

# **Chapter 1**

## **General introduction and objectives**

## **INTRODUCTION**

### **Chemical stressors in the coastal and marine environment**

In recent years, sources, types and levels of contaminants in the marine environment have increased as a consequence of anthropogenic activities worldwide (Halpern et al., 2008). It has been hypothesized that some of these contaminants can negatively affect primary producers at the base of the pelagic food chain. Estuarine and coastal waters are among the most productive regions on the planet and account for approximately one third of all marine biological productivity (Wallace et al., 2009). These areas with the highest primary production will also be the areas that receive relatively high contaminant inputs. Taking both the ecological importance and the contaminant load into account, there is very limited scientific knowledge concerning the effects of contaminants on primary producers, e.g., microalgae in the estuarine and coastal waters (Hylland and Vethaak, 2011).

The major sources of contaminants in estuarine and coastal waters are inputs from river loads (Laane et al., 2006), urban and agricultural runoff (Emelogu et al., 2013) and municipal and industrial discharges (Vethaak et al., 2005), but there are also substantial contributions from other sources, for example antifouling applications, ballast water discharges and atmospheric deposition (Ducrotoy et al., 2000). Recently an overview was given of the multitude and variety of newly detected contaminants from domestic, commercial, and industrial use, e.g., artificial sweeteners, perfluorinated compounds, pharmaceuticals, hormones, drinking water disinfection by-products, sunscreen/UV filters, flame-retardants, siloxanes, musks, algal toxins, pesticides and transformation products (Richardson and Ternes, 2011). Given the worldwide growth in human population and industrial activities, it can be assumed that the chemical pressure on estuarine and coastal waters will increase further (Laane et al., 2012). Contaminants in estuarine and coastal waters are a major concern, especially contaminants affecting microalgae at the base of the pelagic food chain.

### **European regulations**

Contaminants are defined in the European legislation as: “substances (i.e. chemical elements and compounds) or group of substances that are toxic, persistent and liable to get bio-accumulated and other substances or groups of substances which give rise to an equivalent level of concern” (Directive 2000/60/EC). The European Water Framework Directive (WFD) establishes a legal framework to protect and restore clean water across Europe and ensure its long-term and sustainable use (Directive 2000/60/EC). By targeting priority substances, the

WFD focuses on individual contaminants or groups of contaminants that present significant risks to or via the aquatic environment. Among the 45 chemicals categorized as priority substances, 22 are designated as priority hazardous substances due to their persistence, bioaccumulation and toxicity (Directive 2013/39/EU). The Marine Strategy Framework Directive (MSFD) establishes a common basis for the protection and management of Europe's seas and identifies four marine regions: the Baltic Sea, the North East Atlantic Ocean, the Mediterranean Sea and the Black Sea (Directive 2008/56/EC). Member States will need to coordinate the implementation of the MSFD with their actions on the WFD since the two pieces of legislation are closely linked. The MSFD aims to achieve Good Environmental Status (GES) of the European's marine waters by 2020 and to protect the resource based upon which marine-related economic and social activities depend. The WFD reduces pollution of land-based sources from reaching Europe's seas to improve marine conditions. The directive also protects coastal waters as well as transitional waters such as estuaries and coastal lagoons. Together the two directives provide a complete structure for the protection and management of Europe's freshwater and marine waters. The aim of the WFD is to achieve good ecological and chemicals status in all surface and groundwater bodies by 2015 and to prevent any further deterioration of status.

The OSPAR Convention is the current legal instrument guiding international cooperation on the protection of the marine environment of the North-East Atlantic. There are currently 42 substances or groups of substances on the list of chemicals for priority action. The objective of the OSPAR Hazardous Substances Strategy (OSPAR Agreement 2003–2021) is to prevent pollution of the maritime area by continuously reducing discharges, emissions, and losses of hazardous substances, with the ultimate aim of achieving concentrations in the marine environment near background values for naturally occurring substances and close to zero for synthetic substances.

The International Council for the Exploration of the Sea (ICES) – a global organization for enhanced ocean sustainability – has as its main objective to increase the scientific knowledge of the marine environment and its living resources and to use this knowledge to provide advice to competent authorities, e.g. OSPAR and the European Union.

