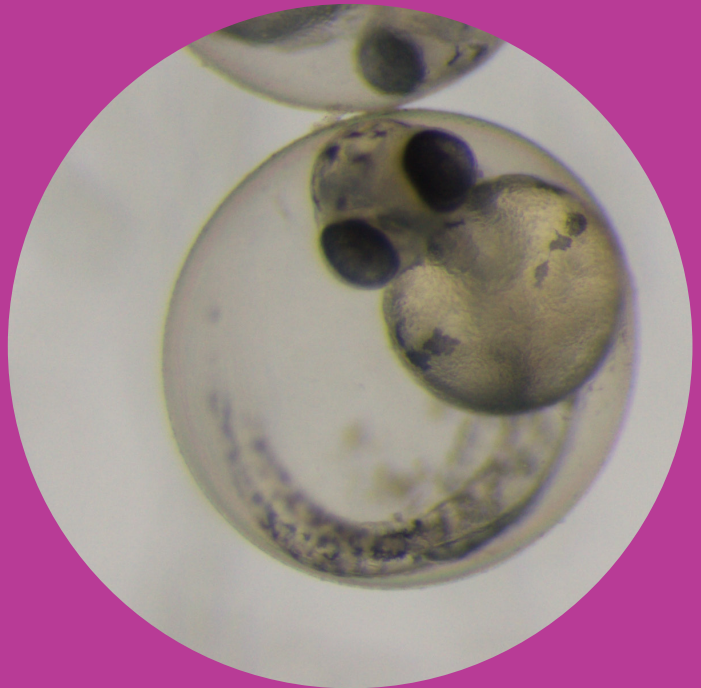


# Toxicity and emerging contaminants – Effect-based assessment of complex environmental samples

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Pia Välitalo



# Toxicity and emerging contaminants – Effect-based assessment of complex environmental samples

**Pia Väitalo**

A doctoral dissertation completed for the degree of Doctor of Science (Technology) to be defended, with the permission of the Aalto University School of Engineering, at a public examination held at the lecture hall T2 of the school on 26 July 2019 at 12:00.

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**Abstract**

At present, approximately 100 000 anthropogenic chemicals are in daily use and new chemicals are frequently developed. Some of these chemicals are released into the environment during their lifecycle. Currently, only a small fraction of chemicals that are present in the environment is monitored creating a so-called "tip of the iceberg" problem. Not all the chemicals that are present in the environment cause harmful effects and the challenge is to identify those chemicals that are harmful to human health and the environment. This problem is well demonstrated related to water pollution and water quality monitoring. For example, wastewater effluents contain a complex mixture of chemicals, including emerging contaminants such as hormones and pharmaceuticals, which can have toxic properties in the aquatic environment. Currently, these types of chemicals are not monitored.

In this thesis, a more holistic, effect-based approach for the assessment of environmental samples is applied. Effect-based tools are sum parameter based, thus unknown chemicals, transformation products and mixture effects are taken into account. The focus of this thesis is on wastewater but aquatic environments are discussed more widely as similar approaches can be applied to different sample matrices. Influent and effluent quality of eight Finnish wastewater treatment plants (WWTPs) was investigated regarding toxicity and emerging contaminants. Removal efficiency of typical Finnish WWTPs was assessed by application of effect-based tools.

These results show that the application of effect-based tools for the assessment of complex environmental samples, such as wastewaters or sediments, can provide valuable information that is not achieved with current methods for monitoring. The results indicate that bioassay batteries for water quality assessment should include a large variety of assays covering multiple endpoints and test organisms. Based on the results, estrogenic activity, thyroid disruption, and embryotoxicity are relevant endpoints related to the assessment of wastewater quality. Effluents may also cause adverse effects on fish embryos or induce genotoxic effects.

The results demonstrate that the removal of emerging contaminants and toxicity is incomplete with conventional treatment methods. More advanced treatment methods are needed to further improve water quality and control the discharge of chemicals to our environment.

Overall, application of effect-based tools together with chemical analysis will help us achieve a safer environment and higher quality of monitoring.

**Keywords** toxicity, emerging contaminants, effect-based assessment, wastewater treatment, micropollutants

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Toksisuus ja orgaaniset haitta-aineet – Monimutkaisten ympäristönäytteiden vaikutusperusteinen arviointi

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Tällä hetkellä arviolta noin 100 000 synteettistä kemikaalia on päivittäisessä käytössä ja uusia kemikaaleja kehitetään jatkuvasti. Osa näistä kemikaaleista päätyy ympäristöön niiden elinkaaren aikana. Tällä hetkellä vain pientä osaa ympäristöön päätyvistä kemikaaleista monitoroidaan, muodostaen niin kutsutun "jäätävän huippu" –ongelman. Kaikki ympäristöön päätyvät kemikaalit eivät ole vaarallisia, joten haasteena on tunnistaa ne kemikaalit, jotka ovat haitallisia ihmisten terveydelle ja ympäristölle. Vesien pilaantuminen ja vedenlaadun seuranta ovat hyvää esimerkki "jäätävän huippu" –ongelmasta. Esimerkiksi jätevedet sisältävät monimutkaisten kokteilin erilaisia orgaanisia haitta-aineita, kuten hormoneja ja lääkkeitä, jotka voivat olla myrkyllisiä vesiympäristössä. Tällä hetkellä tämän tyyppisiä haitta-aineita ei monitoroida.

Tässä työssä käytetään kokonaisvaltaisempaa, vaikutusperusteista lähestymistapaa ympäristönäytteiden arvioinnissa. Vaikutusperusteiset menetelmät huomioivat myös tuntemattomat kemikaalit, muuntumistuotteet ja seosvaikutukset. Yhdyskuntajätevedet ovat työn keskiössä, mutta vesiympäristöä käsitellään laajemmin, koska samanlaisia lähestymistapoja voidaan soveltaa erilaisiin näyttematriiseihin. Tutkimuksessa arvioitiin kahdeksan suomalaisen jätevedenpuhdistamon vedenlaadun myrkyllisyyden ja orgaanisten haitta-aineiden osalta. Tyypillisten suomalaisten jätevedenpuhdistamojen puhdistustehokkuutta arvioitiin soveltamalla vaikutusperusteisia menetelmiä.

Tutkimuksessa havaittiin, että vaikutusperusteisten menetelmien soveltaminen monimutkaisten ympäristönäytteiden, kuten jätevesien tai sedimenttien, arvioinnissa voi tarjota arvokasta lisätietoa, jota ei saavuteta nykyisillä seurantamenetelmillä. Vaikutusperusteisen menetelmän tulisi sisältää useita eri organismeihin ja toksikologisiin vaikutusmekanismeihin perustuvia biologisia testimenetelmiä. Hormonitoimintaa häiritsevät vaikutukset, genotoksisuus ja myrkylliset vaikutukset kalojen alkioihin ovat jäteveden laadun arvioinnin kannalta olennaisia arvioitavia vaikutusmekanismeja, sillä suurimmassa osassa analysoiduista näytteistä havaittiin kyseisiä vaikutuksia.

Tutkimuksessa havaittiin, että perinteiset jäteveden puhdistustekniikat eivät kokonaisuudessaan poista orgaanisia haitta-aineita tai toksisuutta. Sekä haitta-aineita että toksisuutta havaittiin puhdistetuissa jätevesinäytteissä. Paremman vedenlaadun saavuttamiseksi tarvitaan monitoroinnin lisäksi kehittyneempiä puhdistusmenetelmiä. Kaiken kaikkiaan vaikutusperusteisten työkalujen käyttö kemiallisten analyysien tukena voi auttaa meitä saavuttamaan tehokkaammat ja kokonaisvaltaisemmat työkalut monitorointiin ja ympäristön kemikalisoitumiseen liittyviin ongelmiin.

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Espoo, July 2019

*Pia*

# Contents

<b>1</b>	<b>Introduction</b>	<b>8</b>
1.1	Chemicals in the environment	8
1.2	Water pollution and wastewater treatment	9
1.3	Water quality monitoring	11
1.3.1	Emerging contaminants	12
1.3.2	Toxicity and effect-based methods	13
<b>2</b>	<b>Objectives and scope of this thesis</b>	<b>16</b>
<b>3</b>	<b>Materials and methods</b>	<b>18</b>
3.1	Selection of sampling sites	18
3.2	Sample processing	19
3.3	Bioassays	20
3.4	Chemical analysis	22
<b>4</b>	<b>Main findings and discussion</b>	<b>23</b>
4.1	Tools for the assessment of complex environmental samples	23
4.1.1	Selection of the approach	23
4.1.2	Bioassays – responsiveness and applicability	26
4.1.3	Chemical analysis – challenges and analytical choices	28
4.2	Influent and effluent quality of eight Finnish WWTs	29
4.2.1	Wastewater toxicity	29
4.2.2	Detection of emerging contaminants	31
4.3	Removal of toxicity during wastewater treatment	33
4.4	Practical implications and future needs	35
<b>5</b>	<b>Contribution to the fundamental questions</b>	<b>37</b>

# List of publications

This doctoral dissertation consists of a summary and of the following papers which are referred to in the text by their roman numerals.

- I.** **Välitalo, P.**, Perkola, N., Seiler, T. B., Sillanpää, M., Kuckelkorn, J., Mikola, A., Hollert, H., & Schultz, E. (2016). Estrogenic activity in Finnish municipal wastewater effluents. *Water Research*, *88*, 740-749.
- II.** Dahl, M., Survo, S., **Välitalo, P.**, Kabiersch, G., Alitalo, O.S., Penttinen, O.P., Rantalainen, A.L. (2019). Identification of toxicants from a highly C10–C40 contaminated sediment influenced by the wood industry: Petroleum hydrocarbons or biogenic organic compounds? *Environmental Toxicology and Chemistry*, *9999*, 1-11.
- III.** **Välitalo, P.**, Massei, R., Heiskanen, I., Behnisch, P., Brack, W., Tindall, A. J., Pasquier, D., Küster, E., Mikola, A., Schulze, T., & Sillanpää, M. (2017). Effect-based assessment of toxicity removal during wastewater treatment. *Water Research*, *126*, 153-163.
- IV.** **Välitalo, P.**, Kruglova, A., Mikola, A., & Vahala, R. (2017). Toxicological impacts of antibiotics on aquatic micro-organisms: a mini-review. *International Journal of Hygiene and Environmental Health*, *220*(3), 558-569.

