction test with TCC was conducted from 30.06. - 28.07.2014 and results are shown in figure 19. The mean control embryo numbers in the lowest food dose of 62.5 µg/snail x day was 12.7. The reproductive output of control snails in the higher food doses of 250 µg and 500 µg/snail x day was higher with 15.6 and 16.7, respectively. TCC exposure led to a significant increase of the reproductive performance by feeding the snails with 62.5 µg (LOEC: 0.226 µg/L) or the standard dose of 250 µg (LOEC: 0.04 µg/L). By feeding 500 µg/snail x day, no enhancement of the embryo numbers is detectable anymore. Only at the highest test concentration of 3.64 µg TCC/L a significant reduction of the embryo numbers was observed, which also is also applicable to the other two food doses. These results demonstrate that a higher food dose is able to mask reproduction-increasing effects of substances.

Figure 19: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks exposure to triclocarban concentrations and merged negative and solvent control (crosses: replicate mean; line: treatment mean) and fed with food doses of 62.5 µg/snail x day (A), 250 µg/snail x day (B) and 500 µg/snail x day (C). Asterisks indicate significant differences compared to control (Dunnett´s test), ★ = p ‹ 0.05, n = 6 replicates.

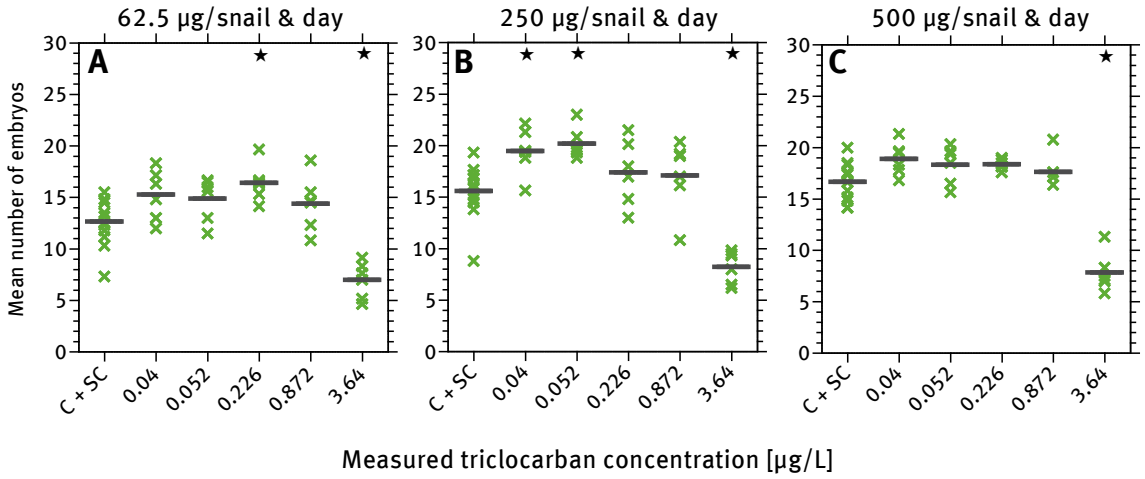


Figure 20 shows the energy contents (protein, glycogen and lipid contents) of snails after exposure to five TCC concentrations and the three different amounts of food. The mean energy content (as the sum of energy contents of proteins, glycogen and lipids) of the snails at test start (T_0) was 1.40 J/mg snail. Thereof, snails contained 0.44 J/mg proteins, 0.27 J/mg glycogens and 0.70 J/mg lipids. By feeding the snails for 28 days with 62.5 µg and 250 µg/snail x day TetraPhyll®, the mean energy content was 1.30 J/mg and 1.28 J/mg, respectively. At the highest food dose a mean energy content of 1.10 J/mg was measured, which is a significant reduction of 21% compared to test start. By having a look at the TCC exposure groups, no significant effect on the protein or the glycogen content could be observed. In contrast, a significant increase in the lipid content was determined with increasing TCC concentrations for all food doses (LOEC: 0.02 - 0.052 µg/L). This enhancement cannot be linked with the increased reproduction of the snails, as a toxic effect on the embryo numbers was observed in the highest test concentration of TCC. The results of the lipid contents in TCC exposed snails lead to the assumption that TCC could represent an obesogen. Pollutants are designated as obesogens, when they are able to promote obesity and disturb the homeostasis of lipids in vertebrates (GRÜN 2010). This hypothesis would need to be verified in further reproduction tests with *P. antipodarum* and already known obesogens.

Figure 20: Energy content in J/mg snail (mean with standard deviation) of protein (grey), glycogen (green) and lipid contents (blue) of *P. antipodarum* after four weeks exposure to triclocarban concentrations and merged negative and solvent control and fed with food doses of 62.5 µg/snail x day (A), 250 µg/snail x day (B) and 500 µg/snail x day (C). Asterisks indicate significant differences compared to control (Dunnett´s test), ★ = p ‹ 0.05, n = 6 replicates.

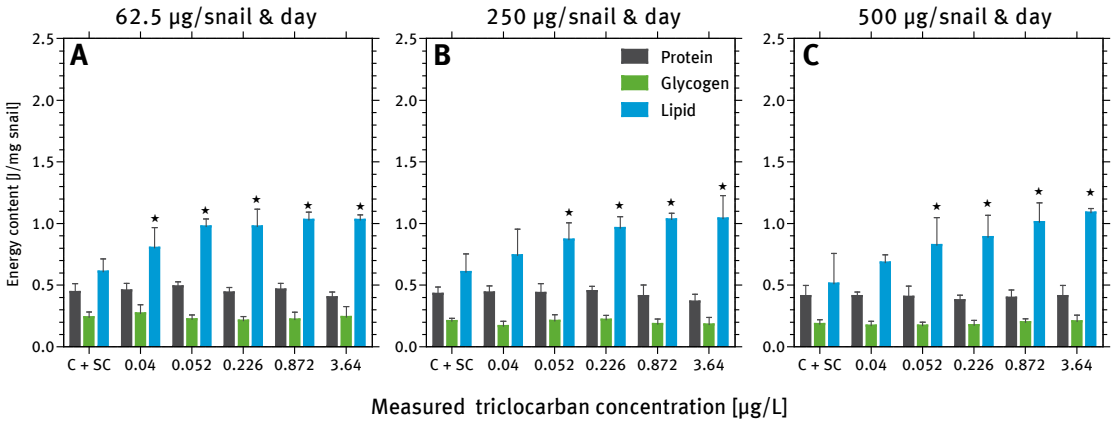


Figure 21 shows the results of the reproduction tests with Cd and the different amounts of food, which were carried out between 22.09. - 20.10.2014. The mean embryo numbers of control snails, which were fed 62.5 µg/snail x day, were 11.5. The embryo numbers of the two higher amounts of food was significantly increased to 18.7 (250 µg) and 17.3 (500 µg). These results also indicate an influence of the food dose on the reproduction of the snails. In Cd exposed snails the mean number of embryos decreased with increasing Cd concentrations, independent from food dose. NOEC values range between 1.05 µg/L (500 µg) and 4.65 µ/L (62.5 µg and 250 µg). The calculated effect concentrations are summarized in table 10. Effect concentrations for Cd are in similar ranges for all tested food doses. The EC_{10} and EC_{50} values range between 3.35 µg/L to 3.49 µg/L and 8.80 µg/L to 12.0 µg/L, respectively.

Figure 21: Total embryo numbers of *P. antipodarum* after four weeks exposure to measured cadmium concentrations (crosses: replicate mean; line: treatment mean) and fed with food doses of 62.5 µg/snail x day (A), 250 µg/snail x day (B) and 500 µg/snail x day (C). Asterisks indicate significant differences compared to control (Dunnett´s test), ★ = p ‹ 0.05, n = 6 replicates.

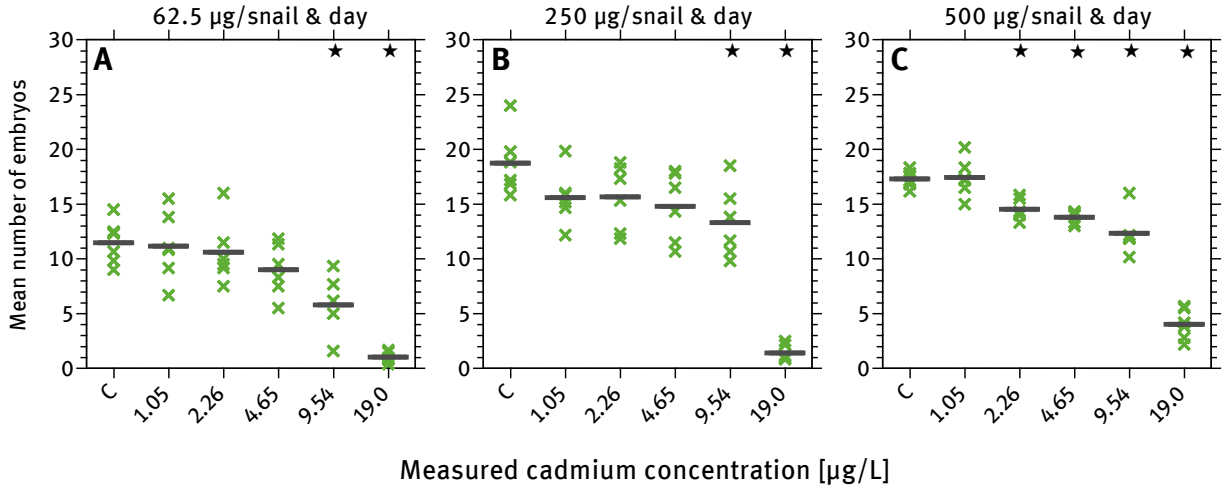


Table 10: Calculated effect concentrations (EC_{10} and EC_{50} with 95%-confidence intervals) for the total embryo numbers of *P. antipodarum* after four weeks exposure to measured cadmium concentrations and different food doses.

	62.5 µg/snail x day	250 µg/snail x day	500 µg/snail x day
EC_{10}	3.35	3.49	3.42
(95%-CI)	(1.98 - 5.66)	(2.77 - 4.38)	(2.44 - 4.78)
EC_{50}	8.80	10.5	12.0
(95%-CI)	(6.98 - 11.1)	(8.33 - 13.1)	(10.4 - 13.9)

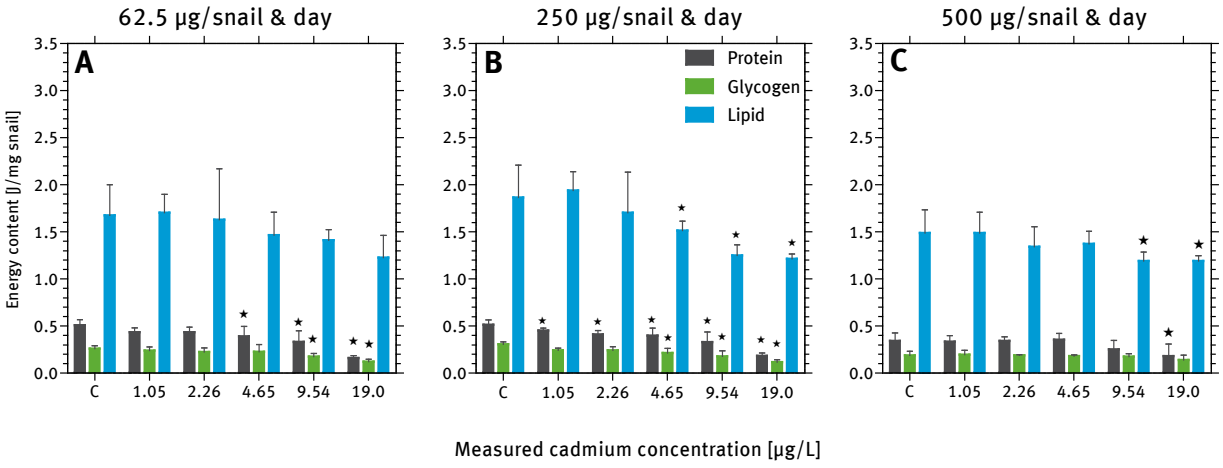
Before the start of the reproduction test with *P. antipodarum* with Cd and the different food doses, the mean energy content was 2.44 J/mg. Thereof, snails contained 0.46 J/mg proteins, 0.24 J/mg glycogens and 1.74 J/mg lipids which is about 1.74-fold higher compared to the energy content of T_0 animals in the first test with TCC. Especially, the lipid contents showed a 2.49-fold increase. This observation is in accordance with a study by GUST ET AL. (2011). They investigated variation of the energy status in a natural population of *P. antipodarum* throughout one year. In early spring the energy content increased, while it decreased during summer at the peak of reproduction. In autumn the energy content increased again. This increase might probably be a built-up of energy reserves for the winter months when food is limited. To verify annual variations of the energy status of laboratory reared snails, further investigations are necessary.

Feeding the snails 250 µg/snail x day resulted in an energy content in control snails of 2.68 J/mg after 28 days. A reduction of the energy content of the control snails was detected at the highest tested food dose with 2.02 J/mg snail. The energy content of the control snails fed with the lowest food dose was on the same level as during the start of the test (T_0). Figure 22

shows the energy contents (protein, glycogen and lipid contents) of snails after exposure to five Cd concentrations under the three feeding scenarios. By comparison of the controls of the different food doses, a significant reduction of the protein and the glycogen content could be detected at 500 µg/ snail x day. This might be due to a weak growth of fungus in the test vessels caused by some leftovers of food.

The results also show that the fitness of the snails is negatively affected by an exposure towards Cd with a declining trend of protein, glycogen and lipid in all feeding scenarios. This effect might be due to a mobilization of the energy reserves for detoxification (RADWAN AND MOHAMED 2013).

Figure 22: Energy content in J/mg snail (mean with standard deviation) of protein (grey), glycogen (green) and lipid contents (blue) of *Potamopyrgus antipodarum* after four weeks exposure to measured cadmium concentrations and fed with different food doses of 62.5 µg/snail x day (A), 250 µg/snail x day (B) and 500 µg/snail x day (C). Asterisks indicate significant differences compared to control (Dunnett´s test), ★ = p ‹ 0.05, n = 6 replicates.



Snails fed with a food dose of 62.5 µg/snail x day showed the lowest increase of reproduction compared to the brood stock. Additionally, reproduction stimulating as well as toxic effects could be mapped. Therefore, a food dose of 62.5 µg/snail x day seems to be the most appropriate food dose for the snails and should be recommended in the draft test guideline for the reproduction test with *P. antipodarum*.

4.3.4 Summary: Identification of an ideal reproduction range

A series of experiments was conducted and all available historical data of reproduction tests with *P. antipodarum* were evaluated to identify the ideal range of embryo numbers in control snails to allow for both, the assessment of reproduction-toxic and reproduction-increasing effects of chemicals.

The sensitivity of snails towards OP at 10°C and 13°C was determined. Tests with OP and EE_2 as positive control were conducted in February and April 2012 at both temperatures. Neither at 10°C nor at 13°C a significant increase of the embryo numbers could be observed after expo-

sure towards OP and EE_2. Because it turned out to be almost impossible to maintain a stable culture at 10°C and 13°C, it was not possible to conduct more reproduction tests with *P. antipodarum* at these temperatures.

Historical in-house data from Goethe University Frankfurt were analysed to determine an ideal reproduction range for testing. In most cases, embryo numbers in control groups varied between 10 and 20. It was also noteworthy that control embryo numbers were usually lower at test start (T_0) than 28 days later. The analysis revealed that the increase of embryo numbers during the course of the 28 d test was greater, the lower the embryo numbers were at T_0. The maximum increase of embryo numbers in control snails was 160%. Besides the embryo numbers in negative controls, the positive control (EE_2) also showed considerable variations. Especially, if embryo numbers in the negative control are already high, a further and statistically significant increase under exposure to EE_2 is unlikely. This analysis indicates that it becomes increasingly unlikely to detect a significant increase of embryo numbers in snails under exposure to EE_2 if embryo numbers in the negative control exceed a mean value of 10.

Excessive food supply is one potential factor that may have contributed to the increase of embryo numbers in control snails. To investigate the influence of this factor reproduction tests were performed with three different the amounts of food. The aim of the study was to identify the food dose, which allows the determination of toxic as well as stimulating effects of test chemicals on the embryo numbers. Cd and TCC were used as test chemicals. Besides embryo numbers the energy reserves (body protein, glycogen and lipid contents) of the snails were assessed. Embryo numbers increased at all three food doses significantly. However, at a food dose of 62.5 µg/snail x day, in both tests the lowest increase of the embryo numbers in control snails was detected. The different food doses did not affect the energy content of control snails, showing that snails fed with the lowest food dose (62.5 µg/snail x day) did not starve. The test with TCC demonstrates that a higher food dose is able to mask the reproduction-increasing effect of a test chemical. TCC exposure did not have an impact on the protein or glycogen content. In contrast, a significant increase in the lipid content was found with increasing TCC concentrations for all food doses. In Cd-exposed snails the mean number of embryos decreased with increasing Cd concentrations, independent from food dose with similar effect concentrations. The results also show a declining trend of protein, glycogen and lipid levels in Cd-exposed snails for all three feeding scenarios.

The analysis of historical data shows that the embryo numbers in controls varied between 10 and 20. Furthermore the reproduction increased over the course of the 28 d experiments in most cases. This increase was smaller when food dose was reduced to 62.5 µg/snail x day. The analysis also revealed that a significant increase of embryo numbers in snails under exposure to EE_2 can only be observed if embryo numbers in the negative control are not too high. A correlation analysis showed that it is getting increasingly unlikely to detect a significant increase of embryo numbers in snails under exposure to EE_2 if embryo numbers in the negative control exceed a mean value of 10. With a food dose of 62.5 µg/snail x day reproduction stimulating as well as toxic effects could be mapped. Therefore, the recommended food dose in the draft test guideline for the reproduction test should be 60 - 80 µg/snail x day to give a sparse of flexibility

to the experimenter. Thus, the test is suitable to assess reproduction-toxic and reproduction-increasing effects of chemicals.

4.4 Identification of a reproduction increasing substance for validation studies

4.4.1 Reproduction tests with 4-tert-octylphenol

Due to the instability of BPA, which had been tested in a ring test with four laboratories during the former project (project code 3708 61 402, SIERATOWICZ AND OEHLMANN 2011), reproduction tests with OP were performed to find an alternative, more stable reproduction increasing substance for validation. Throughout one year (from November 2011 until October 2012) six reproduction tests with OP in a nominal concentration range between 1 and 100 µg/L were performed (for test design description see section 1.3). EE_2 (25 ng/L) was used as a positive control and DMSO was used as solvent at a concentration of 10 µL/L. The aim of this study was to analyse if a possible seasonal reproductive pattern could mask reproduction increasing effects of endocrine disruptors in ecotoxicological tests.

Figure 23 shows the results of the reproduction tests conducted in November 2011 (Fig. 23A), February 2012 (Fig. 23B) and April 2012 (Fig. 23C). In these three reproduction tests mean control embryo numbers were between 8.57 and 12.5. EE_2 caused significant increases in embryo numbers in November and February. In April a slight but not statistically significant increase in embryo numbers was caused by EE_2. OP also caused an increase in embryo numbers at 3 and 10 µg/L in November and April with peak embryo numbers at 10 µg/L. At higher concentrations the reproductive output decreased to embryo numbers comparable to the control groups. In February a significant increased embryo number was detected at all concentrations above 3 µg/L.

Figure 23: Results of reproduction test conducted in November 2011 (A), February 2012 (B) and April 2011 (C). Total embryo numbers (weighted mean + standard deviation) of *Potamopyrgus antipodarum* after four weeks of exposure to five concentrations of 4-tert-octylphenol (OP, green), 17α-ethinylestradiol (EE_2, blue) and merged solvent control with negative control (C + SC, grey, 4 replicates (8 for merged controls) with 10 snails each). Asterisks indicate significant differences compared to control (Dunnett´s test), ★ = p ‹ 0.05, ★★ = p ‹ 0.01, ★★★ = p ‹ 0.001.

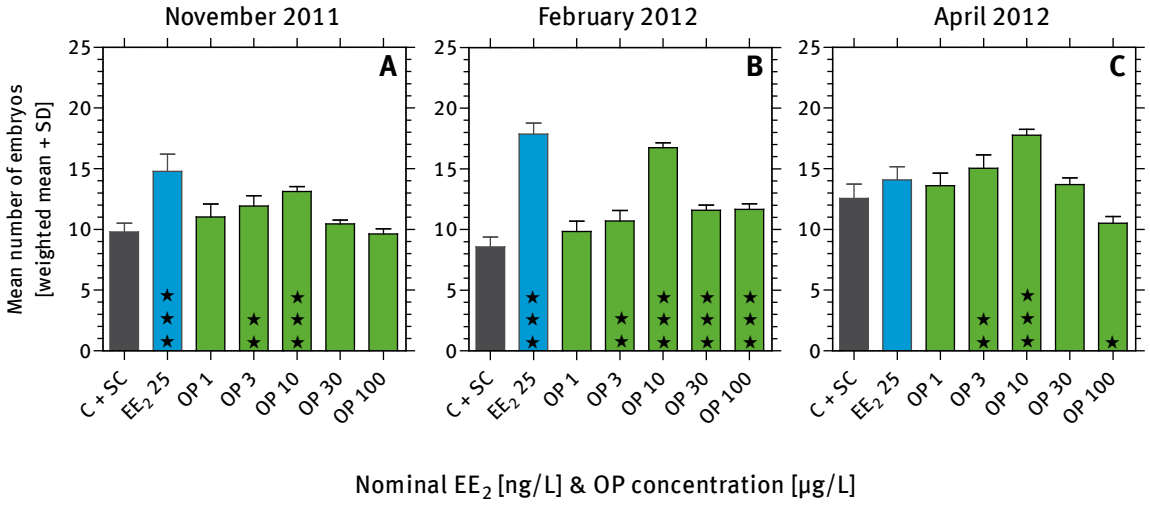
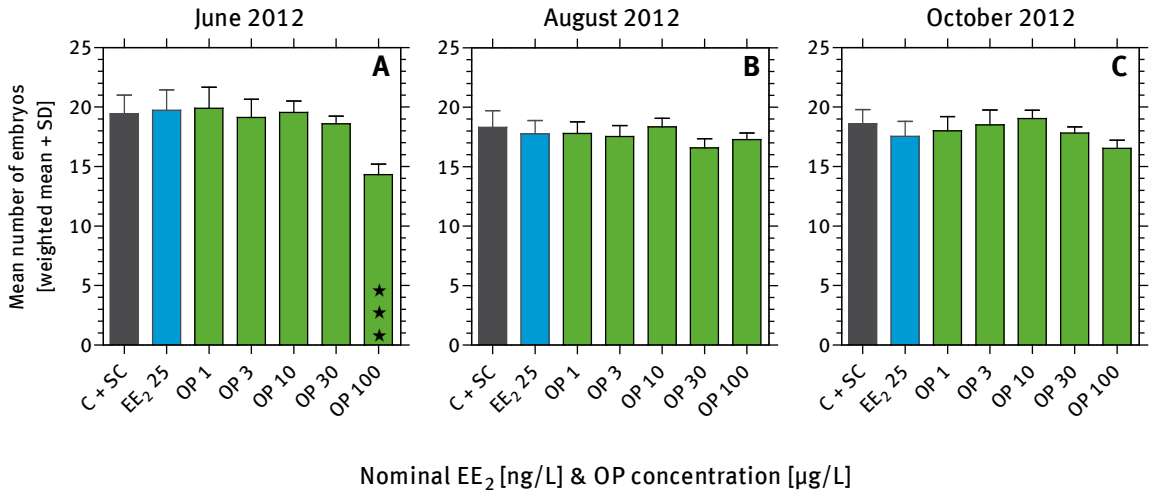


Figure 24 shows the results of the reproduction tests conducted in June 2012 (Fig. 24A), August 2012 (Fig. 24B) and October 2012 (Fig. 24C). In these three reproduction tests mean control embryo numbers were considerably higher compared to the tests conducted in winter and spring (Fig. 23). They were between 18.3 and 19.4. Neither EE_2 nor OP caused reproduction increasing effects in the snails.

A reason for the different results compared to the first three tests might be the high embryo numbers in control groups. Taking into account the conditions favouring the positive assessment of reproduction increasing substances (see section 4.3.2) it was not probable to assess a reproduction increasing effect. The production of embryos should not be too high to get valid, substance exposure related effects.

A probable reason for the higher embryo numbers in summer and autumn is the seasonal variability in snails that was still observed in the culture during the first three tests between November 2011 and April 2012 (see section 3.1.2). These variations could no longer be observed since autumn/winter 2012 and embryo numbers varied between 5.48 and 11.7 embryos per snail. Embryo numbers in following reproduction tests resulted in control reproduction rates considerably higher than at the beginning of the test (T0). This issue is probably caused by differences between the food amounts under culture and test conditions and has been discussed in section 4.3.3.

Figure 24: Results of reproduction test conducted in June (A), August (B) and October 2012 (C). Total embryo numbers (weighted mean + standard deviation) of *Potamopyrgus antipodarum* after four weeks of exposure to five concentrations of 4-tert-octylphenol (OP, green), 17α-ethinylestradiol (EE_2, blue) and merged solvent control with negative control (C + SC, grey, 4 replicates (8 for merged controls with 10 snails each). Asterisks indicate significant differences compared to control (Dunnett´s test), ★★★ = p ‹ 0.001.



4.4.2 Actual exposure concentrations of 4-tert-octylphenol

In the first and the last week of the tests, samples from OP exposure groups, solvent and negative control were taken for chemical analysis. Therefore, on day 0 and day 24 water samples were taken from freshly prepared exposure media and on day 3 and day 28 from old water which was pooled from every replicate of one treatment group. Chemical analysis of OP was performed via High Performance Liquid Chromatography (HPLC, Dionex Corporation) combined with Chromeleon software (6.60, SP2). In the first three tests the limit of quantification (LOQ) and the limit of detection (LOD) were 0.18 µg/L and 0.09 µg/L, respectively. For the tests conducted from June to October LOQ and LOD were 74.2 µg/L and 24.5 µg/L, respectively.
For substance enrichment samples were loaded on cartridges (Oasis® HLB, 6cc (200 mg) Extraction Cartridges; Waters Corporation) according to WAGNER ET AL. (2008) and stored at -21°C. For OP measurements the cartridges were eluted with 4 mL acetonitrile. 1 mL of this eluate was transferred to a vial for HPLC measurement according to ZHAO ET AL. (2009).

The results of the chemical analyses including calculated time weighted mean concentrations (TWM) according to annex 6 of OECD guideline 211 (OECD 2012b) are shown in tables 11 – 15. Measured OP concentrations were similar among the different reproduction tests with exception of the test conducted in November 2011. Measurements of samples taken during this test were considerably higher (TWM: 78.3 – 93.6 µg/L) than in the tests performed afterwards. In these tests TWMs are in a range between 34.0 and 73.8 µg/L. This may derive from a different evaluation method which has been optimised after the first reproduction tests. Overall after 3 or 4 days a general degradation of OP was observed. This may also to be caused by the development of a biofilm on the vessel walls. OP log K_{ow} is 3.96 – 4.21 (DUFT ET AL. 2003b). Further-

more, adsorption to vessel walls, food particles and permeation into snails is probable. The degradation of OP is also enhanced by aeration of the exposition medium (YING AND KOOKANA 2003).

Table 11 Results of OP analyses in exposure media in the reproduction test conducted in November 2011.

OP nominal concentration [µg/L]	Measured concentrations [µg/L]				Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh medium	Day 3 old medium	Day 24 fresh medium	Day 28 old medium		
Negative control	No sample	No sample	No sample	No sample	-	-
Solvent control	No sample	No sample	No sample	No sample	-	-
1.00	0.80	0.81	0.76	0.89	0.82	82.0
3.00	0.95	2.86	2.94	2.69	2.35	78.3
10.0	7.44	10.2	9.80	7.91	8.80	88.0
30.0	28.4	29.9	28.9	12.5	27.9	93.0
100	106	80.2	101	88.8	93.6	93.6

Table 12: Results of OP analyses in exposure media in the reproduction test conducted in February 2012.

OP nominal concentration [µg/L]	Measured concentrations [µg/L]				Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh medium	Day 3 old medium	Day 24 fresh medium	Day 28 old medium		
Negative control	< LOD	< LOD	< LOD	< LOD	-	-
Solvent control	< LOD	< LOD	< LOD	< LOD	-	-
1.00	0.68	0.13	0.59	0.17	0.34	34.0
3.00	2.72	1.57	2.58	0.07	1.30	43.3
10.0	10.2	7.07	10.2	1.33	6.15	61.5
30.0	32.5	17.2	32.3	7.58	20.1	67.0
100	103	31.6	102	43.4	65.5	65.5

LOD: Limit of detection

Table 13: Results of OP analyses in exposure media in the reproduction test conducted in April 2012.

OP nominal concentration [µg/L]	Measured concentrations [µg/L]				Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh medium	Day 3 old medium	Day 24 fresh medium	Day 28 old medium		
Negative control	< LOD	< LOD	< LOD	< LOD	-	-
Solvent control	< LOD	< LOD	< LOD	< LOD	-	-
1.00	0.38	0.01	1.09	0.44	0.37	37.0
3.00	2.66	0.01	3.23	0.84	1.03	34.3
10.0	9.64	1.90	10.4	3.91	5.55	55.6
30.0	28.0	10.7	31.7	18.9	20.9	69.7
100	96.6	39.1	103	54.6	69.0	69.0

LOD: Limit of detection

Table 14: Results of OP analyses in exposure media in the reproduction test conducted in June 2012.

OP nominal concentration [µg/L]	Measured concentrations [µg/L]				Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh medium	Day 3 old medium	Day 24 fresh medium	Day 28 old medium		
Negative control	< LOD	< LOD	< LOD	< LOD	-	-
Solvent control	< LOD	< LOD	< LOD	< LOD	-	-
1.00	0.82	0.23	0.97	0.65	0.65	65.4
3.00	3.01	1.32	3.31	1.58	2.22	73.8
10.0	6.99	4.20	1.67	5.42	4.17	41.7
30.0	25.1	12.7	22.8	17.7	19.3	64.3
100	80.8	45.0	100	46.2	66.0	66.0

LOD: Limit of detection

Table 15: Results of OP analyses in exposure media in the reproduction test conducted in October 2012.

OP nominal concentration [µg/L]	Measured concentrations [µg/L]				Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh medium	Day 3 old medium	Day 24 fresh medium	Day 28 old medium		
Negative control	< LOD	< LOD	< LOD	< LOD	-	-
Solvent control	< LOD	< LOD	< LOD	< LOD	-	-
1.00	0.42	0.68	0.46	0.14	0.38	38.0
3.00	1.88	1.66	2.72	0.87	1.68	56.1
10.0	9.57	3.43	10.6	3.55	6.27	62.7
30.0	24.2	10.1	32.1	8.90	17.3	57.6
100	103	22.5	115	45.5	66.0	66.0

LOD: Limit of detection

4.4.3 Effects of 4-tert-octylphenol on expression levels of the estrogen receptor and vitellogenin in *Potamopyrgus antipodarum*

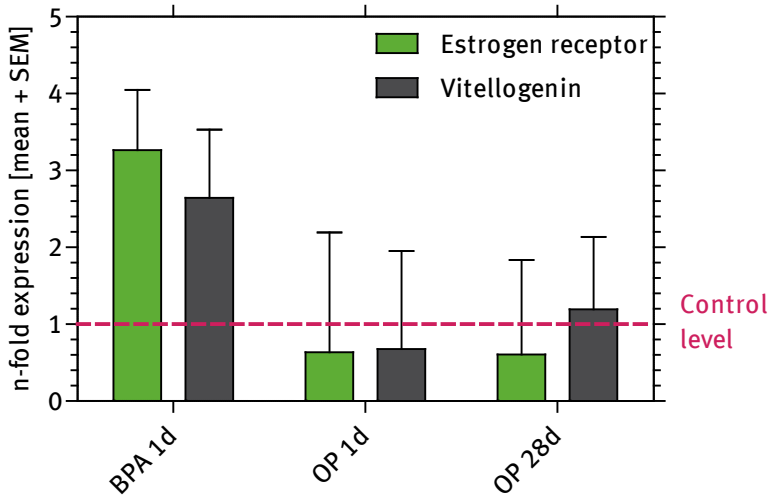
Additionally to the effects on apical endpoints such as embryo numbers and survival of snails, the effects of OP on expression levels on selected target genes were investigated. With these experiments an endocrine-mediated mode of action of OP in *P. antipodarum* was analysed. While it is known that OP reacts as an agonist of the human estrogen receptor (WHITE ET AL. 1994), comparably studies with the mudsnail are not available. STANGE ET AL. (2012) identified the transcript of the estrogen receptor orthologue in this species. By using quantitative real-time-polymerase chain reaction (qPCR) STANGE ET AL. (2012) could demonstrate that the transcription of the estrogen receptor-coding gene was up-regulated by exposure to EE_2 and BPA. An increased expression of the estrogen receptor orthologue and an increasing effect on reproduction of the snails after exposure to OP would be a strong indication for an endocrine mediated mode of action in the snails.

During the OP tests conducted in November 2011 (see section 4.4.1, fig. 23), three additional exposure groups (negative control, solvent control and 30 µg OP/L) with 6 replicates including 20 snails each were used. After 1 day and after 28 days exposure, snails from three replicates each were homogenized and total RNA was isolated with RNeasy plant mini kit (Qiagen). DNA (desoxyribonucleic acid) was digested with Baseline-ZERO™ (Epicentre®) according to the manufacturer's protocol. 2 µg of total RNA were used for cDNA (complementary DNA) synthesis with Sprint™ RT Complete kit (Takara-Clontech). qPCR was performed with the StepOne Real-Time PCR System (Applied Biosystems). Specific primers for the target genes estrogen receptor (ER), vitellogenin (Vtg) and the reference gene 28S ribosomal RNA (ribonucleic acid, not hormone regulated) were analysed. By means of fluorescence measurements with SYBR Master Mix (Applied Biosystems) the quantity of cDNA was measured. Relative quantification of the amplified targets followed the comparative ΔΔC method (PFAFFL 2001). Within this test an additional positive control of 40 µg BPA/L was tested because SIERATOWICZ ET AL. (2011) observed a

significant increase of embryo production at this concentration. After 1 day exposure snails from this group were homogenized and total RNA was isolated as described above.

Figure 25 shows the results of mRNA (messenger RNA) expression of the target genes ER and Vtg in *P. antipodarum* after exposure to BPA and OP. While BPA causes an alteration of the transcription of both genes, the results show that an OP concentration of 30 µg/L did not significantly modulate ER and Vtg expression neither after short-term exposure (1 day) nor after longer exposure (28 days). A significant increase in reproductive output at this OP concentration did not appear (Fig. 23).

Figure 25: Analyses of 4-tert-octylphenol (30 µg/L) and Bisphenol A (40 µg/L) exposed specimens of *Potamopyrgus antipodarum*. Expression of estrogen receptor and vitellogenin mRNA after 1 day and 28 days exposure. n = 3.



The results confirm that BPA is capable to modulate gene regulation in *P. antipodarum*. This supports the assumption that the increase in reproduction of the snails after exposure to BPA is endocrine modulated. In contrast, the test results could not clarify the mode of action of OP in snails. To answer this question, more OP concentrations should be tested and mRNA expression of the ER and Vtg should be measured. The involvement of other receptors in hormone-actions is also conceivable.

4.4.4 Summary: Identification of a reproduction increasing substance for validation studies

To find an alternative, more stable reproduction increasing chemical for validation studies six reproduction tests with OP were performed throughout one year. The results of the reproduction tests were contradictory. While OP or EE_2 caused a significant increase of embryo numbers in the first three tests, no effects on reproduction occurred in the other three tests. A possible explanation could be the differing embryo numbers in control between the two test sets. In the first three reproduction tests mean control embryo numbers were between 8.57 and 12.5. In contrast higher embryo numbers varying between 18.3 and 19.4 were observed in control snails of the other three tests. As already described in section 4.3.2 it is unlikely to assess re-

production-increasing effects of substances when embryo numbers in controls are already high.

Furthermore, experiments were conducted to analyse an endocrine-mediated mode of action of OP in *P. antipodarum*. It is known that OP acts as an agonist at the ER of humans and other vertebrates and that it induces Vtg in oviparous vertebrate species. Therefore, the expression of the target genes for ER and Vtg was analysed in *P. antipodarum*. An increased expression of the ER orthologue and an increasing effect on reproduction of the snails after exposure to OP would be a strong indication for an endocrine mediated mode of action in the snails. The results show that while BPA causes an alteration of the transcription of both genes an OP concentration of 30 µg/L did not significantly modulate ER and Vtg expression neither after short-term exposure (1 day) nor after longer exposure (28 days). However, OP did also not cause a significant increase of embryo numbers in snails during this reproduction test. It turned out that OP is not suitable for the use as a reproduction-increasing substance in validation studies with *P. antipodarum*.

4.5 Development of a full life cycle test with *Potamopyrgus antipodarum*

For the development of a full life cycle (FLC) test with *P. antipodarum* two test scenarios were designed and will be presented in this section.

