High-throughput EDA – automated approaches to directly link fractionation, biotesting and identification

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ABSTRACT

The large number of chemicals used worldwide increases the potential chemical threats discharged into our environment. Understanding the impact of such compounds is a major challenge for environmental scientists. Effect-directed analysis (EDA) has been developed as a tool to try to identify chemical hazards to the environment. EDA uses chemical tools (for the extraction, fractionation, and analysis of samples) together with biological tools (as bioassays) to find the cause of an observed effect. EDA can be very powerful but the extensive material, the high number of fractions generated, and the expertise required by such an intensive study can be an obstacle to their implementation.

This thesis tested different techniques for each of the EDA steps and three EDA studies were performed. The aim of the thesis is to propose ways to increase EDA throughput and to facilitate the use of bioassays, chemical fractionation and analysis together. For the thesis, two bioassays were further developed: one was automated (to look for compounds inhibiting the acetylcholine esterase, AChE) while another one was miniaturised (to detect potential thyroid hormone disrupters). Automation and miniaturisation increased the assays' throughput in order to facilitate their use in EDA studies. The automated bioassay was successfully used in an EDA study to identify AChE inhibitors discharged from off-shore oil and gas production platforms. This study allowed the identification of two new AChE inhibitors present in such discharges. As suspected by previous published research, these compounds contain both aromatic rings (butylated hydroxytoluene and 4-phenyl-1,2-dihydronaphthalene) and are reported in produced water discharge for the first time. Two other EDA studies were carried out to identify algal growth inhibitors and algal photosynthesis inhibitors in a landfill leachate and a wastewater treatment plan effluent. From these studies, a list of identified toxicants was established. Pesticides as well as pharmaceuticals and compounds used in personal care products were identified showing the potential risk to the environment from such discharges. The two EDA studies confirmed the already published data by identifying these compounds in landfill leachate and wastewater treatment plan effluent.

From the knowledge gained with the different studies, a generic protocol to perform enhanced throughput EDA studies on different samples is proposed. A generic solid-phase extraction has been suggested to combine extraction and preliminary fractionation of the compounds. Three high-throughput bioassays to assess the presence of AChE inhibitors, of thyroid hormone disrupters, and of algal growth inhibitors could be performed. A secondary fractionation of the toxic fraction(s) should then be carried out by high-performance liquid chromatography (HPLC) and the HPLC toxic fraction(s) analysed by liquid and solid chromatography coupled to mass spectrometers. This thesis showed the possibility of performing enhanced-throughput EDA as a powerful tool to identify new chemical hazards even if such studies are not 100 % successful every time.

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Automated high-throughput in vitro screening of the acetylcholine esterase inhibiting potential of environmental samples, mixtures and single compounds (**2016**), *Jean Froment, Knut Erik Tollefsen, Kevin V. Thomas, Ecotoxicology and Environmental Safety 130, 74-80, http://dx.doi.org/10.1016/j.ecoenv.2016.04.005.*

Paper II

Identification of petrogenic produced water components as acetylcholine esterase inhibitors (**2016**), *Jean Froment, Katherine Langford, Knut Erik Tollefsen, Inger Lise N. Bråte, Steven J. Brooks, Kevin V. Thomas, Environmental Pollution 215, 18-26, http://dx.doi.org/10.1016/j.envpol.2016.05.004.*

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Miniaturization of a transthyretin binding assay using a fluorescent probe for high throughput screening of thyroid hormone disruption in environmental samples (**2017**), *Xiyu Ouyang and Jean Froment, Pim Leonards, Guttorm Christensen, Knut Erik Tollefsen, Jacob de Boer, Kevin V. Thomas, Marja Lamoree. Chemosphere 171, 722-728, http://dx.doi.org/10.1016/j.chemosphere.2016.12.119.*

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Abbreviations

AChE – acetylcholine esterase

CA – concentration addition

CAS – chemical abstracts service

DMSO – dimethyl sulfoxide

ECHA – European chemical agency

ECOSAR - Ecological Structure Activity Relationships

EDA – effect-directed analysis

ER – estrogen receptor

FITC - fluorescein isothiocyanate

FITC-T_4 – fluorescent labelled thyroxine

GC – gas chromatography

GC-HR-MS – gas chromatography coupled to a high resolution mass spectrometer

HPLC – high-performance liquid chromatography

IA – independent action

LC – liquid chromatography

LC-QToF-MS – liquid chromatography coupled to a quadripole time of flight mass spectrometer

LVSPE – large volume solid phase extraction

MoA – mode of action

MS – mass spectrometry

NIST – National Institute Standards and Technology

NP-HPLC – normal-phase HPLC

PAH – polycyclic aromatic hydrocarbon

PAM – pulse amplitude modulation

PS II – photosystem II

QSAR – quantitative structure-activity relationship

REACH – registration, evaluation, authorisation and restriction of chemicals

RIA – radioimmunoassay

RP-HPLC – reversed-phase HPLC

RSD – relative standard deviation

SPE – solid phase extraction

TNB - 5-thio-2-nitrobenzoic acid

ToF – time of flight

TTR – transthyretin

T_4 - thyroxine

UHPLC – ultra-high performance liquid chromatography

WFD – water framework directive

WWTP – wastewater treatment plant

I. Introduction

1. Effect-directed analysis (EDA) of complex environmental mixtures

More than 109 million individual compounds are registered at the Chemical Abstracts Service (CAS, http://www.cas.org/content/counter), and in Europe around 14,400 chemicals were registered in December 2014 at the European Chemical Agency (ECHA) as part of the REACH regulation (Registration, Evaluation, Authorisation and Restriction of Chemicals). For the REACH regulation, chemicals produced at a volume of more than a ton per year and "chemicals of concern" are registerd for use in Europe. This provides a clear idea of the very high number of chemicals used across the world and the potential for them to be released into the environment. Different procedures are available to evaluate and monitor the impacts of chemicals in the environment. For example, in the European Water Framework Directive (WFD) targeted chemical analysis is used in parallel with bio-indicators to assess the chemical and ecological status of a water body (European Commission, 2007). Targeted chemical analysis, with preselected compounds is usually performed to assess the chemical pressure on a water body. These analyses can provide a good overview of the quantities of targeted dangerous chemicals but do not include any biological effects data to assess the harm caused by any toxicants. Effect-directed analysis (EDA) uses bioassays to detect a specific biological effect caused by the chemicals present in a sample followed by the chemical fractionation of the sample in order to reduce the complexity of the sample matrix (Figure 1, Brack, 2003). Once fractionation has been performed in a satisfactory manner so that the cause of the biological effect is isolated, non-target chemical analysis is then performed to try to identify the compounds present in the toxic fractions. Fractionation of the sample allows easier chemical analysis and a better identification of the unknown compounds. EDA has been used as a powerful tool to identify unexpected causes of biological effects such as synthetic steroids (Desbrow *et al.*, 1998; Creusot *et al.*, 2014) or estrogenic compounds (Schmitt *et al.*, 2012). Improving EDA throughput would enable its implementation on a larger scale by reducing its complexity. Finally, simplifying EDA could also help scientists to understand the impact of toxicants on the environment by identifying the main stressors and not only the targeted ones (Brack *et al.*, 2016).

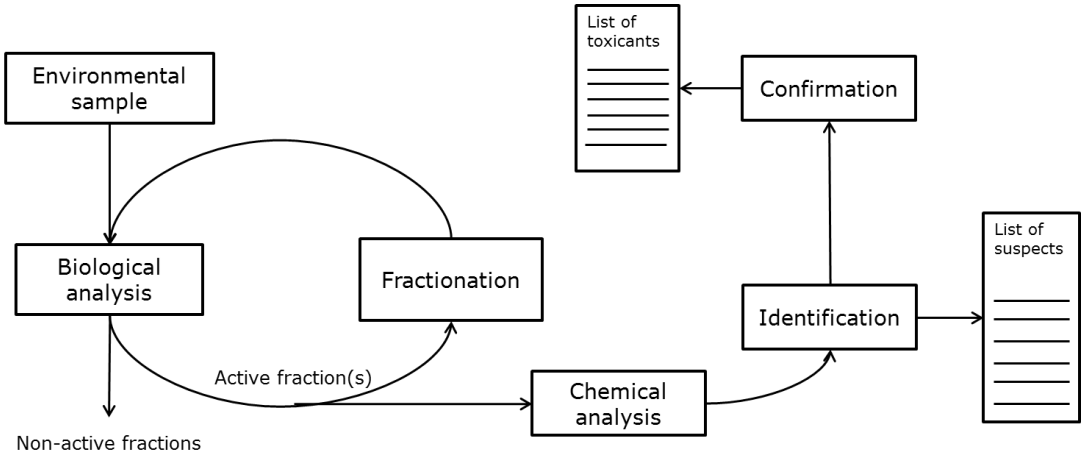

Figure 1: Scheme of effect-directed analysis of complex mixtures (adapted from Brack, 2003)

2. Biological tools for effect-directed analysis

The choice of bioassay for EDA studies is of utmost importance. Indeed, different bioassays describe different effects that may be caused by different types of chemicals. This choice can be made based on the known toxicity of a tested sample where the cause may be unknown; or because the sample has never been tested with the assay and there is a high probability to discover new impacts on the environment by the chemicals present. This section presents the background of the three assays used in the thesis to assess the impact of contaminants on three different endpoints. The acetylcholine esterase (AChE) inhibition assay was used to assess potential disturbances in the nervous system, the pulse amplitude modulation (PAM) fluorometry assay was used to assess inhibition of photosynthesis in primary producers, and the transthyretin (TTR) assay was used to assess endocrine disturbance associated with the TTR, and all three will be therefore further described in the following section.

2.1. Acetylcholine esterase (AChE) inhibitors

AChE is a key enzyme in the nerve signal transmission and nervous system functionality. Neurotransmission is the process by which neurotransmitters (signalling molecules) released by pre-synaptic neurons, bind and activate post-synaptic neurons and thereby facilitate transmission of nervous signals. AChE is involved in the regulation of such neurotransmission by catalysing the hydrolysis of the neurotransmitter acetylcholine into choline and acetate at the cholinergic synapses. Inhibition of the enzyme leads to an accumulation of non-degraded acetylcholine causing an overstimulation of the acetylcholine receptors. This overstimulation can cause what is called the “cholinergic syndrome” (with

various effects on the nervous system). When they occur, problems in the respiratory centres are the most lethal ones (Costa, 2006). The AChE assay to detect AChE inhibiton was first developed by Ellman *et al.* in 1961 and adapted for its use in 96-well plates by Galgani and Bocquene (1991). This assay has subsequently been used to detect the AChE inhibitory potential from various compounds (Kang and Fang, 1997; Nunes-Tavares *et al.*, 2002; Di Tuoro *et al.*, 2011) but also to detect AChE inhibitors in various types of environmental samples (Hamers *et al.*, 2000; Arduini *et al.*, 2006; Holth and Tollefsen, 2012). AChE inhibition has been classified as a "specific biomarker of organophosphorus and carbamate pesticides" (Assis *et al.*, 2010). These compounds are very toxic to most organisms because of their ability to inhibit enzymes such as AChE. Designed to suppress the abundance of selected organisms, pesticides inhibiting AChE are usually also toxic for non-targeted species (Bocquené and Galgani, 1998). Furthermore, it has been recently shown that other type of compounds, such as polychlorinated biphenyls or polycyclic aromatic hydrocarbon (PAHs), can inhibit AChE in environmental samples (Kang and Fang, 1997; Kais *et al.*, 2015). This assay was chosen for the development of a new EDA procedure because of the effects AChE inhibitors can have on organisms such as fish and seabirds (Bocquené and Galgani, 1998; Oropesa *et al.*, 2007) and furthermore because apart from selected pesticides (such as organophosphates and carbamates), only a few classes of compounds have been previously tested. The overall goal being that including this assay in EDA procedures could potentially discover new environmental AChE inhibitors.

2.2. Algal toxicants

Algae are dominant primary producers in aquatic ecosystem and adverse effects on them can potentially lead to adverse effects at a higher trophic level (Fleeger *et al.*, 2003). Bioassays assessing adverse effects on algae have subsequently been used for many decades (Nyholm and Källqvist, 1989) and remain relevant for environmental studies (Książyk *et al.*, 2015). A popular assay is the algal growth inhibition test which is a non-specific assay use to detect alteration of the algae growth rate (Nyholm and Källqvist, 1989; Geis *et al.*, 2000). The PAM fluorometry assay is a specific assay that uses the fluorescence properties of chlorophyll a to evaluate the photosynthesis efficiency (photosystem II, PS II) of algae (Kooten and Snel, 1990). This assay has proven to be a powerful tool in assessing the toxicity of chemicals to algae as well as detecting the effects of chemicals on algae by chemicals in complex environmental mixtures (Booij *et al.*, 2014; Sjollema *et al.*, 2014a). So far, only herbicides

have shown a response with this assay because of their specific action on the PS II but it could be possible for other types of compounds to alter algae photosynthesis. For this thesis, the PAM assay was used as a high-throughput assay to detect photosynthesis inhibitors in various environmental samples. A miniaturised version of the algal growth inhibition test was also used to assess the impact of a wastewater treatment plant (WWTP) effluent on aquatic primary producer (Geis *et al.*, 2000).

