Element accumulation and levels of four biomarkers in common frog (*Rana temporaria*) tadpoles in two sedimentation ponds and a naturally occurring pond

Akkumulering av grunnstoffer og nivåer av fire biomarkører i rumpetroll av vanlig frosk (*Rana temporaria*) i to rensebasseng samt en naturlig forekommende dam

Susanne Lund Johansen

NORWEGIAN UNIVERSITY OF LIFE SCIENCES
DEPARTMENT OF ECOLOGY AND NATURAL RESOURCE MANAGEMENT, INA
MASTER THESIS 60 CREDITS 2013

Preface

The present master's thesis was written as part of a two years master's degree in Natural Resource Management, by the Department of Ecology and Natural Resource Management (INA) at the Norwegian University of Life Sciences (UMB). The thesis was funded by the Norwegian Public Roads Administration (NPRA).

I have been so lucky to have no less than four great supervisors. Postdoctoral research associate Lene Sørlie Heier by the Department of Plant and Environmental sciences (IPM) was officially my main supervisor, while Senior Principal Engineer/Associate Professor Sondre Meland (NPRA/IPM), Professor Bjørn Olav Rosseland (INA) and Professor Lindis Skipperud (IPM) was co-supervisors. I wish to thank you all for giving me good advices and always taking your time to counsel me. In particular I want to thank Lene for teaching me analysis of metallothionein and assisting me whenever I needed help in the laboratory, and Sondre for good counselling during the field work.

I gratefully acknowledge researcher Eivind Farmen at The Norwegian Institute for Water Research (NIVA) for teaching me how to analyze protein, 7-Ethoxyresorufin *O*-deethylase (EROD), glutathione *S*-transferase (GST) and reduced glutathione (GSH) plus how to process the tadpole samples prior to the analyses. His assistance enabled me to do far more analyses than originally intended, which I am very thankful for.

Many thanks are given to Principal Engineer Karl Andreas Jensen (IPM) for analysis of trace elements in water and tadpole samples by inductively coupled plasma mass spectrometry (ICP-MS), and Principal Engineer Solfrid Lohne for teaching me how to decompose samples and prepare them for ICP-MS analysis. I also thank PhD student Siri Lie Olsen (INA) for invaluable help with the R statistics software.

Finally, many thanks are given to Camilla Gulbrandsen for proofreading and to Tor Arne Svanqvist for keeping me company during field work and being such a supporting and encouraging boyfriend.

Ås, 12 May 2013

Susanne Lund Johansen

.

Abstract

During the last decades there has been an increasing awareness of pollution in tunnel wash water and highway runoff, and its ecotoxicological effects. The objectives of the present thesis were to investigate trace element accumulation and the levels of the biomarkers metallothionein (MT), 7-Ethoxyresorufin *O*-deethylase (EROD), glutathione *S*-transferase (GST) and reduced glutathione (GSH) in common frog (*Rana temporaria*) embryos and tadpoles inhabiting Vassum and Skullerud sedimentation ponds along E6 in southeast Norway. A naturally occurring rainwater pond was also included in the study. Tadpoles and water samples were collected weekly in May and June 2012 and analyzed for 34 elements, including several metals of environmental concern. The chemical analyses detected total chromium (Cr), copper (Cu) and zinc (Zn) concentrations in the two sedimentation ponds high enough for the water quality to be classified as 'poor' or 'very poor' at several samplings, according to the classification system developed by the Climate and Pollution Agency. However, the highest concentrations of the majority of the trace elements were identified in the naturally occurring rainwater pond, probably due to impact from an abandoned shooting range nearby. For practically all of the rest of the elements the highest concentrations were detected in Vassum, while more moderate concentrations of most elements were measured in Skullerud.

Frog embryos and tadpoles in all three ponds showed a significant time dependent trace element accumulation. The statistics were performed using the first principal component site scores derived from a principal component analysis (PCA) on tadpole tissue element concentrations as representative values for overall element accumulation (henceforth referred to simply as 'overall tissue element concentrations'). The overall tissue element concentrations reached the highest levels in Vassum tadpoles although the highest total and dissolved water concentrations of most elements were detected in the naturally occurring pond. No significant correlation was found between trace element concentrations in water and tadpoles, respectively.

The levels of MT, EROD, GST and GSH varied significantly with time in tadpoles in all three ponds. Overall tissue element concentrations could not explain the variation in MT, EROD or GSH as no significant correlation was found between overall tissue element concentrations and these biomarkers. However, significant positive correlation was found between MT and tissue lead (Pb) concentrations when testing this separately. Significant positive correlation was also found between overall tissue element concentrations and GST, and between GST and tissue cadmium (Cd) concentrations, and GST and tissue Pb concentrations when testing this separately. Hence, the results suggest that overall tissue element concentrations, as well as tissue Cd and Pb concentrations, can possibly explain some of the variation in GST. Altogether, the lack of an adequate reference group makes it difficult to conclude whether any of the biomarkers are induced above basal level.

The results suggest that *R. temporaria* tadpoles growing up in the sedimentation ponds as well as the naturally occurring pond may be adversely affected by contaminants in the water.

Controlled exposure studies or field studies including a proper reference group are needed to identify the basal level of biomarkers in tadpoles and demonstrate any departures from the natural variation.

Sammendrag

De siste tiårene har det vært økt oppmerksomhet rundt de økotoksikologiske effektene av forurensning i tunnelvaskevann og avrenningsvann fra veg i dagen. Formålet med denne masteroppgaven var å undersøke akkumuleringen av metaller og andre grunnstoffer i embryoer og rumpetroll av vanlig frosk (*Rana temporaria*) i to rensebasseng langs E6 på Østlandet, samt i en naturlig forekommende dam. Dessuten ble nivåene av de fire biomarkørene metallotionein (MT), 7-etoksyresorufin *O*-deetylase (EROD), glutation *S*-transferase (GST) og redusert glutation (GSH) også målt i individene. Rumpetroll og vannprøver ble samlet inn ukentlig i mai og juni 2012, og analysert for 34 grunnstoffer, blant annet flere metaller som ofte er miljømessig problematiske. De kjemiske analysene viste at de totale vannkonsentrasjonene av krom (Cr), kobber (Cu) og sink (Zn) i de to rensebassengene på et eller flere tidspunkter var høye nok til at vannkvaliteten ble klassifisert som 'dårlig' eller 'svært dårlig' etter Klima- og forurensningsdirektoratet (Klif) sitt klassifiseringssystem for miljøkvalitet. De høyeste konsentrasjonene av de aller fleste metaller og andre grunnstoffer ble målt i den naturlig forkommende dammen. Dette skyldes sannsynligvis påvirkning fra et nedlagt skytefelt i nærheten. De høyeste konsentrasjonene av praktisk talt alle de resterende grunnstoffene ble målt i Vassum rensebasseng, mens mer moderate konsentrasjoner ble målt for de fleste grunnstoffene i Skullerud rensebasseng.

I alle tre dammene var det en signifikant akkumulering av metaller og andre grunnstoffer (for enkelhetsskyld kun referert til som metaller heretter) i froskeembryoer og rumpetroll over tid. Statistikken ble utført ved å bruke *site scores* skaffet til veie ved prinsipalkomponentanalyse (PCA) som representative verdier for den generelle konsentrasjonen av metaller i rumpetrollene. Herfra vil begrepet 'generell metallkonsentrasjon i rumpetroll' referere til disse verdiene. Den generelle metallkonsentrasjonen i rumpetroll nådde de høyeste nivåene i individer fra Vassum rensebasseng, til tross for at de høyeste totale og løste konsentrasjonene av de fleste metaller ble målt i vann fra den naturlig forekommende dammen. Det ble ikke funnet noen signifikant korrelasjon mellom generelle metallkonsentrasjoner i vann og rumpetroll.

Nivåene av de fire biomarkørene MT, EROD, GST og GSH varierte signifikant over tid i rumpetroll fra alle de tre dammene. Generell metallkonsentrasjon i rumpetroll kunne ikke forklare variasjonen i verken MT, EROD eller GSH, ettersom det ikke var noen signifikant korrelasjon mellom disse parameterne. Det ble imidlertid funnet signifikant korrelasjon mellom MT og konsentrasjonen av bly (Pb) i rumpetroll, da dette ble testet separat. Det var signifikant positiv korrelasjon mellom GST og generell metallkonsentrasjon i rumpetroll, og også mellom GST og kadmium (Cd), og mellom GST og Pb, i rumpetroll. Resultatene antyder at metallkonsentrasjon i rumpetroll muligens kan forklare deler av variasjonen i GST. For alle biomarkørene gjør mangelen på en referansegruppe det vanskelig å konkludere om hvorvidt noen av dem er indusert over basalnivået.

Resultatene antyder at rumpetroll av vanlig frosk som vokser opp i de to rensebassengene, samt i den naturlig forekommende dammen, kan være negativt påvirket av antropogen

forurensning i vannet. Kontrollerte eksponeringsstudier i laboratorium, eller feltstudier med en god referansegruppe, er nødvendig for å kunne identifisere basalnivåer for biomarkørene i rumpetroll, og for å kunne demonstrere eventuelle avvik fra den naturlige variasjonen.

Table of contents

1 Introduction

The road transport in Europe has grown almost continuously the last decades (Monsrud et al. 2011). The same trend is evident in Norway, as both the traffic load and the road network have increased dramatically in the post-war period (Monsrud et al. 2011; Statens vegvesen 2011). A functioning transport system is an essential part of our society, but the traffic growth also has environmental costs. Traditionally, the main environmental concern of road traffic has been air pollution and noise (e.g. Finkelstein et al. 2004), but research done the last decades has shown that highway runoff and tunnel wash water also contribute to the spreading of pollution into the environment (e.g. Meland et al. 2010a; Norrström & Jacks 1998; Sriyaraj & Shutes 2001). Engine exhaust, de-icing chemicals, tunnel wash detergents, and the wearing of asphalt, road equipment, tyres, and vehicle body create a cocktail of anthropogenic pollutants that may be carried to water bodies near the road with rain or snowfall (Meland et al. 2010c; Preciado & Li 2006; Westerlund et al. 2003; Westerlund & Viklander 2006). In recent years, there has been a growing awareness of the adverse effects of non-point source pollution, i.e. pollution that cannot be localized to a specific point of discharge, such as highway runoff (Kayhanian et al. 2008; Sansalone & Buchberger 1996). This is evident in the implementation of the EU Water Framework Directive. The directive was implemented by law in Norway in 2007 and aims to ensure good quality status for all European water bodies within 2021 (The European Parliament and the Council of the European Union 2000). The status "good quality" includes chemical as well as ecological and hydrological parameters, and the directive emphasizes the importance of mitigating pollution from diffuse sources, such as highway runoff, in achieving this goal.

In Norway, the Norwegian Public Roads Administration (NPRA) has the sectorial environmental responsibility for monitoring the pollution from road traffic and mitigating any negative impact on the aquatic environment (Statens vegvesen 2008). To prevent the spreading of pollutants in highway runoff to receiving waters, NPRA started to construct sedimentation ponds along roads with heavy traffic in the 1990s (Meland 2010). Today there are about 150 ponds in Norway that remove particle bound pollution by sedimentation so the water draining into the recipient is cleaner. Unintentionally, many of the ponds have turned out to be an attractive habitat for organisms such as aquatic insects and amphibians. As many natural ponds and wetlands have been destroyed by human activity, highway storm water ponds may possibly provide additional habitat and contribute positively to biodiversity (Brand & Snodgrass 2010; Le Viol et al. 2009; Le Viol et al. 2012). However, it is also possible that they may constitute ecological traps (Snodgrass et al. 2008), as the water may be toxic to the organisms. Hence, the ponds' role in pollution retention may conflict with their role as habitat. Whether sedimentation ponds represents sources or sinks for biodiversity probably depends on the species in focus and is a field of increasing research (McCarthy & Lathrop 2011).

Several studies have investigated the ecotoxicological effects of highway runoff on amphibians. While some species seem to reproduce successfully in sedimentation ponds

(McCarthy & Lathrop 2011), lethal and sublethal effects have been reported on other species. For instance Snodgrass et al. (2008) observed 100 per cent mortality for larvae of the species *Rana sylvatica* that had been exposed to storm water pond sediment in the laboratory, and Brand et al. (2010) documented increased mortality for newly hatched *Hyla versicolor* embryos. Reports of sublethal effects include a field study documenting metal accumulation in tadpoles of common frog (*Rana temporaria* L. 1758) (Damsgård 2011), and reduced size at metamorphosis for tadpoles exposed to storm water sediments (Snodgrass et al. 2008), but also increased size at metamorphosis combined with earlier metamorphosis (Brand et al. 2010). There have been reports of significant zinc accumulation in froglets that had been exposed to weathered tyre debris from egg to metamorphosis (Camponelli et al. 2009), and Casey et al. (2005) reported tissue metal concentrations that could indicate bioconcentration in the gut coils of tadpoles inhabiting storm water ponds in Maryland, USA.

The objective of this thesis was to investigate the accumulation of trace elements and levels of the four biomarkers metallothionein (MT), 7-Ethoxyresorufin *O*-deethylase (EROD), glutathione *S*-transferase (GST) and reduced glutathione (GSH) in common frog tadpoles growing up in two sedimentation ponds plus one naturally occurring rainwater pond in southeast Norway. Metallothionein was measured because it is a commonly applied biomarker of metal exposure, while measurement of GST and GSH were of interest since they are common biomarkers of oxidative stress caused by metals as well as organic contaminants. EROD is a commonly used biomarker of organic contaminants, and was measured because the organic contaminants polycyclic aromatic hydrocarbons (PAH) are prominent pollutants in highway runoff. The literature on biomarkers in amphibians is growing and includes field studies (Cooper & Fortin 2010; Murphy et al. 2006; Othman et al. 2012) as well as controlled exposure studies (Huang et al. 1998; Kostaropoulos et al. 2005; Loumbourdis et al. 2007; Papadimitriou & Loumbourdis 2002). However, to the author's knowledge no studies on biomarkers have been conducted on amphibians in sedimentation ponds.

Initially, the naturally occurring pond was intended to represent a reference site, and trace element accumulation and biomarker concentrations in tadpoles were to be compared to those of the tadpoles in the sedimentation ponds. Unfortunately, the pond turned out to be polluted by trace elements such as lead and antimony from an abandoned shooting range nearby. A preliminary water sample analysis did not reveal this, probably due to dilution of the trace element concentrations in melt water and rain during early spring. Consequently, the focus of the thesis was changed to investigate the temporal accumulation of trace elements and variation in biomarker levels, without regarding the naturally occurring pond as a reference site.

The problems to be addressed in this thesis are:

- Do the frog embryos and tadpoles in the three ponds accumulate trace elements with time?
- Can trace element concentrations in water explain variation in trace element concentrations in tadpole tissue?

- Do the levels of MT, GST, GSH and CYP450, measured as EROD activity, vary temporally in tadpoles at an early life stage development?
- Can trace element concentrations in tadpole tissue explain variation in levels of MT, GST, GSH and EROD activity, at different samplings?

Investigation of any relationship between trace element concentrations in tadpole tissue and EROD was of interest because of an assumed correlation between organic and inorganic contaminants in highway runoff. Hence, any correlation between tissue element concentrations and EROD would most likely be a reflection of correlation between PAHs and EROD. Since PAHs are readily metabolized in living organisms they are difficult to measure accurately in tissue, and consequently correlation with trace elements was tested as a substitute.

2 Background

2.1 How pollution is spread by highway runoff

Pollution deposited on the road surface will sooner or later be washed off by rain showers or snowmelt and end up in the soil or water bodies along the road. Runoff processes on impervious surfaces are distinguished from those on vegetated surfaces and bare soil by the phenomenon called "first flush" (Deletic 1998). It refers to the assumption that the initial part of the runoff during a storm carries the most concentrated load of pollution and may also have the most toxic effect on biota (Barbosa & Hvitved-Jacobsen 1999; Kayhanian et al. 2008). The water that runs off later contains lower concentrations, as the first masses of water have "cleansed" the road. Vegetated surfaces are not cleansed as easily, hence the phenomenon does not apply here. There is controversy regarding whether the first flush phenomenon really exists, even for impervious surfaces, and if so, when it becomes apparent (Deletic 1998; Färm 2002).

Concentrations of e.g. certain metals may be considerably higher in highway runoff than background levels (Meland et al. 2010c), and snowmelt-induced runoff is often more polluted than rainfall-generated runoff (Sansalone & Buchberger 1996; Westerlund et al. 2003; Westerlund & Viklander 2006). The reason for this is that pollution may accumulate in the snow pack during winter, and because of low temperatures, there is little or no runoff until spring. When spring comes, the snow may melt over a short period, carrying water with high concentrations of contaminants into the recipient (Sansalone & Buchberger 1996; Westerlund & Viklander 2006).

Pollution that accumulates on the road surface inside tunnels will not be washed away by precipitation. Hence, to maintain clear sight and clean roads with good friction, the tunnels must be washed regularly. Tunnels are washed 2 – 12 times a year, depending on the size and traffic load (Statens vegvesen 2010). Tunnel wash water will normally contain higher concentrations of contaminants than runoff from open road areas because pollution is allowed to accumulate for a longer time before it is washed away (Meland 2010; Meland et al. 2010a). In addition to the debris, dust, and contaminants derived from car and road wearing, the tunnel wash water contains detergents, usually at a concentration of 0.5 – 1.0 per cent (Meland et al. 2010b), that may also pose a risk to biota in receiving waters (Corneliussen et al. 2007).

2.2 Sedimentation ponds

In order to mitigate the spread of pollution from highway runoff to the ambient environment, the NPRA constructs sedimentation ponds along roads in Norway with high traffic load. The first ponds were built in the 1990s, and the type called wet detention ponds are the most

common (Meland 2010). These have a permanent pool of water and remove pollution by sedimentation of particle bound contaminants. Removal efficiency is strongly dependent on the detention period of the water, which depends on the precipitation and the dimensioning of the pond (Åstebøl 2005). The potential optimal cleansing is often not achieved during prolonged storms or snow melt periods. An important drawback of wet detention ponds is that they have relatively low removal efficiencies for dissolved pollutants and mobile low molecular mass species (Meland 2010). An unintentional side effect of the sedimentation ponds is that organisms such as aquatic insects and amphibians have adopted them as habitat and breeding grounds. In some cases, they may be important refugees for biodiversity and provide additional habitat for pollution tolerant species in areas where natural wetlands have been destroyed (Le Viol et al. 2009; Le Viol et al. 2012). However, for species less tolerant to pollution, the ponds may represent ecological traps as the organisms perceive them as an attractive habitat while the water is in fact toxic to them or their offspring (McCarthy & Lathrop 2011; Snodgrass et al. 2008). In Norway, time dependent accumulation of metals in *Rana temporaria* tadpoles living in a sedimentation pond has been reported (Damsgård 2011), and Zn accumulation in *Rana sylvatica* tadpoles exposed to weathered tyre material have been reported from USA (Camponelli et al. 2009). There is also a growing literature on the adverse effects on tadpoles due to de-icing salt, e.g. increased frequency and severity of deformities (Hopkins et al. 2013; Sanzo & Hecnar 2006), increased mortality and reduced time to metamorphosis (Sanzo & Hecnar 2006) and reduced swimming speed and more sluggish movements of tadpoles (Collins & Russell 2009; Denoël et al. 2010).

2.3 Pollutants in highway runoff and tunnel wash water

Highway runoff contains a cocktail of organic and inorganic compounds that may be toxic to living organisms. Table 1 shows the specific origin of the different pollutants in highway runoff and tunnel wash water.

Table 1. Contaminants in highway runoff and tunnel wash water. Table modified after Meland (2010). Contaminants included in this study are written in bold letters.

Source		Contaminant*	References
Vehicle	Brakes	**Ba**, **Cu**, **Fe**, **Mo**, **Na**, **Ni**, **Pb**, **Sb**	(Dongarrà et al. 2009; McKenzie et al. 2009; Sternbeck et al. 2002; Thorpe & Harrison 2008)
	Tyres (incl. studded tyres)	**Al**, **Zn**, **Ca**, **Cd**, **Co**, **Cu**, **Mn**, **Pb**, W, hydrocarbons, **PAH** (pyrene, fluoranthene, benzo(ghi)perylene)	(Glaser et al. 2005; Karlsson & Viklander 2008; Lindgren 1998; McKenzie et al. 2009; Ravindra et al. 2008; Sternbeck et al. 2002; Thorpe & Harrison 2008)
	Catalytic converters	Pt, Pd, Rh	(Ek et al. 2004; Whiteley & Murray 2005)
	Vehicle body	**Cr**, **Fe**, **Zn** (steel)	(Taylor & Robertson 2009)
	Combustion	**Ag**, **Ba**, **Cd**, **Cr**, **Co**, **Mo**, **Ni**, V, **Sb**, **Sr**, **Zn**, **PAH** (naphthalene), MTBE, BTEX	(Brown & Peake 2006; Desta et al. 2007; Glaser et al. 2005; Lin et al. 2005; Marr et al. 1999; Ravindra et al. 2008; Wang et al. 2003; Weckwerth 2001)
	Oil and petroleum spill, dripping, used lubricant oil	**PAH** (LMM)	(Ravindra et al. 2008; Wang et al. 2000)
Non-vehicle	Road surface (asphalt, bitumen)	**Al**, **Ca**, **Fe**, **K**, **Mg**, **Na**, **Pb**, **Si**, **Sr**, Ti, **PAH** (mix of HMM and petrogenic LMM, chrysene)	(Brandt & de Groot 2001; Brown & Peake 2006; Sternbeck et al. 2002; Thorpe & Harrison 2008)
	De-icing and dust suppression	**Ca**, **Mg**, **Na**, **Cl**, ferro-cyanide (anticaking agent)	(Aldrin et al. 2008; Novotny et al. 2008; Ramakrishna & Viraraghavan 2005; Viklander et al. 2003)
	Road equipment (e.g. crash barriers, traffic signs, etc.)	**Zn** (galvanized steel)	(Thorpe & Harrison 2008)
	Tunnel wash detergents	Tensides	(Meland et al. 2010b)

***Abbreviations: Ag=silver, Al=aluminium, Ba=barium, BTEX=benzene, toluene, ethylbenzene and xylenes, Ca=calcium, Cd=cadmium, Cl=chlorine, Co=cobalt, Cr=chromium, Cu=copper, HMM=high molecular mass species, K=potassium, LMM=low molecular mass species, Mg=magnesium, Mn=manganese, Mo=molybdenum, MTBE=methyl tert-butyl ether, NA=sodium, Ni=nickel, Pb=lead, Pd=palladium, Pt=platinum, Rh, rhodium, Si=silicon, Sr=strontium, Ti=thallium, Zn=zinc.**

The most prominent contaminants in highway runoff are polycyclic aromatic hydrocarbons (PAH) and metals and metalloids (Meland 2010). In the northern hemisphere road salt, primarily sodium chloride (NaCl), is an important constituent of highway runoff, as it is used as a de-icing agent during winter. Sodium chloride may have negative impact on biota in several ways. First, increased salinity and density may cause strong stratification in the water body, preventing thermocline mixing and leading to hypoxic conditions at the bottom (Marsalek 2003). Second, high concentrations of chloride may be toxic to fresh water organisms due to disruption of the osmoregulation (Marsalek 2003). Third, increased salt concentrations enhance mobilization and the potential for bioavailability of metals from soils and sediment by cation exchange processes and chloride (Cl) complexation, and consequently their potential toxicity to biota in sedimentation ponds or recipients increases (Bäckström et al. 2004; Norrström & Jacks 1998).

2.3.1 Metals and metalloids

Highway runoff often contains large amounts of metals and metalloids due to wearing of tyres, brakes, vehicle body, asphalt and road equipment (Meland 2010). Especially copper (Cu) and zinc (Zn) have been found to be major causes of toxicity in such runoff (Kayhanian et al. 2008), but also cadmium (Cd), mercury (Hg) and lead (Pb) are of great concern (Napier et al. 2008). Metals and metalloids, henceforth referred to as metals for simplicity, are elements occurring virtually everywhere in the environment, either from natural or anthropogenic sources (Fairbrother et al. 2007). Although they are natural substances they may have considerable toxic effects on aquatic organisms if they reach high enough concentrations. For instance, metals may generate reactive oxygen species (ROS) that cause oxidative stress potentially resulting in DNA damage and lipid peroxidation (Lushchak 2011; Watanabe et al. 2003).

It is common to distinguish between nutritionally essential and non-essential metals. Nutritionally essential metals are required for the physiology and metabolism of an organism to function normally (Walker 2006). This group includes the macronutrients calcium (Ca), potassium (K), magnesium (Mg) and sodium (Na), which are required in relatively high amounts, and micronutrients like Cu, Zn and nickel (Ni), which are required in smaller amounts (Fairbrother et al. 2007). Essential metals exhibit a dose-response relationship with an optimal intermediate dose called the "window of essentiality" (Fig. 1), and with deficiency effects occurring at too low doses and toxic effects occurring at too high doses (Fairbrother et al. 2007; Hopkin 1993). Non-essential metals are those that are not required for maintaining a physiological function, although some may be beneficial at very low concentrations (vanadium (V) and arsenic (As) in animals) (Fairbrother et al. 2007). Non-essential elements such as Pb, antimony (Sb) and Cd have no beneficial effects at all, and are toxic above certain levels (Walker 2006).

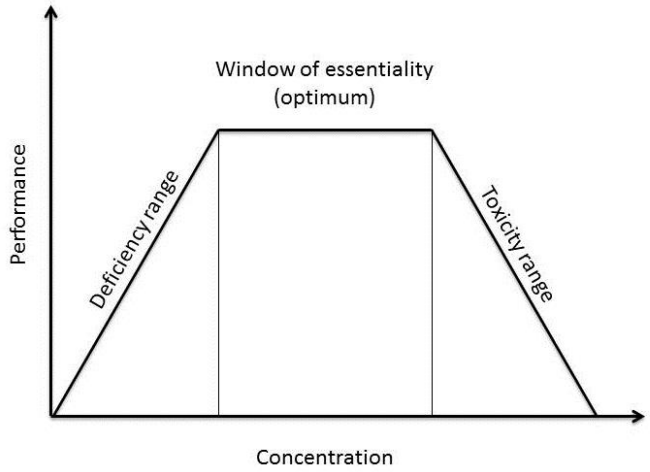

Figure 1. Schematic diagram showing the relationship between the amount of an essential element available for uptake by an organism, and the organism's performance (growth, survival, fecundity, etc.). Modified after Hopkin (1993).

An important feature of metals is that they cannot be created or destroyed by chemical or biological processes. However, they can be transformed into other physicochemical forms called species (Chapman & Wang 2000). Free metal ions, dissolved complexes with e.g. humic ligands, and metals in the form of inorganic sulphide solids, are examples of different species (Chapman & Wang 2000; Fairbrother et al. 2007). The composition of different species of a metal under certain environmental conditions is called *speciation*. The speciation is, among other factors, dependent on pH, redox potential, ionic strength and availability of important complexing ligands such as Cl ions (important for e.g. Cd complexation) or organic matter (very important for Cu complexation) (Fairbrother et al. 2007; VanLoon & Duffy 2011). These factors determine the nuclear composition, electronic state, oxidation state and structure of the metal complex or molecule (Chapman & Wang 2000), which in turn is crucial for the bioavailability of the metal (see section 2.3.2 Bioavailability and biological complexation).

Since metals are non-biodegradable, they cannot be decomposed into less toxic substances by metabolism (Walker 2006). Hence, organisms have evolved other mechanisms to protect themselves against the toxic effect of metals. This involves metal-binding proteins, such as metallothionein and ferritin, and storage of metals in intracellular granules (Chapman & Wang 2000; Fairbrother et al. 2007). This will be further addressed in section 2.4 Biomarkers.

2.3.2 Bioavailability and biological complexation

There is some confusion on the definition of bioavailability in the literature. Fairbrother et al. (2007) describes it as "the extent to which bioaccessible metals absorb onto, or into, and across biological membranes of organisms", while Chapman (2008) define bioavailability as "the portion of a substance that is immediately available for uptake by organisms". Bioaccessible metals may be defined as the fraction of metals present in the ambient environment that may be available for biological uptake in the long term (Chapman 2008).

Usually, low molecular mass species (LMM) such as the free metal ion are the species assumed to cause toxicity because they are more bioavailable than high molecular mass species (HMM) (Fairbrother et al. 2007; Meland 2010). Metals bound in complexes and polymers are assumed to be too large to cross a biological membrane and hence they are of less concern. According to the biotic ligand model, toxicity of a metal occurs when free metal ions reacts with the physiological active binding sites at a biotic receptor, for instance the surface of a gill (Di Toro et al. 2001). The gill may be regarded as a biotic ligand that forms a complex with the metal. pH is assumed to be the most important factor for the behaviour of metals in water, as metals are usually highly soluble and bioavailable at acid conditions while complexation with carbonate and hydrogen complexes increases with increasing pH (Fairbrother et al. 2007). The presence of major cations like Na, Ca and Mg in the water is also of great importance for the toxicity. The reason is that they compete for the binding sites at the biotic receptor and therefore may reduce the uptake of metals (Fig. 2) (Di Toro et al. 2001).

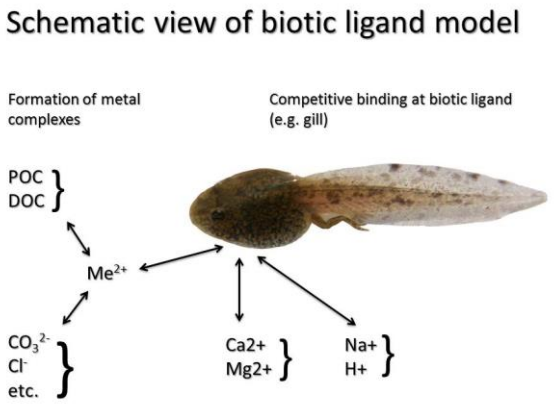

Figure 2. Schematic showing the importance of cations and complexing agents for the toxicity of metals. Particulate organic carbon (POC), dissolved organic carbon (DOC) and carbonate (CO_3^{2-}) bind metals in complexes and hence reduce their bioavailability, while the presence of cations such as Mg and Ca may reduce the bioavailability due to competitive binding at the gills. Modified after Paquin et al. (2000).

2.3.3 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons are organic planar molecules that consist of three or more aromatic rings (benzene rings). They are produced by geological processes in the Earth's crust and during incomplete burning of organic material (Walker 2006). They are present in highway runoff due to oil spill and emissions from burning of fossil fuels in combustion engines (Meland 2010). Road traffic is an important source of PAHs to the environment, and its contribution is increasing due to increase in traffic load and increasing numbers of diesel vehicles on the roads (Napier et al. 2008; Van Metre et al. 2000). Examples of prominent PAHs from combustion are fluoranthene, pyrene, benzo(*a*)anthracene, chrysene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene and benzo(*a*)pyrene (Van Metre et al. 2000). Polycyclic aromatic hydrocarbons are highly lipophilic and in principle inert. They must undergo biotransformation by enzymes in the body in order to become water soluble, before they can be excreted. Many PAHs have carcinogenic and mutagenic properties. However, it is often the metabolic products rather than the original compound that damage the DNA, as the biotransformation creates reactive metabolites that are able to bind to the DNA (Walker 2006).

