UNIVERSITY OF GOTHENBURG

DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

# ECOTOXICOLOGICAL ASSESSMENT OF CONTAMINATED SEDIMENTS IN SKUTBOSJÖN, SWEDEN

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# Abstract

Sediments can function both as sinks for chemicals released into the environment as well as secondary sources of contaminants when compounds get remobilized to the water column. The aim of this thesis was to investigate sediment toxicity of Skutbosjön to aquatic organisms by (*i*) assessing gene expression and enzymatic activity related to detoxification and oxidative stress in juvenile perch, (*ii*) examining embryotoxicity in zebrafish and (*iii*) measuring enzymatic activity associated with detoxification processes in freshwater snails collected from the study sites. The Skutbosjön treatments induced prominent effects on embryonic stages of zebrafish in the form of delayed and reduced hatching rates, with statistically significant differences seen when compared to control groups. Alterations in reproductive parameters might result in lowered biological fitness and furthermore, a lowered developmental rate leads to prolongation of the time period that the organisms remain in a critical stage, thus making examinations of sublethal effects such as these ecologically important. The study results also showed an elevated CYP1A gene expression and EROD activity in perch exposed to sediment from Skutbosjön. However, no statistically significant differences were found in biomarker responses between the different treatments for perch or snails.

The obtained results are indicative of a low bioavailability of the particle-bound compounds. However, the fact that a lack of statistical significance is not equal to a lack of biological significance must be emphasized. No conclusions of the actual effects for wild aquatic organisms in Skutbosjön can be drawn based only on the study results.

**Keywords:** *sediment toxicity, sublethal effects, gene expression, enzymatic activity, embryotoxicity*

# Sammanfattning

Sediment kan agera både som sänkor för kemikalier som släppts ut i miljön och som sekundära föroreningskällor när ämnen remobiliseras till vattenkolumnen. Syftet med detta arbete var att undersöka toxicitet av sediment från Skutbosjön för vattenlevande organismer genom att *(i)* mäta genuttryck och enzymatisk aktivitet relaterade till detoxifikation och oxidativ stress i juvenila abborrar, *(ii)* undersöka embryotoxicitet hos zebrafisk samt *(iii)* mäta enzymatisk aktivitet associerad till detoxifikationsprocesser i sötvattenssnäckor som samlats in från studieplatserna. Exponering för sediment från Skutbosjön inducerade påtagliga effekter på embryonala stadier av zebrafiskar i form av fördröjd och reducerad kläckning, med påvisade statistiskt signifikanta skillnader vid jämförelse med kontrollgrupper. Inverkan på reproduktiva parametrar kan resultera i lägre biologisk fitness, och dessutom kan en lägre utvecklingshastighet leda till förlängning av tidsperioden då en organism befinner sig i ett kritiskt stadium, vilket gör undersökningar av subletala effekter som dessa ekologiskt viktiga. Vidare visade studieresultaten förhöjt genuttryck av CYP1A och ökad EROD-aktivitet hos abborrar som exponerats för sediment från Skutbosjön. Dock fanns inga statistiskt signifikanta skillnader i biomarkör-responser mellan de olika behandlingarna varken för abborrar eller snäckor.

De erhållna resultaten indikerar en låg biotillgänglighet hos de partikelbundna ämnena. Dock måste det understrykas att en avsaknad av statistisk signifikans inte nödvändigtvis är lika med en avsaknad av biologisk signifikans. Inga slutsatser om de faktiska effekterna för naturligt förekommande organismer i Skutbosjön kan dras baserat endast på resultaten från denna studie.

# 1 Introduction

## 1.1 Society's chemical dependency

Chemicals are essential for the maintenance and development of the modern society (ECHA, n.d.). Sectors such as agriculture, industrial manufacturing, energy production and health care are to a large extent dependent on the use of chemicals (UNEP, 2012). However, this widespread use results in a considerable number of potential environmental and human exposure scenarios. Emissions occur during each step of the chemical life cycle, from the extraction of raw material to the manufacturing, use and disposal of the chemical products (UNEP, 2013). Regardless of the original source, aquatic systems often function as the main recipients and final destinations of chemical compounds once released into the environment via effluents, runoff or atmospheric deposition (Di Giulio & Hinton, 2008), where they may accumulate in sediments and persist for substantial periods of time (Chovanec et al., 2003). Additionally, sediments can act as secondary sources of contamination when compounds get remobilized to the water column as a result of e.g. resuspension, bioturbation and dredging (Brinkmann et al., 2015). As the anthropogenic activities intensifies in order to meet the demands of a growing global population, aquatic organisms are increasingly exposed to complex mixtures of xenobiotics (i.e. foreign compounds) with potential adverse impacts on ecosystem health as a result (Adams, 1992; Goksøyr & Förlin, 1992).

## 1.2 The Water Framework Directive

Numerous of chemical compounds are associated with properties such as persistence and the ability of long-distance transportation, making aquatic chemical pollution a transboundary issue. This is considered in the Water Framework Directive (WFD) 2000/60/EG, that was adopted in the European Union in 2000 and covers the freshwater systems, groundwater and coastal waters of Europe (Jones & Gomes, 2014; EEA, 2018). The implementation of the WFD introduced a common, international framework with a novel approach to the management and protection of aquatic systems that requires cross-border cooperation (European Commission, 2019b; Giakoumis & Voulvoulis, 2018). The aims of the directive were to achieve "good ecological and chemical status" by 2015 (Jones & Gomes, 2014). Due to natural variability, no absolute standards can be set for the determination of the ecological status, which instead focus on the quality of the biological community and the hydrological and chemical characteristics. The chemical status, on the other hand, is based on the compliance of pre-decided quality standards for chemical compounds (European Commission, 2019b). In 2019, it was reported that all of the surface water bodies that were included in the monitoring programs in Sweden failed to achieve good chemical status (European Commission, 2019a).

## 1.3 Biomonitoring

Biomonitoring can be defined as the study of biological responses in order to investigate the impacts following anthropogenic stressors (Kerans, 1994) and serves as a useful and

complementary tool to chemical analysis in water quality management (Kuklina et al., 2013). Although chemical analysis of contaminant levels in environmental compartments is of great importance for ecosystem health evaluations, its limitations are acknowledgeable and include e.g. the inability to predict the actual effects on organisms following exposure. Consequently, bioanalysis and effect-based assessments are required to gain an adequate understanding of the chemico-biological interactions (Vasseur & Cossu-Leguille, 2003; Bervoets & Blust, 2003; van der Oost et al., 2003). A biological approach often involves using biomarkers, defined as "a change in a biologic response measured in biologic systems ranging from molecular through biochemical, cellular and physiologic responses to behavioral changes, and which can be related to an exposure to, or toxic effect of, an environmental chemical or chemicals" (Everaarts et al., 1994). Common parameters to investigate are growth, reproduction and enzymatic activity (van der Oost et al., 2003). Monitoring and tools for early warning evaluations are essential in the context of environmental risk assessment (Mayer et al., 2008). The prediction and detection of adverse effects in natural populations and ecosystems involves great difficulties and uncertainties, and can become obvious first after longer time periods. Once the actual effects are determined, there is an overwhelming risk of irreversibility and, thus, mitigating measures or strategies for risk reduction might be insufficient (van der Oost et al., 2003). Biomarkers are used in monitoring programs of both marine and limnic waters within the European Union, e.g. in the Regional Seas Conventions. Furthermore, their potential as tools in monitoring programs under the Water Framework Directive (WFD) as a linkage of the chemical and ecological status assessments has been raised. European countries such as Denmark, Sweden, Norway and the United Kingdom are regularly using physiological and biochemical biomarkers in fish for environmental biomonitoring (European Union, 2014; Tairova et al., 2012; Förlin et al., 2019). Monitoring programs in Sweden have primarily focused on eelpout (*Zoarces viviparus*) and perch (*Perca fluviatilis*) (Sturve, pers. comm., 2020).

## 1.4 Xenobiotic biotransformation and detoxification

As mentioned above, all toxicant-induced effects seen at higher levels of biological organization are preceded by interactions at a biochemical level. When an organism is exposed to a xenobiotic compound, a two-phase biotransformation process is initiated. The phase I reactions involve oxidation, reduction and hydrolysis, that either adds or exposes functional groups of the compound. This facilitates further biotransformation reactions, while also often increasing the polarity and, hence, the water solubility in order to make the xenobiotic more easily excreted. The major phase I system is the cytochrome P-450 (CYP) monooxygenase, which is induced by organic compounds such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and dioxins. This system consists of a variety of distinct enzymes with certain substrate specificities, divided into families (e.g. CYP1, CYP2 and CYP3) and subfamilies (e.g. CYP1A and CYP1B). The chemically altered phase I products can either be directly eliminated from the cell, or enter into the phase II reactions. This phase involves conjugation, i.e. the addition of polar, endogenous groups such as amino acids or glutathione, which further facilitates the excretion of the xenobiotic (Newman, 2015; Di Giulio & Hinton, 2008).

Another molecular system of importance is the antioxidant defense system against oxidative stress (Di Giulio & Hinton, 2008). Oxidative stress can result in damage of various biomolecules and occurs due to an imbalance between the production of reactive oxygen species (ROS) and the ability of ROS detoxification by antioxidants (e.g. glutathione, vitamin C and vitamin E) and antioxidant enzymes (e.g. superoxide dismutase and catalase). ROSs include e.g. superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$), which are produced as by-products of oxygen metabolism and various other processes, as well as induced by xenobiotic exposure (Newman, 2015; Almroth et al., 2019; Pizzino et al., 2017). The ROSs can directly and indirectly alter the balance of oxidants and antioxidants, i.e. the redox status, of a cell and consequently drive the redox status to a more oxidized state. To retain the equilibrium, a number of redox couples are of great importance. An example is GSH/GSSG, which is the reduced and oxidized states of the previously mentioned antioxidant glutathione. ROSs can oxidize GSH into GSSG, and the reduction of GSSG back to GSH is catalyzed by glutathione reductase (Di Giulio & Hinton, 2008). Furthermore, the phase II enzyme glutathione-S-transferase (GST) can catalyze conjugation of GSH to compounds produced from xenobiotics following biotransformation processes, thus making GSTs important mediators in the defense system against oxidative stress (Tsuchida, 2002; Ranson & Hemingway, 2005).

The activities of the enzymes involved in various detoxification processes can function as biomarkers when examining biological responses following contaminant exposure. A common biomarker of exposure is the measurement of ethoxyresorufin-*O*-deethylase (EROD) activity, which detects the induction of primarily the CYP1A subfamily (Whyte et al., 2000). Induction of the egg yolk protein precursor vitellogenin (VTG) in blood plasma of fish following exposure to e.g. xenoestrogenic compounds is another widely used biomarker (Schwaiger & Negele, 1998; Hutchinson et al., 2006). Furthermore, effects can be detected at a genetic level by measuring gene expression. When a receptor is activated by the presence of a xenobiotic compound, the receptor can bind to specific DNA sequences in the cell nucleus, thereby promoting the transcription of target genes and generating messenger RNA (mRNA) production. When exported to the cell cytoplasm, the mRNA information is translated in order to synthesize the corresponding proteins. By using analytical methods such as quantitative polymerase chain reaction (qPCR), gene expression biomarkers can be applied to e.g. quantify the amount of mRNA codifying for the enzymatic activity of CYP1A. Hence, the measurement of gene expression is a very sensitive biomarker with the ability of detecting induction of detoxification processes that are not measurable on a protein level (Piña et al., 2007).

## 1.5 Study site

Due to the potential of sediments acting as both sinks and secondary sources of chemical compounds, assessments of the effects for aquatic organisms following exposure to contaminated sediments are of great ecological importance. Two of the main biological groups that can be determined in the context of biological sediment quality assessments are benthic and pelagic organisms (in particular fish) (Naturvårdsverket, 2008). Benthic, sediment-dwelling organisms such as gastropods are highly exposed to particle-bound substances due to

their feeding habits, which include the ingestion of sediment-associated particles, organic detritus, algae and bacteria (Ma et al., 2010). Furthermore, fish are frequently used as indicator organisms due to their abundance in aquatic systems and their important ecological roles in the trophic food chain (Di Giulio & Hinton, 2008; van der Oost et al., 2003). In biomonitoring of lakes, perch is one the most commonly used test species (WSP, 2017). Perch is a stationary species that stays within the same water area during their whole life cycle, thus making it suitable for assessments of contaminants associated to a specific area (Viktor, 2017).

The study site of this thesis, Skutbosjön, is located in Finspång Municipality in Östergötland County, Sweden. It is a small waterbody that belongs to the larger lake Dovern, which in turn is connected to the lake Glan (see Fig. 1). Skutbosjön, Dovern and the lake Bönnern (located upstream of Skutbosjön) are collectively called Finspångsån. The area surrounding Skutbosjön has an extensive industrial history, beginning at the 16$^{th}$ century. The past and current industries include mainly metal manufacturing companies, involved in e.g. smelting for metal extraction and turbine production processes. Skutbosjön and Dovern are recipients for industrial wastewater discharges and municipal sewage treatment effluents (see Fig. 2).

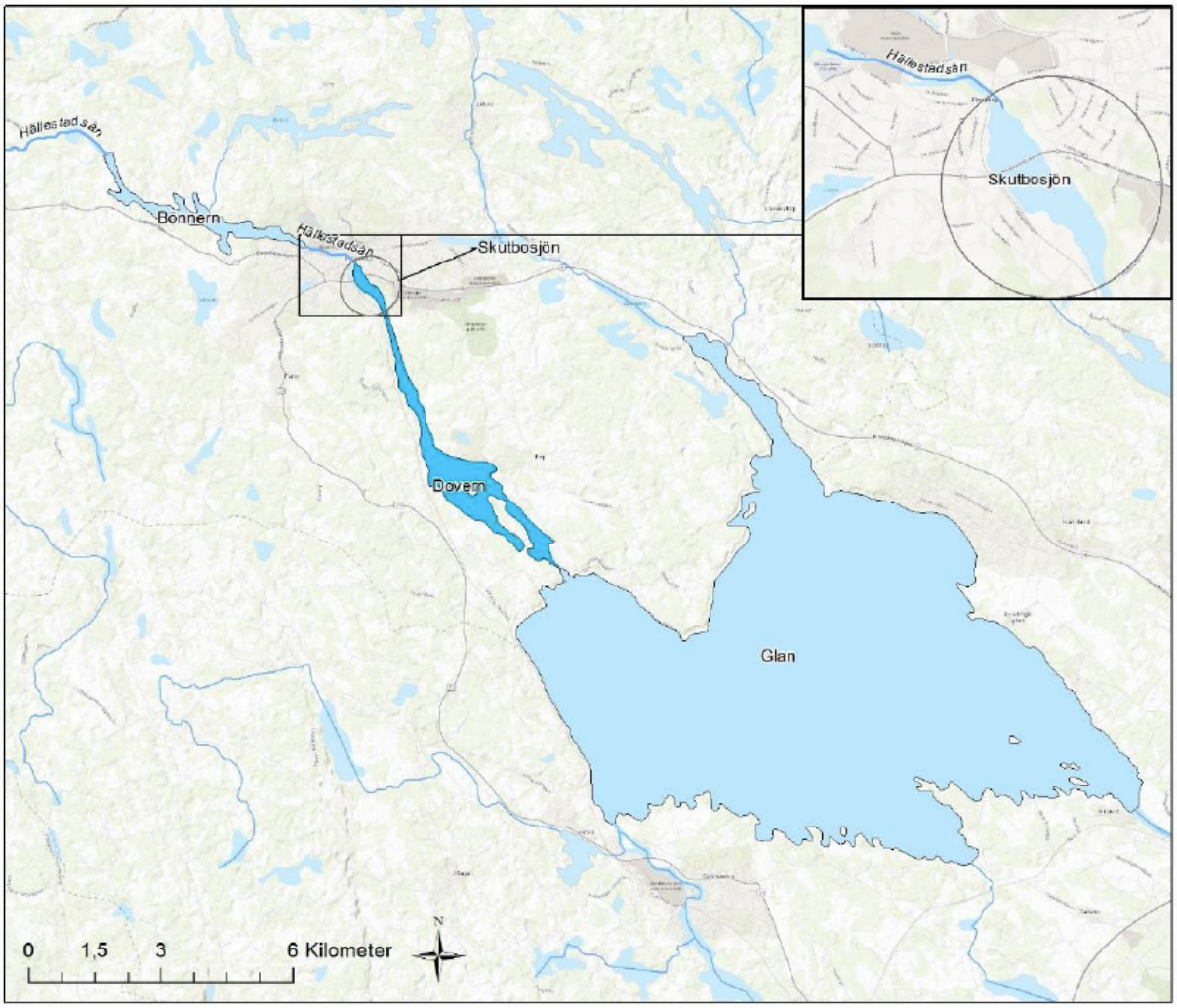


**Fig. 1.** Regional overview of the lake system that Skutbosjön is a part of. Figure source: WSP, 2017 (Copyright ESRI BaseMap, VISS 20171128).

