Assessment of effects of chemical contaminants in dredged material on marine ecosystems and human health

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Assessment of effects of chemical contaminants in dredged material on marine ecosystems and human health

Cornelis Anton Schipper

Thesis
Submitted in partial fulfillment of the requirements for the degree of doctor at Wageningen University by the authority of the Rector Magnificus Prof.dr. M.J. Kropff, in the presence of the Thesis Committee appointed by the Doctorate Board to be defended in public on Tuesday 8 December 2009 at 1.30 PM in the Aula
Title: Assessment of effects of chemical contaminants in dredged material on marine ecosystems and human health

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With references, with summaries in Dutch and English

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Chapter 1

General introduction

In the Netherlands, the coastal environment is dominated by the estuaries of several major European rivers, including the Rhine, Meuse and Scheldt, which are the receiving waters from major industrial areas. Sedimentation of suspended particles in delta areas like those in the Netherlands necessitates dredging of waterways and harbours to prevent obstruction of important shipping routes. Hundreds of millions of tonnes of sediment are removed worldwide each year. Dredged material is subsequently disposed of, often by disposal at sea. The Netherlands disposes of more than 25 million cubic metres of sediment a year in the North Sea (Schipper et al., 2009a). The main effects of the disposal of dredged sediment at sea are related to physical and chemical disturbance of the local environment. Sedimentation of dredged material at the dumping site and further downstream influences the local sediment, enhances concentrations of suspended particulate matter, and may cause toxicity due to toxic chemicals present in the dredged material (Stronkhorst et al., 2003a; Lauwaert et al., 2006).

Effects due to sedimentation and enhanced concentrations of suspended particulate matter

Dredged sediments contain a relatively high content of organic matter. This organic matter provides a food supply for benthic organisms. An adverse effect; however, could be large increases in opportunistic species in response to organic enrichment (Blanchard and Feder, 2003). In addition, it has been shown that during periods of dumping increased suspended particulate matter (SPM) concentrations may interfere with food intake of filter-feeding benthic organisms, but that after dumping has taken place rapid recolonization of the benthic community occurred (Van Dalfsen and Essink, 2001; Stronkhorst et al., 2003b). Furthermore, dredged sediment disposal can adversely affect the benthic community if the sediment structure of the dredged sediments differs too much from the sediment structure of the natural occurring sediments at the disposal site.

Presence of toxic chemicals in the dredged sediments

Over 100,000 chemical can be found in the aquatic environment, but little is known about the toxicity of most of these chemicals. To identify chemicals, which pose a risk to the aquatic ecosystem or human health, hazard, exposure and risk assessment procedures have been applied (Thain et al., 2008). In determining whether ecologically relevant endpoints are disrupted, the availability of the toxic chemicals ultimately influencing exposure is an important factor (Stronkhorst and Van Hattum, 2003a). Dredging activities imply that more toxic chemicals may be introduced into a local environment than would result from natural sediment transport fluxes and contaminant transport in the North Sea. In addition to the physical effects after disposal of dredged material, the potential adverse effects of as yet unmeasured compounds, and mixtures of chemicals present in dredged material is not assessed in current risk assessment procedures.
(Munns et al., 2002). It is well recognized that harbour sediments are often contaminated with persistent organic pollutants (POPs), including polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), tributyltin (TBT), mineral oil and metals, which are of concern for the receiving marine systems (Stronkhorst and Van Hattum, 2003a; Alvarez-Guerra et al., 2007a). However, in the Dutch coastal zone, also biological effects of newly emerging chemicals are observed (Laane et al., 2006). Such newly emerging chemicals include potentially harmful priority substances such as brominated flame retardants like polybrominated diphenyl ethers (PBDEs), perfluorinated chemicals such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), toxic congeners of polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzo-furans (PCDFs), polychlorinated naphthalenes (PCNs), and phthalates (EU, 2008). To minimize ecological effects of open-water disposal of dredged sediments, knowledge of the presence and possible adverse effects of emerging chemicals in sediments is needed, since for these chemicals generally no action levels exist, that would enable judgment of the safety of dredged material based on the chemical contents. Examples of possible adverse effects of chemicals present in sediment are TBT induced effects to benthic gastropods, such as common whelk *Buccinum undatum* and periwinkle *Littorina littorea*, that could be related to emissions from ships along main shipping routes and to disposed dredged material (Ten Hallers-Tjabbes and Van Hattum, 1995; Schipper et al., 2008a). Vethaak et al. (1996) reported the presence of liver nodules in dab, *Limanda limanda*, and flounder, *Platichthys flesus*, a phenomenon that was correlated to the presence of PAHs in the surface sediments. POPs such as PCBs have been detected in fish and in many types of wildlife, with some of the highest levels found in the Harbour seal, *Phoca vitulina*, in Europe (Leonards et al., 2008). The rapid decline of the harbour seal population in the 1970s was ascribed to human pressure and exposure of the seals to environmental PCB-like compounds that were shown to induce immuno- and reprotoxic effects (Mees and Reijnders, 1994). Therefore, it is important to identify chemicals present in dredged sediment which are hazardous towards the aquatic ecosystem or human health and characterize their dose response behavior for relevant organisms. Obviously in practice, to conduct of risk assessments for all these chemicals and all these species is not feasible, and would still not include mixture effects. One approach to identify the toxicity of the mixture of unmeasured chemicals present in dredged material is by effect-based bioassays, quantifying the biological effect of a whole mixture rather than that of each single compound.

Given the potential environmental consequences of chemical exposure by the disposal of dredged material worldwide, it is vital that we improve our understanding of its impact on the environment. Through an improved understanding we can minimize the risks associated with the disposed dredged materials.

### Possible applications and types of bioassays needed to determine toxicological impact from dredged sediment

Chemical and biological analysis of dredged material indicates potential hazards and provides a means to quantify the risk when also bioavailability is taken into account and ecologically relevant endpoints are being studied. The estimated risk and current sediment quality criteria, however need to be validated by performing local impact assessment upon licensed disposal of dredged harbour sediment. Bioassays with local sediment may provide insight into the bioavailability and mixture effects of chemicals from the dredged material. In addition, a wide variety of bioindicators can be used to determine the effects of sediment-associated chemicals and to
highlight adverse effects overlooked by the risk assessment for the local ecosystem at or beyond the disposal site (Thain et al., 2008). This might facilitate improved ecological risk assessment (ERA) of dredged sediments for the receiving marine coastal ecosystem. Sediment-associated persistent, bioaccumulating and toxic (PBT) compounds in dredged material mostly accumulate downstream of the disposal site due to transport of the contaminants bound to small particles (Laane et al., 1999; Sonneveldt and Laane, 2001; Stutterheim, 2002). To identify effects of the chemicals, bioassays and biomarkers are needed that relate ambient sediment chemistry data to specific potential adverse biological effects. For this purpose in vitro bioassays can be used to quantify the total toxic potency of organic chemicals inducing toxic effects through a shared mechanism of toxicity that is specific to certain groups of compounds. The applicability of bioassays and biological effect assessments requires that the role of each method is understood, and that assessment criteria are available. Bioassays are used to determine the toxic potential of emerging chemicals, or a complex environmental matrix such as dredged material (SEDNET, 2007a) consisting of a mixture of chemicals.

The most important types of bioassay used in risk assessment of dredged material are in vivo, in situ and in vitro bioassays. In in vivo bioassays, whole organisms are exposed to mostly whole samples of dredged materials although sediment extracts could be used as well. Quantification of toxic endpoints could be combined with biomarker measurements for sublethal parameters that could also be applied in environmentally exposed organisms, and ideally the internal effect levels would be measured as well allowing comparison with field levels. For in vitro bioassays, proteins, intact cells or tissues are exposed to usually sediment extracts, although there are some cases where bacteria are exposes to full sediment. Such bioassays mostly measure responses that are specific to a well-known mode of action, such as expression of a specific gene, inhibition of a specific enzyme, or binding to specific proteins. An example can be found in studies identifying the potential impact of contamination with dioxins or compounds with a dioxin-like mode of action, by the combination of in vitro and in vivo bioassays based on Ah-receptor responses. There is a need to improve exposure prediction and bioassay and biomarker availability for invertebrates and vertebrates at higher trophic levels in marine ecosystem that can be coupled to guidelines for effect-based monitoring and the evaluation of risk management of dredged material.

History of management approaches, discussion on inclusion of bioassays

Dredged material disposal is regulated in accordance to different environmental conventions and agreements on marine pollution prevention. In Europe, disposal of dredged material is based on three conventions: London Convention (1972), Oslo-Paris Convention (OSPAR, 1992), and Helsinki Convention (HELCOM, 1992). The purposes of these conventions are to limit environmental pollution resulting from disposal of dredged material in the marine environment of the North East Atlantic (North Sea) and the Baltic Sea area. Countries have set into force dredged material guidelines for disposal of dredged material into the sea (Alvarez-Guerra et al., 2007a). In USA, the Inland Testing Manual (US EPA, 1998) and the Green book (US EPA, 1991) provide guidance for the evaluation of the disposal of dredged material into marine waters. Many countries have set their own Action Levels (ALs) for determining whether dredged material may be disposed of at sea, based on determination of a number of conventional contaminants (OSPAR, 2004a; Alvarez-Guerra et al., 2007b). The Sediment Quality Guideline (SQG) provides the acceptable levels of a number of conventional contaminants in the dredged sediment (Long
General introduction

et al. 2000, 2006; MacDonald et al., 2000; Alvarez-Guerra et al., 2007b). The uptake of POPs in organisms is estimated on the basis of any increase in the equilibrium partitioning between sediment and organisms in the exposure medium (Reichenberg and Mayer, 2005). The procedures, however, generally focus on direct effects on the organisms at the disposal location and not on long-term environmental effects at distant downstream locations or on possible recovery of the environment. The issue of the prediction and prevention of impacts of disposal of dredged sediment on species and their habitat has been discussed at OSPAR (2004a), Dutch-German Exchange (DGE) (Manz et al., 2007), SEDNET (SedNet 2007a, 2007b, 2007c) and ICES (2000). The assessment procedure for sediment and dredged material can be improved (SedNet, 2007b) by taking the environmental situation at the dredging locations into consideration, when selecting the disposal location (DelValls et al., 2004; Manz et al., 2007). Methods to investigate the direct effects of dredged sediment disposal on the organisms at the disposal location and the surroundings, on long-term environmental effects, or on possible recovery of the environment are investigated and discussed in this thesis.

In the 1990s sediment toxicity methods were developed (US EPA, 1991; US EPA, 1998; ASTM, 1998; Schipper and Stronkhorst, 1999; SEDNET, 2007a) for dredged material management. In the Netherlands, a permit for disposal into the coastal environment is required from the Ministry of Transport, Public Works and Water Management. Disposal of dredged material is regulated through the national Sea Water Act and the Surface Water Act. To control and assess the quality of sediment, comparison between chemical concentrations and environmental SQGs (Sediment Quality Guidelines) are made. Action limits (ALs) are set based on the SQGs assuming a two-fold dilution factor. Above these ALs, disposal at sea is not permitted. With the introduction of bioassays for assessing dredged material in 2004, SQGs in the Netherlands were supplemented with some toxicity assays and the so-called chemical-based Uniform Content Test (UCT) was replaced by the Chemical Toxicity Test (CTT) approach (Fig. 1) (Staatscourant, 2004; Schipper and Schout, 2004). Based on the results of the application of the toxicity tests which were sometimes hard to interpret, it was decided that bioassays cannot themselves lead to the disqualification of particular batches of dredged material (Schipper and Klamer, 2006; Manz et al., 2007). Since 2007 the design of the CTT and the ultimate choice of bioassays included in the CTT are directed at an alert function of the bioassays, which are mostly used to characterize the potential hazard.

Figure 1: Historical development of approaches to determine the quality of sediments in the Netherlands (for explanation see text).

Discussion on inclusion of bioassays

The most problematic features of in vivo bioassays, hampering their application in SQGs are: the lack of specificity, interference with the response by sample constituents, and poor reproducibility resulting in a too high probability of false positive or false negative results in sediment toxicity tests (Kohn et al., 1994; McElhiney et al., 1998; Stronkhorst et al., 2003b; Postma et al., 2000; Lahr
et al., 2003; Van Hattum and Kruseman, 2005). However, bioassays can provide useful information on (new) effects and they are suitable for assessing ecological quality at disposal sites. Depending on the goal of the test, these disadvantages of bioassays are compensated for by the fact that they take into account the complexity of mostly unknown chemical mixtures, and the bioavailability and potential bioaccumulation of chemicals. Bioassay responses can also help to indicate yet unknown toxic compounds via an iterative process of toxicity identification and evaluation (TIE) (Burgess, 2000). However, the enormous diversity of types and qualities of bioassays and diverse applications made the appreciation and selection of bioassay inconsistent and interpretation of the results problematic within the framework of risk assessment of sediment toxicity for licensing purposes.

Objectives of the thesis

Given the complexity of contaminated sediments, rationally chosen and applied bioassays and bioindicators for hazard, risk and impact assessment are needed that can predict and detect the biological effects of contaminants in combination with chemical analyses (Chapman and Mann, 1999). This thesis focuses on the applicability of in vitro and in vivo bioassays and bioindicators as tools for evaluating the effects of complex chemical mixtures in the process of deciding whether dredged harbour sediments can be safely disposed of at sea. To this end three objectives have been defined:

1. Analyze the main factors determining success and failure when applying bioassays for hazard, risk and impact assessments of dredged sediments.
2. Develop a rationale for the inclusion of bioassays in hazard, risk and impact assessments of dredged sediments.
3. Establish a comprehensive set of bioassays and biomarkers that will provide added value to the risk management process when considering the disposal of dredged sediments.

Combining the information obtained in these newly defined bioassays with existing chemical analyses may facilitate definition of not only the hazard but also the risk posed by the dredged sediment.

The tendency in the USA and Europe is to use objective regulatory assessment frameworks based on SQGs, combined with a reliable risk-based licensing system for disposal of dredged sediments. Most SQGs are based on chemical guidelines (Alvarez-Guerra et al., 2007a), since chemical analyses of classical sediment contaminants are less expensive than toxicity tests. Moreover, results from toxicity measurements of dredged material have been difficult to interpret due to uncertainty related to the performance of the bioassays. The three objectives defined above for the present thesis are unique in terms of their application with dredged material in the Netherlands, since they will facilitate implementation of sediment management based on risk assessment and not only on chemical analysis and thus hazard assessment. The thesis will evaluate the promises and pitfalls of bioassays, selected based on rational choices when used in the risk management processes for specifically distinguished applications related to the licensing system, and the risk and impact assessment of the disposal of dredged material. Given the examples already available, it is expected that both in vitro and in vivo bioassays as well as bioindicators can provide valuable contributions to the risk management of dredged material. The key factor to successful application is the insight into the uncertainty and predictability of bioassays for specific applications in risk assessments of dredged material.
Approach of the thesis

This thesis focuses on the applicability of *in vitro*, *in vivo* bioassays and bioindicators as tools for evaluating the effects of complex chemical mixtures in the process of deciding whether dredged harbour sediments can be disposed of at sea without serious adverse effects on marine ecosystem and human health. It considers the North Sea delta area in order to determine a comprehensive approach for the application of both *in vitro* and *in vivo* bioassays for hazard assessment, advanced risk assessment, and location-specific ecological impact assessment for dredged harbour sediments. To aid in the selection of appropriate, robust and reliable *in vitro* and *in vivo* bioassay and bioindication methods for these specific purposes, the uncertainty, predictability and specificity of the bioassays have been explored and the applicability in combination with other analyses is discussed. The focus of the chosen examples is on bioassays and bioindicators for the relatively well studied dioxin-like contaminants and TBT.

Several studies were conducted to determine the overall environmental impact of dredged sediments in terms of adverse effects on ecosystem health. In Chapter 2, the uncertainty and predictability of *in vitro* and *in vivo* bioassays and bioindicators are explored. The chapter presents a retrospective analysis to explore the applicability of fish biomarkers and sediment bioassays along contaminated salinity transects focussing on biomarker responses in European flounder (*Platichthys flesus*) as the sentinel fish species. It is demonstrated that especially specific mechanism-based biomarkers and *in vitro* bioassays with sediment extract seem most promising and in the next chapter one of these specific mechanism-based *in vitro* toxicity tests was studied in more detail. Chapter 3 discusses the applicability of the *in vitro* DR-CALUX bioassay in the licensing system for disposal of dredged sediment at sea. The example illustrates that bioassay performance criteria for the analysis of complex mixtures of pollutants in sediment samples are both applicable and needed for implementation of bioassays in legislation. The bioassay thus evaluated in chapter 3 is an example of an *in vitro* bioassay with cleaned sediment extract, whereas in the next chapter an *in vivo* bioassay with full sediment was developed for specific ecotoxicological testing in marine environments. Chapter 4 demonstrates the successful optimization of an *in vivo* bioassay with cultured instead of randomly collected heart urchins (*E. cordatum*). The *in vivo* bioassay performed with these animals under controlled laboratory conditions resulted in an indication of an ecologically relevant effects of exposure to toxicants in contaminated sediments with greatly reduced variability in response. In the next chapter the studies were extended to perform an ecological risk assessment using all lines of evidence including chemical analysis, *in vivo* bioassays in the laboratory but also *in situ* ecological biomarkers.

Chapter 5 illustrates a site-specific impact assessment of TBT originating from antifouling paint and present in marine as well as harbour sediments. TBT is an endocrine-disrupting chemical and extremely toxic to marine life such as gastropod species where it causes intersex and population decline.

Finally, Chapter 6 discusses pitfalls and promises for *in vitro* and *in vivo* bioassays and bioindicators as tools in a licensing system with optimised guidelines for deciding whether dredged harbour sediments can be disposed of at sea. Given the complexity of polluted sediments, integrated assessment methods are needed that can detect the relevant toxic chemical mixtures that may pose a risk for ecosystem and human health to be prevented by dredged material risk management.

Chapter 7 presents the summary and conclusions on this integrated approach.
Chapter 2

A retrospective analysis to explore the applicability of fish biomarkers and sediment bioassays along contaminated salinity transects

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ICES Journal of Marine Science. 66, 1-17, 2009

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Abstract: Biological effects monitoring in estuarine environments is complex due to strong gradients and fluctuations in salinity and other environmental conditions, which may influence contaminant bioavailability, and the physiology and metabolism of the organisms. In order to select the most robust and reliable biological effect methods for monitoring and assessment programmes, a large-scale field study was conducted in two estuarine transects in the Netherlands. The locations ranged from heavily polluted harbour areas (the ports of Rotterdam and Amsterdam) to cleaner coastal and freshwater sites. Assessment methods used included a variety of biomarkers in flounder (Platichthys flesus) and a range of in vitro (sediment extracts) and in vivo bioassays. Multivariate statistical analysis was applied to investigate correlations and relationships between various biological effects and contaminant levels in flounder liver or sediments. Several biological methods seemed to be too much affected by salinity differences for routine use in estuaries. The most discriminative biomarkers in the study were hepatic metallothionein content and biliary 1-OH-pyrene in fish. Mechanism-based in vitro assays DR-CALUX and ER-CALUX applied to sediment extracts for screening of potential toxicity were much more responsive than in vivo bioassays with macro invertebrates using survival as an endpoint.

Introduction

Biological effects monitoring is an important element of programmes which aim to assess the quality of the environment, since it can demonstrate links between bioavailable contaminants and organism health. It can be used not only to identify particular sites and geographical areas where environmental quality is poor, but also to identify substances, or mixtures of substances, that have not been previously identified as harmful. Over the past few decades, biological effect research and monitoring programmes have focused mainly on the marine environment. This has resulted in the development and validation of a broad range of biological effect techniques (e.g. in vitro screening tests, in vivo bioassays and biomarkers) to assess marine and coastal contamination (Stebbing et al., 1992a; 1992b; Hylland, 2002; Diamant and von Westerhagen, 2003; Thain et al., 2008). Following appropriate quality assurance and control procedures, several of these techniques have now been adopted in international monitoring programmes, such as the OSPAR Joint Assessment Monitoring Programme (JAMP) (Hill et al., 1993; ICES, 2004; Thain et al., 2008). Recently, however, there has been an increasing effort to apply these techniques to biota from contaminated estuarine environments (Chapman and Wang, 2001). Estuaries are a notoriously complex environment for biological effects monitoring and risk assessment, however. Salinity can interfere with the interpretation of the contamination and biological results, either as a factor in its own right, or as a confounding factor, obscuring correlations. There are also strong fluctuations and gradients in temperature, pH, dissolved oxygen, redox potential and particle composition (Chapman and Wang, 2001). The applicability and sensitivity of conventional biomarkers and bioassays in complex estuarine environments has not yet been fully assessed.

The principal objective of the present field study was to test and compare the available biological effect monitoring techniques in order to select the most robust and reliable methods for further use in monitoring programmes for complex estuarine environments. The study area comprised pollution gradients associated with two east-west regional transects in the Dutch delta extending from freshwater upstream to saltwater closer to the North Sea. The transects were situated in the Amsterdam and Rotterdam port areas with major influence from the river Rhine. The study included a wide range of biological responses including fish biomarkers, in vitro bioassays, in vivo bioassays and accompanying chemical analysis. Fish biomarkers and gross health
indices were measured in flounder, *Platichthys flesus*. This flatfish was used as a sentinel species because 1) it occurs from entirely saline to freshwater environments (it is euryhaline), 2) it is a bottom-dwelling fish that lives on soft substrata in intimate contact with sediments (e.g. Vethaak and Jol, 1996; Matthesien and Law, 2002), and 3) it has been selected as a suitable species for use in national and international monitoring of the estuarine and marine environments (JAMP, 1998a,b). The biomarkers chosen in flounder were cytochrome P450-1A content (CYP1A), metallothioneins (MT), glutathione-S-transferase activity (GST), superoxide dismutase activity (SOD), catalase activity (CAT) and several other biomarkers for oxidative stress, vitellogenin (VTG) in plasma of male fish, 1-OH-pyrene in bile, acetyl cholinesterase activity (AChE) and RNA/DNA ratio. *In vivo* sediment bioassays were conducted with invertebrate species such as the amphipod *Corophium volutator*, the burrowing heart urchin *Echinocardium cordatum* and the polychaete *Arenicola marina*. In addition, a number of *in vitro* screening assays were applied for toxicity characterisation of sediment extracts. These included the Microtox Solid Phase® bioluminescence inhibition assay, the reporter gene assays DR-CALUX and ER-CALUX for dioxin-like and estrogenic effects respectively, and the recombinant Yeast Estrogen Screen (YES) assay.

A detailed account of chemical analysis of sediment and flounder liver along the two transects has previously been published by De Boer et al. (2001). Results of estrogenic activity in sediment extracts using the ER-CALUX assay (Legler et al., 2002), and with NADP(H)-dependent radical oxygen species (ROS) biomarkers in flounder (Livingstone et al., 2000), have been published separately but are included in our overall analysis. Despite the fact that the large-scale field study was conducted more than a decade ago (1996), we felt the findings were worth publishing because test protocols are still valid and few data seem to be available in this area.

**Methods and materials**

**Study area**

The sample locations are shown on the map in Figure 1. Euromonding (Port of Rotterdam) and the North Sea Canal (Port of Amsterdam) are well-known polluted areas. The sites in the North Sea are situated at the end of the estuarine harbour areas, except for Noordwijk (site 6), which is a typical coastal location. Two relatively clean sites, the Oesterput in the Eastern Scheldt estuary (site 14) and IJsselmeer near Enkhuizen (site 12), were also sampled and used as saltwater and freshwater reference sites respectively. In the Port of Rotterdam transect the salinity gradient is continuous whilst in the Port of Amsterdam transect the salinity gradient is interrupted and more complex due to canalisation. The numbers of the sampling sites correspond with the site numbers reported previously for the results of the chemical sediment and flounder liver analyses (De Boer et al., 2001). The transects were sampled from 19 to 26 September 1996. This is the time of the year when flounder are considered to be indicative of the contamination status of the capture location, because they have been there continuously for several months (Vethaak and Jol, 1996).

**Sampling**

At each site water, sediment and flounder (*P. flesus*) tissue were sampled, stored and transported to various laboratories for analysis. Environmental abiotic factors (e.g. depth, tempera-
ture, salinity) were recorded. Sediment samples were collected using a Van Veen grab operated from a ship. At each location a total of 40 l of sediment was sampled and stored in PVC barrels at 4°C. After two weeks they were homogenised during 20 min in an IKA-WERK RW-20 stirrer. From locations presented in Figure 1, all sediments were homogenised and subsamples were taken and used for both chemical and biological analyses. Subsamples were taken and sent by mail, to the various laboratories for chemical analysis and testing in bioassays, where they were kept at 4°C, until analysis within 2 weeks. Pore water extracts were prepared as described by Murk (1996). Flounder were captured with a fyke net at the same location where the sediments were collected. Only flounder measuring 20 cm or more were kept and placed in tanks containing water from the sampling sites. After anaesthetisation 30 specimens per site were sacrificed with a blow to the head. This was done within 12 h of capturing. The sex, length, body weight, liver weight and gonad weight of the fish were recorded and tissue samples were sub-divided for chemical and biomarker analyses. The body weight was measured after removal of the viscera. The otoliths of the fish were removed for age determination. The whole brain, blood serum, the gonads, bile, muscle tissue and part of the liver were taken from each individual fish and stored in liquid nitrogen at -70°C before distribution for biomarker analysis. Two pooled samples of flounder liver from both sexes (nos. 1-15 and 16-30) were used for a few additional biomarkers. The same two pools and one pooled sample of female flounder livers only (nos. 31-45) were

Figure 1: Distribution of sampling sites along two major estuarine areas in the Netherlands; sample locations at Port of Rotterdam transect (Euromonding including the New Waterway shipping lane) and the Port of Amsterdam (IJmonding including the North Sea Canal)
A retrospective analysis to explore the applicability of fish biomarkers and sediment bioassays.