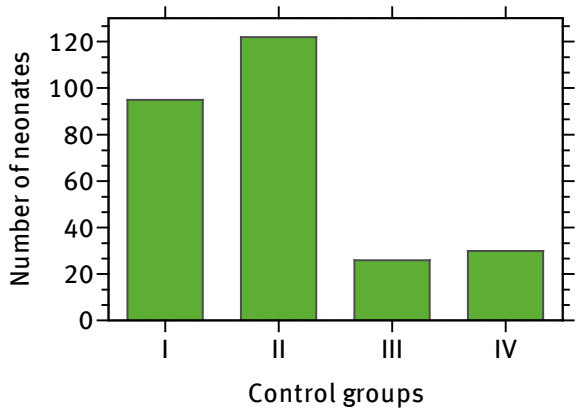
4.5.1 Test design 1 of a full life cycle test with *Potamopyrgus antipodarum*

Test design 1 of a FLC test with *P. antipodarum* comprises three phases. Firstly, the F0-generation is exposed in a partial life cycle (PLC) test over 28 days (phase 1). Therefore, 200 adult snails are introduced into five litres glass aquaria filled with four litres of DRP-medium. Four replicates per exposure group should be used for the test. After 28 days of exposure, adult snails are removed from the test system and in a second phase (phase 2), released neonates are allowed to grow up until they reach sexual maturity. The achievement of sexual maturity is regularly checked by a sample of ten individuals per replicate. A minimum of 10 snails per replicate from F1-generation should survive to assess reproduction in phase 3 of the test.

4.5.1.1 First approach of a full life cycle test with control groups

A first approach of a FLC test with *P. antipodarum* according to test design 1 was conducted in October 2011. Here, a control group with four replicates was used to evaluate the test design. The aquaria were kept at 16 ± 1°C and at a light:dark regime of 16:8 h. Water was renewed twice per week and snails were fed with finely ground TetraPhyll® (2 µg/snail & day, Tetra GmbH, Melle, Germany). After 28 days, adult snails were removed from the test system and after 28 additional days (day 56 of the entire experiment) surviving juveniles per replicate were counted. Figure 26 shows the total number of juveniles of each control group after 56 days. The number of surviving neonates between the four control replicates differs considerably. Most neonates are counted in replicate I and II, 95 and 122 snails, respectively. In replicate III and IV only a number of 26 and 30 juveniles could be found, respectively. Due to the low number of snails, the FLC test had to be stopped. A possible explanation for the insufficient number of living juveniles could be a high mortality caused by the absence of a suitable food source.

Figure 26: Total number of living juvenile snails of *P. antipodarum* after 56 days under control conditions in four replicates (aquaria) of a full life cycle test.



4.5.1.2 Feeding experiment with neonates of *Potamopyrgus antipodarum*

A probable cause for the high mortality of neonates in the FLC test (described in section 4.5.1.1) was that juvenile snails of *P. antipodarum* may need another food source than TetraPhyll®. Therefore, a feeding experiment with neonates and different food types was conducted. The test lasted for 56 days at a temperature of 16 ± 1°C with a light:dark regime of 16:8 h. Up to 24 h old juvenile snails were used for testing. Three different food types with three replicates each were tested. Here, finely ground Tetraphyll® (Tetra GmbH, Melle, Germany), finely ground Sera® Spirulina (Sera GmbH, Heinsberg, Germany) and green algae of *Scenedesmus acutus var. acutus* were offered as food sources and fed *ad libitum*. 100 mL glass beakers were filled up with 50 mL of DRP medium and 15 neonates were introduced to each test vessel. Two times per week water was changed and food remains were removed under a stereo microscope to ensure that no neonate snails were damaged. The endpoint of the test was mortality.

The results of the feeding experiment are shown in figure 27. After 56 days feeding with green algae only 11.1% of the juvenile snails died, whereas high mortalities of 93.3% and 88.9% were observed in the group fed with Sera® Spirulina and TetraPhyll®, respectively.

Figure 27: Mortality (mean and standard error) in juvenile *Potamopyrgus antipodarum* after 56 days of feeding different food sources (green algae, Sera® Spirulina, TetraPhyll®). Asterisks indicate significant differences between tested food groups (Fisher's exact test), ★★★ = p ‹ 0.001 (n = 3).

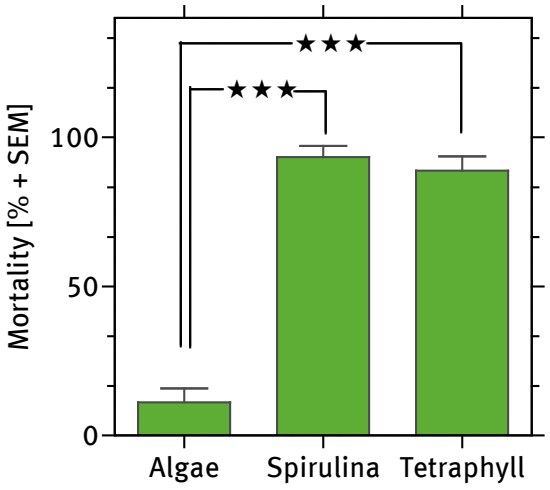
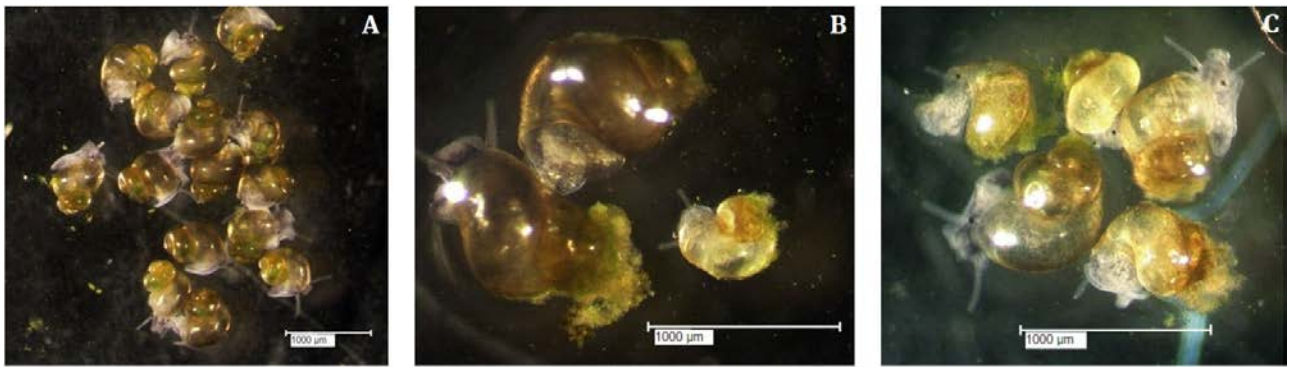


Figure 28 shows some of the surviving snails after 56 days of feeding different food types. Neonates fed with green algae (Fig. 28A) showed a better agility and fitness than those of the other feeding groups. It is noteworthy that growth and development of neonates varied within Sera® Spirulina group (Fig. 28B) and TetraPhyll® group (Fig. 28C). Some of the snails remained comparably small and their shell was nearly transparent. Furthermore, it was observed that the shell of snails fed with TetraPhyll® was quite fragile.

Figure 28: Juvenile snails of *Potamopyrgus antipodarum* after 56 days feeding with green algae (A), Sera® Spirulina (B) or Tetraphyll® (C).



With this feeding experiment it could be shown that TetraPhyll®, which is the standard food type in the reproduction test with adult *P. antipodarum*, and Sera® Spirulina are not suitable as a food source for neonates and juveniles in the FLC test. Therefore, juvenile snails of *P. antipodarum* should be fed with green algae to ensure survival and growth.

As a conclusion from test design 1, aquaria are no suitable vessels for a FLC test with *P. antipodarum*. As freshly hatched neonates are only between 450 and 500 µm in size, small test vessels are sufficient and allow the observation of juvenile snails under a stereo microscope. Also, the feeding experiment demonstrated that there are differences in food preferences between adult and juvenile snails of *P. antipodarum*. Therefore, the snails should be fed with green algae and Tetraphyll® when conducting a FLC test.

Furthermore, test design 1 is not ideal as many adult snails are needed in phase I. To optimise the test design, a FLC test should start with up to one week old juvenile snails and a defined number of neonates should be introduced into the test system.

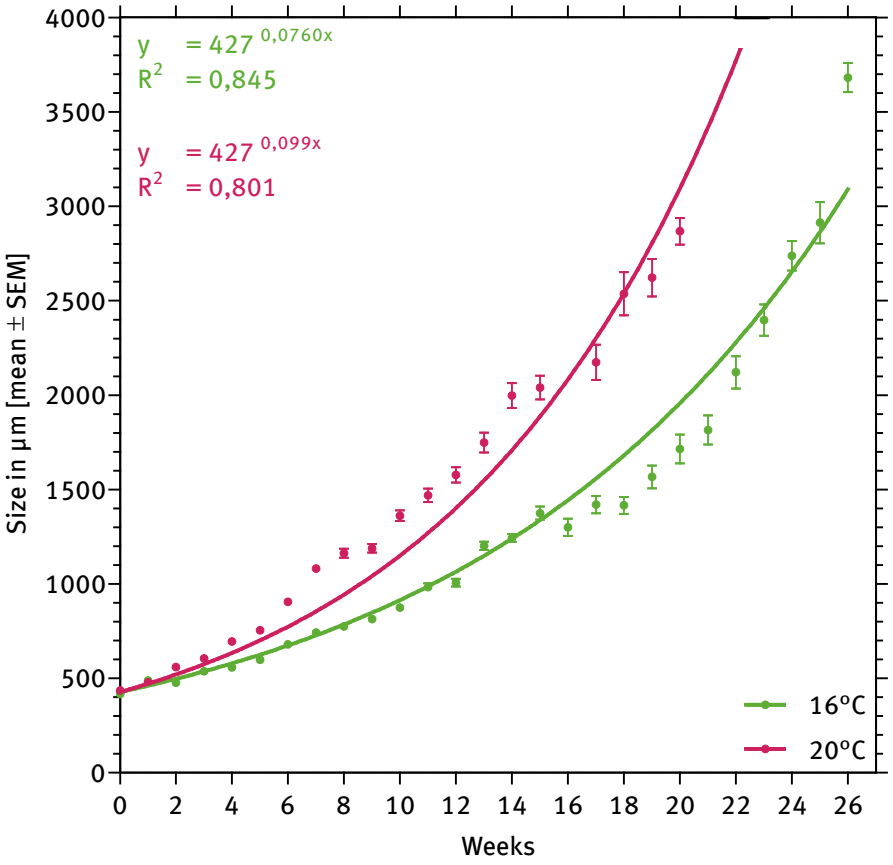
4.5.2 Growth test with juvenile *Potamopyrgus antipodarum* at 16°C and 20°C

To estimate the test duration of a FLC test with *P. antipodarum*, growth tests with newly hatched juvenile snails were conducted over 20 and 26 weeks, respectively. Tests were performed at 16°C and 20°C to assess an influence of temperature on the growth of the neonates. 100 of released juvenile snails (age: 24 h) were held in crystallizing dishes filled with 400 mL medium. Four replicates were used for each temperature and snails were fed with finely ground Tetraphyll® *ad libitum*. At test start green algae *S. acutus var acutus* were applied into test vessels. Under a stereo microscope water was changed twice per week and remains of food leftovers were removed. Once a week, shell length of 10 animals per replicate was measured.

The development of the mean shell height of the snails at 16°C and 20°C is shown in figure 29. Results demonstrate an influence of temperature on the growth of the snails. At the standard temperature for the PLC test with adults (16°C) juvenile snails need 26 weeks to reach mean adulthood (size ≥ 3.5 mm) while at a temperature of 20°C the time period can be shortened to about 22 weeks as juvenile snails grow faster at a higher temperature. Indeed, the experiments also showed that juveniles grow differently in the same replicate. While some of the snails had already reached the minimum size for a PLC test (> 3.5 mm), others remained small. According

to TABORSKY (2006), a limited amount of food can be responsible for a reduced growth rate. Thus, smaller individuals might have found food flakes worse compared to larger snails. Another explanation could be the density of snails in test vessels. The numbers of living snails differed between replicates due to the natural mortality (GOSSELIN & QIAN 1997) of the neonates. In studies with other snail species it was demonstrated that the individual growth rate is negatively influenced by high densities (BAUR AND BAUR 1990, COPE AND WINTERBOURN 2004, DILLON 2000, THOMAS AND BENJAMIN 1974).

Figure 29: Nonlinear growth curves (lines) for mean shell height in mm (dots: 4 replicates with 10 snails each) of juvenile *Potamopyrgus antipodarum* at 16°C (green) and 20°C (red).

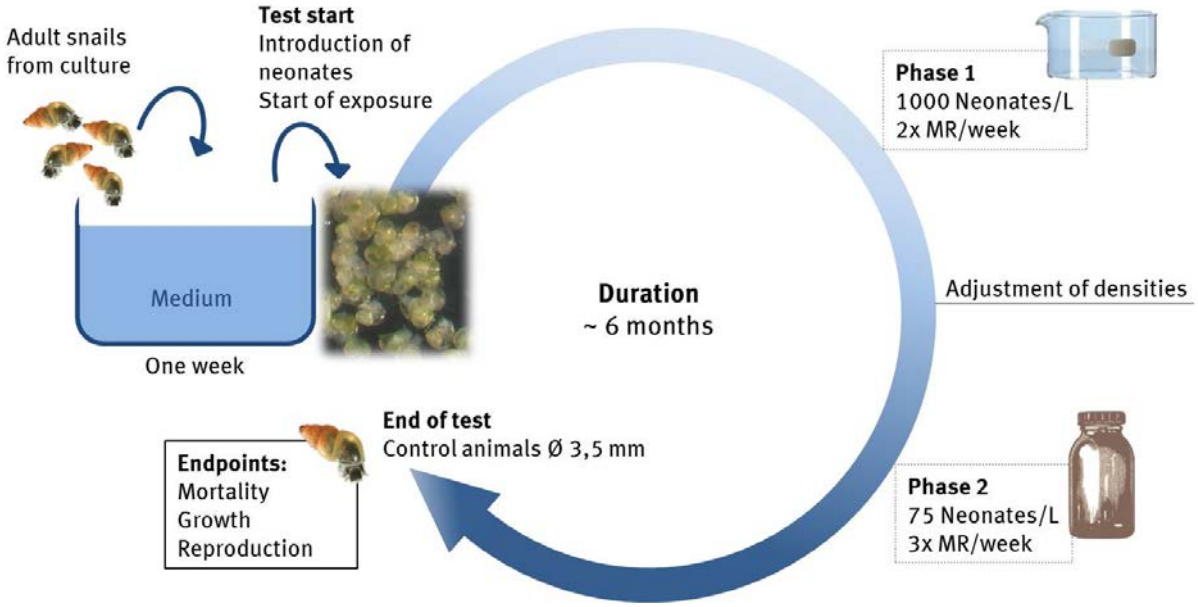


4.5.3 Test design 2 of a full life cycle test with *Potamopyrgus antipodarum*

The test design 2 differs from test design 1 as it starts with the early life stage of *P. antipodarum* to avoid the consumption of high numbers of adult snails. A schematic description of the test design is given in figure 30. First, adult snails from a laboratory culture are transferred into fresh medium and allowed to reproduce for one week. Afterwards, released juvenile snails are introduced into the test system to start the exposure. Crystallizing dishes with four replicates per exposure group are used as test vessels and snails are introduced at a density of 1000 neonates/L. The snails are fed *ad libitum* with *S. acutus var. acutus*. After reaching a mean shell length of 1.5 mm, juveniles are transferred into 500 mL glass beakers (filled with 400 mL me-

dium) and density is adjusted to 75 juvenile snails/L. From then on, snails are additionally fed with fine grounded TetraPhyll® (0.1 mg/snail & day). The test ends when animals in control groups reach a mean size of 3.5 mm. Endpoints of the test are mortality, growth and embryo numbers of the snails.

Figure 30: Scheme of a full life cycle test with *Potamopyrgus antipodarum* (test design 2), MR = medium renewal.

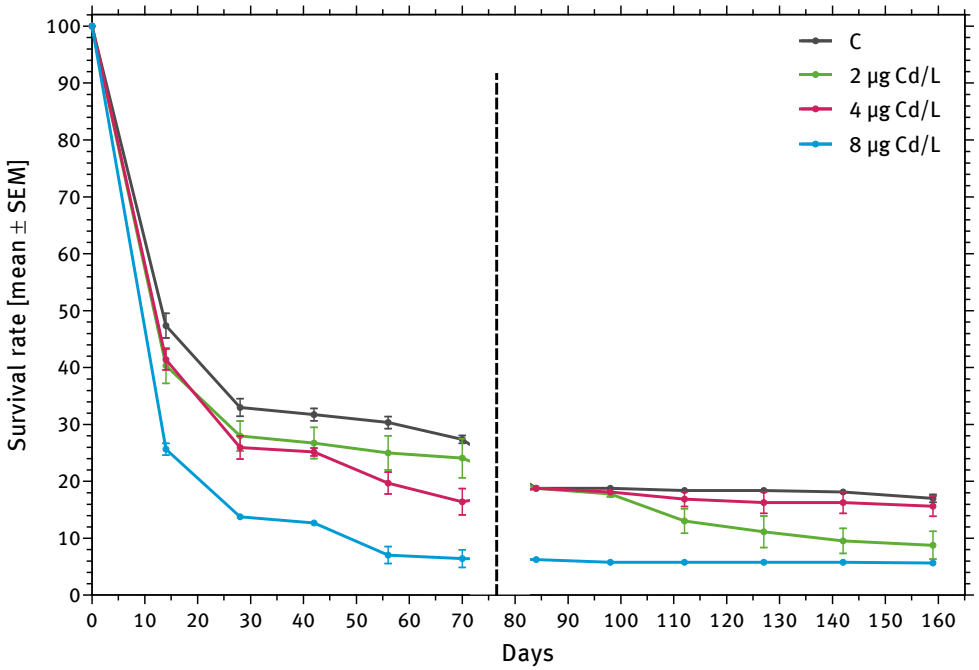


4.5.3.1 Full life cycle test with *Potamopyrgus antipodarum* - design 2

A FLC test according to test design 2 (described in section 4.5.3) was conducted in 2014. Cd (applied as Cd chloride) was chosen as test chemical at 3 nominal concentrations (2, 4 and 8 µg/L). The test lasted 159 days.

Figure 31 shows the survival rate over the test period. High mortality was observed in all exposure groups at test start. In controls and Cd exposed groups more than 50% of juveniles died in the first two weeks of the experiment. Up to day 70 survival rate in control was 27.3% and decreased with increasing Cd concentrations. At the highest test concentration (8 µg/L) survival rate was significantly reduced to 6.41%. Thereafter, density was adjusted (according to description of test design 2) to 75 neonates/L or to 25 neonates/L for 8 µg/L (marked by dotted line in figure 31). After the adjustment of densities on day 70, mortality at 2 µg/L increased significantly starting at day 98. A probable cause for the high mortalities throughout the test is the development of fungus in the test vessels.

Figure 31: Survival rate over 159 days in the full life cycle test with *P. antipodarum* in control and cadmium exposure groups. Dotted line marks the adjustment of density on day 84 to 75 snails/L in all exposure groups, except for 8 µg/L. Here, density was adjusted to 25 snail/L. Number of replicates: until day 70: n = 4, from day 84: C & 2 µg/L: n = 5; 4 µg/L: n = 3; 8 µg/L: n = 4.



The growth of the snails in the FLC test with Cd is shown in figure 32. At test start, mean shell length was 0.5 mm. Juveniles grew continuously during the experiment. After 159 days mean shell length in controls was 3.8 mm. Snails in the 8 µg/L exposure group were smaller compared to controls. This was not statistically significant. After 159 days of exposure to 8 µg Cd/L snails exhibited a mean size of 3.3 mm. No significant effects of Cd on growth were found in the FLC test.

Figure 32: Shell length (mean with standard deviation) over 159 days in the full life cycle test with *Potamopyrgus antipodarum* in control and cadmium exposure groups. Number of replicates: until day 70: n = 4, from day 84: C & 2 µg/L: n = 5; 4 µg/L:n = 3; 8 µg/L: n = 4.

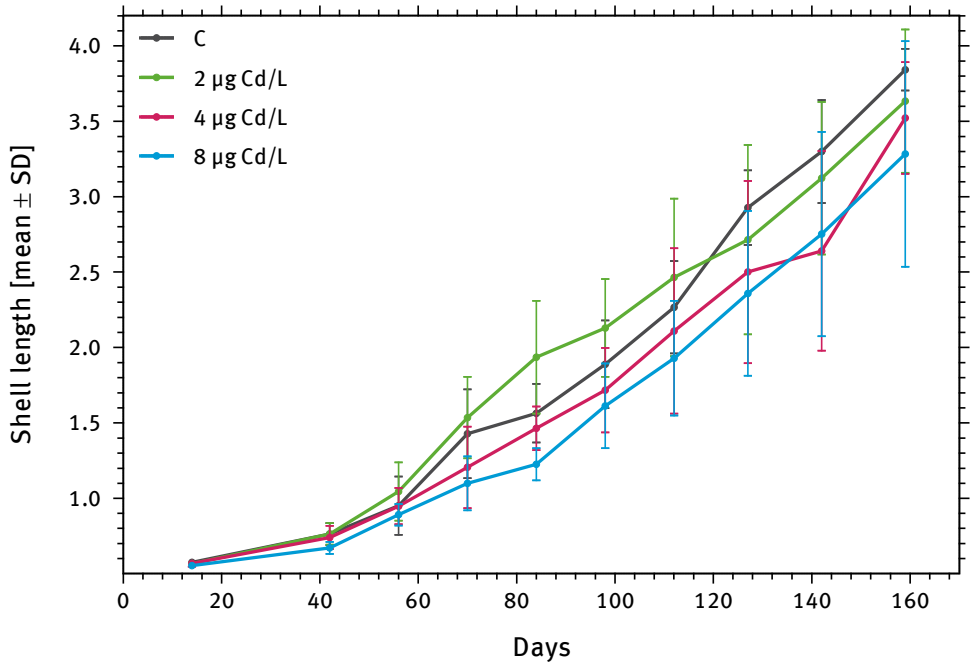


Figure 33 shows the mean number of embryos per female after 159 days of exposure to Cd in the FLC test with *P. antipodarum*. The mean embryo number in controls was 18.2 and decreased with increasing Cd concentrations. 17.5 embryos were found in the snails at 2 µg/L. At 4 µg/L and 8 µg Cd/L the number of embryos was reduced to 8.15 and 6.38, respectively.

Figure 33: Total embryo numbers of *P. antipodarum* after 159 days of exposure to cadmium (crosses: replicate mean; line: treatment mean). Asterisks indicate significance compared to control (Dunnett), ★ = p ‹ 0.05, n = 4 replicates.

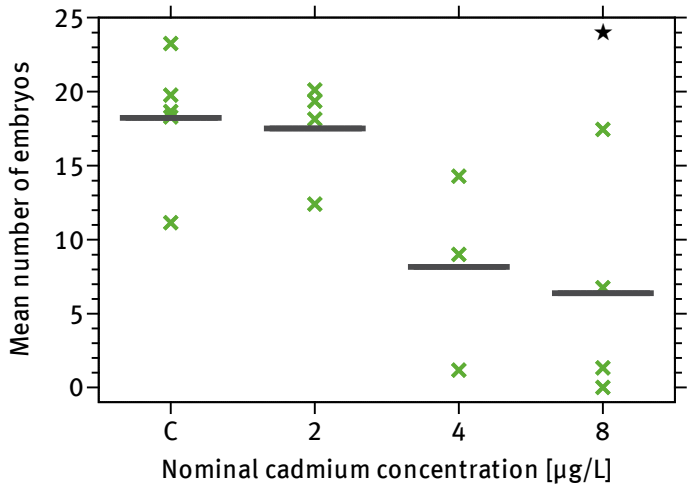


Table 16 summarizes the calculated effect concentrations for the endpoints mortality, growth and reproduction in the FLC test with *P. antipodarum*. Effect concentrations for mortality were evaluated at three different time points. Obviously LC values decreased with test duration. LC_{10} and LC_{50} on day 70 are 1.89 µg/L and 4.77 µg/L, respectively. After 159 days exposure calculated EC_{10} and EC_{50} values for the endpoint reproduction are 0.915 µg/L and 3.16 µg/L.

Table 16: Effect concentrations (NOEC, LOEC, EC_{10} and EC_{50} with 95%-confidence intervals) for mortality, growth and reproduction in the full life cycle test with *Potamopyrgus antipodarum* after exposure to cadmium.

Endpoint	Day	NOEC	LOEC	EC_{10} (95%-CI)	EC_{50} (95%-CI)
Mortality	28	2.00	4.00	2.49 (1.05 - 5.94)	7.20 (5.61 - 9.25)
	70	2.00	4.00	1.89 (0.939 - 3.81)	4.77 (3.51 - 6.49)
	159	-	2.00	n.c.	n.c.
Growth	159	-	-	0.339 (n.c.)	-
Reproduction	159	4.00	8.00	0.915 (0.134 - 6.23)	3.16 (1.49 - 6.69)

n.c.: not calculable

A comparison of this data with calculated effect concentrations from reproduction tests in validation 1 (see section 5.1.4.4) indicates that effect concentrations in the FLC test are slightly lower. In validation II NOEC and LOEC values differed between 4.24 to 11.1 µg/L and 9.03 to 20.8 µg/L, respectively. The mean EC_{10} and EC_{50} values in the five laboratories participating in validation II were 7.96 µg/L and 15.5 µg/L, respectively. The calculated effect concentrations for the endpoint reproduction in the FLC test are up to 8.70-fold (EC_{10} value) lower compared to results of validation II.

4.5.4 Comparison of the sensitivity to tributyltin and cadmium between juveniles and adults of *Potamopyrgus antipodarum*

As juvenile stages of many mollusc species are reported to be more sensitive to toxicants and EDCs than adult stages (AGUIERRE-SIERRA ET AL. 2011, BAUER ET AL. 1997), a comparison of the sensitivity between juvenile and adults of *P. antipodarum* was made. Therefore, adult snails and newly hatched neonates were exposed to cadmium (Cd, as Cd chloride) and tributyltin (TBT) for 28 days and apical endpoints like growth (FLC), reproduction (PLC) and mortality (FLC, PLC) were compared in parallel in a reproduction test with adults and in a juvenile growth test.

Reproduction tests with adult snails were conducted according to the DRP (OECD, 2010; see also section 1.3) at a temperature of 16 ± 1°C with a light:dark regime of 16:8 h. Snails were exposed to nominal Cd concentrations of 1.56, 3.13, 6.25, 12.5, 25 and 50 µg/L and to nomi-

nal TBT concentrations of 25, 65, 160, 400 and 1000 ng Sn/L. As solvent for TBT glacial acetic acid was used at a concentration of 10 µL/L (0.001%). Additionally, a negative control and a solvent control were tested. The mean number of embryos in the brood pouch per snail and also the mortality served as endpoints for the reproduction tests.

For the juvenile growth tests up to 24 h old neonates were used. Endpoints of these tests were growth and mortality. Newly hatched juveniles were placed into glass vials (25 mL DRP-medium) and exposed to the identical nominal Cd and TBT concentrations as in the reproduction tests with adults. Each exposure group, including water and solvent controls, consisted of ten replicates containing two individuals each. The experiment was conducted for 28 days at 16 ± 1°C and a light:dark regime of 16:8 h. Renewal of test medium was done twice per week and juveniles were fed with *S. acutus var. acutus*. Juvenile growth was assessed via shell length every week. Specific growth rates (SGR) from week 0 to four were calculated according to KAUFMANN (1981):

$$SGR = \frac{\ln S_2 - \ln S_1}{t_2 - t_1}$$

S_1 and S_2 represent the shell length at week 0 (t_1) and week four (t_2), respectively.

4.5.4.1 Chemical analysis of Cd and TBT

Samples for the analytical measurement of TBT and Cd were taken in the first and the last week of the test. Therefore, samples from all replicates of a given exposure group were taken before and after renewal of the exposure media, including negative control and solvent control, to calculate TWM concentrations.

Chemical analysis of TBT was performed by gas chromatography according to DIN EN ISO 17353-F13 (2005) (Agilent 7890A with Agilent 5975C). The LOD (referring to the cation of the organotin compound (OTC)) was 2 ng OTC/L, the LOQ was 5 ng OTC/L (equates to 0.82 ng and 2.05 ng TBT-Sn/L, respectively).

25 mL of Cd samples were acidified with 125 µL nitric acid (Suprapur®, Merck KGaA, Darmstadt, Germany). Here, only samples from control, the highest and the lowest Cd concentration were taken. Analysis was performed via inductively coupled plasma mass spectrometry according to DIN 38406 E 29 (1996) (Varian820ICP-MS) and the LOD was 0.002 µg/L, the LOQ was 0.008 µg/L.

Table 17 summarises the measured TBT concentrations and also the calculated TWM concentrations. In solvent control no TBT was detected. The TWM concentrations were between 30.8% and 88.5% of nominal concentrations. Therefore, TWM concentrations were used to calculate effect concentrations.

Table 17: Measured TBT concentrations in exposure media (in ng as Sn/L) of solvent control and TBT exposure groups.

TBT nominal concentration [ng Sn/L]	Measured concentrations [ng Sn/L]				Time weighted mean [ng Sn/L]	% of nominal concentration
	Day 0 fresh medium	Day 3 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	-	-
25.0	33.6	11.5	26.2	10.7	18.6	74.4
65.0	67.2	19.3	37.7	9.87	27.8	42.8
165	109	19.7	118	14.8	50.8	30.8
400	455	326	185	77.0	229	57.3
1000	885	889	459	1290	838	88.5

LOD = 0.82 ng Sn/L

The results of the analytical measurement and calculated TWM concentrations for Cd are shown in table 18. Only on day 28 (old medium) Cd was detected in control group with a concentration of 0.01 µg/L. TWM concentrations were 94.2% and 111% of nominal concentration. Therefore, nominal concentrations were used for EC_x calculation. As both the lowest and highest Cd test concentrations were sufficiently verified these circumstances could be extrapolated to the three in-between test concentrations 3.13, 6.25, and 12.5 µg/L.

Table 18: Measured Cd concentrations in exposure media (in µg/L) of negative control and nominal Cd concentrations of 1.56 µg/L and 50.0 µg/L.

Cd nominal concentration [µg/L]	Measured concentrations [µg/L]				Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh medium	Day 3 old medium	Day 25 fresh medium	Day 28 old medium		
Negative control	< LOQ	< LOQ	< LOQ	0.01	-	-
1.56	2.29	1.27	2.23	1.27	1.47	94.2
50.0	75.8	32.3	93.5	65.9	55.7	111

LOQ = 0.008 µg/L

4.5.4.2 Biological responses

Effect concentrations of Cd in the growth test with juveniles and in the reproduction test with adult *P. antipodarum* are provided in table 19. In the reproduction test with adults mortality in control was 1.25%. Calculated LC_{10} and LC_{50} were 14.1 µg/L and 38.2 µg/L, respectively. The mean embryo number in control was 11.5 and a significant decrease of the mean embryo numbers were observed at concentrations of 12.5, 25.0 and 50.0 µg/L. Here, embryo numbers varied between 0.85 (50.0 µg/L) and 2.40 (12.5 µg/L). Calculated EC_{10} and EC_{50} values for the endpoint reproduction were 9.73 and 11.3 µg/L, respectively.

Control mortality of juvenile snails in the growth test was 25.0%. Juvenile survival was significantly affected at 25.0 and 50.0 µg Cd/L. All snails exposed to these concentrations died during the test. Calculated LC_{10} and LC_{50} were 12.1 and 15.0 µg/L, respectively. After 28 days of expo-

sure to Cd, SGR was significantly reduced at concentrations of 6.25 and 12.5 µg/L. SGR could not be assessed in the two highest test concentrations due to 100% mortality of juvenile snails. Calculated EC_{10} and EC_{50} for the endpoint SGR were 1.16 and 3.82 µg/L, respectively.

Table 19: Effect concentrations for cadmium in µg/L (EC_{10} and EC_{50} with 95%-confidence intervals) for mortality and specific growth rate (SGR) for juvenile *Potamopyrgus antipodarum* and for the endpoints mortality and reproduction for adults.

Cadmium [µg/L]	Juvenile snails		Adult snails	
	Mortality	SGR	Mortality	Reproduction
EC_{10} (95%-CI)	12.1 (8.10 - 18.1)	1.16 (0.446 - 3.04)	14.1 (6.45 - 30.8)	9.73 (0.946 - 100)
EC_{50} (95%-CI)	15.0 (2.82 - 79.5)	3.82 (2.62 - 5.56)	38.2 (29.4 - 49.6)	11.3 (4.50 - 28.4)
NOEC	12.5	3.13	6.25	6.25
LOEC	25.0	6.25	12.5	12.5

The calculated TBT effect concentrations for the juvenile growth test and the reproduction test with adult *P. antipodarum* are summarized in table 20. In the reproduction test with adult snails mortality in controls was 2.50%. A significantly increased mortality occurred at the highest test concentration of 838 ng Sn/L, where 87.5% of the snails died during the test. The calculated LC_{10} and LC_{50} value were 278 and 499 ng Sn/L, respectively. The mean number of embryos per female in the control and solvent control was 18.9 and 19.3, respectively and did not differ significantly. A significant decrease of embryos was observed at 27.8 ng Sn/L and higher concentrations. The calculated EC_{10} and EC_{50} were 12.7 and 124 ng Sn/L, respectively.

Mortality in the control group of the juvenile growth test was 30% and increased with increasing TBT concentrations up to 85% mortality at the highest test concentration. Calculated LC_{10} and LC_{50} were 11.4 and 194 ng Sn/L (table 20), respectively. Also shell length of juvenile *P. antipodarum* was negatively affected by the exposure to TBT. SGR decreased with increasing TBT concentrations and resulted in an EC_{10} and EC_{50} of 5.91 and 175 ng Sn/L, respectively.

Table 20: Effect concentrations for tributyltin in ng Sn/L (EC_{10} and EC_{50} with 95%-confidence intervals) for mortality and specific growth rate (SGR) for juvenile *Potamopyrgus antipodarum* and for the endpoints mortality and reproduction for adults.

TBT [ng Sn/L]	Juvenile snails		Adult snails	
	Mortality	SGR	Mortality	Reproduction
EC_{10} (95%-CI)	11.4 (1.03 - 126)	5.88 (1.38 - 25.0)	278 (183 - 424)	12.7 (5.73 - 28.3)
EC_{50} (95%-CI)	194 (57.1 - 655)	175 (85.1 - 359)	499 (394 - 633)	125 (77.8 - 200)
NOEC	27.8	-	229	18.6
LOEC	50.8	18.6	838	27.8

The experiments with adult and juvenile *P. antipodarum* demonstrate that Cd and TBT have significant impacts on survival, reproduction and growth of the snails. The results indicate that juvenile snails react slightly more sensitive towards the exposure of Cd and TBT although generally 95% confidence intervals overlap so that differences are not statistically significant.

4.5.5 Summary: Development of a full life cycle test with *Potamopyrgus antipodarum*

Within the project two test designs for a FLC test with *P. antipodarum* were developed. Test design 1 starts with the exposure of adult snails and released juvenile snails are allowed to grow up until they reach sexual maturity. A first test with a control group including four replicates was performed. The test had to be stopped because of the low number of surviving neonates. A possible explanation for the high mortality is that TetraPhyll® is not a suitable food source for juveniles. Therefore, a feeding experiment with three different food sources was conducted and mortality of juvenile snails was assessed. Neonates fed with TetraPhyll® or Sera® Spirulina suffered from high mortalities. The lowest mortality occurred in the group of neonates fed with green algae. The test showed that TetraPhyll®, which is the standard food type in the reproduction test with adult *P. antipodarum*, and Sera® Spirulina are not suitable as a food source for neonates and juveniles in the FLC test. Therefore, juvenile snails of *P. antipodarum* should be fed with green algae to ensure survival and growth.

Test design 2 starts with up to one week old neonates of *P. antipodarum*. A FLC test with *P. antipodarum* was performed and Cd was chosen as test chemical. In controls and Cd exposed groups more than 50% of juveniles died in the first two weeks of the experiment probably because of the development of fungi in the test vessels. After 159 days of exposure Cd had no significant effect on the growth of the snails. For the endpoint reproduction the effect concentrations were in similar ranges compared to the 28 days reproduction test with adult snails. A comparison of results for juveniles and adults of *P. antipodarum* revealed that juvenile snails are slightly more sensitive towards the exposure of Cd and TBT.

The FLC experiments demonstrate that particular care is required when working with *P. antipodarum* early life stages. Due to the long test duration and high mortality during the

first weeks, it is difficult to complete such a test successfully. Neonates in a FLC test were slightly more sensitive towards an exposure to Cd compared to adult snails in the reproduction test. However, test design 2 has to be further optimised (e.g. reducing mortality at the beginning of the FLC test) and juvenile *P. antipodarum* should be exposed to further chemicals to answer the question if neonates are more sensitive compared to adults. Only if a significantly higher sensitivity of snails can be demonstrated for a number of test compounds in the FLC test compared to the reproduction test, the additional time and effort of the FLC test is justified.

Based on these investigations a draft SOP for a FLC test with *P. antipodarum* was compiled (see annex 8.2).