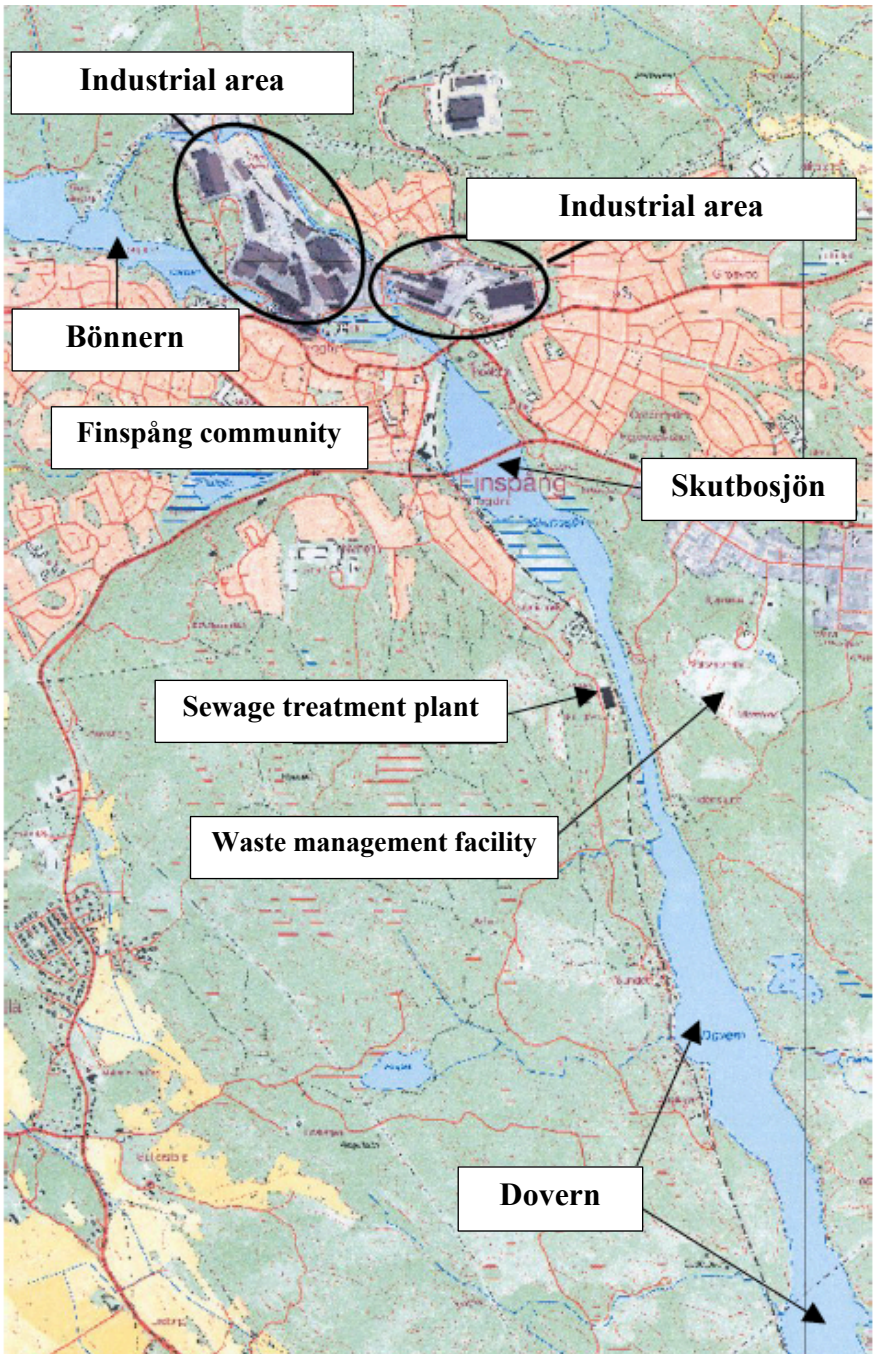


**Fig. 2.** Map illustrating the locations of the industrial areas, the municipal sewage treatment plant and a waste management facility in relation to Skutbosjön and Bönnern. Original figure source: Länsstyrelsen, 2004.

Previous investigations of the sediment in Skutbosjön have concluded that the concentrations of metals (especially copper, chromium, mercury, nickel and zinc), PAHs and PCBs are highly elevated (WSP, 2010). Additionally, a high frequency of mouthpart deformities in Chironomid larvae in Skutbosjön have been found, which is a commonly used indicator of sublethal effects caused by toxicants (WSP, 2017).

## 1.6 Aim of the thesis

The aim of this thesis was to investigate sediment toxicity of the lake Skutbosjön to aquatic organisms by *(i)* assessing mRNA expression of cytochrome P450 1A (CYP1A), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD) and vitellogenin (VTG) as well as ethoxyresorufin-*O*-deethylase (EROD) activity in juvenile perch following three and seven weeks exposure to field-collected sediment samples, *(ii)* examining embryotoxicity in zebrafish exposed to whole-sediment samples and sediment extracts and *(iii)* measuring enzymatic activity of glutathione-S-transferase (GST) in freshwater snails collected from the study sites.

# 2 Method

## 2.1 Sediment sampling

Sediment samples were collected using an Ekmanhuggare from two sites in Skutbosjön (see Fig. 3A) and two sites in the reference site Hällestadsån (inlet to Bönnern) (see Fig. 3B), which is located upstream of Skutbosjön and the industrial areas and thus affected to a much lesser extent by the past industrial activities. All the sediment samples were transported to the University of Gothenburg the same day as they were collected and stored in plastic containers in darkness at 4°C.

Additionally, samples from the top two centimeters of sediment were collected from the two sites in Skutbosjön and from reference site 1, by using a Kajak Corer sediment sampler. Surface water samples were collected from Skutbosjön site 1 and reference site 1 with a Limnos PL water sampler. These samples were taken as a part of the environmental monitoring program by the County Administrative Board of Östergötland and sent for chemical analysis to an accredited laboratory.


**Fig. 3.** Sampling sites in (A) Skutbosjön and (B) the reference site Hällestadsån. Original figure source: Esri ArcGIS.

## 2.2 Perch experiments

Juvenile European perch, *Perca fluviatilis*, (Fig. 4) with a mean weight of 19.3 g were purchased from Vadstena Fisk AB (Vadstena, Östergötland County) and acclimated to the laboratory conditions for five weeks prior to the start of the experiments. The perch were fed thawed red mosquito larvae (producer Akvarieteknik) three times a week, with an amount representing 2 % of the average fish body weight.

**Fig. 4.** European perch (*Perca fluviatilis*).

### 2.2.1 Experimental setup and sampling of perch

The experimental setup for the sediment exposures of perch is illustrated in Fig. 5A and 5B. The sediment samples were thoroughly stirred and randomly distributed to aquariums (9.5 x 58 cm) in amounts of 1.6 L, corresponding to a sediment layer of 3 cm and a water volume of approximately 5 L. The amount of sediment used was based on previous sediment exposure studies (see Koglin et al., 2016, Kobayashi et al., 2010 and Di Gulio et al., 1993). The fish were kept under a 14:10 h light:dark cycle and each aquarium was supplied with an aerator and a water flow of approximately 24 L $h^{-1}$, with a temperature of 12°C.

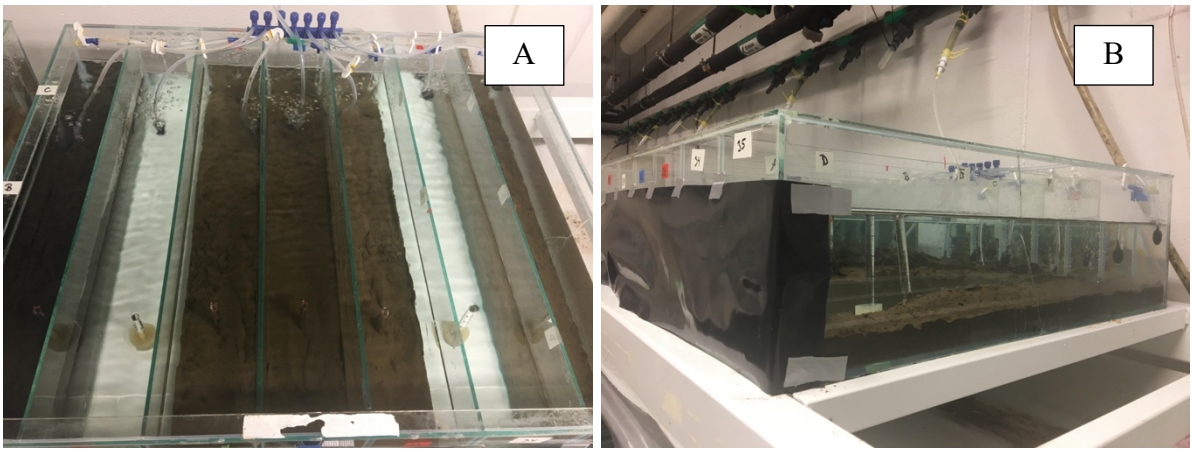


**Fig. 5.** Setup for the sediment exposures of perch.

At the start of the experiment, two randomly selected perch were placed in each aquarium. A total of 70 individuals were included and exposed to either freshwater only (negative controls) or sediment from Skutbosjön site 1, Skutbosjön site 2, reference site 1 or reference site 2 (14 fish per treatment) for three or seven weeks. All fish were starved for one week prior to the end of the exposures and euthanized with a sharp blow to the head. Upon sampling, the fork length (i.e. the distance between the tip of the snout and the end of the middle caudal fin of the fish), total weight and the liver weight of each individual was noted. Additionally, the liver and gill arches were excised. Each liver was divided into three pieces and shock-frozen in liquid nitrogen along with the gill samples. After the sampling, liver and gill samples were stored in -80°C until further analysis.

### 2.2.2 Hepatic and gill EROD activity

*Homogenisation*

Liver and gill samples were homogenised in cold 0.1 M Na/P-phosphate buffer containing 0.15 M KCl (28.65 g $Na_2HPO_4$ * $12H_2O$, 2.72 g $KH_2PO_4$ and 11.2 g KCl brought up to one litre with Millipore water) using sonication (*Branson Digital Sonifier*). After being homogenised, the samples were centrifuged at 10 000 x g for 20 minutes at 4°C (using a *Fisher Scientific accuSpin Micro 17R*) and the supernatants (S9 fractions) were collected with a pipette, divided into aliquots and kept in a -80°C freezer.

*Spectrofluorometric analysis of EROD activity*

The measurement of ethoxyresorufin-O-deethylase (EROD) activity was performed according to a method described by Förlin et al. (1994), using spectrofluorometry at 530 nm (excitation) and 585 nm (emission) with ethoxyresorufin as a substrate and rhodamine as a standard. A 0.1 M sodium-phosphate buffer was prepared by mixing 0.2 M $Na_2HPO_4$ * $12H_2O$ (35.81 g/500 ml Millipore water) and 0.2 M $NaH_2PO_4$ * $H_2O$ (5.52 g/200 ml Millipore water) until a pH of 8.0 was obtained, after which the solution was diluted with Millipore water 1:1. Furthermore, 96.5 g of the buffer was mixed with 1 ml of 50 µM ethoxyresorufin in methanol and 1940 µl of the mixture was distributed into each of the measuring cuvettes used for the spectrofluorometric analysis with a *PTI QuantaMaster 400*. Additionally, 50 µl of S9 fraction samples was added to each cuvette. Right before the start of each the measurement, 10 µl of 10 mM β-Nicotinamide adenine dinucleotide 2´-phosphate reduced tetrasodium salt hydrate (NADPH) (8 mg dissolved in 1 ml cold sodium-phosphate buffer) was pipetted into the cuvette in order to initiate the reaction. A cuvette containing 8 µM rhodamine B (100 µl Rhodamine standard 3 in 1,9 ml sodium-phosphate buffer) functioned as a substrate. Lastly, an aliquot of each sample was diluted with Millipore water (1:40 for liver samples and 1:60 for gill samples) and kept in -20°C for protein measurements.

Protein measurements of the diluted liver and gill samples were performed according to Lowry (1991). One solution A (20 g $Na_2CO_3$, 4 g NaOH and 500 ml Millipore water) and one solution

B (1.63 g $CuSO_4$ * $5H_2O$, 7.68 g K-Na-tartrat, 2 g NaOH and 500 ml Millipore water) was prepared, as well as Bovine Serum Albumin (BSA) standard solutions with the concentrations 0.1, 0.3, 0.5, 0.7 and 0.9 mg/ml. To 96-well microtiter plates, 25 µl of each sample and the BSA standards were added, followed by 100 µl of a solution A and B mixture (25 ml solution A and 1 ml solution B) to each well. The plates were incubated for ten minutes at room temperature, after which 100 µl of diluted Folins reagent (1.25 ml Folins reagent and 23.75 ml Millipore water) were pipetted into the wells. After an additional incubation of 30 minutes at room temperature, the protein concentrations were measured with spectrophotometry (*Molecular Devices Spectra Max 190*) at 750 nm.

For calculating the EROD activity (expressed in pmol/mg protein x min), the following formula was used:

$$\text{EROD activity} = \frac{\frac{0.8}{\left(\frac{\text{standard}}{3}\right)} * 60 * \text{slope}}{(\text{amount of sample } (ml) * \text{ protein } (mg/ml) * 1000)}$$

Where:

| | | |
|---|---|---|
| 0.8 | = | a factor corresponding to nmol rhodamine in the standard sample |
| standard | = | the average value obtained from the spectrofluorometric analysis for the rhodamine standard, divided by three due to the fact that it has been shown to have a value three times higher than the resorufin substrate |
| 60 | = | a factor for time (60 seconds) |
| slope | = | the linear increase of resorufin (counts per second) |
| protein | = | the protein concentration, measured according to Lowry (1991) |

### 2.2.3 Hepatic gene expression analysis using qPCR

*RNA extraction and cDNA synthesis*

Total RNA was extracted from the perch liver tissues by using a *RNeasy Plus Mini Kit (Qiagen)* according to the manufacturer's instructions. The RNA concentration was measured with a *NanoDrop 2000c (Thermo Scientific)* to ensure successful elution. Samples were then kept in -80°C until the following step of transcribing RNA to complementary DNA (cDNA), by using an *iScript cDNA Synthesis Kit (Bio Rad)*. Aliquots of the samples were diluted to 15 µl with nuclease-free water in order to obtain a concentration of 1000 ng of RNA, and thereafter mixed with 4 µl iScript Reaction Mix and 1 µl iScript Reverse Transcriptase. The mixtures were incubated in a thermal cycler *(My Cycler, Bio Rad)* with the following protocol: priming for 5 minutes at 25°C, reverse transcription for 20 minutes at 46°C and Reverse Transcriptase inactivation for 1 minute at 95°C. The cDNA samples were stored in -20°C until the measurements of gene expression using qPCR.

*qPCR analysis*

For the analysis of hepatic gene expression, five target genes were chosen (see Table 1): CYP1A which is involved in the phase I detoxification system, the oxidative stress-related genes GR, CAT and SOD, and lastly VTG, which is induced by xenoestrogenic exposure. Additionally, UBI and GAPDH were used as reference genes in order to normalize the data. Prior to the qPCR analysis, a Mastermix consisting of SsoAdvanced Universal SYBR Green (Bio-Rad) and gene-specific reverse and forward primers was prepared. SYBR Green has the ability to bind to double-stranded DNA and emit light upon excitation (Rebouças et al., 2013). To each well in a 96-well microtiter plate, 4 µl of cDNA samples and 6 µl Mastermix was added. After being centrifuged at 10 000 RPM for 2 minutes in a *Beckman Coulter Allegra 25 R Centrifuge*, the plate was placed in a *BIO RAD CFX Connect Real-Time System* for qPCR analysis. The protocol included DNA denaturation for 3 minutes at 95°C followed by an amplification cycle for 10 seconds at 95°C and 30 seconds of the gene-specific annealing temperature (see Table 1), repeated 40 times. As the amplification reactions progressed over the course of the analysis, the levels of fluorescence were measured. The obtained data were expressed in Cq values, i.e. the number of amplification cycles needed to detect a signal above the background noise. The data were normalized against the reference genes and backtransformed from the logarithmic form, after which the relative mRNA expression of each gene was determined.

**Table 1.** Reference genes (*UBI* and *GAPDH*) and target genes (*CYP1A, GR, CAT, SOD* and *VTG*) as well as their respective annealing temperature and primer sequence used for qPCR analysis in perch liver tissue.