2.4 Biomarkers

The scientific literature is not entirely consistent on the definition of a biomarker (van der Oost et al. 2003). Peakall (1994) defines it as a biological response to an environmental chemical that gives a measure of exposure or toxic effects, ranging all the way from the molecular level to the functioning of ecosystems. Gestel and Brummelen (1996) on the other hand, define a biomarker as a biological response to an environmental chemical at only the

sub individual level, indicating that the normal status in the organism is disturbed. Others comprise biological responses at the individual level or below, including biochemical, physiological, histological, morphological and behavioural responses (Walker 2006). The last-mentioned will be employed here.

Biomarkers are useful in that they are considered intermediates between the mere presence of environmental pollution and adverse effects at a higher level in the ecosystem (van der Oost et al. 2003). Their identification may give information of adverse effects of contaminants before the whole organism or population is irreversibly affected, and hence they may function as early warning signals of effect (Sparling et al. 2010; van der Oost et al. 2003). In this way they make it possible to implement mitigation measures at an early stage and at sublethal levels.

It is common to distinguish biomarkers of exposure, which indicate that the organism is exposed to a toxicant but does not enable a grading of the adverse effects, from biomarkers of toxic effect, which can be related to an actual health impairment (Hagger et al. 2006; van der Oost et al. 2003; Walker 2006). However, Peakall and Walker (1994) claim that the division may be misleading as all biomarkers are to some degree a biochemical effect of an exposure. All the biomarkers investigated in the present thesis may be classified as biomarkers of exposure, although they are also to some degree related to the health of the organism, particularly GST and GSH which are important in the defence against oxidative stress. The biomarkers included in this thesis have the advantages that they are easy to standardize, they are contaminant-related and associated to the organism's health (Hylland et al. 2006). However, their disadvantage is that their ecological relevance might be limited. Even if high concentrations of e.g. MT or EROD are measured in an organism, it does not necessarily imply adverse effects on ecologically relevant endpoints such as reproduction or survival.

Several factors cause uncertainty and difficulties of comparing populations when studying biomarkers. Sex, developmental stage, ambient temperature and season may have great effect on the activity of a biomarker in amphibians (Sparling et al. 2010). Hence, the induction of a certain biomarker may vary between different populations, even when the exposure to a xenobiotic is equal.

2.4.1 Metallothionein (MT)

Metallothionein is a family of cysteine rich proteins that sequesters trace metals and prevents them from damaging cells and organelles (van der Oost et al. 2003). It is present in most types of tissue in vertebrates, but especially in those responsible for uptake, storage and excretion such as the liver (Hylland et al. 2006; van der Oost et al. 2003). The metal binding capacity of MT owes to the sulfhydryl (S-H) content of cysteine. The biomarker is widely used as an indicator of exposure to metal pollution. Although Cd is considered the primary MT inducer, it also sequesters other metals such as Cd, Cu, Zn, Hg, cobalt (Co), Ni, and silver (Ag) (van

der Oost et al. 2003). One MT molecule is able to bind 7 divalent metal cations (e.g. Cd^{2+} or Zn^{2+}) (Cai & Stillman 1988; Hylland et al. 2006).

2.4.2 7-Ethoxyresorufin *O*-deethylase (CYP1A activity)

In animals, organic, lipophilic xenobiotics such as PAH and polychlorinated biphenyls (PCBs) are metabolized by a two-phase enzymatic metabolism (Walker 2006). During Phase I, oxygen is added to the compound to make it more polar and water soluble, and consequently it is more easily excreted. Iron rich enzymes of the system called cytochrome P450 monooxygenase, particularly the subfamily CYP 450 1A, are important for Phase I biotransformation. 7-Ethoxyresorufin *O*-deethylase (EROD) is a specific CYP450 1A enzyme activity that is widely used as a biomarker of exposure to PAH (van der Oost et al. 2003). The enzymes are found in the endoplasmic reticulum (ER) in a variety of tissues, primarily in that of the liver. The EROD activity is determined by quantifying the increase in the amount of resorufin produced, measured as increase in fluorescence over time (van der Oost et al. 2003).

2.4.3 Glutathione *S*-transferase (GST)

During Phase I biotransformation, oxidation creates functional groups in the xenobiotic that enables it to be conjugated to an even more polar compound with reduced glutathione (GSH). The conjugation process is part of the Phase II biotransformation, and it is catalysed by an enzyme family called glutathione *S*-transferase (GST) (van der Oost et al. 2003). Glutathione *S*-transferases are important in the defence against oxidative damage. Their activity is primarily used as a biomarker of exposure to organic pollutants such as PAHs, PCBs, dioxins and organochlorine pesticides (van der Oost et al. 2003), but it has also been suggested that they may be used as a biomarker of metals like Pb and Cd (Othman et al. 2012; Wright et al. 1998) or other metals that can induce oxidative stress.

2.4.4 Reduced glutathione (GSH)

Glutathione is an intracellular low-molecular-weight thiol found in both plants and animals (Kamencic et al. 2000; Sies 1999). It is the main defence mechanism in aerobic cells against oxidative stress and participates actively in the neutralization of ROS (Wang & Ballatori 1998). Besides, it may be conjugated with Phase I biotransformation metabolites which can finally be excreted through the bile or kidney (Sies 1999; van der Oost et al. 2003). Glutathione may be conjugated with xenobiotics either spontaneously or catalysed by GST (Fig. 3) (Wang & Ballatori 1998). Glutathione is commonly applied as a biomarker of oxidative stress caused by metals as well as organic pollutants.

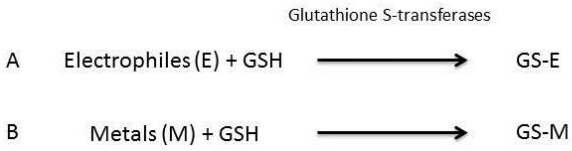

Figure 3. Schematic showing conjugation of electrophiles (e.g. reactive Phase I metabolites) with glutathione (GSH) catalysed by glutathione *S*-transferases (GSTs) (A), and spontaneous conjugation of GSH with metals (B). Modified after Wang and Ballatori (1998).

2.5 The study species – the common frog (*Rana temporaria*)

The common frog (*Rana temporaria*) (Fig. 4) is widely distributed all over Scandinavia and is the most common amphibian in Norway (Dolmen et al. 2004). Amphibians worldwide are in decline regarding their numbers as well as species composition (Beebee & Griffiths 2005; Dolmen 2008; Skei 2006). The decline is probably due to several factors such as habitat destruction, anthropogenic acidification, pesticides, increased UV-B radiation, disease and introduction of alien species. *Rana temporaria* has also experienced local declines, but in general it seems to have large and stable populations (Dolmen et al. 2004; Kuzmin et al. 2009). The species is a habitat generalist (eurytopic), and in Norway they are spawning at altitudes of at least 1000 m a.s.l. (Dolmen 2008).

Figure 4. Adult common frog (*Rana Temporaria*). Foto: Kjell Isaksen.

Both eggs and larvae are dependent on freshwater for development. The mating and spawning usually takes place in small lakes and ponds in April – June, depending on altitude and latitude (Dolmen 2008). The common frog is philopatric, which means that the adults usually return to their birthplace to spawn and use the same pond every year (Kauri 1981; Savage 1962). A female may lay 400 – 6000 eggs which are surrounded by jelly that swells after fertilization. The fully swollen jelly consist of about 0.3 % protein and salts from the water, and 99.7 % water (Savage 1962). Larval development is highly dependent on degree-days as

they are poikilotherms (Riis 1991), and the lower limit for development is 5 – 6 °C (Kauri 1981). Successful reproduction is also dependent on water quality parameters such as pH, ionic strength (NaCl) and Ca content (Dolmen et al. 2004).

The larvae breathe with external gills the first days after hatching, but these are soon overgrown with operculum (Gosner 1960). They feed on pelagic algae, detritus, etc. (Dolmen 2010). The larvae usually metamorphosizes in July – October and then leaves the pond (Dolmen 2008). During the metamorphosis the gills and tail are completely resorbed, the lungs develop, the skin thickens, the mouth parts are transformed and the forelegs emerge (Kauri 1981; Sparling et al. 2010). The adult frogs spend most of their time on land, except in the spawning time. Both larvae and adults have permeable skin through which they can breathe and process water. This is an important route of uptake of waterborne contaminants in tadpoles, in addition to the uptake through food and breathing with gills, and makes them susceptible to pollution in water (Sparling et al. 2010).

In this thesis individuals not yet hatched, roughly speaking Gosner stages 1 – 20, are referred to as embryos (Gosner 1960). Hatched individuals, i.e. Gosner stage 21 and onwards, are referred to as larvae or tadpoles.

3 Materials and methods

3.1 The study sites

All three ponds investigated in this study are located in southeast Norway (Fig. 5). Both sedimentation ponds are of the type wet detention ponds.

Figure 5. Map of the area around the Oslo fjord in southeast Norway showing the locations of the sedimentation ponds at Skullerud and Vassum, and the naturally occurring pond at Prinsdal.

3.1.1 Vassum sedimentation pond

Vassum sedimentation pond is located by the highway E6 in the municipality of Frogn, County of Akershus (Fig. 6 and Fig. 8). It was built in year 2000 and the annual average daily traffic (AADT) was 29 000 vehicles in 2011 (Statens vegvesen 2012a). The pond receives tunnel wash water from three tunnels (the Nordby tunnel, the Smiehagen tunnel and the Vassum tunnel) in addition to water from 1.7 ha of open road area (Meland et al. 2010a). The recipient of the cleansed water is the river Årungselva, which is a locally important river for brown trout (*Salmo trutta*). The pre-sedimentation pond is concreted, and separated from the main pool by a mound. The sedimentation pond was emptied and cleansed the autumn 2011 (Meland 2012). A study carried out by Snilsberg et al. (2002) showed a removal efficiency of 50 per cent for Zn, 75 per cent for Cu and 90 per cent for total suspended solids. There is a large diversity of Dytiscidae in the pond, and frogs are usually spawning there in spring (Fig. 7) (Ole Wiggo Røstad, Department of Ecology and Natural Resource Management at the Norwegian University of Life Sciences, personal communication, October 2012).

Figure 6. Orthophoto showing the E6 junction at Vassum and the sedimentation pond by the acceleration lane.

Figure 7. *Rana temporaria* eggs in the waterline near the outlet of Vassum sedimentation pond. Photo: Susanne Lund Johansen.

Figure 8. Vassum sedimentation pond seen from the inlet. Photo: Susanne Lund Johansen.

3.1.2 Skullerud sedimentation pond

Skullerud sedimentation pond is located by E6 in Oslo municipality, County of Oslo (Fig. 9 and Fig. 10). The pond was built in 1999 simultaneously with a general upgrade of the stretch of highway (Åstebøl 2005). The recipient of the cleansed water is Ljanselva, which is locally important for fish (including brown trout), wildlife and recreation. The pre-sedimentation pond is closed while the main pool is open. The pond receives road runoff from 2.2 ha of asphalted surface and the AADT was 64 000 vehicles in 2011 (Statens vegvesen 2012b). The removal efficiency of contaminants such as metals, oil and PAHs varies from 60 – 90 per cent depending on the chemical (Åstebøl 2005). Common frogs are usually spawning in the pond in spring (Fig. 11), and there is also minnow (*Phoxinus phoxinus*) and a diversity of aquatic insects in the pond (Damsgård 2011).

Figure 9. Orthophoto showing the E6 junction at Skullerud and the sedimentation pond underneath the bridge.

Figure 10. The sedimentation pond at Skullerud seen from the outlet. Photo: Susanne Lund Johansen.

Figure 11. Common frogs (*Rana temporaria*) mating in Skullerud sedimentation pond in April 2012. Photo: Susanne Lund Johansen.

3.1.3 The pond at Prinsdal

The pond at Prinsdal is located in the southern part of Oslo municipality, County of Oslo, in the edge zone between an abandoned shooting range and a mixed forest. A vast number of frogs are spawning there every spring (Fig. 12). The pond is assumed to be shielded from road runoff due to its location high in the terrain and far from roads. However, it is probably affected by metal pollution from ammunition, although the shooting range has been abandoned since 2007 (see Table 2). The pond is smaller and shallower than both the sedimentation ponds and has no outlet, and it often dries up during dry summers (Strand 2006).

Figure 12. Common frogs (*Rana temporaria*) spawning in the pond at Prinsdal in April 2012. Photo: Susanne Lund Johansen.

3.2 Field work

Water samples and frog embryos and tadpoles were collected weekly from the three ponds during May and June 2012. The sampling started in week 18 at Prinsdal and in week 19 at the two sedimentation ponds, and proceeded to week 24 at all sites. There was always 6 – 8 days between each sampling. Three water samples were collected in 50 mL BD Falcon tubes at approximately 10 cm depth at each sampling point: one for analysis of total trace element concentrations, one for dissolved trace element concentrations and one for analysis of total organic carbon (TOC), dissolved organic carbon (DOC) and anions. Samples for analysis of dissolved trace element concentrations were filtrated *in situ* with VWR sterile syringe filters (0.45 µm cellulose acetate membrane). Samples for total and filtered trace element concentrations were acidified with 5 % ultrapure nitric acid (HNO_3) prior to storage. All water samples were stored dark and chill in a fridge at the laboratory until analysis. At each

sampling point a 1000 mL glass bottle (dark) was filled with water for analysis of PAHs. In addition, general water quality parameters were measured at each sampling point using Extech ExStik II EC510 (pH, conductivity and temperature) and Extech DO600 Oximeter (dissolved oxygen).

Frog eggs, and hatchlings basking on top of the eggs, were collected with a small plastic box, while larger tadpoles were collected with a hand net. Subsequently they were put on 2 mL VWR CryoTubes. The jelly surrounding the black vitellus was removed prior to conservation in CryoTubes. To ensure enough tissue material for analysis, approximately forty eggs were put on each tube at the first sampling. The number decreased at subsequent samplings as the larvae grew bigger, and at the last samplings each tube contained only one – three tadpoles. Six CryoTubes were filled for each of the tissue analyses at each sampling to provide six replicates. When a tube was filled it was snap frozen *in situ* on a tank containing liquid nitrogen. Tadpoles sampled at the two last samplings at Vassum sedimentation pond had grown too large for the tubes and were wrapped in aluminium foil instead (glossy side in). All samples were stored in an ultra-low temperature freezer (-82 °C) at the laboratory until analysis.

In both sedimentation ponds the eggs were laid in one or two clusters in a relatively confined part of the pond, by the outlet. Eggs were collected randomly from these clusters. In both ponds many of the tadpoles stayed in proximity to where the eggs had been, when they hatched. Consequently, the tadpoles were mostly collected from the same spot as the eggs. The water samples were also collected from this same spot. At Prinsdal the eggs where spawned across most of the surface. Here, eggs and larvae where collected from several random places in the pond at each sampling, while water samples were collected from one and the same spot each time.

Determination of species was done *in situ* at Skullerud and Prinsdal during spawning. The common frog resembles the moor frog (*Rana arvalis*), that also occur in the area around the Oslo fjord. The common frog can be recognised on its call during mating. Besides, the common frog also has a smaller and softer metatarsal tubercle than the moor frog (Dolmen 2008). At Vassum, the species was identified based on the labial tooth rows of the tadpoles (Fig. 13) because the adult frogs had already left the pond.

Figure 13. The mouth parts of a the common frog (*Rana temporaria*) tadpole sampled at Vassum in week 22, 2012. The four lower labial tooth rows are characteristic of *R. Temporaria* in contrast to *Rana arvalis* that only has three. Photo: Susanne Lund Johansen.

3.3 Analyses of water samples

Except when specified, processing and analysis of water samples were performed by the author.

3.3.1. Total and dissolved trace element concentrations in water

Water samples for analysis of total and dissolved element concentrations were decomposed in Milestone UltraCLAVE (MLS GmbH, Leutkirch im Allgäu, Germany) to remove organic material. All samples were added additional HNO_3 so that they contained 10 % acid in total (included the acid added prior to storage). Internal standard consisting of 20 µg/L of rhodium (Rh), indium (In), tellur (Te) and thallium (Tl) dissolved in 2 % HNO_3 were diluted 10 x and added all samples. Subsequently, the samples were analysed for a number of elements by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 8800 QQQ ICP-MS, Santa Clara, CA, USA). The ICP-MS analysis was performed by Principal Engineer Karl Andreas Jensen at Department of plant and environmental sciences (IPM) at the Norwegian University of Life Sciences (UMB)**.** The elements analysed were sodium (Na), magnesium (Mg), aluminium (Al), silicon (Si), phosphorus (P), sulphur (S), potassium (K), calcium (Ca), scandium (Sc), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), strontium (Sr), molybdenum (Mo), silver (Ag), cadmium (Cd), tin (Sn), antimony (Sb), barium (Ba), lanthanum (La), cerium (Ce), europium (Eu), gadolinium (Gd), ytterbium (Yb), lutetium (Lu), lead (Pb), thorium (Th) and uranium (U). Analytical method blanks and the house standard 1643h, which largely is a copy of the certified reference material 1643e (National Institute of Standards and Technology, Gaithersburg, USA), were also analysed for quality control.

Limit of detection (LD) equalled 3 x the standard deviation of 6 blanks and limit of quantification (LQ) equalled 10 x the standard deviation of 6 blanks (see appendix 1.1 for exact values). The measured values of the certified reference material (CRM) were in good agreement with the certified values, the per cent difference being ≤ 12 % for all elements (appendix 2.1).

3.3.2 Anions and total and dissolved organic carbon

Analysis of anions, TOC and DOC was performed by Principal Engineer Johnny Kristiansen at IPM, UMB**.** Total organic carbon and DOC were measured using the instrument Shimadzu Total Organic Carbon analyzer (TOC-Vcpn, Shimadzu, Tokyo, Japan). The anions analysed were Cl^- and sulphate (SO_4^{2-}), and they were determined by ion-exchange chromatography (Lachat 5000, Loveland, CO, USA) using Dionex column AS22 and Dionex

suppressor. Blanks and a control sample for the relevant analysis range were added for analytical quality control.

3.3.3 Polycyclic aromatic hydrocarbons (PAH)

Water samples collected in 1000 mL dark glass bottles were analysed for the 16 PAHs naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz(*a*)anthracene, chrysene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*a*)pyrene, dibenz(*a,h*)anthracene, benzo(*ghi*)perylene and indeno(*123cd*)pyrene. The analysis was conducted at ALS Laboratory Group Norway AS at Skøyen in Oslo (see appendix 1.3 for LQs).

3.4 Analyses of tadpole tissue

Except when specified, processing and analysis of tadpole samples were performed by the author.

3.4.1 Trace element concentration in tadpoles

The frog embryos and larvae were decomposed in UltraCLAVE to generate homogeneous samples, remove organic material and enable analysis of trace element content. The embryos and larvae were transferred from the CryoTubes into separate Teflon tubes. Internal standard (the same as the one used for the water samples, but not diluted) was added prior to decomposition, in addition to 5 mL HNO_3 resulting in a final HNO_3 concentration of 10 % after dilution. Analytical method blanks and the certified reference materials 1577b Bovine liver (National Institute of Standards and Technology, Gaithersburg, USA), 8415 Whole egg powder (National Institute of Standards and Technology, Gaithersburg, USA), and Dorm-3 (National Research Council, Ottawa, Canada) were also analysed to provide quality control. Principal Engineer Karl Andreas Jensen (IPM, UMB) performed the ICP-MS. The tissue samples were analysed for the same elements as the water samples.

Limit of detection equalled 3 x the standard deviation of 9 blanks and limit of quantification equalled 10 x the standard deviation of 9 blanks (see appendix 1.2 for exact values). The analysis of CRM showed measured values of the elements in good accordance with the certified values (appendix 2.2). The mean per cent difference was $\leq$ 13 % for all elements except Sn and Cr. Hence, Sn and Cr were excluded from the statistical analysis.

3.4.2 Biomarkers

3.4.2.1 Metallothionein (MT)

Tracer technique using the radioactive isotope ^{109}Cd was applied in order to detect any MT in the frog larvae. There are two important assumptions behind the use of radioactive tracers: first, one assumes that the radioactive isotope is chemically identical to any stable isotope of the same element (Choppin et al. 2002). Second, it is assumed that the radioactivity does not affect the chemical and physical properties of the radioactive isotope (Choppin et al. 2002). Based on this, the radioactive isotope is expected to behave identically with a stable isotope of the same element when introduced to for instance a biological system. This is the foundation for using radioisotopes to study the uptake, binding, etc. of an element in an organism.

The method applied in this thesis is similar to the method described by Bartsch et al. (1990) and modified by Olsvik et al. (2001). Prior to analysis, the frog embryos and tadpoles were homogenized in 5 mM Tris-HCl homogenisation buffer (pH 7.4) (1:5 w/v) with 15 strokes using a Potter-Elvehjem homogenizer. Subsequently, the homogenate was centrifuged at 10 000 g for 12 minutes at 3 °C. The samples were kept on ice during homogenization and other handling to ensure a temperature of 4 °C or lower at all times. After centrifugation, 100 µL of the supernatant was transferred to a new Eppendorf tube and frozen at -82 °C until analysis.

For analysis of MT, 100 µL acetonitrile was added to 100 µL sample and vortexed. After 3 minutes of incubation at room temperature 1 mL premade buffer A (10mM Tris-HCl, 85 mM NaCl, pH 7.4) and 40 µL tracer solution was added. The sample was vortexed and the tracer was incubated for 5 minutes. The tracer was prepared by adding radioactive ^{109}Cd and Cd-acetate in solution (2 mg/mL) containing stable Cd isotopes to buffer A. After incubation, 100 µL of the complex binder Chelex-100 resin (Bio-Rad, Hercules, CA, USA) was added and the sample was rotated slowly for 15 minutes. The Chelex was washed with washing buffer beforehand (10mM Tris-HCl, 1M NaCl, pH 7.4), and kept in 60 % suspension with buffer A. After rotation, the sample was centrifuged at 12 000 rpm for 5 minutes at 4 °C. Subsequently 0.9 mL of the supernatant was transferred into a 20 mL plastic vial and the gamma radiation emitted from the sample was measured on a sodium iodide (NaI) automatic gamma counter (Wallac, Perkin Elmer, Wizard 3, 1480 automatic gamma counter).

The activity of the sample in the plastic vials reflects the amount of ^{109}Cd bound to MT in the sample, and hence the amount of MT present. Superfluous Cd is assumed to be bound to the Chelex and left in the precipitate after centrifugation. Chelex has a relatively strong binding capacity of metals, but slightly lower than that of MT. Therefore, Chelex binds only free metal species, and does not pull metal atoms away from the MT (Cai & Stillman 1988).

Background samples were prepared and analysed to measure the Cd binding efficiency of Chelex, and blank samples were analysed to measure the amount of Cd added to the samples.

The MT concentration was calculated assuming a binding capacity of 7 Cd atoms per MT molecule, and given in nmol/g wet weight.

3.4.2.2 7-Ethoxyresorufin O-deethylase (CYP1A activity)

Prior to EROD analysis the embryos and tadpoles were processed on ice with refrigerated buffers. Each sample was added 2.5 mL 0.1 M potassium-phosphate buffer (pH 7.8) (approximately 1:2.5 w/v) containing KCL (0.15 M), dithiothreitol (DTT) (1 mM), glycerol (5% v/v) and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) and homogenized with 15 strokes at 1000 rpm using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10 000 g for 30 minutes at 4 °C. The supernatant (S9 fraction) now containing both the cytosolic and the microsomal fraction was transferred to new centrifuge tubes and centrifuged once more at 50 000 g for 120 minutes at 4 °C to obtain the S100 microsomal and cytosolic fraction. The supernatant containing the cytosolic fraction was transferred to 5 mL tubes for mixing, prior to freezing in Nunc 96-deep well plates in three aliquots at -80 °C. The pellet containing the microsomal fraction was resuspended in 0.75 mL 0.1 M potassium-phosphate buffer (pH 7.8) containing KCl (0.15 M), DTT (1 mM), EDTA (1 mM) and glycerol (20% v/v), and homogenized with 10 strokes at 1000 rpm. Finally the microsomal fraction was transferred to Nunc 96-well plates in three aliquots and frozen at -80 °C.

The protein concentration in the microsomal fraction were analysed applying a modified version of Lowry's method (Lowry et al. 1951) using the DC Protein Assay Kit I from Bio-Rad according to the producer's protocol (Bio-Rad, Hercules, CA, USA). The microsomal samples were diluted 1:3 with 0.1 M Tris buffer (pH 8.0). Four different dilutions of Bio-Rad's Bovine gamma globulin standard were prepared to make a linear regression model for protein concentration and absorbance. Subsequently, 10 µL of diluted samples, standards and blanks were pipetted in triplicates into 96-well microtiter plates (Sarstedt AG & Co, Nümbrecht, Germany). Each well was added 25 µL of Bio-Rad's reagent A and 200 µL of Bio-Rad's reagent B. The plates were incubated for 15 minutes and absorbance was read in a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 750 nm using the SoftMax pro software. Protein concentration was calculated based on the regression model derived from the standard curve.

The EROD assay was performed in a spectrophotometry room to ensure that the 7-ethoxyresorufin reagent was shielded from direct light as it is light sensitive. Microsomal samples were diluted in 0.1 M potassium-phosphate buffer (pH 8.0) to obtain protein concentrations between 1 – 2 mg/mL. A resorufin standard curve was prepared by making 8 different dilutions from 0 to 0.64 µM from a 10 µM stock solution. The resorufin standard (Sigma-Aldrich, St. Louis, MO, USA) was calibrated by measuring the absorbance of the 10 µM concentration at 572 nm. Subsequently, 20 mL 7-ethoxyresorufin reagent (Sigma-Aldrich, St. Louis, MO, USA) per plate was prepared by adding 0.75 mL 7-ethoxyresorufin for every 50 mL of buffer. The reagent was calibrated by measuring the absorbance at 450

nm. A NADPH solution was prepared by dissolving NADPH (Sigma-Aldrich, St. Louis, MO, USA) in buffer. The final concentration of NADPH solution in the wells should be 2.4 mM. Thereafter, 275 µL of the resorufin standards was pipetted in duplicates, and 50 µL of sample was pipetted in triplicates, into 96-well microtiter plates. Blanks and a cross plate reference sample made of cod liver were added to each plate for analytical quality control. Finally, 200 µL 7-ethoxyresorufin reagent and 25 µL NADPH solution was added all wells, except those containing resorufin standards. The plates were read immediately (8 readings) in microplate reader (Victor 1420 Multilabel Counter, PerkinElmer, Waltham, MA, USA) at excitation 530 nm and emission 590 nm.

The EROD activity was determined from the slopes of the measured fluorescence (FU), by relating FU to the resorufin standard curve. Finally, the EROD activity was related to the protein concentration of each sample, to give the EROD activity in pmol/min/mg microsomal protein. After calculation all plates were multiplied with a correction factor given by the performance of the reference sample to correct for variation in readings of the reference.

3.4.2.3 Glutathione S-Transferase activity (GST)

The cytosolic fraction obtained during the processing of tissue for EROD analysis was thawed and protein content was analysed as described in section 3.4.2.2 7-Ethoxyresorufin O-deethylase (CYP1A activity), except the samples were diluted 1:10 (Fig. 14). Subsequently, 50 µL of blanks and diluted samples was pipetted in triplicates into 96-well microtiter plates. One of the tadpole cytosol samples was utilized as cross plate reference. A solution consisting of 25 mL 1 mM glutathione solution and 500 µL 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) solution (dissolved in 2 mL DMSO) was prepared, and 200 µL of this was added each well. The plates were read immediately in microplate reader at 340 nm and monitored through 5 minutes.

GST activity was calculated from the slopes of absorbance increase over the 5 minutes of measuring, and related to protein concentration, by using this formula:

$$\frac{\Delta OD_{340nm}\ min^{-1} \times dilution\ factor}{Protein\ (mg/mL) \times path\ length\ (cm) \times \varepsilon} = nmol\ min^{-1}\ mg\ cytosolic\ protein^{-1}$$

After calculation all plates were multiplied with a correction factor given by the performance of the reference sample to correct for variation in readings of the reference.

Figure 14. Analysis of protein concentration in tadpole cytosol prior to the glutathione *S*-Transferase (GST) assay. Photo: Eivind Farmen.

3.4.2.4 Reduced glutathione (GSH)

Reduced glutathione was measured in the cytosolic fraction of the samples by conjugation of monochlorobimane (mBCl) (Life Technologies Ltd, Paisley, UK) to the sample GSH, forming a stable and fluorescent product (Kamencic et al. 2000). All samples were diluted 1:10 to obtain protein concentration between 0.1 and 1 mg/mL and pipetted in four replicates into 96-well microtiter plates (50 µL in each well). Two of the four replicates were added 0.1 pmol GSH (spike) to correct for quenching (reduction of the fluorescence due to high concentrations of protein). A GSH standard curve was prepared by making 8 different dilutions from 0 to 100 µM from a 10 mM stock solution, and pipetted in triplicates (50 µL) into the microtiter plates. Finally, 50 µL of the reaction buffer, consisting of Trisbuffer pH 7.8 with 200 µM mBCl and 1U/mL of equine GST (Sigma-Aldrich, St. Louis, MO, USA) was added all wells. The plates were incubated in darkness for 16 hours and read the morning after in microplate reader at 486 nm emission and 405 nm excitation.

Concentration of reduced GSH activity was determined by relating measured FU to the GSH standard curve. A correction factor for quenching was derived by calculating the difference between the expected spike FU and the spike FU observed in the spiked samples. Finally GSH was related to the cytosolic protein concentration of each sample, to give the GSH activity in nmol/mg protein.

3.5 Statistics and calculations

All statistical analysis was conducted using the software R version 2.15.2 (R Core Team 2012). Elements in the ICP-MS results with more than 15 per cent of the values below LD were excluded from the analysis. This pass for the elements Ag, S, Se and Sn in the water data and Co, Cr, Eu, Lu, Ni, Sb, Sn and Yb in the tadpole tissue data. For the remaining element concentrations below LD, a value equal to half the LD was employed in the statistical analysis, as suggested by United States Environmental Protection Agency (U.S. Environmental Protection Agency 2000). All values below LQ were included in the statistics despite the uncertain accuracy associated with their low values.

3.5.1 Multivariate statistics

The primary ICP-MS data set was large, consisting of several spatial and temporal measurements of 34 elements. Hence, the ordination technique principal component analysis (PCA) was applied to reduce the number of variables, identify any patterns in this data set and reduce the risk of type 1 error associated with performing a large number of unviariate statistical tests. The data were log transformed (log(x+1)) prior to analysis to reduce the importance of extreme values.

Principal component analysis is an unconstrained analysis (also called indirect gradient analysis) with many response variables and no explanatory variables. The analysis detects the ordination axes that correspond to the greatest variability in the data set (Lepš & Šmilauer 2003). The first principal component (henceforth referred to as PCA axis 1 or the PC 1 axis) describes the most of the variation; the second principal component (henceforth referred to as PCA axis 2 or the PC 2 axis) describes the second most, and so on. The number of axes equals the number of variables, and all of them are uncorrelated. Usually, only the first axis, and sometimes the second, is interpreted as they account for the bulk of the variation in the data set.

The results of the PCA analysis are presented in ordination diagrams called biplots. It is the relative positions and directions of the objects that is important for the interpretation, while the absolute values of the coordinates have no meaning (Lepš & Šmilauer 2003). The quantitative environmental variables (elements, in this case) are displayed as arrows pointing in the direction of which the value of the variable increases (Lepš & Šmilauer 2003). The angle between two arrows indicates the degree of correlation between the variables. Arrows pointing in the same direction represents elements that are predicted to exhibit high correlation. An angle of 90 means there is no correlation, while arrows pointing in opposite directions are negatively correlated.