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# Author's contribution

- I.** Pia Välitälo took part in planning of the study together with the E. Schultz and M. Sillanpää. P. Välitälo took part in the sampling, sample treatment, laboratory analyses and data analyses. She was responsible for writing the manuscript and is the corresponding author. N. Perkola took part in the chemical analysis of the samples and J. Kuckelkorn provided training for the ER-CALUX testing. All of the authors provided comments for the manuscript.
- II.** Pia Välitälo took part in planning of the genotoxicity testing. She took part in the laboratory analyses and data analyses of the genotoxicity testing. She took part in writing the manuscript. M. Dahl had the main responsibility for planning the study, performing the experiments and writing the manuscript. M. Dahl is the corresponding author.
- III.** Pia Välitälo took part in planning of the study together with the M. Sillanpää and T. Schulze. P. Välitälo performed the sampling, sample treatment, most of the laboratory analyses and data analyses. She was responsible for writing the manuscript and is the corresponding author. R. Massei provided training for the fish embryo testing together with E. Küster. I. Heiskanen assisted in the umuC testing and NRR retention testing. A. Tindall and D. Pasquier took part in planning and performing the REACTIV and XETA assays. All of the authors provided comments for the manuscript.
- IV.** Pia Välitälo planned the scope of the mini-review together with A. Kruglova. Pia Välitälo had the main responsibility for writing the manuscript and is the corresponding author. A. Kruglova reviewed the data on antibiotics of high concern and on bacteria involved in wastewater treatment process, wrote the relevant chapters of the review. P. Välitälo reviewed all of the toxicological data and wrote the relevant chapters. All of the authors provided comments for the manuscript.

# List of abbreviations

BAT	Best Available Technique
BPA	Bisphenol-A
CALUX	Chemical-Activated Luciferase Gene Expression
E1	Estrone
E2	17 $\beta$ -estradiol
E3	Estriol
EBT	Effect-based trigger value
EDA	Effect-Directed Analysis
EDCs	Endocrine disrupting compounds
EE2	17 $\alpha$ -ethinylestradiol
EEA	European Environment Agency
ELISA	Enzyme-linked Immunosorbent Assay
ESI	Electrospray Ionization Source
FET	Fish Embryo Toxicity Test
GC-FID	Gas Chromatography – Flame Ionization Detector
GC-MS	Gas Chromatography–Mass Spectrometry
GC-TOF-MS	Gas Chromatography coupled to Time-of-Flight Mass Spectrometry
LC-HRMS	Liquid chromatography–High Resolution Mass Spectrometry
LC-MS/MS	Liquid chromatography coupled to Tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
LVSPE <sub>50</sub>	Automated Large Volume Solid Phase Extraction Device

MBR	Membrane Bioreactor
NRR	Neutral Red Retention assay
REACH	Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals
REF	Relative Enrichment Factor
REP	Relative Estrogenic Potency
PAHs	Polycyclic Aromatic Hydrocarbons
SPE	Solid-phase Extraction
SRT	Sludge Retention Time
T <sub>3</sub>	Triiodothyronine
TIE	Toxicity Identification Evaluation
WFD	Water Framework Directive
WHO	The World Health Organization
WWTP	Wastewater Treatment Plant

# 1 Introduction

## 1.1 Chemicals in the environment

Chemicals are everywhere. Our environment is comprised of chemicals, the air we breathe is a mixture of chemicals, and even we are made of a complex system of chemicals. Chemicals are the vital building blocks of our daily lives and our society depends on them. Nevertheless, some manmade or natural chemicals can be harmful to our environment and health. This is usually the case, when chemicals end up in the wrong place at the wrong time and at the wrong concentration. A classic example of this is the Indian vulture crisis, where the vulture population experienced a rapid population decline due to exposure to a common anti-inflammatory drug diclofenac, which turned out to be lethal to vultures (Green et al., 2004).

The first pharmaceutical residues in the environment were detected in the 1970s due to the establishment of sensitive and selective analytical methods (Ternes et al., 2001). Since then, thousands of studies have been published on the presence of harmful substances in the environment. At present, approximately 100 000 anthropogenic chemicals are in daily use and new chemicals are frequently developed. During manufacturing, transportation, consumption and disposal these chemicals can be released into the environment. Only a miniscule fraction of all the chemicals present in the environment is monitored or regulated based on the assumption that the selected chemicals pose the highest risk to the ecosystem or human health. However, our knowledge is very limited as a comprehensive assessment of the complete chemical universe is merely impossible creating a so-called “tip of the iceberg” problem. Not all the chemicals that are present in the environment cause harmful effects and the challenge is to identify potentially hazardous substances from complex environmental samples and to recognize what kind of effects they have on human health and the environment. The fundamental questions are:

- What are the sources and pathways of harmful chemicals to the environment?
- What kind of effects harmful chemicals have on the environment and human health?
- How can the discharge or transfer of harmful chemicals be avoided or controlled?

- How do we ensure that pollutants are present in the environment at a safe level?
- How can the safe levels of chemicals be monitored?

Scientists from different disciplines, such as ecotoxicology, chemistry and engineering, try to answer these questions by identifying pollutants, determining concentrations, developing biological tests and discovering toxicological pathways. However, these fundamental questions are difficult to answer, because the subject is indescribably complex. We do not know the identity or characteristics of all the chemicals in our environment, thus we do not have information on what kind of harmful effects they may pose. We monitor, but we are not sure if we are monitoring enough chemicals or all of the relevant chemicals. In addition, chemicals can transform and form complex chemical cocktails. As science evolves, new methods are developed, and more knowledge is gained, we get closer to finding the answers to the fundamental questions.

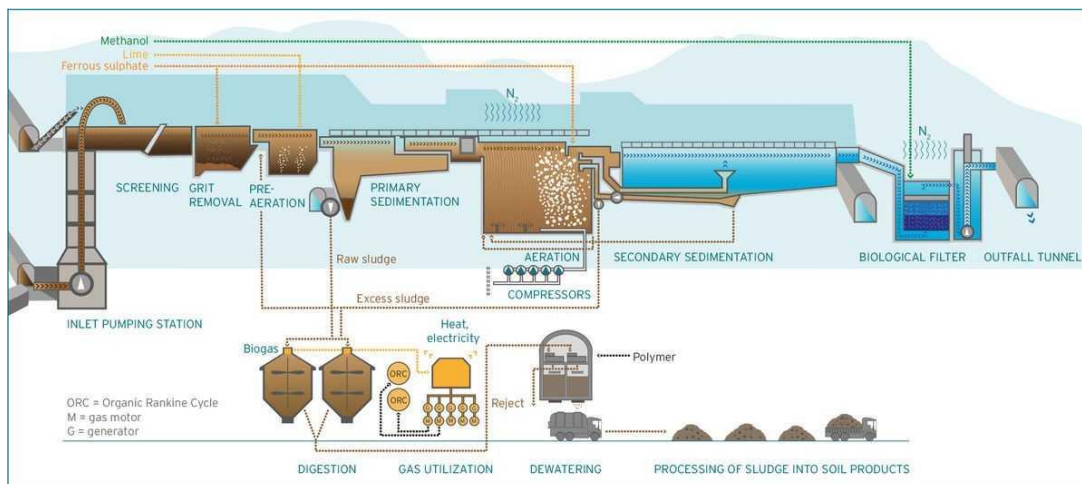
## 1.2 Water pollution and wastewater treatment

Some of the fundamental questions scientists try to answer are: What are the sources and pathways of harmful chemicals to the environment? How can the discharge or transport of harmful chemicals be avoided or controlled? The chemical cocktail in our environment is the result of air, soil, land and water pollution. Water pollution is one of the most serious ecological threats we face today. Harmful pollutants can be discharged or leaked into water bodies through various pathways and sources, such as industry, households, agriculture, transportation, atmospheric deposition and waste management. Once a water source is polluted, it is difficult or even impossible to remove the contaminants and some contaminants may persist long after harmful discharges have stopped.

In Europe, around 40 % of surface waters are in good ecological status and 33 % are in good chemical status according an assessment carried out by the European Environment Agency (EEA Report, No. 7/2018). EEA estimates that the main reasons for failure to achieve good chemical status are atmospheric deposition and discharges from urban waste water treatment plants. The priority substances causing failure to achieve good chemical status are metals, flame retardants, polycyclic aromatic hydrocarbons (PAHs), pesticides, surfactants and plasticizers.

Currently, it is well known that wastewater effluent is one of the primary pathways of organic pollutants to the aquatic environment (Luo et al., 2014; Petrie et al., 2015; Tran et al., 2018). Wastewaters contain a complex mixture of household chemicals, pharmaceuticals, hormones, personal care products, surfactants and other regulated and non-regulated compounds. Some efforts have been made to control chemical discharges via wastewater effluents by developing advanced treatment methods and operational conditions at the wastewater treatment plants (WTTs). However, many of these are not globally applied in practice as only a few countries such as Switzerland or Germany have upgraded their treatment processes on a larger scale.

Even though, the techniques for wastewater treatment have developed over the last decades, most treatment plants rely on conventional treatment processes. Typically, conventional wastewater treatment plants consist of mechanical and biological treatment often coupled with additional chemical treatment (Figure 1). Initially these processes were designed for the removal of suspended solids, pathogens and nutrients, with no particular consideration of organic micropollutants such as hormones, pharmaceuticals or surfactants. Elimination mechanisms in conventional wastewater treatment can be physical, chemical, and/or biological. Typically, sorption, biodegradation, photodegradation and phytoremediation are the most crucial mechanisms for the removal of organic pollutants (Garcia-Rodríguez et al., 2014). Some of these mechanisms are relatively effective in removing certain contaminants, such as ibuprofen, ketoprofen and caffeine (Jelic et al., 2011; Luo et al., 2014; Tran et al., 2018). However, it is well known that the removal of various micropollutants is incomplete during the conventional treatment process; due to which pollutants are regularly detected in WWTP effluent samples globally (Deblonde et al., 2011; Loos et al., 2013; Petrie et al., 2015; Tran et al., 2018).



**Figure 1.** One example of a wastewater treatment process (WWTP 5 in Paper I), combining mechanical, biological and chemical treatment steps. (Figure from HSY)

In general, additional tertiary treatment steps vary from the more robust processes such as sand filtration to a combination of multiple steps such as activated carbon treatment and ozonation, which have proven to be efficient in removing some specific emerging contaminants (Esplugas et al., 2007; Ahmed et al., 2017; Guillosoy et al., 2019). However, the downside is that these upgrades can be costly and require high levels of energy and chemical consumption. In addition, the removal efficiency is typically calculated based on a limited amount of chemicals, circling back to the initial “tip of the iceberg” problem.