**Genes used for qPCR analysis in perch liver tissue**

| Gene name | Abbreviation | Annealing temp (°C) | Primer sequence (5´-3´) |
|---|---|---|---|
| Ubiquitin | *UBI* | 58 | F: ACGTACACCGGTGGAAATCA<br>R: ACGTTCTCAATGGTGTCGCT |
| Glyceraldehyde-3-phosphate dehydrogenase | *GAPDH* | 62 | F: CTGTCGGCAAAGTCATCCCT<br>R: CCAGAATGCCCTTCATGGGT |
| Cytochrome P450 1A | *CYP1A* | 60 | F: GCAGTGAAACAGTTCGGCAG<br>R: GTGCTGAAGGCCAGACTCTT |
| Glutathione reductase | *GR* | 60 | F: ACGATTGTCACCCAAGACCC<br>R: ATCTCGCCGATGTTCAGTCC |
| Catalase | *CAT* | 60 | F: GAAAGACCCCGACATGGTGT<br>R: CAGTTTGAAGGTGTGCGAGC |
| Cu/Zn-superoxide dismutase | *SOD* | 60 | F: CCAGCGGGACCGTTTATTTTG<br>R: TTGTGGGGATTGAAGTGAGGG |
| Vitellogenin | *VTG* | 60 | F: GTGGATCCCTGCAGTACGAG<br>R: AAATCTGGCCACACGTAGCA |

## 2.3 Fish Embryo Acute Toxicity (FET) test with zebrafish

### 2.3.1 Zebrafish breeding and egg collection

The standardized Fish Embryo Acute Toxicity (FET) test with zebrafish (OECD 236) is a method to examine acute toxicity of chemicals on embryonic stages of fish (OECD, 2013). The high correlation with conventional acute fish toxicity tests has established the FET tests as a robust alternative (Braunbeck et al., 2014). In order to determine the embryotoxicity of the sediment samples from Skutbosjön and the reference site Hällestadsån, adult AB zebrafish (*Danio rerio*) raised at the Department of Biological and Environmental Sciences at the University of Gothenburg (kept at 28°C and a 14:10 h light:dark cycle) were used. The day before the start of each FET test, zebrafish were placed in breeding tanks (five or six tanks in total with two pairs in each tank, 50/50 males and females) and left overnight to breed upon light onset. The following morning, fertilized eggs were collected approximately 2-3 hours post fertilization (hpf) and transferred to embryo medium. The embryo medium was prepared by dissolving 4.9 g $MgSO_4$ x $7H_2O$, 0.41 g $KH_2PO_4$, 0.12 g $Na_2HPO_4$, 2.9 g $CaCl_2$ x $2H_2O$, 0.75 g KCl and 17.5 g NaCl in 1 litre of Millipore water, and thereafter diluting the stock 1:20 with Millipore water. At 6 hpf, the zebrafish embryos have passed the 50%-epiboly stage (Kimmel et al., 1995), which facilitates the determination of fertilized and healthy eggs. Consequently, viable, non-deformed and fertilized eggs from a minimum of three breeding tanks (i.e. six pairs of adult zebrafish) were selected at approximately 6 hpf, pooled together to account for inter-population variability and immediately distributed to the different sediment treatments to start the exposure.

### 2.3.2 Experimental design

Two different assays were included in the study: sediment extract and whole-sediment exposure. The sediment extracts were prepared by adding 200 ml of thoroughly mixed sediment into a bottle containing 250 ml of embryo medium and thereafter placing the closed bottle on a shaker (*GFL Shaker*) at the max setting (estimated to approximately 40 rpm) for one week in darkness at 4°C, to simulate water movements in a field setting. After one week, the medium was filtered using filter paper (*Munktell Analytical Filter Papers*). These extracts were assumed to contain the bioavailable and water-soluble fractions of the particle-bound compounds in the sediment samples. The extracts were then diluted with embryo medium to obtain five different concentrations (3, 6, 9, 12 and 15 ml extract per total exposure volume of 15 ml) of the four treatments (Skutbosjön site 1, Skutbosjön site 2, reference site 1 and reference site 2). Embryo medium was used as a negative control. As a positive control, 50 μM copper ions were used which previously has been shown to result in a 100 % mortality rate in zebrafish embryos (Thit et al., 2017). In the whole-sediment assay, the different concentrations tested were obtained by mixing contaminated sediment from Skutbosjön with sediment from the reference site. As two sites in Skutbosjön and two sites in the reference site were sampled, the different exposure scenarios included (A) sediment from Skutbosjön site 1 mixed with sediment from reference site 1, (B) sediment from Skutbosjön site 1 mixed with sediment from reference site 2, (C)

sediment from Skutbosjön site 2 mixed with sediment from reference site 1 and (D) sediment from Skutbosjön site 2 mixed with sediment from reference site 2. The five different concentrations tested were 2, 4, 6, 8 and 10 mg sediment per total weight of 10 mg. The negative and positive controls were the same as for the extract exposure, but an additional negative control of clean, artificial sediment of similar grain size (Sigma Clean Sediment #2, containing 10 % clay, 50 % sand and 40 % silt) was included in the whole-sediment assay, to ensure that the effects seen could be linked to the particle-bound substances. The FET tests were conducted in three independent replicates, i.e. three replicates of sediment extract exposures and three replicates of whole-sediment exposures. Zebrafish embryos were exposed in triplicates in plastic Petri dishes with ten embryos in each dish ($n$ = 30) and incubated at 27 ± 1 °C under static conditions until 96 hpf.

### 2.3.3 FET test observations

Every 24 hours, outcomes in different indicators of lethality were recorded (any positive outcome in one of the four indicators means that the embryo is considered dead):

(1) Coagulation of fertilized eggs. Healthy and viable embryos are transparent, whereas coagulated embryos are milky white.
(2) Lack of somite formation. Somites are masses of mesoderm that eventually will develop dermis, skeletal muscle and vertebrae (OECD, 2013). Under normal conditions, approximately 20 somites are formed at 24 hpf. A lack of somite formation indicates a general retardation of development.
(3) Non-detachment of the tail. Under normal conditions, the tail of the embryo is detached from the yolk during the first 24 hours.
(4) Lack of heartbeat. The heartbeat in embryos is visible after 48 hours.

Additionally, hatching rates in eggs were recorded after 48 hours. Observations were recorded by using a stereo microscope (*Nikon SMZ-10A*). Due to the dark backgrounds in the whole-sediment assays and the transparency of the embryos, observations of all of the four indicators of lethality was not possible. Hence, the only response variables included for those exposures were coagulation of fertilized eggs and hatching rates.

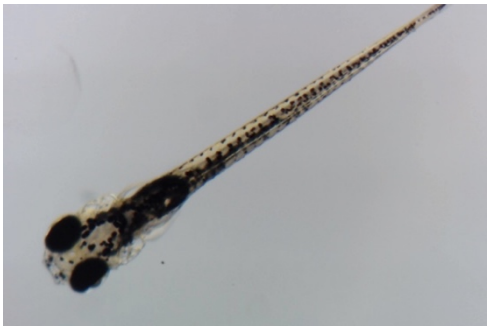

**Fig. 6.** Zebrafish at 96 hours post fertilization (hpf).

## 2.4 Enzymatic activity of GST in freshwater snails

Freshwater snails of a taxonomically unconfirmed species (Fig. 7) found in abundance in Skutbosjön and the reference site were collected and brought to the laboratory, where they were kept in water from their respective lake overnight. The following day, the gastropods were de-shelled, shock-frozen in liquid nitrogen and stored in -80°C. The mean weight ± standard error of the de-shelled individuals was 2.48 ± 0.13 g.

**Fig. 7.** The taxonomically unconfirmed freshwater snail species used for the measurements of enzymatic activity of glutathione-S-transferase (GST).

As preparation for the measurement of enzymatic activity of glutathione-S-transferase (GST), thawed gastropods were homogenised in cold 0.1 M Na/P-phosphate buffer containing 0.15 M KCl (28.65 g $Na_2HPO_4$ * $12H_2O$, 2.72 g $KH_2PO_4$ and 11.2 g KCl brought up to one litre with Millipore water) using an electronic stirrer (*Heidolph RZR 2051*). Following this, the homogenized tissue samples were placed in a *Beckman J2-21M/E Centrifuge* and centrifuged at 10 000 x g for 20 minutes in 4°C. From each sample, the supernatant (S9 fraction) was collected with a pipette, divided into aliquots and kept in a -80°C freezer.

Furthermore, 0.1 M sodium-phosphate buffer was prepared by mixing 0.2 M $Na_2HPO_4$ * $12H_2O$ (35.81 g/500 ml Millipore water) and 0.2 M $NaH_2PO_4$ * $H_2O$ (5.52 g/200 ml Millipore water) until a pH of 7.5 was obtained, after which the solution was diluted with Millipore water 1:1. Thawed samples were diluted ten times with Na/P-phosphate buffer and 10 µl of each sample were pipetted to a 96-well microtiter plate. In a fume hood, 100 mM of 1-chloro-2,4-dinitrobenzene (CDNB) dissolved in dimethylsulfoxid (DMSO) (0.08104 g CDNB in 4 ml DSMO) were diluted 50 times with 1 mM reduced form of glutathione (GSH) dissolved in 0.1 M sodium-phosphate buffer (0.0615 g GSH in 200 ml room tempered sodium-phosphate buffer). To obtain a 50 x dilution, 120 µl of 100 mM CDNB was mixed with 5880 µl of 1 mM GSH solution and stirred for five seconds on a magnetic stirrer. Following this, 190 µl of the solution was added into each well and the microtiter plate was placed in a spectrophotometer (*Molecular Devices Spectra Max 190*) and the absorbance was measured (350 nm). Lastly, an aliquot of each sample was diluted 1:40 with Millipore water and kept in -20°C for protein measurements. The protein measurements were performed according to Lowry (1991), as described in the section *2.2.2 Hepatic and gill EROD activity*.

To calculate the GST activity, the following formula was used:

$$\text{GST activity} = \left(\frac{\text{slope} * 0.001 * 0.2}{9.6 * 0.01 * 0.59 * \text{protein } (mg/ml)}\right) * 10$$

Where:

| | | |
|---|---|---|
| slope | = | absorbance value (mOD) |
| 0.001 | = | multiplied with the slope to obtain the OD value (mOD * mOD → OD) |
| 0.2 | = | sample volume in the well (ml) |
| 9.6 | = | extinct coefficient |
| 0.01 | = | amount of cytosol (ml) |
| 0.59 | = | light path (cm) |
| protein | = | the protein concentration, measured according to Lowry (1991) |
| 10 | = | the dilution factor of the samples |

## 2.5 Statistical analysis

All the statistical analysis was performed in IBM SPSS Statistics 26 with a significance level of 0.05. To establish whether the data fulfilled the assumptions of a parametric test, a Shapiro-Wilks test for normality and a Levene's test for equal variances were made. For normally distributed data with equal variances, a one-way ANOVA was used in order to check for statistically significant differences between treatment groups. Not normally distributed data were analyzed with a Kruskal-Wallis test (a one-way non-parametric ANOVA). In the case of normally distributed data that violated the assumption of equal variances, a Welch's ANOVA was performed. For the statistical analysis of the FET tests with zebrafish, significance values were adjusted by the Bonferroni correction for multiple tests due to the large amount of treatment groups.

Lastly, for the analysis of potential time-dependent differences within treatment groups in the perch experiments, an independent samples t-test were used for data fulfilling the assumptions of a parametric test. If the assumptions were not met, a non-parametric Mann-Whitney U test was applied.

# 3 Results

## 3.1 Chemical analysis of sediment and water samples

Seen in Table 2 are all of the compounds selected for chemical analysis of sediment and water samples collected from Skutbosjön and the reference site Hällestadsån. In Table 3, a selection of the results obtained from the chemical analysis of the sediment samples collected from the two sites in Skutbosjön and reference site 1 as well as the results from the chemical analysis of the water samples from Skutbosjön site 1 and reference site 1 is shown. The obtained data for copper, tributyltin (TBT), anthracene and fluoranthene in the sediment samples have been normalized against the total organic carbon (TOC) content, in order to obtain the bioavailable values. In comparison with the reference site, considerably higher concentrations of the PAHs anthracene and fluoranthene, the metals copper and zinc as well as the dioxins/furans octachlorodibenzodioxin (OCDD) and octachlorodibenzofuran (OCDF) was found in the sediment samples from Skutbosjön. Additionally, the concentrations of copper in both of the Skutbosjön sites and the concentration of anthracene in Skutbosjön site 2 are exceeding the environmental quality standards (EQS) defined by the Swedish Agency for Marine and Water Management (marked with an asterisk in Table 3). Regarding the water samples, both the Skutbosjön and the reference site samples were found to exceed the EQS for fluoranthene, copper and zinc. An exceedance of the EQS for arsenic was also found for the reference site. The full chemical analysis reports with data for all compounds can be seen in Appendix I.

**Table 2.** All of the compounds selected for the chemical analysis of sediment and water samples from Skutbosjön and the reference site.

| Chemical group | Compounds analysed in sediment samples | Compounds analysed in water samples |
|---|---|---|
| **Metals** | Arsenic, lead, cadmium, copper, zinc, chromium (hexavalent) | Calcium, magnesium, aluminum, arsenic, barium, lead, cadmium, cobalt, copper, chromium, manganese, nickel, strontium, zinc, chromium (hexavalent), mercury |
| **Pesticides** | Trifluralin | Aldrin, DDT, dieldrin, endosulfan-alpha, endosulfan-beta, HCH-alpha, HCH-beta, HCH-delta, HCH-gamma (lindan), isodrin, isoproturon, chlorfenvinphos |
| **Phenols** | *n/a* | 4-n-nonylphenol, 4-tert-octylphenol, pentachlorophenol |
| **Phtalates** | *n/a* | Di-(2-ethylhexyl)phtalate |
| **Aliphatic halogen compounds** | *n/a* | Chlorparaffines C10-C13, SCCP |
| **Chlorobenzenes** | *n/a* | Hexachlorobenzene |
| **Dioxins/furans** | 2378 TCDD, 12378 PeCDD, 123478 HxCDD, 123678 HxCDD, 123789 HxCDD, 1234678 HpCDD, OCDD, 2378 TCDF, 12378 PeCDF, 23478 PeCDF, 123478 HxCDF, 123678 HxCDF, 123789 HxCDF, 234678 HxCDF, 1234789 HpCDF, OCDF, I-PCDD/F-TEQ Lower Bound, I-PCDD/F-TEQ Upper Bound, WHO-PCDD/F-TEQ LB, WHO-PCDD/F-TEQ UB | *n/a* |
| **Organometallics** | Tributyltin | Tributyltin |
| **Polyaromatic hydrocarbons** | Acenaphthene, acenaphthylene, naphthalene, anthracene, phenanthrene, fluoranthene, fluorene, pyrene, benzo(a)anthracene, benzo(a)pyrene, benzo(ghi)perylene, chrysene + triphenylene, dibenz(a,h)anthracene, indeno(1,2,3-cd)pyrene, PAH sum cancerogenic, PAH sum others, benzo(b+k)fluoranthene, PAH-H sum, PAH-L sum, PAH-M sum | Naphthalene, anthracene, fluoranthene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(ghi)perylene, indeno(1,2,3-cd)pyrene |
| **Per- and polyfluorinated substances** | *n/a* | PFBS, PFPeS, PFHxS, PFHpS, PFOS linear, PFOS branched, PFOS total, PFDS, PFPeA, PFHxA, PFHpA, PFOA linear, PFOA branched, PFOA total, 4:2 FTS, 6:2 FTS, 8:2 FTS, PFBA, PFNA, PFDA, PFUnDA, PFDoDA, PFOSA, HPFHpA, H4-PFUnDA, sum 11 PFAS |

**Table 3.** Results from the chemical analysis of the top two centimeters of sediment in the two sample sites in Skutbosjön and reference site 1, as well as the results from the chemical analysis of water samples from Skutbosjön site 1 and reference site 1. Included in the table are also the environmental quality standards (EQS) defined by the Swedish Agency for Marine and Water Management (HVFMS 2019:25). The asterisks mark an exceedance of the EQS and DS is the abbreviation for dry substance. Data for copper, TBT, anthracene and fluoranthene in the sediment samples have been normalized against the total organic carbon (TOC) content, in order to obtain the bioavailable values.

| Sediment samples | | | | | |
|---|---|---|---|---|---|
| **Compound** | **Unit** | **Reference site 1** | **Skutbosjön site 1** | **Skutbosjön site 2** | **EQS** |
| Anthracene | µg/kg DS | <3 | 12.92 | 219.51* | 24 |
| Fluoranthene | µg/kg DS | 14.79 | 100 | 1585.27 | 2000 |
| Copper | mg/kg DS | 26.76 | 133.33* | 463.41* | 52 |
| Zinc | mg/kg DS | 160 | 490 | 490 | *n/a* |
| Tributyltin | µg/kg DS | <1 | 0.71 | 1.59 | 1.6 |
| Octachlorodibenzo-dioxin (OCDD) | ng/kg DS | 66 | 190 | 380 | *n/a* |
| Octachlorodibenzo-furan (OCDF) | ng/kg DS | 7.3 | 39 | 95 | *n/a* |

| Water samples | | | | |
|---|---|---|---|---|
| **Compound** | **Unit** | **Reference site** | **Skutbosjön site 1** | **EQS** |
| Fluoranthene | ng/L | 0.47* | 0.77* | 0.12 |
| Copper | µg/L | 2.1* | 2.8* | 0.5 |
| Zinc | µg/L | 5.7* | 6.3* | 5.5 |
| Arsenic | µg/L | 0.57* | 0.52 | 0.55 |

## 3.2 Perch experiments

### 3.2.1 Morphometric data

Table 4 is showing the morphometric data obtained from the sampling of perch exposed to whole sediment samples from Skutbosjön and the reference site during three and seven weeks. The fork length (the distance between the tip of the snout and the end of the middle caudal fin of the fish), total weight and the liver weight of each individual was noted, after which the condition factor and liver somatic index was calculated as follows:

$$\text{Condition factor} = 100 * \frac{\text{total weight } (g)}{\text{length}^3\ (cm)}$$

$$\text{Liver somatic index} = 100 * \frac{\text{liver weight } (g)}{\text{total weight } (g)}$$

No statistically significant differences were found between treatments for either of the exposures and no time-dependent differences within each treatment could be seen ($p > 0.05$).

