Bioaccumulation of lanthanum in marbled crayfish (*Procambarus sp.*)

Master Thesis

Eyerusalem Goitom

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Table of content

Lists of figures

Lists of tables

Abstract

The novel lanthanum modified clay, Phoslock® is a promising tool for remediating the persisting problems of eutrophication. Phoslock® is highly efficient in removing phosphorus from the water column and in preventing phosphorus released from the sediment. The active ingredient of Phoslock® which binds phosphate is the Rare Earth Element lanthanum. In 2008, after the application of Phoslock® in Lake Rawbraken The Netherlands, much higher lanthanum concentration was observed in analysed whole crayfish. It is unknown what the potential accumulation of lanthanum is in specific tissues of the crayfish. In order to find out the potential accumulation of lanthanum in specifc tissues we carried out a bioaccumulation assay experiment with the adult marbled crayfishes. The crayfishes were grouped into control and Phoslock® groups. The Phoslock® group was continously exposed to 1000mg l^{-1} solutions of Phoslock® while the control group was held in clean copper free water for 14-28 days. Ten crayfishes from each group were sacrificed at day 0, after 14 and 28 days of exposure. At the end of 14 and 28 days, the crayfishes were transferred to clean copper free water for 4 days to empty the gut. All crayfishes were dissected into their carapace, gills, ovaries, hepatopancreas and abdominal muscle, and the concentration of lanthanum in each tissue was determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The result showed that there was a strong significant difference in bioaccumulation of lanthanum between control and Phoslock® groups. The order of magnitude for lanthanum bioaccumulation in tissues was gills > hepatopancreas > carapace > abdominal muscle > ovaries with the maximum values 316.74, 109.59, 17.43, 3.68, and 5.50 µg g^{-1} respectively. The study showed that the crayfish has a potential for accumulation of lanthanum and, gills and hepatopancreas are the potential organs for the accumulation of lanthanum.

Keywords: Eutrophication, Phoslock®, Bioaccumulation, Lanthanum, Carapace, Gills, Ovaries, Hepatopancreas, Abdominal muscle

1. Introduction

1.1. Eutrophication

Eutrophication is the process of over-enrichment of surface waters with nutrients leading to increased phytoplankton productivity and subsequently, to a deteriorated under water light regime. As a result of eutrophication surface water may shift from macrophyte dominated system to a system dominated by algal blooms (Scheffer, 2004; Aertebjerg *et al.,* 2003; Chorus and Bartram, 1999). Amplified inputs of nutrients from agricultural runoff, industrial effluent and municipal sewer system are the main causative factors and the negative outcomes of eutrophication have increased in freshwater habitats for decades. Nitrogen and phosphorus are key nutrients, which can limit aquatic primary production. As observed by many researches over- enrichment of surface waters with these nutrients promote eutrophication (Scheffer,2004; Paerl, 1988, 2009; Smith, 1998, 2003).

In standing waters eutrophication lead to blooms of toxic cyanobacteria, which in turn cause a decrease in biodiversity (Smith, 2003; Paerl, 1988). Associated with surface scums, low levels of oxygen are observed, which are held responsible for fish kills (Smith, 1998; Paerl, 2009; Carpenter, 2008; Dittmann and Wiegand, 2006). This is due to the fact that, when cyanobacteria and algae reach the peak in their growth they have usually consumed all nutrients and start to die off. The decay of algal matter may lead to the oxygen depletion in the water, which in turn can cause secondary problems such as fish kills from lack of oxygen and release of toxic substance or phosphates that were previously bound to oxidized sediment (Chorus and Batram, 1999). Furthermore, cyanobacteria are a threat to aquatic wildlife as well as human health (Paerl, 1988; Chorus and Bartram, 1999; Reynolds, 1987), hence render surface water unfit for use.

In eutrophic lake filamentous and colony forming cyanobacteria often dominate in late summer and autumn in which they become inedible by size, low quality food and toxic for zooplankters such as Daphnia (Chorus and Bartram, 1999). This greatly alters the ecological integrity of fresh water resources which may lead to a decline in macro invertebrate abundance, composition and species richness including fish species (Oberholster *et al.,* 2009). It is obvious that controlling the inputs of these nutrients is the best way to mitigate eutrophication problems.

In aquatic environments it is accepted that phosphorus control is more sensible than that of nitrogen (Schindler *et al.,* 2008; Carpenter, 2008; and Likens (1972, as cited in Paerl, 2009). Because, some cyanobacteria are able to escape nitrogen limitation by fixing atmospheric nitrogen (Chorus and Bartram, 1999; Schindler *et al.,* 2008). In addition to that, nitrogen can not be removed chemicallly because, inorganic and organic forms of nitrogen are relatively more soluble and available than phosphorus (Gunnars and Blomquist, 1997).

Herve (2000, as cited in Ross *et al.,* 2008), showed that only one gram of phosphorus is required for every seven grams of nitrogen for the formation of the organic matter created in the process. This designates that, small reduction in phosphorus can lessen large degree of reduction in cyanobacteria growth. Accordingly, Phosphorus (P) control is critically important to mitigate eutrophication problems. In order to attain this objective different management tools were developed. Among eutrophication management tools, lanthanum- modified bentonite clay, Phoslock® is an effective tool which is highly efficient in removing or binding phosphorus from the water column and

preventing the release of phosphorus from the sediment (Akhurst *et al.,* 2004; Ross *et al.,* 2008; Douglas *et al.,* 1999).

There are many studies which concur on the effectiveness of Phoslock® and its ability to remove the filterable reactive phosphorus (FRP) from the water column as well as its ability to prevent the release of FRP from the sediment by more than 90% (Douglas *et al*., 1999; Ross *et al.,* 2008; Akhurst *et al.,* 2004; Robb *et al.,* 2003). Nevertheless, mitigation of eutrophication problems (for example algal blooms) by Phoslock® will only be effective in combination with control of nutrient inputs to the water body from sources (Robb *et al.,* 2003).

1.2. The Cyano team research on Phoslock®

In 2008, novel 'flock and lock', combination of low-dose flocculent and lanthanum enriched bentonite clay (Phoslock®) was applied to Lake Rauwbraken, The Netherlands to remediate persisting problems of cyanobacteria and high phosphorus (P) concentration in lake. The aim was to flocculate the algal biomass to the bottom of the lake using flocculent and then use Phoslock® to capture any orthophosphate in the water column as well as phosphorus released from the sediment. The treatment had an immediate and sustained effect on the lake's appearance and ecological status. That is, filamentous cyanobacteria and all phosphorus were rapidly removed from the water column and strongly reduced P- release from the sediment. Moreover, the concentration of chlorophyll-a reduced to a very low levels of 2 µg l^{-1}. As a result, the lake was able to reopen for swimming soon after the application (Van Oosterhout and Lurling, 2010).

1.3. Characteristics of Phoslock®

Phoslock® is developed by the Common Wealth Scientific and Industrial Research Organization (CSIRO), Australia. It is composed of bentonite clay (95%) and Lanthanum (5%). It can be applied to water bodies including lakes, drinking water reserviours, and aquaculture ponds to eliminate excess FRP by precipitation of lanthanum phosphate ($La^{3+} + PO_4^{3-} \rightarrow LaPO_4 \cdot nH_2O$), reducing the amount of algal blooms (www.Phoslock®.com). Phoslock® has been applied to water bodies in different countries such as The Netherlands, Australia, Germany, UK, Italy, South Africa and New Zealand to mitigate eutrophiation problems (www.Phoslock®.com).

Dosing of Phoslock® application to water bodies depends on the bioavailable and total amount of phosphorus present in the water body, hydrological properties of water such as inflows and runoff, release of phosphates from the sediment as well as chemical properties of water (for example, alkalinity of the water). According to Groves (2010), Phoslock® applied at the rate of 100: 1, Phoslock® to FRP can eliminate the bioavailable phosphorus from the water column.

Phoslock® can be applied as slurry or granules form. When Phoslock® is applied to a water body, it moves downwards, while binding FRP in the water column and settles at the bottom. After settling on the bottom it forms approximately a 3 mm thick layer. This process effectively binds and intercepts the release of FRP from the sediment (Ross *et al.,* 2008; Douglas *et al.,* 1999; Van Oosterhout and Lurling, 2010; Akhurst *et al.,* 2004). Moreover, no adverse effects of Phoslock® are reported so far. Phoslock® does not affect the conductivity and pH of water body (NICNAS, 2001; Ross *et al.,* 2008). Nevertheless, the adsorption capacity of Phoslock® for FRP can be influenced by pH, presence of humic acids (the humic substance may compete with the binding site for FRP in the

bentonite by forming complexes with lanthanum), and particle size of the bentonite in the product Phoslock® (Ross *et al.,* 2008; NICNAS, 2001).

Ross *et al.* (2008) showed that the effectiveness of Phoslock® in removing phorsphorus decreased as the pH increased from 7 to 9. This implies that high pH (> 9) value can reduce the performance of Phoslock®. This could be due to the formation of hydroxyl species of lanthanum ions; loss of the binding site for phosphorus on the Phoslock® surface due to hydroxylation (Ross *et al.,* 2008).

1.4. Occurrence and Distribution of Rare Earth Elements

Rare Earth Elements (REEs) are abundant resources in China that constitute 80% of the world's known REEs reserves is in this country (Zhu *et al*.,2002) and used more extensively in agriculture, forestry, animal husbandry and aquaculture. As a consequence, the aquatic environment receiving much more amount of REEs as dissolved substance which have higher bioavailability for the organisms (Sun *et al*., 1997). In natural water bodies the bioavailability of REEs can be influenced by organic ligands, water hardness, pH and humic acids (Sun *et al*., 1997). Among these factors organic ligands and humic acids play great role in influencing the bioavailability of REEs through forming complexes in the aquatic ecosystem.

REEs are used for industrial and agricultural purposes, for example, the fertilizer used in china is Rare Earth nitrate mixture, the main components are lanthanum, cerium, praseodymium and neodymium Brown *et al*. (1990, as cited in Xu *et al*., 2002), lanthanum is used as active ingredient for the manufacturing of Phoslock® (NICNAS, 2001), and lanthanum carbonate has been used in the medical industry for preparing pharmaceutical drugs (Afsar and Groves, 2009). This use is constantly increasing, which consequently leads to scattering elements in the environment.

1.5. Chemical Properties of Lanthanides

Lanthanides (Ln^{3+}) are rare earth elements with atomic number 57 - 71 including (lanthanum; atomic number 57) to (Lutetium; atomic number 71) in the periodic table which have similar chemical properties and vary in relative atomic number from 57-71 (Evans, 1983; Zhu *et al*., 2002). Furthermore, they have about the same atomic radius and similar chemical properties with metallic ions such as Ca^{2+}, Mg^{2+}, Fe^{2+}, and Mn^{2+}. Lanthanides have the ability to substitute for a large number of metallic ions due to their high affinity to a given binding site (Evans, 1983).

1.6. Physiochemical property of Lanthanum

Lanthanum (La) is the rare earth element which is relatively abundant in the earth crust compared to other rare earth elements (REEs) having 139.9 & 57 molecular weight and atomic number respectively. It is the most electro- positive of REEs and uniformly trivalent, and has similar chemical properties to the alkaline earth elements (Das *et al.,* 1998).

During the preparation of Phoslock®, the lanthanum ions are exchanged with the clay surface adsorbed exchangeable cations (Ross *et al*., 2008). As a result lanthanum is locked in the clay and therefore supposed to be not bioavailable or/ and if lanthnaum reacts with phosphate is not also bioavailable. Lanthanum has a strong affinity for orthophosphate (Das *et al.,* 1988) and forms a higly stable mineral known as Rhabdophane ($La^{3+} + PO_4^{3-} \leftrightarrow LaPO_4 \cdot nH_2O$) Douglas *et al.* (2000, as cited in Ross *et al.,* 2008). As a consequence, the release and the bioavailabilty of lanthanum ion can be reduced. However, not all lanthanum is locked in the clay and some may be released by diffusion

into the water varying from 0.001% (Lurling and Tolman, 2010), to 0,02% (NICNAS, 2001). This could be due to the fact that lanthanum release from the bentonite clay is elevated at high ionic strength (alkaline condition), which can be attributed to the high re exchange capacity of lanthanum with sodium ion or calcium ion (NICNAS, 2001).This implies lanthanum release is dependent on the composition of water or media.

1.7.Toxicity of Lanthanum

It is generally assumed that the use of lanthanum is environmentally safe, lanthanum salts are not listed as harmful substances: lanthanum carbonate is prescribed in high dosage (up to 1000 mg day^{-1}) to patients suffering kidney failure (Mehrotra *et al*., 2008), meaning the compound is an approved and safe medication. However, the safety of lanthanum in human may greatly depend on the sophisticated excretion system of humans, which lower animals may lack. Further, the observed increased lanthanum in several biota, including crayfish (*Orconectes limosus)* (Van Oosterhout and Lurling personal comm.), after the Flock and Lock application in Lake Rauwbraken (April 2008) contradicts the idea that lanthanum is not bioavailable. According to Lürling and Tolman (2010), substantial impacts on *Daphnia magna* were observed from the presence of lanthanum in a culture medium containing phosphorus. This was most likely due to precipitation of Phoslock® particles bound algal matter that drastically reduced food availabilty (Lurling and Tolman, 2010).

Dissolved or free ion forms of lanthanum concentration can be toxic to some aquatic organisms depending on its concentration and application rate as well as chemical composition of water or media (NICNAS, 2001; Akhurst *et al*., 2004; Douglas *et al*., 2008; Barry and Meehan, 2000). According to Douglas (2008), commented on Akhurst *et al.* (2004), over-application of Phoslock® (8mm layer) may lead to overwhelming sensitivity of the benthic biota and can also generate undesirable ecological consequences such as impair respiration as a result of suffocation.

Short term toxicity (96hr) study of Phoslock® in crayfish (*Procambarus acutus*) Fasola *et al.* (2010), and long term toxicity in marbled crayfish (28days exposure) Wijnmalen *et al.* (2010), showed that in all concentrations (100, 330, 1000, 3300,10000 mg l^{-1}) & (100, 500, 1000, 2500 mg l^{-1}) respectively, Phoslock® had no effect on survival of both *Procambarus acutus* and marbled crayfish (*Procambarus sp.*). In crayfish exposed for 15 days to 1 g l^{-1} Phoslock®, lanthanum concentration was 24.2 times higher than in the controls (Fasola *et al*., 2010). This study indicates lanthanum can be taken up by crayfish and thus potentially accumulate in the body of the crayfish.