The scientific evidence is strong that the discharge of WWTP effluents can lead to adverse effects in the receiving aquatic environments. A well-known example

is the development of intersex fish in receiving aquatic ecosystems leading to population declines (Jobling et al., 2002; Bjerregaard et al., 2006). In addition, elevated levels of plasma vitellogenin concentrations and the presence on ovo-testis have been observed in male flounder in several United Kingdom estuaries (Allen et al., 1999; Kirby et al., 2004).

The effects of municipal and industrial wastewater discharges have been investigated also in Finland to some extent. Municipal or industrial effluents are mainly discharged to the Baltic Sea or lakes. The large paper and pulp industry has had a significant effect on the receiving waterbodies, thus research on wastewater toxicity in Finland has focused on the industrial wastewaters with only a few studies that assess municipal wastewater discharges. Karels and Niemi (2002) investigated the effects of pulp and paper mill effluents to fish community responses at Lake Saimaa and they demonstrated that the community structure and abundance may be affected by nutrient loading and the toxicity of paper mill effluents. Turja et al., (2015) demonstrated that exposure to treated WWTP effluent induced pro-oxidant, genotoxic and lysosomal effects in mussels in the Baltic Sea. Pessala et al., (2004) detected genotoxic and estrogenic effects in Finnish municipal and industrial wastewater effluents. Based on these previous investigations, WWTP effluents in Finland may be toxic, thus further research is needed.

### 1.3 Water quality monitoring

It has been known for a long time that certain chemicals are hazardous to human health and the environment and that these chemicals should be monitored. In order to control the discharge of chemicals to the environment and to assure safe levels of exposure, monitoring approaches and water quality parameters are set in legislation. Quality standards are placed to answer the question of “How do we ensure that pollutants are present in the environment at a safe level?” The level of exposure or chemical burden is considered safe if the given limit values or quality standards are met.

To answer the question of “How can the safe levels of chemicals be monitored?”, the primary solution has been to create different lists of selected hazardous chemicals in order to conceptualize monitoring. Some examples of these lists are restricted and authorized chemicals (REACH Regulation, 1907/2006/EC) and priority pollutants (EU Water Framework Directive (WFD), 2000/60/EC). The WFD sets out the primary strategies against water pollution, including the list of priority substances, which consists of substances that are known to present significant risks to the aquatic environment. The list has 33 priority substances or compound groups and eight other pollutants for which environmental quality standards were set. These 33 substances were selected amongst those presenting a significant risk to or via the aquatic environment. Substances are prioritized for action based on risk assessment procedures, which take into account the intrinsic hazard of the substance, level of environmental contamination, production volumes and use patterns.

Nevertheless, previous studies on chemical mixtures have demonstrated that the current regulatory single-chemical threshold values may not serve as the best option for the protection against complex mixture exposure (Carvalho et al., 2014; Altenburger et al., 2018). Carvalho et al. (2014) demonstrated that there is a need for precautionary actions on the assessment of chemical mixtures even if individual toxicants are detected below single-chemical threshold values. Several studies have shown that the observed bioactivity of environmental water samples can only partly be explained by the analyzed pollutants (Maletz et al., 2013; Escher et al., 2013; König et al., 2017) indicating that unknown chemicals, transformation products and mixture effects play a significant role. Multiple case studies of extracted surface water samples (Reineke et al., 2002; Zhao et al., 2011; König et al., 2017, Neale et al., 2017; Hashmi et al., 2018; Rosenmai et al., 2018) or wastewater samples (Smital et al., 2011; Fang et al., 2012; Maletz et al., 2013; Leusch et al., 2014; Itzel et al., 2018) using combinations of chemical and biological analysis have demonstrated that bioassays can provide valuable additional information for water quality monitoring. In addition, the use of effect-based methods was recently recommend for a review of the regulatory framework of the WFD (Brack et al., 2017).

### **1.3.1 Emerging contaminants**

Not all chemicals of concerns are regulated, such as the group of emerging contaminants. Typically, emerging contaminants are commonly detected in environmental samples, but at relatively low concentrations. The group of emerging contaminants comprises of a mixed array of organic pollutants, such as surfactants, pesticides, disinfection by-products, endocrine disruptors, pharmaceuticals and personal care products, of which endocrine disruptors and pharmaceuticals are described in more detail in this Chapter.

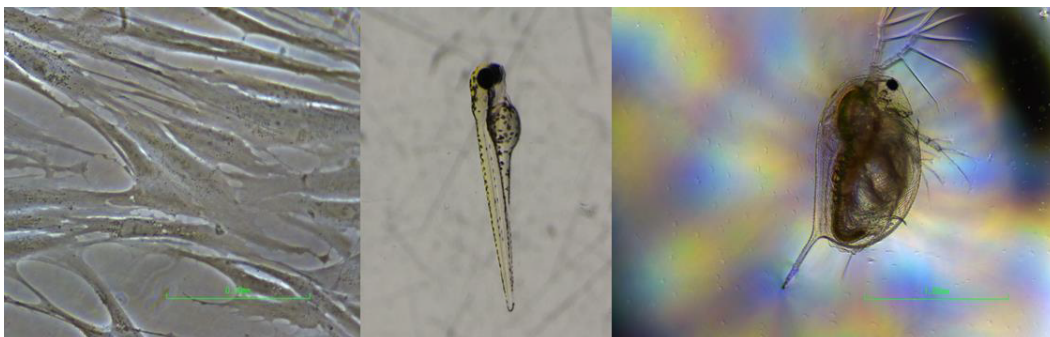
Endocrine disrupting compounds (EDCs) are chemicals that can interfere with the endocrine system. The World Health Organization (WHO, IPCS 2002) defines an endocrine disruptor as “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations.” It is crucial to understand that hormones act at very low concentrations within the endocrine system and the whole system is based on subtle changes in excretion. Thus, EDCs have the ability to be active already at low concentrations. Such concentrations have been detected globally in environmental samples. Close to 800 chemicals have been identified or suspected to be capable of interfering with the endocrine system (WHO, 2012). These include natural hormones and man-made chemicals such as phthalates, parabens and bisphenols (Giulivo et al., 2016). For example, bisphenol A has been linked to the proliferation of breast cancer cells, oxidative stress and development of prostate cancer (Wetherill et al., 2007; Tarapore et al., 2014; Di Donato et al., 2017).

A number of pharmaceuticals and personal care products contain chemicals of emerging concern due to their potential hazardous effects to the environment. The most common pharmaceuticals include anti-inflammatory drugs, antibiotics,

antiepileptic drugs, cardiovascular pharmaceuticals, phyco-stimulants and estrogens and other hormonal compounds (Li, 2014). Antibiotics were introduced in the late 1930s and ever since, their development, production and use have increased. Today, antibiotics are found ubiquitously, and they are considered “pseudo-persistent” due to their continuous release to the environment. Antibiotics and other pharmaceuticals play a crucial role in the management of diseases; however, their bioactive properties raise concerns on their toxicity to non-target organisms. Wide dissemination of antibiotics at low concentrations mainly in the aquatic environment is evident. Concerning levels of antibiotics have been detected in wastewater influents and effluents, surface waters, seawater, groundwater and even drinking water (Santos et al., 2010; Li 2014; Wang et al., 2016).

### 1.3.2 Toxicity and effect-based methods

In addition to chemical composition, water quality can be assessed from the perspective of toxicity. If so, water quality is determined by an effect-based approach, such as bioassays, biomarkers or ecological methods (Figure 2). With this kind of approach, the fundamental question to be answered is what kind of effects harmful chemicals have on the environment and human health?



**Figure 2.** Examples of test organisms (cells, fish embryos and water fleas) applied for effect-based approaches.

Bioassays *in vitro* measure effects at the subcellular level, such as receptor activation or DNA damage (Wernersson et al., 2015). *In vitro* bioassays can be applied to many different matrixes, such as passive samplers, concentrated extracts, effluents or biological tissues. In addition, typically only small sample volumes are required and the exposure time is relatively short compared to *in vivo* or *in situ* tests. In most cases, *in vitro* assays are considered sensitive with high responsiveness, as effects can be detected at low levels. Also the analytical costs are relatively low, especially considering the number of pollutants they respond to. The greatest limitation or disadvantage related to *in vitro* assays, is that the studied systems are highly simplified. Thus, creating the linkage to higher and more complex levels of the biological organization may be challenging.

*In vivo* bioassays are based on whole living organisms, which are exposed to environmental samples or concentrated extracts. The assays can be short-term or long-term tests. The analysed effects are typically not as specific as in the *in vitro* assays. Some examples of such effects are mortality, embryo development, effects on growth, physiological changes, immobilization or bioluminescence. *In vivo* assays respond to a variety of pollutants and different types of toxicity depending on the method and test organism, thus the designed test battery is recommended to consist of species from different trophic levels and taxonomic grounds (Wernersson et al., 2015).

Although effect-based approaches can provide us with vital information on effects, such methods are not adopted in water quality monitoring to their full potential. So far, the established techniques for the biological water quality monitoring rely on phytoplankton, macrophytes, benthic invertebrate, phytobenthos, and fish (EU Water Framework Directive, 2000/60/EC). Recently some suggestions have been made to include batteries of bioanalytical assays that enable comprehensive assessment of impact of mixture effects on water quality and to combine the test batteries with chemical analytical tools to further develop causal links between effects and the chemicals responsible for the observed mixture effects (Altenburger et al., 2015; Brack et al., 2017). One example of such an approach is Effect-Directed Analysis (EDA).

EDA is based on a combination of bioassays, fractionation procedures and chemical analysis (Brack, 2003). These methods are applied to identify hazardous compounds in complex environmental mixtures, such as wastewaters, sediments or surface waters (Brack et al., 2008). The basic steps of an EDA process according to Wernersson et al. (2015) are: 1) testing samples or extracts with the selected bioassays. 2) If effects are detected, the sample is fractionated according to physico-chemical properties of the suspected chemical components. 3) The different fractions are tested with the same bioassays. The mixture may require multiple fractionation steps to further reduce complexity. 4) The key toxicants are identified and quantified by chemical analysis in active fractions. 5) Finally, the contribution of the identified key toxicants to the observed effects are estimated.