Samples from the different ponds at different times are shown in the diagram as points. Points close to the coordinate system of origin are predicted to have a value close to the mean value.

A point close to the extremity of an arrow is predicted to have an above-mean value of the element that the arrow represents, while a point projecting in the opposite direction of an arrow is predicted to have a below-mean value (Lepš & Šmilauer 2003). Points close to each other have much in common regarding element composition while points distant from each other are dissimilar.

When conducting a PCA, the R software gives a summary where the site scores and species scores are presented. The species scores indicate to what extent an element is correlated with the different axes, e.g. the PC 1 axis. The site scores of the PC 1 axis gives an estimate of the relative concentration level of elements best correlated with the PC 1 axis in each sample. That is, the site scores give an indication of which sampled that have the highest overall element concentrations and which samples that have the lowest. Since the PC 1 axis usually accounts for a large portion of the variation, the PC 1 axis site scores can be regarded as a good representative for the overall element concentration levels. In the current thesis the PC 1 axis site scores were utilized further in inferential statistics to represent the overall levels of element concentrations in each sample.

3.5.2 Univariate statistics

Principal component analysis is only a descriptive statistical method. In order to test any hypotheses it must be followed by inferential statistical tests, such as correlation analyses and analyses of variance. One-way analysis of variance was conducted on the PC 1 axis site scores for tadpole tissue element concentrations and biomarker data to test for any significant temporal differences in concentrations. For simplicity, the phrase ‘overall tissue element concentrations’ refer to the PC 1 axis site scores of tissue concentrations in the description of the statistics below.

Kruskal-Wallis test and pairwise Wilcoxon post hoc test were carried out on all three data sets for overall tissue element concentrations, the MT data set for Vassum, and the GST and GSH data set for Prinsdal, as these were not normally distributed. The GSH data set for Prinsdal was log transformed to meet the Kruskal-Wallis assumption of equal variance.

ANOVA, followed by the post hoc test Tukey’s honestly significant difference (Tukey’s HSD), were performed on the MT data sets for Prinsdal and Skullerud, the EROD data set for Vassum, and the GST and GSH data sets for Skullerud and Vassum. The MT data set for Skullerud, the EROD data set for Vassum, and the GST and GSH data sets for Vassum and Skullerud, were log transformed to meet the ANOVA assumptions of normal distribution and equal variance. The GST data sets for Skullerud and Vassum were square root transformed.

There was a problem with non-normal distribution and heteroscedasticity (unequal variance) in the EROD data for Prinsdal and Skullerud. Transformations helped to some extent ((log (x+2)) and (square root (x+2)) respectively), but the heteroscedasticity was still an issue.

Hence Welch's one-way test was performed on these data sets after transformation, as this test does not assume equal variance (Dalgaard 2008).

Pearson correlation test was performed to check whether there was correlation between total or dissolved element concentrations in water (PC 1 site scores), and overall tissue element concentrations. The water data sets were log transformed (log(x+2)) to meet the assumptions of normal distribution and equal variance.

Correlation analyses were also performed to test for any correlation between overall tissue element concentrations, and levels of the four biomarkers MT, EROD, GST and GSH. As the MT datasets were not normally distributed, the non-parametric Spearman method was conducted. Correlation between MT and Cu, Zn, Cd and Pb was also tested separately. Pearson correlation test was performed on the EROD data, the GSH data and the GST data. The EROD and GSH data were log transformed (the EROD data log(x+2)) prior to the correlation test.

In all statistical tests the significance level was set to $p = 0.05$.

Negative values of EROD activity was calculated for many of the samples. Negative activities are impossible so these results only means the value is below limit of detection. Hence, in the box plots these numbers were replaced with the half of the lowest measured positive value. In the statistical tests the original data with negative values were used to avoid extremely non-normal distribution and heteroscedasticity.

3.5.3 Bioconcentration factors (BCF)

A bioconcentration factor (BCF) for each element was calculated by this formula:

$$\frac{\text{Mean element concentration in tadpoles (µg/g)}}{\text{Dissolved element concentration in water (µg/L)}} = \text{BCF}$$

The BCF is a measure of the concentration of an element in an organism relative to the concentration in water, assuming uptake only from water. The mean of the biological replicates was used to avoid pseudoreplication since there was only one water sample from each sampling (Lepš & Šmilauer 2003). Finally, a PCA was performed on the BCFs to get an overview of potential patterns.

4 Results and discussion

In the present thesis a suite of different elements were determined in water and frog embryos and tadpoles. In addition, the levels of the four biomarkers MT, EROD, GST and GSH were measured. The total data material is fairly large and complex, thus the discussion will focus on the most important trends and patterns, and the key factors influencing pond water quality, biological uptake of trace elements and levels of the biomarkers. Note that the sampling started one week earlier at Prinsdal than at the sedimentation ponds.

4.1 Water quality characterization

The values of different water quality parameters and element concentrations at Vassum, Skullerud and Prinsdal in different samples in May and June 2012 are presented in Table 2, 3 and 4. Some of the trace elements are included in the five-parted classification system for environmental quality developed by the Climate and Pollution Agency (Climate and Pollution Agency 2012). In table 2, 3 and 4 it is indicated by colour shading which class the water belongs to, according to the total concentration of these elements (assuming soft water when classifying according to Cd). Blue colour denotes the natural state (class I), green colour denotes good water quality (class II), yellow indicates moderate water quality (class III), orange indicates poor water quality (class IV) and red colour means very poor water quality (class V) (see appendix 5 for the concentrations that define the class limits). No colour shading means there is not developed any classification criteria for the trace elements in Norway.

For some elements, dissolved concentrations were higher than total concentrations, which was contradictory to the expected relationship. In the cases where the difference was small, the elevated dissolved concentrations may be attributed to sources of error in the sampling method or to measuring uncertainty. However, for Sb at all ponds and Zn at Prinsdal, the difference was large and seemed systematic. The other elements analysed in the same particular samples did not exhibit the same pattern, so there has probably not been any confusion of samples. It is difficult to explain this phenomenon. It might be that the syringe filters contaminated the water with Sb and Zn, but this was not tested. Consequently, the dissolved concentrations of Sb and Zn must be interpreted with care.

Table 2. General water quality parameters measured by the outlet of Vassum sedimentation pond at the different samplings in May and June 2012 (week 19 to week 24). Dissolved element concentrations are given in brackets. The colour shading indicates which class the water belongs to according to the classification system for environmental quality developed by the Climate and Pollution Agency. Blue = class I, green = class II, yellow = class III, orange = class IV, red = class V (Climate and Pollution Agency 2012).

Variable	Unit	Week 19	Week 20	Week 21	Week 22	Week 23	Week 24	Mean ± SEM[A)]
TOC	mg/L	2.5	4.1	4.5	5.4	6.1	6.3	4.8 ± 0.58
DOC	mg/L	2.3	3.8	4.0	5.2	5.8	6.2	4.6 ± 0.59
Cl	mg/L	280	483	586	852	866	937	667 ± 106
SO^4	mg/L	14	31	30	29	30	27	27 ± 2.6
pH		7.5	7.8	7.5	7.4	7.6	8.1	7.6 [B)]
Temp.[C)]	°C	14	11	20	20	18	19	17 ± 1.5
Cond.[D)]	µs/cm	1010	1747	2050	2980	3070	3250	2351 ± 364
Dissolved oxygen	mg/L	8.1	7.8	6.1	4.9	5.2	5.3	6.2 ± 0.56
Sum 16 PAHs	µg/L	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Na	mg/L	180 (180)	300 (290)	340 (340)	520 (520)	560 (540)	580 (600)	413 ± 67 (412 ± 68)
Mg	mg/L	3.4 (2.4)	4.7 (4.4)	4.9 (4.9)	6.1 (6.1)	6.7 (6.6)	7.0 (6.7)	5.5 ± 0.56 (5.2 ± 0.67)
Al	µg/L	4000 (54)	510 (49)	240 (27)	400 (40)	230 (53)	1100 (33)	1080 ± 598 (43 ± 4.6)
Si	µg/L	11000 (430)	1400 (<370)	810 (<370)	1100 (<370)	560 (<370)	3100 (1000)	2995 ± 1642 (382 ± 132)
P	mg/L	0.22 (0.0084)	0.045 (0.012)	0.033 (0.013)	0.062 (0.030)	0.054 (0.037)	0.35 (0.038)	0.13 ± 0.053 (0.023 ± 0.0055)
S	mg/L	5.5 (5.2)	13 (12)	11 (11)	11 (11)	10 (10)	9.9 (9.4)	10 ± 1.0 (10 ± 1.0)
K	mg/L	4.0 (2.7)	5.0 (4.8)	5.8 (5.8)	7.8 (7.7)	6.9 (6.9)	7.7 (7.3)	6.2 ± 0.6 (5.9 ± 0.77)
Ca	mg/L	17 (14)	30 (30)	31 (32)	37 (35)	37 (37)	47 (37)	33 ± 4.1 (31 ± 3.6)
Sc	µg/L	0.95 (<0.021)	0.096 (0.025)	0.065 (0.021)	0.088 (0.029)	0.061 (0.033)	0.24 (0.041)	0.25 ± 0.14 (0.028 ± 0.0034)
Cr	µg/L	8.9 (0.50)	1.4 (0.42)	0.80 (<0.25)	0.85 (0.27)	0.53 (0.41)	1.8 (0.32)	2.4 ± 1.3 (0.36 ± 0.040)
Mn	µg/L	71 (11)	24 (5.4)	24 (14)	100 (87)	97 (89)	140 (97)	76 ± 19 (51 ± 18)
Fe	µg/L	3500 (84)	590 (86)	370 (120)	800 (420)	860 (580)	1600 (620)	1287 ± 474 (318 ± 103)
Co	µg/L	1.8 (0.17)	0.74 (0.38)	0.38 (0.24)	0.50 (0.32)	0.47 (0.36)	0.91 (0.38)	0.80 ± 0.22 (0.31 ± 0.035)
Ni	µg/L	5.8 (0.96)	2.6 (2.2)	2.5 (2.2)	2.0 (1.7)	1.7 (1.6)	2.8 (1.6)	2.9 ± 0.60 (1.7 ± 0.19)
Cu	µg/L	25 (5.1)	11 (9.5)	7.0 (6.1)	4.2 (2.3)	3.2 (2.1)	7.0 (1.8)	10 ± 3.3 (4.5 ± 1.2)
Zn	µg/L	94 (17)	69 (58)	39 (32)	20 (10)	15 (8.9)	45 (8.7)	47 ± 12 (22 ± 8.0)
As	µg/L	0.41 (0.17)	0.31 (0.25)	0.33 (0.29)	0.77 (0.71)	0.77 (0.76)	1.0 (0.85)	0.60 ± 0.12 (0.51 ± 0.12)
Se	µg/L	<0.27 (<0.27)	<0.27 (<0.27)	<0.27 (<0.27)	<0.27 (<0.27)	<0.27 (<0.27)	<0.27 (<0.27)	<0.27 (<0.27)
Sr	µg/L	85 (72)	160 (150)	160 (160)	220 (220)	220 (230)	250 (240)	183 ± 24 (179 ± 26)
Mo	µg/L	3.0 (2.1)	3.5 (3.3)	3.2 (3.2)	3.3 (3.3)	3.8 (3.7)	3.9 (4.0)	3.5 ± 0.14 (3.3 ± 0.26)
Ag	µg/L	0.033 (0.029)	0.032 (<0.029)	<LD (<LD)	<0.029 (<LD)	<0.029 (<LD)	0.074 (<LD)	<0.029 (<LD)
Cd	µg/L	0.047 (<0.015)	<0.015 (<0.015)	<0.015 (<0.015)	<0.015 (<0.015)	<0.015 (<LD)	0.029 (<LD)	0.020 ± 0.0064 (<0.015)
Sn	µg/L	1.7 (<LD)	<0.86 (<0.86)	<0.86 (<0.86)	<0.86 (<0.86)	<0.86 (<0.86)	0.96 (<0.86)	<0.86 (<0.86)
Sb	µg/L	4.8 (2.4)	1.7 (2.9)	1.4 (1.8)	2.7 (3.1)	2.9 (3.1)	2.8 (3.1)	2.7 ± 0.49 (2.7 ± 0.22)
Ba	µg/L	50 (<26)	40 (35)	40 (39)	68 (65)	76 (75)	87 (78)	60 ± 8.1 (52 ± 10)

(continued)

Table 2 continued.

Variable	Unit	Week 19	Week 20	Week 21	Week 22	Week 23	Week 24	Mean ± SEM[A)]
La	µg/L	5.7 (0.31)	0.58 (0.081)	0.33 (0.054)	0.61 (0.14)	0.42 (0.19)	1.4 (0.21)	1.5 ± 0.85 (0.16 ± 0.038)
Ce	µg/L	13 (0.68)	1.2 (0.15)	0.66 (0.098)	1.2 (0.28)	0.87 (0.38)	2.9 (0.40)	3.3 ± 2.0 (0.33 ± 0.085)
Eu	µg/L	0.16 (0.0085)	0.018 (<0.0057)	0.012 (<0.0057)	0.020 (0.0086)	0.017 (0.011)	0.044 (0.011)	0.045 ± 0.023 (0.0082 ± 0.0011)
Yb	µg/L	0.23 (<0.0084)	0.029 (0.0086)	0.017 (<0.0084)	0.023 (0.0087)	0.022 (0.0087)	0.060 (0.011)	0.064 ± 0.034 (0.0080 ± 0.00088)
Gd	µg/L	0.83 (0.035)	0.087 (0.014)	0.045 (0.010)	0.091 (0.027)	0.074 (0.035)	0.22 (0.038)	0.22 ± 0.12 (0.027 ± 0.0048)
Lu	µg/L	0.029 (<0.0022)	0.0043 (<0.0022)	<0.0022 (<0.0022)	0.0038 (<0.0022)	0.0029 (<0.0022)	0.0091 (<0.0022)	0.0085 ± 0.0042 (<0.0022)
Pb	µg/L	2.0 (0.11)	0.54 (0.15)	0.3 (0.079)	0.54 (0.23)	0.48 (0.33)	1.2 (0.31)	0.84 ± 0.26 (0.20 ± 0.043)
Th	µg/L	0.56 (0.030)	0.089 (<0.020)	0.049 (<0.020)	0.069 (<0.020)	0.051 (0.020)	0.22 (<0.020)	0.17 ± 0.082 (<0.020)
U	µg/L	0.94 (0.73)	2.1 (2.0)	1.6 (1.4)	1.4 (1.3)	1.6 (1.6)	1.8 (1.7)	1.6 ± 0.16 (1.5 ± 0.18)

[A)] SEM = Standard error of the mean.
[B)] For pH, the median value is given instead of the mean value due to logarithmic scale.
[C)] Temp. = temperature.
[D)] Cond. = conductivity.

Table 3. General water quality parameters measured by the outlet of Skullerud sedimentation pond at the different samplings in May and June 2012 (week 19 to week 24). Dissolved element concentrations are given in brackets. The colour shading indicates which class the water belongs to according to the classification system for environmental quality developed by the Climate and Pollution Agency. Blue = class I, green = class II, yellow = class III, orange = class IV, red = class V (Climate and Pollution Agency 2012).

Variable	Unit	Week 19	Week 20	Week 21	Week 22	Week 23	Week 24	Mean ± SEM[A)]
TOC	mg/L	4.2	6.0	5.6	5.5	5.9	6.0	5.5 ± 0.28
DOC	mg/L	4.0	5.8	5.2	5.5	5.7	5.7	5.3 ± 0.29
Cl	mg/L	111	9.2	26	54	73	97	62 ± 16
SO^4	mg/L	22	5	11	24	22	20	17 ± 3.1
pH		8.4	7.8	7.3	7.6	7.4	7.8	7.7 [B)]
Temp.[C)]	°C	12	13	18	15	11	15	14 ± 1.0
Cond.[D)]	µs/cm	544	81	176	354	411	493	343 ± 74
Dissolved oxygen	mg/L	9.3	9.6	7.7	10	6.1	6.1	8.2 ± 0.74
Sum 16 PAHs	µg/L	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Na	mg/L	68 (65)	6.5 (6.3)	17 (17)	34 (35)	44 (44)	60 (60)	38 ± 10 (38 ± 9.5)
Mg	mg/L	6.4 (6.2)	1.4 (1.3)	2.4 (2.2)	5.0 (5.2)	5.1 (5.2)	5.4 (5.3)	4.3 ± 0.79 (4.2 ± 0.81)
Al	µg/L	1900 (120)	450 (120)	210 (66)	620 (74)	44 (20)	60 (17)	547 ± 286 (70 ± 19)
Si	µg/L	5600 (1100)	1800 (960)	720 (<370)	1600 (<370)	<370 (<LD)	<370 (<370)	1699 ± 826 (491 ± 175)
P	mg/L	0.12 (0.013)	0.037 (0.011)	0.10 (0.048)	0.049 (0.014)	0.033 (0.015)	0.034 (0.016)	0.062 ± 0.016 (0.020 ± 0.0057)
S	mg/L	7.6 (7.5)	<3.3 (<3.3)	3.6 (3.4)	8.2 (7.8)	7.3 (7.5)	7.0 (6.8)	5.9 ± 1.0 (6.0 ± 1.0)
K	mg/L	3.4 (2.8)	0.83 (0.71)	1.2 (1.1)	2.4 (2.2)	2.1 (2.2)	2.2 (2.0)	2.0 ± 0.37 (1.8 ± 0.32)
Ca	mg/L	28 (27)	6.9 (6.8)	12 (12)	26 (25)	25 (25)	25 (25)	20 ± 3.6 (20 ± 3.5)
Sc	µg/L	0.37 (0.034)	0.088 (0.046)	0.053 (0.028)	0.12 (0.027)	0.020 (0.015)	0.03 (0.020)	0.11 ± 0.054 (0.028 ± 0.0044)
Cr	µg/L	7.2 (3.5)	1.7 (0.38)	1.4 (1.0)	1.5 (0.73)	0.48 (0.42)	0.95 (0.76)	2.2 ± 1.0 (1.1 ± 0.48)
Mn	µg/L	66 (7.0)	15 (4.2)	20 (12)	20 (17)	19 (18)	17 (16)	26 ± 8.0 (12 ± 2.3)
Fe	µg/L	1600 (110)	390 (110)	220 (77)	510 (100)	120 (77)	130 (84)	495 ± 230 (93 ± 6.4)
Co	µg/L	0.77 (0.12)	0.19 (0.054)	0.12 (0.087)	0.24 (0.10)	0.078 (0.070)	0.088 (0.080)	0.25 ± 0.11 (0.085 ± 0.0094)
Ni	µg/L	3.0 (0.92)	0.89 (3.6)	0.88 (0.67)	1.3 (0.88)	0.93 (0.83)	0.97 (0.81)	1.3 ± 0.34 (1.3 ± 0.46)
Cu	µg/L	13 (6.9)	3.1 (7.0)	4.5 (3.7)	5.5 (3.8)	3.8 (3.6)	4.9 (4.3)	5.8 ± 1.5 (4.9 ± 0.66)
Zn	µg/L	53 (7.8)	9.7 (3.8)	10 (8.9)	12 (7.5)	4.9 (4.5)	6.0 (6.7)	16 ± 7.5 (6.5 ± 0.81)
As	µg/L	0.44 (0.23)	7.4 (0.22)	0.27 (0.24)	0.36 (0.37)	0.31 (0.32)	0.34 (0.33)	1.5 ± 1.2 (0.29 ± 0.026)
Se	µg/L	<0.27 (<0.27)	<0.27 (<0.27)	<0.27 (<0.27)	<0.27 (<0.27)	<LD (<0.27)	<0.27 (<0.27)	<0.27 (<0.27)
Sr	µg/L	120 (110)	24 (24)	44 (42)	100 (110)	100 (110)	110 (110)	83 ± 16 (84 ± 16)
Mo	µg/L	2.7 (2.5)	0.46 (0.38)	1.1 (1.0)	2.5 (3.0)	2.2 (2.2)	2.1 (2.0)	1.8 ± 0.36 (1.8 ± 0.40)
Ag	µg/L	<0.029 (<LD)	<0.029 (<LD)	<0.029 (<LD)	<0.029 (<LD)	<LD (<LD)	<LD (<LD)	<0.029 (<LD)
Cd	µg/L	0.034 (<0.015)	<0.015 (<0.015)	0.019 (<0.015)	0.022 (<0.015)	<0.015 (<0.015)	<0.015 (<0.015)	0.018 ± 0.0039 (<0.015)
Sn	µg/L	0.97 (<0.86)	<0.86 (<0.86)	<0.86 (<0.86)	<0.86 (<LD)	<0.86 (<LD)	<LD (<LD)	<0.86 (<LD)

(continued)

Table 3 continued.

Variable	Unit	Week 19	Week 20	Week 21	Week 22	Week 23	Week 24	Mean ± SEM[A)]
Sb	µg/L	1.4 (2.0)	0.32 (0.92)	0.88 (2.4)	0.68 (1.7)	0.66 (1.3)	0.77 (0.76)	0.79 ± 0.15 (1.5± 0.26)
Ba	µg/L	38 (<26)	<26 (<LD)	<26 (<26)	<26 (<26)	<26 (<26)	<26 (<26)	<26 (<26)
La	µg/L	1.6 (0.18)	0.61 (0.29)	0.31 (0.16)	0.78 (0.16)	0.12 (0.086)	0.11 (0.057)	0.59 ± 0.230 (0.16 ± 0.033)
Ce	µg/L	2.9 (0.26)	1.1 (0.38)	0.46 (0.20)	1.4 (0.22)	0.16 (0.11)	0.16 (0.068)	1.03 ± 0.43 (0.21 ± 0.045)
Eu	µg/L	0.045 (0.0061)	0.018 (0.0091)	0.0099 (0.0064)	0.02 (<0.0057)	<0.0057 (<0.0057)	<0.0057 (<0.0057)	0.017 ± 0.0062 (<0.006)
Yb	µg/L	0.077 (0.013)	0.034 (0.019)	0.022 (0.015)	0.035 (0.012)	0.010 (0.0085)	0.010 (<0.0084)	0.031 ± 0.010 (0.013 ± 0.0017)
Gd	µg/L	0.21 (0.027)	0.093 (0.047)	0.048 (0.031)	0.10 (0.026)	0.021 (0.015)	0.019 (0.011)	0.082 ± 0.029 (0.026 ± 0.0052)
Lu	µg/L	0.011 (<0.0022)	0.0049 (0.0029)	0.0029 (<0.0022)	0.0049 (<0.0022)	<0.0022 (<0.0022)	<0.0022 (<0.0022)	0.0045 ± 0.0014 (<0.002)
Pb	µg/L	1.5 (0.17)	0.40 (0.083)	0.23 (0.085)	0.43 (0.11)	0.12 (0.08)	0.10 (<0.073)	0.46 ± 0.21 (0.10 ± 0.017)
Th	µg/L	0.28 (0.033)	0.070 (0.037)	0.050 (0.026)	0.097 (0.024)	<0.020 (<0.020)	<0.020 (<0.020)	0.088 ± 0.040 (0.023 ± 0.0048)
U	µg/L	1.8 (1.6)	0.25 (0.23)	0.45 (0.41)	1.6 (1.7)	0.87 (0.83)	0.74 (0.70)	0.95 ± 0.25 (0.91 ± 0.25)

[A)] SEM = Standard error of the mean.
[B)] For pH, the median value is given instead of the mean value due to logarithmic scale.
[C)] Temp. = temperature.
[D)] Cond. = conductivity.

Table 4. General water quality parameters measured at Prinsdal at the different samplings in May and June 2012 (week 18 to week 24). Dissolved element concentrations are given in brackets. The colour shading indicates which class the water belongs to according to the classification system for environmental quality developed by the Climate and Pollution Agency. Blue = class I, green = class II, yellow = class III, orange = class IV, red = class V (Climate and Pollution Agency 2012).

Variable	Unit	Week 18	Week 19	Week 20	Week 21	Week 22	Week 23	Week 24	Mean ± SEM[A)]
TOC	mg/L	4.7	5.4	5.1	6.4	12	10	33	11 ± 3.9
DOC	mg/L	4.6	5.4	5.1	6.5	9.4	9.4	14	7.8 ± 1.3
Cl	mg/L	2.9	2.4	2.2	2.0	2.2	2.2	0.8	2.1 ± 0.20
SO^4	mg/L	1.7	1.8	1.9	1.3	0.6	0.7	1.0	1.3 ± 0.20
pH		6.1	6	6.2	6.1	6.3	6.2	5.7	6.1 [B)]
Temp.[C)]	°C	5.9	13	12	18	12	11	15	12 ± 1.4
Cond.[D)]	µs/cm	26	25	24	26	36	30	26	28 ± 1.6
Dissolved oxygen	mg/L	6.9	7.0	7.5	3.1	4.0	3.0	1.5	4.7 ± 0.90
Sum 16 PAHs	µg/L	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Na	mg/L	1.7 (1.7)	1.7 (1.7)	1.7 (1.7)	1.7 (1.9)	2.1 (2.1)	2.4 (2.3)	2.5 (2.1)	2.0 ± 0.092 (1.9 ± 0.092)
Mg	mg/L	0.50 (0.53)	0.43 (0.43)	0.46 (0.45)	0.50 (0.51)	0.97 (0.91)	0.84 (0.81)	1.2(0.73)	0.70 ± 0.11 (0.62 ± 0.072)
Al	µg/L	170 (120)	170 (130)	290 (110)	210 (130)	970 (230)	840 (230)	4300 (890)	993 ± 565 (263 ± 106)
Si	µg/L	2600 (3000)	2400 (2200)	2000 (1800)	1600 (1600)	4600 (3200)	3900 (2900)	8400 (2200)	3643 ± 888 (2414 ± 235)
P	mg/L	0.034 (0.010)	0.012 (0.0066)	0.048 (0.010)	0.025 (0.014)	0.17 (0.033)	0.095 (0.033)	0.35 (0.18)	0.10 ± 0.046 (0.041 ± 0.024)
S	mg/L	<LD (<3.3)	<LD (<LD)	<LD (<LD)	<LD (<LD)	<LD (<LD)	<LD (<LD)	<3.3 (<LD)	<LD (<LD)
K	mg/L	0.37 (0.33)	0.32 (0.31)	0.36 (0.37)	0.29 (0.26)	0.76 (0.50)	0.58 (0.39)	1.4 (0.30)	0.58 ± 0.15 (0.35 ± 0.030)
Ca	mg/L	2.3 (2.2)	2.2 (2.2)	2.3 (2.3)	2.7 (2.6)	3.8 (4.2)	2.5 (2.8)	3.5 (3.0)	2.8 ± 0.24 (2.8 ± 0.27)
Sc	µg/L	0.091 (0.080)	0.091 (0.072)	0.11 (0.053)	0.10 (0.083)	0.30 (0.16)	0.28 (0.17)	1.1 (0.41)	0.30 ± 0.14 (0.15 ± 0.047)
Cr	µg/L	0.48 (0.41)	0.41 (0.35)	0.69 (0.39)	0.63 (0.51)	1.8 (0.76)	1.4 (0.82)	5.7 (2.1)	1.6 ± 0.71 (0.76 ± 0.23)
Mn	µg/L	25 (35)	22 (17)	13 (69)	41 (36)	460 (460)	230 (230)	400 (340)	170 ± 73 (170 ± 67)
Fe	µg/L	490 (360)	330 (230)	540 (690)	750 (510)	11000 (4700)	6200 (3200)	41000 (29000)	8616 ± 5610 (5527 ± 3965)
Co	µg/L	0.51 (0.59)	0.43 (0.35)	0.30 (1.0)	0.85 (0.83)	6.7 (6.7)	2.3 (2.5)	6.3 (5.0)	2.5 ± 1.1 (2.4 ± 0.94)
Ni	µg/L	1.9 (2.0)	2.0 (1.9)	2.3 (1.9)	3.0 (2.7)	6.0 (5.0)	4.1 (3.9)	10 (7.4)	4.2 ± 1.1 (3.5 ± 0.78)
Cu	µg/L	8.5 (6.2)	16 (13)	14 (9.7)	15 (13)	9.9 (8)	4.3 (4.1)	12 (6.6)	11 ± 1.6 (8.7 ± 1.3)
Zn	µg/L	10 (8.5)	10 (13)	13 (14)	13 (33)	14 (29)	5.6 (39)	11 (16)	11 ± 1.1 (21 ± 1.3)
As	µg/L	0.21 (0.18)	0.22 (0.21)	0.25 (0.27)	0.35 (0.33)	1.1 (0.73)	0.75 (0.60)	3.3 (2.7)	0.88 ± 0.42 (0.72 ± 0.34)
Se	µg/L	<0.27 (<0.27)	<LD (<0.27)	<0.27 (<0.27)	<0.27 (<0.27)	<0.27 (<0.27)	<0.27 (<0.27)	0.49 (0.33)	<0.27 (<0.27)
Sr	µg/L	8.7 (9.1)	8.4 (8.4)	8.7 (8.7)	9.9 (9.8)	18 (19)	12 (13)	21 (16)	12 ± 1.9 (12 ± 1.6)
Mo	µg/L	0.27 (<0.16)	<0.16 (<0.16)	<0.16 (<LD)	<0.16 (<0.16)	<0.16 (<0.16)	<0.16 (<0.16)	0.59 (0.39)	0.19 ± 0.072 (<0.16)
Ag	µg/L	<0.029 (<0.029)	<0.029 (<0.029)	<0.029 (<0.029)	<0.029 (<0.029)	0.064 (0.033)	0.045 (<0.029)	0.12 (0.066)	0.044 ± 0.014 (<0.029)

(*continued*)

Table 4 continued.

Variable	Unit	Week 18	Week 19	Week 20	Week 21	Week 22	Week 23	Week 24	Mean ± SEM[A)]
Cd	µg/L	0.020 (<0.015)	0.023 (0.020)	0.029 (0.020)	0.025 (0.025)	0.038 (0.018)	0.032 (<0.015)	0.065 (0.025)	0.033± 0.006 (0.020+/ 0.0016)
Sn	µg/L	<0.86 (<0.86)	<0.86 (<LD)	<LD (1.5)	<LD (<LD)	<LD (<LD)	<LD (<LD)	<LD (<LD)	<LD (<0.86)
Sb	µg/L	1.7 (1.8)	3.3 (3.2)	2.3 (3.5)	2.1 (3.4)	0.7 (5.4)	0.34 (6.7)	1.0 (2.6)	1.6 ± 0.39 (3.8 ± 0.64)
Ba	µg/L	<26 (<26)	<26 (<26)	<26 (<26)	<26 (<26)	<26(<26)	<26 (<26)	51 (19)	<26 (<26)
La	µg/L	0.49 (0.43)	0.51 (0.49)	0.61 (0.43)	0.63 (0.59)	2.6 (1.3)	1.9 (1.4)	13 (7.7)	2.8 ± 1.7 (1.8 ± 1.0)
Ce	µg/L	1.1 (1.0)	1.1 (1.1)	1.3 (1.0)	1.4 (1.3)	5.5 (2.9)	4.0 (3.0)	23 (16)	5.3 ± 3.0 (3.8 ± 2.1)
Eu	µg/L	0.022 (0.019)	0.021 (0.020)	0.024 (0.017)	0.027 (0.026)	0.084 (0.049)	0.064 (0.052)	0.32 (0.21)	0.080 ± 0.041 (0.056 ± 0.026)
Yb	µg/L	0.062 (0.060)	0.058 (0.055)	0.069 (0.047)	0.072 (0.072)	0.18 (2.0)	0.13 (0.11)	0.47 (0.32)	0.15 ± 0.056 (0.38 ± 0.27)
Gd	µg/L	0.11 (0.10)	0.10 (0.11)	0.13 (0.088)	0.13 (0.13)	0.44 (0.26)	0.32 (0.24)	1.5 (1.1)	0.39 ± 0.191 (0.29 ± 0.14)
Lu	µg/L	0.0076 (0.0083)	0.0078 (0.0079)	0.0091 (0.0077)	0.0094 (0.010)	0.026 (0.018)	0.016 (0.014)	0.06 (0.041)	0.019 ± 0.0072 (0.015 ± 0.0045)
Pb	µg/L	1.4 (0.72)	1.7 (1.2)	2.2 (1.4)	2.6 (1.5)	7.1 (3.2)	2.8 (1.4)	16 (9.2)	4.8 ± 2.0 (2.7 ± 1.1)
Th	µg/L	0.074 (0.075)	0.078 (0.064)	0.10 (0.046)	0.12 (0.092)	0.38 (0.23)	0.34 (0.20)	1.4 (0.83)	0.36 ± 0.18 (0.22 ± 0.11)
U	µg/L	0.048 (0.043)	0.056 (0.052)	0.059 (0.041)	0.059 (0.056)	0.13 (0.078)	0.17 (0.11)	0.55 (0.34)	0.15 ± 0.068 (0.10 ± 0.041)

[A)] SEM = Standard error of the mean.
[B)] For pH, the median value is given instead of the mean value due to logarithmic scale.
[C)] Temp. = temperature.
[D)] Cond. = conductivity.