1.8. Effect of Rare Earth Elements (REEs) on Aquatic organism

Bioaccumulation of rare earth elements (REEs) is affected by characteristics and concentration of the REE compound, specific properties of the aquatic species and physiochemical factors of the surroundings (Qiang *et al*., 1994)**.** Bioaccumulation outcomes are an overall combination of factors like uptake, metabolism and excretion processes. The entry of dissolved REEs into the body of the organism might occur via different routes including gills, alimentary tract and carapace. However, the passage of entry depends on the kind of species (Qiang *et al*., 1994).

Bioaccumulation of representative elements such as light (Lanthanum), medium (Gadolinium) and heavy (Yttrium) REEs in Carps (*Cyprinus carpio*) was reported by Qiang *et al*. (1994). The Carps (*Cyprinus carpio*) exposed up to 45 days to the solutions containing 0.50 mg l^{-1} of lanthanum, gadolinium and yttrium, the pattern of bioaccumulation for all REEs in the tissues was internal

organs > gills > Scale > muscle (Table 1). Internal organs were the first potential site for bioaccumulation of REEs followed by the gills. By contrast, REEs accumulation by muscle was minimal. However, values of La were slightly higher in the skeleton and gills, and a bit lower in muscle and internal organs. Besides very low accumulation, neither synergetic nor antagonistic effects were seen in Carps after being exposed to the rare earth elements (Qiang *et al*., 1994).

Table 1 Literature values for bioaccumulation of REEs in Carps tissues

Tissue	Element	Group	Bioaccumulation values (µg/g wet weight)					
			5 day	10 day	17 days	24 days	31 days	45 days
Scales	La	A	0.86	1.30	1.76	2.50	2.71	2.82
		D	0.93	1.60	2.05	2.74	2.28	2.91
	Gd	B	0.50	1.77	1.41	2.13	2.42	2.30
		D	0.57	1.56	1.31	2.49	1.86	2.37
	Y	C	0.43	0.91	1.30	1.75	1.52	1.89
		D	0.49	0.82	1.41	1.68	1.59	1.54
Muscle	La	A	0.42	1.04	1.19	1.59	1.06	1.29
		D	0.30	0.70	0.86	1.39	1.02	1.23
	Gd	B	0.17	0.72	1.01	1.48	1.67	1.59
		D	0.23	0.83	1.18	1.42	1.28	1.30
	Y	C	0.09	0.24	0.16	0.35	0.59	0.48
		D	0.13	0.32	0.39	0.65	0.48	0.44
Gills	La	A	2.62	4.92	4.53	6.75	8.92	6.97
		D	3.32	4.79	5.07	6.12	5.58	7.03
	Gd	B	2.56	2.81	4.28	5.64	5.27	5.33
		D	1.86	2.37	3.97	4.38	4.20	5.58
	Y	C	1.87	2.32	2.94	3.95	3.41	3.70
		D	2.01	2.48	2.84	3.89	3.36	4.02
Internal organs	La	A	10.00	24.20	19.50	21.90	36.20	38.90
		D	16.10	31.50	37.70	34.40	45.60	41.40
	Gd	B	13.20	23.80	23.40	37.80	41.10	42.30
		D	18.40	33.70	36.30	49.90	52.70	48.50
	Y	C	6.82	15.90	19.30	24.50	22.70	23.60
		D	11.40	18.00	19.10	27.50	21.60	25.00

*Individual REE solutions were used in Group A-C, mixed REE solution were used in group D. (Qiang *et al*., 1994)

1.9. Mechanism of Metal Uptake by Aquatic Invertebrate

1.9.1. Trace metals

Trace metals are metals in extremely small quantities that reside in or are present in animal and plant cells and tissues. They are a necessary part of good nutrition, although they can be toxic if ingested in excess quantities. Examples of trace metals include iron, manganese, zinc, copper and etcetera (www.biology-online.org).

Aquatic invertebrate take up and accumulate trace metals in their tissue whether the metals are essential or not for metabolism (Rainbow, 2002**).** Uptake and accumulation of trace metals by aquatic organisms originate either from the surrounding medium or from food sources (Rainbow, 1997). The concentration of trace metals accumulated in the body tissues varies with the relative bioavailability of metals in the environment and type of the invertebrate involved (Rainbow and Wang, 2001; Rainbow and White, 1990; Rainbow, 2007).

The effects of trace metal accumulation depend on the particular physiology of the invertebrate species (Rainbow, 2002). Species differ in their use of trace metals for metabolic purposes as well as

their excretion or accumulate in the body (Depledge and Rainbow, 1990; Canli and Atli, 2003). Trace metals typically have an affinity for sulphur and nitrogen (Niebore and Richardson, 1980). Because amino acids (proteins) contain sulphur and nitrogen, they have many potential sites for trace metals (Rainbow, 1997). The binding of a trace metal to amino acids may interfere with the normal metabolic role of the protein it constitutes, indicating the potential toxicity of trace metals (Rainbow, 2002).

1.9.2. Heavy metals

Heavy metals refer to any metallic chemical element that has a relatively high density and is toxic at low concentration. Examples of heavy metals include mercury (Hg), cadmium (Cd), arsenic (As), chromium (Cr), and etcetera (Appenroth, 2010).

In aquatic invertebrate, there are three general categories of responses to heavy metals. First, the animal may regulate the metal by excreting all the metals which it has in excess to metabolic needs. Second, the organism may impound the metal permanently and excrete very little. The third category is storing of metals that are successively removed from the body. Hence, body load may reduce if the exposure ends **(**Rainbow, 2002). Crayfish would seem to fall into this third category, especially with regard to heavy metals for instance, cadmium (Rodin and Shivers 1987).

Crayfish have two organs that can involve in heavy metal detoxification and depuration such as hepatopancreas and the green gland. However, this pattern depends on the biological importance of the metals. For example, the biologically important heavy metal like Fe into hepatopancreas while, the one's with no known biological importance like lead into green gland (Roldan and Shivers, 1987). According to Ahearn *et al*. (2004), the gills of crayfish may also be involved in detoxification and depuration. As crustaceans, a crayfish body surface is covered with a cuticle which is primarily permeable unless impermeable by calcification. Impermeable cuticle will act as a site for passive adsorption or attachment of dissolved trace metals (Rainbow, 1997). However, such metals will not be transported into the body of crustaceans because the cell membrane of the epithelium below the cuticle forms a barrier to the passage of metals into the other body parts**.**

While, there are some investigations on the potential accumulation of lanthanum in the whole body of the crayfish (Fasola *et al.,* 2010; Wijnmalen *et al.,* 2010) no previous study on bioaccumulation of lanthanum in the specific tissue of the crayfish was found. Therefore, this study was amied to investigate the potential bioaccumulation of lanthanum in a specifc tissues and its comparative accumulation among the selected tissues of the crayfish. According to our study we define uptake and bioaccumulation as follows :

Uptake is the process by which the crayfish absorbing or taking up of the test subtance, lanthanum to its body from the surrounding medium.

Bioaccumulation is the increase in build- up of lanthanum in the tissues of crayfish (Phoslock® group) relative to the amount of lanthanum in the control crayfish.

2. Research objectives, questions and hypothesis

2.1 Objectives

- To investigate bioaccumulation of lanthanum in specific tissues of marbled crayfish (*Procambarus sp.*)
- To analyse the comparative accumulation of lanthanum among carapace, gills, ovaries, hepatopancreas and abdominal muscle.

2.2. Research questions

- Is there any difference between the lanthanum concentrations in the crayfish tissues exposed to Phoslock® as compared to the controls?
- Is there any difference in lanthanum accumulation among the different tissues of marbled crayfish (*Procambarus sp.)*?

2.3. Hypothesis

-H_o *(null hypothesis*)

- There is no difference between the lanthanum concentrations in the tissues of the Phoslock® group as compared the controls.
- There is no difference in lanthanum accumulation between different tissues.

-H_a *(alternative hypothesis*)

- There is high accumulation of lanthanum in the tissues treated with Phoslock®.
- The accumulation of lanthanum is different among the tissues of marbled crayfish.

3. Materials and methods

3.1. Test organism

Sixty-four (60 experimental and four replacement animals) adult marbled crayfishes (*Procambarus sp*.) body length (from tip of rostrum to tip of teleson) 50-55mm, body weight 3.0- 4.3 g were collected from cultured condition, Alterra laboratory.

The marbled crayfish has no scientific name yet, they are genetically identical to *Procambarus alleni* and very similar to *Procambarus fallax* (Vogt, 2008). Marmorkrebs (German) or Marbled crayfishes (English), are a cambarid species with unknown geographical location. They appeared in late 1990 in the Germany aquarium trade after which they became rapidily popular among aquaries due to their beautiful '' marmorated '' coloration, rapid reproduction and easily handling.

Marbled crayfish are parthenogenetic crayfish, which are all females and the only decapod crustaceans reproducing asexually. This makes them a fast reproducing species and can establish their generation quickly (Vogt *et al*., 2004).

Marbled crayfish reproduce genetically identical offspring and can grow to a total length of approximately 12 cm. Adult marbled crayfish produce upto 270 eggs every 4-8 weeks and reach sexual maturity at age of 25-35 weeks (Vogt, 2004). The embryonic period between spawning and hatching lasts 2-3 weeks depending on temperature. These species have incredible potential as a model organism for research purpose specially in epigenetics, environmental epigenomics and in stem cell research and regeneration. Furthermore, these species have some value for applied biologists, like as a toxicological test species (Vogt, 2008). Because, they are direct and short germ development, stereotyped cell lineage in early development, numerous morphological traits that are easy to analyse. Inaddtion to that, they have broad behavoiural range and, most importantly genetic identity of batch mates and step wise alteration of phenotype by moulting (Vogt *et al*., 2004; Vogt, 2008; Seitz *et al.,* 2005).

Marbled crayfishes are best cultured at temperature of 18-25^{o}C. Maximum growth is obtained at 25^{o}C and maximum survival at 20^{0}C. Higher and lower temperatures affect the growth and metabolic activity of the crayfish (Seitz *et al*., 2005). They can eat almost everything and be fed with pellet (Vogt *et a*l., 2004).

3.2. Crayfish anatomy

Like all crustaceans, a crayfish has a fairly hard exoskeleton that covers its body. As shown in the Figure 1 its body is divided into two main parts, the cephalothorax and the abdomen. The cephalotorax consists of the cephalis (head) region and the thoracic region. The part of the exoskeleton that covers the cephalothorax is called carapace. The lateral fold of the carapace is formed by a longitudinal double fold of the integumen, which encloses a cavity between it and the side of thorax. This lataral fold constitute the branchiostegite, and the cavity encloses is called the branchial chamber, because it impacted the gills (Sowash, 2009; Whitehouse and Grove, 1974) .

The abdomen is located behind the cephalothorax and consists of six clearly divided segments. The cecephalothorax consists of 13 segments. Each segments of both the cephalothorax and the abdomen contains a pair of appendages. The head (or cephalic region has five pairs of appendages.

The antennules are organs of balance, touch, and taste. The mandibles, or jaws are used to crush foods by moving from side to side. Two pairs of maxillae hold solid food, scratch it, and pass it to mouth, the second pair of maxillae also helps to draw water over the gills. Of the eight parts of appendages on the cephalothorax, the first three are maxillipeds, which hold food during eating. The chelipeds are the large claws that the crayfish uses for defense and to capture prey. In the abdomen, the first segments each have a pair of Swimmerets, which create water current and function in reproduction. The sixth segment contains a modified pair of uropods. In the middle of uropods is a structure called teleson, which bears the anus (Sowash, 2009; Whitehouse and Grove, 1974).

The digestive galnd (hepatopancreas) of the crayfish is located immediately behind the proventriculus. They consist a mass of short tubles of a yellowish – brown color when fresh and cream- colored in preserved specimen. The tubules secrete digestive fluid and it is belived that much of the finely divided food may enter the tubules and be digested there (Sowash, 2009; Whitehouse and Grove, 1974).The green glands are the excretory organs of the crayfish and are found, one on each side at the extreme front end of the body. It is consist of two main parts; a thin – walled, bladder – like portion from which the duct to the exterior arises, and a dense rounded green mass of grandular tissue. The green glands excrete waste through pores at the base of antenna (Sowash, 2009; Whitehouse and Grove, 1974).

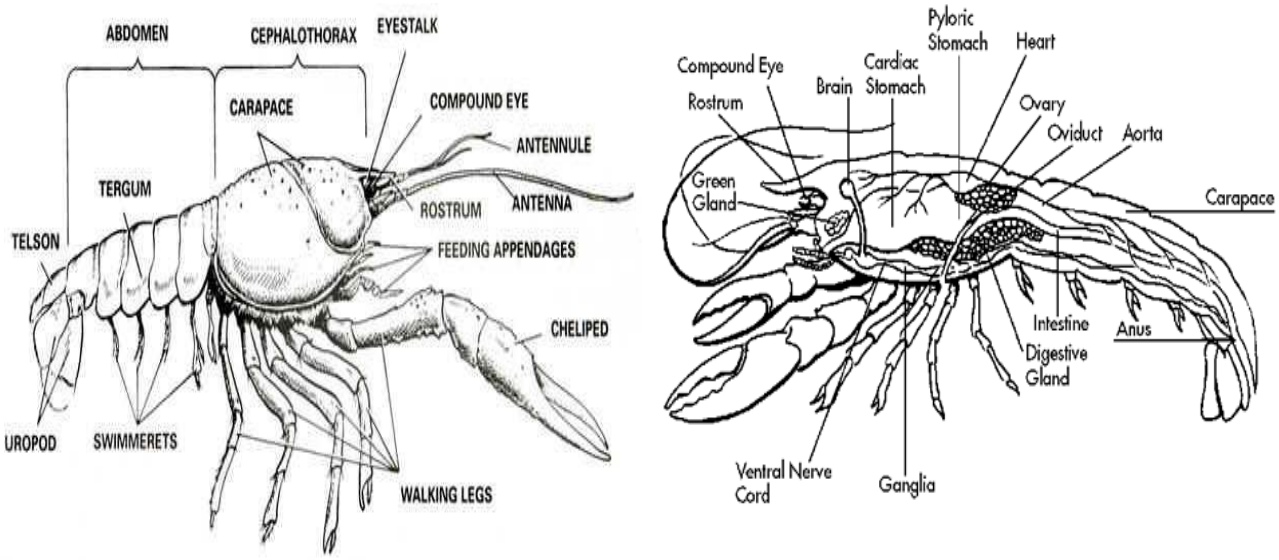

Figure 1. External and internal anatomy of the crayfish (Sowash, 2009)

3.2. Experimental settings

The test vessels or experimental units were placed inside a temperature control water bath, at plankton laboratory, Aquatic Ecology and Water quality group (AEW), Wageningen University (Figure 2).