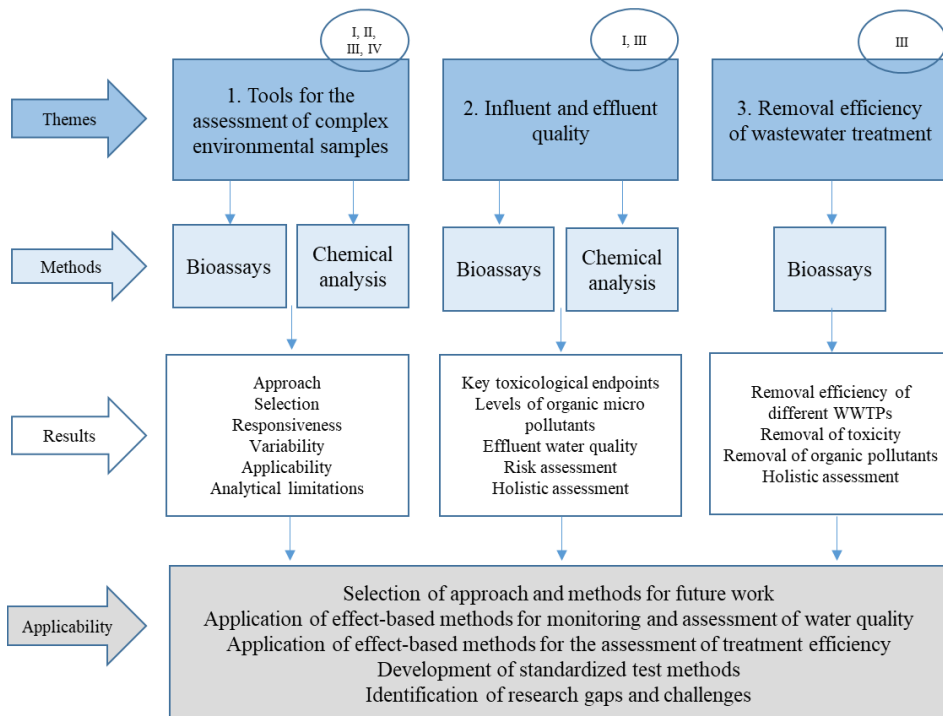
Several other effect-based approaches exist, such as Toxicity Identification Evaluation (TIE), metabolomics, proteomics and next-generation sequencing methods (Prasse et al., 2015). The selected approach depends on the study questions, sample type and available resources. Regardless of the selected approach, it is a challenge to link the effects observed in laboratory tests to actual risks. For example, the estimation of risks posed by wastewater effluent to the receiving waters is challenging due to factors such as flowrate and dilution, which affect the actual risk. In the receiving waters effluents are diluted, but the discharge is constant. Different methods have been developed to assess bioassay results in a risk context. Effect-based trigger (EBT) values were developed to assess whether the detected effects in bioassay are at an acceptable level in terms of risks (Jarošová et al., 2014; Van der Oost et al., 2017; Leusch et al., 2017; Escher et al., 2018). The limit of detection in a highly sensitive *in vitro* bioassay is not directly connected to the adversity of effects at a higher level of organisms or in the receiving environments. Thus, especially for monitoring purposes threshold values for bioassay

results indicating poor water quality are needed. Bioassays together with EBT values have the potential to be applied to assess water quality in monitoring purposes. However, lack of data, standardization of methods and demands for bioassay data quality are still limiting the use of EBT values in a harmonized manner.

## 2 Objectives and scope of this thesis

The purpose of this thesis was to provide new information to help us answer some of the fundamental questions listed in the first chapter of this thesis. These questions are answered from the perspective of effect-based tools for the assessment of complex environmental samples, wastewater quality and assessment of removal efficiency at typical Finnish WWTPs. The driving force behind the whole study was to try to evolve the current list-based "tip of the iceberg" approach toward a more holistic approach that would consider effects and the complexity of the samples. The focus of this thesis is on wastewater but aquatic environments are discussed more widely related to the tools and approaches as they can be applied to different complex sample matrices.

The thesis concentrates on the following themes presented in Figure 3.



**Figure 3.** The main themes addressed in this thesis. Roman numerals refer to the publications which are related to each theme.

### **Theme 1: Tools for the assessment of complex environmental samples**

Both biological methods and chemical analysis were applied in this thesis for the assessment of complex environmental samples, such as influent, effluent, sediment and surface waters. Depending on the selected approach, these methods were applied on their own or combined together. Specific research questions related to this theme were:

- How to select the approach and what drawbacks and advantages are related to different methods?
- How responsive or sensitive are the selected methods?
- How applicable are the selected methods and the approach overall for the selected samples and the research objectives?

These questions are strongly related to all of the studies presented in this thesis (Papers I-IV). Several complex matrices are discussed, as Papers I and III focus on wastewater influent and effluent, Paper II on contaminated sediment and Paper IV on aquatic environments in general.

### **Theme 2: Influent and effluent quality**

Quality of municipal WWTP influent (Paper III) and effluent (Papers I, III) were assessed in this thesis regarding toxicity and pollutants. Specific research questions related to this theme were:

- What are the key toxicological endpoints related to influent and effluent quality?
- Which emerging contaminants are detected at concerning levels?
- What does a holistic assessment of influent and effluent samples reveal about the current state of effluent quality at Finnish WWTPs?

### **Theme 3: Removal efficiency of wastewater treatment**

Removal efficiency of typical Finnish WWTPs was assessed by application of effect-based tools (Paper III). Specific research questions related to this theme were:

- What is the removal efficiency of different WWTPs related to toxicity?
- Can we see reoccurring trends based on the holistic approach applied in this thesis?

## 3 Materials and methods

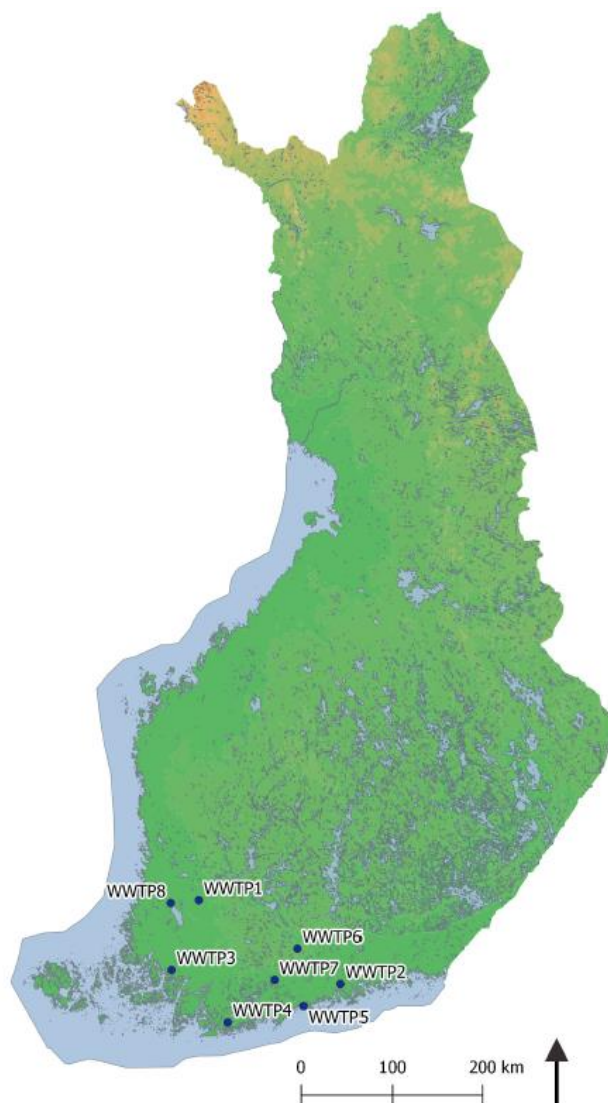
The materials and methods applied in this thesis are briefly summarized here and described more in detail in the Papers.

### 3.1 Selection of sampling sites

In the wastewater studies (Papers I, III), the selection of typical full-scale wastewater treatment plants was crucial for the holistic approach of this thesis. The aim was to select treatment plants that represent typical wastewater treatment plants in Finland, with the most commonly applied treatment processes. The idea was that the selected WWTPs form a representative sampling of WWTPs in Finland. The selected treatment plants vary from small, localized WWTPs to the largest wastewater treatment plant in the country. In addition, the treatment plants vary in the share of industrial loading, tertiary treatment configurations, operational conditions and receiving water bodies. Altogether eight WWTPs were sampled, of which seven were included in all of the studies. More detailed information on the WWTPs are presented in the Papers I and III.

In the sediment study (Paper II), two near-surface samples of sediment samples were taken from the log pond of a plywood mill in south Finland. The sediment of the pond was known to be contaminated with hydrocarbons, but it was unclear whether they originated from the hydraulic fluid of the log hoist or the wood extractives. Thus, the sediment from the log pond was ideal for an EDA study.

The selected WWTPs and their locations are presented in Figure 4. The log pond sediments were collected at a site located in Southern Finland (exact location is confidential).

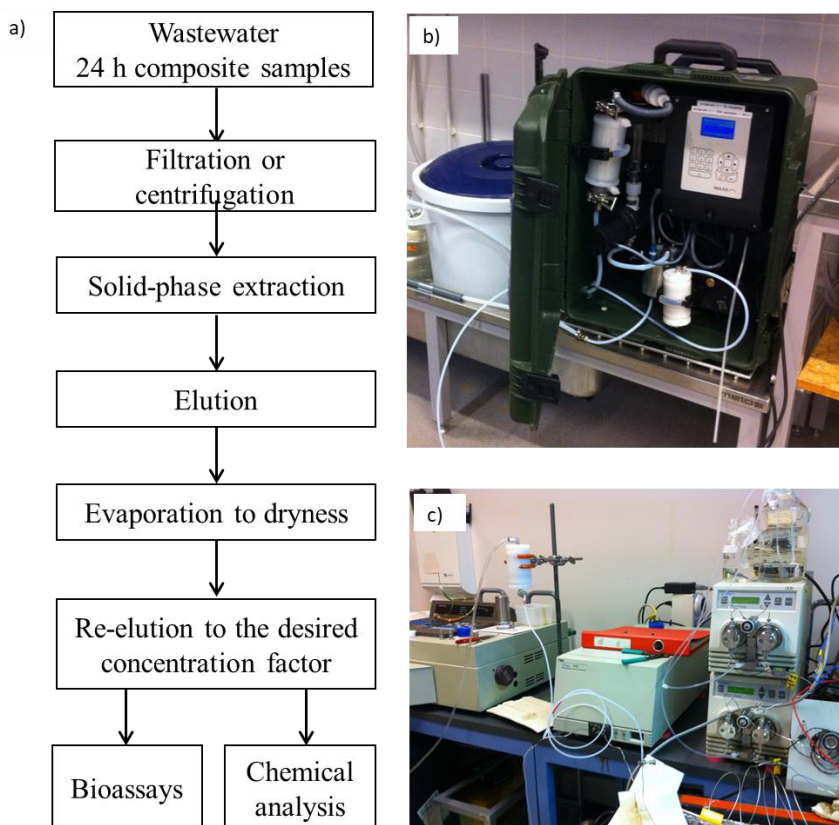


**Figure 4.** Locations of the WWTPs sampled in this thesis. (Figure: The National Land Survey of Finland Topographic Database. CC 4.0 licence. General map 1:1 M. Downloaded 26.3.2019. The material has been edited.)

