Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Chemistry

Oda Siebke Løge

Use of digestive gland explant cultures from blue mussels (*Mytilus edulis*) to assess biomarker effects of single and co-exposure of environmental contaminants

Impacts of urban development in Tromsø

Master's thesis in Analytical Chemistry
Supervisor: Øyvind Mikkelsen
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Preface

This master thesis in Analytical Chemistry was carried out as a part of the study program in Chemical Engineering and Biotechnology at the Norwegian University of Science and Technology (NTNU). The project was in collaboration with Akvaplan-niva in Tromsø. Field work and exposure of explants were conducted by Akvaplan-niva in April 2018, and preparation and analyses of samples were carried out at the Norwegian Institute of Air Research (NILU) at the Fram Centre, Tromsø, in November 2019.

Tromsø, February 27, 2020

Oda Løge

Oda Siebke Løge

Acknowledgements

Working on this project has given me valuable laboratory experience, and a steep learning curve within the field of ecotoxicology.

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Abstract

Strong urbanization and increased marine traffic around Tromsø are increasing the pressure on local marine ecosystems. Finding suitable methods for monitoring the impacts of human activities on marine populations is crucial. Invertebrates, such as bivalves, have been shown to be useful as bioindicators of environmental change, and appropriate models for marine ecotoxicology. The use of *ex vivo* methods allows for studying specific cellular responses, and have an experimental design with high ecological relevance.

In this study, digestive glands of blue mussels (*Mytilus edulis*) were exposed to a selection of environmental compounds expected to be found outside Tromsø, to evaluate their toxic impacts. Two experiments were carried out, exposing explants to *m*-xylene and BDE-99 in the first experiment, and $CdCl_2$ and estradiol-17-β in the second experiment. Dopamine levels were detected and quantified in all 48 samples (limit of quantification (LOQ) = 52 pg/mg). The levels of serotonin were quantifiable only in 9 of the 48 samples (LOQ = 150 pg/mg). The extraction yielded variable recoveries, 1.0-45.2% for dopamine and 12.4-79.7% for serotonin.

The results indicate a synergistic effect from co-exposure of *m*-xylene and BDE-99, with 1.6-fold higher dopamine levels compared to single *m*-xylene exposure, and 2.2-fold higher dopamine levels compared to single BDE-99 exposure. In addition, co-exposure of $CdCl_2$ and estradiol-17-β reduced the level of dopamine 2.2-fold and 1.8-fold respectively, suggesting a possible interaction of cadmium on the estrogen receptor. The altered levels of dopamine and serotonin in bivalves co-exposed to these chemicals demonstrates possible impacts of co-exposure to chemical mixtures within the marine environment surrounding Tromsø.

Sammendrag

Urbanisering og økt sjøtrafikk rundt Tromsø øker presset på lokale marine økosystemer. Det er avgjørende å finne egnede metoder for å overvåke virkningene av menneskelige aktiviteter på havbestandene. Virvelløse dyr, som muslinger, har vist seg å være nyttige som bioindikatorer for miljøendringer, og passende modeller for marin økotoksikologi. Bruken av *ex vivo*-metoder gjør det mulig å studere spesifikke cellulære responser, og har et eksperimentelt design med høy økologisk relevans.

I denne studien ble fordøyelseskjertlene i blåskjell (*Mytilus edulis*) utsatt for et utvalg av miljøforbindelser som forventes å bli funnet utenfor Tromsø, for å evaluere deres potensielle giftige påvirkninger. To eksperimenter ble utført, hvor eksplanter ble eksponert for *m*-xylen og BDE-99 i det første eksperimentet, og $CdCl_2$ og østradiol-17-β i det andre eksperimentet. Dopaminnivåer ble påvist og kvantifisert i alle 48 prøver (kvantifiseringsgrense (LOQ)=52 pg/mg). Nivåene av serotonin var bare kvantifiserbare i 9 av de 48 prøvene (LOQ=150 pg/mg). Ekstraksjonen ga varierende ekstraksjonsutbytte, 1.0-45.2% for dopamin og 12.4-79.7% for serotonin.

Resultatene indikerer en synergistisk effekt fra sameksponering av *m*-xylen og BDE-99, med 1.6 ganger høyere dopaminnivå sammenlignet med ren *m*-xylen-eksponering, og 2.2 ganger høyere dopaminnivå sammenlignet med ren BDE-99-eksponering. I tillegg reduserte sameksponering av $CdCl_2$ og østradiol-17-β nivået av dopamin henholdsvis 2.2 ganger og 1.8 ganger, noe som antyder en mulig interaksjon av kadmium på østrogenreseptoren. De endrede nivåene av dopamin og serotonin i muslinger som er eksponert for disse kjemikaliene, viser mulige effekter av sameksponering for kjemiske blandinger i det marine miljøet rundt Tromsø.

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Acronyms and abbreviations

ACN	Acetonitril.
BFR	Brominated flame retardant.
Cd	Cadmium.
CE	Cultured explant.
CEC	Compounds of emerging concern.
CI	Confidence interval.
CID	Collision-induced dissociation.
DMSO	Dimethyl sulfoxide.
E2	Estradiol-17-β.
ECD	Electrochemical detection.
EDC	Endocrine disruptor.
EP	Emerging pollutant.
ER	Estrogen receptor.
ESI	Electrospray ionization.
HPLC	High performance liquid chromatography.
ISTD	Internal Standard.
LC	Liquid chromatography.
LC-MS	Liquid chromatography-mass spectrometry.
LC-MS/MS	Liquid chromatography-tandem mass spectrometry.
LOD	Limit of detection.
LOQ	Limit of quantification.
MeOH	Methanol.
MIP	Molecularly Imprinted Polymer.
MIP-SPE	Molecularly Imprinted Polymer-Solid Phase Extraction.

MP	Mobile phase.
MS	Mass spectrometry.
MS/MS	Tandem mass spectrometry.
PBDE	Polybrominated diphenyl ether.
PC	Priority compound.
Q1	First quadrupole.
Q2	Collision cell.
Q3	Third quadrupole.
RPLC	Reversed-phase liquid chromatography.
SD	Standard deviation.
SEM	Standard error of mean.
SP	Stationary phase.
SPE	Solid Phase Extraction.
UHPLC	Ultra-high pressure liquid chromatography.
Vtg	Vitellogenin.

1 Introduction

1.1 Background

Increased urbanization of Tromsø has resulted in more anthropogenic stressors, such as shipping traffic, tourism and wastewater emissions, to the local marine environment. With this intensification of marine traffic and urbanization, the release of a cocktail of contaminants to the marine environment puts pressure on the local ecosystems. As there is only primary treatment in the waste water treatment plants in Tromsø (Vasskog, Berger, Samuelsen, Kallenborn, & Jensen, 2006), most chemicals are released directly in the sea, mixing with chemicals from spills and leakages from marine traffic and run-offs from industrial activities. This complex mixture of contaminants may have potentially toxic effects, and developing monitoring programs for investigating impacts of such chemical mixtures on the marine ecosystems is crucial.

However, studying cocktail effects brings several analytical and methodological challenges, including both the experimental design and the interpretation of results. Identifying the chemicals responsible for the observed effects might be difficult, as well as understanding the diverse molecular mechanisms of the mixed pollutants. Accordingly, there is a need to develop new biological tools as alternatives to *in vivo* exposure studies. Recent development of *ex vivo* methods allows for studies with high ecological relevance, using explants from living animals to study effects on a cellular basis (Eide, Karlsen, Kryvi, Olsvik, & Goksøyr, 2014; Gerbron, Geraudie, Rotchell, & Minier, 2010). Previous studies have shown the relevance of blue mussels (*Mytilus edulis*) as a sentinel species for ecotoxicological studies (Beyer et al., 2017). Their biological and ecological characteristics make them ideal for pollution monitoring, and are consistent with the criterias formulated by the OSPAR commission (O. Commission et al., 1999).

In this study, blue mussels have been used to assess biomarker effects of single and co-exposure of selected environmental contaminants. Digestive gland explant cultures have been exposed to chemicals expected to be found in the marine environments in Tromsø, and the level of dopamine and serotonin was measured to investigate possible endocrine effects of cocktail contamination. Dopamine and serotonin are neurotransmitters important

in hormone regulation, particularly in the developmental stages. Up- or downregulation of the levels of these neurotransmitters could impact the development of bivalves, and thus be a risk to population development. The results of this study will contribute to provide more data for risk assessment of marine ecosystems.

1.2 Objectives

This project is a part of the WASTECALL flagship project at the Fram centre in Tromsø, with an overall goal to characterize the impacts of human activities on local marine ecosystems. The objectives of the present study was to (1) use digestive gland explant cultures from blue mussels (*Mytilus edulis*) to study biomarker effects of single and co-exposure of environmental contaminants, (2) evaluate a new analytical method for neurotransmitter measurement in bivalves, and (3) assess the importance of *ex vivo* tools as supplement to *in vivo* methods.

2 Theory

In this section, some model contaminants expected to be found in the marine environment around Tromsø will be introduced, along with a brief introduction to the *ex vivo* system and analytical methods used for analysis of neurotransmitters in explant cultures of the digestive glands of blue mussels (*Mytilus edulis*).

2.1 Urban development, emerging pollutants (EPs) and priority substances (PSs)

There is a growing concern regarding the impact of climate changes, urbanization and industrial development on marine ecosystems. The demand for limited water resources is increasing according to the exponential growth in the human population (Kolpin et al., 2002). The increasing urbanization in Tromsø have lead to more shipping traffic, tourism and a growing population. The intensification of human activities has contributed to an increase in the population in Tromsø with almost 25% the last 15 years (SSB) (Sentralbyrå,

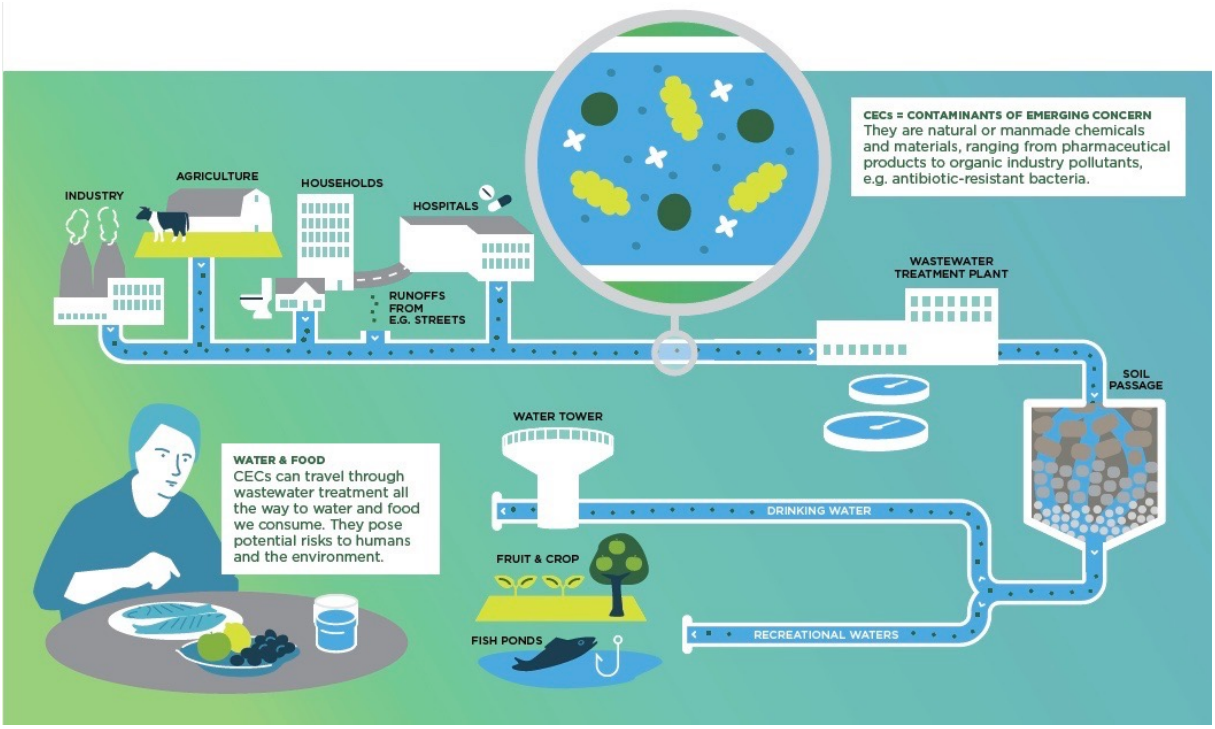

Figure 1: Different pathways of emerging pollutants via wastewater treatment plants to the aquatic environment. Illustration: Water JPI (JPI, 2018).

n.d.). With only mechanical sieving and no chemical or biological degradation of the sewage in Tromsø (Vasskog et al., 2006), there is an increasing release of chemicals into the marine environment. Together with spills from marine traffic and activity, there is a complex mixture of chemicals potentially impacting the local marine ecosystems. Developing monitoring programs to understand impacts of single contaminants and their cocktail effects on marine ecosystems and human health is crucial.

To protect the surface waters and limit the release of emerging contaminants, EU established a framework in the field of water policy, by Directive 2000/60/EC (E. Commission, 2000). It was amended by Directive 2008/105/EC, including for the first time a list of 33 priority substances (PSs) (E. Commission, 2008). Several updated versions have been published later, with the complete list being published in Directive 2015/495/EC, including a Watch List as a guideline of substances that need monitoring data worldwide (E. Commission, 2015; Sousa, Ribeiro, Barbosa, Pereira, & Silva, 2018). Substances included in this Watch List are referred to as emerging pollutants (EPs). This is a designation that can be attributed to contaminants that just recently have raised concerns about their human health or ecological impacts (JPI, 2018). They may have been present in the environment for some years, or appeared recently, but they are not regulated and therefore not included in any routine monitoring programs. Examples of EPs, also known as chemicals of emerging concern (CEC), include plastic additives, industrial chemicals, persistent organic chemicals, pharmaceuticals and personal care products (Allan, Jenssen, & Braaten, 2018). These pollutants enter the aquatic environments mainly through runoffs from agriculture, households, industrial activities and hospitals through wastewater treatment plants, see Figure 1, but also directly from spills from marine activities like tourism and shipping. EPs are found all over the world, both in freshwater and marine environments (Schwarzenbach et al., 2006; Schwarzenbach, Egli, Hofstetter, Von Gunten, & Wehrli, 2010).

2.1.1 Endocrine disruptors

Many of the compounds released in the marine environment have the ability to mimic the action of hormones, or influence their concentration in tissues, and are known as

endocrine disruptors (EDCs) (Gagné & Blaise, 2003). The endocrine system is one of the three important integrating and regulatory systems in the human body, in addition to the nervous and immune systems (Zhu, Kusaka, Sato, & Zhang, 2000). EDCs disrupt the normal actions of endogenous hormones, either resulting in an agonist or an antagonist endocrine response (Colborn, Vom Saal, & Soto, 1993). Hundreds of chemicals have been proved or are presumed to have endocrine disruptive effects, such as pharmaceuticals, industrial chemicals and several environmental pollutants (Zhu et al., 2000).