3.1. Thyroid hormones disrupters

Thyroid hormones are produced by the thyroid gland and spread into the blood by binding to proteins such as TTR and thyroxine-binding globulin. Thyroid hormones are responsible for the regulation (growth and development) of an organism. Effects on thyroid hormones can therefore affect organisms' development, especially in juveniles ones. Furthermore, xenobiotic compounds have proven to interact with thyroid hormones (Boas *et al.*, 2006). One mode of thyroid hormone interference is blocking the biding between the thyroid hormone and the transport protein and by doing so reducing the transport from the site of production to the site of conversion to the active form triiodothyronine (T_3). The TTR binding assay has therefore been developed to assess the disruption of the binding between thyroxine (T_4) and TTR by different xenobiotics (Lans *et al.*, 1993; Weiss *et al.*, 2009b). Organic contaminants such as polychlorinated biphenyls, dioxins, furans, flame retardants, or their metabolites have been shown to disrupt the thyroid hormonal system by interacting with the transport proteins or thyroid hormone metabolizing enzymes (e.g. Brouwer *et al.*, 1998; Meerts *et al.*, 2002; Hamers *et al.*, 2006). Their impact on wildlife has even been reported in remote areas of the world, such as the Svalbard Islands (Bytingsvik *et al.*, 2013). This assay, traditionally used a radioactive ligand to detect T_4 binding to the TTR, was recently improved by Ren and Guo (2012) by using a fluorescent ligand that allows fluorescence measurement of the T_4 bonded to the TTR (Smith, 1977). For this thesis, the protocol has been modified for its use in microwell plates, further enhancing the assays throughput and making it directly compatible with chemical fractionation and analysis for easier use in the context of complex studies such as EDA.

3. Analytical tools for effect-directed analysis

EDA studies use analytical tools for two purposes; fractionation of the sample matrix, and identification of the responsible compounds in the toxic fractions. This section provides an

overview of the available tools for each step with the theory behind the ones used for the present thesis.

3.1. Chemical tools for the fractionation step

Fractionation of a complex mixture allows the separation of the chemicals present according to their specific properties in order to create a simpler matrix. Reducing the number of chemicals is primordial in order to facilitate the non-targeted identification of the active fractions while conserving the responses observed during the bioassays. Liquid chromatography (LC) has been the most commonly reported fractionation technique used in a context of EDA. This technique allows rapid and efficient fractionation while keeping the molecules in a solvent compatible for the bioassay (or transferable to a compatible one). High-Performance Liquid Chromatography (HPLC) is the most frequently used technique for EDA studies (e.g. Grung *et al.*, 2011; Creusot *et al.*, 2014; Fetter *et al.*, 2014) as it is available in most environmental laboratories and compatible with semi-preparative columns (allowing higher injection volumes than the analytical columns for a better link with the bioassay, Fang *et al.*, 2014). HPLC also allows fractionation of the molecules according to different properties by choosing different stationary phases. Normal-phase (NP) and reversed-phase (RP) HPLC can both be used for EDA. NP-HPLC uses a polar stationary phase and a non-aqueous mobile phase and is efficient to separate non-polar and polar chemicals. RP-HPLC uses a non-polar stationary phase and an aqueous (and at least partially polar) mobile phase. RP-HPLC is the most commonly used technique, usually with a gradient of water and methanol or acetonitrile as mobile phase (e.g. Hewitt *et al.*, 2000; Grung *et al.*, 2007; Lübcke-von Varel *et al.*, 2012). NP-HPLC can be a powerful tool to fractionate compounds in sediment extracts for example (Kaisarevic *et al.*, 2009; Fang *et al.*, 2014). Examples of stationary phases used in EDA studies are given in the Table 1.

Table 1: Examples of reversed-phase (RP) and normal-phase (NP) HPLC columns used in effect-directed analysis studies.

	Stationary phase	Mobile phase	Type of sample	Reference
RP-HPLC	C18	Methanol/Water	Wastewater	Grung *et al.*, 2007
	C18	Methanol/Water	Liver extract	Hewitt *et al.*, 2000
	C18	Methanol/Phosphate buffer	Water	Schulze *et al.*, 2010
NP-HPLC	Aminopropyl	Hexane/ Dichloromethane	Sediment	Regueiro *et al.*, 2013
	Diol	Dichloromethane/ Methanol	Sediment porewater	Fang *et al.*, 2014
	Nitrophenylpropyl, cyanopropyl, and porous graphitised carbon (in series)	Hexane	Sediment	Kaisarevic *et al.*, 2009

As a preliminary fractionation step, simpler techniques such as solid phase extraction (SPE) or soxhlet extraction can also be used (the fractions being eluted with different solvent, e.g., Thomas *et al.*, 1999; Zaja *et al.*, 2013). This allows a simple fractionation (usually resulting in just a few fractions) at the same time as the sample is extracted. Since fractionation starts at the same time as extraction, the throughput of the study is therefore enhanced. After testing the resulting fractions, a finer fractionation step can be used on the toxic fraction (or fractions if more than one fraction shows a biological effect). Another way of avoiding repeated fractionation steps is to perform a unique and very fine fractionation. This was the approach chosen by Booij *et al* (2014) when they identified photosynthesis inhibitors in Dutch surface water samples. In this study only one fractionation step was performed by ultra-performance liquid chromatography, collecting a single fraction every twenty seconds directly in a microplate with the help of a fraction collector. Following extraction, water samples were directly fractionated into 150 fine fractions making non-targeted analysis possible in each fraction where a biological response was detected.

3.2. Chemical tools for the identification step

Chemical analysis of the toxic fractions aims to identify all the compounds present in these fractions. The chemical analysis in EDA is therefore non-targeted. Different approaches and instruments can be used for this purpose (such as the nuclear magnetic resonance spectroscopy) but the most common one is mass spectrometry (MS). MS can be coupled with both LC and gas chromatography (GC). The choice of chromatographic technique, as well as the type of MS, is essential in terms of type of compounds possible to identify. For example,

if polar or relatively large compounds, such as many pharmaceuticals (or their transformation products), are suspected to be the origin of the biological effect, then LC-MS is an appropriate technique to identify such compounds. Because of this particularity, some EDA studies combine LC and GC in order to optimise the identification step (e.g. Grung *et al.*, 2007). Different MS are available, having also different characteristics. Integrating analysers (such as the Time of Flight (ToF) and Orbitrap) can detect all ions in a specified range whilst non-integrating analysers (such as quadrupoles and ion traps) can only detect previously specified ions. This is why in EDA studies, ToF and Orbitrap are the most commonly used MS. The ToF-MS analyse the particles by measuring their mass-to-charge ratio which is determined by their ToF between two points (the ion source and the ion detector for example). The advantage of ToF-MS is the low limits of detection (between 1 and 10 pg) achievable when performing a full scan analysis (which is very interesting for the identification of unknown compounds). An Orbitrap mass spectrometer measures the frequency in which ions oscillate around the inner electrode (characteristic to their mass-to-charge). In EDA studies, ToF-MS have been successfully coupled to both GC (*e.g.* Thomas *et al.*, 2009) and LC (*e.g.* de Hoogh et al., 2006). Thus far Orbitrap spectrometers in EDA studies have only been coupled to LC systems (*e.g.* Hogenboom *et al.*, 2009; Weiss *et al.*, 2011). When a compound is truly unknown, the process to find its structure is tedious and complex (Krauss *et al.*, 2010). Therefore, to simplify this step another approach is usually used: identification of the compounds by matching mass spectrum in large library databases. This approach is fast and easy to use for non-experts but it assumes that the compounds present in the toxic fractions are already in library. When using this technique, truly unknown compounds cannot be identified. Huge databases are available today (such as the National Institute Standards and Technology, or NIST, database containing more than 200 000 compounds) but even with such big number of molecules registered, usually lot of peaks do not have a sufficient match with another spectrum to identify them.

Following successful identification a list of candidates responsible for the effect can be established. The last step of the EDA is to link these candidates to the observed effect. This step is important and can be divided into several phases (Brack *et al.*, 2008): the first phase is to make sure that the compounds identified through library search are really the right ones (the analytical confirmation) and the other phase is to investigate the toxicity of the confirmed compounds and the hazardous of such compounds in a realistic scenario. Although it is

usually difficult to explain all the effect observed at first, the EDA approach can be very successful. For example, Thomas *et al.* (2002) identified 99% of the androgenic activity observed in estuaries in the United Kingdom.

4. High-throughput effect-directed analysis

EDA studies are typically time consuming and difficult to carry out because of the complexity of the approach in integrating both biological and chemical tools. To facilitate EDA studies, high-throughput protocols have been developed using different approaches. Booij *et al.* (2014) for example combined a very fine fractionation step (fractions were collected every 20 seconds by reversed-phase ultra-high performance liquid chromatography with a fraction collector suitable for 96-well plates) with a rapid and directly compatible bioassay in order to assess algal photosynthesis efficiency (the PAM fluorometry assay). Indeed, with this assay the PS II efficiency of microalgae can be quickly measured in each well of the plate where the fractions were collected. This allowed the fractionation and biotesting of several samples to be achieved in a short time as well as a successful identification step. The advantage of very fine fractionation is to produce in one step fractions simple enough to analyse by non-target chemical analysis (when most of the EDA studies use at least two fractionation steps). More recently, Oyang *et al.* (2016) also adopted a unique fractionation step by two dimentional LC. Furthermore, by splitting the flow rate after the two LC columns, the chemical identification of the compounds was performed online. This resulted in a high-throughput EDA study which identified new AChE inhibitors in a WWTP effluent. Another way to enhance the throughput of an EDA study would be to perform the bioassay analysis on-line. For example, De Jong *et al.* (2006) developed an on-line AChE assay following RP-HPLC fractionation and prior to the MS detector. Falck *et al.* (2010) also developed an approach using an on-line bioassay post HPLC column to detect the inhibition of the mitogen-activated protein kinase p38. With their protocol, the detection of the PS II inhibitors was done simultaneously by splitting the flow between the assay reactor and the MS detector. If these protocols with on-line bioassays have advantages (higher throughput with a direct identification of the toxic compounds) they haven't been implemented in EDA thus far. The first drawback to this type of protocol is that bioassays with long incubation times are difficult to use with such set-up, thus limiting the type of assay that can be used.

II. Aim and objectives

EDA studies require advanced biological and chemical tools. They are therefore time-consuming and complicated to perform. The main aim of the present thesis was to use existing tools and to develop new ones in order to facilitate high-throughput EDA. The first approach used was to develop high-throughput and automated bioassays compatible with EDA. This step led to the development of two different bioassays: the AChE assay and the TTR binding assay. For this step, new protocols were developed, tested, and compared to already existing ones. In order to prove the usefulness of such protocols, the AChE assay was then used for an EDA study on produced water samples. Different EDA approaches have also been tested and will be compared in the present thesis. An EDA study with a fine and unique fractionation step coupled with a high-throughput assay (the PAM fluorometry assay) has been used while another EDA study have been performed with an algae growth inhibition test coupled to both LC and GC-MS. The secondary aim of the thesis is to propose a high-throughput EDA protocol adaptable for various samples based on the experience from all the EDA studies performed.

III. Methods

1. Effect-directed analysis protocols

This chapter will give an overview of the EDA protocols followed for the different studies and the following sections will describe the methods in detail.

First, the EDA of produced water (Paper II) was carried out with an open column liquid-solid chromatography fractionation followed by an HPLC fractionation together with the newly automated AChE assay. Identification of the compounds in the toxic fractions was performed by GC-HRToF-MS (GCT Premier, Waters, USA) followed by analytical confirmation.

The EDA carried out on a landfill leachate sample in order to identify compounds toxic to microalgae (Paper IV) was performed using a simple fractionation by SPE (generating three fractions) followed by a fine UHPLC fractionation yielding 88 fractions. During this study, the PAM fluorometry assay was used to detect PS II inhibition. The chemical analysis of the toxic fractions was performed by on-line LC-micrOToF-MS (ESI, Bruker Daltonics, Bremen, Germany) as well as off-line GC-HR-ToF-MS.

For the last EDA study, a large volume SPE (LVSPE) device was used to take a waste water treatment plant (WWTP) effluent. The LVSPE cartridges were eluted at different pH and followed by a SPE fractionation generating 9 fractions. The toxic fraction was further fractionated by HPLC (on a C18 column) and chemical analyses were carried out by both LC and GC-MS. A miniaturised bioassay to detect algal growth inhibitor was performed to identify compounds inhibiting algal growth (Paper V).

2. Sample sites

Different samples were taken and tested for the thesis. Most of the samples were taken from different locations in Norway. For papers I and IV, three discharge samples were used: a landfill leachate, a tunnel construction run-off, and a WWTP effluent. The leachate sample was collected in July 2013 from a municipal waste (Lindum) located in the Drammen municipality. The site receives solid waste from a large municipal area (residual waste, screening and sand trap wastes, asbestos containing waste, blow sand, compost). The various origins of contaminants make such sample highly polluted with different types of compounds (Slack *et al.*, 2005; Salem *et al.*, 2008). The WWTP effluent sample was collected in July

2013 from VEAS (Vestfjorden Avløpsselskap) at Slemmestad (Norway). The sample was taken following chemical and biological treatment and before being discharge into the environment. Such discharge is known to contain various organic compounds coming from human activities (Kolpin *et al.*, 2002). Finally, a sample from a tunnel construction site was taken in Espa (60.3450N 11.16228E, near the lake Mjøsa in Norway). The use of explosives can contaminate this water and therefore it is treated onsite by 6 different sedimentation basin The sample was taken after all the treatments just before discharge into the lake. This type of sample is not frequently studied but had already shown some potential impacts (Weideborg *et al.*, 2001; Vikan and Meland, 2013).