Individual crayfish were housed in plastic test boxes to avoid cannibalism or a density effect. They were fed one commercial fish food pellet which was obtained from Alterra laboratory, Wageningen University Research centre (approximately 12 mg) per crayfish, two times per week. For acclimation purposes, the crayfishes were held in clean copper free water for 7 days prior experimentation.

During the course of the experiment four additional text boxes containing 4 crayfishes (2 exposed and 2 controls) were also kept as replacement animals.

3.3. Principle of the experiment

The experiment was consisted of two phases; the *uptake (exposure)* and the *clarification (excretion) phase.*

The *uptake or exposure phase* is the time during which the test organisms were exposed continuously to the test substance, Phoslock®. The duration for the uptake phase was for 14 - 28 days. During this phase, the Phoslock® groups were placed to the test chambers with the Phoslock®. While the control group were held under similar experimental conditions such as pH, temperature, aeration but without Phoslock® (Figure 4).

For the excretion (clarification) phase, the crayfishes were transferred to new vessels containing copper free water devoid of Phoslock® for 4 days to make their gut content empty in order to control the contamination of other tissues with lanthanum that can be excreted from the gut. Hence, contaminations of specimens by gut content were controlled.

3.3. Bioaccumulation Procedure

3.2.1. Preparation

Sixty-four transparent rectangular plastic test vessels with cover were collected from the plankton laboratory (AEW). The boxes (lids) were drilled in order to make two holes for allowing air coming in and out. The test boxes filled with one litre of copper free water were placed in the water bath with aeration tubes, and maintained at a temperature of 19-20^{o}C (Figure 2) & with no light since crayfishes are nocturnal animals. Copper free water used for the experiment was collected from Gaia laboratory, Wageningen University Research Centre. The chemical variables of the water used were as follows: the pH and conductivity of the water was 7.5 and 194.5 µs cm^{-1} respectively. Phoslock® batch Rauwbraken, 2008 was chosen for the experiment.

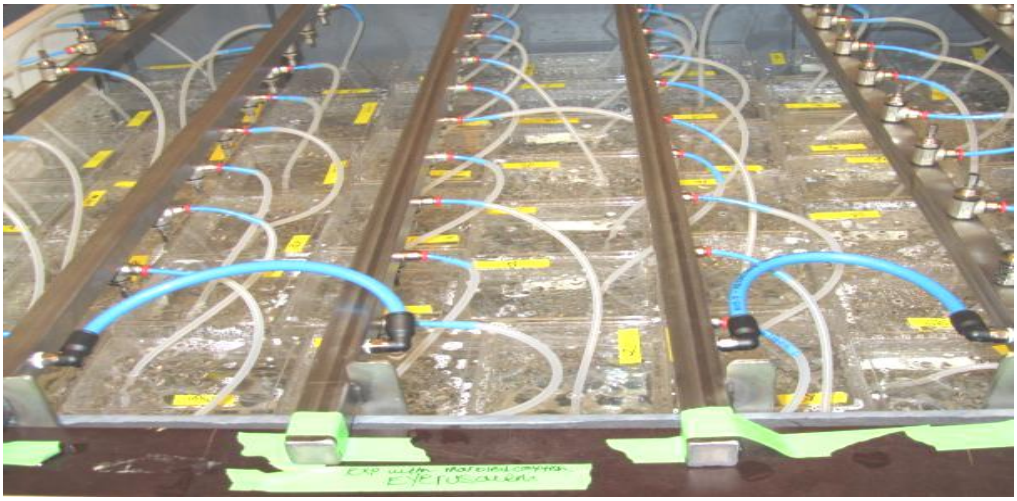

Figure 2. Experimental units in water bath compartment with aeration tubes

3.2.2. Stratification and Randomisation Procedure

To ensure equal body size amongst the treatment groups the crayfish stratified according to body size and randomly assigned to the treatments (30 controls and 30 Phoslock® groups). Stratification was performed by assigning a number to each crayfish, according to its body length. Thus, the largest crayfish received number 1, the smallest received number 60. Then a randomly selected coin (euro) was obtained and treatments we allocated (heads = for control and tail for Phoslock® groups). Starting at number one in the crayfish list the coin was tossed. The outcome assigning the specimen to either control and Phoslock®group, and the next in line to Phoslock® or control groups. After this, procedure was repeated for specimen number 3, etcetera.

The crayfishes (both control and Phoslock® groups) were grouped (split up) in to three batches based on time (t=0, t=14 & t=28 days). First, the data on the body length of each crayfishes were filled in a spreadsheet (Microsoft Excel, 2010), and the crayfishes were randomly split up in two three batches (t=0, t=14 and t=28). Subsequently, the mean value for the total body length of each batches were calculated, and stratification were performed to make a balance on the average body length between the batches. Right after randomisation and prior to experimentation 10 crayfishes (t=0 samples) from each group (control and Phoslock® groups) were picked up based on the above arrangement.

3.2.3. Exclusion Criteria

The rule of thumb during the experiment was as follows: - Dead animals were discarded and animals that bore eggs during acclimation period were replaced.

3.2.4. Phoslock® dosing and replenishing

After acclimation period, animals in the Phoslock® group were exposed to 1000 mg l^{-1} Phoslock® (Rauwbraken batch, 2008). The Phoslock® was added as slurry which was obtained by suspending granular Phoslock® in copper free water. The control group was kept in test boxes with clean copper free water. All test boxes were aerated during the experiment. The water was completely renewed weekly in order to prevent toxicity due to accumulation of waste, that is the Phoslock® group were renewed with a new solution of Phoslock® (1000 mg l^{-1}) while, the controls were with clean copper free water. In the course of replenishment, first controls were replenished followed by the Phoslock® group to prevent cross contamination between as well as within the groups. Figure 3 shows the turbidity of the water in test boxes after Phoslock® application.

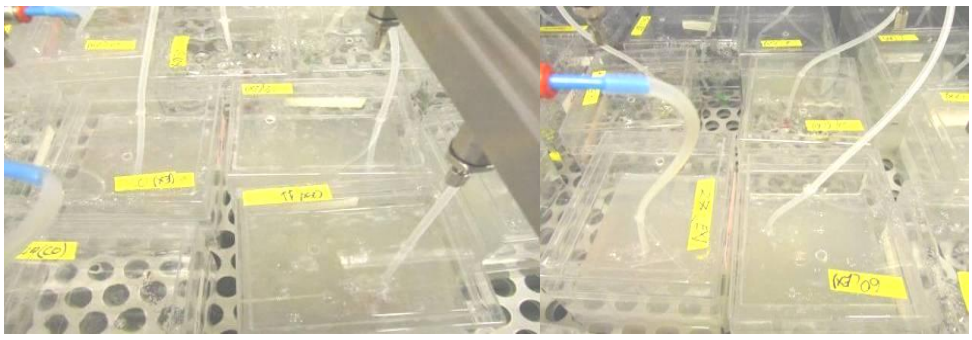

Figure 3.Turbidity after Phoslock® application

3.2.5. Water quality variables

The water quality variables, dissolved oxygen (mg l^{-1}), pH, electric conductivity (µs cm^{-1}), temperature (^{o}C), and turbidity (NTU) were measured two times per week using Oxy Guard Handy Gamma oxygen meter, WTW 320 pH meter, Cond 315i WTW conductivity meter and HACH 2100P turbidity meter respectively. These variables were recorded to ensure safety of the crayfish and to ensure that no other difference between our treatments occurred than the intended effect of Phoslock®. During measurement always first controls followed by Phoslock® group, and the probes and tubes were rinsed with demi and copper free water in order to prevent cross contamination.

3.2.6. Sampling of experimental unit

Water sampling

For ammonia and lanthanum analysis, overlying water samples were taken from each test box and copper free water used for the experiment. Sampling was done weekly before replenishing as well as after 4 days gut cleaning period.

Test organism

Ten crayfishes from each group of test (10 from control and 10 from Phoslock® groups) were sacrificed at day 0, after 14 and 28 days of exposure (Figure 4) as well as 2 from replacement animals for testing lanthanum accumulation. The coming out eggs obtained from 8 crayfishes during the course of experiment was also harvested. Prior to dissection all animals were transferred into clean copper free water for 4 days to empty the gut content.

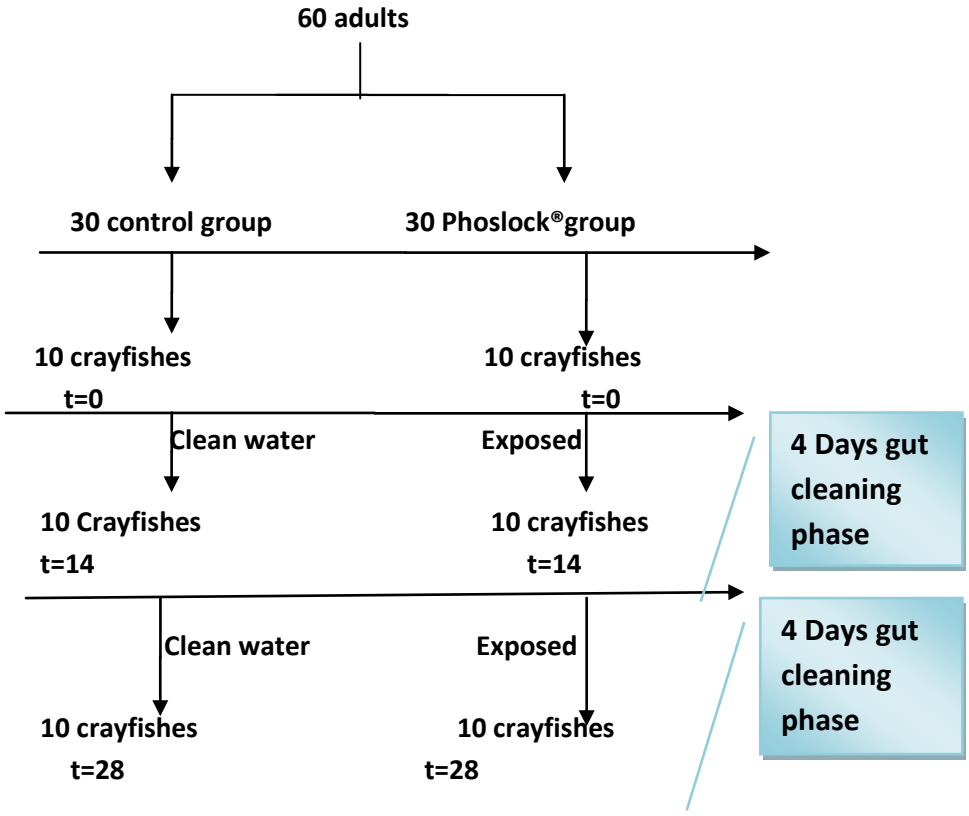

Figure 4. Principle of the experiment and sampling scheme of the test organism

3.3. Analytical methods

Water samples

The water samples were filtered with Whatman membrane filter (cellulose nitrate), 0.45 µm, and ammonia concentration was determined using a scalar continuous flow analyzer. Filterable lanthanum concentration in water samples was determined by ICP-MS in the Chemical- Biological Soil Laboratory of the Department of Soil Sciences (Wageningen University Research Centre).

Tissues samples

Dissection

Prior to dissection, the total body weight and body length of all animals was measured, and the crayfishes were rinsed two times with copper free water in order to remove the attached Phoslock® from the body surface of the crayfish. After that, the animals were placed in a small plastic bag to avoid cross contamination and buried in ice for 30-40 minutes to euthanize them (Figure 5).

The euthanized animals were removed from the ice box and immersed in hot tap water for 2 minutes to kill them. Subsequently, the specimens were dissected to obtain the tissues such as carapace, gills, ovaries, hepatopancreas and abdominal muscle.

Figure 5. Dissection of crayfish

Drying

All the 318 samples (310 tissues/62crayfishes), and the coming out eggs (from 8 crayfish) were placed separately in coulter cup, weighed and kept in a freezer (-20^{o}C). Subsequently, the samples were freeze- dried (-58 ^{o}C) for 24 hrs. (Figure 6), and the dry weight of the tissues and coming out eggs were recorded.

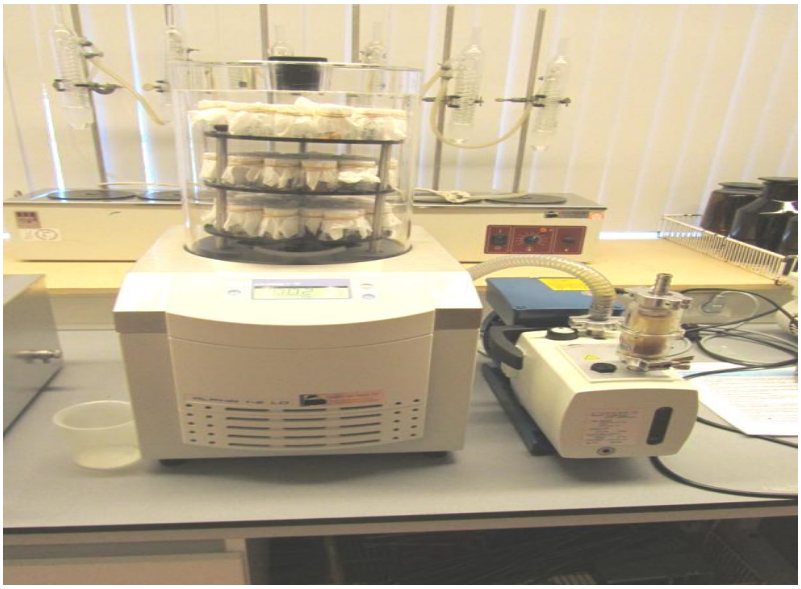

Figure 6. Tissue samples in a dry Freezer

Crushing

The dried samples were crushed (first control followed by Phoslock® groups) using pestle and mortar to obtain the powder and the crushed samples were weighed using micro balance and kept in precision tubes for destruction (Figure 7). In between each sample the pestle and mortar were cleaned with 80% ethanol and demi water using tissue paper to prevent contamination between and within the groups.