The most studied EDCs in the aquatic environment are by far estrogen mimics (Gagné & Blaise, 2003). Estrogens in municipal effluents have been found to increase the number of intersexed animals and females after periods of chronic exposure (Van Aerle et al., 2001). Several EDCs are found to bind the estrogen receptors (ER) in tissues and induce the vitellogenins (Vtgs) in marine and freshwater bivalves (Gagné, Marcogliese, Blaise, & Gendron, 2001; Gagné, Blaise, Salazar, Salazar, & Hansen, 2001). The ER is a nuclear receptor activated by E2. When activated by estradiol-17-β (E2), ER dimerizes so it may bind to specific DNA sequences and stimulate transcription of specific genes (Guevel et al., 2000). The ER is composed of several domains, serving specific roles. Disruption of functions in the different domains may block or activate their feedbacks, altering the normal hormonal responses. Figure 2 and Figure 3 shows the sequence of the ER, and an example of an endocrine disruption adsorption, respectively. Serotonin and dopamine are hormones and neurotransmitters that have been shown to be important for several stages of reproduction. However, there are few studies on the mechanism of action on these hormones when exposed to cocktails of contaminants from municipal effluents, shipping and tourism (Gagné & Blaise, 2003).

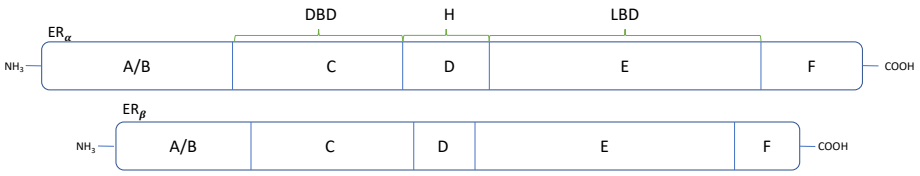

Figure 2: The two nuclear estrogen receptors (ER_α and ER_β), with functional domains. DBD = DNA binding domain; H = hinge (linker region between DBD and LBD); LBD = ligand binding domain.

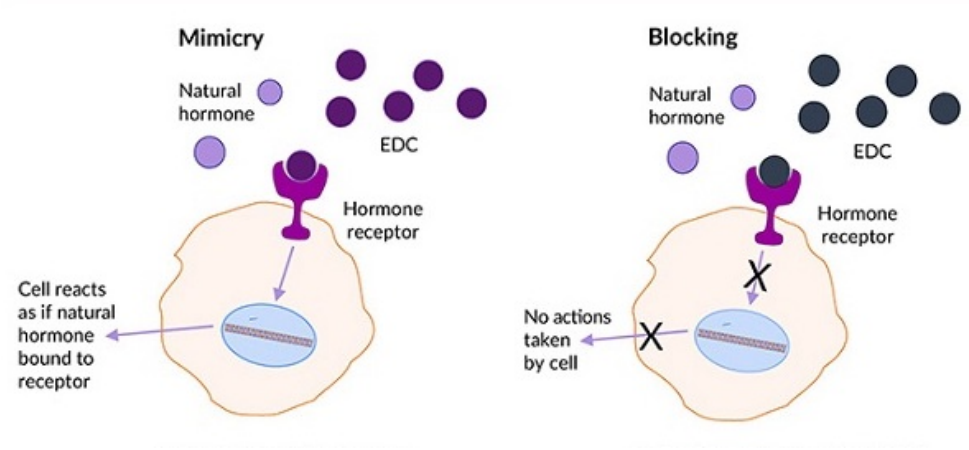

Figure 3: Schematic diagram of an endocrine disruption adsorption. EDCs mimicking hormones binds to hormone receptors and trick the cell into action. With hormones blocking the hormone receptors, the signalling pathway is not turned on, and the cell remains the same. Illustration: Alpco (Brand, n.d.).

Cocktail effects are the result of combined exposure to chemicals, such as endocrine disruptors. The effect may be additive (chemicals "act together", no enhancing or diminishing of actions), synergistic (effects greater than additive) or antagonistic (effects falling short of additivity). Because the combined effect does not necessarily relate to the effect profile of individual compounds, the cocktail effects are often difficult to predict (Kortenkamp,

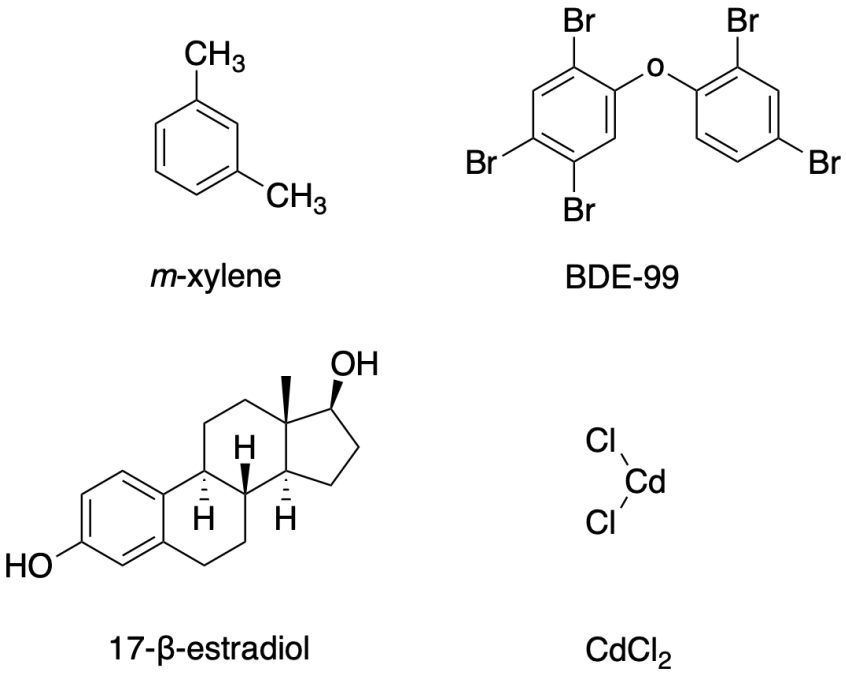

Figure 4: Molecular structure of selected chemicals in this study.

2007). The chemicals selected for this study are all endocrine disruptors expected to affect the reproduction of organisms, and includes BTEXs (xylene), persistent organic pollutants (BDE-99), trace metals (Cadmium) and estrogens (estradiol-17-β) (Figure 4). Exposure to both single and combined chemicals have been tested to investigate possible cocktail effects.

2.1.2 Cadmium

Cadmium (Cd) is a trace metal, an environmental pollutant, and is listed on EUs list of PSs (Henson & Chedrese, 2004; E. Commission, 2008). It is widely used in industry for processes such as stabilization in plastics, protection of steel from corrosion and as a component in batteries and alloy manufactures, as well as a constituent in tobacco smoke (Gerbron, Geraudie, Xuereb, Marie, & Minier, 2015; Chedrese, Piasek, & Henson, 2006). It enters the marine environment through atmospheric deposition, waste water effluents and industrial discharges. Cd has a long half-life, between 15 to 30 years (Sugita & Tsuchiya, 1995), mainly due to the low excretion rate from the body, and is shown to bioaccumulate through ecosystems, leading to high concentrations in organisms in the upper trophic levels (Nasreddine & Parent-Massin, 2002). It has no documented beneficial biological functions, but is shown to have toxic effects in both humans and animals (Henson & Chedrese, 2004).

A review of Cd accumulation in different aquatic species suggested the following ranking as Cd bioaccumulators: mollusks > crustacea > teleostomii fish (Frazier, 1979). Although the test species were collected at different geographical regions, the results are consistent with the observation of field studies. Frazier et al. (1979) found four factors affecting the bioaccumulation of Cd; the geographical region, seasonal alterations of Cd, water temperature in contaminated environments and salinity (Frazier, 1979). Blue mussels are known to accumulate high concentrations of Cd, by filtering particles from the water as they feed (Hervé-Fernández et al., 2010). Cd have been found to mostly be stored in the soft parts of the mussels, with some fractions adsorbed onto the shells (96.3 and 3.2%, respectively) (Hervé-Fernández et al., 2010). As Cd often is found as $CdCl_2$ in the aquatic environment, this is the compound used for exposure in this project.

2.1.3 Estradiol-17-β

Estrogen is a steroid hormone responsible for the development and regulation of the female reproductive system (Dai & Liu, 2017). Estrogens are used as medicines, and are released to the environment mainly through sewage treatment plants and as effluents from livestock feedlots. Environmental estrogen pollution is a well documented issue, with E2 as one of the priority estrogens. E2 is recognized as a major contributor to estrogen effects on the female reproductive system (Dai & Liu, 2017), and is listed on EUs list of PSs (E. Commission, 2008).

Mussel exposure to exogenous E2 have demonstrated a significant accumulation in the tissues, including digestive glands (Janer, Lavado, Thibaut, & Porte, 2005). In the digestive glands, E2 is mainly metabolised to fatty acid esters (Janer et al., 2005). The digestive gland of blue mussels have shown to be a target for the action of E2, and demonstrates the importance of this hormone for the lysosymal function, lipid and glucose metabolism and for oxidative stress (Janer, Lavado, Thibaut, & Porte, 2004).

2.1.4 BDE-99

Polybrominated diphenyl ethers (PBDEs) are additives in brominated flame retardants (BFRs), and are commonly used in a wide array of products and materials (Gustafsson, Björk, Burreau, & Gilek, 1999). PBDEs are found on EUs list of PSs (E. Commission, 2008), as well as being banned under the Stocholm Convention of POPs (22, 2004). Release to the environment is mainly through emissions from manufacturing processes, recycling wastes and leakage from waste disposal sites, and reaches the marine environments via riverine inputs and atmospheric deposition.

BDE-99 is one of the most commonly used PBDEs, and is of concern due to its very high bioaccumulation. Gustafsson et al. (1999) compared the bioaccumulation kinetics of selected PBDEs in blue mussels, and found especially high bioaccumulation factor for BDE-99 (Gustafsson et al., 1999). Uptake from sediments is assumed to be an important pathway for PBDEs in benthic species.

2.1.5 *m*-xylene

BTEX is a group of volatile organic compounds, consisting of benzene, toluene, ethylbenzene and xylenes. These EPs are constituents of petroleum and solvents, and are commonly found as air contaminants (Bolden, Kwiatkowski, & Colborn, 2015). Road traffic, oil spills, improper discharge from petrol and industrial activity can lead to contamination in soil and ground water, but due to the compounds high volatility, the background concentrations of BTEXs in groundwater are usually relatively low (Seagren & Becker, 2002). Xylene is an aromatic hydrocarbon within the group of BTEX.

The xylenes (m,o,p) have high volatility and low factors of accumulation. In addition, they are easily biodegraded by microorgnisms (Jori et al., 1986). Therefore, the impact of xylenes on the aquatic environment is considered to be limited. However, there are few studies on the effects of xylenes co-exposed with other contaminants.

2.2 *Ex vivo* studies

A major issue concerning the globally increasing need of chemical safety assessment, is the reliance of today's testing strategies on live animals. In response to increasing testing demands and requirements, and concern regarding the chemicals to interact with the endocrine systems, several programs have been initiated to develop new guidelines for screening and testing of chemicals in vertebrates (OECD 1998, 2002; EDSTAC 1998, EDSP). These programs are increasingly recommending replacement of certain *in vivo* studies with *ex vivo* methods (Hecker et al., 2011). *Ex vivo* means out of living organism, and unlike *in vivo* methods, the study is performed in a lab using only parts of an organism. For an explant approach, a small piece of tissue is freshly dissected from a living animal, allowing for studies of microscale units with similar biochemical properties as the organs inside the organism. *In vitro* means outside the body, and refers to laboratory studies performed in test tubes. Both *ex vivo* and *in vitro* experiments take place outside of a living organism, but while *in vitro* methods might be more reactive in terms of biological response, *ex vivo* methods are more complex in terms of cell diversity, and therefore closer to *in vivo* conditions.

Ex vivo systems can not replace *in vivo* studies on living animals, but can give valuable information and increase our knowledge of the mechanisms of EDCs, as *ex vivo* tests focus on effects of chemicals on cell and organ levels (Gerbron et al., 2010). In this way, *ex vivo* assays avoid interactions and indirect effects of the whole body. Other advantages of *ex vivo* assays are the reproducability, sensitivity, cost effectiveness (Gerbron et al., 2010), animal welfare concerns associated with toxicity testing, and the biological relevance of the cell metabolism to humans (Gray Jr et al., 1997). Using digestive glands and gonad tissues from Icelandic scallops (*Chlamys islandica*), an *ex vivo* approach for assessing estrogenic effects has been successfully developed. Preliminary results have shown good viability of explants up to 96 hours (<70%), in addition to specific dose-response curve after exposure to model compounds (Cd, BDE-99 and *m*-xylene), both single and cocktail effects (Manuscript in preparation, Flagship project Number 8483).

2.2.1 Study species

The blue mussel *(Mytilus edulis)* is a marine bivalve mollusc in the family Mytilidae. It has a wide distribution in the northern hemisphere, and is found in a wide range of microhabitats, mainly due to its tolerance to environmental varieties (Gosling et al., 1992).

Bivalve molluscs are useful as bioindicators of environmental change, due to their abundance and widespread distribution, high filtration rate and their high accumulation abilities and low rate of biotransformation for both organic and inorganic toxicants (Quinn, Costello, Dorange, Wilson, & Mothersill, 2009). Previous studies have shown the relevance of blue mussels as a sentinel species for temperate and Arctic ecotoxicological studies, including *in situ*, *in vivo* and *in vitro* studies (Goldberg, 1975; Farrington et al., 2016).

2.2.2 Explants of digestive glands

The histology and fine structure of the digestive glands in bivalves have been studied extensively. The digestive glands consist of several blind-ending epithelial tubulus, the epithelium consisting of digestive and basophilic cells (Faggio, Tsarpali, & Dailianis, 2018). The digestive cells are responsible for intra-cellular digestion processes, while the ba-

sophilic cells are important for enzyme-production and secretion (Faggio et al., 2018). Additionally, the digestive cells are important for the detoxification and sequestration of toxic metals and organic pollutants (Pagano et al., 2017), suggesting them as a potential "early warning tool" for adverse biological effects of xenobiotics. Thus, the digestive glands are commonly used to evaluate how environmental stressors, like contaminant exposure, affect organisms (Canesi et al., 2007; Moore, 1988).

Cells and tissues in cultures are primarily used for analysis of the mechanism of toxicity on individual cells isolated from the organism (Quinn et al., 2009). Isolated cells from different bivalve mollusc species have been used in several toxicological studies on digestive glands and other tissues (Le Pennec & Le Pennec, 2003; Chelomin, Zakhartsev, Kurilenko, & Belcheva, 2005). By using cultured explants (CE), the original structure of the tissue is retained, allowing also for studying important cell signalling.