For the Paper II, produced water samples (5L) were taken from two offshore stations (Gullfaks B and Statfjord C) in the Norwegian sector of the North Sea just before been discharged into the sea. Produced water has shown the potential to inhibit AChE (Holth and Tollefsen, 2012) and the samples were therefore selected to perform an EDA to identify AChE inhibitors in such discharge.

Finally, the only sample collected outside of Norway was a WWTP effluent from Brno-Modřice (in Czech Republic, 49.12447N, 16.62697E) and was collected in mid-August 2014 (Paper V). As described earlier, WWTP effluents can contain various organic contaminants and this particular effluent was selected because of its effect observed on algae (Paper V).

3. Sample extraction

The water samples used for this thesis were extracted following two main protocols. The produced water samples were extracted by solid phase extraction (SPE) on an octadecylsilane (C18) cartridge (Paper II). This extraction (as well as the first fractionation by open column chromatography) was performed the same way as Holth and Tollefsen (2012) who detected the presence of AChE potential inhibitors in produced water from platforms in the North Sea.

The other water samples were extracted either on-site or in the laboratory following collection by the same principle. The samples extracted on-site (Paper V) where extracted with the help of a large volume solid phase extraction (LVSPE) device (also used by Liška *et al.*, 2015). With this device, the water (50L) was pumped through three SPE cartridges in series: the first was a Chromabond HR-X (8 g) aimed to retain mainly neutral compounds. The second cartridge was a Chromabond HR-XAW (4 g) to retain mainly acidic compounds while the

third cartridge (Chromabond HR-XCW, 4 g) was used in order to retain basic compounds. The cartridges were eluted in the laboratory with a solution of methanol and ethylacetate for the HR-X cartridge, a solution of methanol containing 1.7% formic acid for the HR-XAW cartridge, and a solution of methanol with 1.7% ammonia for the HR-XCW cartridge. The other water samples were extracted following the same principle but in laboratory following collection of the samples (Papers I, III, and IV) and not using the LVSPE device in situ. The cartridges (Chromabond HR-X, HR-XAW, and HR-XCW, 200 mg, 100 mg, and 100 mg respectively) were connected in series and on all occasions, 1L of water was extracted. The cartridges were eluted with the same elution solvent mixtures as described earlier. Pictures of both LVSPE device and the cartridges set-up in laboratory are shown in the Figure 2. This approach was carried out in order to fractionate the samples and start isolating compounds inducing a response in the chosen bioassay (Liška *et al.*, 2015).

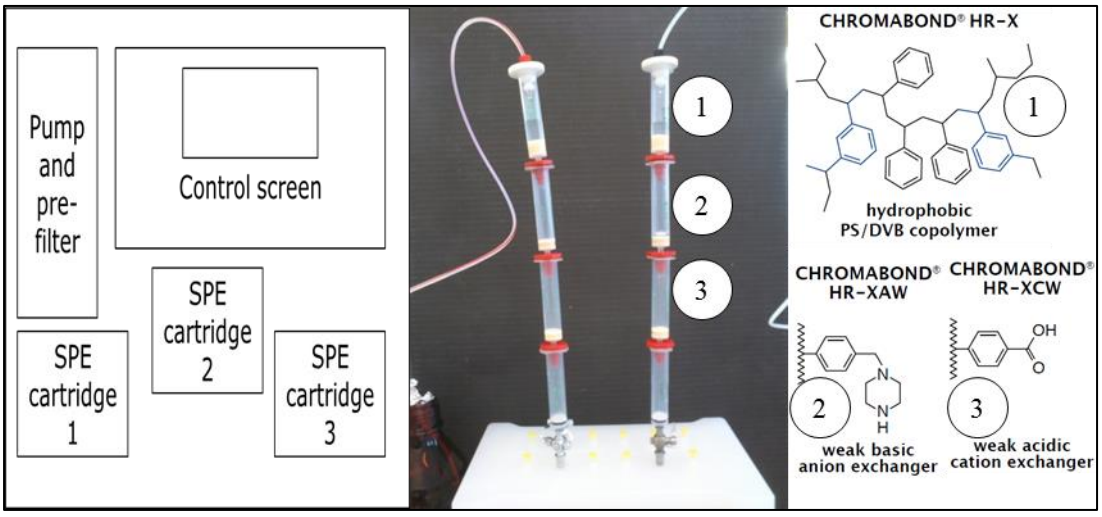

Figure 2: schematic representation of the large volume solid phase extraction (LVSPE) sampling device (left), picture of the solid phase extraction cartridges set-up (middle) made to mimic the LVSPE in laboratory, and sorbent details (right). In both cases three sorbents were used: an hydrophobic copolymer (Chromabond HR-X, #1), an anion exchanger (Chromabond HR-XAW, #2), and a cation exchanger (Chromabond HR-XCW, #3).

Following extraction, aliquots of the extracts were transferred into a bioassay compatible solvent dependent on the bioassay used: dimethyl sulfoxide (DMSO) for the AChE inhibition assay and the TTR binding assay, and f/2 medium for the PAM fluorometry assay.

4. Fractionation procedures

In the present thesis, different fractionation techniques have been used for the different EDA studies performed. The produced water extracts were first fractionated using the same technique used by Holth and Tollefsen (2012): open column liquid solid chromatography using silica gel and alumina oxide (which were heated overnight and deactivated by the addition of water, 5% for silica and 1.5% for alumina). Three fractions were eluted with three different solvents (hexane, dichloromethane, and methanol). The fraction exhibiting the greatest activity was then further fractionated by semi-preparative HPLC using a phenyl-hexyl stationary phase (10 mm x 250 mm of length, Phenomenex, Inc., Torrance, USA) with a gradient of methanol and water (starting with 75% methanol to finish with 100% methanol over 30min before having 5 mins at the starting conditions, Paper II). HPLC (or UHPLC) fractionation was also used in all EDA studies performed for the present thesis. For the study on PS II inhibitors (Paper IV), UHPLC fractionation was carried out on a C18 column with a gradient starting with 10% acetonitrile and 90% water to increase until 100% acetonitrile in 40 min (and stayed at this composition for 10 minutes). 20µL of extract was injected and 88 fine fractions were collected directly into a 96-well microplate by using an automated fraction collector. After addition of 50 µL of glycerol at 35mg/mL in each well, the UHPLC solvents were evaporated using a CentriVap concentrator (Labconco, Kansis City, USA) at 40°C for 6 h. After evaporation, 30 µL f/2 medium was added to each well for the bioassay. For Paper V the HPLC fractionation was preceded by an SPE fractionation step where the extract was separated according to polarity (following the same protocol as Thomas *et al.*, 1999). SPE C18 cartridges (C18-Sep- Pak®, 1g, Waters, Ireland) were used and several extractions were carried out in series. The first SPE fraction was eluted using only pure water while the second fraction was extracted with 25% of methanol, 75% of water. SPE fraction 3 was eluted with 50% of methanol and 50% of water. The methanol contents of the solutions used for the elution of the fractions 4, 5, 6, 7, 8, and 9 equals to 75%, 80%, 85%, 90%, 95%, and 100%, respectively. After evaporation under a gentle nitrogen stream, fine HPLC fractionation was then performed on a Thermo Hypersil (Phenomenex, Værløse, Denmark) BDS C18 column (250x10 mm, particle size 5µm). A gradient of methanol and water was applied for 35 minutes (starting with 60% of methanol, until 100% of methanol in 30 minutes and 5 minutes at 100 % of methanol) and fractions were collected every 30 seconds which provided 31 fractions.

5. Biological tools developed for EDA

Two bioassays were further developed to enhance their throughput: the AChE inhibition assay and the TTR binding assay. And the PAM fluorometry assay was also used for the EDA study on PS II efficiency inhibitors.

5.1. The acetylcholine esterase (AChE) inhibition assay

For the AChE inhibition assay, the production of 5-thio-2-nitrobenzoic acid (TNB, catalysed by the AChE) is followed by absorbance measurements at 405 nm. At the beginning of the experiment, AChE in tris-buffer is incubated for 30 minutes at room temperature with the inhibitor or the extract tested in DMSO. For this experiment the incubated enzyme and inhibitors (or extracts) were split in triplicates. The production of TNB started when acetylthiocholine (ATC) and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were mixed together. The enhancement of absorbance was followed for 10 minutes with a microplate reader (EMax microplate reader with SoftMax Pro 5® software; Molecular Devices, LLC, Silicon Valley, CA, United States). When present, AChE inhibitors will decrease the production of TNB and therefore the absorbance intensity. The new protocol developed for this thesis used an automated workstation (Figure 3, Paper I).

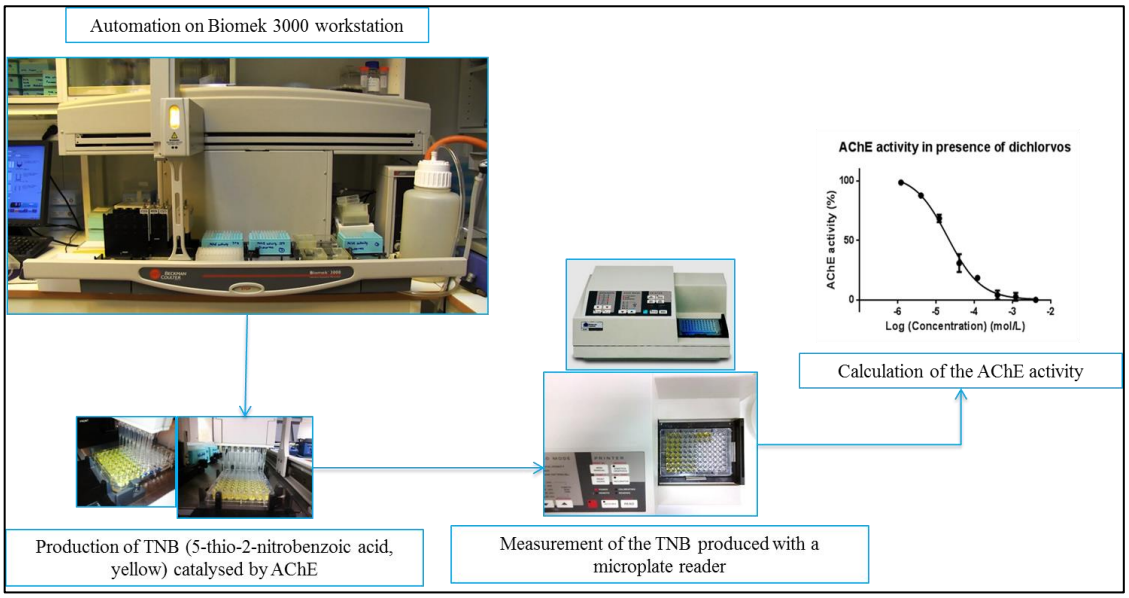

Figure 3: schematic representation of the high-throughput AChE inhibition assay developed for this thesis.

5.2. The transthyretin (TTR) binding assay

The TTR binding assay was further developed for its use in micro-well plates using a fluorescence probe. The aim of this improvement was to enhance the assay throughput and reduce the assay cost when compared to the traditional protocol using a radioactive ligand or a fluorescent probe in cuvette (Paper III). For this assay, fluorescent labelled T_4 (FITC-T_4) was synthesised by using a binding reaction between T_4 and fluorescein isothiocyanate (FITC) in a pyridine/water/triethylamine medium (9:1.5:0.1, v/v/v) for one hour at 37°C following the protocol published by Smith (1977). Following a series of purification steps, the final product (FITC-T_4) was freeze-dried for 48 h (under 0.7 mbar, at -20 °C). Before its use, the FITC-T_4 was dissolved in Tris-NaCl buffer and its concentration measured by absorbance at 490 nm using a Smartspec 3000 spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). For the experiment, 96 well plates were used. In the well, the FITC-T_4 was incubated with TTR for 5 minutes at room temperature. When incubated with TTR, the fluorescent intensity produced by the bonded FITC-T_4 was enhanced making it possible to calculate the amount of FITC-T_4 bonded with TTR. After incubation, the tested chemical or extract was added. A thyroid hormone disrupter able to bind to TTR will decrease the fluorescence intensity making it possible to measure its impact on TTR (Ren and Guo, 2012). The fluorescence intensity was monitored at 490 nm for the excitation and 518 nm for the emission using a Varioskan Flash multimode plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

5.3. The Pulse Amplitude Modulation (PAM) fluorometry assay

The PAM fluorometry assay used was the same as Sjollema *et al.* (2014b): microalgae *Dunaliella tertiolecta* were incubated 4.5h with extracts or HPLC fractions before the effective photosystem II efficiency (ΦPSII) was measured using a WATER-PAM detection device (Heinz Walz GmbH, Effeltrich, Germany). The minimum and maximum fluorescence (F and F'm respectively) were determined and ΦPSII was calculated as followed:

$$\varphi PSII = \frac{F'm - F}{F'm} \qquad \text{(Eq.1)}$$

This protocol has been developed to detect herbicides harming microalgae in water samples and has also been used in intensive studies (Booij *et al.*, 2014).