5 Fourth project part: Validation studies

One aim of the project was to develop a standard test method guideline with a selected mollusc species according to the specifications of the OECD. Goethe University Frankfurt designed the draft standard operating procedure (SOP) for the reproduction test with *Potamopyrgus antipodarum* based on literature and experimental investigations. The validation studies were performed to validate the proposed test conditions and identify issues in performing the draft guideline.

In total, 17 partners (including the ring test conducted within the predecessor project "ValMolRepro I" (project code: 3708 61 402, SIERATOWICZ AND OEHLMANN 2011)) participated in four validation exercises for the reproduction tests coming from academia, government or industry in 10 countries. In table 21 the participating laboratories are presented in alphabetical order. This sequence is not identical with laboratory codes used here.

Table 21: Participating laboratories involved in the validation exercises of the reproduction test with *Potamopyrgus antipodarum*.

Partner	Involved staff	Country
AstraZeneca	G. Le Page, R. Brown	United Kingdom
BASF SE	S. Hartmann, L. Weltje	Germany
Bayer Crop Science	K. Kuhl, D. Faber, E. Bruns	Germany
CEFAS - Centre for Environment, Fisheries & Aquaculture Science	C. Askem, T. Hutchinson, A. Smith	United Kingdom
ECT Oekotoxikologie GmbH	N. Graf, E. Heusner, P. Egeler	Germany
FERA - Department for Environment, Food & Rural Affairs	J. Turton, R. Benstead	United Kingdom
Fraunhofer IME	M. Moenig, M. Lutter, M. Teigeler	Germany
Goethe University Frankfurt a. Main	C. Geiß, K. Ruppert, U. Schulte-Oehlmann, J. Oehlmann	Germany
ibacon GmbH	A. Hengsberger, C. Boerschig, A. Seeland-Fremer	Germany
INRA - National Institute for Agricultural Research	M. Coke, M. Collinet, V. Ducrot	France
NIVA - Norwegian Institute for Water Research	M. Hultman, A. Macken	Norway
University of Antwerp	I. Planojevic, C. Schmitt	Belgium
University of Aveiro	P. Sanchez-Marin, C. Miguez	Portugal
University of Helsinki	P. Kajankari, O.-P. Penttinen	Finland
University of Iowa	C. Tucci, M. Neiman	United Stated of America
University of Southern Den-	B. F. Holbech, K. L. Kinnberg,	Denmark

mark	H. Holbech	
University of Vigo	T. Tato, R. Beiras	Spain

The chemical analyses of exposure media in validation 1 and validation 3 were performed by chemlab GmbH, Bensheim Germany. The University of Southern Denmark performed the analytical measurements in validation 2.

5.1 Validation 1

5.1.1 Organisation of the validation test

Eight laboratories participated in validation 1, which was realized in 2013. They were provided with snails for the test, salts for preparing the medium and also with the test substances.

Cd and TBT were chosen as test chemicals (see section 5.1.2.2). Five of the partners conducted the test with both substances and five laboratories only with TBT.

5.1.1.1 Snail production, biological quality checking and shipping

Snails used for validation 1 were taken from a laboratory culture (haplotype t, morphotype "Warwick A" according to STÄDLER ET AL. 2005) at Goethe University which was built up with specimens from the river Lumda in Hesse, Germany. The laboratory culture runs under standardized conditions at a temperature between 15°C and 17°C with a light/dark regime of 16:8 h. Before shipping to participants, size of the snails was determined (3.5 – 4.5 mm) and the reproductive output was checked.

Table 22 summarizes the shipping and acclimation duration of the animals and gives an overview of the test schedules. Post-shipping mortality did not appear in partner laboratories.

Table 22: Shipping, acclimation and test schedules for the partner laboratories in validation 1. Laboratories 2A and 2J performed the tests twice (as indicated by the letters a and b at the end of the codes).

Partner	2Aa	2Ab	2E	2F	2G	2H	2I	2Ja	2Jb	2K
Snail shipping date	-	-	19/6/13	16/7/13	7/8/13	20/8/13	20/8/13	20/8/13	5/2/14	5/8/13
Snails received	-	-	20/6/13	18/7/13	7/8/13	21/8/13	21/8/13	21/8/13	6/2/14	5/8/13
Number of snails sent	-	-	550	550	550	550	350	350	350	350
Acclimation duration	-	-	18 d	46 d	19 d	68 d	28 d	26 d	18 d	14 d
Test starting date	7/8/13	18/3/14	8/7/13	2/9/13	26/8/13	28/10/13	18/9/13	16/9/13	24/2/14	19/8/13
Test ending date	5/8/13	15/4/14	5/8/13	30/9/13	23/9/13	25/11/13	16/10/13	14/10/13	24/3/14	16/9/13

5.1.2 Implementation of the 28-day reproduction test

5.1.2.1 Principle of the test

Adult females of *P. antipodarum* are exposed in a 28-day-reproduction test to a test chemical with different concentrations. The test substance is spiked into the water and the snails are subsequently introduced into the test beakers. Survival is regularly determined and dead snails are removed from beakers. Renewal of exposure water and controls takes place three times per week. Animals are fed with finely ground Tetraphyll® after every water renewal. After 28 days snails are quick-frozen in liquid nitrogen and stored at -20°C until evaluation. The mortality and the total number of embryos in the brood pouch per female are determined.

5.1.2.2 Chemicals

Cd was used as Cd chloride (CAS-No.: 10108-64-2, Sigma-Aldrich®, Germany) and was provided from a single batch to partners by Goethe University. Five of the partners conducted the reproduction test with Cd.

In validation 1 the same nominal cadmium concentrations were chosen as already used in the first ring test conducted within the previous project "ValMolRepro I" (SIERATOWICZ AND OEHLMANN 2011):

1.56 µg/L, 3.13 µg/L, 6.25 µg/L, 12.5 µg/L, 25 µg/L.

TBT was used as tributyltin chloride (CAS-No.: 1461-22-9, Sigma-Aldrich®, Germany) and was provided from a single batch to partners by Goethe University. For this test chemical glacial acetic acid was used as solvent at a concentration of 10 µL/L. An additional solvent control was considered in the test. The following nominal concentrations were used for the test, whereby the concentrations refer to TBT-Sn:

10 ng/L, 25 ng/L 65 ng/L 160 ng/L 400 ng/L 1000 ng/L

All laboratories tested in a concentration range from 10 to 400 ng TBT-Sn/L, except for the two repeat studies in laboratories 2A and 2J (tests 2Ab and 2Jb). Laboratory 2J had problems with preparing of the TBT-stock solutions for the first test run (2Ja) which resulted in 500-fold lower concentrations than required. Because a statistically significant reduction of embryo numbers occurred only at the highest tested concentration in most of the reproduction tests, laboratories 2A and 2J repeated the reproduction test with *P. antipodarum* in a concentration range from 25 up to 1000 ng TBT-Sn/L.

5.1.2.3 Experimental conditions

Experimental conditions and instructions were given in the draft SOP. A semi-static test design is applied with medium renewals three times a week for all exposure groups and controls.

Snails were exposed in closable 500 mL beakers with aerated 400 mL test medium (see table 23). Each test concentration and the control were tested in four replicates with ten snails each. Snails were fed with finely ground TetraPhyll® (0.25 mg per animal and day; Tetra GmbH, Melle, Germany).

Biological raw data per female (shell length, embryo numbers) as well as water parameters were summarized in a Microsoft Excel® sheet for further data evaluation by the coordinating partner laboratory (Goethe-University).

Table 23: Summary of experimental conditions in validation 1 of the reproduction test with *Potamopyrgus antipodarum*.

Test duration	28 days
Test water	Reconstituted water (with 0.3 g Tropic Marin® salt and 0.18 g $NaHCO_3$ per 1 litre de-ionised water) water quality requirements: pH 7.5 – 8.5, conductivity 770 ± 100 µS/cm, oxygen concentration >60% ASV (air saturation value)
Test vessels	500 mL glass beakers with lids
Water renewal	3 times per week
Temperature	16 ± 1°C
Light intensity	500 ± 100 lx
Water sampling	From all test concentrations and controls water was sampled over two renewal intervals for TBT and over four renewal intervals for cadmium
Photoperiod	16:8 h L:D
Food source	Fine grounded Tetraphyll®
Feeding	0.25 mg/animal and day
Snails origin	Laboratory culture, which was built up with snails from Lumda Hesse, Germany
Test snails size	3.5 – 4.5 mm
Snails density	10 snails per 400 mL medium (4 replicates per tested concentration)
Core test endpoints	Mortality, reproduction

5.1.3 Chemical analysis and biological data analysis

In the first and in the last week of the test, samples from TBT exposure groups and solvent control were taken for chemical analysis. Therefore, on day 0 and day 25 water samples were taken from freshly prepared exposure media and on day 2 and day 28 from old water which was pooled from every replicate of one treatment group. Samples were stored in HDPE-bottles at 4°C until analysis.

Chemical analysis of TBT was performed via gas chromatography according to DIN EN ISO 17353 – F13 (2005) at chemlab GmbH in Bensheim, Germany. The limit of quantification (LOQ) and the limit of determination (LOD) for this method are 2.05 and 0.82 ng TBT-Sn/L, respectively.

For the chemical analysis of Cd, samples of exposure groups and control were taken every week. 25 mL of fresh contaminated test medium were sampled from the test vessels before (2 or 3 days old) and after (fresh) medium renewal for each test concentration, including controls,

bottled into 50 mL tubes and acidified with 125 µL nitric acid (65%, suprapure®, Merck KgaA, Darmstadt, Germany). Chemical analysis of Cd was performed via inductively coupled plasma mass spectrometry according to DIN EN ISO 1729-2 (2005) at chemlab GmbH Bensheim, Germany. The LOQ and the LOD for this method are 0.5 and 0.03 µg Cd/L, respectively. Time-weighted mean (TWM) concentrations of the chemicals were calculated according to Annex 6 of OECD guideline 211 (OECD 2012b).

Biological raw data were reported by the participating laboratories using an Excel® spread sheet which had been provided by Goethe University. From the raw data means were calculated for biological parameters (shell height, embryo numbers). Effect concentrations were calculated by Dunnett's test (NOEC, LOEC) or by a non-linear regression using a four parameter logistic equation (EC_{10}, EC_{50}).

5.1.4 Results of validation 1

5.1.4.1 Compliance with validity criteria

For a test to be valid the conditions were chosen based on available guidelines for freshwater invertebrates and as proposed in OECD (2010). For the purpose of validation 1, the following validity criteria should be fulfilled:

- Mortality in the controls should not exceed 20%,
- Dissolved oxygen saturation must have been at least 60% of the air saturation value (ASV) and
- Water temperature should be 16 ± 1°C throughout the test.

All laboratories achieved adequate oxygen saturation values. The mean ASV for oxygen ranged from 86.9% (laboratory 2K) to 102% (laboratory 2I). Laboratory 2Jb did observe mortality in the solvent control. 30% of the snails died throughout the test. Except for laboratory 2K, all of the participants achieved the defined temperature scale and the mean temperature was between 15.4°C and 17.0°C. The mean temperature of laboratory 2K was 19.0°C. Therefore, the tests from laboratories 2Jb and 2K were not valid and test results from these laboratories are not considered in the following evaluation of reproduction data.

5.1.4.2 Physico-chemical parameters

Table 24 summarizes the means of the physico-chemical parameters for all laboratories. The pH values ranged between 7.95 (laboratory 2E) to 8.34 (laboratory 2F) which is in the determined range of 7.5 - 8.5. Also the measured conductivity was similar among all partners.

Table 24: Mean physico-chemical parameters in validation 1 run of the reproduction test with *Potamopyrgus antipodarum*. n.r.: not received.

Laboratory	pH			Conductivity [µS/cm]			Temperature [°C]			O_2 saturation [%]		
	mean	SD	n	mean	SD	n	mean	SD	n	mean	SD	n
2Aa	8.26	0.700	144	811	58.8	144	15.4	0.386	144	95.0	5.99	144
2Ab	8.13	0.900	84	788	31.3	84	16.7	0.311	84	93.8	7.01	84
2E	7.95	0.707	144	774	22.5	24	15.4	0.344	24	101	1.26	24
2F	8.39	0.680	155	819	31.3	108	16.1	0.709	156	98.5	4.99	155
2G	8.34	0.670	156	755	44.5	156	16.2	0.405	156	95.0	4.28	156
2H	8.06	0.660	156	711	64.4	156	15.8	0.559	156	90.6	6.34	156
2I	8.24	0.890	85	668	37.5	85	17.0	0.498	85	102	5.05	85
2Ja	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
2Jb	8.24	0.863	91	762	34.3	91	15.6	0.279	91	93.4	7.90	70
2K	8.31	0.870	91	719	128	91	19.0	1.05	91	86.9	10.4	91

5.1.4.3 Actual exposure concentrations of cadmium

The results of the analytical analyses of Cd for all laboratories are shown in tables 25 - 29. The measured concentrations are similar among partners although nominal concentrations could not always be reached. In total, 76.9% of the nominal concentrations were achieved. Because of this, measured TWM concentrations were used to calculate effect concentrations. Initial concentrations varied between 69.6% and 304% of nominal concentrations. After two or three days values ranged between 3.27% and 88.4%. The difference between nominal and measured concentrations might be due to adsorption of Cd on test vessel walls, food particles or direct uptake of cadmium by animals (JENSEN ET AL. 2001, PASCOE ET AL. 1990). In some of the water samples at laboratory 2E, 2G and 2H the measured concentrations of Cd were below the LOQ. To calculate TWM concentrations the half of the LOQ (0.25 µg/L) was assumed according to the recommendation of OECD (2000).

The measured concentrations of Cd in controls were below the LOQ (0.5 µg/L), except for one sample from laboratory 2E. Here, at day 7 the measured concentration was 0.9 µg/L, which could not be rediscovered two days later (day 9).

Noticeable are the concentrations of laboratory 2G e.g. on day 0, where up to 6.6-fold of nominal concentrations were achieved. An explanation for this could be a mistake in the preparation of spiking solutions. Nevertheless, these abnormalities in concentrations could not be observed on the other sampling days and were included in the calculation of TWM.

Table 25: Results of cadmium analyses [µg/L] in exposure media from laboratory 2Aa in validation 1 of the reproduction test with *Potamopyrgus antipodarum*. *Italic: not included in TWM calculations.*

Cd nominal concentration [µg/L]	Measured concentrations [µg/L]								Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
Negative control	‹ LOQ	‹ LOQ	‹ LOD	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	-	-
1.56	1.3	0.7	1.0	0.7	1.2	0.8	1.1	1.6	1.07	68.6
3.13	2.6	1.6	2.4	1.6	2.3	1.8	2.6	1.9	2.09	66.8
6.25	5.8	3.7	5.5	3.8	5.1	3.8	5.4	4.1	4.62	73.9
12.5	12.0	7.7	11.3	8.2	10.8	8.8	11.6	16.5	11.1	88.8
25.0	No sample	*17.1*	23.0	17.9	20.8	18.9	24.2	19.4	20.8	83.2

LOQ = 0.5 µg/L

Table 26: Results of cadmium analyses [µg/L] in exposure media from laboratory 2E in validation 1 of the reproduction test with *Potamopyrgus antipodarum*. Bold: half of the LOQ.

Cd nominal concentration [µg/L]	Measured concentrations [µg/L]								Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
Negative control	‹ LOQ	‹ LOQ	0.9	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	-	-
1.56	1.4	0.25	1.4	0.9	1.4	1.0	1.5	0.9	1.06	67.9
3.13	2.7	0.25	3.0	1.9	2.7	2.2	3.1	2.0	2.14	68.3
6.25	5.7	0.25	5.6	4.1	6.2	4.1	6.2	3.9	4.24	67.8
12.5	11.0	1.1	11.3	7.5	12.6	10.2	13.3	8.1	9.03	72.2
25.0	22.4	1.8	22.9	15.4	25.5	20.5	26.1	15.2	17.8	71.3

LOQ = 0.5µg/L

Table 27: Results of cadmium analyses [µg/L] in exposure media from laboratory 2F in validation 1 of the reproduction test with *Potamopyrgus antipodarum*. *Italic: not included in TWM calculations.*

Cd nominal concentration [µg/L]	Measured concentrations [µg/L]								Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
Negative control	‹ LOQ	No sample	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	-	-
1.56	1.3	0.7	1.3	0.9	1.3	0.7	1.5	0.9	1.06	67.9
3.13	2.9	1.5	2.8	2.4	3.0	1.9	2.9	1.9	2.37	75.7
6.25	5.9	3.4	5.9	4.3	6.1	3.6	5.9	3.6	4.74	75.8
12.5	12.1	7.1	12.0	9.4	*12.1*	No sample	12.0	10.6	10.6	84.8
25.0	23.6	16.4	23.4	23.9	24.0	16.9	24.0	17.6	21.0	84.0

LOQ = 0.5 µg/L

Table 28: Results of cadmium analyses [µg/L] in exposure media from laboratory 2G in validation 1 of the reproduction test with *Potamopyrgus antipodarum*. Bold: half of the LOQ.

Cd nominal concentration [µg/L]	Measured concentrations [µg/L]								Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
Negative control	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	-	-
1.56	7.5	0.6	1.3	0.9	0.25	0.8	1.8	1.0	1.41	90.4
3.13	3.4	1.6	3.1	2.2	2.2	1.9	3.8	1.9	2.48	79.2
6.25	41.5	2.8	26.4	3.7	5.5	3.8	5.4	4.5	8.42	135
12.5	26.5	7.1	8.4	8.7	13.8	7.4	6.5	9.6	10.1	80.9
25.0	14.1	15.9	43.0	16.9	15.8	17.5	25.9	19.0	15.9	63.5

LOQ = 0.5 µg/L

Table 29: Results of cadmium analyses [µg/L] in exposure media from laboratory 2H in validation 1 of the reproduction test with *Potamopyrgus antipodarum*. Bold: half of the LOQ.

Cd nominal concentration [µg/L]	Measured concentrations [µg/L]								Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
Negative control	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	-	-
1.56	1.2	0.25	1.5	0.9	1.5	0.6	1.4	0.7	0.95	60.9
3.13	2.3	1.0	3.0	2.7	2.4	1.2	3.3	1.9	2.21	70.6
6.25	4.8	1.5	7.4	6.1	6.2	2.8	6.0	4.3	4.78	76.5
12.5	9.7	4.4	13.4	10.0	11.5	6.0	14.0	6.4	9.19	73.5
25.0	20.3	10.6	25.8	19.5	24.1	14.0	25.2	13.1	18.6	74.4

LOQ = 0.5 µg/L

5.1.4.4 Biological responses to cadmium

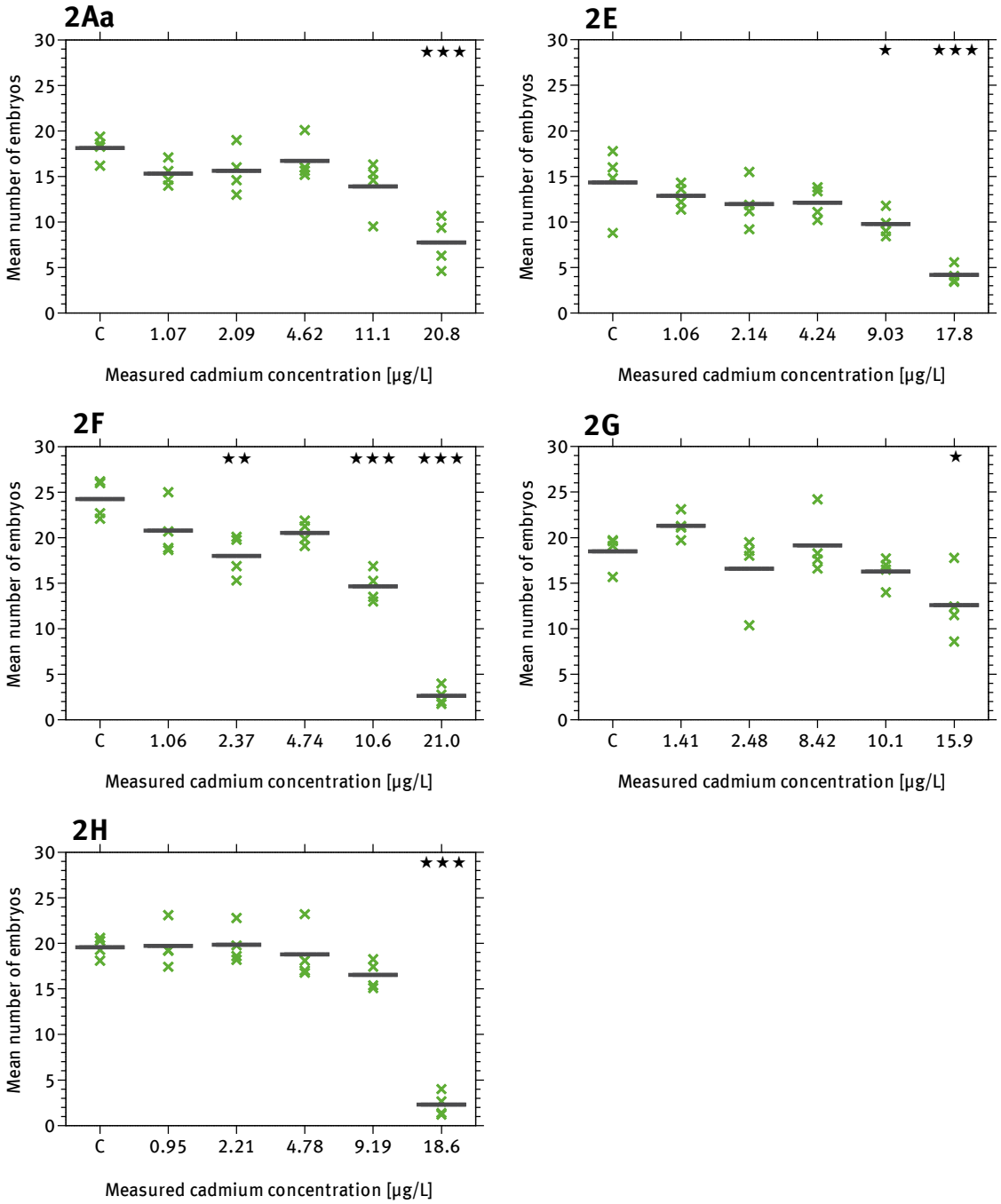
Mortality

In laboratories 2Aa, 2E, 2G and 2H the maximal observed mortality was 5.1% throughout the test. In laboratory 2F a mortality of 37.5% occurred at the highest test concentration (21.0 µg/L), which was significant compared to control group (Fisher's exact test, $p < 0.0001$). The maximal mortality at the other cadmium concentrations was 7.5%.

Reproduction

In figure 34 the total embryo numbers of *P. antipodarum* after four weeks of exposure from all participating laboratories are shown. All of them observed a significant reduction in embryo numbers with increasing Cd concentrations. Laboratory 2Aa, 2G and 2H found comparable NOEC (11.1 µg/L, 10.1 µg/L, 9.19 µg/L) and LOEC (20.8 µg/L, 15.9 µg/L, 18.6 µg/L) values, whereas laboratory 2E and 2F showed slightly lower values (NOEC: 4.24 µg/L, 4.74 µg/L; LOEC: 9.03 µg/L, 10.6 µg/L). Snails thus showed the highest sensitivity towards Cd in these two laboratories. Indeed, the animals of laboratory 2F might have been in a bad condition, because only in this laboratory a significant mortality at the highest test concentration (21.0 µg/L) was observed or the results might be influenced by any other unknown factor.

Figure 34: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks exposure to measured cadmium concentrations at laboratories 2Aa, 2E, 2F, 2G and 2H (crosses: replicate mean; line: treatment mean). Asterisks indicate significant differences compared to control (Dunnett´s test). ★ = p‹0.05, ★★ = p‹0.01, ★★★ = p ‹ 0.001, n = 4 replicates.



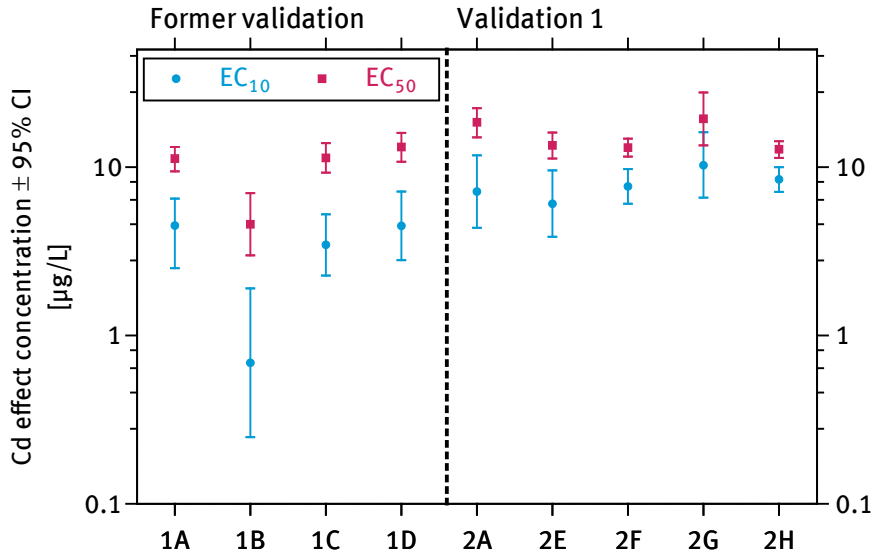
The good inter-laboratory reproducibility becomes apparent in the calculated effect concentrations (see table 30 and figure 35). The EC_{10} values ranged from 6.10 µg/L to 10.3 µg/L and the EC_{50} from 12.8 µg/L to 18.5 µg/L.

In figure 35 the effect concentrations including the 95%-confidence intervals for Cd from each laboratory in a first ring test conducted in the previous project (SIERATOWICZ AND OEHLMANN 2011) and validation 1 are shown. Taken into account that laboratory 1B might be an exception, most of the partners overlap with their 95%-confidence intervals which demonstrates the robustness of the test. The higher sensitivity of snails in laboratory 1B is probably due to a mixing of the originally shipped snail batch which suffered from high mortality after arrival, indicating a poor health status, with a second batch of snails shipped afterwards. These mixed cohorts were used for the testing with Cd in laboratory 1B. SIERATOWICZ ET AL. (2013) demonstrated in tests with *P. antipodarum* and Cd that a previous stress factor results in increased sensitivity of the snails to the test chemical.

Table 30: Effect concentrations (EC_{10} and EC_{50} with 95%-confidence intervals, NOEC, LOEC) for the total embryo numbers of *Potamopyrgus antipodarum* in validation 1 based on time-weighted mean concentrations of cadmium [µg/L].

	2Aa	2E	2F	2G	2H
EC_{10}	7.19 (4.37 - 11.8)	6.10 (3.88 - 9.59)	7.74 (6.3 - 9.78)	10.3 (6.61 - 16.2)	8.47 (7.15 - 10.0)
EC_{50}	18.5 (15.1 - 22.5)	13.5 (11.3 - 16.0)	13.1 (11.6 - 14.8)	19.4 (13.5 - 27.9)	12.8 (11.4 - 14.3)
NOEC	11.1	4.24	4.74	10.1	9.19
LOEC	20.8	9.03	10.6	15.9	18.6

Figure 35: Calculated effect concentrations (EC_{10} in blue, EC_{50} in red) values (µg/L) including 95% confidence intervals of the reproduction test with *Potamopyrgus antipodarum* with cadmium from all participating laboratories of the first ring test conducted in the previous project ValMolRepro I (SIERATOWICZ AND OEHLMANN 2011) and validation 1.



5.1.4.5 Actual exposure concentrations of tributyltin

The results for chemical analyses (Tab. 31 - 39) show that measured TBT concentrations were below nominal concentrations in most of the laboratories. Initial concentrations varied between 6.31% and 285% of nominal concentrations. Calculated TWM values varied between 10.1% and 121% of the nominal concentrations. Average TWM for all laboratories was 42.2%. An explanation for the low exposure concentrations could be the degradation of TBT to monobutyltin and dibutyltin during the exposure. Another reason might be the adsorption of the substance to the vessel wall and to food particles due to the low solubility of TBT and/or by direct uptake by the snails. In the test 2Ja, no TBT could be found (not shown) because of a mistake during stock solution preparation. In all laboratories no TBT could be detected in the solvent controls.

Table 31: Results of tributyltin analyses [ng Sn/L] in exposure media from laboratory 2Aa in validation 1 of the reproduction test with *Potamopyrgus antipodarum*.

TBT nominal concentration [ng Sn/L]	Measured concentrations [ng SnL]				Time weighted mean [ng Sn/L]	% of nominal concentration
	Day 0 fresh medium	Day 2 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	-	-
10.0	3.69	2.87	5.33	4.10	4.11	41.1
25.0	27.5	25.4	71.3	11.9	30.5	121
65.0	41.8	31.1	21.3	33.6	30.7	47.2
160	58.6	52.5	50.8	62.7	56.1	35.1
400	229	122	123	57.8	120	29.9

LOQ = 2.05 ng Sn/L

Table 32: Results of tributyltin analyses [ng Sn/L] in exposure media from laboratory 2Ab in validation 1 of the reproduction test with *Potamopyrgus antipodarum*.

TBT nominal concentration [ng Sn/L]	Measured concentrations [ng SnL]				Time weighted mean [ng Sn/L]	% of nominal concentration
	Day 0 fresh medium	Day 2 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	-	-
25.0	33.6	11.5	26.2	10.7	18.6	74.4
65.0	67.2	19.3	37.7	9.84	27.8	42.7
160	109	19.7	118	14.8	50.8	31.8
400	455	326	185	77.0	229	57.2
1000	885	889	459	1291	838	83.8

LOQ = 2.05 ng Sn/L

Table 33: Results of tributyltin analyses [ng Sn/L] in exposure media from laboratory 2E in validation 1 of the reproduction test with *Potamopyrgus antipodarum*.

TBT nominal concentration [ng Sn/L]	Measured concentrations [ng SnL]				Time weighted mean [ng Sn/L]	% of nominal concentration
	Day 0 fresh medium	Day 2 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	-	-
10.0	No sample	*29.9*	4.10	6.97	5.40	50.4
25.0	No sample	*41.8*	9.43	65.2	28.8	115
65.0	11.1	109	51.2	19.3	36.8	56.6
160	54.1	220	103	63.9	96.3	60.2
400	107	541	219	99.6	198	49.6

LOQ = 2.05 ng Sn/L; *Italics*: not included in TWM calculations.

Table 34: Results of tributyltin analyses [ng Sn/L] in exposure media from laboratory 2F in validation 1 of the reproduction test with *Potamopyrgus antipodarum*.

TBT nominal concentration [ng Sn/L]	Measured concentrations [ng SnL]				Time weighted mean [ng Sn/L]	% of nominal concentration
	Day 0 fresh medium	Day 2 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	-	-
10.0	2.46	2.05	2.46	3.28	2.61	26.1
25.0	5.36	4.10	3.67	5.74	4.66	18.6
65.0	4.10	6.56	13.5	10.7	9.30	14.3
160	11.1	7.79	26.2	16.0	16.1	10.1
400	44.7	85.7	20.9	36.5	41.8	10.5

LOQ = 2.05 ng Sn/L

Table 35: Results of tributyltin analyses [ng Sn/L] in exposure media from laboratory 2G in validation 1 of the reproduction test with *Potamopyrgus antipodarum*.

TBT nominal concentration [ng Sn/L]	Measured concentrations [ng SnL]				Time weighted mean [ng Sn/L]	% of nominal concentration
	Day 0 fresh medium	Day 2 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	-	-
10.0	6.15	2.46	4.51	4.51	4.30	43.0
25.0	10.7	21.3	10.2	11.1	12.5	50.1
65.0	21.3	7.79	24.6	108	39.2	60.3
160	44.3	25.0	61.9	34.4	41.4	25.8
400	118	84.4	158	147	132	32.9

LOQ = 2.05 ng Sn/L

Table 36: Results of tributyltin analyses [ng Sn/L] in exposure media from laboratory 2H in validation 1 of the reproduction test with *Potamopyrgus antipodarum*.

TBT nominal concentration [ng Sn/L]	Measured concentrations [ng SnL]				Time weighted mean [ng Sn/L]	% of nominal concentration
	Day 0 fresh medium	Day 2 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	-	-
10.0	2.05	**2.05**	4.51	3.69	3.04	30.4
25.0	6.15	2.87	2.00	11.1	7.75	31.0
65.0	20.9	16.8	32.0	7.38	17.6	27.0
160	32.0	2.87	95.1	23.8	35.7	22.3
400	88.5	11.5	216	74.6	94.9	23.7

LOQ = 2.05 ng Sn/L; Bold: half of the LOQ.

Table 37: Results of tributyltin analyses [ng Sn/L] in exposure media from laboratory 2I in validation 1 of the reproduction test with *Potamopyrgus antipodarum*.

TBT nominal concentration [ng Sn/L]	Measured concentrations [ng SnL]				Time weighted mean [ng Sn/L]	% of nominal concentration
	Day 0 fresh medium	Day 2 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	-	-
10.0	6.15	6.56	9.43	4.10	6.39	63.9
25.0	6.15	12.7	5.33	16.0	9.43	37.7
65.0	29.1	13.9	13.5	13.1	16.2	25.0
160	121	22.5	34.4	16.4	38.0	23.7
400	180	37.3	97.5	28.3	69.7	17.4

LOQ = 2.05 ng Sn/L

Table 38: Results of tributyltin analyses [ng Sn/L] in exposure media from laboratory 2Jb in validation 1 of the reproduction test with *Potamopyrgus antipodarum*.

TBT nominal concentration [ng Sn/L]	Measured concentrations [ng SnL]				Time weighted mean [ng Sn/L]	% of nominal concentration
	Day 0 fresh medium	Day 2 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	-	-
25.0	10.2	4.51	9.02	8.61	8.08	32.3
65.0	28.3	11.9	15.2	27.0	19.9	30.6
160	22.5	43.9	16.8	23.8	24.9	15.6
400	98.4	29.1	57.4	68.4	60.4	15.1
1000	475	161	357	447	356	35.6

LOQ = 2.05 ng Sn/L

Table 39: Results of tributyltin analyses [ng Sn/L] in exposure media from laboratory 2K in validation 1 of the reproduction test with *Potamopyrgus antipodarum*.

TBT nominal concentration [ng Sn/L]	Measured concentrations [ng SnL]				Time weighted mean [ng Sn/L]	% of nominal concentration
	Day 0 fresh medium	Day 2 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	‹ LOQ	‹ LOQ	‹ LOQ	‹ LOQ	-	-
10.0	14.8	5.74	10.2	4.10	7.84	78.4
25.0	6.56	7.38	5.74	4.92	5.97	23.9
65.0	79.1	11.5	108	25.0	48.0	73.8
160	136	75.0	256	76.2	130	81.3
400	296	170	340	154	232	58.0

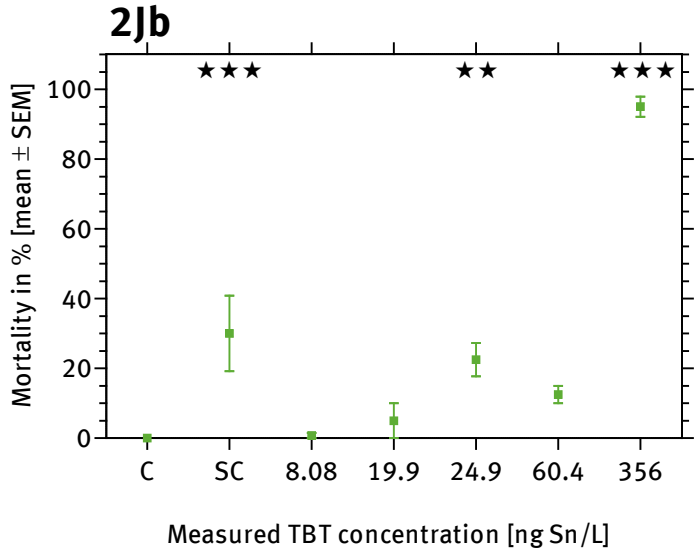
LOQ = 2.05 ng Sn/L

5.1.4.6 Biological responses to tributyltin

Mortality

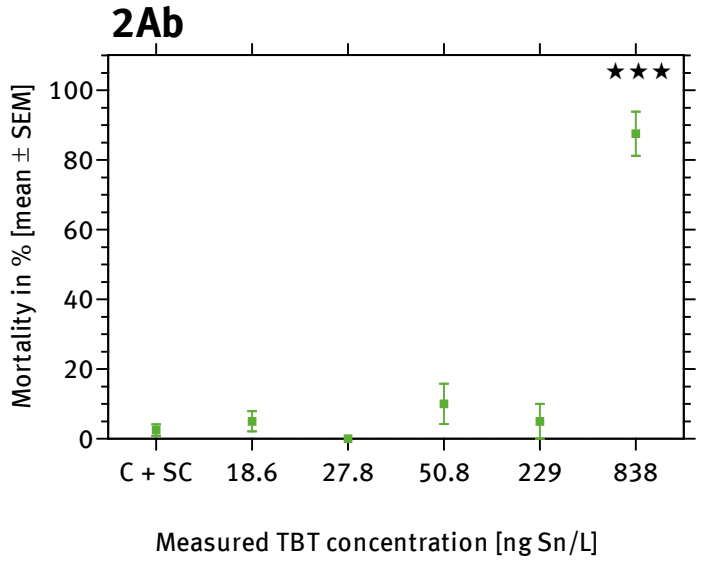
The mortality of control snails was homogenous between the laboratories and did not exceed 5.0%, except in laboratory 2Jb. There a mortality of 30.0% was observed in the solvent control which is significant compared to the negative control (Fig. 36). Therefore, laboratory 2Jb did not meet the validity criterion for mortality (max. 20%) and was excluded from the evaluation of reproduction data.