2.2.3 Neurotransmitters as biomarkers for endocrine disruption

Biomarkers can be defined as measurements of tissues, cells or body fluids that indicate the presence or response of contaminants (Livingstone, Chipman, Lowe, Minier, & Pipe, 2000). Because they have the potential to anticipate changes at higher levels of biologic organizations, such as in populations or ecosystems, they work as short-term indicators of long-term biological effects (Cajaraville et al., 2000). Among biomarkers used to study endocrine disruption, vitellogenin is widely used and has shown to be a reliable biomarker for xenoestrogenic exposure (Sumpter & Jobling, 1995). The neurotransmitters dopamine and serotonin are associated with the sexual differentiation, fertilization, gamete development and spawning stages of reproduction (Gagné & Blaise, 2003), but are far less studied as biomarkers for endocrine disruption.

Neurotransmitters are chemical messengers transmitting signals across synapses in the nervous system. A change in the release of or effectiveness of neurotransmitters may contribute to rapid behavioural events (Hull, Muschamp, & Sato, 2004). Dopamine levels have been found to decrease after injection of E2 in the gonad of sea scallops (*Patinopecten yessoensis*), with levels increasing after the active spawning period (Osada & Nomura, 1989). Dopamine was found to inhibit spawning activity in serotonin treated zebra mus-

sels (*Dreissena polymorpha*), indicating stimulation of the spawning activity by serotonin while being negatively controlled by dopamine (Fong, Noordhuis, & Ram, 1993). Serotonin has also been found to implicate the spawning activity and fertilization in zebra mussels, when treatment with serotonin uptake-inhibitors reduced spawning and the fertilization of oocytes (Hardege, Duncan, & Ram, 1997).

2.3 Molecularly Imprinted Polymer-Solid Phase Extraction (MIP-SPE)

Molecular imprinting is a technique used for producing chemically selective binding sites for a given compound, its analogues or for a single enantiomer (Mayes & Mosbach, 1997). Formation of a complex between a template molecule and polymerizable functional monomers is achieved by utilizing non-covalent interactions like hydrogen bonds or ion pair interactions, or reversible covalent interactions, Figure 5 (Mayes & Mosbach, 1997). The formed molecularly imprinted polymer (MIP) is then irradiated with UV-light or subjected to heat to initiate polymerization, stabilizing the complex within a rigid, highly cross-linked polymer. Extraction of the template leaves the selective binding sites, shaped after the original template (Mayes & Mosbach, 1997).

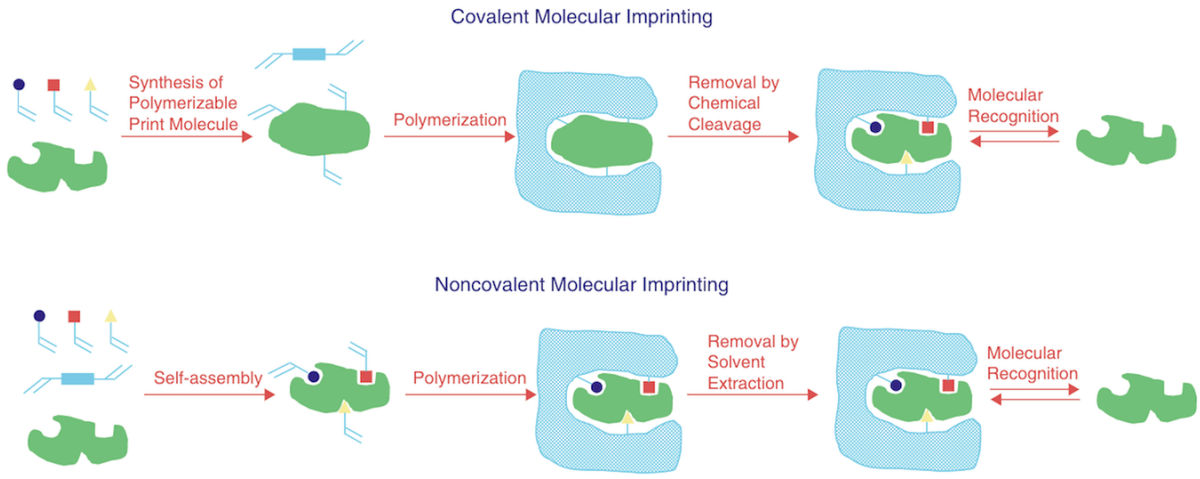

Figure 5: Schemes of the covalent and non-covalent molecular imprinting. Illustration: Yoshikawa et al. (2011) (Yoshikawa et al., 2011).

One of the most interesting applications of MIPs is as sorbents in solid-phase extraction (SPE), Figure 6. SPE is an extraction method that uses a solid phase and a liquid

phase to isolate an analyte from a solution. It is used to clean up the sample before a chromatographic or other analytical method for quantification of the amount of analyte in the sample (Żwir-Ferenc & Biziuk, 2006). The SPE is usually conditioned with a solvent before the solution with the sample is loaded into the SPE (Moldoveanu & David, 2015). Conditioning the SPE is necessary for retention of the analytes to occur. A solvent is passed through the column to wet the sorbent, activating the bonded phase to interact with the sample matrix. With the SPE container under weak vacuum, the stopcocks are opened to pass the sample through the sorbent bed, and the effluent is collected in test tubes (Moldoveanu & David, 2015; Żwir-Ferenc & Biziuk, 2006). There are different variations of the procedure. For cleanup purposes, a basic procedure is the selective retention on the solid phase of interfering compounds from a solution, and collection of analytes for further sample preparation or analysis. Another procedure is retention of the analytes in the solid phase, and rinsing of the interfering compounds, before elution and collection of the analytes. A combination of the two procedures is also possible (Moldoveanu & David, 2015). There are several advantages of SPE. It is less time consuming than liquid-liquid extraction, it uses less toxic solvents and there is a possibility

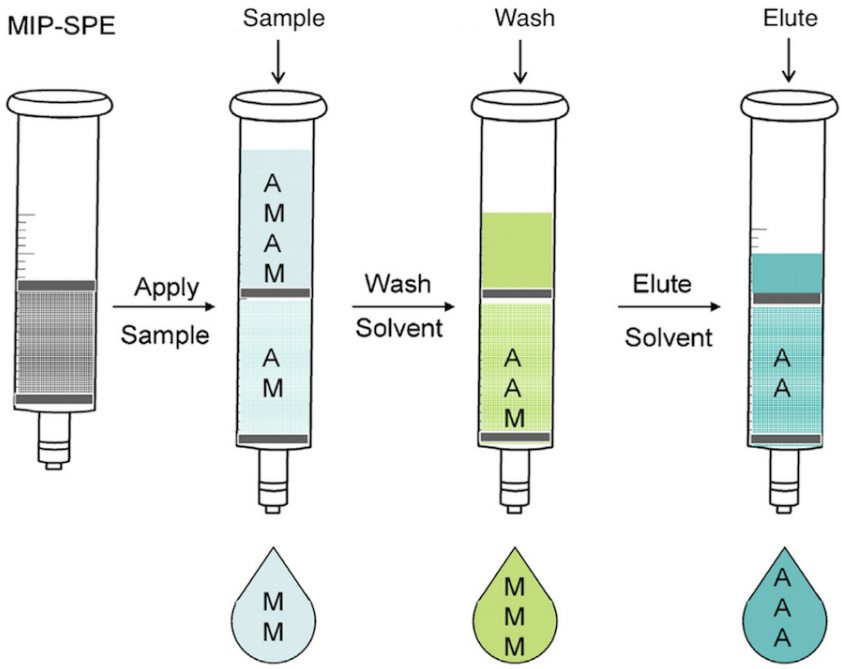

Figure 6: The flowchart of a general MIP-SPE procedure. M is the matrix and A is the analyte. Illustration: Yin et al. (2014) (Yin et al., 2014).

of automation (Andersson, 2000). Despite these features, the classical SPE sorbents, like C_{18} or size-exclusion, do not have selectivity towards target analytes (Sanagi et al., 2013).

The use of MIPs in SPE has been developed to improve the selectivity, and allows both the analyte to be pre-concentrated, and for other compounds present in the sample matrix to be removed (Mayes & Mosbach, 1997). When selectivity or retention cannot be achieved in stationary phases in traditional SPEs, MIP-SPEs are advantageous, as they are designed to capture the specific target molecule of interest. Due to the strong interactions, it is possible to wash with a higher percentage of organic solvent for removal of co-extracted matrix without risking loss of analyte. Due to their low K_{ow}, dopamine and serotonin are easily removed during washing of the SPE. This is can be avoided with MIP-SPE. The first reported study on MIP-SPE used a pentamidine selective MIP to demonstrate on-line sample enrichment of a spiked urine sample (Sellergren, 1994). Later, MIP-SPE has been used for extracting compounds from biological samples, such as biofluids and tissues, and environmental samples, like water and soil.

2.4 Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Liquid Chromatography-Mass Spectrometry (LC-MS) combines separation of analytes based on chemical interactions with both the mobile and stationary phase as well as detection using mass spectrometry (MS). It is used for analysis of non-volatile and labile organic, inorganic or biochemical compounds in samples of environmental or biological origin (Brima, Jenkins, & Haris, 2006). The principle of LC is to pump analytes through an analytical column to separate the analytes according to their interactions with the stationary phase (SP) and mobile phase (MB) (Niessen & Tinke, 1995). The system transfers the separated components from the LC column to the MS ion source through an electrospray interface (Niessen & Tinke, 1995). Figure 7 demonstrates a schematic overview of the LC-MS/MS system with a triple quadrupole mass analyzer.

The LC, or high performance liquid chromatography (HPLC) technique, developed in the 1970s, was first considered the normal phase LC. It contained a polar SP and a non-polar

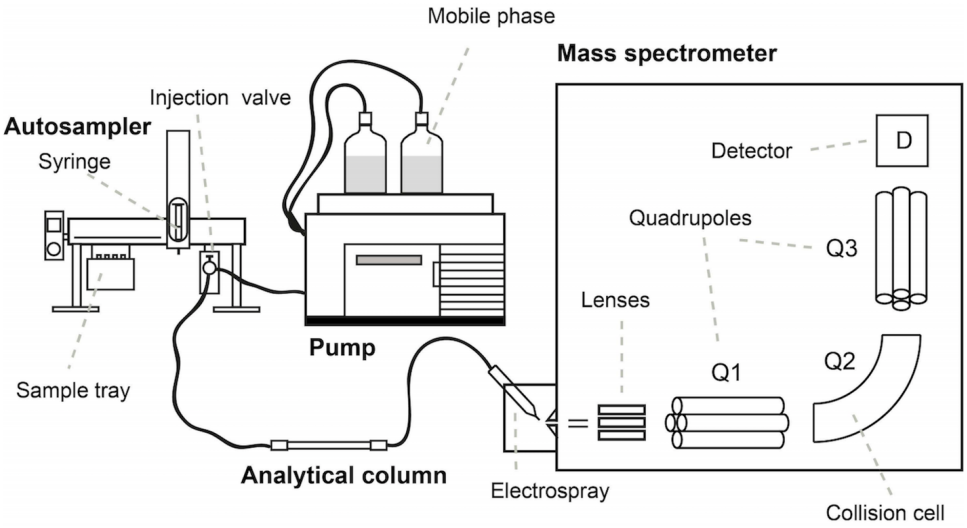

Figure 7: Schematic overview of the LC-MS/MS system with a triple quadrupole mass analyzer. Illustration: Ostman et al. (2018) (Östman, 2018).

mobile phase (MP). Reversed-phase liquid chromatography (RPLC) technique switches the polarity, where the MP is polar and the SP is non-polar (Poole & Lenca, 2017). Today, RPLC is the most widely used of the LC separation modes, accounting for approximately 75-90% of all separation by LC (Poole & Lenca, 2017). The mechanism of RPLC is complex, retaining sample components through non-specific hydrophobic interactions with the SP. For polar solutes, increasing the polarity of the MP will result in less retention to the non-polar SP (Robards, Haddad, & Jackson, 1994). In recent years, the ultra-high pressure liquid chromatograph (UHPLC) have become the standard HPLC platform (Dong & Zhang, 2014). It provides several performance enhancements compared to the HPLC, like lower dwell volumes and system dispersion and a "greener" technology (i.e. less organic solvent, smaller sample amounts, faster analysis time). Regardless, the most important advantage is that the band broadening of analyte compounds is greatly reduced, allowing more efficient separation. UHPLC is often used for method development, as it has quicker run time and responses to changes in column or MP conditions (Dong & Zhang, 2014).

2.4.1 Ionization

Detection of the analytes is achieved by the coupling of MS to the LC. As a direct introduction of the liquid mobile phase to the high vacuum MS would result in production of a large amount of gas, ionization at atmospheric pressure of compounds eluting from the LC is necessary (Vogeser & Parhofer, 2007). Several ionization methods working at atmospheric pressure have been developed, with electrospray ionization (ESI) being the most widely applied (Vogeser & Parhofer, 2007).

The principle of ESI is to use electrical energy to assist ions from the solution to a gaseous phase, before introduction into the MS (Ho et al., 2003). The transfer of ions from solution to gas phase includes dispersal of a fine spray of charged droplets and solvent evaporation followed by ion ejection from the charged droplets. A continuous stream of sample solution is passed through a quartz or a stainless steel capillary tube, maintained at a high voltage (2.5-6 kV) (Ho et al., 2003). This, together with a nebulising gas (e.g. nitrogen), generates a spray of highly charged droplets, which continues down a pressure and potential gradient toward the analyser region of the MS (Ho et al., 2003). The droplets are continued to reduce in size by evaporation of the solvent, assisted by an elevated temperature or another stream of nitrogen drying gas. Hence, the radius of the droplets are reduced and the surface charged density is increased (Ho et al., 2003). The electrical field strength within the droplets will eventually reach a critical point, making it possible for the ions on the surface of the droplets to eject into the gas phase. Finally, emitted ions are sampled by the sampling skimmer and transferred to the MS, Figure 8 (Ho et al., 2003).

Coupled to an LC, the ESI makes it possible to analyse molecules of various sizes and polarities in complex samples. Together with the MS, complicated sample purification is simplified, and it provides a rapid analysis with high sample throughput (Ho et al., 2003).

2.4.2 Mass spectrometry

There are several different mass separation devices, but they all serve the same purpose; to allow the analyte ions of a particulate mass-to-charge ratio to pass through the detector,

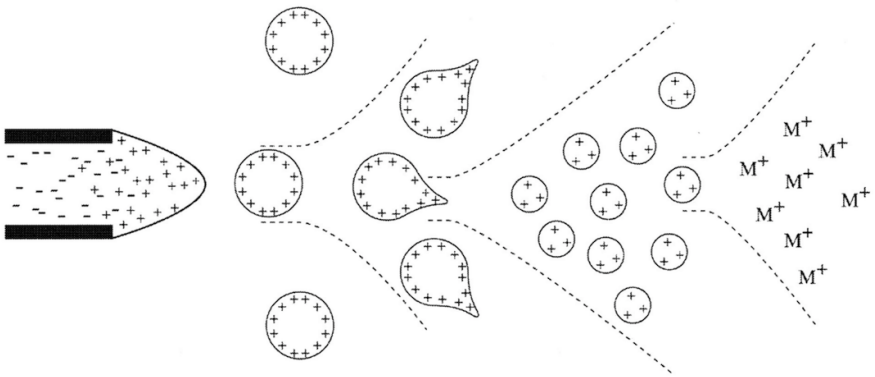

Figure 8: Mechanism of electrospray ionization. Illustration: Ho et al. (2003) (Ho et al., 2003).

while ions produced from sample matrix or other analytes not of interest are filtered out (Thomas, 2013). The most common separators are the quadrupole analyzers.