**Table 4.** Morphometric data for perch exposed during three and seven weeks to sediment collected from the Skutbosjön sites, the reference sites and freshwater only (NC = negative control) ($n = 7$). The data is shown as mean values ± SE. No statistically significant differences were found between treatments and no time-dependent differences could be seen within treatments ($p > 0.05$).

| | | Length (cm) | Weight (g) | Liver weight (g) | Condition factor (g/cm$^3$) | Liver somatic index |
|---|---|---|---|---|---|---|
| **Three weeks exposure** | NC | 12.17 ± 0.68 | 20.04 ± 3.35 | 0.24 ± 0.02 | 1.05 ± 0.01 | 1.42 ± 0.22 |
| | Reference site 1 | 11.04 ± 0.39 | 14.82 ± 1.59 | 0.22 ± 0.02 | 1.08 ± 0.01 | 1.49 ± 0.12 |
| | Reference site 2 | 10.87 ± 0.50 | 14.03 ± 2.08 | 0.19 ± 0.03 | 1.05 ± 0.01 | 1.29 ± 0.04 |
| | Skutbosjön site 1 | 10.80 ± 0.56 | 14.12 ± 2.19 | 0.17 ± 0.03 | 1.06 ± 0.02 | 1.27 ± 0.07 |
| | Skutbosjön site 2 | 10.29 ± 0.44 | 11.86 ± 1.72 | 0.18 ± 0.02 | 1.05 ± 0.02 | 1.57 ± 0.12 |
| **Seven weeks exposure** | NC | 11.13 ± 0.55 | 14.30 ± 2.29 | 0.17 ± 0.03 | 0.99 ± 0.01 | 1.20 ± 0.10 |
| | Reference site 1 | 12.06 ± 0.65 | 17.80 ± 2.74 | 0.19 ± 0.02 | 0.98 ± 0.03 | 1.11 ± 0.08 |
| | Reference site 2 | 10.91 ± 0.49 | 13.84 ± 2.05 | 0.15 ± 0.02 | 1.02 ± 0.01 | 1.15 ± 0.09 |
| | Skutbosjön site 1 | 11.90 ± 0.39 | 17.62 ± 1.76 | 0.21 ± 0.02 | 1.03 ± 0.04 | 1.24 ± 0.11 |
| | Skutbosjön site 2 | 11.41 ± 0.56 | 15.60 ± 1.82 | 0.17 ± 0.02 | 1.02 ± 0.02 | 1.14 ± 0.05 |

### 3.2.2 Hepatic gene expression

Shown in Table 5 and Fig. 8A-E are the results from the gene expression analysis of CYP1A, GR, CAT, SOD and VTG for perch liver samples, following exposure to sediment samples for three and seven weeks. No statistically significant alterations in gene expression was found for any of the examined genes between treatments ($p > 0.05$). However, a significant temporal time trend between the three and seven week exposure duration could be observed for the expression of CYP1A in perch exposed to sediment from both of the reference sites ($p = 0.014$ and 0.029) as well as from Skutbosjön site 1 ($p = 0.002$). The same time-dependent differences for the three sites was shown also for GR expression ($p = 0.011$, 0.002 and 0.001, respectively).

The obtained data from the gene expression of VTG should be interpreted with caution since a number of outliers have been excluded. A large induction of the expression of VTG is usually seen for sexually mature females, but no correlation between the outliers and gender could be established. Additionally, no correlation was found between the outliers and treatment groups.

**Table 5.** Relative mRNA expressions of CYP1A, GR, CAT, SOD and VTG in perch liver samples ($n$ = 7 for all groups with the exception of negative controls (NC) for the seven weeks exposure period, where $n$ = 6). Data is shown as mean values ± SE. No statistically significant differences were found between treatments ($p > 0.05$), but a significant time-dependent differences could be seen within treatments for CYP1A and GR expression in perch exposed to sediment from the two reference sites and Skutbosjön site 1.

| | | **Average relative mRNA expression** | | | | |
|---|---|---|---|---|---|---|
| *Gene* | *Exposure duration* | NC | Ref. site 1 | Ref. site 2 | Skutbosjön site 1 | Skutbosjön site 2 |
| CYP1A | Three weeks | 303.50 ± 33.65 | 292.02 ± 21.97 | 310.02 ± 32.44 | 301.04 ± 15.27 | 386.74 ± 56.73 |
| | Seven weeks | 404.37 ± 36.08 | 433.26 ± 44.06 | 428.08 ± 34.92 | 519.53 ± 43.37 | 543.36 ± 57.35 |
| GR | Three weeks | 6.87 ± 0.65 | 7.61 ± 0.75 | 7.08 ± 0.59 | 7.05 ± 0.66 | 8.63 ± 0.76 |
| | Seven weeks | 9.52 ± 1.08 | 11.99 ± 1.16 | 12.06 ± 1.06 | 14.08 ± 1.45 | 11.07 ± 0.96 |
| CAT | Three weeks | 440.62 ± 73.45 | 431.45 ± 36.66 | 413.20 ± 36.81 | 430.89 ± 26.98 | 495.52 ± 53.13 |
| | Seven weeks | 436.25 ± 38.65 | 495.21 ± 42.52 | 561.78 ± 97.11 | 490.92 ± 64.59 | 453.58 ± 39.62 |
| SOD | Three weeks | 612.75 ± 98.98 | 525.55 ± 29.28 | 627.12 ± 49.79 | 519.08 ± 51.36 | 675.42 ± 136.99 |
| | Seven weeks | 474.52 ± 43.27 | 536.24 ± 77.36 | 638.40 ± 75.54 | 565.08 ± 63.91 | 566.60 ± 64.65 |
| VTG | Three weeks | 0.07 ± 0.04 | 0.11 ± 0.04 | 0.09 ± 0.02 | 0.07 ± 0.02 | 0.07 ± 0.03 |
| | Seven weeks | 0.13 ± 0.06 | 0.09 ± 0.03 | 0.14 ± 0.08 | 0.16 ± 0.07 | 0.16 ± 0.04 |

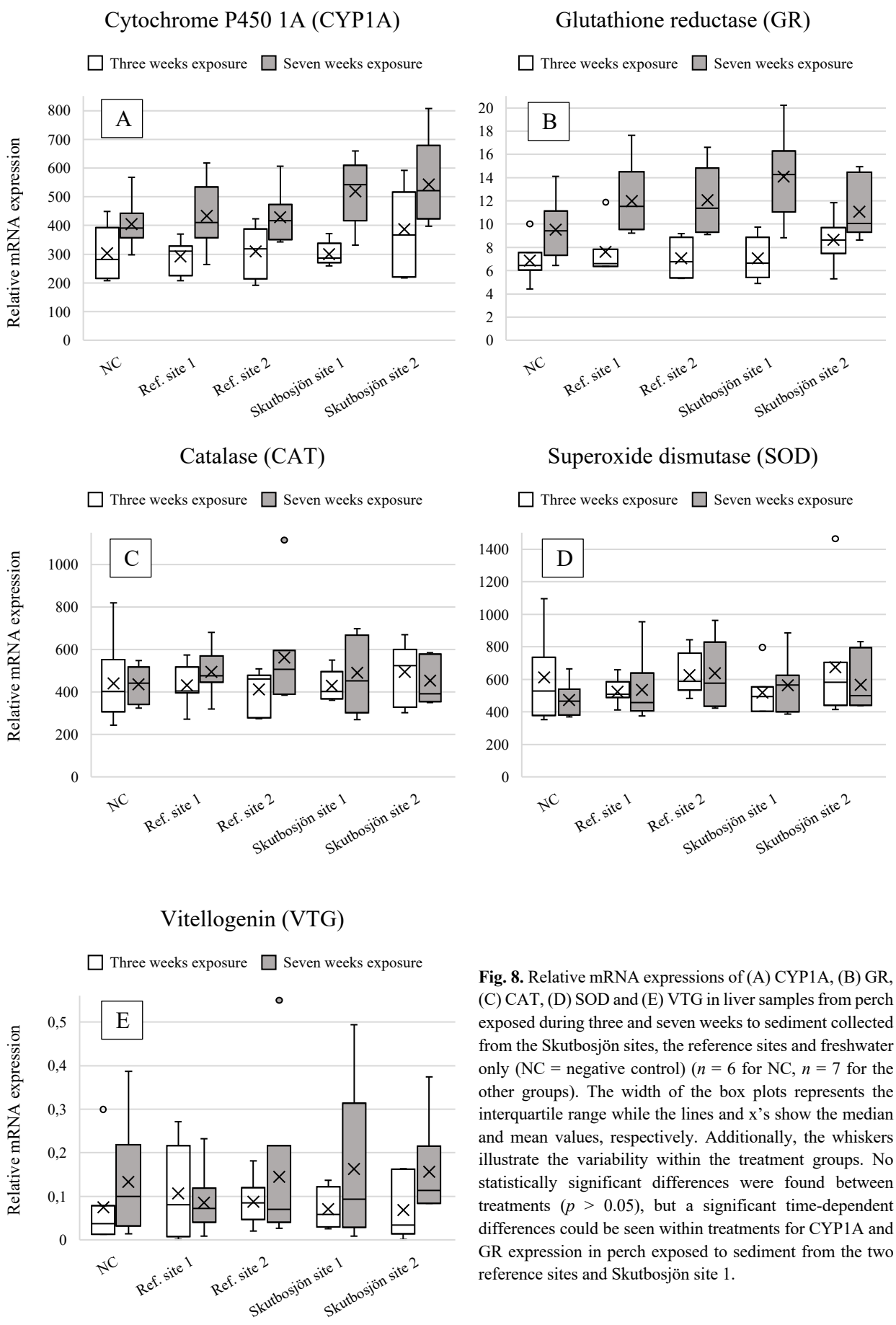


**Fig. 8.** Relative mRNA expressions of (A) CYP1A, (B) GR, (C) CAT, (D) SOD and (E) VTG in liver samples from perch exposed during three and seven weeks to sediment collected from the Skutbosjön sites, the reference sites and freshwater only (NC = negative control) ($n$ = 6 for NC, $n$ = 7 for the other groups). The width of the box plots represents the interquartile range while the lines and x's show the median and mean values, respectively. Additionally, the whiskers illustrate the variability within the treatment groups. No statistically significant differences were found between treatments ($p$ > 0.05), but a significant time-dependent differences could be seen within treatments for CYP1A and GR expression in perch exposed to sediment from the two reference sites and Skutbosjön site 1.

### 3.2.3 Gill and hepatic EROD activity

The highest induction of hepatic and gill EROD activity was found in perch exposed to the sediment collected from the sites in Skutbosjön, as can be seen in Table 6 and Fig. 9A and 9B, although no statistically significant differences between treatments were found ($p > 0.05$). An indication of a temporal trend of an increasing EROD activity over time can be seen for the liver samples in all treatments (see Fig. 9B). However, statistically significant time-dependent differences were found only for the negative control and for the perch exposed to sediment from Skutbosjön site 1 ($p = 0.01$ and 0.05, respectively).

**Table 6.** Average gill and hepatic EROD activity in perch exposed during three and seven weeks to sediment collected from the Skutbosjön sites, the reference sites and freshwater only (NC = negative control) ($n = 7$). The data is shown as mean values ± SE. No statistically significant differences were found between treatments ($p > 0.05$), but a significant time-dependent increase in hepatic EROD activity could be seen for the negative controls and the perch exposed to sediment from Skutbosjön site 1 ($p = 0.01$ and 0.05, respectively).

| | **Average gill EROD activity** | | **Average hepatic EROD activity** | |
|---|---|---|---|---|
| | **Three weeks** | **Seven weeks** | **Three weeks** | **Seven weeks** |
| NC | 0.30 ± 0.12 | 0.49 ± 0.09 | 106.19 ± 5.94 | 142.90 ± 10.00 |
| Reference site 1 | 0.62 ± 0.11 | 0.57 ± 0.11 | 109.16 ± 7.71 | 164.18 ± 32.47 |
| Reference site 2 | 0.44 ± 0.08 | 0.69 ± 0.13 | 118.32 ± 11.09 | 145.31 ± 16.89 |
| Skutbosjön site 1 | 1.44 ± 0.35 | 1.06 ± 0.19 | 129.71 ± 13.32 | 204.44 ± 33.99 |
| Skutbosjön site 2 | 0.59 ± 0.23 | 0.86 ± 0.19 | 144.32 ± 33.26 | 198.95 ± 22.15 |

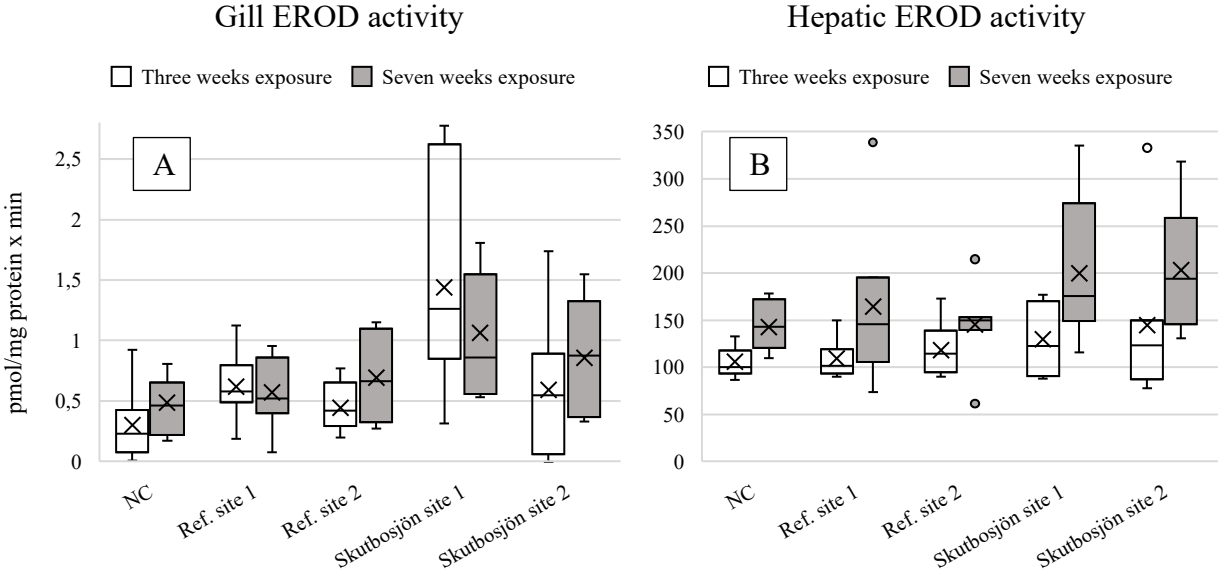


**Fig. 9.** EROD activity in (A) gill and (B) liver samples from perch exposed during three and seven weeks to sediment collected from the Skutbosjön sites, the reference sites and freshwater only (NC = negative control) ($n = 7$). The width of the box plots represents the interquartile range while the lines and x's show the median and mean values, respectively. Additionally, the whiskers illustrate the variability within the treatment groups. No statistically significant differences were found between treatments ($p > 0.05$), but a significant time-dependent increase in hepatic EROD activity could be seen for the negative controls and the perch exposed to sediment from Skutbosjön site 1 ($p = 0.01$ and 0.05, respectively).