Figure 7. Crushed tissues for micro destruction

Destruction

All crushed tissue and fish food samples were digested in micro destruction block (Figure 8) with the combination of Ultrex HNO_3 (65%) and H_2O_2 (30%), following the protocol suggested by Griethuysen *et al*. (2000), (Appendix 6, Protocol 2).

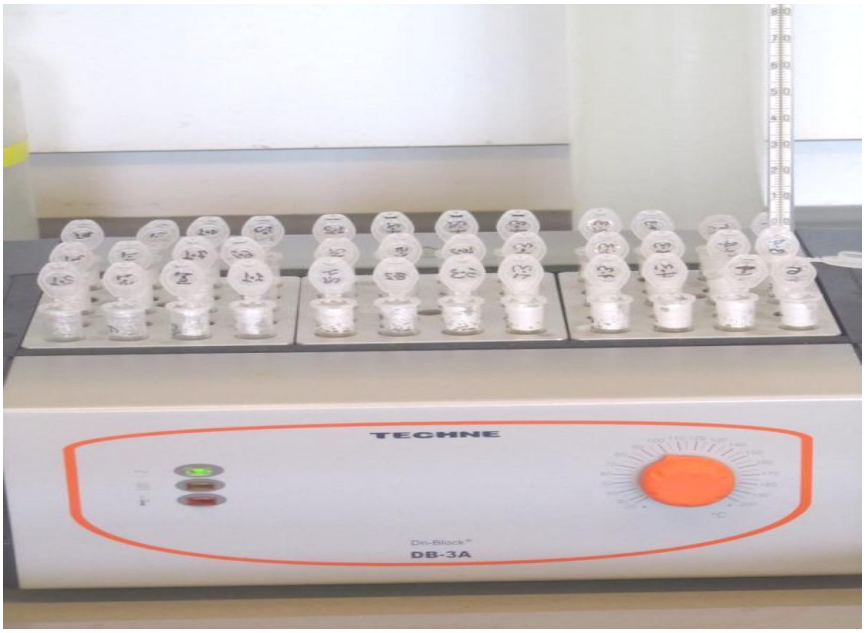

Figure 8. Micro destruction block containing crayfish tissue samples

Lanthanum concentration in 300 tissue samples was determined by ICP-MS in the Chemical-Biological Soil Laboratory of the Department of Soil Sciences (Wageningen University Research Centre).

Fish food

Three samples from the fish food were sampled in order to determine the source of lanthanum for bioaccumulation. The samples were crushed and processed as the same as the tissues.

3.4. Data analysis

Water quality variables, dissolved oxygen, conductivity, pH, turbidity, temperature, and ammonia were compared between groups by Mann -Whitney- U test to reveal possible differences between the two treatment groups. Filterable lanthanum (µg l-1) concentration in the water samples was analysed using t- test to show the difference between two treatments. The amount of lanthanum in the selected tissues of the crayfish was first calculated using the equation:

A = (C * V)/ DW in which,

A = Amount of lanthanum in the tissues (µg g^{-1}DW)

C = Concentration of lanthanum (µg l^{-1}) in the sample

V = Volume of sample dilution (litre)

DW = Dry weight of the tissue used for micro destruction (gram)

The difference between treatments was analysed using Kruskal-Wallis test and the difference among the tissue was compared using Wilcoxon Signed Ranks test. We used non-parametric test for water quality variables (dissolved oxygen, conductivity, pH, temperature, turbidity & ammonia) and for crayfish tissue because; the measurements were not normally distributed even with transformation (Appendix 3,Table 7). We used Shapiro-Wilk method to test normality. The data were analysed using statistical package SPSS, version – 17.

4. Results

4.1. Water quality variables

Over the whole experiment the dissolved oxygen concentration (DO) ranged from 8.53 to 8.95 mg l^{-1}. There were minor differences between control and Phoslock® groups (Table 2), which only reached statistical significance between two groups in week 3. The difference between two groups during this week was found only 0.07 mg l^{-1} (Table 2). Dissolved oxygen (DO) ranged from 8.5-8.9 mg l^{-1} in both control and Phoslock® groups (Table2). Higher DO values were recorded at week 4 followed by week 2 and 3.

Conductivity value throughout the experiment ranged from 168.0 - 198.8 µs cm^{-1}. Conductivity was higher at week 3 and 4. Conductivity value was higher throughout the study for control group (Table 2). The mean difference between two groups ranged from 0.72- 20.38 µs cm^{-1} (Table 2), which only reached significant difference between control and Phoslock® groups in week 3 and week 4. The difference between two groups during these weeks was found 7.37 – 7.56 µs cm^{-1} (Table 2). The pH value (7.88 – 8.09) was not different between treatments in week 1 and 4. However, there was a minor difference (0.08) between groups in week 2 and 3 (Table 2). In the course of experiment the mean temperature ranged 19 - 20^{o}C (Table 2). The value of temperature between groups was quite similar throughout the experiment. Temperature of week 1 and 2 was higher than week 3 and 4 (Table 2).

Table 2. Water quality variables mean (± SD) and mean difference(MD) of dissolved oxygen (mg l^{-1}), conductivity (µs cm^{-1}), pH, and temperature (^{o}C) for both control (CG) and Phoslock® groups (PG) throughout the experiment (standard deviation values are given in parenthesis).

Variables	Week1			Week2			Week3			Week4		
	CG	PG	MD	CG	PG	MD	CG	PG	MD	CG	PG	MD
DO2(mg/l)	8.63 (0.18)	8.53 (0.23)	0.09	8.8 (0.04)	8.79 (0.03)	0.01	8.85 (0.04)	8.77 (0.04)	0.07	8.93 (0.07)	8.95 (0.05)	0.02
EC(µs/cm)	168 (26.65)	188.39 (20.29)	20.38	172.71 (23.88)	171.99 (22.96)	0.72	190.76 (4.97)	183.20 (7.68)	7.56	198.80 (9.02)	191.44 (4.41)	7.37
pH	7.88 (0.17)	7.91 (0.14)	0.02	8.03 (0.06)	7.96 (0.07)	0.07	8.09 (0.02)	8.01 (0.04)	0.08	8.05 (0.04)	8.05 (0.03)	0
Temp(oC)	20.17 (0.02)	20.18 (0.04)	0.01	20.10 (0.05)	20.09 (0.03)	0.01	19.9 (0.06)	19.96 (0.04)	0.05	19.70 (0.06)	19.75 (0.05)	0.08

During the course of the experiment turbidity was lowest in the control group, the highest for the Phoslock® group. The mean value of turbidity ranged from 0.66 to 0.87 NTU and from 72.88 – 165.06 NTU in control and Phoslock® groups, respectively (Appendix 1, Table 3). There was a strong significant difference between two groups (Appendix 1, Table 3). The maximum turbidity value was recorded in week 2 (Figure 9).

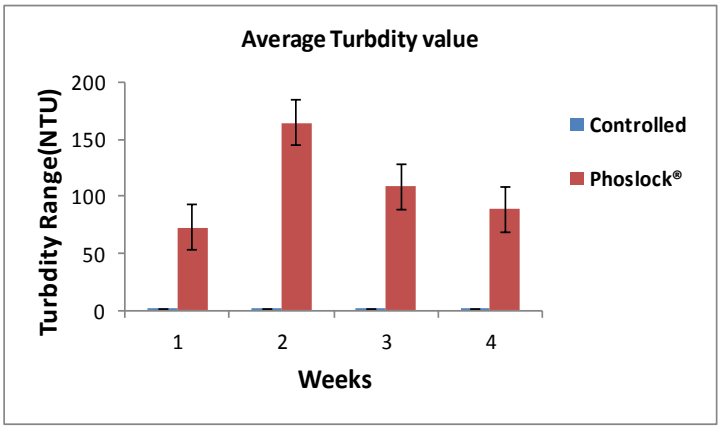

Figure 9. Average turbidity value in control and Phoslock® groups during experiment

Concentration of ammonia in overlying water samples was lower in control group, while it was higher in Phoslock® group throughout the experiment (Figure 10). Ammonia concentration between two groups ranged from 0.11 – 1.24 mg l^{-1} and the difference was significant in week 2 (Appendix 1, Table 5).

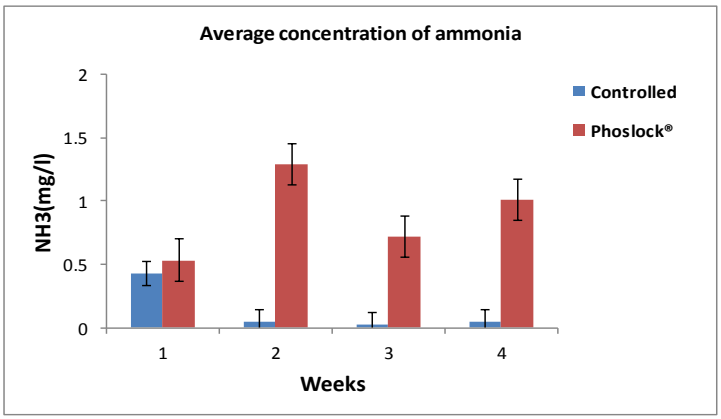

Figure 10. Average ammonia concentrations in control and Phoslock® groups

4.2. Lanthanum in water and crayfish tissue samples

4.2.1. Lanthanum in water samples

Figure 11 represents the mean concentration of filterable lanthanum in clean copper free water (CFW), control and Phoslock® experimental units. Filterable lanthanum concentration was low in

CFW and controls (0.04 µg l^{-1}) and, high in Phoslock® treatments (16 µg l^{-1}). Filterable lanthanum concentration was different between controls and Phoslock® groups (Appendix 1, Table 6).

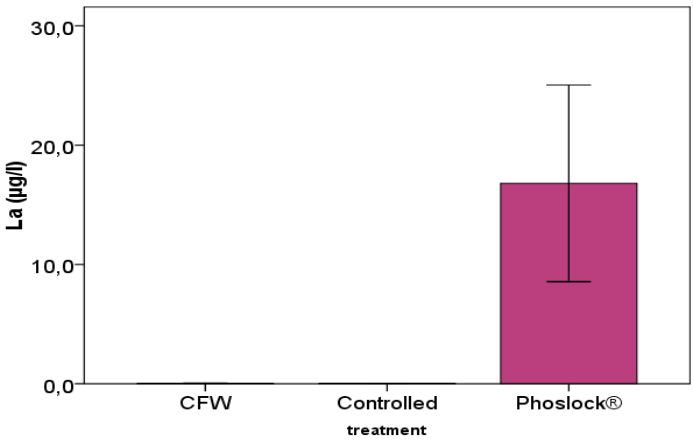

Figure 11. Average filterable lanthanum concentrations (µg l^{-1}) in CFW, Control and Phoslock® experimental units during the exposure period.

Figure 12 represent the amount of lanthanum excreted after 4 days gut cleaning phase. The filterable lanthanum concentration in the overlying water after 4 days gut cleaning period for control group was lower (0.03 µg l^{-1}) than Phoslock® group (0.75 - 2.58 µg l^{-1}) (Appendix 1, Table 4).

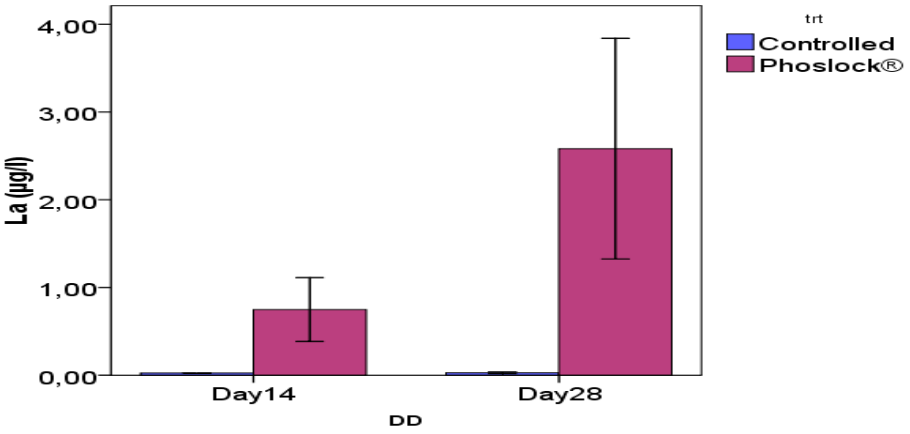

Figure 12. Average filterable lanthanum concentrations after 4 days gut cleaning period.

4.2.2. Lanthanum in Crayfish tissue samples

Figure 13 represent the average amount of lanthanum in the selected tissues at day 0, after 14 and 28 days of exposure. Lanthanum concentration in control group tissues was 0.55, 1.80, 0.50, 0.53 and 0.09 μg g^{-1} in carapace, gills, ovaries, hepatopancreas and abdominal muscle respectively. Whereas in Phoslock® group was 15.56, 255.79, 3.14, 72.29, and 5.78 μg g^{-1} in carapace, gills, ovaries, hepatopancreas and abdominal muscle respectively.

The average amount of lanthanum at day 0 samples of both control and Phoslock® groups ranged from 0.04 – 0.9 μg g^{-1} for all the selected tissues (Appendix 3, Table 8). The lanthanum content in a dry fish food was 0.11 μg g^{-1}. The difference in lanthanum concentration between the control and Phoslock® groups was statistically significant at α level 0.05 (Appendix 3, Table 9). No mortality was observed in the Phoslock® group while only 1 crayfish was died from the control group.