The quadrupole MS analyzer consists of four cylindrical rods in which both a direct and alternating current are applied, creating an electric field. Depending on the voltage applied to the rods, the electric field will only allow ions of a certain mass to charge ratio to pass. The quadrupoles have relatively high throughput, but low resolution (Skoog, West, Holler, & Crouch, 2013). For low resolution systems, the mass tolerance allowed to pass would typically be 1 m/z. The high selectivity achieved by the MS is due to the unique fragmentation of each molecule. The fragmentation pattern works like a fingerprint to identify the compounds, but matrix interferences from remaining matrix compounds producing the same analyte mass is still possible.

Tandem mass spectrometry (MS/MS) helps avoid matrix interference that can still occur in single quaropole MS systems by selectively monitoring an ion transition of a given chemical. In a tandem quadrupole system, two quadrupole mass filters are combined, with target analyte molecules transferred to a controlled fragmentation in a collision cell (Q2) after the first quadrupole (Q1), Figure 7. The process of fragmentation in the Q2 is known as collision-induced dissociation (CID), creating daughter ions related to the molecular structure of the parent ions from the Q1 (Ho et al., 2003). Finally, the third quadrupole (Q3) can selectively target the daughter ions that are generated from the analytes parent ion. By monitoring this selective ion transition, many matrix interferences can be removed, helping reduce noise introduced to the overall signal and

increasing sensitivity and selectivity.

2.5 Identification and quantification of analytes

After chromatographic analysis, identification and quantification of the target analytes is necessary. One way to confirm the identity of analytes is to inject standard solutions under identical analytical conditions. By comparing retention times and response of the peak in the standard solution chromatogram with the sample chromatogram, peaks may be assigned. To avoid peak mis-assignment, it is important to match the concentration of the standard solutions with the sample solution. For further identification, mass spectrometers (MS) can be used by comparing the molecular structure of the target analyte with fragments from the MS-spectra (Skoog et al., 2013).

Further, analytes are quantified to determine their concentrations in the sample. As the response from the detector depends on both the analyte and the matrix from which it comes, standards are usually used to calibrate the instrument response. In the internal standard calibration, a known amount of a reference compound is added to all samples, standards and blanks. The response signal is then the ratio of the analyte signal to the reference compound signal. The reference compound is known as the internal standard (ISTD). It is a compound with similar chemical properties as the analyte, and is added to the sample prior to sample preparation. Isotopically labeled standards are ideal, as they have identical structures as the analyte and only differs in mass, allowing for separation from target analytes using MS. A calibration curve is prepared by plotting the ratio of responses against the concentration ratio. The concentration of the analyte may then be calculated by obtaining the concentration ratio that corresponds to the peak area ratio, and multiply by the concentration of the added ISTD. The ISTD method can compensate for some types of errors, like sample loss, variation in injected sample volume, volume correction of solvent, matrix suppression or enhancement and other errors influencing both the analyte and the ISTD to the same proportional extent (Skoog et al., 2013).

Finally, several steps have to be performed to make sure the quantification is reliable and the quality of the performed work is adequate.

2.5.1 Limit of detection and limit of quantification

The limit of detection (LOD) is the lowest concentration of analyte that can be detected with a given analytical method. The limit of quantification (LOQ) is the lowest concentration of an analyte that can be determined with acceptable accuracy and precision under the stated conditions. These parameters can be estimated using several different methods, depending on the matrix and analytical method used.

In this study, the lower calibration standard variance was used to calculate the LOD and LOQ, by

$$\mathrm{LOD[pq/\mu L]} = 3 \times \mathrm{SD} \tag{1}$$

and

$$\mathrm{LOQ[pq/\mu L]} = 10 \times \mathrm{SD} \tag{2}$$

where SD is the standard deviation of the lowest quantification standard (1 pg/µL).

2.5.2 Accuracy

Accuracy may be expressed using extraction recovery. The recovery is a measure of how much of the internal standard that is left after the extraction. Extraction recovery of added internal standards assesses both analyte losses and the impact of ion suppression/enhancement effects. As previous results from testing the same method have showed that recoveries lower than 25% may artificially elevate the response, this is used as a limit during discussions on recovery.

2.5.3 Precision

Precision says how well replicate measurements agree with one another, and is usually expressed as a standard deviation (SD). The arithmetic mean can be calculated using

$$\overline{x} = \frac{\sum x_i}{n} \tag{3}$$

where $\sum x_i$ is the sum of the replicate measurements and n is the total number of measurements. The standard deviation can then be obtained from

$$SD = \sqrt{\frac{\sum (x_i - \overline{x})^2}{n - 1}} \tag{4}$$

Precision may also be expressed using standard error of mean (SEM), obtained from

$$SEM = \frac{SD}{\sqrt{n}} \tag{5}$$

3 Materials and methods

Three male blue mussels (*Mytilus edulis*) were collected at Tromsø Island on 26th of April 2018. Adult specimens were collected from a floating peer (69°38'25.5"N 18°56'33.8"E) and brought back to the laboratory where they were immediately prepared for the experiment. The transportation time between the sampling site and the laboratory was less than 10 minutes. Explants were placed in an incubator, and the time between the collection of the specimens in the field and the start of the experiment was less than 2 hours. Viability of the explants were completed on the 3rd and 4th of May 2018. Samples were prepared and analyzed at Norwegian Institute for Air Research (NILU), Fram Centre, Tromsø, during a three week period in November 2019 (08/11/19-21/11/19). A map of the sampling site is shown in Figure 9.

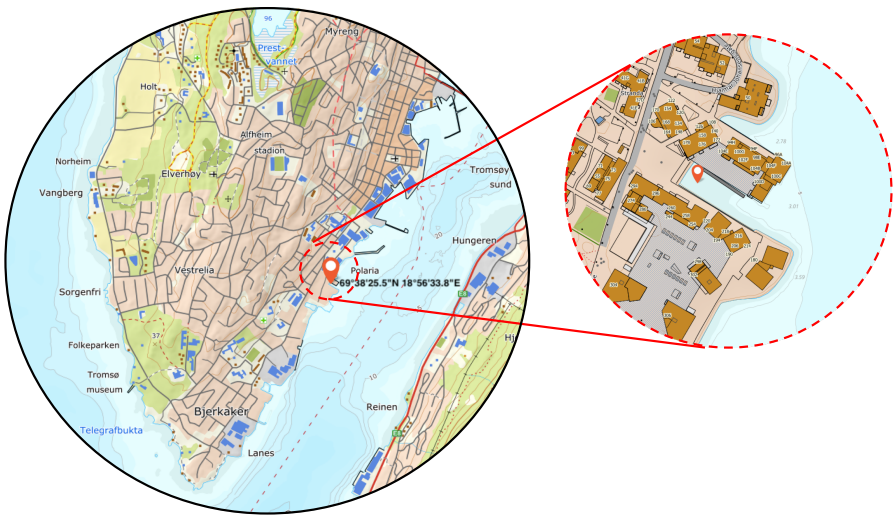

Figure 9: Position of blue mussel sampling in Tromsø (69°38'25.5"N 18°56'33.8"E).

3.1 Standards, chemicals and materials

A complete list of standards, chemicals and materials can be found in Appendix, Table A.1, Table A.2 and Table A.3. Standards of dopamine and serotonin were purchased from CHIRON AS (Trondheim, Norway). Standards and chemicals were of trace analysis

grade.

All equipment used during field and laboratory work was cleaned thoroughly before use to reduce possible contamination of samples. All glass- and metal-ware were washed in a laboratory dishwasher, and stopcocks used for extraction were cleaned in methanol (MeOH) in an ultrasonic bath (USC-TDH, VWR International).

3.2 Cultured explants (CE) of digestive glands of bivalves

For the experimental tests, three individuals with similar size and gonad development were used. Digestive glands were removed under sterile conditions before being rinsed in filtered sea water containing 1% antibiotic. The tissue was cut by hand using a scalpel mounted with a blade (number 24). Rectangular pieces of about 3 mm^3 were cut in a petridish containing fresh culture media (Leibovitch L15 with 1% antibiotic), and maintained on ice. To avoid bias in biological responses due to the sex of the animals, only males were used for this study. The distinction between sex was not always possible by visual observations, so confirmation of the sex has been done under a binocular microscope to ensure the presence of testicular cells in the mantle tissue. About 96 to 144 of satisfying explants were obtained from 3 digestive glands of blue mussels (weighing about 0.6 to 1.2 g). Then the explants were randomly placed into each well of the 24 well plate containing 1 mL of fresh sterile culture media (Leibovitch L15 with 1% antibiotic). Plates were incubated at 5 ℃ under gentle agitation (40 rpm) for 72 hours.

3.3 Exposure to chemicals

After 24 hours preincubation, the medium of each well was removed and replaced by the appropriate amount of fresh medium associated with the test compounds (*m*-xylene, BDE-99, $CdCl_2$ and 17-β-estradiol) at different concentrations, using 3 explants per condition. Six blank samples were not exposed to any test compounds. Stock solutions of test compounds were made in dimethyl sulfoxide (DMSO) and then diluted in ethanol. Final concentrations of ethanol and DMSO in the culture medium were 0.1%. The media were collected after 48 hours of incubation and stored at -80 ℃, before being used for the measurement of dopamine and serotonin levels.

All explants were exposed to three different concentrations, denoted C1, C2 and C3, indicating a logaritmic increase of concentrations. Co-exposure for both experiments was conducted with C2 concentrations of all single chemicals. Concentrations and exposure design is presented in Appendix, Figure A.1.

3.4 Viability of explants

The viability of the explants was assessed by the measurement of nonspecific esterase activity using a FDA-hydrolysis assay (Larsson & Nygren, 1989; Tutundjian, Minier, Le Foll, & Leboulenger, 2002). After being weighed, explants were sonicated in 1 mL culture medium. Extracts were then diluted (1 of 9), and 200 µL were transferred in a 96 well plate (four replicates per sample). Fifteen mM of FDA were added to each well before reading of the plate (excitation filter, 485 nm; emission filter, 530 nm). The absorbance was measured for 30 minutes every 2.5 minutes to determine the initial speed of the FDA hydrolysis.

3.5 Sample preparation

The method for neurotransmitter extraction was based on the method for selective phase extraction of catecholamines in blood using molecularly imprinted polymers developed by AFFINISEP®. Using this method, the expected result is a clean-up and a pre-concentration of the sample at trace levels.

3.5.1 Molecularly Imprinted Polymer-Solid Phase Extraction (MIPs-SPE)

The explants were placed in centrifuge test tubes. Next, 10 µL internal standard mixture (ISTD, 10 ng/µL ^{13}C-dopamine/d_4-serotonin in MeOH) were spiked on the tissues and 990 µL MeOH-H_2O (50/50, v/v), acidified to 1% formic acid, was added to the test tubes. The tubes where homogenized with a Precellys instrument (Precellys 24 tissue homogenizer, Bertin Instruments) for 2 × 20 s, followed by centrifugation (Jouan A14 centrifuge, ThermoFisher Scientific) for 10 minutes at 13000 rpm. Supernatant (200 µL) was diluted to 10 mL by adding phosphoric acid (H_3PO_4)–sodium hydroxide (NaOH) buffer (pH=7, 10 mM ionic strength, 9,8 mL) , and NaOH (1 M) was added dropwise (1-2

drops) until pH=7.

For clean-up of explant tissue extracts, MIP-SPE cartridges (AFFINIMIP® SPE) were connected to a vaccum manifold system. First, cartridges were equilibrated by washing with H_3PO_4–NaOH buffer (3 mL, 2 drops/s) and then MilliQ water (3 mL, 2 drops/s). Sample dilutions (5 mL, 1 drop every 2 s) were then added to the equilibrated SPE columns. After sample loading, MIP-SPE columns were washed of matrix interferents by first adding MilliQ water (3 mL, 1 drop/s) and then MeOH-H_2O (15/85, v/v, 2 mL, 1 drop/s), followed by partially drying of the cartridges by applying vacuum for 30 s. The last interferents were washed with MeOH (1 mL, 1 drop/s). Analytes were then collected by eluting with MeOH-AcOH (95/5, v/v, 1.5 mL, 1 drop/s) and evaporated until dryness in a MiVac (Genevac). Samples were reconstituted in 0.1% formic acid (200 µL), and transferred to autosampler vials through glass wool filtering.

3.6 Preparation of calibration standards

A 1 ng/µL and a 10 ng/µL stock solution of serotonin and dopamine was prepared by mixing of single compound standard solutions and dilution with MeOH. Calibration standards of 1, 5, 10, 25 and 50 pg/µL were prepared by dilution of the stock solutions with 0.1% formic acid. A constant amount of ISTD (50 µL) was added to each calibration standard solution.

3.7 Instrumental analysis

Neurotransmitter analysis was carried out using ultra-high pressure liquid chromatography tandem mass spectrometry (UPLC-LC-MS/MS) on a Thermo Scientific Vantage MS/MS (Vantage TSQ, Thermo Fisher Scientific Inc., Waltham, MA, USA). A 10 µL sample aliquot was injected on a Waters Acquity UPLC HSS 3 T column (2.1×100 mm, 1,8 µm) (Waters Corporation, Milford, MA, USA) equipped with a Waters Van guard HSS T3 guard column (2.1×5 mm, 1.8 µm) (Waters Corporation, Milford, MA, USA). Separation of dopamine and serotonin utilized an initial mobile phase of 0.1% formic acid and acetonitrile (ACN) in a 98:2 ratio at constant flow rate of 0.3 mL/min for 0.5 min. The mobile phase composition was changed to 92% formic acid and 8% ACN over

1.25 minutes and held for 2 minutes until the elution of dopamine (1.15 min) and serotonin (2.57 min). Column was then flushed with ACN for 3 minutes followed by gradual reconditioning of column to starting conditions for 7.5 minutes.

Every calibration standard and sample was run twice, and a calibration curve was run both at the beginning and at the end of the sequence to assess changes in the instrument response over time. Chromatograms were processed by the computer software Thermo Scientific^{TM} Dionex^{TM} Chromeleon^{TM} 7 Chromatography Data System software (version 7.2.10).

Mass transitions used for analysis are listed in Table 1, and conditions for the LC-MS/MS system are listed in Table 2. Argon was used as a collision gas.

Table 1: Mass transitions used for analyses. Col.e. = Collision energy.

Compound		**Parent** (m/z)	**Product** (m/z)	**Col.e.** (V)	**S-lens** (V)
Dopamine	*quant.*	154.10	137.07	47	10
	qual.		91.06		23
$^{13}C_6$-Dopamine	*quant.*	160.00	143.06	55	10
	qual.		96.05		24
Serotonin	*quant.*	177.10	160.10	47	11
	qual.		115.06		28
D_4-Serotonin	*quant.*	181.00	164.08	56	10
	qual.		118.03		27

Table 2: Conditions for the LC-MS/MS analysis.