6. Analytical tools.

Different analytical techniques were used for the EDA studies performed in the present thesis: GC coupled to high resolution time of flight mass spectrometer (GC-HRToF-MS, carried out on a gas chromatography system, GCT Premier, from Waters, Milford, MA, USA, coupled to a high-resolution mass spectrometer), LC coupled to a quadrupole time of flight mass spectrometer (LC-QToF-MS, carried out on an Acquity UPLC System coupled to a Xevo™ G2-S QTof mass spectrometer from Waters, Milford, MA, USA), and a UHPLC system connected to a micrOTOF time of flight MS (Bruker Daltonics, Bremen, Germany). All the techniques were used to identify compounds present in the toxic fractions by non-target analysis. Different approaches were followed in all cases. For the GC analyses the peaks were selected manually following background substraction, and the mass spectrum extracted. This spectrum was then used to look for matches in the National Institute of Standards and Technology (NIST, Gaithersburg, Marylend, USA) library. For LC analysis the peaks were selected automatically using the MarkerLynx tool found in the MassLynx software (Waters, Manchester, UK). This tool allowed the selection of all the peaks above the desired intensity threshold. Non-toxic fractions were also injected and their peak of interests selected with MarkerLynx on the LC. This allowed a statistical analysis of the peaks by principal component analysis to discriminate peaks only occurring in the toxic fractions from the others. These peaks were then compared to the STOFF-IDENT database (Huckele and Track, 2013) for matches using their retention time and accurate masses.

7. Data interpretation and statistical treatment

All data obtained from the different bioassays was analysed using the software GraphPad Prism 6 (GraphPad software, La Jolla California, USA). During the automation of the AChE inhibition assay the relative standard deviations (RSDs) have been calculated by dividing the standard deviation by the mean value of the IC50 (concentration where 50% of inhibition has been observed). The RSD intra-assay (using the data from three different experiments) and inter-assay (using the data from replicates of the same experiment) were used to understand the changes caused by the automation. For the AChE inhibitors (dichlorvos and paraoxon), statistical comparison of differences between the different concentration response curves' parameters (slope, top, bottom, and IC50) obtained manually and with the automated protocol were carried out with a F-test ($p \leq 0.05$).

Data generated by the chemical analyses was processed using the MassLynx software (Waters, Manchester, UK) for both LC and GC.

7.1. Combined toxicity assessment

Simple mixtures of selected pesticides (dichlorvos and the pesticide metabolite paraoxon) were tested using the AChE inhibition assay. The results were compared to two prediction models in order to understand their mode of action (MoA) on the enzyme when the compounds are exposed in combination. The models used are the CA model (concentration addition model, which suggest that the compounds have a similar mode of action, Löewe and Muischnek, 1926) and the IA model (independent action model, which assumes that the compounds have a different mode of action, Bliss, 1939). With the CA model, the total predicted effect concentration of the mixture inducing an effect $EC_{x(mix)}$ is calculated as follows:

$$EC_{x(mix)} = \left(\sum_{i=1}^{n} \frac{pi}{ECxi}\right)^{-1} \qquad \text{(Eq. 2)}$$

Where pi is the relative fraction of the compound i in the mixture and $ECxi$ the concentration of substance i needed to induce the effect x when tested alone.

With the IA model, the effect of the mixture (E_{mix}) is calculated as follows:

$$E_{mix} = 1 - \prod_{i=1}^{n}(1 - Ei) \qquad \text{(Eq. 3)}$$

In this equation n is the number of compounds in the mixture, and Ei the effect of the substance i when tested alone.

To assess the difference between model predictions and experimental data of the mixtures, the model deviation ratio (or MDR) was calculated as follows:

$$MDR = \frac{IC50pred}{IC50obs} \qquad \text{(Eq.4)}$$

IC50pred is the predicted IC50 and IC50obs is the IC50 obtained experimentally. If the MDR is between 0.5 and 2, the combined toxicity of the mixture is assumed to be within the range expected based on experimental variance, whereas a ratio below 0.5 indicate that the chemicals in the mixture act by antagonism and above 2 indicate that the interaction is

synergistic (Belden *et al.*, 2007). Finally, to assess the fit between the model predictions and experimental data, statistical comparisons were performed: first, the normality of the data was tested with a Shapiro-Wilk test. Then, to detect significant differences between predicted and experimental data, a Kruskal-Wallis test together with a Dunn's post hoc test was carried out.

7.2. Toxicity prediction

For papers IV and V, the toxicity to algae of the identified compounds had to be estimated. For that purpose, the Ecological Structure Activity Relationships (ECOSAR) model from the U.S. Environmental Protection Agency was used as well as the US EPA Ecotox database (http://cfpub.epa.gov/ecotox/) and the available scientific peer-reviewed literature. From the collected data, the best candidates were selected and prioritized using expert knowledge.

IV. Main Results

This section will present the main results obtained during the thesis.

1. Enhanced throughput bioassays (Papers I and III)

Two bioassays were improved for use in EDA studies through using two different approaches: one was automated while the other was miniaturised. The TTR binding assay was miniaturised and the AChE assay was further developed and validated for its use on an automated workstation (Paper I) for easier and faster experiments with comparable sensitivity and specificity.

For the AChE inhibition assay, responses obtained for two known AChE inhibitors (dichlorvos and paraoxon) were compared for when the assay was performed manually and when using the automated workstation.. The concentration response curves (CRCs) obtained did not show any significant differences ($p = 0.02$ for dichlorvos and $p = 0.03$ for paraoxon) and their IC50s were similar with both protocols. A binary mixture of the two inhibitors was tested with the assay in order to assess the possibility of testing simple mixtures. The CRC obtained, as well as the analytical treatment, showed that the CA model best described the mixture behaviour. Automation had several advantages with faster analysis, an easier to handle procedure, and better repeatability than when the assay was performed manually (with lower relative inter- and intra-assay standard deviations, or RSDs). The resulting improvements could be very useful in the context of intensive studies generating lot of samples or fractions to test such as EDA.

Another option for improving the throughput of an assay is to develop a new simpler protocol for easier and faster analysis while maintaining sensitivity. Paper III proposed a new protocol for the TTR binding assay using a florescent probe for its use in microplates. The sensitivity of the new protocol was tested with compounds known to interfere with the T_4-TTR bond and their EC50s obtained with the new protocol compared to published data. For the most active compounds (having an EC50 < 100 nM with the radio-ligand assay) the sensitivity of the new protocol is about one order of magnitude lower whilst for the other compounds the sensitivity is close to the published data. For example, 4-OH-CB-107 (2,3,3',4',5-pentachloro-4-biphenylol) and triclosan showed a higher IC50 with an order of magnitude of 1 compared to

IC50s measured with the radioactive ligand (with measured IC50s of 0.24 and 0.93 µM for 4-OH-CB-107 and triclosan compared to their published IC50s of 0.024 and 2.84 µM respectively, Meerts *et al.*, 2002; Weiss *et al.*, 2015). Furthermore, PFOS (perfluorooctanesulfonic acid) and 6-OH-BDE-47 (6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether) displayed similar IC50s with the new protocol (Hamers *et al.*, 2006; Weiss *et al.*, 2009b). This protocol presents several advantages: first of all it is much faster, indeed the experiment can be performed in few minutes while with the purification steps the radioactive ligand require around 24 hours. This protocol is also cheaper, with much easier experiments easier to perform and require a simpler equipment. Indeed, the specific training required by the use of a radioactive ligand is eliminated in the new protocol as well as all the purifications steps. Finally, the herring gull egg analyses in Paper III show that the assay is applicable to measure T_4-TTR binding disruptions in environmental samples. The improvements carried out in paper III make this assay suitable for extensive studies and usable in a larger scale. The improvements obtained for the two described assays are summarised in the Table 2.

Table 2: Summary of the improvements made to the two bioassays.

Bioassay	Way of enhancing throughput	Strength from the new protocol	Test chemicals	Environmental samples tested
AChE inhibition assay (Paper I)	Automation on an automated workstation	Faster experiment Lower internal standard deviation Comparable sensitivity	Dichlorvos Paraoxon	Landfill leachate WWTP effluent Tunnel construction site
TTR binding assay (Paper III)	Miniaturisation for its use in microplates and use of a fluorescent ligand	Faster experiment No use of radio-active ligand Acceptable sensitivity Easier and cheaper experiment	2,3,3',4',5-pentachloro-4-biphenylol 6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether Tetrabromobisphenol A perfluorooctanoic acid Triclosan perfluorooctanesulfonic acid 2, 4, 6-tribromophenol Mono (2-ethylhexyl) phthalate	Herring gull eggs

2. EDA of produced water (Paper II)

An EDA study of produced water was performed in order to identify AChE inhibitors known to be present in such discharges (Holth and Tollefsen, 2012). Prior to performing the EDA study an attempt was made to identify new AChE inhibitors that are frequently found in produced water. Three polycyclic aromatic hydrocarbons (PAHs), three alkylphenols, and a mixture of naphthenic acids were for the first time tested to assess their impact on AChE. Two PAHs (phenanthrene and chrysene) and the three alkylphenols (2,6-dimethyphenol, 4-ethylphenol, and 4-propylphenol) showed the potential to inhibit AChE. Interestingly, the mixture made with the two PAHs followed the two mixture toxicity prediction models (without any significant differences from CA and IA). The mixture of naphthenic acids showed an ability to inhibit AChE at concentrations found in produced water discharges. The prediction models suggested that the present mixture of alkylphenols could affect AChE but at concentrations over the solubility limit (at concentrations higher than 0.1 M). The results also suggested that PAHs and naphthenic acids may contribute to the AChE inhibition observed with produced water discharge. An EDA study was subsequently performed to investigate whether this was the case and whether other compounds may also be responsible for these effects. The EDA study performed on produced water showed that the specific compounds identified as potential toxicity drivers were not responsible for the effect observed for the produced water sample tested. One of the selected produced water sample exhibited the same potential of inhibiting AChE as described by Holth and Tollefsen (2012). The medium polar fraction (obtained by open column liquid solid chromatography and eluted with dichloromethane) was indeed the most potent fraction. This fraction was therefore selected for further fractionation (by HPLC) and for a complete EDA study. Out of the 31 HPLC fractions, 5 of them showed the presence of potential AChE inhibitors. In one of these toxic fractions, four compounds were identified and analytically confirmed. From these candidates, two of them (butylated hydroxytoluene, and 4-phenyl-1,2-dihydronaphthalene) are able to cause AChE inhibition at low concentrations. Finally, saithe (*Pollachius virens*) caught near two offshore platforms showed lower AChE activity than those collected from a location assumed to not be affected by emission from oil or gas production platforms. Targeted analysis of saithe for the newly identified AChE inhibitors detected butylated hydroxytoluene, thus showing its potential to accumulate in aquatic organisms. However, the cause for the reduced enzymatic activity in the tissues was not clearly identified.

3. EDA of a landfill leachate to identify algal PS II inhibitor (Paper IV)

This study was performed to identify PS II inhibitors present in various discharges. Three different samples (a landfill leachate, a WWTP effluent, and a tunnel construction site run-off) were fractionated and analysed for PS II inhibitory potential using the PAM fluorometry assay. The sample from a landfill in Norway was selected for further EDA in order to identify the photosynthesis inhibitors present, since it appeared to contain potential photosynthesis inhibitors. From the 88 UHPLC fractions generated, 28 of them exhibited PS II inhibition greater than 80 % with low standard deviation (<10%). In these toxic fractions several compounds were detected but following testing and analytical confirmation, only one of them (imazamethabenz-methyl) showed the potential to give a response with the PAM fluorometry assay and only at very high concentrations (with an EC50 of 390 mg/L). Since this compound could not provide an explanation for the observed toxicity, additional chemical analyses by GC-HRToF-MS were performed. This additional step allowed the identification of more compounds suspected to have a role in the effect observed. These suspected toxicants could not be tested with the PAM fluorometry assay but their potential toxicity to algae was investigated using the Ecological Structure Activity Relationships (ECOSAR) model from the U.S. Environmental Protection Agency. From these results, two compounds (butyl stearate and bis(1,1,3,3-tetramethylbutyl) disulphide) were qualified as toxic to algae at low concentration while three other chemicals (thiophene-2-methanethiol, (1-bromo-2-methyl-1-propenyl)benzene, and 2-benzylquinoline) could also impact algae especially in concentrated samples such as landfill leachate. The study showed the possibility to use an already established protocol for EDA with a different type of sample with its limitation. Indeed, the on-line chemical analysis was tested in order to enhance the throughput of the study, but the lack of explanation of the observed effect on algae made necessary the use of an additional chemical analysis step by gas chromatography. From the identified compounds, the presence of such variety of compounds shows the complexity of landfill leachate and the numerous possible sources to contaminate the leachate recipient environment (Slack *et al.*, 2005). This enhanced throughput EDA study already proved to be efficient to identify hazards to microalgae in water bodies (Booij *et al.*, 2014) and now in a municipal landfill leachate. The same protocol could also be followed with another bioassay compatible with microplates (such as the AChE inhibition assay, or the miniaturised TTR binding assay) in order to identify other toxicants in complex environmental samples.