## 3.3 Embryotoxicity in zebrafish (*Danio rerio*)

### 3.3.1 Extract exposure

The most prominent effect observed in the Fish Embryo Acute Toxicity (FET) tests with zebrafish eggs exposed to sediment extracts, were delayed and reduced hatching rates for eggs exposed to extracts made from sediment samples from reference site 2 and from the two sites in Skutbosjön (see Fig. 10). Particularly pronounced was this sublethal effect in the sediment samples from Skutbosjön site 2, where an average hatching rate of solely 15.56 ± 8.84 % was seen for the highest concentration of extract at 96 hpf, i.e. at the end of the test. The equivalent values for the negative control, reference site 1 and 2 and Skutbosjön site 1 were 91.11 ± 3.51 %, 83.33 ± 8.98 %, 47.78 ± 11.03 % and 56.67 ± 7.64 %, respectively. At 72 hpf, the average hatching rates in the highest extract concentrations were found to be 91.11 ± 3.51 % for the negative control, 80.00 ± 10.00 % and 37.78 ± 11.76 % for reference site 1 and 2, respectively. For Skutbosjön site 1 and 2, the average hatching rates were 30.00 ± 8.82 % and 3.33 ± 2.36 %, respectively.

Hatching rates are recorded at 48 hpf and onwards. At that point in time, statistically significant differences between hatching rates were found for the Skutbosjön site 2 extracts in comparison with the negative control for the three highest concentrations out of the total five, i.e. 9, 12 and 15 ml per total volume ($p$ = 0.029, 0.023 and 0.011, respectively). For the Skutbosjön site 1 extracts, the concentrations of 6, 9 and 15 ml per total volume resulted in statistically significant differences when compared with the negative control ($p$ = 0.033, 0.029 and 0.011, respectively). Additionally, statistically significant differences could be seen for the reference site 2 extracts against the negative control in the concentrations 12 and 15 ml per total volume ($p$ = 0.023 and 0.011, respectively).

Differences in hatching rate at 72 hpf in comparison with the controls indicate a delay in hatching. At 72 hpf, the Skutbosjön site 2 extracts caused statistically significant differences in hatching rates for the four highest concentrations of 6, 9, 12 and 15 ml per total volume, when compared to the negative control ($p$ = 0.02, 0.001, 0.000 and 0.000, respectively) and reference site 1 ($p$ = 0.002, 0.000, 0.000 and 0.000, respectively). Statistically significant differences for Skutbosjön site 1 extracts were found for the two highest concentrations (12 and 15 ml per total volume) when compared to the negative control ($p$ = 0.003 and 0.028, respectively) and for the concentrations of 6, 9 and 12 ml per total volume in comparison to reference site 1 ($p$ = 0.029, 0.039 and 0.003, respectively). Lastly, statistically significant differences were found for reference site 2 extracts against the negative control and reference site 1 for the concentrations of 9 ml per total volume ($p$ = 0.028 and 0.012, respectively) and 12 ml per total volume ($p$ = 0.002), as well as for the concentration of 6 ml per total volume when compared to reference site 1 ($p$ = 0.016).

At the end of the exposure time (96 hpf), a similar pattern as for the 48 and 72 hpf readings of hatching rates was seen, with statistically significant differences for the three highest concentrations of 9, 12 and 15 ml per total volume of the Skutbosjön site 2 extracts in

comparison with the negative control ($p$ = 0.001, 0.000 and 0.000, respectively) and reference site 1 ($p$ = 0.000, 0.000 and 0.001, respectively). At 6 ml per total volume, the Skutbosjön site 2 extracts resulted in statistically significant differences against reference site 1 ($p$ = 0.001). For the other extracts, statistically significant differences were seen for Skutbosjön site 1 in the concentrations of 6 and 9 ml per total volume when compared to reference site 1 ($p$ = 0.036 and 0.027, respectively), for reference site 2 in the concentrations of 6 and 12 ml per total volume against reference site 1 ($p$ = 0.025 and 0.002, respectively) and, lastly, for reference site 2 against the negative control for the concentration of 12 ml per total volume ($p$ = 0.002).

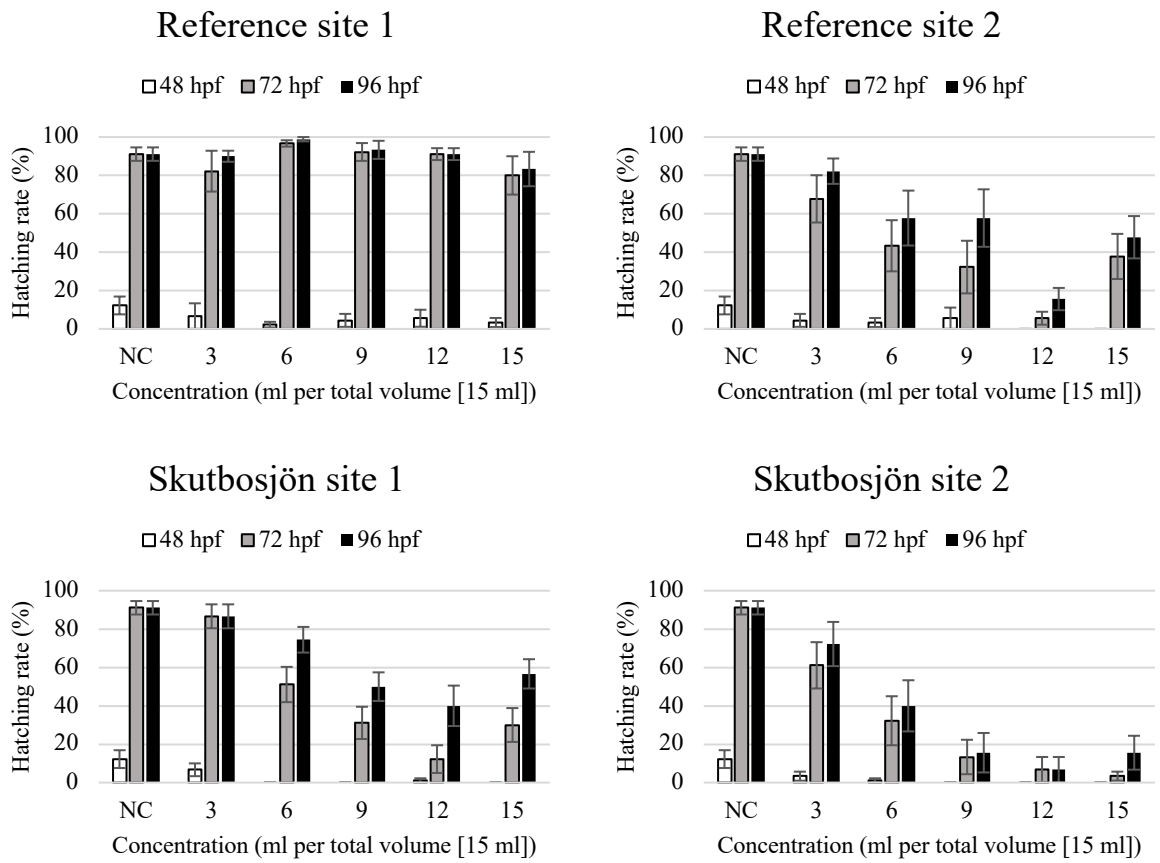


**Fig. 10**. Hatching rates at 48, 72 and 96 hpf in zebrafish eggs exposed to sediment extracts made from sediment samples from the Skutbosjön sites and the reference sites, presented as mean values ± SE from three independent experiment replicates ($n$ = 30). NC = negative control, i.e. embryo medium only.

Exposure to sediment extracts did not lead to statistically significant lethal effects in any of the exposures. Positive outcomes were only recorded for the endpoint coagulation of fertilized eggs (see Fig. 11), i.e. in one in the four indicators of lethality (the rest being lack of somite formation, lack of detachment of the tail-bud from the yolk sac and lack of heartbeat). However, no statistically significant differences were found between treatments ($p > 0.05$), indicating that the recorded coagulation of eggs had no correlation to the exposure scenarios. Furthermore, the standard errors shown in Fig. 11 are relatively large, reflecting a high irregularity of mean values within the treatments and subsequently making the results less representative.

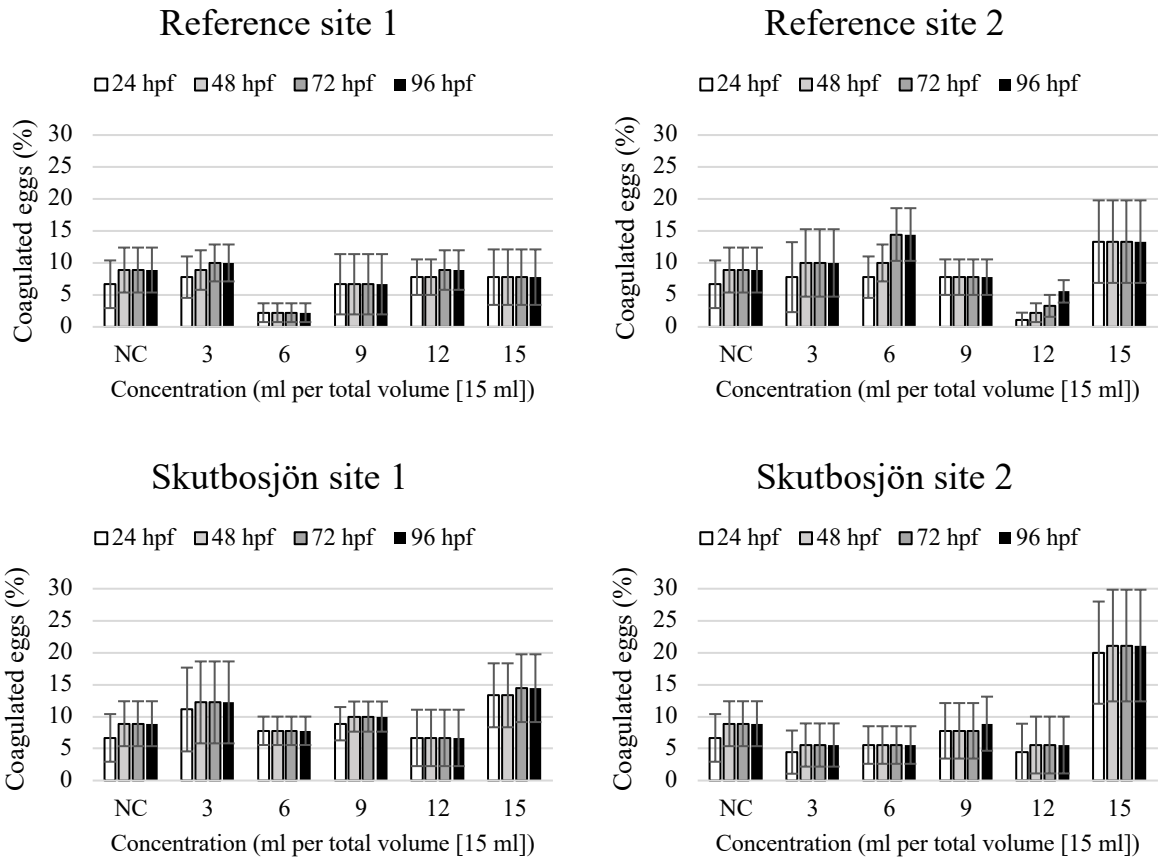


**Fig. 11**. Coagulation of fertilized eggs at 24, 48, 72 and 96 hpf in zebrafish eggs exposed to sediment extracts made from sediment samples from the Skutbosjön sites and the reference sites, presented as mean values from three independent experiment replicates ± SE ($n$ = 30). NC = negative control, i.e. embryo medium only. Not shown in the figure is the positive control (50 µM copper ions), which induced a 100 % mortality at 24 hpf.

The criteria ensuring the validity of the test results were fulfilled in the extract assay, with an overall survival of 91.1 % of embryos in the negative control at 96 hpf, a mortality rate of 100 % in the positive control at 96 hpf and a hatching rate of 91.1 % in the negative control at 96 hpf.

### 3.3.2 Whole-sediment exposure

As with the sediment extract exposures, the most pronounced effect observed in the zebrafish exposed to whole-sediment were delayed and reduced hatching rates (see Fig. 12 and Fig. 14). This sublethal effect was seen most clearly in the Skutbosjön site 2 treatment, where the average hatching rate for the highest concentration (i.e. only sediment from Skutbosjön site 2) at 96 hpf was 0 %. The equivalent value for the negative control and the artificial sediment exposure at 96 hpf was 100.00 % and 98.89 ± 1.11 %, respectively. When exposed to sediment from Skutbosjön site 1, reference site 1 and reference site 2, the average hatching rates were found to be 61.11 ± 9.04 %, 88.89 ± 3.89 % and 46.67 ± 9.86 %, respectively, at the end of the exposure.

Also at 72 hpf, the average hatching rate for the Skutbosjön site 2 treatment was 0 %. This can be compared to the negative control and the artificial sediment treatments, where an average hatching rate of 100 % and 97.78 ± 2.22 %, respectively, was seen. For Skutbosjön site 1 and reference site 1 and 2, the equivalent values were 2.22 ± 1.47 %, 64.44 ± 9.44 % and 18.89 ± 11.11 %, respectively.

Regarding the statistics, all of the concentrations of the four sediment mixtures (Skutbosjön site 1 mixed with reference site 1, Skutbosjön site 1 mixed with reference site 2, Skutbosjön site 2 mixed with reference site 1 and Skutbosjön site 2 mixed with reference site 2) resulted in statistically significant differences for hatching rates in comparison with the negative control at 48 hpf ($p = 0.024$), since no eggs had hatched in any of the sediment treatments. At 72 hpf, the highest concentrations of sediment from Skutbosjön site 1, Skutbosjön site 2 and reference site 2 (i.e. only sediment from the respective site, no mixtures) resulted in statistically significant differences when compared to the negative control and the artificial sediment ($p = 0.000$ for the Skutbosjön sites and $p = 0.001$ and 0.003 for reference site 2). Furthermore, statistically significant differences were found for all of the concentrations of Skutbosjön site 2 mixed with both reference site 1 and 2 against the negative control and the artificial sediment exposure ($p = 0.000$). Additionally, the concentrations of 4, 6 and 8 mg per total weight of both of the Skutbosjön site 2 mixtures induced statistically significant differences when compared to reference site 1 ($p = 0.045$, 0.039 and 0.022, respectively). For the Skutbosjön site 1 and reference site 2 mixture, statistically significant differences could be seen against the negative control and the artificial sediment for all of the concentrations ($p = 0.000$ for the concentrations of 2 mg per total weight up to 6 mg per total weight, and $p = 0.002$ and 0.003 for the concentration of 8 mg per total weight). Lastly, the Skutbosjön site 1 and reference site 1 mixture of 4 mg per total weight resulted in statistically significant differences when compared to both of the Skutbosjön sites mixtures ($p = 0.038$). This was also seen for the concentration of 8 mg per total weight for the same mixture, against the negative control ($p = 0.014$) and the artificial sediment ($p = 0.026$).

At 96 hpf, a similar statistical pattern as for the 72 hpf readings could be seen for the Skutbosjön site 2 treatments regarding hatching rates. The highest concentrations of Skutbosjön site 1, Skutbosjön site 2 and reference site 2 sediment (i.e. only sediment from the respective site) resulted in statistically significant differences when compared to the negative control and the artificial sediment ($p = 0.018$ and 0.039 for Skutbosjön site 1, $p = 0.000$ for Skutbosjön site 2 and $p = 0.002$ and 0.006 for reference site 2). For Skutbosjön site 2, a statistically significant difference could also be seen when compared to the reference site 1 sediment ($p = 0.002$). In all of the other concentrations of Skutbosjön site 2 sediment mixed with both of the reference sites, statistically significant differences were found in comparison with the negative control and the artificial sediment ($p = 0.000$ for all except for the reference site 1 mixture of 2 mg per total weight, where $p = 0.003$ and 0.007). All of the concentrations also resulted in statistically significant differences when compared to reference site 1, with the exception of the lowest concentration of 2 mg per total weight for the reference site 1 mixture ($p = 0.030$, 0.005 and 0.003 for the concentrations of 4, 6 and 8 mg per total weight for the reference site 1 mixtures, and $p = 0.014$, 0.009, 0.005 and 0.003 for the concentrations of 2, 4, 6, and 8 mg per total weight

for the reference site 2 mixtures). For the Skutbosjön site 1 treatments, only the mixtures with reference site 2 in the two lowest concentrations of 2 and 4 mg per total weight resulted in statistically significant differences against the negative control and the artificial sediment ($p$ = 0.001 and 0.002 for the concentration of 2 mg per total weight and $p$ = 0.004 and 0.008 for the concentration of 4 mg per total weight). Moreover, statistically significant differences could be seen for the Skutbosjön site 1 and reference site 1 mixtures in comparison with the mixture of Skutbosjön site 2 and reference site 1, as well as the mixture of Skutbosjön site 2 and reference site 2 for the concentrations of 4 mg per total weight ($p$ = 0.003 and 0.001), 6 mg per total weight ($p$ = 0.007) and 8 mg per total weight ($p$ = 0.026). Lastly, the lowest concentration of 2 mg per total weight of the Skutbosjön site 1 and reference site 1 mixture were statistically significant against the Skutbosjön site 1 and reference site 2 mixture ($p$ = 0.031) as well as the Skutbosjön site 2 and reference site 2 mixture ($p$ = 0.002).