Separation conditions	
Instrumentation	Thermo Scientific quaternary Accela 1250 pump
Column	Waters Acquity UPLC HSS 3 T (2.1×100 mm, 1,8 µm)
Guard column	Waters Van guard HSS T3 (2.1×5 mm, 1.8 µm)
Mobile phase	A: 0.1% formic acid, B: acetonitril
Column temperature	40 ℃
Flow rate	0.3 mL/min
Injection volume	10 µL
MS conditions	
Instrumentation	Thermo Scientific TSQ Vantage
Ionization conditions	ESI
Polarity	Positive
Spray voltage	3000 V
Vaporization temperature	260 ℃
Sheath gas pressure	60 a.u.
Auxiallary gas pressure	5 a.u.
Capillary temperature	230 ℃
Collision gas pressure	1.5 mTorr
Declustering voltage	2 V
Data processing	
Software	Thermo Scientific^{TM} Dionex^{TM} Chromeleon^{TM} 7 Chromatography Data System

3.8 Identification and quantification of analytes

UHPLC-MS/MS data was acquired using Thermo Scientific^{TM} Dionex^{TM} Chromeleon^{TM} 7 Chromatography Data System software (version 7.2.10).

Analytes were quantified using internal standard calibration with a five point calibration curve. Carbon labeled dopamine ($^{13}C_6$-dopamine) and deuterated serotonin (D_4-serotonin) was used as ISTD. Calibration curves were established by analyzing standard solutions of varying concentrations of dopamine and serotonin (1-50 pg/µL) mixed with a constant amount of ISTD (50 µL).

Validation of the analysis was carried out as described in section 2.5. The LOD and LOQ values for dopamine and serotonin was calculated using Equation 1 and 2. Results are expressed as mean ± SD or mean ± SEM with n=3 or n=2. The SD and SEM values were calculated using Equation 4 and 5.

3.8.1 Statistical analysis

Data was imported to Statistica 6, where normal distribution of data was assessed using a Shapiro-Wilk's W test ($p \leq 0.05$). Homogeneity of variance was assessed using Levene's test ($p > 0.05$). Comparisons between different exposure groups and concentrations were then made using a one-way analysis of variance (ANOVA) followed by multiple independent group's comparison (Tukey test, $p \leq 0.05$). No significant difference between groups were observed.

4 Results and discussion

4.1 Viability of explants

Results showed that viability of explants incubated in medium culture was over 60% until incubated for 72 hours, Figure 10. Thus, culturing the explants for up to 72 hours was considered suitable. Viability of explant cells exposed to different concentrations of *m*-xylene, BDE-99, $CdCl_2$ and estradiol-17-β were similar to the control, except for BDE-99 C3 treated explants (<60% of initial activity). Figures are shown in Appendix, Figure A.3 and Figure A.4.

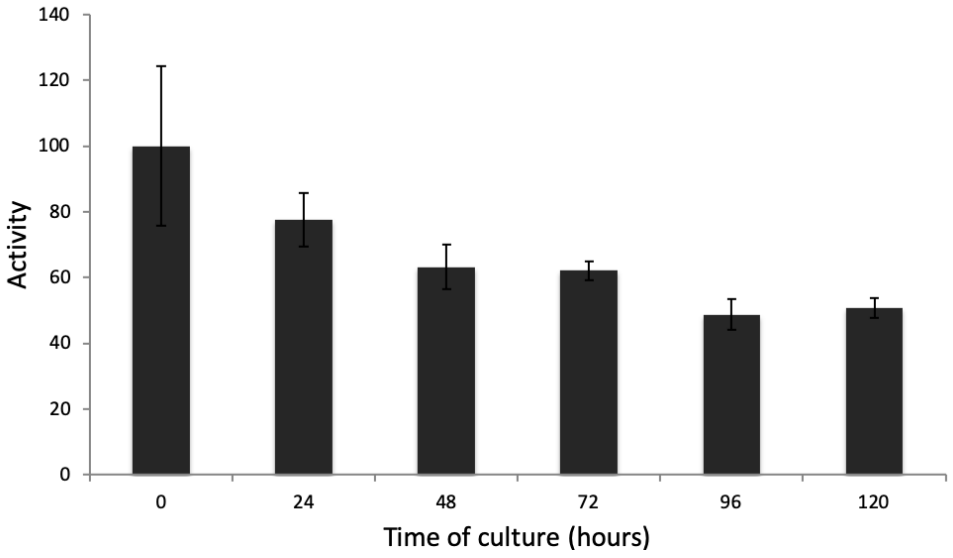

Figure 10: Viability of explants using FDA hydrolysis activity for different culture times. Results are given as a percentage of the control FDA hydrolysis activity ± S.E.M.

4.2 Validation of LC-MS analysis

All samples were analyzed using UHPLC-MS/MS. Typical chromatograms of dopamine and serotonin, with their isotopically labeled isotopes, are shown in Figure 11. Calibration curves were established for both dopamine and serotonin and are presented in Appendix, Figure A.2.

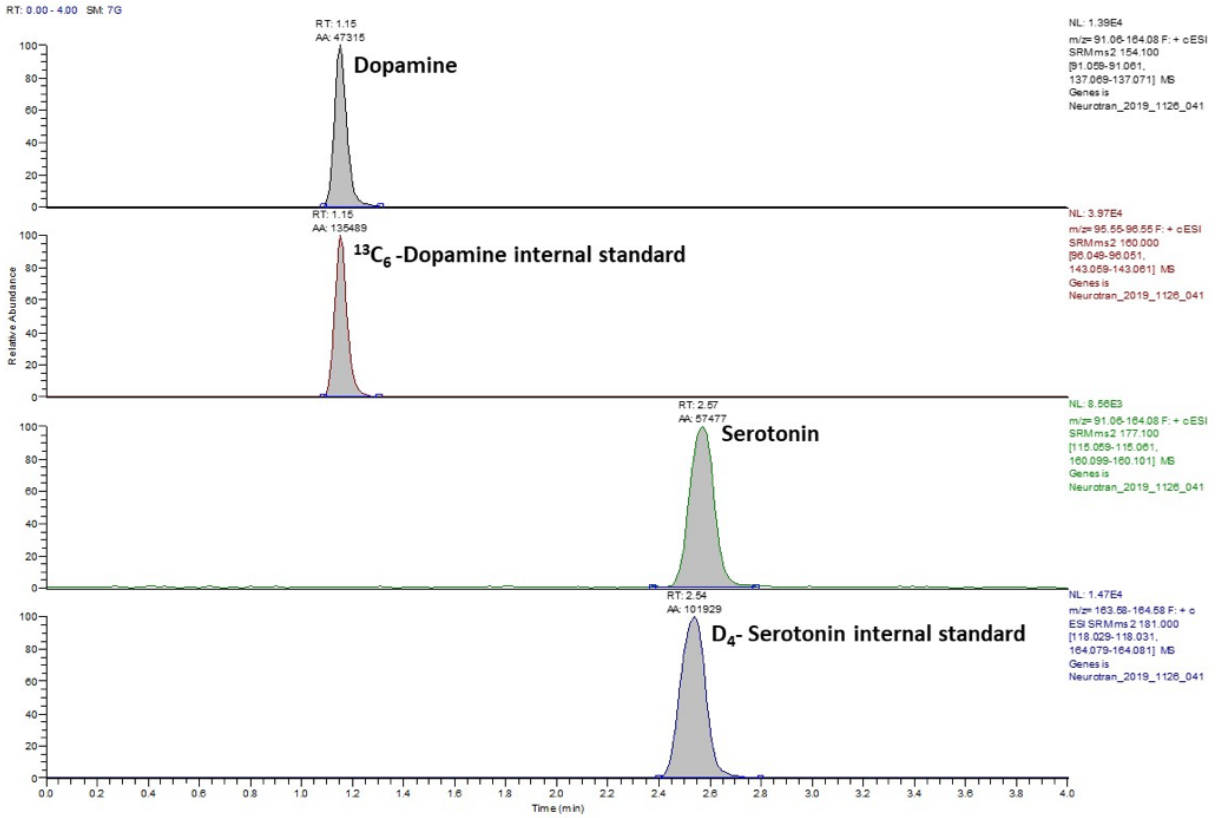

Figure 11: Typical chromatogram from the UHPLC analysis. Chromatrogram shows signals from dopamine and serotonin and their isotopically labeled internal standards.

4.2.1 LOD and LOQ

The LOQ values are reported in Table 3 to Table 6. LOD values were 15 pg/mg and 45 pg/mg for dopamine and serotonin respectively. All concentrations of dopamine were above LOQ, while only 7 of the 48 samples resulted in responses above LOQ for serotonin. Samples sizes were between 3 and 10 mg, with a mean of 5.46 mg, and the low responses of serotonin may be due to small sample sizes. Response of serotonin is obtained for some of the smallest samples (3 mg), but this can be due to exposure, coincidence or sample sizes. Larger sample masses would give higher detection frequency, due to more injected analyte.

4.2.2 Accuracy

The accuracy was estimated using recovery, and is reported in Table 3 to Table 6. Earlier work during the WASTECALL project (unpublished work) has shown that the sample

matrix usually suppresses the signal by around 20%, and this may be the reason for low recoveries observed in some samples. Although the ISTD corrects for extraction losses and ion suppression, if the ISTD response is too low (<25%), previous results (unpublished work) have showed that it may artificially elevate the signal, resulting in too high reported concentrations.

The recovery for dopamine was generally lower than for serotonin, with 28 of 48 samples having recovery lower than 25%. This is probably due to the higher polarity of dopamine than serotonin. Dopamine has two hydroxygroups whereas serotonin only has one. This makes dopamine more hydrophilic, and thus might be easier eluted with matrix interferents during washing with water, resulting in lower recoveries. Even though only 9 of the 48 samples had too low recoveries (<25%) for serotonin, some improvements of the extraction method are recommended (Section 4.4).

The LC-MS analysis of the samples were run in two batches, with the two exposure experiments in separate analysis (Blank1-BDE.C3.3: batch 1, CDCL.C1.1-E2.C3.3: batch 2, Table 3 to Table 6). Out of the 21 samples in batch 2, only 3 samples had recovery above 25% for dopamine, and 8 out of the 9 samples with low recovery of serotonin was in batch 2. Most likely there is a factor during the second analysis contributing to lower recovery. However, calibration curves for both analyses were almost identical, and showed no difference in the impact of ion suppression. This indicates that losses likely occurred during the extraction procedure. The elution of the samples may have been too fast, leaving less time for the samples to interact with the solid phase. Previous studies have demonstrated altered conformation of polymer chains in the structure of MIPs from change in pH, solvent, temperature and ionic strength (Piletsky et al., 2002; Turner et al., 2004). Altered conformation causes a change in the size and shape of the template-binding pockets, and disrupts the binding of target compounds (Turner et al., 2004). Therefore, if the pH of the sample or buffer solution was measured inaccurately, this might result in lower affinity for dopamine in the MIPs solid phase, resulting in low recoveries.

Replicas with low recoveries and unlikely high responses in batch 1 were removed from further calculations, as they would give a false impression on the effects on dopamine and serotonin levels. As many samples had low recoveries in the second round of analysis, their

responses were generally higher than for the first batch. However, removing all samples with recoveries lower than 25% would leave an insignificant amount of samples, preventing calculations of standard deviation and confidence intervals. Additionally, not all samples with low recoveries had artificially high responses. Thus, for the second batch, all samples with similar responses have been accounted for, regardless of recoveries. Consequently, it would be insufficient to compare the responses of the two exposure experiments with one another, as the overall levels of dopamine will be higher in the experiment with $CdCl_2$ and E2 than *m*-xylene and BDE-99.

4.2.3 Precision

Precision is reported as standard deviation. As mentioned, some of the responses were artificially high, due to low recoveries. These replicas were removed, resulting in a lower standard deviation for some of the samples. Removing replicas increases the confidence interval and thus decreases the probability of detecting possible significant different effects. For future studies, number of replicas should be increased to 5 per sample to achieve required statistical strength with respect to outliers. The mean of reported concentrations with and without the outliers are shown in Appendix, together with their respective SDs, Figure A.5 and Figure A.6.

All samples in the first round of LC-MS analysis were run twice to evaluate the repeatability. As responses were approximately the same, all samples were only analyzed once in the second batch.

4.3 Levels of neurotransmitters in digestive gland explants

Dopamine and serotonin was detected and quantified in all 48 samples and in 9 of the 48 samples, respectively. Table 3 to Table 6 present the concentrations of dopamine and serotonin measured in every sample, together with sample sizes, LOQ values and recoveries. The sample ID indicates exposure chemicals and the type of treatment (XYL=xylene, C1=lowest concentration of exposure etc.) and number of replica (3 parallels). All reported concentrations were above calculated LOQ. Sample concentrations with extraction recoveries less than 25% are presented in italics. Two of the blank EtOH samples had an

error in extraction procedure, and are reported as not detected (n.d.). As co-exposure was conducted with the C2 concentrations of all chemicals, discussions comparing co-exposure to single exposure is based on C2 treated explants.

As many of the samples were in the range below LOQ for levels of serotonin, analysis of larger sample sizes could be carried out. To achieve higher recovery of internal standards of dopamine, improvement of extraction method might be necessary. These corrections were not tested due to the limited time frame of the project, but suggestions for method improvements is discussed in section 4.4.

Table 3: Overview of sample results (part 1).

		Dopamine			Serotonin		
Sample ID	Sample mass [mg]	Conc [pg/mg]	LOQ [pg/mg]	Recovery [%]	Conc [pg/mg]	LOQ [pg/mg]	Recovery [%]
Blank1	6.49	n.d.[a]	52	n.o.[b]	n.d.	150	n.o.
Blank2	6.72	n.d.	52	n.o.	n.d.	150	n.o.
Blank3	5.81	538	52	36.8	<LOQ[c]	150	69.9
Blank4	5.59	392	52	29.5	<LOQ	150	54.9
Blank5	4.74	*665*	52	*8.5*	<LOQ	150	49.8
Blank6	4.71	*745*	52	*10.9*	<LOQ	150	51.1
XYL.C1.1	5.99	437	52	31.7	<LOQ	150	72.4
XYL.C1.2	8.07	238	52	43.3	185	150	70.1
XYL.C1.3	4.97	274	52	52.0	<LOQ	150	79.7
XYL.C2.1	5.50	*1164*[d]	52	*17.7*	<LOQ	150	51.0
XYL.C2.2	6.18	806	52	27.8	<LOQ	150	40.4
XYL.C2.3	5.45	569	52	45.2	<LOQ	150	62.3

[a] n.d. denotes not detected

[b] n.o. denotes not obtained

[c] Values are below the limit of quantification

[d] Values in italic denotes that the values have recovery <25%

Table 4: Overview of sample results (part 2).