The order of magnitude of tissue concentration of lanthanum was gills > hepatopancreas > carapace > abdominal muscle > ovaries. The most considerable bioaccumulation of lanthanum was obtained in the gills followed by hepatopancreas. There was significant difference in lanthanum accumulation between tissues obtained except between hepatopanceas and carapace; abdominal muscle and ovaries after 14 days of exposure as well as between abdominal muscle and carapace; hepatopancreas and gills after 28 days of exposure (Appendix 4, Table 11). Higher concentration of lanthanum was measured in the tissues obtained after 28 days of exposure. The concentration of lanthanum measured in the carapace, gills, hepatopancreas, and ovaries of the crayfish after 14 days of Phoslock® exposure were not significantly different from those after 28 days of exposure. Whereas, significance difference only in abdominal muscle was obtained (Appendix 3, Table 10).

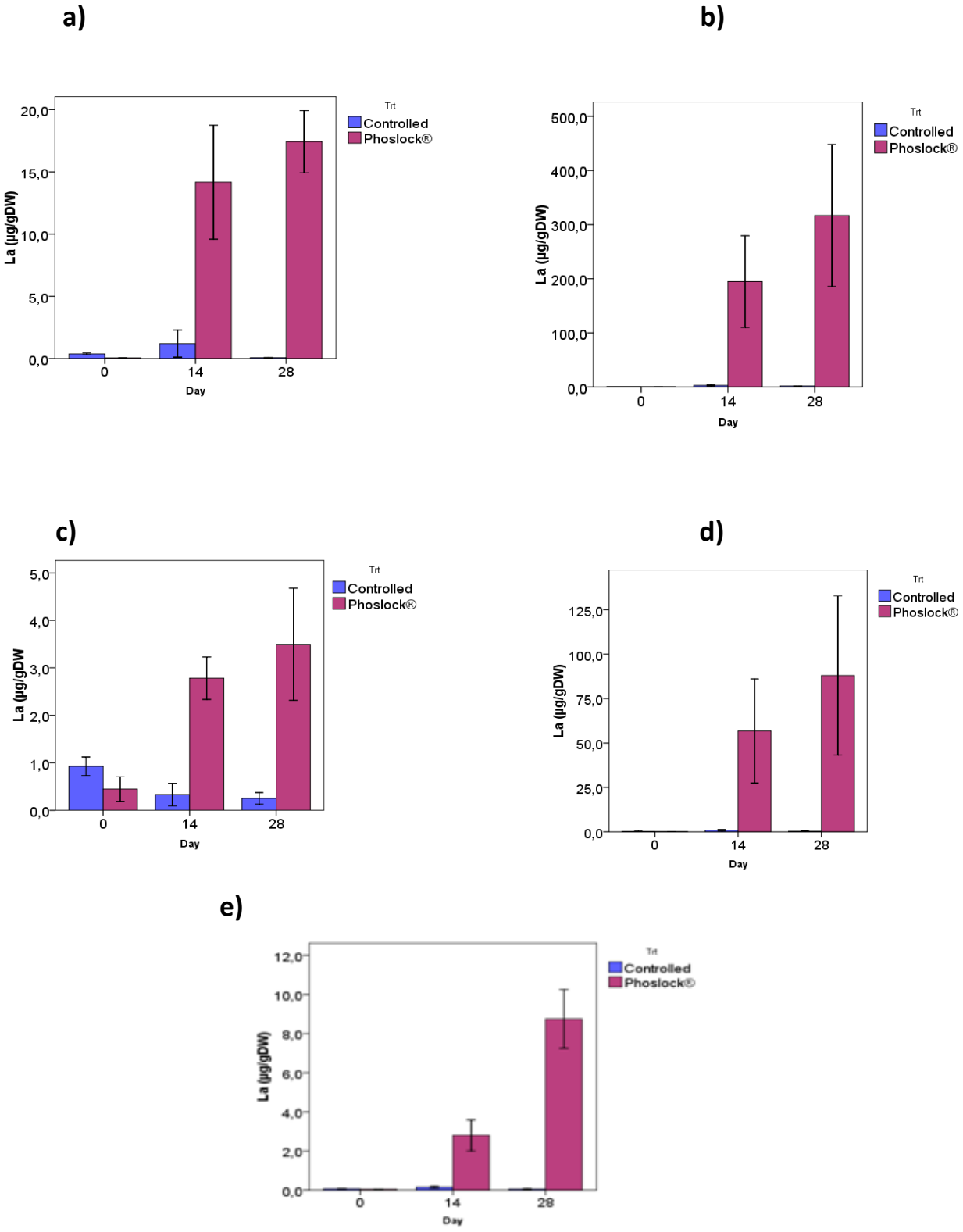

Figure 13. Average amount of lanthanum (µg g^{-1}) in Carapace (a), Gills (b), Ovaries (c), Hepatopancreas (d) and Abdominal muscle (e) of Marbled crayfish (control and Phoslock® groups) at day 0, after 14 and 28 days of exposure.

5. Discussion

5.1. Water quality variables

Although statistically significant difference between control and Phoslock® groups was obtained, the observed differences were too small to have an ecological meaning. All the water quality variables value in both treatments encompass the optimal ranges where the crayfish can be best cultured and live without any ecological stress (Seitz *et al*., 2005; Powell and Watts, 2006; Trouilhe *et al*., 2007).

Values of turbidity in Phoslock® group was higher than the control group. This is explained by the presence of Phoslock® (Van Oosterhout and Lurling, 2010; Fasola *et al*., 2010). The higher value of turbidity (Figure 9) recorded in week 2 was due to the fact that turbidity was measured after the other water quality variables such as dissolved oxygen, conductivity and pH (stirring with oxygen, condcutivity and pH probes made the water turbid). Overall higher turbdity was caused by the activity of the crayfish. Although statistically no significant difference in ammoina concentration between control and Phoslock® groups was obtained, higher concentrations of ammonia in Phoslock® group were measured (Appendix 1, Table 3). This might be due to bentonite effect (Lurling personal comm.). Nevertheless, the concentration obtained in these groups during the experiment was below the amount which can cause toxicity to the crayfish (Meade and Watts, 1995; Arthur *et a*l., 1997).

5.2. Lanthanum in crayfish tissues

The presence of lanthanum in controls indicate that the background value which originates from the environment. Lanthanum is found in the earth crust naturally and can discharge to fresh water ecosystem from different routes (Qiang *et al*., 1994; Evans, 1983; Zhu *et al*., 2002). Our result was in agreement with the results reported by Fasola *et al*. (2010) and Wijnmalen *et al*. (2010). Huge amount of lanthanum was measured in the crayfish tissues exposed to Phoslock®. Statistically, there was a strong significant difference between the tissues exposed to Phoslock® and controls. Thus, the accumulation of lanthanum in the crayfish tissues is dependent on the amount and availability of lanthanum in the water medium. The values of lanthanum accumulation in gills and hepatopancreas were greater than the values in the carapace, abdominal muscle and ovaries. This implies that the accumulation of lanthanum by these two organs was tissue specific.

There is lack of data on the bioaccumulation of lanthanum in the selected tissues of the crayfish so far. Hence, the comparison of our result with studies on bioaccumulation of other metals in crayfish and other species body tissues, and that comparing is thus not straightforward.

Qiang *et al*. (1994) showed that (Carps exposed to 0.5 mg l^{-1} of lanthanum) gills of the Carps were the potential organs for lanthanum accumulation (Table 1), which is in agreement with our study. Many authors confirm a higher accumulation of dissolved metals in gills and hepatopancreas (Christopher *et al*., 2001; Naqvi *et al*., 1998; Guner, 2007, 2010; Anderson *et al*., 1997; Pastor *et al*., 1988).The gills of crayfish are in direct contact with water and surrounding environment and thus, act as a gate for the entry of dissolved metal in to the body and, can readily absorb the dissolved metals (Naqvi *et al*., 1998; Torreblanca *et al*., 1989). Besides, the gills may also be involved in detoxification and depuration of metals (Ahearn *et al*., 2004).

Following gills, the amount of lanthanum in hepatopancreas was also quite high. Similar finds for lead, cadmium, and copper were attributed to the role of hepatopancreas in many physiological processes such as secretion of digestive juices, absorption and storage of digested food, primary site for nutrient absorption, uptake and metabolism of organic chemicals and detoxification (Roldan and Shivers 1987; Christopher *et al*., 2001; Jewell and Winston, 1989). The lower amount of lanthanum in carapace might be due to the influence of moulting since, most of the participants (crayfishes) during the experiment undertaken moult. Lanthanum presence in carapace could play a major role as a possible elimination mechanism through moulting.

The lower lanthanum concentration in abdominal muscle is in accordance with studies on other metals (Copper, cadmium and lead) by Naqvi *et al*., 1998; Guner, 2007, 2010; Anderson *et al*., 1997; Naghshbandi *et al*., 2007, which also show the lowest concentrations of the metals in abdominal muscle. Even though, only a small amount of lanthanum presence in ovaries was found it indicates build-up of lanthanum concentration in the ovaries which may influence the reproduction of crayfish.

In our study we also analysed the amount of lanthanum excreted in overlying water after 4 days of gut cleaning period. The increase in lanthanum in this water to be caused by excretion of lanthanum by the crayfish and that this concentration would probably have been higher if the depuration period would have lasted longer. Thus, the observed concentration of lanthanum in the tissues might be lower. Although the crayfishes are able to excrete lanthanum in laboratory test, the situation in the field is different. Because, the lanthanum excreted from the body of the crayfish will bound again to the Phoslock® settles down on the bottom of the sediment. Thus, the crayfishes are not able to escape from the exposure in the field condition.

6. Conclusion and Recommendation

6.1. Conclusions

- The crayfishes exposed to Phoslock® accumulate more lanthanum than the controls.
- Lanthanum accumulates most in gills and hepatopancreas, and also present in carapace, abdominal muscle and ovaries but with lower concentration.

6.2. Recommendations

- The gills and hepatopancreas of the crayfish can be used to detect lanthanum presence in aquatic ecosystem where Phoslock® applied. However, the ability of the crayfish to depurate lanthanum should be investigated by providing longer depuration period to address the suitability of these organs for long term bio monitoring.
- Though no adverse effect of lanthanum was observed in this study, its non-lethal effect on reproduction, growth, development, metabolic rate, damage on organs and its threshold level should be inspected.
- The presence of lanthanum in ovaries might indicate the possibility of lanthanum transfer from mothers to the offspring through eggs. Further research is needed to verify transfer of accumulated lanthanum from mothers to the offspring through eggs.

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8. Appendices

Appendix 1. Descriptive and statistical test analysis for Water quality variables

Table 3. Results of Descriptive statistics for dissolved oxygen, conductivity, pH, temperature, turbidity and ammonia in control and Phoslock® experimental units at week 1, week 2, week 3 and week 4

Dissolved Oxygen(mg/l)

Week	Controlled group				Phoslock® group				Difference
	N	Mean	Median	Std. Deviation	N	Mean	Median	Std. Deviation	Mean
1	10.00	8.63	8.65	0.18	10.00	8.53	8.60	0.23	0.09
2	10.00	8.80	8.80	0.04	10.00	8.79	8.80	0.03	0.01
3	10.00	8.85	8.85	0.04	10.00	8.77	8.75	0.04	0.07
4	10.00	8.93	8.95	0.07	10.00	8.95	8.95	0.05	0.02

Electric Conductivity (µs/cm)

Week	Controlled group				Phoslock® group				Difference
	N	Mean	Median	Std. Deviation	N	Mean	Median	Std. Deviation	Mean
1	10.00	168.01	174.75	26.65	10.00	188.39	196.20	20.29	20.38
2	10.00	172.71	179.83	23.88	10.00	171.99	179.68	22.96	0.72
3	10.00	190.76	191.18	4.97	10.00	183.20	185.93	7.68	7.56
4	10.00	198.80	196.68	9.02	10.00	191.44	189.98	4.41	7.37

pH

Week	Controlled group				Phoslock® group				Difference
	N	Mean	Median	Std. Deviation	N	Mean	Median	Std. Deviation	Mean
1	10.00	7.88	7.92	0.17	10.00	7.91	7.96	0.14	0.02
2	10.00	8.03	8.04	0.06	10.00	7.96	7.96	0.07	0.07
3	10.00	8.09	8.09	0.02	10.00	8.01	8.02	0.04	0.08
4	10.00	8.05	8.07	0.04	10.00	8.05	8.05	0.03	0.00

Temperature (^{o}C)

Week	Controlled group				Phoslock® group				Difference
	N	Mean	Median	Std. Deviation	N	Mean	Median	Std. Deviation	Mean
1	10.00	20.17	20.15	0.02	10.00	20.18	20.20	0.04	0.01
2	10.00	20.10	20.10	0.05	10.00	20.09	20.10	0.03	0.01
3	10.00	19.90	19.90	0.06	10.00	19.96	19.95	0.04	0.05
4	10.00	19.70	19.70	0.06	10.00	19.78	19.75	0.05	0.08

Turbidity (NTU)

Week	Controlled group				Phoslock® group				Difference
	N	Mean	Median	Std. Deviation	N	Mean	Median	Std. Deviation	Mean
1	10.00	0.87	0.72	0.39	10.00	72.88	63.45	38.77	72.01
2	10.00	0.72	0.74	0.16	10.00	165.06	139.93	108.02	164.34
3	10.00	0.66	0.60	0.19	10.00	108.88	109.28	48.76	108.22
4	10	0.70	0.64	0.21	10.00	88.70	96.85	43.17	88.00

Ammonia (mg l^{-1})

Week	Controlled group				Phoslock® group				Difference
	N	Mean	Median	Std. Deviation	N	Mean	Median	Std. Deviation	Mean
1	10.00	0.43	0.13	0.63	10.00	0.53	0.06	0.76	0.11
2	10.00	0.05	0.05	0.02	10.00	1.29	0.93	1.42	1.24
3	10.00	0.02	0.01	0.04	10.00	0.72	0.02	1.12	0.69
4	10	0.04	0.03	0.04	10.00	1.01	0.05	1.37	0.97

Table 4. Mean and and standard devaition for filterable lanthnaum concentration during the exposure and after 4 days of gut cleaning period in control and Phoslock® experimental units.