The air temperatures increased over the sampling period, which was expectable as the field work took place in springtime (Fig. 15 A). There were several days with rain during the whole period, but the bulk of the precipitation fell during the first half of the sampling period (Fig. 15 B).

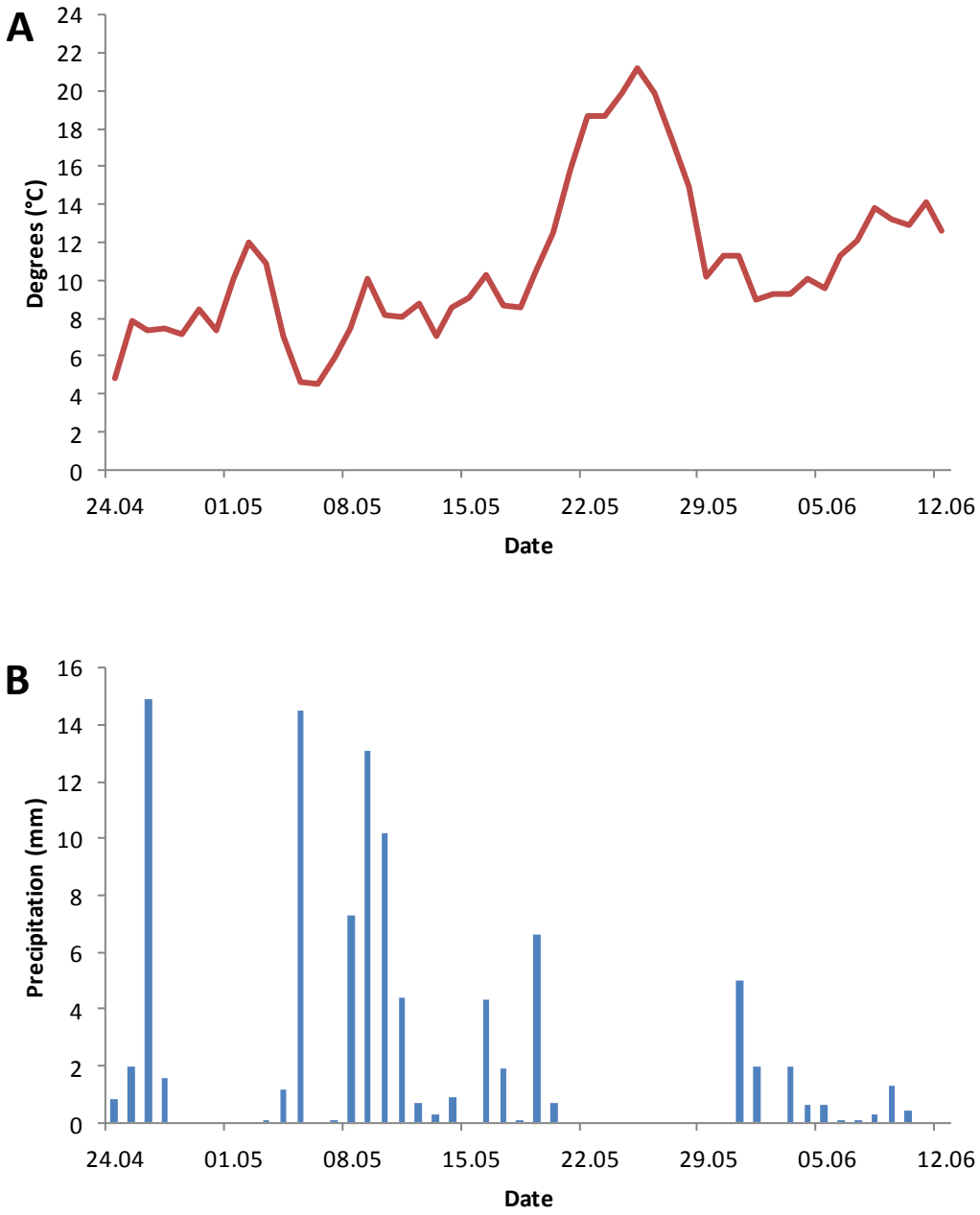

Figure 15. The mean 24 hours temperatures (A) and the precipitation (B) measured at the field station for agroclimatic studies at Sørås (municipality of Ås, County of Akershus) during the sampling period (1 May – 12 June 2012), in addition to data for the 7 days before the field work started. The data are assumed to be fairly representative for the weather conditions for all three ponds, although the field station is situated approximately 6 km from Vassum sedimentation pond, 19 km from the pond at Prinsdal and 23 km from Skullerud sedimentation pond. Modified after Hansen and Grimenes (2013).

The range of the measured DOC concentrations were 2.3 to 6.2 mg/L in Vassum, 4.0 to 5.7 mg/L in Skullerud and 4.6 to 14 mg/L in Prinsdal. The mean measured concentrations of both TOC as well as DOC were higher in Prinsdal than the two sedimentation ponds. This stands to reason, as the pond is shallow and situated in the edge zone of a forest where it receives a lot of organic material from plants. This may also explain why the median measured pH was

more than one unit lower at Prinsdal than in the sedimentation ponds. Decomposition of plant material has an acidifying effect due to generation of organic and inorganic acids (Brady et al. 2010).

The conductivity was considerably higher in the sedimentation ponds than in Prinsdal, the mean being 12 times higher in Skullerud and 85 times higher in Vassum. The high conductivity was most likely due to road salt, which was also indicated by their relatively high Cl and Na concentrations. The Cl concentrations ranged from 280 – 937 mg/L at Vassum and 9.2 – 111 mg/L at Skullerud. This is in good agreement with other measurements of Cl concentrations in highway runoff in Norway (Amundsen & Roseth 2004; Damsgård 2011; Åstebøl et al. 2002; Åstebøl 2005). There was a consistent temporal increase in Cl and Na concentrations in Vassum sedimentation pond, and a similar trend in Skullerud, with the exception of the first sampling. The increasing concentrations may be explained by evaporation from the ponds, as increasing air temperatures during the sampling period coincided with relatively little precipitation in the second half of the sampling period. In contrast to particle bound contaminants, NaCl is highly water soluble and mobile and will not readily sediment (this is particularly true for the chloride ion) (Amundsen et al. 2008). Hence, evaporation may lead to increased NaCl concentrations in the water.

The highest mean total and dissolved concentrations of the macronutrients Na, Mg, K, Ca, and the trace elements Zn, Sr, Mo, Ba and U, were identified in Vassum. The highest mean total concentrations of Al, Cr and Sb were also measured in water from this pond. Considerably lower total and dissolved concentrations were identified for most elements in Skullerud, with the exception that the highest mean total concentrations of As, and the highest dissolved concentrations of Sc and Cr, were identified here. The difference between the two ponds is in agreement with earlier studies (Damsgård 2011; Meland et al. 2010a; Meland et al. 2010c) and is most likely due to Vassum receiving tunnel wash water in addition to runoff from open road area, while Skullerud only receives runoff from open road. Tunnel wash water often contains higher concentrations of pollution than storm water runoff does, due to the long term accumulation of contaminants on the road surface between washing (Meland 2010). At one or more times the total water concentrations of Cr, Cu and Zn were high enough for the water quality to be classified as ‘poor’ at Skullerud according to the Climate and Pollution Agency’s classification system. At one or more times this was also true for the concentrations of Cr and Cu at Vassum, while the Zn concentrations justified classifying the water quality as ‘very poor’ at two occasions.

In Vassum, several trace elements such as Al, Cr, Fe, Co, Zn, Sb, La and Ce, were measured at higher concentrations in week 19 than later in the period. This may be explained by snowmelt episodes before the field work started or rainfall before or in the beginning of the sampling period, carrying loads of pollution accumulated on the road as demonstrated by Sansalone and Buchberger (1996). Sedimentation of particle bound trace elements during the period may also be an explanation, indicating that the pond functions as intended.

The mean total concentrations of trace elements of environmental concern such as Cr, Ni, Cu, Zn, Cd and Pb were slightly lower in the two sedimentation ponds than earlier measurements

of their outlet water (Damsgård 2011; Åstebøl 2005). It is difficult to compare element concentrations in highway runoff across sites, since several factors such as meteorological conditions, drainage area and AADT influence their concentrations. Nevertheless, to give a pointer of the relative pollution levels in Vassum and Skullerud it is worth mentioning that the mean total concentrations of Cu, Zn and Pb in both ponds were considerably lower than measurements of highway runoff in Canada from two highways with comparable AADT (Preciado & Li 2006), and also lower than measurements from a sedimentation pond in Sweden by a highway with lower AADT (Färm 2002).

Surprisingly, very high concentrations of several elements were detected in the pond at Prinsdal. In fact, the highest mean total concentrations for 17 elements and the highest mean dissolved concentration for 21 elements (out of 34 measured) were identified here. These included among others total and dissolved concentrations of Mn, Fe, Co, Ni, Cu, Pb and all the measured lanthanides (La, Ce, Eu, Yb, Gd and Lu), and dissolved concentrations of Al and Sb. Consequently, the pond could not be regarded as a reference site as originally intended. The high concentrations were most likely due to contamination from the abandoned shooting range nearby, or some other influences in the catchment. The link to the shooting range was particularly evident in the high concentrations of Pb and Sb, which are commonly used in ammunition (Heier et al. 2010). At Prinsdal, total concentrations of Pb and Sb reached levels of 16 and 3.3 µg/L respectively, which are in good accordance with studies of runoff from shooting ranges in Norway (Heier et al. 2009; Heier et al. 2010). There was a noteworthy increase in overall element concentrations over time, probably due to the pond drying up towards summer (Strand 2006). There was considerably less water in the pond in the last samples than at the earlier ones, probably because of little precipitation in the second half of the sampling period, which dramatically increased the concentrations even though there was presumably little supply of trace elements from external sources during the sampling period.

All values of the 16 PAHs were below limits of quantification in all ponds.

The temporal variations in total and dissolved concentrations of the metals Cu, Zn, Cd and Pb in the three ponds are visualized in Fig. 16.

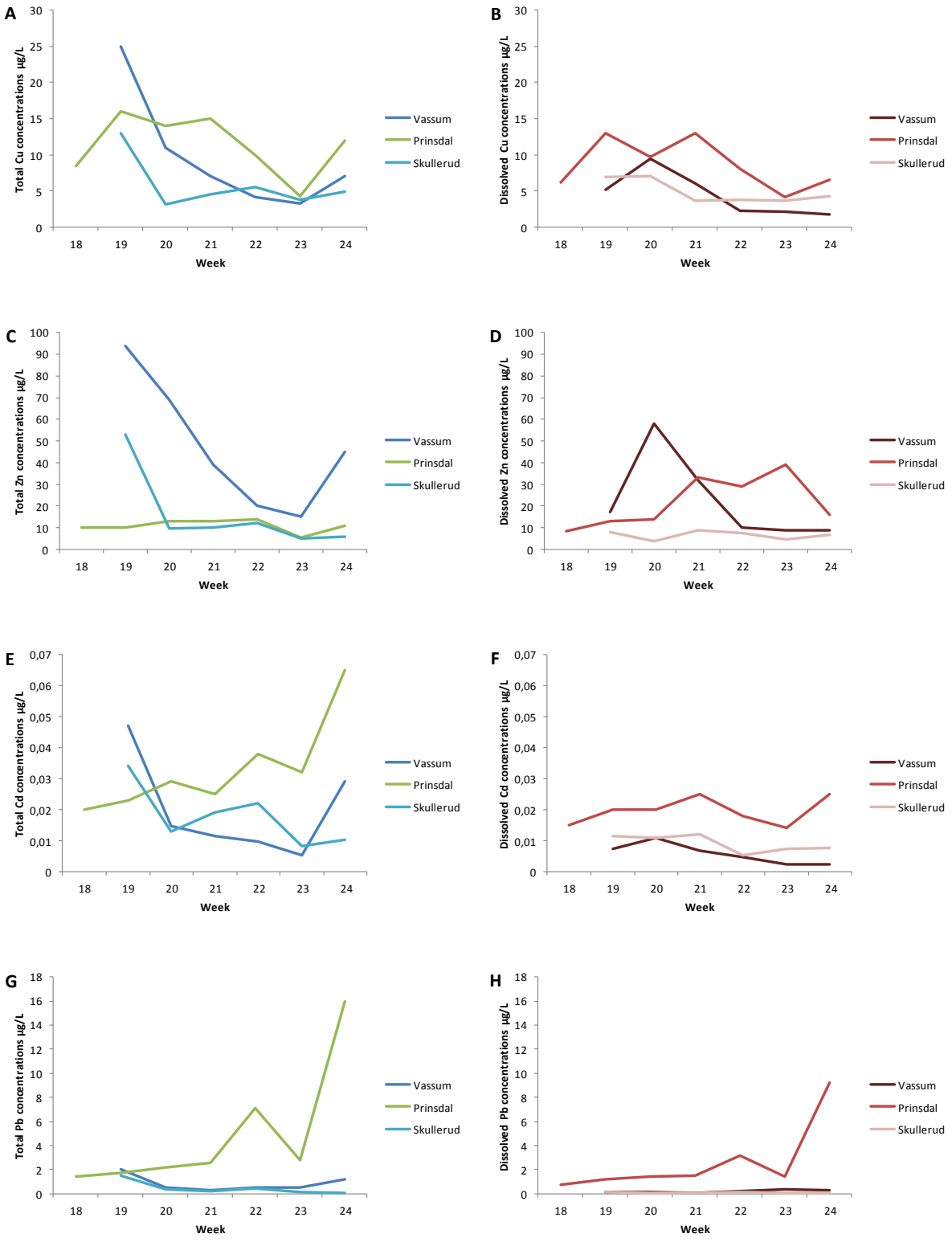

Figure 16. The temporal variations in total (A) and dissolved (B) Cu, total (C) and dissolved (D) Zn, total (E) and dissolved (F) Cd and total (G) and dissolved (H) Pb concentrations at Vassum and Skullerud sedimentation ponds, plus the naturally occurring pond at Prinsdal. Water samples were collected weekly in May and June 2012. Note that sampling at Prinsdal started one week earlier than at the two other ponds. All concentrations are given in µg/L.

A principal component analysis was performed on both total and dissolved water concentrations to get an overview of potential patterns in the data material. The biplots in Fig. 17 show how the three ponds are grouped in relation to the first and second principal component axes.

For the total concentrations, the elements that correlated best with the PC 1 axis were Al, Si, P, Sc, Cr, Mn, Fe, Co, Ni, Cu, Cd, La, Ce, Eu, Yb, Gd, Lu, Pb and Th. The PC 1 axis accounted for 53 per cent of the variation in the data. The PC 2 axis accounted for 28 per cent of the variation and the elements that correlated best with this axis were the macronutrients Na, Mg, K and Ca, plus the trace elements Zn, Sr, Mo, Sb, Ba and U. Arsenic correlated better with another axis.

For dissolved concentrations, Al, Si, Sc, Mn, Fe, Co, Ni, Cd, La, Ce, Eu, Yb, Gd, Lu, Pb and Th were best correlated with the PC 1 axis, which accounted for 58 per cent of the variation. The elements Na, Mg, P, K, Ca, As, Sr, Mo, Ba and U on the other hand, correlated best with the PC 2 axis, which accounted for 21 per cent of the variation in the dataset. The trace elements Cr, Cu, Zn and Sb correlated better with other axes.

For total water concentrations, Prinsdal (red points) had several samplings with mean or above-mean values of elements that correlated best with the PC 1 axis, while Vassum (green points) had more samples with concentrations below-mean values. On the other hand: All samples at Vassum showed above-mean concentrations of elements that correlated best with the PC 2 axis while all samples from Prinsdal showed below-mean values.

For dissolved concentrations, all Prinsdal samples showed above-mean values for elements that correlated best with the PC 1 axis, while all Vassum samples showed below-mean values for these elements.

For both total and dissolved concentrations, almost all Skullerud samples (blue triangles) exhibited below-mean values for elements that correlated best with the PC 1 axis. For elements that were best correlated with the PC 2 axis the pattern is less apparent, as about half-and-half of the samples exhibited above- and below-mean values.

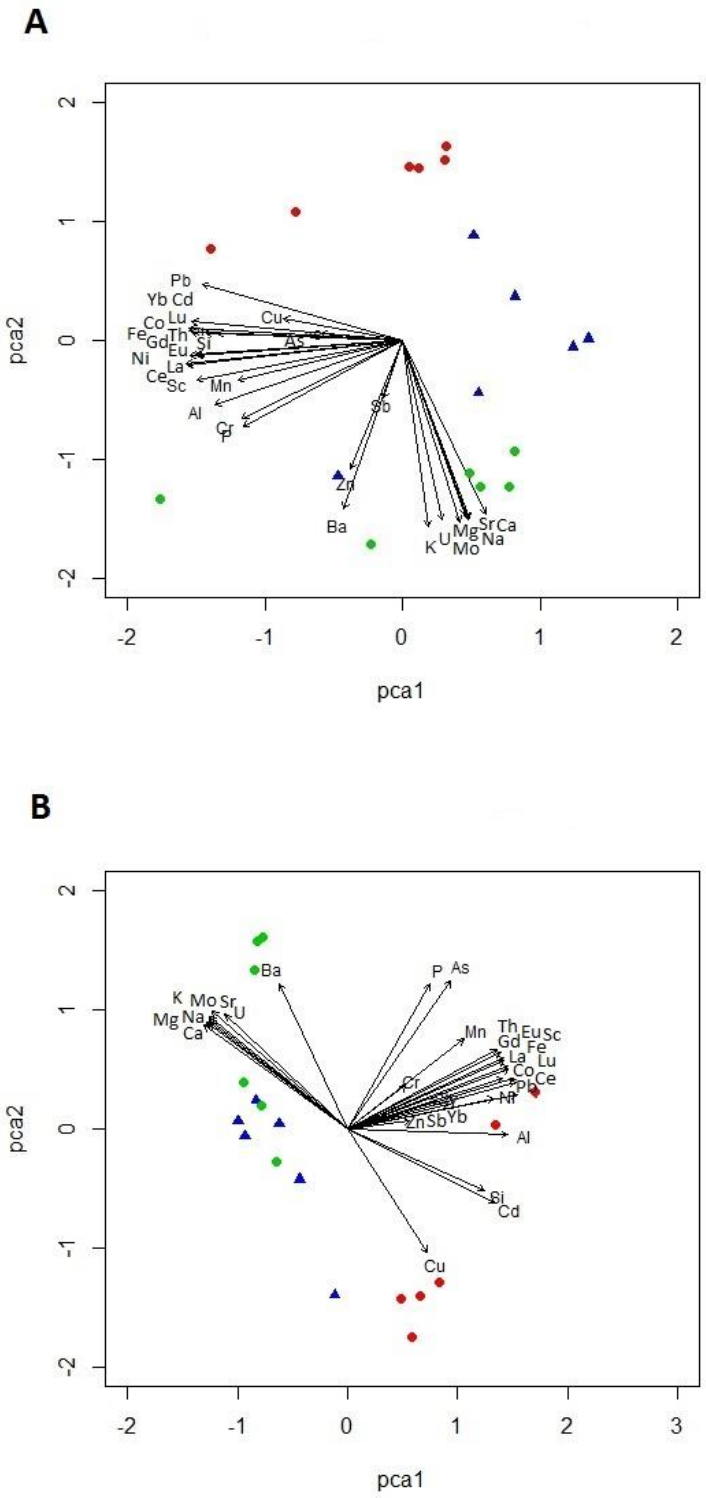

Figure 17. Biplots displaying the results of principal component analysis (PCA) conducted on total (A) and dissolved (B) element concentrations in water from Vassum (green circles), Skullerud (blue triangles) and Prinsdal (red circles). A: The PC 1 axis accounted for 53 % of the variation while the PC 2 axis accounted for 28 %. B: The PC 1 axis accounted for 58 % of the variation while the PC 2 axis accounted for 21 %. Trace elements are displayed as arrows pointing in the direction of which the value of the element increases. Points close to the coordinate system of origin have a predicted value close to the mean value. A point close to the extremity of an arrow has an above-mean predicted value of that element. Points (samples) close to each other have similar trace element composition.

The temporal variations in levels of overall water element concentrations in the samplings relative to each other, are displayed in Fig 18. The site scores of the PC 1 and PC 2 axes are used as representatives for the overall levels of element concentrations as they account for the bulk of the variation in the data. The graphs can be regarded as a synthesis of the variation in the elements seen in Fig. 16, in addition to many other elements. During the whole sampling period, Prinsdal had the highest levels of both total and dissolved element concentrations that correlated best with the PC 1 axis, except from total concentrations in week 19 (nothing can be concluded for week 18 since the sampling had not yet started at Skullerud and Vassum). Vassum generally had the highest levels of total and dissolved element concentrations that correlated best with the PC 2 axis.

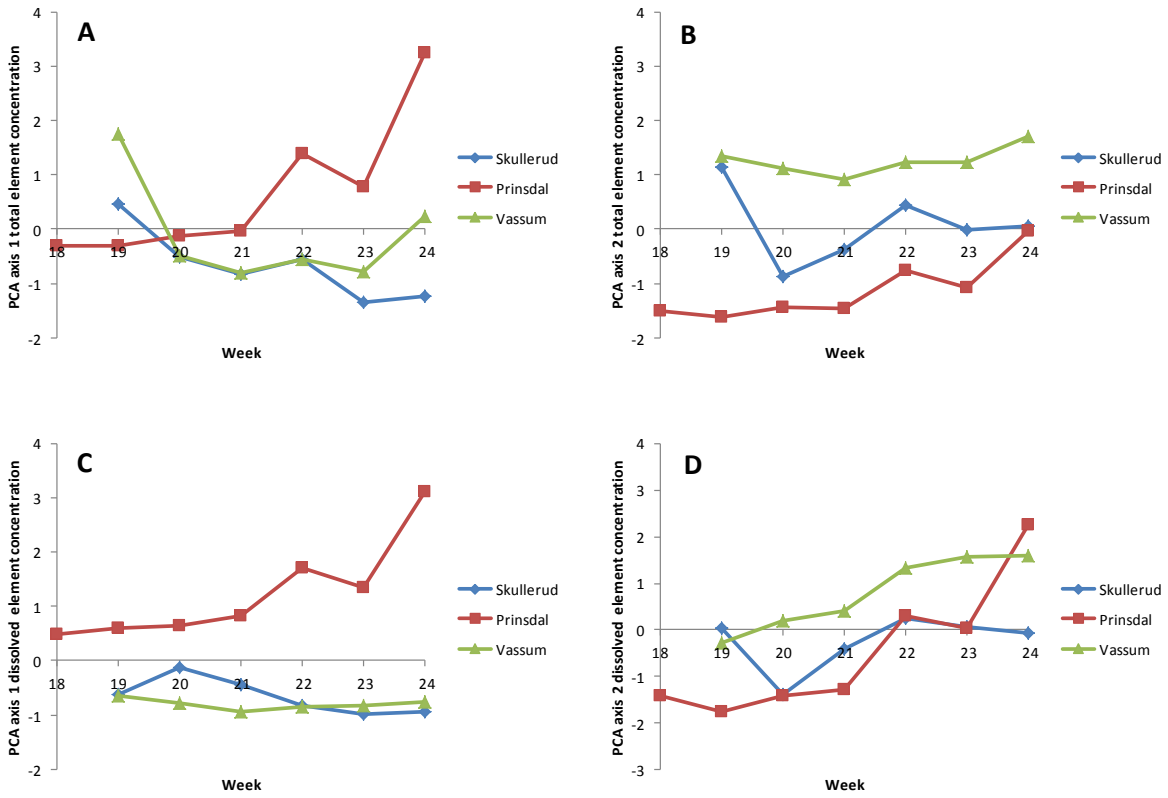

Figure 18. Total (A and B) and dissolved (C and D) element concentrations in water presented as principal component analysis (PCA) site scores. The site scores for total element concentration (both PCA axis 1 and 2) were transformed (multiplied with -1) to enable an intuitive visualization, as the PCA returned increasing values as increasingly negative (see biplot Fig. 17 A). A: PCA axis 1 site scores for total concentrations (accounted for 53 % of the variation in total concentrations). B: PCA axis 2 site scores for total concentrations (accounted for 28 % of the variation in total concentrations). C: PCA axis 1 site scores for dissolved concentrations (accounted for 58 % of the variation in dissolved concentrations). D: PCA axis 2 site scores for dissolved concentrations (accounted for 21 % of the variation in dissolved concentrations).

4.2 Analyses of tadpole tissue

The trace element tissue concentrations and the biomarker levels of the frog embryos and tadpoles were analysed in order to identify any impact of xenobiotics in the water on the organisms. The embryos and tadpoles from the different ponds were not at the exact same developmental stage at all times. This complicated comparison across sites, since there may be differences in trace element accumulation and biomarker levels due to differences in developmental stage. Hence, results will primarily be discussed according to temporal trends within each pond.

4.2.1 Trace element accumulation

The whole-body wet weight concentrations of the 34 elements measured in tadpole tissue are presented in table 5, 6 and 7. For simplicity, all concentrations will be referred to as tissue concentrations, although it cannot be ruled out that parts of the concentrations stem from sediment and undigested food in the gut, since the digestive tract was not removed. However, this is probably not relevant for the individuals of the prefeeding Gosner stages 1 -25 (Gosner 1960) sampled in week 19 at Vassum, 19 and 20 at Skullerud and 18 and 19 at Prinsdal.

Table 5. Whole-body wet weight concentrations of different elements measured by ICP-MS in tadpole tissue sampled in Vassum sedimentation pond in May and June 2012 (week 19 to 24), given as the mean of 6 biological replicates ± standard error of the mean (SEM).

Element	Unit	Week 19	Week 20	Week 21	Week 22	Week 23	Week 24
Na	g/kg	0.66 ± 0.017	1.1 ± 0.042	1.6 ± 0.16	1.9 ± 0.12	2.0 ± 0.070	1.8 ± 0.065
Mg	g/kg	0.086 ± 0.0039	0.28 ± 0.011	0.41 ± 0.039	0.68 ± 0.093	0.66 ± 0.053	0.72 ± 0.063
Al	mg/kg	19 ± 5.3	530 ± 28	880 ± 84	1300 ± 190	1300 ± 115	1300 ± 138
Si	mg/kg	39 ± 11	310 ± 23	380 ± 67	300 ± 17	220 ± 11	240 ± 11
P	g/kg	1.5 ± 0.065	0.61 ± 0.023	0.55 ± 0.066	0.56 ± 0.043	0.78 ± 0.024	0.96 ± 0.066
S	g/kg	0.61 ± 0.028	0.41 ± 0.013	0.40 ± 0.040	0.57 ± 0.049	0.73 ± 0.034	0.75 ± 0.034
K	g/kg	0.90 ± 0.033	0.78 ± 0.037	0.89 ± 0.097	1.0 ± 0.092	1.3 ± 0.031	1.4 ± 0.049
Ca	g/kg	0.077 ± 0.0053	0.30 ± 0.019	0.49 ± 0.052	0.89 ± 0.11	1.3 ± 0.073	1.9 ± 0.12
Sc	mg/kg	0.0051 ± 0.0018	0.17 ± 0.012	0.28 ± 0.026	0.41 ± 0.060	0.40 ± 0.033	0.46 ± 0.052
Cr	mg/kg	0.044 ± 0.016	1.4 ± 0.085	2.1 ± 0.21	3.5 ± 0.53	3.3 ± 0.32	3.7 ± 0.44
Mn	mg/kg	0.62 ± 0.13	13 ± 0.80	26 ± 2.9	44 ± 8.1	62 ± 7.0	47 ± 5.6
Fe	mg/kg	37 ± 7.4	650 ± 37	1200 ± 105	2100 ± 303	2000 ± 169	2200 ± 229
Co	mg/kg	<LD	0.36 ± 0.017	0.63 ± 0.063	1.0 ± 0.14	1.1 ± 0.063	1.1 ± 0.11
Ni	mg/kg	<0.066	0.68 ± 0.032	1.1 ± 0.10	1.8 ± 0.24	1.7 ± 0.14	1.8 ± 0.19
Cu	mg/kg	1.0 ± 0.058	4.1 ± 0.18	6.0 ± 0.59	11 ± 1.2	10 ± 0.94	9.6 ± 0.92
Zn	mg/kg	23 ± 1.4	29 ± 1.0	48 ± 4.3	74 ± 9.0	73 ± 6.9	69 ± 5.9
As	mg/kg	0.031 ± 0.0074	0.079 ± 0.0038	0.14 ± 0.014	0.21 ± 0.028	0.27 ± 0.016	0.29 ± 0.015
Se	mg/kg	0.26 ± 0.026	<0.14	<0.14	0.15 ± 0.012	0.18 ± 0.0087	0.20 ± 0.015
Sr	mg/kg	0.15 ± 0.023	1.4 ± 0.082	2.3 ± 0.23	4.0 ± 0.54	4.2 ± 0.35	5.5 ± 0.42
Mo	mg/kg	0.018 ± 0.0056	0.13 ± 0.0068	0.22 ± 0.020	0.34 ± 0.024	0.38 ± 0.025	0.35 ± 0.021
Ag	mg/kg	0.016 ± 0.0017	0.010 ± 0.00071	0.011 ± 0.0013	0.014 ± 0.0017	0.013 ± 0.0014	0.015 ± 0.0013
Cd	mg/kg	<0.0051	0.0094 ± 0.00040	0.018 ± 0.0024	0.022 ± 0.0033	0.023 ± 0.0023	0.020 ± 0.0018
Sn	mg/kg	<LD	<0.27	0.37 ± 0.031	0.68 ± 0.059	0.62 ± 0.078	0.38 ± 0.070
Sb	mg/kg	<0.012	0.12 ± 0.0042	0.15 ± 0.021	0.14 ± 0.036	0.15 ± 0.032	0.096 ± 0.022
Ba	mg/kg	0.31 ± 0.050	3.6 ± 0.19	5.6 ± 0.55	9.3 ± 1.3	10 ± 0.82	10 ± 0.83
La	mg/kg	0.018 ± 0.0064	1.1 ± 0.25	1.7 ± 0.17	3.0 ± 0.48	2.8 ± 0.24	3.4 ± 0.39
Ce	mg/kg	0.036 ± 0.013	2.3 ± 0.52	3.5 ± 0.34	5.9 ± 0.95	5.6 ± 0.48	6.8 ± 0.76
Eu	mg/kg	<0.00082	0.027 ± 0.0032	0.042 ± 0.0043	0.070 ± 0.011	0.071 ± 0.0065	0.082 ± 0.0088
Yb	mg/kg	<0.0014	0.042 ± 0.0048	0.066 ± 0.0065	0.093 ± 0.014	0.090 ± 0.0076	0.10 ± 0.010
Gd	mg/kg	0.0025 ± 0.00089	0.12 ± 0.016	0.21 ± 0.020	0.33 ± 0.051	0.32 ± 0.027	0.37 ± 0.039
Lu	mg/kg	<LD	0.0054 ± 0.00062	0.0087 ± 0.00088	0.012 ± 0.0018	0.011 ± 0.00097	0.013 ± 0.0014
Pb	mg/kg	<0.018	0.48 ± 0.024	0.80 ± 0.077	1.2 ± 0.18	1.2 ± 0.10	1.3 ± 0.14
Th	mg/kg	0.0025 ± 0.00076	0.23 ± 0.055	0.32 ± 0.035	0.50 ± 0.081	0.46 ± 0.044	0.53 ± 0.061
U	mg/kg	0.0017 ± 0.00029	0.15 ± 0.0071	0.17 ± 0.019	0.22 ± 0.016	0.24 ± 0.020	0.20 ± 0.016

Table 6. Whole-body wet weight concentrations of different elements measured by ICP-MS in tadpole tissue sampled in Skullerud sedimentation pond in May and June 2012 (week 19 to 24), given as the mean of 6 biological replicates ± standard error of the mean (SEM).