		Dopamine			Serotonin		
Sample ID	Sample mass [mg]	Conc [pg/mg]	LOQ [pg/mg]	Recovery [%]	Conc [pg/mg]	LOQ [pg/mg]	Recovery [%]
XYL.C3.1	4.63	582	52	45.6	<LOQ[a]	150	71.6
XYL.C3.2	5.81	*461*[b]	52	*14.7*	<LOQ	150	54.0
XYL.C3.3	5.99	*1174*	52	*7.5*	<LOQ	150	42.2
XYL-BDE.C2.1	5.89	*972*	52	*5.7*	<LOQ	150	39.9
XYL-BDE.C2.2	6.23	*2344*	52	*1.0*	<LOQ	150	*14.5*
XYL-BDE.C2.3	4.51	1296	52	27.2	787	150	36.4
BDE.C1.1	3.35	979	52	27.9	346	150	27.8
BDE.C1.2	4.68	527	52	34.6	<LOQ	150	58.2
BDE.C1.3	7.88	549	52	36.4	<LOQ	150	51.9
BDE.C2.1	4.96	600	52	33.5	241	150	49.8
BDE.C2.2	9.71	484	52	32.5	157	150	51.8
BDE.C2.3	7.51	*469*	52	*21.6*	<LOQ	150	40.5

[a]Values are below the limit of quantification

[b]Values in italic denotes that the values have recovery <25%

Table 5: Overview of sample results (part 3).

		Dopamine			Serotonin		
Sample ID	Sample mass [mg]	Conc [pg/mg]	LOQ [pg/mg]	Recovery [%]	Conc [pg/mg]	LOQ [pg/mg]	Recovery [%]
BDE.C3.1	5.33	477	52	41.6	184	150	54.2
BDE.C3.2	5.28	*514*[a]	52	*14.8*	<LOQ[b]	150	51.1
BDE.C3.3	5.32	*747*	52	*7.3*	<LOQ	150	52.9
CDCL.C1.1	5.93	*562*	52	*14.3*	<LOQ	150	56.8
CDCL.C1.2	4.68	940	52	35.1	<LOQ	150	29.0
CDCL.C1.3	5.56	*1003*	52	*10.5*	<LOQ	150	*23.1*
CDCL.C2.1	6.50	*1675*	52	*9.2*	<LOQ	150	27.6
CDCL.C2.2	6.83	*2072*	52	*10.5*	<LOQ	150	*24.3*
CDCL.C2.3	3.93	*2289*	52	*7.2*	<LOQ	150	39.4
CDCL.C3.1	4.95	*1527*	52	*9.8*	<LOQ	150	*16.6*
CDCL.C3.2	7.65	*1041*	52	*11.5*	<LOQ	150	26.8
CDCL.C3.3	4.75	*554*	52	40.8	299	150	60.9

[a] Values in italic denotes that the values have recovery <25%

[b] Values are below the limit of quantification

Table 6: Overview of sample results (part 4).

		Dopamine			Serotonin		
Sample ID	Sample mass [mg]	Conc [pg/mg]	LOQ [pg/mg]	Recovery [%]	Conc [pg/mg]	LOQ [pg/mg]	Recovery [%]
CDCL-E2.C2.1	4.71	*628*[a]	52	*20.7*	<LOQ[b]	150	32.1
CDCL-E2.C2.2	3.33	647	52	35.6	<LOQ	150	59.5
CDCL-E2.C2.3	3.09	*1444*	52	*9.8*	<LOQ	150	*20.1*
E2.C1.1	5.78	*1083*	52	*8.6*	<LOQ	150	25.5
E2.C1.2	4.67	*2998*	52	*4.5*	<LOQ	150	*13.6*
E2.C1.3	6.12	*1250*	52	*7.0*	<LOQ	150	27.3
E2.C2.1	4.87	*1238*	52	*10.0*	<LOQ	150	*22.5*
E2.C2.2	2.95	*1834*	52	*14.8*	<LOQ	150	*18.1*
E2.C2.3	4.81	*1954*	52	*6.6*	<LOQ	150	*12.4*
E2.C3.1	5.32	*1161*	52	*7.2*	<LOQ	150	29.8
E2.C3.2	5.65	*870*	52	*17.3*	<LOQ	150	57.5
E2.C3.3	5.26	*1282*	52	*8.7*	<LOQ	150	28.3

[a]Values in italic denotes that the values have recovery <25%

[b]Values are below the limit of quantification

4.3.1 Exposure to *m*-xylene and BDE-99

Explants of digestive glands exposed to an increasing concentration of *m*-xylene were found to have increasing levels of dopamine from C1 to C2 (Figure 12). Still, no significant differences were found between the exposure groups and the blank (95% confidence interval (CI)). Concentrations of dopamine exposed to different concentrations of BDE-99 were approximately in the same order of magnitude, indicating no effect of BDE-99 concentration on level of dopamine. For the co-exposure of *m*-xylene and BDE-99, the level of dopamine increased, with 1.6-fold higher levels compared to single *m*-xylene exposure, and 2.2-fold higher levels compared to single BDE-99 exposure. Thus, co-exposure of these chemicals, with no observed effects after single exposure, increased the magnitude of the measured response. This illustrates that by only studying single exposure, the physiological effects may be under-evaluated if no effect is observed. Therefore, testing co-exposure of single chemicals showing no effect is important.

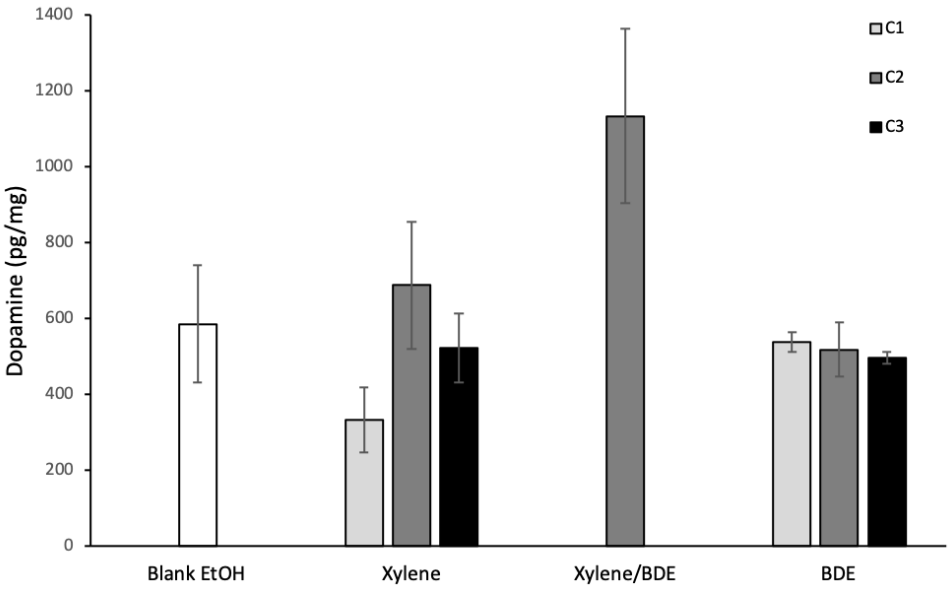

Figure 12: Concentration of dopamine in explants exposed to different concentrations of single *m*-xylene and BDE-99 and co-exposure of C2 concentrations of both. Results are given as mean ±SD. Darker grey values indicate higher concentration of exposure compounds.

As only 9 of 48 samples gave responses of serotonin above LOQ, these results cannot be used to draw any conclusions, but together with the results of dopamine, they may contribute to indicate some trends. The concentration of serotonin in explants co-exposed

to *m*-xylene and BDE-99 are higher than all single contaminant exposed explants, and 4-fold higher than C2 BDE-99 treated explants (Figure 13). This trend is similar as for dopamine concentrations, indicating a synergistic effect of mixing *m*-xylene with BDE-99. As no blank samples were above the limit of quantification for serotonin, the effect of single exposure is unknown.

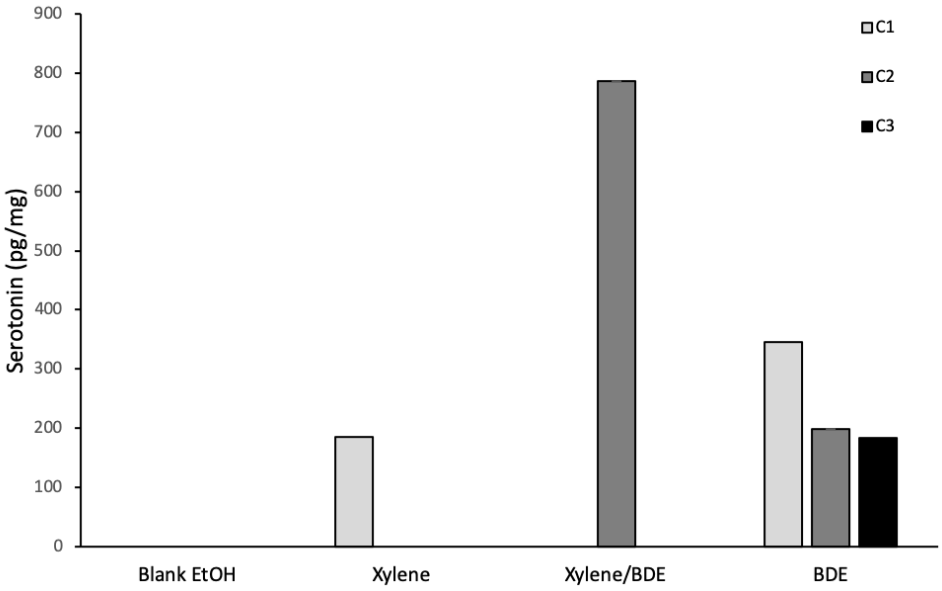

Figure 13: Concentration of serotonin in explants exposed to different concentrations of single *m*-xylene and BDE-99 and co-exposure of C2 concentrations of both. Results are given as concentration of the the single replicas with recovery <25%, and mean if possible (two replicas were quantifiable only for C2 treatment of BDE). Darker grey values indicate higher concentration of exposure compound.

BDE-99 was found to have no effects on dopamine levels, for none of the exposure concentrations. It has been proposed by Opperhulzen et al. (1985) that organic molecules with a cross-section area greater than 9.5 Å are restricted from passing through biological membranes, due to their large sizes (Opperhulzen et al., 1985). With a cross-section area of 9.6 Å (Stapleton, Letcher, & Baker, 2004), BDE-99 might be too large to pass through the membranes of the digestive gland, resulting in no interference with the levels of dopamine. Anyway, co-exposure induced the level of both dopamine and serotonin. The presence of *m*-xylene might aid the dissolution of BDE-99 in the culture media, and thus increase its exposure to the explants compared to single exposure of BDE-99. Another reason for the absent impact from BDE-99 exposure might be the chosen culture

media. With a hydrophilic culture media, the hydrophobic BDE-99 will not dissolve and are likely to precipitate out of the solution. This will result in no or limited exposure to the explants. The same might explain the limited impact of *m*-xylene exposure.

In most cases, co-exposure of two chemicals are based on a theory or expectation to demonstrate a mechanism of action, i.e. mixing of two chemicals expected to respond oppositely to demonstrate an antagonistic effect (effect of contrasting actions) (Gerbron et al., 2015). Other experiments base their theories on mixing chemicals expected to be found together, such as two pharmaceuticals. However, marine organisms, such as bivalves, are exposed to a various mix of very different chemicals, not necessarily connected or correlated in any way. The effects of such co-exposures are not often studied, but they are important to understand their potentially complex mechanisms of action. No other ecotoxicological studies combining *m*-xylene and BDE-99 was found. Thus, the results can not be compared to any previous reported results. Even though the results in this study are not significant (95% CI), enclosed they are an example of gene expression where co-exposure does have an effect on estrogenic activities. As this is the first study to highlight this synergistic effect, more studies to understand the associated mechanism of action, and to better understand what factors contributes to the observed effect are of high importance.

4.3.2 Exposure to $CdCl_2$ and E2

For both $CdCl_2$ and E2, the concentration of dopamine increased from C1 to C2, and decreased for C3 treated explants (Figure 14). Concentrations were in the same order of magnitude for C1 and C3 for both $CdCl_2$ and E2. No significant differences were observed between the groups (25% CI). As only 3 of the samples in this experiment had recoveries above 25%, the overall levels of dopamine may be artificially high. However, outliers were removed and the remaining responses should be comparable. Lower levels of dopamine were observed after co-exposure of $CdCl_2$ and E2, with levels reduced 2.2-fold and 1.8-fold respectively.

Explants exposed to single $CdCl_2$ and E2 showed greater levels of dopamine for all treated explants, compared to the non-exposed control. Increased levels of dopamine after ex-

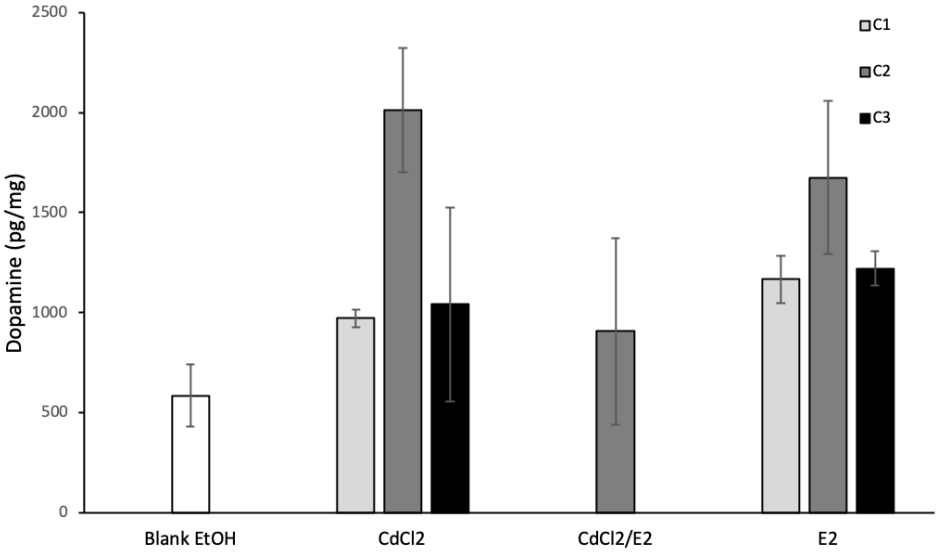

Figure 14: Concentration of dopamine in explants exposed to different concentrations of single $CdCl_2$ and 17-β-estradiol and co-exposure of C2 concentrations of both. Results are given as mean concentration ±SD. Darker grey values indicate higher concentration of exposure compounds.

posure to Cd for 12 hours in digestive glands of mussels were also observed by Almeida et al. (2003) (Almeida, Bainy, Medeiros, & Di Mascio, 2003). Injections of E2 in the freshwater mussel (*Elliptio complanata*) have showed decreased levels of both dopamine and serotonin in the gonad and nerve ganglia (Gagné & Blaise, 2003). However, the contrasting results compared to this study might be due to differences in test species, tissues, concentrations or other exposure design factors. Furthermore, as most samples exposed to $CdCl_2$ and E2 had low recoveries, the levels of dopamine might be unnaturally high compared to the blank sample. Thus comparing groups within this experiment, excluding the blank, might be more accurate.