Figure 36: Mortality (mean with standard error) of *Potamopyrgus antipodarum* after four weeks exposure to measured tributyltin concentrations at laboratory 2Jb. Asterisks indicate significant differences compared to negative control (C) (Fisher's exact test). ★★ = p‹0.01, ★★★ = p ‹ 0.001, n = 4 replicates.



In experiments with a maximum nominal concentration of 400 ng TBT-Sn/L mortality in TBT exposed snails did not exceed 5.0% in laboratories 2Aa, 2E, 2F, 2G, 2H and 2I. In laboratory 2Ab (Fig. 37) with a maximum tested nominal concentration of 1000 ng TBT-Sn/L mortality increased up to 87.5% at the highest tested concentration ($p < 0.0001$).

Figure 37: Mortality (mean with standard error) of *Potamopyrgus antipodarum* after four weeks exposure to measured tributyltin concentrations at laboratory 2Ab. Asterisks indicate significant differences compared to merged solvent control and negative control (C + SC) (Fisher's exact test). ★★★ = $p < 0.0001$, n = 4 replicates.



Reproduction

Figure 38 shows the results of the reproduction tests with *P. antipodarum* of all participating labs which met the validity criteria. All laboratories found a concentration-dependent decrease of embryo numbers in the brood pouch of *P. antipodarum* under exposure to TBT, although with slightly different effect concentrations. Laboratories 2Aa, 2Ab, 2F, 2G, 2H and 2I provided comparable NOECs (30.7, 18.6, 16.1, 39,2, 35.7 and 38.0 ng Sn/L) and LOECs (56.1, 27.8, 41.8, 41.4, 94.9 and 69.7/L). The results from laboratory 2E resulted in considerably higher effect concentrations with a significant reduced number of embryos at 198 ng TBT-Sn/L (LOEC).

Figure 38: Embryo numbers of *P. antipodarum* after exposure to TBT at laboratories 2Aa, 2Ab, 2E, 2F, 2G, 2H and 2I (crosses: replicate mean; line: treatment mean). Significance compared to merged solvent and negative control (C + SC): ★ = p<0.05, ★★ = p<0.01, ★★★ = p < 0.001, n = 4 replicates, (Dunnett)

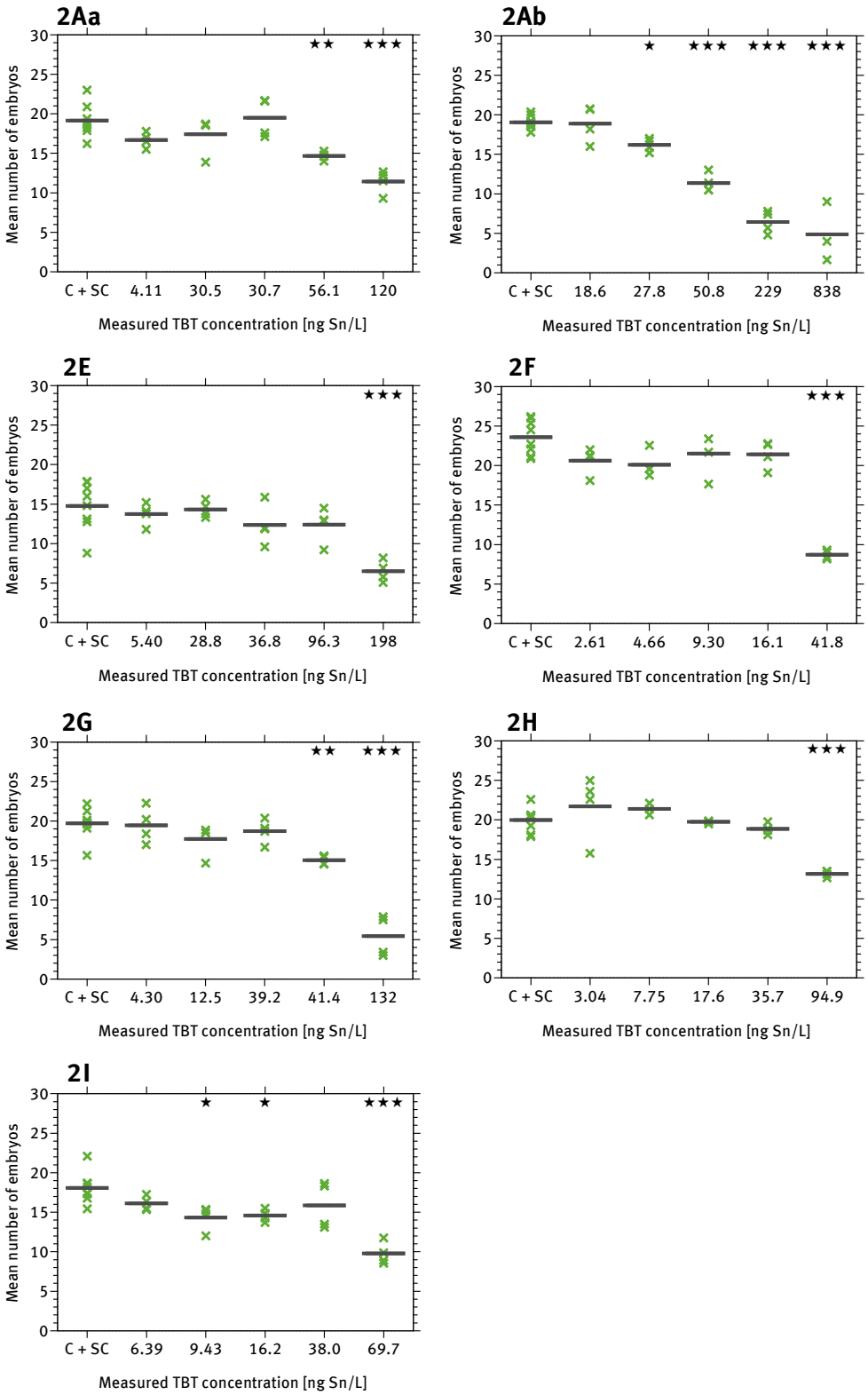
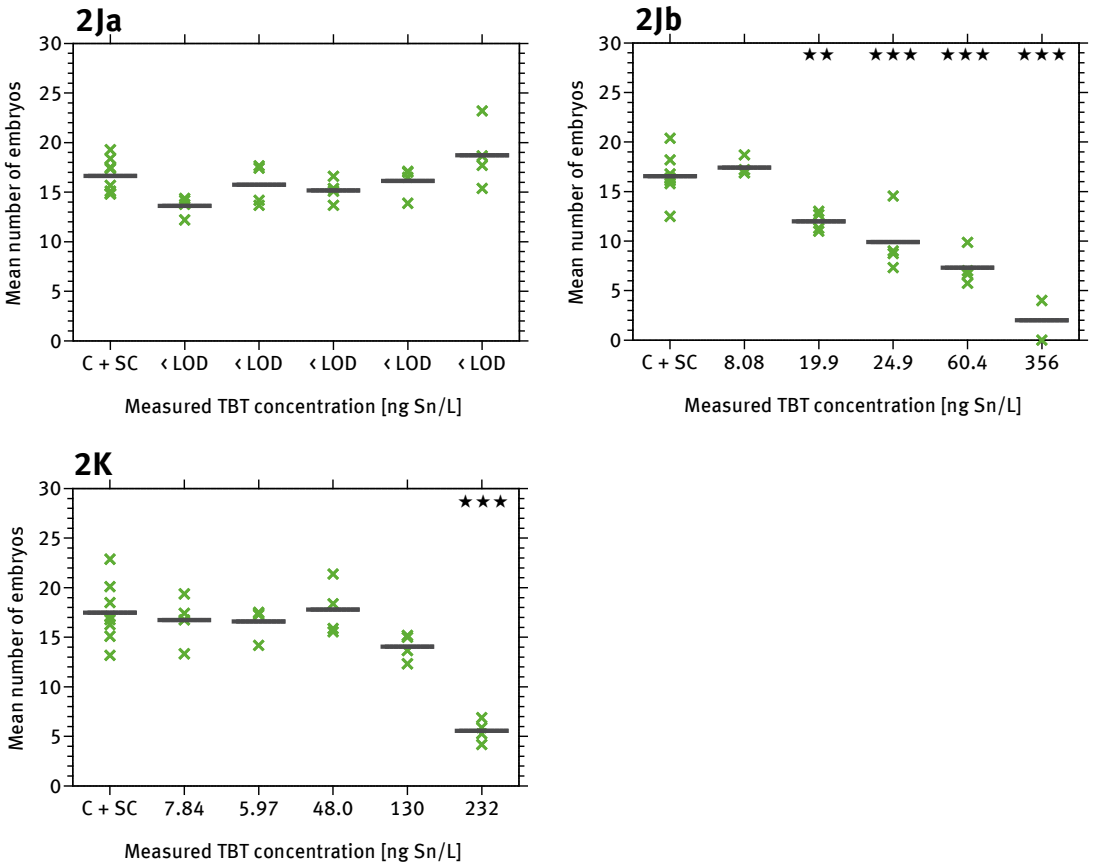


Figure 39 shows the results of the laboratories which did not achieve the validity criteria or in which no TBT could be detected in exposure groups. The embryo numbers of snails in the experiment at laboratory 2Ja were all on the same level, as no TBT exposure occurred there. In the second experiment (2Jb), exposed snails showed a high sensitivity towards the TBT exposure with a NOEC of 8.08 ng Sn/L. This high sensitivity might be due to a poor health status of the animals, as snails suffered from high mortality (see fig. 36). Laboratory 2K was not able to reach a mean temperature between 15°C and 17°C, despite that the results from laboratory 2K are comparable with the results of the valid laboratories. A significant reduction was observed at the highest test concentration of 232 ng Sn/L (LOEC).

Figure 39: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks exposure to measured tributyltin concentrations at non-valid laboratories 2Ja, 2Jb and 2K (crosses: replicate mean; line: treatment mean). Asterisks indicate significant differences compared to merged solvent control and negative control (C + SC) (Dunnett´s test). ★★ = p<0.01, ★★★ = p < 0.001, n = 4 replicates.

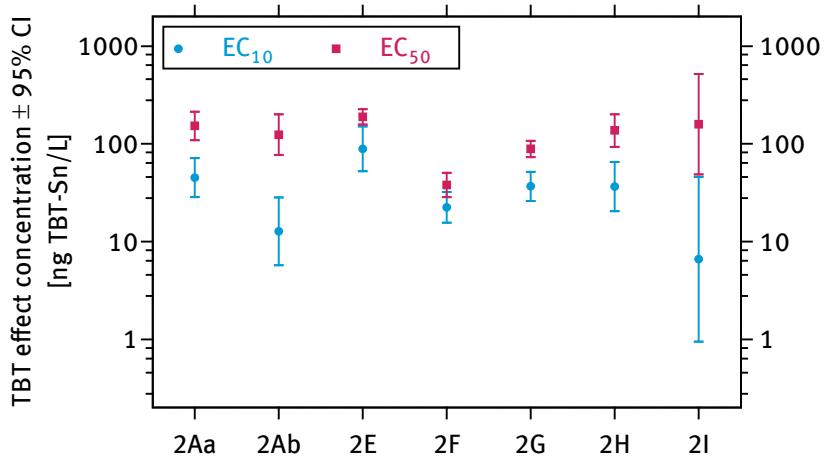


The good match of results for TBT between valid laboratories is also reflected by the calculated EC_{10} and EC_{50} values (tab. 40, fig. 40). The findings from laboratory 2E resulted in higher EC_{10} values whose 95%-confidence intervals only overlapped with the EC_{10} confidence interval of laboratory 2H. Laboratory 2F showed lowest effect concentrations with an EC_{50} of 37.2 ng Sn/L.

Table 40: Effect concentrations (EC_{10} and EC_{50} with 95%-confidence intervals, NOEC, LOEC) for the total embryo numbers of *Potamopyrgus antipodarum* in validation 1 based on time-weighted mean concentrations of tributyltin [ng Sn/L]. *Italics: data from laboratories with non-valid test results.*

	2Aa	2Ab	2E	2F	2G	2H	2I	2Jb	2K
EC_{10}	45.0 (28.4 - 71.2)	12.7 (5.73 - 28.3)	89.1 (53.2 - 149)	22.4 (15.6 - 32.3)	36.8 (26.1 - 51.7)	36.5 (20.4 - 65.0)	6.62 (0.95 - 45.9)	-	-
EC_{50}	153 (109 - 213)	124 (77.0 - 200)	188 (157 - 226)	37.9 (28.5 - 50.4)	88.8 (73.5 - 107)	137 (93.2 - 200)	159 (48.8 - 519)	-	-
NOEC	30.7	18.6	96.3	16.1	39.2	35.7	38.0	*8.08*	*130*
LOEC	56.1	27.8	198	41.8	41.4	94.9	69.7	*19.9*	*232*

Figure 40: Calculated effect concentrations (EC_{10} in blue, EC_{50} in red) [ng Sn/L] including 95% confidence intervals of the reproduction test with *P. antipodarum* with tributyltin from all participating and valid laboratories of validation 1.



5.1.5 Comparison of present results with published and grey literature

The effect concentrations for Cd obtained in the ring tests are in good accordance with published data. SIERATOWICZ ET AL. (2011) determined 28 days EC_{10} and EC_{50} values of 1.30 µg/L (95% CI: 0.662 – 2.55 µg/L) and 11.5 µg/L (95% CI: 8.58 – 15.4 µg/L), respectively. DORGELO ET AL. (1995) performed a ten weeks test with Cd and reported a significantly decreased number of released juveniles and a delayed production of young snails after five to six weeks already at the lowest nominal test concentration of 25 µg Cd/L.

The effect concentrations for TBT obtained in the ring tests are in good accordance with published data. Exposure to TBT, conducted *i.a.* in experiments during the EU-project COMPRENDO (EU project code: EVK1-CT-2002-00129) resulted in an identical response pattern. The total embryo numbers significantly decreased resulting in an EC_{50} of 115 ng Sn/L, an EC_{10} of 37.8 ng

Sn/L, and a LOEC of 50 to 100 ng Sn/L after 8 weeks exposure (SCHULTE-OEHLMANN 1997, DUFT ET AL. 2007).

Compared to other invertebrates, e.g. *Daphnia magna*, *P. antipodarum* shows a higher sensitivity for TBT. The NOEC for TBT in *D. magna* reproduction is 456 ng Sn/L (OBERDÖRSTER ET AL. 1997); a LOEC could not be assessed due to high mortality at the highest test concentration of 912 ng Sn/L ($LOEC_{mortality}$). MATHIJSSEN-SPIEKMAN ET AL. (1989) also assessed higher effect concentrations in a 21 day reproduction test with TBT-oxide and *D. magna*. The detected LOEC for reproduction was 1.8 µg TBTO/L (≙ 716 ng TBT-Sn/L), the NOEC 1.0 µg TBTO/L (≙ 398 ng TBT-Sn/L).

5.1.6 Conclusions from validation 1

In summary, it can be stated that the results of the reproduction tests with Cd showed a good agreement among participating laboratories. The mean values (with coefficient of variation) for EC_{10}, EC_{50}, NOEC and LOEC from these five laboratories are 7.96 µg/L (19.7%), 15.5 µg/L (20.8%), 7.87 µg/L (40.2%) and 15.0 µg/L (33.8%), respectively. If results of the first ring test conducted in the previous project ValMolRepro I (1B excluded, SIERATOWICZ AND OEHLMANN 2011) are also considered, than the mean values for EC_{10}, EC_{50}, NOEC and LOEC are 6.53 µg/L (35.5%), 14.2 µg/L (21.8%), 6.45 µg/L (50.5%) and 12.6 µg/L (42.2%), respectively with a minimum of a 1.72-fold difference (EC_{50} values) and a maximum of a 3.91-fold difference (LOEC values).

Furthermore, also the results of the reproduction tests with TBT showed a good accordance among partners. Effect concentrations show a minimum of a 4.78-fold difference (LOECs) and a maximum of a 13.5-fold difference (EC_{10} values). The mean values (with coefficient of variation) for EC_{10}, EC_{50}, NOEC and LOEC values from these laboratories are 35.6 ng Sn/L (76.9%), 127 ng Sn/L (39.3%), 39.2 ng Sn/L (68.3%) and 75.7 ng Sn/L (77.0%), respectively. In addition no significant mortality occurred during the experiments except for the highest additionally tested concentration of 838 ng Sn/L at laboratory 2Ab.

Some uncertainties concerning the measured concentrations might have influenced the results, because TWM have been calculated based on only two measuring intervals, due to the high costs of analytical measurements.

All in all it can be concluded, that the reproduction test with *P. antipodarum* turned out to be a well suited tool for the investigation of reproductive toxicants and also for endocrine disrupting chemicals such as TBT.

5.2 Validation 2

5.2.1 Organisation of the validation test

Eight laboratories participated in the second ring test with *P. antipodarum* which started in June 2014. The partners were provided with snails, salts for preparing the medium and also the test substances, except laboratory 3P. Trenbolone (TR) and prochloraz (PCZ) were chosen as test chemicals, which had been used as reference chemicals in other recent OECD validation tests.

5.2.1.1 Snail production, biological quality checking and shipping

Snails used for the experiments came from the same laboratory culture which has been run under the same conditions as described in section 5.1.1.1 for validation 1. Only laboratory 3P used animals from their own laboratory culture. These snails were acclimatized for 28 days to the proposed medium. Normally these snails are cultured in a different medium.

Table 41 summarizes the shipping and acclimation duration and gives an overview of the test schedules. Post shipping mortality did not occur in the partner laboratories.

Table 41: Shipping, acclimation and test schedules for the partner laboratories in validation 2.

Partner	3A	3D	3H	3L	3M	3N	3O	3P
Snail shipping date	-	25/06/14	30/07/14	16/07/14	16/06/14	29/07/14	11/08/14	-
Snails received	-	27/06/14	31/07/14	17/07/14	17/06/14	30/07/14	12/08/14	-
Number of snails sent	-	500	500	500	500	500	500	-
Acclimation duration	-	23 d	18 d	18 d	13 d	14 d	20 d	28 d
Test starting date	07/07/14	21/07/14	18/08/14	04/08/14	30/06/14	13/08/14	01/09/14	16/06/14
Test ending date	04/08/14	28/08/14	15/09/14	01/09/14	28/07/14	10/09/14	29/09/14	14/07/14

5.2.2 Implementation of the 28-day reproduction test

5.2.2.1 Principle of the test

The principle of the reproduction test with *P. antipodarum* and the test procedure is the same as in validation 1 (see section 5.1.2.1). Only the number of replicates and the number of introduced snails per replicate are modified (see section 5.2.2.3).

5.2.2.2 Chemicals

As test chemicals TR (CAS-No.: 10161-33-8, Sigma-Aldrich®, Germany) and PCZ (CAS-No.: 67747-09-5, Sigma-Aldrich®, Germany) were chosen. For both substances DMSO was used as a

solvent at a concentration of 10 µL/L. Therefore, an additional solvent control group was considered. TR is a synthetic androgen and was tested at the following nominal concentrations:

10 ng/L, 30 ng/L, 100 ng/L, 300 ng/L, 1000 ng/L.

PCZ belongs to the imidazoles and acts as a fungicide. This chemical was tested at the following nominal concentrations:

3.2 µg/L, 10 µg/L, 32 µg/L, 100 µg/L, 320 µg/L.

5.2.2.3 Experimental conditions

The experimental conditions were the same as in validation 1. Only the number of replicates was enhanced to six and the number of snails per replicate was six to increase the statistical power of the test. Experimental conditions are summarized in table 42.

Table 42: Summary of the experimental conditions in validation 2 of the reproduction test with *Potamopyrgus antipodarum*.

Test duration	28 days
Test water	Reconstituted water (with 0.3 g Tropic Marin® salt and 0.18 g $NaHCO_3$ per 1 litre de-ionised water) water quality requirements: pH 7.5 – 8.5, conductivity 770 ± 100 µS/cm, oxygen concentration › 80% ASV (air saturation value)
Test vessels	500 mL glass beakers with lids
Water renewal	3 times per week
Temperature	16 ± 1°C
Light intensity	500 ± 100 lx
Water sampling	From all test concentrations (trenbolone & prochloraz) and solvent control water was sampled over four renewal intervals
Photoperiod	16:8 h L:D
Food source	Fine grounded Tetraphyll®
Feeding	0.25 mg/animal and day
Snails origin	Laboratory culture, which was built up with snails from Lumda Hesse, Germany
Test snails size	3.5 – 4.5 mm
Snails density	6 snails per 400 mL medium (6 replicates per tested concentration)
Core test endpoints	Mortality, reproduction

5.2.3 Chemical analysis and biological data analysis

Every week of the test, samples from PCZ and TR exposure groups and solvent control were taken before (2 or 3 days old) and after (fresh) medium renewal for chemical analysis. Samples from old medium were pooled from every replicate of one treatment group. Samples were stored in HDPE-bottles at -20°C until analysis.

TR and PCZ samples were analysed by LC-MS-MS (Agilent 1200 series triples quadrupole) with detection limits (LOD) of 0.39 ng/L and 1.56 µg/L, respectively. TR samples were extracted on solid-phase columns with methyl-testosterone as internal standard before analysis whereas PCZ was analysed directly from filtered samples at University of Southern Denmark.

TWM concentrations of the chemicals were calculated according to Annex 6 of OECD guideline 211 (OECD 2012b).

Biological raw data were reported by the participating laboratories using an Excel® spread sheet which had been provided by Goethe University. From the raw data means were calculated for biological parameters (shell height, embryo numbers). Effect concentrations were calculated by Dunnett's test (NOEC, LOEC) or by a non-linear regression using a four parameter logistic equation (EC_{10}, EC_{50}).

5.2.4 Results of validation 2

5.2.4.1 Compliance with validity criteria

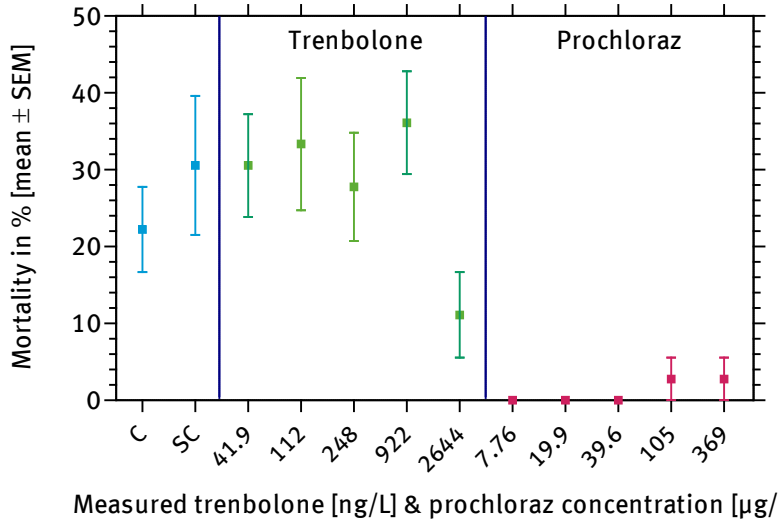
For a test to be valid the conditions were chosen based on available guidelines for freshwater invertebrates and as proposed in OECD (2010) and already used in validation 1. The validity criteria were amended by a biological criterion requiring a minimum mean embryo number in the controls:

- mortality in the controls should not exceed 20%,
- mean embryo number per snail in the controls should be ≥ 5,
- dissolved oxygen saturation must have been at least 60% of the air saturation value (ASV) and
- water temperature should be 16 ± 1°C throughout the test.

All laboratories achieved adequate oxygen saturation values. The mean ASV for oxygen ranged from 93.6% (laboratory 3N) to 99.6% (laboratory 3O). All participants achieved the required temperature with a mean temperature between 15.7°C and 16.4°C. Laboratory 3H exceeded the validity criterion for the maximum control mortality in the negative control (22.2%) and the solvent control (30.6%) (Fig. 41). In the exposure groups with TR in laboratory 3H mortality of snails was in the same range. In PCZ exposed snails no mortality was observed. The high mortality in control groups and TR exposed snails was probably caused by fungus growth in the vessels during the test. Hence, the fungicide PCZ prevented fungal growth and therefore reduced snail mortality. In the draft test guideline a weekly change of the test vessels is proposed. Due to a lack of glass beakers, laboratory 3H was not able to change the test vessels every week. The remained food in the test vessels might have promoted fungal growth. In laboratory 3P the mean embryo numbers in the controls were 1.08 and thus below the validity criterion of ≥ 5. A possible explanation for the low embryo numbers of snails in laboratory 3P could be that the acclimation period to the medium used in this ring test was too short as this laboratory used their own laboratory culture of *P. antipodarum* which is normally cultured with tap water. Already prior test start (T_0) the mean embryo number of 20 snails was 1.00.

Hence, the tests from laboratories 3H and 3P were not valid and test results from these laboratories are not considered in the following evaluation of reproduction data.

Figure 41: Mortality (mean with standard error) of *Potamopyrgus antipodarum* after four weeks exposure to trenbolone (green) and prochloraz (red) concentrations at laboratory 3H. n = 6 replicates.



5.2.4.2 Physico-chemical parameters

Table 43 summarizes the means of the physico-chemical parameters for all laboratories. The pH values ranged between 7.58 (laboratory 3P) to 8.44 (laboratory 3L) which is in the required range of 7.5 - 8.5. The same applies to the values for conductivity which is similar among all partners. Mean temperatures in all laboratories were within the validity criterion (16 ± 1°C).

Table 43: Mean physico-chemical parameters of all laboratories in validation 2 of the reproduction test with *Potamopyrgus antipodarum*. n.r.: not received.

Laboratory	pH			Conductivity [µS/cm]			Temperature [°C]			O_2 saturation [%]		
	mean	SD	n	mean	SD	N	mean	SD	n	mean	SD	n
3A	8.28	0.69	145	791	39.9	145	16.2	0.61	145	96.1	3.06	145
3D	8.26	0.68	156	750	23.2	156	15.9	0.44	156	99.3	1.41	156
3H	8.33	0.68	156	725	37.3	156	15.9	0.21	156	94.4	4.20	144
3L	8.44	0.67	156	718	23.6	156	16.5	0.34	156	98.4	2.31	156
3M	8.11	0.65	156	751	30.2	156	16.4	0.85	157	99.4	4.07	156
3N	8.24	0.66	156	722	18.6	156	15.8	0.34	156	93.6	6.95	156
3O	8.16	0.68	144	818	15.1	145	16.3	0.52	140	99.6	1.75	145
3P	7.58	0.74	156	n.r.	n.r.	n.r.	15.7	0.57	157	n.r.	n.r.	n.r.

5.2.4.3 Actual exposure concentrations of trenbolone

The results of the analytical analyses of TR for all laboratories are shown in tables 44 - 51. In total, 152% of the nominal concentrations were achieved (except 3H and 3P). Because of this, measured TWM concentrations were used to calculate effect concentrations. Initial concentrations varied between 41.6% and 704% of nominal concentrations. After two or three days values ranged between 8.98% and 398%. In all laboratories, with the exception of laboratory 3N, the measured concentrations were higher than nominal concentrations. The most likely reason is a technical failure during weighing the substances. Especially the highest exposure concentrations were considerably higher compared to nominal. In laboratory 3N the measured concentrations varied between 50% and 105% of nominal concentrations.

The measured concentrations of TR in controls were below the LOD (0.39 ng/L), except for samples from laboratory 3M and 3L. In laboratory 3M TR was detected in all control samples indicating a continuous exposure of control snails to the test compound. The calculated TWM was 14 ng/L. In laboratory 3L TR was detected in five out of eight samples. The measured concentrations varied between 1.25 – 33.2 ng/L; a TWM could not be calculated.

Surprisingly, the measured concentrations of TR in laboratory 3P were extremely high. Here, up to 6957% of the nominal concentrations were achieved.

Table 44: Results of trenbolone analyses [ng/L] in exposure media from laboratory 3A in validation 2 of the reproduction test with *Potamopyrgus antipodarum*.

TR nominal concentration [ng/L]	Measured concentrations [ng/L]								Time weighted mean [ng/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
SC	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	-	-
10.0	11.8	12.6	16.0	14.9	9.46	14.3	13.4	14.5	13.3	133
30.0	31.0	33.3	36.1	48.2	28.3	32.5	32.9	34.4	34.4	115
100	134	151	150	163	115	118	130	109	132	132
300	327	417	335	387	385	406	355	376	372	124
1000	1743	1148	1154	1206	1534	1429	1398	1395	1373	137

LOD = 0.39 ng/L; SC: solvent control

Table 45: Results of trenbolone analyses [ng/L] in exposure media from laboratory 3D in validation 2 of the reproduction test with *Potamopyrgus antipodarum*.

TR nominal concentration [ng/L]	Measured concentrations [ng/L]								Time weighted mean [ng/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
SC	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	-	-
10.0	20.3	15.4	19.5	16.8	23.6	15.0	25.4	19.9	19.7	197
30.0	71.5	42.7	69.2	44.1	61.0	43.1	70.1	44.7	55.1	184
100	493	23.0	690	428	*429*	No sample	759	122	350	350
300	2159	1161	2433	1217	2406	1678	2573	1612	1882	627
1000	5123	3600	5698	3952	4832	3406	7043	4218	4763	476

LOD = 0.39 ng/L; SC: solvent control; Italics: not included in TWM calculations.

Table 46: Results of trenbolone analyses [ng/L] in exposure media from laboratory 3H in validation 2 of the reproduction test with *Potamopyrgus antipodarum*.

TR nominal concentration [ng/L]	Measured concentrations [ng/L]								Time weighted mean [ng/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
SC	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	19.8	‹ LOD	-	-
10.0	59.2	30.6	62.8	44.7	57.6	22.9	56.9	21.4	42.0	420
30.0	147	149	166	89.2	171	32.8	17.6	50.1	112	373
100	495	248	479	282	428	58.6	432	20.2	248	248
300	1210	1008	1043	1716	753	757	712	545	922	307
1000	700	3381	3104	2602	3300	2813	3319	2460	2644	264

LOD = 0.39 ng/L; SC: solvent control

Table 47: Results of trenbolone analyses [ng/L] in exposure media from laboratory 3L in validation 2 of the reproduction test with *Potamopyrgus antipodarum*.

TR nominal concentration [ng/L]	Measured concentrations [ng/L]								Time weighted mean [ng/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
SC	22.8	‹ LOD	‹ LOD	1.25	14.8	33.2	2.42	‹ LOD	-	-
10.0	40.9	38.1	36.6	27.6	43.5	25.1	36.4	16.2	31.6	316
30.0	123	54.2	115	70.4	114	41.2	80.8	46.2	75.5	252
100	309	94	259	185	397	296	250	89.2	217	217
300	569	238	688	529	587	530	695	178	469	156
1000	4663	1635	5204	3985	5468	3688	2252	1136	3205	321

LOD = 0.39 ng/L; SC: solvent control

Table 48: Results of trenbolone analyses [ng/L] in exposure media from laboratory 3M in validation 2 of the reproduction test with *Potamopyrgus antipodarum*.

TR nominal concentration [ng/L]	Measured concentrations [ng/L]								Time weighted mean [ng/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
SC	2.93	2.71	23.5	12.1	17.8	37.4	12.2	11.3	14.0	-
10.0	22.3	25.8	30.1	25.7	41.7	24.6	29.3	23.1	27.4	274
30.0	64.9	34.0	54.9	59.5	57.5	39.6	59.7	38.9	50.2	167
100	223	185	179	164	201	168	164	131	173	173
300	523	344	426	378	501	390	360	321	396	132
1000	1899	1049	1918	1271	1374	1297	1283	960	1335	134

LOD = 0.39 ng/L; SC: solvent control

Table 49: Results of trenbolone analyses [ng/L] in exposure media from laboratory 3N in validation 2 of the reproduction test with *Potamopyrgus antipodarum*.

TR nominal concentration [ng/L]	Measured concentrations [ng/L]								Time weighted mean [ng/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
SC	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	-	-
10.0	9.45	7.63	9.03	5.65	9.35	3.23	10.3	14.9	8.92	89.2
30.0	23.4	19.1	21.1	14.5	26.6	5.13	19.4	4.48	14.9	49.7
100	91.5	76.8	142	46.3	41.6	27.8	101	17.0	60.9	60.9
300	304	246	377	85.9	388	44	290	26.9	177	59.0
1000	2266	1583	1899	680	1397	355	1242	201	1046	105

LOD = 0.39 ng/L; SC: solvent control

Table 50: Results of trenbolone analyses [ng/L] in exposure media from laboratory 3O in validation 2 of the reproduction test with *Potamopyrgus antipodarum*.

TR nominal concentration [ng/L]	Measured concentrations [ng/L]								Time weighted mean [ng/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
SC	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	-	-
10.0	16.0	15.0	9.0	17.0	22.0	17.0	17.0	20.0	16.7	167
30.0	41.0	39.0	41.0	35.0	44.0	35.0	35.0	39.0	38.4	128
100	156	152	175	136	213	183	196	153	170	170
300	617	542	533	388	592	485	418	465	496	165
1000	2345	1921	1778	1975	1709	1669	2123	2563	2043	204

LOD = 0.39 ng/L; SC: solvent control

Table 51: Results of trenbolone analyses [ng/L] in exposure media from laboratory 3P in validation 2 of the reproduction test with *Potamopyrgus antipodarum*.

TR nominal concentration [ng/L]	Measured concentrations [ng/L]								Time weighted mean [ng/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
SC	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	-	-
10.0	1153	1218	2401	892	68.0	440	61.0	96.0	640	6400
30.0	2440	2342	7796	4977	149	440	241	372	2087	6957
100	9575	11615	21420	5116	565	493	1166	1263	5399	5399
300	3866	5007	51414	36332	1368	1733	5079	5036	12662	4221
1000	49564	459	129538	7073	3009	653	17511	1325	10258	1026

LOD = 0.39 ng/L; SC: solvent control

5.2.4.4 Biological responses to trenbolone

Mortality

In laboratories 3A, 3D, 3M and 3O no mortality was observed in any of the exposure groups. In laboratory 3L the maximal observed mortality was 8.34% in the solvent control. In laboratory 3N a mortality of 2.78% occurred at the test concentration of 15 ng/L.

Reproduction

None of the participating laboratories found a concentration-dependent decrease of the embryo numbers in the brood pouch of *P. antipodarum* (Fig. 42). Only laboratory 3A did observe significant reduced embryo numbers at the two lowest concentrations, which were not detected at higher test concentrations. Laboratory 3L found a significant effect on reproduction of the solvent DMSO with a decreased number of embryos.

Figure 42: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks exposure to measured trenbolone concentrations at valid laboratories 3A, 3D, 3L, 3M, 3N and 3O (crosses: replicate mean; line: treatment mean). Asterisks indicate significant differences compared to merged solvent control and negative control (Dunnett´s test) or compared to solvent control and negative control at laboratory 3L (unpaired t-test). ★ = p<0.05, n = 6 replicates.