Lanthanum (µg l^{-1}) water samples before during the exposure

treatment	N	Mean	Std. Deviation
CFW	4	0.04	0.03
Controlled group	16	0.04	0.03
Phoslock® group	16	16.80	32.95
Total	36	7.49	23.17

Lanthanum (µg l^{-1}) in water samples after 4 days gut cleaning period

Day	Controlled group			Phoslock® group		
	N	Mean	Std	N	Mean	Std
14	4	0.03	0.00	4.00	0.75	0.73
28	4	0.03	0.02	4.00	2.58	2.51
Total	8	0.03	0.01	8.00	2.51	1.97

Table 5. Mann- Whitney U- test values for dissolved oxygen, conductivity, pH, temperature, turbdity and ammonia between control and Phoslock® groups in week 1, week 2, week 3, and week 4

Test Statistics[b,c]

	DO	Ec	pH	Turb	Temp	NH3
Mann-Whitney U	34,000	26,000	48,500	,000	37,000	47,000
Wilcoxon W	89,000	81,000	103,500	55,000	92,000	102,000
Z	-1,223	-1,814	-,114	-3,781	-1,091	-,227
Asymp. Sig. (2-tailed)	,221	,070	,910	,000	,275	,821
Exact Sig. [2*(1-tailed Sig.)]	,247[a]	,075[a]	,912[a]	,000[a]	,353[a]	,853[a]

a. Not corrected for ties
b. Week1
c. Grouping Variable: Treatment

Test Statistics[b,c]

	DO	Ec	pH	Turb	Temp	NH3
Mann-Whitney U	45,500	46,000	20,500	,000	40,000	17,000
Wilcoxon W	100,500	101,000	75,500	55,000	95,000	72,000
Z	-,548	-,302	-2,232	-3,780	-,835	-2,495
Asymp. Sig. (2-tailed)	,584	,762	,026	,000	,404	,013
Exact Sig. [2*(1-tailed Sig.)]	,739[a]	,796[a]	,023[a]	,000[a]	,481[a]	,011[a]

a. Not corrected for ties
b. Week=2
c. Grouping variable: Treatment

Test Statistics[b,c]

	DO	Ec	pH	Turb	Temp	NH3
Mann-Whitney U	11,500	23,000	,000	,000	24,500	29,000
Wilcoxon W	66,500	78,000	55,000	55,000	79,500	84,000
Z	-3,014	-2,041	-3,790	-3,780	-1,988	-1,587
Asymp. Sig. (2-tailed)	,003	,041	,000	,000	,047	,112
Exact Sig. [2*(1-tailed Sig.)]	,002[a]	,043[a]	,000[a]	,000[a]	,052[a]	,123[a]

a. Not corrected for ties
b. Week=3
c. Grouping variable: Treatment

Test Statistics[b,c]

	DO	Ec	pH	Turb	Temp	NH3
Mann-Whitney U	44,000	18,000	43,500	,000	15,500	29,000
Wilcoxon W	99,000	73,000	98,500	55,000	70,500	84,000
Z	-,481	-2,419	-,495	-3,781	-2,749	-1,587
Asymp. Sig. (2-tailed)	,631	,016	,620	,000	,006	,112
Exact Sig. [2*(1-tailed Sig.)]	,684[a]	,015[a]	,631[a]	,000[a]	,007[a]	,123[a]

a. Not corrected for ties
b. Week=4
c. Grouping Variable: Treatments

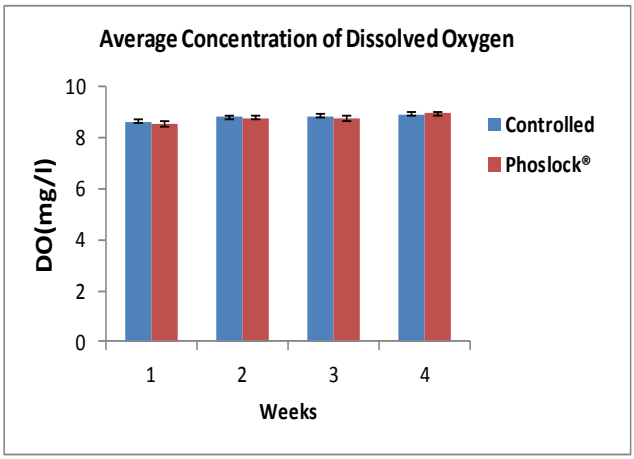
Table 6. Independent t-test results for filterable lanthanum in water samples during the exposure and after 4 days gut cleaning period for both control and Phoslock® groups

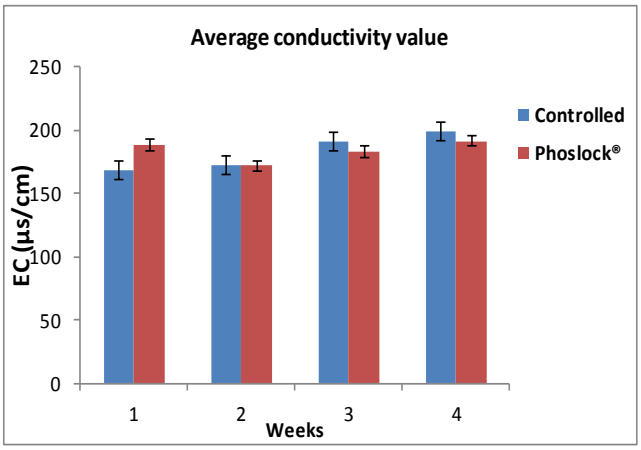
Independent Samples Test

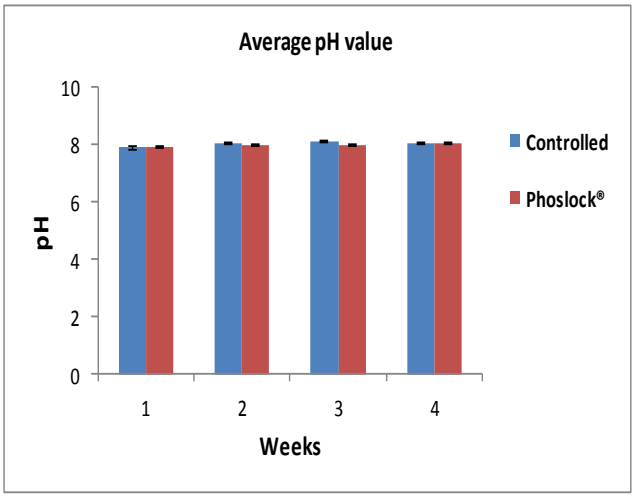
		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Log Lanthanum after depuration	Equal variances assumed	3.87	0.07	-7.20	14.00	0.00	-1.56	0.22	-2.03	-1.10
	Equal variances not assumed			-7.20	8.35	0.00	-1.56	0.22	-2.06	-1.07

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
logLawa	Equal variances assumed	2,263	,143	-14,580	30	,000	-2,27559	,15607	-2,59433	-1,95685
	Equal variances not assumed			-14,580	22,781	,000	-2,27559	,15607	-2,59862	-1,95256







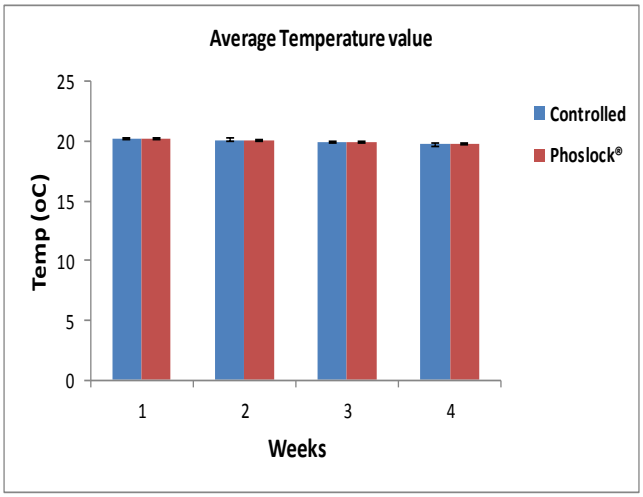
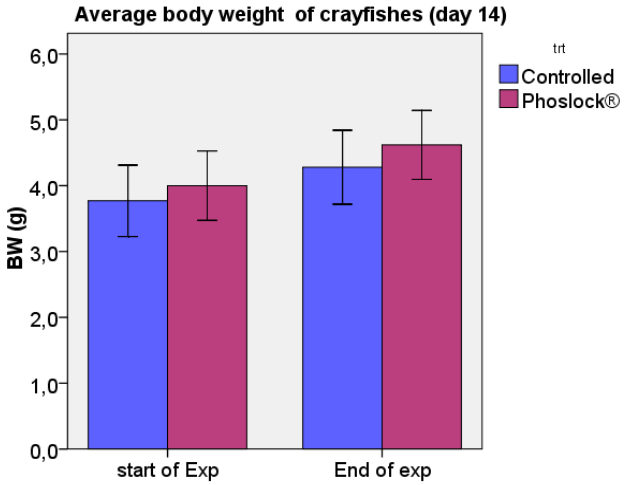
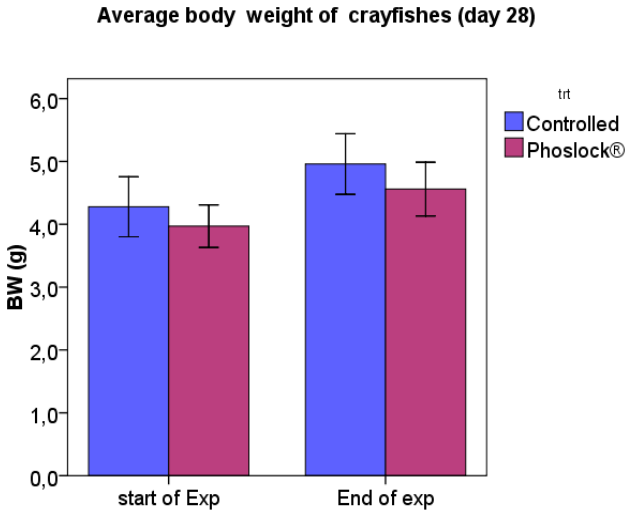
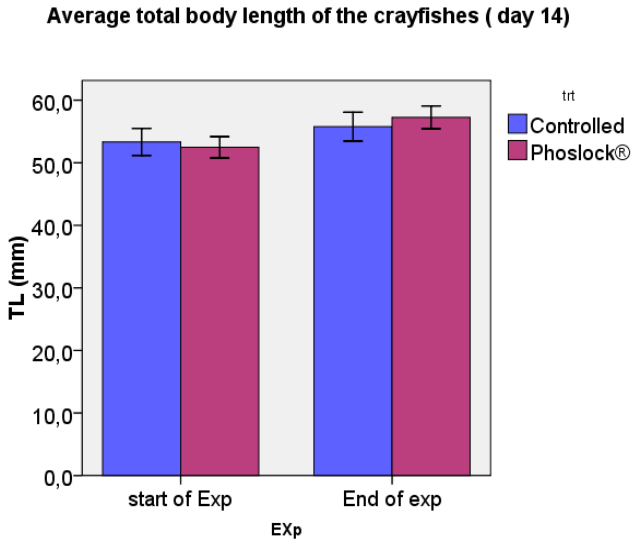


Figure 14. Average values of dissolved oxygen, conductivity, pH, and temperature for both control and Phoslock® groups in week 1, week 2, week 3, and week 4

Appendix 2. Total body weight and total body length of the crayfish at start and end of the experiment







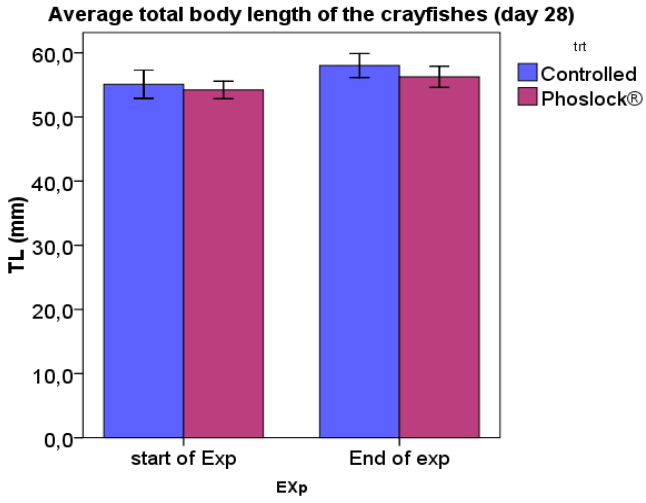


Figure 15. Average total body weight and body length of the crasyfihes at the start and end of the experiment (at the end of 14 and 28 days).