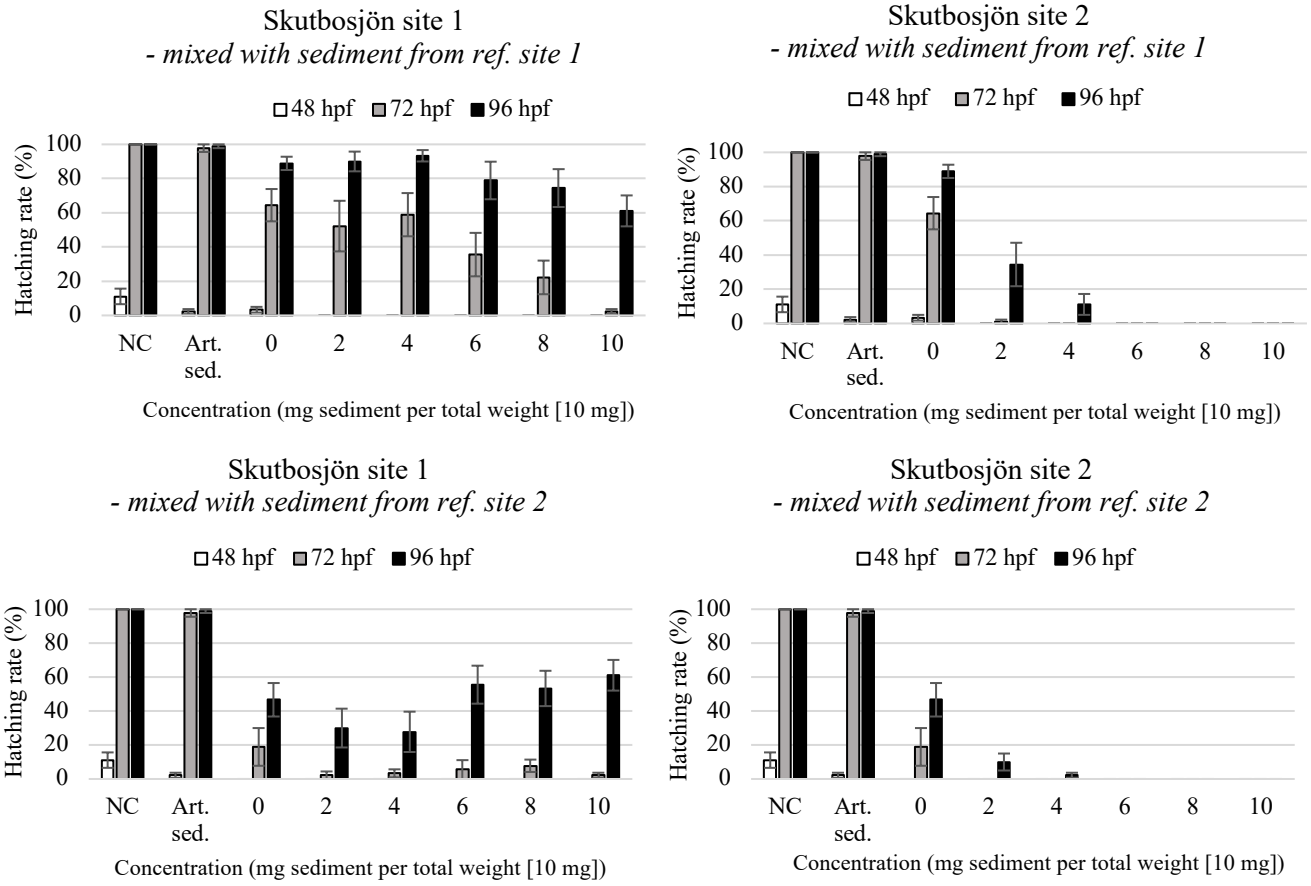


**Fig. 12**. Hatching rates at 48, 72 and 96 hpf in zebrafish eggs exposed to sediment samples from the Skutbosjön sites and the reference sites, presented as mean values ± SE from three independent experiment replicates ($n$ = 30). A concentration of 0 mg sediment per total weight represents sediment from the reference site only. NC = negative control, i.e. embryo medium only, artificial sediment = clean, artificially made sediment.

The exposure to whole-sediment samples did not lead to statistically significant lethal effects in any of the exposures, except for the Skutbosjön site 1 sediment mixed with reference site 2 for the endpoint coagulation of fertilized eggs at 96 hpf (see Fig. 13). For that mixture, the concentration of 6 mg per total weight resulted in statistically significant differences when compared to the negative control and to the Skutbosjön site 2 sediment mixed with reference site 1 ($p$ = 0.019).

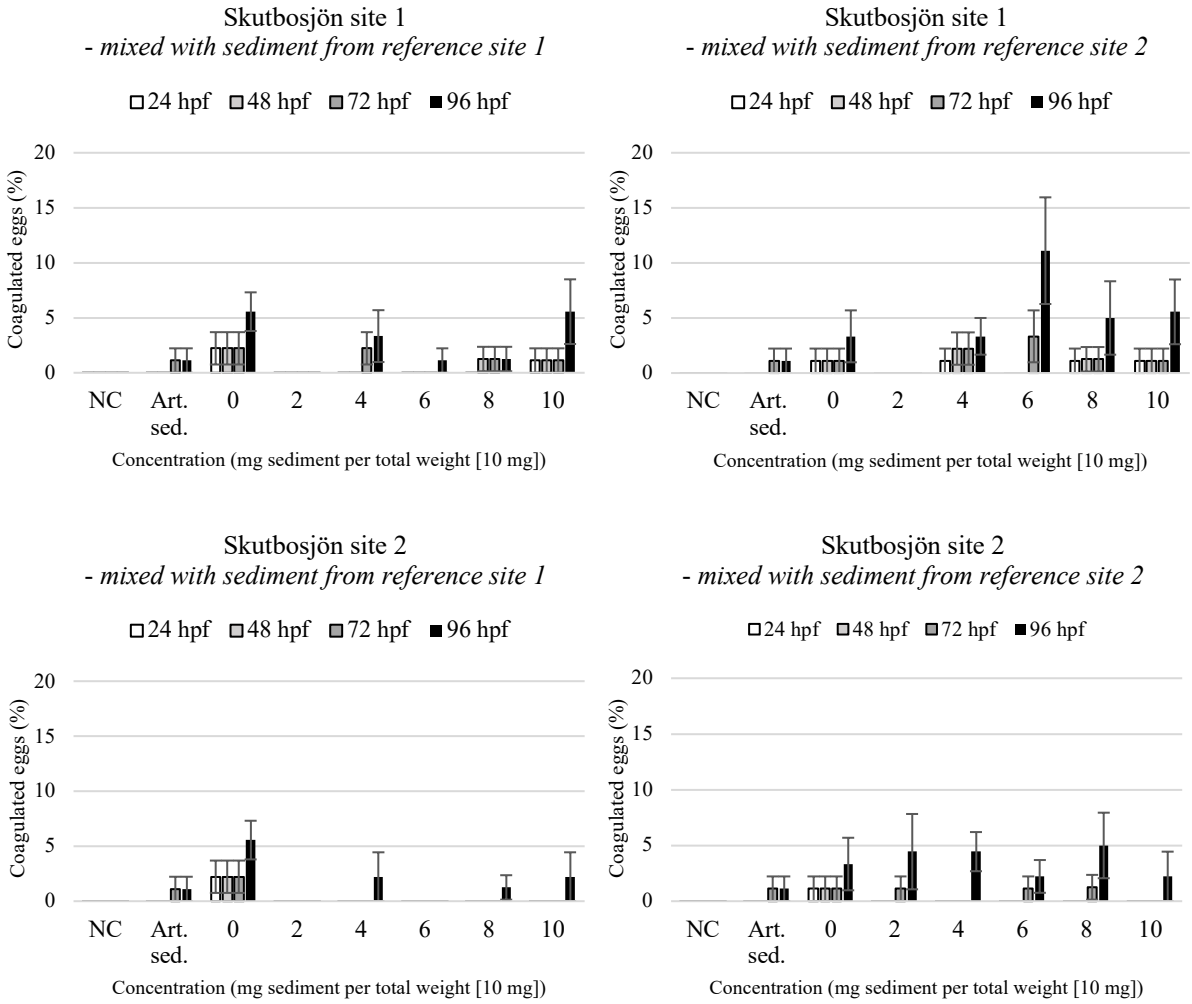


**Fig. 13.** Coagulation of fertilized eggs at 24, 48, 72 and 96 hpf in zebrafish eggs exposed to whole-sediment mixtures from the Skutbosjön sites and the reference sites, presented as mean values from three independent experiment replicates ± SE ($n$ = 30). A concentration of 0 mg sediment per total weight represents sediment from the reference site only. NC = negative control, i.e. embryo medium only, artificial sediment = clean, artificially made sediment. Not shown in the figure is the positive control (50 µM copper ions), which induced a 100 % mortality at 24 hpf.

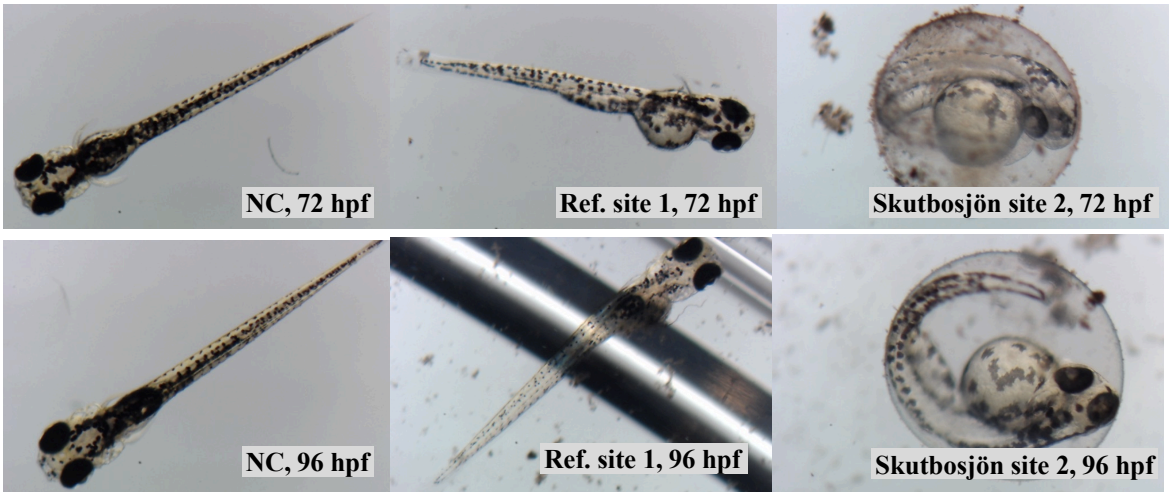


**Fig. 14.** Zebrafish exposed to freshwater only (NC) as well as whole-sediment samples from reference site 1 and Skutbosjön site 2, at 72 and 96 hpf.

The criteria ensuring the validity of the test results were fulfilled in the whole-sediment assay, with an overall survival of 100 % of embryos in the negative control at 96 hpf, a mortality rate of 100 % in the positive control at 96 hpf and a hatching rate of 100 % in the negative control at 96 hpf.

## 3.4 Enzymatic activity in freshwater snails

Shown in Fig. 15 is the enzymatic activity of GST in freshwater snails ($n$ = 14) collected from the reference site Hällestadsån and at Skutbosjön, with average values of 0.37 ± 0.02 and 0.41 ± 0.03 mmol/mg/min, respectively. No statistically significant difference was found between the two exposure scenarios ($p$ = 0.982).

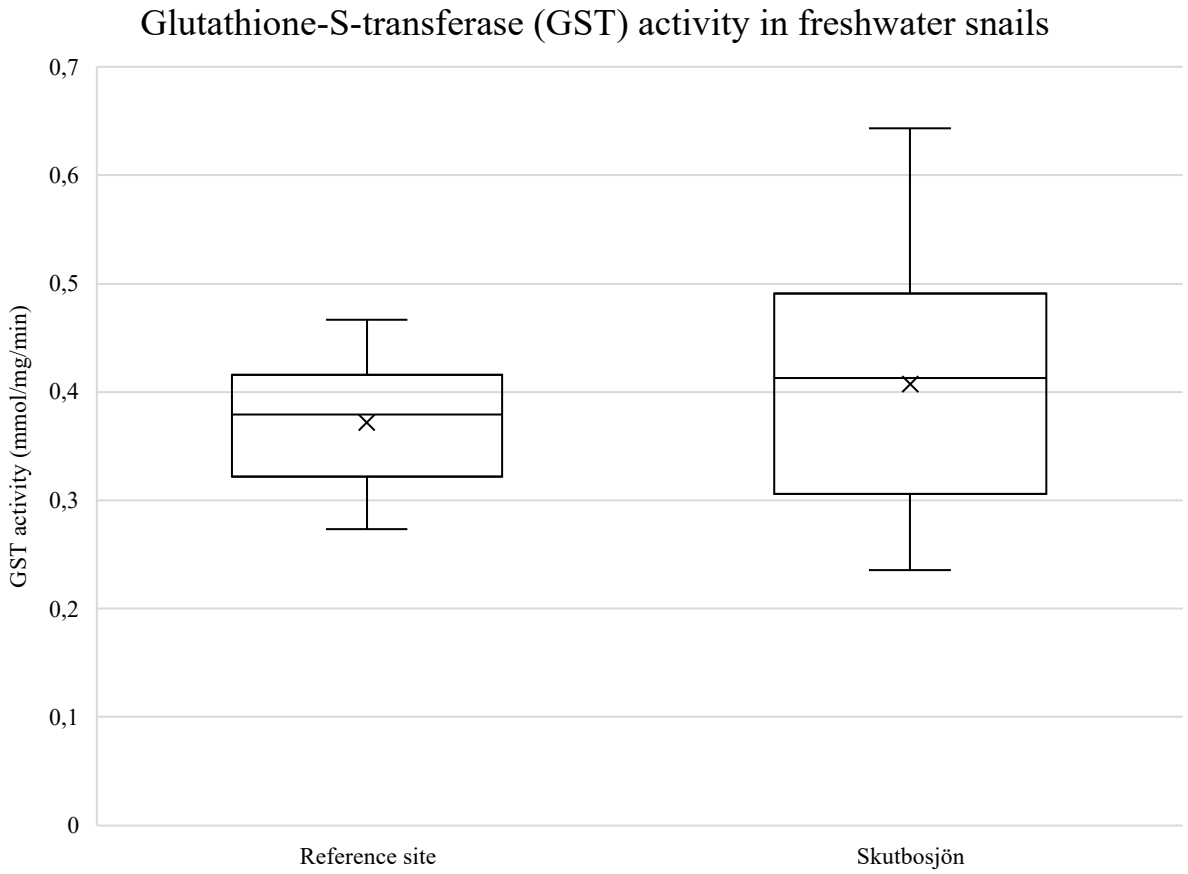


**Fig. 15.** GST activity in freshwater snails collected from the reference site and at Skutbosjön ($n$ = 14). The width of the box plots represents the interquartile range while the lines and x's show the median and mean values, respectively. Additionally, the whiskers illustrate the variability within the treatment groups. No statistically significant difference was found between the two sites ($p$ = 0.982).

# 4 Discussion

This thesis aimed to investigate sediment toxicity of the lake Skutbosjön to aquatic organisms by assessing gene expression and enzymatic activity related to detoxification and oxidative stress in juvenile perch, examining embryotoxicity in zebrafish and, lastly, measuring enzymatic activity associated with detoxification processes in freshwater snails collected from the study sites.

## 4.1 Chemical analysis of sediment and water samples

The chemical analysis of the sediment samples from the two sites in Skutbosjön showed elevated levels of PAHs, dioxins/furans and metals in comparison with the reference site (see Table 3). Additionally, exceedances of the environmental quality standards for anthracene and copper could be established. Several of these substances (or the group of substances they belong to) are included in the Water Framework Directive list of priority substances (European Commission, 2019c), meaning they have been shown to be of major concern for European waters (European Commission, 2020). This applies for anthracene, fluoranthene, TBT, OCDD (belonging to the group polychlorinated dibenzo-p-dioxins) and OCDF (belonging to the group polychlorinated dibenzofurans). However, the results from the chemical analysis cannot be said to fully reflect the chemical contents of the sediment samples used for the perch and zebrafish exposures. The samples sent for chemical analysis were taken with a technique where only the top two centimeters of sediment are collected, whereas the samples used for the different exposure scenarios also consist of sediment from deeper parts of the lakebed. Consequently, the data can only be interpreted as indicative of the actual chemical content. Although this thesis focus on sediment toxicity, the data from the chemical analysis of the collected water samples must also be considered in the broader context of risk evaluation. Most noticeable in the obtained water data were the concentrations of fluoranthene, copper and zinc, which all exceeded the EQS for both Skutbosjön and the reference site (see Table 3).