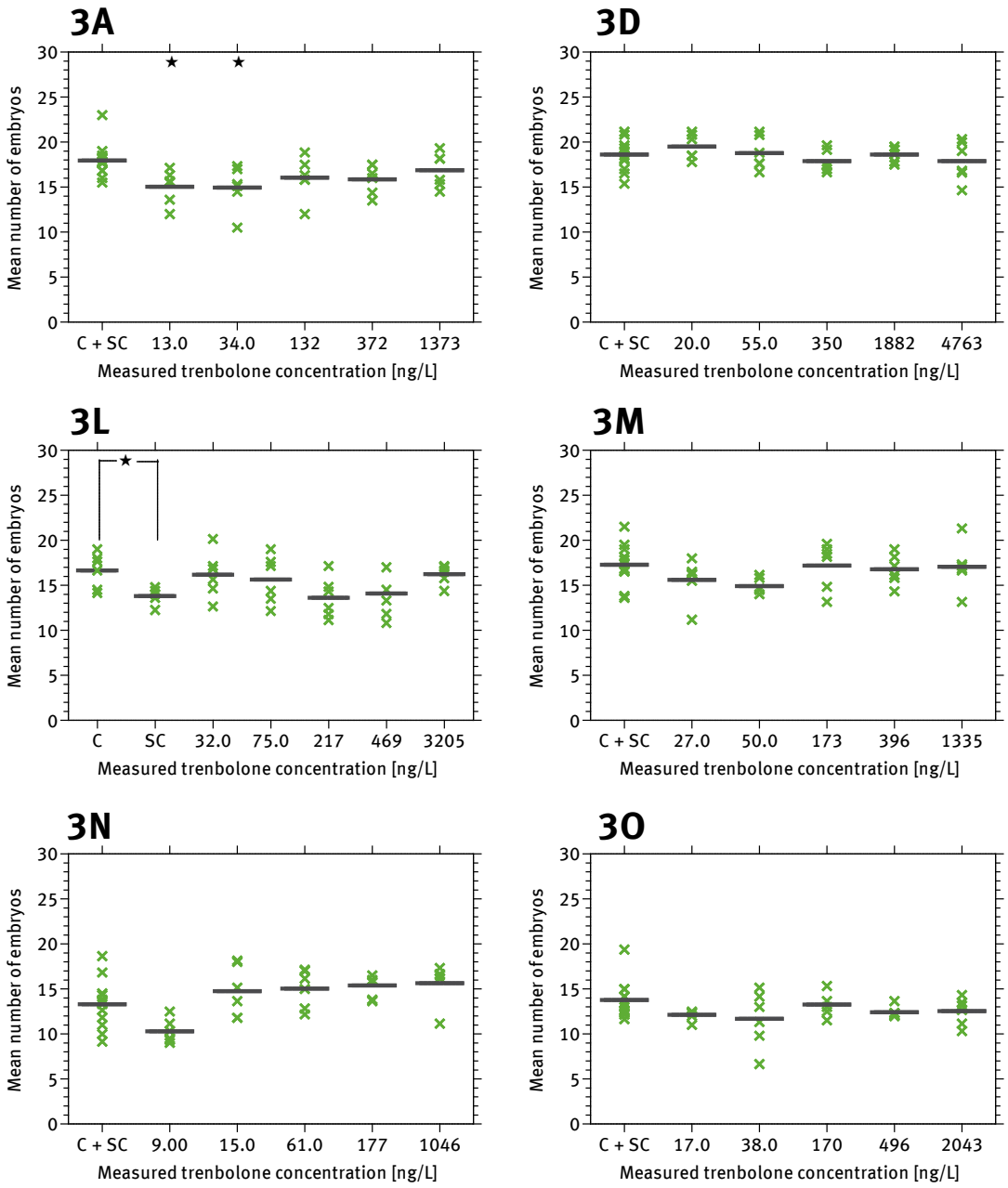
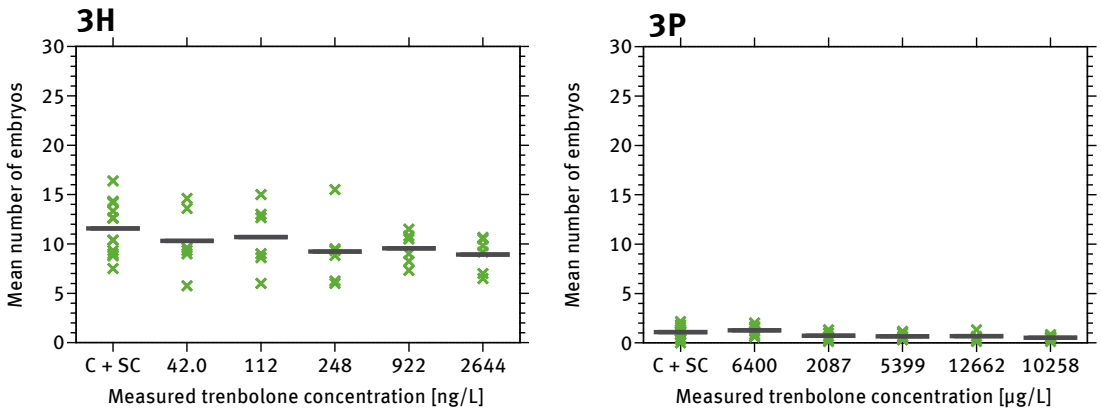


Figure 43 shows the results of the reproduction tests at laboratories 3H and 3P which did not achieve the validity criteria. In spite of the fungus growth as an additional stressor in the test vessels of laboratory 3H, TR was not able to modulate the reproductive output of the snails. In laboratory 3P the mean embryo numbers in the controls were 1.08 and thus below the validity

criterion of ≥ 5 (see section 5.2.4.1 for discussion). The mean embryo numbers in TR exposed groups were also on the level of the control.

Figure 43: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks exposure to measured trenbolone concentrations at non-valid laboratories 3H and 3P (crosses: replicate mean; line: treatment mean). n = 6 replicates.



5.2.4.5 Actual exposure concentrations of prochloraz

The results of the analytical measurement of PCZ for all laboratories are shown in tables 52 - 59. In total, 273% of the nominal concentrations were achieved (except 3H and 3P). Therefore, TWM concentrations were used to calculate effect concentrations. Initial concentrations varied between 153% and 617% and after two or three days values varied between 169% and 506%. Measured concentrations of PCZ in controls were below the LOD which was 1.56 µg/L except for laboratories 3D, 3L and 3N. PCZ was measured at a maximum concentration of 9.63 µg/L during the last two water renewal intervals in the controls of laboratory 3D (table 53). In laboratory 3L PCZ was measured in every solvent control sample, indicating a continuous exposure to the test compound (table 55). Here, a TWM concentration of 9.98 µg/L was calculated. Therefore, laboratory 3L was excluded from the evaluation of the ring test. For laboratory 3N only on day 2 a positive finding with a measured concentration of 1.20 µg/L was found in the control. PCZ was also detected in the solvent controls of laboratories 3H and 3P which both reported non-valid results. In laboratory 3H the maximum measured PCZ concentration in the solvent control was 0.907 µg/L, whereas measured concentrations of PCZ in solvent control of laboratory 3P varied between 0.058 to 8.18 µg/L.

Table 52: Results of prochloraz analyses [µg/L] in exposure media from laboratory 3A in validation 2 of the reproduction test with *Potamopyrgus antipodarum*.

PCZ nominal concentration [µg/L]	Measured concentrations [µg/L]								Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
Solvent control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	-	-
3.20	11.7	13.6	9.47	11.0	8.39	11.8	7.97	10.1	10.3	322
10.0	29.5	26.9	25.8	24.5	21.3	28.0	25.9	34.5	27.3	273
32.0	50.0	54.5	51.1	47.4	47.4	51.7	55.2	51.7	51.4	161
100	221	254	214	221	225	259	227	219	229	229
320	551	567	513	493	629	599	675	602	585	183

LOD = 1.56 µg/L

Table 53: Results of prochloraz analyses [µg/L] in exposure media from laboratory 3D in validation 2 of the reproduction test with *Potamopyrgus antipodarum*.

PCZ nominal concentration [µg/L]	Measured concentrations [µg/L]								Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
Solvent control	< LOD	< LOD	< LOD	< LOD	4.00	9.34	9.63	9.32	-	-
3.20	25.2	25.7	32.8	23.3	49.4	34.5	31.6	30.7	31.4	981
10.0	51.9	49.1	53.1	78.9	73.9	64.6	53.4	49.5	58.2	582
32.0	47.8	55.8	54.4	66.4	71.6	65.9	40.1	35.9	52.8	165
100	237	240	257.0	245	337	392	277	186	266	266
320	537	516	561	718	781	659	379	291	529	165

LOD = 1.56 µg/L

Table 54: Results of prochloraz analyses [µg/L] in exposure media from laboratory 3H in validation 2 of the reproduction test with *Potamopyrgus antipodarum*.

PCZ nominal concentration [µg/L]	Measured concentrations [µg/L]								Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
Solvent control	< LOD	< LOD	0.231	< LOD	0.052	0.607	0.907	0.230	-	-
3.20	10.2	1.47	11.2	13.7	7.71	4.87	7.79	7.93	7.76	243
10.0	26.1	13.0	26.4	26.4	18.0	18.6	17.0	18.1	19.9	199
32.0	52.2	30.3	52.6	59.7	35.5	39.1	35.2	24.9	39.6	124
100	104	66.3	108	103	145	139	134	62.7	105	105
320	552	221	596	273	363	348	358	347	369	115

LOD = 1.56 µg/L

Table 55: Results of prochloraz analyses [µg/L] in exposure media from laboratory 3L in validation 2 of the reproduction test with *Potamopyrgus antipodarum*.

PCZ nominal concentration [µg/L]	Measured concentrations [µg/L]								Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
Solvent control	0.290	10.3	6.58	15.1	14.6	14.9	10.4	12.6	9.98	-
3.20	7.57	13.3	8.90	19.2	15.2	11.6	17.8	14.1	13.5	422
10.0	21.3	29.9	28.2	31.0	23.9	21.2	20.9	23.5	24.6	256
32.0	44.8	48.4	46.2	45.0	39.3	38.4	40.3	39.7	42.5	133
100	195	176	171	178	140	145	149	143	160	160
320	468	443	455	402	308	344	341	322	379	118

LOD = 1.56 µg/L

Table 56: Results of prochloraz analyses [µg/L] in exposure media from laboratory 3M in validation 2 of the reproduction test with *Potamopyrgus antipodarum*.

PCZ nominal concentration [µg/L]	Measured concentrations [µg/L]								Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
Solvent control	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	-	-
3.20	15.8	19.3	22.8	22.7	27.3	23.2	22.5	26.4	22.7	709
10.0	28.8	32.9	33.5	35.4	35.0	33.7	30.1	34.5	32.9	329
32.0	55.2	60.1	55.6	57.7	71.7	57.0	55.2	57.0	58.3	182
100	302	330	314	325	307	303	284	290	305	305
320	702	733	702	713	593	620	503	548	626	196

LOD = 1.56 µg/L

Table 57: Results of prochloraz analyses [µg/L] in exposure media from laboratory 3N in validation 2 of the reproduction test with *Potamopyrgus antipodarum*.

PCZ nominal concentration [µg/L]	Measured concentrations [µg/L]								Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
Solvent control	‹ LOD	1.20	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	-	-
3.20	8.74	8.79	11.4	10.7	10.6	10.9	10.5	11.3	10.4	325
10.0	18.6	17.9	26.7	24.9	24.8	22.4	23.4	24.7	23.0	230
32.0	40.5	37.7	51.7	45.2	23.1	24.5	48.7	45.7	40.4	126
100	160	154	235	208	209	197	184	203	194	194
320	375	375	561	466	502	498	491	471	468	146

LOD = 1.56 µg/L

Table 58: Results of prochloraz analyses [µg/L] in exposure media from laboratory 3O in validation 2 of the reproduction test with *Potamopyrgus antipodarum*.

PCZ nominal concentration [µg/L]	Measured concentrations [µg/L]								Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
Solvent control	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	‹ LOD	-	-
3.20	13.9	19.4	14.6	12.1	11.3	13.2	6.69	8.47	11.9	372
10.0	23.3	26.7	26.8	19.9	18.1	22.4	17.3	19.6	21.3	213
32.0	41.4	43.7	35.1	35.3	33.2	43.3	29.4	38.5	40.0	125
100	169	214	155	197	156	217	147	222	183	183
320	488	522	475	491	449	513	460	518	489	153

LOD = 1.56 µg/L

Table 59: Results of prochloraz analyses [µg/L] in exposure media from laboratory 3P in validation 2 of the reproduction test with *Potamopyrgus antipodarum*. n.r.: not received.

PCZ nominal concentration [µg/L]	Measured concentrations [µg/L]								Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh	Day 2 old	Day 7 fresh	Day9 old	Day 14 fresh	Day 16 old	Day 25 fresh	Day 28 old		
Solvent control	6.15	8.18	‹ LOD	0.293	0.0582	2.85	n.r.	n.r.	-	-
3.20	30.8	14.7	16.1	13.9	18.3	19.7	n.r.	n.r.	18.6	581
10.0	55.7	76.0	39.8	30.0	44.8	41.8	n.r.	n.r.	47.8	478
32.0	87.6	119	74.3	62.9	75.9	67.7	n.r.	n.r.	80.9	253
100	463	596	439	305	389	n.r.	n.r.	n.r.	447	447
320	501	668	873	474	n.r.	n.r.	n.r.	n.r.	617	193

LOD = 1.56 µg/L

5.2.4.6 Biological responses to prochloraz

Mortality

None of the participating laboratories with valid results did observe mortalities in the negative control group. The maximum observed mortality in solvent control was 8.34%. Only laboratory 3M observed a significant mortality compared to control groups at a concentration of 22.7 µg/L (8.33%) and 626 µg/L (11.1%).

Reproduction

Figure 44 shows the test results for PCZ of all partners which met the validity criteria. All laboratories found a decrease of embryo numbers with increasing concentrations of PCZ. Laboratories 3A, 3M, 3N and 3O found comparable NOEC (27.3 µg/L, 32.9 µg/L, 40.4 µg/L and 21.3 µg/L) and LOEC (51.4 µg/L, 58.3 µg/L, 194 µg/L and 40 µg/L) values.

The findings from laboratory 3L resulted in considerably higher NOEC and LOEC values (compared to solvent control) of 160 µg/L and 379 µg/L, respectively. Here, the contamination of PCZ and TR in the control groups has to be taken into account. Therefore, the results of laboratory 3L are excluded from the final evaluation. The animals used for the reproduction test in laboratory 3D showed the highest sensitivity for PCZ. Already at the lowest test concentration (31.4 µg/L) the number of embryos was significantly reduced.

Figure 44: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks exposure to measured prochloraz concentrations at valid laboratories 3A, 3D, 3L, 3M, 3N and 3O (crosses: replicate mean; line: treatment mean). Asterisks indicate significant differences compared to merged solvent control and negative control (Dunnett´s test) or compared to solvent control and negative control at laboratory 3L (unpaired t-test). ★ = p<0.05, ★★ = p<0.01, ★★★ = p<0.001, n = 6 replicates.

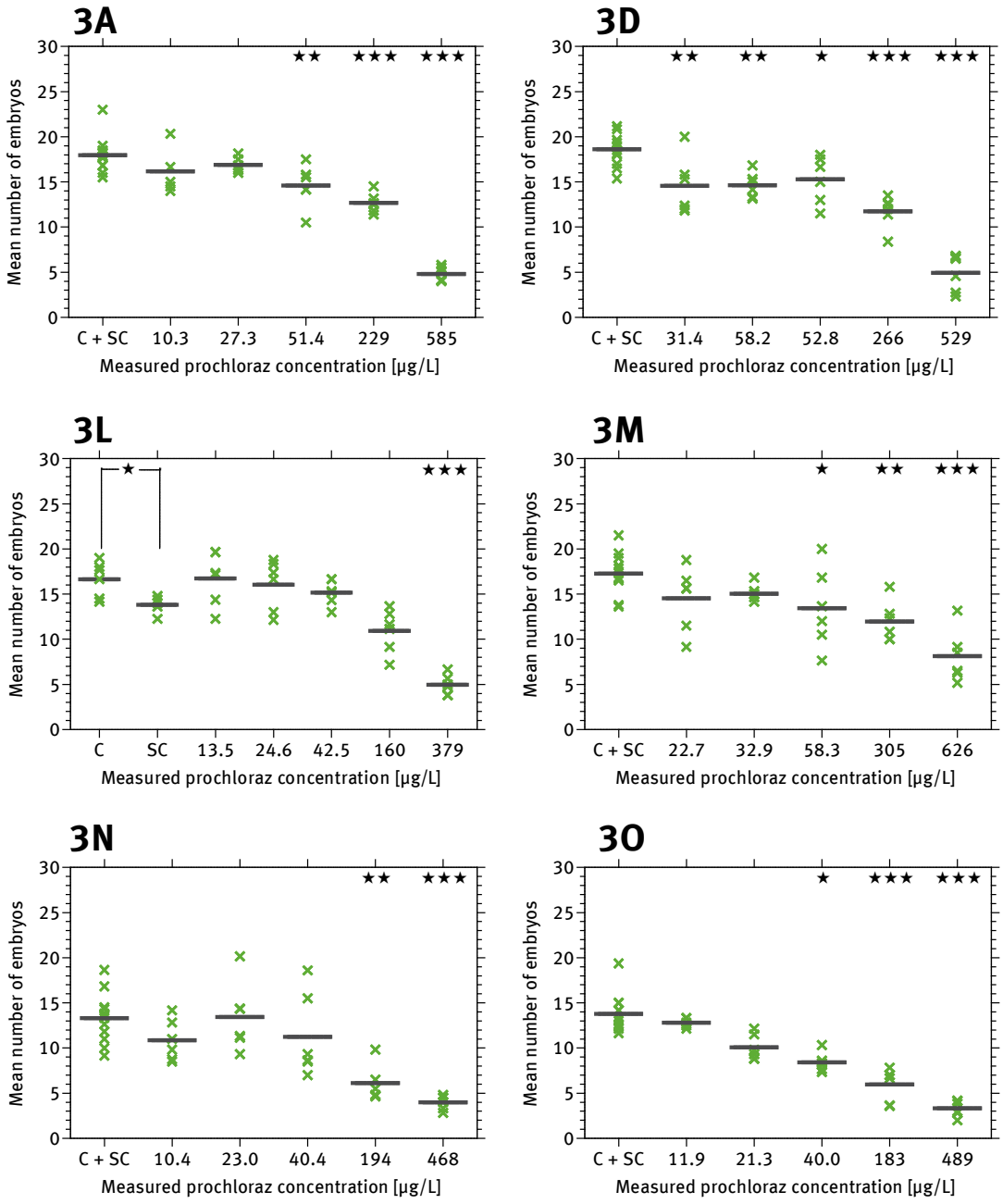


Figure 45 shows the results of the reproduction tests with *P. antipodarum* at laboratories 3H and 3P which did not achieve the validity criteria. The results of laboratory 3H are in accordance with the findings from laboratories with valid test results. The NOEC and LOEC values are 39.6 µg/L and 105 µg/L, respectively. The embryo numbers in the reproduction test conducted

at laboratory 3P are particularly low compared to the other laboratories. Nevertheless, a significant effect was observed at the three highest test concentrations (80.9 µg/L, 447 µg/L and 617 µg/L). Despite the non-valid tests, similar effect concentrations could be detected compared to the laboratories which have fulfilled the validity criteria of the test.

Figure 45: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks exposure to measured prochloraz concentrations at non-valid laboratories 3H and 3P (crosses: replicate mean; line: treatment mean). Asterisks indicate significant differences compared to merged solvent control and negative control (Dunnett´s test). ★ = p‹0.05, ★★ = p‹0.01, ★★★ = p‹0.001, n = 6 replicates.

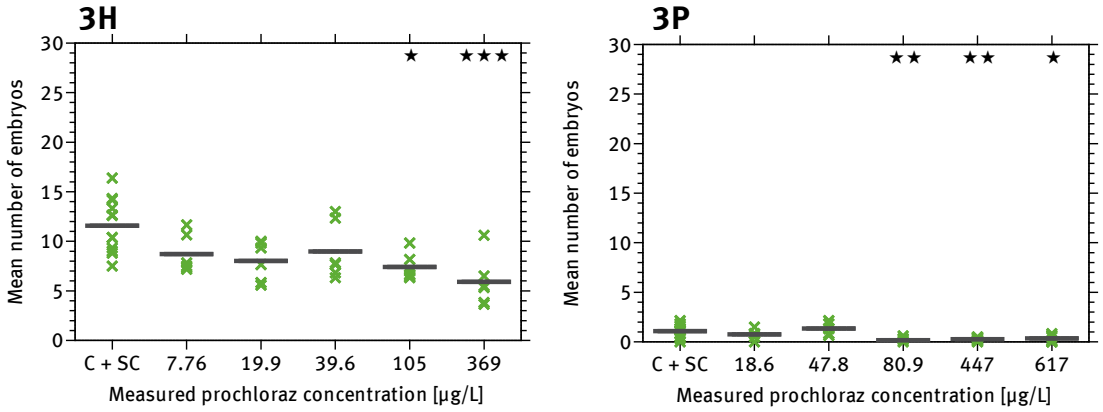
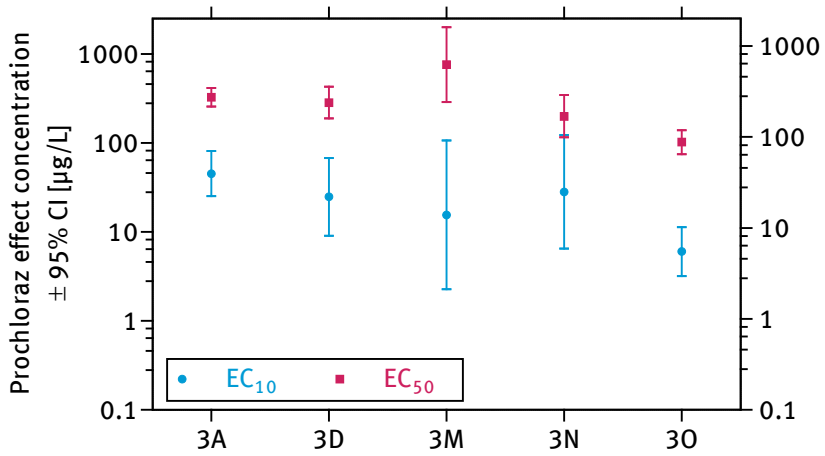


Table 60 and figure 46 summarize the calculated effect concentrations based on measured concentrations from all participating laboratories providing valid data. The good match of results is reflected by the EC_{10} and EC_{50} values. The EC_{10} values from all laboratories overlap with their 95%-confidence intervals. The EC_{10} ranges from 15.6 µg/L (laboratory 3M) to 45.4 µg/L (laboratory 3A) and the EC_{50} from 103 µg/L (laboratory 3O) to 763 µg/L (laboratory 3M).

Table 60: Effect concentrations (EC_{10} and EC_{50} with 95%-confidence intervals, NOEC, LOEC) for total embryo numbers of *Potamopyrgus antipodarum* in validation 2 based on time-weighted mean concentrations of prochloraz in µg/L. *Italics: data from laboratories with non-valid test results.*

	3A	3D	3M	3N	3O	*3L*	*3H*	*3P*
EC_{10}	45.4 (25.3 - 81.5)	24.9 (9.11 - 67.8)	15.6 (2.27 - 107)	28.3 (6.50 - 123)	6.04 (3.20 - 11.4)	-	-	-
EC_{50}	327 (257 - 416)	285 (190 - 429)	763 (289 - 2015)	200 (116 - 346)	103 (75.3 - 140)	-	-	-
NOEC	27.3	-	32.9	40.4	21.3	*160*	*39.6*	*47.8*
LOEC	51.4	31.4	58.3	194	40.0	*379*	*105*	*80.9*

Figure 46: Calculated effect concentrations (EC_{10} in blue, EC_{50} in red) [µg/L] including 95% confidence intervals of the reproduction test with *Potamopyrgus antipodarum* with prochloraz from all participating and valid laboratories of validation 2.



5.2.5 Comparison of present results with published and grey literature

Snails exposed to TR in the tested concentration range did not show concentration dependent effects on reproduction. The assessed sensitivity differs from sensitivity data assessed with other species: Fecundity of the fathead minnow in a short-term screening assay was significantly reduced by exposure to measured TR test concentrations ≥27 ng/L (ANKLEY ET AL. 2003). In another study of HOLBECH ET AL. (2006) juveniles of *Danio rerio* were exposed to TR-acetate. The sex ratio of exposed fish was significantly altered to an all-male population from exposure to 9.7 ng/L and above. In a study by OLMSTEAD ET AL. (2012) *Xenopus tropicalis* showed a significant shift in sex ratio toward males at 78 ng/L. In comparison to fish and amphibians *P. antipodarum* is less sensitive when exposed to TR.

The effect concentrations for PCZ obtained in this ring test are in good accordance to other species. Compared to studies with fish, *P. antipodarum* shows a comparable sensitivity towards PCZ. THORPE ET AL (2011) investigated the sexual differentiation mode of the fathead minnow and zebrafish and found a LOEC of 100 µg/L for fathead minnow and 320 µg/L for zebrafish by a decreasing proportion of females, respectively. In another study of ZHANG ET AL. (2008) *Oryzias latipes* was exposed towards PCZ for ten days. The EC_{50} value for the number of laid eggs per fish was 30 µg/L. Compared to other invertebrates the mudsnail also shows a comparable sensitivity to PCZ. The assessed EC_{10} and EC_{50} for *Daphnia magna* in a 21-day reproduction test is 154 µg/L and 286 µg/L, respectively (HASSOLD AND BACKHAUS 2009).

5.2.6 Conclusions from validation 2

In conclusion the findings from all partners showed a good agreement between the laboratories. Also the numbers of embryos in the control groups were in the same range among partners (with a coefficient of variation of 14.3%). Only two out of eight laboratories did not achieve the validity criteria. In both cases the biological criteria (maximum control mortality or minimum

embryo numbers in snails of the control groups) were not met. In laboratory 3H the high mortailty was caused by fungal growth in the test vessels, most probably because test vessels were not changed as foreseen in the SOP for the validation exercise. In laboratory 3P the embryo numbers in control snails were below 5. The snails used by this laboratory came from an own culture in tap water in contrast to the culturing of *P. antipodarum* at Goethe University in reconstituted water. Probably, the acclimation period to the test medium was too short.

None of the participating laboratories observed an effect of TR on the reproduction of *P. antipodarum* which also represents a consistent result for this validation run.

Furthermore also the results of the reproduction tests with PCZ showed a good match among partners. The mean values (with coefficient of variation) for EC_{10}, EC_{50}, NOEC and LOEC from laboratories 3A, 3D, 3M, 3N and 3O are 24.1 µg/L (61.3%), 336 µg/L (75.7%), 30.5 µg/L (26.7%) and 75.0 µg/L (89.7%). Moreover, also the results of the non-valid laboratories are in good accordance with the results of the valid laboratories. The NOEC in laboratory 3H and 3P was 39.6 and 47.8 µg/L, respectively.

The effect concentrations show a minimum of a 1.90-fold difference (NOECs) and a maximum of a 7.52-fold difference (EC_{10} values). Overall it can be concluded that the robustness and the inter-laboratory reproducibility of the reproduction test with *P. antipodarum* with TR and PCZ could be demonstrated.

5.3 Validation 3

5.3.1 Organisation of the validation test

Four laboratories participated in the third ring test with *P. antipodarum* which started in September 2014. The partners were provided with snails, salts for preparing the medium and also the test substances. Triclocarban (TCC) and triclosan (TCS) were chosen as potentially reproduction-enhancing test chemicals, because in pre-tests with *P. antipodarum* a concentration-dependent increase of embryo numbers compared to controls was observed at Goethe University.

5.3.1.1 Snail production, biological quality checking and shipping

Snails used for the experiments came from the same laboratory culture which has been run under the same conditions as described in section 5.1.1.1 for validation 1 and validation 2.

Table 61 summarizes the shipping and acclimation duration and gives an overview of the test schedules. Post shipping mortality did not occur in the partner laboratories.

Table 61: Shipping, acclimation and test schedules for the partner laboratories in validation 3.

Partner	4A	4C	4G	4L
Snail shipping date	-	09/10/14	16/10/14	02/09/14
Snails received	-	09/10/14	16/10/14	03/09/14
Number of snails sent	-	500	500	500
Acclimation duration	-	32 d	25 d	15 d
Test starting date	06/10/14	10/11/14	10/11/14	17/09/14
Test ending date	03/11/14	08/12/14	08/12/14	15/10/14

5.3.2 Implementation of the 28-day reproduction test

5.3.2.1 Principle of the test

The principle of the reproduction test with *P. antipodarum* and the test procedure is the same as in validation 2 (see section 5.2.2.1).

5.3.2.2 Chemicals

As test chemicals TCC (CAS-No.: 101-20-2, Sigma-Aldrich®, Germany) and TCS (CAS-No.: 3380-34-5, Sigma-Aldrich®, Germany) were chosen. For both substances DMSO was used as a solvent at a concentration of 10 µL/L. Therefore, an additional solvent control group was considered. TCC and TCS are used as antimicrobial agents and both were tested at the following nominal concentrations:

0.1 µg/L, 0.3 µg/L, 1 µg/L, 3 µg/L, 10 µg/L.

5.3.2.3 Experimental conditions

The experimental conditions were the same as in validation 2. Only the amount of food was adjusted to 62 µg/snail x day in accordance to the findings of the feeding experiments with *P. antipodarum* (see section 4.3.3). Experimental conditions are summarized in table 62.

Table 62: Summary of the experimental conditions in validation 3 of the reproduction test with *Potamopyrgus antipodarum*.

Test duration	28 days
Test water	Reconstituted water (with 0.3 g Tropic Marin® salt and 0.18 g $NaHCO_3$ per 1 litre deionised water) water quality requirements: pH 7.5 – 8.5, conductivity 770 ± 100 µS/cm, oxygen concentration › 60% ASV (air saturation value)
Test vessels	500 mL glass beakers with lids
Water renewal	3 times per week
Temperature	16 ± 1°C
Light intensity	500 ± 100 lx
Water sampling	From all test concentrations (triclocarban & triclosan) and solvent control water was sampled over three renewal intervals
Photoperiod	16:8 h L:D
Food source	Fine grounded TetraPhyll®
Feeding	62.5 µg/animal and day
Snails origin	Laboratory culture, which was built up with snails from Lumda Hesse, Germany
Test snails size	3.5 – 4.5 mm
Snails density	6 snails per 400 mL medium (6 replicates per tested concentration)
Core test endpoints	Mortality, reproduction

5.3.3 Chemical analysis and biological data analysis

In the first, the third and in the last week of the test, samples from TCC and TCS exposure groups and solvent control were taken for chemical analysis. Therefore, on day 0, day 14 and day 25 water samples were taken from freshly prepared exposure media and on day 2, day 16 and day 28 from old water which was pooled from every replicate of one treatment group. Samples were stored in HDPE-bottles at 4°C until analysis.

Chemical analysis of TCC and TCS was performed by liquid chromatography-mass spectrometry (LC/MS, Agilent HPLC 1200 series with triple quadrupole mass spectrometer 6410, Santa Clara, USA) after solid-phase extraction (SPE), based on HALDEN AND PAULL (2005) at chemlab GmbH in Bensheim, Germany. SPE cartridges (Strata-x 33 u polymeric reversed phase, 200 mg/3 mL, Phenomenex, Aschaffenburg, Germany) were conditioned with 5 mL of 1:1 acetone:methanol mixture and 5 mL ultrapure water. Up to a nominal concentration of 1 µg/L, including solvent

control, 200 mL of water samples were extracted. At nominal concentrations of 3 and 10 µg/L 100 mL of water samples were extracted. After sample loading, cartridges were dried in a desiccator overnight. For elution 2 x 4 mL 1:1 methanol:acetone mixture with 10 mM acetic acid were used. Samples volumes were reduced under a constant nitrogen flow of 500 µL. The LOD for both substances was 0.04 µg/L.

TWM concentrations of the chemicals were calculated according to Annex 6 of OECD guideline 211 (OECD 2012b).

Biological raw data were reported by the participating laboratories using an Excel® spread sheet which had been provided by Goethe University. From the raw data means were calculated for biological parameters (shell height, embryo numbers). Effect concentrations were calculated by Dunnett's test (NOEC, LOEC).

5.3.4 Results of validation 3

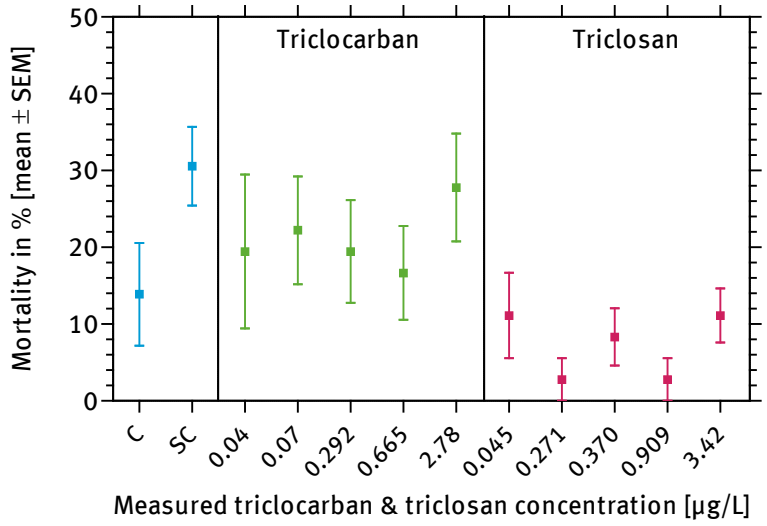
5.3.4.1 Compliance with validity criteria

For a test to be valid the conditions were chosen based on available guidelines for freshwater invertebrates and as proposed in OECD (2010). For the purpose of validation 3, the following validity criteria should be fulfilled:

- mortality in the controls should not exceed 20%,
- mean embryo number per snail in the controls should be ≥ 5,
- dissolved oxygen saturation must have been at least 60% of the air saturation value (ASV) and
- water temperature should be 16 ± 1°C throughout the test.

All laboratories met the given validity criteria of the water temperature and the dissolved oxygen saturation. The water temperature ranged between 15.3°C (laboratory 4A) and 16.5°C (laboratory 4C). The measured oxygen saturation was between 95.8% (laboratory 4A) and 100% (laboratory 4L). Laboratory 4G exceeded the validity criterion for the maximum control mortality in the solvent control (30.6%) for unknown reasons (Fig. 47). In the negative control the mortality was 13.9%. Also in the TCC exposed groups an increased mortality was observed with a maximum of 27.8% at the highest test concentration. In the TCS groups a maximum of 11.1% of snails died during the test. Hence, the tests from laboratory 4G were not valid and test results from this laboratory are not considered in the following evaluation of reproduction data.

Figure 47: Mortality (mean with standard error) of *Potamopyrgus antipodarum* after four weeks exposure to measured triclocarban (green) and triclosan (red) concentrations at laboratory 4G. n = 6 replicates.



5.3.4.2 Physico-chemical parameters

Table 63 summarizes the means of the physico-chemical parameters for all laboratories. The pH values ranged between 8.20 (laboratory 4C) to 8.43 (laboratory 4L) which is in the required range of 7.5 - 8.5. The same applies to the values for conductivity which were similar among all laboratories.

Table 63: Mean physico-chemical parameters of all laboratories in validation 3 of the reproduction test with *Potamopyrgus antipodarum*.

Laboratory	pH			Conductivity [µS/cm]			Temperature [°C]			O_2 saturation [%]		
	mean	SD	n	mean	SD	n	mean	SD	n	mean	SD	n
4A	8.36	0.693	145	825	45.3	145	15.3	0.767	145	95.8	1.61	144
4C	8.20	0.684	145	721	15.9	146	16.5	0.478	146	98.0	2.47	146
4G	8.35	0.691	145	738	32.7	145	16.0	0.327	145	99.2	0.822	145
4L	8.43	0.039	144	702	30.7	144	16.3	1.37	144	100	2.00	144

5.3.4.3 Actual exposure concentrations of triclocarban

The results of the analytical measurement of TCC for all laboratories are shown in tables 64 - 67. In total, 26.6% of the nominal concentrations were measured analytically. Therefore, TWM concentrations were used for the calculations of effect concentrations. The initial concentrations varied between 7.94% and 90.0% and after two or three days values varied between 4.25% and 82.1% of nominal concentrations. In all laboratories the measured concentrations

of TCC in solvent control groups were below the LOD of 0.04 µg/L. In some of the water samples from laboratory 4L the measured concentrations of TCC were below the LOD. To calculate TWM concentrations the half of the LOD (0.02 µg/L) was used according to the recommendation of OECD (2000). The analytical measurements of TCC at the lowest tested concentration (0.10 µg/L) and/or at 0.3 µg/L at laboratory 4C and 4G were mostly below the LOD (< 0.04 µg/L). Therefore, according to the recommendation of OECD (2000), the LOD (0.04 µg/L) was used for this exposure groups to calculate effect concentrations.

Table 64: Results of triclocarban analyses [µg/L] in exposure media from laboratory 4A in validation 3 of the reproduction test with *Potamopyrgus antipodarum*.

TCC nominal concentration [µg/L]	Measured concentrations [µg/L]						Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh medium	Day 2 old medium	Day 14 fresh medium	Day 16 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	-	-
0.1	0.040	0.0821	0.0900	0.040	0.0544	0.0620	0.058	58.0
0.3	0.173	0.117	0.112	0.040	0.214	0.0887	0.121	40.3
1	0.561	0.256	0.178	0.312	0.450	0.311	0.340	34.0
3	0.350	0.398	0.541	0.463	0.603	0.766	0.542	18.1
10	3.03	1.46	3.29	1.54	3.06	2.90	2.55	25.5

LOD = 0.04 µg/L

Table 65: Results of triclocarban analyses [µg/L] in exposure media from laboratory 4C in validation 3 of the reproduction test with *Potamopyrgus antipodarum*.

TCC nominal concentration [µg/L]	Measured concentrations [µg/L]						Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh medium	Day 2 old medium	Day 14 fresh medium	Day 16 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	-	-
0.1	< LOD	< LOD	< LOD	0.0562	0.0581	< LOD	0.04	-
0.3	0.040	< LOD	< LOD	< LOD	0.103	0.0584	0.04	-
1	0.141	0.110	0.0794	0.0425	0.200	0.217	0.142	14.2
3	0.371	0.367	0.253	0.234	0.397	0.509	0.368	12.3
10	1.27	1.16	0.968	1.33	2.81	1.77	1.64	16.4

LOD = 0.04 µg/L

Table 66: Results of triclocarban analyses [µg/L] in exposure media from laboratory 4G in validation 3 of the reproduction test with *Potamopyrgus antipodarum*.

