D4.4 Report/scientific articles on development of new bioassays for monitoring sub-lethal effects on chemically mediated behaviours in gastropods and crustaceans.

Stockholm University and RISE Research Institutes of Sweden

Project acronym: BONUS CHANGE

Project title: Changing antifouling practices for leisure boats in the Baltic Sea

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Background and aim

The key theme chosen by the Bonus CHANGE consortium was Bonus theme 4.1 – Governance structures, policy performance and policy instruments and the subthemes addressed by the Bonus CHANGE are 1.4 – Multilevel impacts of hazardous substances and 2.2 – Meeting the multifaceted challenges in linking the Baltic Sea with its coast and catchment. Hence, the Bonus CHANGE consortium is convinced and dedicated to deliver scientific results that can help improve policy performance and policy instruments to reduce to a minimum the spread of hazardous (toxic) biocides from antifouling paints used on leisure boats in the Baltic Sea.

The overall aim of work package 4 (WP4) is to provide scientific data on antifouling performance and ecotoxicity of currents antifouling (AF) biocides to convince boat owners to use environmentally friendly antifouling techniques. An important objective of the CHANGE project is to develop and use relevant test organisms and study characteristics in these organisms for early signs of effects of hazardous compounds or of mixtures of contaminants. In this deliverable, belonging to task 4.3, we have focused on developing a range of new bioassays in the lab to **directly measure effects** from hazardous substances present in antifouling paints, and in particular Cu and Zn. The goal has been to create more realistic exposure scenarios mimicking *in situ* conditions in harbours using species relevant for the Baltic Sea. The following exposure routes were used: sediment, contaminated food, and indirect water exposure via panels coated with anti-fouling paint (Table 1). The methods have focussed on new sensitive endpoints on avoidance behaviour and foraging. Advanced video-monitoring behavioural assays have been

used to establish sub-lethal effects on behaviours related to the overall fitness of the organism in gastropods and crustaceans. The aim is that these methods will better fulfil the needs for taking measures to reach the goals of WFD, MSFD, and BSAP for predictions and mitigations of environmental impacts of hazardous substances. Moreover, we anticipate that the methods for measurements of toxicity described herein will be better suited to the purpose than existing assays and methods for environmental impact studies. Although all methods need further development and validation, we foresee that in the future, our methodologies will have the potential of becoming established tools for measurements of mixture toxicity in different environmental matrices and for monitoring purposes.

Table 1. *Organisms and endpoints used in pilot studies, and development and set up of new bioassays.*In the pilot experiments the suitability of three gastropod species was evaluated.*

Endpoint	*Monoporeia affinis*	*Theodoxus fluviatilis (gastropods)*	Biofilm	Barnacles
Foraging		X		
Movement		X		X
Growth			X	
Avoidance	X	(X)		X
Mortality	X	X*	X	
Metal uptake		X*		

The Baltic Sea – a unique and particularly sensitive sea area

The Baltic Sea is a world unique brackish water body and is the youngest of the World's Seas – formed some 10,000-15,000 years ago after the last Ice Age. In fact, the Baltic Sea is one of the planet's largest brackish waters, governed by special hydrographical and climatic conditions. It is composed of high salinity seawater from the North East Atlantic and fresh water from rivers and streams draining from an area four times larger than the Baltic Sea itself.

This highly sensitive and interdependent marine ecosystem gives rise to unique flora and fauna. The Baltic Sea hosts species of various origins and environmental tolerances. These immigrated to the sea some 10,000 to 15,000 years ago or have been introduced to the area over the relatively recent history of the system. The Baltic Sea has only one known endemic species. In general, but not in all organism groups, high sub-regional total species richness is associated with elevated salinity. Salinities in the Baltic Sea varies from the South to the North spanning from ~ 20 PSU in the South where high salinity water from the NE Atlantic enters the sea through the Kattegatt and the Öresund and Bälten (the Sound and the Belts) to ~ 2 PSU in the Bothnian Sea in the North. However, in comparison with fully marine areas the Baltic Sea supports fewer species (Ojaveer et al. 2010). A system made up of so few species is not very stable, and is very susceptible to such pressures as fishing, habitat destruction, and pollution. The Baltic ecosystem

is immature and still evolving since it reached its current form and salinity level only 2000 years ago, and changes such as land uplift still continue, particularly in the northern areas (HELCOM).

On average, the water—and all the contaminants discharged from the catchment area with 85 million people—remains in the Baltic for decades. The input of freshwater from the catchment is larger than the in-flow of saline water from the North Sea. This causes strong stratification of the water column, which at times leads to hypoxia or anoxia at the sea floor. Nevertheless, the occasional in-flows of saline water bring along well-oxygenated water, which breathes life into the deeps of the Baltic Sea.

Due to the special hydrographical, biological and climatic conditions, the Baltic Sea is vulnerable. Marginal ecosystems such as the Baltic Sea can be of great conservation value because they may harbour unique genetic variation and even novel species. Indeed, a new species of macro algae has evolved inside the Baltic Sea (Wennerström et al. 2013). At the same time, the dense human population of the Baltic drainage area imposes threats to its aquatic biota via eutrophication, habitat destruction, and overfishing (Ducrotoy and Elliott 2008).

Over the past 100 years, the natural environment of the Baltic Sea has degraded dramatically. The Baltic Sea is one of the most threatened marine ecosystems on the planet. Decades of human activities in and around the Baltic Sea continue to affect its sensitive environment negatively and impacts can be observed over the entire sea area. The Baltic Sea is one of the most used and polluted seas in the world. One of the key threats to the well-being of the Baltic Sea ecosystem is the waterborne transport and discharges and airborne emissions of excessive amounts of nutrients and hazardous substances. The greatest source of eutrophication-causing nutrients and a significant source of hazardous substances are from land-based human activities (Helsinki Convention and Helsinki Commission, (HELCOM)).

The HELCOM has worked for over 40 years to improve the environmental status of the Baltic Sea through regional cooperation between Member States in the Baltic Sea area. In 2007, the HELCOM adopted the Baltic Sea Action Plan which visions and goals include that hazardous substances should be reduced to near natural levels.

The Baltic Sea is also classified as a Particular Sensitive Sea Area under the IMO. A **Particularly Sensitive Sea Area (PSSA)** is an area that needs special protection through action by IMO because of its significance for ecological, socio-economic or scientific reasons, and may be vulnerable to damage by international maritime activities.

Leisure boating and antifouling

Leisure boating is contributing to the high levels of contaminants in the Baltic Sea. A staggering 3-3.5 million boats have their homeports in the countries bordering the Baltic. In particular, the

use of toxic leaching antifouling paints that aim to deter settlement of marine organisms on constructions in the sea, continuously adds to the supply of biocides in the coastal ecosystem.

Marine biofouling is the colonization and subsequent growth of sessile organisms on all manmade surfaces in the sea, including boat hulls. Marine biofouling is made up of a wide range of organisms, i.e., slime-forming microorganisms, algae and invertebrates. The barnacle is considered to be the most serious fouler because of great difficulties in removing barnacle base plates from boat hulls. Biofouling increases drag and weight and thereby fuel consumption. Biofouling also decreases vessel manoeuvrability. Consequently, biofouling is a safety issue and a continual economic and technical problem for leisure boat owners.

Fouling prevention methods

The first method of choice for leisure boat owners is the use of traditional toxic biocide paints. Biocide-based paints kill-off fouling organisms and dominate the market of antifouling products. A typical biocide-based paint erodes slowly over time, giving rise to a slow, but controlled release of biocides in the water. Leisure boat marinas or harbours are sites of contaminant accumulation, due to their semi-enclosed nature, which allows only limited water circulation. Waste oil, fuel, paints and solid waste are the main contaminants in such locations (Aly et al., 2013). In particular, antifouling (AF) paints are releasing toxic heavy metals such as copper (Cu) and zinc (Zn) into the water and studies have shown a high accumulation of these metals in sediment and biota from harbours (Aly et al., 2013; Paradas and Amado Filho, 2007). Thus, it is important to develop tools that can be used for assessing the effects of such complex exposures.

In order to get a more comprehensive view of the potential risks in harbours, we have developed new bioassays that are more sensitive and we have focussed on assessing foraging and other behavioural responses. Organisms from different trophic levels were used, namely algae, crustaceans, and molluscs. As some organisms are new in the context of assessing toxicity, we also had to gain experience in culturing/rearing at laboratory conditions as well as establish baseline toxicity data.

Behaviour in ecotoxicology

Behaviour is defined as the ’cumulative interaction of a variety of biotic and abiotic factors that represents the animal’s response to internal (physiological) and external (environmental, social) factors and relates one organism to another’ (Dell’Omo, 2002). These responses can within an ecotoxicological framework, be used as early warning signals. The advantage with behavioural responses is that they are an integrated result of biochemical and physiological processes, reflecting changes at higher levels of biological organisation, which allows a comprehensive assessment of responses the surrounding environment, including various to stressors such as

contaminants (Hellou 2011). Contaminants may affect organisms directly, by altering their physiology, but also indirectly, through chemically-induced changes in their environment. Already in 1966 Warner et al. suggested that behavioural patterns should be used as a proxy for sub-lethal toxicity in wild organisms. Still little integrative work (Fig.1.) in behavioural ecotoxicology has been done (Peterson et al. 2017). The authors found this surprising since behaviour is; an indicator of multiple levels of biological outcomes, among the most sensitive indicators of exposure, and is an excellent early warning tool. Behavioural studies can also be non-invasive and relatively inexpensive, which is important from both an ethical and economical point of view.

Behavioural changes are also amongst the most sensitive indicators of shifts in environmental quality and may offer fast and non-destructive tests (Gerhardt, 2007) and responses can be up to 1000 times more sensitive than conventional LC50 tests (Hellou et al. 2008, Robinsson 2009). Moreover, changes in behaviour are not only a result of exposure to contaminants, but may in turn affect exposure by changing contaminant uptake rates. In nature, the exposure is complex as the organisms are exposed to multiple contaminants simultaneously, often in combination with other stressors such temperature or light variability. In the behavioural experiments performed within this task, organisms were exposed to Cu and Zn solely or in combinations. In addition, four different booster biocides were examined; copperpyrithione, zincpyrithione and diuron, and chlorothalonil. Diuron (N-(3,4-dichlorophenyl)-N,N-dimethyl-urea), is a phenylurea herbicide that acts on photosynthetic membranes by blocking the photosystem II electron transfer, thus inhibiting photosynthesis (Allen et al., 1983). Diuron has been detected in the Baltic Sea at concentrations of 1.8 µg/L (SWECO, 2009). Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile) is a broad-spectrum organo-chloric fungicide mainly used to prevent fungi infections and control fungal foliar diseases (Van Scoy & Tjeerdema, 2014).

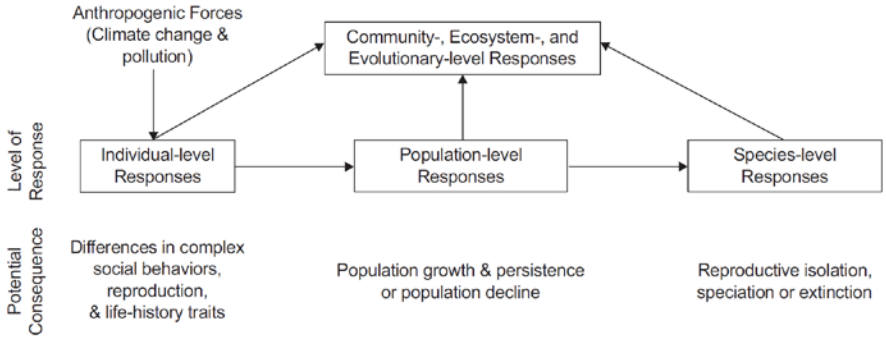


Figure 1. *Individual level responses to anthropogenic stress, e.g. pollution, can affect social behaviours, reproduction, and life history traits at the individual level. This stress at the individual level may affect responses at higher biological organisation levels. (From Peterson et al 2017.)*

Gastropod experiments

Acute toxicity in gastropods

To gain basic knowledge on gastropod responses to contaminants such as metals we carried out some pilot experiments, which provided valuable information into this deliverable as well as to D4.5, "Report on development of in situ bioassays for studying sub-lethal effects at marinas and natural harbours".

To obtain baseline data on several gastropod species, two bachelor projects were designed, albeit the focus was acute toxicity rather than behaviour. These baseline information constitued the starting point for experimental part on gastropods of both D4.4 and D4.5. The focus of these pilot experiments were:

1. Species selection and culturing of organisms
2. Uptake and depuration patterns
3. Assess acute toxicity from mixture toxicity experiments

Test organisms

Three different gastropods were selected: *Lymnaea stagnalis, Radix balthica*, and *Theodoxus fluviatilis.* The freshwater pulmonate *Lymnaea stagnalis* is widely spread in the northern hemisphere, and they have an important role in consumption and decomposition of aquatic plants and epiphytes (Berrie 1965)*. L. stagnalis* has been suggested as a good indicator species

for heavy metal pollution and it is exceptionally sensitive to metals (Co, Pb, Ni and Cu) in chronic exposure experiments (Grosell et al 2006; De Shamphelaere et al 2008; Schlekat et al 2010; Brix et al 2011). The wandering snail, *Radix balthica*, and the river nerite, *Theodoxus fluviatilis* are two common snails in the Baltic Sea. The lymnaeid *R. balthica* is a highly adaptive pulmonate snail that tolerates salinity up to 14 psu and pH between 5.8 and 9.9. (Wetter-Schultes, 2013). It has been found at depths of 0.2 to 2 m, but can live down to 10 m (Økland, 1964). *T. fluviatilis* is often found in the same environments as *R. balthica*. It is a common snail in the Baltic Sea, found in areas with salinities up to 18 ppt (Skoog, 1978), and in abundances of 200 -1000 individuals/m^2 (Zettler, 2004). The nerite *T. fluviatilis* inhabits rocky coastal areas, has a lifespan of ca 2-3 years and plays an important role in the ecosystem by grazing benthic diatoms and algae and by serving as food source to fish or birds. None of the species are vessel fouling organisms and hence they were tested in these experiments as a non-target organism representative of the Baltic Sea ecosystem. Below you will find a resumé of our experiences culturing these organisms in the laboratory.

Culturing snails and in particular *Theodoxus fluviatilis*

Snails are quite robust to changes in light and temperature, but in order to stress them as little as possible and keep them in a healthy condition, we suggest that the protocol below is followed.

Collection

In spring, when potential ice cover has melted, snails crawl up to shallower depths. Their natural habitat is directly on rocks and on *Fucus* sp. stands and they are easily picked by hand directly from the shore or by collecting rocks or *Fucus* sp. from a small boat.

Transport

Try to maintain ambient temperature during transport. Keep a lid on your container to prevent escape and use aeration if necessary.

Acclimatisation

When brought directly from the field, make sure the room temperature and light cycle are similar to the conditions that prevailed at the sampling site. If possible use water from the same habitat as the snails were collected in during the first week in the lab (> 90 %), after which a gradual dilution with water from the 'brackish water tap' can be done).

Acclimatisation to higher salinities can be done gradually without an obvious effect on survival, sampling from 2.3 psu and increasing by 0.2-0.5 units per day until 6 psu was done for the study published in (Bighiu et al. 2017). Acclimatisation to higher temperatures can also be done gradually.

Feeding

Fucus vesiculosis (bladderwrack) collected from the same location as the snails is a good substrate for periphyton that the snails usually feed on. In addition, concentrated (old) stocks of green algae and diatoms from the regular cultures were added about once a week. For this, most of the supernatant water was discarded from the flasks containing the diatoms, in order to avoid adding

too much salty water to the snail aquarium. For one aquarium (15L) containing about 400 snails, 2-3 pieces *of F. vesiculosis* and 1-2 additions of additional algae per week is sufficient.

Cultivation setup

A clear aquarium is better than a bucket as it allows easier observations of important changes (e.g. water quality or mass mortalities, etc.). Small rocks were added into the aquarium in order to create a substrate similar to the natural one. A cuttlefish bone was added as a source of calcium. The aquarium was permanently aerated using a pump. Note that it is important to keep the aquarium covered with a lid in order to prevent the escape of the snails, which tend to align at the water surface. In addition, the lid will slow down evaporation, which otherwise leads to snail death by desiccation (particularly important to remember when carrying out experiments with smaller volumes of water; these snails do not go back into the water as the level drops, they stay on top and dry to death!). Therefore, it is important to check the aquarium 1-2 times per week and add water if necessary (and measure salinity with regular intervals). It is often necessary to push the snails away from the edges of the aquarium in order not to smash them when closing the lid.

Water exchange

Before emptying the aquarium, save a few litres (2-3) of the surface water which looks clear. This will be used as an inoculum for the new batch of water (it contains the good bacteria, etc). Make sure to bring a bucket of water to the right temperature first (fix a day in advance). Most of the living snails will be attached on the walls of the aquarium, the rocks, the bladderwrack or the bone and thus, the water can be poured out without significantly disturbing or losing living snails (remove the stones first, to prevent them from smashing the snails). Depending on the aspect of the water and the snail density, the water can be exchanged 1-2 times a month or more seldom.

Cultures of Lymnaea stagnalis and Radix balthica

Specimen of *Radix balthica* was sampled on two different occasions but did not survive in the laboratory for more than ~3 weeks. *R. balthica* laid eggs in the laboratory, though the hatching was not successful.

Lymnaea stagnalis is quite robust to the laboratory environment. The procedure for culturing this species is similar to the one for *Theodoxus*, except that sand is needed for a proper trituration of the food in the gizzard. The food consists mainly of lettuce or thin slices of carrots (it has a different type of radula than *Theodoxus*). The newly laid egg sacks are visible on the walls of the aquarium and can be carefully detached using something thin and soft. The egg sacks should be transferred to new aquaria, to prevent the adults from eating the newly hatched juveniles.

The main reason for choosing *Theodoxus* over *Lymnaea* for the experiments in the BONUS CHANGE project was that the first one covers a wider salinity range in the Baltic Sea. However, the small size and the presence of operculum result in little biomass and more work-intensive dissection in *Theodoxus*. This means that several individuals might have to be pooled for some physiological or chemical analyses, whereas individual measurements may be obtained for *L. stagnalis,* which are considerably larger.

Mixture toxicity experiments

A brief description of the experiments reported in Appendix 1 follows. Toxicity experiments were performed using the following substances: Cu, Zn and the booster biocides diuron and chlorothalonil. Booster substances are added to antifouling paints to enhance the effect of the main substance.

Single substances

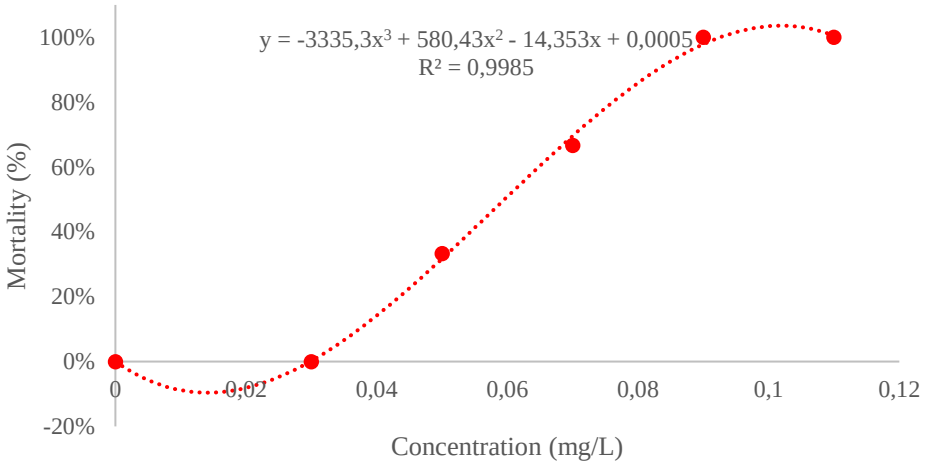
Acute toxicity tests were conducted in triplicates for each of the chemicals individually, the duration of which was 96 hours and the endpoint mortality and each test included one *R. balthica*. Three controls with the test water and three solvent controls with the test water and the solvent (DMSO). After 24, 48, and 72 hours, respectively, the test solutions were renewed. The snails were considered dead if there was no movement after having been gently poked with the pointy end of a plastic spoon. The mortality was noted, and 96-h LC_{50} values were calculated by graphical interpolation for all chemicals but chlorothalonil, which caused 100 % mortality in all concentrations. Results and attempts to estimate LC_{50} of the other biocides, i.e., Cu, Zn and diuron are shown below in Fig. 2.

Binary mixtures

The binary mixtures tested were Cu-Zn, Cu-diuron, Cu-chlorothalonil, Zn-diuron, Zn-chlorothalonil, and diuron-chlorothalonil. Due to an unfortunate mass mortality of *R. balthica* in the lab culture, this test was conducted with *T. fluviatilis* instead. The mixture concentrations were supposed to be based solely on the 96-h LC_{50} values derived from the single toxicity test, but with the change of species, some adjustments were made.

The binary mixtures were tested in ratios of 1:4, 1:1 and 4:1, by taking 0.2, 0.5 and 0.8 times the 96-h LC_{50} of one chemical, and 0.8, 0.5 and 0.2 times the 96-h LC_{50} of the other chemical, correspondingly. The ternary mixtures were Cu-Zn-diuron, Cu-Zn-chlorothalonil, Cu-diuron-chlorothalonil, and Zn-diuron-chlorothalonil. These were tested in ratios of 1:1:1; 0.33 times the 96-h LC_{50} for each chemical. The quaternary mixture, Cu-Zn-diuron-chlorothalonil, was tested at a ratio of 1:1:1:1, 0.25 times the 96-h LC_{50} for each chemical.

a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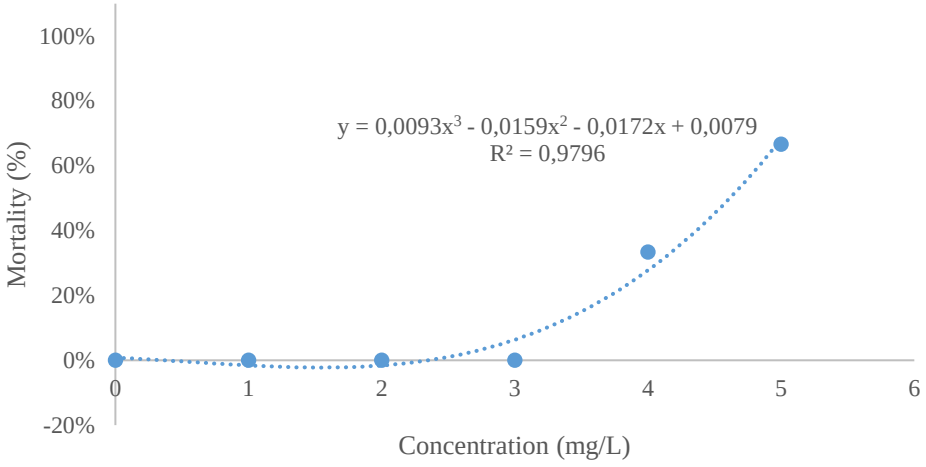
R² = 0,9985
100%
80%
60%
40%
20%
0%
-20%
Mortality (%)
0
0,02
0,04
0,06
0,08
0,1
0,12
Concentration (mg/L)

b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R² = 0,9796
100%
80%
60%
40%
20%
0%
-20%
Mortality (%)
0
1
2
3
4
5
6
Concentration (mg/L)

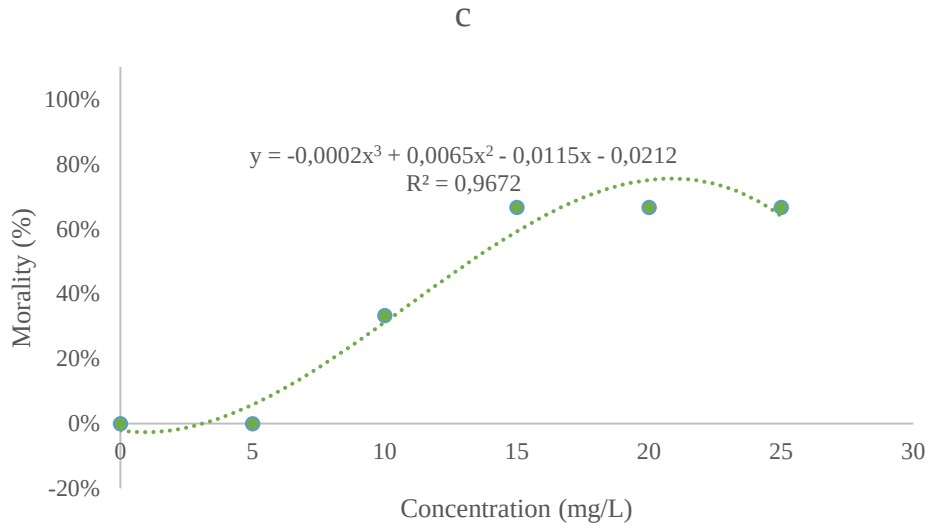


Figure 2. *Concentration-response (mg/L-percentage mortality) curves for the chemicals tested: a) Cu, b) Zn, and c) diuron. With 3rd degree polynomial trend lines for all. (From Svedberg 2016.)*

Conclusion

T. fluviatilis was easier to keep under laboratory conditions compared to R. balthica and was thus selected as test species in the further behavioural tests as well as in the caging experiments reported in D4.5. In the single substance experiments, relevant concentration ranges were found for all substances but chlorothalonil. The mixture toxicity results were very complex and showed both antagonistic and synergistic effects depending on substance and concentration. Mixtures of the same substances expressed different types of toxic effects when the concentrations varied. However, the majority (74 %) of the toxic effects produced by the mixtures were synergistic.

Metal uptake and depuration experiments

To increase our knowledge on uptake of complex mixtures we investigated if there is a difference in uptake and depuration for the freshwater snail *Lymnaea stagnalis* of copper, zinc and cadmium, if they are in single, binary or tertiary mixtures. *L. stagnalis* was used due to its bigger size, to have enough biomass for metal analyses. This experiment is presented in detail in Appendix 2.

In brief, the experiments were conducted during six days divided into a three-day uptake and a three-day depuration phase. The design consisted of seven different single and mixed metal exposures using Cu, Zn and Cd (Table 2). The experiment was divided in two parts; single

substance uptake experiments defining no observed effect concentrations (NOECs) and multiple substances experiments assessing mixture effects. As a precaution, the concentrations of the single metal used were based on 70 % of the NOEC obtained from part I of the experiment. Single metal concentrations were set to equal one fraction toxic unit. The concentration in binary mixture was 0.5 of the single metal concentration and the tertiary mixture was 0.33 of the single metal concentration (Table 2). These fractions were chosen to achieve a combined toxicity of less than one toxicity fraction unit for binary and tertiary mixture (Table 2). At the following times: 24, 48, 72, 96, 120, and 144 hours, one snail was randomly picked out from each treatment.

Table 2. *The nominal metal concentration in each test system; each fraction of metal contributing to the metal mixture and the total combined fraction of all metals in the mixture (From Ahlström 2016)*

	Nominal metal concentration µg/l			Fraction			Combined fraction
	Cu	Zn	Cd	Cu	Zn	Cd	1
Cu only	70	-	-	1	-	-	1
Zn only	-	2100	-	-	1	-	1
Cd only	-	-	350	-	-	1	1
Cu+Zn	35	1050	-	0.5	0.5	-	1
Cu+Cd	35	-	175	0.5	-	0.5	1
Zn+Cd	-	1050	175	-	0.5	0.5	1
Cu+Zn+Cd	23.11	700	115.5	0.33	0.33	0.33	1
Clean water	-	-	-	-	-	-	-

Conclusion

In single metal exposures, the uptake was significantly correlated to the size of the snails. Moreover, Cu can have a negative effect on the uptake of Zn and Cd in binary mixtures. For the tertiary mixtures, it is more difficult to conclude interactions between the metals.

Behavioural studies on gastropods

Rationale

The main idea for the behavioural experiments on gastropods was to subject individuals to pre-contaminated biofilm and record their behavioural responses. The behavioural responses focussed on are related to movement and foraging. With this concept, we can assess potential impact on both the biofilm, the grazing gastropod as well as the interaction between them. The responses observed on an individual level can help us to predict effects on the community level (Petersson et al. 2017).

Pilot studies on the behaviour of *Theodoxus fluviatilis*

We worked along two different tracks, using natural biofilm on stones assessing the foraging by stable isotope technique or culturing biofilm on tiles assessing the foraging by filming and image analyses. Along the second track a series of pilot experiments were performed to gain experience in growing biofilm, establish exposure concentrations and exposure time, developing an optimal filming procedure, and selecting a suitable image analysis software.

Natural biofilm approach

The set-up approach was inspired by (Lesutiene et al. 2014) and included natural biofilm on small stones. Small stones were collected from the bottom of a reference site. On each stone periphyton was visible. In the lab all periphyton, but a standardized circular area was removed. All stone was submerged in a 0.04 mM solution containing sodium bicarbonate NaH_3CO_3. The stones were picked up after 24 h and the status, in terms of photosyntethic efficiency of the biofilm was assessed by a Pulse Amplitude Modulator (PAM). The incorporation of stable isotopes in the biofilm was followed by addition of contaminants. A full factorial set of combinations of Cu, Zn, and diuron as well as nutrients to the biofilm and lastly the snails were placed on the rocks and could feed on the various biofilm treatments. After the foraging, the snails were dried and sent to analysis of stable isotope analysis. The incorporation of stable isotope into the biofilm differed greatly depending on biofilm thickness, which caused high variability between snails from the same treatment, which in turn defeated all attempts to find differences between treatments. This concept has, however, great potential but is a PhD-project of its own.

Cultured biofilm approach

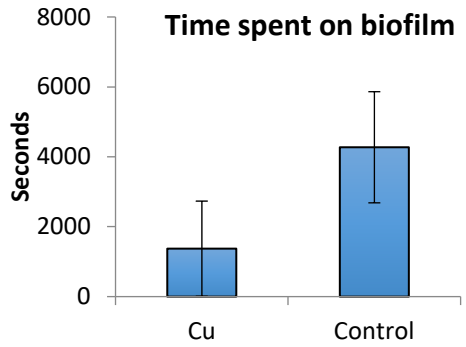
Biofilm growth

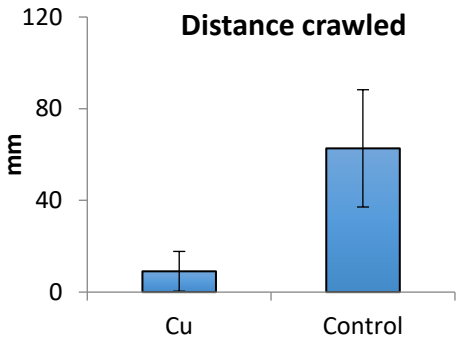
Small tiles were obtained by cutting ceramic tiles into squares (3.5 x 3.5 cm). The tiles were washed and then dried. To facilitate biofilm growth, we immersed the tiles in an albumin solution after which we transferred them to plastic tanks containing 30 L brackish water. Commercially available plant fertilizer (Blomstra) was added at three occasions (ca 40-75 mL per tank). The tanks were covered with transparent lids and kept outdoors under natural sunlight conditions. A visible layer of natural biofilm was established after a few weeks.

Biofilms are known to accumulate metals. To ensure that the metal exposure did not kill the biofilm, we exposed biofilm to a range of Zn and Cu concentrations for 48 h. We then assessed the photosynthetic activity by a Pulse Amplitude Modulator (PAM). No difference in photosynthetic activity was observed depending on metal exposure in the tested exposure range.

Results

In small behavioural experiments we examined exposure time and exposure concentration, number of snails per tile, software etc. to optimise the experimental setup. These experiments were the backbone of the final gastropod behaviour experiment (Appendix 3). Prior to this behavioural experiment, the biofilm had been exposed to Cu (100µg/L) for 48 h. The results obtained showed clear differences in snail behaviour depending on if they were exposed to copper-spiked biofilm or control biofilm (Fig. 3.) The main findings were avoidance of the exposed biofilm in comparison to control biofilm and more active snails in the controls.





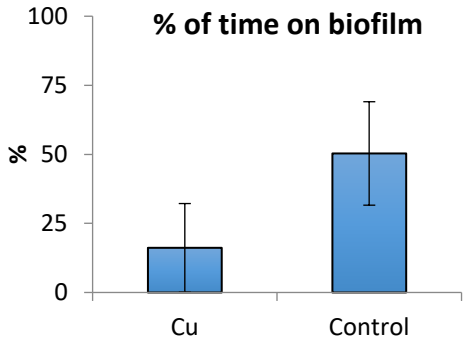


Figure 3. *Results from the pilot experiments testing behavioural responses of T. fluviatilis to Cu exposed biofilm. Prior to behavioural experiment, the biofilm had been exposed to Cu (100 µg/L) for 48h.*

Final experiment on the behaviour of *T. fluviatilis* as a response to Cu- or Zn-spiked biofilms

Experimental design

The details of this study are presented in Appendix 3. In brief, we exposed the cultured biofilm on tiles to high and low concentrations of Cu and Zn and two combined solutions. Five replicates of each treatment were exposed to metals during 96 h. The experimental setup also included procedure controls, which consisted of a treatment with no tile (just a snail in control water), a treatment with no biofilm (clean tile and snail) and a treatment with control biofilm exposed to non-starved snails. With the exception of the non-starved snails, all snails were starved for 24 h prior experiment. The automated and quantitative photographic system developed in the pilot study was used. In this system, snails were allowed to move freely in a Petri dish with a ceramic tile with biofilm (Figure 4.). Their movement was recorded by a camera every 15 sec (over a 3h and 35min period). The images were then used in Tracker software (Version 4 .94). The behavioural endpoints recorded were time to first active movement and crawling distance covered (separated by low speed movement, characterized mainly by exploratory movements at speeds lower than 0.033 mm/sec, and higher speeds (faster than 0.033 mm/sec)). In addition to movement, the foraging was also estimated. The feeding was quantified in terms of area of biofilm scraped from the tiles, using image analysis in Image J v2. In addition, a small subsample from all the biofilms was taken for live/dead cell staining. For this, the biofilm was scraped with a needle and mixed with 5 µL of 1 mM TO-PRO™ -1 Iodide stain (Invitrogen™) and incubated in darkness for 15 min. The stained biofilm was then observed under a fluorescence microscope and pictures were taken using the camera previously described. The ratio between red (alive) and green (dead) cells was then estimated from the pictures using Image J v2.