In The Netherlands, Rijkswaterstaat, which is part of the Ministry of Infrastructure and the Environment, monitors the status of contaminants in the Dutch surface waters in the context of the OSPAR convention, the MSFD and the WFD.

## **Effect-Directed Analysis**

In the past, monitoring to assess the “impact” of hazardous substances has been based primarily on chemical measurements of concentration. This was because the questions being asked concerned concentrations of such substances in water and such measurements were possible. However, in order to more fully assess the status of the estuarine and coastal waters, questions about the bioavailability of hazardous substances and their impact on marine organisms or processes are now being posed.

Effect-Directed Analysis (EDA) combines analytical chemistry with bioassays to isolate and ultimately identify the compounds in a complex sample that are responsible for the effects observed. Typically, in EDA extracts of environmental samples are assessed with bioassays to observe a response. If a response is observed, the extracts are fractionated according to the physicochemical properties of the compounds in order to decrease the complexity of the extract. The fractions thus obtained are assessed separately with the same bioassay. Next, compounds in active fractions are identified with chemical analysis. Finally, the identified compounds are confirmed chemically and biologically and the contribution of the identified compound to the measured effect is quantified to be certain that major toxic contributors have not been overlooked.

In the last 10 years, EDA has been discussed in Europe in the context of the WFD as a way to cope with the continuously increasing number of emerging pollutants entering European waters as well as the legacy contaminants already present (Burgess et al., 2013). Non-target analysis in EDA offers the possibility of identifying new compounds and degradation products, which are not included in target lists of the WFD or OSPAR.

A major challenge in EDA is dealing with the large numbers of data produced from non-target analysis (Weiss et al., 2011). High-resolution mass spectrometry software and extensive spectral libraries are needed to support identification and models providing fragmentation- and retention-based classifiers have to be developed to improve structure elucidation (Schymanski et al., 2012). Isolation and identification of individual contaminants out of the thousands of chemical compounds present in a typical environmental sample often demand several fractionation steps and therefore high-throughput methods (e.g., a rapid bioassay and sophisticated fractionation techniques) are preferred in EDA (Burgess et al., 2013).

Out of the multitude of bioassays used to observe impacts on marine organisms, the Pulse Amplitude Modulation (PAM) fluorometry assay is one of the most sensitive bioassays in aquatic toxicity testing (Juneau et al., 2002). Another approach to assess chemical toxicity to marine organisms is system biology studies, e.g., metabolomics. Metabolomics can provide additional information to traditional bioassay endpoints in phytotoxicity assessment (Kluender et al., 2009).

In traditional bioassays, e.g., the PAM assay, only one typical endpoint is detected, whereas with metabolomics hundreds of metabolites can be detected representing hundreds of different endpoints.

### **Passive sampling and spot sampling**

Passive sampling systems have been developed to extract trace levels of complex mixtures of contaminants from water over extended periods, enable the determination of their time-weighted average (TWA) concentrations in water, and provide a method of estimating the potential exposure of aquatic organisms. Passive samplers are used for pre-concentration of contaminants to increase the sensitivity of the measurements. Spot or grab sampling, e.g., collecting water at a specific time point, provides a snapshot of the situation at the instant of sampling. Fluctuations associated with episodic events will be missed by spot sampling. Moreover, when contaminants are present at ultra-trace concentrations, large volumes of water often need to be collected by spot sampling in order to reach the analytical limits of detection (LOD) required to assess potential exposure to organisms (Emelogu et al., 2013). In order to calculate TWA concentrations in the passive samplers, the uptake rate must be calibrated for all compounds to be monitored. Uptake of chemicals depends on their physico-chemical properties, but also on the sampler design and material and is influenced by environmental variables such as temperature, flow rate, turbulence and bio fouling of the sampler surface. In most cases, uptake rates for these chemicals are controlled by transport within the boundary layer that is present at the surface of the samplers (Huckins et al., 2006) which is based on free flow (according to the Fick's first law of diffusion) of analyte molecules from the water phase to a collecting passive sampler. TWA concentrations can only be determined when sampling occurs in the kinetic phase. If sampling is in the equilibrium phase, then analytes collected by the samplers can also be lost if environmental concentrations reduce. Performance reference compounds (PRCs) can be used to quantify the influence of environmental conditions on the uptake rate and kinetics (Vermeirssen et al., 2013). PRCs are compounds, which are not present in the surface water, e.g. labeled standards, and are preloaded in the passive sampler. From the release of PRCs during deployment, the uptake rate and kinetic phase can be determined. For passive samplers standard operation procedures are not yet available, whereas for spot water sampling well validated method protocols have been developed.