### 3.2 Sample processing

Regarding the wastewater studies (Papers I and III), typically, samples need to be pre-concentrated in some way to detect observable effects with the bioassays or avoid problems with the sensitive, chemical analytical devices. I employed two different approaches, both based on solid-phase extraction (SPE) for sample treatment in this thesis. In the first approach (Paper I), the extraction process was optimized for estrogenic compounds and sample-specific recoveries were applied

in order to achieve results that are accurate and more reliable. I prioritized high level of accuracy as the detected concentration were used to calculate the contribution of the analysed chemicals to the observed estrogenic activity. The second approach (Paper III) aimed at quantity over quality. The overall goal of the sampling approach was to achieve large sampling volumes, select an extraction material that is as broad as possible in order to capture the highest possible cascade of pollutants and still achieve a reasonable level of accuracy. Regardless of the SPE protocol, the principles of the sample treatment were similar (Figure 5).



**Figure 5.** a) The key steps of sample treatment process applied in this thesis. The automated large volume solid phase extraction device (LVSP50) applied in Paper III (Figure 5b) and the elution step following the solid-phase extraction (Figure 5c).

In the sediment study (Paper II), samples were extracted by Soxhlet extraction and fractionated according to Grote et al. (2005) with minor modifications. Compounds were separated into five fractions using open-column chromatography with solvents of increasing polarity.

### 3.3 Bioassays

The bioanalytical tools applied in this thesis include *in vitro* and *in vivo* assays.

In the wastewater studies (Papers I, III), I selected the following endpoints to be included in the test batteries: cytotoxicity, androgenic and estrogenic activity, thyroid disruption, genotoxicity, embryotoxicity, acute toxicity and long-term toxicity (Table 1). The selected test organism form a diverse group consisting of genetically modified cells, bacteria, biomolecules, transgenic eleuthero-embryos, fish embryos and aquatic invertebrates. The test battery contained standardized test systems and bioassays that have been recently developed. For example, the ELISA-E2 immunosorbent assay and the transgenic eleuthero-embryonic models were novel approaches for wastewater testing.

The toxicity of the sediment samples (Paper II) was assessed using a biotest battery that included two in vivo acute toxicity tests, an estrogenicity test, and a genotoxicity test. The applied bioassays are summarized in Table 1.

**Table 1.** The bioanalytical tools applied in this thesis for influent and effluent samples.

Bioassay	Type	Toxicological endpoint	Influent samples	Effluent samples	Sediment	Reference	Paper
NRR-retention test (RTL-W1 cells)	<i>In vitro</i>	Acute cytotoxicity	x	x		Lee et al., 1993	III
AR-CALUX®	<i>In vitro</i>	Androgenic activity	x	x		Van der Linden et al., 2008	III
ER-CALUX®	<i>In vitro</i>	Estrogenic activity	x	x		Van der Linden et al., 2008	I, III
ELISA-E2 immunosorbent assay	<i>In vitro</i>	Estrogenic activity		x		no reference available	I
YES assay	<i>In vitro</i>	Estrogenic activity			x	Leskinen et al., 2005	II
p53-CALUX®	<i>In vitro</i>	Genotoxicity	x	x		Van der Linden et al., 2014	III
umuC assay	<i>In vitro</i>	Genotoxicity	x	x	x	ISO 13829 (2000)	II, III
<i>V.fischeri</i> inhibition assay	<i>In vivo</i>	Acute toxicity		x		ISO 11348-3, DIN 38412	I
<i>Aliivibrio fischeri</i> toxicity test	<i>In vivo</i>	Acute toxicity			x	BioTox™ Kit	II
Zebrafish embryo toxicity	<i>In vivo</i>	Embryotoxicity (survival, sublethal effects)		x		OECD TG 236 (2013)	III
Rapid estrogen activity in vivo (REACTIV) medaka assay	<i>In vivo</i>	Estrogenic activity		x		Spirhanzlova et al., 2016	III
<i>Xenopus</i> embryonic thyroid assay (XETA)	<i>In vivo</i>	Thyroid disruption		x		Fini et al., 2007	III
<i>D.magna</i> acute toxicity	<i>In vivo</i>	Acute toxicity		x	x	ISO 6341:2012, OECD Test 202 (2004)	I,II
<i>D.magna</i> long term toxicity	<i>In vivo</i>	Long term toxicity		x		ISO 1706:2000	I

In addition to the wastewater analysis, the available bioanalytical methods and test organisms for the toxicological assessment of antibiotics in aquatic environment were assessed in a mini-review (Paper IV). The differences between different species within the same taxonomic group were also assessed.

### **3.4 Chemical analysis**

The focus in the wastewater studies (Papers I, III) was on estrogenic compounds concerning the chemical analysis. The chemical analysis is based on LC-MS/MS and LC-HRMS. The estrogenic compounds (Paper I) were analyzed with an Acquity ultra performance liquid chromatograph coupled to a Xevo TQ mass spectrometer with an electrospray ionization (ESI) source. Sample and compound specific recoveries were applied. The contribution of the analyzed chemicals to the observed effects were calculated based on the chemical concentrations and relative estrogenic potency of each substance.

In the sediment study, the focus was on mineral oils and non-target analysis. The mineral oil (C<sub>10</sub>–C<sub>40</sub>) content was analyzed according to the standard ISO 16703 (2004) by using a Gas Chromatography – Flame Ionization Detector (GC-FID) for quantification of total petroleum hydrocarbons. Non-target analysis of sediment and fresh oil sample fractions was carried out on a gas chromatograph time of flight mass spectrometer (GC-TOF-MS). Quantification of wood extractive concentrations was performed using gas chromatography mass spectrometer (GC-MS).

## 4 Main findings and discussion

The main findings of this thesis are presented according to the main themes presented in Figure 1.

### 4.1 Tools for the assessment of complex environmental samples

- *How to select your approach and what drawbacks and advantages are related to different approaches and the methods?*
- *How responsive or sensitive are the selected methods?*
- *How applicable are the selected methods and the approach overall for the selected samples and the research objectives?*

#### 4.1.1 Selection of the approach

Several different approaches for the assessment of complex environmental samples, such as municipal wastewater (influent, effluent) and contaminated sediments, are applied in this thesis depending on the research questions and aims of each study. Each approach tackles the fundamental research questions, but from a different angle depending on the aim of the study. In an ideal world, the selected approach would be accurate, reliable, easy, quick, comprehensive, specific, cheap and holistic at the same time. However, in reality that is not possible and each approach is a compromise between these elements. The boundaries are determined by the specific research questions and aims of each study. An overview of the different approaches applied in this thesis with an assessment of their advantages and disadvantages is presented in Table 2.

**Table 2.** Overview on the approaches applied in this thesis and their advantages and disadvantages and applicability.

Paper	Approach	Pros	Cons	Applicability
<b>I</b>	Assessing estrogenic potency by combining biotests and chemical analysis. One toxicological endpoint in focus, targeted chemical analysis. Application of sample and compound specific recoveries. Calculating contribution of detected chemicals to the observed effects.	<ul style="list-style-type: none"> <li>- Reliability</li> <li>- Accuracy</li> <li>- Specificity</li> <li style="padding-left: 20px;"><i>Endpoint,</i></li> <li style="padding-left: 20px;"><i>Sample</i></li> <li style="padding-left: 20px;"><i>treatment</i></li> <li>- Identification of key toxicants</li> </ul>	<ul style="list-style-type: none"> <li>- Laborious</li> <li>- Time consuming</li> <li>- Initial data needed on suspected toxicants</li> <li>- Limits of quantification can be too high</li> </ul>	Studies where one or two specific endpoints are of interest and prior information on key toxicants and data that enables to link the data together (e.g REP values) are available.
<b>II</b>	Identification of unknown key toxicants via effect-directed analysis. Combination of biotests and chemical analysis (targeted and non-targeted).	<ul style="list-style-type: none"> <li>- Enhanced probability of identifying key toxicants, even unknown chemicals</li> <li>- Reliability</li> <li>- Accuracy</li> <li>- Data on effects and chemical composition</li> </ul>	<ul style="list-style-type: none"> <li>- Highly laborious</li> <li>- Time consuming</li> <li>- Extensive sample treatment process</li> <li>- Requires a lot of testing and analytics</li> </ul>	Studies where unknown key toxicants are identified and causal links between effect-data and chemical analytical data are defined. Not for screening purposes, but for sites, that are known to be polluted.
<b>III</b>	Holistic assessment of influent and effluent toxicity and removal efficiency. Focus on effect-based water quality assessment, various toxicological endpoints.	<ul style="list-style-type: none"> <li>- Comprehensive-ness</li> <li>- Large data set</li> <li>- Allows to observe trends, “big picture”</li> <li>- Automation for sample treatment</li> <li>- Focus on effects, no initial data needed on suspected toxicants</li> </ul>	<ul style="list-style-type: none"> <li>- Requires large sample volumes and automated extraction</li> <li>- A lot of testing</li> <li>- No information on chemicals, only effects</li> </ul>	Studies where comprehensive assessment with multiple endpoints is the objective. Holistic assessment, which allows to observe bigger trends regarding effects.
<b>IV</b>	Review of available analytical methods for assessing toxicological impacts of antibiotics for aquatic micro-organisms. Focus on effects and effect concentrations.	<ul style="list-style-type: none"> <li>- Comparability</li> <li>- Large amounts of data</li> <li>- Overview on available methods and their sensitivity</li> </ul>	<ul style="list-style-type: none"> <li>- Requires going through multiple data sets</li> <li>- Based on previous studies, requires critical assessment</li> </ul>	Background information for selecting test methods and comparing them. Identification of knowledge gaps.

For example, the greatest advantage of the approach applied in Paper I was the production of highly reliable results, based on sample and compound specific recoveries. After all, one of the key findings of the study was that the recoveries may vary notably between different samples when dealing with complex samples matrices, such as wastewater effluent. However this kind of approach would not be applicable for the study presented in Paper III, where the amount of analyzed endpoints is so large that sample and compound specific recoveries would not be

reasonable. Thus, expressions like “quality over quantity” or “the more the merrier” become essential aspects to consider when making decisions on the most applicable approaches.