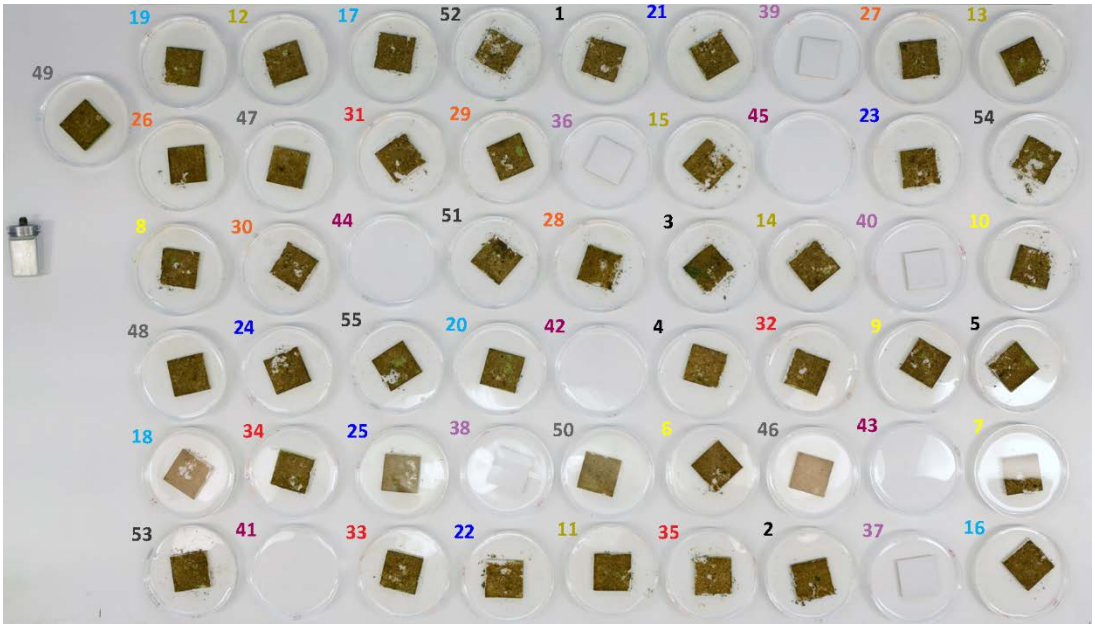


Figure 4. *Setup of the snail behavioural experiment. Snail movement was simultaneously recorded in all Petri dishes by a camera mounted 1.5 m above the setting.*

Results

The ratio between live and dead cells, obtained from image analysis of stained biofilms, was the lowest for the procedure control biofilm that was not grazed by snails. This ratio differed significantly from that in the procedure control biofilm grazed by unstarved snails (ANOVA, *Welch's F* (2,48.4) = 11.51, $P < 0.001$). Although no clear evidence of interactions between Cu and Zn was found, cell viability in low and high concentrations of metal mixtures was slightly higher than in the remaining treatments. Significant differences were detected between the high concentration of metal mixtures and 3 out of 4 single metal treatments (ANOVA, *Welch's F* (6,86.6) = 4.54, $P < 0.001$), ().

Effects on the condition of the biofilm, expressed as the ratio between live and dead cells, were found between grazed and un-grazed tiles and the biofilm not exposed to snail grazing presented the lowest cell viability ratio, which could indicate that the condition of the biofilm was improved by grazing. In the mixed Cu and Zn treatments, cell viability was slightly higher compared to the other treatments. Some effects on snail behaviour were also found, but they were less pronounced compared to the pilot experiments. The time when a snail first started to actively move (>0.033 mm/sec), was significantly different in snails from control and clean tile treatments compared to snails on exposed biofilm. Although the total distance crawled by *T. fluviatilis* was similar across the metal treatments, the speed varied. In the control treatment, the distance crawled by the snails at higher speeds (>0.033 mm/sec) was significantly greater than the

distance covered by snails exposed to biofilm spiked either with low Cu concentrations or with Cu and Zn mixed at low concentrations. If the total distance covered by each snail is taken into account, the percentage of total movement at higher speeds (>0.5mm/15sec) was higher in snails from the control treatment when compared to the Cu treatments or the mixed treatments. Approximately 24 ± 6 % of the movement done by snails in the control treatment was performed at speeds higher than 0.033 mm/sec; which was higher than those measured for any of the snails exposed to metal contaminated food. In conclusion, short-term exposure to dissolved Cu and Zn at ppb levels did not promote a quick and extensive alteration in the quality of the biofilm. In contrast to the pronounced effects of the pilot experiments, responses of *T. fluviatilis* to dietary exposure to both single and combined Cu and Zn were also constricted and consisted of impacts on initial movement behaviour and alteration of crawling speed patterns. These differences indicate that small methodological in the experimental setup affects the outcome of the experiment and needs further evaluation.

Experiments on crustaceans

Behaviour of amphipods exposed to contaminated sediments

Avoidance or preference behaviour in response to sub-lethal levels of toxic substances has been reported in several species, such as earthworms (Reinecke et al., 2002) and amphipods (Wiklund et al., 2006). This series of experiments is a further development of a preference test method that was first developed and used in Wiklund et al. (2006). In the new experiments, we enhanced the method by testing: the effect of food in combination with contaminants; simultaneous exposure to multiple contaminants in a range of concentrations, as well as the influence of experiment duration. All in all, six different experiments were performed. The organism used in the experiments was the amphipod *Monoporia affinis*, which is a benthic key species in the Baltic Sea. *M. affinis* is a glacial relict occurring in both brackish water and freshwater environments. It is the most productive macrofauna species on soft bottoms in greater Swedish lakes such as Lake Mälaren and the Baltic Sea. The high abundance, ecological importance and high lipid content (Wiklund et al., 2003), make it a relevant test species. As many other amphipods, it is proven sensitive to contaminants (Sundelin, 1983; Sundelin and Eriksson, 1998).

Experimental design

Six different experiments were performed and experiment one was repeated to improve the experimental setup. In the experiments, we tested the avoidance/preference of *M. affinis* to

sediments spiked with combinations of Cu, Zn, zinc pyrithione (ZnPt) and copper pyrtithione (CuPt) at different concentrations (Table 3). In experiment one we also examined the effect of food addition in combination with the contaminants. The organisms and the sediments were collected in the Baltic Sea outside of Askö field station (58°49'08.5"N, 17°38'07.2"E). The sediment were sieved through a 1.0 mm mesh size net, to remove larger particles and macrofauna, before it was mixed with sand and water to get a dry weight of about 70 to 80%. The copper sediment and the zinc sediment were made five weeks before the trials to allow the chemicals to equilibrate in the sediment. Due to the fast degradation of the pyrithiones, sediments with CuPt and ZnPt were made a couple of days before the respective experiment. Stock solutions of the highest concentrations (1 µg/g dw CuPt and Zn Pt, 100 µg/g dw copper and 400 µg/g dw zinc) were made. These sediments were then diluted with control sediments to achieve the desired concentrations. CuPt and ZnPt CuPt and ZnPt were dissolved in 5 mL acetone followed by ultrasound during ten minutes before sediment addition. All sediment-contaminant mixtures were slowly stirred during 24h. All beakers containing CuPt and ZnPt were covered in aluminium foil to prevent degradation from light. To avoid differences in acetone concentration, a corresponding amount of acetone–water mixture was added to all batches. The acetone concentration in each batch was less than 1 per-mille. Petri dishes for respective experiment were prepared as described below (Table 3). The petri dishes were randomly put in each aquarium and the sediments were allowed to settle for 2 h (Figure 5.). The aquaria were gently filled with water before the flow through system was connected. After the aquaria were filled 150 individuals of *M. affinis* were added to each aquarium. In each experiment, we used nine aquaria. The experiment was performed at 8°C with controlled light conditions, corresponding to Scandinavian winter in terms of light intensity and duration. Three samplings per experiment was performed, after 96, 192, 288 hours. Three replicates were terminated at each occasion. All petri dishes were immediately removed from respective aquarium and the number of individuals in each petri dish in each aquarium, were counted. This procedure was repeated at each sampling. Free-swimming individuals were counted to assess potential mortality. The response in the ZnPt and CuPt treatments was not consistent and we suspected degradation of the pyrithiones. Thus we performed a short-term experiment in complete darkness, sampling performed after 2, 4 and 8 hours.

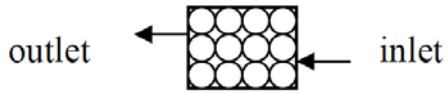


Figure 5. *The experimental set up, 10 petri dishes filled with prepared sediment were randomly placed in each aquarium. To each aquarium 150 individuals of M. affinis were added.*

Table 3. *An overview of substances and concentrations in the experiments.*

	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 5
Experiment 1	Cu: 25 µg g^{-1} Zp: 1µg g^{-1}	- -	- -	- -	- -
Experiment 2	CuPt: 0,1 µg g^{-1} ZnPt: 0,1 µg g^{-1}	0,25 µg g^{-1} 0,25 µg g^{-1}	0,5 µg g^{-1} 0,5 µg g^{-1}	0,75 µg g^{-1} 0,75 µg g^{-1}	1,0 µg g^{-1} 1,0 µg g^{-1}
Experiment 3	Cu: 25 µg g^{-1} Zn: 100 µg g^{-1}	50 µg g^{-1} 200 µg g^{-1}	75 µg g^{-1} 300 µg g^{-1}	100 µg g^{-1} 400 µg g^{-1}	- -
Experiment 4	Zn: 20 µg g^{-1} Zp: 0,1 µg g^{-1}	50 µg g^{-1} 0,25 µg g^{-1}	100 µg g^{-1} 0,5 µg g^{-1}	150 µg g^{-1} 0,75 µg g^{-1}	200 µg g^{-1} 1,0 µg g^{-1}
Experiment 5	Cu: 10 µg g^{-1} CuP: 0,1 µg g^{-1}	25 µg g^{-1} 0,25 µg g^{-1}	50 µg g^{-1} 0,5 µg g^{-1}	75 µg g^{-1} 0,75 µg g^{-1}	100 µg g^{-1} 1,0 µg g^{-1}
Experiment 6 Short exposure	CuPt: 0,1 µg g^{-1} ZnPt: 0,1 µg g^{-1}	0,25 µg g^{-1} 0,25 µg g^{-1}	0,5 µg g^{-1} 0,5 µg g^{-1}	0,75 µg g^{-1} 0,75 µg g^{-1}	1,0 µg g^{-1} 1,0 µg g^{-1}

Results and conclusion

All results are graphically described in detail in Appendix 4. The main results are the following: No effect of exposure time on the preference response was found. The effect of a fresh alga addition did not affect the amphipod preference. In experiments 1-5 we found an increasing avoidance with increasing concentrations in a dose response relationship for Cu and Zn but not for CuPt or ZnPt. This was an indication of degradation of the pyrithiones and when a short term experiment performed in darkness using only CuPt and ZnPt a similar dose response relationship as for Cu and Zn was obtained. In the short-term experiment in darkness with pyrithiones similar dose response patterns were obtained. In conclusion, the experimental set first tested by Wiklund et al. 2006 was also successful in a more complex setting. The amphipod *M. affinis* is able to respond to sediment contaminants at low doses. We also found that a short experimental duration (<24 h) is sufficient to get a distinct behavioural response. This simple setup is a cost-effective approach to test effects of contaminants in sediments on an organism relevant for the Baltic Sea.

Development of bioassays for studying sub-lethal effects of copper and zinc on barnacle behaviour

Introduction

Barnacles are considered one of the most problematic fouling organisms for boat owners (Aldred & Clare 2008). However, they are also important in the coastal ecosystem, where they provide a link between the water column and the benthic community by filtering out plankton from the water column and converting it to organic matter for sediment dwelling species (Foster 1987). Barnacles are also food for benthic species including starfish, whelks and crabs. Furthermore, their calcareous shells, when empty, provide a three-dimensional habitat where many small animals find shelter. Therefore, despite being one of the major fouling organisms on boats, it is still important to minimize the environmental impact from antifouling paints on non-target communities of benthic organisms, including barnacles, in shallow coastal environments. Since most antifouling paints used today, involve a slow but steady release of biocides into the water, there is great potential of effects on other barnacles than those trying to attach to the hull.

Scope and application

The aim with this work was to develop a bioassay for studying effects of copper and zinc on barnacles in a context that is closely connected to the field exposure that barnacle larvae are exposed to in the marine environment. The idea was to take a starting point in exposure to submerged surfaces coated with an antifouling paint containing copper and zinc but also study effects from the biocides that are release out into the water phase. Most studies using coated surfaces of antifouling paints focus only on the paint efficacy, rather than the effects of the painted surface on the fouling organisms. Instead, bioassays on ecotoxicological effects are often performed in static petri dishes, mainly focussing on the biocide *per se*, rather than effects of the total paint composition. In this new bioassay, we aimed to explore ways of studying these aspects together, trying to simulate a more realistic scenario, where marine organisms are exposed to painted boat hulls in shallow coastal bays. Furthermore, we chose to look at behaviour, which in other organisms, has been suggested to be a more sensitive endpoint (compared to e.g. survival) when it comes to ecotoxicological effects that may affect the dynamics of marine ecosystems (e.g. Bownik, A. 2017).

Choice of test organism

Barnacles have a complex life cycle where adults release free-swimming nauplius larvae, which go through six naupliar metamorphoses before hatching into a non-feeding cyprid larvae (Fig.

6A). The whole series of metamorphoses from released nauplius larvae to cyprids takes less than a week. The cyprid larvae then normally settles within a week, but sometimes it takes longer, depending on environmental parameters such as prevailing wind and water currents and temperature conditions. It has previously been documented that cyprid larvae display a wide range of complex swimming and surface exploratory behaviors, which is associated with finding a suitable substrate to settle on (Maleschlijski et al. 2015). They are also easy to maintain in laboratory culture (Berntsson et al. 2000), which makes them suitable for ecotoxicological studies and bioassays (Fig 6B).

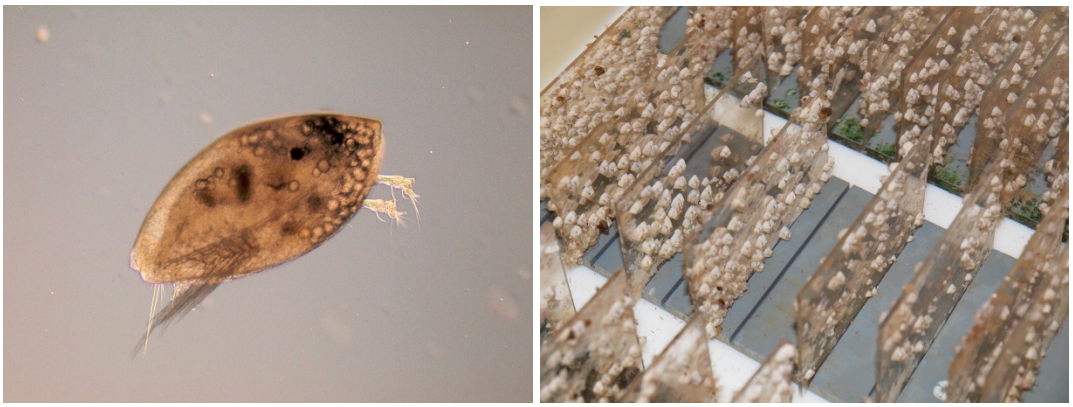


Figure 6. *A) A close-up image of a cyprid larvae; B) Barnacles growing on panels in the flow through culture facility at Tjärnö, Sweden; (Photo: Anna-Lisa Wrange and Jonathan Havenhand).*

Most ecotoxicological studies on copper (Cu) and barnacles so far have focused on effects on nauplius swimming behaviour and phototaxis (Romano et al. 2010; Gall et al. 2013). Thomason et al. (2002) suggested that cyprid search behavior might serve as an indicator of the efficacy of anti-fouling paints and coatings based on field bioassays. Quantitative studies of cyprid swimming behaviour in the lab using motion analysis have been shown to yield useful baseline information and provide a better understanding of general search and settlement behaviour exhibited by cyprid larvae (e.g. Amsler et al 2006; Maleschlijski et al. 2015). However, no studies have previously used motion analysis to study effects of copper (or in combination with zinc) on cyprid behaviour.

Effects of copper-based antifouling paints on barnacle settlement using flow cells

Aim

In the first study, the aim was to use a newly developed flow-through laboratory system to screen for preliminary toxic effects of antifouling coatings containing Cu and Zn on barnacles.

Most eco-toxicological bioassays that are performed to support the authorization of antifouling compounds are based on static petri dish studies, where organisms are exposed to biocides dissolved in water (Rittschof et al. 1992; Briand et al. 2009). This design becomes problematic if you instead want to investigate effects of a paint/coating containing and releasing biocides, since biocides will accumulate in the bulk water and hence confound the results, thus not providing information on realistic responses under field conditions. Therefore, it was suitable to use a flow-through system similar to the one recently published (Pansch et al. 2017) to get a first indication of which concentrations of Cu in AF paints that could be used to study sub-lethal effects on cyprid swimming behaviour.

Setup of flow cell assay

Flow cell characteristics

The general design of the flow cell has recently been published (Pansch et al. 2017). In brief, filtered seawater is pumped into a large header tank and distributed through tubing into 24 flow cells (Fig. 7), with room for two panels in each. The bioassay setup has several specific advantages compared to static systems:

- The design of the flow cell prevents cyprids from settling on other surfaces than the exposed test panels.
- A constant flow of water through the cell prevents accumulation of biocides or other substances within the bulk water.
- It allows for adjustment of the water level within the flow cells to achieve more even settlement of larvae across a surface.

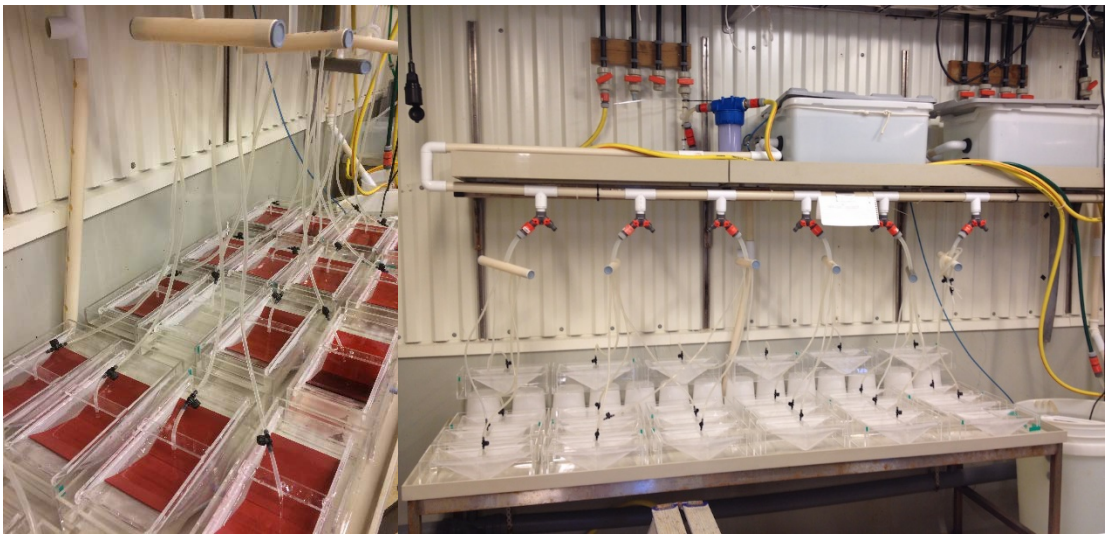


Figure 7. *Set up for the barnacle settlement experiments using the flow through chambers at the Lovén Centre for Marine Sciences, Tjärnö, Sweden.*

Treatment preparations

- PMMA panels (110 x 110 mm) were coated with two layers of paint (approx. 40-90 µm depending on treatment, thicker when including Zn), applied using a fine roller and left to dry for 24 hours in between layers.
- The treatments used were clean PMMA and paints with 0%, 2.6% or 8.5% (weight) Cu_2O, combined with 0% or 20% ZnO (a total of seven treatments). Three replicates were prepared for each treatment (with 2 panels per replicate). The treatments were chosen based on that they have been used within the BONUS CHANGE project previously to study antifouling efficacy of low copper containing paints and release rates of copper and zinc under field conditions (unpublished data from 2016; Lindgren et al. *accepted*)
- The painted panels were pre-soaked in flowing seawater (salinity 20 PSU; temperature 20 °C) in separate aquaria at a flow rate of approximately 9 L per hour for one week before being used in the assays, allowing the paint systems to potentially approach a more steady-state release of Cu (a procedure often used in other studies of release rates and also, the release rates (µg/cm^2/day) of copper from the European paint manufacturers CEPE mass balance model for calculating release rates of Cu uses values after 14 days of initial release, because then the release is assumed to enter a steady state release rate.).
- Two panels from each treatment were placed opposite each other in each flow cell (Fig.7).
- Cyprid larvae of the barnacle *Balanus (Amphibalanus) improvisus* (Darwin) were obtained in May 2017 from a stock of adult individuals maintained at the Lovén Centre for Marine Science (Strömstad, Sweden) and reared in batch culture as described by Wrange et al. (2014).
- Larvae (120 cyprids/flow cell) were added to each chamber and the total number of settled barnacles was recorded on each panel after 7 days. The number of larvae was chosen based on the total amount of available larvae at that time, and is within the range of what is suitable for using in the flow cell (Pansch et al. 2017).

Results from settlement study

The experiment showed that a paint with 8.5% Cu on its own had a negative effect on settlement, whereas zinc (20%) on its own did not affect settlement significantly (Fig. 8). However, the only treatments that were statistically different in the analyses were the Cu8.5%/Zn20% compared to the Cu2.8%/Zn0 (ANOVA: df_5, F=3.63, 0=0.03). This may have to do with low number of replicates (n=3) and high variation found between replicates. In any case, similar to previous findings in the

BONUS CHANGE project (Lindgren et al. *submitted*), we can see that the addition of Zn on its own may reduce settlement of barnacles to some extent. Also, Lindgren et al (submitted) show that Zn increases the release rate of Cu, which is indicated in the present results too, as shown by lower settlement on the paints containing both Cu and Zn.

Even though very low or no settlement was observed in the highest copper concentration tested (8.5% Cu, both with and without Zn), swimming larvae were still observed in the bulk water after several days, indicating that the release of Cu from the paint was not instantly lethal during short-term exposure (one week). This indicated that the paint containing 8.5% Cu could be suitable for further investigations of sub-lethal effects on swimming behaviour in the next step.

Settlement on the lower Cu treatment (2.8%) was not significantly different from the control treatment, indicating that barnacles could settle on this paint. However, the barnacles were not monitored over an extended period time, which could potentially reveal negative chronic effects such as reduced growth, reproduction and long-term survival, when settling on low Cu containing paints. Furthermore, the experiments showed that painted panels in general had significantly lower settlement compared to PMMA panels (ANOVA: df_6, $F=16.73$, $p<0.01$), indicating that possibly surface structure, colour or other chemical components in the paint may have affected settlement.

NOTE: It should also be noted that the general settlement rate on the control panels was overall relatively low (<40%) which may be due to poor larvae quality (which may vary as a result of e.g. reduced quality of food for the barnacle larvae, Ogemark, M., pers. comm.). Since larvae were added in relatively high numbers each time (by pipetting small volumes of water containing many larvae), the activity of all individual larvae was not checked, hence some may have been dead or in bad condition at the start of the experiment (despite careful overall inspection). Reducing the number of larvae and checking that all larvae are swimming actively prior to adding them to the experiments may solve this issue. Preferably, these experiments should also be repeated with multiple batches of larvae to control for batch variation, since it is known to occur and may influence (Pansch et al. 2013).

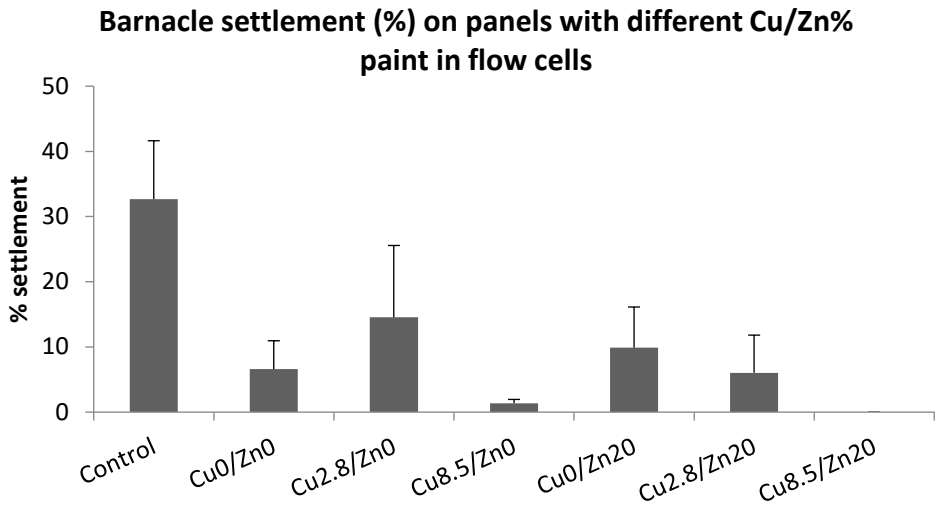


Figure 8. *Barnacle settlement (% of initial larvae added to each flow cell) on panels painted with different paints containing copper and zinc. Control (= transparent PMMA panels). The remaining bars are presented as paints containing 0, 2.8 or 8.5 weight-% copper oxide combined with 0, 10 or 20 weight% zinc oxide. N=3 and error bars represent standard deviations.*

Motion analysis of barnacle larval swimming behaviour when exposed to antifouling paints containing copper and zinc

Aim

In the second part, we aimed to develop a new bioassay focusing on sub-lethal effects of Cu and Zn on barnacle cyprid swimming behaviour. Swimming behaviour of larvae in different treatments was recorded using computerized motion analysis.

Experimental design and treatment preparation

- The treatments chosen for developing and testing a method to evaluate sub-lethal effects using motion analysis were; A) Control PMMA panels, B) panels painted with the generic Boero paint (Boero Group Inc.) without added biocides, C) and finally the generic paint but with addition of 8.5% Cu and 20% Zn. These were chosen based partly on the initial flow cell studies.
- Experimental treatment water was prepared by placing (two weeks pre-soaked in flowing seawater 20psu) painted panels (and control PMMA panels) in full 6L aquaria (six panels from each treatment in the same aquaria) where they were left to soak for seven days in seawater (20psu, 20°C).
- One litre of the “leaching water” was collected in a glass bottle and stored dark and cold

until the next day when the experiments were performed.

- In contrast to other ecotoxicological studies on Cu, we chose in this first study to, not only use Cu that has been dissolved in water, but rather use a "true field scenario", with soaking water from a surface coated with an AF paint, which contains copper but also zinc and other paint components that the larvae would be exposed to in the field.

NOTE: However, the assay could also be used to test direct effects of Cu (or other compounds) on swimming behaviour in cyprids. Since only pilot studies were conducted, water samples from each treatment were not sent for chemical analysis (including metals but also dissolved organic compounds etc.). However, this would be included when the bioassay is to be run again, to verify that the concentrations are correctly estimated.

Exposure and filming of larval behaviour

- Cyprid larvae were obtained from the same barnacle culture as mentioned above, in May 2016.
- Cyprids were stored in the dark at 10°C for 48h prior to use.
- Recordings of cyprid behaviour were performed in sterile polystyrene 12-well plates (Nunc^{TN}) as experimental arenas (Fig. 9B).
- The general set-up was to supply each well with 4 mL of the treatment water at room temperature and then add approximately 10 cyprids to each well. Nine replicate wells were prepared for each treatment.
- The cyprids were exposed to treatment water for 4h respectively 22h and then their swimming behaviour was filmed (repeated measures of the same wells). The times were chosen to simulate short-term and long-term exposure, without having larvae start settling
- After filming, the cyprids were left in the petri dishes to settle and the number of settled barnacles was checked one week after experiments were first initialized.
- The behaviour that cyprids displayed in response to the different treatments was recorded using a Ximea camera (xiQ usb3 camera) connected to an Olympus stereomicroscope (SZX16) with a macro lens (Fig. 4A).
- The films were recorded using the software obtained with the camera (Ximea CamTool) but other softwares were also tested (Streampix).
- The experimental arenas were placed underneath the digital camera in dark field illumination to enhance contrast and optimize detection of the cyprids.
- Movement of the multiple individuals in a dish was tracked continuously for 10-30 s with a capturing rate of 60-100 images per s, at a resolution of 1.3 megapixels.

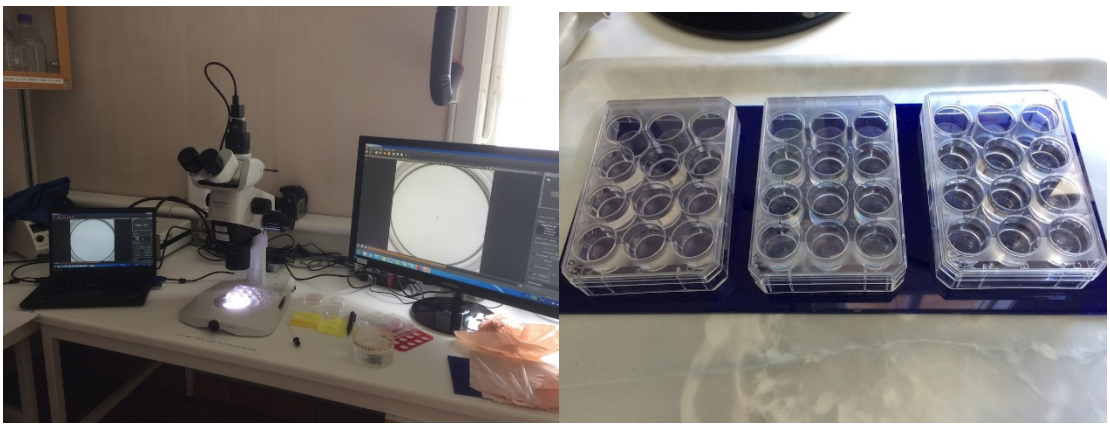
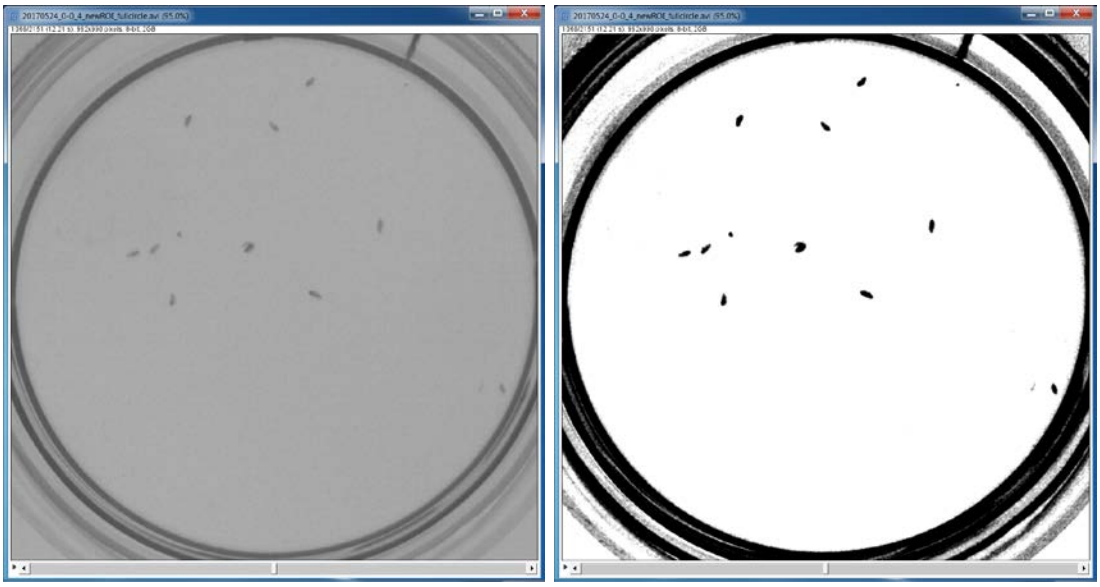


Figure 9. *Experimental setup for filming the cyprid larvae in 12 well plates.*

Analyses of film material

- The films were analyzed using a Motion Analysis Software (Fiji ImageJ plugin: TrackMate; Tinevez et al. 2017). Figure 10 shows some of the steps in processing and analyzing the films with larvae.
- From the software it was also possible to obtain displacement, as well as mean, maximum and minimum speed for each larvae. An average of all moving larvae in each replicate was calculated. The % of active (moving) vs. passive (non-moving) larvae was also noted by visual inspection of each movie.
- The tracked positions (x and y coordinates) of the multiple cyprids in a recording sequence were also obtained from the software.



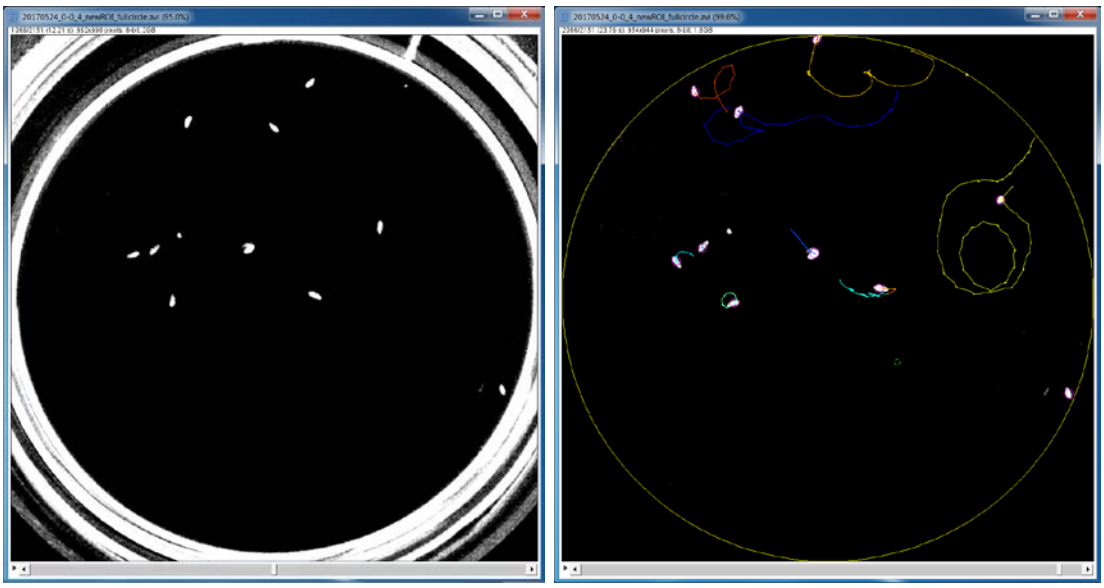


Figure 10. *Procedure for processing and analyzing films of swimming cyprid larvae in petri dishes using the TrackMate software. A) the original film, B) adjusted image for best detection of larvae, C) inverted image, D) Tracking of individual larvae over time.*

Step by step procedure using TrackMate:

The films were analysed using a Motion Analysis Software (Fiji ImageJ plugin: TrackMate; Tinevez et al. 2017). From the software it was also possible to obtain displacement, as well as mean, maximum and minimum speed for each larva. An average speed of all moving larvae in each replicate was calculated (excluding larvae which did not move at all). The proportion of active (moving) vs. passive (non-moving) larvae was also noted by visual inspection of each movie. The tracked positions (x and y coordinates) of the multiple cyprids in a recording sequence were also obtained from the software. Here is a detailed step-by-step protocol of the processing of the film sequences of barnacle behaviour using the softwares ImageJ and TrackMate (Tinavez et al. 2017).

1. Open ImageJ FIJI.
2. File -> Open… -> select film sequence (avi)
3. AVI reader: select “Convert to grey scale” (unclick “use virtual stack”) – here it is also possible to select equal nbr of frames used (set 1-X…)
4. If needed: Image -> adjust… -> Brightness/Contrast (make it more black/white), *it is also possible to remove background, check Image J manual…*
5. Edit -> Invert (to get black background)
6. Select circle tool, hold SHIFT, mark area -> Image -> Crop; Edit -> clear outside
7. Plugins -> Tracking -> Trackmate -> Swap Z&T? -> Yes
8. Calibration settings: default

9. Select a detector: LoG detector
    a. Estimated blob diameter: 10-16 (depends on image quality)
    b. Threshold: 5-7
    c. Preview (NB: check several times in different frames)
10. Note nbr of larvae in movie _____and nbr of spots detected _____
    a. *Problem with larve being detected twice, can be solved by adjusting blob diameter, but risk of loosing other larvae too…*
11. Click next -> Detecting starts… (<1 min)
12. *(To add or remove spots: place mouse over spot and press D (delete) or A (add) – however this seems to only remove the spot from one frame???)*
13. Initial thresholding -> next…
14. Hyperstack Displayer –> next…
15. Set filters on spots – here it is possible to exclude certain spots if needed by choosing settings in the roll down menu.
16. Simple LAP tracker (without merging or splitting of tracks)
    a. Linking max distance: 60 pixel
    b. Gap-closing max distance: 60 pix
    c. Gap closing max frame gap: 5 pix
17. Set filters on tracks (set color by Track ID) – check how many tracks are detected
18. Display tracks -> set color by track ID
19. Click: Analysis -> give three csv files:
    a. Track statistics (table with all tracks summarized)
    b. Links in track statistics
    c. Spots in track statistics
20. Save these files and import them into excel
21. Compare number of tracks with number of larvae in movie, remove tracks that are not correct, mark tracks that are larvae not moving
    Most tracks should start at Frame 0, but not always…

<u>Output parameters</u>:
Track displacement (track stats) – gross displacement
Track X and Y at beginning and end (spot stats) – use to calculated net displacement, but can also be used as input data for Matlab
Track mean speed (as well as max and min)

<u>Notes to facilitate import of file to excel</u>:
1. Import csv file to excel (spot stats file)
2. NB: data file suitable for settings of "." as comma (ok in mac, not in PC – needs changing in case it is used on PC)
3. Data -> Analysis -> Pivot table
4. Row labels: FRAME
5. Column labels: TRACK ID
6. Values: Position X, Position Y.