Appendix 3. Normality, descriptive and statistical test results for lanthanum in tissue samples for control and Phoslock® groups

Table 7. Normality test results for control and Phoslock® tissues before data transformation(a), after log (b), SQRT (c), double SQRT (d), Arsin (e), and Arsin *SQRT (f) transformations.

a)

	Trt	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
carapace	1	0.43	18.00	0.00	0.34	18.00	0.00
	2	0.18	18.00	0.13	0.89	18.00	0.03
Gills	1	0.29	18.00	0.00	0.73	18.00	0.00
	2	0.26	18.00	0.00	0.69	18.00	0.00
ovaries	1	0.27	18.00	0.00	0.81	18.00	0.00
	2	0.21	18.00	0.04	0.86	18.00	0.01
Hepatopancreas	1	0.35	18.00	0.00	0.69	18.00	0.00
	2	0.32	18.00	0.00	0.63	18.00	0.00
Muscle	1	0.25	18.00	0.00	0.60	18.00	0.00
	2	0.21	18.00	0.04	0.83	18.00	0.00

b)

	Trt	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
logcarapace	1	0.13	18.00	,200*	0.90	18.00	0.05
	2	0.30	18.00	0.00	0.78	18.00	0.00
loggills	1	0.17	18.00	,200*	0.93	18.00	0.23
	2	0.28	18.00	0.00	0.80	18.00	0.00
logovaries	1	0.14	18.00	,200*	0.91	18.00	0.09
	2	0.25	18.00	0.01	0.84	18.00	0.01
logHP	1	0.27	18.00	0.00	0.91	18.00	0.09
	2	0.27	18.00	0.00	0.85	18.00	0.01
logMuscle	1	0.14	18.00	,200*	0.90	18.00	0.05
	2	0.22	18.00	0.02	0.82	18.00	0.00

c)

	Trt	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
SQRTcara	1	0.27	18.00	0.00	0.56	18.00	0.00
	2	0.21	18.00	0.04	0.88	18.00	0.03
SQRTgills	1	0.24	18.00	0.01	0.85	18.00	0.01
	2	0.18	18.00	0.11	0.89	18.00	0.04
SQRTova	1	0.16	18.00	,200*	0.88	18.00	0.02
	2	0.13	18.00	,200*	0.95	18.00	0.43
SQRTHP	1	0.33	18.00	0.00	0.81	18.00	0.00
	2	0.17	18.00	0.16	0.86	18.00	0.01
SQRTMus	1	0.19	18.00	0.07	0.76	18.00	0.00
	2	0.20	18.00	0.06	0.90	18.00	0.05

d)

	Trt	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
SSqrtcara	1	0.20	18.00	0.05	0.74	18.00	0.00
	2	0.23	18.00	0.01	0.83	18.00	0.00
SSqrtgills	1	0.21	18.00	0.04	0.90	18.00	0.05
	2	0.20	18.00	0.05	0.89	18.00	0.03
SSqrtova	1	0.14	18.00	,200*	0.90	18.00	0.07
	2	0.18	18.00	0.14	0.92	18.00	0.14
SSqrtHP	1	0.31	18.00	0.00	0.86	18.00	0.01
	2	0.18	18.00	0.11	0.91	18.00	0.09
SSqrtmus	1	0.17	18.00	,200*	0.84	18.00	0.01
	2	0.22	18.00	0.03	0.87	18.00	0.02

e)

	Trt	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
ARscara	1	0.26	6.00	,200*	0.87	6.00	0.22
	2	0.29	5.00	,200*	0.89	5.00	0.38
ARsgills	1	0.21	6.00	,200*	0.96	6.00	0.80
	2	0.34	5.00	0.06	0.80	5.00	0.09
ARsova	1	0.32	6.00	0.06	0.74	6.00	0.02
	2	0.45	5.00	0.00	0.60	5.00	0.00
ARsHP	1	0.18	6.00	,200*	0.95	6.00	0.77
	2	0.33	5.00	0.07	0.83	5.00	0.14
ARSMus	1	0.42	6.00	0.00	0.61	6.00	0.00
	2	0.20	5.00	,200*	0.97	5.00	0.90

f)

	Trt	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
ARSQcara	1	0.20	6.00	,200*	0.93	6.00	0.57
	2	0.24	5.00	,200*	0.95	5.00	0.73
ARSQgills	1	0.20	6.00	,200*	0.97	6.00	0.86
	2	0.29	5.00	0.19	0.88	5.00	0.31
ARSQova	1	0.23	6.00	,200*	0.84	6.00	0.12
	2	0.43	5.00	0.00	0.65	5.00	0.00
ARSQHP	1	0.17	6.00	,200*	0.95	6.00	0.74
	2	0.32	5.00	0.11	0.86	5.00	0.22
ARSQMus	1	0.37	6.00	0.01	0.69	6.00	0.01
	2	0.19	5.00	,200*	0.97	5.00	0.89

Table 8. Results of descriptive statistics for the amount of lanthanum in all the selected tissues of the crayfish

Amount of lanthanum in Carapace (µg g^{-1}DW)

		Treatments					
		Controlled			Phoslock® group		Difference
Days	N	mean	STD	N	mean	STD	mean
0	6	0.38	0.18	6.00	0.06	0.03	0.32
14	6	1.20	2.67	6.00	14.18	11.22	12.98
28	6	0.07	0.05	6.00	17.43	6.13	17.36
Total	18	0.55	1.53	18.00	10.56	10.41	10.01

Amount of lanthanum in Gills (µg g^{-1}DW)

		Treatments					
		Controlled			Phoslock® group		Difference
Days	N	mean	STD	N	mean	STD	mean
0	6	0.70	0.32	6.00	0.26	0.19	0.44
14	6	2.99	2.83	6.00	194.83	206.97	191.85
28	6	1.73	1.05	6.00	316.74	321.08	315.01
Total	18	1.80	1.91	18.00	170.61	246.79	168.81

Amount of lanthanum in Ovaries (µg g^{-1}DW)

		Treatments					
		Controlled			Phoslock® group		Difference
Days	N	mean	STD	N	mean	STD	mean
0	6	0.93	0.48	6.00	0.45	0.63	0.48
14	6	0.33	0.59	6.00	2.78	1.10	2.45
28	6	0.25	0.30	6.00	3.50	2.89	3.25
Total	18	0.50	0.54	18.00	2.24	2.17	1.74

Amount of lanthanum in Hepatopancreas (µg g^{-1}DW)

		Treatments					
		Controlled			Phoslock® group		Difference
Days	N	mean	STD	N	mean	STD	mean
0	6	0.29	0.32	6	0.11	0.09	0.18
14	6	0.97	1.07	6	56.75	71.84	55.78
28	6	0.35	0.30	6	88.03	109.59	87.68
Total	18	0.53	0.70	18	48.29	80.33	47.76

Amount of lanthanum in Abdominal Muscle (µg g^{-1}DW)

		Treatments					
		Controlled			Phoslock® group		Difference
Days	N	mean	STD	N	mean	STD	mean
0	6	0.07	0.01	6.00	0.04	0.01	0.02
14	6	0.14	0.13	6.00	2.80	1.94	2.66
28	6	0.06	0.03	6.00	8.75	3.68	8.70
Total	18	0.09	0.08	18.00	3.87	4.37	3.78

Table 9. Kruskal Wallis test results between control and Phoslock® groups for all the selected tissues at day 0, after 14 and 28 days of exposure.

Test Statistics[a,b,c]

	Cara	Gills	Ova	HP	Mus
Chi-Square	8,308	5,026	2,564	2,564	7,410
df	1	1	1	1	1
Asymp. Sig.	,004	,025	,109	,109	,006

a.Day=0
b. Kruskal Wallis Test
c. Grouping variable: Treatment

Test Statistics[a,b,c]

	Cara	Gills	ova	HP	Mus
Chi-Square	7,410	8,308	8,308	8,308	8,308
df	1	1	1	1	1
Asymp. Sig.	,006	,004	,004	,004	,004

a. Day=14
b. Kruskal Wallis Test
c. Grouping variable: Treatment

Test Statistics[a,b,c]

	Cara	Gills	Ova	HP	Mus
Chi-Square	8,308	8,308	8,308	8,308	8,308
df	1	1	1	1	1
Asymp. Sig.	,004	,004	,004	,004	,004

a. Day=28

b. Kruskal Wallis Test

c. Grouping variable: Treatment

Table 10. Kruskal Wallis test for the Phoslock® group after 14 and 28 days of exposure

Test Statistics[a,b,c]

	carapace	Gills	ovaries	Hepatopancreas	Abdominal muscle
Chi-Square	1.26	1.64	0.10	0.64	7.41
df	1.00	1.00	1.00	1.00	1.00
Asymp. Sig.	0.26	0.20	0.75	0.42	0.01

a.Treatment = Phoslock® group

b.Kruskal Wallis test

c. Grouping variable= after 14 and 28 days

Appendix 4. Multiple comparison of Crayfish tissues

Table 11. Descriptive and Wilcoxon Signed Ranks test results for control and Phoslock® groups at day 0, after 14 and 28 days of exposure

Descriptive Statistics[a]

	N	Mean	Std.	Minimum	Maximum
Carapace	6	0.38	0.18	0.17	0.60
Gills	6	0.70	0.32	0.47	1.20
Ovaries	6	0.93	0.48	0.32	1.38
Hepatopancreas	6	0.29	0.32	0.11	0.94
Abdominal muscle	6	0.07	0.01	0.05	0.08

a.Treatment = control, Day=0

Test Statistics[c,d]

	Gi-Ca	Ov-Ca	H-Ca	Mu-Ca	Ov-Gi	HP-Gi	Mu-Gi	HP-Ov	Mu-Ov	Mu-HP
Z	-1,572[a]	-1,992[a]	-,943[b]	-2,201[b]	-,943[a]	-2,201[b]	-2,201[b]	-2,201[b]	-2,201[b]	-2,201[b]
Asymp. Sig. (2-tailed)	.116	.046	.345	.028	.345	.028	.028	.028	.028	.028

Ca= Carapace, Gi= Gills, Ov= Ovaries, HP= Hepatopancreas, Mu= Abdominal muscle

a.Based on negative ranks

b.Based on positive ranks

c. Control at day=0

d.Wilcoxon Signed Ranks test

Descriptive Statistics[a]

	N	Mean	Std. Deviation	Minimum	Maximum
Carapace	6	1.20	2.67	0.03	6.64
Gills	6	2.99	2.83	0.38	7.57
Ovaries	6	0.33	0.59	0.02	1.51
Hepatopancreas	6	0.97	1.07	0.05	2.74
Abdominal Muscle	6	0.14	0.13	0.03	0.39

a.Treatment = control, after 14 days

Test Statisticsc,d

	Gi -Ca	Ov-Ca	HP-Ca	Mu-Ca	Ov- Gi	HP-Gi	Mu-Gi	HP-Ov	Mu-Ov	Mu- HP
Z	-1,363[a]	-,314[b]	-,524[a]	-,105[a]	-1,782[b]	-1,572[b]	-2,201[b]	-1,572[a]	-,105[a]	-1,153[b]
Asymp. Sig. (2-tailed)	0.173	0.753	0.600	0.917	0.075	0.116	0.028	0.116	0.917	0.249

Ca= Carapace, Gi= Gills, Ov= Ovaries, HP= Hepatopancreas, Mu= Abdominal muscle

a.Based on negative ranks

based on positive ranks

c. Control after 14 days

d.Wilcoxon Signed Ranks test

Descriptive Statistics[a]

	N	Mean	Std. Deviation	Minimum	Maximum
Carapace	6	0.07	0.05	0.03	0.18
Gills	6	1.73	1.05	0.64	3.47
Ovaries	6	0.25	0.30	0.03	0.82
Hepatopancreas	6	0.35	0.30	0.09	0.90
Abdominal muscle	6	0.06	0.03	0.04	0.11

a.Treatment = control, Day=28

Test Statistics[c,d]

	Gi-Ca	Ov-Ca	HP-Ca	Mu-Ca	Ov- Gi	HP-Gi	Mu-Gi	HP-Ov	Mu-Ov	Mu - HP
Z	-2,201[a]	-1,782[a]	-2,201[a]	-,734[b]	-2,201[b]	-2,201[b]	-2,201[b]	-,734[a]	-1,572[b]	-2,201[b]
Asymp. Sig. (2-tailed)	0.028	0.075	0.028	0.463	0.028	0.028	0.028	0.463	0.116	0.028

Ca= Carapace, Gi= Gills, Ov= Ovaries, HP= Hepatopancreas, Mu= Abdominal muscle

a.Based on negative ranks

b.Based on positive ranks

c. Control after day 28

d.Wilcoxon Signed Ranks test

Descriptive Statistics[a]

	N	Mean	Std. Deviation	Minimum	Maximum
Carapace	6	0.06	0.03	0.02	0.12
Gills	6	0.26	0.19	0.07	0.61
Ovaries	6	0.45	0.63	0.03	1.53
Hepatopancreas	6	0.11	0.09	0.03	0.25
Abdominal muscle	6	0.04	0.01	0.03	0.05

a.Treatment = Phoslock® group, Day=0

Test Statistics[c,d]

	Gi-Ca	Ov-Ca	HP-Ca	Mu-Ca	Ov-Gi	HP-Gi	Mu-Gi	HP-Ov	Mu- Ov	Mu- HP
Z	-2,201[a]	-1,153[a]	-1,572[a]	-1,153[b]	-,105[a]	-1,992[b]	-2,201[b]	-,734[b]	-1,572[b]	-1,572[b]
Asymp. Sig. (2-tailed)	0.028	0.249	0.116	0.249	0.917	0.046	0.028	0.463	0.116	0.116

Ca= Carapace, Gi= Gills, Ov= Ovaries, HP= Hepatopancreas, Mu= Abdominal muscle

a.Based on negative ranks

b.Based on positive ranks

c. Phoslock® group at day=0

d.Wilcoxon Signed Ranks test

Descriptive Statistics[a]

	N	Mean	Std. Deviation	Minimum	Maximum
Carapace	6	14.18	11.22	2.11	35.09
Gills	6	194.83	206.97	51.46	600.15
Ovaries	6	2.78	1.10	1.51	4.26
Hepatopancreas	6	56.75	71.84	4.72	199.95
Abdominal Muscle	6	2.80	1.94	1.13	6.20

a.Treatment =. Phoslock® group after 14 days

Test Statistics[c,d]

	Gi- Ca	Ov -Ca	HP-Ca	Mu-Ca	Ov-Gi	HP-Gi	Mu-Gi	HP-Ov	Mu-Ov	Mu - HP
Z	-2,201[a]	-2,201[b]	-1,572[a]	-2,201[b]	-2,201[b]	-2,201[b]	-2,201[b]	-2,201[a]	-,524[b]	-2,201[b]
Asymp. Sig. (2-tailed)	0.028	0.028	0.116	0.028	0.028	0.028	0.028	0.028	0.600	0.028

Ca= Carapace, Gi= Gills, Ov= Ovaries, HP= Hepatopancreas, Mu= Abdominal muscle

a.Based on negative ranks

b.Based on positive ranks

c. Phoslock® group after 14 days

d.Wilcoxon Signed Ranks test

Descriptive Statistics[a]

	N	Mean	Std. Deviation	Minimum	Maximum
Carapace	6	17.43	6.13	7.99	23.78
Gills	6	316.74	321.08	125.21	962.05
Ovaries	6	3.50	2.89	0.84	7.74
Hepatopancreas	6	88.03	109.59	19.21	306.34
Abdominal muscle	6	8.75	3.68	4.43	13.59

a.Treatment = Phoslock® group, Day=28

Test Statistics[c,d]

	Gi-Ca	Ov-Ca	HP-Ca	Mu-Ca	Ov-Gi	HP-Gi	Mu-Gi	HP- ov	Mu-Ov	Mu- HP
Z	-2,201[a]	-2,201[b]	-2,201[a]	-1,782[b]	-2,201[b]	-1,572[b]	-2,201[b]	-2,201[a]	-2,201[a]	-2,201[b]
Asymp. Sig. (2-tailed)	0.028	0.028	0.028	0.075	0.028	0.116	0.028	0.028	0.028	0.028

Ca= carapace, Gi= Gills, Ov= Ovaries, HP= Hepatopancreas, Mu= Abdominal muscle

a.Based on negative ranks

based on positive ranks

c. Phoslock® group after 28 days

d.Wilcoxon Signed Ranks test

Appendix 6. Laboratory Protocol

Protocol 1

1. Experimental Set up
   - Prepare test vessels (60 Transparent plastic) and make two holes in each vessel for air coming in and coming out using driller.
   - Wash them first with hot tap water and then with demi water and copper free water let them to dry and at the end label them 1- 60 using water proof tape and permanent marker.
   - Arrange the aeration valves and tubes with aeration stones in water bath
   -
2. Collection of the experimental unit and water media

2.1. Water collection

- Collect copper free water from Gaia laboratory.
- Take copper free water sample and measure the pH, Temperature , conductivity and dissolved oxygen using pH meter: pH 315i WTW with pH electrode, conductivity meter, Oxyguard Handy Polaris respectively.
- Fill 1liter of copper free water in measuring cylinder(1000ml) and pour it in each test vessels and mark on each test vessels to monitor the volume(loss due to evaporation) of water during experiment.
- Place them randomly in a water bath (57 of them in compartment four and 3 in compartment 2), the water bath is maintained at a temperature of 19-20^{o}c.
- Place aeration tubes in each test vessels and adjust the aeration pumps to check whether it works properly.