Co-exposing $CdCl_2$ and E2 was tested to study the anti-estrogenic activity of $CdCl_2$. As $CdCl_2$ can bind to the estrogen receptor (ER), co-exposure of these chemicals will indicate if they compete or not, and if the mechanism of action involves ER or other pathways. Results show a decrease in dopamine levels, compared to explants treated with C2 concentrations of single $CdCl_2$ and E2. This suggests that Cd could have inhibitory properties after ER activation by E2, also suggested by Gerbron et al. (2015) after observing the same effect on Vtg levels in liver explants of roach (*Rutilus rutilus*) co-exposed to $CdCl_2$ and E2 (Gerbron et al., 2015). It was suggested by Guevel et al. (2000)

that Cd could act on the C-terminal domain of the ER without inhibiting the binding of E2, but by decreasing the interaction of the activated ER with the DNA, reducing ER transcriptional activity (Guevel et al., 2000). However, the mechanism of endocrine disruption causing adverse outcomes in bivalves is yet to be established. Even though an ER homologous to vertebrates have been identified in mussels, it does not bind EDCs (Blalock et al., 2018). Therefore, similar observed effects in vertebrates and invertebrates are likely to occur through different pathways and mechanisms. Additionally, $CdCl_2$ alone seems to induce dopamine, and interpreting and confirming the complexity of mechanisms of action of $CdCl_2$ is difficult. In some conditions, $CdCl_2$ might act estrogenic, and when associated with E2, it might act anti-estrogenic. As only one concentration of the chemicals was used for co-exposure, observation of the dose-response curve of anti-estrogenic activity cannot be confirmed.

Even though no conclusion can be drawn, due to low recoveries and few samples, they indicate that exposure to $CdCl_2$ and E2 may alter the normal metabolism of serotonin and dopamine, both involved in several biological processes in bivalves. Chemicals acting through ER-pathways are likely to cause the observed effect.

4.3.3 Impact of urban development on marine ecosystems

The observed effects of co-exposure to these chemicals indicate their possible impacts on organisms in the marine ecosystems. More tourism, shipping traffic and urban development in Tromsø will increase the presence of such contaminants, and new chemicals will likely emerge. There is a lack of knowledge concerning the cocktail effects of environmental contaminant mixtures on marine ecosystems. Especially in Tromsø, where water treatment only consists of a primary filtration process, allowing the complex mixture of chemicals to enter the marine environment (Evenset, Jørgensen, & Christensen, 2009; Warner, Nøst, Andrade, & Christensen, 2014).

Dopamine and serotonin have shown to be important for several processes in bivalves and other marine organisms. Giard et al. (1995) showed that dopamine evoked the release of α-amylase from the stomach-digestive gland complex of the scallop (*Pecten maximus*), suggesting the importance of dopamine in feeding and digestive processes of

the scallop (Giard, Favrel, & Boucaud-Camou, 1995). A review on selective lesioning techniques to elucidate the role of dopamine and serotonin in the feeding system of the pond snail (*Lymnaea stagnalis*) also suggested the importance of dopamine for basic feeding response to food to occur. They also pointed out the importance of serotonin in the feeding behaviour (Kemenes, 1997). Studies on the freshwater mussels (*Elliptio complanata*) exposed to wastewater effluents have showed an increased number of females in the exposed group, and altered levels of dopamine and serotonin in nerve tissues, both involved in sexual differentiation in bivalves and fish (Blaise et al., 2003; Gagné & Blaise, 2003). Serotonin also has a key role in bivalves by being responsible for gamete releases during spawning.

The results in this study demonstrates that analysis of dopamine and serotonin in tissues of mussels potentially could serve as biomarkers for contamination in marine environments. The observed altering in dopamine and serotonin levels in exposed blue mussels is concerning. It is important to investigate the impacts of increased human activities in Tromsø on the release of a cocktail of environmental contaminants and to better understand their impacts on marine ecosystem.

4.4 Method improvements

The *ex vivo* method using cultured explants has previously been tested on liver explants from roach (Gerbron et al., 2010), and developed for Icelandic scallop (Manuscript in preparation, Flagship project Number 8483). In the present study, this method was tested on blue mussels. Viability of exposed explants was between 78 and 107% for exposure to different concentrations of $CdCl_2$ and E2 and between 58 and 135% for exposure to different concentrations of *m*-xylene and BDE-99, Figure A.3 and Figure A.4. To be able to confirm a dose-response curve of anti-estrogenic activity, co-exposure of all concentration treatments (C1, C2, C3) should be carried out. Further, changing the environment of the blue mussels during sampling may contribute to a change in levels of dopamine and serotonin. This will be similar in all samples, including blanks, but is worth taking into account when comparing to other studies, or during future studies. Another factor that should be tested is the actual exposure concentrations in the culture media.

Extraction of the culture media could be carried out for analysis of the contaminants of interest to determine exposure concentrations, and to make sure similar exposure is occurring across all replicas.

The analytical method for measuring levels of neurotransmitters in tissue was based on a method of analysis of plasma.(Claude, Morin, & Denoroy, 2014) An acidic solution was added to the explants after spiking with ISTD. To neutralize this acidic solution before extraction, it was dissolved in phosphate buffer (pH=7) and adjusted with NaOH (1 M) to pH=7. Cartridges were conditioned with phosphate buffer (pH=7) to activate the acidic groups of the imprinted polymer. This creates strong specific electrostatic interactions between neurotransmitter molecules and MIP during the washing-step (Claude, Nehmé, & Morin, 2011). Elution was carried out with a polar and protic organic mixture (MeOH-AcOH, 95/5 v/v). The extraction yielded low recoveries (1.0-45.2% for dopamine and 12.4-79.7% for serotonin) and poor detection of serotonin (39 of 48 samples<LOQ=150 pg/mg). This suggests for several method improvements. Trying other extraction techniques or sorbents, improving the MIPs-SPE procedure, or change the detection method are all initiatives that can be tested.

Other sorbents for SPE have been reported for the clean-up step with good recoveries of dopamine, such as C18, cation-exchange, alumina (Zhu et al., 2000), and by immunosorbents (Kim, Jeon, Paeng, & Paeng, 2008). Among these, an enzyme-linked immunosorbent assay (ELISA) has resulted in the best performance of highly selective extractions of dopamine (Kim et al., 2008). It is based on the antigen-antibody principle, retaining the analyte of interest by highly selective interactions (Bouri, Lerma-García, Salghi, Zougagh, & Ríos, 2012). However, it is expensive to produce, has very strict conditions for proper use, it can only extract a low amount of molecules, and can be used only a limited amount of times. Cation-exchange sorbents have also been reported to yield high recoveries of dopamine (Zhang, Wu, Chow, Tam, & Rios, 2016). High extraction yields and good reproducability was obtained for pre-treatment of plasma for the determination of catecholamines, using cationic exchange cartridges with carboxypropylic adsorbent (Raggi, Sabbioni, Nicoletta, Mandrioli, & Gerra, 2003). Compared to other sorbents used for SPE, it is more rapid and feasible, and testing cationic exchange cartridges

for neurotransmitters in tissue samples could be interesting. However, the specificity of the extraction is improved using MIPs, with analyte-supporting interactions depending on the spatial arrangement of functional groups, in addition to acid-base properties (Claude et al., 2014).

Recoveries between 79-96% for dopamine and serotonin have been reported using the MIPs-SPE method on serum (Claude et al., 2014). The serum was diluted in water (1/10, v/v) before extraction, followed by approximately the same extraction procedure as in this study. This suggests that the problem might be the treatment of the tissue before extraction. As mentioned, previous studies have demonstrated the dependence on changes in pH on the structure of MIPs (Turner et al., 2004). Cathecolemaines are also unstable in basic conditions. Thus, some changes to better control the pH might improve the selectivity of dopamine. The pH of the sample solution was controlled with pH paper before extraction. The scale on the pH-paper is not very precise (resolution=0.5 pH unit), and inaccurate reading of the pH paper may lead to variations in the pH of the sample solution for extraction. If the pH of the sample solution is mis-read with 0.5 pH units, resulting in a more basic solution, dopamine might be oxidized by dissolved oxygen, forming degradation products. This will reduce the recovery of extracted internal standards. More accurate pH-measurement may be used. Alternatively, by adding pure MeOH after spiking with ISTD, the solution can be evaporated until dryness. Then, dissolving the sample in water instead of buffer solution is possible. This way, the pH during the extraction only depends on the buffer used for conditioning of the column and thus is easier to control. In addition, it will reduce the ionic strength of the solution, which also have an impact on the MIPs interaction with the analytes.

Some studies have reported good results using "dilute and shoot" (Poh et al., 2019; Almeida et al., 2003). The samples are diluted in ISTD and injected directly into the LC-MS, without any sample clean-up. This method may reduce the loss of analyte and low recoveries during sample preparation. Almeida et al. (2003) tested this for the analysis of dopamine and serotonin levels in muscles and digestive glands of the brown mussel (*Perna perna*) (Almeida et al., 2003). Tissue was homogenated in mobile phase, centrifuged, and the supernatant was filtered and injected to the HPLC column. Dopamine and serotonin

was identified based on retention time and by spiking samples with standards, but chromatogram indicates several interfering peaks with similar retention times as dopamine. As there is no clean-up procedure before analysis, this method might involve more impurities, resulting in higher ion suppression. The solution may be diluted to minimize signals from interferents, but might also suppress signals of analytes below detection limits for small sample sizes, which is the case in this study. Electrochemical detector (ECD) combined with HPLC requires minimal sample preparation, and have demonstrated high selectivity and low detection limits for dopamine and serotonin (Tor-Agbidye, Yamamoto, & Bowyer, 2001). It uses an amperometric detection whose oxidative potential is optimized for catecholamines (Tareke, Bowyer, & Doerge, 2007). However, the limited sample preparation allows for more interference from unretained matrix components. This limits ECD when analyzing dopamine, as several electroactive interferents, such as uric acid and ascorbic acid, usually is present in concentrations hundred to thousand times higher than dopamine in biological tissues, and have very similar oxidation potentials (Sajid et al., 2016). Therefore, with its high sensitivity and selectivity, and unique ability to remove matrix interferents by monitoring ion transition, MS/MS detection is probably the best option for the simultaneous detection of neurotransmitters.

Testing some of these suggestions for method improvements might result in higher recoveries, and more accurate and precise analyses.

4.5 Linking *ex vivo* and *in vivo*

New tools for *ex vivo* methods in bivalves have been tested in this study. As mentioned, *ex vivo* methods are valuable for investigating specific responses, as it focuses on effects of chemicals on cell and organ levels (Gerbron et al., 2010). Thus, such methods avoids interactions and indirect effects of the whole body, and also has better reproducability, sensitivity and cost effectiveness compared to *in vivo* methods (Gerbron et al., 2010). Additionally, *ex vivo* methods make it possible to exclude influence of typical responses from environmental changes, such as temperature, air exposure/re-submersion, reproductive cycle and time of day, which also affect the level of dopamine and serotonin in the body (Almeida et al., 2003). Using explants from organs also allows for fewer test individuals,

as several samples can be obtained from one individual.

However, there are several challenges and limitations using *ex vivo* studies. Most xenoestrogenic compounds are found in low concentrations in the environment and have low physiological effects (Gerbron et al., 2010). To be able to measure their potential impacts, establishing the explant culture conditions to maintain an optimal response of the biomarker is essential, but difficult. Additionally, as *ex vivo* methods focus on cell and organ levels, they do not reflect the complexity of the endocrine system or effects of interactions between organs and their feedback loops (Gerbron et al., 2015). Tissue distribution, pharmacokinetics and biotransformation of chemicals occurring *in vivo* are often not accounted for by *ex vivo* assays.

Studies using both *ex vivo* an *in vivo* tools to assess endocrine disruption potency have demonstrated both similar and different responses. Similar estrogenic effects of Cd have been reported in fish, using both *in vivo* exposure of the whole fish and *ex vivo* exposure of liver explants (Gerbron et al., 2015). Hinfray et al. (2011) found contrasting inhibitory action of clotrimazole on 11-KT production in zebrafish (*Danio rerio*) in *ex vivo* and *in vivo* studies (Hinfray et al., 2011). This emphasizes the importance of studying endocrine potency using both *ex vivo* and *in vivo* methods, as it will provide a more comprehensive overview of the mechanistic responses. By using the *ex vivo* method in this study, it was possible to highlight potential endocrine disruption after exposure to chemicals more cost effective, with fewer test individuals and with higher reproducability than *in vivo* methods. Still, *in vivo* studies will be interesting and important to investigate if exposure to whole organisms will result in similar effects.

5 Conclusion

New tools for *ex vivo* methods in bivalves and a new analytical method for neurotransmitter measurement in bivalves have been developed during the WASTECALL flagship project, and tested in this study. Viability of explants of digestive glands from blue mussels incubated in medium culture was over 60% until incubated for 72 hours. Explant cells exposed to different concentrations of the model contaminants were similar to the control, with good viability (78-135%). The UHPLC-MS/MS analysis made it possible to identify and quantify dopamine and serotonin in all 48 samples and 9 of the 48 samples, respectively. The MIPs-SPE procedure was shown to be suitable for clean-up and pre-concentration of the samples, but yielded low recoveries, especially for dopamine, with recovery lower than 25% for 28 of 48 samples.

The levels of dopamine exposed to single *m*-xylene and BDE-99 showed no difference compared to the blank. Co-exposure of both chemicals indicated a synergistic effect for both dopamine and serotonin, and gave 1.6-fold and 2.2-fold higher dopamine levels compared to single *m*-xylene and BDE-99 exposure, respectively. Serotonin levels were 4-fold higher than single exposure to BDE-99. This is the first study to demonstrate a synergistic effect between *m*-xylene and BDE-99, and more studies are needed to understand the associated mechanism of action. Single exposure of $CdCl_2$ and estradiol-17-β altered the level of dopamine in a dose-dependent manner. Co-exposure of both $CdCl_2$ and estradiol-17-β reduced the level of dopamine, compared to single exposure, 2.2-fold and 1.8-fold, respectively. No significant difference between exposure groups and different concentrations were observed, and further investigations are needed to ascertain and elucidate the observed effects. These results suggest the need to increase the numbers of studies working on co-exposure. Additionally, the study demonstrates that analysis of dopamine and serotonin in tissues of mussels could serve as biomarkers for contamination in aquatic environments, although more studies are needed to understand the impact of the complex mixture of contaminants on marine ecosystems.

6 Recommendations for further work

Future studies should aim on optimizing the MIPs-SPE procedure, to increase the internal standard extraction recoveries. Trying other solvents to easier control the pH during extraction, or testing other sorbents for the SPE, such as cation-exchange, might be useful. Larger sample sizes are essential to be able to identify and quantify serotonin.

To be able to confirm a dose-response curve of anti-estrogenic activities, co-exposure of all concentration treatments should be carried out. In addition, extraction of the culture media could be carried out for analysis of the contaminants of interest to make sure similar exposure is occurring across all replicas. It could be interesting to carry out the same exposure of contaminants in an *in vivo* experiment, to be able to compare the observed effects. The impact of exposure of whole organisms could yield different responses in dopamine and serotonin levels than exposure of explant cells. Measuring the levels of the model contaminants used in this study in the sea and sediments in Tromsø could also be interesting, to evaluate the actual level of exposure to marine ecosystems.