4. Identification of algal growth inhibitors in treated wastewater by effect-directed analysis (Paper V)

This study was performed to identify algal growth inhibitors present in the discharge from a WWTP in the Czech Republic. A miniaturised assay to measure algal growth in 96-well microplates was used as described by Geis *et al.* (2000). Two of the nine SPE fractions of the toxic LVSPE eluted fraction showed a high growth inhibition with the assay used. Since these SPE fractions were eluted one after the other, they were recombined and further fractionated by HPLC (which generated 31 HPLC fractions). From these HPLC fractions, three of them showed significant effect on algae. The potent fractions were analysed by LC-ToF-HR-MS and GC-ToF-HR-MS following the protocol described in the method section. Additional two-dimensional GC non-target analysis was also performed on the toxic fractions. From the chemicals identified in the toxic fractions, 25 have been assessed as potential algae growth inhibitors. Chemical analysis of the same effluent taken a year before and without any SPE and HPLC fractionations identified 10 toxicants to algae, with 2 of them also present in the EDA toxic HPLC fractions. This study showed the purpose of the chemical fractionation in order to facilitate non-targeted chemical screening. Finally, it also showed the importance of monitoring WWTP effluents, not only by targeted chemical analysis but also with bioassays to assess the overall toxicity of such discharge.

5. Summary of the EDA studies performed

Three different environmental samples have been analysed using EDA approach in order to identify toxicants (Table 3). As described in the previous section, different extraction and fractionation techniques were performed as well as different bioassays and chemical analysis techniques. The number of identified compounds increased when both LC- and GC-MS instruments were used for the chemical screening. The three bioassays were selected for their enhanced-throughput and because environmental samples similar to the ones studied already showed responses with these particular assays. From the three assays used, only one is an *in vitro* assay (the AChE assay) whilst the two others use primary producers (algae).

Table 3: Summary of the EDA studies performed

Paper	Matrix	Fractionation technique	Bioassay performed	Chemical analysis carried out	Study conclusion
II	Produced water discharge	• open-column liquid solid chromatography • RP-HPLC	Automated AChE inhibition assay	GC-HR-ToF-MS	Two new AChE inhibitors identified (butylated hydroxytoluene and 4-phenyl-1,2-dihydronaphthalene)
IV	Landfill leachate	• 3 SPE cartridges • RP-UHPLC	PAM fluorometry assay	On-line LC-micrOToF-MS and off-line GC-HR-ToF-MS	10 compounds suspected to contribute to the toxicity
V	WWTP effluent	• 3 SPE cartridges • SPE fractionation • RP-HPLC	Miniaturised algae growth inhibition assay	LC- and GC-ToF-HR-MS GCxGC-MS	25 identified compounds suspected to contribute to the toxicity

V. Discussion

In this section, the environmental responses measured for the thesis are summarized and contextualised within existing research in this field. Subsequently, all of the methods used to enhance EDA throughput are discussed according to the different steps included in the EDA approach (bioassay, chemical fractionation, and chemical analysis) before discussing the possibility of performing high-throughput EDA.

Three different bioassays were performed within the thesis, measuring different endpoints in five different environmental samples with the effects summarised in Table 4. This led to three EDA studies being performed. An EDA study was performed on produced water discharged from off-shore oil and gas production platform in order to identify AChE inhibitors. Another was performed on a municipal landfill leachate to identify algal PS II inhibitors, and a third study was performed on a WWTP effluent in order to identify algal growth inhibitors.

Table 4: Summary of the environmental responses measured during the thesis.

Thesis paper	Sample	Target	Effect measured	EDA performed?
I	Landfill leachate	AChE activity	11% inhibition	No
II	Produced water	AChE activity	69% inhibition	Yes (2 AChE inhibitors identified)
III	Herring gull eggs	TTR binding	Between 11% and 91% binding inhibition	No
IV	Landfill leachate	Algae PS II efficiency	12% inhibition	Yes (10 toxicants to algae identified)
V	WWTP effluent	Algal growth	100% inhibition	Yes (25 toxicants to algae identified)

In the following sections, the effects measured in the samples as well as the identified chemicals will be discussed.

1. Characterising the cause of AChE inhibition in produced water and landfill leachate samples

The occurrence of AChE inhibitors was evaluated in a landfill leachate and two produced water discharges (Papers I and II). In the most toxic fractions 11% and 69% of the enzyme activity was inhibited in the landfill leachate and the produced water samples, respectively. For the produced water samples, the highest response was observed in the medium polar

fraction (around 70% inhibition observed in Paper II). This is similar to a previous study by Holth and Tollefsen (2012) where they reported around 60 % inhibition in the medium polar fraction (referred to as the "aromatic fraction" in their study). However, when analysing complex environmental samples the co-extraction of dissolved organic matter should be considered. Neal and Escher (2013) showed that dissolved organic matter can mask the effect of AChE inhibitors. It may therefore not be possible to detect inhibitors with the AChE inhibition assay in certain martices even if they are present should they be extracted together with dissolved organic matter. This could be one reason as to why the total produced water extract did not significantly inhibit AChE with inhibiton first detected following the first fractionation (Holth and Tollefsen, 2012; Paper II). Different changes in enzymatic kinetics have been reported in presence of the toxic fractions in the two papers (with a reduced maximum hydrolysis rate, $V_{max,}$ and an unchanged Michaelis-Menten constant, $K_{M,}$ in presence of the toxic fractions). The two studies (Holth and Tollesfen, 2012; Paper II) show that it is more likely that different types of AChE inhibitors (with dissimilar MoAs) are released into the North Sea from off-shore oil and gas production platforms. This suggests that traditional (with the same MoA as organophosphates and carbamates) and un-traditional inhibitors (with other MoAs such as PAHs or antidepressant and antipsychotic drugs) are released into the environment. AChE inhibition has previously been measured in produced water (Holth and Tollefsen, 2012) as well as in municipal landfill leachate (Tsarpali and Dailianis, 2012) but to our knowledge no previous identification of the inhibitors has been performed in these types of effluents. In this study, two AChE inhibitors (butylated hydroxytoluene, and 4-phenyl-1,2-dihydronaphthalene) were identified in produced water effluent following EDA (Table 5). As hypothesised by Holth and Tollefsen (2012) these compounds contain aromatic rings but surprisingly do not possess the structural characteristics of traditional AChE inhibitors such as organophosphates and carbamates (Fukuto, 1990). Indeed, the two identified compounds do not feature any carbamate ester, carbamic acid, or phosphate ester functional groups. 4-phenyl-1,2-dihydronaphthalene was estimated to be responsible for 8% of the overall toxicity of the produced water sample on AChE while butylated hydroxytoluene for 4%. AChE inhibitors can be harmful for aquatic organisms (Bocquené and Galgani, 1998; Oropesa *et al.*, 2007) and compounds other than organophosphates have previously been reported to inhibit AChE. For example, Frasco *et al.* (2005) reported that metals can inhibit AChE and Kang and Fang (1997) showed that PAHs

can also potentially be AChE inhibitors. Other compounds containing aromatic rings (such as antidepressant drugs) have shown their potential to inhibit the enzyme (Nunes-Tavares *et al.*, 2002). Nunes-Tavares *et al.* (2002) showed that these inhibitors do not act as competitive inhibitors unlike the traditional AChE inhibitors. The same MoA was described for the two compounds identified by EDA (Paper II). The two identified compounds are similar to other compounds found in produced water (i.e. contain aromatic rings, McCormack *et al.*, 2001) and one of them (butylated hydroxytoluene) is most likely to be an injected chemical (chemicals injected in the natural reservoir to ensure safer operation and better recovery of the oil). Indeed, butylated hydroxytoluene is used to prevent the oxidation of fluids such as oil. This compound has not been reported in other produced water discharges but is known to be use in synthetic oil as well as in personal care products such as skin-whitening creams (Xiang *et al.*, 2015; Galimany-Rovira *et al.*, 2016). In general, butylated hydroxytoluene is also used as additive in food and plastics for its antioxidant properties (Williams *et al.*, 1999; Tombesi and Freije, 2002). Tombesi and Freije (2002) showed its potential to migrate from the plastic to the drinking water and its effect on helath is contradictory depending on the study (Williams *et al.*, 1999). Thus far, 4-phenyl-1,2-dihydronaphthalene was not reported in any environmental studies but was reported as biproduct or transitional product during the synthesis of organic compounds such as pharmaceuticals (Kashima *et al.*, 1991). It is therefore possible to hypothesis that this compounds was not intentionally injected to the reservoir but was naturally present in the extracted oil.

The occurrence of known produced water components in fish, such as alkylphenols (and their metabolites), has previously been reported (Beyer *et al.*, 2011; Beyer *et al.*, 2012), showing the potential of compounds discharged from off-shore platforms to impact aquatic organisms. In Paper II, the AChE activity measured in fish tissue from a platform-free location and from fish caught near two platforms in the North Sea were compared. Results showed lower activity in saithe caught near the platforms compared to saithe form the platform-free location. Chemical analysis of the tissue was carried out to investigate if the occurrence of butylated hydroxytoluene, and 4-phenyl-1,2-dihydronaphthalene could explain the low AChE activity observed near the off-shore platforms. The two compounds were not detected in fish caught near the off-shore platforms but butylated hydroxytoluene was detected in a fish from the platform-free location showing its ability to find its way into fish. So far the only demonstrated impact of the oil and gas industry on AChE activity of organisms is via

accidental oil spill (Oropesa *et al.*, 2007). Indeed, Oropesa *et al.* (2007) reported lower AChE activity in seabirds such as guillemot and razorbill that were exposed to the Prestige oil spill in Galicia than in unexposed seabirds. However, Paper II reasserts the potential of EDA for the identification of toxicants by combining biotesting and chemical analysis. Investigating further the risk caused by the two identified compounds on the possible organisms exposed (such as fish) and measuring the concentration of these compounds in other produced water discharges would be useful to assess the potential risks caused by these compounds. Nevertheless, as this study showed, non-traditional AChE inhibitors are also potential toxicants for aquatic organisms and confirms that the identification of AChE inhibitors is important and that effect-based techniques such as EDA can be very useful in this perspective. Other effect-based studies have identified various toxicants in produced water. For example, Thomas *et al.* (2009) successfully identified naphthenic acids as estrogen receptor agonists and PAHs and alkylphenols as androgen receptor antagonists. Furthermore, EDA studies to identify AChE inhibitors have though been successfully performed on other samples. For example, Ouyang *et al.* (2016) identified the pharmaceuticals tiapride, amisulpride, and lamotrigine as AChE inhibitors in a WWTP effluent.

Table 5: AChE inhibitors identified by EDA in produced water

Name	CAS number	Class	Known use
Butylated hydroxytoluene	128-37-0	Phenol-derivate	Prevent oil oxidation, food and plastic additive
4-phenyl-1,2-dihydronaphthalene	7469-40-1	PAH	Undocumented

2. Algal growth inhibitors and PS II inhibitors

Two different EDA studies were performed to identify toxicants to algae in different samples. The objective was to test different EDA protocols as well as different bioassays in order to help understand in which areas EDA throughput could be enhanced. Compounds toxic to algae were detected in the landfill leachate sample as well as in WWTP effluent (Papers IV and V). In both cases, different endpoints were measured (PS II efficiency and growth inhibition) and an attempt made to identify the causes of the observed effects (Table 4). Being aquatic primary producers, negative effects on algae can impact organisms from higher trophic levels (Fleeger *et al.*, 2003). Bioassays using algae were developed and standardised

because they are easy to implement, inexpensive, and have a good sensitivity (Nyholm and Källqvist, 1989). For these reasons, algal bioassays are commonly included in EDA studies (e.g. Schulze *et al.*, 2010; Booij *et al.*, 2014).

Ten compounds that were toxic to algae were identified in Paper IV (Table 6) and their toxicity predicted using the ECOSAR model. This model was chosen for its availability and its applicability with compounds toxic to algae. Although not specific to PS II inhibition (but assess the general toxicity to algae), this model has previously been proven to efficiently predict the toxicity to algae of various class of compounds (Sanderson *et al.*, 2003). Whilst this model can have difficulties predicting the algal toxicity of compounds such as aldehydes, Sanderson *et al.* (2003) showed that in general the model was a good indicator of the impact a compound can have on algae. That is why, in the absence of the possibility to perform biological confirmation with the PAM fluorometry assay, this model provided an overview of the toxicity of the compounds identified by non-target screening to algae. The list of the identified compounds is detailed in Table 6. Some of the identified compounds are registered by ECHA (thiophene-2-methanethiol, bis(1,1,3,3-tetramethylbutyl) disulphide, 1-bromo-3-methylcyclohexane, and butyl stearate) and their toxicity has therefore already been investigated (some are toxic to the respiratory system for example) but never previously to algae. The structures of these compounds are not very similar to traditional PS II inhibitors (Booij *et al.*, 2014; Sjollema *et al.*, 2014b) as they lack some specific structural characteristics (such as the triazine heterocycle) and in most of the cases being smaller. These compounds are used by the food, pharmaceutical and cosmetic industry. Other compounds such as pesticides (imazamethabenz-methyl, benzofuran,2,5-diacetyl-6-methoxy-, and 4-ethyl-4H-1,2,4-triazole) and an additive of wood treatment solutions (2-benzylquinoline) were also detected. From the identified compounds, two of them (imazamethabenz-methyl and 1-bromo-3-methylcyclohexane) have already been reported in environmental samples such as ground water and WWTP influent in the USA and in France (Table 6). Their presence in a landfill leachate shows their potential to reach the environment. However, for this study, effluent from a WWTP was also analysed and did not show any significant effect on the PAM fluorometry assay. It is indeed possible to think that the threat to algae observed in the leachate sample (which is send to the WWTP studied) was removed by the different treatments.