A number of passive sampler types, e.g. silicone rubber sheets and polar organic integrative samplers (POCIS), are available for sampling of various contaminants from water. Passive samplers can be successfully combined with the PAM assay to assess the toxic effects of water

samples (Escher et al., 2006). In this thesis work, passive samplers were employed in estuarine and coastal waters in order to accumulate a broad range of contaminants, without aiming to quantify these, whereas spot sampling was used for quantitative analysis of contaminants.

## **Metabolomics**

Metabolomics is the study of the endogenous low molecular weight metabolites within a cell, tissue or biofluid (Viant, 2007). Metabolomics can be used to characterize the interaction of the organism with its environment. The change in patterns of metabolites within an organism can provide insight in the organism's response to toxicity and identify metabolomic pathways involved in such processes. By detecting metabolites in organisms, information can be obtained on the energetic, reproductive or oxidative status of the organism. Therefore, metabolomics is a potentially strong tool to study organisms and their interaction with the environment, such as adaptation to anthropogenic and natural stressors (Viant, 2008).

Our non-target metabolomics profiling study focused on primary metabolites, for example aminoacids, fatty acids and sugars, which are involved in growth, development, and reproduction of organisms. Primary metabolites are typically formed during the growth phase as a result of energy metabolism. By using multivariate analysis, metabolites with significant differences between groups are identified and can be related to their metabolomic pathway.

Metabolomics using UPLC/QTOF-MS-based metabolic profiling with multivariate statistical analysis was successfully applied to explore the changes in metabolic profiles of microalgae in response to stress conditions (Chen et al., 2013). The effectiveness of metabolomics as a new tool for toxicological assessment has also already been assessed. A metabolomics approach in algae revealed multiple metabolomic markers, responding to exposure of prometryn, and providing additional information to traditional endpoints in phytotoxicity assessment (Kluender et al., 2009). Metabolomics offers considerable potential for rapid assessment of the metabolic status of marine organisms, is capable of multi-species investigations, and can provide molecular information that is closely related to whole organism physiology and function (Viant, 2007).

A challenge in metabolomics is the amount and complexity of data and identification of metabolites, which require sophisticated analytical techniques, e.g. high-resolution mass spectrometry combined with multivariate analysis, and availability of libraries and databases. In this thesis, non-target metabolomics was used to explore the mode of action of microalgae exposed to the herbicide diuron, one of the compounds identified and confirmed in our EDA study (Chapter 3). Metabolomic profiling does not focus on target metabolites but instead

characterizes groups of metabolites, which makes it possible to discover unexpected metabolic responses (Bundy et al., 2009).

### **Pulse Amplitude Modulation fluorometry bioassay**

To assess toxicity of single compounds or complex field samples to microalgae the PAM assay, based on chlorophyll *a* measurement, is a frequently used technique. Fundamentally, chlorophyll *a* fluorescence is a method of measuring the amount of absorbed energy used in photochemical processes (photosynthesis), thus providing insight into the organism's overall 'health' (Ralph et al., 2007). The PAM assay was successfully applied to measure the impacts of photosystem II (PSII) inhibiting compounds on microalgae (Magnusson et al., 2008; Escher et al., 2006; Schreiber et al., 2002).

The PAM assay is a rapid, non-invasive and non-destructive bioassay (Ralph et al., 2007). Since the inhibition of photosynthesis in PSII can translate to reduced growth rates and biomass of microalgae, PAM fluorescence-based techniques can consequently be recommended as a suitable, ecologically relevant tool for assessing toxic impacts (Magnusson et al., 2008). The PAM bioassay is a quick bioassay, since it provides information within several hours on the photosynthetic activity of the algae in contrast to standardized growth inhibiting test (ISO 8692:2012, ISO 10253:2006, OECD 201), which requires at least 72 hours (Sjollema et al., 2014a).