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A Appendix

A.1 Standards, chemicals and materials

Table A.1: List of standards and chemicals used for the experiments.

Standards		**Producer**
$^{13}C_6$-dopamine		CHIRON AS
D_4-serotonin		CHIRON AS
Dopamine hydrochloride		Sigma Aldrich
Serotonin hydrochloride		Sigma Aldrich
Chemicals	**Purity**	**Producer**
2-Phenoxyethanol		Sigma Aldrich
Ag nanoparticles		Sigma Aldrich
Acetic acid	99-100%	VWR International
Acetonitrile	for liquid chromatography	Merck KGaA
Antibiotic		Sigma Aldrich
BDE-99		Sigma Aldrich
Cadmium chloride		Sigma Aldrich
Dimethyl sulfoxide		Sigma Aldrich
Estradiol-17-β		Sigma Aldrich
Formic acid	98-100%	Merck KGaA
Fullerene 60		Sigma Aldrich
Leibovitch L15		Sigma Aldrich
m-Xylene		Sigma Aldrich
Methanol	for liquid chromatography	Merck KGaA
Natrium hydroxid	pellets for analysis	Merck KGaA
ortho-Phosphoric acid	85%	Merck KGaA
Triton		Sigma Aldrich

Table A.2: List of materials used for the preparation and analyses of explants.

Material	**Specification**	**Producer**
Glass Pasteur Pipettes	150 mm	VWR International
Chromacol 03-FISV vials	300 µL	Thermo Scientific
Affinimip®SPE cartridges	50c-3mL	Affinisep
Centrifuge tubes	15 mL	Supelco
Solid cap	18 mm, PTFE Liner	Supelco
Homogenization vials		VWR International
Homogenization beads		VWR International
Centrifuge tubes	15 mL	VWR International
Multipipette E3	1 µL-50 mL	Eppindorf
Finnipipette F2	Various sizes	Thermo Scientific
Vacuum manifold		VWR International
Stopcocks		VWR International
Beakers	Various sizes	VWR International
Erlenmeyer flasks	Various sizes	VWR International
Spatulas	Metal	
Scalpel	Blade nr 24	
Sigma® cell culture plate	48 wells	Sigma Aldrich
Sigma® cell culture plate	96 wells	Sigma Aldrich

Table A.3: List of instruments used for the preparation and analyses of explants.

Instrument	**Specification**	**Producer**
Quaternary Accela 1250 pump		Thermo Scientific
Acquity UPLC HSS 3 T	2.1×100 mm, 1.8 µm	Waters Corporation
Van guard HSS T3	2.1×5 mm, 1.8 µm	Waters Corporation
TSQ Vantage		Thermo Scientific
Dionex^{TM} Chromeleon^{TM} 7 CDS	Version 7.10.3	Thermo Scientific
Vortex mixer MELB1719		Merck eurolab
Precellys 24 tissue homogenizer		Bertin Instruments
Jouan A14 centrifuge		Thermo Scientific
Ultrasonic cleaner USC-TDH		VWR International
Refrigerated MiVac Speed Trap		Genevac
MiVac Quattro Pump		Genevac
MiVac Centrifugal Concentrators		Genevac
BP211D Analytical Balance		Sartorius

A.2 Exposure design

	1	2	3	4	5	6
A	**Blk EtoH**	**Blk EtoH**	**Blk EtoH**	**Blk EtoH**	**Blk EtoH**	**Blk EtoH**
B	XYLC1	XYLC1	XYLC1	CdClC1	CdClC1	CdClC1
C	XYLC2	XYLC2	XYLC2	CdClC2	CdClC2	CdClC2
D	XYLC3	XYLC3	XYLC3	CdClC3	CdClC3	CdClC3
E	**Coexp C2**	**Coexp C2**	**Coexp C2**	**Coexp C2**	**Coexp C2**	**Coexp C2**
F	BDEC1	BDEC1	BDEC1	E2C1	E2C1	E2C1
G	BDEC2	BDEC2	BDEC2	E2C2	E2C2	E2C2
H	BDEC3	BDEC3	BDEC3	E2C3	E2C3	E2C3
	Plate 1 Biomarkers			**Plate 3 Biomarkers**		
	m-xylene and BDE-99			$CdCl_2$ and estradiol-17-β		

C (ng/ml)	$CdCl_2$	BDE-99	*m*-xylene	estradiol
C1	18,332	56,469	17,823	27,238
C2	183,32	564,69	178,23	272,38
C3	18332	5646,9	1782,3	2723,8

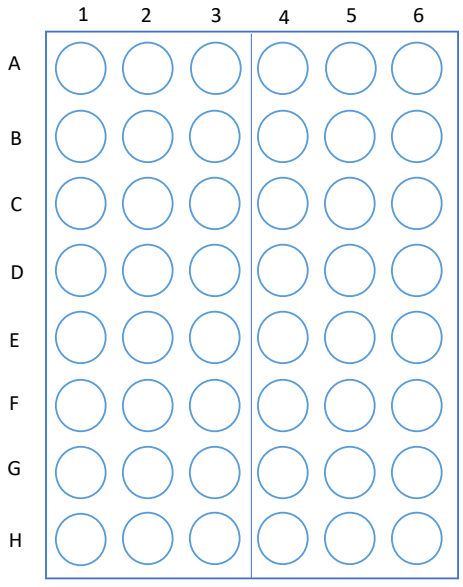

Figure A.1: Exposure design and concentration of chemicals for exposure of explants. The first table shows how explants were placed and exposed in the wells, and the second table shows the concentrations of added contaminants in three different treatments, C1, C2 and C3.

A.3 Calibration curves

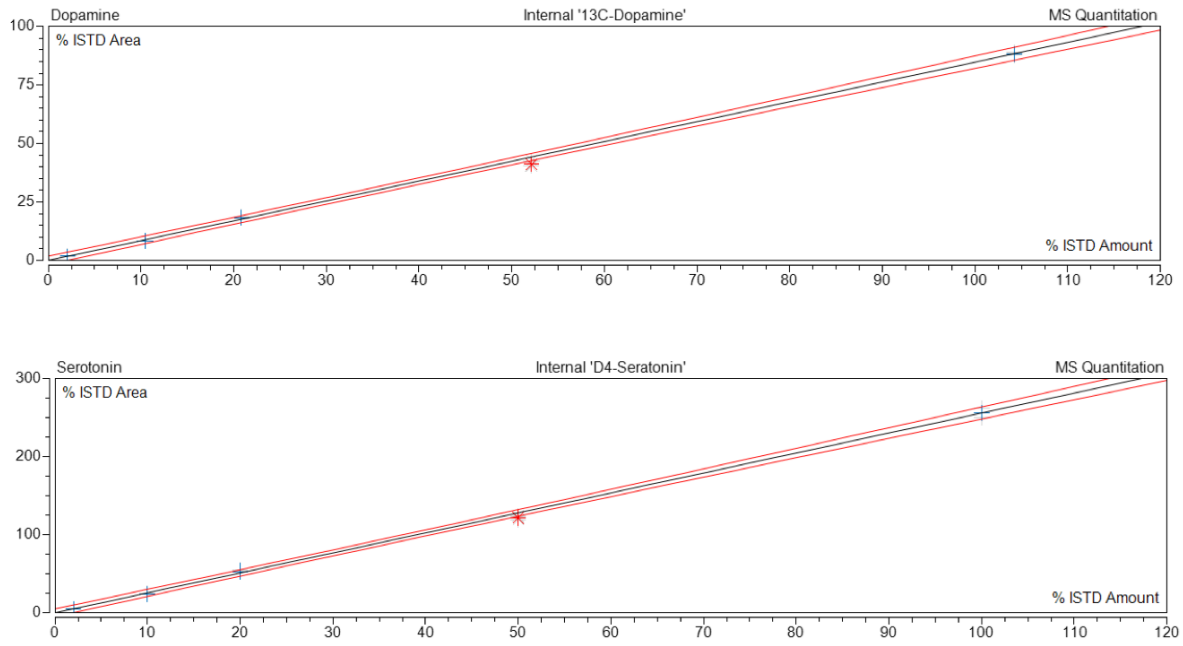

Figure A.2: Calibration curve for dopamine (top) and serotonin (bottom). Upper and lower red lines represent the 99.5% confidence interval. Each standard was run a total of 4 times over the entire sequence: Standard concentrations dopamine (pg/µL): 1.04, 5.25, 10.5, 26.25, 52.55. Standard concentrations serotonin (pg/µL): 1.05, 5.2, 10.4, 26, 52.

A.4 Viability of explants

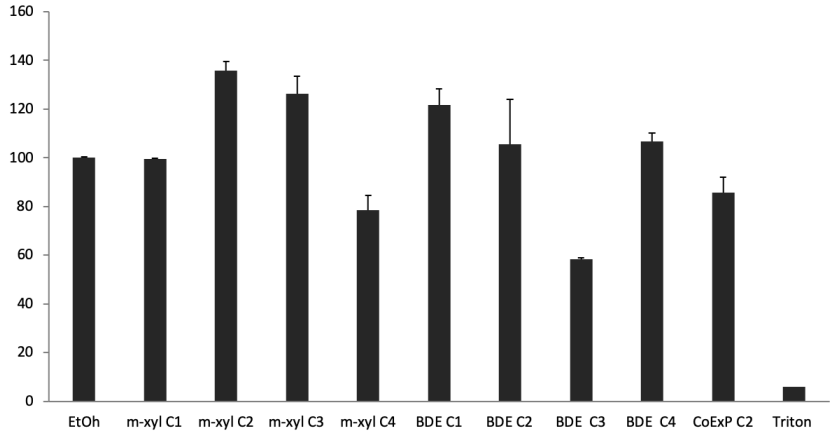

Figure A.3: Viability of the digestive gland explants exposed to different concentrations of single *m*-xylene and BDE-99 and co-exposure. Results are given as a percentage of the control FDA hydrolysis activity ±SEM.

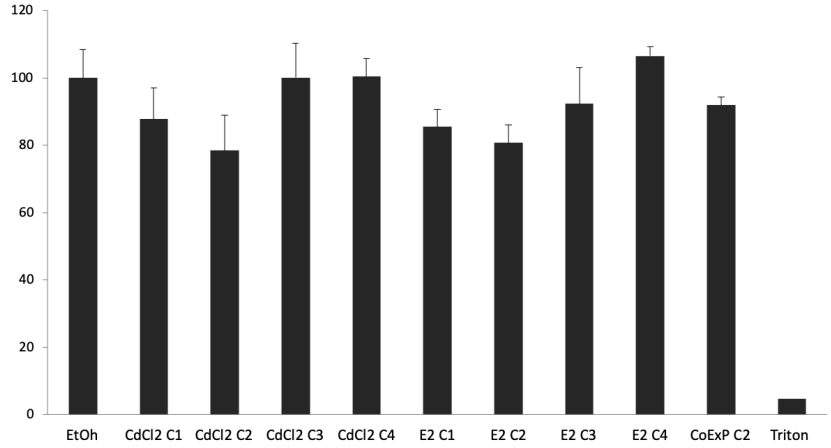

Figure A.4: Viability of the digestive gland explants exposed to different concentrations of single $CdCl_2$ and 17-β-estradiol and co-exposure. Results are given as a percentage of the control FDA hydrolysis activity ±SEM.

A.5 Level of neurotransmitters with outliers

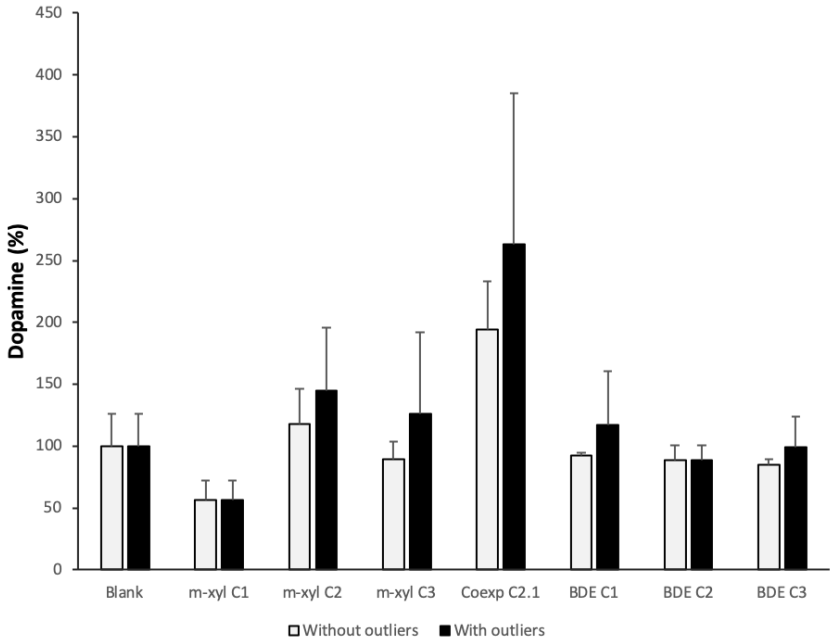

Figure A.5: Concentration of dopamine in explants exposed to both different concentrations of single *m*-xylene and BDE-99 and co-exposure. Results are given as a percentage of the mean compared to the blank ±SD. Light grey values are the mean after exclusion of outliers, while black values are the mean of all replicas.

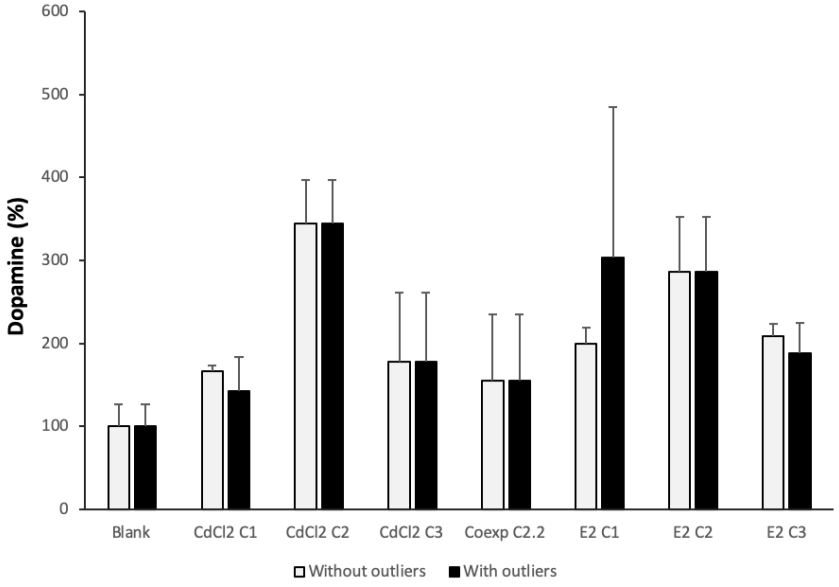

Figure A.6: Concentration of dopamine in explants exposed to both different concentrations of single $CdCl_2$ and 17-β-estradiol and co-exposure. Results are given as a percentage of the mean ±SD. Light grey values are the mean after exclusion of outliers, while black values are the mean of all replicas.

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