7. Mark which tracks you want to include (remove the rest)
8. Result: File with data for Matlab?
9. There is also a possibility to chose “Next”+Next instead -> Select an action: Export tracks to XML file -> to use directly in Matlab
10. Read TrackMate manual for how to connect Matlab to ImageJ…

Potential for more in depth analysis method

Furthermore, we aimed to get more detailed quantitative measurements of cyprids swimming and surface-exploratory behaviour, through analysis of the output data (X Y coordinates) using a custom-written Matlab© software.

The original Matlab routine (which was developed by Prof. Jon Havenhand, University of Gothenburg several years ago) has the potential to provide several metric variables to characterize differences in the structure of cyprids movement paths between different treatments including:

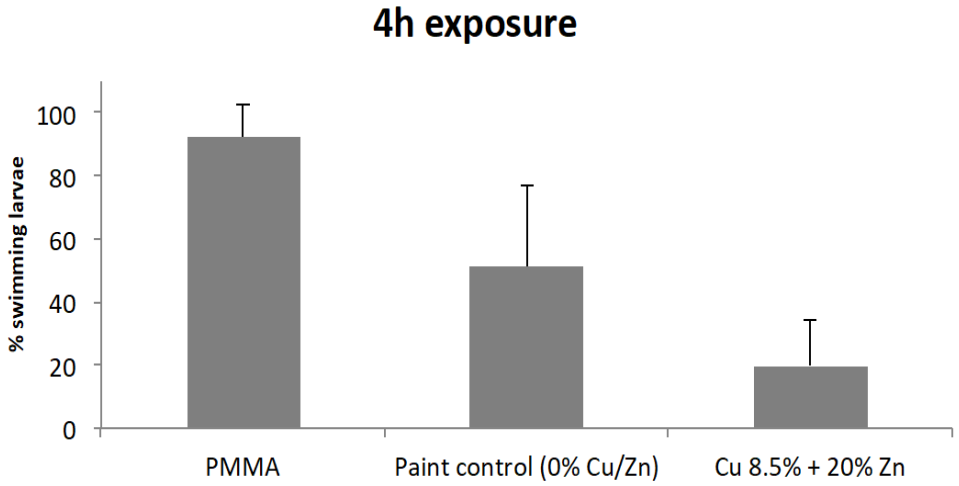
- **total distance** (average total move length of track, mm),
- **swimming velocity** (average move speed, mm.s-1),
- **angular velocity** (mean absolute turn angle per s, ± radians.s-1),
- **rate of change of direction** (RCDI, mean amount of turning per s, |radians|.$^{s-1}$)
- **sinuosity** (mean statistical distribution of the angular velocity per mm between successive turns, radians.mm$^{-0.5}$).

NOTE: However, due to problems updating the Matlab routine and getting the analyses to work, results are not available from this method yet. But future collaboration has been discussed with Professor J.R. Havenhand and colleagues at the University of Gothenburg to further obtain more results using this method.

Results from behaviour studies

Due to limited time at the field station facility and unpredictable and variable larval supply from the barnacle culture at the field station (Lovén Centre of Marine Sciences at Tjärnö, Sweden) during May 2017, only pilot studies were finalized, but showing good potential for further development and usage as a bioassay for studying sub-lethal effects of Cu and Zn as well as other biocides. Detailed results from these pilot experiments will be made available for other researchers who are interested in taking the results further (there are on-going discussions with other research colleagues as mentioned above).

Our results from the filming show that, based on visual inspection of each entire movie of 30 seconds from different treatments (since full TrackMate analysis has not been able to be completed), there was a significantly lower number of cyprid larvae that were swimming already after 4h exposure to the Cu/Zn treatment, compared to the control treatment (PMMA). Furthermore, larvae swam faster in the control treatment compared to the Cu treatment although full analysis of the movies will be required to confirm this observation. Interestingly, there was also an effect of the antifouling paint without biocides after 4h (Fig. 11B), however this difference was not found after 22h exposure (Fig. 11B). This needs further investigation.



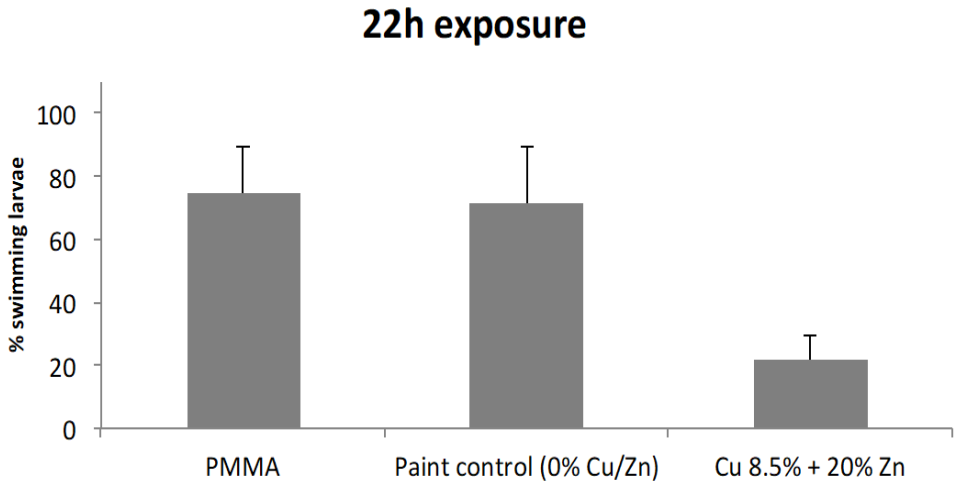


Figure 11. *Percentage of larvae that are actively swimming after, A) 4 hours and B) 22 hours, in the different treatments based on visual inspection of each movie (30 sec). PMMA = control, Paint control = paint without added copper and zinc, and Cu 8.5%+20%Zn = paint with the addition of 8.5% copper and 20% zinc.*

Settlement of larvae following the behavioural assay

To see if the effects on swimming behaviour also could be correlated to settlement, the multiwall dishes were kept in room temperature with a semi-sealing lid on (to avoid high evaporation, and settlement (number of larvae that settle) was noted after one week.

However, settlement in the multi-dish plates was extremely low (<10% for all treatments) after one week in all treatments, although personal observations indicated that larvae in the control treatments (with no biocides) were more actively swimming during several days following the filming, compared to in the Cu treatment, where many larvae died within a few days.

NOTE: Delays in settlement may be associated with poor larval quality. But another plausible explanation could be that the larvae got stuck in the water surface tension, a phenomenon that has been observed previously (Qiu et al. 2008; Di Fino et al. 2014). Therefore, an improvement of the bioassay would be to use completely filled wells with sealing lids, to remove the surface tension where larvae otherwise can get stuck.

Further development of the bioassay

Quite some efforts were made towards testing and evaluating different softwares, both for filming (Streampix and Ximea CamTool) and for motion analysis, including different plug-ins in ImageJ (e.g. CASA, WrmTrck, TrackMate, etc.), in order to find one that was suitable for barnacle larvae size and shape, as well as the various types of complex swimming behaviour that cyprids display. Therefore, only preliminary data are available and more work is needed before presenting a robust bioassay protocol that is useful to test sub-lethal dose response effects of Cu and other biocides on barnacles.

<u>Further development of this assay could include:</u>

- Further optimization of experimental setup, using filled containers with tight fitting lids to avoid larvae getting stuck in the surface tension when filming larval behaviour.
- Optimising the settings for the analysis using the more advanced Matlab routine (provided by Havenhand), to get a more complex picture of how behaviour may be affected by Cu
- Soaking several paints with different copper release rates and confirming bioavailable

copper content (as well as DOC which may reduce Cu availability) through chemical analyses of the water treatments.

- Re-running the exposures of cyprid larvae to a wider range of treatments and comparing swimming behaviour
- Evaluating the role of batch variation (using repeated batches of larvae from the barnacle culture) in response to contaminants such as Cu, to get a more general understanding of the effects on barnacles
- Testing different mixtures of copper, zinc and other common biocides used in AF paints to quantify effects on swimming and settlement behaviour.
- Potentially also expose nauplii life stages to the same paint leakage water "cocktail" to get an idea of the in situ effects that current antifouling paints on leisure boats may cause.

Conclusions behavioural assays examining single substance and mixture toxicity

The task of developing new bioassays, using both new and previously known test organisms using new and more relevant exposure matrices in comparison to standard toxicity tests, has been challenging. A vast number of pre-experiments have been performed in order to select test species, evaluate uptake and depuration kinetics as well as to optimise test conditions and experimental settings. In each respective assay, the level of development varies depending on the complexity of the assay itself as well as with what kind of equipment that needs to be further reined.

The assays are based on a wide range of experimental setup principles. Both flow-through systems and static exposure set-ups have been used. Several exposure routes have been applied; water, sediment, biofilm and artificial boat hulls. We have classified their potential of becoming future methods assessing toxicity in three classes (Table 4).

Table 4. *The usefulness of organisms and endpoints of the bioassay concepts tested in BONUS CHANGE, green = high potential, yellow = intermediate, red = low potential.*

Endpoint	*Monoporeia affinis*	*Theodoxus fluviatilis*	Biofilm	Barnacles
Foraging				
Movement				
Growth				
Avoidance				

The baseline experiments on gastropods helped us select *T. fluvivatilis* as a test organism in both bioassay and in situ experiments. This species has many advantages: it is common both in the Baltic Sea and in freshwater all over Europe, it is easy to keep in the lab, it has sexual reproduction making it suitable in a wide range of different experiments. In the acute tests we found that the mixture toxicity patterns are very complex. Although synergistic effects were the most common response, antagonistic effects were also observed. All effects were both substance and concentration specific. The uptake and depuration kinetics were also complex as Cu lowered the uptake of both Zn and Cd. In addition, we found differences between metals during the depuration phase. Snails were able to reduce their body burden of copper while no depuration of Cd was found.

The behavioural pre-experiments using *T. fluviatilis* indicated that combining foraging and movement could be a successful path to assess toxicity. We found strong effects between snails exposed to Cu –spiked biofilm compared to controls. However, in the main experiment we observed less profound effects indicating a complexity in the interactions between biofilm and grazer. There were small differences in the experimental setups between the pre-experiment and the main experiment suggesting that the experimental setup in itself did not cause the large difference in response. Even if there is room for optimising and for improvements, we infer that studies combining biofilm and grazers have a potential (Table 3) as bioassays for understanding sub-lethal, behavioural effects of environmental contaminants.

All assays on crustaceans showed promising results in terms of measurable effects from relevant exposure scenarios, such as exposure to low concentrations of contaminants. These assays have the potential to be applied to other environmental stressors, not just contaminants originating from AF paints (Table 3).

The choice to use a “true field condition scenario” in the barnacle test instead of testing the effect of single dissolved compounds as is done in traditional bioassay toxicological studies increases the complexity and thereby reducing the chance of pinpointing an effect to a particular contaminant. We used panels coated with an antifouling paint that were soaked in water to simulate a cocktail of copper, zinc and other paint components that the larvae would be exposed to in the water under field conditions. Having said this, the assay could however be combined with tests of direct effects of dissolved Cu or Zn on swimming/search behaviour in order to investigate more precisely what metal components elicit which kind of behavioural response. Although the main focus in these studies was barnacles, the behavioural assays could easily be adapted to fit other sessile organisms with free-swimming larvae such as tunicates and mussels. Both the flow cell approach looking at settlement and the behavioural assay on swimming responses are promising tools to study sub-lethal effects of biocides on marine organisms. However, further work is needed to establish a more robust protocol. The results from the pilot

studies on barnacles strongly indicate that difference in swimming behaviour were elicited in larvae exposed to Cu/Zn treatments as compared to controls, however further analyses are required to confirm this (Table 3). Although only pilot studies on barnacles were finalised during the project and more work is required to obtain a solid protocol, using motion analysis of barnacle larvae behaviour has great potential of becoming a standard method in bioassays. Barnacles are relatively easy to culture, and their larvae have complex swimming and search behaviours, which are an important part in determining where the larvae will settle (a crucial part for survival).

The goal in this task has been to create bioassays using more realistic exposure scenarios mimicking *in situ* conditions in harbours using species relevant for the Baltic Sea. By mimicking *in situ* conditions, we have also increased the natural variability in all systems, decreasing the chance of detecting significant effects. In the consortium, we have a solid experience in working with crustaceans and the outcome of the behavioural assays in this task might reflect this fact. In the experiments using crustaceans we foresee a higher potential than in the work with gastropods and biofilm. The future challenge is to create robust protocols and validate the most promising methods. Validation will include factors such as exposure temperature, light condition, experimental duration. However, turning a promising assay into a standardised test accepted by OECD or SIS is a process that takes many years to achieve. At the Department of Applied Environmental Science and Analytical Chemistry, we have the experience of taking an assay from a promising method to an internationally validated guideline (e.g. ISO 10710, ISO 18220, SIS (1991).

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Appendices

Appendix 1 Svedberg, L. 2016. Mixture Toxicity of Antifouling Substances to Brackish Water Snails. Bachelor's thesis, 15 HE credits, Environmental Science, 2016:3, Stockholm University.

Appendix 2. Ahlström, D. 2016. Bachelor thesis Influence of Heavy Metal Mixture on Uptake and Depuration by freshwater pond snail *Lymnaea stagnalis* Bachelor's thesis, 15 HE credits Environmental science, 2016:2, Stockholm University.

Appendix 3. Ferreira Behavioural responses of Theodoxus fluviatilis to metal-contaminated biofilms Manuscript will be submitted to Plos One.

Appendix 4. Bighiu, M. Wiklund AKE. 2017. Method and results compilation from a behavioural study of the amphipod *Monoporeia affinis* exposed to spiked sediments.

Deliverable 4.4 Report/scientific articles on development of new bioassays for monitoring sub-lethal effects on chemically mediated behaviours in gastropods and crustaceans.

Appendix 1

Mixture Toxicity of Antifouling Substances to Brackish Water Snails

Lina Svedberg

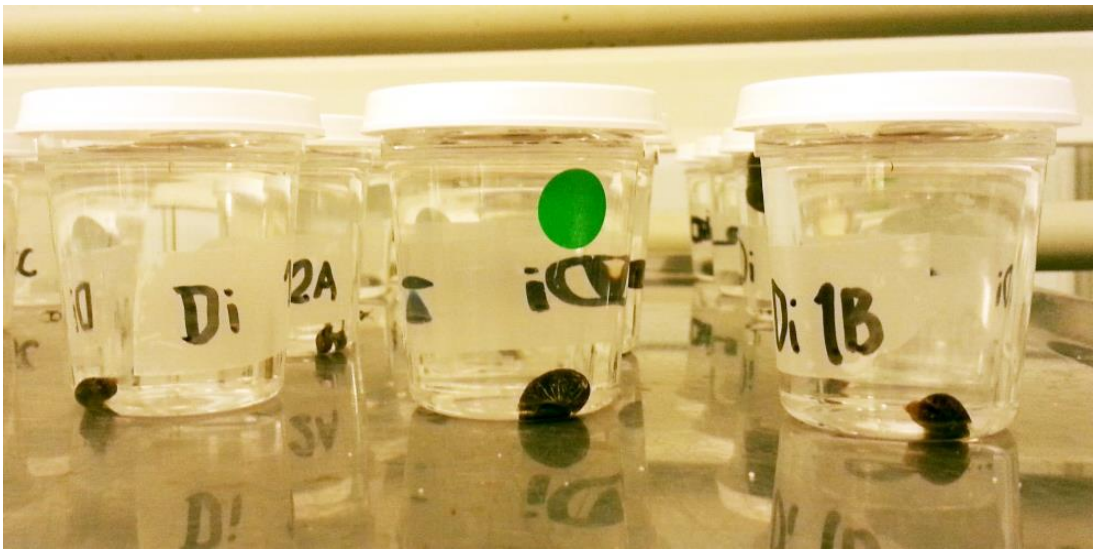
Bachelor's thesis, 15 HE credits in Environmental Science,

2016: 3

Stockholm University

Mixture Toxicity of Antifouling Substances to Brackish Water Snails

Lina Svedberg
Bachelor's thesis, 15 HE credits
Environmental Science, 2016:3



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Department of Environmental Science and Analytical Chemistry

Mixture Toxicity of Antifouling Substances to Brackish Water Snails

Lina Svedberg

Maria Bighiu & Ann-Kristin Eriksson Wiklund

Abstract

Chemicals are often released into the environment due to human activities, for example their use in antifouling paints. They often come in mixtures and the combined toxic effect of these may be different from the effects they would produce if present alone. The combined toxicity can be equal to the total toxicity of its components (additive), or the mixtures can create effects lower (antagonistic) or higher (synergistic) than additive. The present study investigated the toxicity of the antifouling substances copper, zinc, diuron and chlorothalonil, and their mixtures, to the brackish water gastropods *Radix balthica* and *Theodoxus fluviatilis*. The obtained LC_{50} values for the individual chemicals were 0.06, 4.5, 12.2 and 1.0 mg/L, respectively. All three chemical interactions were seen in the mixtures, some in line with what has been shown before. The majority of the observed effects were synergistic, which highlights the importance of studying mixture toxicity, and further investigations are needed to obtain larger ecological relevance and broader knowledge in this field.

Keywords

Mixture toxicity, antifouling, copper, zinc, diuron, chlorothalonil, *Radix balthica*, *Theodoxus fluviatilis*

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Introduction

Due to human activities, chemicals are often released into the environment where they may cause harm to various organisms and communities (Zhou et al., 2006). The anthropogenic routes of chemicals to the environment are many, but to the aquatic environment, one source is from antifouling paints. It is not uncommon for antifouling paints to leach or be scrubbed off of boat hulls (Kiaune & Singhasemanon, 2011). Organotin-based antifouling agents (e.g. tributyltin, TBT) were restricted in the 1980s due to their damaging impact on the environment and its organisms (Adamson & Brown, 2002), such as imposex in snails (Walker et al., 2012). Since then, the use of alternative antifouling agents has increased (Konstantinou & Albanis, 2004); however, these alternatives are not harmless either. Some of them are metal-based, using e.g. copper (Cu) or zinc (Zn), and some contain organic booster biocides, to increase the effect (Koutsaftis & Aoyama, 2007).

Chemicals often come in mixtures, meaning organisms are exposed to many pollutants at the same time (De Zwart & Posthuma, 2005). When in mixtures, chemicals may pose combined effects, different from those they would produce if present singly (Pan et al., 2015). These combined effects can be additive, i.e. the toxicity of the mixture is approximately equal to the total toxicity of its components; each chemical expresses the same toxicity in a mixture as it would if tested alone (Walker et al., 2012). Some chemicals may interact with each other, however, and create effects greater or less than additive. The latter is called antagonism, which is when the mixture toxicity is lower than the toxicity of the most toxic component alone at the same concentration (Pan et al., 2015). If the effect is greater than additive, it is called synergism, i.e. the components of the mixture produce a higher toxic effect in the mixture than they would if present singly (Cedergreen, 2014). The effect that a mixture produces may depend on its components and the concentration ranges tested (Meyer et al., 2015).

Cu is an essential trace metal, occurring naturally in the environment. It is needed in low concentration for all organisms to function properly, as it participates in many enzymatic syntheses and activities, e.g. iron absorption and haem synthesis (Barceloux & Barceloux, 1999a). At higher concentrations, however, it is toxic, as it then interferes with biological functions i.e. interacts with enzymes. At these concentrations it can be used as a pesticide in antifouling paints, but at the same time non-target organisms may be affected (Kiaune & Singhasemanon, 2011), and it has been seen to cause various lethal and sublethal effects in several invertebrate species (Voulvoulis et al., 1999). The main mechanism of copper toxicity is assumed to be the causing of oxidative stress, but it also inhibits protein function by binding to sulfhydryl groups on proteins and disrupting their structure (Wendt, 2013). Some other adverse effects that Cu can exert is inhibition of ATPase enzyme activity in brine shrimp (Katranitsas et al., 2003) and impairment of ion- and osmoregulation in fish (Blanchard & Grosell, 2005; McIntyre et al., 2008). It has been detected in the Baltic Sea at concentrations up to 2 μg/L (Herbert et al., 2009). Zn is another essential trace metal and it is a co-factor in many cellular processes, e.g. DNA synthesis, reproduction, and behavioural responses (Barceloux & Barceloux, 1999b). At higher concentrations, Zn may be harmful to non-target organisms. It has, for example, been seen to block the development of brine shrimp larvae (Bagshaw et al., 1986) and inhibit the reproduction in fathead minnows (Brungs, 1969). In 2009, concentrations of Zn found in the Baltic Sea were up to 280 μg/L (Herbert et al., 2009).

A common organic booster biocide in antifouling paints is diuron (N-(3,4-dichlorophenyl)-N,N-dimethyl-urea). It is a phenylurea herbicide that acts on photosynthetic membranes by blocking the photosystem II electron transfer, thus inhibiting photosynthesis (Allen et al., 1983). The solubility and half-life of diuron in water is 42 mg/L and 33 days, respectively (USEPA, 2003). It has been widely used as weed-control, but its use in antifouling paints is what makes it a direct source to coastal waters and the organisms living there (DeLorenzo & Fulton, 2012). Some effects of diuron on non-target organisms that have been studied are adverse effects on the development of fathead minnows (Call et al., 1987), and mortality and species loss in meiobenthic communities (Gallucci et al., 2015). Diuron levels has been detected in the Baltic Sea at levels of 1.8 µg/L (SWECO, 2009). Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile) is another organic booster biocide that is commonly used in antifouling paints. It is a broad-spectrum organochloric fungicide mainly used to prevent fungi infections and control fungal foliar diseases (Van Scoy & Tjeerdema, 2014). In addition, it is used to protect wood, and as a biocide against e.g. insects, bacteria, algae, and spiders (DeLorenzo & Fulton, 2012). The water solubility of the fungicide is 0.9 mg/L, and its aqueous half-life is 8-9 days (Caux et al., 1996). Chlorothalonil acts on fungi by inactivating sulfhydryl enzymes in cells (Sherrard et al., 2003). It also reduces cellular thiols such as glutathione, which leads to disruption of energy production (Gallagher et al., 1992), and may cause oxidative stress as glutathione is known to break down reactive oxygen species (ROS) (Cedergreen, 2014). It has been seen to affect non-target organisms, e.g. inhibiting the growth of macrophyte species (Belgers et al., 2009) and reducing larval growth and settlement in marine invertebrates (Bellas, 2006).

Earlier studies have investigated the combined toxic effects of the binary mixtures of these chemicals on different organisms. Meyer et al. (2015) presented in their study the synergistic and additive effects of Cu and Zn in mixtures to *Daphnia magna*. Previous studies have shown that Cu can act antagonistically to Zn uptake in phytoplankton and macroalgae (Luoma, 1983). Cu-diuron mixtures can act either additive or slightly antagonistic on the growth of *Lemna minor*, and it is suggested that the antagonism is caused by diuron preventing oxidative stress induced by Cu (Teisseire et al., 1999). Mixtures of the same substances have also showed synergistic effects on the growth of the marine alga *Chaetoceros gracilis*, whereas Zn-diuron indicated addition when following the concentration addition (CA) model, which assumes additive joint actions. The same study also used the model of probabilities, which is based on the correlation of an effect caused by one chemical with the probability of an organism to be affected by it. When following this, Cu-diuron mixtures showed additive effects in all concentrations except for one where there was synergism. Zn-diuron mostly had synergistic effects, but when the Zn concentration was at its highest, antagonistic effects were observed (Koutsaftis & Aoyama, 2006). Mixtures of diuron and chlorothalonil have been found to be antagonistic, slightly antagonistic, or partially additive to the brine shrimp (Koutsaftis & Aoyama, 2007).

Gastropoda is the second most species-rich animal class (Ponder & Lindberg, 2008), meaning they may be representative as common non-target organisms that could be affected by chemicals in coastal waters. The wandering snail, *Radix balthica*, and the river nerite, *Theodoxus fluviatilis* are two common snails in Swedish inland waters and the Baltic Sea. The lymnaeid *R. balthica* is a highly adaptive pulmonate snail that tolerates salinity up to 14 psu and pH between 5.8 and 9.9. (Wetter-Schultes, 2013). It has been found at depths of 0.2 to 2 m, but can live down to 10 m (Økland, 1964). The species has not been widely used before in the

study of chemical mixtures, which is why it would be interesting to use in the present study. *T. fluviatilis* are often found in the same environments as *R. balthica*, but in coastal waters, it can live down to 60 m depths (Zettler et al., 2004). It has been found at salinities up to 18 psu in the Baltic Sea (Bunje, 2005), and pH ranging from 7.0 to 8.4 (Carlsson, 2000; Lucey et al., 1992).

While mixture toxicity studies are becoming more common, most ecotoxicological studies have focused on effects on organisms caused by single compounds (Enserink et al., 1991). This, however, has been needed as it has laid the groundwork for the study of mixture toxicity. The present study proposes to investigate the toxicity of metals (copper and zinc) and organic biocides used in antifouling agents (diuron and chlorothalonil), and their mixtures, to the brackish water snails *R. balthica* and *T. fluviatilis*, and assess the effects of these mixtures (antagonism, addition, and synergism).

Method

Experiment

Test organisms

Snails of the species *R. balthica* and *T. fluviatilis* were collected on the 2nd and 16th of November 2015, respectively, from rocks in Brunnsviken. The snails were brought back to lab where each species was kept in separate containers with aeration for three weeks to acclimatise to laboratory conditions. The salinity was controlled continuously and adjusted with a mixture of brackish water from the Baltic Sea and charcoal-filtered freshwater (from here onwards referred to as *test water*) to be held constant at 2.7 psu. During this time, the snails were fed with duckweed (*Lemna spp.*), collected from a nearby pond.

Chemical analysis

Water samples were taken from Brunnsviken and were sent for chemical analysis to see the concentrations of dissolved copper and zinc.

Preparation of solutions

Four chemicals were tested in the present study: the metals Cu and Zn, and the organic biocides chlorothalonil and diuron. Stock solutions of each chemical were prepared to a concentration of 1 g/L, using Milli-Q water to dissolve $CuCl_2 \cdot H_2O$ and $ZnCl_2$, and due to the low water solubility and half-life of diuron and chlorothalonil, dimethyl-sulfoxide (DMSO) was used to dissolve them. The stocks were stored for a few days before the tests. The experimental concentration series (table 1) were determined based on LC_{50} (Lethal Concentration 50 %, i.e. the concentration at which 50 % of the population dies) values from earlier studies (table S1) and then prepared by diluting stock solutions with the test water of a salinity around 2.7 psu.

Table 1. Concentration series for each of the chemicals in the initial 96-h LC_{50} test

	Concentration series (mg/L)				
Copper	0.030	0.050	0.070	0.090	0.11
Zinc	1.0	2.0	3.0	4.0	5.0
Diuron	5.0	10	15	20	25
Chlorothalonil	2.0	4.0	8.0	16	32

Single toxicity

Acute toxicity tests were conducted for each of the chemicals individually, the duration of which was 96 hours and the endpoint mortality. The tests were conducted in triplicates, each including one *R. balthica*, with three controls with the test water and three solvent controls with the test water and the solvent (DMSO) used for chlorothalonil and diuron. The experimental concentrations were prepared by diluting the stock solutions with test water. Salinity was measured in the lowest and highest concentrations of each chemical, and pH in all concentrations. 40 ml of each chemical of each concentration was poured into 50 ml plastic beakers. One individual of *R. balthica* was randomly picked and gently dabbed with a tissue to remove excess water which could add volume to the test solution. The shell length of each snail was measured before it was placed in one of the 50 ml beaker. Then, perforated lids were put on each beaker. The snails were not fed during the test.

After 24, 48, and 72 hours, respectively, the test solutions were renewed. The snails were considered dead if there was no movement after having been gently poked with the pointy end of a plastic spoon. The mortality was noted, and 96-h LC_{50} values were calculated by graphical interpolation for all chemicals.

Mixture toxicity

Due to an unfortunate mass mortality of *R. balthica* in the lab culture, the mixture toxicity test was conducted with *T. fluviatilis* instead. The mixture concentrations were supposed to be based solely on the 96-h LC_{50} values derived from the single toxicity test, but with the change of species, this was not entirely possible and some adjustments were made based on previous experiments for Zn, where the value was lowered. Cu did not need to be adjusted because of the similar ranges in earlier experiments, and no data was available for diuron and chlorothalonil so they were not adjusted either.

The binary mixtures tested were Cu-Zn, Cu-diuron, Cu-chlorothalonil, Zn-diuron, Zn-chlorothalonil, and diuron-chlorothalonil. They were tested in ratios of 1:4, 1:1 and 4:1, by taking 0.2, 0.5 and 0.8 times the 96-h LC_{50} of one chemical, and 0.8, 0.5 and 0.2 times the 96-h LC_{50} of the other chemical, correspondingly. The ternary mixtures were Cu-Zn-diuron, Cu-Zn-chlorothalonil, Cu-diuron-chlorothalonil, and Zn-diuron-chlorothalonil. These were tested in ratios of 1:1:1; 0.33 times the 96-h LC_{50} for each chemical. The quaternary mixture, Cu-Zn-diuron-chlorothalonil, was tested in a ratio of 1:1:1:1, 0.25 times the 96-h LC_{50} for each chemical.

The test concentrations were prepared by diluting the stock solutions with the test water. The set up was the same as for the 96-h LC_{50} test, with one individual in each container, three replicates, three controls and three solvent controls, but given the smaller body size of *T. fluviatilis* compared to *R. balthica*, 30 ml containers were used.

After 24, 48, and 72 hours, respectively, the test solutions were renewed. To determine mortality, the snails were placed in clean water for 2-3 hours when the test had ended, and if they had not moved within that time, they were considered dead.

Data processing and statistical analyses

Single toxicity

Linear regressions were fitted for the single chemical toxicity tests to establish correlations between concentration and mortality. To investigate whether the shell length affected the mortality of the snails, logistic regressions were conducted.

All data processing and graphical analyses were made using Microsoft Excel 2013. The linear regression analyses were conducted in R v.3.2.1 and R Studio v.0.99.467, and SPSS v.23 was used for the logistic regressions.

Mixture toxicity

To assess the mixture toxicity, calculations were made based upon the equations derived either from the linear or the polynomial regression lines. From the concentration of one chemical in the mixture, the percentage mortality that concentration would produce if the chemical was present singly was calculated. Then, this value was compared to the percentage mortality of the mixture. If the latter was higher or lower than the predicted value, the effect was considered either synergistic or antagonistic, respectively. If the percentage mortality of the mixture was equal to the predicted individual mortality, the effect was considered to be additive.

Results

Single toxicity

Table 2. Results from the chemical analysis of the Brunnsviken water samples

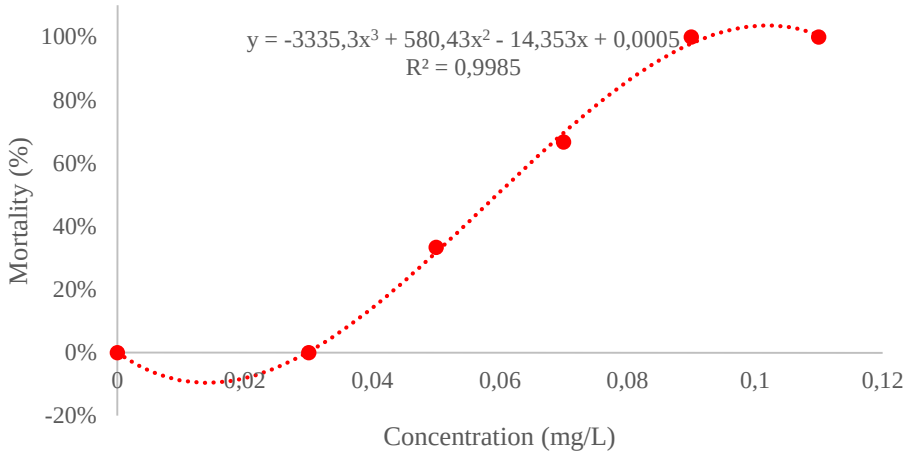
Chemical	Concentration (mg/L)
Cu	0.00154
Zn	0.0223

The measured pH in all samples was (mean ± SD) 8.01 ± 0.12 and the salinity was 2.68 ± 0.01 psu. Mortality increased with higher concentration for Cu (linear regression, $p = 0.00194$) and diuron (linear regression, $p = 0.01008$), but not for Zn (linear regression, $p > 0.05$) and chlorothalonil (linear regression, $p > 0.05$, table 3). The LC_{50} values for the individual chemicals were: 0.060 mg/L for Cu (figure 1a), 4.5 mg/L for Zn (figure 1b), 12.2 mg/L for diuron (figure 1c), and 1.0 mg/L for chlorothalonil (figure 1d). Highest mortality was caused by chlorothalonil, where all individuals in all concentrations died (figure 1d).