### **Aim of this thesis**

There are still significant gaps in our ability to assess the environmental status of estuarine and coastal waters in an integrative manner (Borja et al., 2013). A major challenge is the development of methodologies and indicators that can summarize, integrate and simplify complex data, while being easily understandable by the public, media, resource users, and decision-makers (Borja et al., 2008).

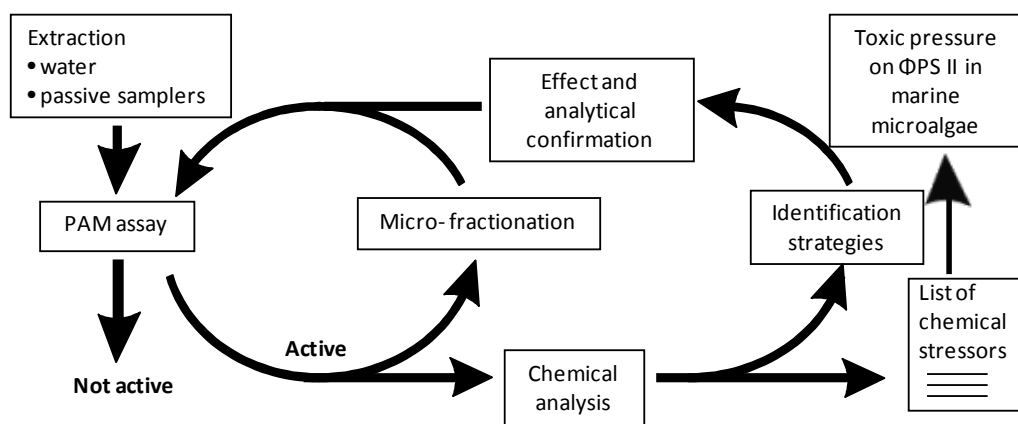
The research described in this thesis was performed to identify the main chemical contributors to the toxic pressure on microalgae in Dutch estuarine and coastal waters and to establish the toxic pressure on microalgae exerted by these compounds under environmental conditions. To this end, different extractions tools were investigated with regard to their suitability to extract a broad range of compounds from the Dutch estuarine and coastal waters that affect the PSII efficiency. Next, an enhanced throughput EDA method was developed to identify and confirm the main contributors to the PSII efficiency on microalgae. The third research question of the thesis work was to explore metabolomics as an additional tool for ecotoxicity assessment. The

fourth research question was to quantify the toxic pressure of the compounds identified in EDA on marine microalgae at several locations in the Dutch estuarine and coastal waters.

### Outline of this thesis

In order to successfully identify compounds in the Dutch estuarine and coastal waters that affect the PSII efficiency in microalgae, four commonly used extraction tools were compared: Spot water sampling using two different types of solid phase extraction cartridges, and passive samplers employing silicone rubber sheets and POCIS (Chapter 2). Extracts obtained with these extraction techniques were subjected to the PAM assay to investigate which extraction tool was most suitable for further research. In Chapter 3, extracts from passive samplers were used for further identification and determination of photosystem II inhibitors. A 96-well plate microfractionation technique using ultra performance liquid chromatography was used in an EDA setup to decrease the complexity of the passive sampler extracts, followed by effect assessment using the PAM assay and chemical analysis of biologically active fractions using high-resolution mass-spectrometry. Special focus was given to the enhancement of throughput in EDA in order to assist in the identification process of the main photosynthesis inhibitors of pelagic microalgae in the Dutch estuarine and coastal waters. The outline of this EDA study is shown in Figure 1.1.

Figure 1.1. Outline of the EDA workflow used in this thesis.



To expand the scope of traditional bioassay testing in EDA, the use of metabolomics as an end point was explored employing complementary analytical techniques for the identification of the mode of action pathway responsible for the effect of diuron on the microalga *Dunalliella tertiolecta* (Chapter 4).



Finally, the environmental relevance of the compounds identified in the Dutch estuarine and coastal waters affecting PSII efficiency in microalgae was investigated (Chapter 5). For the quantification of contributions from contaminants identified, summation of toxic units as derived from the individual components was used to estimate the integrated toxic pressure on PSII efficiency in microalgae. The last chapter provides a general discussion and concluding remarks regarding the results of this thesis and suggestions to improve monitoring and regulation of contaminants in estuarine and coastal waters.