### Table 1: Gross indices for general health and pollution biomarkers in flounder (*Platichthys flesus*) measured during the estuarine field study in the Netherlands.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Acronym</th>
<th>Type of biomarker</th>
<th>Indicative of</th>
<th>Organ/tissue used in study</th>
<th>No. of fish analysed per site</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gross indices</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Condition factor</td>
<td>CF</td>
<td>General condition</td>
<td>Nutritional status &amp; environmental pollutants</td>
<td>Whole animal</td>
<td>17-30</td>
</tr>
<tr>
<td>Gonadosomatic index</td>
<td>GSI</td>
<td>Reproduction</td>
<td>Reproductive activity</td>
<td>Gonad (females only)</td>
<td>0-13</td>
</tr>
<tr>
<td>Hepatosomatic index</td>
<td>HSI</td>
<td>Liver disease, nutritional status</td>
<td>PCBs, OCPs, BKME, PCDDs, PAHs</td>
<td>Liver</td>
<td>4-30</td>
</tr>
<tr>
<td>Liver protein content</td>
<td>LivProt</td>
<td>General condition</td>
<td>Nutritional status Environmental pollutants</td>
<td>Liver</td>
<td>6-15</td>
</tr>
<tr>
<td><strong>Biochemical &amp; enzyme markers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome P450 1A content</td>
<td>CYP1A</td>
<td>Phase I biotransformation enzyme</td>
<td>PAHs, PCBs, PCDDs, PCDFs</td>
<td>Liver tissue</td>
<td>6-15</td>
</tr>
<tr>
<td>Glutathione S-transferase activity</td>
<td>GST</td>
<td>Phase II biotransformation enzyme</td>
<td>PAHs, PCBs, OCPs, PCDDs</td>
<td>Cytosol liver tissue (l.t.)</td>
<td>6-15</td>
</tr>
<tr>
<td>Superoxide dismutase activity</td>
<td>SOD</td>
<td>Oxidative stress parameter</td>
<td>Paraquat, PCDFs, HCB, BKME</td>
<td>Liver tissue</td>
<td>1-15</td>
</tr>
<tr>
<td>Catalase activity</td>
<td>CAT</td>
<td>Oxidative stress parameter</td>
<td>PCBs, BKME, PAHs, DNOC, TCB</td>
<td>Liver tissue</td>
<td>0-11</td>
</tr>
<tr>
<td>Malondialdehyde content</td>
<td>MDA</td>
<td>Oxidative stress parameter</td>
<td>Lipid peroxidation inducers</td>
<td>Liver tissue</td>
<td>6-15</td>
</tr>
<tr>
<td>2-Keto-4-methylbutyric acid</td>
<td>KMBA&lt;sub&gt;cyt&lt;/sub&gt;</td>
<td>Oxidative stress parameter</td>
<td>OCPs, nitraromatics, PAHs</td>
<td>Cytosol l.t. pooled</td>
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</tr>
<tr>
<td></td>
<td>KMBA&lt;sub&gt;inst&lt;/sub&gt;</td>
<td>Oxidative stress parameter</td>
<td>OCPs, nitraromatics, PAHs</td>
<td>Mitochondrial fraction l.t. pooled</td>
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</tr>
<tr>
<td></td>
<td>KMBA&lt;sub&gt;micr&lt;/sub&gt;</td>
<td>Oxidative stress parameter</td>
<td>OCPs, nitraromatics, PAHs</td>
<td>Microsomal fraction l.t. pooled</td>
<td></td>
</tr>
<tr>
<td>Western blot of oxidized proteins</td>
<td>WOP</td>
<td>Oxidative stress parameter</td>
<td>Pro-oxidant contaminants</td>
<td>Microosomal fraction l.t. pooled</td>
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<tr>
<td>Metallothionein content</td>
<td>MT</td>
<td>Stress protein</td>
<td>Cd, Cu, Zn, Hg, oxidative stress</td>
<td>Liver tissue</td>
<td>6-15</td>
</tr>
<tr>
<td>1-Hydroxy pyrene content</td>
<td>1-OHpyr</td>
<td>Biotransformation product</td>
<td>PAHs</td>
<td>Bile fluid</td>
<td>6-15</td>
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<tr>
<td>Acetyl cholinesterase activity</td>
<td>AChE&lt;sub&gt;brain&lt;/sub&gt;</td>
<td>Neurotoxic parameter</td>
<td>Organophosphate and carbamate pesticides</td>
<td>Brain tissue</td>
<td>13-15</td>
</tr>
<tr>
<td></td>
<td>AChE&lt;sub&gt;muscle&lt;/sub&gt;</td>
<td>Neurotoxic parameter</td>
<td>Organophosphate and carbamate pesticides</td>
<td>Muscle tissue</td>
<td>10-15</td>
</tr>
<tr>
<td>Vitellogenin content</td>
<td>VTG</td>
<td>Reproductive &amp; endocrine parameter</td>
<td>(Xeno-)estrogenic compounds</td>
<td>Blood plasma of male fish</td>
<td>0-13</td>
</tr>
<tr>
<td>RNA and DNA</td>
<td>RNA/DNA</td>
<td>Growth, metabolic state</td>
<td>Environmental pollutants</td>
<td>Liver tissue</td>
<td>2-12</td>
</tr>
<tr>
<td>DNA adduct number</td>
<td>DNAadd</td>
<td>Genotoxic parameter</td>
<td>PAHs &amp; other environmental mutagens</td>
<td>Liver tissue</td>
<td>5-6</td>
</tr>
<tr>
<td><strong>Histological markers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vacuolation (presence)</td>
<td>Vac</td>
<td>Metabolism, general condition</td>
<td>Environmental pollutants</td>
<td>Liver tissue</td>
<td>16-32</td>
</tr>
<tr>
<td>Glycogen content</td>
<td>Glyc</td>
<td>Nutritional status</td>
<td>Environmental pollutants</td>
<td>Liver tissue</td>
<td>16-32</td>
</tr>
<tr>
<td>Fibrillar structures (presence)</td>
<td>Fibr</td>
<td>Possible genotoxic parameter</td>
<td>Genotoxic compounds</td>
<td>Liver tissue</td>
<td>16-32</td>
</tr>
<tr>
<td>Cytochrome P450 1A (presence)</td>
<td>CYP1Ahs</td>
<td>Phase I biotransformation enzyme</td>
<td>PAHs, PCBs, PCDDs, PCDFs</td>
<td>Liver tissue</td>
<td>16-32</td>
</tr>
</tbody>
</table>

* BKME = bleach kraft pulp mill effluent; DNOC = 4,6-dinitro-o-cresol; l.t. = liver tissue
* a Van der Oost et al. (2003)
* b Inhibition rather than induction by marked compound(s) reported
* c Livingstone et al. (2000)
analysed chemically for organic contaminants and trace metals as described by De Boer et al. (2001). All samples for biomarker analysis were sent to various laboratories in and outside the Netherlands by express mail. They were stored on dry ice during transportation, except for the formalin-fixed liver samples used for histopathology.

The sediment from Amerikahaven (site 10) was found to contain unexpectedly low contaminant levels during sampling in 1996 (see also De Boer et al., 2001). This was attributed to repeated dredging activity. The sediment was therefore sampled a second time in September 1997 at a non-dredged site. Analysis of this sediment showed considerably higher contaminant levels. These results are considered more representative of this location and were therefore used instead of the 1996 data in the multivariate statistical analysis of biomarker data. Sediment bioassays were however conducted with the material collected in 1996 and these data for location no. 10 were used for multivariate analysis when sediment chemistry was included.

As part of this field study, relevant quality assurance/quality control (QA/QC) criteria and guidelines (SETAC, 1993; JAMP, 1998a,b) have to be set to insure the quality of data generated during the assessments. The development of QA/QC criteria for this study involved conducting a series of replicate bioassays with each of the methods. Samples tested included a control sediment, contaminated sediments and reference toxicants. Based on the results of the bioassay replicates, the variability associated with the tests was quantified and we were able to determine what we considered acceptable QA/QC criteria for these methods.

### Gross health indices, biomarkers, and bioassays

The fish biomarkers and other indices measured during this study are presented in Table 1. The table also lists the acronyms used here, the type of biomarker, the substances that induce the biomarker (Van der Oost et al., 2003), and the ranges of the number of fish samples analysed per individual biomarker. Several biomarkers, namely hepatic glutathione S-transferase (GST), superoxide dismutase (SOD) and acetyl cholinesterase (AChE) activity in muscle tissue were analysed by two laboratories. The utility of these biomarkers appeared to be method specific, not lab-specific. The units used to present the biomarker results sometimes vary from one laboratory to another.

The condition factor (CF), hepatosomatic and gonadosomatic indices (HSI and GSI) were calculated as follows: $CF = 100 \times \frac{\text{body weight (g)}}{\text{length}^3 \text{(cm)}}$; $\text{HSI} = 100 \times \frac{\text{liver weight (g)}}{\text{body weight (g)}}$; $\text{GSI} = 100 \times \frac{\text{gonad weight (g)}}{\text{body weight (g)}}$. References to the methods used for biomarker analysis are summarized in Table 2. Methods with references and expression of units for the in vivo bioassays with invertebrate species, in vitro bioluminescence and reporter gene assays used in this study are also presented in Table 2.

### Statistical analysis

The univariate response data on all standard biomarker data were analysed, including analysis of variance for unbalanced design, using Genstat v7.1 statistical software (VSN, 2003). In addition, a-priori pairwise t-tests were performed with the mean reference value, using the pooled variance estimate from the ANOVA. The real value data were not transformed. The average values for the KMBA and WOP biomarkers were not based on different flounder captured at the sites, but on replicate measurements of pooled liver tissue. The nominal response data of the immunohistochemical biomarkers (classification of effects) were analysed by means of a Monte
A retrospective analysis to explore the applicability of fish biomarkers and sediment bioassays

Table 2: References for the methods used to measure biomarkers in flounder (Platichthys flesus) and to conduct bioassays

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Gross indices</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condition factor</td>
<td>CF</td>
<td>Text</td>
</tr>
<tr>
<td>Gonadosomatic index</td>
<td>GSI</td>
<td>Text</td>
</tr>
<tr>
<td>Hepatosomatic index</td>
<td>HSI</td>
<td>Text</td>
</tr>
<tr>
<td>Liver protein content</td>
<td>LivProt</td>
<td>Text</td>
</tr>
<tr>
<td>Biochemical &amp; enzyme markers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome P450 1A content</td>
<td>CYP1A</td>
<td>Celander and Forling (1992)</td>
</tr>
<tr>
<td>Glutathione S-transferase activity</td>
<td>GST lab1</td>
<td>Habig et al. (1974); Wilbrink et al. (1991)</td>
</tr>
<tr>
<td>Glutathione S-transferase activity</td>
<td>GST lab2</td>
<td>Leaver et al. (1993)</td>
</tr>
<tr>
<td>Superoxide dismutase activity</td>
<td>SOD lab 3</td>
<td>Sazuka et al. (1989)</td>
</tr>
<tr>
<td>Superoxide dismutase activity</td>
<td>SOD lab 4</td>
<td>Mc Cord and Fridovich (1988)</td>
</tr>
<tr>
<td>Catalase activity</td>
<td>CAT</td>
<td>Clairbone, 1985</td>
</tr>
<tr>
<td>Malondialdehyde content</td>
<td>MDA</td>
<td>Esterbauer and Cheeseman (1990)</td>
</tr>
<tr>
<td>2-Keto-4-methylbutyric acid</td>
<td>KMBA&lt;sub&gt;ext, ext, micr&lt;/sub&gt;</td>
<td>Lemaire and Livingstone (1997); Livingstone et al. (2000)</td>
</tr>
<tr>
<td>Western blot of oxidized proteins</td>
<td>WOP&lt;sub&gt;micr&lt;/sub&gt;</td>
<td>Fessard and Livingstone (1998); Keller et al. (1993)</td>
</tr>
<tr>
<td>Metallothionein content</td>
<td>MT</td>
<td>Eaton and Toal (1982)</td>
</tr>
<tr>
<td>1-Hydroxy pyrene content</td>
<td>1-OHpyr</td>
<td>Ariese et al. (2005)</td>
</tr>
<tr>
<td>Acetyl cholinesterase activity</td>
<td>AChE&lt;sub&gt;brain&lt;/sub&gt;</td>
<td>Ellman et al. (1961); Sturm et al. (1999)</td>
</tr>
<tr>
<td>Acetyl cholinesterase activity</td>
<td>AChE&lt;sub&gt;muscle&lt;/sub&gt; lab 5</td>
<td>Ellman et al. (1961); Sturm et al. (1999)</td>
</tr>
<tr>
<td>Acetyl cholinesterase activity</td>
<td>AChE&lt;sub&gt;muscle&lt;/sub&gt; lab 6</td>
<td>Bocquené et al. (1990)</td>
</tr>
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<td>Vitellogenin content</td>
<td>VTG</td>
<td>Allen et al. (1999a, 1999b)</td>
</tr>
<tr>
<td>DNA adduct number</td>
<td>DNA add</td>
<td>Reddy and Randerath (1986)</td>
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<td>Ribonucleic acid</td>
<td>RNA</td>
<td>Munro and Fleck (1966); Cesarone et al. (1979); Labarca and Puig (1980)</td>
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<tr>
<td>RNA/DNA ratio</td>
<td>RNA/DNA</td>
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</tr>
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<td>Histological markers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vacuolation (presence)</td>
<td>Vac</td>
<td>Grinwis et al. (2000, 2001)</td>
</tr>
<tr>
<td>Glycogen content</td>
<td>Gly</td>
<td>Grinwis et al. (2000, 2001)</td>
</tr>
<tr>
<td>Fibrillar structures (presence)</td>
<td>Fibr</td>
<td>Grinwis et al. (2000, 2001)</td>
</tr>
<tr>
<td>Cytochrome P450 1A (presence)</td>
<td>CYP1Ahis</td>
<td>Grinwis et al. (2000, 2001)</td>
</tr>
</tbody>
</table>

**In vivo bioassays**

- *C. volutator* (10d)  
  ASTM (1988); Schipper and Stronkhorst (1999)
- *E. cordatum* (14d)  
  Bowmer (1993); Schipper and Stronkhorst (1999)
- *A. marina* (10d)  
  Thain and Bifield (2001)
- *C. elegans*  
  Donkin and Williams (1995)

**In vitro bioassays**

- Microtox SP<sup>a</sup>  
  Schipper and Stronkhorst (1999); Ringwood et al. (1997)
- Mutatox  
  SDI (1996); Schipper and Stronkhorst (1999)
- DR-CALUX  
  Murk et al. (1996); Schipper and Stronkhorst (1999); Stronkhorst et. al (2002)
- ER-CALUX  
  Schipper and Stronkhorst (1999); Legler et al. (2002)
- YES  
  Routledge and Sumpter (1996); Legler et al. (2002)
Table 3: Average values (± pooled SE of the mean) for different health parameters and pollution biomarkers in flounder (*Platichthys flesus*) and salinity at the time of sampling.

<table>
<thead>
<tr>
<th>Site no.</th>
<th>Salinity (%)</th>
<th>Average age (yr)</th>
<th>Average weight (g)</th>
<th>Average length (cm)</th>
<th>CF</th>
<th>GSI</th>
<th>HSI</th>
<th>SG</th>
<th>LivProt</th>
<th>CYP1A (units/mg pms prot.)</th>
<th>GST (μmol/min mg pms prot.) (lab 1)</th>
<th>GST (μmol/min/mg pms prot.) (lab 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.6</td>
<td>2.43</td>
<td>210</td>
<td>27.3</td>
<td>1.03 ± 0.02</td>
<td>1.21 ± 0.22</td>
<td>1.56 ± 0.11</td>
<td>19.6</td>
<td>121 ± 7</td>
<td>47.7 ± 3.10</td>
<td>0.293 ± 0.043</td>
<td>0.561 ± 0.031</td>
</tr>
<tr>
<td>3</td>
<td>25.1</td>
<td>2.26</td>
<td>177</td>
<td>25.9</td>
<td>0.97 ± 0.02</td>
<td>1.56 ± 0.25</td>
<td>11.5</td>
<td>118 ± 7</td>
<td>32.5 ± 3.33</td>
<td>0.230 ± 0.054</td>
<td>0.399 ± 0.036</td>
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<tr>
<td>4</td>
<td>31.0</td>
<td>2.48</td>
<td>250</td>
<td>29.4</td>
<td>1.01 ± 0.03</td>
<td>-</td>
<td>-</td>
<td>33.9</td>
<td>128 ± 7</td>
<td>35.1 ± 3.21</td>
<td>0.375 ± 0.054</td>
<td>0.452 ± 0.032</td>
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<tr>
<td>6</td>
<td>31.6</td>
<td>2.37</td>
<td>267</td>
<td>29.1</td>
<td>1.04 ± 0.02</td>
<td>-</td>
<td>1.93 ± 0.14</td>
<td>45.8</td>
<td>176 ± 7</td>
<td>40.3 ± 3.10</td>
<td>0.443 ± 0.042</td>
<td>0.493 ± 0.031</td>
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<tr>
<td>7</td>
<td>12.0</td>
<td>3.37</td>
<td>229</td>
<td>27.8</td>
<td>1.03 ± 0.02</td>
<td>1.24 ± 0.20</td>
<td>1.71 ± 0.09</td>
<td>1.1</td>
<td>166 ± 7</td>
<td>37.7 ± 3.10</td>
<td>0.301 ± 0.042</td>
<td>0.373 ± 0.031</td>
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<td>9</td>
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<td>271</td>
<td>29.6</td>
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<td>1.41 ± 0.11</td>
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<td>136 ± 11</td>
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<td>0.298 ± 0.061</td>
<td>0.388 ± 0.049</td>
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<td>10</td>
<td>20.0</td>
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<td>1.28 ± 0.51</td>
<td>1.24 ± 0.10</td>
<td>-16.2</td>
<td>138 ± 7</td>
<td>51.3 ± 3.10</td>
<td>0.327 ± 0.049</td>
<td>0.415 ± 0.031</td>
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<tr>
<td>11</td>
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<td>4.17</td>
<td>218</td>
<td>27.2</td>
<td>1.02 ± 0.02</td>
<td>1.91 ± 0.27</td>
<td>1.32 ± 0.11</td>
<td>-14.6</td>
<td>155 ± 7</td>
<td>34.8 ± 3.10</td>
<td>0.374 ± 0.057</td>
<td>0.322 ± 0.031</td>
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<td>12Fref</td>
<td>&lt;2.0</td>
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<td>209</td>
<td>27.0</td>
<td>1.03 ± 0.02</td>
<td>2.03 ± 0.24</td>
<td>2.04 ± 0.09</td>
<td>-</td>
<td>143 ± 7</td>
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<td>1.58 ± 0.27</td>
<td>1.57 ± 0.11</td>
<td>-</td>
<td>182 ± 7</td>
<td>16.9 ± 3.10</td>
<td>0.316 ± 0.042</td>
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<table>
<thead>
<tr>
<th>Site no.</th>
<th>SOD mean (U/mg prot.) (lab 3)</th>
<th>Total SOD (units/mg pms protein) (lab 4)</th>
<th>CAT (mg/min/mg prot.)</th>
<th>MDA (nmol TMA/mg prot.)</th>
<th>KMBA&lt;nsub&gt;_cya&lt;/nsub&gt; (pmol ethylene/ min/mg prot.)</th>
<th>KMBA&lt;nsub&gt;_mit&lt;/nsub&gt; (pmol ethylene/ min/mg prot.)</th>
<th>KMBA&lt;nsub&gt;_mic&lt;/nsub&gt; (pmol ethylene/ min/mg prot.)</th>
<th>WOP&lt;nsub&gt;_mic&lt;/nsub&gt; (A.U.)</th>
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</thead>
<tbody>
<tr>
<td>2</td>
<td>132 ± 12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.1 ± 6.5</td>
<td>-</td>
<td>5.67 ± 0.88&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>95.9 ± 9.90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>983 ± 126</td>
<td>46.7 ± 9.91&lt;sup&gt;aa&lt;/sup&gt;</td>
<td>169 ± 6.99</td>
</tr>
<tr>
<td>3</td>
<td>136 ± 15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.8 ± 7.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85 ± 13</td>
<td>5.61 ± 0.92&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>123.3 ± 9.90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1856 ± 126</td>
<td>55.2 ± 9.91&lt;sup&gt;aa&lt;/sup&gt;</td>
<td>174 ± 6.99</td>
</tr>
<tr>
<td>4</td>
<td>125 ± 15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.7 ± 6.9&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>100 ± 12</td>
<td>4.34 ± 0.96</td>
<td>95.1 ± 9.90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1843 ± 126</td>
<td>112.2 ± 9.91&lt;sup&gt;aa&lt;/sup&gt;</td>
<td>165 ± 6.99</td>
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<tr>
<td>6</td>
<td>123 ± 12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.6 ± 6.5</td>
<td>-</td>
<td>2.72 ± 0.92</td>
<td>113.9 ± 9.90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2425 ± 126</td>
<td>135.0 ± 9.91&lt;sup&gt;aa&lt;/sup&gt;</td>
<td>160 ± 6.99</td>
</tr>
<tr>
<td>7</td>
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<td>79.0 ± 25.0</td>
<td>109 ± 11</td>
<td>2.14 ± 0.82</td>
<td>71.7 ± 9.90</td>
<td>1190 ± 126</td>
<td>55.4 ± 9.91&lt;sup&gt;aa&lt;/sup&gt;</td>
<td>156 ± 6.99</td>
</tr>
<tr>
<td>9</td>
<td>121 ± 18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49.4 ± 11.2</td>
<td>99 ± 14</td>
<td>6.23 ± 1.30&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>77.8 ± 9.90</td>
<td>1264 ± 126</td>
<td>31.7 ± 9.91&lt;sup&gt;aa&lt;/sup&gt;</td>
<td>193 ± 6.99&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
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<td>64.8 ± 7.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>98 ± 12</td>
<td>7.25 ± 0.82&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>91.8 ± 9.90</td>
<td>1990 ± 126</td>
<td>80.2 ± 9.91&lt;sup&gt;aa&lt;/sup&gt;</td>
<td>165 ± 6.99</td>
</tr>
<tr>
<td>11</td>
<td>127 ± 16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.6 ± 6.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>138 ± 13&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.10 ± 0.82</td>
<td>85.8 ± 9.90</td>
<td>1121 ± 126</td>
<td>127.0 ± 9.91&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>168 ± 6.99</td>
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<tr>
<td>12Fref</td>
<td>148 ± 14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.2 ± 6.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>109 ± 11</td>
<td>3.28 ± 0.38</td>
<td>74.5 ± 9.90</td>
<td>934 ± 126</td>
<td>52.9 ± 9.91&lt;sup&gt;aa&lt;/sup&gt;</td>
<td>165 ± 6.99</td>
</tr>
<tr>
<td>14Sref</td>
<td>100 ± 12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.7 ± 6.5</td>
<td>87 ± 11</td>
<td>2.41 ± 0.82</td>
<td>70.8 ± 9.90</td>
<td>928 ± 126</td>
<td>96.0 ± 9.91&lt;sup&gt;aa&lt;/sup&gt;</td>
<td>157 ± 6.99</td>
</tr>
</tbody>
</table>

<sup>a</sup> pairwise comparison on the basis of replicate measurements rather than replicate fish

<sup>b</sup> significantly different from saltwater reference site (Sref) at p<0.05

<sup>ab</sup> significantly different from Sref at p<0.01

<sup>a</sup> significantly different from freshwater reference site (Fref) at p<0.05

<sup>ab</sup> significantly different from Fref at p<0.01

Fref = freshwater reference site

Sref = saltwater reference site
Table 3: continued.