In Paper V, a WWTP effluent showed a strong effect on algal growth with the first LVSPE eluted fraction showing a total inhibition (100%) of the tested algae. From the EDA study performed 25 identified toxicants were selected as potential causes for the effect. Some of the compounds present in the study are commonly included in environmental studies such as S-metolachlor or terbuthylazine for example (Halfon *et al.*, 1996; Mai *et al.*, 2013). Whilst these compounds and other identified pesticides (such as pencycuron, flurochloridone, and phenothrin) are expected to inhibit algal growth, other compounds such as pharmaceuticals (such as 3-hydroxytamoxifen, crotarbital, and secobarbital) were also identified and suspected to contribute to the measured toxicity. Although the toxicity of these particular pharmaceuticals has not been previously investigated on algae, similar type of compounds (such as clofibric acid, diclofenac, ofloxacin, and sulfamethoxazole) have previously showed their potential to inhibit algal growth (Ferrari *et al.*, 2004). It is known that pharmaceuticals present in WWTP effluent have a residential or hospital origin while pesticides have an agricultural or residential origin (Kolpin *et al.*, 2002). These chemicals are a potential hazard for organisms, like in this case for algae. From the 25 identified compounds 16 of them were reported in environmental samples such as surface water, wastewater, or ground water (Table 6). The EDA of WWTP effluents has allowed the identification of numerous toxicants across Europe. For example, Grung et al. (2007) identified PAHs, alkyl substituted PAHs, nitro-polycyclic aromatic compounds, carbazoles and alkyl substituted carbazoles as CYP1A inducers in a WWTP effluent from Croatia. Furthermore, Aerni *et al.* (2004) identified estrogens (such as estrone, estradiol, and ethinylestradiol) in five WWTP effluents in Switzerland. From the compounds identified in Paper V (Table 6), three of them were identified as potential principal contributor to the toxicity based on the ECOSAR model and the published literature: terbutryn, terbuthylazine, and prometryn. These compounds are herbicides and toxic to algae at low concentrations and have been reported present in surface water samples (Coupe *et al.*, 2005; Quednow and Püttmann, 2007; Bottoni *et al.*, 2013).

Table 6: Summary of the compounds toxic to algae identified by effect-directed analysis.

Name	CAS number	Matrix	Biological effect	Known use	Occurance in the environment
Imazamethabenz-methyl	81405-85-8	Landfill leachate	Confirmed PS II inhibitor	Herbicide	Detected in soil and ground water in USA (Aichele and Penner, 2005)
Benzofuran, 2,5-diacetyl-6-methoxy-	23840-15-5	Landfill leachate	Suspected PS II inhibitor	Pesticide	No previous report
thiophene-2-methanethiol	6258-63-5	Landfill leachate	Suspected PS II inhibitor	Food additive	No previous report
(1-Bromo-2-methyl-1-propenyl)benzene	5912-93-6	Landfill leachate	Suspected PS II inhibitor	Undocumented	No previous report
2-Benzylquinoline	1745-77-3	Landfill leachate	Suspected PS II inhibitor	Use in wood treatment	No previous report
6-Acetyl-2,5-dihydroxynaphthoquinone	13378-90-0	Landfill leachate	Suspected PS II inhibitor	Undocumented	No previous report
Bis(1,1,3,3-tetramethylbutyl) disulphide	29956-99-8	Landfill leachate	Suspected PS II inhibitor	Use in the synthesis of thin film	No previous report
4-Ethyl-4H-1,2,4-triazole	43183-55-7	Landfill leachate	Suspected PS II inhibitor	Fungicide	No previous report
1-bromo-3-methylcyclohexane	13905-48-1	Landfill leachate	Suspected PS II inhibitor	Undocumented	Detected in WWTP influent in France (Nguyen *et al.*, 1994)
Butyl stearate	123-95-5	Landfill leachate	Suspected PS II inhibitor	Use in cosmetics and personal care products as lubricants	No previous report
Terbutryn	886-50-0	WWTP effluent	Suspected algal growth inhibitor	Herbicide	Detected in surface waters in Germany (Quednow and Püttmann, 2007)

Table 6 (continue)

Name	CAS number	Matrix	Biological effect	Known use	Occurance in the environment
Terbuthylazine	5915-41-3	WWTP effluent	Suspected algal growth inhibitor	Herbicide	Detected in soil, runoff water, and surface waters in Italy and Spain (Bottoni *et al.*, 2013; Calderon *et al.*, 2016)
Prometryn	7287-19-6	WWTP effluent	Suspected algal growth inhibitor	Herbicide	Detected in surface water in USA (Coupe *et al.*, 2005)
2-(2-heptadec-8-enyl-2-imidazolin-1-yl)ethanol	95-38-5	WWTP effluent	Suspected algal growth inhibitor	Industrial formulation of lubricant additives, lubricants, functional fluids and greases	No previous report
Acetochlor	34256-82-1	WWTP effluent	Suspected algal growth inhibitor	Herbicide	Detected in water and human urine in USA (Freeman and Rayburn, 2006; Barr *et al.*, 2007)
6-deisopropylatrazine	1007-28-9	WWTP effluent	Suspected algal growth inhibitor	Degradation product of herbicide	Detected in surface water and groundwater in Italy (Bottoni *et al.*, 2013)
Flurochloridone	61213-25-0	WWTP effluent	Suspected algal growth inhibitor	Herbicide	Detected in soil and plant in Serbia (Marković *et al.*, 2010)

Table 6 (continue)

Name	CAS number	Matrix	Biological effect	Known use	Occurance in the environment
Carbendazim	10605-21-7	WWTP effluent	Suspected algal growth inhibitor	Fungicide	Detected in soil, sediments and surface water in the Netherlands (Hogendoorn *et al.*, 2000)
Caffeine	58-08-2	WWTP effluent	Suspected algal growth inhibitor	Psychoactive drug	Detected in surface water and WWTP effluent in Barbados (Edwards *et al.*, 2015)
3,4-dichlorophenylurea	3567-62-2	WWTP effluent	Suspected algal growth inhibitor	Metabolite of herbicide diuron	Detected in soil in UK (Gooddy *et al.*, 2002)
Phenothrin	26002-80-2	WWTP effluent	Suspected algal growth inhibitor	Component of aerosol insecticides for domestic use	Detected in inddor air in Japan (Yoshida, 2009)
Triallyl cyanurate	101-37-1	WWTP effluent	Suspected algal growth inhibitor	Intermediate in the production of other chemicals such as rubbers, resins and polymers	No previous report
Deethylatrazine	6190-65-4	WWTP effluent	Suspected algal growth inhibitor	Degradation product of the herbicide atrazine	Detected in ground water and surface water in USA (Rollag *et al.*, 1996; Townsend and Young, 2000)
S-Metolachlor	87392-12-9	WWTP effluent	Suspected algal growth inhibitor	Herbicide	Detected in water samples in Greece (Thomatou *et al.*, 2011)

Table 6 (continue)

Name	CAS number	Matrix	Biological effect	Known use	Occurance in the environment
Resmethrin	10453-86-8	WWTP effluent	Suspected algal growth inhibitor	Insecticide	Detected in sediment in USA (Hladik and Kuivila, 2012)
1,3-Dimethyluracil	13401-18-8	WWTP effluent	Suspected algal growth inhibitor	Metabolite of methylxanthines	No previous report
3-hydroxytamoxifen	82413-20-5	WWTP effluent	Suspected algal growth inhibitor	Synthetic antiestrogen	No previous report
Crotarbital	1952-67-6	WWTP effluent	Suspected algal growth inhibitor	Pharmaceutical	No previous report
Pencycuron	66063-05-6	WWTP effluent	Suspected algal growth inhibitor	Fungicide	Detected in surface water in Japan (Tanabe and Kawata, 2009)
Temurin	2309-49-1	WWTP effluent	Suspected algal growth inhibitor	Metabolite of methylxanthines	Not reported
Secobarbital	76-73-3	WWTP effluent	Suspected algal growth inhibitor	Pharmaceutical	Detected in water and WWTP effluent in Spain (Arbeláez *et al.*, 2015)
Levometiomeprazine	7009-43-0	WWTP effluent	Suspected algal growth inhibitor	Pharmaceutical	No previous report
Thiamylal	77-27-0	WWTP effluent	Suspected algal growth inhibitor	Pharmaceutical	No previous report
Azithromycin	83905-01-5	WWTP effluent	Suspected algal growth inhibitor	Antibiotic	Detected in water in USA (Panditi *et al.*, 2013)
Methohexital	151-83-7	WWTP effluent	Suspected algal growth inhibitor	Pharmaceutical	No previous report

3. Thyroid hormone disrupters

In Paper III, the TTR binding disruption of different classes of selected compound (such as bactericides, brominated flame retardants andsurfactants) was confirmed using the newly developed fluorescent ligand. The disruption of the thyroid hormonal system is known to interfere with the growth and development of young organisms (Brouwer *et al.*, 1998; Boas *et al.*, 2006). Xenobiotics interfering with TTR-T_4 binding have previously been detected in wildlife as well as in remote areas (Gutleb *et al.*, 2010; Gabrielsen *et al.*, 2015). Furthermore, TTR binding disrupting compounds such as polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins, and polychlorinated dibenzofurans were detected in sea gull eggs, supporting the previous finding of such compounds in organisms (Pusch *et al.*, 2005), which confirms the hypothesis that TTR binding inhibitors are discharged into the environment. Indeed, Pusch *et al.* (2005) detected pollutants such as polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins, and polychlorinated dibenzofurans in sea gull eggs in Norway and Ucán-Marín *et al.* (2009) showed that these compounds (i.e. BDE47 and CB187 and their oxygenated-substituted analogs) can interfere with TTR-T4 binding in sea gulls (with some compounds capable of almost full bidning inhibition at high concentrations). In Paper III, thyroid hormone disrupters were detected in both a remote area (Musvær Island) and a more densly populated area (Reiaren Island). Nevertheless, a higher inhibition of TTR-T_4 binding was measured in samples from the populated area showing the potential impact of the pollution on the development of wild organisms. The same pattern has been reported with aryl hydrocarbon receptor agonists for the same samples (Muusse *et al.*, 2014).

4. EDA: a tool increasingly used internationally

Studies integrating both chemical and biological data are increasingly important for environmental scientists (e.g. Macova *et al.*, 2011; Leusch *et al.*, 2014; Jonker *et al.*, 2015). In Europe, it was supported by the implementation of the Water Framework Directive which proposes to assess both the chemical and biological status of every water body on the continent (European Parliament, 2000). By definition, EDA combines biological and chemical data and has therefore been suggested as a tool to help identify principal stressors in European water bodies (Brack *et al.*, 2007). The European project "Novel tools in Effect-directed Analysis for identifying and monitoring emerging toxicants on a European scale" (or "EDA-

EMERGE", http://www.ufz.de/eda-emerge/), of which this thesis is one of the thirteen PhD projects (Brack *et al.*, 2013), aimed at developing biological, chemical, or hyphenated tools which could be used in EDA studies. In this context, new biological (e.g. Paper I; Paper III) and chemical tools (e.g. Ouyang *et al.*, 2015) were developed within the network. The project helped developed existing assays for their use in EDA (Di Paolo *et al.*, 2015; Paper I; Paper III) as well as integrating new techniques such as metabolomics (Tufi *et al.*, 2015). Efforts were made to facilitate the identification of specific chemicals in toxic fractions (Hu *et al.*, 2016; Muz *et al.*, 2017). Overall, EDA-EMERGE further developed tools for the improved EDA of environmental samples. Across Europe, other projects such as the SOLUTIONS project also aim to develop effect-based tools (such as EDA) to help assessing the impact of complex chemical mixtures on water quality (Altenburger *et al.*, 2015).

Over the past years, EDA has helped identify toxicants that were not originally suspected to be the cause of the observed toxicity. For example, Creusot *et al.* (2014) used EDA to identify synthetic steroids (such as dexamethasone, spironolactone, and 6-alpha-methylprednisolone) downstream of a pharmaceutical company as major contributors for the reproductive alterations observed in surrounding fish even if these particular compounds were not suspected as major toxicants. EDA has also helped prove that suspected toxic compounds can indeed be very harmful even in complex environmental mixtures. For example, studies from Desbrow *et al.* (1998), Thomas *et al.* (2002), and Houtman *et al.* (2006) identified 17β-estradiol as a major contributor to the general estrogenicity observed. Furthermore, effect-based studies have helped scientists to identify the possible sources of environmental contamination. For example, Aerni *et al.* (2004) found estrogens in five Swiss WWTP effluents showing the discharge of endocrine disrupting compounds in our environment by human activities. Using EDA, Paper V showed also that a Czech WWTP effluent containing compounds toxic to algae was discharged in the environment. Effect-based fractionation coupled to non-targeted chemical analysis can also be used to detect toxic compounds in food samples. For example, Morlock *et al.* (2014) identified six endocrine disrupting compounds in propolis samples and spices by EDA.