Element	Unit	Week 19	Week 20	Week 21	Week 22	Week 23	Week 24
Na	g/kg	0.19 ± 0.0088	0.36 ± 0.032	1.1 ± 0.031	1.4 ± 0.032	1.4 ± 0.068	1.7 ± 0.056
Mg	g/kg	0.052±0.0039	0.086 ± 0.0098	0.11 ± 0.0056	0.29 ± 0.010	0.22 ± 0.019	0.29 ± 0.014
Al	mg/kg	<2.7	10 ± 2.0	100 ± 10	780 ± 27	540 ± 58	820 ± 51
Si	mg/kg	<11	25 ± 5.7	220 ± 15	310 ± 17	310 ± 24	380 ± 25
P	g/kg	0.96 ± 0.080	1.9 ± 0.22	0.92 ± 0.029	0.70 ± 0.0081	0.56 ± 0.035	0.63 ± 0.034
S	g/kg	0.45 ± 0.029	0.77 ± 0.097	0.41 ± 0.010	0.42 ± 0.0081	0.33 ± 0.037	0.46 ± 0.025
K	g/kg	0.37 ± 0.030	0.73 ± 0.063	1.1 ± 0.036	1.0 ± 0.022	0.85 ± 0.055	1.1 ± 0.056
Ca	g/kg	0.056±0.0023	0.043 ± 0.0053	0.12 ± 0.0042	0.36 ± 0.0089	0.35 ± 0.034	0.49 ± 0.032
Sc	mg/kg	<0.0013	0.0023± 0.00052	0.024 ± 0.0026	0.21 ± 0.0068	0.15 ± 0.014	0.21 ± 0.013
Cr	mg/kg	<0.021	0.038 ± 0.014	0.20 ± 0.022	1.8 ± 0.066	1.5 ± 0.28	2.1 ± 0.15
Mn	mg/kg	0.25 ± 0.018	0.63 ± 0.10	4.2 ± 0.64	33 ± 1.6	32 ± 3.6	28 ± 3.4
Fe	mg/kg	9.2 ± 0.95	22 ± 2.9	100 ± 10	780 ± 25	600 ± 59	790 ± 55
Co	mg/kg	<LD	<LD	<LD	0.38 ± 0.0089	0.31 ± 0.026	0.38 ± 0.026
Ni	mg/kg	<LD	<0.066	0.11 ± 0.010	0.87 ± 0.040	0.61 ± 0.066	0.89 ± 0.058
Cu	mg/kg	0.68 ± 0.055	1.3 ± 0.16	1.1 ± 0.074	5.0 ± 1.2	5.6 ± 2.4	4.2 ± 0.30
Zn	mg/kg	21 ± 2.2	34 ± 5.5	18 ± 0.45	24 ± 1.8	13 ± 1.3	18 ± 1.1
As	mg/kg	0.15 ± 0.056	0.15 ± 0.11	0.048 ± 0.0050	0.14 ± 0.0024	0.12 ± 0.0095	0.14 ± 0.0087
Se	mg/kg	<0.14	0.18 ± 0.023	<0.14	<0.14	<0.14	<0.14
Sr	mg/kg	0.18 ± 0.0097	0.092 ± 0.013	0.29 ± 0.025	1.5 ± 0.049	1.3 ± 0.12	1.9 ± 0.080
Mo	mg/kg	0.0089± 0.00069	0.014 ± 0.003	0.026 ± 0.0019	0.18 ± 0.0077	0.13 ± 0.026	0.23 ± 0.0086
Ag	mg/kg	<0.0062	0.019 ± 0.0033	0.0068± 0.00049	0.013 ± 0.00060	0.0096 ± 0.0011	0.012 ± 0.00067
Cd	mg/kg	<LD	<0.0051	<0.0051	0.026 ± 0.00066	0.021 ± 0.0039	0.037 ± 0.0025
Sn	mg/kg	<LD	<LD	<LD	<0.27	<0.27	<0.27
Sb	mg/kg	<LD	<LD	0.028 ± 0.0074	0.081 ± 0.0027	0.067 ± 0.0057	0.092 ± 0.0031
Ba	mg/kg	0.12 ± 0.014	0.26 ± 0.035	1.1 ± 0.12	7.2 ± 0.25	5.4 ± 0.52	8.5 ± 0.51
La	mg/kg	0.0021± 0.00065	0.0096 ± 0.0023	0.09 ± 0.011	0.95 ± 0.019	0.97 ± 0.087	0.98 ± 0.079
Ce	mg/kg	0.0038 ± 0.0013	0.019 ± 0.0045	0.18 ± 0.021	1.8 ± 0.045	1.9 ± 0.14	1.9 ± 0.12
Eu	mg/kg	<LD	<0.00082	0.0025± 0.00030	0.023 ± 0.00060	0.020 ± 0.0017	0.026 ± 0.0014
Yb	mg/kg	<LD	<0.0014	0.0045± 0.00059	0.043 ± 0.00087	0.036 ± 0.0027	0.046 ± 0.0023
Gd	mg/kg	<LD	0.0011± 0.00028	0.012 ± 0.0016	0.12 ± 0.0024	0.11 ± 0.0081	0.12 ± 0.0075
Lu	mg/kg	<LD	<LD	<0.00083	0.0053± 0.00017	0.0046± 0.00032	0.0057± 0.00031
Pb	mg/kg	<LD	<0.018	0.072 ± 0.0085	0.61 ± 0.022	0.49 ± 0.054	0.71 ± 0.042
Th	mg/kg	<LD	0.0018± 0.00067	0.022 ± 0.0022	0.19 ± 0.0058	0.22 ± 0.014	0.19 ± 0.012
U	mg/kg	0.0025± 0.00016	0.0019± 0.00076	0.034 ± 0.0041	0.29 ± 0.016	0.24 ± 0.068	0.48 ± 0.045

Table 7. Whole-body wet weight concentrations of different elements measured by ICP-MS in tadpole tissue sampled in the naturally occurring pond at Prinsdal in May and June 2012 (week 18 to 24), given as the mean of 6 biological replicates ± standard error of the mean (SEM).

Element	Unit	Week 18	Week 19	Week 20	Week 21	Week 22	Week 23	Week 24
Na	g/kg	0.30 ± 0.025	0.33 ± 0.029	0.95 ± 0.090	1.3 ± 0.031	1.7 ± 0.017	1.6 ± 0.021	1.5 ± 0.045
Mg	g/kg	0.095± 0.0062	0.057± 0.0043	0.087± 0.0080	0.12 ± 0.0073	0.16 ± 0.0049	0.21 ± 0.0048	0.47 ± 0.029
Al	mg/kg	<2.7	3.2 ± 1.1	64 ± 12	340 ± 44	550 ± 40	850 ± 30	2200 ± 149
Si	mg/kg	<LD	<11	160 ± 23	350 ± 22	360 ± 106	270 ± 18	410 ± 23
P	g/kg	2.5 ± 0.20	1.1 ± 0.078	0.90 ± 0.054	0.65 ± 0.0093	0.58 ± 0.016	0.63 ± 0.0031	0.66 ± 0.016
S	g/kg	1.0 ± 0.085	0.43 ± 0.030	0.39 ± 0.024	0.35 ± 0.0037	0.32 ± 0.0083	0.34 ± 0.0021	0.38 ± 0.0091
K	g/kg	0.93 ± 0.058	0.63 ± 0.047	1.0 ± 0.067	0.94 ± 0.019	0.95 ± 0.019	1.1 ± 0.017	1.5 ± 0.070
Ca	g/kg	0.03± 0.0019	0.035± 0.0025	0.085± 0.0062	0.14 ± 0.0042	0.18 ± 0.0056	0.21 ± 0.0017	0.39 ± 0.021
Sc	mg/kg	<LD	<0.0013	0.014± 0.0024	0.073± 0.0087	0.12 ± 0.0080	0.19 ± 0.0056	0.46 ± 0.030
Cr	mg/kg	0.043± 0.027	<LD	0.076 ± 0.014	0.44 ± 0.049	0.72 ± 0.048	1.1 ± 0.042	3.0 ± 0.20
Mn	mg/kg	0.59 ± 0.053	1.6 ± 0.11	5.7 ± 0.60	9.8 ± 0.83	19 ± 0.43	24 ± 1.8	29 ± 2.5
Fe	mg/kg	22 ± 1.7	13 ± 2.0	120 ± 16	510 ± 43	1400 ± 26	1200 ± 21	1900 ± 99
Co	mg/kg	<LD	<LD	<0.2	0.28 ± 0.020	0.51 ± 0.013	0.54 ± 0.019	0.81 ± 0.043
Ni	mg/kg	<LD	<LD	0.11 ± 0.013	0.42 ± 0.037	0.72 ± 0.025	0.81 ± 0.024	1.7 ± 0.11
Cu	mg/kg	1.8 ± 0.23	0.79 ± 0.052	1.3 ± 0.13	4.1 ± 0.17	3.5 ± 0.19	2.2 ± 0.060	3.1 ± 0.095
Zn	mg/kg	42 ± 2.9	18 ± 1.4	16 ± 0.95	11 ± 0.40	7.9 ± 0.19	8.1 ± 0.12	11 ± 0.43
As	mg/kg	0.015± 0.0054	0.016± 0.0028	0.027± 0.0017	0.076± 0.0045	0.12 ± 0.0031	0.13 ± 0.0043	0.18 ± 0.0079
Se	mg/kg	0.36 ± 0.035	0.15 ± 0.011	<0.14	<0.14	<0.14	<0.14	<0.14
Sr	mg/kg	0.040 ± 0.0040	<0.035	0.12 ± 0.017	0.41 ± 0.033	0.71 ± 0.046	1.0 ± 0.035	2.4 ± 0.17
Mo	mg/kg	0.020± 0.0049	0.0049 ± 0.00037	0.0090 ± 0.0013	0.040± 0.0076	0.054± 0.0015	0.073± 0.0015	0.11 ± 0.0051
Ag	mg/kg	0.033 ± 0.0067	0.011 ± 0.00094	0.0095 ± 0.00077	0.014 ± 0.00052	0.021 ± 0.00067	0.035± 0.0014	0.041± 0.0021
Cd	mg/kg	<0.0051	<0.0051	<0.0051	0.0098 ± 0.00062	0.013 ± 0.00040	0.017 ± 0.00079	0.022 ± 0.00084
Sn	mg/kg	<LD	<LD	<LD	<LD	<LD	<LD	<LD
Sb	mg/kg	<LD	<LD	0.032± 0.0043	0.11 ± 0.0054	0.086 ± 0.013	0.026± 0.0014	0.022± 0.0017
Ba	mg/kg	0.24 ± 0.036	0.13 ± 0.016	0.9 ± 0.15	3.6 ± 0.35	5.6 ± 0.39	8.1 ± 0.26	20 ± 1.3
La	mg/kg	0.0028 ± 0.0010	0.0034 ± 0.0014	0.071 ± 0.011	0.34 ± 0.034	0.58 ± 0.033	0.84 ± 0.031	1.7 ± 0.092
Ce	mg/kg	0.0053 ± 0.0020	0.0077 ± 0.0031	0.16 ± 0.025	0.72 ± 0.079	1.1 ± 0.042	1.6 ± 0.049	3.3 ± 0.19
Eu	mg/kg	<LD	<LD	0.0020 ± 0.00029	0.0093 ± 0.00090	0.015 ± 0.00056	0.019 ± 0.00054	0.043± 0.0026
Yb	mg/kg	<LD	<LD	0.0035 ± 0.00057	0.017± 0.0017	0.029± 0.0015	0.034± 0.0011	0.076± 0.0049
Gd	mg/kg	<LD	<0.0011	0.0099 ± 0.0017	0.046± 0.0042	0.074± 0.0028	0.094± 0.0035	0.20 ± 0.011
Lu	mg/kg	<LD	<LD	<0.00083	0.0022 ± 0.00023	0.0035 ± 0.00018	0.0044 ± 0.00014	0.0098 ± 0.00056
Pb	mg/kg	<0.018	0.039± 0.016	0.63 ± 0.095	2.6 ± 0.23	2.6 ± 0.095	2.0 ± 0.082	2.9 ± 0.18
Th	mg/kg	<LD	<0.0014	0.012± 0.0022	0.064± 0.0073	0.11 ± 0.0076	0.16 ± 0.0043	0.39 ± 0.025
U	mg/kg	<LD	<LD	0.0052 ± 0.00086	0.027± 0.0024	0.036± 0.0023	0.053± 0.0016	0.11 ± 0.0078

For certain trace elements of environmental concern there was a considerable accumulation in tadpoles, as tissue concentrations exhibited a 100-fold increase or more during the sampling period. In Skullerud tadpoles this was true for Al, Sc, Cr and Mn. In Vassum tadpoles Mn and Mo increased a 100-fold over the sampling period, while at Prinsdal this pass for Al, Sc, As and Pb. In all three ponds there was also a 100-fold increase in tissue concentrations of the lanthanides La, Ce and Gd. However, the maximum concentrations were 3.4 mg La/kg and 6.8 mg Ce/kg in Vassum tadpoles, which was considerably higher than in Skullerud (0.98 mg La/kg and 1.9 mg Ce/kg) and Prinsdal (1.7 mg La/kg and 3.3 mg Ce/kg). In Vassum there was also a 100-fold increase in concentrations of Eu and a strong increase in Yb that was not found in tadpoles in the two other ponds. The higher accumulation of lanthanides in Vassum tadpoles is interesting as these elements are being increasingly utilized in the automobile industry (Goonan 2011). Hence, it is reason to believe that organisms inhabiting sedimentation ponds are exposed to these elements. However, the highest total and dissolved water concentrations of these elements were measured in Prinsdal (see section 4.1 Water quality characterization).

A principal component analysis was performed on the dataset for tadpole tissue element concentrations to identify potential patterns. The PC 1 axis accounted for 66 per cent of the variation in the data set and the elements Na, Mg, Al, Ca, Sc, Mn, Fe, Cu, As, Sr, Mo, Cd, Ba, La, Ce, Gd, Pb, Th and U correlated best with this axis. The elements P, S, Zn and Se correlated best with the PC 2 axis, which accounted for 15 per cent of the total variation. Silver correlated better with another axis than the first and the second. The majority of Vassum tadpole samples showed mean or above-mean concentrations of elements that correlated best with the PC 1 axis, as well as elements that correlated best with the PC 2 axis (Fig. 19). Among the tadpole samples from Skullerud and Prinsdal, considerably fewer samples showed above-mean values of any elements.

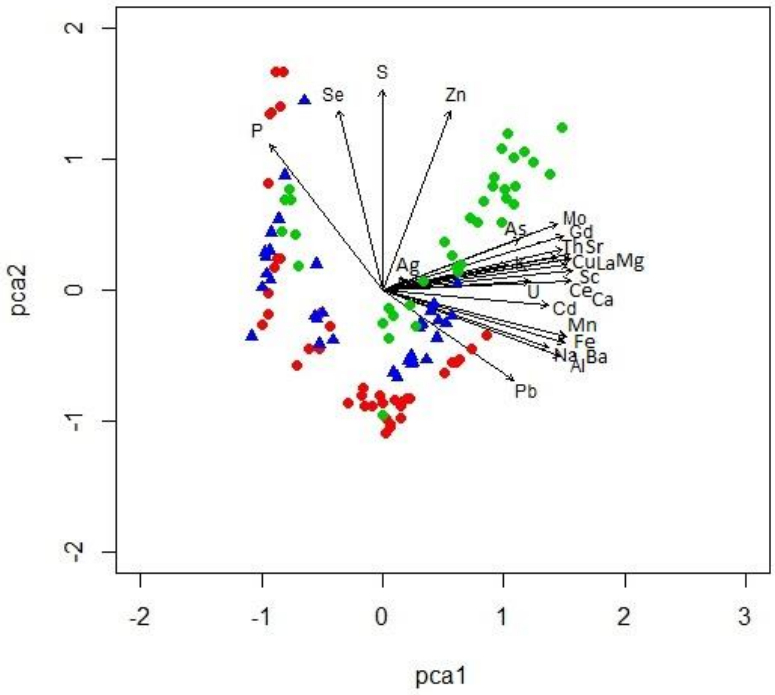

Figure 19. Biplot displaying the results of principal component analysis (PCA) conducted on element concentrations in tadpole tissue at Vassum (green circles), Skullerud (blue triangles) and Prinsdal (red circles). The PCA axis 1 accounted for 66 % of the variation in metal concentrations while axis 2 accounted for 15 %. Tissue trace elements are displayed as arrows pointing in the direction of which the value of the element increases. Points (samples) close to the coordinate system of origin have a predicted value close to the mean value. A point close to the extremity of an arrow has an above-mean predicted value of that element. Points close to each other show tadpole samples with similar tissue element composition.

For tadpoles in all ponds there was a time dependent accumulation of the trace elements that correlated best with the PC 1 axis (Fig. 20). Kruskal-Wallis/pairwise Wilcoxon tests and ANOVA/Tukey's HSD tests revealed that several samplings were significantly different from each other, as indicated by the significance letters in the box plots. Accordingly, the increases in tadpole tissue trace element concentrations were statistically significant. The results are in agreement with a field study by Kelepertzis et al. (2012) who showed that *Pelophylax kurtmuelleri* tadpoles growing up in contaminated areas accumulated considerable amounts of metals. The whole-body metal concentrations were the highest ever reported in tadpole tissue and reached dry weight concentrations of 182 mg Cu/kg, 11 460 mg Zn/kg, 82 mg Cd/kg and 4490 mg Pb/kg (Kelepertzis et al. 2012). If assuming a dry weight to wet weight ratio of 5, which is common for e.g. fish and bivalves (Consortium for Risk Evaluation with Stakeholder Participation 2006), these levels are far higher than those in the present thesis. A field study of *Rana temporaria* tadpole populations in an area in the Alps with little direct human impact documented accumulation of Al, Cd and Pb (Hofer et al. 2005) The metals in the water were assumed to stem from atmospheric deposition and geological processes (Hofer et al. 2005). Tissue concentrations of Al, Cd and Pb were given in dry weight but if assuming a dry weight

to wet weight ratio of 5, the concentrations reported were in the lower range of the current results.

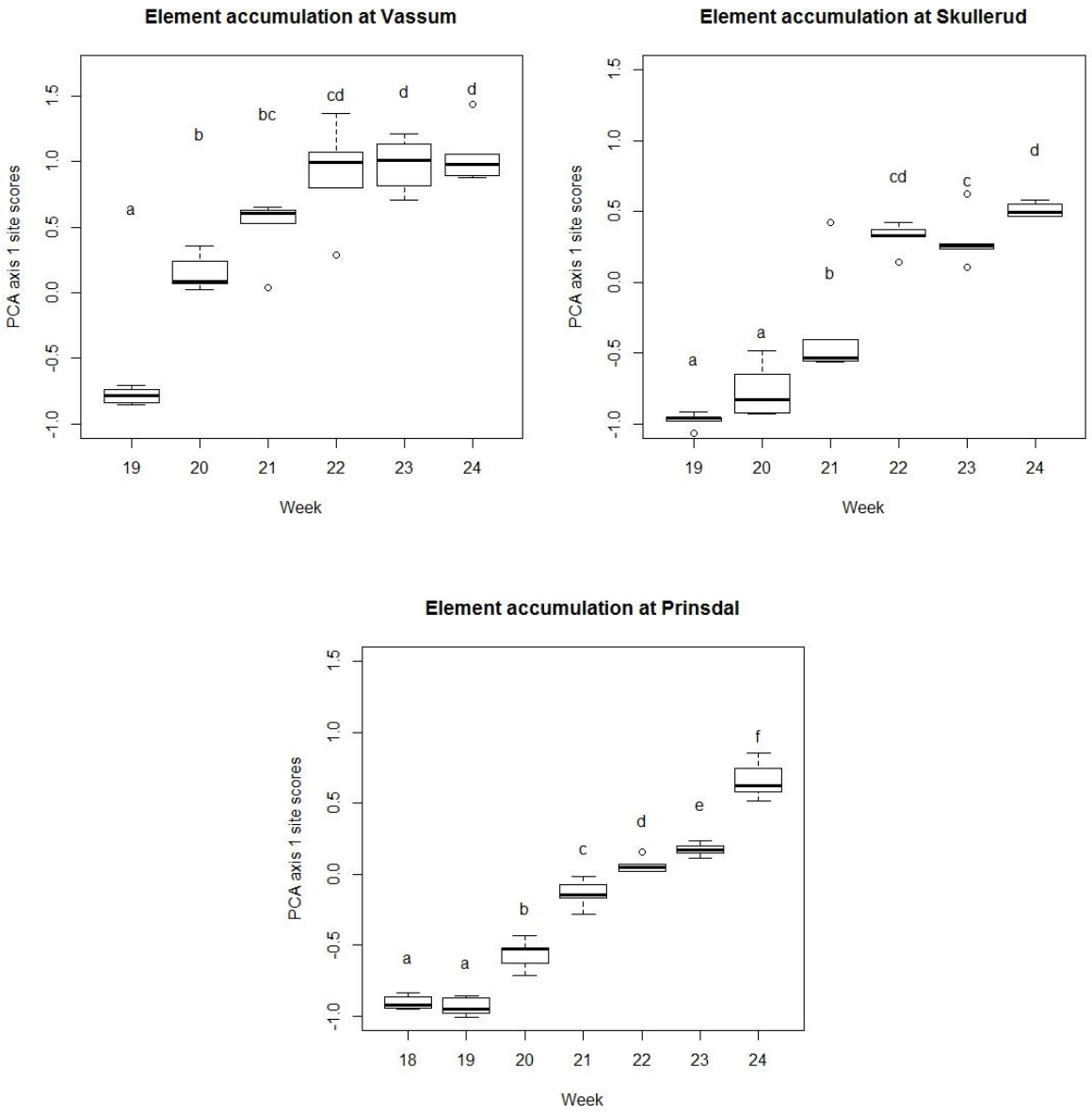

Figure 20. Box plots showing time dependent element accumulation in tadpoles presented as the principal component analysis axis 1 (PCA axis 1) site scores obtained by PCA on element concentrations in tadpole tissue. Each sample constitutes 6 biological replicates. A box constitutes the middle 50 % of the sample values and the remaining 50 % are situated between the box and the whiskers. Circles represent outliers from this setup. The black lines across the boxes show median values. Different letters indicate significant differences between the samplings within a pond according to Tukey's HSD or pairwise Wilcoxon post hoc tests (significance level set to p=0.05). The letters does not denote any difference between samples across sites.

Laboratory experiments exposing amphibians to metal contaminated water have shown considerable time dependent accumulation of metals such as Cr, Cu, Zn and Cd where the skin probably is an important route for uptake (Dobrovoljc et al. 2012; Loumbourdis et al.

2007; Papadimitriou & Loumbourdis 2002; Papadimitriou & Loumbourdis 2003). The tadpoles investigated in the present thesis may have taken up trace elements through the permeable skin and gills, as well as by ingestion of food and sediment. When comparing with results from other studies one should keep in mind that significant species-specific differences have been observed regarding metal accumulation in tadpoles and susceptibility to contamination (Snodgrass et al. 2004; Snodgrass et al. 2008).

Pearson correlation tests showed no significant correlation neither between total nor dissolved trace element concentrations in water, and trace element concentration in tadpoles, when looking at elements best correlated with the PC 1 axis ($r = -0.15$, $p = 0.55$ for total concentrations vs. tissue concentrations, $r = -0.38$, $p = 0.11$ for dissolved concentrations vs. tissue concentrations). Consequently, other factors must be considered to explain the bioaccumulation. Sediment concentrations of elements may be one explanation factor. Tadpoles are omnivores that eat plenty of sediment, and have been referred to as benthic "bulldozers" as they disturb the sediment while foraging (Flecker et al. 1999; Ranvestel et al. 2004). Hence, they are highly exposed to pollution in the sediment and strong correlation has been found in other studies between sediment and tadpole tissue concentrations of trace elements (Kelepertzis et al. 2012; Sparling & Lowe 1996). Unfortunately, sediment was not sampled for this thesis. However, previous measurements of Vassum and Skullerud sedimentation ponds detected higher sediment concentrations of trace elements at Vassum than at Skullerud (Damsgård 2011), which is interesting as the highest tissue element accumulation was measured in tadpoles from Vassum in the current results.

The element concentrations in tadpole tissue reached the highest levels in the tadpoles from Vassum, despite Prinsdal having the highest total and dissolved concentrations for most of these elements. ANOVA and Tukey's HSD performed on the sampling with the highest element concentration from each pond (i.e. the last sampling) showed that Vassum concentration levels were significantly different from the two other ponds ($p = 0.0022$ for Vassum vs. Prinsdal, and $p < 0.001$ for Vassum vs. Skullerud). Skullerud and Prinsdal were not significantly different from each other ($p = 0.18$). A possible explanation for the lower bioaccumulation at Prinsdal relative to Vassum is the high concentrations of DOC at Prinsdal (the mean DOC concentration being 7.8 mg/L at Prinsdal compared to 4.6 mg/L at Vassum) providing strong complexation of trace elements and hence making them less bioavailable (Di Toro et al. 2001), especially at the later samplings.

The tadpoles' growth and metabolism patterns may provide another explanation for the higher trace element accumulation in Vassum. The mean weight of the tadpoles at Vassum apparently exceeded that of the tadpoles in the two other ponds at the late samplings (Fig. 21), although this was not statistically tested. They also had a considerably larger body size at most of the samplings, which is evident to some degree by counting the squares on the graph paper in the two last pictures in Fig. 22, 23 and 24, respectively.

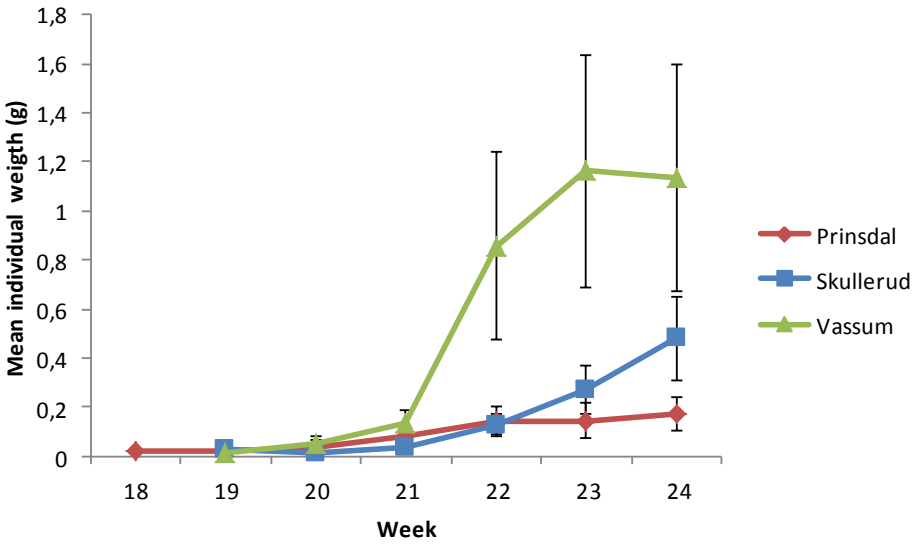

Figure 21. The mean wet weight of the tadpoles at the different samplings, calculated by dividing the total weight of the individuals on randomly picked CryoTubes, by the number of individuals on the tube and thereafter calculating the mean of the estimated individual weight of tadpoles from 3 – 8 different tubes (i.e. *n* varies from 3 – 8). Error bars denotes standard error of the mean (SEM).

Several factors may have influenced this development. First, Vassum is highly sun exposed, in contrast to Prinsdal, that is situated shadily by the forest edge, and Skullerud that is partly roofed over by the highway and surrounded by more vegetation. Besides, the pond at Vassum is smaller than Skullerud so it takes shorter time to heat. Consequently, the overall water temperature is probably higher at Vassum than the two other ponds, providing a higher sum of degree-days. This is to some extent reflected in the temperature measurements, which were highest at Vassum at all samplings except one (the mean temperature at Vassum was 5 C° higher than at Prinsdal and 3 C° higher than at Skullerud). However, to actually demonstrate the difference, a temperature logger should have been applied instead of the single-point measurements that were taken during the field work for this thesis. Hence a higher sum of the degree-days at Vassum cannot be demonstrated, only assumed.

A higher sum of the degree-days would most likely accelerate the tadpole development, growth and metabolism, as they are poikilotherm. In addition, a laboratory study showed that tadpoles exposed to storm water pond sediment with elevated road salt concentrations grew more rapidly and metamorphosized at a larger size, than tadpoles of the control groups (Brand et al. 2010). It was suggested that this may be due to the water being isotonic with regard to their body fluids, saving the tadpoles for energy demanding osmoregulation. Hence, more energy could be allocated to growth. It is possible that this mechanism is in work in the Vassum tadpoles. Their high growth rate may explain their higher tissue trace element concentrations compared to the two other ponds, because fast growing organisms need high food intake which may increase the tadpoles' ingestion of potentially contaminated food and sediment. Köck et al. (1996) also explained increasing metal accumulation in Arctic char (*Salvelinus alpinus*) during summer by increasing temperatures. They suggested that one of

the mechanisms behind temperature driven metal accumulation may be increased gill ventilation leading to higher processing of water and higher metal uptake (Köck et al. 1996).

The mere accumulation of trace elements does not necessarily imply adverse health effects, as tissue concentrations may include trace elements involved in essential processes or stored in inert forms (e.g. bound to MT), as well as those causing damage (Wood 2012). Still, increasing trace element concentrations in tadpoles during spring is worth notice as they may be remobilized and potentially cause toxic effects during the metamorphosis as the organs undergo extensive reconstruction (Hofer et al. 2005). In that respect, the trace element accumulation observed in the current thesis may be potentially harmful to the tadpoles. The digestive tract was not removed or emptied in tadpoles in the present thesis. If this had been done, the results may have been different. This ought to be considered in any further research.