## 4.2 Perch experiments

### 4.2.1 Biological responses following sediment exposure

A number of morphometric indices were measured in the perch upon sampling, including length, weight, liver weight, condition factor and liver somatic index. Apart from an on average higher length and total weight in the control fish of the three week exposure experiment in comparison to the other treatments (see Table 4), no group was particularly distinctive and no statistically significant differences were found between treatments or exposure durations ($p > 0.05$), indicating a comparable general physiological health for all individuals.

Furthermore, no statistically significant differences could be seen between the treatment groups in hepatic mRNA expression of the genes examined (CYP1A, GR, CAT, SOD and VTG) or in EROD activity ($p > 0.05$). However, a statistically significant temporal time trend between the

three and seven week exposure duration was found for the expression of CYP1A in perch exposed to sediment from both of the reference sites ($p$ = 0.014 and 0.029) as well as from Skutbosjön site 1 ($p$ = 0.002). The same time-dependent differences for these three sites was shown also for GR expression ($p$ = 0.011, 0.002 and 0.001, respectively), indicating that prolonged exposure periods might result in more prominent biological effects on a genetic level. Regarding EROD activity, an indication of a temporal trend of an increasing EROD activity over time can be seen for the liver samples in all treatments (see Fig. 9B). However, statistically significant time-dependent differences were found only for the negative control and for the perch exposed to sediment from Skutbosjön site 1 ($p$ = 0.01 and 0.05, respectively). Although statistically significant on an enzyme level, this temporal trend was supported on a genetic level solely for Skutbosjön site 1 (see Section *3.2.2 Hepatic gene expression*), indicating that the significant time-dependent difference seen in the negative controls might be due to other reasons than the exposure scenarios.

Generally, a higher variability within treatments was seen for the perch exposed to sediment from Skutbosjön in relation to the reference site (see Fig. 8-9). Variability in biological responses between individuals receiving the same treatment is commonly observed in tests involving living organisms even during controlled laboratory conditions, and can be explained by the inherent biological differences that exists between individuals of the same species. Factors such as body weight, gender, state of health and nutritional status all might influence how chemical compounds are absorbed, distributed and metabolized in an organism and, thus, result in variability in biological responses (Food Standards Agency, 2007; National Research Council, 2000). Rather than a significant induction of e.g. enzymatic activity, an elevated variability can often be seen within groups following low-dose exposure to toxicants (Sturve, pers. comm., 2020). In addition to the individual differences in sensitivity, there is a possibility of exposure heterogeneity within treatment groups in sediment-based studies. Although the sediment in the present study were stirred thoroughly before being distributed into the aquariums, slightly different exposure scenarios due to chemical "hot-spots" in the samples could potentially contribute to the variabilities.

As mentioned in section *3.1 Chemical analysis of sediment and water samples*, the chemical analysis of the sediment samples from the sites in Skutbosjön showed elevated levels of PAHs, dioxins/furans and metals in comparison with the reference site. Additionally, PCB-7 has previously been established to be one of the main contaminants in the sediments of Skutbosjön (WSP, 2017; Länsstyrelsen, 2004). The obtained results in this study indicate that the concentrations of chemical compounds in the sediment samples are not high enough to induce measurable biological responses in the current experimental set-up, and/or that the particle-bound compounds might have a low bioavailability. The uptake and bioconcentration (bioaccumulation from water) of fish to organic compounds such as PAHs and PCBs is strongly linked to the hydrophobic properties of the compounds, as well as e.g. exposure duration and metabolic activity of the individuals (Djomo et al., 1996). To quantify the bioavailability of the contaminants was not included in the aim of this thesis, but it is relevant to consider in the broader context of risk evaluations. Out of the PAHs included in the chemical analysis of the Skutbosjön sediment samples, anthracene and fluoranthene were found in the highest

concentrations. In a previous study by (Djomo et al., 1996), uptake of sediment-adsorbed anthracene in rainbow trout (*Oncorhynchus mykiss*) were shown to be relatively high, but the compound was also rapidly metabolized. Brinkmann et al. (2015) measured the presence of the PAH metabolites 1-hydroxypyrene, 1-hydroxyphenathrene and 3-hydroxybenzo[a]pyrene in rainbow trout (*Oncorhynchus mykiss*) following contaminated sediment exposure, and found concentrations of the PAH metabolites to rapidly increase during the first week of exposure but then remain relatively constant during the rest of the 90 days exposure. Furthermore, the same study measured the uptake and accumulation of six i-PCBs, 12 dl-PCBs and 17 different PCDDs/PCDFs leaching from contaminated sediments and found a significant uptake of these compounds, although to a lesser extent than the PAHs (Brinkmann et al., 2015). Lastly, the bioconcentration of PCDDs/PCDFs with four or more chlorine atoms have been shown to be less than for the PCDDs/PCDFs with fewer (Opperhuizen & Sijm, 1990), which suggests that the dioxins/furans found in the highest concentrations in the Skutbosjön sediment samples (octachlorodibenzodioxin, OCDD, and octachlorodibenzofuran, OCDF) might be tightly bound to the sediment particles. In consideration to these previous findings, the exposure duration of sediment toxicity tests is suggestively of considerable importance for the obtained results.

In 2014, wild perch in Skutbosjön were sampled and analyzed for the presence of PCB-7 in muscle tissue. An average of 5.5 ng/g wet weight for smaller individuals (15-20 cm) and 4.5 ng/g wet weight for larger individuals (20-30 cm) were found, which is approximately double the amount found in fish from the reference lake Bönnern (with corresponding values of 3.0 and 1.8 ng/g wet weight) and demonstrates the bioavailability of PCB-7 (WSP, 2017). Contrary to e.g. PCBs, PAHs are, as previously mentioned, generally relatively quickly metabolized by fish and accumulated to a much lesser extent, making chemical analyses of PAH contents in tissues less representative (Hanson et al., 2009). However, the same perch from Skutbosjön that were analysed for PCB-7 in 2014, were also analysed for the presence of the PAHs anthracene and fluorene. Anthracene and fluorene were found in doses of 1.8 and 58 ng/g lipid weight, respectively, which can be compared with the average values of 0.19-1.2 ng/g lipid weight of anthracene and 4.8-13 ng/g lipid weight of fluorene that were found in 20 Swedish lakes in 2009 (WSP, 2017). An alternative approach to quantifying PAH doses can be to conduct fluorescence measurements in the bile fluid, which are indicative of the PAH uptake (Hanson et al., 2009). In terms of metals, previous investigations have concluded that the main metals found in Skutbosjön (copper, nickel and zinc) are tightly bound to the sediment particles and therefore considered to be of low bioavailability. Based on calculation modelling which estimates the bioavailable fractions of dissolved metals, the average values for copper, nickel and zinc were found to not exceed the quality standards defined by HVMFS 2013:19 (estimated bioavailable fractions of copper, nickel and zinc = 0.04-0.13, 0.16-0.36 and 0.45-1.1 μg/L, respectively, with the corresponding quality standards being 0.5, 4.0 and 5.5 μg/L) (WSP, 2017).

### 4.2.2 Experimental design

The tested approach in this study in order to evaluate sediment toxicity could be further developed. The amount of sediment per kg of fish was relatively low, and it is possible that a

larger amount could have induced measurable biological responses. Moreover, it was not possible to keep the experimental conditions constant throughout the exposure durations. A varying level of stress between the test organisms was visually observed during e.g. feeding. While some individuals kept still when someone approached the aquariums, other were noticeably stressed and began to rapidly swim around and stir up the sediment particles which then, consequently, were brought out of the aquarium with the water flow. Another aspect of importance is the feeding and nutritional status of the perch. During the acclimation period prior to the start of the experiments, the fish were offered standard fish pellets. Since all individuals refused to eat pellets, they were fed red mosquito larvae instead. However, a very varied interest in food was noted and while some fish ate all of the larvae they were given, others ignored them and it remains unclear if the larvae were consumed or not. Alternatively, the rapid swimming behavior of stressed individuals caused larvae to get flushed out with the water flow. This is relevant to consider since the nutritional status can affect the toxicity of a waterborne chemical and, hence, have impact on the study results. A poor nutritional status will have negative effects on the metabolic rate, which is of very high importance for the uptake, metabolism and elimination of a toxicant. Feed deprivation, type of diet and protein content can alter the enzymatic activity of the detoxification system in teleost fish (Lanno et al., 1989). Consequently, there is a possibility that the restrictive food intake might have impacted the results.

A risk within fields such as ecotoxicology, is to place too much emphasis on whether an observed effect is statistically significant or not. As discussed by Chapman et al. (2002), just like a recorded statistical significance does not automatically imply ecological importance, a lack of proven significance can never be said to *not* exert biologically relevant effects to some extent. There are major difficulties and uncertainties associated with estimating ecological relevance and extrapolating results from toxicity tests performed in a laboratory to actual effects seen in the natural environment. Hence, there is often a tendency to rely on whether an effect is statistically significant or not (Chapman et al., 2002). A high p-value (i.e. a non-significant one) might be due to a low number of *n* or a high variability within treatments, resulting in an insufficient amount of evidence to be able to reject a null hypothesis (Steel et al., 2013). These aspects are relevant to consider in the context of this study, where a general lack of statistical significance was observed. No conclusions of the actual effects for wild aquatic organisms in Skutbosjön can be drawn based only on the obtained study results. An adjusted experimental design (e.g. an increased amount of sediment used for exposures, altered exposure durations and/or a larger number of test species) could lead to results of both higher statistical as well as ecological significance.

### 4.2.3 Potential CYP1A inhibition due to antagonistic effects

Despite the dissimilarities between the exposure conditions in the lab and the natural conditions in the field, the perch in this study were exposed to complex mixtures of compounds much alike those in Skutbosjön. The interactions between substances in chemical mixtures aggravates the estimations of biological effects following exposure, due to the possibilities of synergistic or antagonistic effects. Synergism means that the actual effect is higher than simply the sum of

the predicted, individual effects of the mixture components, while antagonism results in the opposite, i.e. reduced observed effects than the sum of effects of the individual compounds (Newman, 2015). Antagonism might be an explanation to why such a low CYP1A activity was seen in the Skutbosjön treatments, despite the high concentrations of organic compounds found in the sediment which usually induce activity. Also found in the sediment samples was TBT, which have been shown to inhibit the expression and function of CYP1A in fish (Di Gulio & Hinton, 2008). TBT is a lipophilic compound, which to a large extent is sediment-bound in the aquatic environment (Dingle Pope, 1998). However, the stirring that occurred in the aquariums in the perch experiments because of the movement patterns of the stressed individuals could, suggestively, have facilitated the resuspension of TBT from the sediment, thus making the compound more bioavailable. The active monooxygenase system in fish can oxidize TBT into hydroxylated derivates followed by phase II-related enzyme mediated conjugation and, consequently, facilitate elimination of TBT. In contrast to e.g. molluscs who tend to accumulate TBT due to their low cytochrome P450 content, fish have the ability to, to a certain extent, more rapidly metabolize TBT (Lee, 1996). This might be a possible explanation to why a significant time-dependent difference was seen for the perch exposed to sediment from Skutbosjön site 1. Since there was no inflow of additional TBT, the perch might have been given the opportunity to metabolize TBT which potentially might result in an increase of CYP1A activity over time.

## 4.3 Fish Embryo Acute Toxicity (FET) test with zebrafish

### 4.3.1 Lethal and sublethal effects following exposure

A low and statistically non-significant mortality rate with positive outcomes for the endpoint coagulation of fertilized eggs could be seen for all of the tested sediments in comparison with negative control, for both the whole-sediment and extract exposures. The coagulation of eggs could at least partly be explained by limitations in the ability to collect viable and healthy, fertilized eggs due to inexperience. This is further supported by the general decrease of the amount of coagulated eggs across treatments seen for each experimental replicate when inspecting the raw data (not included in the thesis), indicating an increased ability of collecting health eggs with training.

However, pronounced sublethal effects in the form of delayed and reduced hatching rates following incubation with whole-sediment samples and sediment extracts from Skutbosjön were found. The average hatching rate from three independent replicates in embryos exposed to whole-sediment samples from Skutbosjön site 2 was found to be 0 %, consequently resulting in unexceptional failure of reaching the developmental stage expected at 96 hpf. The equivalent value for the Skutbosjön site 1 exposure was 61.11 ± 9.04 %. Since an average hatching rate of 100 % and 98.89 ± 1.11 % for the negative control and the artificial sediment, the effects can most likely be linked to the sediment-bound chemical compounds. For the reference sites, the average hatching rates at 96 hpf were 88.89 ± 3.89 % and 46.67 ± 9.86 % for site 1 and 2, respectively. In the sediment extract exposures, the average hatching rates were found to be 56.67 ± 7.64 % and 15.56 ± 8.84 % for the Skutbosjön site 1 and site 2 exposures, respectively.

For the negative control and reference site 1 and 2, the average hatching rate were 91.11 ± 3.51 %, 83.33 ± 8.98 % and 47.78 ± 11.03 %, respectively.

Reduced and/or delayed hatching rates in zebrafish following exposure to sediments contaminated with organic compounds and metals have been observed in previous studies (see e.g. Massei et al., 2019 and Schiwy et al., 2015). The hatching of fish eggs is stimulated by a combination of the hatching enzyme chorionase, muscular contractions, active embryonic water uptake and increased pressure in the perivitelline space between the outer chorionic membrane and the inner vitelline membrane in teleost eggs (Dave & Xui, 1991; Hisaoka, 1958). Delayed or reduced hatching due to chemical exposure can be explained by e.g. lowered developmental rates or affected enzymatic function of chorionase (Johnson et al., 2007). Hagenmaier (1974) extracted chorionase from rainbow trout eggs and reported chorionase inhibition due to copper exposure. Furthermore, a lowered developmental rate might result in detrimental effects for a developing organism due to a prolongation of the time period that the individual remains in a critical stage, which increases the risk of harmful effects occurring (Newman, 2015). Alterations in reproductive parameters such as hatching success, might result in lowered fitness. To examine sublethal effects related to e.g. reproduction can be considered at least as important as investigating acute or chronic lethal effects (Newman, 2015) and, as stated by Hollert et al. (2003), delayed or reduced hatching rates can represent a more sensitive endpoint to examine than pure lethality. A prolonged exposure duration in the FET tests of this thesis could have determined to what extent the eggs eventually hatched, to thereby examine the viability of the embryos and enable a further investigation of reproductive impacts.

### 4.3.2 Whole sediment vs extract exposure

Several previous studies have examined the embryotoxic effects from extracts made by extracting chemical compounds from sediment samples using organic solvents such as acetone (Strmac et al., 2002; Kosmehl et al., 2008; Hollert et al., 2003). The major drawback of using these types of extracts is their tendency to overestimate toxicity, since they most likely mainly contain high concentrations of compounds that are non-water soluble. Consequently, the bioavailability of particle-bound compounds is not considered, which results in lower ecological relevance. Therefore, whole-sediment assays are generally preferred as they are more representative (Hollert et al., 2003; Kosmehl et al., 2008). It should be noted that while whole-sediment assays primarily expose eggs to water-soluble and bioavailable fractions, hydrophobic compounds can also reach the embryos via the direct contact to the sediment, but the exposure is more limited than extracts made by organic solvents (Kosmehl et al., 2008).

No direct comparison of toxicity between the whole-sediment vs extract exposure scenarios can be done, since the amount of sediment used for the whole-sediment exposures were not of the same proportions as the ones used to prepare the extracts. However, the practical benefits of the extracts combined with the fact that they, similarly to the whole-sediment assays, are considering bioavailability and demonstrably have the ability to induce embryotoxic effects, allows them to be preferred in FET tests of similar experimental setups like in this study. The major disadvantage with the whole-sediment exposures were the difficulties of performing the

daily recordings of outcomes for the lethal endpoints. Several endpoints had to be excluded because they simply could not be examined against the dark sediment backgrounds. These difficulties could have been overcome by temporarily transferring each egg to a petridish containing only medium, but the experimental design with the consequently large amount of eggs made this set-up non-feasible since it would result in major delays and, hence, non-consistency in the timing of the readings between replicates and treatments.