Also, in Paper II the approach was selected based on the original research questions, where the aim was being able to identify unknown key toxicants using the EDA approach. The results of this study revealed that standard test methods, such as mineral oil analysis, are not applicable to sediment samples which contain a lot of co-extracting organic material and contamination may be overestimated. This study demonstrated that approaches like EDA are suitable for such purposes. EDA could also be applied to wastewaters or surface water samples or other complex environmental mixtures. EDA is especially applicable in cases when it is suspected that toxic effects are caused by unknown or unexpected chemicals. However, EDA also has its limitations. For example, volatile organic compounds are difficult to extract, fractionate and biotest (Brack, 2003). EDA is more suitable for pre-identified “hot-spots” for pollution like the log pond in Paper II rather than for screening purposes like Paper III.

Regarding effect-based approaches, the amount of available test methods is a challenge when deciding on the battery of tests. The selection of toxicological endpoints is a crucial step and pre-determines the identified toxicants in approaches like the EDA. There are tens of different tests methods only for estrogenicity, such as ER-CALUX, YES assay, ER-GeneBLazer and HeLa-9903 assay, only to mention a few. Addition of different modes of action, endpoints or test species has to do with defining the boundaries for your test battery. Ideally, you would be able to cover all relevant endpoints, multiple species and levels of biological systems. Obviously, in real life this is limited by time, money, know-how, equipment and other restrictions. One practical example of a limiting factor may be required sample volumes, especially if sampling is challenging (e.g. influent and clogging during extraction). Typically, cell-based bioassays require less sample than *in vivo* methods like the fish embryo toxicity test or *D.magna* tests. Thus, *in vitro* assays may be preferential in cases where sample volumes are limited. Overall, the biological test battery can be built based on the following criteria or ideals:

- The sensitivity to different toxicants varies between organisms, thus multi-taxa assessment enhances the comprehensive assessment of toxicant effects (Guillén et al., 2012; Di Paolo et al., 2016).
- Modes of action can be divided into non-specific, specific and reactive toxicity. These groups target different types of contaminants and toxicity. Test battery covering all three groups, would provide a good starting point for optimal coverage of relevant modes of action. (Escher & Leusch, 2011)
- Mechanism-specific bioassays provide information on modes-of-action that are specifically of concern, e.g. endocrine disruption, mutagenicity or photosynthesis inhibition (Escher & Leusch, 2011; Di Paolo et al., 2016). Including a range of specific modes of action facilitates optimal coverage of relevant modes of action.

Another important aspect related to the research approach, is choosing how and what to sample. For example, in this thesis the quantity of wastewater treatment plants is clearly prioritized over having multiple samples from one or two WWTPs. Sampling multiple WWTPs allows to identify trends that occur regardless of treatment processes and provides a holistic view on the most commonly applied treatment processes or occurring toxicities and contaminants. However, seasonal or time-dependent variations are not covered and possible outliers are harder to detect.

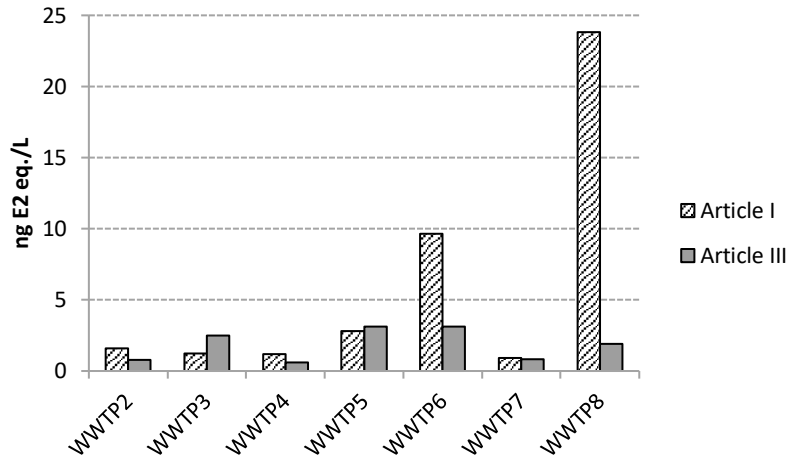
#### **4.1.2 Bioassays – responsiveness and applicability**

The responsiveness of the bioassays used in this thesis varied depending on the method, sample and endpoint. Interestingly, the results varied within the same endpoint depending on the mode of action or even species. For example, the results of the ELISA-E2 immunosorbent assay were approximately 10 fold higher than those determined with chemical analysis or ER $\alpha$ -CALUX<sup>®</sup> (Paper I). Previous studies have demonstrated that sensitivity of the selected bioassays can vary. For example, Leusch et al. (2010) demonstrated differences in the amplitude of the response between different bioassays applied for the detection of estrogenic activity. However, this could be explained by the mechanisms of action of the chemicals (e.g. receptor mediated genomic events vs. nongenomic estrogenic effects).

Majority of the same WWTPs were sampled for Paper I and Paper III and tested with the ER-CALUX<sup>®</sup> assay. The results between the two different sampling events are quite similar (Fig. 6). The variation in estrogenic potency of the effluent samples is relatively small concerning WWTP 2, WWTP 3, WWTP 5 and WWTP 7. However, the results in Paper I are two times higher for WWTP 6 and twelve times higher for WWTP 8. Previous studies have demonstrated that the estrogenic activity of wastewater effluents may vary substantially on a daily or seasonal basis (Martinovic et al., 2007; Jin et al., 2008; Martinovic-Weigelt et al., 2013). Temporal changes in the estrogenic activity of wastewater effluents may be affected by temperature, rainfall, process conditions, population demographics and influent composition (Hemming et al., 2004). Regarding WWTP 8 the differences in estrogenic activity between the two sampling periods, are likely due to major upgrades done at the treatment plant (process optimization, increase of capacity) and fluctuations in the share of industrial loading. In addition temperature and weather conditions may affect the results.

Genotoxic effects were detected only in the test with metabolic activation with the p53-CALUX<sup>®</sup>, whereas in the umuC assay genotoxicity was observed in the test without metabolic activation. In addition, the results obtained with the transgenic medaka embryos were similar to those obtained with ER-CALUX<sup>®</sup>, however slight differences were observed. Also, the sensitivity of the green algae species for testing antibiotic toxicity, depends highly on the test substance. All of these results indicate that the assays respond in a different way to some of the compounds present in the samples and some compounds may act as antagonists, due to which

some of the results (especially receptor-based assays) may be underestimated. Analyzing both agonism and antagonism in parallel may result in a more accurate assessment (Neale and Leusch, 2015). Each method has its limitations and advantages, thus a combination of several approaches provides a more comprehensive assessment.



**Figure 6.** Estrogenic activity of effluent samples analyzed with the ER-CALUX assay. Effluent samples were collected from seven WWTPs sampled in Paper I and Paper III. WWTPs are named according to Paper I.

The responsiveness of the assays varies, but novel *in vivo* methods, such as the medaka assay, and receptor-based methods, such as the CALUX assays, have proven to be more responsive compared to some of the standardized methods, such as *V.fischeri* or *D.magna* tests. Each method has its limitations and advantages, thus a combination of several approaches provides a more comprehensive assessment. Recent studies on assembling a bioanalytical test battery for water quality monitoring agree that ideally the selection should be based on effects typically found in water and the battery should include a wide range on environmentally relevant modes of action (Altenburger et al., 2015; Neale et al., 2017). At the end, the final compositions of the test battery is a compromise between the desire to cover all possible endpoints, practicability and the purpose of the assessment. For routine monitoring properties such as robustness, low sample volume requirements and efficiency, play a key role (Escher and Leusch, 2011). For a comprehensive assessment of water quality, it is recommended that the test battery includes tests for different adverse outcome pathways, e.g. metabolisms (activation or detoxification), binding to hormone receptors, inhibition of enzymes, DNA damage or mutagenicity, p53 mediated DNA repair, cell death and organisms responses (Macova et al., 2011; Altenburger et al., 2015; Neale et al., 2017). The bioassays selected for this thesis cover many of these pathways, however endpoints

like metabolism (activation or detoxification) or disruption of cellular homeostasis, are not covered. In addition, immunotoxicological effects (Salo et al., 2007) and oxidative stress responses (Lungu-Mitea et al., 2018) could be considered in the future.

#### **4.1.3 Chemical analysis – challenges and analytical choices**

The chemical analytical methods applied in this thesis were selected based on the compounds of interest, sample matrix, applicability and research questions. For example in Paper I the focus was on estrogenic compounds, where the sample treatment process was optimized for natural and synthetic hormones by selecting the most applicable extraction material and methods. On the other hand, in Paper III, the focus was on catching as many organic pollutants as possible, thus a “universal” extraction material and multistep elution process was applied. The approach in Paper III also required large sample volumes, due to which an automated large volume solid phase extraction device (LVSPE50) device was applied. Wastewater is a complex sample matrix, which is reflected in the results of this thesis. For example, in Paper I the limit of quantification (LOQ) was relatively high for many of the analysed hormones, especially when considering the low concentrations at which these chemicals are likely present in wastewaters. Nevertheless, hormones and other EDCs can cause adverse effects already at very low concentrations. Thus, more sensitive analytical methods are needed to be able to analyse compounds present at low concentrations.

In addition to LOQs, the extraction methods applied in this thesis do not consider particle bound contaminants due to filtration steps prior to extraction. The work presented in this thesis is focused on water-soluble contaminants. Thus, toxicity, pollutant concentrations or removal efficiencies may be underestimated.

In Paper II a combination of target and non-target methods were applied following multiple fractionation procedures. Fractionation allows for the sequential reduction of complexity and the non-toxic fractions are separated from the toxic fractions. Fractionation can be done based on different physicochemical properties and in this case it was based on polarity. The chosen sample treatment steps and analytical methods affect the observed chemical content, as potential monoterpenes were lost during solvent exchange and resin acids or sterols may have been underestimated due to derivatization step. However, as for bioassays, compromises are always required in these types of studies and overall the chosen analytical methods and fractionation processes were suitable for the detection of unknown key toxicants.