<table>
<thead>
<tr>
<th>Site no.</th>
<th>MT (μg/mg pms prot.)</th>
<th>1-OH pyr (ng/mL)</th>
<th>AChEbrain (ng/min/mg prot.)</th>
<th>AChEmuscle (ng/min/mg prot.)</th>
<th>AChEmuscle (U/min/mg prot.)</th>
<th>VTG in males (μg/mL)</th>
<th>RNA/protein (μg/ng)</th>
<th>RNA/DNA ratio (μg/μg)</th>
<th>DNAadd (nr./10^8 nucleotides)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.55 ± 0.18s</td>
<td>50 ± 29</td>
<td>128 ± 5eff</td>
<td>54 ± 5eff</td>
<td>8347 ± 941</td>
<td>0.1 ± 426</td>
<td>0.116 ± 0.077</td>
<td>9.19 ± 1.67</td>
<td>13.0 ± 3.2</td>
</tr>
<tr>
<td>3</td>
<td>1.76 ± 0.19eff</td>
<td>70 ± 37</td>
<td>105 ± 5</td>
<td>91 ± 5</td>
<td>9458 ± 941</td>
<td>0.1 ± 426</td>
<td>0.140 ± 0.071</td>
<td>6.86 ± 0.83s</td>
<td>14.0 ± 3.2</td>
</tr>
<tr>
<td>4</td>
<td>1.99 ± 0.17ss</td>
<td>37 ± 45</td>
<td>105 ± 5</td>
<td>60 ± 5eff</td>
<td>6949 ± 941eff</td>
<td>-</td>
<td>0.118 ± 0.032</td>
<td>7.96 ± 0.89</td>
<td>20.1 ± 3.5s</td>
</tr>
<tr>
<td>6</td>
<td>1.40 ± 0.17</td>
<td>32 ± 33</td>
<td>127 ± 5s</td>
<td>65 ± 5s</td>
<td>7738 ± 974</td>
<td>551 ± 337</td>
<td>0.138 ± 0.167</td>
<td>7.52 ± 0.71</td>
<td>15.2 ± 3.2</td>
</tr>
<tr>
<td>7</td>
<td>2.14 ± 0.17ss</td>
<td>365 ± 31ss</td>
<td>83 ± 5s</td>
<td>61 ± 5eff</td>
<td>7537 ± 941</td>
<td>1.8 ± 337</td>
<td>0.078 ± 0.054</td>
<td>5.83 ± 0.75s</td>
<td>15.4 ± 3.2</td>
</tr>
<tr>
<td>9</td>
<td>2.27 ± 0.26s</td>
<td>79 ± 35</td>
<td>71 ± 5s</td>
<td>60 ± 5s</td>
<td>10558 ± 1214</td>
<td>0.9 ± 476</td>
<td>0.094 ± 0.047</td>
<td>6.62 ± 0.79s</td>
<td>14.4 ± 3.2</td>
</tr>
<tr>
<td>10</td>
<td>1.37 ± 0.17</td>
<td>117 ± 39s</td>
<td>87 ± 5s</td>
<td>72 ± 5eff</td>
<td>9295 ± 974</td>
<td>2.2 ± 476</td>
<td>0.088 ± 0.035</td>
<td>6.22 ± 0.75s</td>
<td>15.8 ± 3.2</td>
</tr>
<tr>
<td>11</td>
<td>2.61 ± 0.17ss</td>
<td>103 ± 45s</td>
<td>75 ± 5s</td>
<td>86 ± 5</td>
<td>9800 ± 941</td>
<td>0.4 ± 476</td>
<td>0.098 ± 0.030</td>
<td>7.73 ± 0.96</td>
<td>16.3 ± 3.5</td>
</tr>
<tr>
<td>12Fref</td>
<td>1.07 ± 0.18</td>
<td>7.9 ± 31</td>
<td>109 ± 5s</td>
<td>91 ± 5</td>
<td>9290 ± 1010</td>
<td>962 ± 318</td>
<td>0.050 ± 0.021</td>
<td>6.64 ± 0.83s</td>
<td>17.3 ± 3.5</td>
</tr>
<tr>
<td>14Sref</td>
<td>1.18 ± 0.17</td>
<td>21 ± 29</td>
<td>97 ± 5s</td>
<td>90 ± 5</td>
<td>9393 ± 974</td>
<td>0.2 ± 264</td>
<td>0.069 ± 0.023</td>
<td>9.51 ± 0.68s</td>
<td>11.0 ± 3.2</td>
</tr>
</tbody>
</table>

* significantly different from saltwater reference site (Sref) at p<0.05
* significantly different from Sref at p<0.01
* significantly different from freshwater reference site (Fref) at p<0.05
* significantly different from Fref at p<0.01
Fref = freshwater reference site
Sref = saltwater reference site
A retrospective analysis to explore the applicability of fish biomarkers and sediment bioassays

Carlo test (Manly, 1997) using the chi^2 statistic (Snedecor and Cochran, 1967) because of the many low-number (or zero) observations. In the simulations, the number of observations per location was kept fixed. For each test, 10,000 simulations were performed and pairwise comparisons were also made using this method.

Multivariate techniques are increasingly being used to link field concentrations of chemicals with bioassay responses (Shaw and Manning, 1996; Del Vals et al., 1997; Ter Braak, 1995; Van den Brink et al., 2003; Van den Brink and Kater, 2006). In this paper principal component analysis (PCA), a frequently used ordination technique (Ter Braak and Smilauer, 2002), was used to correlate concentrations in sediment and fish with each other and with the responses of biomarkers and bioassays. PCA triplot was used for exploratory data analysis. Since these data were collected in a field-monitoring programme, no conclusions can be drawn as to causality. PCA gives a correlation plot which serves as a graphical summary of the data set showing the correlations between all variables. In PCA, imaginary, latent explanatory variables are calculated from the data set which best explain the variation in concentrations of chemicals in the sediments from the different sampling sites. The first two latent variables are normally used to construct an ordination diagram. This diagram provides an overview of mutual correlations between ‘species’ (here, the concentrations of chemicals in sediments or fish) on the one hand and passive ‘environmental’ variables (here, concentrations in fish, bioassay or biomarker responses) on the other (Van den Brink et al., 2003). For a more elaborate description of PCA plot interpretation the reader is referred to Ter Braak (1995). For further explanation of the use of PCA and related methods to analyse ecotoxicological and biomonitoring data, see Van den Brink et al. (2003).

Four different PCA analyses were performed to:

- correlate the sediment concentrations of chemicals at the different sampling sites with the response of biomarkers,
- correlate the sediment concentrations of chemicals at the different sampling sites with the concentrations of chemicals in fish,
- correlate the fish concentrations of chemicals at the different sampling sites with the response of biomarkers and gross health indices,
- correlate the sediment concentrations of chemicals at the different sampling sites with the response of in vitro and in vivo bioassays.

In all analyses, chemical concentrations were transformed using a natural logarithm. Concentrations below the limit of detection were replaced by concentrations at half the limit of detection. Parameters with more than two missing data were not included in the analysis (i.e. GSI, Mutatox). Since salinity may interfere with the response of biomarkers, it was decided to add the salinity measurements (see Table 3) to the chemical data used in the multivariate analysis of biomarker results. This was deemed pointless for the analysis of the bioassay data because seawater was added during all tests or the animals were exposed to extracts (nematodes). The input for the histopathology and immunohistochemical parameters was the weighted average effect class per location. The PCA triplot analyses were performed using the software program Canoco for Windows 4.5 (Ter Braak and Smilauer, 2002) by centring and standardising the concentrations of the chemicals. The results were presented in correlation diagrams (Van den Brink et al., 2003). The significance of the correlation between the separate biomarkers and bioassays with the concentrations of the chemicals in the two matrices was tested using Monte Carlo permutation tests applying the redundancy analysis (RDA, a constrained form of PCA) option (Van den Brink and Kater, 2006).
Results

Salinity and contaminants in sediments and flounder

The salinity of the water above the sediments during sampling at each site is presented in Table 3. As expected, the salinity gradient was less distinct in the Port of Amsterdam transect than in the Port of Rotterdam transect. The concentrations of contaminants in sediments and flounder liver as previously published by the De Boer et al. (2001) are summarized in Tables 4a and 4b. The highest levels of polychlorinated biphenyls (PCBs), several organochlorine pesticides (OCPs), polybrominated diphenylethers (PBDEs) and organotins (e.g. TBT and TPhT) were found in the industrialized harbour areas in the low salinity segment of each transect. The contamination level generally decreased and salinity increased towards the marine end of the estuaries, but there are specific differences between both transects. For example, fairly high concentrations of PBDE and cadmium were found in flounder liver from the Port of Rotterdam, and elevated levels of PAHs were found in sediments at most sites from the Port of Amsterdam transect (see Table 5).

Gross health indices in flounder

The first part of Table 3 shows the results of the biological parameters and gross health indices of flounder captured at the different sites. There were considerable differences in the age composition of the flounder samples > 20 cm. Flounders from the sites in the Port of Amsterdam transect were older than those from the Port of Rotterdam transect (average age range 3.6 to 4.4 and 2.3 to 2.5 years respectively). The Splitsingsdam location (site 3) yielded not only the youngest specimens but, compared to the reference sites, the flounder with the lowest weight and lowest average length. Compared to the freshwater reference site (IJsselmeer near Enkhuizen; site 12) average condition factors were slightly but significantly reduced at two polluted sites, site 3 (Splitsingsdam) and site 10 (Amerikahaven). In general the flounder seem to have a fairly similar average condition factor and none of the locations deviated from the saltwater reference site in the Eastern Scheldt estuary.

The average gonadosomatic indices (GSI) in female flounder and hepatosomatic indices (HSI) (both sexes) at the saltwater and freshwater reference locations differ significantly from each other (p<0.01). Average GSI was significantly decreased at sites 2 (Benelux tunnel) and 7 (IJmuiden), but only compared to the freshwater reference site where flounder had the greatest average GSI of all locations where gonad weights were measured (not sampled at sites 3, 4 and 6). The difference in average HSI between the two reference locations was also significant (p<0.01). Average HSI values at all other sites also differed significantly from one or both references, though these index values were all within the range between the two reference sites.

Biomarkers in flounder

The average values of the biomarker measurements in flounder are also shown in Table 3. For several of the biomarkers, there was a distinct and significant difference between the average values at the saltwater and freshwater reference sites. This was the case for CYP1A, protein content in the liver, GST measured by lab 1 (GST at the saltwater reference site was not measured by lab 2), SOD analysed by lab 3, microsomal 2-Keto-4-methiolbutyric acid (KMBAmic), RNA/DNA ratio (differences with p<0.01), AChEbrain and VTG in male fish (all with p<0.05).
Table 4a: Summary of chemicals measured in the sediments of the Port of Rotterdam and Port of Amsterdam sites (according to De Boer et al., 2001)

<table>
<thead>
<tr>
<th>Sites</th>
<th>Units</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>7</th>
<th>9</th>
<th>10*</th>
<th>11</th>
<th>12</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum Organotins (MBT, DBT, TBT, MPT, DPT, TPhT)</td>
<td>μg Sn/kg dry wt</td>
<td>106</td>
<td>110</td>
<td>52</td>
<td>43</td>
<td>237</td>
<td>170</td>
<td>14</td>
<td>73</td>
<td>206</td>
<td>8</td>
</tr>
<tr>
<td>Cu</td>
<td>mg/kg dry wt</td>
<td>47</td>
<td>38</td>
<td>21</td>
<td>20</td>
<td>77</td>
<td>40</td>
<td>18</td>
<td>140</td>
<td>41</td>
<td>10</td>
</tr>
<tr>
<td>As</td>
<td>mg/kg dry wt</td>
<td>16</td>
<td>15</td>
<td>14</td>
<td>17</td>
<td>29</td>
<td>17</td>
<td>11</td>
<td>29</td>
<td>41</td>
<td>14</td>
</tr>
<tr>
<td>Cd</td>
<td>mg/kg dry wt</td>
<td>2.34</td>
<td>1.46</td>
<td>0.88</td>
<td>0.81</td>
<td>1.92</td>
<td>0.90</td>
<td>0.24</td>
<td>3.02</td>
<td>3.16</td>
<td>2.90</td>
</tr>
<tr>
<td>Hg</td>
<td>mg/kg dry wt</td>
<td>0.69</td>
<td>0.53</td>
<td>0.34</td>
<td>0.30</td>
<td>2.43</td>
<td>0.54</td>
<td>0.08</td>
<td>- 1.24</td>
<td>0.65</td>
<td>0.16</td>
</tr>
<tr>
<td>Cr</td>
<td>mg/kg dry wt</td>
<td>110</td>
<td>93</td>
<td>92</td>
<td>100</td>
<td>142</td>
<td>76</td>
<td>78</td>
<td>109</td>
<td>121</td>
<td>75</td>
</tr>
<tr>
<td>Zn</td>
<td>mg/kg dry wt</td>
<td>295</td>
<td>231</td>
<td>162</td>
<td>238</td>
<td>587</td>
<td>269</td>
<td>88</td>
<td>648</td>
<td>719</td>
<td>422</td>
</tr>
<tr>
<td>Ni</td>
<td>mg/kg dry wt</td>
<td>28</td>
<td>27</td>
<td>21</td>
<td>20</td>
<td>41</td>
<td>37</td>
<td>35</td>
<td>41</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>Pb</td>
<td>mg/kg dry wt</td>
<td>72</td>
<td>69</td>
<td>57</td>
<td>88</td>
<td>272</td>
<td>85</td>
<td>34</td>
<td>255</td>
<td>234</td>
<td>72</td>
</tr>
<tr>
<td>Sum 13 PAHs (Ant, BaA, Bap, BbF, BeP, BghiPe, BkF, Chr, dBahA, Fen, Flu, Pyr, InP)</td>
<td>mg/kg dry wt</td>
<td>6132</td>
<td>4343</td>
<td>4774</td>
<td>1313</td>
<td>11463</td>
<td>7726</td>
<td>673</td>
<td>11269</td>
<td>11660</td>
<td>2013</td>
</tr>
<tr>
<td>Sum 7 PCBs (28, 101, 118, 138+163, 153, 180)</td>
<td>μg/kg dry wt</td>
<td>89.5</td>
<td>54.5</td>
<td>38.2</td>
<td>23.7</td>
<td>57.6</td>
<td>33.4</td>
<td>6.8</td>
<td>82.1</td>
<td>84.6</td>
<td>41.4</td>
</tr>
</tbody>
</table>

* sediment was sampled a second time at a non-dredged site in this harbour
Table 4b: Summary of chemicals measured in flounder *Platichthys flesus* liver (pooled) from the Port of Rotterdam and Port of Amsterdam sites (according De Boer et al., 2001)

<table>
<thead>
<tr>
<th>Units</th>
<th>Sites 2</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>7</th>
<th>9</th>
<th>10</th>
<th>10*</th>
<th>11</th>
<th>12</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum 28 PCBs (28, 31, 44, 47, 49, 52, 56, 66+95, 87, 97, 99, 101, 105, 110, 118, 128, 137, 138, +163, 141, 149 151, 153, 156, 170, 180, 187, 194, 202, 206)</td>
<td>μg/kg lipid weight</td>
<td>11545</td>
<td>6596</td>
<td>3115</td>
<td>1635</td>
<td>2927</td>
<td>6587</td>
<td>5455</td>
<td>n.a.</td>
<td>6173</td>
<td>1578</td>
</tr>
<tr>
<td>Sum OCPs (Aldrin, Endrin, Dieldrin, Lindane gamma Heptachlor epoxide, Hexachlorobenzene)</td>
<td>μg/kg lipid weight</td>
<td>392.4</td>
<td>242.6</td>
<td>184</td>
<td>148.8</td>
<td>152.2</td>
<td>218.3</td>
<td>159</td>
<td>n.a.</td>
<td>111</td>
<td>92.1</td>
</tr>
<tr>
<td>Cd</td>
<td>μg/kg lipid weight</td>
<td>0.133</td>
<td>0.054</td>
<td>0.107</td>
<td>0.065</td>
<td>0.034</td>
<td>0.031</td>
<td>0.028</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.006</td>
</tr>
<tr>
<td>Cr</td>
<td>μg/kg lipid weight</td>
<td>0.005</td>
<td>0.02</td>
<td>0.005</td>
<td>0.005</td>
<td>0.07</td>
<td>0.005</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.005</td>
<td>0.002</td>
</tr>
<tr>
<td>Hg</td>
<td>μg/kg lipid weight</td>
<td>0.18</td>
<td>n.a.</td>
<td>0.06</td>
<td>0.03</td>
<td>0.05</td>
<td>0.05</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.015</td>
<td>0.16</td>
</tr>
<tr>
<td>Zn</td>
<td>μg/kg lipid weight</td>
<td>50</td>
<td>n.a.</td>
<td>66</td>
<td>43</td>
<td>40</td>
<td>41</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>29</td>
</tr>
<tr>
<td>Sum of brominated flame retardants (2,4,2',4'-TBDE, 2,4,5,2',4'-PeBDE)</td>
<td>μg/kg lipid weight</td>
<td>304</td>
<td>149</td>
<td>&lt;62</td>
<td>&lt;36</td>
<td>&lt;44</td>
<td>81</td>
<td>&lt;62</td>
<td>n.a.</td>
<td>123</td>
<td>&lt;40</td>
</tr>
</tbody>
</table>

n.a. = not analysed
A retrospective analysis to explore the applicability of fish biomarkers and sediment bioassays

For these biomarkers, parameter values at non-reference sites were rarely significantly different from both freshwater and saltwater reference sites, though some difference might be expected. The first exception to this was CYP1A in the liver, which still showed a significant increase at three other sites (site 2, Beneluxtunnel; site 6, Noordwijk; site 10, Amerikahaven) compared to both (significantly different) reference sites. The second exception was brain AChE, but both significant increases and significant decreases compared to the two references were observed for this biomarker.

With only one freshwater and one saltwater reference site, biomarkers that did not seem to be affected by differences in salinity and showed significant and unambiguous responses at several non-reference sites were GST2, SOD3 (1 of 2 labs), MDA, cytosolic and mitochondrial KMBA, Western blot of microsomal oxidized proteins (WOP), MT, 1-OH pyrene, AChE in muscle tissue (2 labs) and DNA adducts. The average VTG content of the flounder varied considerably from site to site, but the difference between the reference sites was large and significant. However, for VTG, the variation is three orders of magnitude greater than the mean. Some biomarkers showed limited power to discriminate between polluted and less polluted sites (i.e. a significant response at only one polluted location). These were DNA adducts, AchEmuscle2, CAT and WOP. However, for the latter two, the increases are highly significant (p<0.01) and occur at two of the most polluted sites (11 and 9 respectively; see Table 4).

The results of the histopathological and immunohistochemical biomarker analyses are shown in Table 5. Vacuolation of the liver tissues differed significantly between the saltwater and freshwater reference sites. The average degree of vacuolation was highest at the saltwater site. Compared to this location, vacuolation was significantly lower (p<0.05) at sites 4 (Loswal Noord), 9 (Buitenhuizen) and 11 (Oranjesluis). Glycogen content barely differed between sites. Only flounder from site 10, Amerikahaven, had a somewhat greater quantity of PAS positive material in their hepatocytes. Fibrillar structures in hepatocytes did not differ significantly between sampling locations. Average immunoreactivity against CYP1A was low in flounder from all sites, except at North Sea Canal sites 10 (Amerikahaven) and 11 (Oranjesluis) where the frequency of higher classes was significantly increased compared to both reference sites. Site 10 was also the site with the greatest average CYP1A content in flounder liver homogenate.

In vivo bioassays

Most of the in vivo bioassays conducted with the whole sediments from the locations in the study showed barely any differences between sites (Table 6). Endpoints and tests that appeared particularly indiscriminative were survival of C. volutator (in tests performed by two different laboratories), survival of A. marina, and survival and reburrowing behaviour of field heart urchin E. cordatum. The total number of casts produced by A. marina during the bioassays varied greatly between sediments from different locations, and also between the two references. The same was found for the average dry weight of C. volutator at the end of the 10d experiments. Most in vivo whole sediment bioassays were not replicated and therefore could not be statistically tested. Significant reproductive effects with nematode C. elegans were observed at sites 2, 3, 6, 7, 9 and 11 for female nematodes exposed to sediment extracts for 24 hours prior to egg laying. The magnitude of the effects followed the degree of contamination on the different gradients. Interestingly, although the total number of offspring was not significantly reduced at location 11 (Oranjesluis in the Port of Amsterdam), their development typically halted in stage J3 and was not completed.
A retrospective analysis to explore the applicability of fish biomarkers and sediment bioassays

Table 5: Frequency distribution of numbers of flounder livers in histological effect classes. Explanation of the classes in the main text.

<table>
<thead>
<tr>
<th>Site no.</th>
<th>Vacuolation</th>
<th>Glycogen content</th>
<th>Fibrillar structures</th>
<th>CYP1A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Class 0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>1</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>0</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>0</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>1</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>0</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>12</td>
<td>0</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>10</td>
<td>14</td>
<td>0</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>11</td>
<td>15</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Fref</td>
<td>17</td>
<td>0</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Sref</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>9</td>
</tr>
</tbody>
</table>

s = significantly different from saltwater reference site (Sref) at p<0.05
ss = significantly different from Sref at p<0.01
f = significantly different from freshwater reference site (Fref) at p<0.05
ff = significantly different from Fref at p<0.01

Table 6: Results of in vivo bioassays with sediment or sediment pore water from estuarine and coastal locations in the Netherlands and a freshwater and saltwater reference site.

<table>
<thead>
<tr>
<th>Site no.</th>
<th>Corophium volutator</th>
<th>Arenicola marina</th>
<th>Echinocardium cordatum</th>
<th>C. elegans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average survival (%)(lab 7)</td>
<td>Average dry wt (μg/ind.) (lab 7)</td>
<td>Average survival (%) (lab 8)</td>
<td>Average total cast number (lab 8)</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>93</td>
<td>514</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>82</td>
<td>513</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>85</td>
<td>522</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>94</td>
<td>437</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>94</td>
<td>337</td>
<td>97</td>
<td>100</td>
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<tr>
<td>10</td>
<td>94</td>
<td>394</td>
<td>97</td>
<td>100</td>
</tr>
<tr>
<td>11</td>
<td>80</td>
<td>365</td>
<td>93</td>
<td>80</td>
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<tr>
<td>12Fref</td>
<td>83</td>
<td>399</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>14Sref</td>
<td>97</td>
<td>512</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* = a significant effect (p<0.05) compared to the control
** = a significant effect (p<0.01) compared to the control
*** = a significant effect (p<0.001) compared to the control

In vitro bioassays

The bacterial Microtox tests and the other in vitro bioassays clearly indicated differences in sediment toxicity between locations (Table 7). In the Microtox SP® assay inhibitory effects were found in sediment extracts from the Port of Amsterdam transect (i.e. TU values greater than 20 at sites 7 and 11). The highest response in the Mutatox® assay was found at Oranjesluis (site 11) in the Port of Amsterdam. The reference values from the Mutatox® assay at this site were below
the detection limit. A high response was also found at Oranjesluis (site 11) and Buitenhuizen (site 9) with the 6h (indicating toxic PAHs) and 48h (indicating dioxin-like toxicity) results of the DR-CALUX. Other locations where toxic equivalent (TEQ) values in the 6h, 24h and 48h DR-CALUX were greater than the saltwater references at Splitsingsdam (site 3) and Buitenhuizen (site 9). EEQs in the sediment extracts measured with the ER-CALUX varied by almost one order of magnitude. The greatest estrogenic activity (EEQ>10 pmol/g dry weight) was found in sediments from the interregional locations at Port of Rotterdam (sites 2 and 3), Noordwijk (site 6) and Ijmuiden Harbour (site 7). EEQs in the sediment extracts measured using the YES were one order of magnitude higher than the references at locations Splitsingsdam (site 3) and Ijmuiden Harbour (site 7), but lower at Loswal Noord (site 4), Buitenhuizen (site 9) and Amerikahaven (site 10).

Multivariate analysis

The results of the principle component analysis (PCA) of internal pollutant concentrations in flounder liver at the sampling locations plotted against the concentrations of the same substances in the sediments are shown in Figure 2. There is a clear distinction between the more polluted sites on the right site of the triplot and the cleaner locations on the left. Figure 2 also shows that the pattern of most individual PCB congeners in flounder liver is strongly correlated with the distribution of these PCBs in the sediment. The only exception to this is PCB 105. There is a weaker correlation between the concentrations of HCB in flounder liver and measured concentrations of these contaminants in the sediments, and none at all in the case of metals (Zn, Cd, Cr and Hg).

A number of biomarkers correlated to some extent with the chemical compounds that were measured in flounder liver tissue. Those explaining >10% of the variance are shown in Figure 3. Biomarkers and health parameters that correlated significantly (p < 0.10) - either positively or negatively - with contamination were HSI (p = 0.015), LivProt (p = 0.027), GST (measured by lab 1; p = 0.058), MDA (p = 0.076), WOPmicr (p = 0.079) and VTG (p = 0.080). There was no correlation between VTG values in male fish and the sediment estrogenic activity. HSI, LivProt and GST decreased with higher concentrations of many higher PCBs. MDA and WOPmicr were positively correlated with high body burdens of lower PCBs and several other organochlorine contaminants.

Figure 4 shows a similar triplot of biomarkers and health indices at the various sites against sediment concentrations of measured contaminants. Biomarkers and health indices that correlated significantly (p < 0.10) with pollutants in the sediments were 1-OH-pyr (p = 0.022), MT (p = 0.045), HSI (p = 0.053), SOD (lab4; p = 0.066), CYP1A (p = 0.070) and CYP1Ahis (p = 0.098). These parameters also correlated with the degree of contamination in the sediments. CYP1A and CYP1Ahis corresponded well with higher levels of PCBs (and with each other) and 1-OH-pyrene with high levels of PAHs. MT correlated with high levels of organotin compounds but not with metals such as Zn and Cd.

The flounder health indices and biomarkers in multivariate analysis that explained less than 10% of the observed variance were CF (Figs. 3 and 4), GST (lab 2) (Fig. 3), SOD (measured by labs 3 and 4) (Fig. 3), CAT (Fig. 3), MDA (Fig. 4), KMBA (mitochondrial, microsomal and in cytosol) (Figs. 3 and 4), WOPmicr (Fig. 4), 1-OH-pyrene (Fig. 3), AchEmuscle (labs 5 and 6) (Fig. 4), AchEbrain (Fig. 3), RNA/DNA (Fig. 3), DNAadd (Figs. 3 and 4), Glyc (Figs. 3 and 4), Fibr (Fig. 4) and CYP1Ahis (Fig. 3).
A retrospective analysis to explore the applicability of fish biomarkers and sediment bioassays

Table 7: Results of Microtox tests and in vitro bioassays.

<table>
<thead>
<tr>
<th>Site no.</th>
<th>MSP</th>
<th>Mutatox (with clean-up dry sediment)</th>
<th>DR-CALUX</th>
<th>Pore water ER-CALUX on polar fraction sediment</th>
<th>YES on 10× diluted sediment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TU Average LOEC (mg/ml)</td>
<td>6h-TEQ (pg/g)</td>
<td>24h-TEQ (pg/g)</td>
<td>48h-TEQ (pg/g)</td>
</tr>
<tr>
<td>2</td>
<td>&lt;20</td>
<td>31.8</td>
<td>2.5</td>
<td>16</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>&lt;20</td>
<td>10.5</td>
<td>16</td>
<td>78</td>
<td>79</td>
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<tr>
<td>4</td>
<td>&lt;20</td>
<td>47.8</td>
<td>2.6</td>
<td>10</td>
<td>dl</td>
</tr>
<tr>
<td>6</td>
<td>&lt;20</td>
<td>191</td>
<td>0.24</td>
<td>3</td>
<td>dl</td>
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<tr>
<td>7</td>
<td>12</td>
<td>65.4</td>
<td>7</td>
<td>23</td>
<td>15</td>
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<tr>
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<td>&lt;20</td>
<td>DI</td>
<td>32</td>
<td>87</td>
<td>65</td>
</tr>
<tr>
<td>10</td>
<td>&lt;20</td>
<td>DI</td>
<td>2.7</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
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<td>2.6</td>
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<td>DI</td>
<td>8.1</td>
<td>36</td>
<td>37</td>
</tr>
<tr>
<td>14 Ref</td>
<td>&lt;20</td>
<td>DI</td>
<td>3.9</td>
<td>29</td>
<td>20</td>
</tr>
</tbody>
</table>

dl = detection limit

Figure 5 displays the PCA triplot for the sediment bioassay responses versus the measured concentrations of pollutants in sediments. Most in vivo bioassays correlate negatively with pollutants in a general sense, i.e. survival (C. volutator), number of casts (A. marina) and reburrowing behaviour (E. cordatum) increase with decreasing contamination, although not significantly. The bioassay C. volutator showed a slightly decreased survival rate at site 11, which is associated with sediment containing high concentrations of organotins, PAHs and PCBs. The survival of E. cordatum correlates significantly (p= 0.031) with increasing contamination, although survival at all sites was barely affected, if at all (Table 6). The heart urchin showed only a slightly decreased survival rate at site 10, which is associated with sediment contamination by PCBs (Fig. 5).