2.2. Marbled crayfish collection

- Collect the crayfishes from cultured condition, Alterra laboratory using small fish net
- Select 60 experimental and 4 replacement animals adult crayfishes with proper size, put them in a bucket with copper free water and brought to the plankton laboratory.
- First measure the total length (from tip of rostrum to end of teleson) and measure the total body weight of the animals.
- Keep the measured animals in the test vessels and feed them (1 pellet fish food for each fish) twice weekly with a proper aeration.

3. Assigning in to groups
   - First the data on their body weight and total length put in excel sheet and sort it out based on their body size.
   - The crayfishes were assigned in to two groups(30 control and 30 expose) by tossing a coin based on their body size(stratification) and label in each vessels ‘’**CO’’** for control groups and **’’EX’’** for exposed(treated groups) and prior to treatment, after 1 week acclimation period 10 crayfishes from both groups were sampled to baseline(t=0) lanthanum analysis.
4. Replacement animals
   - During acclimation period the egg bearing animals and dead animals were replaced by new ones by measuring their body length and weight which were collect from Alterra laboratory. However, during real experiment only the dead ones were replaced.

- During the course of the experiment, I kept 5 replacement crayfishes (3 crayfishes were maintained as control and the rest 2 were exposed with 1g/l of Phoslock®.

5. Replenish the water media
   - The water in each vessels were completely replenished weekly with clean copper free water for controls, and with 1gm(1000mg/l) of Phoslock® solution for Phoslock® groups. First I replenished the controls and then after the exposed ones to prevent cross contamination.
   - During the experiment I also refill copper free water for those vessels that lost water due to evaporation.
6. Exposing
   - First I prepare coulter cups with lids
   - Prior measuring I close the door because the balance is really sensitive so if the door is open it is difficult to get the right amount.
   - Calibrate the balance by pressing the cal- button, and then press T- button to make it zero.
   - Put the cutler cup without lid in a balance and close the door of balance, then press T- button and wait until Zero number was displayed.
   - Clean sampling spoon with tissue paper to avoid contamination with others and take Phoslock® from the plastic bottle using spoon and place it in coulter cup which was in the balance.
   - Measure approximately 1g of Phoslock® batch Rawbraken, 2008 in a coulter cup and write the weight of Phoslock® in each cup. Because, when I measure Phoslock® I couldn't get exactly 1 gram.
   - After 1 week acclimation period the Phoslock® group, 20 crayfishes were exposed with 1000mg (1g) Phoslock® and the rest were maintained as a control group.
   - During exposing, take little sample of water from each boxes one by one using coulter cup which contain Phoslock® and cover the lid shake well until the Phoslock® mixes with the water and add the solution in each boxes. At the same time the amount of Phoslock® added in each box were recorded.
7. Experimental conditions and measurements

7.1. pH measurement
   - First I calibrate the pH electrode with buffer 7.0 and 4.0. and keep the electrode in KCl with cover
   - After Calibration I rinse the electrode with demi water and shake gently and rub with tissue paper
   - First I measure the controls and then Phoslock® group and between each measurement I cleaned up the probe with demi water to prevent cross- contamination.

7.2. Dissolved oxygen
   - First I calibrate the oxygen probe with screw (100% in air).
   - Rinse with demi water and shake gently and clean up with tissue paper and then immerse in each test box (first in controls and then in Phoslock® groups the same procedure as pH).

- During measuring I gently move the probe because when you place the probe in the water the probe will consume oxygen itself.
- Dissolved oxygen is measure in mg/l

7.3. Conductivity and Temperature

- The same procedure is used to measure as dissolved oxygen and pH
- The temperature is also measured using conductivity meter

7.4. Turbidity measurement

- First I calibrated the turbidity meter using demi water in a cuvette (calibration should be 0.2NTU). Fill the cuvette Demi water and put in turbid meter, close the cover, press power button and then cal- button, after that press Read- button.
- Take overlying water sample from each boxes one by one using cuvette (First controls and then Phoslock® group and rinse the cuvette with demi water between each measurement to prevent contamination.

N.B. pH, Dissolved oxygen, conductivity, temperature and oxygen were measured two times per week.

8. Sampling of experimental unit (crayfish), water media and food for analysis

8.1. Sampling of crayfish

- At time t=0, t=14, t=28 ; 10 crayfishes from each groups will be harvested and transferred to clean copper free water for 4 -6days depuration period.
- After 4 days depuration period the animals will be sacrificed to obtained the tissues

8.2. Water sampling for ammonium and Lanthanum analysis

- First I prepare sampling bottles(250ml), put them(including lids) in washing machine to clean them
- Dry them in oven(drying oven)
- -Label them; date, code of the sample, and type of analysis
- Before replenishing, take overlying water sample using cleaned small beakers and pour to the sampling bottles (first from controls and then exposed or treated ones to control cross contamination).
- Keep in a freezer until filtering them to prevent loss especially ammonium.
- Prior filtering I prepare 50ml sampling bottles, wash them in washing machine and dry and defrost the samples (collects from the freezer and keeps them outside.
- Label the bottles; date, code of the sample, Filtered and type of analysis (Lanthanum and ammonium).
- Thereafter, Clean the vacuum water filter with demi water

- Prepared the membrane filter (Whatman membrane filter, cellulose nitrate) 0. 45micrometer and put in.
- Rinse it with Demi water
- First shake the samples to have uniform sample and pour
- Filter first controls and then treated as well as between each sample I used new filter for each and rinse the instrument with Demi water to prevent contamination.
- After filtering keep the samples again in a freezer till analysis.
- 50ml of unfiltered samples also kept in a freezer for total lanthanum analysis.
- Ammonium was first analysed in auto analyser and then lanthanum in ICP- MS.
- During replenishing one copper free water sample taken and filtered for Lanthanum analysis.

8.3. Food sampling

- Fish food was sampled, grounded and destructed to check lanthanum in it.

9. Dissection of crayfish

- Prepare, weigh coulter cups with lids and label them (date, code of the sample, type of tissue (for example HP for hepatopancreas, etcetera)
- 3 crayfishes from each group per day were sacrificed for dissection and washed with copper free water prior sacrificing to remove the attached lanthanum on the surface of the body.
- Each animal were put in a small plastic bag to prevent contamination, label and buried in ice for 15 – 30 minute to euthanize them.
- The specimens were kept in hot tap water for 2minute to kill them.
- Dissection of crayfish to obtain the tissues(First open carapace using small scissor and blades, lift up and place it in coulter cup using forceps , remove gills(right and left, remove the muscles below the carapace carefully and then heart after the heart was removed take off the ovaries carefully using forceps, hepatopancreas(digestive gland), and then cut the head part, remove the abdominal carapace, after that carefully remove the intestine and at the end remove the abdominal muscle), keep the each tissues separately in a coulter cup, weigh (coulter cup with lids and wet tissue), and kept in a freezer(-18^{o}c & -20^{o}c) prior to drying.

10. Preparation for analysis

- The samples in a freezer were collected, remove the lids (first controls and then exposed ones; the lids also labelled (date, code of sample, type of tissue) and kept the control lids and treated ones separately to avoid cross- contamination.
- Covered the samples with pieces of tissue papers and put in a dry freezer (-60^{o}c) for 24 hr. to remove the moisture content (dry them).
- The dry weight of each tissue were measured and recorded.

- The dry samples were crushed using Mortar and Pestle to obtain the powder.
- Weigh the crushed samples and kept in a precision tubes for micro destruction (see the protocol below which was suggested by Griethuysen et al. (2000).
- At the end the destructed samples were sent to soil laboratory and lanthanum in the destruct samples were analysed by inductively coupled plasma mass spectrometry (ICP-MS).

Protocol 2.

Preparation for Identification of trace metals in Macro fauna and Zooplankton

Protocol number: C8. E6 Project:SSEO

Date: version 1, August 22, 2000

Authors: C.Van Griethuysen, C.T.A. Moermond, J. Van Baren

Introduction

The trace metals content in benthic and pelagic macro fauna is determined after a digestion of the tissue with a combination of HNO_3 and H_2O_2. Bioaccumulation of trace metals can be assessed in this way. In the rest of this protocol, oligochaetes will be the target species, but the method can also be used for bulk macro fauna and zooplankton samples. The method is developed at the UvA (Aquatic Ecology and Eco toxicology (. Some of the materials and all reagents used are derived from the UvA.

Methods/ Measurements

- About 20mg of dry samples is weighed exactly on the analytical balance into the precision tips.
- 200ul of Ultrex HNO_3 (65%) is added to the sample and it is placed on the heated destruction block for 2hrs at 94^0C . Before the addition to the first samples, the pipette point is pre-rinsed with HNO_3.
- When the HNO_3 of the previous step is almost evaporated, repeat the previous step (addition of 200ul HNO_3.
- The destruction block is cooled down till 65-70^0C, when the HNO_3 of the previous step is almost evaporated.
- Then, 100ul H_2O_2 (30-35%) is added to the samples. If the reaction is very strong, take the sample out of the destruction block and/or add some Nano pure water (as less as possible).
- The temperature is increased to 94^0C for three hours.
- When the samples are completely evaporated, they can be taken out of the destruction block(the moment, at which complete evaporation is reached, can differ for different samples)
- 2ml of Nano pure water is added to the samples. Before dilution the samples must be thoroughly mixed with a vortex apparatus. When there is long period of time between filling up and diluting, Vortexing directly after filling up is desirable in addition to vortexing before dilution.
- Samples are diluted1:10(ml of sample, 9ml of 0.1M of Ultrex HNO_3.) by means of a diluter.
- Diluted solutions are measures on ICP-MS, (detection limit roughly 5ppbat 10 times dilution) at the soil Science and plant nutrition Group.

Equipment

- Destruction block for 60 samples
- Reaction vials(Eppendorf precision tips, 2ml
- Automatic pipette 50- 200ul, vortex apparatus, dilutor apparatus
- ICP-MS(Elan 6000, Perkin Elmer)

Chemicals

- HNO_3. conc.(65% approximately 14.3M), Ultrex quality(UvA)
- H_2O_2. 30-35%, Ultrex quality(UvA)

Special Precautions

In this analysis, strong acids are used. Therefore, always work with a laboratory coat and work in a fume hood. When making reagents, also use gloves and safety glasses.

Power analysis for lanthanum accumulation in some of tissues

Tissue = carapace Day= 14 α=0.05

N	mean1(C)	mean2(P)	Cstd	β
6	1.2	14.18	10.31	0.59
8	1.2	14.18	10.31	0.71
10	1.2	14.18	10.31	0.8
12	1.2	14.18	10.31	0.87
14	1.2	14.18	10.31	0.91

Tissue = carapace Day= 28 α=0.05

N	mean1(C)	mean2(P)	Cstd	β
6	0.07	17.43	9.96	0.86
8	0.07	17.43	9.96	0.94
10	0.07	17.43	9.96	0.97
12	0.07	17.43	9.96	0.99

Tissue = Gills Day= 14 α=0.05

N	mean1(C)	mean2(P)	Cstd	β
6	2.99	194.83	171.79	0.49
8	2.99	194.83	171.79	0.61
10	2.99	194.83	171.79	0.7
12	2.99	194.83	171.79	0.78
14	2.99	194.83	171.79	0.84

Tissue = Gills Day= 28 α=0.05

N	mean1(C)	mean2(P)	Cstd	β
6	1.73	316.74	271.89	0.52
8	1.73	316.74	271.89	0.64
10	1.73	316.74	271.89	0.74
12	1.73	316.74	271.89	0.81
14	1.73	316.74	271.89	0.87

Tissue = HP Day= 14 α=0.05

N	mean1(C)	mean2(P)	Cstd	β
6	0.97	56.75	56.53	0.4
8	0.97	56.75	56.53	0.51
10	0.97	56.75	56.53	0.6
12	0.97	56.75	56.53	0.68
14	0.97	56.75	56.53	0.74
16	0.97	56.75	56.53	0.8

Tissue = HP Day= 28 α=0.05

N	mean1(C)	mean2(P)	Cstd	β
6	0.35	88.03	86.92	0.42
8	0.35	88.03	86.92	0.52
10	0.35	88.03	86.92	0.62
12	0.35	88.03	86.92	0.7
14	0.35	88.03	86.92	0.76
16	0.35	88.03	86.92	0.81

Keys:
N= sample size
Mean 1= mean value for control group
Mean 2= mean value for Phoslock® group
CStd= common standard deviation
HP= Hepatopancreas
β= Power value

N.B. The actual sample size was 6 for all the tissues.