Most of the literature on the combination of bioassays and non-targeted analysis have focused on effects such as mutagenicity, aryl hydrocarbon receptor-mediated effects, endocrine disruption and green algae (Brack *et al.*, 2016), but other types of effects can also be

investigated providing the assay's compatibility with chemical fractionation and identification (which is a major challenge to perform EDA). Enhancing the throughput of a bioassay can be a way of facilitating its use in EDA (Paper I and Paper II). This thesis showed that various fractionation techniques, bioassays, and identification techniques could be used for EDA (Table 3). For example, widely used fractionation techniques such as RP-HPLC and SPE can efficiently fractionate organic compounds in a complex sample (Paper V). Finally, combining the use of both LC- and GC-MS techniques for the non-targeted analysis is a good way to ensure sufficient results without the need of special expertise to identify unknown compounds (Paper IV, Paper V).

5. High-throughput bioassays: the solution for intensive studies

In the context of EDA, the choice of bioassay influences which type of compounds will be identified (and which organism is potentially affected). Furthermore, for each sample, chemical fractionation can quickly generate a large number of fractions that all have to be tested (e.g. Lübcke-von Varel *et al.*, 2012; Zaja *et al.*, 2013). Therefore, scientists performing EDA or other large biological studies are more likely to use *in vitro* assays due to the medium to high throughput screening capacity of these assays. *In vivo* assays have the advantage of better describing what could happen in living organisms while *in vitro* assays are more specific and usually easier to implement in a laboratory (as it does not require specific animal maintenance). Furthermore, *in vitro* assays usually require less sample volume and display higher sensitivity than in vivo assays, making them possible to use even after a fine fractionation step. Due to the integrative nature of many *in vivo* toxicity endpoints, these assays can be difficult to use for identification of specifically-acting compounds as may involve multiple MoA leading to the same toxicity endpoint. Indeed, linking the observed effect to bioactive compounds is more challenging than with *in vitro* assays with a defined MoA and chemical specificity. Cell-free *in vitro* assays (such as the AChE inhibition assay and the TTR binding assay) are in general easier to developed (to automate or miniaturised for example) compare to cell-based assays since automated mechanical and liquid handling may affect the cell viability. However, cell-based assays have the strength to use a biological barrier (the cell) making them more representative of what could happen than cell-free assays. Nevertheless, some *in vitro* assays can also be demanding and requires adaptations in order to facilitate their use (Papers I and III). If automation can be a way to enhance the throughput of

bioassays (e.g. Risch *et al.*, 2006; Brinkmann and Eisentraeger, 2008), adaptation of the protocol for easier use (for its use in microplates for example) can also be the solution (e.g. Galgani and Bocquene, 1991; Paper III). The development of bioassays in microplates has several advantages. Firstly, the format allows testing of a large number of samples or conditions with the help of a microplate reader to generate the data. Furthermore, this format can be directly linked with chemical fractionation with the help of a special fraction collector (e.g. Paper IV; Ouyang *et al.*, 2016). Finally, the cost of a bioassay can be lowered when the assay is simplified, does not require keeping animals in the laboratory, and most of the time when it is downscaled as well (as it requires less solvent use and more samples can be run in a day). For example, in the Paper III the TTR binding assay has been miniaturised and thus allowing a significant increase in assay throughput. Traditionally, this assay has been performed by using a radioactive probe (Lans *et al.*, 1993), which requires special precautions (with specific dedicated spaces in laboratories as well as specifically trained scientists) and several purification steps in order to dissociate the radioactive T_4 bonded to TTR to the unbounded radioactive T_4. To overcome these challenges the old principle of creating a fluorescent T_4 by reaction with the fluorescent probe FITC (Smith, 1977) has been recently used (Ren and Guo, 2012). For this thesis the protocol has been adapted for its use in 96-well plates. Adaptation implied a miniaturisation of the existing protocol performed in a cuvette for its use in microplates with all the advantages discussed below. Enhancing bioassays' throughput could also be useful to screen a high amount of samples (such as drinking water or recycled water) to assess their potential toxicity. The AChE inhibition assay was for example part of the bioassay battery used by Leusch *et al.* (2014) to assess the toxicity of recycled water from nine Australian water reclamation plants. Using the automated protocol as well as other automated bioassay could be used to more frequently to monitor the toxicity of such samples.

6. Rapid chemical fractionation procedures

For the present thesis different approaches have been tested to fractionate complex environmental samples. One approach started the fractionation simultaneously with the extraction either during the sampling (Paper V) or in the laboratory when grab samples were taken (Paper IV). The idea is to use the principle of SPE itself: different cartridges with different sorbents will retain different types of compounds (Poole, 2003). In Papers IV and V,

water samples passed through three cartridges before elution of each cartridge with different solutions. This procedure generates a few fractions but a significant amount of non-toxic compound can easily be eliminated and thus facilitating the execution of the next fractionation step (typically performed by HPLC). One SPE cartridge can also be used as support for generating several fractions (e.g. Thomas *et al.*, 1999; Paper V). This way is another approach to easily reduce the complexity of the newly generated toxic fractions. The only effect-directed study using SPE as fractionation technique is from Thomas *et al.* (1999) where they demonstrated that organic compounds such as atrazine, phenanthrene, nonylphenol can be efficiently fractionated using this technique. The use of this technique could help studies such as EDA as preliminary fractionation technique as it is easy to carry out and being efficient (Paper V). Chromatographic techniques (such as HPLC and UHPLC) remain the easiest way to fractionate complex environmental samples. This fractionation methods are widely used in studies combining bioassays and chemical fractionation as they are easy to perform, efficient, and already available in most laboratory. As described in the introduction part, both NP- and RP-HPLC have been use in effect-directed studies with the RP-HPLC being the most popular technique. For example, Hewitt *et al.* (2000) and Grung *et al.* (2007) used a RP-HPLC with a C18 stationary phase and a mobile phase of Methanol and water to successfully fractionate organic compounds such as PAHs, carbazoles, polychlorinated dioxins, and furans. The same approach was therefore used in the Paper V which also studied a WWTP effluent as in the study from Grung *et al.* (2007). Weiss *et al.* (2009a) used successfully both RP- and NP-HPLC to fractionate anti-androgens from androgens as potential endocrine disruptors. This study shows the potential of combining different fractionation techniques in order to identify toxicants in complex environmental mixtures. Faster than the commonly used HPLC, UHPLC can also be used for rapid fractionation. Furthermore, when linked to a microplate fraction collector, very fine fractions can be collected in a very short time window (e.g. Paper IV). For example, Booij *et al.* (2014) used this technique to perform EDA of surface water samples from the Netherlands which generated 150 fractions. In addition, HPLC and UHPLC systems can be used together in the same EDA study to perform a very fine fractionation step. For example, Ouyang *et al.* (2016) identified AChE inhibitors in a WWTP effluent using a two dimensional fractionation step with a HPLC system as first dimension and a UHPLC system as second dimension. This fractionation protocol generated 384 fractions in one step, making any additional fractionation unnecessary. The UHPLC technique has the advantage of

facilitating linkage between bioassay screening and the fractionation as well as facilitating non-targeted chemical analysis of fractions identified as toxic. A potential problem with this approach comes from the very fine fractions: it is indeed possible that after such fractionation, the combined effect of multiple active compounds of the total sample may not be detectable by the bioassay in a single fraction. This is the case when the mixture effects (such as additivity or synergy) are of high relevance and in this scenario the EDA approach cannot be successfully used. For this thesis another chromatographic technique has also been used to generate simple fractions: open column liquid solid chromatography (Paper II). This technique is not the fastest way to fractionate a complex sample but showed to be efficient in some cases (Holth and Tollefsen, 2012). It is indeed still used when scientists want to fractionate organic compounds in discharges related to the oil and gas industry. For example, Adams *et al.* (2014) used open column chromatography with a silica gel stationary phase to fractionate hydrocarbons in an effect-based study to identify compounds toxic to rainbow trout embryos in fuel oil. Hong *et al.* (2015) also used the open column chromatography approach to fractionate PAHs in crude oil and oil-contaminated sediments in an EDA study to identify aryl hydrocarbon receptor active compounds. The only fractionation type used in EDA studies that was not tested in the present thesis was preparative gas chromatography (Meinert *et al.*, 2007). This technique is interesting when high resolving power is needed, for example, to fractionate isomers with different biological effects but require special equipment which is not commonly available.

7. Identification of the toxicant(s)

Identifying compounds responsible for the observed biological effect is typically the most challenging task when performing EDA. For the chemical analysis two main techniques have been used during the present thesis: GC and LC-MS. These two techniques are the most commonly used when non-target analysis are performed on environmental samples (Hernandez *et al.*, 2012). As shown in Figure 4 the type of chromatography used can influence the type of compound detected (for example, GC would be the best option to detect more lipophilic compounds). More results are obtained when LC-MS and GC-MS analysis are performed together (e.g. Papers IV, and V). For example, Grung *et al.*(2007) identified steroids (such as 17β-estradiol or progesterone) present in toxic fractions by LC-MS and

additional analysis by GC-MS allowed the identification of alkylphenols and methylparaben also in the toxic fractions.

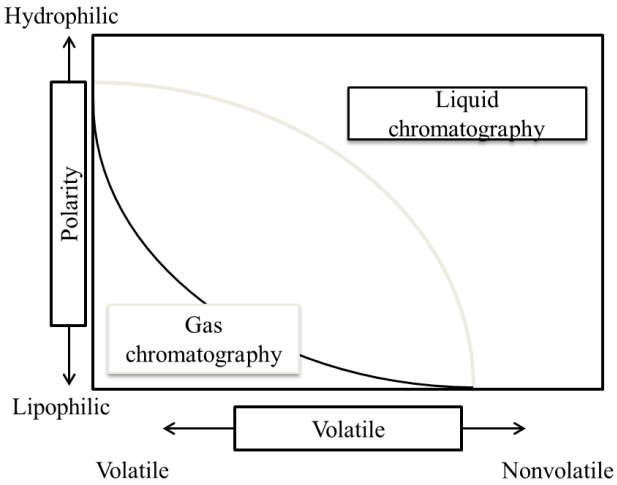

Figure 4: polarity/volatility diagram with the application ranges of gas and liquid chromatography (adapted from Giger, 2009).

When using MS, two main options are available to identify a peak from which the mass spectrum has been collected: identification either by manual interpretation or by library search. The manual interpretation is tedious and requires expert knowledge (Kind and Fiehn, 2010). Schymanski *et al.* (2008) developed an alternative to the manual interpretation of mass spectrum and library match by combining MOLGEN-MS modules and the NIST database to generate possible structures for unknown compounds. The majority of environmental studies including non-target analysis use comprehensive strategies with a library search in order to identify unknown peaks (Krauss *et al.*, 2010). Using this approach allows the rapid identification of numerous pick but only a few of them would be analytically and biologically confirmed. For example, in surface water samples, Booij *et al.* (2014) identified 54 compounds by database matches and concluded that from these candidates six of them (mainly pesticides) were responsible for the observed toxicity. To ensure a good success rate, strategies can also be developed to identify compounds responsible for a specific effect. For example, Bataineh et al. (2010) proposed a protocol to identify partially polar mutagenic polyaromatic compounds in sediment extracts by using RP-HPLC, high-resolution MS and the PubChem database. The method showed the power to identify mutagenic compounds which are structurally similar to the 55 selected model compounds. For this thesis, two main

strategies have been followed to identify compounds in the toxic fractions. With the GC-MS analysis a manual peak picking was performed (which can be tedious but ensure the selection of only relevant peaks) together with a search for best matches in the NIST database (Papers II, IV, and V). While with the LC-MS, statistical tools have been used to discriminate peaks only occurring in toxic fractions together with a search in the STOFF-IDENT database (Paper V) or a search in a pesticide-specific database (Paper IV). The workflow is summarised in the Figure 5. In the thesis papers, different water samples were analysed using both techniques and the best results were achieved when combining both.

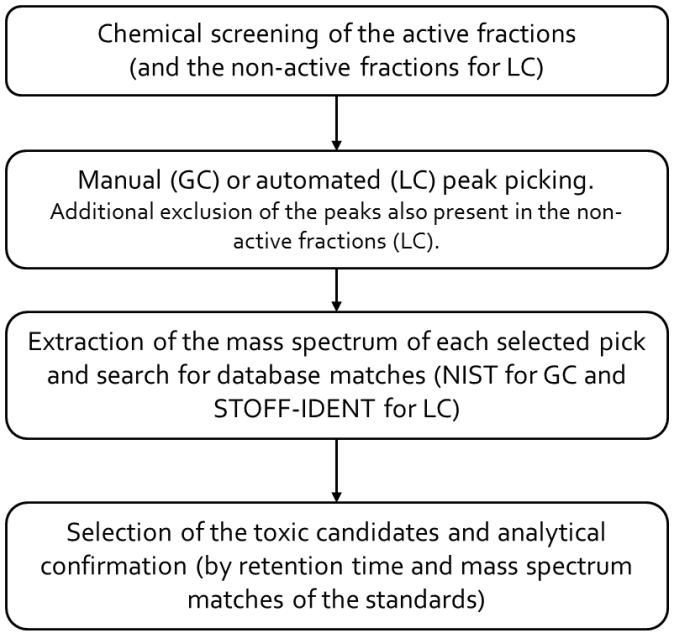

Figure 5: Workflow followed for the chemical identification carried out by GC- and LC-MS for the thesis.