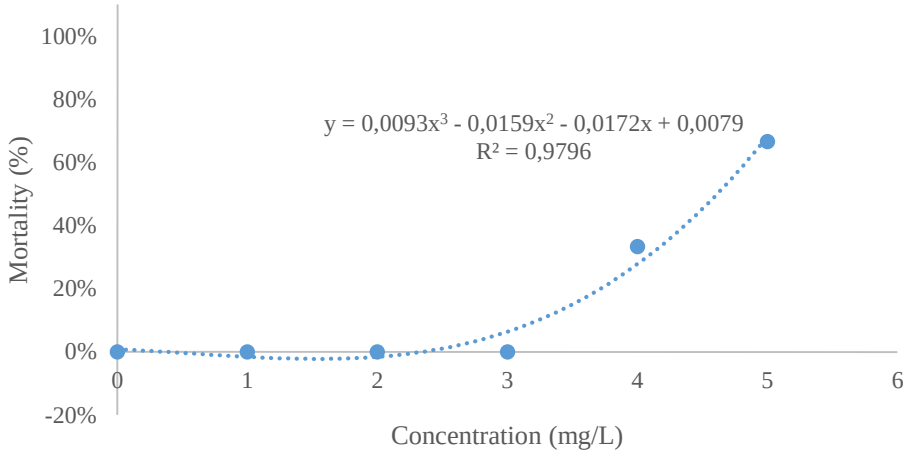
Table 3. Results from the linear regression on the log-transformed concentrations and percentage mortality

	Estimate	Std. Error	t value	Pr(>|t|)	R square	F-statistics (1 and 4 df)
Cu	1.9241	0.1864	10.32	0.00194 **	0.9726	106.6
Zn	0.8232	0.4038	2.038	0.1342	0.5807	4.1555
Diuron	1.0354	0.1778	5.824	0.01008 *	0.9188	0.01008
Chlorothalonil	1.433e-16	8.271e-17	1.732e+00	0.1635	0.5294	3.375

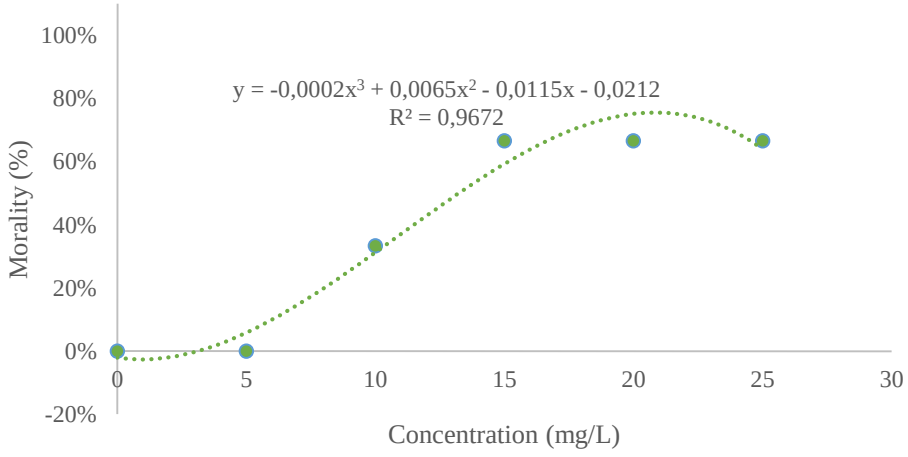
a


y = -3335,3x³ + 580,43x² - 14,353x + 0,0005
R² = 0,9985
100%
80%
60%
40%
20%
0%
-20%
Mortality (%)
0
0,02
0,04
0,06
0,08
0,1
0,12
Concentration (mg/L)

b


y = 0,0093x³ - 0,0159x² - 0,0172x + 0,0079
R² = 0,9796
100%
80%
60%
40%
20%
0%
-20%
Mortality (%)
0
1
2
3
4
5
6
Concentration (mg/L)

c


y = -0,0002x³ + 0,0065x² - 0,0115x - 0,0212
R² = 0,9672
100%
80%
60%
40%
20%
0%
-20%
Morality (%)
0
5
10
15
20
25
30
Concentration (mg/L)

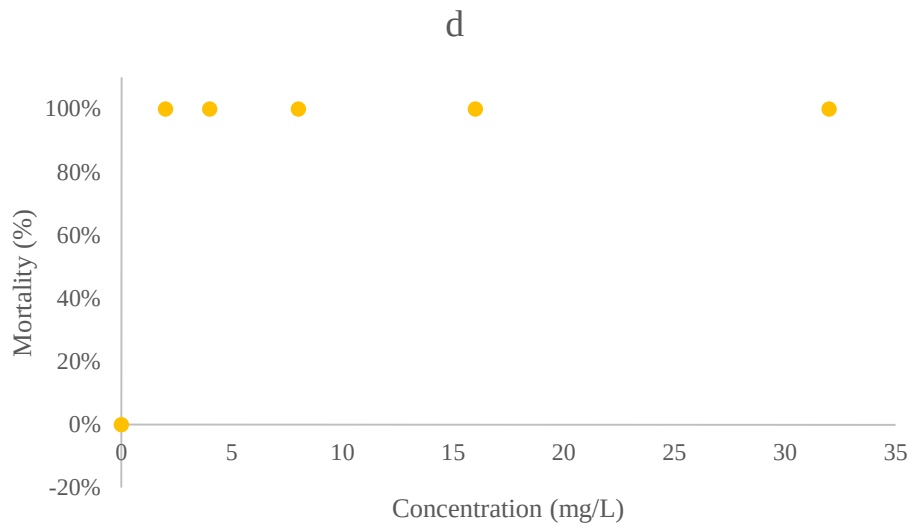


Figure 1. Concentration-response (mg/L-percentage mortality) curves for the chemicals tested: a) Cu, b) Zn, c) diuron, and d) chlorothalonil. With 3rd degree polynomial trend lines for all except chlorothalonil, where no trend line could be fitted.

The snails *R. balthica* used in the present study had a larger size range (7-20 mm) than lymnaeid snails used in previous studies (20 ± 3 mm, table S1), but shell length was not correlated with mortality (logistic regression, p >> 0.05, data not shown).

Mixture toxicity

The measured pH in all samples was (mean ± SD) 7.72 ± 0.12, and salinity was 2.71 ± 0.04 psu. The adjusted LC_{50} value for Zn was 3.0 mg/L. The equations from the linear regression were used for Cu (equation 1, $R^2 = 0.9726$) and diuron (equation 2, $R^2 = 0.9188$) to calculate the predicted toxicity:

$$\%\, mortality = 1.9241x + 2.9025 \text{ (equation 1)}$$

$$\%\, mortality = 1.0354x - 0.6876 \text{ (equation 2)}$$

In which x is the log-transformed concentration of the single chemical in the mixture. To achieve a higher R^2-value for Zn, the 3rd degree polynomial regression was used (equation 3, $R^2 = 0.9796$). Due to the inability to fit a trend line to chlorothalonil, no calculations were made on it, but it was included in its mixtures in the comparisons with the other substances.

$$\%\, mortality = 0.0093x^3 - 0.0159x^2 - 0.0172x + 0.0079 \text{ (equation 3)}$$

In which x is the concentration of the single chemical in the mixture. Copper and zinc had synergistic effects in all mixtures except the 4:1, where there was slight antagonism. The 4:1 Cu-diuron mixture also showed antagonism, but in the 1:4 and 1:1 mixtures, the effects were additive. In all Cu-chlorothalonil mixtures, as well as all ternary and the quaternary mixtures, synergistic effects were seen (figure 2a). Zn-diuron and Zn-chlorothalonil all had synergistic effects, with the exception of Zn-diuron 1:4, where addition was seen (figure 2b). The diuron-chlorothalonil 4:1 mixture had slightly antagonistic effects, but the 1:4 and 1:1 mixtures were synergistic (figure 2c). Overall, 74 % of the mixtures proved to have synergistic effects, while antagonism and addition each occurred in 13 % of the mixtures (table S2).

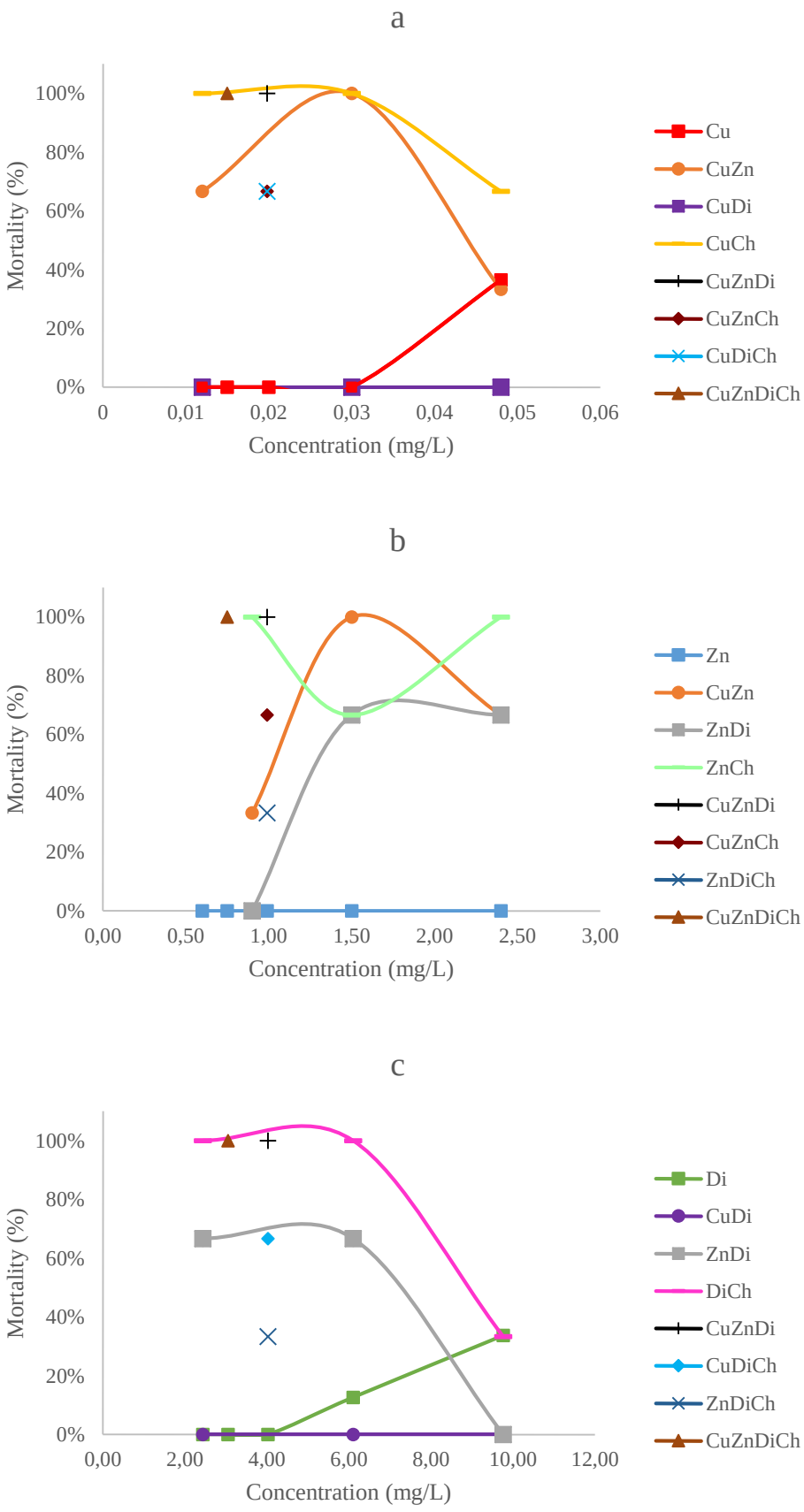


Figure 2. The effects in percentage mortality caused by the mixtures compared to the chemical alone at the same concentration, for a) Cu, b) Zn, and c) diuron.

Discussion

The 96-h LC_{50} values obtained for Cu, Zn and diuron in the present study (0.06, 4.5 and 12.2 mg/L, respectively, figure 2a, b, c) were all in line with the values collected from literature (0.058, 3.5 and 15.3 mg/L, respectively, table S1). This was the case despite the wider size range of *R. balthica* in this study compared to lymnaeid snails in the previous studies (table S1), but the size had no correlation with mortality (logistic regression, $p >> 0.05$, data not shown). The LC_{50}s of Cu and Zn were higher than the levels found in their natural habitat, Brunnsviken (0.00154 and 0.0223 mg/L, respectively, table 2). They were also higher than concentrations that have been detected in various areas of the Baltic Sea (Herbert et al., 2009). However, LC_{50} values are used in risk assessments mainly to represent the worst case scenario, and at lower concentrations, other toxic effects than mortality may be seen.

Since all snails in every replicate died in the single toxicity test for chlorothalonil (figure 1d), the concentration range used in the test must have been far off. Few studies have been published on the toxicity of chlorothalonil to molluscs, even less so to gastropods, thus it could be discussed that the reference values (table S1) were not suitable. The lowest concentration in the range used in the present study was precautionary lower than the values gathered from literature, and still the mortality was surprisingly high in comparison. No previous toxicity data was available for *R. balthica* or any member of its family though, so one explanation could be that this species is more sensitive to chlorothalonil than the ones used in earlier studies. Ideally, a single toxicity test with lower concentrations should have been made to obtain a correct LC_{50} value, although the limited time frame of this bachelor's project as well as the mass mortality of the *R. balthica* prevented this. With the change of species to *T. fluviatilis*, it would also be ideal to conduct a new single toxicity test to attain correct values for the new species. However, the LC_{50} values obtained in the single toxicity tests, and the adjustments made to them, seemed to fit the change of species in the mixture toxicity tests.

In the mixture toxicity tests to *T. fluviatilis*, the same substances in varying concentrations expressed different effects, which has also been seen in previous studies (Meyer et al., 2015). In two of the Cu-Zn mixtures, synergistic effects were found (figure 2a, b), which is consistent with what has been studied with *D. magna* (Meyer et al., 2015). The antagonistic effects that Cu can have on the uptake of Zn (Luoma, 1984) is a possible explanation to the slight antagonism seen in the 4:1 Cu-Zn mixture (figure 2a), which contained the highest Cu concentration. The possibility of Cu to prevent the uptake of Zn should increase when the concentration of the former is higher, so this may be the case here. However, when adding diuron or chlorothalonil, or both chemicals, synergism occurs again.

Effects caused by diuron-chlorothalonil mixtures were additive in the 1:4 and 1:1 mixtures, but slightly antagonistic in the 4:1 mixture (figure 2c). The same effects were seen in the corresponding mixtures of Cu and diuron (figure 2a). These results are in line with those of the combined effects of Cu-diuron mixtures on *L. minor*, where the slight antagonism possibly was due to diuron blocking copper-induced oxidative stress (Teisseire et al., 1999). Chlorothalonil may also increase the occurrence of ROSs as it depletes the ROS scavenger glutathione (Cedergreen, 2014), and diuron might prevent oxidative stress in this case also. The antioxidant properties of diuron could be a possible reason to the slight antagonism in the present study; however, the duration of this test might have been too short for any major ROS-caused damage to arise.

The additive effects found in the 1:4 and 1:1 mixtures of Cu and diuron (figure 2a) are in line with studies on the brine shrimp, that mostly showed additive effects after being exposed to Cu-diuron mixtures (Koutsaftis & Aoyama, 2006). Zn-diuron mixtures in the same study indicated synergism, which was also seen in the present study for the 1:1 and 4:1 mixtures (figure 2b). Cu-chlorothalonil mixtures were all synergistic (figure 2a), which could perhaps be explained by the ability of both chemicals to bind to sulfhydryl groups on proteins (Wendt, 2013; Sherrard et al., 2003). As no previous studies have been made on the ternary and quaternary mixtures of these chemicals, something worth noting is that in this study, all of those mixtures had synergistic effects (figure 2a-c). Organisms are often exposed to chemical mixtures in the environment (De Zwart & Posthuma, 2005), which makes it worrisome seeing the increased toxicity of the mixtures in the present study. The chemicals are more toxic in low concentrations in mixtures than they are alone at the same concentrations. This emphasizes the relevance of mixture toxicity as a field of study, since they may pose threats to the environment, where even more substances are present than the ones used in this study.

In conclusion, further studies are needed on the mixture toxicity of these, and other, antifouling substances. Mixtures of the same substances caused different types of chemical interactions when the concentrations varied. The slight antagonistic effects seen in binary mixtures containing diuron could be due to its previously studied ability to prevent oxidative stress. It would be interesting to further investigate toxicant-induced oxidative stress to gastropods in a longer study, and the possible influence of diuron on it. The majority (74 %) of the interactions produced by the mixtures were synergistic, which possibly makes mixture toxicity an important focus in future studies, one approach of which could be to study the toxicity of these mixtures to other species than those used in the present study. Another possible area to explore further would be mixture toxicity on multiple species, perhaps using species from different trophic levels and classes, in order to achieve a more ecologically relevant overview of the toxicity of mixtures.

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Appendix

Table S1. LC_{50} values from literature for the chemicals used in the present study. The shadowed areas are lymnaeid snails. (NR = not reported, FW = freshwater)

Chem	LC_{50} (µg/L)	Time (h)	Test organism	Class	Salinity	Size/age	Reference
Cu	87	96	*Haliotis rubra*	Gastropod	NR	23-35 mm/ 1.5 y	Gorski & Nugegoda, 2009
	570	96	*Meretrix casta*	Bivalve	25 psu	33-40 mm	Kumaraguru et al., 1980
	2101	24	*Cerithedia cingulata*	Gastropod	30 psu	23 mm	Ramakritinan et al., 2012
	1152	48	*Cerithedia cingulata*	Gastropod	30 psu	23 mm	Ramakritinan et al., 2013
	1052	72	*Cerithedia cingulata*	Gastropod	30 psu	23 mm	Ramakritinan et al., 2014
	521	96	*Cerithedia cingulata*	Gastropod	30 psu	23 mm	Ramakritinan et al., 2015
	137	24	*Modiolus philippinarum*	Bivalve	30 psu	10 mm	Ramakritinan et al., 2016

76	48	*Modiolus philippinarum*	Bivalve	30 psu	11 mm	Ramakritinan et al., 2017
38	72	*Modiolus philippinarum*	Bivalve	30 psu	12 mm	Ramakritinan et al., 2018
23	96	*Modiolus philippinarum*	Bivalve	30 psu	13 mm	Ramakritinan et al., 2019
902	12	*Radix luteola*	Gastropod	FW	19.2-22.5 mm	Mathur et al. 1981
491	24	*Radix luteola*	Gastropod	FW	19.2-22.5 mm	Mathur et al. 1982
341	48	*Radix luteola*	Gastropod	FW	19.2-22.5 mm	Mathur et al. 1983
172	96	*Radix luteola*	Gastropod	FW	19.2-22.5 mm	Mathur et al. 1984
81	24	*Radix luteola*	Gastropod	FW	19-23 mm	Khangarot & Ray, 1988
52	48	*Radix luteola*	Gastropod	FW	19-23 mm	Khangarot & Ray, 1988
27	72	*Radix luteola*	Gastropod	FW	19-23 mm	Khangarot & Ray, 1988
27	96	*Radix luteola*	Gastropod	FW	19-23 mm	Khangarot & Ray, 1988
30.7	96	*Lymnaea stagnalis*	Gastropod	FW	7 days	Brix et al., 2011
24.9	96	*Lymnaea stagnalis*	Gastropod	FW	19-23 mm/ juvenile	Ng et al., 2011
34.22	96	*Pomacea paludosa*	Gastropod	FW	2 days	Rogevich et al., 2008
42.77	96	*Pomacea paludosa*	Gastropod	FW	14 days	Rogevich et al., 2008
44.88	96	*Pomacea paludosa*	Gastropod	FW	30 days	Rogevich et al., 2008
141.7	96	*Pomacea paludosa*	Gastropod	FW	60 days	Rogevich et al., 2008
182	96	*Pomacea paludosa*	Gastropod	FW	120 days	Rogevich et al., 2008
102	24	*Lymnaea acuminata*	Gastropod	FW	1.8-2.3 mm	Khangarot et al., 1982
49	48	*Lymnaea acuminata*	Gastropod	FW	1.8-2.3 mm	Khangarot et al., 1982
39	72	*Lymnaea acuminata*	Gastropod	FW	1.8-2.3 mm	Khangarot et al., 1982
34	96	*Lymnaea acuminata*	Gastropod	FW	1.8-2.3 mm	Khangarot et al., 1982
820	24	*Melanoides tuberculata*	Gastropod	FW	15-20 mm	Shuhaimi-Othman et al., 2012
390	48	*Melanoides tuberculata*	Gastropod	FW	15-20 mm	Shuhaimi-Othman et al., 2012
210	72	*Melanoides tuberculata*	Gastropod	FW	15-20 mm	Shuhaimi-Othman et al., 2012

	140	96	*Melanoides tuberculata*	Gastropod	FW	15-20 mm	Shuhaimi-Othman et al., 2012
	8540	96	*Babylonia areolata*	Gastropod	34.74 psu	Adult	Vedamanikam & Hayimad, 2013
	4980	96	*Babylonia areolata*	Gastropod	34.74 psu	Juvenile	Vedamanikam & Hayimad, 2013
Zn	4900	24	*Haliotis rubra*	Gastropod	NR	23-35 mm/ 1.5 y	Gorski & Nugegoda, 2009
	1730	96	*Haliotis rubra*	Gastropod	NR	23-35 mm/ 1.5 y	Gorski & Nugegoda, 2009
	59101	24	*Cerithedia cingulata*	Gastropod	33 psu	23 mm	Ramakritinan et al., 2012
	31229	48	*Cerithedia cingulata*	Gastropod	33 psu	23 mm	Ramakritinan et al., 2012
	16389	72	*Cerithedia cingulata*	Gastropod	33 psu	23 mm	Ramakritinan et al., 2012
	8990	96	*Cerithedia cingulata*	Gastropod	33 psu	23 mm	Ramakritinan et al., 2012
	17520	96	*Babylonia areolata*	Gastropod	34.74 psu	Adult	Vedamanikam & Hayimad, 2013
	15190	96	*Babylonia areolata*	Gastropod	34.74 psu	Juvenile	Vedamanikam & Hayimad, 2013
	10230	12	*Radix luteola*	Gastropod	FW	19.2-22.5 mm	Mathur et al., 1981
	8190	24	*Radix luteola*	Gastropod	FW	19.2-22.5 mm	Mathur et al., 1981
	7730	48	*Radix luteola*	Gastropod	FW	19.2-22.5 mm	Mathur et al., 1981
	613	96	*Radix luteola*	Gastropod	FW	19.2-22.5 mm	Mathur et al., 1981
	7000	24	*Radix luteola*	Gastropod	FW	19-23 mm	Khangarot & Ray, 1988
	3800	48	*Radix luteola*	Gastropod	FW	19-23 mm	Khangarot & Ray, 1988
	3800	72	*Radix luteola*	Gastropod	FW	19-23 mm	Khangarot & Ray, 1988
	1680	96	*Radix luteola*	Gastropod	FW	19-23 mm	Khangarot & Ray, 1988
	2181	24	*Radix luteola*	Gastropod	FW	19.2-22.5 mm	Khangarot & Ray, 1987
	1540	48	*Radix luteola*	Gastropod	FW	19.2-22.5 mm	Khangarot & Ray, 1987
	1140	72	*Radix luteola*	Gastropod	FW	19.2-22.5 mm	Khangarot & Ray, 1987
	1100	96	*Radix luteola*	Gastropod	FW	19.2-22.5 mm	Khangarot & Ray, 1987

	2E+05	24	*Nassarius obsoletus*	Gastropod	20 psu	19-24 mm	Eisler & Hennekey, 1977
	50000	96	*Nassarius obsoletus*	Gastropod	20 psu	19-24 mm	Eisler & Hennekey, 1977
	7400	168	*Nassarius obsoletus*	Gastropod	20 psu	19-24 mm	Eisler & Hennekey, 1977
	16130	24	*Lymnaea acuminata*	Gastropod	FW	1.8-2.3 mm	Khangarot et al., 1982
	11740	48	*Lymnaea acuminata*	Gastropod	FW	1.8-2.3 mm	Khangarot et al., 1982
	11150	72	*Lymnaea acuminata*	Gastropod	FW	1.8-2.3 mm	Khangarot et al., 1982
	10490	96	*Lymnaea acuminata*	Gastropod	FW	1.8-2.3 mm	Khangarot et al., 1982
	33970	24	*Melanoides tuberculata*	Gastropod	FW	15-20 mm	Shuhaimi-Othman et al., 2012
	13150	48	*Melanoides tuberculata*	Gastropod	FW	15-20 mm	Shuhaimi-Othman et al., 2012
	4730	72	*Melanoides tuberculata*	Gastropod	FW	15-20 mm	Shuhaimi-Othman et al., 2012
	3900	96	*Melanoides tuberculata*	Gastropod	FW	15-20 mm	Shuhaimi-Othman et al., 2012
	1400	96	*Gyraulus sp*	Gastropod	FW	NR	Mebane et al., 2012
	1451	96	*Gyraulus sp*	Gastropod	FW	NR	Mebane et al., 2012
Diu	33200	24	*Lymnaea spp.*	Gastropod	FW	NR	Christian & Tate, 1983
	30300	48	*Lymnaea spp.*	Gastropod	FW	NR	Christian & Tate, 1983
	28600	72	*Lymnaea spp.*	Gastropod	FW	NR	Christian & Tate, 1983
	15300	96	*Lymnaea spp.*	Gastropod	FW	NR	Christian & Tate, 1983
Chl	5940	96	*Mytilus edulis*	Bivalve	29.98-31.2 psu	59 mm	Ernst et al., 1991
	34780	96	*Mya arenaria*	Bivalve	29.98-31.2 psu	52 mm	Ernst et al., 1991
	837	96	*Mercenaria mercenaria*	Bivalve	NR	Juvenile	DeLorenzo et al., 2004
	9000	48	*Semisulcospira libertina*	Gastropod	NR	NR	Hashimoto & Nischiuchi, 1981
	15000	48	*Indoplanorbis exustus*	Gastropod	NR	NR	Hashimoto & Nischiuchi, 1981

	37000	48	*Physa acuta*	Gastropod	NR	NR	Hashimoto & Nischiuchi, 1981
	280	96	*Lampsilis siliquoidea*	Bivalve	NR	Juvenile	Bringolf et al., 2007

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Table S2. Effects caused by the different mixtures (ant = antagonism, add = addition, syn = synergism)

Concentration in mixture (mg/L)				**Ratio**	**Predicted single compound mortality (%)**			**Mixture mortality (%)**	**Effect**
Cu	***Zn***	***Di***	***Ch***		***Cu***	***Zn***	***Di***		
0,012	2,400			1:4	0	0		66,7	Syn
0,030	1,500			1:1	0	0		100	Syn
0,048	0,600			4:1	36,5	0		33,3	Ant*, **
0,012		9,760		1:4	0		33,7	0	Add*
0,030		6,100		1:1	0		12,6	0	Add*
0,048		2,440		4:1	36,5		0	0	Ant*
0,012			0,800	1:4	0			100	Syn
0,030			0,500	1:1	0			100	Syn
0,048			0,200	4:1	36,5			66,7	Syn
	0,600	9,760		1:4		0	33,7	0	Add*
	1,500	6,100		1:1		0	12,6	66,7	Syn
	2,400	2,440		4:1		0	0	66,7	Syn
	0,600		0,800	1:4		0		100	Syn
	1,500		0,500	1:1		0		66,7	Syn
	2,400		0,200	4:1		0		100	Syn
		2,440	0,800	1:4			0	100	Syn
		6,100	0,500	1:1			12,6	100	Syn
		9,760	0,200	4:1			33,7	33,3	Ant**
0,020	0,990	4,026		1:1:1	0	0	0	100	Syn
0,020	0,990		0,330	1:1:1	0	0	0	66,7	Syn
0,020		4,026	0,330	1:1:1	0	0	0	66,7	Syn
	0,990	4,026	0,330	1:1:1	0	0	0	33,3	Syn
0,015	0,750	3,050	0,250	1:1:1:1	0	0	0	100	Syn

** Two results, but the one chosen has the best fit, i.e. the highest R^2: Cu > Zn > diuron.*
*** Slightly antagonistic.*

Deliverable 4.4 Report/scientific articles on development of new bioassays for monitoring sub-lethal effects on chemically mediated behaviours in gastropods and crustaceans.

Appendix 2

Influence of Heavy metal mixture on uptake and depuration

by freshwater pond snail *Lymnaea stagnalis,*

Daniel Ahlström

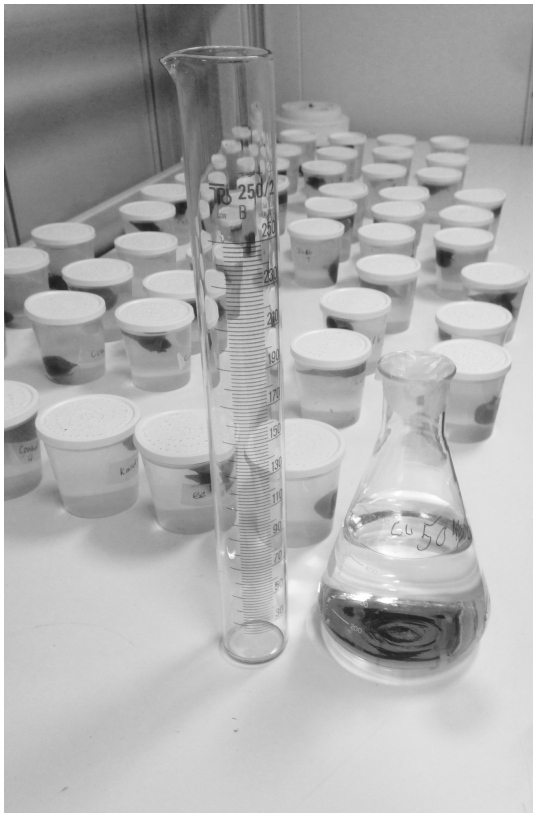
Bachelor's thesis, 15 HE credits, Environmental science, 2016:2

Stockholm University

Influence of Heavy metal mixture on uptake and depuration by freshwater pond snail *Lymnaea stagnalis*

Daniel Ahlström

Bachelor's thesis, 15 HE credits
Environmental science, 2016:2



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Department of Environmental Science and Analytical Chemistry

Influence of Heavy metal mixture on uptake and depuration by freshwater pond snail *Lymnaea stagnalis*

Daniel Ahlström

Maria Bighiu & Ann-Kristin Eriksson Wiklund

ABSTRACT

In the aquatic environment, trace metals such as the essential copper and zinc, and the non-essential cadmium are naturally occurring at low levels. Organisms have taken advantage of the essential metals and developed specific regulation mechanisms regarding uptake and depuration. A common source for increased levels copper and zinc is antifouling paints, and for cadmium runoff from use of fertilizers in agriculture. Aquatic organisms' uptake of ions is mainly from the water. *Lymnaea stagnalis* is freshwater gastropod that has been implied by several studies to be sensitive to single metal exposure, and that it is good at accumulating metals. In the present study, a 96 hours acute toxicity test and an uptake and depuration experiment were conducted. The acute toxicity test gave the following LC_{50} for copper, zinc and cadmium: 175, 3400 and 1200 μg/g respectively. For uptake it was found in this study that the length of the snail and uptake of metals in single exposure over time had a significant correlation. In binary mixtures, it seems that copper has an effect on the uptake and lowered the concentration of zinc and cadmium. During depuration, *L. stagnalis* can eliminate the accumulated concentrations of copper and zinc back to background levels, but none such outcome was found for cadmium.

Key Words

Lymnaea stagnalis, Metal, Toxicity, Mixture, Uptake, Depuration, Copper, Zinc, Cadmium

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Appendix 22

INTRODUCTION

In the environment, trace metals are naturally occurring from rocks and soil via weathering (Kabata-Penials 2010). Organisms take advantage of this and use them for essential biological processes such as metabolism, oxygen transport, and macromolecular structures (Deplegde 1990). Every organism has an optimum concentration window for essential trace metals that has to be maintained for normal functions. Low levels could cause different deficiencies and too high levels will create toxicity. This optimum window could be narrow between what is optimal and what is toxic (Walker et al 2012). Copper (Cu) and zinc (Zn) are important essential trace metals and macronutrients for many species. Cu is important in the antioxidant system and too high levels are toxic for the cells and can lead to oxidative stress (Nevitt et al 2011; Walker et al 2012). Zn is important for major metabolic pathways as a cofactor or stabilisation of enzymes (Valle & Alud 1990; Walker et al 2012). Too high concentrations of Zn can become neurotoxic and interfere with different signal pathways, and also interfere with the reactive oxygen species (ROS) detoxification process (Bishop et al 2007; Sensi et al 2004). Cadmium (Cd) is a non-essential trace metal that is naturally occurring in the environment at low levels, but also can enter the aquatic environment via runoffs from the agricultural use of fertilizers or from factories (Zhenli et al 2005). Besides causing toxicity to organisms at low levels, cadmium has been shown to induce deficiency of essential trace metals and affect their homeostasis. By competing for the active site with essential trace metals, Cd can interfere with many different metal dependent proteins (Nzegue et al 2011; Walker et al 2012). The availability of metals in an aquatic environment can be affected by physical factors such as alkalinity, hardness of water, pH, dissolved oxygen, temperature, organic substances (DOC) etc. All these factors can either increase or reduce the toxicity of the metal (Hoang & Tong 2015; Khangarot & Ray 1987; Wang 1987). Also the tolerance, size, nutrient and life stage of the organism has importance, which gives the big variety between reactions among different species (Chubbad et al., 2011; Wang 1987). Recently it has been found that genetic variation is also of great importance for different tolerances within a species (Côte et al 2015). All these different factors contribute to the complexity when studying metal toxicity (Louma 1983).

The uptake of metals can be from both food and from free dissolved ions in the water (Louma 1983). For Cu, Zn and Cd, it is the uptake from dissolved ions in the water that is the major control of ion levels in aquatic organisms (Louma 1983). The organisms have developed specific pathways for the uptake of essential metals. The pathways could be divided into three general groups: uptake by diffusion, create a complex of polar form, and endocytosis (Louma 1983, Walker et al. 2012). Cu influx to cells is mainly through specific cell membrane channels (Nevitt et Al 2012). Zn is transported into the cell via the proteins belonging to the ZIP super family. For non-essential metals such as Cd, the uptake occurs passively with similar pathways as for several different essential metals (Nzegue et al 2011). Studies have indicated that in the presence of Zn, ZIP family proteins in the cell membrane can increase the influx of Cd in to the cell (He et al 2009).

It is important for the organism to have mechanisms to regulate the levels of metals, especially if metal levels are becoming excessive in the surrounding environment of the organism. The regulation is done by encapsulation of metals in insoluble forms in granules which are either stored or excreted via faeces (Walker et al. 2012). It is important that this is

done fast to prevent the metals to enter cells and interfere with biological processes (Desouky 2005). The regulation and detoxification of metals are usually done in the digestive gland or kidneys (Walker et al 2012). There are different types of granules. Cu, Zn and Cd belong to class B metals, which have a preference to bind to ligands that have sulphur or nitrogen as a donor atom. They will bind to metalloproteins in the cytoplasm and can be integrated in granules called Type B (Dillinger & Berger 1993, Dallinger 1996). The process of elimination is an energy cost for the organism, that also increases the stress for the individual. It will reduce the fitness, and could finally lead to death (Walker et al 2012).

To investigate how metal toxicity affects the aquatic environment, tests with single metals on one species at a time are far the most common (Norwood et al 2003). Most frequently used is acute toxicity testing. Two common parameters in toxicity testing are Lethal Concentration 50 (LC_{50}) and it is the concentration that kills 50% of the population. The "No Observed Effect Concentration" or NOEC is the concentration before any effect is shown in the tested population (Walker et al 2012). In the environment, it is rarely only one metal that it is present to cause a toxic effect. It is more often a mix of different metals and other pollutants that together can, for example, exert their toxicity (Pan 2014). A mix of pollutants can act in different ways: additive (the effect of two pollutants express the same toxicity in a mixture as tested alone), synergistic (a pollutant become more potentiated by another pollutant and the effect is larger than predicted), or antagonistic (when one pollutant inhibits the effect of another) (Cedergren 2014; Walker et al 2012). Earlier studies indicate that the combination of Cu, Zn and Cd have either synergistic or antagonistic effects, rather than additive effects (Pan 2014). When testing binary mixtures of Cu+Zn, Cu+Cd and Zn+Cd, synergistic effects were shown on *Daphnia magna*, *Penaeus stiferus* and *Gobiocypris rarus* (Cedergren 2014). When studying the organism *Hyella azteca* it was found that Cu, Zn and Cd can influence each other both in uptake and bioconcentration when in binary mixtures (Shuhaimi-Othman & Pascoe 2006). Cu, Zn and Cd in tertiary mixtures are not well explored but Meyer et al (2015) found that effects can be additive, synergistic and antagonistic. In general, it is still a gap in understanding the effects of binary and tertiary metal mixture toxicity (Norwood et al 2003).

The freshwater pulmonate *Lymnaea stagnalis* is widely spread in the northern hemisphere, and in the ecosystem, they have an important role in consumption and decomposition of aquatic plants and epiphytes (Berrie 1965). Pulmonates such as *L. stagnalis* lack gills and use aerial respiration as their primary source of breathing (Jones 1961). This has lead to the main transportation of ions being across the skin. The transport of ions is focused mainly around the area of the foot which is covered in mucus that can act as a weak negative ion exchanger (Schlichter 1982). Gastropods are found to be good for monitoring the levels of metals in the aquatic environment (Gundacker 1999; Khangarot et al 1982; Lau et al., 1998; Shuhaimi-Othman et al 2012). *L. stagnalis* has been suggested as a good indicator species for heavy metal pollution due to their great capacity of accumulating heavy metals (V- Balogh et al 1988). They are also relatively large organisms for analyses and they are geographically widespread (Berrier 1965; Desoukey 2005). Several studies imply that *L. stagnails* is exceptionally sensitive to metals (Co, Pb, Ni and Cu) in chronic exposure (Grosell et al 2006; De Shamphelaere et al 2008; Schlekat et al 2010; Brix et al 2011). It has also been shown that *L. stagnalis* is among the most sensitive organisms to Cu in acute exposure (Brix et al 2011; Ng et al 2011.), but less sensitive towards chronic Zn exposure (De Schamphelaere & Janssen 2010). For Cd, it has been found that *L. stagnalis* is tolerant to levels up to 2500 µl/L acute

exposure under 48 h (Coeurdassier et al 2004). Also, the incorporation of Cd can lower the naturally occurring levels of Zn in the snail (Présing et al 1993). Trace metals are mostly accumulated in the digestive gland and the liver, but they can be found in most tissues of *L. stagnalis* (Desouky 2005). The sensitivity to metals and other pollutants indicate *L. stagnalis* to be a good test organism in ecotoxicology (Bandow 2012).