The results of the Microtox SP® assay did not correlate significantly with sediment contamination. However, individual sediment response results at site 7 and site 11 showed a moderate response. The results for the DR-CALUX correlated significantly with the sediment concentrations (p = 0.011), especially with PAHs, organotins and some metals (Fig. 5). There was a difference between the correlations of the responses in the DR-CALUX after 6h, 24h and 48h incubation with sediment extract at sites 3,9 and 11 or pore water extracts at all sites. This suggests PAHs are dominant in the sediment extract, and the cells were not able to metabolize them within 24 hours. The ER-CALUX response increased with rising levels of the more chlorinated PCB congeners in the sediment, but this was not significant (p = 0.205). The YES assay did not correlate with PCB congeners in the sediment.

Discussion

Contaminants in sediments and flounder

The Port of Amsterdam transect yielded four locations in the estuarine system with highly polluted sediment (sites 7, 9, 10 and 11). The chemical concentrations there were higher than at the sites in the Port of Rotterdam transect (Table 4a). PCB and trace metal concentrations in Rotterdam harbour sediments and TBT, triphenyltin (TPhT) and PAH concentrations in all sedi-
ments exceeded the Dutch Maximum Permissible Concentration (MTC) environmental quality standard (the concentration that theoretically protects 95% of the species in ecosystems (De Boer et al., 2001). In Euromonding, including the New Waterway shipping lane (see Fig 1), the lower concentrations of contaminants found may have been due to intensive dredging, since nearly 20 million cubic metres of sludge has been disposed of in the open sea each year. Moreover, tides appeared to transport sediment-associated contaminants westwards (Stronkhorst et al., 2003c).

The contamination ranking from high to low sediment concentrations of metals (copper, zinc, mercury, chromium, nickel and lead), sum-PCB and sum-PAH was as follows in the Port of Amsterdam locations: Oranjesluis (site 11), IJmuiden (site 7), Amerikahaven (site 10; 1997) and Buitenhuizen (site 9). De Boer et al. (2001) concluded that in flounder liver the highest concentrations of PCBs, several OCPs and PBDEs were found at the inland side of the Port of Amsterdam and Port of Rotterdam transects (Table 4b). While relative concentrations may be inferred from the location on the PCA plots, PCA triplot calculations express the relative covariation between variables, not their absolute value. Overall, the PCA triplot showed that there is a positive correlation between sediment contamination and the body burden in flounder at the sites (Fig. 2). The PCB concentrations in most sediments correlate weakly with body burden in pooled flounder livers (correlation coefficient; \( r^2 = 0.4036 \)). Interestingly, the highest levels of PCB contamination in sediments were observed at the Amsterdam transect locations IJmuiden (site 7), Buitenhuizen (site 9), Amerikahaven (site 10) and Oranjesluis (site 11), whereas the body burdens from Buitenhuizen and IJmuiden were higher and lower respectively compared to matched sediments. The PCA triplot (Fig. 2) clearly shows that flounder caught at Buitenhuizen (site 9) showed no correlation between sediment contamination and body burden, indicating that flounder may be locally migratory. This suggests that flounder sampled at Buitenhuizen (site 9) are probably not representative of this location, but may be migrants from the more highly polluted locations IJmuiden (site 7) or Oranjesluis (site 11). PCBs and trace metal concentrations in flounder liver were not higher than the literature data (Kopecka et al., 2006). PCBs accumulate in fatty tissues such as the liver. Since the levels of most individual PCB congeners closely reflected the pattern in the sediments, equilibrium between flounder and their surroundings may be readily achieved for these substances. PCB105, the only measured congener whose levels in sediment and flounder corresponded less well, is known to be readily metabolized in fish (Boon and Duinker, 1985). In our study organic contaminants and metal concentrations were measured in liver tissue. The correlation of liver contamination with sediment contamination was much better for PCBs than for metals and HCB. In the case of metals this may be caused by the fact that contaminants were only measured in flounder livers. Certain metals may accumulate more efficiently in other tissues such as the kidneys and gills (Storelli and Marcotriagiano, 2001; Camusso et al., 1995).

**Gross indices in flounder**

Gross indices of health, such as GSI (high values indicate increased reproductive activity) and HSI (high values indicate good nutritional status, but this may also be caused by increased liver activity due to exposure to organic pollutants), and CF (high values indicate good nutritional status) in flounder can be used to underpin evaluations of more specific biomarker measurements. And although small differences in these indices between sites are generally difficult to relate to a specific cause, when many minor differences are taken into account they can together provide clues to contaminant effects on populations (Kleinkauf et al., 2004a). The indices measured in our study (CF, GSI and HSI) were not however able to distinguish well between more polluted sites and seemed unrelated to concentrations of PCBs, PAHs and trace metals.
A retrospective analysis to explore the applicability of fish biomarkers and sediment bioassays

Figure 2: PCA triplot showing the correlation between chemical contaminants in estuarine sediments and their levels in flounder (“environmental” variables). The horizontal first axis displays 74% of the variation in contaminants in the sediment, the vertical second axis another 12%. Contaminants in flounder: arrows; contaminants in sediments: crosses; sampling locations: filled circles. Abbreviations of substances are explained in Annex 1.

Figure 3: PCA triplot showing the correlation between chemical contaminants in flounder liver (and salinity) and the response of the biomarkers. 12% of the total variance was captured by the covariable age. The horizontal first axis displays 74% of the remaining variation in contaminants in the fish, the vertical second axis another 9%. Only biomarkers that explained 10% or more of the total variance are shown. Biomarkers: arrows; contaminants in flounder: crosses; sampling locations: filled circles. Abbreviations of substances are explained in Annex 1 and fig 2.
A retrospective analysis to explore the applicability of fish biomarkers and sediment bioassays

**Figure 4**: PCA triplot showing the correlation between chemical contaminants in sediments and the response of the biomarkers. 12% of the total variance was captured by the covariable age. The horizontal first axis displays 65% of the remaining variation in contaminants in the fish, the vertical second axis another 14%. Only biomarkers that explained 10% or more of the total variance are shown. Biomarkers: arrows; contaminants in flounder: crosses; sampling sites: filled circles. Abbreviations of substances are explained in Annex 1 and fig 2.

**Figure 5**: PCA triplot showing the correlation between the chemical contaminants in estuarine sediments and the response of bioassays. The horizontal first axis displays 74% of the variation in contaminants in the sediment, the second vertical axis another 10%. Bioassays: arrows; contaminants in sediments: crosses; sampling sites: filled circles. Abbreviations of substances are explained in Annex 1 fig 2.
A retrospective analysis to explore the applicability of fish biomarkers and sediment bioassays

It is well known that populations of flounder display a growth rate depending on the environmental conditions, particularly temperature, salinity and diet (Fonds et al., 1992; Drevs et al., 1999). Females grow faster than males. Growth rates are higher in Dutch fresh and brackish waters than in marine waters and differences in growth rates occur between different areas and estuaries (Van Leeuwen and Vethaak, 1988). The observed differences in age at length data of flounder between the two transects may be caused by the possibly less favourable environmental conditions in the semi-closed Port of Amsterdam area. Differences in somatic growth rate may confound the biomarker responses that are linked to somatic growth, such as RNA/DNA ratios in flounder liver tissue in the present study or RNA in crustaceans (Korsloot et al., 2004). The potential confounding effects of factors such as age, growth, gender and diet on biomarker responses should therefore be investigated (Mayeux, 2004; Kleinkauf et al., 2004a).

Certain skin diseases of flounder are known to be general indicators of stress, including chemical contaminants in estuarine environments (Vethaak and Jol, 1996). However, skin lesions such as ulcers were not monitored in our study, because such indices require larger sample sizes. However, a parallel study conducted in the Port of Amsterdam transect in September 1996 showed that up to 6.5% of flounder were afflicted with skin ulcers and a significant correlation was found between the odds for ulcers and the concentration of PCBs and cadmium in flounder liver (Pieters et al., 2000).

Biomarkers in flounder

Average parameter values per biomarker often varied from one site to another. In general, biomarker responses were less strong in flounder captured in the relatively clean coastal areas (sites 4, 6 and 14) and at the freshwater reference site 12 (see Fig. 3 and Fig. 4). So sets of combinations of biomarkers in flounder can be used to distinguish between cleaner and more polluted estuarine areas. Several biomarkers such as MDA, KMBAm, KMBAc, KMBAmic, WOP, Glyc and Fib were not able to distinguish more polluted sites and lost track of the distribution in sediment of PCBs, PAHs and metals. Though the biomarkers CYP1A, GST (lab 1), SOD (lab3), KMBAmic, AChEbrain, VTG and RNA/DNA responses differed between the two freshwater and saltwater reference sites, the differences were not necessarily due to salinity. This implies that these particular biomarkers may be less suitable for use in estuarine environments where salinity may fluctuate on a daily or seasonal basis or where fish may migrate between areas. The biomarker responses at the IJmuiden location (site 7) do not show a clear correlation with PCB sediment contamination (Fig. 3) since, compared to sediment at this site, they showed relatively low PCB body burdens. CYP1A content in flounder liver varied between locations, but the largest difference was found between the saltwater and freshwater reference sites (Table 3). CYP1A content in liver tissue seemed to be influenced by (extreme) salinity differences, but still showed significant increases at some polluted locations compared to both the freshwater and saltwater reference site. In this study the CYPT1A results showed no correlation with contaminant levels of PCBs and PAHs in sediments. It is unfortunate that the results for CYP1A content cannot be compared to its activity, as EROD activity was not determined in this study. However, most of these studies (Eggens et al., 1995; Rotchell et al., 2001; Kirby et al., 2004) were not conducted over an entire transect, from freshwater to saltwater, as in the present study.

The biotransformation enzyme GST showed significant and unambiguous decreased responses compared to references but it did not correlate with contamination levels for PCBs and PAHs. Since it appeared to be influenced by salinity differences it would seem to have little value as an estuarine biomarker.
A retrospective analysis to explore the applicability of fish biomarkers and sediment bioassays

Oxidative enzymes CAT, SODlab4, MDA in liver tissue, KMBA in liver cytosol/mitochondria and WOP appeared to be unaffected by salinity (Table 3) and showed significant differences between locations in univariate statistical analysis. The responses of oxidative enzymes WOP and MDA in multivariate analysis (Fig. 3) were positively correlated with high body burdens of lower PCBs and several organochlorine contaminants. The concentrations of MDA obscured the biological significance in relation to chemical exposure at sites 2, 3, 9 and 10. It may be that multistress status influences malondialdehyde production (Huggett et al., 2002). SOD and CAT are both biomarkers for oxidative stress, albeit for a different part of the chain of reactions that such compounds provoke (Van der Oost et al., 2003). For SOD, the two labs involved used different methods. Univariate analysis proved that a significant difference for CAT was found only at Oranjesluis (site 11); compared to the reference it correlated with high contamination levels for PCBs and PAHs in the Port of Amsterdam transect. The biomarkers KMBA (mitochondrial, microsomal and in cytosol) did not correlate with the chemical compounds measured in flounder liver tissue and explained less than 10% of the observed variance of contaminants in sediments.

The MT biomarkers in flounder liver correlate with gradient of contamination in both sediment and fish (Fig. 3, Fig. 4). This may be an indicator of specific stress by organotins, although the expected correlation was not found for metals such as Zn and Cd. Surprisingly, there was a good correlation between the concentration of MT and chemical exposure to total organotin in sediment ($r^2=0.7304$). In a few studies it has been shown that, in fish, detoxification of TBT is induced by metalloproteins (Kawano et al., 1996; Padros et al., 2000).

The OH-pyrene biochemical marker varied greatly among individual flounder. Averages for several biomarkers differed only marginally, if at all, between locations (Table 3). The OH-pyrene biomarker did not show correlation with the biota PAH levels (Fig. 3). However, in PCA triplot (Fig. 4) the relationship between OH-pyrene and high concentrations of PAHs in sediment correlates ($r^2=0.4949$) at sites 7, 10 and 11. This is clearly not a significant result, although the 1-OH pyrene value of site 7 (IJmuiden) seemed to be an outlier, since the 1-OH pyrene value measured was extremely high compared to flounder liver, which showed low body burden. Moreover, pairwise univariate comparison of individual biomarker data showed that for several biomarkers there were considerable and often significant differences in the average response between flounder captured at the freshwater and saltwater reference sites (Table 3). The positive experiences with the biomarkers MT in the liver and OH-pyrene during the present study with some of the more established biomarkers for specific groups of compounds were in agreement with the findings of other studies in which they were measured in flounder: MT in liver (Goksøyr et al., 1996; Padros et al., 2000; Rotchell, 2001; George et al., 2004) and 1-OH-pyrene in bile (Richardson et al., 2001; Ruddock et al., 2002; Vethaak et al., 1996).

AChE$_{\text{brain}}$ induction correlated with the body burden of total pesticides in 15 pooled flounder livers at the Benelux tunnel (site 2) and Noordwijk (site 6) locations. The highest concentrations of several OCPs (total 150–400 $\mu$g/kg$^{-1}$ lipid weight) were found in flounder from the Rotterdam transect (De Boer et al., 2001). The Rotterdam transect (sites 6 to 2) is characterised by acetylcholinesterase inhibition (mainly brain), which may reflect contamination by pesticides in this transect. The concentrations of total OCPs in SPM varied in the Rotterdam and Amsterdam transects from below the detection limit to 1.8 mg kg$^{-1}$ and 1.6 mg kg$^{-1}$ respectively. Rotterdam showed relatively higher levels of OCPs from upstream, caused by one of the major sources of OCP residues in the rivers Meuse and Rhine. The contamination might be classified as historical, or industrial centres might still be emitting these compounds (Voorspoels et al., 2004).
One biomarker for which the results proved difficult to interpret during the study was VTG in male flounder. The reason for this phenomenon is still unknown. In the UK, VTG in male flounder has proven to be an excellent and very specific biomarker for the effects of estrogenic contamination in estuaries (Allen et al. 1999a; Kleinkauf et al., 2004b).

In the present study RNA/DNA ratios in flounder liver tissue did not correlate with contaminants in the flounder or sediments. Nucleic acid contents can rise over a season, influenced by biotic and abiotic factors such as fish size, somatic growth, temperature effects and salinity. This is in contrast with studies in which the ratio of RNA to DNA in fish has been positively correlated with long-term growth and is therefore used in environmental sciences as a parameter for growth rate (Bulow, 1987). This biomarker was found to be suitable for assessing the general condition of fish (Rooker and Holt, 1996; Suthers et al., 1996) and growth rates were found to be negatively correlated to sublethal contamination levels.

DNA adducts in flounder liver tissue were found to differ slightly but not significantly between sampling locations, and these effects were not PAH exposure-related. This is not surprising, since it is consistent with the finding that this parameter is not discriminating for locations based on an analysis of variance calculated on the values for individual fish. However, in a study in two UK estuaries the average number of DNA adducts at the four UK sampling sites was positively correlated with average biliary 1-OH-pyrene concentrations (Lyons et al., 1999)

Previous analysis showed that CYP1A immunoreactivity in flounder could be induced in laboratory experiments by exposure to substances such as TCDD and PCB-126 (Grinwis et al., 2000). The present study demonstrated that this induction of CYP1A immunoreactivity can be found in liver from flounder. The other histopathological biomarkers in the present study proved either difficult to interpret (vacuolation) or relatively insensitive to contaminants at the locations in this study (glycogen content, fibrillar structures).

In vivo bioassays

The finding that the in vivo sediment bioassays C. volutator, E. cordatum, A. marina and C. elegans showed little response to the contaminated sediments from sites 2, 7, 9, 10 and 11 (Table 6) seems remarkable in retrospect (Van den Brink and Kater, 2006). The bioassay with the amphipod C. volutator has since been included in the set of standard bioassays that is used for routine assessment of polluted harbour sediments using the Chemical Toxicity Test (CTT) in the Netherlands (Schipper and Schout, 2004) and the burrowing heart urchin E. cordatum has long been under consideration for this purpose. Both showed only significant effects upon exposure to heavily polluted harbour sediments at the Oranjesluis site (site 11). However, the relation with bioavailability of the contaminants (Reichenberg and Mayer, 2006), measured contaminants in those sediments is not always evident (Stronkhorst et al., 2003a).

The A. marina bioassay is routinely used in the UK, but only in more saline conditions (Thain and Bifield, 2001). The survival of this polychaete in our study was hardly affected by the sediments tested during the study, but the number of casts produced varied considerably (Table 6). Significant effects with the above sediment in vivo bioassays used here have been demonstrated in other studies, not only in Dutch harbour sediments, but also marine and estuarine environments (Matthiessen et al., 1998; Kater et al., 2001; Stronkhorst, et al., 2003b).

The nematode C. elegans assay did show a location- and gradient-related response for reproduction and development (at sites 2, 3, 7, 9, and 10). This assay seems independent of the salinity of a sample, and even of the source of the sample (biotic or abiotic), as long as extracts
are used. The results obtained with the C. elegans assay are however difficult to extrapolate to field conditions, partly because this is not a native benthic marine or estuarine species.

**In vitro bioassays**

The Microtox Solid Phase®, Mutatox, DR-CALUX, ER-CALUX and YES in vitro assays all revealed some differences between the sediments tested in this study (Table 7). The PCA overall analysis showed that levels of PAHs, PCBs, trace metals and organotins in the sediment correlate strongly with the in vitro bioassays. The most discriminative in vitro bioassays in the present study were DR-CALUX, ER-CALUX and Microtox SP®. Microtox SP® did not however find the expected correlations for PAHs. The DR-CALUX response was sometimes higher after 48h incubation time. This suggests that the effects are greater after longer exposure times, indicating that the response due to PCBs or dioxins/furans and the concentrations were too high for the test cells to metabolize them all within 24h (Hamers et al., 2000).

There is no significant correlation between the ER-CALUX bioassay and the vitellogenin biomarker, given the doubtful statistical validity of the vitellogenin data. The ER-CALUX was more sensitive than the YES assay. All of the above in vitro tests have been applied successfully to field sediments from marine, estuarine and freshwater environments (Stronkhorst et al., 2002; Johnson and Long, 1998; Legler et al., 2003; Houtman et al., 2004; Thomas et al., 2004). The Mutatox® assay for genotoxicity showed differences between locations, but was not applied to sediments from either the saltwater or freshwater reference sites. Suitable sediment bioassays are the Microtox® Solid Phase bioluminescence assay and the in vitro reporter gene assays DR-CALUX and ER-CALUX. The Microtox SP® and DR-CALUX have been included in the battery of standard bioassays used to assess Dutch harbour sediments with the CTT (Schipper and Schout, 2004). In general, Microtox SP® and DR-CALUX results hinted that the most toxic sediment collected during the study came from site 11 (Oranjesluis) (see Tables 5 and 6). This is in line with the results of the chemical analysis (De Boer et al., 2001).

**Conclusion**

The results of this study show that body burden in benthic fish at least partly fingerprints the chemical characteristics of the sediment from the sites along the salinity transects where they were sampled. The most discriminative biomarkers and bioassays were those based on types of biological effects for specific groups of compounds, which gave more straightforward results than gross indices and biomarkers of general damage or condition. Mechanism-based in vitro assays DR-CALUX and ER-CALUX applied to sediment extracts for screening of potential toxicity were much more responsive than in vivo bioassays with macro invertebrates using survival as an endpoint. However, no clear responses were found between induction of Microtox SP® by PAHs, metallothioneins by metals such as Zn and Cd, 24h- and 48h-DR-CALUX by PCBs or HCB. The utility of some biotransformation and oxidative stress biomarkers in detecting differences between sites or transects was also very limited. These comparisons may, however, have been blurred by the effects of salinity on the bioavailability of contaminants such as metals, shortcomings in the extraction and clean-up methodology and, in the case of some biomarkers, by the migratory behaviour of flounder. Based on this study, a tentative list of appropriate biomarkers in flounder for monitoring and assessment of chemical contaminants in estuarine environments would include induction of hepatic metallothionein content (indicating exposure to organotins) and concentration of 1-OH-
pyrene in bile (an indicator of exposure to PAHs). Mechanism-based in vitro assays DR-CALUX and ER-CALUX applied to sediment extracts for screening of potential toxicity were much more responsive than in vivo bioassays with macro invertebrates using survival as an endpoint.

This is undoubtedly an area requiring further investigation. In the past years the Working Group on Biological Effects of Contaminants of the International Council for the Exploration of the Sea (ICES, 2008) and field surveys such as the EU BEEP project in the Baltic Sea (Lethonen et al., 2006) have been calling for more research on the effects of interfering factors such as salinity affecting biomarker responses in marine and estuarine fish and bioassays to help fill this knowledge gap.

Acknowledgements

This study was initiated and coordinated by the National Institute for Coastal and Marine Management (RIKZ) of the Directorate-General of Public Works and Water Management (RWS) in the Netherlands. Timco van Brummelen and Marco van Wieringen (RWS) provided background documents. Piet den Besten (RWS-RIZA, Lelystad, the Netherlands) and Joost Stronkhorst (RWS-RIKZ, The Hague, the Netherlands) wrote the minutes of the workshop. John Stegeman of the Biology Department of Woods Hole Oceanographic Institution, Woods Hole, MA, USA, kindly provided the monoclonal CYP1A antibody. Johan Jol, Jan Pieters and Peter Schout of RWS-RIKZ, the Netherlands, took part in the sampling and conducted bioassays. Thanks to Valérie Fessard for carrying out all the WOP analyses. Georgos Martakis of Alterra in Wageningen, the Netherlands, performed univariate statistical analyses.

Annex 1

Abbreviations of substances used in PCA triplots (Figs. 2-5). Abbreviation of biomarkers and bioassays see table 1 and 2.

HCH  hexachlorocyclohexane
DDD  dichlorodiphenyldichloroethane
DDE  dichlorodiphenyldichloroethylene
DDT  dichlorodiphenyltrichloroethane
QCB  pentachlorobenzene
HCB  hexachlorobenzene
HCBD  hexachlorobutadiene
OCS  octachlorostyrene
β-hepo  heptachloroepoxide
TBDE  2,4,2,4'-tetrabromodiphenylether
PeBDE  2,4,5,2,4'-pentabromodiphenylether
TBT  tributyltin
DBT  dibutyltin
MBT  monobutyltin
TPhT  triphenyltin
DPT  diphenyltin
MPT  monophenyltin
Ant  anthracene
A retrospective analysis to explore the applicability of fish biomarkers and sediment bioassays

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Chapter 3

Intra- and interlaboratory calibration of the DR CALUX® bioassay for the analysis of dioxins and dioxin-like chemicals in sediments

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*Environmental Toxicology and Chemistry, Vol. 23, No. 12, 2781–2789, 2004*

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Abstract: In the Fourth National Policy Document on Water Management in the Netherlands (Ministerie van Verkeer en Waterstaat, 1998), it is defined that in 2003, in addition to the assessment of chemical substances, special guidelines for the assessment of dredged material should be recorded. The assessment of dredged material is based on integrated chemical and biological effect measurements. Among others, the DR CALUX® (dioxin responsive–chemically activated luciferase expression) bioassay has tentatively been recommended for inclusion in the dredged material assessment. To ensure the reliability of this bioassay, an intra-and interlaboratory validation study, or ring test, was performed, organized by the Dutch National Institute for Coastal and Marine Management (RIKZ) in cooperation with BioDetection Systems BV (BDS). The intralaboratory repeatability and reproducibility and the limit of detection (LOD) and quantification (LOQ) of the DR CALUX bioassay were determined by analyzing sediment extracts and dimethyl sulfoxide (DMSO) blanks. The highest observed repeatability was found to be 24.1%, whereas the highest observed reproducibility was calculated to be 19.9%. Based on the obtained results, the LOD and LOQ to be applied for the bioassay are 0.3 and 1.0 pM, respectively. The interlaboratory calibration study was divided into three phases, starting with analyzing pure chemicals. During the second phase, sediment extracts were analyzed, whereas in the third phase, whole sediments had to be extracted, cleaned, and analyzed. The average interlaboratory repeatability increased from 14.6% for the analysis of pure compound to 26.1% for the analysis of whole matrix. A similar increase in reproducibility with increasing complexity of handlings was observed with the interlaboratory reproducibility of 6.5% for pure compound and 27.9% for whole matrix. The results of this study are intended as a starting point for implementing the integrated chemical–biological assessment strategy and for systematic monitoring of dredged materials and related materials in the coming years.