8. Is routine EDA possible?

The first obstacle to overcome in order to perform an EDA study is the instrumentation and the required expertise in the different fields within the environmental sciences. Indeed, the combination of bioassays and chemical tools often makes it difficult for laboratories to perform EDA studies. However, when these challenges are overcome it is possible to implement EDA protocols. The choice of the bioassay is a critical step. It is possible to use the same bioassay for different samples and find different causes to the same effect (Booij *et*

al., 2014; Paper IV), but having the ability to perform different assays can increase the EDA studies' relevance. When talking about routine studies, a key factor to consider is the possibility to analyse numerous samples in a short time. Therefore, it is important to choose high-throughput assays that are easy to perform, low in cost, and possible to link with the chemical steps included in EDA (e.g. Papers I, and III). But it would be also beneficial to select robust assays that have different selectivity (not only cell-based and cell-free *in vitro* assays but maybe also *in vivo* assays). Regarding the fractionation step, if the collection of the sample with a device combining different SPE cartridges can help remove some non-active compounds, the simple fractionation on a SPE C18 cartridge (eluted at different methanol concentrations) looks efficient enough as a preliminary fractionation step for an EDA study (Thomas *et al.*, 1999; Paper V). This technique followed by an additional HPLC fractionation could be a good generic option when organic compounds are suspected to be the cause of the effect. The analysis by both LC and GC-MS (if possible) would give good chance of obtaining some results. Even if the library search cannot permit the identification of truly unknown compounds, this procedure would really make possible routine EDA in comparison with the manual interpretation of the mass spectrum. Furthermore, by combining both LC and GC-MS, different libraries can be used, increasing the potential of identifying the toxicants. That is why several studies already performed their chemical screening with both instruments (e.g. Grung *et al.*, 2007; Schmitt *et al.*, 2012; Paper V). In summary, when the instruments are available, it is possible to follow a generic protocol to perform relatively fast EDA studies on different type of samples. But the confirmation step (to confirm the presence of the toxicant in the sample and assess its toxicity) would be determinant on the throughput and the success of the all study (Brack *et al.*, 2008). Indeed, after narrowing down a list of suspected toxicants, they should be purchased and injected in the analytical instrument used (to confirm that the right compound has been identified) as well as tested with the assay (to measure their toxicity). Another way to develop routine EDA could be to implement a powerful and unique chemical analysis step directly on-line with a very fine fractionation step. This is what Ouyang *et al.* (2016) developed with a two dimensional LC fractionation step coupled with an on-line ToF-MS. Although very efficient to enhance EDA throughput it seems difficult to implement additional chemical analysis using another technique in order to ensure a good success rate when analysing different samples. From this thesis the suggestion of "routine EDA" protocol could be as followed: firstly, the extraction of a liquid sample can be

performed by SPE. Three different bioassays could be run on the extract using easy to handle and rapid assays such as the AChE inhibition assay (Paper I), the fluorescent TTR binding assay (Paper III), and the algal growth inhibition assay (Paper V). All these assays can assess different effects and are developed for their use in microplates (making possible the analysis of a large number of generated fractions at once). If a sample shows some effect in (at least) one of the selected assays, the first fractionation step could be performed by SPE while the toxic SPE fraction(s) could be subjected to additional fractionation by HPLC. Active HPLC fraction(s) could finally be analysed by both LC and GC-MS from which a list of suspected toxicants could be drawn up. All the possible standards from this list would then have to be purchased in order to confirm their presence in the sample (and if possible to measure their concentration) as well as assessing their toxicity. Once the EDA protocol is set up and all the necessary material available, this protocol could be carried out by the same operator on different type of samples to identify toxicants. A workflow for this routine EDA protocol is proposed in the Figure 6. Another way to implement routine EDA would be to develop on-line bioassays in parallel of the fractionation and chemical screening. Several studies have already shown the potential of on-line EDA (e.g. Falck *et al.*, 2010; Jonker *et al.*, 2015) which could allow the analysis of different samples in a very short time. A drawback to such studies is the difficulty of performing different assays as not all the bioassay could be implemented directly on-line with the chemical analysis and fractionations. Furthermore, as shown in the Paper IV, EDA can benefit from the use of various chemical screening instruments instead of only one, and this would also difficult when dealing with on-line EDA. Finally, when trace compounds have to be detected by EDA, it is possible to use passive samplers for their extraction (such as polar organic integrated samplers as used by Booij *et al.*, 2014). The workflow in the Figure 6 could be then be followed as suggested.

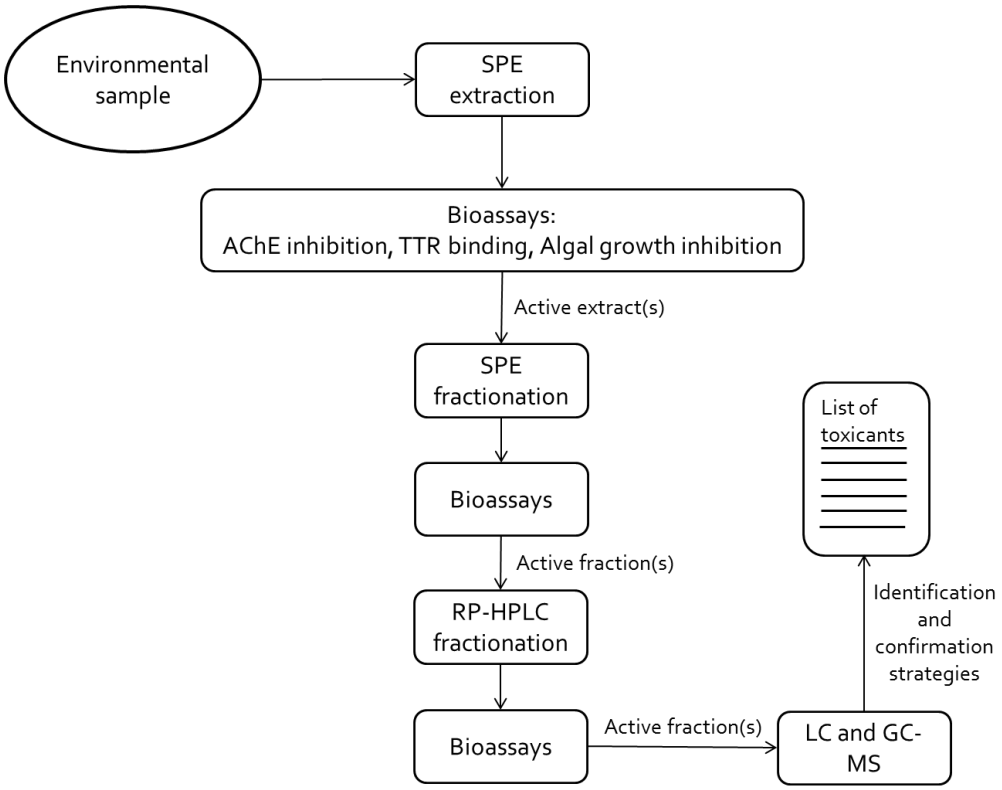

Figure 6: Proposed EDA routine workflow based on the thesis results.

9. Future work

Two EDA studies from this thesis could not be concluded properly (Papers IV and V). In these cases the toxicity of the identified compounds has to be further investigated, if possible by testing the compounds on the bioassay (Paper V). For Paper IV, the PAM fluorometry assay is unfortunately not available anymore in any institutes this thesis was carried out but other assays measuring inhibition of algal photosynthesis should be performed with the identified compounds (Azov and Goldman, 1982). Finally, in both studies an estimation of the contribution to the overall toxicity of the confirmed toxicents should then be carried out.

The EDA approach could help identify chemical hazards released into the environment. In Europe, EDA could be the next step of the WFD to implement in order to understand poor ecological states in water bodies reported in a good chemical state, such as recommended by Brack *et al.*(2016). For example, EDA or other methods integrating bioassays together with chemical screening could be an option to use in Saxony, Germany, where 85% of the water bodies have been described in a bad (or poor) ecological state and where targeted chemical

analysis only found 16% of the water bodies in a bad chemical state (European Comission, 2012). Furthermore, when new discharges due to human activity are reported as potentially harmful for the environment, EDA could be performed in order to identify the threat and help the responsible for the discharge to decrease its impact on the environment. To help the implementation of EDA in laboratories, a generic EDA protocol that should be usable with different samples would have to be suggested as proposed in Section V.8 (Figure 6). The proposed EDA routine workflow suggested here should be tested by taking different samples and using the three proposed bioassays or other relevant bioassays. Several aspects of this workflow could be improved. A good addition to the three suggested bioassays would be for example an *in vitro* cell based assay to assess the presence of estrogenic compounds such as the ER-CALUX assay. For example, Houtman *et al.* (2006) used this assay to identify estrogenic compounds from an harbour by EDA. From the three assays suggested from this thesis two of them are *in vitro* cell-free assays (the AChE inhibition assay and the TTR binding assay) while the miniaturised algal growth inhibition assay is an *in vivo* as discussed in the section V.5. Furthermore, different bioassays could be improved in order to increase their throughput and facilitate their use in studies combining bioassays and chemical analysis. For example, other *in vivo* assays and cell-based *in vitro* to detect endocrine disrupting compounds could be also be improved and implemented. Assays using human cells would be a good way to assess the risk to human health caused by pollution (Brand *et al.*, 2013). Furthermore, the extraction step could also be improved and automated procedures could be tested in order to facilitate EDA studies (Hutchinson *et al.*, 2007). Finally, in order to use the EDA approach with sediments, the bioavailability of the compounds has to be taking into account. If the suggested workflow could be directly applicable for liquid samples, it would be a good idea to use bioaccessibility-directed extraction techniques (such as mild solvent extraction, extraction with biotic or biomimetic fluids, and desorption into water with subsequent adsorption to a competitive adsorbant) when analysing sediment samples (Brack *et al.*, 2009). This sediment-specific extraction could then be linked with the proposed fractionation, chemical analysis, and bioassays in the Figure 6. The implementation of other on-line EDA (with on-line bioassay, and chemical fractionation and analysis) could also be a way to facilitate such approach. But to enhance the success rate of high-throughput EDA, efforts could now be made to improve identification and confirmation strategies during the non-target analysis. This step is indeed decisive for EDA success rate and integrative tools

could help such study (Brack *et al.*, 2008; Dévier *et al.*, 2011). From the MS peak picking selection to the list of suspected candidates, an automated workflow for example could help improve EDA throughput as well as making it easier to perform for non-MS experts. Finally, the improvement of MS databases (with the inclusion of more compounds for example) could also help to increase EDA success rate. For example, in Europe, efforts have been made through the Norman Network and should be further supported to improve databases such as MassBank and STOFF-IDENT. In general, from this thesis and other European studies (Brack *et al.*, 2007) a guideline could be suggested to the European Commission to use a generic EDA protocol which could be performed by most environmental laboratories across Europe in order to strengthen the WFD.

VI. Conclusions

Two bioassays were further developed in order to enhance their throughput: the AChE inhibition assay was automated (Paper I) and the TTR binding assay was adapted for its use with a fluorescent probe in microplates (Paper III). These enhanced throughput assays were subsequently used to facilitate studies that required intensive biotesting such as EDA (e.g. paper II). Different types of EDA studies were performed in order to test different ways of performing such studies. Different degrees of success were achieved showing that EDA does not guarantee 100% success. As hypothesised by Holth and Tollefsen (2012) the AChE inhibitors identified by EDA in Paper II (4-phenyl-1,2-dihydronaphthalene and butylated hydroxytoluene) contain aromatic rings and their occurrence in off-shore oil and gas production platform discharges was reported for the first time. From Paper IV, different compounds suspected to alter algae PS II efficiency were identified in a landfill leachate. From these suspects a pesticide (imazamethabenz-methyl) as well as several compounds used in personal care products or food additive (such as butyl stearate or thiophene-2-methanethiol) have been reported. In Paper V, the EDA performed on a WWTP effluent allowed the identification of several toxic compounds, mainly pesticides (such as diuron, terbutryn, or atrazine) but also pharmaceuticals and industrial compounds (such as oxytetracycline, or azithromycin). Such compounds have already been reported in WWTP effluents making them potential hazards to our environment (Kolpin *et al.*, 2002; Hernando *et al.*, 2006). In general, in order to maximise the EDA success rate, effort can be placed on toxicant identification and confirmation. Nevertheless, the different EDA procedures followed in the present thesis show that different paths are possible in order to perform enhanced throughput studies. Indeed, different fractionation procedures are possible (by open column chromatography, SPE, or HPLC for example). Chemical analysis was performed by LC-micrOToF-MS, LC-ToF-MS, and GC-HRToF-MS, as well as on-line coupled to fractionation (Paper IV) and off-line. Based on the experiences gained with the different studies, this thesis proposes a generic protocol for rapid and complete EDA to identify organic toxicants in various samples. Implementing such routine EDA procedure would help scientists to identify the as yet unknown causes of observed detrimental effects.

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