One source of metals to the aquatic environment is antifouling paints. These paints often contain metals and are used on boat hulls to prevent attachment of fouling organism (Fernandez-Alba et al 2002). These paints used to earlier include Cd, but today they consist of combinations that contain Cu or Zn and a biocide. It is indicated that these metals also leak out to aquatic environment and affect non-target organisms. (Konstantinou & Albanis 2004; Singh & Turner 2008; Tomas 2010; Valkris et al 2003).

The aim of this bachelor thesis is to investigate if there is any difference in uptake and depuration for the freshwater snail *Lymnaea stagnalis* of copper, zinc and cadmium, if they are in single, binary or tertiary mixtures.

MATERIALS AND METHOD

Two different experiments have been conducted: Part I, an acute toxicity test for 96 hours and Part II, an uptake and depuration experiment. From the acute toxicity test the LC_{50} and NOEC were conducted. The uptake and depuration experiment was conducted in two phases using the same organism.

Experiment

Snail culture

Lymnaea stagnalis were collected form a pond using a hop net in the Bergius Botanical Gardens next to Stockholm University on the following dates: 2, 11, 12 and 16 of November 2015. The snails were kept in a temperate room at 17°C in two aerated 50 L plastic buckets filled with water collected from the pond. They were feed a diet of *Lemna minor* that was gradually switched over to *Lactuca sativa.* After a three-day settlement period, the water was switched gradually over a seven-day phase to water that was filtered freshwater. The water was communal water from lake Mälaren and it had passed through two particle filter and one charcoal filter. The filtered freshwater was used for both experiment part I and part II.

Background metal levels

For measurement of the background level of metals, five snails were randomly selected from the snail culture. They were measured, weighed and washed in EDTA 10 μM solution for five minutes. The liquidation of the snails was done by the use of liquid nitrogen. Once the snails were dead, they were placed in pre-weighed aluminium cups and dried in an oven at 60°C for 48 hours. The shell and the soft tissue were separated and the dried snail soft tissue was grinded to a fine powder by hand using acid washed mortle and pestle made of ceramic. The powder was then transferred in to plastic zip lock bag. An analysis was done with XRF to detect the level of metals.

XRF

X-ray fluorescence (XRF) is a technique that uses the high energy in form of short wavelength X-rays to produce a fluorescent light from an element. This happens when atoms in an element become excited, the X-rays will eject electron or electrons from the atom. Around the atom there are different electron orbitals, and the energy from X-rays may eject electrons from the inner orbitals. When electrons are removed from an atom it will become instable, and if it is possible, an electron from an outer orbital will "drop" in to take the ejected electrons place. When dropping in, energy in form of a photon will be released that is equal to the energy between the orbitals, and every element has their own characteristic energy release between the orbitals. The fluorescent photon energy can be used by the XRF to determine which element and how much of the element that is present (Beckhoff et al 2007).

The instrument used was a portable Delta XRF (Olympus). The soil module was used for measurements with three X-ray beams: one of 30, one of 40 and one of 50 keV. Several certified samples were used as references: SiO_2(blank), Nist 2710a, Nist 2711a, CCRMP TILL 4 and PACS-3. They were re-run every 20th sample. The running time for each beam was 40 seconds and each sample was beamed three times after a small anti-clockwise movement. Both for investigating the background metal levels and metal analysis in uptake and depuration experiment the XRF technique was used. The limits of detection were the following Cu 6 μg/g, Zn 3.8 μg/g and Cd 3.2 μg/g.

A comparison between the X-ray absorbance of plastic cups, zip lock bags and mylar film was performed in order to decide which is the most suitable for analysing the snail samples (i.e. the material with the lowest absorption was chosen). The background grinded snails were used as reference and they were scanned with XRF in one of the container at a time. The XRF scanning procedure was done as described above.

Part I Acute toxicity test (96h)

55 adult *L. stagnalis* with shell length of 39.42 ± 4.86 mm (average ± standard deviation) were randomly selected from the snail culture. They were starved one day before to avoid the accumulation of faeces in the test water. This was done to prevent accumulation of metals to the faeces, which could have reduced the overall metal concentration in the test water. Acute toxicity tests were conducted for the following single metals: Cu, Zn and Cd, in the concentration series seen in Table 1. As a control test solution filtered fresh water was used. The test consisted of three replicates for each metal concentration and four replicates for the control. Acid washed 170 ml plastic beakers, which had lids with premade air holes were used as test beakers. One snail was transferred into each beaker. Metal concentrations were prepared by diluting stock solutions (1000 μg/L) of copper sulphate ($CuSO_4$ H_2O), zinc chloride ($ZnCl_2$) and cadmium following the concentration series in Table 1. The tests were performed under static conditions and the solutions were renewed every day, and no food was provided. The experiment was carried out in temperature-controlled room at 17°C and the light interval was 12:12 light and dark. The pH was measured in each beaker daily using a Mettler Toledo Five easy pH meter. To determine if a snail was dead a plastic tweezers was used to touch the foot of the snail and lack of response indicated death. This was performed each day and dead snails were removed. At the end of experiment all snails were rinsed in EDTA solution of 10 μM for 5 min. Surviving snails were first stunned with carbonated water

for five minutes and frozen in at –30°C. Snails that died before the end of the experiment were treated in the same procedure except that they were not placed in carbonated water.

Table 1. Nominal concentrations used in the acute 96 h toxicity test

	1	2	3	4	5
Cu (µg/L)	25	50	100	200	400
Zn (µg/L)	1000	2000	3000	4000	5000
Cd (µg/L)	500	1000	1500	2000	2500
Clean water	-	-	-	-	-

Part II Uptake and depuration

The experiment was conducted during six days divided into a three-day uptake and an equally long depuration phase. The design consisted of seven different single and mixed metal exposures using Cu, Zn and Cd (Table 2). As a precaution, the concentrations for the single metal used were based on 70 % of NOEC obtained from the part I experiment. Single metal concentrations were set to equal one fraction toxic unit. The concentration in binary mixture was 0.5 of the single metal concentration and the tertiary mixture was 0.33 of the single metal concentration (Table 2). This so that the combined toxicity should not be more than one toxicity fraction unite for binary and tertiary mixture (Table 2). The metal concentrations were prepared by diluting stock solutions of 1000 (µg/L) of copper sulphate ($CuSO_4$ H_2O), zinc chloride ($ZnCl_2$) and cadmium (Table 2). All treatments consisted of three replicates except the control which had four. The experiment was conducted in a thermo constant room at 20°C with a photoperiod of 12:12 light and dark. The pH was measured everyday using Mettler Toledo Five easy pH meter.

A total of 175 adult *L. stagnalis* with a shell length of 42.03 ± 4.86 mm were kept in a two aerated 50 L plastic buckets. Before starting the experiment all snails were moved over to one 50 L plastic bucket. From the bucket seven snails were randomly selected and transferred into an acid washed 1000 ml plastic beaker that was filled with 900 ml of solution. Every snail was fed daily a disc of *L. sativa* measuring 17mm in radius. During the daily renewal of solutions, food and faeces were removed to avoid accumulation of metals in the organic substance and also to reduce the risk of oxygen deprivation due to decomposition. At the following times: 24, 48, 72, 96, 120, and 144 hours, one snail was randomly picked out from each treatment. The snails were weighed, measured, and washed in EDTA solution of 10 µM for five minutes. They were transferred in to carbonated water five minutes until they were stunned. They were moved over to pre-weighed aluminium cups and dried in an oven at 60°C for 72 hours. The snails were washed in EDTA first to minimize the time from being stunned until they were put in the oven. Before starting the depuration phase the remaining snails were washed in filtered fresh water for five minutes. They were then transferred into acid washed 800 ml glass aquariums that were filled with 500 ml of filtered fresh water. Renewal of water, food and removal of faeces followed the same procedure as for the uptake phase.

All dried snails were put into a desiccator for at least 48 hours. They were then weighed with the aluminium cup. The shell and soft tissue was separated and the soft tissue was grinded by hand using acid washed mortle and pestle made of ceramic. The fine grinded powder was transferred into pre weight plastic zip lock bag (60x80x0.5mm). Between grinding of the

samples for the same day, the mortle and pestle was washed with a 70% ethanol solution and between grinding of the different days the pestle was acid washed.

Table 2. The nominal metal concentration in each test system, each fraction of metal contributed to the metal mixture that and the total combined fraction of all metals in the mixture

	Nominal metal concentration µg/l			Fraction			Combined fraction
	Cu	Zn	Cd	Cu	Zn	Cd	1
Cu only	70	-	-	1	-	-	1
Zn only	-	2100	-	-	1	-	1
Cd only	-	-	350	-	-	1	1
Cu+Zn	35	1050	-	0.5	0.5	-	1
Cu+Cd	35	-	175	0.5	-	0.5	1
Zn+Cd	-	1050	175	-	0.5	0.5	1
Cu+Zn+Cd	23.11	700	115.5	0.33	0.33	0.33	1
Clean water	-	-	-	-	-	-	-

XRF analyses

The same procedures as described for background levels measurement were conducted for the XRF analyses.

Chemical analyses

Water was collected from the pond at the Bergian Garden and was sent to chemical analyses for determination of background metal levels in the water.

Data processing and statistical analyses

Part I Acute toxicity test (96 h)

All data was processed in Microsoft Excel 2011, and LC_{50} and NOEC were determined by graphical interpolation.

Part II Uptake and depuration

All data was processed in Microsoft Excel 2011, all data were $\log_{10}$ transformed. The metal levels from the background snails were used as starting point in all treatments. A graphical evaluation was used to decide which container to use for XRF analyses. An ANCOVA was performed with SPSS regarding the uptake of single metals over time depending on the length of the snails.

RESULTS

Part I Acute toxicity test (96 h)

The average measured pH and standard deviation were the following: control solutions 7.84 ± 0.027, Cu 7.7385 ± 0.047, Zn 7.702 ± 0.032 and Cd 7.05 ± 0.37. The survival in the control was 100% after 96 h.

For Cu, the LC_{50} was 175 µg/L and the NOEC was 100 µg/L, and after the 96 h toxicity test 100% of the individuals were dead in the highest concentration 400 µg/L.. For the next highest concentration (200 µg/L), 66% of the individuals were dead. Already after 72 h 33%

were dead in the highest concentration (Figure 1 A). After 96h none of the Zn treatment reached 100% mortality, only 66 % were dead in both the highest (5000 µg/L) and next highest (4000 µg/L). The LC_{50} was 3400 µg/L and the NOEC was 3000 µg/L (Figure 1B). For Cd 100% of the snails were dead in 2500 and 2000 µg/L, 66% at 1500 µg/L, and 33% at 1000 µg/L after 96 h. Already after 72 h, 66 % of the individuals were dead at 2000 µg/L and 33% dead at 2500 µg/L. The LC_{50} was 1200 µg/L and the NOEC was 500 µg/L (Figure 1C).

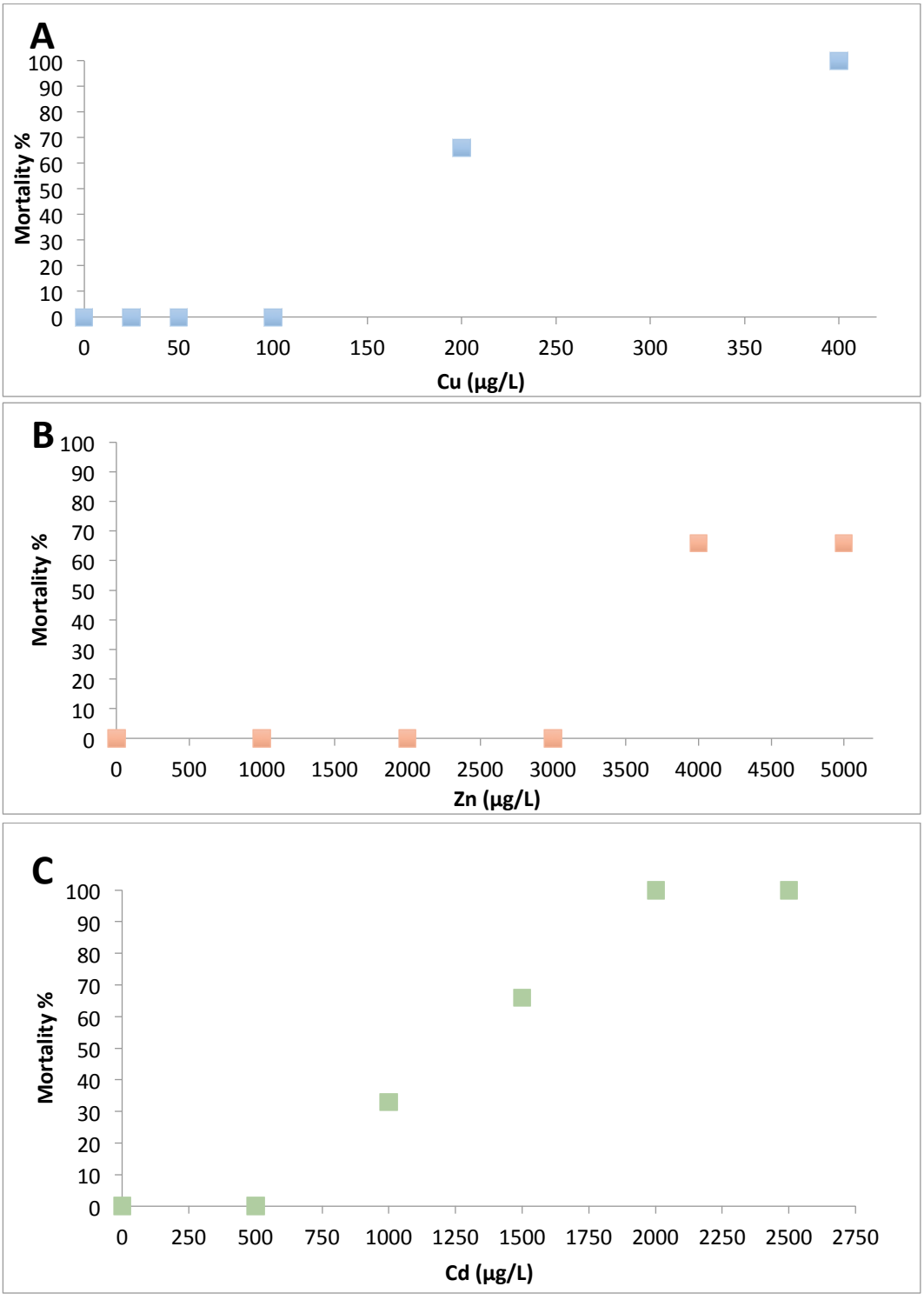


Figure 1a,b,c. Acute toxicity tests (96h) for the following: A Cu, B Zn and C Cd.

Part II Uptake and depuration

The results of the absorption test with XRF on plastic cup, mylar and zip lock bag are shown in figures S2A and S2B indicate that zip lock bags are more suitable than plastic cup for XRF analyses.

Measurements for the three certified XRF standards Nist 2710a (Table S1), Nist 2711a (Table S2) and CRRMP TILL 4 (Table S3) were in the measurement limit of ±20% from validated levels. Any values outside the ±20 % limits are due to human errors.

The detected concentrations of Cu and Zn were found in the background snails but no concentration of Cd (Figure S1). The mean pH during the experiment was 7.64 ± 0.19. The *L. sativa* feed to the snails contained the average Cu of 53.66 ± 22.33 µg/g, Zn 23.333 ± 2.081 µg/g, and no Cd was detected. The metal levels detected in the pond water were Cu 1.2 µg/L, Zn 42.3 µg/L and Cd 0.000323 µg/L. The XRF of the background snail detected Cu and Zn but no Cd was detected (Figure S1). The mean measured Cu and Zn concentrations in *L stagnalis* under control treatment in figure 2, no Cd was detected. All the individuals in the control treatment survived. After five days two individuals had died one in Cu and one in Cu+Zn.

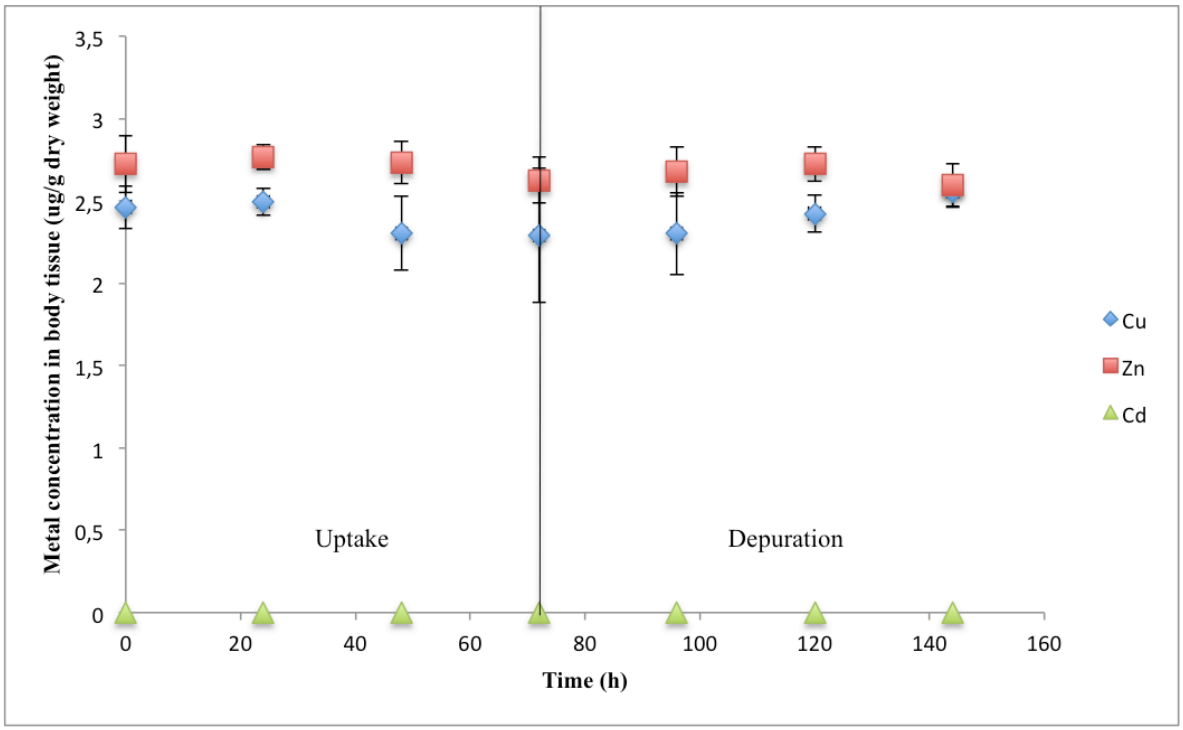


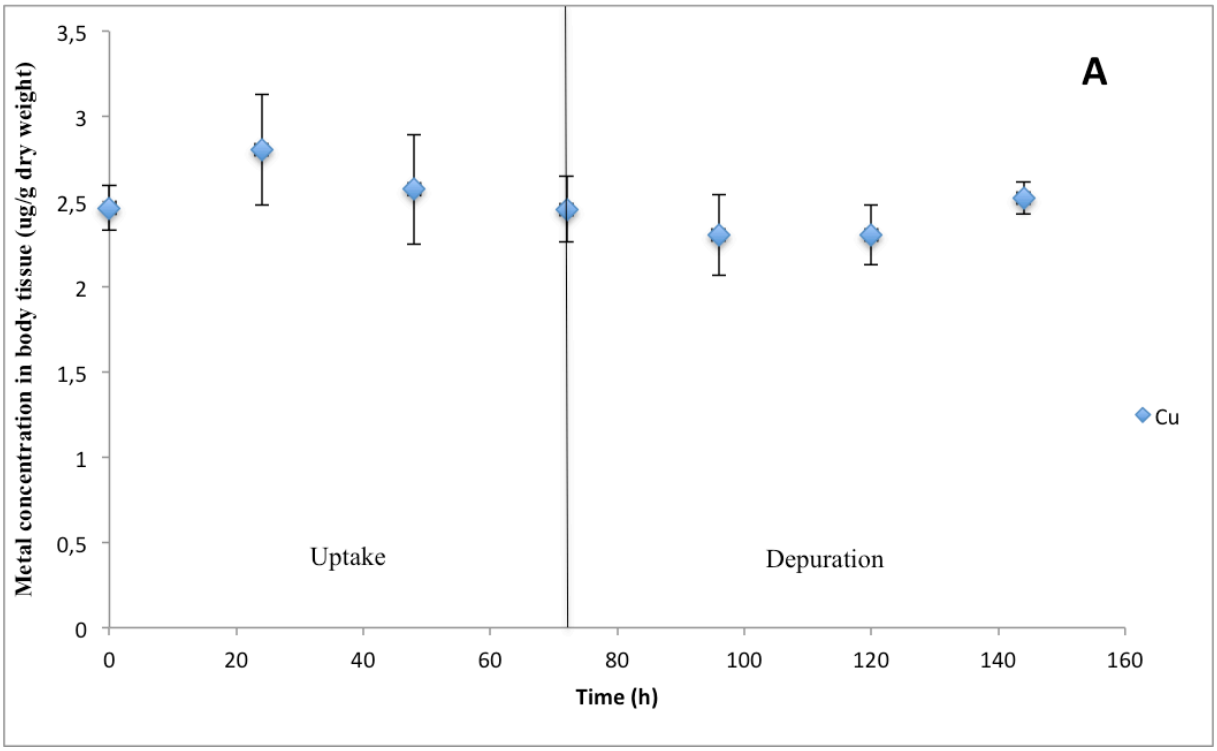
Figure 2. Metal concentration in L. stagnalis under control treatment, with logged concentrations, standard deviation as the error bars and the vertical line at 72 h indicates the shift between uptake an depuration phase.

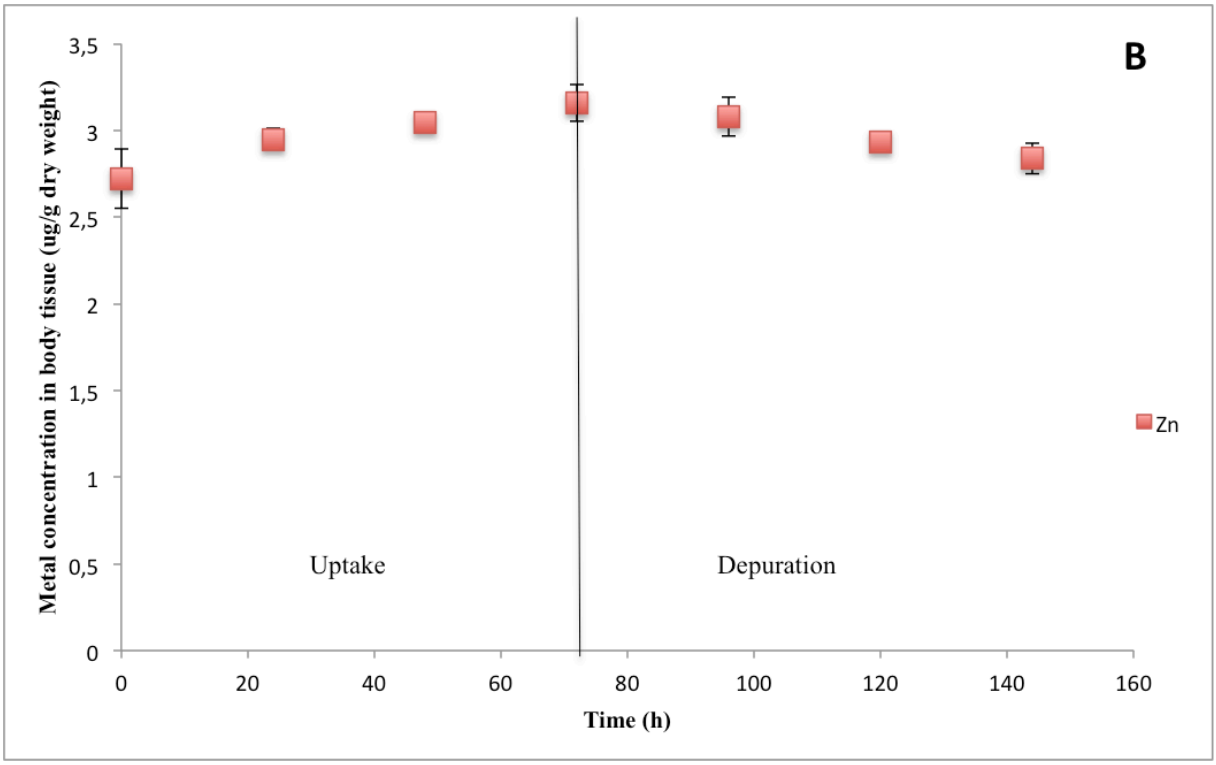
The single metal exposures are represented in figure 3 A Cu, B Zn and C Cd. The average measured concentrations after 72 h uptake (with standard deviation) were: Cu 282.44 ± 122.49 µg/g, Zn 1438 ± 342.54 µg/g and Cd 77.44 ± 38.42 µg/g. The result shows that the concentration of Zn and Cd increase over time. Cu shows fluctuations and the highest average concentration occurs after 24 h 636.33 ± 406.88 µg/g. Cd has it largest uptake the first 24 h.

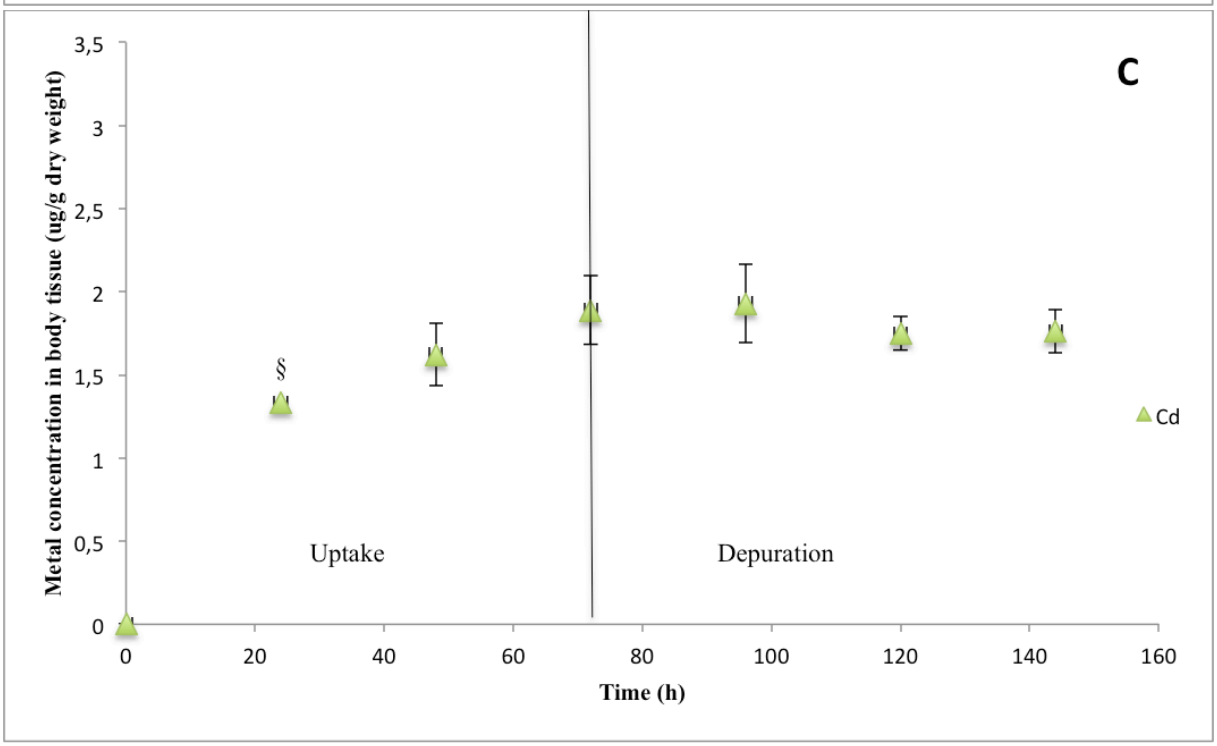
After the depuration period Zn and Cu is almost back to background level, while Cd does not show any depuration after 3 days.

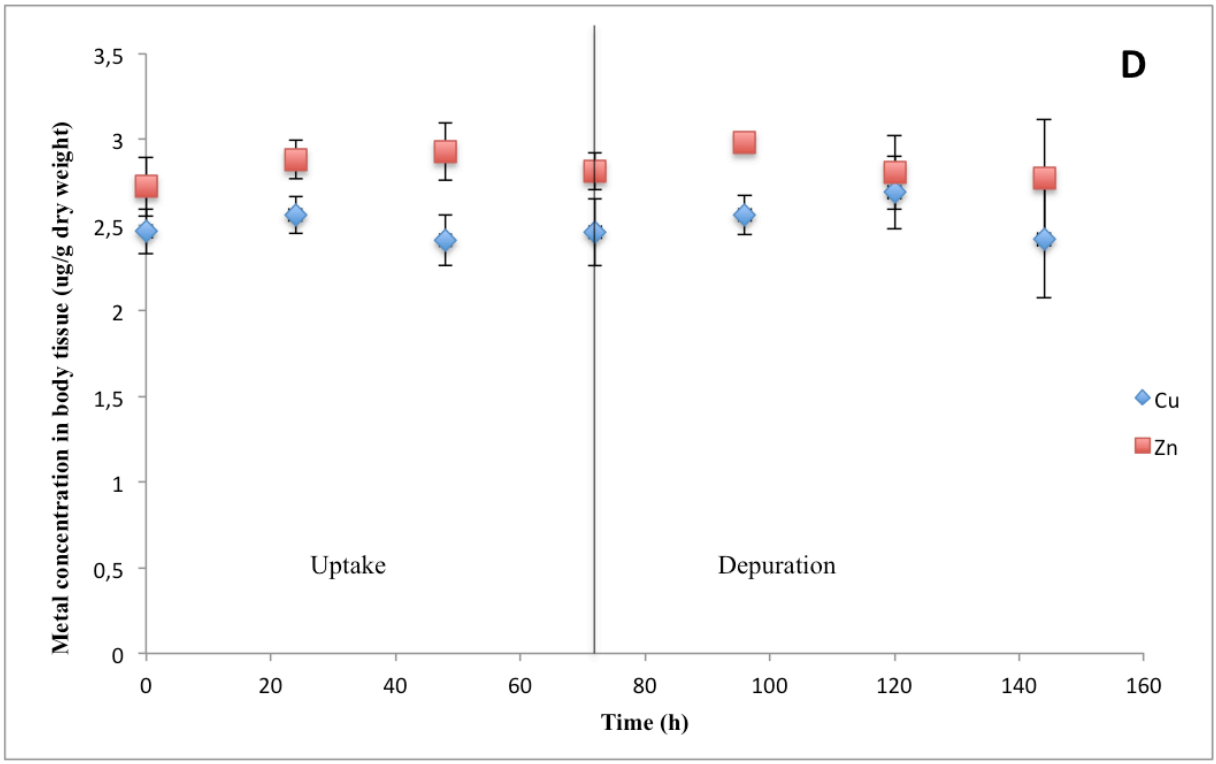
Binary mixtures of Cu+Zn are shown in figure 3D. The average measured concentrations after 72 h uptake were Cu 227.66 ± 136.47 µg/g and Zn 713 ± 55.45µg/g. It appears that Cu is influencing the uptake of Zn which has an increase 53% compared with the control. Both Cu and Zn are back to background levels after the depuration period. Mixtures of Cu+Cd are shown in Figure 3E. The average measured concentrations after 72 h uptake were Cu 447.66 ± 96.62 µg/g and Cd 24.55 ± 2.36 µg/g. Cd seems to have an overall effect on Cu and the average measured Cu concentration after depuration is 7 % less compared with the control. Uptake of Cd is slower compared to the single treatment and Cd does not eliminate at all. The results from the Zn+Cd mixtures are shown in figure 3F. The average measured concentration after 72 h uptake were Zn 1115.55 ± 143.89 µg/g and for Cd the it was 33.77 ± 12.18 µg/g. It seems that Zn influences the uptake of Cd. In this treatment Zn has increased with 163% compared with the control. Zn has depurated back to staring level and Cd does not eliminate at all.

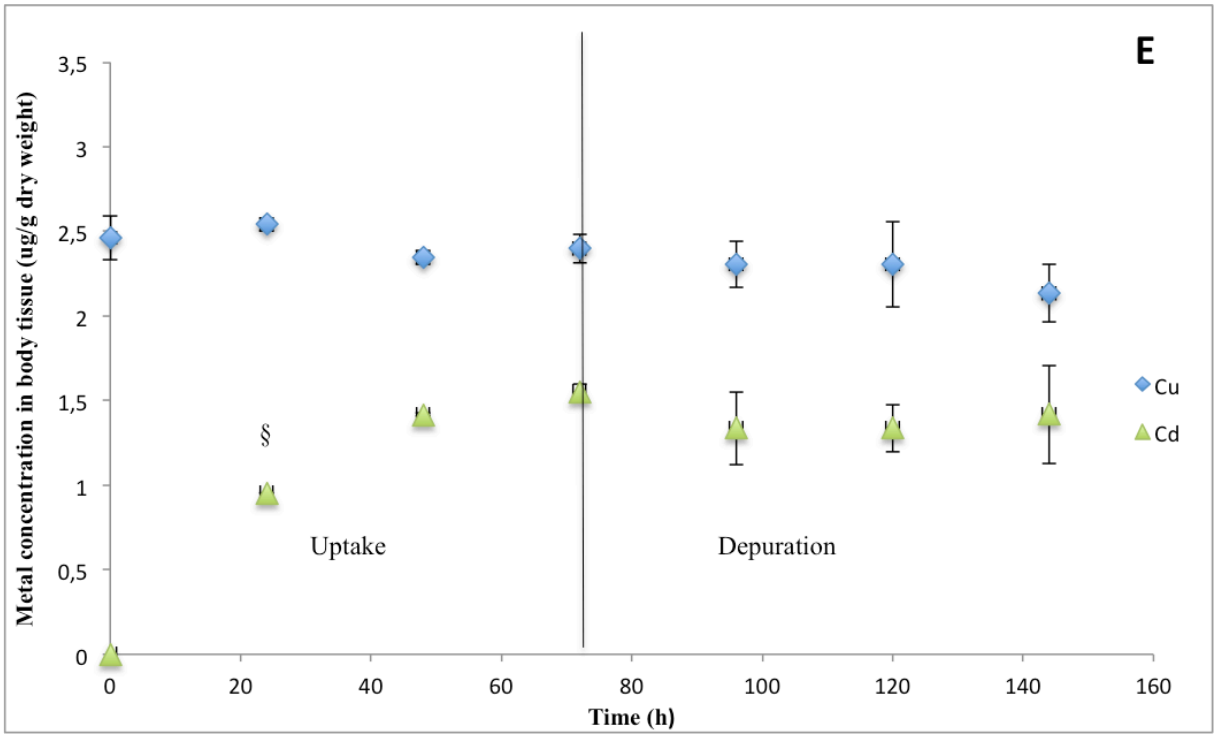
Data from the tertiary mixture Cu+Zn+Cd is shown in Figure 3G. The average measured concentration after 72 h uptake Cu 222.22 ± 9.57 µg/g, Zn 532 ± 45 µg/g and Cd 15.11 ± 4.52 µg/g. Uptake of Cd is slower compared to the binary and single exposure. After the depuration period the average measured Cu level is 50 % lower compared to the control.