## 4.4 Enzymatic activity in freshwater snails

Gastropods are one of the most commonly used groups of invertebrates used in biomonitoring (Chmist et al., 2018) as well as in hazard and risk assessments of water and sediment (Salánki et al., 2003). Previous studies have emphasized the suitability of gastropods as indicator species of ecosystem health due to their ecological importance, considerable part of biodiversity and sensitivity to a wide selection of organic and inorganic compounds (Tallarico, 2015; de Freitas Tallarice et al., 2014; Zheng & Zhou, 2016). In the present study, a freshwater snail species was collected from Skutbosjön and the reference site, after which the GST activity was examined. No statistically significant difference was found between the snails from Skutbosjön and the reference site ($p = 0.982$), with average values of GST activity being $0.41 \pm 0.03$ and $0.37 \pm 0.02$ mmol/mg/min, respectively. Similarly to the effects seen for the perch in this study that were previously discussed under section *4.2.1 Biological responses following sediment exposure*, a higher variability within the treatment group was observed for the snails collected from Skutbosjön (see Fig. 15). As mentioned before, this is often seen following low-dose exposure to toxicants, as opposed to a significant induction of e.g. enzymatic activity (Sturve, pers. comm., 2020).

Previous studies of freshwater snails have measured the induction of enzymatic activities following contaminated sediment exposure (Wu et al. 2020; Li et al., 2018; Ma et al., 2010), demonstrating the ability of biochemical responses in gastropods as reliable biomarkers in the context of sediment toxicity tests. In terms of sample preparations for general biochemical examinations, different approaches seem to be applied when using gastropods as test organisms. While the hepatopancreas tissue (the digestive gland) is often used (see e.g. Wu et al. 2020, Li et al., 2018 and Ma et al., 2010), some studies are performing analysis in whole-body soft tissue (see e.g. Kristoff et al., 2008, Bhagat et al., 2016 and Nmaduka et al., 2018). The present study used de-shelled, whole-body soft tissue for the measurement of GST activity. No literature investigating the differences in biochemical responses when using the digestive gland or the whole-body soft tissue has been found and, consequently, the potential impact that the choice of sample preparation might have on the obtained results remains unclear.

# 5 Conclusion

The sediment samples from Skutbosjön induced prominent sublethal effects on embryonic stages of zebrafish in the form of delayed and reduced hatching rates, with statistically significant differences seen for the Skutbosjön treatments when compared to control groups. Alterations in reproductive parameters might result in lowered biological fitness and furthermore, a lowered developmental rate leads to prolongation of the time period that the organisms remain in a critical stage, thus making examinations of sublethal effects such as these ecologically important. The study results also showed an elevated CYP1A gene expression as well as EROD activity in perch exposed to sediment from Skutbosjön. However, no statistically significant differences were found in biomarker responses between the different treatments for perch and for freshwater snails. A higher variability within treatment groups in the Skutbosjön exposures in relation to the reference site was noted throughout the biomarker measurements, which is commonly observed following low-dose exposure to toxicants.

The obtained results of this study are indicative of a low bioavailability of the particle-bound compounds. This is in accordance with previous investigations of the sediments in Skutbosjön. However, aspects such as the possibility of antagonistic effects by the chemical mixtures as well as the experimental design could have impacted the study results. Furthermore, the fact that a lack of statistical significance is not equal to a lack of biological significance must be emphasized. No conclusions of the actual effects for wild aquatic organisms in Skutbosjön can be drawn based only on the obtained study results. An adjusted experimental design (e.g. an increased amount of sediment used for exposures, altered exposure durations and/or a larger number of test species) could lead to results of both higher statistical as well as ecological significance.

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# Appendix I – Chemical analysis data of sediment and water samples

## SEDIMENT

| Laboratorium | | SYNLAB Linköping | SYNLAB Linköping | SYNLAB Linköping |
|---|---|---|---|---|
| **Resultat** | | | | |
| **Provfakta** | | | | |
| Provets märkning | | Hällestadsån | Skutbosjön 1 | Skutbosjön 2 |
| Koordinater | | 58.73350, 15.67627 | 58.70359, 15.78472 | 58.701299, 15.788634 |
| Provtagningsdjup | m | 2,3 | 7 | 4,5 |
| **Fysikaliska/kemiska egenskaper** | | | | |
| Glödgningsförlust | % av TS | 14,1 | 25,2 | 17,9 |
| Glödgningsrest | % av TS | 85,9 | 74,8 | 82,1 |
| Torrsubstans | % | 10,8 | 7,19 | 13,6 |
| **Metaller i fast material bestämda med ICP/AES** | | | | |
| Arsenik, As | mg/kg TS | 3,8 | 8,1 | 6,7 |
| Bly, Pb | mg/kg TS | 20 | 46 | 47 |
| Kadmium, Cd | mg/kg TS | 0,87 | 1,8 | 1,6 |
| Koppar, Cu | mg/kg TS | 38 | 320 | 760 |
| Koppar, Cu (TOC-normaliserad) | mg/kg TS | 26,76 | 133,33 | 463,41 |
| Zink, Zn | mg/kg TS | 160 | 490 | 490 |
| **Övriga metallanalyser** | | | | |
| Krom sexvärd, Cr6+ | mg/kg TS | <2,3 | <4,5 | <2,1 |
| **Organiska miljöanalyser - Bekämpningsmedel** | | | | |
| Trifluralin | mg/kg TS | <3,63 | <3,6 | - |
| Trifluralin | mg/kg | - | - | <0,5 |
| **Organiska miljöanalyser - Dioxiner/furaner** | | | | |
| 2378 TCDD | ng/kg TS | <0,6 | <0,6 | <0,6 |
| 12378 PeCDD | ng/kg TS | <0,6 | 1,1 | 2,5 |
| 123478 HxCDD | ng/kg TS | <1,5 | 2,7 | 1 |
| 123678 HxCDD | ng/kg TS | <0,6 | 2,9 | 7,7 |
| 123789 HxCDD | ng/kg TS | <0,6 | 1,8 | 4,8 |
| 1234678 HpCDD | ng/kg TS | 7,8 | 26 | 53 |
| OCDD | ng/kg TS | 66 | 190 | 380 |
| 2378 TCDF | ng/kg TS | <0,6 | 2,2 | 5,7 |
| 12378 PeCDF | ng/kg TS | <0,6 | 1,6 | 3,3 |
| 23478 PeCDF | ng/kg TS | <0,6 | 3,2 | 6,7 |
| 123478 HxCDF | ng/kg TS | <0,6 | 3,5 | 9 |
| 123678 HxCDF | ng/kg TS | <0,6 | 2,5 | 6 |
| 123789 HxCDF | ng/kg TS | <0,6 | <0,6 | <0,6 |
| 234678 HxCDF | ng/kg TS | <0,6 | 3,3 | 6,1 |
| | ng/kg TS | 4,4 | 30 | 170 |

| | | | | |
|---|---|---|---|---|
| 1234789 HpCDF | ng/kg TS | <1,5 | 2,7 | 5,5 |
| OCDF | ng/kg TS | 7,3 | 39 | 95 |
| I-PCDD/F-TEQ Lower Bound | ng/kg TS | 0,2 | 5 | 12 |
| I-PCDD/F-TEQ Upper Bound | ng/kg TS | 2 | 5,6 | 12 |
| WHO-PCDD/F-TEQ LB | ng/kg TS | 0,1 | 4,7 | 11 |
| WHO-PCDD/F-TEQ UB | ng/kg TS | 2,1 | 5,4 | 12 |
| **Organiska miljöanalyser - Metallorganiska föreningar** | | | | |
| Tributyltenn | ug/kg TS | <1 | 1,7 | 2,6 |
| Tributyltenn (TOC-normaliserad) | ug/kg TS | <1 | 0,71 | 1,59 |
| **Organiska miljöanalyser - Polyaromatiska föreningar** | | | | |
| Acenaften | mg/kg TS | <0,05 | <0,05 | <0,05 |
| Acenaftylen | mg/kg TS | <0,05 | <0,05 | <0,05 |
| Naftalen | mg/kg TS | <0,03 | 0,04 | 0,1 |
| Antracen | mg/kg TS | <0,03 | 0,04 | 0,05 |
| Antracen | ug/kg TS | <3 | 31 | 360 |
| Antracen (TOC-normaliserad) | ug/kg TS | <3 | 12,92 | 219,51 |
| Fenantren | mg/kg TS | <0,05 | 0,19 | 0,31 |
| Fluoranten | mg/kg TS | <0,05 | 0,4 | 0,55 |
| Fluoranten | ug/kg TS | 21 | 240 | 2600 |
| Fluoranten (TOC-normaliserad) | ug/kg TS | 14,79 | 100,00 | 1585,37 |
| Fluoren | mg/kg TS | <0,05 | <0,05 | <0,05 |
| Pyren | mg/kg TS | <0,05 | 0,36 | 0,48 |
| Benso(a)antracen | mg/kg TS | 0,06 | 0,27 | 0,26 |
| Benso(a)pyren | mg/kg TS | <0,05 | 0,17 | 0,26 |
| Benso(ghi)perylen | mg/kg TS | <0,05 | 0,23 | 0,27 |
| Krysen + Trifenylen | mg/kg TS | <0,05 | 0,28 | 0,38 |
| Dibens(a,h)antracen | mg/kg TS | <0,05 | <0,05 | <0,05 |
| Indeno(1,2,3-cd)pyren | mg/kg TS | <0,05 | 0,19 | 0,27 |
| PAH,summa cancerogena | mg/kg TS | <0,7 | 1,5 | 1,8 |
| PAH,summa övriga | mg/kg TS | <0,9 | 1,3 | 1,8 |
| Benso(b+k)fluoranten | mg/kg TS | 0,09 | 0,58 | 0,66 |
| PAH-H,summa | mg/kg TS | <0,2 | 1,7 | 2,1 |
| PAH-L,summa | mg/kg TS | <0,08 | <0,08 | 0,1 |
| PAH-M,summa | mg/kg TS | <0,13 | 0,99 | 1,4 |
| **Organiska summametoder** | | | | |
| TOC | % av TS | 7,1 | 12 | 8,2 |

# WATER

| Laboratorium | | SYNLAB Linköping | SYNLAB Linköping |
|---|---|---|---|
| **Resultat** | | | |
| **Provfakta** | | | |
| Provets märkning | | Hällestadsån | Skutbosjön 1 |
| Koordinater | | 58.73350, 15.67627 | 58.70359, 15.78472 |
| **Fysikaliska/kemiska egenskaper** | | | |
| Absorbans vid 420 nm, filt | abs/5cm | 0,37 | 0,34 |
| Alkalinitet, HCO3 | mekv/l | 0,25 | 0,3 |
| Konduktivitet 25°C | mS/m | - | - |
| pH vid 20°C | | 6,8 | 6,9 |
| Turbiditet FNU | FNU | - | - |
| **Anjoner** | | | |
| Klorid, Cl | mg/l | 6,9 | 7,2 |
| **Metaller i vatten bestämda med ICP/AES** | | | |
| Kalcium, Ca | mg/l | 10 | 11 |
| Magnesium, Mg | mg/l | - | - |
| **Metaller i vatten bestämda med ICP/MS** | | | |
| Aluminium, Al | µg/l | - | - |
| Arsenik, As | µg/l | 0,57 | 0,52 |
| Barium, Ba | µg/l | - | - |
| Bly, Pb | µg/l | 0,64 | 0,62 |
| Kadmium, Cd | µg/l | 0,026 | 0,025 |
| Kobolt, Co | µg/l | - | - |
| Koppar, Cu | µg/l | 2,1 | 2,8 |
| Krom, Cr | µg/l | - | - |
| Mangan, Mn | µg/l | 59 | 61 |
| Nickel, Ni | µg/l | 1,6 | 1,6 |
| Strontium, Sr | µg/l | - | - |
| Zink, Zn | µg/l | 5,7 | 6,3 |
| **Övriga metallanalyser** | | | |
| Krom sexvärd, Cr6+ | mg/l | <0,02 | <0,02 |
| Kvicksilver, Hg Fluorescense | ng/l | 3 | 2 |
| **Övriga kemiska analyser** | | | |
| Klorofyll a | µg/l | - | - |
| **Organiska miljöanalyser - Bekämpningsmedel** | | | |
| Aldrin | ng/l | <0,5 | <0,5 |
| DDT, summa | ng/l | <3 | <3 |
| Dieldrin | ng/l | <0,5 | <0,5 |
| Endosulfan-alfa | ng/l | <0,5 | <0,5 |
| Endosulfan-beta | ng/l | <0,5 | <0,5 |
| HCH-alfa | ng/l | <0,5 | <0,5 |
| HCH-beta | ng/l | <0,5 | <0,5 |
| HCH-delta | ng/l | <0,5 | <0,5 |

| | | | |
|---|---|---|---|
| HCH-gamma (Lindan) | ng/l | <0,5 | <0,5 |
| Isodrin | ng/l | <0,5 | <0,5 |
| Isoproturon | µg/l | <0,003 | <0,003 |
| Klorfenvinfos | ng/l | <0,5 | <0,5 |
| **Organiska miljöanalyser - Fenoler** | | | |
| 4-n-nonylfenol | µg/l | <0,01 | <0,01 |
| 4-tert-oktylfenol | µg/l | <0,003 | <0,003 |
| Pentaklorfenol | µg/l | <0,015 | <0,015 |
| **Organiska miljöanalyser - Ftalater** | | | |
| Di-(2-etylhexyl)ftalat | µg/l | <0,2 | <0,2 |
| **Organiska miljöanalyser - Halogenerade alifatiska ämnen** | | | |
| Klorparaffiner C10-C13, SCCP | µg/l | <0,03 | <0,03 |
| **Organiska miljöanalyser - Klorbensener** | | | |
| Hexaklorbensen | ng/l | <0,6 | <0,6 |
| **Organiska miljöanalyser - Metallorganiska föreningar** | | | |
| Tributyltenn | ng/l | <0,2 | <0,2 |
| **Organiska miljöanalyser - Polyaromatiska föreningar** | | | |
| Naftalen | ng/l | <3 | <3 |
| Antracen | ng/l | <0,2 | <0,2 |
| Fluoranten | ng/l | 0,47 | 0,77 |
| Benso(a)pyren | ng/l | <0,2 | 0,28 |
| Benso(b)fluoranten | ng/l | 0,39 | 0,67 |
| Benso(k)fluoranten | ng/l | <0,2 | <0,2 |
| Benso(ghi)perylen | ng/l | <0,2 | 0,64 |
| Indeno(1,2,3-cd)pyren | ng/l | <0,2 | 0,36 |
| **Organiska miljöanalyser - PFAS** | | | |
| Perfluorbutansulfonat (PFBS) | ng/l | 0,17 | <0,15 |
| Perfluorpentansulf. (PFPeS) | ng/l | <0,15 | <0,15 |
| Perfluorhexansulfonat(PFHxS) | ng/l | <0,15 | <0,15 |
| Perfluorheptansulf. (PFHpS) | ng/l | <0,15 | <0,15 |
| PFOS, linjär | ng/l | <0,1 | 0,11 |
| PFOS, grenad | ng/l | 0,16 | 0,19 |
| PFOS, total | ng/l | 0,16 | 0,3 |
| Perfluordekansulfonat (PFDS) | ng/l | <1 | <1 |
| Perfluorpentansyra (PFPeA) | ng/l | <0,3 | 0,34 |
| Perfluorhexansyra (PFHxA) | ng/l | 0,24 | 0,28 |
| Perfluorheptansyra (PFHpA) | ng/l | 0,32 | 0,32 |
| PFOA, linjär | ng/l | 0,39 | 0,42 |
| PFOA, grenad | ng/l | <0,15 | <0,15 |
| PFOA, total | ng/l | 0,39 | 0,42 |
| Fluortelomersulfo. (4:2 FTS) | ng/l | <0,15 | <0,15 |
| Fluortelomersulfo. (6:2 FTS) | ng/l | <0,15 | <0,15 |
| Fluortelomersulfo. (8:2 FTS) | ng/l | <1 | <1 |
| Perfluorbutansyra (PFBA) | ng/l | 0,47 | 0,82 |

| | | | |
|---|---|---|---|
| Perfluornonansyra (PFNA) | ng/l | <0,3 | <0,3 |
| Perfluordekansyra (PFDA) | ng/l | <0,3 | <0,3 |
| Perfluorundekansyra (PFUnDA) | ng/l | <1 | <1 |
| Perfluordodekansyra (PFDoDA) | ng/l | <1 | <1 |
| Perfluoroktansulfonami.PFOSA | ng/l | <0,15 | <0,15 |
| 7H-Dodekafl.hept.syra HPFHpA | ng/l | <0,15 | <0,15 |
| H4-PFUnDA | ng/l | <1 | <1 |
| Summa 11 PFAS | ng/l | <2,5 | <2,5 |
| **Organiska summametoder** | | | |
| DOC | mg/l | 20 | 18 |
| TOC | mg/l | 20 | 19 |