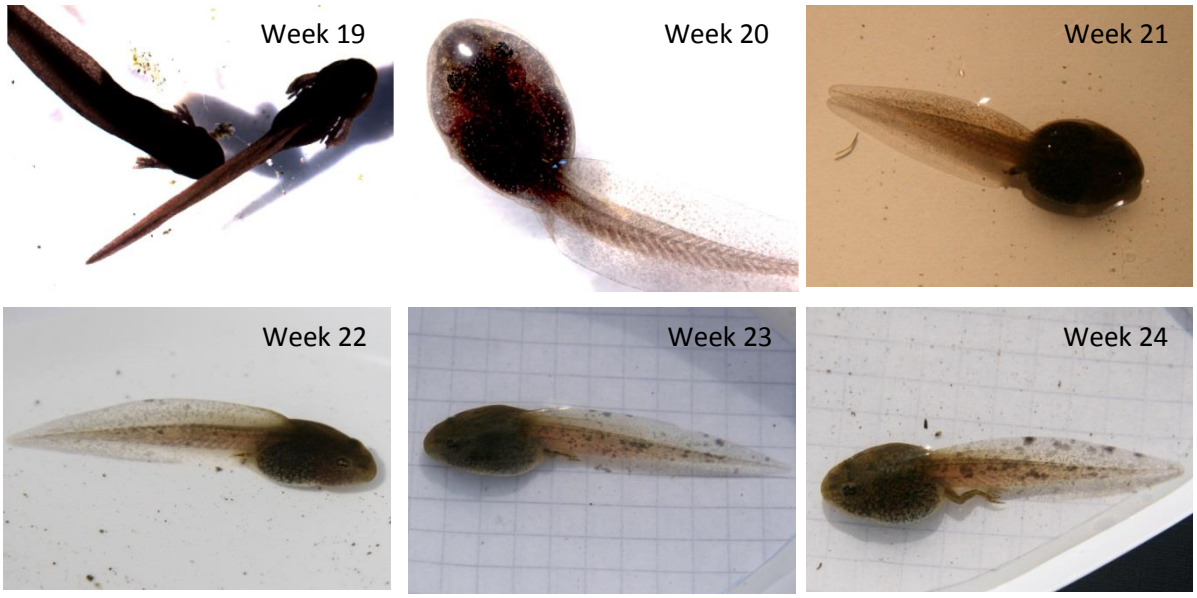

Figure 22. Developmental stages of common frog tadpoles (*Rana temporaria*) sampled in Vassum sedimentation pond from week 19 to 24, 2012.

Figure 23. Developmental stages of common frog tadpoles (*Rana temporaria*) sampled in Skullerud sedimentation pond from week 19 to 24, 2012.

Figure 24. Developmental stages of common frog tadpoles (*Rana temporaria*) sampled in the naturally occurring pond at Prinsdal from week 19 to 24, 2012. Sampling at Prinsdal started in week 18 2012, but photography from this week is missing.

The bioconcentration factors (BCFs) of most elements varied considerably throughout the sampling period (see appendix 3 for exact BCF values). For several metals of environmental concern such as Al, Mn, Fe, Cu, Zn, Cd and Pb, plus for the lanthanides La, Ce and Gd, the BCFs were far higher at Vassum and Skullerud than at Prinsdal. For instance, the BCF for Pb was in the range of 0.019 to 16 at Skullerud and 0.11 to 10 at Vassum, while it was only 0.015

to 1.7 at Prinsdal. A PCA was performed on the BCFs to detect any patterns and the results are displayed in Fig. 25. The elements Al, Sc, Mn, Fe, Cu, As, Cd, La, Ce, Gd, Pb and Th correlated best with the PC 1 axis, which accounted for 48 per cent of the variation in the dataset. The PC 2 axis accounted for 25 per cent of the variation, and the elements that correlated best with this axis were Na, Mg, P, K, Ca, Sr, Mo, Ba and U. An interesting finding was that all Prinsdal samples showed below-mean BCF values for elements best correlated with the PC 1 axis, despite this pond having the highest mean total and dissolved water concentrations of most of these elements. The majority of Vassum samples showed above-mean BCF values for elements best correlated with the PC 1, while Skullerud samples showed BCFs equally distributed between above- and below mean values.

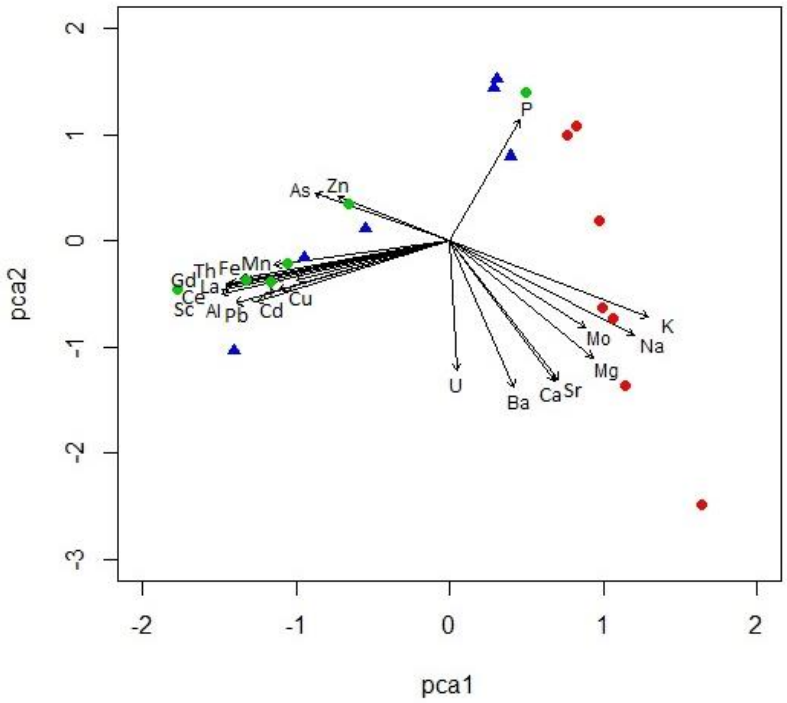

Figure 25. Biplot displaying the results of principal component analysis (PCA) conducted on the bioconcentration factors (BCF) of elements for Vassum (green circles), Skullerud (blue triangles) and Prinsdal (red circles). The PCA axis 1 accounted for 48 % of the variation in the data set, while PCA axis 2 accounted for 25 %. Bioconcentration factors are displayed as arrows pointing in the direction of which the value of the BCF increases. Points close to the coordinate system of origin have a predicted value close to the mean value. A point close to the extremity of an arrow has an above-mean predicted value of that BCF. Points close to each other have similar BCF composition.

The PC 1 axis site scores derived from the PCA performed on the BCFs are displayed in Fig. 26 to visualize the temporal variation in BCFs at the three ponds throughout the sampling period.

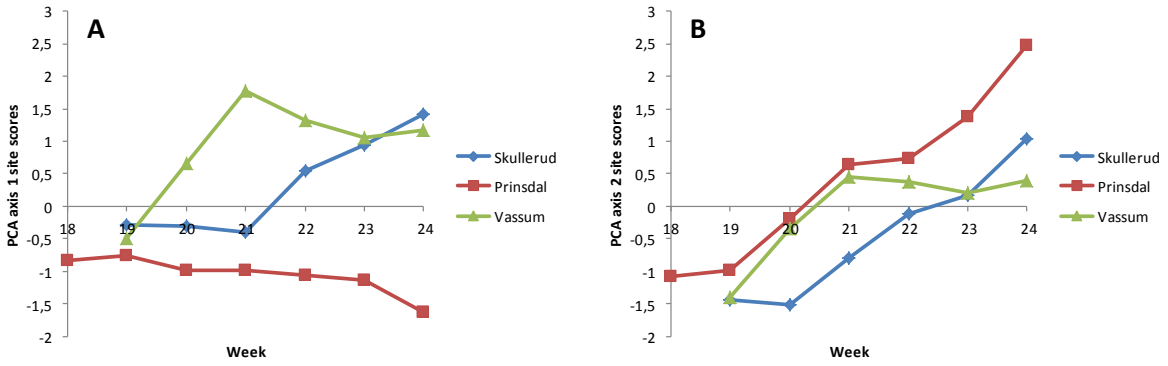

Figure 26. Bioconcentration factors (BCFs), calculated by dividing dissolved element concentrations in water on element concentrations in tadpoles, displayed as the site scores of principal component analysis (PCA) axis 1 (A) and 2 (B). The site scores were transformed by multiplying the values with -1 to enable a more intuitive visualization of the data, as the PCA returned increasing values as increasingly negative (see biplot, Fig. 25).

There is an evident inverse relationship between water concentrations and BCFs, as all Prinsdal samples showed below-mean BCF values for elements best correlated with the PC 1 axis, at the same time as this pond exhibited the highest mean dissolved water concentrations for almost all these elements. Although this may seem counterintuitive, it is a common phenomenon for metal BCFs, giving a high value when the water concentrations are low, and a low value when the water concentrations are high (McGeer et al. 2003). This owes among other factors to the hydrophilic nature of most metals, which makes active uptake by ion channels necessary for them to cross the lipid-rich cells membranes (Wood 2012), such as Cd uptake through channels for Ca uptake in fish gills (Wicklund & Runn 1988). These channels are often saturable and the organisms have a high ability to regulate them by feedback mechanisms (Wood 2012). For instance, a wide range of aquatic organisms show high ability to regulate the essential metal Zn, keeping the internal levels fairly constant independent of the ambient concentrations (McGeer et al. 2003), resulting in very low BCFs at high water concentrations. Such mechanisms may explain the inverse relationship in the present results. Besides, it is an important assumption for the use of BCF that it reflects equilibrium conditions between concentrations in water and the organism, which is difficult to ascertain for conditions in the field (McGeer et al. 2003). It is possible that BCFs calculated here underestimates the real value because the conditions are not in equilibrium, especially at Prinsdal where the concentrations increased strongly in the last samples.

4.2.2 Biomarkers

The concentrations of the biomarkers MT, EROD, GST and GSH in tadpoles in different samples are displayed in Fig. 27. For all ponds and all biomarkers there were statistically significant temporal variations in biomarker levels. Since the embryos and tadpoles were growing and developing almost continuously, some of the differences in biomarker levels may be due to different ability of the organisms to produce stress proteins and induce biomarkers at different developmental stages. Since it was difficult to find biomarker literature on *R. temporaria* direct comparison of biomarker concentrations will be done with studies of other frog species, preferably genus *Rana*, taking certain reservations that there might be species-specific differences in biomarker induction.

All biomarkers were tested for correlation with tadpole tissue element concentrations, to identify any relationships between trace element accumulation and induction of biomarkers. This was done by using the PC 1 site scores derived from the PCA on tadpole tissue element concentrations as representative values for overall element accumulation. Henceforth, the phrase ‘overall tissue element concentrations’ refer to the PC 1 axis site scores of tissue concentrations. The PCA axis 1 accounted for 66 per cent of the variation in tadpole tissue concentrations. Hence the bulk of the variation is accounted for when using this value in further statistics, although it is of course a simplification.

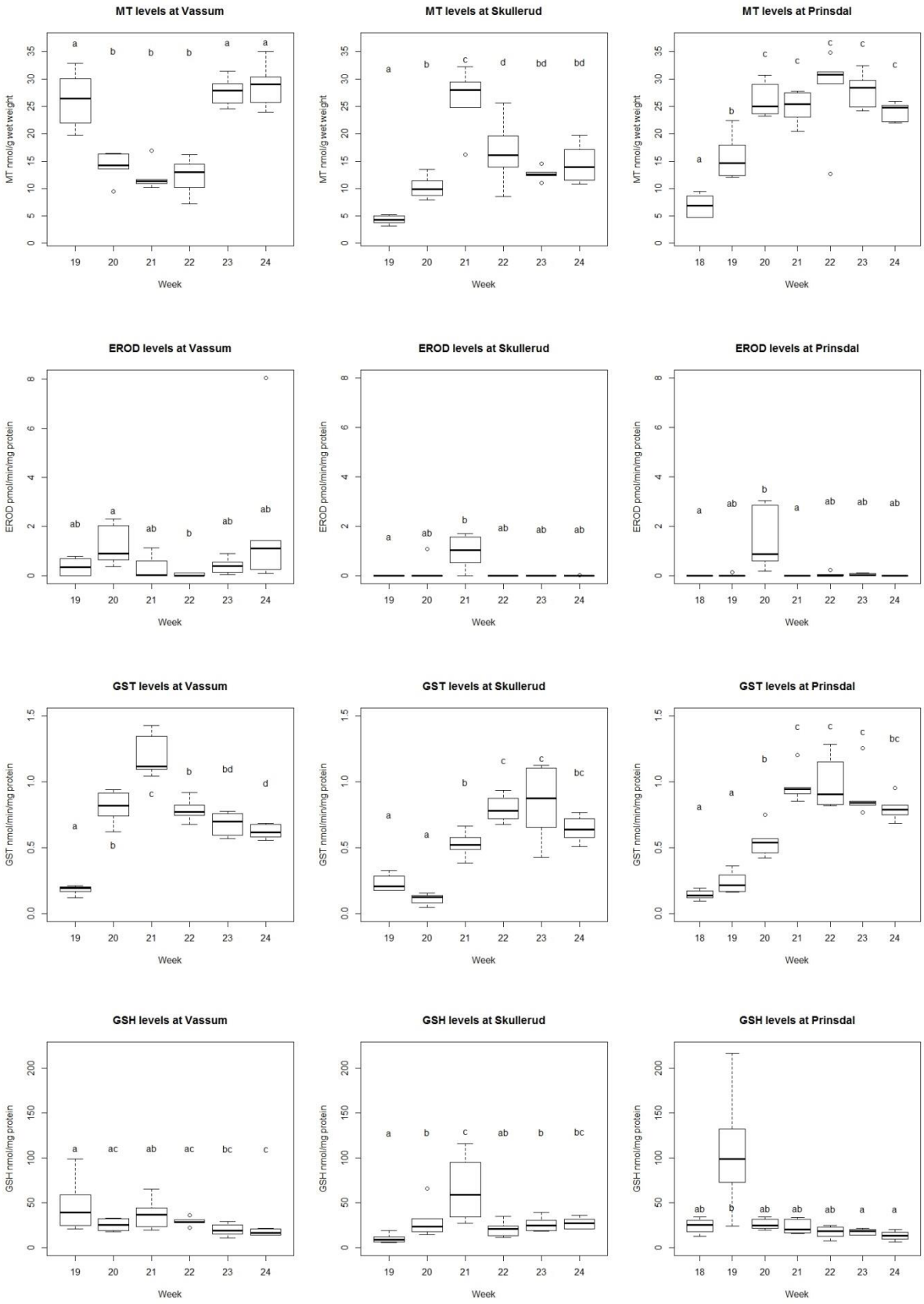

Figure 27. Levels of metallothionein (MT) (nmol/g wet weight), 7-Ethoxyresorufin *O*-deethylase (EROD) (pmol/min/mg protein), glutathione *S*-transferase (GST) (nmol/min/mg protein) and reduced glutathione (GSH) (nmol/mg protein) in tadpoles sampled in May and June 2012. A box constitutes the middle 50 % of the sample values and the remaining 50 % are situated between the box and the whiskers. Circles represent outliers, while the black lines across the boxes show median values. Different letters show significant differences between the samplings within a pond (significance level of p = 0.05). The letters do not denote any difference between samples across sites.

Metallothionein was measured and tested for correlation with overall tissue element concentrations because it is a commonly used biomarker of metal exposure (van der Oost et al. 2003). The tadpoles from the different ponds exhibited very different temporal patterns of MT concentrations. Vassum tadpoles showed a decreasing trend in the beginning from relatively high MT levels at week 19 (median value 26 nmol/g) to about half of that concentration at week 21 (median value 11 nmol/g), while there was an increasing trend at the last samplings. In contrast, both Skullerud and Prinsdal showed an increasing trend at the beginning that was reduced (Skullerud) or levelled out (Prinsdal) at the later samplings. The range of the median values was relatively similar for all sites, being 7 – 31 nmol/g wet weight at Prinsdal, 4 – 28 nmol/g wet weight at Skullerud and 11 – 29 nmol/g wet weight at Vassum. Spearman correlation test did not reveal any significant correlation between overall tissue element concentrations and MT (rho = 0.33, p = 0.17). Correlation between MT and tissue concentrations of Cu, Zn, Cd and Pb was tested separately, as these metals in particular are known to induce MT (van der Oost et al. 2003). The Spearman test showed significant positive correlation between Pb and MT (rho = 0.57, p = 0.01). This may be a result of MT induction by Pb, and is in accordance with Campana et al. (2003) who measured increased hepatic MT levels in the toadfish *Halobatrachus didactylus* after exposure to Pb. However, correlation does not necessarily imply causality. No significant correlation was found for MT and Cu, Zn or Cd (see appendix 6 for rho- and p-values). This is in line with Othman et al. (2012) who reported no significant correlation between hepatic Cd and MT in rice frogs (*Fejervarya limnocharis*). On the other hand, Loumbourdis et al. (2007) reported strong positive correlation between MT and Cd in adult marsh frog (*Rana ridibunda*) exposed to Cr and Cd in a laboratory experiment, and Cooper and Fortin (2010) observed correlation between hepatic MT, Cu and Cd concentrations in a field study of American bullfrogs (*Rana catesbeiana*). Current results may suggest that the Cu, Zn and Cd concentrations in the ponds are too low to induce MT. The values for most of the samplings in the current thesis are higher than induced MT values reported by Papadimitriou and Loumbourdis (2003) for adult female *R. ridibunda*. They measured mean Cu induced MT levels of 5.7 – 8.6 nmol/g wet weight and mean reference values of 2.85 – 3.12 nmol/g wet weight. The relatively high MT levels in the present results strengthen the hypothesis that MT may be induced by Pb.

The EROD (CYP1A) activity was measured because it is a commonly used biomarker of PAHs, which are common pollutants in highway runoff (Meland 2010). The analysis of EROD did not detect CYP1A activity to any great extent. Indeed, at most of the samplings from Skullerud and Prinsdal the EROD activity was not measurable (returned negative mean values, which in practice means zero or negligible concentration). In the box plots these values were replaced with the half of the lowest measured positive value. In the statistical tests the original data with negative values were used. The levels are in accordance with concentrations reported for northern green frog (*Rana clamitans melanota*) tadpoles by Jung et al. (2004) ranging from 0.2 to 0.5 pmol/min/mg protein. The researchers found no correlation between EROD activity and the planar halogenated hydrocarbons (PHHs) that was in focus for the controlled exposure study. Hence the reported concentrations may be regarded as the basal level. It is difficult to establish whether the low concentrations in the current thesis are evidence of the tadpoles not being exposed to PAHs, or if it is due to the biomarker

being inadequate for indicating exposure. Amphibians show lower catalytic activities, and lower and slower induction of the enzymes of the mixed-function oxidase system (MFO) after exposure to xenobiotics, than other animal groups does (Ertl & Winston 1998). Hence CYP1A enzymes may not be sensitive biomarkers in amphibians (Huang et al. 1998; Venturino et al. 2003), and the lack of CYP1A activity may not be an evidence of absence of PAHs. In addition, it has been suggested that tadpoles have less developed CYP450 enzyme activity than adult amphibians (Jung et al. 2004). Certainly, analysis of water samples did not detect any PAHs in any of the ponds, and this may be the reason for the low EROD activity. However, it is still possible that the tadpoles, primarily those in the sedimentation ponds, are exposed to PAHs through ingestion of and contact with sediment. A previous study measured PAH dry weight concentrations of 3.05 mg/kg in the sediment in Vassum sedimentation pond, including several PAHs assumed to be human carcinogens (Meland 2012). The level corresponds to class III in the classification system for environmental quality developed by the Climate and Pollution Agency, characterized by risk of chronic effects by long term exposure (Meland 2012). Polycyclic aromatic hydrocarbons have also been measured in tunnel wash water ending up in Vassum sedimentation pond (Meland et al. 2010b). The tadpoles from Vassum seem to exhibit slightly higher levels of EROD than the others. This may indicate induction by PAH exposure, although this was not tested statistically. On the other hand, the highest median value at Vassum is only 1.1 pmol/min/mg protein, which is well below the concentrations measured in adult amphibians. Murphy et al. (2006) reported EROD concentrations in adult *Rana clamitans* in the range of 5 – 13 pmol/min/mg protein (females) and 13 – 21 pmol/min/mg protein (males) for animals sampled from agricultural and non-agricultural sites. The researchers found no correlation between EROD activity and the herbicide atrazine, which was the contaminant in focus. Rocha-e-Silva et al. (2004) measured EROD levels of 238 nmol/min/mg protein in cane toad (*Bufo marinus*) and 34 nmol/min/mg protein in bullfrog, which is a 30,000-fold greater than the measurements in the current thesis. Test for correlation between EROD and overall tissue element concentrations was performed because of an assumed correlation between tissue element concentrations and PAHs, since sedimentation ponds often contains high concentrations of both trace elements and PAHs (Meland 2012). Polycyclic aromatic hydrocarbons are readily metabolized in living organisms, while trace elements are not. Hence, trace elements are easier to measure accurately in tissue. Consequently, correlation between EROD and overall tissue element concentration was tested as a substitute for correlation between EROD and tissue PAH concentrations. However, no statistically significant correlation between overall tissue element concentrations and EROD activity was found ($r = 0.35$, $p = 0.14$).

Glutathione *S*-transferase was measured and tested for correlation with overall tissue element concentrations because GST is important in the defense against oxidative stress. At all three ponds the GST activities seemed to increase at the early samplings and decrease or level out at the later ones. The median activity ranges was 0.19 – 1.1 nmol/min/mg protein at Vassum, 0.13 – 0.87 nmol/min/mg protein at Skullerud and 0.14 – 0.94 nmol/min/mg protein at Prinsdal. There was significant positive correlation between GST and overall tissue element concentrations ($r = 0.71$, $p < 0.001$). Positive correlation between GST and Pb ($r = 0.81$, $p <$

0.001) and between GST and Cd (r = 0.60, p = 0.0067) was also detected when testing these separately (Fig. 28).

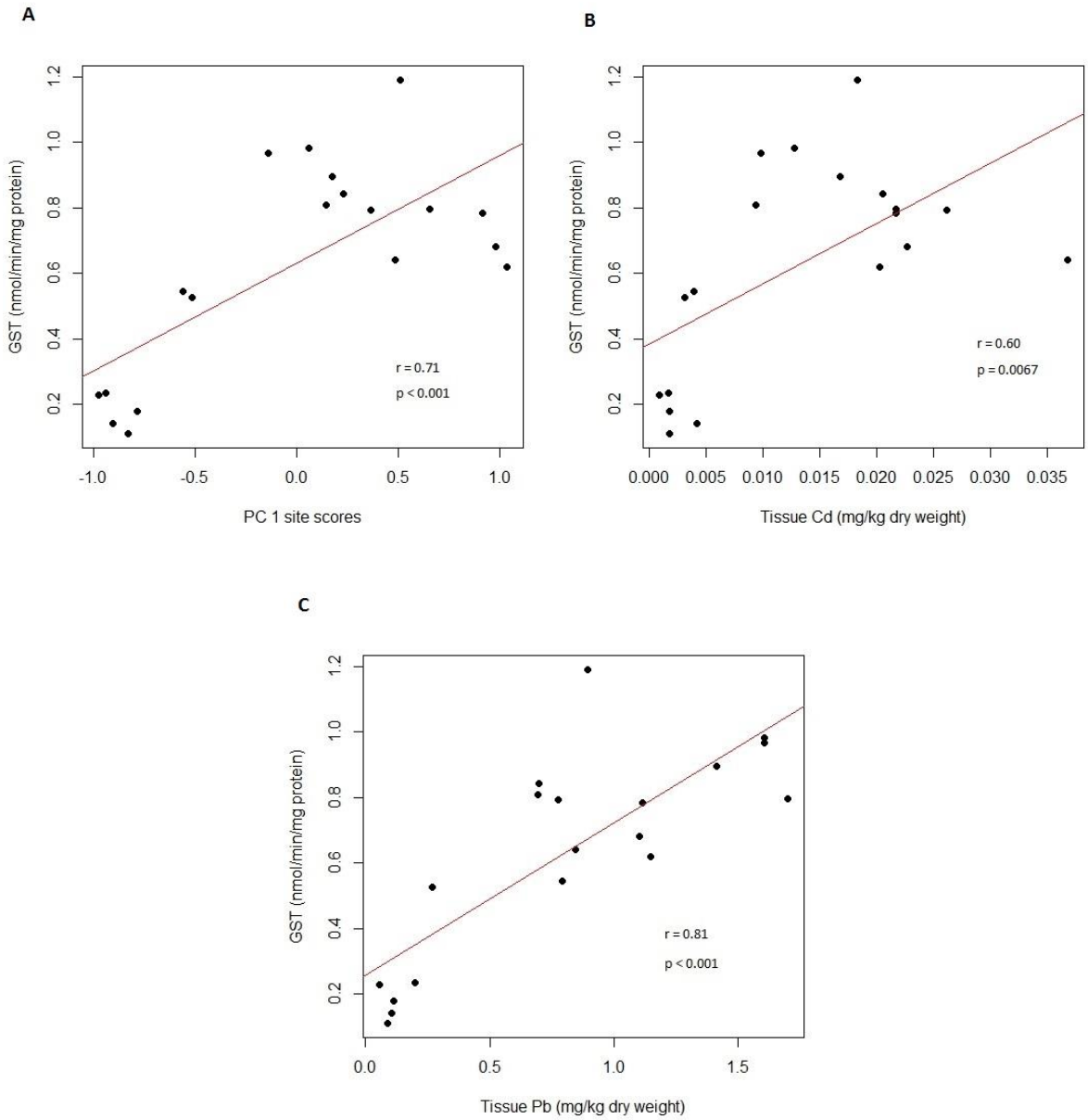

Figure 28. Correlation between glutathione *S*-transferase (GST) activity and overall tissue element concentrations, represented by first principal component site scores (PC 1 site scores) obtained by principal component analysis (PCA) on tissue element concentrations (A). Correlation between GST activity and tissue Cd concentrations (B) and between GST activity and tissue Pb concentrations (C). r = correlation coefficient, p = significance level.

Hepatic GST levels have been measured in rice frogs in the range of 0.20 – 0.33 µmol/min/mg protein at Cd contaminated sites and 0.15 – 0.16 µmol/min/mg protein at a reference site (Othman et al. 2012). This is roughly speaking a 1000-fold greater than the measurements in this thesis, and similar GST activity ranges was reported for marsh frog by

Kostaropoulos et al. (2005). The initial increase in GST activity may indicate GST catalysed conjugation of GSH with xenobiotics, but the low activities compared to other studies may indicate that there is no induction above basal level. However, the low values may be due to differences in life stages as the current thesis investigated tadpoles, while Othman et al. (2012) and Kostaropoulos et al. (2005) studied adult individuals. The strong correlation between GST and overall tissue element concentrations, and between GST and Cd and GST and Pb, may indicate GST being induced by trace element exposure. This is in accordance with Othman et al. 2012 who found strong significant correlation between hepatic GST and Cd in Cd exposed *F. limnocharis*, and Wright et al. (1998) who reported strong correlation between Pb and GST in rats. Based on such observations it has been suggested that GST might be a promising biomarker of exposure to different metals (Othman et al. 2012; Wright et al. 1998), although the literature is not unambiguous as significant negative correlation between hepatic GST and both Cd and Cr in *R. ridibunda* also have been reported (Kostaropoulos et al. 2005). Glutathione *S*-transferase is often regarded a biomarker of organic contaminants (Ahmad et al. 2006; van der Oost et al. 2003; Venturino et al. 2003). In the present thesis overall tissue element concentration may be correlated with concentrations of organic pollutants, as sedimentation ponds often contain high levels of both organic and inorganic contaminants (Meland 2010). For instance, a previous study of the sediment in Vassum sedimentation pond showed high concentrations of both metals and PAHs (Meland 2012). Hence, correlation between GST and overall tissue element concentrations may indirectly represent a correlation with organic pollution, such as PAHs.

Glutathione is a very important antioxidant and is often used as a biomarker of metal induced oxidative stress. The GSH concentrations in tadpoles from all three ponds are in the range of 8.8 to 99 nmol/mg. At Vassum the GSH concentration starts at its maximum level and decreases steadily thereafter. At Prinsdal and Skullerud the GSH concentrations show an increasing trend at the early samplings and decreases after day 8 and 13 respectively. There was a slight negative correlation between GSH and overall tissue element concentrations, although not statistically significant ($r = -0.35$, $p = 0.14$). Papadimitriou and Loumbourdis (2002) performed a controlled exposure study on adult *R. ridibunda* and studied GSH induction following Cu exposure. They reported increased oxidative stress and hepatic GSH levels as a result of Cu exposure. Although GSH is commonly used as a biomarker for oxidative stress with elevated concentrations indicating metal exposure (Kuroshima 1995), decreasing levels may also imply exposure as it may indicate GSH depletion (Papadimitriou & Loumbourdis 2002). This may happen when the availability of reduced GSH decreases to due metal binding or oxidation during conjugation processes, while the regeneration is insufficient. This has been reported for metal exposed freshwater catfish (*Ictalurus melas*) (Elia et al. 2003), metal exposed Antarctic scallop (*Adamussium colbecki*) (Regoli et al. 1998), European eel (*Anguilla anguilla* L.) (Ahmad et al. 2006) and marsh frog (Papadimitriou & Loumbourdis 2002). This mechanism may be at work in the tadpoles in the present thesis, but the lack of any statistically significant positive or negative correlation with overall tissue element concentrations makes it difficult to conclude. Current results may indicate that GSH is the limiting factor for GST activities.

Generally, the lack of a proper reference group makes it difficult to demonstrate whether the measured biomarker levels results from induction by xenobiotics above the basal level. It is also possible that the observed variations in biomarker levels to some extent were due to variations in developmental stage rather than exposure time. A controlled exposure study in a laboratory ensuring comparable developmental stages at all times would be necessary to differentiate between variations due to developmental stage and xenobiotic exposure respectively. This ought to be considered for any further research.

4.3 Tunnel wash event – June 2012

All tadpoles in Vassum sedimentation pond died after washing of the Nordby tunnel 14 – 16 June 2012 (Fig. 29). Over 400 dead individuals were counted, and many of them were undergoing metamorphosis (Johansen & Thygesen 2012). Measured concentrations of selected water quality parameters by the inlet and the outlet of Vassum sedimentation pond during three hours of the washing event, are presented in Table 8. The measurements were conducted as part of an ecological risk evaluation for the Norwegian Public Roads Administration, and not as part of the present thesis. High concentrations of elements such as Na, Cl, Cu and Zn were measured, particularly in the inlet water.

Table 8. Minimum, maximum and mean values of selected water quality parameters during washing of the Nordby tunnel 14 – 16 June 2012. Measurements were taken by the inlet and the outlet of Vassum sedimentation pond every half hour during three hours of the washing (i.e. *n* = 6). Modified after Johansen and Thygesen (2012).