B
Metal concentration in body tissue (ug/g dry weight)
3,5
3
2,5
2
1,5
1
0,5
0
Uptake
Depuration
Zn
0
20
40
60
80
100
120
140
160
Time (h)


C
Metal concentration in body tissue (ug/g dry weight)
3,5
3
2,5
2
1,5
1
0,5
0
§
Uptake
Depuration
Cd
0
20
40
60
80
100
120
140
160
Time (h)


D
Metal concentration in body tissue (ug/g dry weight)
3,5
3
2,5
2
1,5
1
0,5
0
Uptake
Depuration
Cu
Zn
0
20
40
60
80
100
120
140
160
Time (h)


E
Metal concentration in body tissue (ug/g dry weight)
3,5
3
2,5
2
1,5
1
0,5
0
§
Uptake
Depuration
Cu
Cd
0
20
40
60
80
100
120
140
160
Time (h)

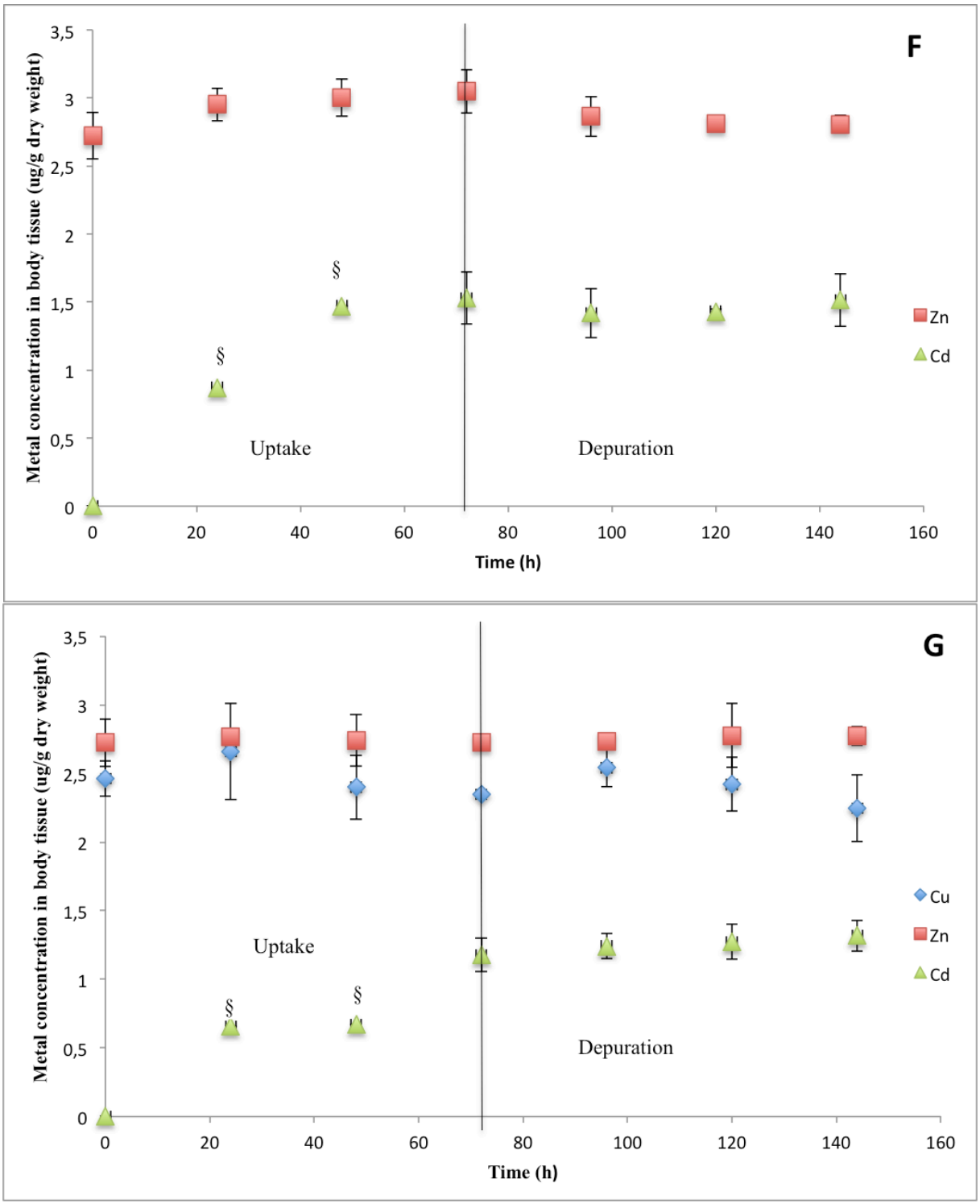
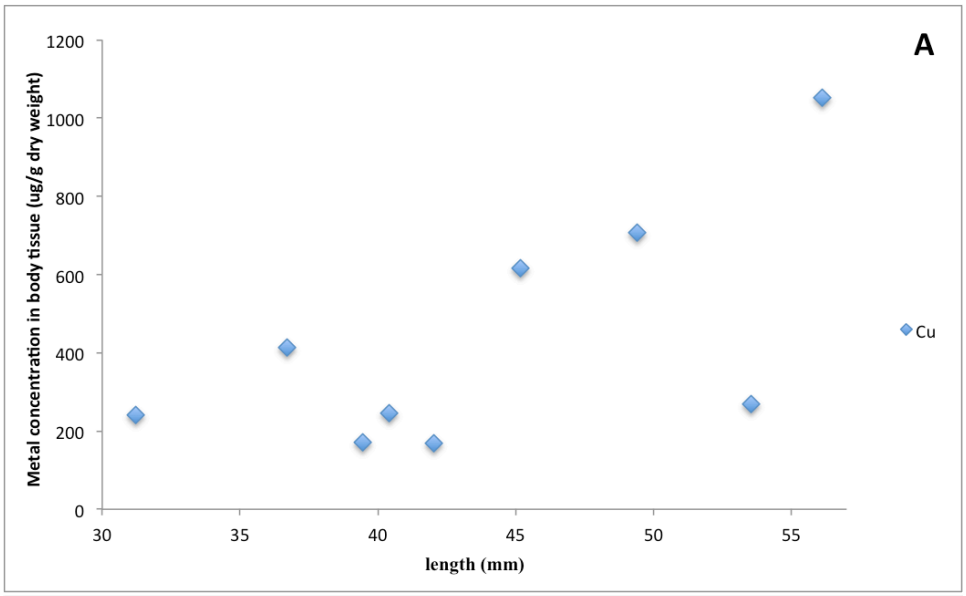
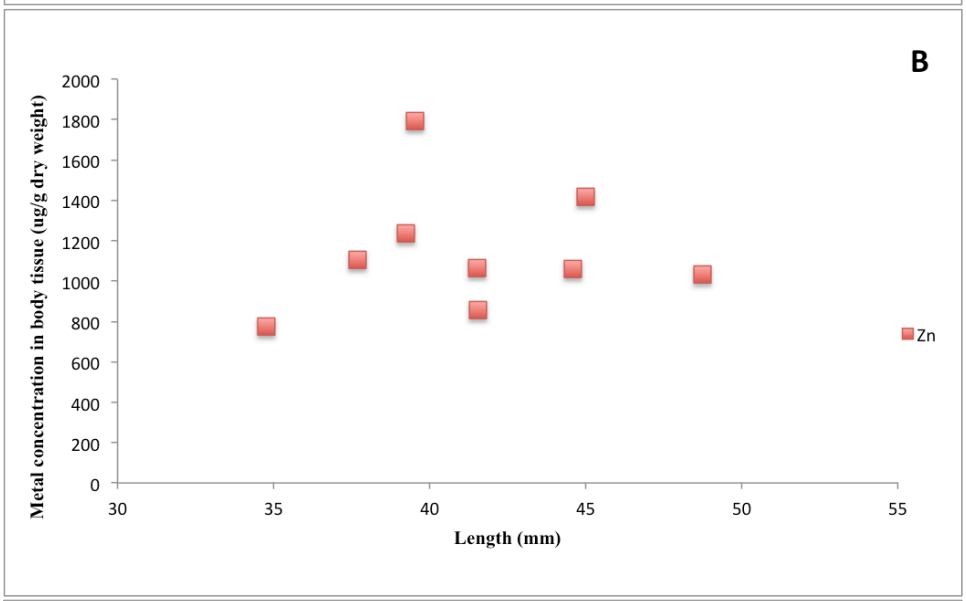


Figure 3 a, b, c, d, e, f. Average concentration of metals in L stagnalis after 3 days uptake and 3 days depuration, concentrations are log_{10}, standard deviation as error bars, the vertical line at 72 h shows the shift from uptake to depuration. The different diagrams are A Cu only, B Zn only, C Cd only, D Cu+Zd, E Cu+Cd, F Zn+Cd and G Cu+Zn+Cd. § indicates that it was not possible to calculate standard deviation due to less than three measurements.

The ANCOVA showed a statistical significance of uptake of metals over time depending on the length of the snails ($P<0.05$). (Figure 3 A, B & C). No such relationship was found for depuration.


A
Metal concentration in body tissue (ug/g dry weight)
1200
1000
800
600
400
200
0
30
35
40
45
50
55
length (mm)
Cu


B
Metal concentration in body tissue (ug/g dry weight)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
30
35
40
45
50
55
Length (mm)
Zn

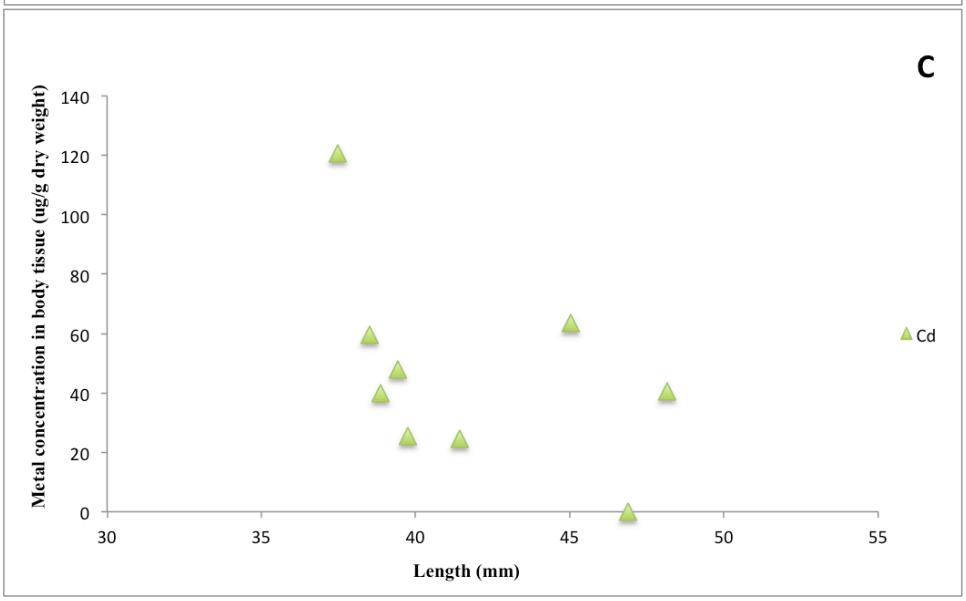

C
Metal concentration in body tissue (ug/g dry weight)
140
120
100
80
60
40
20
0
30
35
40
45
50
55
Length (mm)
Cd

Figure 4 a, b, c. Average metal concentration for uptake phase and the shell length. For A Cu, B Zn and C Cd.

DISCUSSION

Part I Acute toxicity test (96 h)

By studying the figures 1 A, B and C it can be determined that *L. stagnalis* is most sensitive to copper and least sensitive to zinc in this experiment. An LC_{50} (96 h) of 175µg/L for Cu is much higher than what is found in other studies on *L. stagnalis*, Brix et al (2011) found 31µg/L and Ng et al (2011) 24,9 µg/L, and compared to another snail species *Melaonides turberculata* 140 µg L (Shuhaimi-Othman et al 2012).

An LC_{50} (96h) for Zn of 3400 µg/L is lower compared other snail species; *Radix luteloa* had 8100 µg/L (Khangarot & Ray 1987) and *M. turberculata* 3900 µg/L (Shuhaimi-Othman et al. 2012), but higher compared to *Pomacea paludosa* 1074 µg/L (Hoang & Tong 2015).

The LC_{50} (96h) of 1200 µg/L Cd is lower than in a study by Conerdassier et al (2004), where the value was 1545 µg/L. Compared to other snails, the value is lower than *M. turberculata* (1490 µg/L) (Shuhaimi-Othman et al 2012) and higher than for *R luteloa* (872 µg/L) (Khangarot et al 1982). In this study, it is to be found that adult *L, stagnalis* is quite tolerant to acute exposures of Cu, and moderately sensitive to Zn and Cd compared to other species of snails.

The studies by Brix et al (2011) and Ng et al (2011) were both acute exposures (96 h) without food and in both cases, *L. stagnalis* juveniles were used. Juveniles are more sensitive to metals than adults (Coeurdassier et al 2004). For this experiment, only adults were used and size and life stage have been seen to have an effect on the sensitivity an individual has towards metals (Wang 1987). It has been suggested when conducting toxicity testing that different life stages should be taken in consideration when determining the LC_{50} for a species (Coeurdassier et al 2004). Coeurdassier et al (2004) performed experiments with three different life stages of *L. stagnalis* and found that there is a big difference in tolerance for Cd between juveniles and adults. Instead of just conducting acute toxicity tests for a life stage of an organism it would be better to use several different life stages. Côte et al (2015) also stated that genetic variation within the species give a difference in sensitivity towards different metals, and that it should be taken in consideration when conducting toxicity testing. Safety limits should not be biased on only one genetic origin especially for a species such as *L. stagnalis*, that is widespread over different continents. Côte et al (2015) suggest the use of at least four different genetic strains, which would be possible with technique available today.

Different abiotic factors such as temperature has a role for metal toxicity, as studied by Khangarot & Ray (1987), who showed that toxicity increases with increasing temperature. They proved that for *R. luteola*, an increased temperature from 17.5 °C to 22.5°C lowered the Zn LC_{50} with 28%. They link a higher temperature to increased metabolic processes in the organism and a higher oxygen demand. More oxygen will increase the production of ROS. Studies have showed that too high levels of Cu can induce the production of ROS (Nevitt et al 2011) and high levels Zn interfere with the ROS detoxification (Bishop et al 2007). For the acute toxicity test conducted under this study, the temperature was 17°C compared to other

studies that reported standard temperatures 20-22 °C (Brix et al 2011; Ng et al 2011; Coeurdassier et al 2004). But reality is not as simple that only temperature can be used to explaining different LC_{50}s. A study by Tong and Hong (2015) showed that when tested separately, increasing water hardness, pH and DOC levels would lower the toxicity of metals, and in their case they studied Zn on *P. paludosa*. They suggested that increased water hardness gives a protective effect by the competitive inhibition of metals at biotic ligands from the ions of example calcium and magnesium. A higher pH will reduce the level of free bioavailable metal ions in the water and in that way, give a protective effect for the organism. DOC will bind to metal ions which leads to a reduction of the bioavailability and lower concentrations of free metal ions. This displays the complexity of different abiotic factors that can be of a big importance, with how they can modify the abilities of metals and therefore induce or reduce the toxic effect.

Part II Uptake and depuration

For understanding how mixture toxicity acts on an organism it is important to get a greater knowledge about the uptake mechanism and how it differs for different organisms. There is not much information today about how well organism can regulate metal mixtures. When *L. stagnalis* were exposed to single metals in this study, there were increasing concentrations of Zn and Cd in the soft tissue with increasing time compared to the control. This has been seen in other studies regarding *H. azteca* (Shuhaimi-Othman & Pascoe 2006). *L. stagnalis* in this study eliminates the bioconcentrated Cu and Zn over the equal long depuration period and there is no elimination of Cd, which also follows previous studies (Shuhaimi-Othman & Pascoe 2006: Présing et al. 1993). This implies that *L. stagnalis* has good mechanisms for regulation of essential metals.

For the binary mixtures in this study it was indicated that the presence of one metal influenced the concentration of the other metal in the mixture. Cu seems to act antagonistic towards Zn and Cd. In mixtures where Cu is present, the uptake of Zn and Cd is lower compared to the Zn and Cd mixture. It has been suggested in other studies that combinations of Cu, Zn and Cd may either be antagonistic or synergistic (Wang 2014). In a combination of Cu and Zn, it has been found that Cu can have an antagonist effect on the uptake of Zn (Louma 1983). Zn had a positive effect on the uptake of Cd, which has been observed in other studies where the presence of the carrier protein for Zn can also increase the uptake of Cd (He et al 2009). It is suggested that the result found in this present study that it might be some inhibition from Cu in the uptake of Cd and Zn in *L. stagnalis.* An aspect to take in consideration is that the found difference between measuring occasions can be influence of that different individual representing the obtained value at the different measuring occasions.

In this study, there were no clear results for the tertiary mixture, it was hard to evaluate the interactions between each metal. Still, it seems that metals in combination have an effect on the uptake for *L. stagnalis* in the present study. It is similar to what Shuhaimi-Othman and Pascoe (2006) found in their study for *H. Azteca*, where they stated that it was not possible to quantify the effect of interactions between the metals in a tertiary mixture. Myer et al (2015) stated that the effect in complex mixtures could be dependent on the metals in the mixture, the range of the metal concentration and the form of the metal (e.g. ions). The next step for better understanding would be to preform the present study with concentrations series. It could help

to get a clearer perspective over the conducted results. It is a big variety between species if the effect of a metal mixture becomes antagonistic or synergistic and there seems to be no straight pattern (Shuhaimi-Othman & Pascoe 2006).

It is hard to compare between the different systems (single, binary and tertiary) due to the fact that the actual exposure varies due to different concentrations. Meyer et al (2015) suggest that when conducting mixture metal experiment, the exposure design should be to titrate one metal into a constant background concentration of single or binary mixture. In that way, it is possible to test an organism in concentrations ranging from sublethal to above lethal concentrations. They found it helpful to detect any additive patterns and they also state that helps to increase the repeatability of the experiments. It would be interesting to perform similar experiment for other aquatic species such as *L. stagnalis* and se if would help to predict mixture metal toxicity in a better way.

Desouky (2005) found for *L. stagnalis* that all trace metals are accumulated in most tissues over the first 10 days, especially in the digestive gland and kidney. After a 10-day exposure, the non-essential trace metals were redistributed to the digestive gland. The same study also found that the digestive gland can store up to 70 % of the total amount of Zn in the soft body of *L. stagnalis*. In the present study, *L. stagnalis* shows the ability to regulate the levels of Cu and Zn but not Cd. A study done on uptake and depuration on Cd conducted over two months one for each phase on *L. stagnalis* found that there was only a small depuration of Cd between day three and four for the depuration period (Présing et al 1993).

In this study, it was found that there is a significant correlation between the length of the snail and the uptake of metals in in single exposure over time. When investigating four different gastropods, Cubadda et al (2001) found that there is a positive correlation of body weight and accumulation of Cu, Zn and Cd. Body weight and length are often positively correlated with each other. The correlation between time and uptake of metals was found to be significant in several studies (Shuhaimi-Othman & Pascoe 2006; Présing et al. 1993). All these reports indicate that *L. stagnalis* is suitable for both biomonitoring and ecotoxicology testing, which also have been implied by several other studies (Bandow 2012; V- Balogh et al 1988).

Summary and Conclusion

For the acute exposure (96 h) under the conditions of this conducted experiment *L. stagnalis* is less sensitive compared to other snail species for Cu, but to Cd and Zn, it seems to be moderately sensitive. For further investigations, it would be important to include different life stages and also to look at genetic variation within the species. In single metal exposures, the uptake is significantly correlated to the size of the snails. Moreover, Cu can have a negative effect on the uptake of Zn and Cd in binary mixtures. For the tertiary mixtures, it is more difficult to conclude interactions between the metals. For both biomonitoring and toxicology testing, it would be suggested to investigate further on the effects and interactions of complex metal mixtures, in order to get a greater understanding of metal toxicity towards *L. stagnalis* and other aquatic organisms.

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APPENDIX

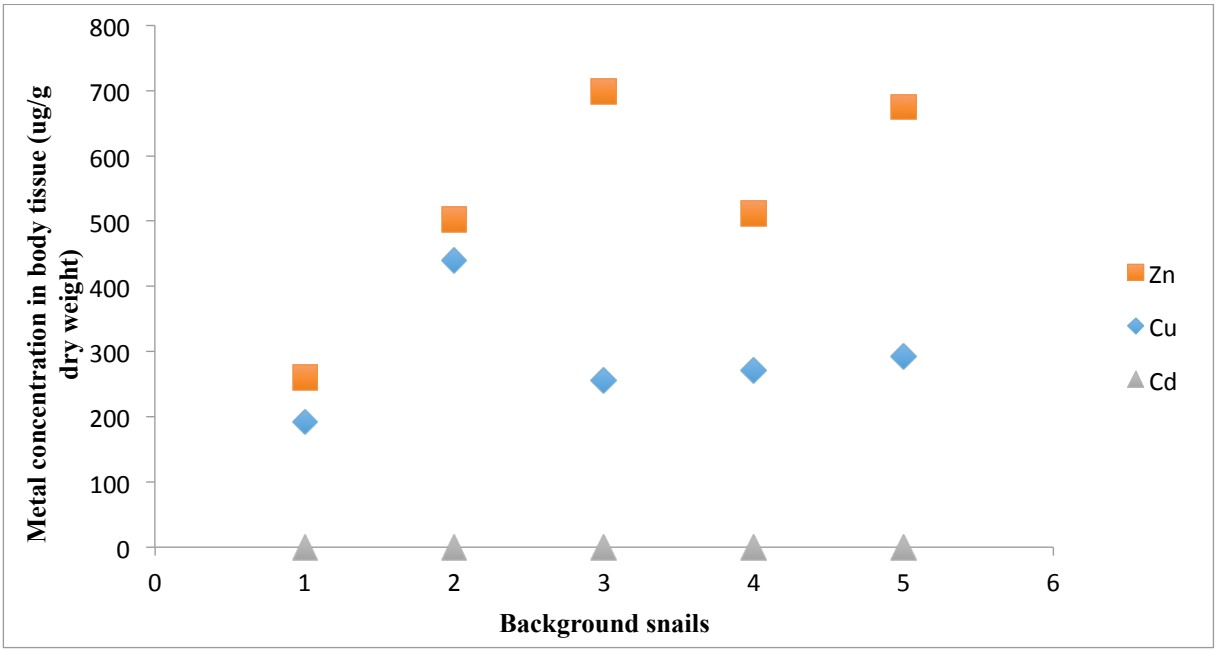
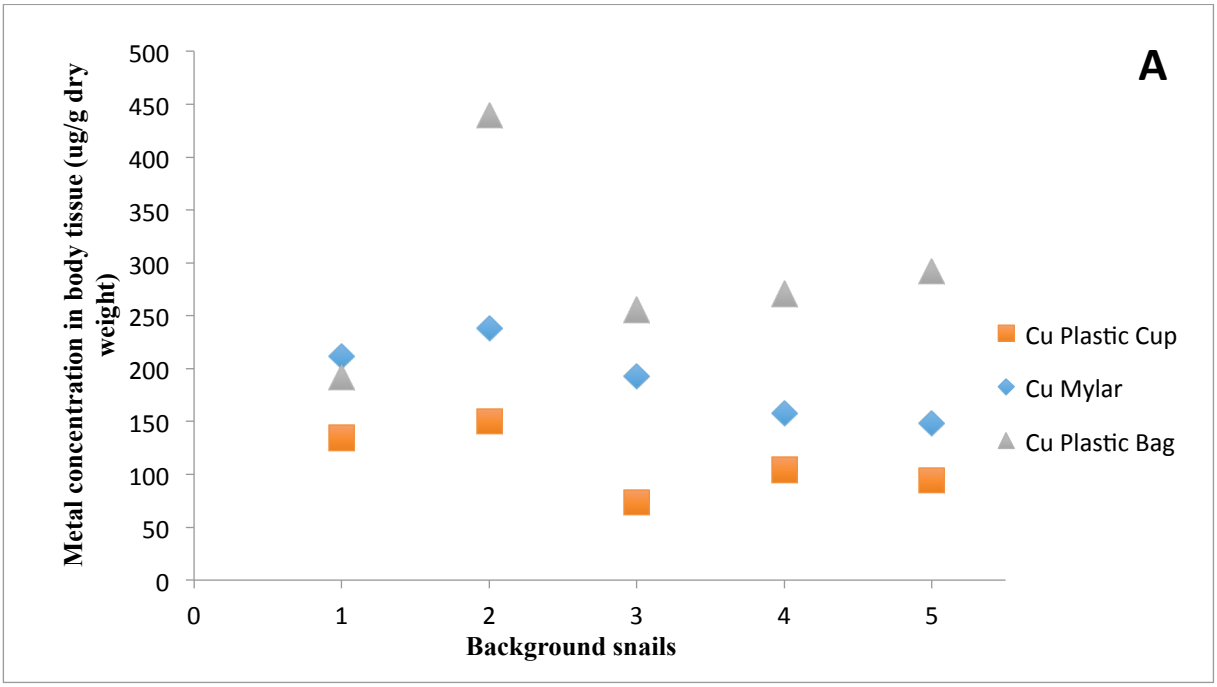


Figure S1. Metal concentrations detected in the five background snails using XRF.



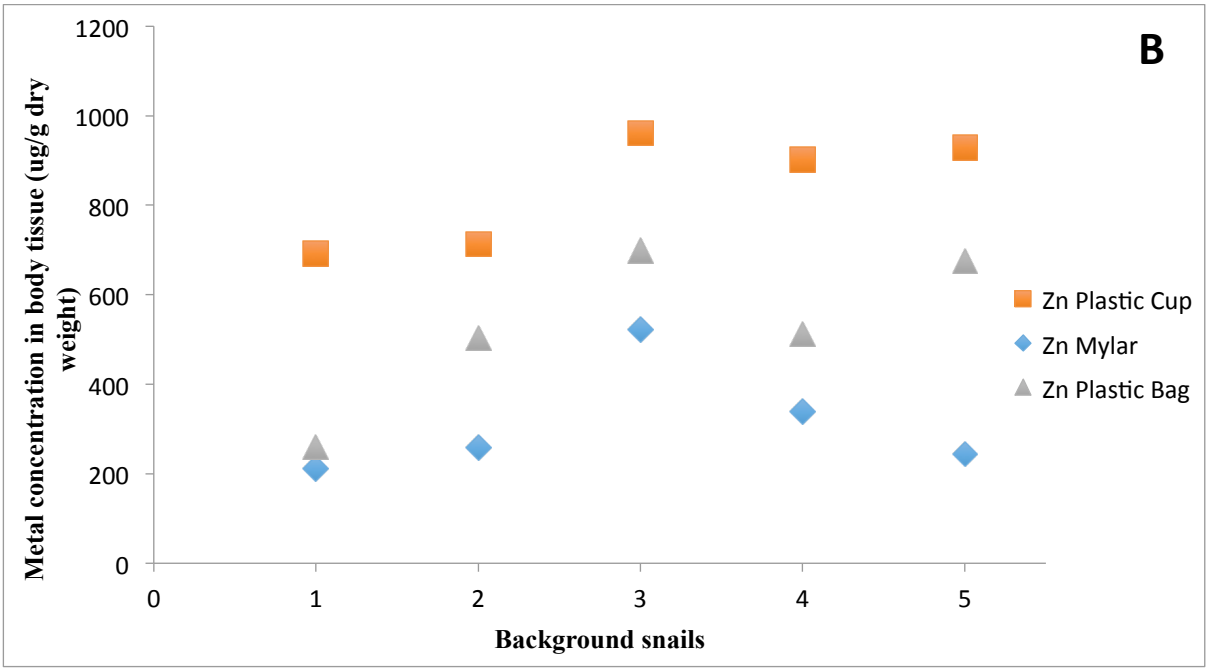


Figure S2 a, b. Difference in measured absorption levels for determination of container for XRF A Cu, B Zn.

Table S1. Measurements with XRF of the certified reference Nist 2710a showing the deviation from the certified material.

Measurement no.	Cu deviation	Zn deviation	Cd deviation
1	10,71%	6,76%	-6,47%
2	5,71%	6,52%	-11,65%
3	13,57%	5,80%	-11,46%
4	7,86%	5,56%	-19,78%
5	7,86%	10,39%	-7,58%
6	7,14%	5,07%	-12,38%
7	-6,43%	-11,35%	-19,04%
8	-7,86%	-6,28%	-17,19%
9	-1,43%	5,07%	-7,58%
10	5,00%	0,24%	-9,24%
11	-2,86%	0,00%	-11,83%

Table S2. Measurements with XRF of the certified reference Nist 2711a showing the deviation from the certified material.

Measurement no.	Cu deviation	Zn deviation	Cd deviation
1	12,95%	13,80%	-13,82%
2	12,81%	12,99%	-22,76%
3	11,75%	12,56%	-32,52%
4	9,59%	11,15%	-29,27%
5	12,95%	14,28%	-20,33%
6	7,37%	7,18%	-21,14%
7	9,12%	9,81%	-1,63%
8	8,60%	11,72%	-34,15%
9	12,40%	12,89%	1,63%
10	9,91%	11,41%	-10,57%

11	11,17%	10,41%	-7,32%

Table S3. Measurements with XRF of the certified reference TILL 4 showing the deviation from the certified material.

Measurement no.	Cu deviation	Zn deviation
1	0,42%	11,43%
2	5,49%	20,00%
3	8,44%	18,57%
4	1,69%	2,86%
5	15,19%	22,86%
6	15,19%	30,00%
7	8,44%	21,43%
8	4,22%	21,43%
9	4,64%	11,43%
10	5,06%	18,57%
11	6,75%	18,57%

Table S4. Measurements with XRF of the non certified reference PACS-3 showing the deviation from the certified material

Measurement no.	Cu deviation	Zn deviation	Cd deviation
1	193,87%	189,10%	258,74%
2	140,80%	131,12%	213,90%
3	109,20%	103,19%	-10,31%
4	148,77%	140,96%	258,74%
5	136,81%	134,04%	213,90%
6	121,47%	113,56%	213,90%
7	351,84%	339,10%	438,12%
8	349,69%	339,36%	438,12%
9	358,59%	366,22%	438,12%
10	248,16%	270,21%	348,43%
11	242,02%	254,79%	348,43%

Deliverable 4.4 Report/scientific articles on development of new bioassays for monitoring sub-lethal effects on chemically mediated behaviours in gastropods and crustaceans.

Appendix 3

Behavioural responses of *Theodoxus fluviatilis* to metal-contaminated biofilms and food absence

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Behavioural responses of *Theodoxus fluviatilis* to metal-contaminated biofilms and food absence

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Abstract

Food can be a vector for transferring contaminants to living organisms. This study aimed to investigate whether the behaviour of organisms changes when exposed to contaminated food, while also exploring behaviour changes due to starvation and food absence. The nerite *Theodoxus fluviatilis* is an abundant and widely spread snail in the Baltic Sea. Individuals collected from a relatively pristine area (58°49'08.5"N 17°38'07.2"E), in April 2017, were fed laboratory cultures of biofilm, spiked with metals at low (11 µg L^{-1} Cu; 69 µg L^{-1} Zn) and high (96 µg L^{-1} Cu; 637 µg L^{-1} Zn) levels, mixtures of these two metals, as well as procedure controls with no food. The photosynthetic efficiency of the biofilm did not differ significantly between any of the treatments. Video tracking was used to study the locomotor and feeding behaviour of the snails. The results indicated that in the absence of food, snails were bolder, as they started to move earlier. The total distance crawled was significantly increased by the lack of food, but was not affected by metal exposure. However, metal treatments seemed to decrease the speed at which snails moved compared to the control. Despite such behaviour, no difference was detected in the amount of food eaten by snails from different treatments, possibly indicating their inability to distinguish clean vs. contaminated biofilm. This might lead to accumulation of high metal levels in the snails, with potentially detrimental effects on predators such as birds and fish.

Keywords

Copper; Zinc; Snails, Behaviour; Feeding; Locomotion.

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Introduction

Behaviour is defined as the 'cumulative interaction of a variety of biotic and abiotic factors that represents the animal's response to internal (physiological) and external (environmental, social) factors and relates one organism to another' (Dell'Omo 2002). Hence, behavioural changes are an integrated result of biochemical and physiological processes, which allows a comprehensive assessment of responses to stressors such as contaminants. Contaminants may affect organisms directly, by altering their physiology, but also indirectly, through chemically-induced changes in their environment such as changes in food quality or abundance (Fleeger et al. 2003). In nature, it is not easy to distinguish between these two types of effects and hence it is not straightforward to assess which environmental compartment is most vulnerable. Changes in animal behaviour may affect predation, feeding rate or mating, which can alter species abundance and community composition (Fleeger et al. 2003).

Behavioural changes are amongst the most sensitive indicators of shifts in environmental quality and may offer fast and non-destructive tests (Gerhardt 2007; Peterson et al. 2017). Moreover, changes in behaviour are not only a result of exposure to contaminants but may in turn affect exposure by changing contaminant uptake rates. The exposure becomes more complex as the organisms are simultaneously exposed to multiple contaminants, which is often the case in nature. Currently, studies on mixture effects on behaviour are scarce.

The Baltic Sea has a history of extensive use of chemicals, which has lead to strong contamination of its marine environment (HELCOM 2017). Significant amounts of heavy metals and persistent organic pollutants (POPs) enter the Baltic Sea through point sources, situated on the coast or inland, land-based diffuse sources and atmospheric deposition (HELCOM 2010). Due to their high toxicity even at low concentrations, mercury, cadmium and lead have long been a cause for concern in the Baltic Sea, with legislation in place to decrease their inputs (HELCOM 2017). More recently, an increase in sediment concentrations of other metals, such as arsenic, cadmium, chromium, cobalt, copper, nickel and zinc, has also been observed, thus, raising concerns about the environmental impact of heavy metals on an already stressed ecosystem (HELCOM 2010).

Metals (Cu and Zn) are the contaminants in focus in this study and represent an interesting group of contaminants, as they are essential in small amounts but become toxic at higher concentrations. Metals may induce behavioural toxicity by altering biochemical processes or by damaging a nerve or epithelial tissues (Dell'Omo 2002). In nature, metals are commonly found in mixtures, and may thus compete with each other for ligands in the environment (e.g. sediment, biofilms) or in the organisms (e.g. gills), which ultimately affects metal bioavailability and toxicity.

Biofilms are ubiquitous in the aquatic environment and play essential ecological roles such as nutrient cycling and primary production (Peters and Traunspurger 2012; Russell et al. 2013). They represent a major fraction of the benthos biomass, therefore being an important food resource for primary consumers, such as benthic grazers (Bustamante et al. 1995; Thompson et al. 2004). Biofilms have a high affinity for binding metals, e.g. a bioconcentration factor of up to 25 000 for Cu (Barranguet et al. 2000). Moreover, high levels of metals can persist in biofilms long after the source has been removed (McElroy et al. 2016). Hence, being widely available on many natural and artificial surfaces, biofilms represent a vector for the transfer of toxic metals to primary consumers (Sanders et al. 1989; Ballance et al. 2001; Tien and Chen 2013). Primary consumers, through grazing activity, can also affect biofilm biomass and alter its species composition by changing the balance between autotrophic and bacterial biomass (Lawrence et al. 2002).

In this context, we investigated the behavioural effects of dietary exposure to Cu, Zn and their mixture on the nerite snail *Theodoxus fluviatilis*, a highly abundant and widespread species in the Baltic Sea that mainly feeds on biofilms containing diatoms, cyanobacteria and green algae (Peters and Traunspurger 2012; Wiklund et al. 2012; Snoeijs-Leijonmalm et al. 2017). More specifically, we evaluated the effects of metal-contaminated biofilm on snail locomotor and feeding behaviour. Our experimental design allowed us to investigate the potential interactive effects between Cu and Zn on the biological responses. In addition, we explored the effects of starvation and absence of food on snail behaviour. We hypothesized that: i) dietary exposure to metal mixtures would affect snail behaviour to a higher extent than exposure to single metals; ii) absence of food would increase snail movement; iii) starved snails would graze on a larger area than unstarved snails.