TCC nominal concentration [µg/L]	Measured concentrations [µg/L]						Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh medium	Day 2 old medium	Day 14 fresh medium	Day 16 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	-	-
0.1	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	0.04	-
0.3	< LOD	0.0650	0.100	0.0651	0.0690	0.0823	0.070	23.3
1	0.118	0.237	0.369	0.264	0.317	0.404	0.292	29.2
3	0.471	0.399	0.850	0.631	0.634	0.928	0.665	22.2
10	1.41	2.21	2.96	2.84	2.74	4.11	2.78	27.8

LOD = 0.04 µg/L

Table 67: Results of triclocarban analyses [µg/L] in exposure media from laboratory 4L in validation 3 of the reproduction test with *Potamopyrgus antipodarum*. Bold: half of the LOD.

TCC nominal concentration [µg/L]	Measured concentrations [µg/L]						Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh medium	Day 2 old medium	Day 14 fresh medium	Day 16 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	-	-
0.1	0.0400	0.0400	0.0400	0.02	0.180	0.02	0.047	47.0
0.3	0.0900	0.150	0.110	0.0700	0.220	0.0900	0.117	39.0
1	0.350	0.270	0.340	0.360	0.260	0.350	0.320	32.0
3	0.850	0.350	0.590	0.640	1.08	0.680	0.681	22.7
10	2.19	1.56	1.56	1.35	1.31	1.19	1.52	15.2

LOD = 0.04 µg/L

5.3.4.4 Biological responses to triclocarban

Mortality

At laboratory 4L no mortality was observed in any of the exposure groups and controls. The maximum mortality was observed at laboratory 4A at the highest test concentration of TCC (2.55 µg/L), where 2.78% snails died.

Reproduction

Figure 48 shows the results of the valid reproduction tests with TCC at laboratory 4A, 4C and 4L. In none of the participating laboratories an increase of the reproduction of the snails could be detected in TCC exposure groups. Laboratory 4A observed a significant decrease of the embryo numbers with increasing TCC concentrations. Laboratory 4L found a significant effect of the solvent DMSO on reproduction with a decreased number of embryos. Compared to the solvent control, this laboratory found a significant decrease of the embryo numbers at the highest test concentration of 1.52 µg/L.

Figure 48: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks exposure to measured triclocarban concentrations at valid laboratories 4A, 4C and 4L (crosses: replicate mean; line: treatment mean). Asterisks indicate significant differences compared to merged solvent control and negative control (Dunnett´s test) or compared to solvent control and negative control at laboratory 4L (unpaired t-test). ★ = p<0.05, ★★★ = p<0.001, n = 6 replicates.

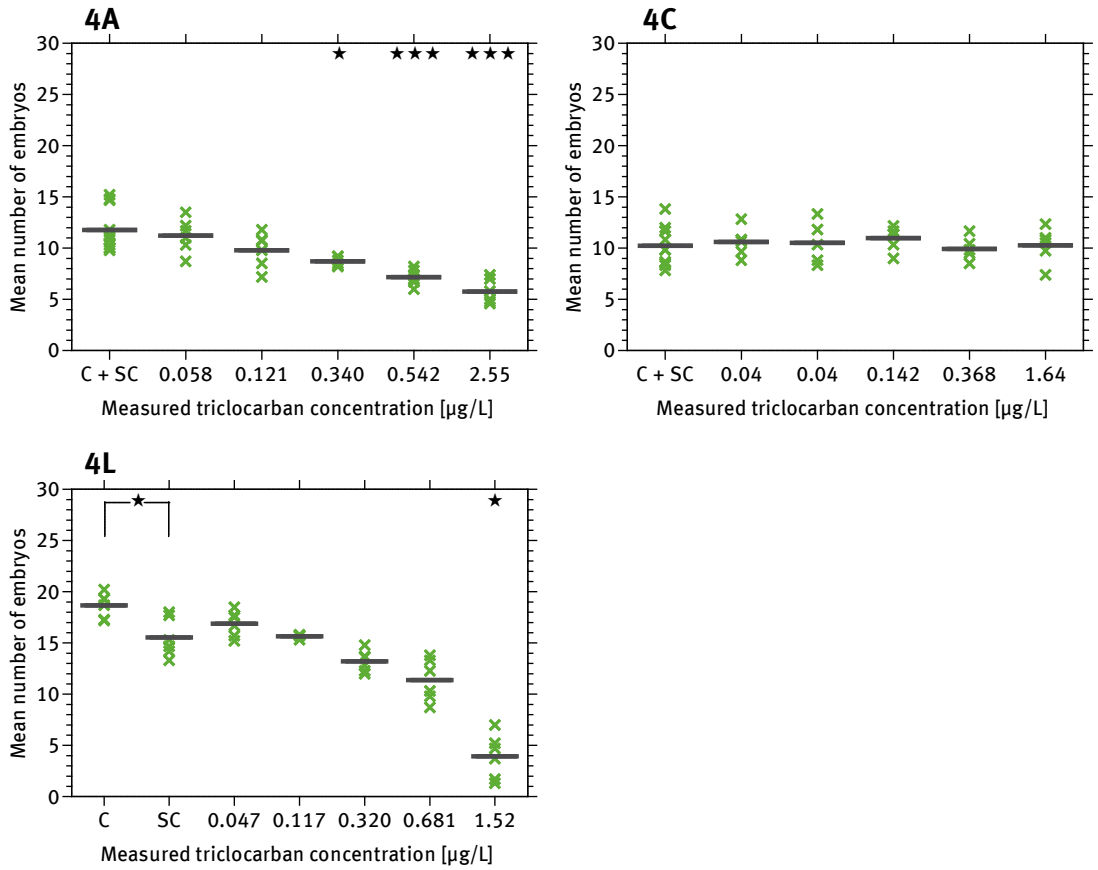
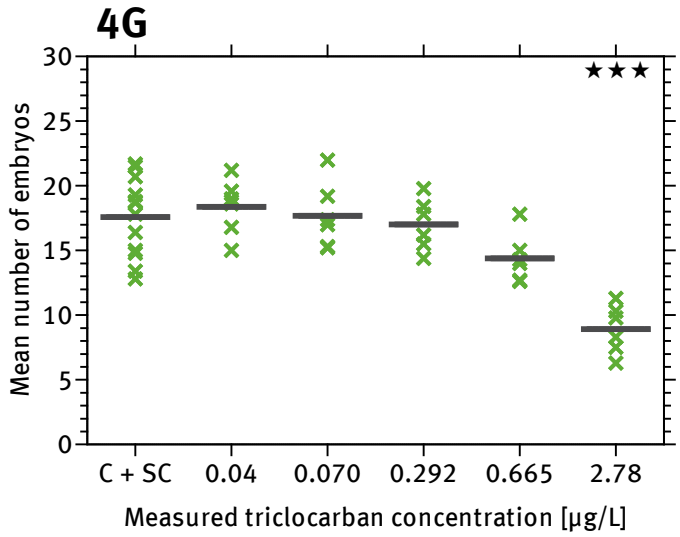


Figure 49 shows the results of the reproduction tests with *P. antipodarum* at laboratory 4G which did not achieve the validity criterion for the maximum control mortality. Besides the high mortality of snails in control and TCC exposure groups, the results of this laboratory are comparable with the other laboratories with valid test results. Here also a significant decrease

in the reproduction of *P. antipodarum* was observed at the highest test concentration of 2.78 µg/L.

Figure 49: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks exposure to measured triclocarban concentrations at the non-valid laboratory 4G (crosses: replicate mean; line: treatment mean). Asterisks indicate significant differences compared to merged solvent control and negative control (Dunnett´s test). ★★★ = p<0.001, n = 6 replicates.



5.3.4.5 Actual exposure concentrations of triclosan

The results of the analytical measurement of TCS for all laboratories are shown in tables 68 - 71. In total, only 31.1% of the nominal concentrations were achieved. Therefore, TWM concentrations were used for the calculations of effect concentrations. The initial concentrations varied between 2.34% and 88.2% and after two or three days values varied between 2.27% and 88.3% of nominal concentrations. In all laboratories the measured concentrations of TCS in solvent control groups were below the LOD of 0.04 µg/L. In some of the water samples at all laboratories the measured concentrations of TCS were below the LOD. To calculate TWM concentrations the half of the LOD (0.02 µg/L) was used according to the recommendation of OECD (2000). The analytical measurements of TCS at the lowest tested concentration (0.10 µg/L) and at 0.3 µg/L at laboratory 4A were mostly below the LOD (< 0.04 µg/L). Therefore, according to the recommendation of OECD (2000), the LOD (0.04 µg/L) was used for this exposure groups to calculate effect concentrations.

Table 68: Results of triclosan analyses [µg/L] in exposure media from laboratory 4A in validation 3 of the reproduction test with *Potamopyrgus antipodarum*. Bold: half of the LOD.

TCS nominal concentration [µg/L]	Measured concentrations [µg/L]						Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh medium	Day 2 old medium	Day 14 fresh medium	Day 16 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	-	-
0.1	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	0.04	-
0.3	< LOD	< LOD	< LOD	0.0400	< LOD	0.100	0.04	-
1	0.02	0.0400	0.02	0.100	0.02	0.340	0.0708	7.08
3	0.02	0.160	0.02	0.240	0.380	0.260	0.180	6.00
10	< LOD	< LOD	0.100	2.57	1.68	1.84	1.36	13.6

LOD = 0.04 µg/L

Table 69: Results of triclosan analyses [µg/L] in exposure media from laboratory 4C in validation 3 of the reproduction test with *Potamopyrgus antipodarum*. Bold: half of the LOD.

TCS nominal concentration [µg/L]	Measured concentrations [µg/L]						Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh medium	Day 2 old medium	Day 14 fresh medium	Day 16 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	-	-
0.1	0.02	0.0475	0.0488	0.0560	0.0882	0.0688	0.057	57.0
0.3	0.129	0.137	0.163	0.254	0.198	0.171	0.175	58.3
1	0.422	0.364	0.558	0.599	0.656	0.482	0.519	51.9
3	0.946	0.686	1.24	0.837	1.21	1.14	1.03	34.3
10	2.42	2.14	3.87	2.07	5.12	3.01	3.17	31.7

LOD = 0.04 µg/L

Table 70: Results of triclosan analyses [µg/L] in exposure media from laboratory 4G in validation 3 of the reproduction test with *Potamopyrgus antipodarum*. Bold: half of the LOD.

TCS nominal concentration [µg/L]	Measured concentrations [µg/L]						Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh medium	Day 2 old medium	Day 14 fresh medium	Day 16 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	-	-
0.1	0.0424	0.0371	0.0538	0.02	0.0354	0.0815	0.045	45.0
0.3	0.121	0.0812	1.24	0.265	0.167	0.124	0.271	90.3
1	0.442	0.293	0.465	0.0227	0.585	0.468	0.370	37.0
3	0.943	0.911	0.0702	0.116	1.93	1.05	0.909	30.3
10	1.23	2.69	0.808	0.928	7.10	5.33	3.42	34.2

LOD = 0.04 µg/L

Table 71: Results of triclosan analyses [µg/L] in exposure media from laboratory 4L in validation 3 of the reproduction test with *Potamopyrgus antipodarum*. Bold: half of the LOD.

TCS nominal concentration [µg/L]	Measured concentrations [µg/L]						Time weighted mean [µg/L]	% of nominal concentration
	Day 0 fresh medium	Day 2 old medium	Day 14 fresh medium	Day 16 old medium	Day 25 fresh medium	Day 28 old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	-	-
0.1	0.0414	0.02	0.0411	0.0400	0.0385	0.0400	0.0364	36.4
0.3	0.0780	0.0554	0.0694	0.0600	0.0683	0.0769	0.0677	22.6
1	0.502	0.112	0.464	< LOD	0.238	0.280	0.220	22.0
3	1.03	0.185	0.632	0.430	0.454	0.398	0.480	16.0
10	1.46	0.737	1.09	0.320	1.28	1.14	0.964	9.6

LOD = 0.04 µg/L

5.3.4.6 Biological responses to triclosan

Mortality

At laboratory 4L no mortality was observed in any of the TCS exposure groups and controls. The maximal observed mortality was 5.56% at laboratory 4A at the lowest test concentration.

Reproduction

Figure 50 shows the results of the valid reproduction tests with TCS at laboratory 4A, 4C and 4L. In none of the TCS exposure groups an increase of the reproduction of the snails could be detected. Laboratory 4A found a significant decrease of embryo numbers at the lowest test concentration, which was not detected at the higher test concentrations. Compared to solvent control, a significant reduction was observed at a TCS concentration of 0.964 µg/L at laboratory 4L. Laboratory 4C did not detect any significant effect on the reproductive output of *P. antipodarum*.

Figure 50: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks exposure to measured triclosan concentrations at valid laboratories 4A, 4C and 4L (crosses: replicate mean; line: treatment mean). Asterisks indicate significant differences compared to merged solvent control and negative control (Dunnett´s test) or compared to solvent control and negative control at laboratory 4L (unpaired t-test). ★ = p‹0.05, ★★★ = p‹0.001, n = 6 replicates.

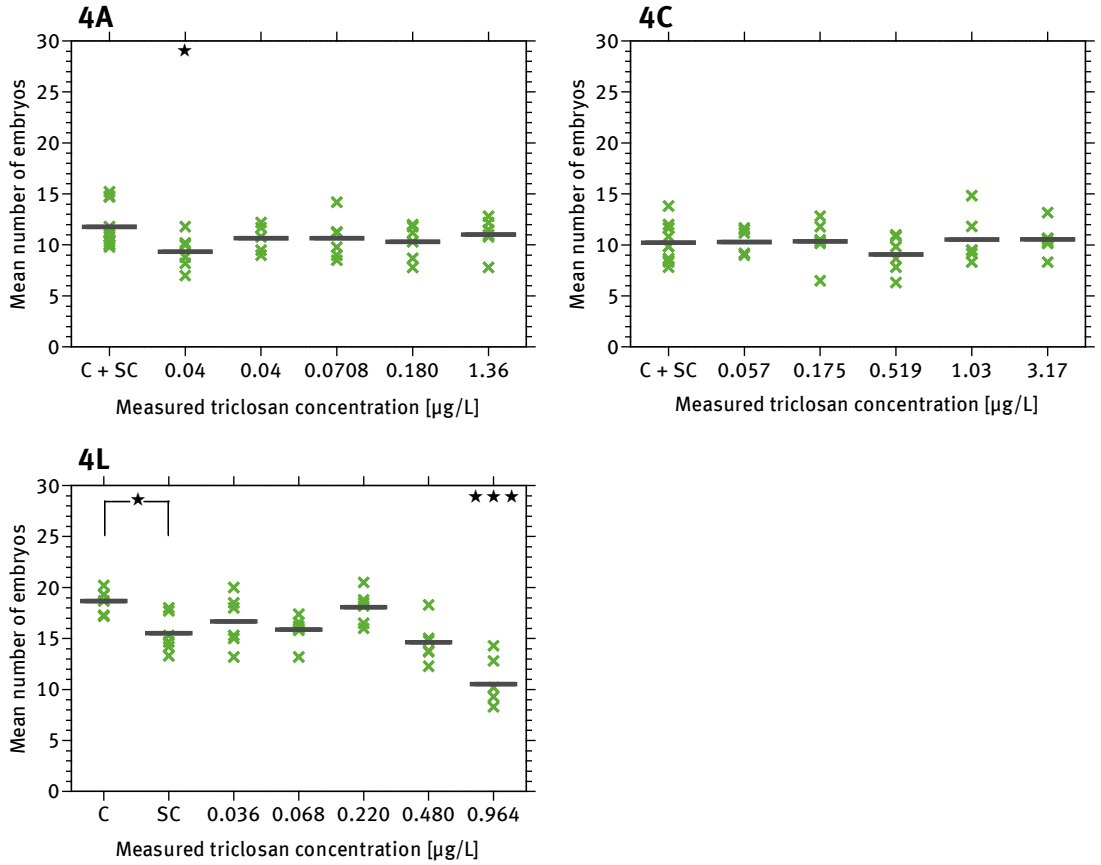
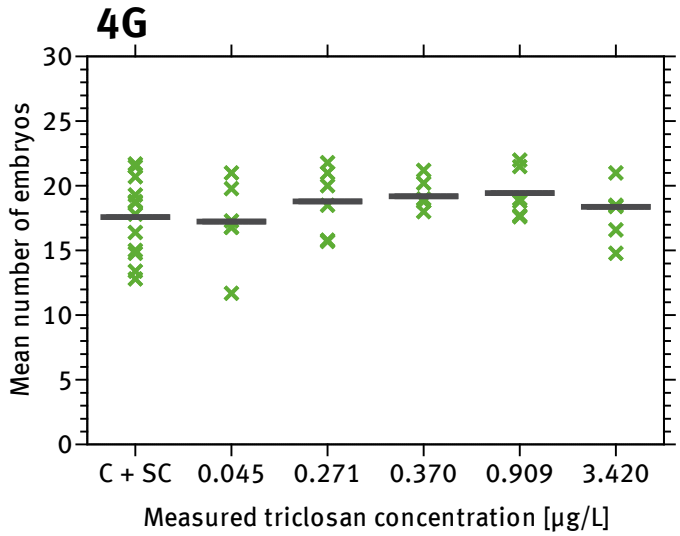


Figure 51 shows the results of the reproduction test with TCS at laboratory 4G, which did not meet the validity criterion of the maximal mortality in controls. As already described for the other laboratories (Fig. 50), no increasing effect of TCS on the embryo numbers of the snails could be detected. The reproduction of TCS exposed snails is on the control level.

Figure 51: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks exposure to measured triclosan concentrations at the non-valid laboratory 4G (crosses: replicate mean; line: treatment mean). n = 6 replicates.



5.3.5 Comparison of present results to published and grey literature

Within validation 3, TCC or TCS exposed snails did not show an increase in the reproductive output. This is in contradiction with already published and grey literature. GIUDICE AND YOUNG (2010) found a concentration-dependent increase in embryo numbers of *P. antipodarum* with NOEC and LOEC values of 0.05 µg/L and 0.2 µg/L, respectively. Also, a significant increase of the embryo numbers caused by the exposure to TCC at a concentration of 0.226 µg/L was observed in the feeding experiments (see section 4.3.3) at the same feeding level (62.5 µg/snail x day). Furthermore, HEIDELBACH (2014) tested TCC and TCS in a reproduction test with *P. antipodarum* and found for TCC an inverted U-shaped concentration response relationship, with a stimulation of reproduction at low concentrations followed by an inhibition at higher test concentrations.

The sensitivity to TCC and TCS in *P. antipodarum* differs from other species. A chronic toxicity test with *D. magna* and TCS resulted in a NOEC after 21 days of 40 µg/L (ORVOS ET AL. 2002). For TCS exposed eggs of *Oryzias latipes* a reduced hatching rate was found at a concentration of 313 µg/L (ISHIBASHI ET AL. 2004). The exposure of *Ceriodaphnia dubia* to TCC resulted in a NOEC of 1.90 µg/L after 8 days (TAMURA ET AL. 2012).

5.3.6 Conclusions from validation 3

For the exposure to TCC, two out of three laboratories with valid test results found a concentration-dependent decrease in the embryo numbers. For the reproduction tests with TCS, only one laboratory observed a significant decrease in the reproduction of snails at the highest test concentration. None of the participating laboratories found an increase of the reproduction of the snails contrary to pre-tests at Goethe University. A possible explanation could be the low meas-

ured concentrations of the test substances in the ring test, probably caused by a mistake or technical failure during weighing of the test chemicals.

The results also demonstrate that the reduced food level does not affect the reproductive output of the snails compared to the other two validation studies but is advantageous because the amount of unconsumed food and therefore the risk of fungus growth and increased snail mortality are reduced.

5.4 Overall conclusions from the validation studies with *Potamopyrgus antipodarum*

The robustness and the reproducibility of the reproduction test with *P. antipodarum* have been demonstrated in three validation exercises and in a ring test performed during the former project ValMolRepro I (SIERATOWICZ AND OEHLMANN 2011) with six test compounds. In total 17 partners from 10 countries participated in these round robins coming from industries, government and academia. Within the four validation studies, 43 reproduction tests have been performed, thereof one laboratory had to repeat the reproduction test with TBT due to very low concentrations of the test substance and five laboratories did not achieve the given validity criteria. Another laboratory had technical issues to satisfy the temperature between 15°C and 17°C. Three laboratories exceeded the validity criterion for the maximum control mortality mostly caused by a growth of fungus. Food remains in the test vessels may promote fungal growth which is the most likely reason for the increased mortality. In the draft test guideline a weekly change of the test vessels is proposed to reduce the danger of fungal growth and of resulting increased mortality of snails. One laboratory did not achieve the minimum embryo numbers of ≥ 5 in control groups in snails coming from a different culture. Here, probably the acclimation period to the medium used in the ring test was too short. Mean embryo numbers of 20 snails were checked prior to the test start (T_0) and were below 5. In the draft test guideline it is recommended to check the ability to reproduce prior test start. The mean embryo number in snails used for testing should be between 5 and 20. Accordingly, these snails should not have been used in a reproduction test. In total, only about 19% of the reproduction tests conducted were not valid. This suggests that the given criteria are appropriate and achievable if the instructions of the test guideline are followed. All partners achieved the proposed physico-chemical parameters.

The actual measured concentrations of TBT, TCC and TCS were obviously below the nominal concentrations. An explanation for the low exposure concentrations of TBT could be the degradation of TBT to monobutyltin and dibutyltin during the exposure. Another reason might be the adsorption of the substance to the walls of the test vessels and to food particles due to the low solubility of TBT in water and/or by direct uptake by the snails. Furthermore, it should be noted that the calculation of TWM concentrations based only on two measuring intervals, due to the high costs. However, the assessed effect concentrations of TBT were comparable among partners. The low measured concentrations of TCC and TCS might explain the lack of a reproduction-increasing effect in *P. antipodarum* during the validation exercise which is in contrast to in-house studies or published data (GUIDICE AND YOUNG 2010, HEIDELBACH 2014).In validation 2 with TR and PCZ measured concentrations exceeded the nominal concentrations by up to factor 1.5 2.7, respectively. A probable cause is a technical failure during weighing of the substances. None of the participating laboratories found a concentration-dependent decrease of the embryo numbers in *P. antipodarum* under exposure to TR. The lack of effects is in line with the results of the validation studies with *L. stagnalis* (OECD 2015c). Here, two laboratories investigated the effects of TR in a 56 day reproduction test and did not observe any effect on the fecundity of the snails up to a measured concentration of 394 ng TR/L.

In the ring test with PCZ all participating laboratories detected a concentration-dependent decrease of the embryo numbers of *P. antipodarum*. Also the laboratories which did not achieve the validity criteria observed comparable NOEC and LOEC values.

For the tested chemicals Cd, TBT, TR and PCZ the inter-laboratory reproducibility of the test has been shown as most of the laboratories detected comparable NOEC, LOEC, EC_{10} and EC_{50} values with overlapping 95%-confidence intervals for the latter, even if difficult to handle substances were chosen as test compounds (e.g. TBT or TR). Furthermore within the ring test conducted in the project ValMolRepro I and validation 1 the repeatability/intra-laboratory reproducibility could be demonstrated as laboratory A repeated the reproduction tests with TBT and Cd.

All in all, the reproduction test turned out to be a practical tool for the risk assessment of chemicals. The test is good manageable and the results are reproducible. At the 10th meeting (10. - 12. December 2014) of the "validation management group on ecotoxicity testing" (VMGeco) of OECD, the experts acknowledged the validation exercises and requested the submission of a draft test guideline for the reproduction test with *P. antipodarum* which will be submitted in June 2015. After an international commenting round and the acceptance by the member states, the test guideline is expected to come into force in 2016. Therefore, the outcome of the project contributes to the further development of test methods and evaluation concepts for the regulation of reproductive-influencing chemicals in REACH, as well as pesticides, pharmaceuticals and biocides and moreover for the assessment of long-term and/or sublethal substance effects on populations and ecosystems. It is the first aquatic non-arthropod-test, which was successfully validated in the frame of the *Conceptual Framework for Endocrine Disrupters* as a level 4 assay (OECD 2012a). Thereby, molluscs are getting considered as a sensitive and ecological important group of invertebrates in the guideline program for the risk assessment of chemicals of the OECD.

The results of the ring tests presented here will also be published by OECD in a validation report (OECD 2015a).

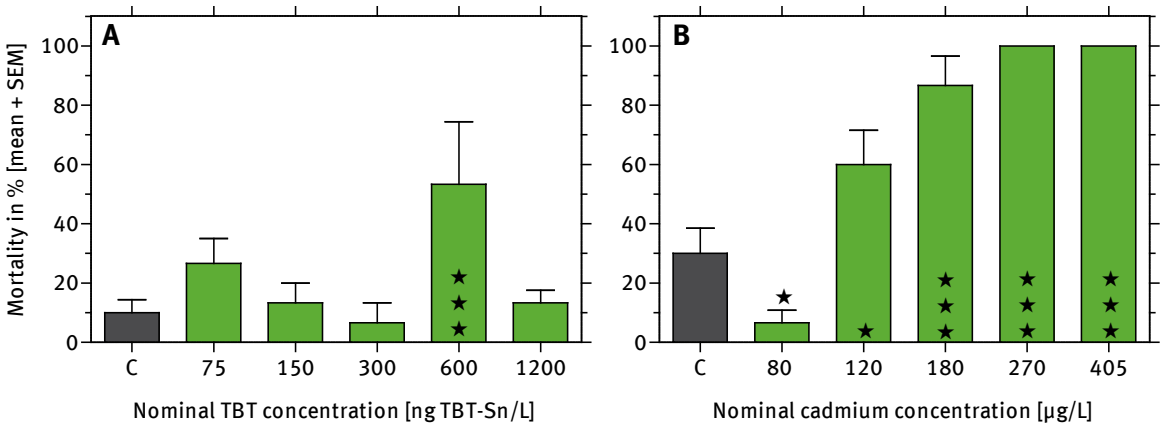
6 Further studies

6.1 Contributions to the validation of the reproduction test with *Lymnaea stagnalis*

In the context of the validation of the reproduction test with the great pond snail *Lymnaea stagnalis*, coordinated by INRA in Rennes (France), Goethe University Frankfurt participated in two international ring tests. The reproduction test lasts for 56 days and the endpoints are mortality, number of clutches per snail and number of eggs per snail. For a test to be valid the following criterions should be met: mortality in controls should not exceed 20% and the mean cumulated individual fecundity in the controls should be at least 4 egg-clutches per individual-day at the end of the test. Furthermore the dissolved oxygen saturation should not drop below 60% and the mean water temperature should be between 19° and 21°C throughout the test (OECD 2015b). Snails were fed with fresh lettuce *ad libitum* and five replicates with six snails each were used. The first validation round was conducted in 2011 with the reproduction-toxic substances TBT and Cd. TBT and Cd were tested in nominal concentration ranges between 75 and 1200 ng Sn/L and 80 and 405 µg/L, respectively.

Figure 52 shows the mortality after 56 days exposure to TBT (Fig. 52A) and Cd (Fig. 52B). Mortality in controls of the TBT experiment was 10% and a significant increased mortality of 53.3% occurred at a concentration of 600 ng Sn/L. At the highest test concentration (1200 ng Sn/L) the mortality was again on the level of control. The mortality in control groups of the Cd experiment was 30% and therefore exceeded the validity criterion. Mortality increased with increasing Cd concentrations and at concentrations of 270 µg/L and 405 µg/L all snails died during the test.

Figure 52: Mortality in % (mean with standard error) of *Lymnaea stagnalis* after eight weeks exposure to tributyltin (A) and cadmium (B). Asterisks indicate significant differences compared to control (Fisher's exact test), ★ = p ‹ 0.05, ★★★ = 0.001, n = 6 replicates.



In figure 53 the number of clutches (Fig. 53A) and the number of eggs (Fig. 53B) per snail after exposure to TBT are summarized. Snails in controls produced 10.1 clutches and 645 eggs over

56 days. The number of clutches and eggs decreased in a concentration-dependent manner. The NOEC for both endpoints was 150 ng Sn/L. The calculated EC_{10} of the number of clutches and the number of eggs per snail had to be extrapolated and was 28.0 ng Sn/L (95%-CI: 6.25 - 125 ng Sn/L) and 32.7 ng Sn/L (95%-CI: 8.83 - 121 ng Sn/L), respectively.

Figure 53: Mean number of clutches (A) and eggs (B) per specimen of *Lymnaea stagnalis* after eight weeks exposure to tributyltin. Asterisks indicate significant differences compared to control (Dunnett´s test), ★ = p ‹ 0.05, ★★ = 0.01, ★★★ = 0.001, n = 6 replicates.

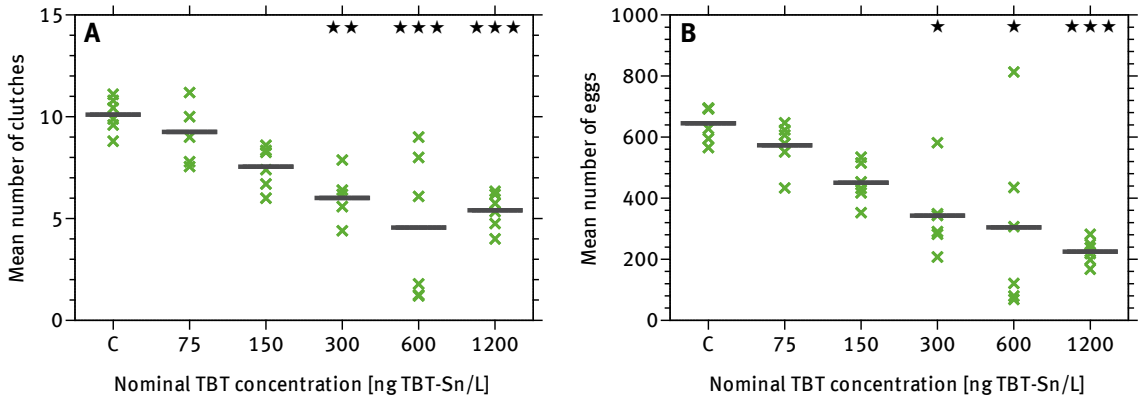
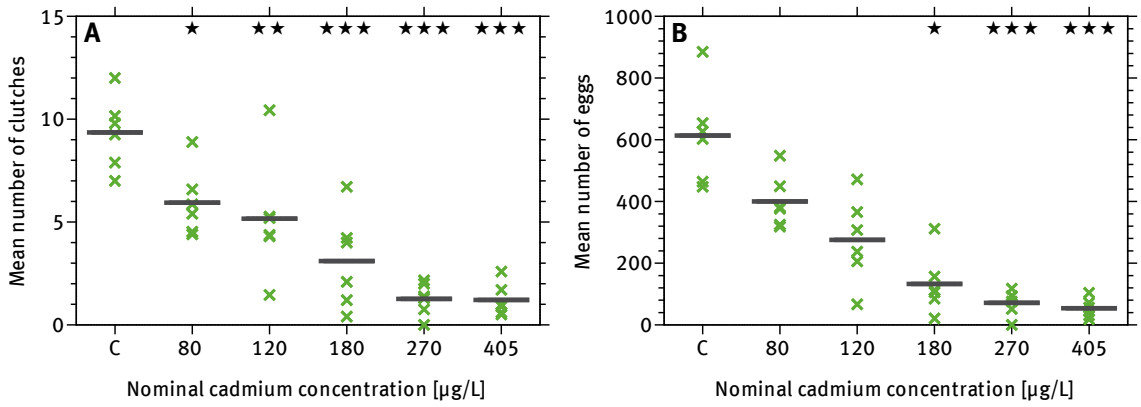


Figure 54 shows the results of the reproduction test with *L. stagnalis* and Cd for the number of clutches (Fig. 54A) and eggs (Fig. 54B). Control snails in the 56 days-test produced an average of 9.35 clutches and 614 eggs. A significant decrease of the number of clutches and eggs was observed after 56 days exposure. Already at the lowest test concentration of 80 µg/L a significant effect occurred. The NOEC for the number of eggs was 120 µg/L. The calculated EC_{10} of the number of clutches and the number of eggs per snail had to be extrapolated and was 35.1 (95%-CI: 18.2 - 67.7 µg/L) and 38.7 µg/L (95%-CI: 24.0 - 62.4 µg/L), respectively.

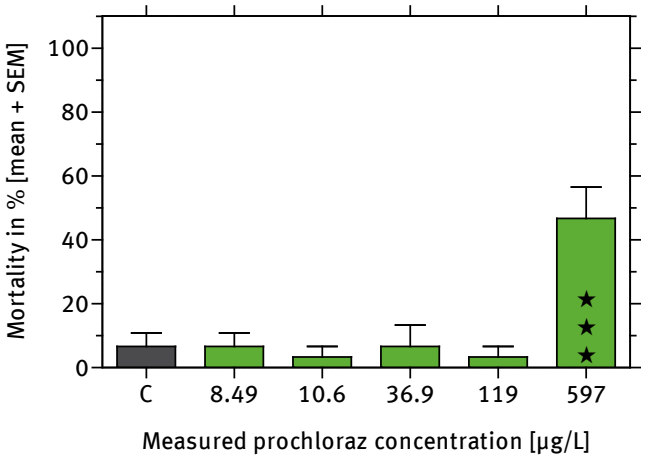
Figure 54: Mean number of clutches (A) and eggs (B) per specimen of *Lymnaea stagnalis* after eight weeks exposure to cadmium. Asterisks indicate significant differences compared to control (Dunnett´s test), ★ = p ‹ 0.05, ★★ = 0.01, ★★★ = 0.001, n = 6 replicates.



A second ring trial of the reproduction test with *L. stagnalis* was conducted in early 2014. Here, PCZ was tested at a nominal concentration range between 10 and 1000 µg/L. Analytical measurements were performed at the University of Southern Denmark and calculated TWM concentrations were 8.49, 10.6, 36.9, 119 and 597 µg/L. The validity criteria were achieved (OECD 2015b).

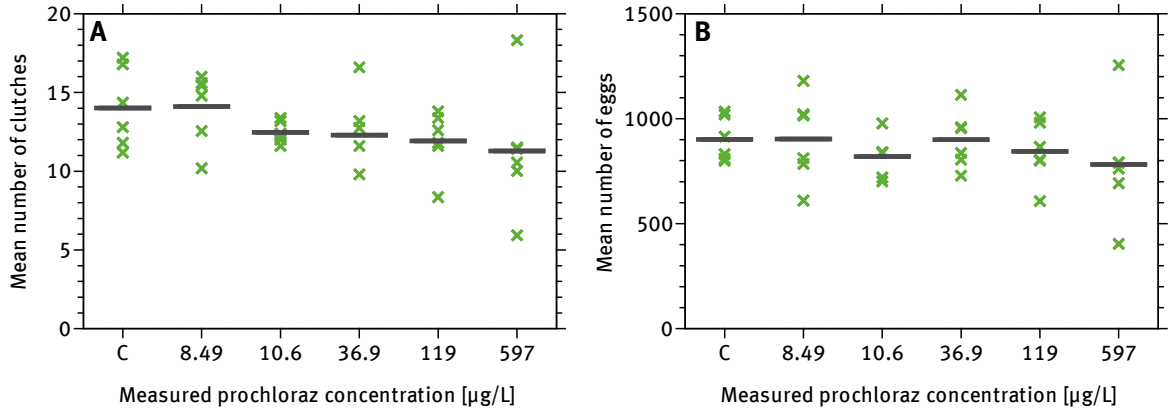
Figure 55 shows the observed mortality of the snails after 56 days exposure to PCZ. Mortality in control was 6.67%. A significant increased mortality was detected at a concentration of 597 µg/L, where 46.7% of the snails died throughout the test.

Figure 55: Mortality in % (mean with standard error) of *Lymnaea stagnalis* after eight weeks exposure to prochloraz. Asterisks indicate significant differences compared to control (Fisher's exact test), ★★★ = 0.001, n = 6 replicates.



In figure 56 the number of clutches (Fig. 56A) and the number of eggs (Fig. 56B) per snail after exposure to PCZ are shown. An average of 14.0 clutches and 902 eggs were produced by control snails. For none of the PCZ exposed groups, a significant effect was found. The number of clutches and eggs in the PCZ exposure groups were all on the level of control.

Figure 56: Mean number of clutches (A) and eggs (B) per specimen of *Lymnaea stagnalis* after eight weeks exposure to prochloraz. n = 6 replicates.



The validation of the reproduction test with *L. stagnalis* has been successfully completed. The results of the validation study with Cd are already published in DUCROT ET AL. (2014). The drawing up of a guideline-proposal of the reproduction test with *L. stagnalis* was acknowledged by the OECD on the 10th meeting of VMGeco in December 2014. The results of the ring tests presented here will also be published in the validation report on the reproduction test with *L. stagnalis* by OECD (2015c).

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8 Annex

8.1 Influence of different food doses on the reproduction of *Potamopyrgus antipodarum* under chronic pollutant exposure

Table 72: Results of the analytical measurements (actual exposure concentrations) of triclocarban in samples of the exposure media and solvent control in the reproduction test with *Potamopyrgus antipodarum* and calculated time weighted mean concentrations.

TCC nominal concentration [µg/L]	Measured concentrations [µg/L]				Time weighted mean [µg/L]	% of nominal concentration
	Day 11 fresh medium	Day 14 old medium	Day 21 fresh medium	Day 23 old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	-	-
0.10	< LOD	< LOD	< LOD	< LOD	0.04	-
0.30	0.0541	0.0400	0.0837	0.0421	0.052	17.3
1.00	0.192	0.155	0.392	0.233	0.226	22.6
3.00	1.30	0.505	0.877	0.961	0.872	29.1
10.0	6.70	1.66	4.14	3.27	3.64	36.4

LOD = 0.04 µg/L

Table 73: Results of the analytical measurements (actual exposure concentrations) of cadmium in samples of the exposure media and solvent control in the reproduction test with *Potamopyrgus antipodarum* and calculated time weighted mean concentrations.