		Inlet water			Outlet water		
	Fraction	Minimum	Maximum	Mean (*n*=6)	Minimum	Maximum	Mean (*n*=6)
DOC (mg/L)		8	192	92	9	24	15
pH		8.2	9.6	9.1[A)]	7.8	8.4	8.3[A)]
Conductivity (ms/cm)		0.0088	7.5	5	3.1	3.3	3.2
Temperature °C		9.7	15	13	13	15	14
Cl (mg/L)	Total	144	2370	1541	891	969	921
Ca (mg/L)	Total	47	114	73	35	37	36
	Dissolved	40	59	49	39	42	40
Mg (mg/L)	Total	6.7	58	30	6.2	6.5	6
	Dissolved	6	16	11	6	6	6
Na (mg/L)	Total	98	1360	899	543	628	597
	Dissolved	111	1440	976	559	614	583
Cd (µg/L)	Total	0.056	2.7	1	0.051	0.056	0.054
	Dissolved	0.047	1.5	0.47	0.0064	0.037	0.018
Cu (µg/L)	Total	23	1010	584	6.9	25	12
	Dissolved	22	307	106	3.3	18	9
Zn (µg/L)	Total	460	20500	11415	66	625	246
	Dissolved	397	9200	4016	41	515	204

[A)] For pH, the median value is given instead of the mean value, due to logarithmic scale.

The exact cause of the tadpoles' death is not certain. The lethal effect of the tunnel wash water may be due to high levels of metals, road salt or detergents, or a combination of these or other factors (Johansen & Thygesen 2012). Although the incident was not directly related to the problems addressed in the present thesis, it was a noteworthy event. It demonstrated that sedimentation ponds, in particular those receiving tunnel wash water, may indeed represent ecological traps for amphibians, as pointed out by Snodgrass et al. (2008) and McCarthy and Lathrop (2011).

Figure 29. Dead *Rana temporaria* tadpoles in Vassum sedimentation pond after washing of the Nordby tunnel 14 – 16 June 2012. All tadpoles in the pond (several hundred) were dead, and many of them were undergoing metamorphosis at that point, as evident by the emerged forelegs on one of the tadpoles in the present picture. Photo: Susanne Lund Johansen.

5 Conclusions

The water quality in Skullerud and Vassum sedimentation ponds was classified as 'poor' or 'very poor' at several samplings according to the Climate and Pollution Agency's classification system due to high total concentrations of Cr, Cu and Zn. The pond at Prinsdal exhibited concentrations of Ni, Cu, Zn and Pb that justified classifying the water quality as 'poor' at several samplings. Polycyclic aromatic hydrocarbons were not detected in any of the ponds. The concentrations of important metals of environmental concern such as Cr, Ni, Cu, Zn, Cd and Pb were slightly lower in Skullerud and Vassum than measurements in earlier studies of the ponds. Surprisingly, the highest concentrations of the majority of the 34 measured elements were identified in Prinsdal. This was probably due to impact from an abandoned shooting range nearby. For practically all of the rest of the elements the highest concentrations were detected in Vassum, while more moderate concentrations of most elements were measured in Skullerud.

The trace elements Al, Cr, Mn, Fe, Mo, La, Ce, Eu and Gd may be of particular ecotoxicological concern in the sedimentation ponds, as they increased strongly in tadpole tissue. In Prinsdal tadpoles particularly Al and Pb accumulated strongly. In all three ponds the frog embryos and tadpoles showed a time dependent accumulation of trace elements. The PC 1 site scores obtained from PCA were used as representative values of the overall tissue trace element levels (referred to as 'overall tissue element concentrations' henceforth). The overall tissue element concentrations reached the highest levels in Vassum tadpoles, despite Prinsdal having the highest total and dissolved water concentrations of most elements. The water element concentrations could not explain the variation in tissue concentrations, as neither total nor dissolved element concentrations in water (represented by PCA axis 1 site scores) showed any statistically significant correlation with overall tissue element concentrations.

The levels of the four biomarkers MT, EROD, GST and GSH showed significant temporal variation in embryos and tadpoles. Metallothionein was positively correlated with Pb. Hence, Pb tissue concentrations can possibly explain some of the MT variation, and may indicate that MT is induced by Pb in tadpoles in the present study. On the other hand, no significant correlation was found between MT and overall tissue element concentrations. Hence, the overall tissue element concentrations could probably not explain the variation in the MT levels. The GSH levels exhibited a decreasing trend in all ponds, which may indicate a depletion of GSH as a result of trace element induced oxidative stress. However, there was no significant correlation between GSH and overall tissue element concentrations. Hence, the overall tissue element concentrations could not explain variation in GSH levels. Strong correlation was found between GST and overall tissue element concentrations, and also between GST and Cd and GST and Pb when testing these separately. This may imply that the organisms experience metal induced oxidative stress, and tissue element concentrations may explain some of the variation in GST activity. The correlation between GST and trace element concentrations in tadpole tissue may suggest that GST is a useful biomarker of trace element exposure. The EROD activity was highest at Vassum where earlier studies have reported considerable concentrations of PAH. Correlation between EROD and overall tissue element

concentrations was tested as a substitute for correlation between EROD and tissue PAH concentrations, since trace elements and PAHs are often correlated in sedimentation ponds and trace elements are easier to measure in tissue. However, overall tissue element concentrations could not explain the variation in EROD, as there was no statistically significant correlation between these parameters.

Altogether, there are indices in the results that *Rana temporaria* tadpoles inhabiting the two sedimentation ponds may be negatively affected by contaminants in highway runoff. The tadpoles at Prinsdal may be negatively affected by contaminants from the abandoned shooting range nearby. The lack of a reference group makes it difficult to conclude if any of the biomarkers are induced above basal levels. Further research in the form of controlled exposure studies in the laboratory, or field studies including a good reference group, is necessary to identify the basal level of biomarkers and to demonstrate any departures from the natural variation. Further investigations are also necessary to identify any links these endpoints and ecologically relevant endpoints such as reproduction and survival.

6 References

Ahmad, I., Pacheco, M. & Santos, M. A. (2006). *Anguilla anguilla* L. oxidative stress biomarkers: An in situ study of freshwater wetland ecosystem (Pateira de Fermentelos, Portugal). *Chemosphere*, 65 (6): 952-962.

Aldrin, M., Hobæk Haff, I. & Rosland, P. (2008). The effect of salting with magnesium chloride on the concentration of particular matter in a road tunnel. *Atmospheric Environment*, 42 (8): 1762-1776.

Amundsen, C. E. & Roseth, R. (2004). Utslippsfaktorer fra veg til vann og jord i Norge. Oslo: Statens vegvesen, Vegdirektoratet. 71 pp.

Amundsen, C. E., French, H., Haaland, S., Pedersen, P. A., Riise, G. & Roseth, R. (2008). Salt SMART Miljøkonsekvenser ved salting av veger - en litteraturgjennomgang. Oslo: Statens vegvesen, Vegdirektoratet. 98 pp.

Barbosa, A. E. & Hvitved-Jacobsen, T. (1999). Highway runoff and potential for removal of heavy metals in an infiltration pond in Portugal. *Science of the Total Environment*, 235 (1-3): 151-159.

Bartsch, R., Klein, D. & Summer, K. (1990). The Cd-Chelex assay: A new sensitive method to determine metallothionein containing zinc and cadmium. *Archives of Toxicology*, 64 (3): 177-180.

Beebee, T. J. C. & Griffiths, R. A. (2005). The amphibian decline crisis: A watershed for conservation biology? *Biological Conservation*, 125 (3): 271-285.

Brady, N. C., Weil, R. R., Brady, N. C. & Weil, R. R. (2010). *Elements of the nature and properties of soils*. Upper Saddle River, N.J.: Prentice Hall. 606 pp.

Brand, A., Snodgrass, J., Gallagher, M., Casey, R. & Meter, R. (2010). Lethal and sublethal effects of embryonic and larval exposure of *Hyla versicolor* to stormwater pond sediments. *Archives of Environmental Contamination and Toxicology*, 58 (2): 325-331.

Brand, A. B. & Snodgrass, J. W. (2010). Value of artificial habitats for amphibian reproduction in altered landscapes. *Conservation Biology*, 24 (1): 295-301.

Brandt, H. C. A. & de Groot, P. C. (2001). Aqueous leaching of polycyclic aromatic hydrocarbons from bitumen and asphalt. *Water Research*, 35 (17): 4200-4207.

Brown, J. N. & Peake, B. M. (2006). Sources of heavy metals and polycyclic aromatic hydrocarbons in urban stormwater runoff. *Science of the Total Environment*, 359 (1–3): 145-155.

Bäckström, M., Karlsson, S., Bäckman, L., Folkeson, L. & Lind, B. (2004). Mobilisation of heavy metals by deicing salts in a roadside environment. *Water Research*, 38 (3): 720-732.

Cai, W. & Stillman, M. J. (1988). Metal binding in metallothioneins: Competition for cadmium and zinc between chelex-100 and metal binding sites in metallothionein. *Inorganica Chimica Acta*, 152 (2): 111-115.

Campana, O., Sarasquete, C. & Blasco, J. (2003). Effect of lead on ALA-D activity, metallothionein levels, and lipid peroxidation in blood, kidney, and liver of the toadfish *Halobatrachus didactylus*. *Ecotoxicology and Environmental Safety*, 55 (1): 116-125.

Camponelli, K. M., Casey, R. E., Snodgrass, J. W., Lev, S. M. & Landa, E. R. (2009). Impacts of weathered tire debris on the development of *Rana sylvatica* larvae. *Chemosphere*, 74 (5): 717-722.

Casey, R. E., Shaw, A. N., Massal, L. R. & Snodgrass, J. W. (2005). Multimedia evaluation of trace metal distribution within stormwater retention ponds in suburban Maryland, USA. *Bulletin of Environmental Contamination and Toxicology*, 74 (2): 273-280.

Chapman, P. M. & Wang, F. (2000). Issues in ecological risk assessment of inorganic metals and metalloids. *Human and Ecological Risk Assessment: An International Journal*, 6 (6): 965-988.

Chapman, P. M. (2008). Environmental risks of inorganic metals and metalloids: A continuing, evolving scientific odyssey. *Human and Ecological Risk Assessment: An International Journal*, 14 (1): 5-40.

Choppin, G. R., Liljenzin, J. O., Rydberg, J. & Choppin, G. R. (2002). *Radiochemistry and nuclear chemistry*. Oxford: Butterworth-Heinemann. 709 pp.

Climate and Pollution Agency. (2012). Utkast til Bakgrunnsdokument for utarbeidelse av miljøkvalitetsstandarder og klassifisering av miljøgifter i vann, sediment og biota. Oslo: Climate and Pollution Agency. 103 pp.

Collins, S. J. & Russell, R. W. (2009). Toxicity of road salt to Nova Scotia amphibians. *Environmental Pollution*, 157 (1): 320-324.

Consortium for Risk Evaluation with Stakeholder Participation. (2006). *Note on: Wet to dry weight conversions for biota from Amchitka and Kiska*: Consortium for Risk Evaluation with Stakeholder Participation. 3 pp.

Cooper, S. & Fortin, C. (2010). Metal and metallothionein content in bullfrogs: Study of a whole watershed impacted by agricultural activities. *Ecotoxicology and Environmental Safety*, 73 (3): 391-399.

Corneliussen, C. H., Meland, S., Hanssen, J. F. & Rosseland, B. O. (2007). Effekter av tunnelvaskemiddel (Remikraft 703) på vekst hos bakterier isolert fra fangdam ved E6. Oslo: Statens vegvesen. 12 pp.

Dalgaard, P. (2008). *Introductory statistics with R*. Second ed. New York: Springer. 363 pp.

Damsgård, M. B. (2011). *Accumulation of heavy metals in benthic invertebrates and frogs from sedimentation ponds receiving runoff from a four lane motorway (E6)* Master's thesis. Ås: Norwegian University of Life Sciences (UMB) Department of Plant and Environmental Sciences (IPM). 80 pp.

Deletic, A. (1998). The first flush load of urban surface runoff. *Water Research*, 32 (8): 2462-2470.

Denoël, M., Bichot, M., Ficetola, G. F., Delcourt, J., Ylieff, M., Kestemont, P. & Poncin, P. (2010). Cumulative effects of road de-icing salt on amphibian behavior. *Aquatic Toxicology*, 99 (2): 275-280.

Desta, M. B., Bruen, M., Higgins, N. & Johnston, P. (2007). Highway runoff quality in Ireland. *Journal of Environmental Monitoring*, 9 (4): 366-371.

Di Toro, D. M., Allen, H. E., Bergman, H. L., Meyer, J. S., Paquin, P. R. & Santore, R. C. (2001). Biotic ligand model of the acute toxicity of metals. 1. Technical Basis. *Environmental Toxicology and Chemistry*, 20 (10): 2383-2396.

Dobrovoljc, K., Falnoga, I., Žnidarič, M., Mazej, D., Ščančar, J. & Bulog, B. (2012). Cd, Cu, Zn, Se, and metallothioneins in two amphibians, *Necturus maculosus* (Amphibia, Caudata) and *Bufo bufo* (Amphibia, Anura). *Biological Trace Element Research*, 150 (1-3): 1-17.

Dolmen, D., Blakar, I. & Skei, J. K. (2004). The distribution of *Rana temporaria* L. (Amphibia) in an acidified and a non-acidified region of Norway *Fauna Norvegica*, 24: 19-29.

Dolmen, D. (2008). *Norske amfibier og reptiler (Feltherpetologisk guide)*. Trondheim: NTNU, Vitenskapsmuseet. 77 pp.

Dolmen, D. (2010). Amphibia and Reptilia. In Kålås, J. A., Viken, Å., Henriksen, S. & Skjelseth, S. (eds). *The 2010 Norwegian Red List for Species.* Norway: Norwegian Biodiversity Information Centre. 480 pp.

Dongarrà, G., Manno, E. & Varrica, D. (2009). Possible markers of traffic-related emissions. *Environmental Monitoring and Assessment*, 154 (1-4): 117-125.

Ek, K. H., Morrison, G. M. & Rauch, S. (2004). Environmental routes for platinum group elements to biological materials - a review. *Science of the Total Environment*, 334–335: 21-38.

Elia, A. C., Galarini, R., Taticchi, M. I., Dörr, A. J. M. & Mantilacci, L. (2003). Antioxidant responses and bioaccumulation in *Ictalurus melas* under mercury exposure. *Ecotoxicology and Environmental Safety*, 55 (2): 162-167.

Ertl, R. P. & Winston, G. W. (1998). The microsomal mixed function oxidase system of amphibians and reptiles: components, activities and induction. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 121 (1–3): 85-105.

Fairbrother, A., Wenstel, R., Sappington, K. & Wood, W. (2007). Framework for metals risk assessment. *Ecotoxicology and Environmental Safety*, 68 (2): 145-227.

Finkelstein, M. M., Jerrett, M. & Sears, M. R. (2004). Traffic air pollution and mortality rate advancement periods. *American Journal of Epidemiology*, 160 (2): 173-177.

Flecker, A. S., Feifarek, B. P. & Taylor, B. W. (1999). Ecosystem engineering by a tropical tadpole: Density-dependent effects on habitat structure and larval growth rates. *Copeia*, 1999 (2): 495-500.

Färm, C. (2002). Evaluation of the accumulation of sediment and heavy metals in a storm-water detention pond. *Water Science and Technology*, 45 (7): 105-112.

Gestel, C. A. M. & Brummelen, T. C. (1996). Incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. *Ecotoxicology*, 5 (4): 217-225.

Glaser, B., Dreyer, A., Bock, M., Fiedler, S., Mehring, M. & Heitmann, T. (2005). Source apportionment of organic pollutants of a highway-traffic-influenced urban area in Bayreuth (Germany) using biomarker and stable carbon isotope signatures. *Environmental Science & Technology*, 39 (11): 3911-3917.

Goonan, T. G. (2011). Rare earth elements - End use and recyclability. Reston, Virginia: U.S. Geological Survey. 15 pp.

Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*, 16 (3): 183-190.

Hagger, J. A., Jones, M. B., Leonard, D. R. P., Owen, R. & Galloway, T. S. (2006). Biomarkers and integrated environmental risk assessment: Are there more questions than answers? *Integrated Environmental Assessment and Management*, 2 (4): 312-329.

Hansen, V. T. & Grimenes, A. A. (2013). Meteorologiske data for Ås 2012. Ås: Institutt for matematiske realfag og teknologi, Universitetet for miljø- og biovitenskap. 17 pp.

Heier, L. S., Lien, I. B., Strømseng, A. E., Ljønes, M., Rosseland, B. O., Tollefsen, K.-E. & Salbu, B. (2009). Speciation of lead, copper, zinc and antimony in water draining a shooting range - Time dependant metal accumulation and biomarker responses in brown trout (*Salmo trutta* L.). *Science of the Total Environment*, 407 (13): 4047-4055.

Heier, L. S., Meland, S., Ljønes, M., Salbu, B. & Strømseng, A. E. (2010). Short-term temporal variations in speciation of Pb, Cu, Zn and Sb in a shooting range runoff stream. *Science of the Total Environment*, 408 (11): 2409-2417.

Hofer, R., Lackner, R. & Lorbeer, G. (2005). Accumulation of toxicants in tadpoles of the common frog (*Rana temporaria*) in high mountains. *Archives of Environmental Contamination and Toxicology*, 49 (2): 192-199.

Hopkin, S. P. (1993). Ecological implications of '95% protection levels' for metals in soil. *Oikos*, 66 (1): 137-141.

Hopkins, G. R., French, S. S. & Brodie Jr, E. D. (2013). Increased frequency and severity of developmental deformities in rough-skinned newt (*Taricha granulosa*) embryos exposed to road deicing salts (NaCl & $MgCl_2$). *Environmental Pollution*, 173: 264-269.

Huang, Y.-w., Melancon, M. J., Jung, R. E. & Karasov, W. H. (1998). Induction of cytochrome P450-associated monooxygenases in northern leopard frogs, *Rana pipiens*, by 3,3',4,4',5-Pentachlorobiphenyl. *Environmental Toxicology and Chemistry*, 17 (8): 1564-1569.

Hylland, K., Ruus, A., Børseth, J. F., Bechmann, R., Barsiene, J., Grung, M., Tollefsen, K. E. & Myhre, L.-P. (2006). Biomarkers in monitoring - a review. Oslo: NIVA. 106 pp.

Johansen, S. L. & Thygesen, H. (2012). *Økologisk risikovurdering av vegavrenning ved bruk av biotisk ligandmodell*. Oslo: Statens vegvesen, Vegdirektoratet. 47 pp. Unpublished manuscript.

Jung, R. E., Karasov, W. H. & Melancon, M. J. (2004). Cytochrome P450 activity in green frogs (*Rana clamitans melanota*) exposed to water and sediments in the Fox River and Green Bay, Wisconsin, USA. *Bulletin of Environmental Contamination and Toxicology*, 73 (6): 955-962.

Kamencic, H., Lyon, A., Paterson, P. G. & Juurlink, B. H. J. (2000). Monochlorobimane fluorometric method to measure tissue glutathione. *Analytical Biochemistry*, 286 (1): 35-37.

Karlsson, K. & Viklander, M. (2008). Polycyclic aromatic hydrocarbons (PAH) in water and sediment from gully pots. *Water, Air, and Soil Pollution*, 188 (1-4): 271-282.

Kauri, H. (1981). Amfibiene. In Frislid, R. & Semb-Johansson, A. (eds) *Norges dyr - tredje bind: fisker, amfibier, krypdyr*, p. 366-381. Oslo: J.W. Cappelens forlag.

Kayhanian, M., Stransky, C., Bay, S., Lau, S. L. & Stenstrom, M. K. (2008). Toxicity of urban highway runoff with respect to storm duration. *Science of the Total Environment*, 389 (2–3): 386-406.

Kelepertzis, E., Argyraki, A., Valakos, E. & Daftsis, E. (2012). Distribution and accumulation of metals in tadpoles inhabiting the metalliferous streams of eastern Chalkidiki, Northeast Greece. *Archives of Environmental Contamination and Toxicology*, 63 (3): 409-420.

Kostaropoulos, I., Kalmanti, D., Theodoropoulou, B. & Loumbourdis, N. (2005). Effects of exposure to a mixture of cadmium and chromium on detoxification enzyme (GST, P450-MO) activities in the frog *Rana ridibunda*. *Ecotoxicology*, 14 (4): 439-447.

Kuroshima, R. (1995). Hepatic metallothionein and glutathione levels in Red Sea bream. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 110 (1): 95-100.

Kuzmin, S., Ishchenko, V., Tuniyev, B., Beebee, T., Andreone, F., Nyström, P., Anthony, B., Schmidt, B., Ogrodowczyk, A., Ogielska, M., et al. (2009). *Rana temporaria. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.1*: IUCN. Available at: http://www.iucnredlist.org/details/58734/0 (accessed: 1 october 2012).

Köck, G., Triendl, M. & Hofer, R. (1996). Seasonal patterns of metal accumulation in Arctic char (*Salvelinus alpinus*) from an oligotrophic Alpine lake related to temperature. *Canadian Journal of Fisheries and Aquatic Sciences*, 53 (4): 780-786.

Le Viol, I., Mocq, J., Julliard, R. & Kerbiriou, C. (2009). The contribution of motorway stormwater retention ponds to the biodiversity of aquatic macroinvertebrates. *Biological Conservation*, 142 (12): 3163-3171.

Le Viol, I., Chiron, F., Julliard, R. & Kerbiriou, C. (2012). More amphibians than expected in highway stormwater ponds. *Ecological Engineering*, 47: 146-154.

Lepš, J. & Šmilauer, P. (2003). *Multivariate analysis of ecological data using CANOCO*. Cambridge: Cambridge University Press. 269 pp.

Lin, C.-C., Chen, S.-J., Huang, K.-L., Hwang, W.-I., Chang-Chien, G.-P. & Lin, W.-Y. (2005). Characteristics of metals in nano/ultrafine/fine/coarse particles collected beside a heavily trafficked road. *Environmental Science & Technology*, 39 (21): 8113-8122.

Lindgren, Å. (1998). *Road construction materials as a source of pollutants*. Doctoral PhD: Luleå University of Technology, Division of Traffic Engeneering, Department of Environmental Engeneering 130 pp.

Loumbourdis, N. S., Kostaropoulos, I., Theodoropoulou, B. & Kalmanti, D. (2007). Heavy metal accumulation and metallothionein concentration in the frog *Rana ridibunda* after exposure to chromium or a mixture of chromium and cadmium. *Environmental Pollution*, 145 (3): 787-792.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the folin phenol reagent *Journal of Biological Chemistry*, 193 (1): 265-275.

Lushchak, V. I. (2011). Environmentally induced oxidative stress in aquatic animals. *Aquatic Toxicology*, 101 (1): 13-30.

Marr, L. C., Kirchstetter, T. W., Harley, R. A., Miguel, A. H., Hering, S. V. & Hammond, S. K. (1999). Characterization of polycyclic aromatic hydrocarbons in motor vehicle fuels and exhaust emissions. *Environmental Science & Technology*, 33 (18): 3091-3099.

Marsalek, J. (2003). Road salts in urban stormwater: an emerging issue in stormwater management in cold climates. *Water Science and Technology*, 48 (9): 61-70.

McCarthy, K. & Lathrop, R. (2011). Stormwater basins of the New Jersey coastal plain: Subsidies or sinks for frogs and toads? *Urban Ecosystems*, 14 (3): 395-413.

McGeer, J. C., Brix, K. V., Skeaff, J. M., DeForest, D. K., Brigham, S. I., Adams, W. J. & Green, A. (2003). Inverse relationship between bioconcentration factor and exposure concentration for metals: Implications for hazard assessment of metals in the aquatic environment. *Environmental Toxicology and Chemistry*, 22 (5): 1017-1037.

McKenzie, E. R., Money, J. E., Green, P. G. & Young, T. M. (2009). Metals associated with stormwater-relevant brake and tire samples. *Science of the Total Environment*, 407 (22): 5855-5860.

Meland, S. (2010). *Ecotoxicological effects of highway and tunnel wash water runoff*. Doctoral PhD. Ås: Norwegian University of Life Sciences, Department of Plant and Environmental Sciences. 161 pp.

Meland, S., Borgstrøm, R., Heier, L. S., Rosseland, B. O., Lindholm, O. & Salbu, B. (2010a). Chemical and ecological effects of contaminated tunnel wash water runoff to a small Norwegian stream. *Science of the Total Environment*, 408 (19): 4107-4117.

Meland, S., Heier, L. S., Salbu, B., Tollefsen, K. E., Farmen, E. & Rosseland, B. O. (2010b). Exposure of brown trout (*Salmo trutta* L.) to tunnel wash water runoff — Chemical characterisation and biological impact. *Science of the Total Environment*, 408 (13): 2646-2656.

Meland, S., Salbu, B. & Rosseland, B. O. (2010c). Ecotoxicological impact of highway runoff using brown trout (*Salmo trutta* L.) as an indicator model. *Journal of Environmental Monitoring*, 12 (3): 654-664.

Meland, S. (2012). Kjemisk karakterisering av sediment fra Vassum sedimenteringsbasseng. *Statens vegvesens rapporter*. Oslo: Vegdirektoratet - Trafikksikkerhet, miljø- og teknologiavdelingen - Miljø. 19 pp.

Monsrud, J., Wethal, A. W., Engelien, E. & Steinnes, M. (2011). Kjøretøypark og infrastruktur. In Brunvoll, F. & Monsrud, J. (eds). *Samferdsel og miljø 2011 - Utvalgte indikatorer for samferdselssektoren*. Oslo Statistisk sentralbyrå. 163 pp.

Murphy, M. B., Hecker, M., Coady, K. K., Tompsett, A. R., Jones, P. D., Newsted, J. L., Wong, H. L., du Preez, L. H., Solomon, K. R., Carr, J. A., et al. (2006). Sediment TCDD-EQs and EROD and MROD Activities in Ranid Frogs from Agricultural and Nonagricultural Sites in Michigan (USA). *Archives of Environmental Contamination and Toxicology*, 51 (3): 467-477.

Napier, F., D'Arcy, B. & Jefferies, C. (2008). A review of vehicle related metals and polycyclic aromatic hydrocarbons in the UK environment. *Desalination*, 226 (1–3): 143-150.

Norrström, A. C. & Jacks, G. (1998). Concentration and fractionation of heavy metals in roadside soils receiving de-icing salts. *Science of the Total Environment*, 218 (2–3): 161-174.

Novotny, E. V., Murphy, D. & Stefan, H. G. (2008). Increase of urban lake salinity by road deicing salt. *Science of the Total Environment*, 406 (1–2): 131-144.

Olsvik, P. A., Gundersen, P., Andersen, R. A. & Zachariassen, K. E. (2001). Metal accumulation and metallothionein in brown trout, *Salmo trutta*, from two Norwegian rivers differently contaminated with Cd, Cu and Zn. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 128 (2): 189-201.

Othman, M. S., Khonsue, W., Kitana, J., Thirakhupt, K., Robson, M., Borjan, M. & Kitana, N. (2012). Hepatic metallothionein and glutathione-*S*-transferase responses in two populations of rice frogs, *Fejervarya limnocharis*, naturally exposed to different environmental cadmium levels. *Bulletin of Environmental Contamination and Toxicology*, 89 (2): 225-228.

Papadimitriou, E. & Loumbourdis, N. S. (2002). Exposure of the frog *Rana ridibunda* to copper: impact on two biomarkers, lipid peroxidation, and glutathione. *Bulletin of Environmental Contamination and Toxicology*, 69 (6): 0885-0891.

Papadimitriou, E. A. & Loumbourdis, N. S. (2003). Copper kinetics and hepatic metallothionein levels in the frog *Rana ridibunda*, after exposure to $CuCl_2$. *BioMetals*, 16 (2): 271-277.

Paquin, P. R., Santore, R. C., Wu, K. B., Kavvadas, C. D. & Di Toro, D. M. (2000). The biotic ligand model: a model of the acute toxicity of metals to aquatic life. *Environmental Science & Policy*, 3, Supplement 1 (0): 175-182.

Peakall, D. (1994). The role of biomarkers in environmental assessment (1). Introduction. *Ecotoxicology*, 3 (3): 157-160.

Peakall, D. B. & Walker, C. H. (1994). The role of biomarkers in environmental assessment (3). Vertebrates. *Ecotoxicology*, 3 (3): 173-179.

Preciado, H. & Li, L. (2006). Evaluation of metal loadings and bioavailability in air, water and soil along two highways of British Columbia, Canada. *Water, Air, & Soil Pollution*, 172 (1): 81-108.

R Core Team. (2012). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Ramakrishna, D. & Viraraghavan, T. (2005). Environmental impact of chemical deicers – a review. *Water, Air, and Soil Pollution*, 166 (1-4): 49-63.

Ranvestel, A. W., Lips, K. R., Pringle, C. M., Whiles, M. R. & Bixby, R. J. (2004). Neotropical tadpoles influence stream benthos: evidence for the ecological consequences of decline in amphibian populations. *Freshwater Biology*, 49 (3): 274-285.

Ravindra, K., Sokhi, R. & Van Grieken, R. (2008). Atmospheric polycyclic aromatic hydrocarbons: Source attribution, emission factors and regulation. *Atmospheric Environment*, 42 (13): 2895-2921.

Regoli, F., Nigro, M. & Orlando, E. (1998). Lysosomal and antioxidant responses to metals in the Antarctic scallop *Adamussium colbecki*. *Aquatic Toxicology*, 40 (4): 375-392.

Riis, N. (1991). A field study of survival, growth, biomass and temperature dependence of *Rana dalmatina* and *Rana temporaria* larvae. *Amphibia-Reptilia*, 12 (3): 229-243.

Rocha-e-Silva, T. A. A., Rossa, M. M., Rantin, F. T., Matsumura-Tundisi, T., Tundisi, J. G. & Degterev, I. A. (2004). Comparison of liver mixed-function oxygenase and antioxidant enzymes in vertebrates. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 137 (2): 155-165.

Sansalone, J. & Buchberger, S. (1996). Characterization of metals and solids in urban highway winter snow and spring rainfall-runoff. *Transportation Research Record: Journal of the Transportation Research Board*, 1523 (1): 147-159.

Sanzo, D. & Hecnar, S. J. (2006). Effects of road de-icing salt (NaCl) on larval wood frogs (*Rana sylvatica*). *Environmental Pollution*, 140 (2): 247-256.

Savage, R. M. (1962). *The ecology and life history of the common frog (Rana temporaria temporaria)*. New York: Hafner Pub. Co. 221 pp.

Sies, H. (1999). Glutathione and its role in cellular functions. *Free Radical Biology and Medicine*, 27 (9–10): 916-921.

Skei, J. K. (2006). *Conservation biology and acidification problems in the breeding habitat of amphibians in Norway*. Doctoral PhD. Trondheim: Norwegian University of Science and Technology, Department of Biology, Faculty of Natural Sciences and Technology. 204 pp.

Snilsberg, P., Roseth, R. & Amundsen, C. E. (2002). Naturbaserte behandlingsanlegg for vegavrenning : undersøkelse av rensegrad og anleggsfunksjon for tre anlegg på ny E6 Korsegården-Vassum i Ås kommune i 2000 og 2001. Ås: Jordforsk. 53 pp.

Snodgrass, J. W., Hopkins, W. A., Broughton, J., Gwinn, D., Baionno, J. A. & Burger, J. (2004). Species-specific responses of developing anurans to coal combustion wastes. *Aquatic Toxicology*, 66 (2): 171-182.

Snodgrass, J. W., Casey, R. E., Joseph, D. & Simon, J. A. (2008). Microcosm investigations of stormwater pond sediment toxicity to embryonic and larval amphibians: Variation in sensitivity among species. *Environmental Pollution*, 154 (2): 291-297.

Sparling, D. W. & Lowe, T. P. (1996). Metal concentrations of tadpoles in experimental ponds. *Environmental Pollution*, 91 (2): 149-159.