Materials and Methods

Biofilm growth

Natural biofilm was cultivated in clear plastic tanks containing 30 L of brackish water (salinity 7 psu, pH 8.15, collected from a non-contaminated area in Studsvik, Sweden at 37 meters depth). Ceramic tiles (35 x 35 mm) were used as substrata and were first conditioned with bovine serum albumin (Sigma Aldrich, 22.2 mg L^{-1}) for 2 h. Nutrients were added to the incubation tanks in the form of commercially available Blomstra fertiliser (51 g L^{-1} N and 10 g L^{-1} P); 40 or 75 mL were added on 4 occasions. The tiles were incubated under natural sunlight conditions for the first 5 months and in the laboratory for the following 5 months (1121 ± 12.5 lux (mean ± SE), *n* = 1062; 10.1 ± 0.01 °C, *n* = 1729; 12h light: 12 h dark). Thus, the experiments were carried out using 10-months old biofilm with apparent even coverage, which was dominated by diatoms.

Snail collection

Theodoxus fluviatilis were collected from Askö (58°49'08.5"N, 17°38'07.2"E), a remote and relatively clean area in the Baltic Sea. The snails were handpicked from *Fucus* spp. fronds and transported to the laboratory in plastic buckets containing 15 L of their ambient water. Acclimatisation in the laboratory occurred for 6 weeks prior to the experiment, during which the snails were fed laboratory cultures of diatoms and green algae.

Biofilm spiking

Metal solutions (Cu as $CuCl_2$ and Zn as $ZnSO_4$, Merck) were prepared in natural brackish water. The water was filtered through 0.45 µm GF/C filters (Millipore) and the metal solutions were left to equilibrate for 72 h. Low and high levels of Cu and Zn, as well as their mixture (Table 1), were used for uptake by the biofilm. The concentration of the metal stocks was verified using ICP-MS (Inductively-Couples Mass Spectrometry) according to SS EN ISO 17294-2:2005. Individual tiles covered by biofilm were placed in plastic Petri dishes (92 mm diameter) containing 45 mL of each metal solution. Five replicate tiles were exposed to metals in each treatment during 96 h. The exposure period was intended to allow metal accumulation by the biofilm, without any significant detrimental impact on the biofilm.

Photosynthetic efficiency by pulse-amplitude modulated fluorometry

The photosynthetic efficiency of photosystem II (PSII) of the biofilm was assessed with a DIVING-PAM Underwater Fluorometer (Heinz Walz GmbH, Effeltrich, Germany) using saturating pulse intensities (800 ms, 4200 µmol photons m^{-2} s^{-1}). Measurements taken on dark-adapted biofilm samples were made at three points in time: before exposure to metals, immediately after exposure to metals and after 96 h of exposure to snail grazing. For each of the measuring procedures, biofilm samples were initially subjected to a period of 15 min in dark conditions, after which the ratio of variable to maximal chlorophyll fluorescence, Fv/Fm (Maximum quantum efficiency of PSII photochemistry, where Fv = Fm – F0, Fm is the maximal fluorescence and F0 is the initial fluorescence in dark-adapted algae) was recorded (Krause and Weis 1991; Baker 2008). In between the measuring procedures, biofilm samples were kept in Petri dishes to prevent dehydration.

Behavioural experiment

Petri dishes containing tiles from the different treatments were positioned on a table in random order. Natural brackish water (pH = 7.31 ± 0.03, *n* = 21), 45 mL containing 25 % water from the snail aquarium, was added to each Petri dish. Each experimental treatment was replicated 5 times (**Error! Reference source not found.**). Besides the

treatments listed in Table 1, the behavioural experiment included several additional controls, henceforth referred to as 'procedure controls': no tile (just a snail in control water), no biofilm (clean tile and snail) and a treatment with unstarved snails feeding on control biofilm. Except for the latter treatment, all the other snails were starved for 24 h prior experiment. The snails were carefully handled using soft plastic tweezers. One snail with closed operculum was placed in the centre of each tile. Snails of similar size (5.07 - 6.93 mm shell length, 6 ± 0.05 mm, n = 50) were used for the experiment; size did not differ significantly between treatments or procedure controls (ANOVA, F (6,28) = 0.61, P = 0.72 and ANOVA, F (3,16) = 1.12, P = 0.39, respectively). A photographic system was then used to analyse gastropod movement in an automated and quantitative manner. In this system, individual snails were allowed to move freely in a Petri dish (92 mm diameter) and their movement was recorded every 15 s (over a 3h and 35 min period) by a digital camera (Canon Mark III camera with Canon EF 24–70mm f/2.8L lens) mounted 1.5 m above the experimental area. The images were then used in Tracker software (version 4 .94), a free video analysis and modelling tool. After manually defining the coordinates of feeding areas, the Tracker software was used to automatically recognize snails and record change of their positions (coordinates) over time. The behavioural endpoints recorded were time to first active movement (mean speed > 0.033 mm s^{-1}, sustained for 15 s) and total crawling distance. The crawling distance at low speed (< 0.033 mm s^{-1}) and higher speed (> 0.033 mm s^{-1}) was also measured. The chosen speeds were based on observations of snail behaviour that showed low speeds to be predominant for short exploratory movements (covering a distance smaller than 1/3 of the mean snail size (≈2 mm) over a minute), while higher speeds were used for active movement. Light and temperature in the exposure room were continuously monitored (Hobo® Data loggers, Onset®) and were 704.8 ± 7.38 lux, n = 44 and 9.5 ± 0.03 °C, n = 44, respectively.

Feeding experiment

The snails were allowed to feed on the biofilm for 96 h (740.7 ± 2.50 lux, n = 432; 9.3 ± 0.01 °C, n = 864; 12 h light: 12 h dark). The feeding was quantified in terms of area of biofilm scraped from the tiles, using image analysis in Image J v2. In order to monitor the status of the biofilm over the 96 h, extra Petri dishes with control water, uncontaminated biofilm and no snails were also included (**Error! Reference source not found.**). These biofilms were also checked with the PAM using the procedure previously mentioned. In addition, a small subsample from all the biofilms was taken for live/dead cell staining. For this, the biofilm was sampled with a needle and mixed with 5 µL of 1 mM TO-PRO™ -1 Iodide stain (Invitrogen™) and incubated in darkness for 15 min. The stained biofilm was observed under a fluorescence microscope and pictures were taken using the previously described camera. The ratio, between red (alive) and green (dead) cells, was then estimated from the pictures by analysing 4 to 6 microscope fields per sample using Image J v2.

Data analysis

The statistical analysis was performed in R version 3.3.3 (R Core Team 2017) and SPSS version 20 (IBM Corp. 2011). One-way analysis of variance (ANOVA) was used to determine whether there were any statistically significant differences between metal treatments for each of the snail responses, as well as between the procedure controls. Significant results from one-way ANOVAs were further investigated with Ryan–Einot–Gabriel–Welsch Q (REGWQ) test. If the homogeneity of variances assumption was not fulfilled, Welch's ANOVA and Games-Howell post-hoc test were used instead. The assumptions for ANOVA were verified by testing the normality of residuals with the Shapiro-Wilk test and homogeneity of variance with Cochran's test. The data were transformed when necessary (square, cubic and

quadratic root transformations or natural logarithm transformations were used for different variables). For the analysis of biofilm photosynthetic efficiency a mixed design ANOVA with 1 between subject factor (metal treatment) with 7 levels and 1 within subject factor (time of measurement) with 3 levels was used. Probabilities for multiple comparisons were corrected using the Bonferroni correction, while the Greenhouse-Geisser Epsilon correction was used to deal with the sphericity of the data.

Results

In general, metal exposure did not lead to adverse responses of biofilm, whereas the effects on snail behaviour were more pronounced, in particular in terms of initial movement and crawling speed patterns. However, no clear evidence of interactions between Cu and Zn in mixed metal treatments, at tested levels, was found on any of the snail behavioural or biofilm endpoints, thereby contradicting our expectations that metal mixtures would have a stronger effect than single metals (hypothesis i). Starvation of snails also did not lead to larger grazed areas, contradicting our hypothesis (iii). In contrast and in agreement with our hypothesis (ii), absence of food did impact snail behaviour, leading to a larger stimulatory effect than metal contamination.

Biofilm condition

Photosynthetic efficiency

No effect of metal treatment was observed across the three measurements of the maximum PS II quantum efficiency, Fv/Fm ratio (ANOVA, F (6,28) = 1.39, P = 0.252). A significant effect of time of the measurement was detected (ANOVA, F (1.5,42.3) = 233.50, P < 0.001). A general increase in Fv/Fm was observed across all treatments from the 1st measurement (carried out after removal of the tiles from the growth tank) to the 2nd measurement (carried out after the terminus of the metal exposure period), (**Error! Reference source not found.**). The lower and more variable Fv/Fm values measured before metal exposure are probably due to exposure to slightly higher light levels (1121 ± 12.5 lux, n = 1062) before the dark adaptation period(Baker and Oxborough 2004). No Fv/Fm increase was observed on the 3rd measuring date (carried out after the behaviour experiment), which had pre-dark adaption light levels similar to the ones used during the 2nd measurement (740.7 ± 2.50 lux, n = 432). No interaction between time of the measurement and metal treatment was observed, (ANOVA, F (9.1,42.3) = 0.442, P = 0.905).

Cell viability

The ratio between live and dead cells, obtained from image analysis of stained biofilms, was the lowest for the procedure control biofilm that was not grazed by snails. This ratio differed significantly from that in the procedure control biofilm grazed by unstarved snails (ANOVA, *Welch's F* (2,48.4) = 11.51, P < 0.001). Although no clear evidence of interactions between Cu and Zn was found, cell viability in low and high concentrations of metal mixtures was slightly higher than in the remaining treatments. Significant differences were detected between the high concentration of metal mixtures and 3 out of 4 single metal treatments (ANOVA, *Welch's F* (6,86.6) = 4.54, P < 0.001), (**Error! Reference source not found.**).

Locomotor behaviour

Initially, time spent in feeding and non-feeding areas was also an endpoint, but could not be determined as snails stayed on the food source for the whole duration of the experiment.

Time to first active movement

Time to first active movement was measured as the time at which a snail first actively moved at a mean speed > 0.033 mm s^{-1} over a 15 s period. The procedure control treatments showed that snails exposed to clean tiles (no food) started to actively move earlier than snails from the control treatment (with food), (ANOVA, F (3,16) = 4.40, P = 0.019). Similar trends, although not statistically significantly, were also observed for snails from the dishes with no tiles and for the unstarved snails (**Error! Reference source not found.**). In the metal treatments, although results are not

statistically different (ANOVA, F (6,28) = 2.05, P = 0.091), snails exposed to spiked biofilm anticipated their first movements compared with snails in the control treatment, who took, on average, at least twice the time to start actively moving (**Error! Reference source not found.**).

Total crawling distance

Theodoxus fluviatilis exposed to clean tiles or no tile (no food treatments) crawled for longer distances than those in the control treatment (ANOVA, F (3,16) = 5.46, P = 0.009), namely 5.2 and 4.6 x the mean distance crawled by snails in the control treatment (**Error! Reference source not found.**). In contrast, the spiking of food with metals did not seem to affect significantly the distances crawled by the gastropods (ANOVA, F (6,28) = 1.10, P = 0.389). The mean distance crawled by snails on the metal-contaminated biofilm was between 103 % and 60 % of the distance crawled by those in the control treatment during the whole period of the experiment (**Error! Reference source not found.**).

Total distance crawled by active movement (speed > 0.033 mm s^{-1}) and its percentage of the total movement

Although the total distance crawled by *T. fluviatilis* was similar across the different metal treatments, the speed at which such distance was covered varied. In the control treatment, the distance crawled by the snails at higher speeds (> 0.033 mm s^{-1}) was significantly greater than the distance crawled by snails exposed to biofilm spiked either with low Cu concentrations or with Cu and Zn mixed at low concentrations (ANOVA, F (6,28) = 2.88, P = 0.026), (**Error! Reference source not found.**). The percentage of total movement at higher speeds (> 0.033 mm s^{-1}) was also higher in snails from the control treatment when compared to previously mentioned treatments (ANOVA, F (6,28) = 3.77, P = 0.007). Approximately 18 ± 1 % (n = 5) of the movement done by snails in the control treatment was performed at speeds higher than 0.033 mm s^{-1}; a percentage that was higher than for any of the snails exposed to metal contaminated food (**Error! Reference source not found.**). However, results from the procedure controls show that in treatments without food snails move the furthest at higher speeds (ANOVA, F (3,16) = 9.33, P < 0.001), (**Error! Reference source not found.**). In the treatments without food, the percentage of active movement was significantly greater than in the control treatment (ANOVA, F (3,16) = 11.49, P < 0.001), corresponding to around 62 ± 12 % (n = 5) and 55 ± 12 % (n = 5) of the total distance crawled, respectively (**Error! Reference source not found.**).). Thus, snails facing food absence not only crawl over greater distances but also do it at greater speeds.

Feeding behaviour

The area of biofilm grazed by snails was similar across the experimental treatments (ANOVA, F (6,28) = 0.44, P = 0.848). Over the 96 h of the experimental period, snails removed on average 0.46 ± 0.14 mm^2 (n = 35) of biofilm, corresponding to ca 765 mg wet weight (**Error! Reference source not found.**). Contrary to our expectations, unstarved snails seemed to feed slightly more than starved control snails (0.88 ± 0.26 mm^2, n = 5), although no significant differences were detected (ANOVA, F (6,28) = 2.81, P = 0.132).

Discussion

The metal sorption characteristics of biofilm affect metal bioavailability and influence metal transfer along the food web (Ancion et al. 2010). Our experimental design explored the effects of both single and combined exposure to Cu and Zn of natural biofilm, as well as the behavioural effects of these metals on a biofilm grazer, the snail *Theodoxus fluviatilis*.

Biofilm quality

Exposure of biofilm to high metal concentrations can lead to inhibition of the biofilm photosynthetic system (Barranguet et al. 2000; Corcoll et al. 2012). Nonetheless, exposure of 10-month old biofilm for 96 h to different Cu and Zn treatments did not lead to changes in the fluorescence parameter measured in our experiment. Biomass development in biofilms, through an accumulation of polysaccharide-rich materials over time, can have a protective action against toxicants (Sabater et al. 2007). Maximum PS II quantum efficiency (Fv/Fm ratio) did not change significantly across different metal treatments and generally remained above 0.6. Such results indicate good photosynthetic performance and are in agreement with values observed in other studies (e.g. Jesus et al. 2005; Tiam et al. 2015).

The biofilm not exposed to snail grazing presented the lowest cell viability ratio, which might indicate that grazing had a stimulating effect on biofilm by removing the top layer of senescent cells. Higher levels of chlorophyll a production in biofilms from grazed areas have also been observed in exclusion experiments with marine snails (Skov et al. 2010). Cell viability varied among treatments, although the metal levels were chosen in a manner that was not intended to impact biofilm condition in a detrimental way. In particular, biofilm exposed to metal mixtures presented the highest cell viability. The presence of Zn and Cu at a wide range of levels has been shown to affect, both positively and negatively, different microorganism and diatom species in biofilm (Soldo and Behra 2000; Ivorra et al. 2002; Vijver et al. 2011). However, in our experiment, it was the simultaneous presence of metals, rather than the individual metals, that led to higher cell viability in the biofilm. The effect of metal mixtures has also been described in the response of other organisms, e.g. response of isopods to single and combined exposure to Cd and Zn (Odendaal and Reinecke 2004) or *Oscillatoria* cyanobacterium exposed to mixtures of Pb, Zn and Cd (Lefcort et al. 2008). Exposure of riverine biofilms to Cu has been shown to induce a shift in the population from diatoms to cyanobacteria, while exposure to Zn promoted a strong reduction in diatoms in fluvial biofilm (Ancion et al. 2010; Xu et al. 2016). The toxicity of metals depends on several factors, such as the chemical speciation of the metals, the concentration and interaction between metals and the exposure duration (Soldo and Behra 2000; Massieux et al. 2004). Such factors have been shown to affect biofilm communities differently, leading to changes in abundance or alteration in the structure and composition of biofilm microbial communities (Paerl and Pinckney 1996; Barranguet et al. 2003). Therefore, it is important to assess the impact of exposure to multiple metals on natural communities, as species with short lifespan can lead to rapid modifications in biofilm community structure following environmental changes.

Snail behaviour

Exposure of aquatic organisms to metals can occur through three main pathways: direct contact with metals in the water phase, in the sediment or by exposure along the food chain (Deb and Fukushima 1999; Clearwater et al. 2002; De Schamphelaere et al. 2004). Assimilation of metals by primary producers like biofilms can promote

bioaccumulation through a food web (Behra et al. 2002; Meylan et al. 2003). Biofilms are an important resource for many invertebrate species (Lawrence et al. 2002; Becker et al. 2011), which may lead to bioaccumulation in primary consumers such as gastropods (Cardwell et al. 2013).

By providing metal-spiked biofilm as food resource to a gastropod, we focused on how metal accumulation in biofilms can impact feeding behaviour of primary consumers. Behaviour, as an integrator of responses on multiple biological levels, can provide early warnings of larger changes that might affect populations or entire communities (Peterson et al. 2017). The first response measured, time to first active movement, was significantly different between snails from control and clean tile (no food) treatments. This result indicates that snails discriminated between biofilm presence and absence. In the absence of biofilm, foraging started almost immediately, while snails placed in control biofilm seemed to initiate grazing before actively moving for the first time. This ability to distinguish between presence and absence of biofilm has been previously reported for other snail species (e.g. Kawata et al. 2001; Schössow et al. 2016). Previous studies have also shown that some snail species can actually employ strategies to select food sources. As an example, work on *Lymnaea stagnalis* shows that this species is able to select biofilm food sources through their odours (Moelzner and Fink 2014) and can sense the presence of metals (Zn and Cd) in their environment via the osphradium (Byzitter et al. 2012). In our study, a tendency for earlier active movement was observed in snails exposed to metal-spiked biofilm, which could indicate that these snails did not settle initially and had bolder behaviour. In contrast, snails from the control treatment were less bold and presented a more varied response.

In terms of total distance crawled, our results clearly show that in areas with no food source snails actively forage. This high foraging intensity was confirmed by the fact that snails started to move almost immediately after the start of the experiment and crawled over longer distances (around 5x more than those from control biofilm) while exploring for a food source at faster speeds. Our results mimic those from studies performed on other snail species (e.g. Kawata et al. 2001; Schössow et al. 2016) and concur with the optimal foraging theory (Charnov 1976).

In experimental treatments where biofilm was present, metal spiking of the food source and starvation of snails did not affect the total distance crawled by snails. Interestingly, the speed used by snails varied, with snails from control treatments moving a great percentage of the distance at higher speeds than those exposed to spiked biofilm. This indicates that snails exposed to spiked biofilm moved more frequently, but at slower speeds. Previous study results also show that metal contamination may alter gastropod activity. Depending on metal identity and concentration, the activity of snails may either decrease or increase (Truscott et al. 1995; Campbell et al. 2000; Hartono et al. 2017). For example, Hartono et al. (2017) illustrated that exposure to fine particulate (PM2.5), containing Al at different concentrations, either can led to hyperactivity or decreased activity of the freshwater snail, *Parafossarulus striatulus*. The alteration of crawling patterns in our experiment also suggests that when food is not available, snails move faster and crawl the greatest distances, presumably in search of a food source. Similar results were previously described for other aquatic snails species (Crisp et al. 1978). Any impairment or even alteration of normal movement patterns, as the ones detected by our experimental design, can have important implications during longer exposures. Such behaviours should be further studied as they can affect predator avoidance and foraging efficiency, which in turn may diminish the energy storage and alter overall fitness levels affecting specimens' survival.

The snail feeding rates observed across treatments were not influenced by differences in snail crawling patterns. Unstarved snails consumed the largest areas of biofilm, although statistically not different from the control treatments, which could indicate a better condition of unstarved snails. In gastropods, feeding involves the combined use of the olfactory system, chemoreceptors and mechanoreceptors to directly locate the food source (Chase 2002). Previous studies have shown that exposure to dissolved Cu can reduce prey searching efficiency of the snail *Polinices sordidus* (Hughes et al. 1987) and reduce time spent feeding by the predatory snail *Nassarius festivus*, while dissolved Zn increased the time spent feeding by *N. festivus* (Cheung et al. 2002). In contrast, exposure of snails to Cu and Zn through spiked biofilm did not affect feeding rates in our experiment. This could be explained by the metal concentrations used for biofilm spiking, which were possibly not high enough to affect feeding behaviour in *T. fluviatilis*. On the other hand, it is also possible that food palatability to *T. fluviatilis* does not change in the presence of Cu and Zn, leading to similar feeding behaviour across treatments. Assuming the last case is valid then an accumulation of such metals in snail tissues from contaminated areas, such as mining or others industrial areas, is expected to be facilitated. Other species of aquatic snails have shown to uptake and accumulate both Zn and Cu in their tissues (Ying et al. 1993; Pyatt et al. 2003; Hoang et al. 2008). Accumulation by primary consumers, such as *T. fluviatilis*, that are key components of aquatic food webs leads to potential additional bioaccumulation along the food chain, as gastropods are important prey for fish and invertebrate predators.

Conclusion

In summary, our results show that short-term exposure to dissolved Cu and Zn at ppb levels did not promote a quick and extensive alteration in the quality of the biofilm. Responses of *T. fluviatilis* to dietary exposure to both single and combined Cu and Zn were also constricted and consisted of impacts on initial movement behaviour and alteration of crawling speed patterns. Such effects may potentially initiate other alterations leading to a decrease in the fitness of the snails. A 24-hour starvation period did not affect the behaviour of *T. fluviatilis*. However, in the absence of food, the snails increased the total distance crawled and altered their crawling speed patterns. Therefore, frequent incorporation of behavioural response variables in ecotoxicological studies is encouraged. Suggestions for future studies include simultaneous use of unstarved snails and snails starved for a longer period, and exposure to a longer experimental period, as the manifestation of behavioural effects may be delayed and perceived long after the source of contaminant exposure is removed.

Compliance with Ethical Standards

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Tables

Table 1

Measured metal concentrations (µg L^{-1}) in the stock solutions used for spiking of the biofilm

Treatment	Cu	Zn
Control	0.7	3.3
Cu low	10.5	2.7
Cu high	96.4	2.6
Zn low	1.1	69.3
Zn high	1.3	636.8
Cu + Zn low	10.7	68.5
Cu + Zn high	94.4	631.5

Figures

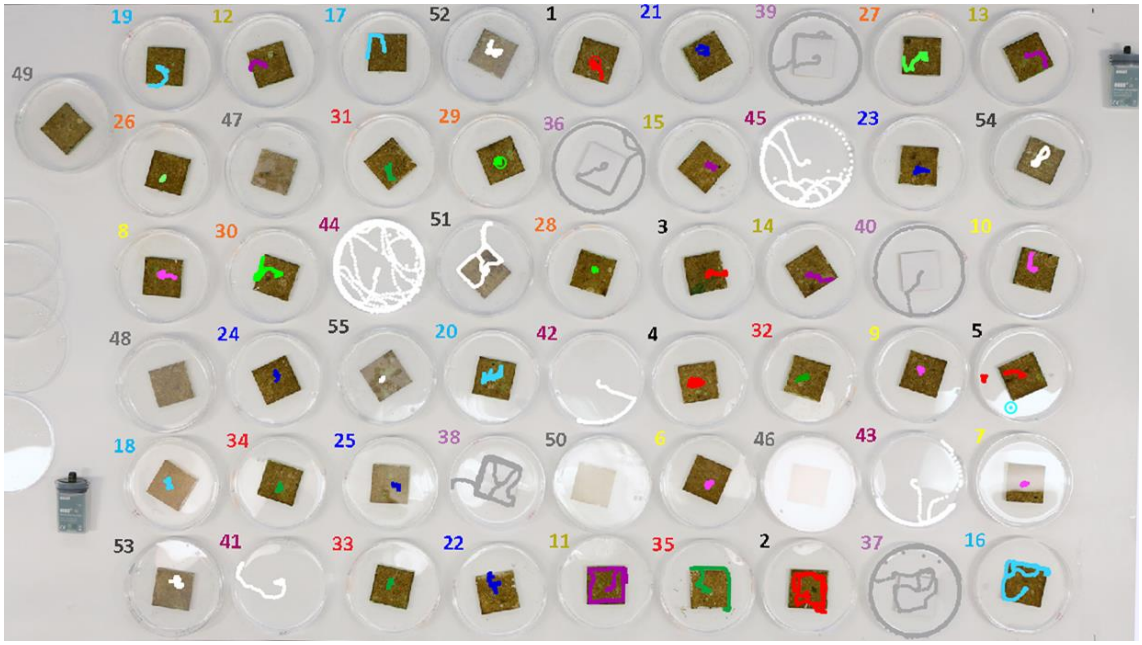

Fig. 1 Experimental setup showing paths of movements after a 3 h and 35 min period. Treatments: 1_5 Control, 6_10 Cu [Low], 11_15 Cu [High], 16_20 Zn [Low], 21_25 Zn [High], 26_30 Cu & Zn [Low], 31_35 Cu & Zn [High], 36_40 No biofilm, 41_45 No tile, 46_50 No Snails, 51_55 Unstarved snails

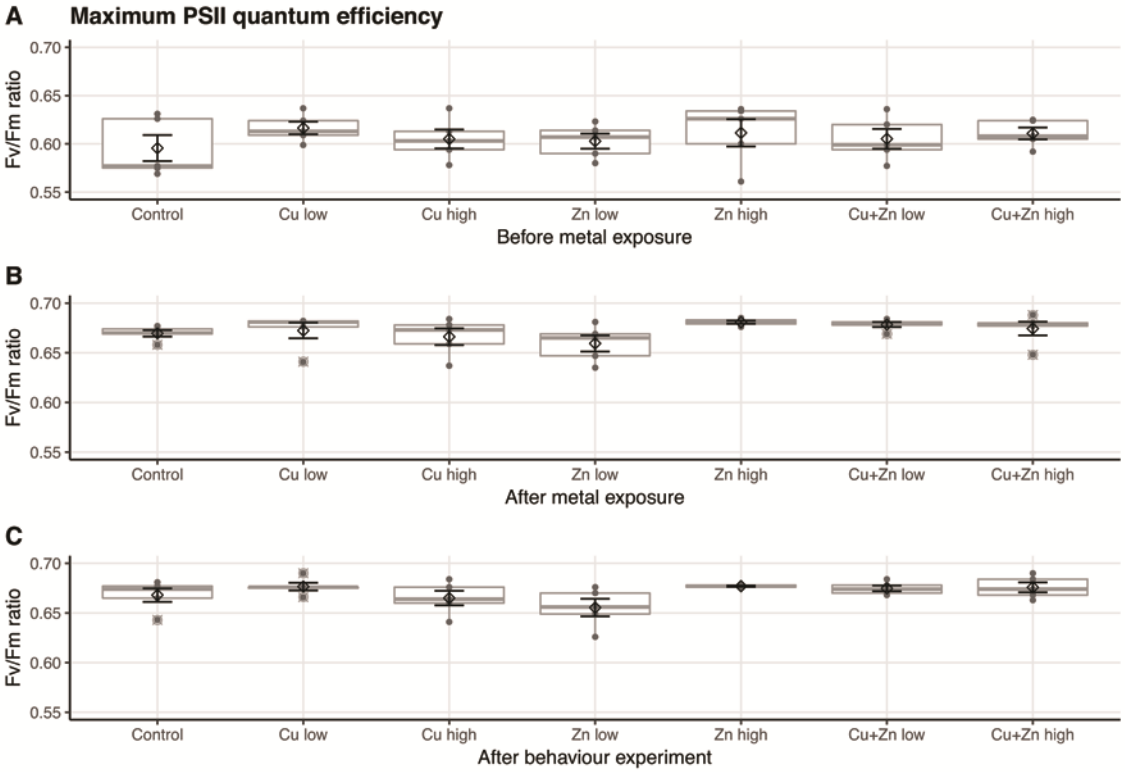

Fig. 2 Maximum PSII quantum efficiency in biofilm exposed to different treatments. a) Measured before metal exposure. b) Measured after metal exposure. c) Measured after behaviour experiment. Box plots and data points are shown in grey (*n* = 5), while means and 1 SE are in black

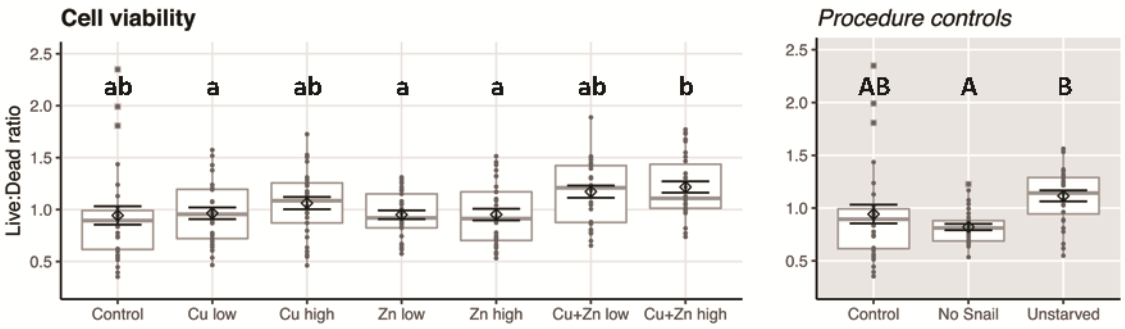

Fig. 3 Ratios between live and dead cells in the biofilm from different metal treatments and in the procedure controls. Box plots and data points are shown in grey (n = 28 to 30), while means and ±1 SE are in black. Different lower case letters, indicating significantly different responses across the different metal treatments, and different upper case letters, indicating significant differences detected across procedure controls, annotate the results of Games-Howell tests (statistically significant at α = 0.05)

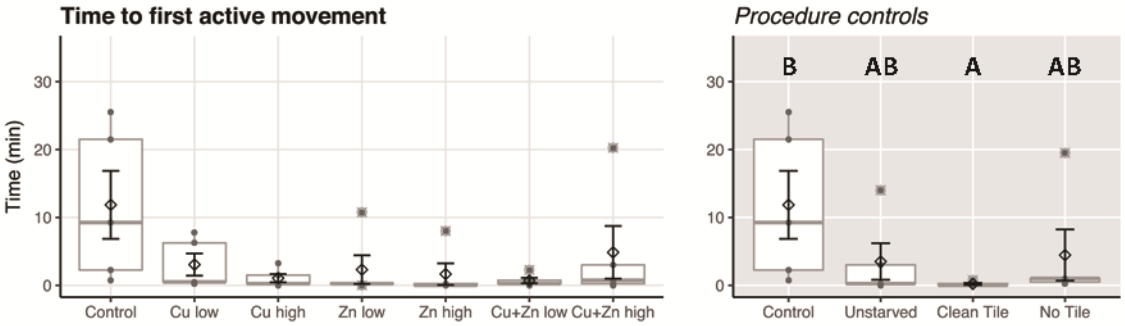

Fig. 4 Time to first active snail movement (mean speed > 0.033 mm s^{-1}, sustained for 15 s). Box plots and data points are shown in grey (*n* = 5), while means and ±1 SE are in black. Different *upper case letters,* indicating significant differences detected across procedure controls, annotate the results of REGWQ tests (statistically significant at α = 0.05)

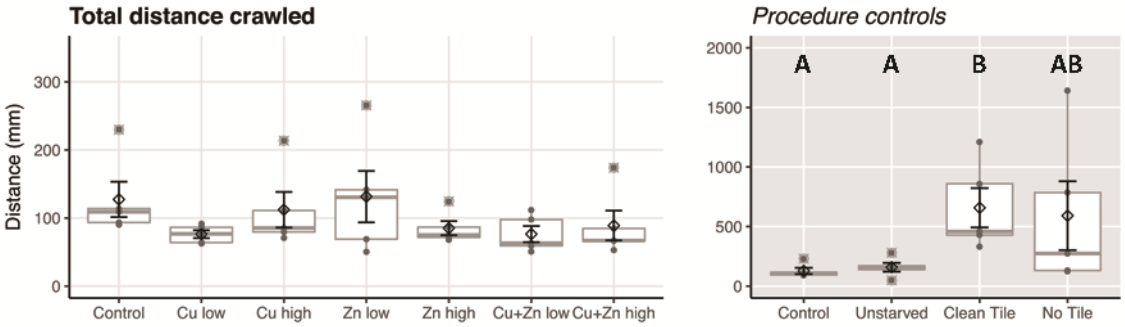

Fig. 5 Total distance crawled by snails during the behaviour experiment (≈ 3.5 h). Box plots and data points are shown in grey (n = 5), while means and ±1 SE are in black. Different upper case letters, indicating significant differences detected across procedure controls, annotate the results of REGWQ tests (statistically significant at α = 0.05). Note the different scales on the Y axes

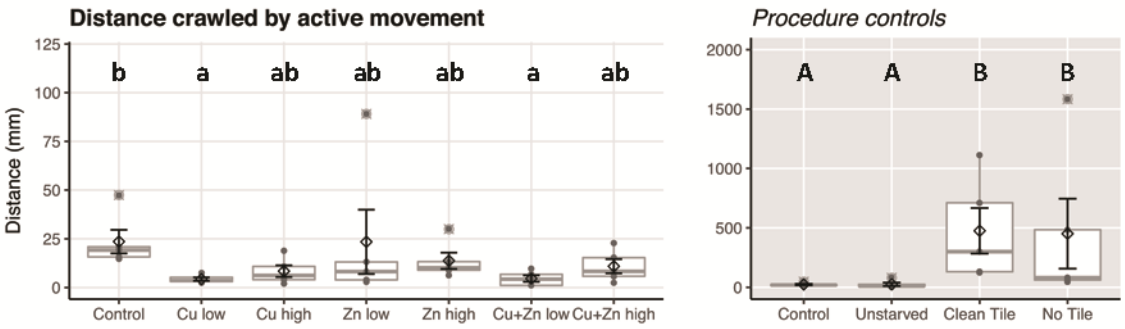

Fig. 6 Distance crawled by snails at speeds higher than > 0.033 mm s^{-1}. Box plots and data points are shown in grey (n = 5), while means and ±1 SE are in black. Different lower case letters, indicating significantly different responses across the different metal treatments, and different upper case letters, indicating significant differences detected across procedure controls, annotate the results of REGWQ tests (statistically significant at α = 0.05). Note the different scales on the Y axes

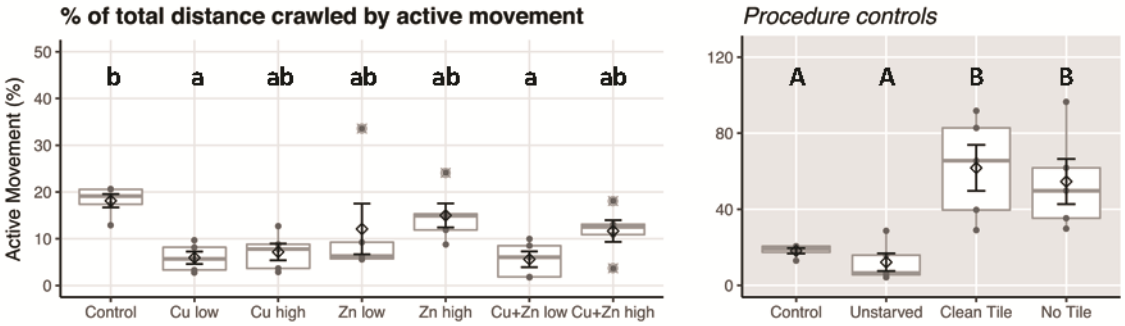

Fig. 7 Percentage of the total distance crawled by snails at speeds higher than > 0.033 mm s^{-1}. Box plots and data points are shown in grey (n = 5), while means and ±1 SE are in black. Different lower case letters, indicating significantly different responses across the different metal treatments, and different upper case letters, indicating significant differences detected across procedure controls, annotate the results of REGWQ tests (statistically significant at α = 0.05). Note the different scales on the Y axes

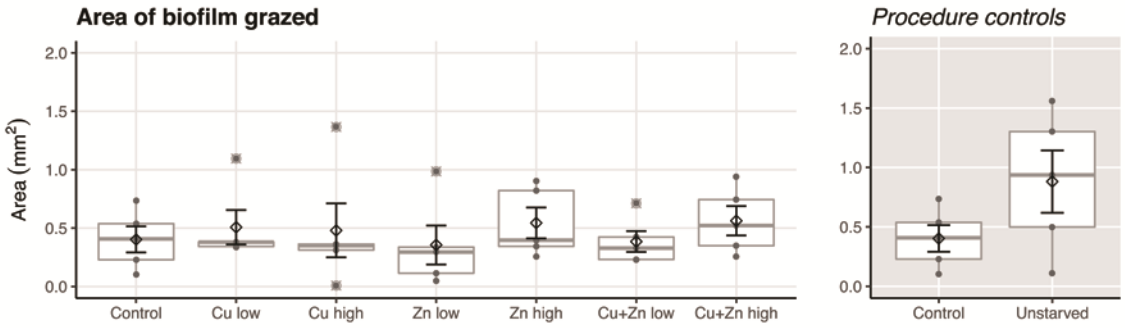

Fig. 8 Area of biofilm grazed by snails during the feeding experiment (96 h). Box plots and data points are shown in grey (n = 5), while means and ±1 SE are in black

Deliverable 4.4 Report/scientific articles on development of new bioassays for monitoring sub-lethal effects on chemically mediated behaviours in gastropods and crustaceans.