Cd nominal concentration [µg/L]	Measured concentrations [µg/L]								Time weighted mean [µg/L]	% of nominal concentration
	Day 2 fresh	Day 4 old	Day 9 fresh	Day 11 old	Day 16 fresh	Day 18 old	Day 25 fresh	Day 28 old		
Negative control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	-	-
1.56	1.3	0.9	1.3	0.9	1.3	0.9	1.3	0.7	1.05	67.3
3.13	2.8	1.9	2.8	1.8	2.8	1.9	2.8	1.5	2.26	72.2
6.25	5.7	4.1	5.7	3.9	5.6	4.1	5.6	3.3	4.65	74.4
12.5	11.4	8.4	11.3	8.0	11.3	8.8	11.6	6.9	9.54	76.3
25.0	23.2	16.4	22.9	16.2	21.8	17.0	22.5	14.4	19.0	76.0

LOD = 0.5 µg/L

8.2 Standard operating procedure of a full life cycle test with *Potamopyrgus antipodarum* - draft version

DRAFT Standard Operating Procedure

Potamopyrgus antipodarum Full Life Cycle Test (version as of June 2015)

INTRODUCTION

1. This Standard Operating Procedure (SOP) is designed to assess potential effects of prolonged exposure to chemicals on growth, reproduction and survival of the freshwater mudsnail *Potamopyrgus antipodarum* over the full life cycle.

2. The measured parameters in this test are mortality and the assessment of growth as the shell length and of reproduction as the total number of embryos in the brood pouch per female without distinction of developmental stages.

3. Before use of the test guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

PRINCIPLE OF THE TEST

4. The primary objective of the test is to assess the effect of chemicals on growth and reproduction of *P. antipodarum* over the entire life cycle. To this end snails are continuously exposed to a concentration range of the test chemical starting with new-borne snails over the entire development phase until they have successfully reproduced (at least in the control group) at a shell length of 3.5 mm. First, adult snails from a laboratory culture are transferred into fresh medium and allowed to reproduce for one week. Phase 1 of the test starts when released new-borne snails of a maximum age of 1 week are introduced into crystallizing dishes at a density of 1000 neonates/L and the test chemical is spiked into the water. Phase 1 of the test ends when control snails reach a mean shell length of 1.5 mm. For the following phase 2 juveniles are transferred into glass beakers and density is adjusted to 75 juvenile snails/L. Phase 2 and the full life cycle test end when animals in control groups reach a mean size of 3.5 mm. Endpoints of the test are mortality, growth and embryo numbers of the snails. Growth is measured by the increase of the shell length over the course of the experiment in regular intervals. Reproduction is examined at the end of the test. After removal of the shell, embryos can easily be seen through the epithelia (see ANNEX 3, Figs. 1B, 2). By opening the brood pouch and subsequently removing the embryos and counting them, the reproductive success of each female is determined (see ANNEX 3, Fig. 1C).

5. The toxic effect of the test chemical on growth and reproduction is expressed as EC_X by fitting a non-linear regression model to test data to estimate the concentration that would cause x% reduction in length of the shell or x% reduction embryo numbers or alternatively as the No Observed Effect Concentration and Lowest Observed Effect Concentration (NOEC/LOEC) value (1). The test concentrations should preferably bracket the lowest of the used effect concentrations (e.g. EC_{10}) which means that this value is calculated by interpolation and not extrapolation.

INFORMATION ON THE TEST CHEMICAL

6. Information on the test chemical which may be useful in establishing the test conditions includes the structural formula, purity of the substance, stability in light, stability under the conditions of the test, pK_a, P_{ow} and results of a test for ready biodegradability (see OECD Test Guidelines 301 and 310).

7. The water solubility and the vapour pressure of the test chemical should be known and a reliable analytical method for the quantification of the substance in the test solutions with reported recovery efficiency and limit of quantification (LOQ) should be available.

REFERENCE SUBSTANCES

8. Suitable reference substances may be tested periodically as a means of assuring that the test conditions are reliable. A toxicant successfully used in previous full life cycle studies with *P. antipodarum* is cadmium chloride with EC_{50} ranges of 100 to 150 µg Cd/L for growth and of 1.5 to 7 µg Cd/L for reproduction (2).

VALIDITY OF THE TEST

9. For a test to be valid the following conditions should be fulfilled:
   - mortality in the controls should not exceed 80% during phase 1 and 20% during phase 2 of the test;
   - the mean number of embryos in controls should be at least 5 embryos per female at the end of the test,
   - the dissolved oxygen content must have been at least 60% of the air saturation value throughout the test and
   - water temperature should be 16 ± 1.5°C throughout the test in both control and exposure groups. On one given measuring date the difference of temperature between test vessels should not be higher than ± 1 °C.

Recommendations for further parameters are provided below (cf. section 14).

DESCRIPTION OF THE METHOD

Apparatus

10. Test vessels and other apparatus which will come into contact with the test solutions should be made entirely of glass or other materials chemically inert to the test chemical. Additionally, the following equipment will be required:

- 2000 mL crystallizing dishes
- 200 mL crystallizing dishes
- 500 mL glass beakers
- glass pipettes
- oxygen meter
- pH meter
- conductivity meter
- stereomicroscope
- climate chambers or temperature regulated room or other adequate apparatus for temperature and lighting control
- dissecting dish and dissecting instruments

Test organism

11. The species to be used in the test is *Potamopyrgus antipodarum* (Gray, 1853). Snails used in previous full life cycle studies belong to haplotype *t* and morphotype "Warwick A" according to Städler et al. (2005, ref. 3) and should be used for testing. Field collected organisms should not be used.

12. Test animals should be laboratory-reared and taken from a parasite-free stock of female snails (i.e. showing no signs of stress such as high mortality, poor fecundity, etc.). The stock snails must be maintained at culture conditions (light, temperature, medium and feeding) similar to those to be used in the test (culturing methods for *P. antipodarum* are described in ANNEX 2).

Test medium

13. Reconstituted (synthetic) water should be used as test medium. The reconstituted water should be prepared with 3 g Tropic Marin® sea salt and 1.8 g sodium hydrogen carbonate ($NaHCO_3$) dissolved per 10 litre deionised water. The reconstituted water should be prepared in a container of sufficient volume, e.g. a 50-litre aquarium, where the water is stored for further use, preferably at ambient temperature in the dark. The reconstituted water has to be aerated for at least 24 hours before use. It should be used within a maximum storage period of 2 weeks.

14. The following water parameters should be achieved and kept:

pH:	8.0 ± 0.5
Oxygen saturation:	> 60% ASV (air saturation value)
Conductivity:	770 ± 100 µS/cm
Light intensity:	500 ± 100 lx

15. Test vessels (200 mL crystallizing dishes in phase 1, 500 mL glass beakers in phase 2) should contain 160 and 400 mL of reconstituted water, respectively. Glass beakers in phase 2 should be replaced weekly while crystallizing dishes should not be replaced during phase 1. Larger volumes may sometimes be necessary to meet requirements of the analytical procedure used for determination of the test chemical concentration, although pooling of replicates for chemical analysis is also allowable. The crystallizing dishes should be covered with a watch glass, the beakers with a lid or gauze.

Test solution

16. Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should be prepared by dissolving the test chemical in the test water by mixing or agitating the test chemical in test medium using mechanical means such as agitating, stirring or ultrasonication, or other appropriate methods. If possible, the use of solvents or dispersants should be avoided.

17. If a solvent is used for the application of the test chemical, a solvent control using the same amount of solvent as in the treatments should be used. The amount of solvent in test concentrations should be as small as possible. If no other information is available, the appropriate amount of the selected solvent has to be determined in a preliminary test and depends on the type of test chemical and the sensitivity of the test organisms towards the selected solvent. Dimethyl sulfoxide (DMSO) or triethylene glycol (TEG) should be used at a maximum concentration of 20 µL/L in the test vessels (= 0.002%) as solvent whenever possible. DMSO and TEG are characterised by a low toxicity for *P. antipodarum* and do not cause biofilm development in the test vessels. In contrast, the use of ethanol as a solvent results often in considerable development of fungi and bacteria even at concentrations as low as 0.003%.

PROCEDURE

Conditions of exposure

Duration

18. The test duration is 150 to 180 days.

Loading

19. Snails used for the production of neonates to be used in phase 1 of the test have to be checked for their ability to reproduce. Therefore, the shell height, and the embryo numbers of 10 snails from the culture batch selected for the test should be measured. The shell height should be between 3.5 and 4.5 mm. The mean number of embryos per snail should be 5 or more. 200 adult snails have to be allocated to each of the six 2000 mL crystallizing dish containing the reconstituted water using tweezers. After one week all adult snails are removed from the crystallizing dishes.

20. For phase 1 of the test 160 neonates have to be allocated randomly to each test vessel (200 mL crystallizing dish) containing the exposure water using 1 mL glass pipettes. Phase 1 has a duration of 70 to 90 days and ends when control snails reach a mean shell length of 1.5 mm. With the end of phase 1 the phase 2 of the tests starts and 30 juvenile snails are allocated randomly to each test vessel (500 mL glass beakers) containing the exposure water using tweezers.

Feeding

21. During the production of neonates (cf. section 19) by adult *Potamopyrgus*, snails are fed *ad libitum* with finely ground TetraPhyll® (60 to 80 µg per animal and day) and an algae suspension of *Scenedesmus acutus* var. *acutus* twice (on day 1 and 4). During phase 1 feeding of neonates should preferably be done *ad libitum* with an algae suspension of *Scenedesmus acutus* var. *acutus* twice per week after the change of the test media. During phase 2 feeding should preferably be done daily, but at least 3 times per week with finely ground TetraPhyll® (60 to 80 µg per animal and day). Deviations from this have to be reported and justified. The food should be applied by preparing a suspension with deionised or distilled water and pipetting in each test vessel. Food suspension should be homogenized by shaking each time before pipetting into a given test vessel. The applied volume should be as little as possible to avoid a dilution of the test concentrations. The suspension should be prepared immediately before use.

Light regime

22. The photoperiod in the culture and the test is L:D = 16:8 hours throughout the test. Light intensity should be 500 ± 100 lx, when testing photo-labile chemicals lower light intensities have to be used. The light source should be positioned above the test vessels. Test vessels should be positioned on a dark surface to avoid the snails from escaping. Deviations from this have to be reported and justified.

Temperature

23. The temperature of the test media should be 16 ± 1.5°C throughout the test. On one given measuring date the difference of temperature between test vessels should not be higher than ± 1°C.

Aeration

24. During phase 1 no aeration of water is provided. In phase 2 water should be aerated through glass pipettes (Pasteur pipettes) connected to an air tubing system. Adjustable valves should be used to ensure continuous and constant air flow.

25. Dissolved oxygen content should be kept above 60% ASV, however the test vessels should be aerated in phase 2 gently to avoid stripping of test chemicals.

Test design

26. At least five concentrations, bracketing effective concentration (e.g. EC_x), with six replicates each should be tested in a geometric series with a factor between concentrations not exceeding 2.2. Prior knowledge on the toxicity of the test chemical (e.g. from range finding studies or other sources like read across, etc.) should help in selecting appropriate test concentrations. Justification should be provided if fewer than 5 concentrations are used. Chemicals should not be tested above their solubility limit in test medium. Before conducting the experiment it is advisable to consider the statistical power of the tests design and using appropriate statistical methods (1). In setting the range of concentrations, the following should be borne in mind:

(i) When EC_x for effects on embryo numbers is estimated, it is advisable that sufficient concentrations are used to define the EC_x with an appropriate level of confidence. Test concentrations used should preferably bracket the estimated EC_x such that EC_x is found by interpolation rather than extrapolation. It is an advantage for the following statistical analysis to have more test concentrations (e.g. 10) and fewer replicates of each concentration (e.g. 3 thus holding the total number of vessels constant) and with 6 control replicates.

(ii) When estimating the LOEC and/or NOEC, the lowest test concentration should be low enough so that the embryo number at that concentration is not significantly different from the control. If this is not the case, the test should be repeated at lower concentrations.

(iii) When estimating the LOEC and/or NOEC, the highest test concentration should be high enough so that the embryo number at that concentration is significantly different from the control. If this is not the case, the test should be repeated with an increased highest concentration unless the maximum required test concen-

tration for chronic effects testing (i.e., 10 mg/L) was used as the highest test concentration in the initial test.

27. If no effects are observed at the highest concentration in the range-finding test (e.g. at 100 mg/L or a concentration equal to the limit of solubility), or when the test chemical is highly likely to be of low/no toxicity based on lack of toxicity to other organisms and/or low/no uptake, the reproduction test may be performed as a limit test (with fewer than five concentrations as the definitive test), using a test concentration of e.g.10 mg/L and the control. 10 replicates should be used for both the treatment and the control groups. A limit test will provide the opportunity to demonstrate that there is no statistically significant effect at the limit concentration, but if effects are recorded a full test will be required. Justifications should be provided if fewer than five concentrations are used.

Controls

28. Control vessels without added test chemical should be included in the test with an appropriate number of replicates, six replicates for the dilution-water control and, if needed, six replicates for the solvent control containing the solvent carrier only.

Test medium renewal

29. The frequency of medium renewal will depend on the stability of the test compound, but should be at least two times per week in phase 1 and three times per week in phase 2. If, from preliminary stability tests or from the physico-chemical properties of the test chemical, the concentration is evaluated not to be stable (i.e. outside the range 80 - 120% of nominal or falling below 80% of the measured initial concentration) over the maximum renewal period (i.e. three days), considerations have to be given to more frequent medium renewals. Alternatively, phase 2 of the test should be conducted under flow-through conditions when testing “difficult chemicals” (i.e. volatile, unstable, readily biodegradable and adsorbing chemicals). Due to the inevitable loss of neonates under flow-through conditions, it is imperative to perform phase 1 of the test under semi-static conditions.

30. The following procedure is used for water renewal. Exposure water is completely removed from the test vessels. During phase 1 the test medium should be siphoned off with a 100 mL glass pipette under a stereomicroscope to prevent the loss of neonates. During phase 2 a sieve can be used to collect the snails which might detach from the glass walls. Test vessels are refilled using water at the test temperature. The test chemical is immediately added to the renewed water using stock solutions. Newly contaminated water is homogenized by manual agitation. Snails are then placed back in the test vessel. Food is provided in the given range (see section 21) to animals once the water renewal and contamination have been completed.

Observations

31. The test vessels should be observed at least three times per week to achieve visual assessment of any abnormal behaviour (e.g. avoidance of water, avoidance of food or lethargy). Any signs of stress should be recorded. If there are snails found in the lid or outside of test water, they have to be transferred back to the medium, immediately.

Mortality

32. Dead snails should be removed from the test vessels and recorded weekly during phase 1 and preferably daily, or at least as frequently as test medium is renewed during phase 2 of the test.

Growth

33. The shell length of neonates and juvenile snails during phase 1 of the test is measured biweekly (every second week). Therefore, ten randomly selected snails per replicate are alive transferred with a glass pipette to a Petri dish under a stereomicroscope and the individual shell length is measured to the nearest 0.01 mm with an ocular micrometer or alternative with an image analysing system. After the measurement snails are re-transferred to their original test vessel. During phase 2 of the test the measurement of shell length is continued biweekly, again for 10 randomly selected snails per replicate, until the mean shell length in the control group has reached 3.5 mm. A precision of 0.05 mm is sufficient in phase 2 and snails can be handled using tweezers.

Reproduction

34. The number of embryos in the brood pouch of all surviving snails per replicate is analysed at the end of phase 2 of the test when control have reached a mean shell length of 3.5 mm. Therefore, the snails should preferably be quick-frozen (in liquid nitrogen or in a -80°C freezer) and stored at -20°C until analysis. Alternatively, the snails can be narcotised for 45 to 90 minutes in a solution of 2.5% magnesium chloride hexahydrate ($MgCl_2$ x 6 H_2O) in deionised or distilled water before dissection.

35. Shell height of snails has to be measured under a stereomicroscope with an ocular micrometer as described before (cf. section 33). The shell of the snails should be cracked carefully with a pair of pincers. Subsequently the snails are placed into a dissecting dish containing a small volume of test medium or tap water. The soft body can be prepared by removing the shell with dissecting needles or pointed tweezers. The brood pouch of the snails should be opened carefully with a dissecting needle, and the embryos have to be counted.

36. If snails did not produce embryos, animals have to be sexed. Males are characterised by a penis in the neck (bottom of the mantle cavity behind the snout and the two ocular tentacles). The influence of the presence of males on the results of the reproduction test is not known because males did never occur in laboratory cultures of haplotype *t* (4).

37. Data are recorded in an appropriate data sheet. The mean and variability parameters such as standard deviation or standard error of the mean, for the shell height and the number of embryos are calculated.

Frequency of analytical determinations and measurements

Concentration of the test chemical

38. During the test, the concentrations of the test chemical are determined at regular intervals. Prior to initiation of the exposure period, proper function of the chemical delivery system across all replicates should be ensured. Analytical methods required should be established, including an appropriate limit of quantification (LOQ) and sufficient knowledge on the substance stability in the test system.

39. In semi-static tests where the concentration of the test chemical is expected to remain within ± 20% of the nominal (i.e. within the range 80 - 120% - see section 29), it is recommended that, as a minimum, the highest and lowest test concentrations are analysed when freshly prepared and the old solution at the time of renewal on one occasion during the first week of the test (i.e. analyses should be made on a sample from the same solution - when freshly prepared and at renewal). These determinations should be repeated at least at monthly intervals thereafter.

40. For tests where the concentration of the test chemical is not expected to remain within ± 20% of the nominal, it is necessary to analyse all test concentrations, when freshly prepared and at renewal. However, for those tests where the measured initial concentration of the test chemical is not within ± 20% of nominal but where sufficient evidence can be provided to show that the initial concentrations are repeatable and stable (i.e. within the range 80 - 120% of initial concentrations), chemical determinations could be reduced in months 2, 3 and 4 of the test to the highest and lowest test concentrations. In all cases, determination of test chemical concentrations need only be performed on one replicate vessel at each test concentration changing systematically between replicates.

41. If a flow-through test is used, a similar sampling regime to that described for semi-static tests is appropriate (but measurement of 'old' solutions is not applicable in this case). However, increasing the number of sampling occasions during the first week (e.g. at least two sets of

measurements) may help to demonstrate that the test concentrations remain stable. In these types of test, the flow-rate of diluent and test chemical should be checked daily.

42. If there is evidence that the concentration of the test chemical has been satisfactorily maintained within ± 20% of the nominal or measured initial concentration throughout the test, then results can be based on nominal or measured initial values, respectively. If the deviation from the nominal or measured initial concentration is greater than ± 20%, results should be expressed in terms of the time-weighted mean (see guidance for calculation in ANNEX 4).

Physico-chemical parameters

43. Water quality parameters such as pH, oxygen saturation, conductivity, temperature, ammonium and total nitrite content should be measured before water renewal in one replicate per exposure group and controls. Additional measurements of nitrate and water hardness should be done if necessary.

DATA AND REPORTING

Treatment of results

44. The main parameters to be evaluated statistically are the mortality and the shell length at the end of phase 1 and phase 2 of the test and additionally the number of embryos per female at the end of phase 2 of the test. For statistical evaluation of the shell length and of the embryo numbers, the mean shell length and the mean embryo number across replicates for each concentration has to be calculated. The standard effect in the reproduction test is a decrease of the mean shell length and/or the embryo number with increasing concentration of the test compound. The endpoint reproduction during phase 2 has been validated exclusively for reproductive toxic chemicals. Reproduction increases may occur in the test and have been observed with chemicals such as ethanol and those with known or suspected estrogenic effects in vertebrates (5 - 8). However, the reproduction test is not suited to proof an endocrine mediated mode of action solely on the basis of a decreased or increased embryo number.

45. Before employing the statistical analysis, e.g. ANOVA procedures, comparison of treatments to the control by Student t-test, Dunnett's test, Williams' test, or stepdown Jonckheere-Terpstra test, it is recommended to consider transformation of data if needed for meeting the requirements of the particular statistical test (1). As non-parametric alternatives one can consider Dunn's or Mann-Whitney's tests. 95% confidence intervals are calculated for mean values per replicate.

46. The number of surviving snails in the untreated controls at the end of phase 1 and phase 2 of the test is a validity criterion, and has to be documented and reported. Also all other detrimental effects, e.g. abnormal behaviour as specified under section 31, abnormal appearance of

embryos and toxicological significant findings should be reported in the final report as well.

ECx

47. ECx-values, including their associated lower and upper 95% confidence limits, are calculated using appropriate statistical methods (e.g. logistic or Weibull function, trimmed Spearman-Karber method, or simple interpolation). To compute the EC_{10}, EC_{50} or any other ECx, the complete data set should be subjected to regression analysis.

NOEC/LOEC

48. If a statistical analysis is intended to determine the NOEC/LOEC appropriate statistical methods should be used according to OECD Document 54 on the Current Approaches in the Statistical Analysis of Ecotoxicity Data: a Guidance to Application (1). In general, adverse effects of the test chemical compared to the control are investigated using one-tailed hypothesis testing at $p < 0.05$.

49. Normal distribution and variance homogeneity can be tested using an appropriate statistical test, e.g. the Shapiro-Wilk test and Levene test, respectively ($p < 0.05$). One-way ANOVA and subsequent multi-comparison tests can be performed. Multiple comparisons (e.g. Dunnett's test) or step-down trend tests (e.g. Williams' test, or stepdown Jonckheere-Terpstra test) can be used to calculate whether there are significant differences ($p < 0.05$) between the controls and the various test chemical concentrations (selection of the recommended test according to OECD Guidance Document 54 (1)). Otherwise, non-parametric methods (e.g. Bonferroni-U-test according to Holm or Jonckheere-Terpstra trend test) could be used to determine the NOEC and the LOEC.

Test report

50. The test report includes the following information:

Test chemical:

- Mono-constituent substance:
  physical appearance, water solubility, and additional relevant physicochemical properties; chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate).
- Multi-constituent substance, UVBCs and mixtures:
  characterized as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.
- analytical method for quantification of the test chemical where appropriate.

Test species:

- scientific name, source and culture conditions.

Test conditions:

- test procedure used (e.g. semi-static or flow-through, volume, loading in number of snails per litre);
- photoperiod and light intensity;
- test design (e.g. test concentration used, number of replicates, number of snails per replicate, etc.);
- method of test chemical pre-treatment and spiking/application;
- the nominal test concentrations, details about the sampling for chemical analysis and the analytical methods by which concentrations of the test chemicals were obtained;
- media characteristics (including pH, conductivity, temperature and oxygen ASV, ammonium concentrations and any other measurements made);
- detailed information on feeding (e.g. type of food, source, amount given frequency of feeding).

Results:

- results from any preliminary studies on the stability of the test chemical;
- the nominal test concentrations and the results of all analyses to determine the concentration of the test chemical in the test vessels; the recovery efficiency of the analytical method, the means of the measured values and the limit of detection should also be reported;
- water quality within the test vessels (i.e. pH, temperature, oxygen ASV and ammonium concentration);
- the full record of shell length by replicate at the end of phases 1 and 2 of the test and of embryo numbers by replicate at the end of the test;
- the number of death among the snails at the end of phases 1 and 2 of the test;
- where appropriate the Lowest Observed Effect Concentration (LOEC) for reproduction (embryo numbers), including a description of the statistical procedures used and an indication of what size of effect could be expected to be detected (a power analysis can be performed before the start of the experiment to provide this) and the No Observed Effect Concentration (NOEC) for reproduction; where appropriate, the LOEC or NOEC for mortality of the animals should also be reported;
- where appropriate, the EC_X for reproduction and confidence intervals (e.g. 95%) and a graph of the fitted model used for its calculation, the slope of the concentration-response curve and its standard error;
- other observed biological effects or measurements: report any other biological effects which were observed or measured (e.g. developmental anomalies of snails) including any appropriate justification;
- an explanation for any deviation from the Test SOP.

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ANNEX 1

DEFINITIONS

For the purposes of this guideline the following definitions are used:

EC_x is the concentration of the test chemical dissolved in water that results in a x per cent reduction in reproduction of *Potamopyrgus* within a stated exposure period.

Lowest Observed Effect Concentration (LOEC) is the lowest tested concentration at which the substance is observed to have a statistically significant effect on reproduction and mortality (at $p < 0.05$) when compared with the control, within a stated exposure period. However, all test concentrations above the LOEC should have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation should be given for how the LOEC (and hence the NOEC) has been selected.

No Observed Effect Concentration (NOEC) is the test concentration immediately below the LOEC, which when compared with the control, has no statistically significant effect ($p < 0.05$), within a stated exposure period.

Limit of detection is the lowest concentration that can be detected but not quantified.

Limit of determination is the lowest concentration that can be measured quantitatively.

Mortality. An animal is recorded as dead when it is immobile, i.e. when it is does not show any reaction after gently touching the foot or the operculum (in case of snails retracted into the shell) with a pair of tweezers.

ANNEX 2

RECOMMENDATIONS FOR CULTURE OF *POTAMOPYRGUS ANTIPODARUM*

INTRODUCTION

1. This Standard Operating Procedure (SOP) describes the laboratory rearing of *Potamopyrgus antipodarum*. The objective is to achieve reproducible results with each experiment conducted under the conditions described. Therefore, it is necessary that snails are kept under standardised conditions. Mortality should be on a low level and the mean number of embryos per snail should be between 5 and 20. To this end a good food supply is to be guaranteed and a distinct population density must not be exceeded.

TEST ORGANISM

Taxonomy

2. *Potamopyrgus antipodarum* (Gray, 1853), the freshwater mudsnail, belongs to the phylum Mollusca, class Gastropoda, order Neotaenioglossa and family Hydrobiidae.

Ecology

3. *P. antipodarum* originates from New Zealand, but has been introduced to other parts of the world. Typical habitats are running waters from small creeks to streams, lakes and estuaries, where its reproduction is often very intensive (1 - 4). The shell height of adult snail averages about 4.0 – 4.5 mm. *P. antipodarum* is predominantly living in freshwater, but it is also able to survive and reproduce in brackish water with a salinity up to 15‰ (5). Mudsnails prefer living in or on soft sediments of standing or slowly flowing water bodies as well as in estuarine areas on the coasts. The species feeds on detritus, algae and bacteria, which are rasped from the surface of plants, stones or the sediment.

Biology

4. In their ancestral distribution area, the populations have an almost balanced ratio of males to females with a sympatric coexistence of biparental and parthenogenetic populations. In other parts of the world populations consist almost entirely of female snails reproducing parthenogenetically. In this way a single snail is capable of establishing an entire population. In Europe, male snails are found only very rarely (6, 7) and did never occur in a long-term laboratory culture. Snails used for the validation exercises belong to haplotype *t* and morphotype "Warwick A" according to Städler et al. (2005, ref. 8).

5. Reproduction occurs all over the year. *P. antipodarum* performs a very distinct kind of brood care. The eggs develop in the anterior part of the pallial oviduct section, which is transformed into a brood pouch. Older embryos are situated in the anterior and younger embryos in the posterior part of the brood pouch. The embryos are released through the female aperture when the egg shell tears open. This kind of reproduction is called ovovivipary (2).

EQUIPMENT, TEMPERATURE AND LIGHT REGIME

Temperature and light regime

6. The culturing of *P. antipodarum* has to be carried out at a water temperature of 16 ± 1°C and a light-dark period of 16:8 hours. The light intensity should be 500 ± 100 lx.

Aquaria and accessories

7. The following equipment is needed:

- Culturing aquaria (e.g. 15 litre; made of glass)
- Storage tank of appropriate volume for reconstituted water (e.g. 50-litre glass aquaria)
- Air pumps
- Flexible air tubes (Teflon-coated)
- Glass pipettes
- Measuring electrodes for conductivity, oxygen and pH
- Test kits for ammonium, nitrite and nitrate measurements in water
- Stereomicroscope
- Cold light source
- Dissecting dish & dissecting instruments

Chemicals, food and products for water conditioning

8. The following compounds and products are needed:

- Sodium hydrogen carbonate ($NaHCO_3$)
- Calcium source (e.g. cuttlebone or calcium carbonate)
- Magnesium chloride hexahydrate ($MgCl_2$ x 6 H_2O) for narcotisation
- Tropic Marin® sea salt (Dr. Biener GmbH, Wartenberg, Germany)
- TetraPhyll® (Tetra GmbH, Melle, Germany)

OPERATING PROCEDURE

Culture medium: Reconstituted water

9. For the culturing of snails reconstituted water is used. To produce reconstituted water 3 g Tropic Marin® sea salt and 1.8 g sodium hydrogen carbonate ($NaHCO_3$) are dissolved per 10 litre deionised water. The reconstituted water is prepared e.g. in a 50-litre aquarium, where the water is stored for further use for up to 2 weeks. The reconstituted water has to be aerated for at least 24 hours before use.

10. The following water parameters should be achieved and kept:

Temperature:	16 ± 1°C
pH:	8.0 ± 0.5
Oxygen saturation:	> 60% ASV (air saturation value)
Conductivity:	770 ± 100 µS/cm

Before the water is used in the culture aquaria, the compliance of these parameters has to be checked.

Population density

11. The population density must not be higher than 100 snails per 1 litre.

Food and feeding

12. The snails are fed with finely ground TetraPhyll® flakes *ad libitum*, preferably daily but at least 3 times a week. The flakes are ground either with a porcelain mortar with pistil or with a coffee mill with a high-grade steel masticator.

Cleaning and care

13. Once a week temperature, pH-value, oxygen saturation, conductivity, ammonium and nitrite concentration of all aquaria in the breeding program have to be measured. Additional measurements of nitrate should be done if necessary.

14. Once per week a partial renewal of the culture water is required. Weekly replacement of at least 50% of the water has been found appropriate. Water renewal is accompanied by removal of feed remains and detritus from the culture vessel. Care must be taken to ensure that juvenile snails are not removed from the aquaria.

15. Before replacing water for the culture aquaria, temperature, pH-value, oxygen saturation, and conductivity of the water in the storage aquarium have to be measured. After a partial change of water a calcium source (e.g. a piece of cuttlebone) should be added to each aquari-

um. When measuring the parameters in the aquaria of the breeding program, all electrodes have to be thoroughly cleaned before used in the next aquarium to prevent a potential transfer of diseases or pathogens.

Monthly registration of embryo numbers

16. Each month the reproduction of 20 adult snails (> 3.5 mm) has to be registered, together with measurements of shell height. Procedure:

- Snails are shock-frozen (in liquid nitrogen or in a -80°C freezer) and stored at -20°C until analysis. Alternatively, the snails are narcotised for at least 45 minutes up to a maximum of 90 minutes in a solution of 2.5% magnesium chloride hexahydrate ($MgCl_2$ x 6 H_2O) in deionised or distilled water.
- Shell height of the snails has to be measured under a stereomicroscope with an ocular micrometer. Data are recorded in a spread sheet.
- The shell of the snails is broken open carefully with a pair of pincers. Subsequently the snails are placed into a dissecting dish containing a small volume of culture water.
- The soft body is exposed by removing the shell with dissecting needles or pointed tweezers.
- The brood pouch of the snails is opened with a dissecting needle and all embryos are removed out of this pouch.
- Then all embryos have to be counted. Data are recorded in a spread sheet. The mean and variability parameters such as the standard deviation or the standard error of the mean for the shell height and the number of embryos are calculated.

ALGAE GROWTH, DISEASES AND MORTALITY

17. If heavy algae growth occurs on the shell of the snails, algae have to be removed manually if possible. If it is not possible to reduce the growth of the algae in this way, affected snails have to be removed from the brood and the aquaria have to be cleaned thoroughly.

18. If dead snails are found in an aquarium they have to be removed. If there is an aquarium with a high mortality (> 20%), the mudsnails in this aquarium have to be observed for several days. If the mortality continues to be high, all snails have to be removed from the breeding program and the aquarium with all equipment has to be cleaned and disinfected thoroughly.

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ANNEX 3

Figure 1: *Potamopyrgus antipodarum* with shell (A) or with partly removed shell and uncovered brood pouch (white) (B) and the embryos extracted from its brood pouch (shelled ones on the right and unshelled ones on the left) (C).

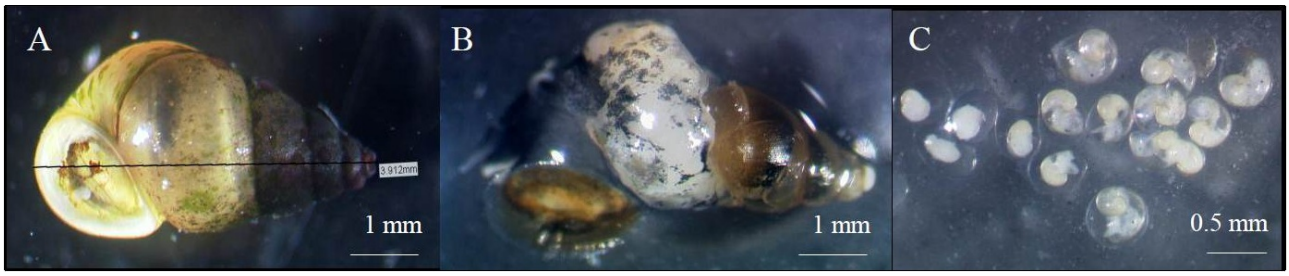
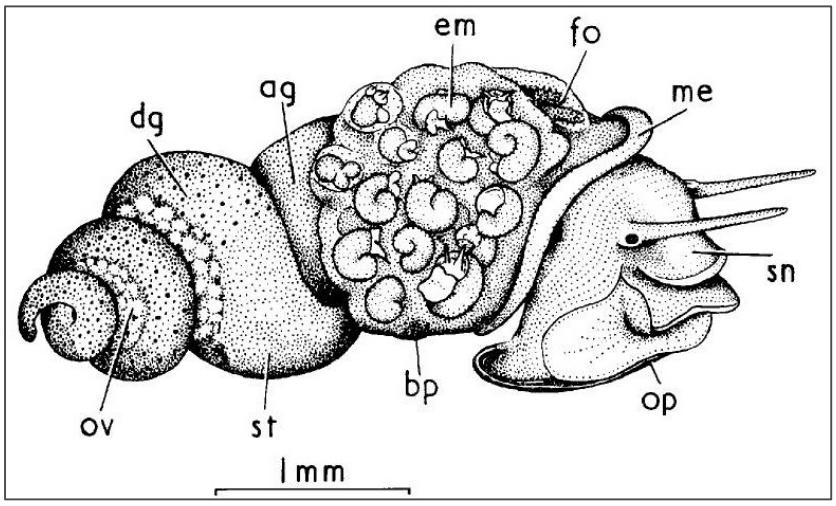


Figure 2: *Potamopyrgus antipodarum*, soft body after removal from the shell (modified after 2); ag = albumen gland, bp = brood pouch, dg = digestive gland, em = embryo, fo = female opening (vagina), me = mantle edge, op = operculum, ov = ovary, sn = snout, st = stomach.



ANNEX 4

Calculation of a time-weighted mean

Time-weighted mean

Given that the concentration of the test chemical can decline over the period between medium renewals, it is necessary to consider what concentration should be chosen as representative of the range of concentrations. The selection should be based on biological considerations as well as statistical ones. For example, if reproduction is thought to be affected mostly by the peak concentration experienced, then the maximum concentration should be used. However, if the accumulated or longer term effect of the toxic substance is considered to be more important, then an average concentration is more relevant. In this case, an appropriate average to use is the time-weighted mean concentration, since this takes account of the variation in instantaneous concentration over time.

Figure 1: Example of time-weighted mean.

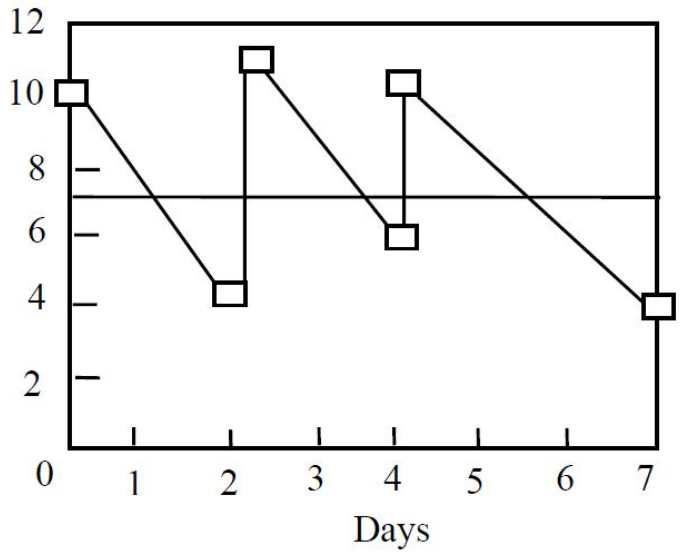


Figure 1 shows an example of a (simplified) test lasting seven days with medium renewal at Days 0, 2 and 4.