Introduction

It is generally accepted that marine sediments form a sink for hydrophobic pollutants such as polychlorinated dibenzo-\textit{p}-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs). Since these classes of compounds tend to bioaccumulate in aquatic organisms inhabiting such polluted areas, they may pose a health risk for aquatic wildlife and also for humans through the consumption of contaminated fish. The occurrence of certain diseases in fish populations has been related to environmental pollutants. Pollution of the aquatic environment has been assumed to contribute (at least in part) to the etiology of fish diseases such as skin and liver tumors and fin rot (Malins \textit{et al.}, 1995; Vethaak, 1993; Vethaak and Wester, 1996). The main source of contamination in the North Sea is through riverine outflows, primarily from the Rhine, Scheldt, and Meuse (The Netherlands), Elbe and Weser (Germany), and Thames (United Kingdom). As a consequence, coastal areas and estuaries are significantly polluted by PCDDs, PCBs, and other persistent organic pollutants (Klamer and Laane, 1991; De Boer, 1995). In order to monitor the extent of contamination, the Dutch government has stated that the assessment of dredged materials will be based on integrated chemical and biological effect measurements (Ministerie van Verkeer en Waterstaat, 1998; Stronkhorst \textit{et al.}, 2002). The DR CALUX® (dioxin responsive–chemically activated luciferase expression) bioassay has been recommended for inclusion in the dredged material assessment for the analysis of dioxins and/or dioxin-like chemicals.
Traditional techniques for the detection and quantitation of PCDDs, PCDFs, and PCBs in sediments involve costly and time-consuming instrumental methods, such as high-resolution gas chromatography separation and mass spectrometry (HRGC/MS), making extensive monitoring of sediments difficult. Although these techniques provide information on the presence and concentration of individual congeners, no direct information is provided on the total biological (toxic) activity of such compounds in complex mixtures. The major advantage of using mechanistic-based bioassays for the assessment of dredged materials, such as the DR CALUX bioassay, is that instead of analyzing specific individual congeners, it determines the total biological (toxic) activity of groups of chemicals with a similar toxic mode of action (Stronkhorst et al., 2002). However, traditional analytical chemical techniques such as HRGC/MS are essential to determine the exact nature of individual congeners present in the samples under investigation.

The DR CALUX bioassay comprises a genetically modified H4IIE rat hepatoma cell line, incorporating the firefly luciferase gene coupled to dioxin responsive elements (DREs) as a reporter gene for the presence of dioxins and/or dioxin-like compounds (Aarts et al., 1993; Aarts et al., 1995; Garrison et al., 1996; Murk et al., 1996). In the DR CALUX bioassay, the induction of luciferase by 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8 TCDD) is dose dependent. Hence, a 2,3,7,8-TCDD calibration curve can be used to quantify the total dioxin and/or dioxin-like content of a sample under investigation. Whereas concentrations of individual polyhalogenated aromatic hydrocarbons (PHAHs) as determined using HRGC/MS have to be multiplied by their respective toxic equivalent factor (TEF) value and added up to give the total 2,3,7,8-TCDD toxic equivalent (TEQ) (Safe, 1987; Safe, 1990; Van den Berg et al., 1998), the DR CALUX bioassay directly measures the arylhydrocarbon receptor (AhR)–related toxic potency of a mixture. The DR CALUX bioassay has been successfully used for the analysis of dioxins and/or dioxin-like chemicals in a wide variety of matrices, such as (human) serum, (human) milk, fish, fish oil, citrus pulp, and so on (Denison et al., 1996; Bovee et al., 1996; Bovee et al., 1998; Murk et al., 1998; Covaci et al., 2001; Laier et al., 2001). In addition, a number of papers have been published describing the validation of the bioassay and describing the correlation between DR CALUX and HRGCMS derived 2,3,7,8-TCDD TEQs (Pauwels et al., 1999; Hoogenboom et al., 2000; Hoogenboom et al., 2001; Besselink et al., 2002).

To ensure the reliability of the DR CALUX bioassay for monitoring dredged materials, the accuracy and repeatability of the DR CALUX bioassay has to be determined. Therefore, both intra- and interlaboratory calibration studies were performed. Six laboratories, located in The Netherlands, the United Kingdom, Japan, and Belgium, were selected to participate. Each of these laboratories analyzed anonymous sediment samples in each of the three phases of the study. The participants were asked to perform the analyses according to supplied protocols. In addition, they were asked to extract and clean up sediments according to the procedure developed and validated by The Netherlands Institute for Fisheries Research (RIVO), in cooperation with the Dutch National Institute for Coastal and Marine Management. The protocol for the analysis of dioxin and/or dioxin-like content in sediment extracts (DR CALUX bioassay) was recently modified by BioDetection Systems BV (BDS) (SOP Dutch National Institute for Coastal Marine Management SPECIE*07). OpdenKamp performed the statistical analyses necessary to interpret the results of this interlaboratory validation study. The results of these studies are presented here and are intended as a starting point for implementation of the DR CALUX bioassay in the assessment of dredged materials for systematic monitoring in the coming years.
Materials and Methods

Chemicals

The 2,3,7,8-TCDD was purchased from LGC Promochem (Wesel, Germany). The PCB 126 and PCB 169 were obtained from Labor Dr. Ehrenstorfer (Augsburg, Germany). Ultraclean DMSO and ethylenediaminetetraacetic acid (EDTA) were obtained from Acros (New Brunswick, NJ, USA). Ultra resianalyzed n-hexane, sodium sulfate, and cleaned and ignited sea sand were from Mallinckrodt Baker B.V. (Deventer, The Netherlands). Silica 60 (63–200 µm) was obtained from Merck (Darmstadt, Germany). Sulfuric acid (95–97%) was from Riedel de Haén (Seelze, Germany). Minimal essential medium (0MEM with phenol red as pH indicator), fetal calf serum (Australian origin), and trypsin were purchased from Gibco, Invitrogen (Breda, The Netherlands). Dithiothreitol (DTT) and Luciferin was from Duchefa Biochemie B.V. (Haarlem, The Netherlands). Coenzyme A (free acid grade I) was from Roche Diagnostics (Mannheim, Germany).

Intralaboratory study

The intralaboratory calibration study was performed by the Institute for Environmental Studies. Sediment was extracted and cleaned up as indicated here. The determination of dioxin and/or dioxin-like content was according to the method indicated under the section DR CALUX analysis. For the intralaboratory study, the following parameters were investigated: limit of detection (LOD), limit of quantitation (LOQ), and reproducibility and repeatability of the bioassay.

Interlaboratory study

Project description. Six laboratories, located in The Netherlands, the United Kingdom, Japan, and Belgium, were selected to participate. Each of these laboratories analyzed blind samples in each of three phases of the study. The participants were asked to perform the analyses according to supplied protocols (see the following discussion). All samples supplied were analyzed three times. In addition, each single sample was analyzed in triplicate. All the laboratories participating in the interlaboratory study for the validation of the DR CALUX bioassay for dioxins and dioxin-like chemicals in sediment had experience in running the DR CALUX bioassay. However, none of the participating laboratories had prior experience with the extraction protocol to be used. Furthermore, the participants were free to use the dilution factor of their choice unless indicated otherwise.

Phase 1. The first phase of the interlaboratory study consisted of the DR CALUX analysis of two defined standard solutions (2,3,7,8-TCDD in DMSO; TCDD/PCB-126/PCB-169 mix in DMSO). The standard solutions were prepared by the Institute for Environmental Studies, Vrije Universiteit Amsterdam, The Netherlands, and sent to the participants. In addition, the participants received a complete concentration range of 2,3,7,8-TCDD in DMSO to be used as a TCDD calibration curve (a total of eight different TCDD concentrations, 0–300 pM 2,3,7,8-TCDD/well, including DMSO as a blank control). This calibration curve was used throughout the whole interlaboratory study. Furthermore, each participant analyzed its own TCDD calibration curve. Participating laboratories received three vials containing a TCDD stock solution in DMSO and three vials containing a TCDD/PCB-126/PCB-169 mix in DMSO. Dilutions of the stock solutions were prepared by the participants in DMSO and tested for dioxin and/or dioxin-like con-
Intra- and interlaboratory calibration of the DR CALUX® bioassay

Phase 2. In the second phase of the study, the participants were asked to analyze three extracted and cleaned sediment samples using the DR CALUX bioassay. Sediments used for extraction and cleanup were freshwater sediments from the Western Scheldt, The Netherlands. The sediment extracts were prepared by the Royal Institute for Fishery Research (RIVODLO), IJmuiden, The Netherlands, according to the protocol given here. Dilutions of the supplied sediment extracts were prepared by the participants in DMSO and tested for dioxin and/or dioxin-like content. On each 96-well microtiter plate, a 2,3,7,8-TCDD standard calibration curve was analyzed. Raw data as well as converted data were used for statistical evaluation.

Phase 3. During phase 3 of the interlaboratory study, participants received an identical sediment sample (freshwater sediment from the Western Scheldt, The Netherlands). The participants were asked to extract and clean up the sediment in three separate sessions according to the supplied protocol. Following extraction and cleanup, the three sediment extracts were analyzed in the DR CALUX bioassay. Participants were not instructed on the dilution to be used. Dilutions of the sediment extracts were prepared by the participants in DMSO and tested for dioxin and/or dioxin-like content. On each 96-well microtiter plate, a 2,3,7,8-TCDD standard calibration curve was analyzed. Furthermore, an appropriate commercially available procedure blank (washed and ignited sea sand; Baker, catalog 0252) was extracted, cleaned, and analyzed using the exact same protocols. Raw data as well as converted data were used for statistical evaluation.

Preparation of samples for the intra- and interlaboratory studies

**Defined standard solutions.** The two defined standard solutions were prepared by dissolving either 2,3,7,8-TCDD or 2,3,7,8-TCDD, PCB 126, and PCB 169 in DMSO. The 2,3,7,8TCDD standard solution contained 2,3,7,8 TCDD in DMSO at a concentration of 7.5 nM. Sample 2 contained a mixture of 2,3,7,8 TCDD, PCB 126, and PCB 169 at concentrations of 5.0, 25, and 250 nM, respectively. The total 2,3,7,8-TCDD TEQ content of this mixture was calculated using both World Health Organization (Paris, France) WHO-TEF values and DR CALUX-relative potency (REP) values (Hosoe et al., 2002) and found to be 10 and 7.5 nM 2,3,7,8-TCDD TEQ, respectively. Overall, 27 individual measurements per sample and per participant were available for data analysis.

**Extraction of sediment samples.** Prior to extraction, sediment samples were freeze-dried and homogenized. Approximately 10 g of dried sediment were placed in a preextracted thimble, and a small piece of silanized glass wool was placed in the thimble on top of the sample to prevent sediment parts from leaving the thimble. The thimble was placed in a Soxhlet setup and extract for 16 h (overnight) with 200 ml hexane/acetone (3/1 v/v). The extracts were concentrated in the rotation evaporator until approximately 5 ml (p = 0.05 bar; T = 40°C) of extract remained. If the extract still contained solid particles, the extract was filtered with diatomaceous earth or sodium sulfate. The extract was transferred to a diatomaceous earth– or sodium sulfate–filled funnel and flushed with 10 ml hexane. The eluted extract was evaporated again in the rotary evaporator until approximately 5 ml of extract remained. The extract was transferred to a cleaned glass tube and concentrated until near dryness. The dried extract was finally redissolved in 3 ml hexane.

**Cleanup of sediment samples.** The extracted sediment samples were cleaned up using a multilayer column. The multilayer glass column consisted of the following materials (from top to bottom): 1 cm water-free sodium sulfate, 1 g silica, 7 g 44% sulfuric acid on silica, 1 g silica, 2 g 33% sodium hydroxide on silica, 1 g silica, 1.5 g 10% silver nitrate on silica, and a small piece of silanized glass wool. After addition of each layer, the column was compacted by tapping
the column. After moistening and preelution with 25 ml of hexane of the column, the complete extract was transferred on the top of the column.

The column was eluted with 130 ml of hexane, after which the eluate was concentrated on the rotation evaporator until approximately 5 ml remained ($p = 0.2$ bar; $T = 40^\circ C$). The concentrated cleaned sediment extract was transferred to a clean glass tube and further concentrated to near dryness under a gentle stream of nitrogen. The extract was redissolved in 50 μl DMSO.

**DR CALUX analysis**

The DR CALUX cells were cultivated in minimal essential medium ($\alpha$-MEM) supplemented with 10% fetal calf serum under standardized conditions ($37^\circ C$, 5% CO2, 100% humidity). The DR CALUX analyses of samples in DMSO of the three phases were performed in 96-well cell culture plates (Greiner). Cells were seeded in 100 μl growth medium and incubated for 24 h under standardized conditions until the cells reached a confluence of at least 95%. An additional 100 μl of growth medium were added to the wells containing the samples in DMSO. The final DMSO concentration in the wells was 0.4%. After 24 h of incubation, the exposure medium was removed, and the cells were rinsed with diluted phosphate buffered saline (demi water/PBS; 1/1, v/v). Thirty microliters of lysis mix (25 mM Tris, 2 mM dithiothreitol [DTT], 2 mM 1,2-diaminocyclohexane-$N,N,N',N'$-tetra-acetic acid [CDTA], 10% glycerol, and 1% Triton x-100 [Sigma, St. Louis, MO, USA] pH 7.8) were added to each well and incubated at 4°C for at least 30 min, after which the microtiter plates were frozen at -80°C for a minimum of 30 min and a maximum of 1 d to lyse the cells.

The luciferase activity was measured using a luminometer equipped with two dispensers. The microtiter plates were thawed and shaken for 2 min at room temperature and placed in the luminometer. One hundred microliters of glow mix (20 mM trycin, 1.07 mM magnesium hydroxide carbonate pentahydrate, 2.67 mM magnesium sulfate, 0.1 mM ethylenediaminetetraacetic acid, 33.3 mM dithiothreitol, 270 μM coenzyme A, 470 μM luciferin) were automatically injected into each well. The light output was recorded on which the reaction was stopped by automatic injection of 100 μl of 0.2-M NaOH.

On each 96-well microtiter plate, a complete 2,3,7,8-TCDD standard concentration range was incubated and analyzed in triplicate. A curve fit of the 2,3,7,8-TCDD standard range was produced for the calculation of DR CALUX TEQ content in the samples tested. The analyzed relative light units (RLU) from the samples were interpolated on the 2,3,7,8-TCDD standard curve, and the DR CALUX TEQ content was quantified between the limit of quantitation (LOQ) and the concentration of 2,3,7,8-TCDD at which 50% of the maximum response is observed (EC50).

**Statistical analysis**

To maintain consistency of statistical analyses, an identical microtiter plate setup was used by all participants, and all samples were analyzed in an identical manner. Both raw data and pretreated data from analyzed samples were submitted to OpdenKamp Registration and Notification for statistical evaluation. Data pretreatment consisted of all necessary calculations to convert the luminosity readings as submitted by the participating laboratories to effective dioxin-receptor activity (pM 2,3,7,8-TCDD TEQ). In addition to the analysis results of the defined samples (phase 1), the cleaned sediment extracts (phase 2), and the complete sediments (phase 3), all participants also submitted the results of the complete 2,3,7,8-TCDD calibration curves for statistical evaluation.
Intra- and interlaboratory calibration of the DR CALUX® bioassay

Intra- and interlaboratory calibration of the DR CALUX® bioassay

Figure 1: Example of a 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) standard calibration curve. The relative light units have been corrected for dimethyl sulfoxide blank. $r^2=0.993$.

Calibration curves were fitted, and EC50 values were derived using the nonlinear regression package pro Fit 5.5 (QuantumSoft, Zürich, Switzerland). The results of the calibration curve measurements were fitted to a sigmoidal dose–response function of the following form with a slope factor of 1:

$$R = R_0 + \frac{R_{\text{max}} - R_0}{1 + 10^{(EC50 - \log C)}}$$

with $C = \text{concentration}$, $R = \text{response}$, $R_0 = \text{control response}$, $R_{\text{max}} = \text{maximum response}$, and $EC50 = \text{the concentration at which 50% response is observed}$. The $R_0$ was fixed in all fits to the response of the control sample at concentration 0; $R_{\text{max}}$ and EC50 were fit by a nonlinear least-squares algorithm (the default Levenberg–Marquardt algorithm).

Analysis of variance (ANOVA) analyses were performed using the general statistical package StatView 5.01 (SAS Institute, Cary, NC, USA). The ANOVAs were calculated as repeated-measures ANOVAs with wells as within factor for phase 1 and with plates as within factor for subsequent phases. Specialized statistics, such as comparison of fits of different calibration curves, were calculated in MATLAB 5.1 (MathWorks, Natick, MA, USA) using custom routines.

Results

Intralaboratory study

For the determination of the limit of detection (LOD) and LOQ, 10 standard 2,3,7,8-TCDD calibration series were analyzed in triplicate using the DR CALUX bioassay. In Figure 1, a typical example of a standard 2,3,7,8-TCDD calibration curve is given. For each individual calibra-
Intra- and interlaboratory calibration of the DR CALUX® bioassay

The LOD was calculated as three times the standard deviation of the DMSO blank (0 pM 2,3,7,8-TCDD), whereas the LOQ was calculated as 10 times the standard deviation of the DMSO blank (Hermans et al., 1998). For the 10 standard 2,3,7,8-TCDD calibration curves, the LOD varied between 0.04 and 0.25 pM 2,3,7,8-TCDD per well. The LOQ varied between 0.12 and 0.88 pM 2,3,7,8-TCDD per well. Finally, an overall LOD and LOQ was calculated as the average of 10 observations plus three times the standard deviation (95% confidence) resulting in a LOD and LOQ of 0.3 and 1 pM 2,3,7,8-TCDD per well, respectively.

For the determination of the repeatability, two sediments originating from coastal areas along the Dutch coastline were extracted and cleaned up. One of the sediments had a low 2,3,7,8-TCDD TEQ content, whereas the second sediment had a relatively high 2,3,7,8-TCDD TEQ content (4.8 and 26 pg 2,3,7,8-TCDD TEQ/g sediment, respectively). The 2,3,7,8-TCDD TEQ content in both extracts was determined by DR CALUX analysis 10 times on the same day. The reproducibility was determined by analyzing a 3-pM 2,3,7,8-TCDD standard and a cleaned sediment extract. Both samples were analyzed on 10 different days and by various persons. The results are summarized in Table 1.

The linearity of response of the bioassay depends on the linearity of the luminometer used. To determine the linearity of response, a concentration range of luciferase was prepared and the activity measured. A good linear correlation between the detected amount of light and the luciferase concentration was found ($r^2 = 0.9997$).

Interlaboratory study

**Phase 1: 2,3,7,8-TCDD calibration curves.** In phase 1 of the ring test, all six laboratories analyzed two calibration curves per microtiter plate, a BDS-supplied calibration curve, as well as a calibration curve prepared in house by the participants themselves. In Table 2, the EC50 values and the coefficients of determination for the curve fits for all participants are summarized. In addition, the 3-pM point of the 2,3,7,8-TCDD calibration curve is given. Differences in EC50 values reported by the participating laboratories are apparent. In particular, participant B reported relatively high EC50 values in both the calibration series provided by the coordinator and the calibration series prepared by participant B themselves.

Both the EC50 values and the 3-pM point of the 2,3,7,8-TCDD calibration curve serve as quality criteria. For each participant, the results for both data points from all 96-well plates analyzed during the presented study were collected and recorded in Shewhart control charts. The Shewhart control chart is used to identify variations on performance of the DR CALUX bioassay brought about by unexpected or unassigned causes. The Shewhart control chart shows the mean of the EC50 and 3-pM control point and the upper and lower control limits. In Figure 2, a typical Shewhart control chart is shown. Over the analysis period, none of the participants exceeded the action levels (AVG ± 3 S).

The results of the multiple analysis of the standard 2,3,7,8-TCDD calibration curves are also be used to determine the per-participant LOD and LOQ taking into account interlaboratory variation (Table 3). This results show that on average, the participants of the calibration study meet the set LOD and LOQ derived from the intralaboratory study.

**Phase 1: Defined standard solutions.** Participants were asked to measure the response of the two standard samples in the DR CALUX bioassay three times in triplicate. The total DR CALUX 2,3,7,8-TCDD TEQ content of both the 2,3,7,8TCDD sample as well as the mixed sample was calculated to be 7.5 nm TEQ. Since the DMSO content during exposure was 0.4% and the samples were diluted seven times by all participants, the expected DR CALUX TEQ...
content per well for both samples was 4.3 pM 2,3,7,8-TCDD TEQ. Overall, 27 individual measurements per sample and per participant were available for data analysis. Averaged results for the concentration of dioxin equivalents per participant and per sample are summarized in Table 4. The DR CALUX results for the dioxin sample are slightly higher than the actual 2,3,7,8-TCDD concentration in the sample. The results for the TCDD/PCB mixed sample are on average lower than the 2,3,7,8-TCDD TEQ content as calculated using CALUX REP values for the individual congeners.

Table 4: Intralaboratory repeatability and reproducibility of the dioxin response–chemically activated luciferase (DR CALUX®) bioassay for sediment extracts; 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD); TEQ = toxic equivalent.

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<td>Sediment 1</td>
<td>Sediment 2</td>
<td>3 pM 2,3,7,8-TCDD</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>-----------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Average</td>
<td>4.03</td>
<td>25.73</td>
</tr>
<tr>
<td>SD</td>
<td>0.97</td>
<td>2.54</td>
</tr>
<tr>
<td>%SD</td>
<td>24.1</td>
<td>9.9</td>
</tr>
</tbody>
</table>

aFor the determination of the repeatability and reproducibility, each sample/extract analysis was analyzed 10 times in triplicate.

bThe repeatability and reproducibility are calculated as percentage standard deviation (%SD).

Table 2: Summary of dioxin responsive–chemically activated luciferase (DR CALUX®) analysis result (pM 2,3,7,8-tetrachlorodibenzo-p-dioxin [2,3,7,8-TCDD]/well) for the 2,3,7,8-TCDD calibration curves.

<table>
<thead>
<tr>
<th>Participant</th>
<th>Calibration curve provided by coordinator</th>
<th>Calibration curve prepared by participant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50 (pM TCDD/well)</td>
<td>r²</td>
</tr>
<tr>
<td>A</td>
<td>6.22</td>
<td>0.997</td>
</tr>
<tr>
<td>B</td>
<td>21.7</td>
<td>0.994</td>
</tr>
<tr>
<td>C</td>
<td>11.0</td>
<td>0.999</td>
</tr>
<tr>
<td>D</td>
<td>13.9</td>
<td>0.999</td>
</tr>
<tr>
<td>E</td>
<td>10.0</td>
<td>0.999</td>
</tr>
<tr>
<td>F</td>
<td>10.9</td>
<td>0.960</td>
</tr>
</tbody>
</table>

aMedian effective concentration. bResult of the 3 pM 2,3,7,8-TCDD calibration concentration prepared by the participants.
Intra- and interlaboratory calibration of the DR CALUX® bioassay

**Phase 2: Sediment extracts.** In phase 2, participants were provided with three extracted sediment samples, all originating from the same batch. The participants were advised to dilute the supplied samples 10× and 30×. Since the three samples were analyzed on three separate plates, nine measurement values per extract dilution were available per participant. The ANOVA results for the sediment extract samples, when analyzed by individual participant, show that significant differences exist between the results obtained per laboratory ($p < 0.0001$) and also between the two dilutions employed ($p = 0.0007$). It was observed that DR CALUX analysis of the 30× diluted samples give higher results than the 10× diluted samples (data not shown). Averaged results for the concentration of 2,3,7,8-TCDD TEQs per participant and per sample (30× diluted) are given in Table 4. Quantitatively Bonferroni–Dunn multiple comparisons indicate that overall (taken over both dilutions), participant C is significantly different from the rest. Although not traceable anymore, and because the results obtained from the three control samples (DMSO blank, 3-pM 2,3,7,8-TCDD control, and internal reference sample) complied with the quality performance criteria for the DR CALUX bioassay, the indication is strong that a dilution error was made by participant C in the supplied sample.

In addition to the sediment extracts, all participants received a procedure blank. The procedure blank is analyzed to check for possible contamination from chemicals and materials used during extraction and/or cleanup. DR CALUX analysis results from this procedure blank for all participants were below the limit of quantitation (1 pM 2,3,7,8-TCDD TEQ/well) and therefore comply with the DR CALUX performance criteria (data not shown).

**Phase 3: Sediment sample.** In phase 3, participants were provided with a single contaminated sediment. The participants were asked to extract and clean up the sediment in three separate sessions using the Soxhlet extraction method. Following extraction and cleanup, the three sediment extracts were analyzed using the DR CALUX bioassay method. Participants were left free to choose their own dilutions. The different dilutions chosen made it impossible to perform variance analyses by participants × dilutions. Analysis of variance by participants indicates that significant differences exist between laboratories (data not shown). Figure 3 shows graphical representations of the results by participants. The results are categorized as 0× to 200× dilutions. Since not all the participants submitted results in higher dilution ranges, these results were not evaluated. However, it should be noted that higher responses were observed by participants at higher dilutions as compared to the 0 to 200 category as presented here. In the lowest dilution category, significant differences ($p = 0.006$) exist between participants, mainly because of low analytical responses for participant F. Averaged results for the concentration of 2,3,7,8-TCDD TEQs per participant and per sediment are given in Table 4.

**Repeatability and reproducibility.** The repeatability of the DR CALUX bioassay was calculated for all samples (2,3,7,8 TCDD sample, mixed sample, sediment extract, and sediment sample) analyzed by the participants over the three phases of the interlaboratory validation study as the relative standard deviations of the obtained results (Table 4). The average repeatability for the participating laboratories ranged from 14.6% for the dioxin sample analysis to 26.1% for the sediment samples that had to be extracted by the participants themselves. It can be seen that the repeatability was lowest for phase 3, during which the participants were asked to extract, clean up, and perform a DR CALUX bioassay on a supplied sediment sample. In Table 4, the reproducibility for the various analyzed samples is also given. The percentage standard deviations over the DR CALUX bioanalysis results for the analyzed samples ranged from 6.5% for the dioxin sample to 27.9% for the supplied sediment sample. Again, the biggest differences in analysis results were observed in the sediment sample that had to be extracted and cleaned up by the participants themselves.
Intra- and interlaboratory calibration of the DR CALUX® bioassay

Standard 2,3,7,8-TCDD calibration curves

Calibration curves were made with a dioxin standard and were used to convert DR CALUX response levels to concentrations expressed as 2,3,7,8-TCDD TEQs. The section of the 2,3,7,8-TCDD calibration curve between the LOQ (1 pM) and the EC50 is used to quantify DR CALUX analysis results. This section is not linear (see Fig. 1). However, when the calibration curve is plotted on a linear–linear scale, the indicated region can be regarded as linear. In addition, the region between the LOQ and EC50 is chosen for quantification of analysis results since this region of the 2,3,7,8-TCDD calibration curve is least sensitive to variations in observed DR CALUX activity.