Appendix 4

Excerpts from the manuscript in progress: Behavioural study of the amphipod *Monoporeia affinis* exposed to sediments spiked with antifouling substances.

Maria Bighiu, Ann-Kristin Eriksson Wiklund

Excerpts from the manuscript in progress, preliminarily entitled:

Behavioural study of the amphipod *Monoporeia affinis* exposed to sediments spiked with antifouling substances.

Maria Bighiu, Ann-Kristin Eriksson Wiklund

2017.

Material and Methods

Six different experiments were performed and experiment one was repeated to improve the experimental setup. In the experiments we tested the avoidance/preference of *M. affinis* to sediments spiked with, combinations of Cu, Zn zinc pyrithione (ZnPt) and copper pyrtithione (CuPt) at different concentrations (Table 2).In experiment one we also examined the effect of food addition in combination with the contaminants. The organisms and the sediments were collected in the Baltic Sea outside of Askö field station. The sediment were sieved through a 1.0 mm mesh size net, to remove larger particles and macrofauna, before it was mixed with sand and water to get a dry weight of about 70 to 80%. Solutions were prepared to achieve the highest concentrations (1 µg/g dw CuPt and Zn Pt, 100 µg/g dw copper and 400 µg/g dw zinc). These sediments were then diluted with control sediments to achieve desired concentrations (Table 2). CuPt and ZnPt were disolved in 5 ml acetone followed by ultrasound during ten minutes before sediment addition. All sediment-contaminant mixtures were slowly stirred during 24h. The copper sediment and the zinc sediment were made five weeks before the experiment start to allow equilibration with sediment. Due to the fast degradation of the pyrithiones, sediments with CuPt and ZnPt were made a couple of days before respective experiment. All beakers containing CuPt and ZnPt were covered in aluminium foil to prevent degradation from light. To avoid differences in acetone concentration, a corresponding amount of acetone–water mixture was added to all batches. The acetone concentration in each batch was less than 1 per mille. Petri dishes were filled with sediments (Table 1) and were then randomly put in each aquarium. (Figure 1.). The dishes were allowed to settle for 2 h before the aquaria were gently filled with water and the flow through system was connected. After the aquaria was filled 150 individuals of *M. affinis* was added to each aquarium. In each experiment, we used nine replicate aquaria. The experiment was performed at 8°C at controlled light conditions corresponding to Scandinavian winter in terms of light intensity and duration. Three samplings per experiment was performed, after 96, 192, 288 hours. Three replicates were terminated at each occasion. All petri dishes were immediately removed from respective aquarium and the number of individuals in each petri dish, in each aquarium were counted. This procedure was then repeated at each sampling. Free-swimming individuals were counted to assess potential mortality. The response in the ZnPt and CuPt treatments was not consistent and we suspected degradation of the pyrithiones. Thus we performed a short term experiment in complete darkness, sampling performed after 2, 4 and 8 hours.

The statistical analyses of animal numbers were performed using a generalized linear model (e.g. Agresti, 2002) assuming a Poisson distribution and using a logarithmic link function. The model included a factor allowing for over-dispersion and allowed for correlation between jar/Petri dishes from the same aquarium. The model included factors for concentration/food type/substance, and the interaction between these factors. The statistical tests are based on a chi-square distribution with one degree of freedom. Based on our statistical model, we estimated relevant differences and contrasts between groups. The calculations were performed using the GENMOD procedure of the SAS Software (ver. 9.4). A multiplicity correction, including all comparisons, was performed according to Holm, (1979).

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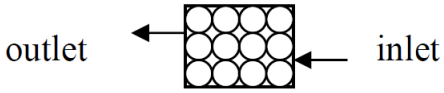

Figure 1. The experimental set up, 10 petri dishes filled with prepared sediment was randomly placed in each aquarium. To each aquarium 150 individuals of *Monoporeia affinis* was added.

Table 2. An overview of substances and concentrations in the experiments.

	Concentration				
Experiment 1	Cu: 25 µg/g Zp: 1µg/g	- -	- -	- -	- -
Experiment 2	CuPt: 0,1 µg/g ZnPt: 0,1 µg/g	0,25 µg/g 0,25 µg/g	0,5 µg/g 0,5 µg/g	0,75 µg/g 0,75 µg/g	1,0 µg/g 1,0 µg/g
Experiment 3	Cu: 25 µg/g Zn: 100 µg/g	50 µg/g 200 µg/g	75 µg/g 300 µg/g	100 µg/g 400 µg/g	- -
Experiment 4	Zn: 20 µg/g Zp: 0,1 µg/g	50 µg/g 0,25 µg/g	100 µg/g 0,5 µg/g	150 µg/g 0,75 µg/g	200 µg/g 1,0 µg/g
Experiment 5	Cu: 10 µg/g CuP: 0,1 µg/g	25 µg/g 0,25 µg/g	50 µg/g 0,5 µg/g	75 µg/g 0,75 µg/g	100 µg/g 1,0 µg/g
Experiment 6 Short exposure	CuPt: 0,1 µg/g ZnPt: 0,1 µg/g	0,25 µg/g 0,25 µg/g	0,5 µg/g 0,5 µg/g	0,75 µg/g 0,75 µg/g	1,0 µg/g 1,0 µg/g

Experiment 1

Cu (25 µg/g dw) and ZnPt (1 µg/g dw) were tested in combination with different food qualities in the form of micro algae. Threee species of cultured alga were used; *Thalassiosira weissflogi, Rhodomonas salina* and *Aphanizomenon sp.* The algae were cultured semi-statically in standard culturing mediums adapted for each species and were harvested every 3-4 day. The cultured algae were allowed to settle and were washed with natural brackish water before carbon content was determined. The carbon content of the algal suspensions was determined by measuring cell density and size and applying standard equations

(Hasle and Fryxell, 1977; Hill and Wetherbee, 1989; Menden-Deuer and Lessard, 2000). Algae corresponding to x mg fresh carbon of each algae species were added. To each aquarium

Experiment 2
CuPt and ZnPt were tested in five different concentrations (Table 1) preparing one Petri dish of each concentration and two controls in every aquarium.
Experiment 3
Cu and Zn were tested in four different concentrations (Table 1) preparing one Petri dish of each concentration and two controls.
Experiment 4
Zn and ZnPt were tested in five different concentrations preparing one Petri dish of each concentration and two controls. (Table 1). In order to get a more differentiated behavioural response the Zn concentration was reduced by 50 % compared to experiment 3.
Experiment 5
Cu and CuPt were tested in five different concentrations (Table 1) preparing one Petri dish of each concentration and two controls.
Experiment 6
CuPt and ZnPt were tested in five different concentrations (Table 1) preparing one Petri dish of each concentration and two controls in every aquarium. The duration was in total 8 h and the exposure was performed in darkness.

Results

A general result from all experiments was that the exposure length did not affect the amphipod preference. Thus, results from all sampling occasions within one experiment were pooled.

Experiment 1
122 of 150 animals survived. There was no significance between the algae treatments when comparing the sum of all survivors in the different algae treatments.

When comparing the contaminant exposures in the combined food and contaminant experiment there was significant difference between ZnPt and copper in all algae treatments (Fig. 2). There was only a significant difference between copper and control in the treatment with sediment only (Fig 2). When comparing zinc-pyrithione and control all the algae treatments apart from the sediment treatment were significantly different (Fig. 3). Comparing the different algae in copper, zinc pyrithione and control there were no significant difference. There were also significant differences between copper and zinc pyrithione and the control treatment.

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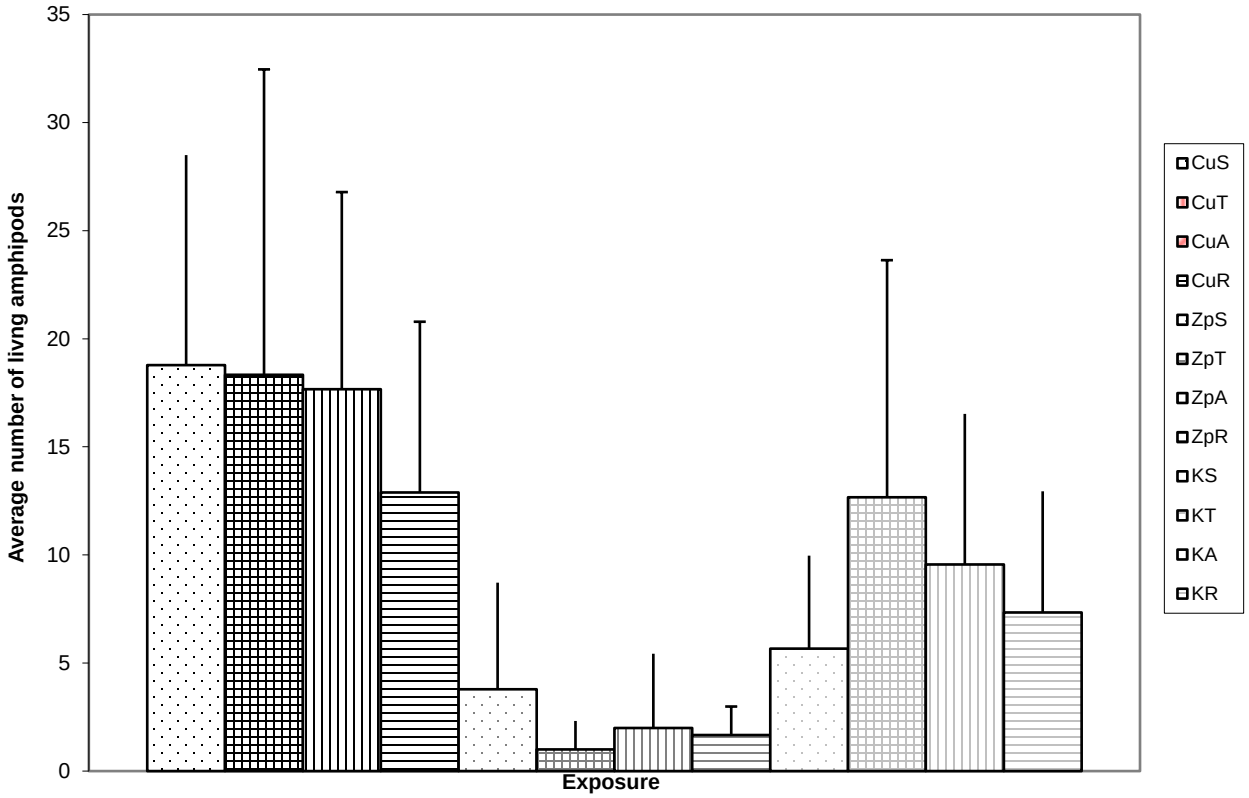

Figure. 2. Numbers of amphipods present in each Petri dish, bars represent mean values of 9 replicates + SD exposed to combinations of Cu or ZnPt and algae. The figure legend on top represents the bar to the left and so on. For details see text. S= sediment only, T= Thalassiosira, A=Aphanisomenon, R=Rhodomonas, K=Control, Cu=Copper, Zp=Zinc pyrithione.

Excerpts from the manuscript in progress, preliminarily entitled:

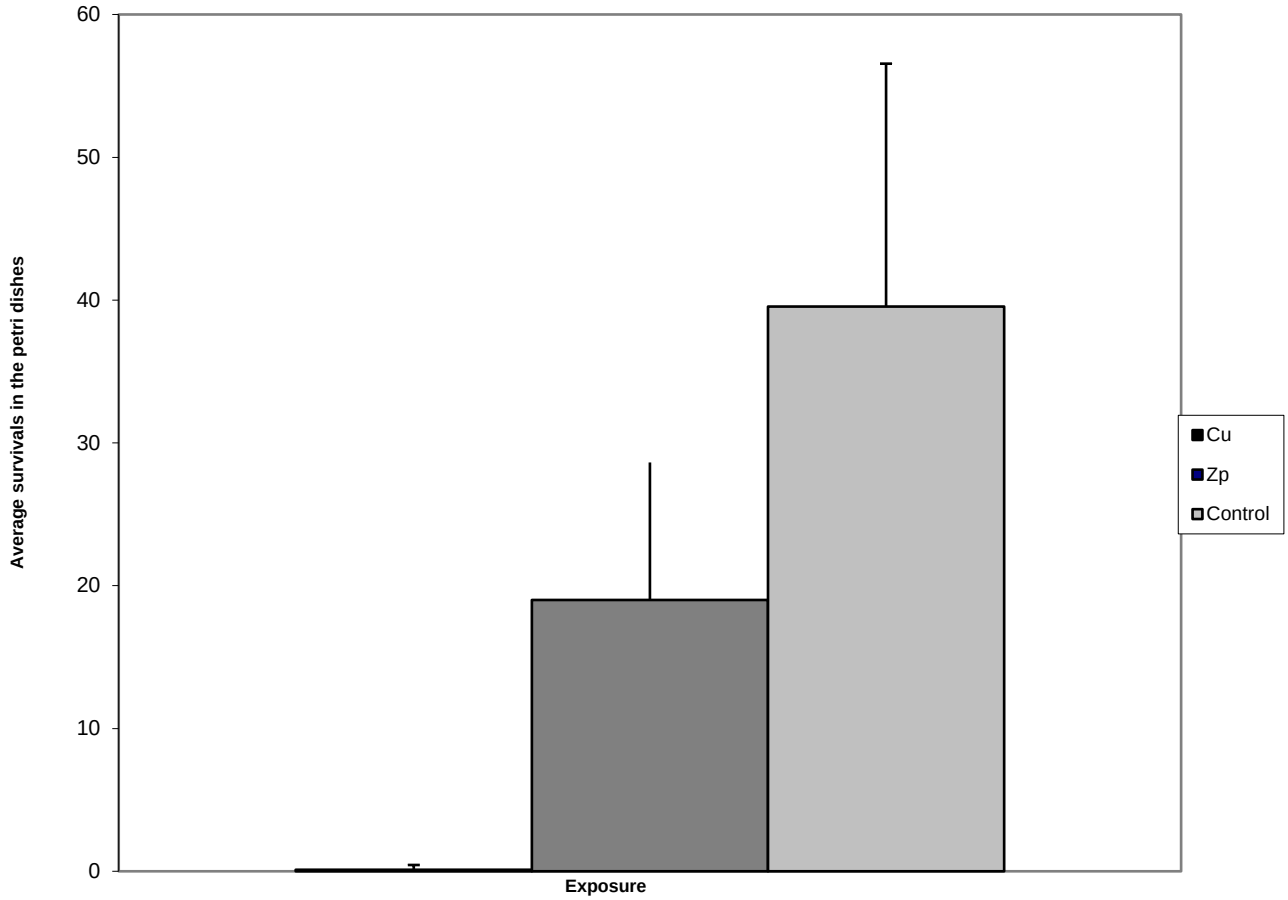

Figure 3. Numbers of amphipods present in each Petri dish, data represent mean value of 27 pooled replicates + SD, irrespective algae treatment.

Experiment 2

73 of 150 animals survived.

No significant difference was observed between the control and the different concentrations of CuPt and ZnPt (Fig. 4)

Excerpts from the manuscript in progress, preliminarily entitled:

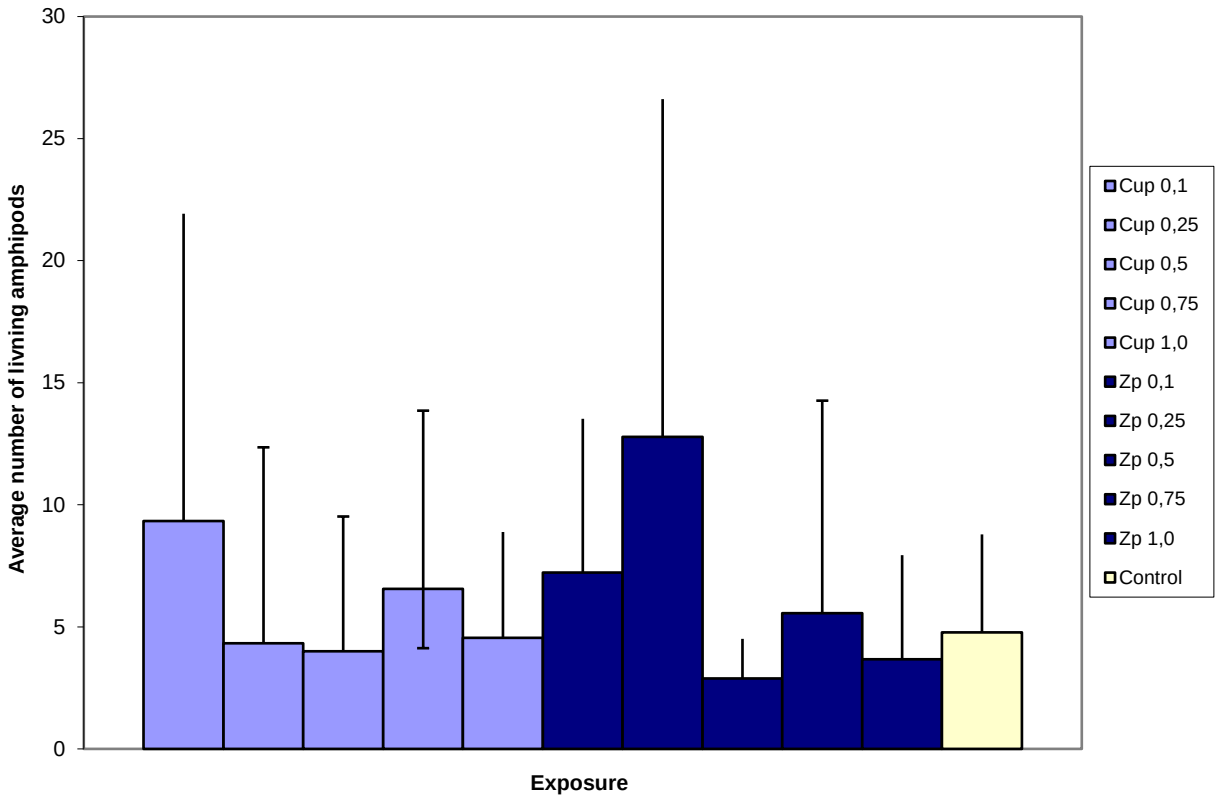

Figure 4. Numbers of amphipods present in each Petri dish, data represent mean value of 9 replicates + SD. Each bar represents a concentration of an exposure, concentrations (µg/g dry weight) increase from left to right.

Experiment 3

73 of 100 animals survived.

When comparing the different concentrations of copper there was significant difference between the preference for concentrations 25 and 100 µg/g dw as well as 50 and 100 µg/g (Fig. 5. There was also significant difference between the highest and lowest zinc concentrations. Significant differences could be seen between the control and 25, 75 and 100 µg/g dw of Cu, and between the control and all the zinc concentrations EC_{50} for copper was 25,4 µg/g dry weight and for zinc 39,4 µg/g dry weight.

Excerpts from the manuscript in progress, preliminarily entitled:

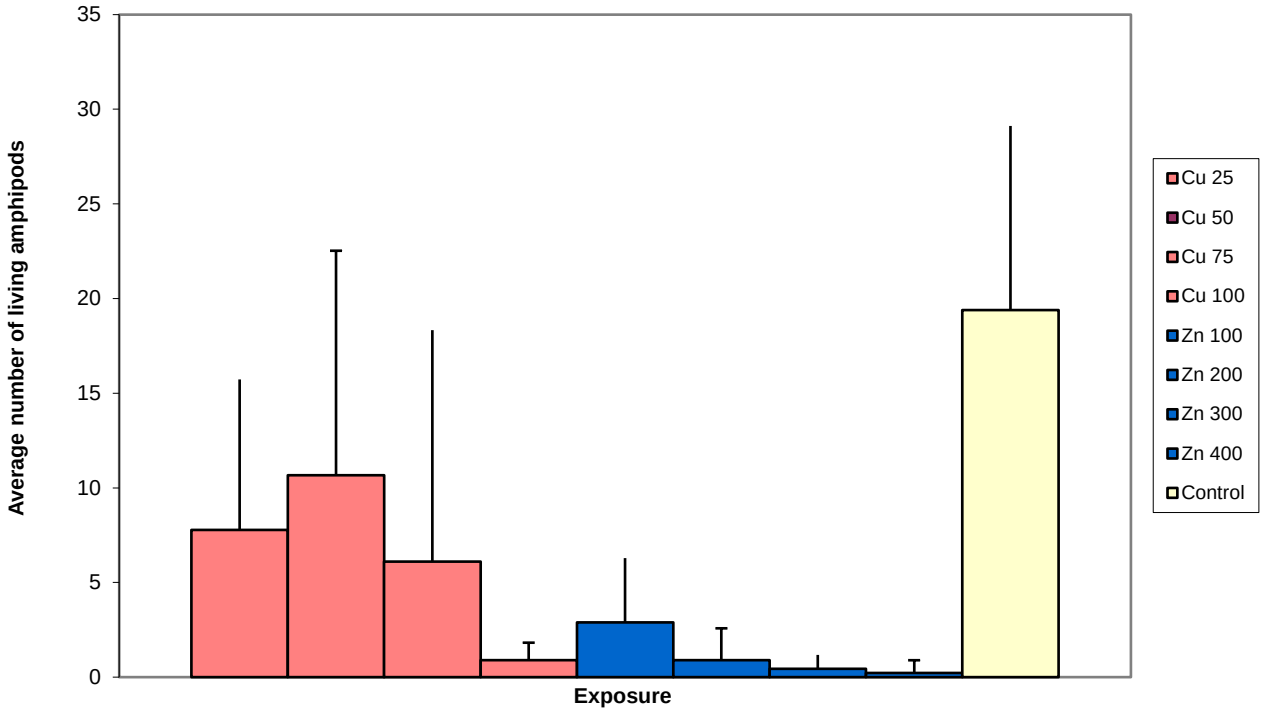

Figure 5. Numbers of amphipods present in each Petri dish, data represent mean values of 9 replicates + SD. Each bar represents an exposure concentration in μg/g dry weight. Concentrations increase from left to right.

Experiment 4

31 of 150 animals survived.

When comparing the preference for sediments spiked with zinc there were significant differences between 20 μg/g dw and 100, 150, and 200 μg/g dw. (Fig. 6). Significant differences could be seen between the control and zinc concentration 50, 100, 150 and 200 μg/g dw and control and zinc pyrithione concentration 0.25 and 0.75μg/g dw (Fig. 6).

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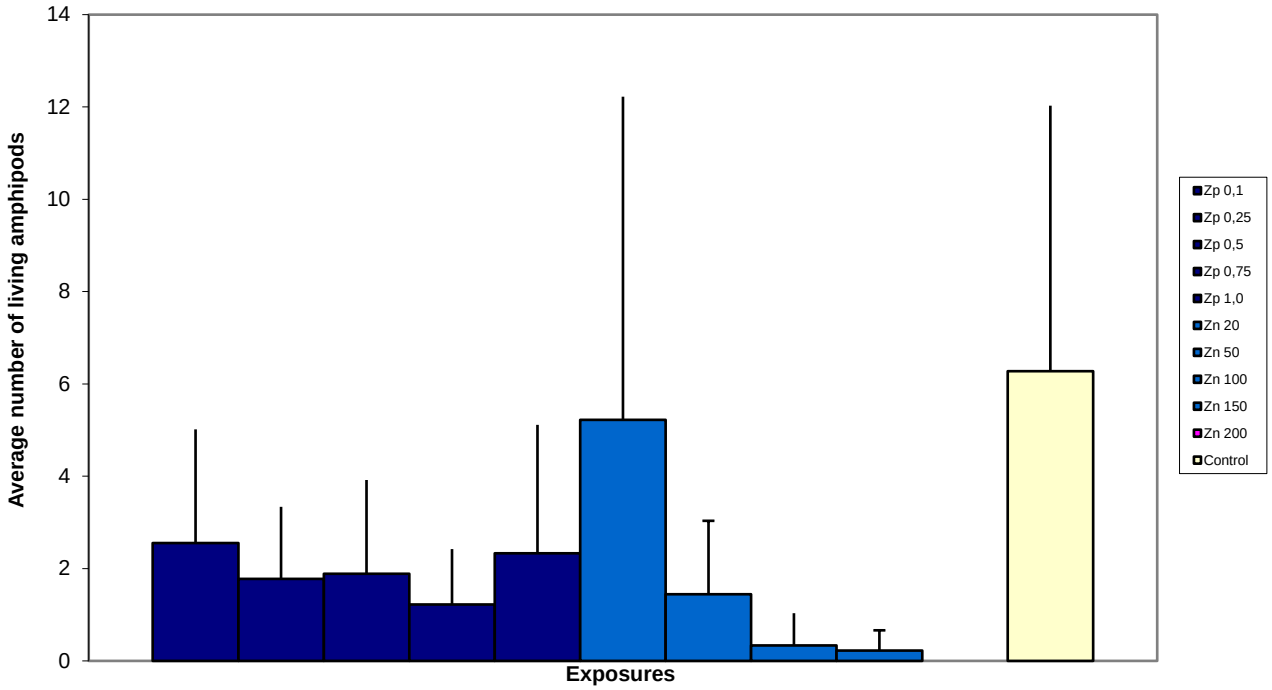

Figure 6. Numbers of amphipods present in each Petri dish, data represent mean value of 9 replicates + SD. Each bar represents a concentration of an exposure, concentrations (µg/g dry weight) increase from left to right.

Experiment 5

23 of 150 animals survived.

There was no concentration dependent difference in amphipod preference for CuPt. (Fig .7). On the other hand, there were significant differences between the copper concentration 10 µg/g dw and 75 and 100 µg/g dw (Fig. 7). No difference in amphipod preference was observed between the control and CuPt, while there were differences between the control and Cu (75 and 100 µg/g dw) (Fig 6).

Excerpts from the manuscript in progress, preliminarily entitled:

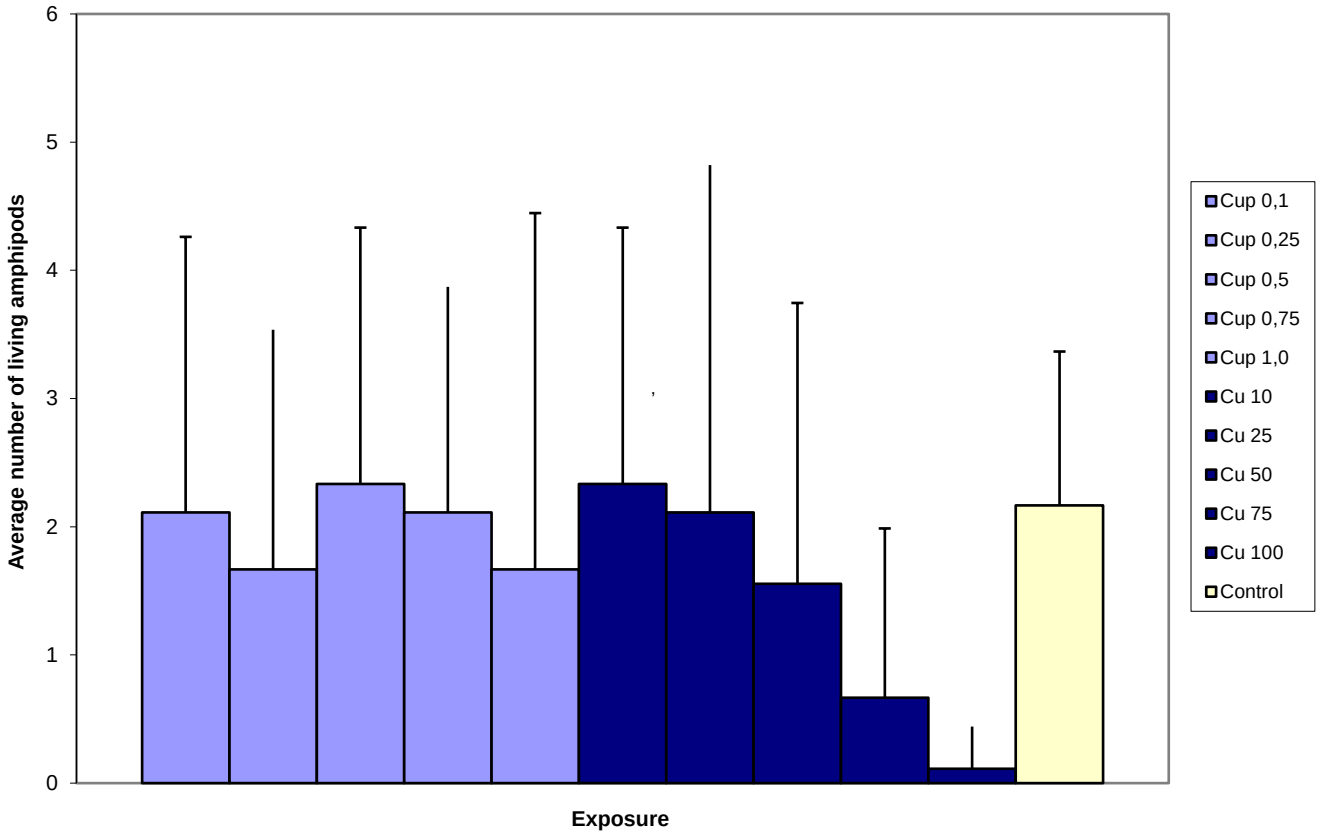

Figure 7. Numbers of amphipods present in each Petri dish, data represent mean value of 9 replicates + SD. Each bar represents a concentration of an exposure, concentrations (μg/g dry weight) increase from left to right.

Experiment 6 short exposure

When comparing the different concentrations in CuPt there was significance difference in amphipod preference between the lowest concentration all but the second lowest concentration (Fig. 8). Due to high variability, there was no differences in preference between the ZnPt concentrations (Fig. 8).

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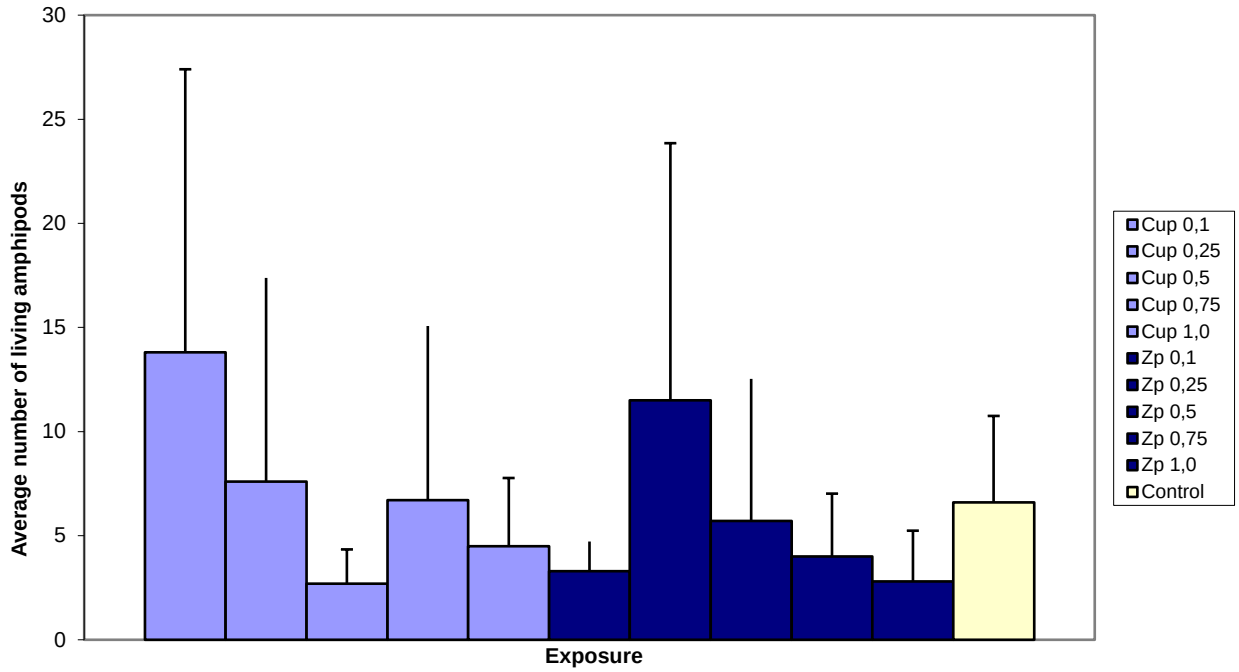

Figure 8. Numbers of surviving amphipods in each Petri dish, data represent mean value of 9 replicates + SD. Each bar represents a concentration of an exposure, concentrations (μg/g dry weight) increase from left to right.

Conclusions

M. affinis is a key stone species on Baltic Sea soft bottoms and used within the Swedish National Monitoring Programme and the indicator "reproduction disorders" of this species is a supplementary HELCOM indicator (HELCOM 2017). Its reproduction has previously been used in long term toxicity at the department Jakobsson et al 2006, 2008; Wiklund et al. 2005) In these series of short term experiments no effect of exposure time on the preference response was found and a short experimental duration (<24 h) was sufficient to get a distinct behavioural response. The effect of a fresh alga addition did not affect the amphipod avoidance to the contaminants. In experiments 1-5 we found an increasing avoidance response with increasing concentrations in a dose response relationship for Cu and Zn but not for CuPt or ZnPt. This was an indication of degradation of the pyrithiones. A short term experiment performed in darkness using only CuPt and ZnPt was performed to test this hypothesis and a similar dose response relationship as for Cu and Zn was obtained. In conclusion, the experimental set up first tested by Wiklund et al. 2006 was also successful in a more complex setting testing multiple factors (several concentrations for and more than one substance) simultaneously. The amphipod *M. affinis* is able to respond to sediment contaminants at low doses. This simple setup is a cost effective approach to test contaminants in sediments to a relevant organism. The method can most likely be adopted to other similar species like the amphipod *Pontoporeia femorata*.

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Excerpts from the manuscript in progress, preliminarily entitled:

HELCOM 2017
http://www.helcom.fi/Core%20Indicators/Reproductive%20disorders_malformed%20embryos%20of%20amphipods%20-%20HOLAS%20II%20component_June%202017.pdf

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