- The zig-zag line represents the concentration at any point in time. The fall in concentration is assumed to follow an exponential decay process.
- The 6 plotted points represent the observed concentrations measured at the start and end of each renewal period.
- The horizontal solid line indicates the position of the time-weighted mean.

The time-weighted mean is calculated so that the area under the time-weighted mean is equal to the area under the concentration curve. The calculation for the above example is illustrated in Table 1.

Table 1: Example for the calculation of the time-weighted mean.

Renewal No.	Days	Conc 0	Conc 1	Ln(Conc 0)	Ln(Conc 1)	Area
1	2	10.000	4.493	2.303	1.503	13.767
2	2	11.000	6.037	2.398	1.798	16.544
3	3	10.000	4.066	2.303	1.403	19.781
Total Days:	7				Total Area: TW Mean:	50.092 7.156

Days is the number of days in the renewal period

Conc 0 is the measured concentration at the start of each renewal period

Conc 1 is the measured concentration at the end of each renewal period

Ln(Conc 0) is the natural logarithm of Conc 0

Ln(Conc 1) is the natural logarithm of Conc 1

Area is the area under the exponential curve for each renewal period. It is calculated by:

$$\text{Area} = \frac{Conc\ 0 - Conc\ 1}{Ln(Conc\ 0) - Ln(Conc\ 1)} \text{ x Days}$$

The time-weighted mean *(TW Mean)* is the *Total Area* divided by the *Total Days*. Of course, for the reproduction test with *Potamopyrgus antipodarum* the table would have to be extended to cover 28 days.

It is clear that when observations are taken only at the start and end of each renewal period, it is not possible to confirm that the decay process is, in fact, exponential. A different curve would result in a different calculation for *Area*. However, an exponential decay process is not implausible and is probably the best curve to use in the absence of other information.

However, a word of caution is required if the chemical analysis fails to find any substance at the end of the renewal period. Unless it is possible to estimate how quickly the substance disappeared from the solution, it is impossible to obtain a realistic area under the curve, and hence it is impossible to obtain a reasonable time-weighted mean.

8.3 OECD draft guideline *Potamopyrgus antipodarum* reproduction test

Adopted:
xx.xx.xxxx

DRAFT

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Potamopyrgus antipodarum Reproduction Test (version as of June 2015)

INTRODUCTION

1. This guideline is designed to assess potential effects of prolonged exposure to chemicals on reproduction and survival of the freshwater mudsnail *Potamopyrgus antipodarum*.

2. The measured parameters in this test are mortality and the assessment of the reproduction as the total number of embryos in the brood pouch per female after 28 days exposure without distinction of developmental stages.

PRINCIPLE OF THE TEST

3. The primary objective of the test is to assess the effect of chemicals on reproduction of *Potamopyrgus antipodarum* assessed by embryo numbers in the brood pouch without the distinction of developmental stages. To this end adult female *P. antipodarum* are exposed to a concentration range of the test substance. The test substance is spiked into the water and adult snails are subsequently introduced into the test beakers. Survival of the snails and reproduction are examined at the end of the test after 28 days of exposure to the test substance. After removal of the shell, embryos can easily be seen through the epithelia (see ANNEX 3, Figs. 1B, 2). By opening the brood pouch and subsequently removing the embryos and counting them, the reproductive success of each female is determined (see ANNEX 3, Fig. 1C).

4. The toxic effect of the test substance on embryo numbers is expressed as EC_x by fitting the data to an appropriate model by non-linear regression to estimate the concentration that would cause x% reduction in embryo numbers or alternatively as the No Observed Effect Concentration and Lowest Observed Effect Concentration (NOEC/LOEC) value (1). The test concentrations should preferably bracket the lowest of the used effect concentrations (e.g. EC_{10}) which means that this value is calculated by interpolation and not extrapolation.

INFORMATION ON THE TEST SUBSTANCE

5. Information on the test substance which may be useful in establishing the test conditions includes the structural formula, purity of the substance, stability in light, stability under the conditions of the test, pK_a, P_{ow} and results of a test for ready biodegradability (see OECD Test Guidelines 301 and 310).

6. The water solubility and the vapour pressure of the test substance should be known and a reliable analytical method for the quantification of the substance in the test solutions with reported recovery efficiency and limit of quantification (LOQ) should be available.

REFERENCE SUBSTANCES

7. Reference substances may be tested periodically as a means of assuring that the test conditions are reliable. Toxicants successfully used in international validation studies are cadmium chloride, tributyltin chloride and prochloraz with EC_{50} ranges of 5 to 20 µg Cd/L, 35 to 190 ng TBT-Sn/L and 100 to 800 µg prochloraz/L (2).

VALIDITY OF THE TEST

8. For a test to be valid the following conditions should be fulfilled:
- mortality in the controls should not exceed 20% at the end of the test;
- the mean number of embryos in controls should be at least 5 embryos per female at the end of the test,
- the dissolved oxygen content must have been at least 60% of the air saturation value throughout the test and
- water temperature should be 16 ± 1°C throughout the test in both control and exposure groups.

Recommendations for further parameters are provided below (cf. section 13).

DESCRIPTION OF THE METHOD

Apparatus

9. Test vessels and other apparatus which will come into contact with the test solutions should be made entirely of glass or other materials chemically inert to the test chemical. Additionally, the following equipment will be required:
- 500 mL glass beakers
- glass pipettes
- oxygen meter
- pH meter

- conductivity meter
- stereomicroscope
- climate chambers or temperature regulated room or other adequate apparatus for temperature and lighting control
- dissecting dish and dissecting instruments

Test organism

10. The species to be used in the test is *Potamopyrgus antipodarum* (Gray, 1853).

11. Test animals should be laboratory-reared and taken from a parasite-free stock of female snails (i.e. showing no signs of stress such as high mortality, poor fecundity, etc.). The stock snails must be maintained at culture conditions (light, temperature, medium and feeding) similar to those to be used in the test (culturing methods for *P. antipodarum* are described in ANNEX 2). Snails used for the validation exercises belong to haplotype *t* and morphotype "Warwick A" according to Städler et al. (2005, ref. 3) and should be used for testing. Field collected organisms should not be used.

Test medium

12. Reconstituted (synthetic) water should be used as test medium. The reconstituted water should be prepared with 3 g Tropic Marin® sea salt and 1.8 g sodium hydrogen carbonate ($NaHCO_3$) dissolved per 10 litre deionised water. The reconstituted water should be prepared in a container of sufficient volume, e.g. a 50-litre aquarium, where the water is stored for further use, preferably at ambient temperature in the dark. The reconstituted water has to be aerated for at least 24 hours before use. It should be used within a maximum storage period of 2 weeks.

13. The following water parameters should be achieved and kept:

pH:	8.0 ± 0.5
Oxygen saturation:	> 60% ASV (air saturation value)
Conductivity:	770 ± 100 µS/cm
Light intensity:	500 ± 100 lx

14. Test vessels should contain 400 mL of reconstituted water. Glass beakers should be replaced weekly. Larger volumes may sometimes be necessary to meet requirements of the analytical procedure used for determination of the test substance concentration, although pooling of replicates for chemical analysis is also allowable. The test vessels should be covered (with a lid or gauze).

Test solution

15. Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should be prepared by dissolving the test substance in the test water by mixing or agitating the test substance in test medium using mechanical means such as agitating, stirring or ultrasonication, or other appropriate methods. If possible, the use of solvents or dispersants should be avoided.

16. If a solvent is used for the application of the test substance, a solvent control using the same amount of solvent as in the treatments should be used. The amount of solvent in test concentrations should be as small as possible. If no other information is available, the appropriate amount of the selected solvent has to be determined in a preliminary test and depends on the type of test substance and the sensitivity of the test organisms towards the selected solvent. Dimethyl sulfoxide (DMSO) or triethylene glycol (TEG) should be used at a maximum concentration of 20 µL/L in the test vessels (= 0.002%) as solvent whenever possible. DMSO and TEG are characterised by a low toxicity for *P. antipodarum* and do not cause biofilm development in the test vessels (2). In contrast, the use of ethanol as a solvent results often in considerable development of fungi and bacteria even at concentrations as low as 0.003%.

PROCEDURE

Conditions of exposure

Duration

17. The test duration is 28 days.

Loading

18. Snails used for the tests have to be checked for their ability to reproduce. Therefore, the shell height, and the embryo numbers of 20 snails from the culture batch selected for the test should be measured. The shell height should be between 3.5 and 4.5 mm. The mean number of embryos per snail should be between 5 and 20.

19. Six adult snails have to be allocated randomly to each test vessel containing the exposure water using tweezers.

Feeding

20. Feeding should preferably be done daily, but at least 3 times per week with finely ground TetraPhyll® (60 to 80 µg per animal and day). Deviations from this have to be reported and justified. The food should be applied by preparing a suspension with deionised or distilled

water and pipetting in each test vessel. Food suspension should be homogenized by shaking each time before pipetting into a given test vessel. The applied volume should be as little as possible to avoid a dilution of the test concentrations. The suspension should be prepared immediately before use.

Light regime

21. The photoperiod in the culture and the test is L:D = 16:8 hours throughout the test. Light intensity should be 500 ± 100 lx, when testing photo-labile substances lower light intensities have to be used. The light source should be positioned above the test vessels. Test vessels should be positioned on a dark surface to avoid the snails from escaping. Deviations from this have to be reported and justified.

Temperature

22. The temperature of the test media should be 16 ± 1°C throughout the test.

Aeration

23. Water should be aerated through glass pipettes (Pasteur pipettes) connected to an air tubing system. Adjustable valves should be used to ensure continuous and constant air flow.

24. Dissolved oxygen content should be kept above 60% ASV, however the test vessels should be aerated gently to avoid stripping of test chemicals.

Test design

25. At least five concentrations, bracketing effective concentration (e.g. EC_x), with six replicates each should be tested in a geometric series with a factor between concentrations not exceeding 2.2. Prior knowledge on the toxicity of the test substance (e.g. from range finding studies or other sources like read across, etc.) should help in selecting appropriate test concentrations. Justification should be provided if fewer than 5 concentrations are used. Substances should not be tested above their solubility limit in test medium. Before conducting the experiment it is advisable to consider the statistical power of the tests design and using appropriate statistical methods (1). In setting the range of concentrations, the following should be borne in mind:

(iv) When EC_x for effects on embryo numbers is estimated, it is advisable that sufficient concentrations are used to define the EC_x with an appropriate level of confidence. Test concentrations used should preferably bracket the estimated EC_x such that EC_x is found by interpolation rather than extrapolation. It is an advantage for the following statistical analysis to have more test concentrations

(e.g. 10) and fewer replicates of each concentration (e.g. 3 thus holding the total number of vessels constant) and with 6 control replicates.

(v) When estimating the LOEC and/or NOEC, the lowest test concentration should be low enough so that the embryo number at that concentration is not significantly different from the control. If this is not the case, the test should be repeated at lower concentrations.

(vi) When estimating the LOEC and/or NOEC, the highest test concentration should be high enough so that the embryo number at that concentration is significantly different from the control. If this is not the case, the test should be repeated with an increased highest concentration unless the maximum required test concentration for chronic effects testing (i.e., 10 mg/L) was used as the highest test concentration in the initial test.

26. If no effects are observed at the highest concentration in the range-finding test (e.g. at 100 mg/L or a concentration equal to the limit of solubility), or when the test substance is highly likely to be of low/no toxicity based on lack of toxicity to other organisms and/or low/no uptake, the reproduction test may be performed as a limit test (with fewer than five concentrations as the definitive test), using a test concentration of e.g.10 mg/L and the control. 10 replicates should be used for both the treatment and the control groups. A limit test will provide the opportunity to demonstrate that there is no statistically significant effect at the limit concentration, but if effects are recorded a full test will be required. Justifications should be provided if fewer than five concentrations are used.

Controls

27. Control vessels without added test substance should be included in the test with an appropriate number of replicates, six replicates for the dilution-water control and, if needed, six replicates for the solvent control containing the solvent carrier only.

Test medium renewal

28. The frequency of medium renewal will depend on the stability of the test compound, but should be at least three times per week. If, from preliminary stability tests or from the physico-chemical properties of the test substance, the concentration is evaluated not to be stable (i.e. outside the range 80 - 120% of nominal or falling below 80% of the measured initial concentration) over the maximum renewal period (i.e. three days), considerations have to be given to more frequent medium renewals. Alternatively, the test should be conducted under flow-through conditions when testing “difficult substances” (i.e. volatile, unstable, readily biodegradable and adsorbing chemicals).

29. The following procedure is used for water renewal. Exposure water is completely removed from the test vessels. A sieve can be used to collect the snails which might detach from the glass walls. Test vessels are refilled using water at the test temperature. The test substance is immediately added to the renewed water using stock solutions. Newly contaminated water is homogenized by manual agitation. Snails are then placed back in the test vessel. Food is provided in the given range (see section 20) to animals once the water renewal and contamination have been completed.

Observations

30. The test vessels should be observed at least three times per week to achieve visual assessment of any abnormal behaviour (e.g. avoidance of water, avoidance of food or lethargy). Any signs of stress should be recorded. If there are snails found in the lid or outside of test water, they have to be transferred back to the medium, immediately.

Mortality

31. Dead snails should be removed from the test vessels and recorded preferably daily, or at least as frequently as test medium is renewed.

Reproduction

32. The number of embryos in the brood pouch of all surviving snails per replicate is analysed after 28 days exposure to the test substance. Therefore, the snails should preferably be quick-frozen (in liquid nitrogen or in a -80°C freezer) and stored at -20°C until analysis. Alternatively, the snails can be narcotised for 45 to 90 minutes in a solution of 2.5% magnesium chloride hexahydrate ($MgCl_2$ x 6 H_2O) in deionised or distilled water before dissection.

33. Shell height of snails has to be measured under a stereomicroscope with an ocular micrometer. Data are recorded in a data sheet example in ANNEX 5.

34. The shell of the snails should be cracked carefully with a pair of pincers. Subsequently the snails are placed into a dissecting dish containing a small volume of test medium or tap water. The soft body can be prepared by removing the shell with dissecting needles or pointed tweezers. The brood pouch of the snails should be opened carefully with a dissecting needle, and the embryos have to be counted.

35. If snails did not produce embryos, animals have to be sexed. Males are characterised by a penis in the neck (bottom of the mantle cavity behind the snout and the two ocular tentacles). The influence of the presence of males on the results of the reproduction test is not known because males did never occur in laboratory cultures of haplotype *t* (4).

36. Data are recorded in an appropriate data sheet (example in ANNEX 5). The mean and variability parameters such as standard deviation or standard error of the mean, for the shell height and the number of embryos are calculated.

Frequency of analytical determinations and measurements

Concentration of the test substance

37. During the test, the concentrations of the test substance are determined at regular intervals. Prior to initiation of the exposure period, proper function of the chemical delivery system across all replicates should be ensured. Analytical methods required should be established, including an appropriate limit of quantification (LOQ) and sufficient knowledge on the substance stability in the test system.

38. In semi-static tests where the concentration of the test substance is expected to remain within ± 20% of the nominal (i.e. within the range 80 - 120% - see section 28), it is recommended that, as a minimum, the highest and lowest test concentrations are analysed when freshly prepared and the old solution at the time of renewal on one occasion during the first week of the test (i.e. analyses should be made on a sample from the same solution - when freshly prepared and at renewal). These determinations should be repeated at least at weekly intervals thereafter.

39. For tests where the concentration of the test substance is not expected to remain within ± 20% of the nominal, it is necessary to analyse all test concentrations, when freshly prepared and at renewal. However, for those tests where the measured initial concentration of the test substance is not within ± 20% of nominal but where sufficient evidence can be provided to show that the initial concentrations are repeatable and stable (i.e. within the range 80 - 120% of initial concentrations), chemical determinations could be reduced in weeks 2, 3 and 4 of the test to the highest and lowest test concentrations. In all cases, determination of test substance concentrations need only be performed on one replicate vessel at each test concentration changing systematically between replicates.

40. If a flow-through test is used, a similar sampling regime to that described for semi-static tests is appropriate (but measurement of 'old' solutions is not applicable in this case). However, increasing the number of sampling occasions during the first week (e.g. at least two sets of measurements) may help to demonstrate that the test concentrations remain stable. In these types of test, the flow-rate of diluent and test substance should be checked daily.

41. If there is evidence that the concentration of the test substance has been satisfactorily maintained within ± 20% of the nominal or measured initial concentration throughout the test,

then results can be based on nominal or measured initial values, respectively. If the deviation from the nominal or measured initial concentration is greater than ± 20%, results should be expressed in terms of the time-weighted mean (see guidance for calculation in ANNEX 4).

Physico-chemical parameters

42. Water quality parameters such as pH, oxygen saturation, conductivity, temperature, ammonium and total nitrite content should be measured before water renewal in one replicate per exposure group and controls. Additional measurements of nitrate and water hardness should be done if necessary.

DATA AND REPORTING

Treatment of results

43. The main parameters to be evaluated statistically are the mortality and the number of embryos per female. For statistical evaluation of the embryo numbers, the mean embryo number across replicates for each concentration has to be calculated. The standard effect in the reproduction test is a decrease of the mean embryo number with increasing concentration of the test compound. The reproduction test has been validated exclusively for reproductive toxic chemicals. Reproduction increases may occur in the test and have been observed with substances such as ethanol (2) and those with known or suspected estrogenic effects in vertebrates (5 - 8). However, the reproduction test is not suited to proof an endocrine mediated mode of action solely on the basis of a decreased or increased embryo number.

44. Before employing the statistical analysis, e.g. ANOVA procedures, comparison of treatments to the control by Student t-test, Dunnett's test, Williams' test, or stepdown Jonckheere-Terpstra test, it is recommended to consider transformation of data if needed for meeting the requirements of the particular statistical test (1). As non-parametric alternatives one can consider Dunn's or Mann-Whitney's tests. 95% confidence intervals are calculated for mean values per replicate.

45. The number of surviving adult snails in the untreated controls is a validity criterion, and has to be documented and reported. Also all other detrimental effects, e.g. abnormal behaviour as specified under 30, abnormal appearance of embryos and toxicological significant findings should be reported in the final report as well.

EC_x

46. EC_x-values, including their associated lower and upper 95% confidence limits, are calculated using appropriate statistical methods (e.g. logistic or Weibull function, trimmed Spearman-Karber method, or simple interpolation). To compute the EC_{10}, EC_{50} or any other EC_x,

the complete data set should be subjected to regression analysis.

NOEC/LOEC

47. If a statistical analysis is intended to determine the NOEC/LOEC appropriate statistical methods should be used according to OECD Document 54 on the Current Approaches in the Statistical Analysis of Ecotoxicity Data: a Guidance to Application (1). In general, adverse effects of the test substance compared to the control are investigated using one-tailed hypothesis testing at $p < 0.05$.

48. Normal distribution and variance homogeneity can be tested using an appropriate statistical test, e.g. the Shapiro-Wilk test and Levene test, respectively ($p < 0.05$). One-way ANOVA and subsequent multi-comparison tests can be performed. Multiple comparisons (e.g. Dunnett's test) or step-down trend tests (e.g. Williams' test, or stepdown Jonckheere-Terpstra test) can be used to calculate whether there are significant differences ($p < 0.05$) between the controls and the various test substance concentrations (selection of the recommended test according to OECD Guidance Document 54 (1)). Otherwise, non-parametric methods (e.g. Bonferroni-U-test according to Holm or Jonckheere-Terpstra trend test) could be used to determine the NOEC and the LOEC.

Test report

49. The test report includes the following information:

Test substance:

- relevant physicochemical properties;
- chemical identification data (name, structural formula, CAS number, etc.) including purity;
- analytical method for quantification of the test substance where appropriate.

Test species:

- scientific name, source and culture conditions.

Test conditions:

- test procedure used (e.g. semi-static or flow-through, volume, loading in number of snails per litre);
- photoperiod and light intensity;
- test design (e.g. test concentration used, number of replicates, number of snails per replicate, etc.);
- method of test substance pre-treatment and spiking/application;
- the nominal test concentrations, details about the sampling for chemical analysis and the analytical methods by which concentrations of the test substances were obtained;

- media characteristics (including pH, conductivity, temperature and oxygen ASV, ammonium concentrations and any other measurements made);
- detailed information on feeding (e.g. type of food, source, amount given frequency of feeding).

Results:

- results from any preliminary studies on the stability of the test substance;
- the nominal test concentrations and the results of all analyses to determine the concentration of the test substance in the test vessels; the recovery efficiency of the analytical method, the means of the measured values and the limit of detection should also be reported;
- water quality within the test vessels (i.e. pH, temperature, oxygen ASV and ammonium concentration);
- the full record of embryo numbers by replicate at the end of the test (see example data sheet in ANNEX 5);
- the number of deaths among the snails (see example data sheet in ANNEX 5);
- where appropriate the Lowest Observed Effect Concentration (LOEC) for reproduction (embryo numbers), including a description of the statistical procedures used and an indication of what size of effect could be expected to be detected (a power analysis can be performed before the start of the experiment to provide this) and the No Observed Effect Concentration (NOEC) for reproduction; where appropriate, the LOEC or NOEC for mortality of the animals should also be reported;
- where appropriate, the EC_X for reproduction and confidence intervals (e.g. 95%) and a graph of the fitted model used for its calculation, the slope of the concentration-response curve and its standard error;
- other observed biological effects or measurements: report any other biological effects which were observed or measured (e.g. growth of snails) including any appropriate justification;
- an explanation for any deviation from the Test Guideline.

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ANNEX 1

DEFINITIONS

For the purposes of this guideline the following definitions are used:

EC_x is the concentration of the test substance dissolved in water that results in a x per cent reduction in reproduction of *Potamopyrgus* within a stated exposure period.

Lowest Observed Effect Concentration (LOEC) is the lowest tested concentration at which the substance is observed to have a statistically significant effect on reproduction and mortality (at $p < 0.05$) when compared with the control, within a stated exposure period. However, all test concentrations above the LOEC should have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation should be given for how the LOEC (and hence the NOEC) has been selected.

No Observed Effect Concentration (NOEC) is the test concentration immediately below the LOEC, which when compared with the control, has no statistically significant effect ($p < 0.05$), within a stated exposure period.

Limit of detection is the lowest concentration that can be detected but not quantified.

Limit of determination is the lowest concentration that can be measured quantitatively.

Mortality. An animal is recorded as dead when it is immobile, i.e. when it is does not show any reaction after gently touching the foot or the operculum (in case of snails retracted into the shell) with a pair of tweezers. (If another definition is used, this should be reported together with its reference).

ANNEX 2

RECOMMENDATIONS FOR CULTURE OF *POTAMOPYRGUS ANTIPODARUM*

INTRODUCTION

1. This Standard Operating Procedure (SOP) describes the laboratory rearing of *Potamopyrgus antipodarum*. The objective is to achieve reproducible results with each experiment conducted under the conditions described. Therefore, it is necessary that snails are kept under standardised conditions. Mortality should be on a low level and reproduction should be between 5 and 20 embryos per female. To this end a good food supply is to be guaranteed and a distinct population density must not be exceeded.

TEST ORGANISM

Taxonomy

2. *Potamopyrgus antipodarum* (Gray, 1853), the freshwater mudsnail, belongs to the phylum Mollusca, class Gastropoda, order Neotaenioglossa and family Hydrobiidae.

Ecology

3. *P. antipodarum* originates from New Zealand, but has been introduced to other parts of the world. Typical habitats are running waters from small creeks to streams, lakes and estuaries, where its reproduction is often very intensive (1 - 4). The shell height of adult snails averages about 4.0 – 4.5 mm. *P. antipodarum* is predominantly living in freshwater, but it is also able to survive and reproduce in brackish water with a salinity up to 15‰ (5). Mudsnails prefer living in or on soft sediments of standing or slowly flowing water bodies as well as in estuarine areas on the coasts. The species feeds on detritus, algae and bacteria, which are rasped from the surface of plants, stones or the sediment.

Biology

4. In their ancestral distribution area, the populations have an almost balanced ratio of males to females with a sympatric coexistence of biparental and parthenogenetic populations. In other parts of the world populations consist almost entirely of female snails reproducing parthenogenetically. In this way a single snail is capable of establishing an entire population. In Europe, male snails are found only very rarely (6, 7) and did never occur in a long-term laboratory culture. Snails used for the validation exercises belong to haplotype *t* and morphotype "Warwick A" according to Städler et al. (2005, ref. 8).

5. Reproduction occurs all over the year. *P. antipodarum* performs a very distinct kind of brood care. The eggs develop in the anterior part of the pallial oviduct section, which is transformed into a brood pouch. Older embryos are situated in the anterior and younger embryos in the posterior part of the brood pouch. The embryos are released through the female aperture when the egg shell tears open. This kind of reproduction is called ovovivipary (2).

EQUIPMENT, TEMPERATURE AND LIGHT REGIME

Temperature and light regime

6. The culturing of *P. antipodarum* has to be carried out at a water temperature of 16 ± 1°C and a light-dark period of 16:8 hours. The light intensity should be 500 ± 100 lx.

Aquaria and accessories

7. The following equipment is needed:

- Culturing aquaria (e.g. 15 litre; made of glass)
- Storage tank of appropriate volume for reconstituted water (e.g. 50-litre glass aquaria)
- Air pumps
- Flexible air tubes (Teflon-coated)
- Glass pipettes
- Measuring electrodes for conductivity, oxygen and pH
- Test kits for ammonium, nitrite and nitrate measurements in water
- Stereomicroscope
- Cold light source
- Dissecting dish & dissecting instruments

Chemicals, food and products for water conditioning

8. The following compounds and products are needed:

- Sodium hydrogen carbonate ($NaHCO_3$)
- Calcium source (e.g. cuttlebone or calcium carbonate)
- Magnesium chloride hexahydrate ($MgCl_2$ x 6 H_2O) for narcotisation
- Tropic Marin® sea salt (Dr. Biener GmbH, Wartenberg, Germany)
- TetraPhyll® (Tetra GmbH, Melle, Germany)

OPERATING PROCEDURE

Culture medium: Reconstituted water

9. For the culturing of snails reconstituted water is used. To produce reconstituted water 3 g Tropic Marin® sea salt and 1.8 g sodium hydrogen carbonate ($NaHCO_3$) are dissolved per 10 litre deionised water. The reconstituted water is prepared e.g. in a 50-litre aquarium, where the water is stored for further use for up to 2 weeks. The reconstituted water has to be aerated for at least 24 hours before use.

10. The following water parameters should be achieved and kept:

Temperature:	16 ± 1°C
pH:	8.0 ± 0.5
Oxygen saturation:	> 60% ASV (air saturation value)
Conductivity:	770 ± 100 µS/cm

Before the water is used in the culture aquaria, the compliance of these parameters has to be checked.

Population density

11. The population density must not be higher than 100 snails per 1 litre.

Food and feeding

12. The snails are fed with finely ground TetraPhyll® flakes *ad libitum*, preferably daily but at least 3 times a week. The flakes are ground either with a porcelain mortar with pistil or with a coffee mill with a high-grade steel masticator.

Cleaning and care

13. Once a week temperature, pH-value, oxygen saturation, conductivity, ammonium and nitrite concentration of all aquaria in the breeding program have to be measured. Additional measurements of nitrate should be done if necessary.

14. Once per week a partial renewal of the culture water is required. Weekly replacement of at least 50% of the water has been found appropriate. Water renewal is accompanied by removal of feed remains and detritus from the culture vessel. Care must be taken to ensure that juvenile snails are not removed from the aquaria.

15. Before replacing water for the culture aquaria, temperature, pH-value, oxygen saturation, and conductivity of the water in the storage aquarium have to be measured. After a partial change of water a calcium source (e.g. a piece of cuttlebone) should be added to each aquari-

um. When measuring the parameters in the aquaria of the breeding program, all electrodes have to be thoroughly cleaned before used in the next aquarium to prevent a potential transfer of diseases or pathogens.

Monthly registration of embryo numbers

16. Each month the reproduction of 20 adult snails (> 3.5 mm) has to be registered, together with measurements of shell height. Procedure:

- Snails are shock-frozen (in liquid nitrogen or in a -80°C freezer) and stored at -20°C until analysis. Alternatively, the snails are narcotised for at least 45 minutes up to a maximum of 90 minutes in a solution of 2.5% magnesium chloride hexahydrate ($MgCl_2$ x 6 H_2O) in deionised or distilled water.
- Shell height of the snails has to be measured under a stereomicroscope with an ocular micrometer. Data are recorded in a spread sheet.
- The shell of the snails is broken open carefully with a pair of pincers. Subsequently the snails are placed into a dissecting dish containing a small volume of culture water.
- The soft body is exposed by removing the shell with dissecting needles or pointed tweezers.
- The brood pouch of the snails is opened with a dissecting needle and all embryos are removed out of this pouch.
- Then all embryos have to be counted. Data are recorded in a spread sheet. The mean and variability parameters such as the standard deviation or the standard error of the mean for the shell height and the number of embryos are calculated.

ALGAE GROWTH, DISEASES AND MORTALITY

17. If heavy algae growth occurs on the shell of the snails, algae have to be removed manually if possible. If it is not possible to reduce the growth of the algae in this way, affected snails have to be removed from the brood and the aquaria have to be cleaned thoroughly.

18. If dead snails are found in an aquarium they have to be removed. If there is an aquarium with a high mortality (> 20%), the mudsnails in this aquarium have to be observed for several days. If the mortality continues to be high, all snails have to be removed from the breeding program and the aquarium with all equipment has to be cleaned and disinfected thoroughly.

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ANNEX 3

Figure 1: *Potamopyrgus antipodarum* with shell (A) or with partly removed shell and uncovered brood pouch (white) (B) and the embryos extracted from its brood pouch (shelled ones on the right and unshelled ones on the left) (C).

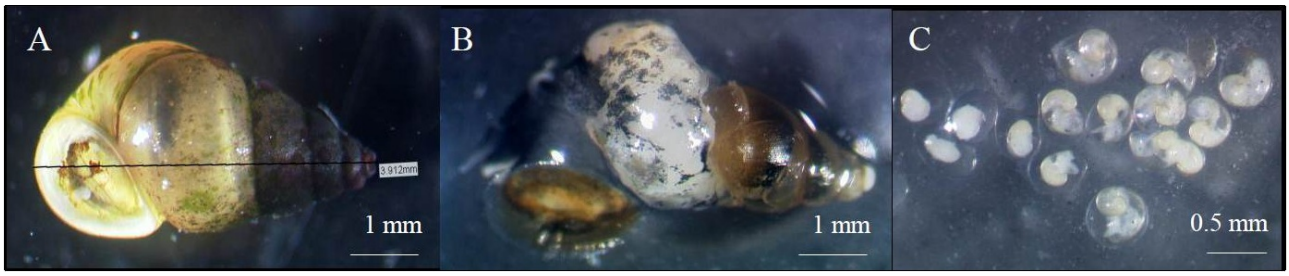
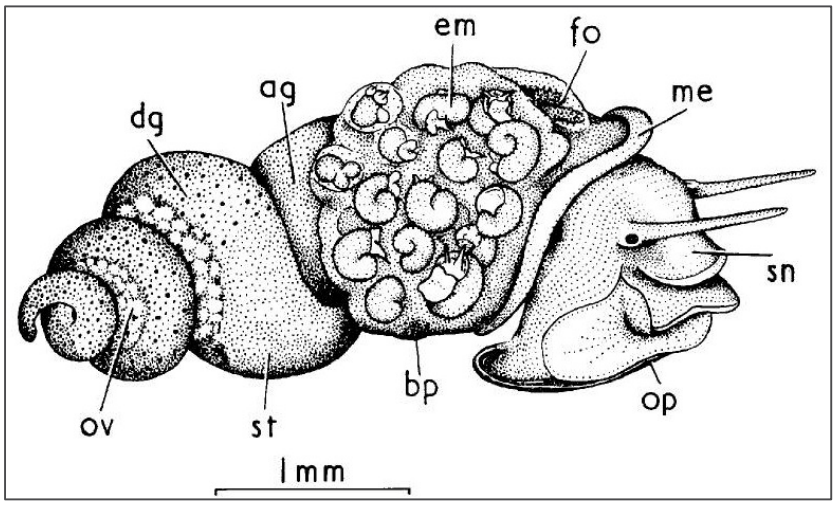


Figure 2: *Potamopyrgus antipodarum*, soft body after removal from the shell (modified after 2); ag = albumen gland, bp = brood pouch, dg = digestive gland, em = embryo, fo = female opening (vagina), me = mantle edge, op = operculum, ov = ovary, sn = snout, st = stomach.



ANNEX 4

Calculation of a time-weighted mean

Time-weighted mean

Given that the concentration of the test substance can decline over the period between medium renewals, it is necessary to consider what concentration should be chosen as representative of the range of concentrations. The selection should be based on biological considerations as well as statistical ones. For example, if reproduction is thought to be affected mostly by the peak concentration experienced, then the maximum concentration should be used. However, if the accumulated or longer term effect of the toxic substance is considered to be more important, then an average concentration is more relevant. In this case, an appropriate average to use is the time-weighted mean concentration, since this takes account of the variation in instantaneous concentration over time.

Figure 1: Example of time-weighted mean.

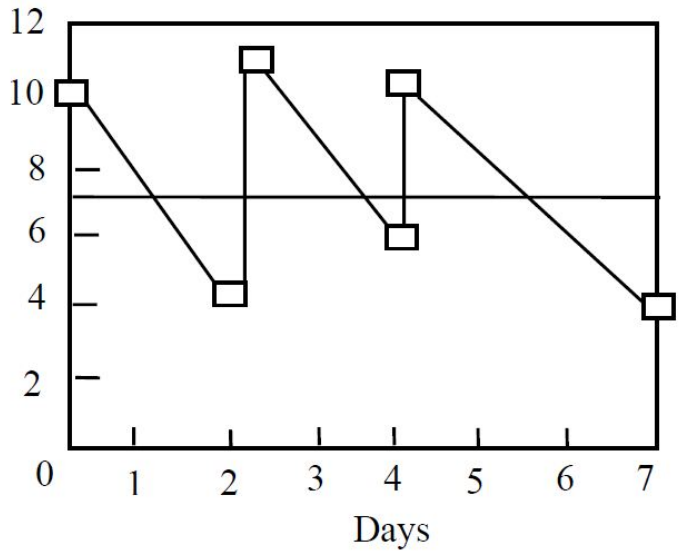


Figure 1 shows an example of a (simplified) test lasting seven days with medium renewal at Days 0, 2 and 4.

- The zig-zag line represents the concentration at any point in time. The fall in concentration is assumed to follow an exponential decay process.
- The 6 plotted points represent the observed concentrations measured at the start and end of each renewal period.
- The horizontal solid line indicates the position of the time-weighted mean.

The time-weighted mean is calculated so that the area under the time-weighted mean is equal to the area under the concentration curve. The calculation for the above example is illustrated in Table 1.

Table 1: Example for the calculation of the time-weighted mean.

Renewal No.	Days	Conc 0	Conc 1	Ln(Conc 0)	Ln(Conc 1)	Area
1	2	10.000	4.493	2.303	1.503	13.767
2	2	11.000	6.037	2.398	1.798	16.544
3	3	10.000	4.066	2.303	1.403	19.781
Total Days:	7				Total Area: TW Mean:	50.092 7.156

Days is the number of days in the renewal period

Conc 0 is the measured concentration at the start of each renewal period

Conc 1 is the measured concentration at the end of each renewal period

Ln(Conc 0) is the natural logarithm of Conc 0

Ln(Conc 1) is the natural logarithm of Conc 1

Area is the area under the exponential curve for each renewal period. It is calculated by:

$$\text{Area} = \frac{Conc\ 0 - Conc\ 1}{Ln(Conc\ 0) - Ln(Conc\ 1)} \text{ x Days}$$

The time-weighted mean *(TW Mean)* is the *Total Area* divided by the *Total Days*. Of course, for the reproduction test with *Potamopyrgus antipodarum* the table would have to be extended to cover 28 days.

It is clear that when observations are taken only at the start and end of each renewal period, it is not possible to confirm that the decay process is, in fact, exponential. A different curve would result in a different calculation for *Area*. However, an exponential decay process is not implausible and is probably the best curve to use in the absence of other information.

However, a word of caution is required if the chemical analysis fails to find any substance at the end of the renewal period. Unless it is possible to estimate how quickly the substance disappeared from the solution, it is impossible to obtain a realistic area under the curve, and hence it is impossible to obtain a reasonable time-weighted mean.

ANNEX 5

EXAMPLE DATA TABLE FOR RECORDING MEDIUM RENEWAL, PHYSICO/CHEMICAL MONITORING DATA AND FEEDING

Experiment No. Date started: Test substance: Nominal conc:

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	
Medium renewal (tick)																													
pH*																													new
																													old
O_2 [mg/L]*																													new
																													old
Temperature [°C]*																													new
																													old
Conductivity [µS/cm]																													new
																													old
Food provided (tick)																													

EXAMPLE DATA TABLE FOR RECORDING POTAMOPYRGUS REPRODUCTION AND MORTALITY

Experiment No. Date started: Test substance: Nominal conc:

	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Replicate 5		Replicate 6	
	Shell height	Embryos	Shell height	Embryos	Shell height	Embryos	Shell height	Embryos	Shell height	Embryos	Shell height	Embryos
1												
2												
3												
3												
4												
5												
6												
Mean												
No. of dead adults												

* Indicate which vessel was used for the experiment box