Table 3: Averaged limits of detection (LOD) and limits of quantitation (LOQ) (pM 2,3,7,8-tetrachlorodibenzo-p-dioxin [2,3,7,8-TCDD]/well) over all experiments, by participant; SD = standard deviation; %SD = percentage standard deviation

<table>
<thead>
<tr>
<th>Participant</th>
<th>LOD</th>
<th>SD</th>
<th>LOQ</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.17</td>
<td>0.09</td>
<td>0.52</td>
<td>0.31</td>
</tr>
<tr>
<td>B</td>
<td>0.41</td>
<td>0.47</td>
<td>0.92</td>
<td>0.92</td>
</tr>
<tr>
<td>C</td>
<td>0.36</td>
<td>0.33</td>
<td>0.95</td>
<td>0.85</td>
</tr>
<tr>
<td>D</td>
<td>0.29</td>
<td>0.07</td>
<td>0.75</td>
<td>0.19</td>
</tr>
<tr>
<td>E</td>
<td>0.43</td>
<td>0.37</td>
<td>1.16</td>
<td>0.91</td>
</tr>
<tr>
<td>F</td>
<td>0.21</td>
<td>0.14</td>
<td>0.61</td>
<td>0.28</td>
</tr>
<tr>
<td>Average</td>
<td>0.31</td>
<td>0.11</td>
<td>0.82</td>
<td>0.24</td>
</tr>
<tr>
<td>%SD</td>
<td>34.8</td>
<td>28.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3: Averaged dioxin response–chemically activated luciferase (DR CALUX®) results by participant for the sediment extracted by the participants (phase 3). Participants were free to choose their own dilution factor for analysis.

Data presented are 0× to 200× diluted samples; 2,3,7,8-TCDD = 2,3,7,8-tetrachlorodibenzo-p-dioxin; TEQ = toxic equivalents.
Table 4: Dioxin responsive–chemically activated luciferase (DR CALUX®) repeatability and reproducibility for the bioanalysis of the defined standard solutions and sediment samples of the various phases of the present interlaboratory validation study.

<table>
<thead>
<tr>
<th>Participant</th>
<th>Dioxin sample</th>
<th>Mixed sample</th>
<th>Sediment extract</th>
<th>Sediment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TCDD TEQ (pM)</td>
<td>Repeatability (%SD)</td>
<td>TCDD TEQ (pM)</td>
<td>Repeatability (%SD)</td>
</tr>
<tr>
<td>A</td>
<td>4.5</td>
<td>9.4</td>
<td>3.7</td>
<td>12.1</td>
</tr>
<tr>
<td>B</td>
<td>4.5</td>
<td>21.0</td>
<td>3.2</td>
<td>13.9</td>
</tr>
<tr>
<td>C</td>
<td>5.1</td>
<td>8.4</td>
<td>4.5</td>
<td>11.3</td>
</tr>
<tr>
<td>D</td>
<td>4.6</td>
<td>1.0</td>
<td>4.3</td>
<td>10.5</td>
</tr>
<tr>
<td>E1</td>
<td>4.5</td>
<td>17.0</td>
<td>4.2</td>
<td>15.5</td>
</tr>
<tr>
<td>E2</td>
<td>4.8</td>
<td>11.4</td>
<td>4.2</td>
<td>12.9</td>
</tr>
<tr>
<td>F</td>
<td>4.2</td>
<td>34.2</td>
<td>4.0</td>
<td>35.9</td>
</tr>
<tr>
<td>Average repeatability (%SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average (pM)</td>
<td>4.6</td>
<td>4.0</td>
<td>34.3</td>
<td>4.0</td>
</tr>
<tr>
<td>Reproducibility (%SD)</td>
<td>6.5</td>
<td>10.5</td>
<td>18.0</td>
<td>27.9</td>
</tr>
</tbody>
</table>
Discussion

The aim of the present study was to identify the DR CALUX bioassay performance criteria for the analysis of PHAHs in sediment samples in order to implement the bioassay in the assessment of dredged materials for systematic monitoring in the coming years. Therefore, both an intra-and interlaboratory validation study was performed.

Because the 2,3,7,8-TCDD calibration curve is used for quantification of analysis results, the stability and quality of the calibration curves is important. Furthermore, the calibration curves themselves are used as a DR CALUX bioassay quality criterion. According to the performance criteria set for the DR CALUX bioassay, the fitted EC50 should be within the range of 6 to 18 pM 2,3,7,8-TCDD; otherwise, the results are rejected. In addition, the EC50 value of 2,3,7,8-TCDD should be constant over a longer time period. Finally, the coefficient of determination ($r$) should be more then 0.95 (Behnisch et al., 2001a). The numerical results of the fit are summarized in Table 2.

Differences in EC50 values between labs are apparent. Since actual fits are based on log (concentration) values and therefore yield log EC50 estimates, differences in these estimates will be exaggerated when transforming these values to EC50s. In addition, high EC50 values reported by participant B are correlated to low relative responses at the low end of the concentration range, up to and including 3.0 pM (individual curve fits not shown). Based on the results of the present study, EC50 values may range between 8.3 and 18.1 pM (based on a relative error of 15.4%). In a number of previous studies, EC50 values in the same range as suggested previously were found (Laier et al., 2001; Pauwels et al., 1999; Murk et al., 1997; Behnisch et al., 2001b; Behnisch et al., 2001c). For the moment, the EC50 value of the 2,3,7,8-TCDD calibration curve is used as a quality control for the 2,3,7,8-TCDD calibration curve. It can be observed that EC50 values may differ between persons performing the DR CALUX bioassay but also between analyses performed by a single person. However, fluctuating EC50 values do not interfere with the final results of a DR CALUX analysis, especially with data quantified below the EC50 of the standard curve. Observed differences in the 2,3,7,8-TCDD calibration curves occur mainly at the high response end of the calibration curves above the EC50. Since the high end of the calibration curve is not used for data interpolation, differences do not significantly influence analysis results. Most probably, the EC50 value is an indication of the quality or condition of the cells rather than a performance criterion. Despite differences between individual calibration curves, the coefficient of determination for the individual analyzed 2,3,7,8-TCDD calibrations curves is high, and the 3-pM 2,3,7,8-TCDD concentration of the 2,3,7,8-TCDD calibration curves prepared by the participants themselves showed good comparability between the participating laboratories. The 3-pM 2,3,7,8-TCDD concentration is used as quality control and is registered on a Shewhart control chart. The calculated average value was 2.91 pM (standard deviation = 0.21). The percentage standard deviation was calculated to be 7.2%.

**WHO-TEFs versus DR CALUX-REPs**

The WHO-TEF values are internationally accepted toxic equivalent factors for dioxins, furans, and dioxin-like PCBs, as stated by the WHO and derived from both in vivo and in vitro studies. The relative toxic potency of dioxins, furans, and dioxin-like PCBs, relative to 2,3,7,8-tetrachlorodibenzop-dioxin (2,3,7,8-TCDD), can also be derived from analyzing the response elicited by various congeners using the DR CALUX bioassay. The potencies found using this method are expressed as CALUX REPs (CALUX relative potencies). The CALUX REP values are actual TEF values for the congeners in the DR CALUX bioassay and represent the...
total toxic potency of all congeners present that show affinity toward the Ah receptor. A number of authors have compared WHO TEFS and DR CALUX REPs (Hosoe et al., 2002; Murk et al., 1997; Behnisch et al., 2001b). Differences between WHO TEFS and DR CALUX REPs are apparent. As a consequence, DR CALUX TEQs differ from HRGCMS-analyzed 2,3,7,8-TCDD TEQs in a given sample because of the difference between WHO TEFS and DR CALUX REPs (Besselink et al., 2002; Besselink et al., 2003). This was demonstrated for the mixed sample from phase 1 analyzed by the participants. Furthermore, differences in DR CALUX–derived TEQs and HRGCMS-derived TEQs can be a result of the fact that by using HRGCMS, only specified dioxin and/or dioxin-like compounds are determined. In contrast, all compounds showing affinity toward the Ah receptor are detected by the DR CALUX bioassay.

**LOD and LOQ**

The LOD and the LOQ of the DR CALUX bioassay were determined by analyzing 10 standard 2,3,7,8-TCDD calibration series. From these analyses, it was concluded that taking into account 95% confidence, a LOD and LOQ of 0.3 and 1 pM 2,3,7,8-TCDD per well, respectively, should be applied. Hence, in case 10 g of sediment are processed and analyzed using 0.4% of DMSO per well, the LOD and LOQ can be calculated to be 0.04 and 0.16 pg 2,3,7,8-TCDD equivalents per gram of sediment. Similar LOD and LOQ were reported by a number of authors (Bovee et al., 1998; Behnisch et al., 2001c).

The participants of the interlaboratory validation study also analyzed multiple standard 2,3,7,8-TCDD calibration curves. From these data, a per-participant LOD and LOQ could be determined. On average, the participants of the calibration study met the set LOD and LOQ derived from the intralaboratory study. Furthermore, analysis of variance indicated that no significant differences in LOD between laboratories could be identified.

**Effect of dilutions**

The sediment extracts were analyzed at 10X and 30X dilutions. Whereas the 10X diluted samples showed an average DR CALUX TEQ content over all participants of 27.3 ± 4.0 pg 2,3,7,8-TCDD TEQ/g sediment, the 30X diluted extract gave a DR CALUX response of 34.9 ± 6.1 pg 2,3,7,8-TCDD TEQ/g sediment. In general, an effect of dilution on the total DR CALUX TEQ content in sediment samples is observed. Although the exact nature for this observation is not known, it is hypothesized that this is due to the presence of various compounds in sediment extracts showing variable affinity toward the Ah receptor. Dose–response curves in the DR CALUX bioassay of individual compounds have been studied and showed obvious differences (Hosoe et al., 2002) both in maximum response and slope of the curve fit.

**Repeatability**

For the determination of the intralaboratory repeatability of the DR CALUX bioassay for sediment samples, two sediment extracts were analyzed 10 times. Each analysis was performed in triplicate. As a prerequisite for a correct triplicate analysis, the percentage standard deviation in the triplicate determination should be below 15%. This is in accordance with the harmonized quality criteria for cell-based bioassay analyses of PCDDs/PCDFs in feed and food as formulated by Behnisch et al. (Behnisch et al., 2001a) and as detailed in European Union directive 2002/69/EC and directive 2002/70/EC. The repeatability for the low-2,3,7,8-TCDD-content sediment
extract was found to be 24.1% whereas in the high-content-sediment extract, the repeatability was shown to be 9.9%.

For each participating laboratory, the repeatability of the DR CALUX bioassay was calculated for the four samples (2,3,7,8-TCDD sample, mixed sample, sediment extract, and sediment sample) analyzed by the participants over the three phases of the interlaboratory validation study. The average repeatability for the participating laboratories ranged from 14.6% for the dioxin sample analysis to 26.1% for the sediment samples that had to be extracted by the participants. In an interlaboratory comparison exercise for the analysis of PCDD/PCDFs in digested sewage sludge using HRGCMS, relative standard deviations for standard solutions varied between 15 and 41% (Stevens et al., 2001). Similar ranges of relative standard deviations were reported in two other round-robin studies for standard solutions: 18 to 61% and 8 to 43% (Tashiro et al., 1990a; Tashiro et al., 1990b). This indicates that the determination of dioxin-like activity in sediment using the DR CALUX bioassay is at least as consistent as the established HRGCMS methods. In addition, the results show that the intraand interlaboratory repeatability is comparable. From the data it can also been seen that the repeatability was lowest for phase 3, during which the participants were asked to extract, clean up, and perform a DR CALUX bioassay on a supplied sediment sample. Since in the third phase, extra steps to the total procedure are introduced (extraction and cleanup), it is very likely that these add to the variability of the total process. Furthermore, as none of the participants had prior experience using the supplied extraction procedure, it can be anticipated that with increasing experience using the supplied extraction protocol, the repeatability will also increase.

Reproducibility

As with the determination of the intralaboratory repeatability, the intralaboratory reproducibility was determined by analyzing a cleaned sediment extract and a 3-pM 2,3,7,8TCDD standard on 10 separate days and by multiple persons. The reproducibility for the 3-pM 2,3,7,8-TCDD standard was found to be 13.8%, whereas the reproducibility for the cleaned sediment extract was shown to be 19.9%. Since the observed reproducibilities are in the range of relative standard deviations for two sediment extracts analyzed in 10-fold on the same day (intralaboratory repeatability), the DR CALUX bioassay can be evaluated as a stable and robust bioanalytical tool.

The interlaboratory results obtained from the analysis of defined standard solutions, but also from the analysis of sediment extracts prepared either by the coordinator of the study or by the participants themselves, also provide a measure of the variation between laboratories. The results show that the interlaboratory reproducibility ranges from 6.5% for the defined dioxin sample to 27.9% for the sediment sample extracted by the participants themselves. As was mentioned before, the reproducibility for this last sample is relatively high and most presumably due to the introduction of extra handlings (extraction and cleanup) to the total procedure. In addition, the fact that not all the participants had prior experience with the extraction protocol to be used could have added to the increase in variability of the process. Furthermore, the dilution factor was not dictated. This also introduces a certain degree of variation. For the reproducibility of the DR CALUX bioassay itself and not caused by differences in operating extraction conditions, the maximum variation between laboratories was observed to be 18.0%. The results for the sediment extract samples can also be used to estimate the method variability for extracts, that is, based on samples of unknown composition. Again, given the intra-as well as the interlaboratory variations observed in this study, it appears justified to conclude that the standard deviation of the means provides a reasonable estimate of the method variability, based on the overall aver-
Intra- and interlaboratory calibration of the DR CALUX® bioassay

Average concentrations (in 2,3,7,8-TCDD TEQs) for a single sediment extract sample, determined at two different dilutions. The largest standard deviation of the means is therefore proposed as the method error for analyzed samples, being 18.0% for sediment extracts and 10.5% for analytical samples.

Conclusion

Several overall conclusions can be drawn based on the statistical evaluation of the data submitted by the participants of the DR CALUX intra-and interlaboratory validation study. First, differences in expertise between the laboratories are apparent based on the results for the calibration curves (both for the curves as provided by the coordinator and for the curves that were prepared by the participants) and on the differences in individual measurement variability. Second, the average results, over all participants, are very close to the “true” concentration, expressed in DR CALUX 2,3,7,8-TCDD TEQs for the analytical samples. Furthermore, the interlaboratory variation for the different sample types can be regarded as estimates for the method variability. The analytical method variability is estimated to be ±10.5% for analytical samples and ±22.0% for sediment extracts. Finally, responses appear dependent on the dilution of the final solution to be measured. This is hypothesized to be due to differences in dose–effect curves for different dioxin responsive element–active substances. For 2,3,7,8-TCDD, this effect is not observed. Overall, based on bioassay characteristics presented here and harmonized quality criteria published elsewhere (Behnisch et al., 2001a), the DR CALUX bioassay is regarded as an accurate and reliable tool for intensive monitoring of coastal sediments.
Chapter 4

Cultivation of the heart urchin *Echinocardium cordatum* and validation of its use in marine toxicity testing for environmental risk assessment

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*Journal of Experimental Marine Biology and Ecology, 364, 11–18, 2008*

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³ Cefas Weymouth Laboratory
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⁵ IMARES, Wageningen IMARES
Abstract: To study environmental risk assessment, echinoderms provide a useful model for ecotoxicological testing. However, limited knowledge of the life history of field collected heart urchins is a problem and the use of cultured urchins has been investigated here. The present study describes a culture method for the heart urchin Echinocardium cordatum under controlled laboratory conditions, providing organisms with a low biological variation. Based on our optimized growth protocol both larvae and juveniles have a growth rate comparable to E. cordatum in the wild. The toxicological response of cultured and field-collected E. cordatum was compared in standard saltwater toxicity bioassays. Using ammonium chloride as a water-soluble reference toxicant, mean 96h LC50 values for cultured heart urchins versus field collected animals were 37.4 ± 7.6 mg NH4+/l (n=5) versus 22.5 ± 4.9 mg NH4+/l (n=19), respectively. Additional toxicity experiments with tributyl tin (TBT) spiked sediments revealed 14d LC50 values of 1,242 (95% confidence interval 986-1,564) and 964 (95% confidence interval 843-1,102) μg Sn/kg dw respectively in cultured and field collected E. cordatum. From this it was concluded that cultured heart urchins are less sensitive to TBT than field collected E. cordatum. Furthermore in whole sediment toxicity tests, survival of cultured sea urchins was higher or at least similar to that of field collected E. cordatum. The increased sensitivity of field urchins compared to cultured urchins in various toxicity tests may be due to multiple environmental stressors reducing their overall performance. Overall it was demonstrated that the use of cultured E. cordatum provides a significant advance for urchin-based bioassays for marine environmental toxicity testing, resulting in a more homogeneous, vital population with experimental data displaying reduced variability.

Introduction

To study hazard or risk assessment in marine environment, echinoderms form a useful model for ecotoxicological testing. Heart urchins naturally live in sandy, silt-rich areas in North Sea and the coastal areas of Japan (Nakamura, 2001) with a relatively high level of organic material and can be kept in the laboratory for extended periods. A range of biological responses of echinoderms showed that parameters such as reburial activity, echinoid development (Basuyaux and Blin, 1998; Lawrence and Bazhin, 1998; Grosjean et al., 1998, 2003) and gonadal maturation (Nakamura, 2001) can be affected by external stressors. Responses in larval and adult echinoderms show good reproducibility making them useful for physiological (Laurin et al., 1988; Duineveld, 1984; Beukema, 1985; Holtman et al., 1996), evolutionary (Kashenko, 1994; Gourmelon, and Ahtiainen, 2007) and developmental studies. The heart urchin Echinocardium cordatum is used for marine bioassays where possible reduced reburial activity and survival due to chemical contamination of sandy and silty marine sediments are assessed (Bowmer, 1993; Daan and Mulder, 1996; Stronkhorst et al., 1999; Brils et al., 2002) and is routinely used for the assessment of sediment quality in coastal European waters and screening of contaminated dredged material that is proposed for open water disposal (Stronkhorst et al., 2003). Sediment toxicity tests with field E. cordatum have been standardized in a standard operating procedure RIKZ/SPECIE (Bowmer, 1993; Schipper & Stronkhorst, 1999), and confounding factors have been described (Postma et al., 2002). Mortality differences among replicates were attributed to high biological variation (Hoeven van der, 2002; Stronkhorst et al., 2003). Subsequently, the bioassay with E. cordatum for hazard assessment of contaminated dredged material has been validated in an inter-laboratory comparison in the Netherlands (Stronkhorst et al., 2004). The mortality results from this inter-laboratory comparison showed a large variability in the coef-
Cultivation of the heart urchin *Echinocardium cordatum*

...efficient of variation (CV) for samples from harbour sediments of 2 to 50%.

For bioassay testing protocols with *E. cordatum* individuals are generally collected from the field. However, the use of field urchins for salt water environmental toxicity testing has several limitations. Field collection of *E. cordatum* is difficult in coastal zones because of heterogeneous population density. Furthermore urchins collected from the field may suffer from acute trauma during collection, which is likely to influence their sensitivity to chemical contaminants in the bioassays and introduce experimental variability. Production of cultured urchins under controlled conditions can mitigate these problems (Fernandez, 1996; Grosjean et al., 1998).

The objective of the present study was to develop and optimize the cultivation process for raising 1 year old adult *E. cordatum* under laboratory conditions and to validate the use of these cultured heart urchins for sediment toxicity testing by comparing their responses with field *E. cordatum* in sediment toxicity studies and to evaluate the effect of additional handling on the biological response.

**Material and Methods**

**Collecting heart urchins**

Mature *E. cordatum* were collected in the Eastern Scheldt or Dutch North Sea coastal zone at a depth of 6 to 11 meters using a modified hydraulic jet Van Veen grab (0.6 m² at a sediment depth of 30 to 40 cm) during two periods, April to June and September to October 2003. Urchins were held in polyester containers measuring 70 x 50 cm, containing 10 cm of sand and 20 cm of seawater at a biomass density of 150-200 organisms per m². Seawater was collected directly from the Eastern Scheldt at high tide and stored in a reservoir. Urchins were acclimatized at 15°C water temperature with full replenishment of seawater daily via a continuous drip feed, with a 16h day/8h night/photoperiod. Acclimatization took place in sediment from the Eastern Scheldt for at least 14 days without additional feeding. Organisms that died during the acclimatization period were removed. Some of the cultured *E. cordatum* specimens (52 weeks old) are preserved and archived at the Department of Zoology, the Netherlands under registration numbers RMNH Echinodermata 6398 - 6399.

**Spawning, fertilization and development of larvae**

Batches of field collected heart urchins were fed with the debris of a mixed culture of algal species *Phaeodatylum tricornutum*, *Skeletonema costatum*, *Thalasiosira pseudonana*, and *Tetraselmis succinea*. Before the first fertilization step the gonadal index (GI) was determined for 3 urchins per spawning group ([gonad weight (g) / total weight (g)] x 100) to assess the production of roe (Kobayashi, 1992). Urchins were induced to spawn by an external signal using 0.5 M KCl, a temperature change from 15°C to 20°C, or a salinity change from 28 to 32 g l⁻¹. Influence of light on spawning was not observed. *E. cordatum* were stimulated to spawn in September 2003, April, June, and September 2004, and in May 2005. The procedure for echinoid fertilization was based on the Environment Canada (1992) method. After 1 hour fertilization the cell membrane was checked microscopically, and fertilized eggs surrounded by cumulus cells were counted. Typical fertilization success is approximately 95%. The developing four-cell stage embryos were transferred to a 2-liter cylindrical container and reared to the larvae phase. Two days after fertilization the larvae were fed with a diet of algae according to Kelly et al. (2000), with densities...
of 1500, 2500, and 4000 *Dunaliella* sp. cells/ml for the 4-, 6- 8- and 10-arm stage respectively. After development into the 10-arm stage the larvae were fed *ad libitum* with mixed diets freshly collected cultures of *T. succinea, D. tertiolecta, T. pseudonan, S. costatum, Isochrysis galbana, Pavlova lutheri,* and *Chaetoceros calcitrans* at 15°C. Their development was monitored and photographed at post fertilization. Just before the larvae reached the metamorphosis stage, they were transferred to aquaria (dimensions 45-70 cm) with a 5 cm layer of clean Eastern Scheldt field sediment containing naturally occurring diatoms as a biofilm. Twelve weeks after fertilization (Table 2), the juvenile urchins were transferred to a second aquarium, and fed twice a week with a mixture of algae and TetraMin®. After metamorphosis, juvenile heart urchins were fed *ad libitum* with freshly collected debris of algal cultures *C. calcitrans, S. costatum* and *T. pseudonan* and artificial fish food TetraMin®. The field sediment was replaced every 4-6 weeks to ensure the availability of algae occurring food. Somatic growth (SG) was monitored with each replacement. The SG rate was calculated as the difference between the final and initial wet weight divided by the time for growth (Spirlet et al., 2001):

\[
SG = \frac{(S_{t_{\text{max}}} - S_0)}{t_{\text{max, mean}}} S
\]

**Sensitivity of cultured and field heart urchins towards ammonium chloride**

The sensitivity of adult cultured and field heart urchins towards ammonium chloride was tested in a 96h water-phase test using ammonium chloride at a concentration range from 1 to 60 mg NH₄⁺/l. Multiple tests were made with different weight classes at different times of year. All tests were carried out with a range of six concentrations, each containing 5 organisms of the same size. After 96 hours the organisms were placed on sediment again; organisms were recorded dead when they did not burrow within 30-minutes.

![Figure 1. Map of locations where sediments were collected at Dutch sites in the Port of Rotterdam at stations Rijnmond RMND 12, RMND 16, RNMD 19, RMND 42 and RNMD 701.](image-url)
Cultivation of the heart urchin Echinocardium cordatum

Sensitivity of cultured and field heart urchins towards sediments

Sediments were collected in the Port of Rotterdam (Figure 1) at stations Rijnmond, respectively RMND 12, RMND 16, RNMD 19, RMND 42 and RNMD 701 using a Van Veen grab. Control sediment was taken from a clean site in the Delta region of the Eastern Scheldt, Oesterput. Sediments collected from the reference site at Oesterput and at polluted sites in the Port of Rotterdam (Table 1) were homogenized and analyzed for sediment grain size texture and bulk chemistry as previously described (Stronkhorst et al., 2004). Both field heart urchins and cultivated heart urchins were tested in a whole sediment toxicity bioassay for 14 days according to RIKZ/SPECIE-03 (Schipper and Stronkhorst, 1999), and all tests were carried out with 4 replicates per treatment, each containing 10 organisms.

The sensitivity of field and cultured heart urchins to tributyltin (TBT) spiked sediment was tested using heart urchins of different weight classes (mean weight field urchin 38 g per individual and mean weight cultured urchins 5 g per individual) according to standard operation procedure RIKZ/SPECIE-03 (Schipper and Stronkhorst, 1999) using TBT-spiked sediment. Control sediment from Eastern Scheldt was spiked with tributyltin (TBT) according to the method described by Bowmer et al. (1993), with a nominal range of six concentrations: 0; 425; 852; 1,705; 5,253; 8,542 and 17,047 μg Sn/kg dry weight. The sediments were allowed to settle 42 days after spiking prior to use in the sediment toxicity test. Actual concentrations of TBT-Sn were determined as described by Stäb et al. (1994).