Chemical analytical results were combined with the results from bioassays in Paper I and II. In Paper I relative estrogenic potencies (REP values) determined in single-substance studies were applied together with chemical concentrations to determine the contribution of each substance to the observed effect. The LOQs in chemical analysis were a limiting factor in evaluating the contribution, emphasizing

ing the importance of developing more sensitive chemical analytical methods. Regardless of the LOQs, the results demonstrated that the observed estrogenic activity was only partly explained by analysed compounds, which indicates that some effects are due to mixture effects and unknown chemicals. In Paper II, bioassays were applied to the different fractions, in order to identify the toxic fractions for further analysis. Wood-originated diterpenic compounds were detected in the most toxic fraction, typical wood extractives such as sesquiterpenes were detected in a genotoxic fraction and triterpenes were detected in a fraction that showed estrogenic potency. However, the focus of this study was not being able to calculate the contribution of these substances to the observed effects, but to direct and guide the analytics to the chemical groups that are likely responsible for toxicity and thus reduce complexity of the sample.

## **4.2 Influent and effluent quality of eight Finnish WWTPs**

- *What are the key toxicological endpoints related to influent and effluent quality?*
- *Which emerging contaminants are detected at concerning levels?*
- *What does a holistic assessment of influent and effluent samples reveal about the current state of effluent quality at eight Finnish WWTPs?*

### **4.2.1 Wastewater toxicity**

The results of this thesis suggest that the removal of toxic effects is incomplete at the WWTPs and wastewater effluents are potentially toxic (Papers I, III). Based on the overall results of this thesis, the key endpoints related to wastewater toxicity in Finland are estrogenic activity, thyroid disruption and fish embryo toxicity (Table 3). These endpoints were activated in effluents from majority of the WWTPs and responses were detected at low sample concentrations indicating high toxic potency.

**Table 3.** Toxicities and their detection frequencies observed in wastewater effluent samples sampled in this thesis (Paper I, III). The responsiveness of each assay is presented according to the lowest sample concentration (REF) where effects were detected.

Bioassay	Type	Toxicological end-point	Detection frequency	Responsiveness (lowest REF)
NRR-retention test (RTL-W1 cells)	<i>In vitro</i>	Acute cytotoxicity	Some samples	<REF5**
AR-CALUX®	<i>In vitro</i>	Androgenic activity	None of the samples	>REF20
ER-CALUX®	<i>In vitro</i>	Estrogenic activity	Most of the samples	<REF1**
ELISA-E2 immunosorbent assay	<i>In vitro</i>	Estrogenic activity	Most of the samples	<REF1**
p53-CALUX®	<i>In vitro</i>	Genotoxicity	Some of the samples	>REF20
umuC assay	<i>In vitro</i>	Genotoxicity	Some of the samples	<REF20
<i>V.fischeri</i> inhibition assay	<i>In vivo</i>	Acute toxicity	None of the samples	*
Zebrafish embryo toxicity	<i>In vivo</i>	Embryotoxicity (survival, sublethal effects)	Most of the samples	<REF2.5**
Rapid estrogen activity in vivo (REACTIV) medaka assay	<i>In vivo</i>	Estrogenic activity	Most of the samples	<REF1**
<i>Xenopus</i> embryonic thyroid assay (XETA)	<i>In vivo</i>	Thyroid disruption	Most of the samples	<REF5**
<i>D.magna</i> acute toxicity	<i>In vivo</i>	Acute toxicity	None of the samples	*
<i>D.magna</i> long term toxicity	<i>In vivo</i>	Long term toxicity	None of the samples	*

\* Raw effluent was used for testing, no effects were observed at the highest test solution.

\*\* Lowest REF tested.

Estrogenic activity was detected in all of the wastewater samples (Papers I, III), practically with all of the different test methods (ER-CALUX, ELISA-E2, medaka assay). The results of this thesis support the findings of previous studies on wastewater toxicity, which have also identified estrogenicity as one of the key toxicological endpoints (Salste et al., 2007; Van Der Linden et al. 2008; Adeel et al., 2017; Leusch et al., 2017; Gehrman et al., 2018; Leusch et al., 2018). With sensitive bioassays, estrogenic potency can be detected even in samples with extremely low chemical contamination after sufficient sample enrichment (Leusch et al., 2018). Thus, it is important to differentiate between an acceptable level and an unacceptable level of effects, for which effect-based trigger values (EBT) have been developed (Jarošová et al., 2014; Escher et al., 2015; Van der Oost et al., 2017). The EEQ values presented in this thesis (Paper I, III) exceed EBT values

determined in previous studies, which vary between 0.2 and 2.0 ng/L EEQ depending on the sample matrix and exposure duration (Jarošová et al., 2014; Escher et al., 2015; Leusch et al., 2017). The results suggest, that the effluents may pose a risk to the receiving waters concerning estrogenic activity.

Thyroid disruption was detected in five effluent samples, indicating significant pro-thyroid effects ( $<LOD-1.34 \mu\text{g T}_3 \text{ eq./L}$ ). Effects were detected in the same samples also in spiked test mode (spiking with  $\text{T}_3$ ), showing antagonistic effects and more complex effects such as disruption of thyroid hormone metabolism ( $<LOD-3.71 \mu\text{g T}_3 \text{ eq./L}$ ). Unlike for estrogenic activity, the results for thyroid disruption were unexpected based on previous studies, where effluents have not shown high potential for thyroid disruption (Jugan et al., 2009; Escher et al., 2013). However, these studies were based on *in vitro* studies, which may be less responsive compared to the *Xenopus* embryonic thyroid assay because thyroid hormone disruptors act via non-receptor based mechanisms of action (Wegner et al., 2016).

Toxic effects were observed in all of the effluent samples tested with the fish embryo toxicity (FET) test. Considerable mortality (20-43 %) were observed at lowest exposure concentrations (REF2.5). In addition, embryos presented also several malformations, such as scoliosis and pericardial edema. The use of fish acute toxicity test in environmental risk assessment is already widely applied in several European countries (Scholz et al., 2008). However, typically raw effluent samples are applied. This study (Paper III) demonstrated that FET test can also be applied to analyze wastewater extracts dissolved in a suitable carrier solvent.

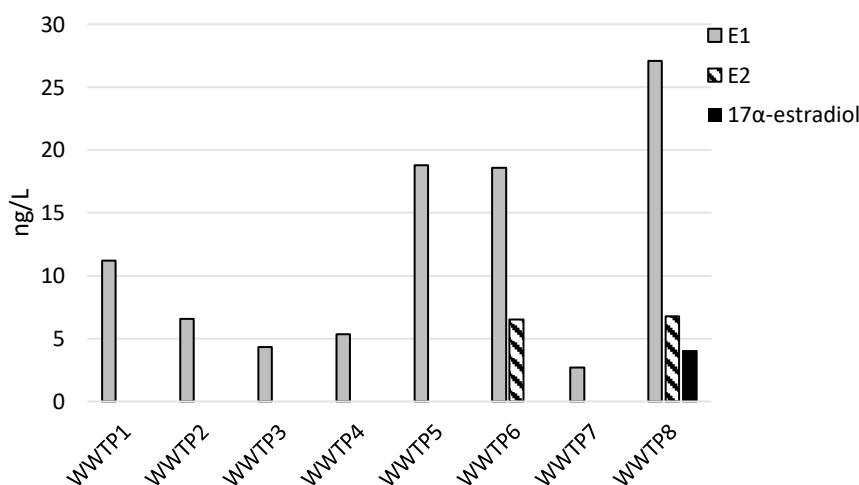
Currently, endpoints like estrogenic activity or thyroid disruption, are not applied in routine monitoring. The most commonly applied methods for monitoring are standardized *in vivo* test with water fleas (*D.magna*), bacteria (*V.fischeri*) and green algae (*P.subcapitata*). For example, these tests are recommended as “best available technique, BAT” for monitoring of industrial wastewaters in the BAT conclusions for waste water and waste gas treatment/ management systems in the chemical sector (EU 2016/902). However, as indicated by the results of this thesis, these methods are not sensitive enough for treated wastewater effluents and do not give any indication on more specific toxicity, such as endocrine disruption or genotoxicity. Thus, test batteries for water quality monitoring should be further enhanced by adding biotests that are specific for certain modes of action, such as estrogenic activity or thyroid disruption. However, toxicity was observed with *D.magna* and *V.fischeri* tests in the sediment study (Paper II), where the sediments were known to be highly polluted. In such cases, these types of tests can be useful indicators of acute toxicity.

#### **4.2.2 Detection of emerging contaminants**

The results of this thesis support the previous findings related to emerging contaminants such as endocrine disrupting compounds and pharmaceuticals. These compounds are not fully removed during wastewater treatment and thus small

traces of these compounds are continuously discharged into aquatic environments. According to the review in Paper IV on maximum and median concentrations of antibiotics found in surface waters and wastewater effluents, antibiotics can be present at concentrations above 10 µg/L. Typically pharmaceuticals and antibiotics are present in wastewaters and surface waters at concentrations below 1 µg/L, but it is not rare that pharmaceuticals are detected at higher levels. Based on the review, highest concentrations have been analyzed for sulfamethoxazole (13.765 µg/L, Ngumba et al., 2016), ofloxacin (7.87 µg/L, Leung et al., 2012), ciprofloxacin (5.93 µg/L, Wei et al., 2012) and enrofloxacin (4.24 µg/L, Wei et al., 2012).

Natural hormones excreted by humans, such as estrone (E1), 17β-estradiol (E2) and 17α-estradiol were detected in WWTP effluents sampled in Paper I (Figure 7). The highest concentrations were analyzed for E1, followed by E2, which can be explained by the human urinary excretion rates of these compounds together with the transformation processes during wastewater treatment (Liu et al., 2009). The synthetic hormone 17α-ethinylestradiol (EE2) has a higher relative estrogenic potency compared to natural hormones, thus it may play a significant role in the estrogenic activity of wastewater effluents. However, EE2 was not detected, probably due to the relatively high limit of quantification (10 ng/L).



**Figure 7.** Levels of natural hormones detected in wastewater effluents sampled in Paper I.

Exceptionally high concentration (956 µg/L) of bisphenol-A (BPA) was detected in effluent sample from WWTP 8. Calculations based on relative estrogenic potency showed that BPA was a significant contributor (37%) to the observed effects in the ER-CALUX assay (Figure 6). The high amount of BPA was likely traced back to two paper product factories that discharge significant amount of industrial

wastewaters into the WWTP and use notable amounts of BPA in their process. Based on the results of this study and previous studies conducted in Finland, concentrations for BPA in effluent samples typically range between 100-600 ng/L. Thus, a more intensive sampling campaign should be performed at WWTP8 to better understand the discharge patterns related to BPA at this treatment plant. It is likely that industrial wastewaters containing high levels of BPA are irregularly discharged to the WWTP.