Sparling, D. W., Linder, G., Bishop, C. A. & Krest, S. K. (eds). (2010). *Ecotoxicology of amphibians and reptiles*. Second ed. Boca Raton: CRC Press. 916 pp.

Sriyaraj, K. & Shutes, R. B. E. (2001). An assessment of the impact of motorway runoff on a pond, wetland and stream. *Environment International*, 26 (5–6): 433-439.

Statens vegvesen. (2008). *The environmental vision*. vegvesen.no: Statens vegvesen. Available at: http://www.vegvesen.no/en/Professional/Environment/Environmental+vision (accessed: 22 March 2012).

Statens vegvesen. (2010). Håndbok 111: Standard for drift og vedlikehold av veger og gater - Høringsutgave 23. mars 2010. Oslo: Statens vegvesen, Vegdirektoratet 125 pp.

Statens vegvesen. (2011). *Trafikkarbeid*. vegvesen.no: Statens vegvesen. Available at: http://www.vegvesen.no/Fag/Trafikk/Nokkeltall+transport/Trafikk (accessed: 20 November 2012).

Statens vegvesen. (2012a). *ÅDT nivå1-punkt Akershus fylke*. Available at: http://www.vegvesen.no/_attachment/62347/binary/621131 (accessed: 11 November 2012).

Statens vegvesen. (2012b). *ÅDT nivå1-punkt Oslo*. Available at: http://www.vegvesen.no/_attachment/62348/binary/670678 (accessed: 11 November 2012).

Sternbeck, J., Sjödin, Å. & Andréasson, K. (2002). Metal emissions from road traffic and the influence of resuspension—results from two tunnel studies. *Atmospheric Environment*, 36 (30): 4735-4744.

Strand, L. Å. (2006). Amfibienes bruk av dammer og tjern i marka, Oslo kommune. Oslo. 24 pp.

Taylor, K. G. & Robertson, D. J. (2009). Electron microbeam analysis of urban road-deposited sediment, Manchester, UK: Improved source discrimination and metal speciation assessment. *Applied Geochemistry*, 24 (7): 1261-1269.

The European Parliament and the Council of the European Union. (2000). *Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy*. Official Journal - 2000 - L 327: EUR-lex. 82 pp.

Thorpe, A. & Harrison, R. M. (2008). Sources and properties of non-exhaust particulate matter from road traffic: A review. *Science of the Total Environment*, 400 (1–3): 270-282.

U.S. Environmental Protection Agency. (2000). Guidance for data quality assessment - Practical methods for data analysis. Washington, DC. 219 pp.

van der Oost, R., Beyer, J. & Vermeulen, N. P. E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology*, 13 (2): 57-149.

Van Metre, P. C., Mahler, B. J. & Furlong, E. T. (2000). Urban sprawl leaves its PAH signature. *Environmental Science & Technology*, 34 (19): 4064-4070.

VanLoon, G. W. & Duffy, S. J. (2011). *Environmental chemistry: a global perspective*. Oxford: Oxford University Press. 545 pp.

Venturino, A., Rosenbaum, E., Caballero De Castro, A., Anguiano, O. L., Gauna, L., Fonovich De Schroeder, T. & Pechen De D'Angelo, A. M. (2003). Biomarkers of effect in toads and frogs. *Biomarkers*, 8 (3-4): 167-186.

Viklander, M., Marsalek, J., Malmquist, P. A. & Watt, W. E. (2003). Urban drainage and highway runoff in cold climates: conference overview. *Water science and technology : a journal of the International Association on Water Pollution Research*, 48 (9): 1-10.

Walker, C. H. (2006). *Principles of ecotoxicology*. Boca Raton, Fla.: CRC Taylor & Francis. 315 pp.

Wang, J., Jia, C. R., Wong, C. K. & Wong, P. K. (2000). Characterization of polycyclic aromatic hydrocarbons created in lubricating Oils. *Water, Air, and Soil Pollution*, 120 (3-4): 381-396.

Wang, W. & Ballatori, N. (1998). Endogenous glutathione conjugates: Occurrence and biological functions. *Pharmacological Reviews*, 50 (3): 335-355.

Wang, Y.-F., Huang, K.-L., Li, C.-T., Mi, H.-H., Luo, J.-H. & Tsai, P.-J. (2003). Emissions of fuel metals content from a diesel vehicle engine. *Atmospheric Environment*, 37 (33): 4637-4643.

Watanabe, M., Henmi, K., Ogawa, K. i. & Suzuki, T. (2003). Cadmium-dependent generation of reactive oxygen species and mitochondrial DNA breaks in photosynthetic and non-photosynthetic strains of Euglena gracilis. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 134 (2): 227-234.

Weckwerth, G. (2001). Verification of traffic emitted aerosol components in the ambient air of Cologne (Germany). *Atmospheric Environment*, 35 (32): 5525-5536.

Westerlund, C., Viklander, M. & Backstrom, M. (2003). Seasonal variations in road runoff quality in Lulea, Sweden. *Water Sci Technol*, 48 (9): 93-101.

Westerlund, C. & Viklander, M. (2006). Particles and associated metals in road runoff during snowmelt and rainfall. *Science of the Total Environment*, 362 (1-3): 143-156.

Whiteley, J. D. & Murray, F. (2005). Autocatalyst-derived platinum, palladium and rhodium (PGE) in infiltration basin and wetland sediments receiving urban runoff. *Science of the Total Environment*, 341 (1–3): 199-209.

Wicklund, A. & Runn, P. (1988). Calcium effects on cadmium uptake, redistribution, and elimination in minnows, Phoxinus phoxinus, acclimated to different calcium concentrations. *Aquatic Toxicology*, 13 (2): 109-121.

Wood, C. M. (2012). An introduction to metals in fish physiology and toxicology: basic principles In Wood, C. M., Farrell, A. P. & Brauner, C. J. (eds) vol. 31a *Homeostasis and toxicology of essential metals*, p. 1-51. New York: Academic Press.

Wright, L. S., Kornguth, S. E., Oberley, T. D. & Siegel, F. L. (1998). Effects of lead on glutathione *S*-transferase expression in rat kidney: A dose - response study. *Toxicological Sciences*, 46 (2): 254-259.

Åstebøl, S. O., Røhr, P. K. & Pedersen, P. A. (2002). Effekter av veisalting på jord, vann og vegetasjon - Rapport for perioden 1998-2001. Oslo: Institutt for plantefag, Norges landbrukshøgskole og Interconsult ASA. 42 pp.

Åstebøl, S. O. (2005). *Overvåking av rensebasseng for overvann fra E6 Skullerudkrysset i Oslo, 2003-2004*, vol. 2005/02. Oslo: Utbyggingavdelingen, Vegdirektoratet. 29 pp.

Appendix 1 Limit of detection (LD) and limit of quantification (LQ) in the ICP-MS data

Appendix 1.1. Limit of detection (LD) and limit of quantification (LQ) for elements analysed by ICP-MS in water samples.

Element	Unit	LD	LQ
Na	Mg/L	0.50	1.6
Mg	Mg/L	0.00040	0.0010
Al	µg/L	2.6	8.7
Si	µg/L	111	371
P	Mg/L	0.0020	0.0050
S	Mg/L	1.0	3.3
K	Mg/L	0.060	0.21
Ca	Mg/L	0.0030	0.010
Sc	µg/L	0.0060	0.021
Cr	µg/L	0.080	0.25
Mn	µg/L	0.011	0.037
Fe	µg/L	1.8	6.1
Co	µg/L	0.0030	0.0090
Ni	µg/L	0.17	0.56
Cu	µg/L	0.18	0.59
Zn	µg/L	0.20	0.65
As	µg/L	0.030	0.11
Se	µg/L	0.080	0.27
Sr	µg/L	0.23	0.78
Mo	µg/L	0.050	0.16
Ag	µg/L	0.0090	0.029
Cd	µg/L	0.0040	0.015
Sn	µg/L	0.26	0.86
Sb	µg/L	0.013	0.042
Ba	µg/L	8.0	26
La	µg/L	0.0010	0.0020
Ce	µg/L	0.0020	0.0050
Eu	µg/L	0.0020	0.0060
Yb	µg/L	0.0030	0.0080
Gd	µg/L	0.0010	0.0020
Lu	µg/L	0.0010	0.0020
Pb	µg/L	0.022	0.073
Th	µg/L	0.0060	0.020
U	µg/L	0.0030	0.011

Appendix 1.2. Limit of detection (LD) and limit of quantification (LQ) for elements analysed by ICP-MS in tadpole tissue.

Element	Unit	LD	LQ
Na	g/kg	0.0030	0.0090
Mg	g/kg	0.00020	0.00060
Al	mg/kg	0.80	2.7
Si	mg/kg	3.0	11
P	g/kg	0.00040	0.0015
S	g/kg	0.0016	0.0055
K	g/kg	0.0012	0.0039
Ca	g/kg	0.0016	0.0055
Sc	mg/kg	0.00040	0.0013
Cr	mg/kg	0.0060	0.021
Mn	mg/kg	0.0040	0.012
Fe	mg/kg	0.12	0.39
Co	mg/kg	0.060	0.20
Ni	mg/kg	0.020	0.066
Cu	mg/kg	0.022	0.072
Zn	mg/kg	0.21	0.70
As	mg/kg	0.0014	0.0047
Se	mg/kg	0.040	0.14
Sr	mg/kg	0.010	0.035
Mo	mg/kg	0.0013	0.0044
Ag	mg/kg	0.0019	0.0062
Cd	mg/kg	0.0015	0.0051
Sn	mg/kg	0.080	0.27
Sb	mg/kg	0.0037	0.0123
Ba	mg/kg	0.0089	0.0295
La	mg/kg	0.00030	0.0012
Ce	mg/kg	0.00040	0.0015
Eu	mg/kg	0.00020	0.00080
Yb	mg/kg	0.00040	0.0014
Gd	mg/kg	0.00030	0.0011
Lu	mg/kg	0.00020	0.00080
Pb	mg/kg	0.0055	0.0183
Th	mg/kg	0.00040	0.0014
U	mg/kg	0.00040	0.0012

Appendix 1.3. Limit of quantification (LQ) for polycyclic aromatic hydrocarbons (PAH) analysed at ALS Laboratory Group Norway at Skøyen (Oslo).

Element	Unit	LQ
Naphthalene	µg/l	<0.100
Acenaphthylene	µg/l	<0.010
Acenaphthene	µg/l	<0.010
Fluorene	µg/l	<0.020
Phenanthrene	µg/l	<0.030
Anthracene	µg/l	<0.020
Fluoranthene	µg/l	<0.030
Pyrene	µg/l	<0.060
Benz(*a*)anthracene*	µg/l	<0.010
Chrysene*	µg/l	<0.010
Benzo(*b*)fluoranthene*	µg/l	<0.010
Benzo(*k*)fluoranthene*	µg/l	<0.010
Benzo(*a*)pyrene*	µg/l	<0.020
Dibenz(*a,h*)anthracene*	µg/l	<0.010
Benzo(*ghi*)perylene*	µg/l	<0.010
Indeno(*123cd*)pyrene*	µg/l	<0.010
Sum PAH-16	µg/l	Not detected

*** Carcinogenic**

Appendix 2 Accuracy of measurement of the certified reference material (CRM)

Appendix 2.1. Certified values compared with measured values of constituent elements in the certified reference material 1643h used in ICP-MS analysis of water samples.

Element		Unit	1643H (I)	1643H (II)	1643H (III)	Mean difference (%)
Na	Measured value	Mg/L	19	19	20	
	Certified value	Mg/L	20.74	20.74	20.74	
	Difference	Mg/L	1.7	1.7	0.74	
	Difference	%	8.4	8.4	3.6	6.8
P	Measured value	Mg/L	2.5	2.5	2.5	
	Certified value	Mg/L	2.5	2.5	2.5	
	Difference	Mg/L	0.0065	0.014	0.025	
	Difference	%	0.26	0.57	1.0	0.61
S	Measured value	Mg/L	2.4	2.4	2.4	
	Certified value	Mg/L	2.5	2.5	2.5	
	Difference	Mg/L	0.13	0.12	0.13	
	Difference	%	5.1	4.8	5.4	5.1
Mg	Measured value	Mg/L	7.7	7.7	7.6	
	Certified value	Mg/L	8.037	8.037	8.037	
	Difference	Mg/L	0.34	0.34	0.44	
	Difference	%	4.2	4.2	5.4	4.6
Al	Measured value	µg/L	140	140	140	
	Certified value	µg/L	141.8	141.8	141.8	
	Difference	µg/L	1.8	1.8	1.8	
	Difference	%	1.3	1.3	1.3	1.3
K	Measured value	Mg/L	2.0	2.0	1.9	
	Certified value	Mg/L	2.034	2.034	2.034	
	Difference	Mg/L	0.034	0.034	0.13	
	Difference	%	1.7	1.7	6.6	3.3
Ca	Measured value	Mg/L	31	31	28	
	Certified value	Mg/L	32.3	32.3	32.3	
	Difference	Mg/L	1.3	1.3	4.3	
	Difference	%	4.0	4.0	13.3	7.1
Cr	Measured value	µg/L	21	21	21	
	Certified value	µg/L	20.4	20.4	20.4	
	Difference	µg/L	0.6	0.6	0.6	
	Difference	%	2.9	2.9	2.9	2.9
Mn	Measured value	µg/L	38	38	38	
	Certified value	µg/L	38.97	38.97	38.97	
	Difference	µg/L	0.97	0.97	0.97	
	Difference	%	2.5	2.5	2.5	2.5

(***continued***)

Appendix 2.1 continued

Element		Unit	1643H (I)	1643H (II)	1643H (III)	Mean difference (%)
Fe	Measured value	µg/L	100	100	100	
	Certified value	µg/L	98.1	98.1	98.1	
	Difference	µg/L	1.9	1.9	1.9	
	Difference	%	1.9	1.9	1.9	1.9
Co	Measured value	µg/L	26	26	26	
	Certified value	µg/L	27.06	27.06	27.06	
	Difference	µg/L	1.1	1.1	1.1	
	Difference	%	3.9	3.9	3.9	3.9
Ni	Measured value	µg/L	61	60	62	
	Certified value	µg/L	62.41	62.41	62.41	
	Difference	µg/L	1.4	2.4	0.4	
	Difference	%	2.3	3.9	0.7	2.3
Cu	Measured value	µg/L	22	22	21	
	Certified value	µg/L	22.76	22.76	22.76	
	Difference	µg/L	0.76	0.76	1.76	
	Difference	%	3.3	3.3	7.7	4.8
Zn	Measured value	µg/L	77	77	76	
	Certified value	µg/L	78.5	78.5	78.5	
	Difference	µg/L	1.5	1.5	2.5	
	Difference	%	1.9	1.9	3.2	2.3
As	Measured value	µg/L	60	61	60	
	Certified value	µg/L	60.45	60.45	60.45	
	Difference	µg/L	0.45	0.55	0.45	
	Difference	%	0.74	0.91	0.74	0.80
Se	Measured value	µg/L	12	12	12	
	Certified value	µg/L	11.97	11.97	11.97	
	Difference	µg/L	0.030	0.030	0.030	
	Difference	%	0.25	0.25	0.25	0.25
Sr	Measured value	µg/L	340	350	330	
	Certified value	µg/L	323.1	323.1	323.1	
	Difference	µg/L	17	27	7	
	Difference	%	5.2	8.3	2.1	5.2
Mo	Measured value	µg/L	120	120	120	
	Certified value	µg/L	121.4	121.4	121.4	
	Difference	µg/L	1.4	1.4	1.4	
	Difference	%	1.2	1.2	1.2	1.2
Ag	Measured value	µg/L	0.95	0.95	0.90	
	Certified value	µg/L	1.062	1.062	1.062	
	Difference	µg/L	0.11	0.11	0.16	
	Difference	%	11	11	15	12

(***continued***)

Appendix 2.1 continued

Element		Unit	1643H (I)	1643H (II)	1643H (III)	Mean difference (%)
Cd	Measured value	µg/L	6	6.1	6	
	Certified value	µg/L	6.568	6.568	6.568	
	Difference	µg/L	0.57	0.47	0.57	
	Difference	%	8.6	7.1	8.6	8.1
Sb	Measured value	µg/L	59	58	57	
	Certified value	µg/L	58.3	58.3	58.3	
	Difference	µg/L	0.70	0.30	1.3	
	Difference	%	1.2	0.51	2.2	1.3
Ba	Measured value	µg/L	610	600	590	
	Certified value	µg/L	544.2	544.2	544.2	
	Difference	µg/L	66	56	46	
	Difference	%	12	10	8.4	10
Pb	Measured value	µg/L	20	20	19	
	Certified value	µg/L	19.63	19.63	19.63	
	Difference	µg/L	0.37	0.37	0.63	
	Difference	%	1.9	1.9	3.2	2.3
Th	Measured value	µg/L	0.88	0.87	0.88	
	Certified value	µg/L	1.0	1.0	1.0	
	Difference	µg/L	0.12	0.13	0.12	
	Difference	%	12	13	12	12
U	Measured value	µg/L	0.90	0.90	0.87	
	Certified value	µg/L	1.0	1.0	1.0	
	Difference	µg/L	0.10	0.10	0.13	
	Difference	%	10	10	13	11

Appendix 2.2. Certified values compared with measured values of constituent elements in the certified reference materials (CRM) used in ICP-MS analysis of the tadpole tissue. The certified values expressed as mass fractions (%) are based on the dry weight of the CRM.

Element		Unit	Egg powder 8415	Egg powder 8415	Egg powder 8415	Dorm-3	Dorm-3	1577b Bovine liver	Mean difference (%)
Na	Certified value	%	0.377	0.377	0.377	-	-	0.242	
	Measured value	%	0.36	0.35	0.36	-	-	0.22	
	Difference	%	4.5	7.2	4.5	-	-	9.1	6.3
P	Certified value	%	1.001	1.001	1.001	-	-	1.1	
	Measured value	%	0.94	0.88	0.86	-	-	1.0	
	Difference	%	6.1	12	14	-	-	9.1	10
S	Certified value	%	0.512	0.512	0.512	-	-	0.785	
	Measured value	%	0.51	0.48	0.47	-	-	0.71	
	Difference	%	0.39	6.3	8.2	-	-	9.6	6.1
K	Certified value	%	0.319	0.319	0.319	-	-	0.994	
	Measured value	%	0.30	0.30	0.30	-	-	0.94	
	Difference	%	6.0	6.0	6.0	-	-	5.4	5.8
Ca	Certified value	%*	0.248	0.248	0.248	-	-	0.116	
	Measured value	%*	0.23	0.23	0.22	-	-	0.12	
	Difference	%	7.3	7.3	11	-	-	3.4	7.3
Mg	Certified value	g/kg	0.305	0.305	0.305	-	-	0.601	
	Measured value	g/kg	0.31	0.29	0.3			0.59	
	Difference	%	1.6	4.9	1.6	-	-	1.8	2.5
Al	Certified value	mg/kg	540	540	540	-	-	-	
	Measured value		560	550	550			-	
	Difference		3.7	1.9	1.9	-	-	-	2.5
Cr	Certified value	mg/kg	0.37	0.37	0.37	1.89	1.89	-	
	Measured value		0.37	0.33	0.70	2.0	1.8	-	
	Difference		0.00	11	89	5.8	4.8	-	22
Mn	Certified value	mg/kg	1.78	1.78	1.78	-	-	11	
	Measured value		1.7	1.6	1.7	-	-	10	
	Difference		4.5	10	4.5	-	-	4.8	6.0
Fe	Certified value	mg/kg	112	112	112	347	347	184	
	Measured value		100	100	110	350	330	190	
	Difference		11	11	1.8	0.90	4.9	3.3	5.4
Co	Certified value	mg/kg	0.012	0.012	0.012	-	-	-	
	Measured value		0.014	0.012	0.014	-	-	-	
	Difference		14	0.00	13	-	-	-	8.9

(*continued*)

Appendix 2.2 continued

Element		Unit	Egg powder 8415	Egg powder 8415	Egg powder 8415	Dorm-3	Dorm-3	1577b Bovine liver	Mean difference (%)
Ni	Certified value	mg/kg	-	-	-	1.28	1.28	-	
	Measured value		-	-	-	1.4	1.3	-	
	Difference		-	-	-	9.4	1.6	-	5.5
Cu	Certified value	mg/kg	2.7	2.7	2.7	15.5	15.5	160	
	Measured value		2.6	2.6	2.6	19	16	160	
	Difference		3.7	3.7	3.7	23	3.2	0.00	6.2
Zn	Certified value	mg/kg	68	68	68	51	51	127	
	Measured value		64	64	63	50	51	130	
	Difference		5.2	5.2	6.7	2.5	0.58	2.4	3.8
As	Certified value	mg/kg	-	-	-	6.88	6.88	-	
	Measured value		-	-	-	7.1	6.5	-	
	Difference		-	-	-	3.2	5.5	-	4.4
Se	Certified value	mg/kg	1.4	1.4	1.4	-	-	-	
	Measured value		1.4	1.3	1.3	-	-	-	
	Difference		0.72	6.5	6.5	-	-	-	4.6
Sr	Certified value	mg/kg	5.63	5.63	5.63	-	-	0.14	
	Measured value		5.5	5.3	5.3	-	-	0.19	
	Difference		2.3	5.9	5.9	-	-	40	13
Mo	Certified value	mg/kg	0.25	0.25	0.25	-	-	3.5	
	Measured value		0.22	0.22	0.20	-	-	3.7	
	Difference		11	11	19	-	-	5.7	12
Cd	Certified value	mg/kg	-	-	-	0.29	0.29	0.50	
	Measured value		-	-	-	0.30	0.29	0.48	
	Difference		-	-	-	3.4	0.00	4.0	2.5
Sn	Certified value	mg/kg	-	-	-	0.066	0.066	-	
	Measured value		-	-	-	0.076	-0.030	-	
	Difference		-	-	-	15.2	145.5	-	80
Pb	Certified value	mg/kg	0.061	0.061	0.061	-	-	0.129	
	Measured value		0.058	0.045	0.055	-	-	0.12	
	Difference		4.9	26	9.8	-	-	7.0	12

*** The unit of the certified and measured values of Ca in CRM 1577b Bovine liver is mg/L.**

Appendix 3 Bioconcentration factors (BCF)

Appendix 3.1 Bioconcentration factor (BCF) at Prinsdal at the seven samplings, calculated by dividing mean element concentration in tadpole tissue on dissolved element concentration in water.

Element	Week 18	Week 19	Week 20	Week 21	Week 22	Week 23	Week 24
Na	0.18	0.20	0.56	0.68	0.80	0.68	0.71
Mg	0.18	0.13	0.19	0.24	0.18	0.26	0.65
Al	0.010	0.024	0.58	2.6	2.4	3.7	2.5
P	247	162	90	46	17	19	3.7
K	2.8	2.0	2.7	3.6	1.9	2.8	5.1
Ca	0.013	0.016	0.037	0.053	0.042	0.074	0.13
Sc	0.0030	0.0099	0.26	0.88	0.72	1.1	1.1
Mn	0.017	0.091	0.082	0.27	0.040	0.11	0.085
Fe	0.060	0.058	0.18	1.0	0.30	0.36	0.06
Cu	0.28	0.061	0.13	0.32	0.44	0.54	0.46
Zn	5.0	1.4	1.2	0.34	0.27	0.21	0.71
As	0.086	0.076	0.10	0.23	0.16	0.21	0.067
Sr	0.0044	0.0036	0.014	0.042	0.037	0.077	0.15
Mo	0.23	0.091	0.37	0.32	0.76	0.65	0.28
Cd	0.28	0.086	0.19	0.39	0.71	1.2	0.87
Ba	0.024	0.011	0.074	0.24	0.30	0.67	1.0
La	0.0064	0.0070	0.17	0.57	0.44	0.60	0.23
Ce	0.0053	0.0070	0.16	0.55	0.39	0.52	0.21
Gd	0.0024	0.0040	0.11	0.36	0.28	0.39	0.18
Pb	0.015	0.033	0.45	1.7	0.81	1.4	0.32
Th	0.0027	0.011	0.27	0.70	0.47	0.78	0.47
U	0.0047	0.0066	0.13	0.48	0.47	0.48	0.33

Appendix 3.2 Bioconcentration factor (BCF) at Skullerud at the six samplings, calculated by dividing mean element concentration in tadpole tissue on dissolved element concentration in water.

Element	Week 19	Week 20	Week 21	Week 22	Week 23	Week 24
Na	0.0029	0.057	0.066	0.040	0.032	0.028
Mg	0.0084	0.066	0.052	0.056	0.042	0.054
Al	0.020	0.086	1.6	11	27	48
P	74	169	19	50	37	39
K	0.13	1.0	0.97	0.46	0.39	0.53
Ca	0.0021	0.0063	0.010	0.014	0.014	0.020
Sc	0.016	0.049	0.84	7.6	9.9	11
Mn	0.035	0.15	0.35	1.9	1.8	1.8
Fe	0.08	0.20	1.3	7.8	7.8	9.4
Cu	0.10	0.18	0.30	1.3	1.6	0.98
Zn	2.6	9.1	2.0	3.2	3.0	2.6
As	0.65	0.67	0.20	0.37	0.37	0.42
Sr	0.0016	0.0038	0.0069	0.014	0.012	0.017
Mo	0.0035	0.036	0.026	0.060	0.057	0.12
Cd	0.078	0.17	0.26	4.9	2.8	4.8
Ba	0.0057	0.066	0.10	0.35	0.29	0.45
La	0.012	0.033	0.56	5.9	11	17
Ce	0.015	0.049	0.88	8.2	18	28
Gd	0.011	0.024	0.38	4.5	7.1	11
Pb	0.019	0.096	0.84	5.5	6.1	16
Th	0.0097	0.049	0.83	8.0	28	18
U	0.0015	0.0081	0.083	0.17	0.28	0.69

Appendix 3.3 Bioconcentration factor (BCF) at Vassum at the six samplings, calculated by dividing mean element concentration in tadpole tissue on dissolved element concentration in water.

Element	Week 19	Week 20	Week 21	Week 22	Week 23	Week 24
Na	0.0037	0.0037	0.0047	0.0036	0.0037	0.0030
Mg	0.036	0.064	0.083	0.11	0.10	0.11
Al	0.35	11	33	33	24	40
P	177	51	42	19	21	25
K	0.33	0.16	0.15	0.13	0.19	0.20
Ca	0.0055	0.010	0.015	0.025	0.035	0.052
Sc	0.28	6.7	13	14	12	11
Mn	0.057	2.5	1.9	0.51	0.70	0.48
Fe	0.44	7.6	9.6	4.9	3.4	3.6
Cu	0.20	0.43	0.98	4.6	4.8	5.3
Zn	1.3	0.49	1.5	7.4	8.2	7.9
As	0.18	0.32	0.49	0.30	0.35	0.34
Sr	0.0021	0.0093	0.014	0.018	0.018	0.023
Mo	0.0086	0.039	0.067	0.10	0.10	0.087
Cd	0.24	0.87	2.7	4.6	10	9.0
Ba	0.017	0.10	0.14	0.14	0.14	0.13
La	0.057	14	32	21	15	16
Ce	0.053	15	35	21	15	17
Gd	0.070	8.9	21	12	9.0	9.7
Pb	0.11	3.2	10	5.4	3.7	4.2
Th	0.084	14	38	40	23	27
U	0.0024	0.077	0.12	0.17	0.15	0.12

Appendix 4 Summaries of principal component analyses (PCA)

Appendix 4.1 Eigenvalues and the importance of components in principal component analysis (PCA) performed on total element concentrations in water.

	Eigenvalue	Proportion explained	Cumulative proportion
PC1	15.8764	0.5292	0.5292
PC2	8.3046	0.2768	0.806
PC3	2.35154	0.07838	0.88442
PC4	1.1431	0.0381	0.9225

Appendix 4.2 Eigenvalues and the importance of components in principal component analysis (PCA) performed on dissolved element concentrations in water.

	Eigenvalue	Proportion explained	Cumulative proportion
PC1	17.4621	0.5821	0.5821
PC2	6.1577	0.2053	0.7873
PC3	2.19051	0.07302	0.86034
PC4	1.28963	0.04299	0.90333

Appendix 4.3 Eigenvalues and the importance of components in principal component analysis (PCA) performed on element concentrations in tadpole tissue.

	Eigenvalue	Proportion explained	Cumulative proportion
PC1	16.4601	0.6584	0.6584
PC2	3.8052	0.1522	0.8106
PC3	1.878	0.07512	0.88573
PC4	0.83453	0.03338	0.91911

Appendix 4.4 Eigenvalues and the importance of components in principal component analysis (PCA) performed on bioconcentration factors (BCFs).

	Eigenvalue	Proportion explained	Cumulative proportion
PC1	10.5547	0.4798	0.4798
PC2	5.4296	0.2468	0.7266
PC3	2.18439	0.09929	0.82585
PC4	1.01218	0.04601	0.87186

Appendix 5 Classification system for environmental quality developed by the Climate and Pollution Agency

The trace element concentrations defining the class limits of the classification system for environmental quality developed by the Climate and Pollution Agency. All values are upper concentration limits, except class V where the given concentrations denotes the lower limit. Values are given in µg/L. Modified after Climate and Pollution Agency (2012).

Metal	Class I Background	Class II Good	Class III Moderate	Class IV Poor	Class V Very poor
Cadmium (hard water)	0,03	0,19	1,5	15	>15
Cadmium (soft water)	0,03	0,08	0,45	4,5	>4,5
Nickel	0,5	1,7	34	67	>67
Mercury	0,001	0,05	0,07	0,7	>0,7
Lead	0,05	1,3	14	57	>57
Zinc	1,5	11	11	60	>60
Copper	0,3	7,8	7,8	78	>78
Arsenic	0,15	4,8	8,5	85	>85
Chromium	0,2	3,4	3,4	360	>360

Appendix 6 Results of the correlation tests

The p-values and Pearson's r/Spearman's rho obtained from the correlation tests. 'Tadpole element concentration' refers to the site scores of the principal component analysis axis 1 (PCA axis 1) obtained by PCA on tadpole tissue trace element concentrations. Results in bold are statistically significant at a 95 % confidence level.

Data	P-value	Degree of correlation
Tadpole element concentration and log(TMW[1)]+2)	0.55	Pearson's r = -0.15
Tadpole element concentration and log(DMW[2)]+1)	0.11	Pearson's r = -0.38
Tadpole element concentration and MT[3)]	0.17	Spearman's rho = 0.33
Tadpole Cu and MT	0.67	Spearman's rho = 0.10
Tadpole Zn and MT	0.17	Spearman's rho = -0.32
Tadpole Cd and MT	0.44	Spearman's rho = 0.19
Tadpole Pb and MT	**0.01**	**Spearman's rho = 0.57**
Tadpole element concentration and log(EROD[4)]+2)	0.14	Pearson's r= 0.35
Tadpole element concentration and GST[5)]	**0.00061**	**Pearson's r = 0.71**
Tadpole Cd and GST	**0.0067**	**Pearson's r = 0.60**
Tadpole sqrt(Pb) and GST	**0.000032**	**Pearson's r = 0.81**
Tadpole element concentration and log(GSH[6)])	0.14	Pearson's r = -0.35

[1)] **TMW = total element concentration in water.**
[2)] **DMW = dissolved element concentration in water**
[3)] **MT = metallothionein**
[4)] **EROD = 7-Ethoxyresorufin *O*-deethylase**
[5)] **GST= glutathione-S-transferase activity**
[6)] **GSH = reduced glutathione.**