The effects of handling stress during collection were studied in experiments with field heart urchins. Multi-stress by handling was tested by careful transfer by hand of field collected sea

Table 1: Chemical analyses of sediment from Port of Rotterdam (Stronkhorst et al., 2004)

<table>
<thead>
<tr>
<th>Physicochemical characteristics</th>
<th>Unit</th>
<th>Oesterput control</th>
<th>RMND 12</th>
<th>RMND 16</th>
<th>RMND 19</th>
<th>RMND 42</th>
<th>RMND 701</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metals</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arsenic</td>
<td>mg/kg dw</td>
<td>9.9</td>
<td>21</td>
<td>29</td>
<td>25</td>
<td>15</td>
<td>27</td>
</tr>
<tr>
<td>Cadmium</td>
<td>mg/kg dw</td>
<td>0.15</td>
<td>2.7</td>
<td>4</td>
<td>6.2</td>
<td>2.2</td>
<td>6.8</td>
</tr>
<tr>
<td>Chromium</td>
<td>mg/kg dw</td>
<td>27</td>
<td>80</td>
<td>120</td>
<td>110</td>
<td>56</td>
<td>160</td>
</tr>
<tr>
<td>Copper</td>
<td>mg/kg dw</td>
<td>8.4</td>
<td>79</td>
<td>60</td>
<td>100</td>
<td>54</td>
<td>130</td>
</tr>
<tr>
<td>Mercury</td>
<td>mg/kg dw</td>
<td>0.02</td>
<td>1.2</td>
<td>1.2</td>
<td>2.1</td>
<td>0.83</td>
<td>2.5</td>
</tr>
<tr>
<td>Nickel</td>
<td>mg/kg dw</td>
<td>17</td>
<td>40</td>
<td>45</td>
<td>56</td>
<td>28</td>
<td>44</td>
</tr>
<tr>
<td>Lead</td>
<td>mg/kg dw</td>
<td>13</td>
<td>110</td>
<td>110</td>
<td>180</td>
<td>78</td>
<td>190</td>
</tr>
<tr>
<td>Zinc</td>
<td>mg/kg dw</td>
<td>76</td>
<td>480</td>
<td>365</td>
<td>610</td>
<td>330</td>
<td>860</td>
</tr>
<tr>
<td><strong>Organic contaminants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mineral oil (C10-C40)</td>
<td>mg/kg dw</td>
<td>40</td>
<td>640</td>
<td>1250</td>
<td>1200</td>
<td>400</td>
<td>3200</td>
</tr>
<tr>
<td>Hexachlorobenzene</td>
<td>mg/kg dw</td>
<td>&lt;0.5</td>
<td>0.0047</td>
<td>0.02</td>
<td>0.0049</td>
<td>0.0092</td>
<td>0.0095</td>
</tr>
<tr>
<td>DDT/DDE/DDD (sum)</td>
<td>mg/kg dw</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>0.02</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>0.016</td>
</tr>
<tr>
<td>PCB s (sum of 7)</td>
<td>mg/kg dw</td>
<td>0.5</td>
<td>0.088</td>
<td>0.1</td>
<td>0.19</td>
<td>0.0697</td>
<td>0.47</td>
</tr>
<tr>
<td>PAHs (sum of 10)</td>
<td>mg/kg dw</td>
<td>0.47</td>
<td>4.7</td>
<td>8</td>
<td>11</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>Tributyltin (TBT)</td>
<td>μg Sn/kg dw</td>
<td>1.3</td>
<td>45.5</td>
<td>100</td>
<td>nd</td>
<td>23</td>
<td>nd</td>
</tr>
</tbody>
</table>

nd = not determined
< = under detection limit
Cultivation of the heart urchin Echinocardium cordatum

urchins onto ‘reference’ Oesterput or ‘polluted’ RMND 42 sediment every 2 days, during a 14 day period giving a total of 6 transfers and comparison of the mortality of the handled urchins to mortality of an unhandled control population on respectively ‘reference’ Oesterput or ‘polluted’ RMND 42 sediment. Four parallel tests were performed, each with 10 heart urchins.

In the whole sediment toxicity bioassay mortality was tested in a 750 cm² aquarium with a 10 cm layer of sediment and covered with 10 cm of filtered seawater with a salinity 32 ± 4 g l⁻¹ at a flow rate of 10 ± 2 L per 24 hours, and a water temperature of 15 ± 2°C. At the end of the 14 days exposure, organisms were recorded dead when they did not burrow within 30 minutes. Potential confounding factors such as salinity, oxygen, concentration of NH₄⁺, and pH of the water phase were monitored to ensure validity criteria as defined by Postma et al. (2002).

Histopathology of heart urchin

Histopathology of intestine and gonads was performed in both field heart urchins (weight approximately 20g) and cultured heart urchins (weight approximately 7g). Excised tissues were fixed in Davidson’s fixative (Shaw and Battle, 1957) and transferred after 24 h fixation to 70% ethanol solution. Tissues were processed using a vacuum infiltration processor embedded in paraffin wax and sectioned using a rotary microtome. Sections were cut at 5 μm and stained with Harris haematoxylin and eosin. Representative micrographs from the digestive tract intestine, stomach, rectum and gonads were taken using a Nikon Eclipse E800 photomicroscope.

Statistics

The mortality data were compared for field and cultured heart urchins using two way ANOVA. Survival data were statistically analyzed in ToxCalc™ using the Trimmed Spearman-Karber point estimate test to determine the lethal concentration LC₅₀.

Results

Spawning, fertilization and development to post larvae, juveniles and sub-adults

Spawning was successful in period April to June when a combination of an induction with KCl and a low pre-temperature period was applied. Influence of high temperatures, salinity and food on spawning was not affected. After spawning, eggs were successfully fertilized by mixing them with diluted sperm cells. Embryos developed from the blastula stage (Fig. 2A) into echinopluteus larvae (Fig. 2B and 2C) within two days (Gosselin and Jangoux, 1998). In addition to induction of spawning, development to metamorphosis larvae (post-larvae stage) (Fig 2C and 2D) appeared to be very critical. Approximately 75 000 sea urchin larvae were obtained in the three separate aquaculture batches. The larvae developed in 2 to 12 days to stages with 2 (Fig 2B), 3 (Fig 2C), 4 (Fig 2D) and 5 pairs (not shown) of spiny arms, and passed metamorphosis at day 35 (Fig 2E). Within 6 weeks after metamorphosis, juveniles reached a size ranging from 2 to 4 mm. Twelve weeks after fertilization 20% of the larvae had successfully developed into juveniles (Table 2). 52 weeks after fertilization, sub-adult E cordatum (Fig 2F, 44 weeks) had a mean diameter of 20 mm and a wet weight of 4.7± 0.2 g. SG results are presented in Table 2, which shows that the SG increases at relatively high rate from week 12 after fertilization onwards.
Cultivation of the heart urchin *Echinocardium cordatum*

**Figures 2A and 2B:** *Echinocardium cordatum*. Blastula stage after 10 hours and echinopluteus larva with 4 arms 48 hours after fertilization at 15 ± 2°C.

**Figures 2C and 2D:** *Echinocardium cordatum*. Echinopluteus larva with 6 pair skeletal arms after 6 days and larva with 8 arms just before postmetamorphic stage 23 days after fertilization at 15 ± 2°C.

**Figures 2E and 2F:** *Echinocardium cordatum*. Postmetamorphic larva stage after 31 days and juvenile heart urchin 44 weeks after fertilization at 15 ± 2°C.
Table 2: Development and growth rate of echinopluteus and juvenile heart urchin E. cordatum in aquaculture under optimized diet condition as described in the results.

<table>
<thead>
<tr>
<th>Development stages</th>
<th>Diet</th>
<th>Post-Fertilization time</th>
<th>Somatic wet weight (g)</th>
<th>Mean somatic growth per development stage (% day⁻¹)</th>
<th>Survival rate E. cordatum (%)</th>
<th>Survival rate P. Lividus ¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo</td>
<td>D. tertiolecta</td>
<td>21d</td>
<td>nd</td>
<td>nd</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Echinopluteus competent</td>
<td>T. succinea, D. tertiolecta, T. pseudonan, S. costatum, I. galbana, P. lutheri, and C. calcitrans</td>
<td>35d</td>
<td>nd</td>
<td>nd</td>
<td>41.2</td>
<td>45.3</td>
</tr>
<tr>
<td>Juvenile</td>
<td>C. calcitrans, S. costatum, T. pseudonan, TetraMin®</td>
<td>12w</td>
<td>0.10 ± 0.04</td>
<td>0.12 ± 0.05</td>
<td>21.7</td>
<td>24.7</td>
</tr>
<tr>
<td>Sub-adult</td>
<td>Replace every 4-6 weeks field sediment with naturally occurring food</td>
<td>52w</td>
<td>4.7 ± 0.2</td>
<td>1.29 ± 0.05</td>
<td>nd</td>
<td>1.2</td>
</tr>
<tr>
<td>Adult</td>
<td>Replace every 4-6 weeks field sediment</td>
<td>83w</td>
<td>16.0 ± 1.2</td>
<td>2.75 ± 0.21</td>
<td>nd</td>
<td>0.6</td>
</tr>
</tbody>
</table>

nd=not determined
¹) Growth conditions Paracentrotus lividus (mean global survival rate%) according to Grosjean, 1998

Laboratory exposure of cultured and field heart urchins in different toxicity tests

The LC₅₀ values for cultured heart urchins exposed to ammonium chloride during 96h were significantly higher (mean 37.4 ± 7.6 mg NH₄+/l) compared to the LC50 values for the field E. cordatum (mean 22.5 ± 4.9 mg NH₄+/l) (Fig 4). Different weight classes of E. cordatum did not differ in their responses to ammonium chloride. No significant effect was observed for time of year when field urchins were collected (data not shown). The LC₅₀ test conditions were in accordance with standard protocol requirements for factors such as salinity, pH, ammonia, and grain size as established by Postma et al. (2002) (data not shown).

In another set of experiments the effect of exposure of field and cultured E. cordatum to tributyltin (TBT) spiked sediments was compared. The results are presented in Fig 5. The measured concentrations of TBT in the spiked sediment after 14 days exposure ranged from 9.6 to 16,646 µg Sn/kg dry weight and consisted of 82 to 98 % of the nominal concentrations. TBT concentrations of 4,346 µg Sn/kg dry weight caused 100% mortality in both field and cultured E. cordatum. Cultured heart urchins are less sensitive to TBT spiked sediments than field urchins. Mortalities were observed at concentrations above 1,525 and 702 µg Sn/kg dw respectively for cultured and field E. cordatum.

The performance of field and cultured E. cordatum was also studied in sediment toxicity tests with polluted sediments RNMD 12, RNMD 16, RNMD 19 and RNMD 701 (Table 1), using mortality (determined by the absence of reburial activity) as endpoint (Fig 6). Both cultured and field collected E. cordatum showed high survival rates and full reburial behaviour in the control sediment. Exposure to sediments from site RNMD 12 caused high mortality compared to control sediment, for both field and cultured E. cordatum. Sediment RNMD 12 resulted in significant mortality (p<0.01) for the field urchins, indicating their higher sensitivity as compared to cultured urchins. Sediment RNMD 701 produced no significant differences between field and cultured E. cordatum. However, in sediments RNMD 16 field collected E. cordatum showed a significantly (p=0.001) higher percentage of non-reburial compared to cultured E. cordatum. Furthermore the results revealed that cultured E. cordatum (maximum CV=9.6%) gave a lower
Cultivation of the heart urchin *Echinocardium cordatum*

**Figure 3A and 3B:** Group of Field E. *cordatum*. Sections through immature ovarian tissues (3A) and high power view of melanin pigment associated with degenerative change (3B).

**Figure 3C and 3D:** Group of Cultured E. *cordatum*. Sections through immature ovarian tissues (3C) and high power view of oocytes of variable size which and with the absence of significant melanin deposition (3D).

statistical variation of sediment toxicity in the polluted sediments than the field heart urchins (maximum CV=18.9%).

Transfer experiments with field *E. cordatum* resulted in higher mortality for the transferred groups (Table 3) compared to the control groups that were not transferred. This effect of handling stress was observed for ‘reference’ Oesterput sediment and also when using moderately toxic dredged material RMND42. The results also reveal that *E. cordatum* moved to RMND42 sediment suffered dramatically from transfer damage (87.5% mortality, significantly higher than in Oesterput sediment; p <0.001). After the experiment there was no clear evidence of missing spines or outer damage to the heart urchins.

**Histopathology of heart urchin**

Histopathology of field and cultured *E. cordatum* was performed to compare the appearance of tissues in the two groups of heart urchins. Although no external visible signs of damage were noted in either field or cultured *E. cordatum*, in field *E. cordatum* evidence of cellular damage
caused by infections and/or other stressors was detected. In the intestine, increased deposition of melanin pigment in the epithelium was observed (Figures not shown), which was not present in cultured *E. cordatum*. Histopathology of field collected *E. cordatum* oocytes (Fig 3A and 3B) also revealed the presence of melanin pigment distribution throughout the tissue, accompanied by degeneration of oocytes. These features were not observed in cultured *E. cordatum* (Fig 3C and 3D). Furthermore, in contrast to the cultured *E. cordatum* the immature testis of field *E. cordatum* showed lipid containing cells and possible degenerate cells containing pigment (Figures not shown). No pathogens were detected in the tissues from field-collected animals.

**Discussion**

The present study describes a new method for culturing the heart urchin *E. cordatum* under controlled laboratory conditions. Juveniles fed with algae layer of mixed cultures *C. calcitrans, D. tertiolecta, S. costatum* and *T. pseudonana* developed most successfully. The somatic growth and survival rate of echinopluteus larvae to juvenile was successful (Table 2); this was in line with growth of *P. lividus* (Grosjean, 1998; Gosselin, 1998; Vaitilingon, 2001). In our study, 1-year-old cultured heart urchins were approximately 20 mm in diameter, and 4.7 g in weight on average. A somatic weight (SW) calculated from 90 field collected heart urchins (Fig. 3) showed a relationship between SW and diameter of heart urchin: SW (g) = 0.68* diameter^2.7 (cm). Field organisms in sandy sediment between 10 and 15 mm in diameter are comparable with an age of about 1 year (Beukema, 1985). This illustrates that cultured *E. cordatum* displayed a growth rate comparable or even higher to that of field organisms, especially when grown on high quality food (Kröncke, 2004).

The performance of cultured organisms in standard toxicity bioassays was much better than that of *E. cordatum* collected from the field. Using cultured heart urchins instead of field heart urchins results in improved reproducibility and lower statistical variation; the CV with field heart urchins was utmost 19% respectively. However the mortality observed in the tests using field heart urchins differs from the findings of Stronkhorst (2004), since the results showed a higher CV for samples from harbour sediments. The LC_{50} for ammonium chloride of cultured *E. cordatum* was significantly higher LC_{50} for cultured *E. cordatum* as compared to field *E. cordatum* 37.4 and 22.5 mg NH₄⁺/l respectively (95% confidence interval 31.0–48.8 and 95% confidence interval 20.0–52.0 mg NH₄⁺/l). This suggests a lower sensitivity towards ammonium chloride toxicity of cultured *E. cordatum* compared to field organisms. This is in line with results from a 72h water-only ammonium chloride sensitivity test with the marine invertebrate *Corophium volutator* where the cultured *C. volutator* had a two times higher LC_{50} value than the field caught *C. volutator*, with LC_{50} values amounting to respectively 85.2 (95% confidence interval 73.5–98.6) mg NH₄⁺/l and 40.3 (95% confidence interval 23.6–68.6) mg NH₄⁺/l (Peters and Ahlf, 2003).

In toxicity experiments with TBT the LC_{50} reflected by the non-reburial activity for field *E. cordatum* was 702 µg Sn/kg dry weight actual TBT concentration of in the spiked sediment, while for cultured *E. cordatum* the LC_{50} was observed at 1,525 µg Sn/kg dw (Fig 5). In this comparative study, cultured and field *E. cordatum* revealed LC_{50} values of 1,242 (95% confidence interval 986-1,564) and 964 (95% confidence interval 843-1,102) µg Sn/kg dw respectively also demonstrates the lower sensitivity of the cultured field urchins as compared to the field organisms. This toxicity for field *E. cordatum* is not in accordance with the studies by Stronkhorst et al. (1999) where LC_{50} of 4,055 ng Sn/g dry weight was observed. The results also show that TBT is moderately toxic to *E. cordatum*.
In a final set of experiments the sensitivity of cultured *E. cordatum* was compared to that of field *E. cordatum* in a whole sediment toxicity test with dredged materials. In this whole sediment toxicity test survival of cultured sea urchins was higher or at least similar to that of field *E. cordatum* (Fig. 4). So, similar to the results obtained in the water-only ammonium chloride LC$_{50}$ test, and the TBT spiked sediment toxicity test these experiments with polluted sediments also suggest a higher sensitivity in toxicity tests of the field as compared to the cultured heart urchins. The presence of increased melanin pigmentation, as detected in the field urchins, is often associated to ‘stress’, including pathogen insult, resulting in cell damage and recycling involving the production of pigments as part of the host defense mechanism (Fox et al., 1966; De Ridder et al., 1984; Bandaranayake, 2006). Histopathological analysis of immature gonads and digestive epithelia of cultured urchins provided no evidence of infections or cellular necrosis. However the histological comparison of field and cultured urchins was difficult since they were of different ages. This may also explain the lower variability in the toxicity tests with cultured organisms. The handling stress experiments indicated that regular transfer of the urchins resulted in a higher mean percentage mortality than that observed for controls for groups maintained on both ‘clean’ reference Oesterput sediment and on polluted RMND42 sediment. However, quantitative data on the effect of handling stress of urchins are still lacking. Increased mortality of heart urchins due to multiple stressors has been reported before (Heugens et al., 2001). In addition to handling stress during toxicity testing, urchin-fishing techniques can cause lethal crushing or sub-lethal trauma (Tegner, 1977; Hauton et al., 2003) resulting in inhibited reburial activity in subsequent bioassays. From this it can be concluded that multiple stressors may be a significant confounding factor in urchin-based bioassays for saltwater environmental toxicity testing.

### Conclusion

The present study describes the cultivation and use of the heart urchin *E. cordatum* for whole sediment toxicity testing. Under laboratory conditions echinopluteus larvae developed successfully after fertilization, from the 2-, 3-, 4- and 5-arm pair stage, via metamorphosis to yield juveniles. Based on our optimized growth protocol both larvae and juveniles of cultured *E. cordatum* have a growth rate at least comparable to field *E. cordatum*. A lower sensitivity to ammonium chloride toxicity of cultured *E. cordatum* compared to field organisms results in improved reproducibility and lower statistical variation. It was also concluded that cultured heart urchins are less sensitive to TBT than field collected *E. cordatum*. Furthermore in whole sediment toxicity tests, survival of cultured sea urchins was higher or at least similar to that of field collected *E. cordatum*. Histopathology provided indirect evidence of the presence of infectious agents in tissues of field-collected urchins whereas these effects were reduced or absent in cultured heart urchins. The increased sensitivity of field urchins compared to cultured urchins
in various toxicity tests may be due environmental multiple stressors reducing their overall performance. Overall it was demonstrated that the use of cultured *E. cordatum* provides a significant improvement for urchin-based bioassays for marine environmental toxicity testing, resulting in a more homogeneous, vital population and experimental data with lower variability. The cultivation of heart urchin *E. cordatum* provides animals for which the life history is known, with freedom of infection and without ecological or collection trauma. Thereby offering a distinct advantage over using field collected heart urchins in marine toxicity testing.

**Acknowledgement**

This study was initiated and coordinated by the National Institute for Coastal and Marine Management (RIKZ) of the Directorate-General of Public Works and Water Management (RWS) in the Netherlands. The cultured heart urchins are archived at the Department of Zoology, National Museum of Natural History Naturalis, Leiden, the Netherlands. We thank Dick Vethaak and Martine van de Heuvel-Greve for critically reviewing of the manuscript. We are grateful to Matthew Green (Cefas) for production of histological sections.
Chapter 5

A weight-of-evidence approach to assessing the ecological impact of organotin pollution in Dutch marine and brackish waters; combining risk prognosis and field monitoring using common periwinkles (Littorina littorea)

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Marine Environmental Research, 66, 231–239, 2008

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Abstract: In the present study an integrated ecological risk assessment based on multiple lines of evidence (LOEs) was evaluated in order to better assess the risk from TBT in Dutch harbours and open coastal waters. On the basis of spatial distributions of measured tributyltin (TBT) concentrations in sediments and suspended matter, predictions of the intersex index (ISI) in *Littorina littorea* and the ecological risk expressed as the Potentially Affected Fraction (PAF) of species were made. The results were compared to actual ISI measurements and presence of *L. littorea* in the field. The PAF calculated on the basis of TBT levels for open coastal waters ranged from 4.2% to 15.3%; for harbours it ranged from 3.5% to 26.9%. Significant intersex levels were observed only in waters where the risk was calculated above 10% PAF. This study suggests that the absence of *L. littorea* from some harbours with high ecological risk values can be explained by high TBT concentrations. A call is made for the use of integrated approaches like weight of evidence (WOE) to help practitioners improve ecological risk assessment.

Introduction

Tributyltin (TBT) was first used as an antifouling agent in the late 1960s. It proved to be very effective, but also extremely toxic to aquatic life (Champ and Seligman, 1996). TBT effects were first recognized in the marine environment in the 1970s and attracted particular attention in the 1980s, when the effects of TBT started to become apparent in the Netherlands and elsewhere (Marquenie and Vethaak, 1989; Champ and Seligman, 1996). In the 1980s, there was a sharp decline in the population of dog whelk (*Nucella lapillus*) in the Delta area of the south-western Netherlands and in the 1990s the occurrence of imposex was demonstrated among whelks (*Buccinum undatum*) in the same area (Mensink et al., 1996) as well as in the open North Sea (Ten Hallers-Tjabbes et al., 1994).

Given the concern about the environmental effects of TBT in the Dutch marine environment a lot of data has been collected in the past. The available data relate to TBT concentrations in water, sediments and suspended matter and field measurements of intersex in, and the distribution and abundance of gastropods. The Chemical Toxicity Test (CTT) has shown TBT to be the most problematic of all the chemicals tested in toxicological terms in assessing whether dredged material may be relocated in the marine environment (Schipper and Schout, 2004). Up to now, in assessing the impact of TBT on the ecosystem, the available information has been evaluated separately. This paper describes the results of an integrated assessment including all the information collected in recent years. Comparable to the sediment quality Triad concept, which uses information from the toxicity line, the biological field line and the chemistry line, this assessment is also based on results of three lines of evidence (LOEs) (Chapman, 1996; Rutgers, 2000). However, instead of using measurements from multiple species in the biological field line, as normally done in the Triad approach, the assessment in the present study uses information from one indicator species only. In the present study the LOEs are primarily represented by measurements of: (i) chemical concentrations (exposure levels), (ii) (eco)toxicological indicators and results from ecotoxicological laboratory experiments, and (iii) abundance of indicator species *Littorina littorea* in the field. The Potentially Affected Fraction of species (PAF) was used to represent the ecological risk and quantify the degree of TBT stress on the ecosystem (Aldenberg et al., 2002). All three LOEs provide indications of the ecological health status related to TBT pollution, though all approaches based on this level include uncertainties:
(i) TBT pollution is directly chemically measured in samples collected in the field. However, the relevance of chemical analysis of sediments to the quality of water bodies is not always clear. Processes such as bioavailability determine the actual relation between chemical concentrations and possible ecological effects. Ecological risk prognosis, using accepted generic procedures, predicts the health status related to the stressor under consideration based on the measured or estimated chemical concentrations assuming equilibrium partitioning and constant bioavailability. The prognosis will provide the likelihood of effects to occur. Whether or not these effects will actually occur depends on location specific conditions.

(ii) Biological effect parameters can be used to assess the potential and actual impacts of TBT on marine coastal life. However, it can be difficult to link biological effects to exposure to a single contaminant. In this study, the degree of intersex index (ISI) in the common periwinkle, *L. littorea*, was selected as an indicator of TBT pollution, as this species is the most common prosobranch species on hard substrates in Dutch coastal waters. Intersex in this species has proven to be sensitive to the degree of environmental organotin pollution in coastal waters (Oehlmann et al., 1998). Although periwinkles are not the most sensitive gastropod and ISI is not a rapid response, measuring ISI levels in *L. littorea* in the field is still a useful way of assessing effective levels of TBT pollution. Several studies have shown that ISI can be used as a biological tool to determine the degree of TBT pollution, which can be expressed as approximate TBT concentrations in seawater (Stroben et al., 1992; Oehlmann et al., 1998). However, the ISI indicator in periwinkles cannot easily be translated to a generic health indicator for the water body under consideration.

(iii) Observations from biological effect monitoring at gastropod level (Ten Hallers-Tjabbes et al., 1994; Mensink, 1996; De Wolf, 2001) are used to assess effects of the distribution and contamination levels in the Dutch marine environment. The abundance of *L. littorea* and the far more sensitive dog whelk, *N. lapillus*, can be used as indicators. TBT contamination has made the dog whelk a rare species in Dutch coastal waters and it is almost entirely absent from harbours and marinas (Harding et al., 1992; Van Moorsel, 1996). TBT potentially accumulates in sediments. However, there is no direct link between sediment concentrations and effects in gastropods, as the preferred habitats of some of the most common gastropods (e.g. *N. lapillus*, *L. littorea*) are hard substrates, including natural substrates such as rock and artificial substrates such as harbour walls, breakwaters, etc. (Oehlmann, 2002).

In this study the results of the LOEs are compared in order to establish their similarity and reduce the uncertainty of the overall assessment. The methodological links between different LOEs must be evaluated so that the results of monitoring effects on *L. littorea* and risk assessment studies focusing on the impacts of TBT can be compared. This paper describes a WOE approach (Smith et al., 2002) focusing on TBT in Dutch harbours and open waters. Focusing on the integration of indicator monitoring and risk prognosis, the present study applies a more holistic approach to impact assessment (Suter II, 2001).
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Materials and Methods

Study area

An integrated ecological risk assessment approach including evaluation of three LOEs is applied to the collected data to assess ecological health status related to TBT pollution in Dutch marine and estuarine waters. Figure 1 shows the harbour locations 1 to 30 and the seven open water areas (A to G) from which chemical, (eco)toxicological and/or ecological data have been included in this study.

Chemistry

The top 5 cm of sediment was sampled using a box core at 120 locations along the Dutch coast between 1998 and 2003. Over this period, 106 locations were sampled twice with an interval of three years. The locations covered the main open North Sea waters, the Wadden Sea, the Eastern Scheldt and estuarine areas such as the Western Scheldt and Ems-Dollard (see Figure 1). TBT was also analysed in sediment from a number of harbour locations from 1999 to 2002 (Table 1).