### 4.3 Removal of toxicity during wastewater treatment

- *What is the removal efficiency of different WWTPs related to toxicity and pollutants?*
- *Can we see reoccurring trends based on the holistic approach applied in this thesis?*

The removal efficiency of the sampled WWTPs to reduce toxicity was calculated by comparing the toxicity of the influent samples and effluent samples, which were collected according to the hydraulic retention time of each WWTP (Paper III). Thus, in theory the “same” water was sampled. The removal was assessed for estrogenic activity (ER-CALUX), genotoxicity (p53-CALUX, umuC), cytotoxicity (NRR assay) and androgenic activity (AR-CALUX). In addition all effluent samples were analyzed for embryotoxicity and thyroid disruption.

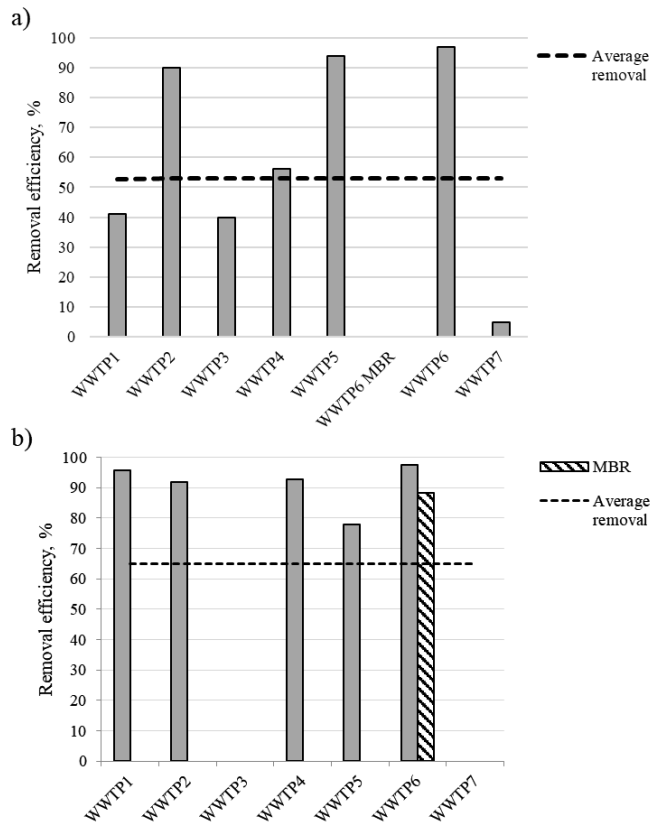
Interestingly, there was no correlation between toxicity removal efficiencies and the presence or absence of advanced tertiary treatment methods. The results suggest that sand filtration does not provide clear advantages related to toxicity removal. However, the results could be different if particle bound contaminants were considered. In addition, other WWTP parameters, such as sludge retention time or nitrification rate, did not correlate with toxicity removal. Most of the previous studies have focused on investigating the correlation between removal efficiencies of individual selected substances and sand filtration, sludge age or other parameters, thus a holistic view on multiple WWTPs and toxicities was missing. Based on the findings of this thesis, the removal efficiency of toxicity is more related to each toxicological endpoint than characteristics of the WWTPs. The toxicological characteristics of the influent samples was similar among all of the sampled WWTPs. Almost all of the samples induced toxic effects in majority of the bioassays. Also the toxicological patterns for the effluent samples were similar between the different WWTPs. The remaining toxicities after treatment were estrogenic activity, thyroid disruption and genotoxicity.

Although, the toxicological removal patterns appear similar between the WWTPs, some variation in removal efficiencies can be seen when looking at the different toxicological endpoints in more detail. On average, the removal for cytotoxicity was more than 50% (Figure 8a). At three of the sampled WWTPs cytotoxic

effects were almost completely removed. However, poor removal was observed at WWTP 7 and the MBR pilot plant at WWTP6. WWTP 7 has the highest industrial loading which could at least partly explain the results as industrial influent may contain more compounds that are not biodegraded during the treatment process. At the MBR pilot no significant reduction in cytotoxic effects was observed. The system was newly installed and the operational parameters might not have been fully optimized, as the results were similar related to other toxicities.

The reduction in estrogenic activity ranged between 0-97%, with the average removal of 63% (Figure 8b). In majority of the WWTPs the removal efficiency was above 90%, however in WWTP3 and WWTP7 estrogenic activity was not removed at all. In these samples, the levels of estrogenic activity were low to begin with, thus seeing any variation between influent and effluent samples is unlikely. These results are supported by previous studies which have applied ER-CALUX to study removal efficiencies of estrogenic activity (Maletz et al, 2013; Gehrman et al., 2018).

Androgenic activity was completely removed during wastewater treatment. A similar trend was observed for genotoxicity, as in adaptive stress response assay (p53-CALUX) all genotoxic effects were completely removed except the MBR pilot effluent, where genotoxic effects were reduced by 16%. However, results from the umuC assay indicate that genotoxic effects are not fully removed. Controversies between these two different methods may suggest that these assays respond to different types of chemicals that may induce genotoxic effects.



**Figure 8.** Removal efficiencies for a) cytotoxicity and b) estrogenic activity based on the results presented in Paper III.

#### 4.4 Practical implications and future needs

##### *Effect-based tools for the assessment of influent and effluent quality*

Circling back to the initial "tip of the iceberg" problem presented in the introduction, it is highly unlikely that monitoring can be continued only based on selected

individual chemicals as awareness and concerns related to transformation products, mixture effects and the role of unknown chemicals are growing. As demonstrated by the results of this thesis, bioassays can provide valuable information on water quality. Effect-based tools are sum parameter based, thus unknown chemicals, transformation products and mixture effects are taken into account. However, development of standardized test systems and effect-based trigger values are needed to further improve the application of effect-based test methods for regular assessment of water quality or monitoring. As demonstrated in Paper IV more information on data variability and differences between test species are needed to improve standardization and harmonization of test systems.

In addition, a consensus on the composition of the biological test battery is needed. The results of this thesis indicate that bioassay batteries for water quality assessment should include a large variety of assays covering multiple endpoints and test organisms. Estrogenic activity, thyroid disruption, and embryotoxicity may be the most relevant endpoints as demonstrated in Paper I and III. However, more holistic assessments covering multiple WWTPs and endpoints are needed to draw further conclusions.

As demonstrated in Paper II, effect-based tools can also be applied for the assessment of other complex environment samples, such as sediments. The results of this study demonstrate that traditional methods for the assessment of pollution may not be sufficient in identifying key toxicants. Approaches, like the EDA, are needed to make the causal links between toxic effects and key toxicants, especially in the case of unknown chemicals. Thus, more research is needed to develop these types of methods to make them more suitable for monitoring purposes.

#### *Assessment of wastewater treatment efficiency*

Some of the chemicals posing the potential to cause adverse effects are vital to human health, thus it is unlikely that their use can be completely restricted. In regard to natural hormones, such restrictions are not even possible because these substances are naturally excreted. Therefore, the need for point source control at the WWTPs is needed and conventional treatment methods should be upgraded. Currently, assessment of treatment efficiency is based on a similar "tip of the iceberg" approach as in monitoring. Treatment efficiency is calculated based on a selection pre-selected substances and mixture effects or transformation products are typically not considered. Based on the results of this thesis, it would be highly beneficial to assess treatment efficiency with effect-based tools. If high removal of toxicity is achieved, also a high removal of the chemicals causing the toxic effects is met. Are the removal rates for single substances even needed to know if toxicity levels are at an acceptable level?

## 5 Contribution to the fundamental questions

The fundamental questions presented in Chapter 1 were studied from the perspective of effect-based tools for the assessment of complex environmental samples, wastewater quality and assessment of removal efficiency at typical Finnish WWTPs. The questions are broad, interdisciplinary and complex, thus these findings only partly answer them. However, we move closer to finding the full answers with every study that tries to at least partly find the answers.

*What are the sources and pathways of harmful chemicals to the environment?*

Based on the findings of this thesis, municipal wastewater treatment plants are potential pathways of pollutants to the environment as treated effluents have toxic properties and contain complex mixtures of organic micropollutants. The significance of treated effluents as a pathway to pollutants compared to other pathways was not investigated, thus any conclusions on that cannot be drawn. Interestingly, effluents have similar ecotoxicological profiles regardless of the WWTP parameters or treatment methods indicating that the removal efficiency is more related to each toxicological endpoint than characteristics of the WWTPs. These findings suggest that the sampled municipal WWTP effluents are quite similar when it comes to toxicity that is related to water soluble chemicals.

*What kind of effects harmful chemicals have on the environment and human health?*

Wastewater effluents or polluted sediments may be toxic to our environment and health, as demonstrated in this thesis. Effluents have endocrine disrupting properties, as they show estrogenic activity and thyroid disruption. Effluents may also cause adverse effects on fish embryos or induce genotoxic effects. Highly polluted sediments may be even acutely toxic. As indicated by the results of this thesis, chemicals that are discharged to our environment can have various toxicological effects and different test systems react to different types of chemicals and toxicities. As more sensitive test systems are developed and new relevant endpoints are identified, valuable information on the effects of chemicals and complex chemical mixtures is gathered. More research is needed to identify key toxicological endpoints related to wastewater effluents and environmental safety.

*How can the discharge or transfer of harmful chemicals be avoided or controlled?*

As indicated by the results of this thesis, wastewater effluents may pose risks to the aquatic environments. Thus, point source control at the WWTPs is crucial. The results of this thesis demonstrate that the removal of emerging contaminants and toxicity is incomplete with conventional treatment methods. More advanced treatment methods are needed to further improve water quality and control the discharge of chemicals to our environment. In addition to treatment techniques, advances in the regulation and monitoring of harmful pollutants are needed. Further research is needed on the applicability of advanced tertiary treatment methods with holistic approaches combining bioassays and chemical analytical methods.

*How do we ensure that pollutants are present in the environment at a safe level?  
How can the safe levels be monitored?*

This is probably the hardest question to answer. Linking the results obtained in laboratory studies to real life conditions is challenging. The results of this thesis indicate that chemical discharges from the industry or municipal wastewaters have toxic properties and contain pollutants that are known to potentially cause adverse effects. Effect-based methods should be applied for monitoring purposes to move towards a more holistic assessment that would also consider sum parameter based effects. EBT values have been developed to estimate whether the observed effects are at a safe and acceptable level. However, calculation of EBT values is not possible for all endpoints at this stage and more data is needed to further improve the application of EBT values. The estrogenic activities observed in the effluents samples in this thesis exceed EBT values determined in previous studies, indicating that effluents may pose a real risk to the receiving waters. Application of effect-based tools together with chemical analysis will help us achieve a safer environment and higher quality of monitoring.

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