TBT in suspended particulate matter (SPM) was measured four times a year at eight locations in four different open water areas (A. Ems-Dollard; B. Wadden Sea; C. North Sea; E. Eastern Scheldt; G. Western Scheldt, Figure 1) along the Dutch coast between 1998 and 2003, giving information on fluctuations between and within years. A further 17 locations (data not shown) from the French-Belgian border to the Ems-Dollard estuary on the Dutch-German border were sampled in 2003. Suspended matter was sampled by pumping surface water through a flow-through centrifuge lined with Teflon plates to collect the suspended matter. At harbour locations 23 and 29 (see Figure 1) the seasonal variation of water-dissolved TBT was sampled from 1999 to 2002.

The sediments were freeze-dried and homogenized using a Retsch planetary ball mill. 1.5 ml acetic acid, 15 ml methanol and 7 ml hexane were added to 1 g of sediment. This mixture was stirred for 30 min, after which 3 ml of 4 M sodium acetate solution (pH=5) was added to obtain optimal ethylation. Then, 4 ml of 5% sodium tetraethyl borate solution was slowly added using a peristaltic pump over 20 min, during which time the mixture was continuously stirred. The mixture was shaken vigorously and 5 ml 10 M sodium hydroxide solution added to remove boroxin formed at ethylation. About 5 ml of water was added, and after shaking and subsequent centrifugation the hexane layer with the ethylated organotin compounds was collected. This hexane extract was cleaned over a column of 4-gramme aluminium oxide (Alumina B-super deactivated with 10% water) using hexane as an eluent. 1 ml of iso-octane was added to the hexane eluate and this extract was then concentrated to a final volume of 0.5 ml using Kuderna-Danish solvent evaporation. The final extracts were analyzed for ethylated organotin compounds by electron impact (EI) GC-MS using selective ion monitoring. The method uses ethylated Quasimeme standards and tetrapropyltin as internal standard. Quality assurance recovery control with carried out with monopropyltin and tripropyltin in each sample and blank control in every analysis batch. Yearly participation in Quasimeme laboratory proficiency exercises for organotins resulting in satisfactory z-scores.

Probabilistic risk assessment methods

The measured TBT-Sn concentrations were used as a basis for a probabilistic risk assessment. In those samples where the fraction organic carbon (f_{oc}) was reported, the sediment and
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Figure 1: Map showing sampled harbours (1-30) and open waters (A-G) along the Dutch coast.

Table 1: Chemical and biological datasets used in this study.

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Area type</th>
<th>Medium</th>
<th>Time range</th>
<th># areas</th>
<th># locations</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBT concentrations</td>
<td>Open water</td>
<td>Suspended matter</td>
<td>1998-2003</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>TBT concentrations</td>
<td>Open water</td>
<td>Sediment</td>
<td>1998-2003</td>
<td>7</td>
<td>123</td>
</tr>
<tr>
<td>TBT concentrations</td>
<td>Harbour</td>
<td>Suspended matter</td>
<td>1999-2002</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>TBT concentrations</td>
<td>Harbour</td>
<td>Water</td>
<td>1999-2002</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>TBT concentrations</td>
<td>Harbour</td>
<td>Sediment</td>
<td>1999-2002</td>
<td>31</td>
<td>270</td>
</tr>
<tr>
<td>ISI values and abundance</td>
<td>Open water</td>
<td>Periwinkle</td>
<td>2002-2005</td>
<td>5</td>
<td>24</td>
</tr>
<tr>
<td>ISI values and abundance</td>
<td>Harbour</td>
<td>Periwinkle</td>
<td>2002-2005</td>
<td>9</td>
<td>18</td>
</tr>
</tbody>
</table>

Suspended matter concentrations from the field surveys were normalised to water concentrations, applying the formulas in the European Technical Guidance Document for Risk Assessment (EC, 2003). The ecological risk expressed as the Potentially Affected Fraction of species (PAF) was calculated in accordance with Aldenberg et al. (2002). This method can be summarised as fol-
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The calculated water concentrations were compiled in a log-normal distribution representing the spatial variation of TBT concentrations in a specific area. The concentration distribution was then compared to the species sensitivity distribution (SSD) for TBT. The SSD describes the average sensitivity of species and the variation among species (Posthuma et al., 2002). The SSD for TBT reported by Lepper (2002) is based on toxicity data for 29 marine and freshwater species representing seven taxonomic groups (algae, annelida, crustacea, echinodermata, insecta, mollusca, pisces). The SSD for TBT-Sn used in this study is provided in the supplementary data for this paper.

The PAF indicates the likelihood that adverse ecological effects will occur as a result of TBT exposure in a particular area. This value can be interpreted as the probability that a randomly selected exposure concentration will exceed species sensitivity. The probabilistic risk assessment was performed on the clustered data for harbours and for open waters, as well as for each harbour and open water separately. Only water systems for which exposure concentrations were measured at two or more locations were selected (all open waters and 19 out of 30 harbours).

The likely range of the intersex indicator (ISI) for \textit{L. littorea} was also calculated based on the dose-response relationship for \textit{L. littorea} as published by Oehlmann (2002) and the spatial distribution of water concentrations. The range of possible ISI values from the 5\textsuperscript{th} and 95\textsuperscript{th} percentile of the exposure concentration distributions were calculated applying Formula 1.

\begin{equation}
\text{ISI} = \frac{3.35}{1 + \exp\left(-\frac{\text{PEC}_{\text{seawater}} - 18.3}{4.73}\right)}
\end{equation}

where:
\begin{itemize}
  \item \text{ISI} = \text{calculated ISI level [-]}
  \item \text{PEC}_{\text{seawater}} = \text{concentration of TBT-Sn in seawater [ng.l}^{-1}]
\end{itemize}

Biological effects and abundance surveys

Periwinkles were sampled from 2002 to 2005 at 42 locations along the Dutch coast, representing the main Dutch open waters areas and harbour locations (Figure 1). Grevelingen (D) and Veersemeer (F) lakes were not included. At the sampling locations the presence of \textit{L. littorea} was recorded at every location randomly selected in 5 to 7 areas of 1 m\textsuperscript{2}. If present, at least 40 individuals of \textit{L. littorea} with a shell height between 15 and 25 mm (approx. 1.5-year-old new adults) were collected. The occurrence of intersex in the periwinkles was analysed within seven days, in accordance with the methods described by Bauer et al. (1995). ISI is expressed as the average intersex stage within the population.

Overview of chemical and biological datasets

Table 1 lists the chemical and biological datasets compiled for this study. The number of ‘areas’ refers to the open waters presented in Figure 1. The number of ‘locations’ refers to different sampled harbours within the areas.
Table 2: Observed and predicted concentrations, ecological risk values and observed and predicted ISI levels in different Dutch open waters.

<table>
<thead>
<tr>
<th>Area</th>
<th># locations</th>
<th>Observed concentration range in sediments and SPM Sn-TBT μg kg⁻¹ dw (min – max)</th>
<th>Calculated concentration range in water Sn-TBT ng l⁻¹ (5 - 95 %tile)</th>
<th>Calculated Ecological Risk as PAF (%)</th>
<th>Calculated predicted ISI range (based on 5 – 95 %tile concentrations)</th>
<th>Observed ISI (min – max)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Open waters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ems-Dollard</td>
<td>10</td>
<td>5.1 – 23.0</td>
<td>0.59 – 2.20</td>
<td>5.13</td>
<td>0.08 - 0.11</td>
<td>0.00</td>
</tr>
<tr>
<td>Grevelingen Lake</td>
<td>5</td>
<td>7.0 – 42.7</td>
<td>0.79 – 3.72</td>
<td>7.29</td>
<td>0.08 - 0.15</td>
<td>n.d.</td>
</tr>
<tr>
<td>North Sea</td>
<td>49</td>
<td>0.2 – 52.5</td>
<td>0.23 – 5.45</td>
<td>6.16</td>
<td>0.07 -0.21</td>
<td>0.00 – 0.09</td>
</tr>
<tr>
<td>Eastern Scheldt</td>
<td>15</td>
<td>0.2 – 24.4</td>
<td>0.10 – 3.87</td>
<td>4.2</td>
<td>0.07 -0.15</td>
<td>0.00 (3x)</td>
</tr>
<tr>
<td>Veersemeer</td>
<td>6</td>
<td>0.2 – 97.9</td>
<td>0.76 – 16.4</td>
<td>13.7</td>
<td>0.08 - 1.35</td>
<td>n.d.</td>
</tr>
<tr>
<td>Western Scheldt</td>
<td>13</td>
<td>7.4 – 160.7</td>
<td>1.73 – 12.8</td>
<td>15.3</td>
<td>0.10 - 0.80</td>
<td>0.00 – 0.15</td>
</tr>
<tr>
<td>Wadden Sea</td>
<td>40</td>
<td>0.2 – 36.6</td>
<td>0.25 – 3.23</td>
<td>4.78</td>
<td>0.07 - 0.13</td>
<td>0.00 (3x)</td>
</tr>
<tr>
<td><strong>All open waters</strong></td>
<td>138</td>
<td>-</td>
<td>-</td>
<td>6.72</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Harbours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schiermonnikoog</td>
<td>2</td>
<td>0.5 – 0.5</td>
<td>0.04 – 1.22</td>
<td>1.56</td>
<td>0.07 – 0.09</td>
<td>n.d.</td>
</tr>
<tr>
<td>Emshaven</td>
<td>5</td>
<td>1.8 – 12</td>
<td>0.29 – 1.71</td>
<td>3.50</td>
<td>0.07 - 0.10</td>
<td>0.04 – 0.10</td>
</tr>
<tr>
<td>Kornwederzand</td>
<td>3</td>
<td>4.8 – 15</td>
<td>0.37 – 1.55</td>
<td>3.61</td>
<td>0.07 - 0.09</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ameland</td>
<td>4</td>
<td>0.5 – 25</td>
<td>0.12 – 3.60</td>
<td>4.21</td>
<td>0.07 - 0.14</td>
<td>n.d.</td>
</tr>
<tr>
<td>Perkpolder</td>
<td>2</td>
<td>3.9 – 24</td>
<td>1.36 – 3.92</td>
<td>8.92</td>
<td>0.09 - 0.15</td>
<td>n.d.</td>
</tr>
<tr>
<td>IJmuiden†</td>
<td>32</td>
<td>0.5 – 980</td>
<td>0.20 – 16.4</td>
<td>10.2</td>
<td>0.07 - 1.35</td>
<td>1.18 – 1.38</td>
</tr>
<tr>
<td>Breskens</td>
<td>2</td>
<td>0.5 – 13</td>
<td>0.29 – 13.8</td>
<td>10.2</td>
<td>0.07 – 0.93</td>
<td>n.d.</td>
</tr>
<tr>
<td>Terneuzen</td>
<td>6</td>
<td>4.3 – 24</td>
<td>1.06 – 9.19</td>
<td>11.8</td>
<td>0.09 - 0.43</td>
<td>n.d.</td>
</tr>
<tr>
<td>Hansweert</td>
<td>2</td>
<td>19 – 37</td>
<td>2.27 – 6.07</td>
<td>12.6</td>
<td>0.11 - 0.23</td>
<td>n.d.</td>
</tr>
<tr>
<td>Delfzijl†</td>
<td>11</td>
<td>6.8 – 240</td>
<td>0.57 – 18.6</td>
<td>13.4</td>
<td>0.08 - 1.73</td>
<td>0.14</td>
</tr>
<tr>
<td>Lauwersoog</td>
<td>6</td>
<td>0.5 – 210</td>
<td>0.15 – 70.3</td>
<td>17.0</td>
<td>0.07 - 3.35</td>
<td>2.58</td>
</tr>
<tr>
<td>Vlissingen</td>
<td>7</td>
<td>3.4 – 180</td>
<td>2.01 – 20.2</td>
<td>18.7</td>
<td>0.10 - 2.01</td>
<td>0.52 – 2.60</td>
</tr>
<tr>
<td>Rijnmond†</td>
<td>124</td>
<td>0.5 – 5700</td>
<td>0.70 – 61.9</td>
<td>21.4</td>
<td>0.08 - 3.35</td>
<td>0.70</td>
</tr>
<tr>
<td>Yerseke</td>
<td>3</td>
<td>16 – 250</td>
<td>1.04 – 56.1</td>
<td>22.4</td>
<td>0.08 - 3.35</td>
<td>0.80 – 1.15</td>
</tr>
<tr>
<td>Den Helder†</td>
<td>12</td>
<td>3.2 – 460</td>
<td>0.93 – 61.6</td>
<td>22.6</td>
<td>0.08 - 3.35</td>
<td>2.86 – 3.00</td>
</tr>
<tr>
<td>Texel</td>
<td>7</td>
<td>4.1 – 190</td>
<td>0.61 – 86.8</td>
<td>23.1</td>
<td>0.08 - 3.35</td>
<td>n.d.</td>
</tr>
<tr>
<td>Scheveningen</td>
<td>8</td>
<td>7 – 370</td>
<td>1.21 – 61.3</td>
<td>23.7</td>
<td>0.09 - 3.35</td>
<td>0.48</td>
</tr>
<tr>
<td>Den Oever</td>
<td>5</td>
<td>4.2 – 110</td>
<td>0.41 – 127.0</td>
<td>24.0</td>
<td>0.07 - 3.35</td>
<td>n.d.</td>
</tr>
<tr>
<td>Harlingen†</td>
<td>15</td>
<td>0.5 – 1700</td>
<td>0.27 – 255.4</td>
<td>26.9</td>
<td>0.07 - 3.35</td>
<td>-</td>
</tr>
<tr>
<td><strong>All harbours</strong></td>
<td>270</td>
<td>-</td>
<td>-</td>
<td>19.1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Where no range was measured, single values are reported in the table.
† No periwinkles were present at any sampling stations.
n.d. = location not sampled
Results

Observed and predicted exposure levels

The observed concentration ranges of TBT in sediment or SPM in Dutch open waters and 19 Dutch harbours are shown in Table 2. The highest observed TBT-Sn concentration in Dutch harbours was a factor 30 higher than the highest TBT-Sn concentrations in open waters. The lowest TBT concentrations were more or less in the same range. In order to normalise the sediment and suspended matter concentrations to water concentrations a value for the $K_{oc}$ (organic carbon partition coefficient) needs to be estimated. From the distribution of measured sediment and
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Water concentrations in Dutch harbours a value for the $K_{oc}$ representative of the Dutch situation was estimated. A value of 5.34 gave the best correspondence between observed and predicted water concentrations in Dutch waters (More information on how this value was obtained can be found in the supplementary data). As no temporal trends were observed in the concentrations of TBT-Sn in either the open waters or the harbours, the average concentration of TBT-Sn was taken to represent the exposure at a sampling location. This average concentration was used for further analysis in the risk predictions. Since the concentration distributions of the normalised sediment samples and SPM samples did not differ significantly (Student’s $t$ test, equal variances, $p=0.42$), the normalised data of the two distributions were pooled. Figure 2 presents the overall exposure concentration distributions for TBT-Sn for 138 open water and 270 harbour locations respectively.

Dedicated exposure concentration distributions were derived for all areas and locations (open waters and harbours), representing the spatial distribution of water concentrations for each area. In Table 2 the 5th and 95th percentile of the distribution represent the range of water concentrations for each area.

Predicted ecological risk

The two distributions presented in Figure 2 were used as input for a probabilistic risk assessment as described by Aldenberg et al. (2002). This resulted in an expected total ecological risk for open waters of 6.7% and 19.1% for harbours. This implies that, on average, for open waters it is likely that 6.7 % of species will be negatively affected as a result of exposure to TBT. For the harbours this fraction is 19.1%. The risk values for the individual open waters and harbours are given in Table 2. Relatively high-risk values were obtained for open waters in the Western Scheldt (G) and Veersemeer (F), while the risk values for the Wadden Sea (B) and the Eastern Scheldt (E) were below 5%. For harbours the highest risk value was predicted at Harlingen in the Wadden Sea (location 8). Four harbours, all small harbours in the Wadden Sea, yielded a risk value equal to or lower than the lowest risk value for the individual open water bodies (see Table 2). The table presents the observed and predicted concentrations and ISI levels in $L$. littorea. The ecotoxicological risk presented as PAF can be interpreted as the characterization of the potential adverse health effects of exposure to environmental hazards. The 5th and 95th percentiles of the exposure concentration distribution in the dedicated open water or harbour were used to calculate the range of ISI values likely to occur in the area. The upper and lower limits of the calculated ISI values are reported in Table 2.

Observed ISI values and species abundance

ISI observations are available for the open waters, except for Grevelingen (D) and Veersemeer (F) lakes, where no ISI sampling was carried out. The observed ISI values for open waters were relatively low. The highest ISI value was observed in the Western Scheldt. Ten harbours were selected for ISI sampling. However, since no periwinkles were found in Harlingen (location 8), ISI observations are available for only 9 harbours. Periwinkles with low abundance were found at the harbours in Delfzijl (location 1), Den Helder (location 14), IJmuiden (location 15) and Rijnmond (location 18). Much higher ISI levels were observed in harbours than in open waters. The highest value (3.0) was observed in Den Helder (location 14). The wide range of ISI levels observed in Vlissingen harbour (location 25) indicates that a high variation in ISI levels can exist within a single harbour.
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Figure 4: Comparison of the observed ISI values and the predicted ISI ranges based on the concentration distribution in the corresponding open waters (A) and harbours (B). The calculated ISI values corresponding to the median and the 5th and 95th percentile of the exposure concentration distribution are also shown. At some sampling locations in harbours periwinkles were rare, while at other locations(†) no periwinkles were observed.

Comparison of the LOE results

To check the correspondence between the maximum ISI levels that can be expected in a harbour and the ecological risk, PAF values have been plotted against the calculated ISI value corresponding to the 95th percentile of the exposure concentration distribution (Figure 3). The 95th percentile was chosen because this value corresponds to the most polluted part of the harbour or water body corresponding to a maximum ISI value that might be encountered during
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Field monitoring. In ecological risk prognosis a Potentially Affected Fraction of 5% of species is generally considered acceptable (e.g. Van Straalen and Denneman, 1989; Aldenberg and Slob, 1993; Newman et al., 2000; Van der Hoeven, 2001; EC, 2003). This study shows that at this 5% level intersex in *L. littorea* is unlikely to occur in the field. But even at higher risk levels, up to approximately 10%, ISI levels in the field are not expected to exceed the ISI signalling level of 0.3 (Oehlmann, 2002). This study showed that high ISI levels up to a maximum of 3.35 are expected in the field only when the predicted risk level exceeds 20%. These results indicate that either the ISI measurements in *L. littorea* are not sensitive enough to assess ecosystem health, or the risk prognosis is too conservative (predicting risks while there are no actual effects).

In order to check whether the conservatism in the risk assessment is a result of the estimated concentrations (chemistry LOE), the calculated ranges of ISI values based on the 5th and 95th percentile of TBT concentrations are compared by the observed ISI values in the field (Ecotoxicity LOE). If there is a good correspondence between calculated and observed ISI, it can be concluded that the concentrations are a proper basis for the risk calculations as well. Figure 4 presents the comparison between calculated and observed ISI for open waters (A) and Dutch harbours (B).

The observed ISI values were relatively low for open waters, often below the 5th percentile of the predicted ISI range based on the TBT concentrations. Given the TBT concentrations in the Western Scheldt (G) and Veersemeer (F), predicted ISI values around 1.0 might be expected. Although intersex was observed at a low level (ISI = 0.15) at one location in the Western Scheldt, the observations never exceeded the signalling level of 0.3 (Oehlmann, 2002). Nearly all observed ISI levels in harbours fall within the ISI ranges corresponding to the median and 95th percentile of the exposure concentrations. The ISI levels observed in IJmuiden (location 15), Den Helder (location 14), Vlissingen (location 25) and Lauwersoog (location 3) corresponded to the upper 25% of the exposure concentrations. At 4 other harbours – Delfzijl (location 1), Yerseke (location 22), Scheveningen (location 17) and Rijnmond (location 18) – the observed ISI levels corresponded to the upper 50% of the exposure concentrations. Maximum intersex levels are predicted for three harbours (locations 8, 14 and 18) where no periwinkles were found at some stations. In IJmuiden (location 15) and Delfzijl (location 1), where periwinkles were in low abundance, the maximum calculated ISI level is around 1.5.

**Discussion**

In the present study a weight-of-evidence approach derived on the basis of three LOEs has been evaluated in order to better assess the risk from TBT in Dutch harbours and open waters. Based on chemical data, risk values were determined according to Aldenberg et al. (2002) and ISI values were calculated based on the dose-response curve reported by Oehlmann (2002). Actual ISI observations were made in the field and the presence of gastropods was recorded. In the overall approach evidence from the different LOEs was combined.

The partition coefficient (K_p) was used for the conversion of TBT concentrations in sediment and SPM to TBT concentrations in water. This K_p for TBT was calculated by multiplying the organic carbon partition coefficient (K_{oc}) with the measured fraction organic carbon (f_{oc}). Consequently, the K_{oc} value has a strong impact on the final results of the risk prognosis. Generally, in risk predictions the lowest K_{oc} value is applied to calculate concentrations in water (EC, 2003). This results in a worst-case water concentration, in accordance with the precautionary principle. However, with literature values for the K_{oc} of TBT ranging from 3.0 – 6.2 (Lepper, 2002), it is more appropriate to base an assessment on local measured values. In this study the...
K$_{oc}$ value was estimated from measured aqueous and sediment concentrations in 30 Dutch harbours evenly distributed over the Netherlands. This resulted in an average K$_{oc}$ value of 5.34 (see also the supplementary data). This outcome is within the range of reported K$_{oc}$ values from the literature (Lepper, 2002).

The highest ISI value, measured in the Western Scheldt (G), still fell within the predicted ISI range. Higher ISI values for samples from the Western Scheldt (G) have, however, been reported by De Wolf et al. (2001 and 2004), showing a decline from 1.3 in 1998 and 2001 to 0.5 in 2002. This decline was confirmed by the surveys reported here, where an ISI of 0.15 was found at the same location in 2002, using only young adults. This is comparable to the ISI value reported by De Wolf et al. (2004), as these were adults that had been exposed some years before the young adults were exposed. In contrast to the trend in the ISI, no downward trend in the sediment-derived water concentrations from 1998 to 2003 was observed in the Western Scheldt (G). We can conclude from the data that observed ISI levels in open waters are all in the lower range of predicted ISI levels.

**Combination of LOE results and WOE**

The results of the risk predictions confirm that *L. littorea* is not the most sensitive species regarding to TBT pollution (Oehlmann *et al.*, 1998; Ospar, 2003). In the field, significant effects on intersex levels are expected for this species only when the risk level exceeds 10%, whereas a risk level of 5% is generally considered acceptable for the protection of ecosystem health (e.g. Van Straalen and Denneman, 1989). High ISI levels, up to a maximum of 3.35, are expected only when the risk level exceeds 20%. At these high ISI levels, up to a maximum of 3.35, the population of *L. littorea* is expected to be completely sterile (Oehlmann, 2002). It may even be argued that a risk level of 5% is far too high for specifically acting toxicants such as TBT. Even though *L. littorea* is not the most sensitive species to TBT pollution, the only species that are more sensitive are other species of prosobranch molluscs. Protecting 95% of species in general would still exterminate the carnivorous neogastropods and also a number of deposit-feeders such as *Scrobicularia plana*, which can be considered as key species for ecosystem function. Therefore for specifically acting toxicants even stricter criteria should be argued for. In absence of other indicator species *L. littorea* can serve as a species for monitoring, however, when observed ISI levels in *L. littorea* do not exceed natural environmental background levels it is still likely that 10% of other species might be affected by TBT contamination.

Both calculated ISI and PAF values are based on the spatial distribution of concentrations. This distribution is derived from TBT concentrations in sediment and SPM. One complicating factor lies in the fact that, in most water systems, periwinkles do not live near the sampled sediment (which is also used to derive the water concentrations), but considerably higher up, on the hard substrates where the macro algae they feed on grow. There might therefore be no direct relationship between TBT in sediment in harbours and ISI values in periwinkles. Instead, the periwinkles are probably predominantly exposed to TBT dissolved in the water and adsorbed to SPM circulating in the water. The fair correspondence between observed and calculated ISI values for *L. littorea* as presented in this study shows that sediment and SPM concentrations, combined with a realistic partition coefficient (K$_{oc}$), can be usefully applied to estimate effective concentrations in the water column.

With this relationship between predicted ISI in periwinkles and the PAF value, the ISI indicator might potentially not only serve as an indicator of TBT pollution but also to represent generic ecological health status. However, this relationship is useful only if many ISI measure-
ments are available for an area, covering the whole distribution of possible ISI values. The wide range of ISI levels observed in Vlissingen harbour (location 25) indicates that a high variation in ISI levels can exist within a single harbour.

For open waters, only two measured ISI values fell within the predicted ISI ranges (North Sea (C)) and Western Scheldt (G), while the rest of the observations were below the calculated ranges. This is presumably a consequence of the asymptotic minimum of 0.07 in Formula 1. Differences in local $K_{oc}$ values may have caused the predicted ISI levels in open waters to exceed the observed ISI values. In this study the $K_{oc}$ is based on measured concentrations in sediment and water from harbours. This is in line with the fact that the correspondence in harbours is much better. It might be possible to derive a dedicated $K_{oc}$ for open waters from the comparison of water and sediment concentrations in open waters, potentially improving the correspondence between predicted and measured ISI levels for these water systems. However, such data are not available. The possible over-estimation of water concentrations in open waters would also imply that the ecological risk values presented in this study have been over-estimated for open waters.

There is good correspondence between observed ISI levels and the predicted ISI ranges for each harbour. Since almost all observed ISI levels fell within the ranges of predicted ISI levels in open waters, it can be concluded that the spatial distribution of TBT concentrations is a good basis for determining predicted ISI levels, and therefore ecological risk.

Periwinkles were found in low abundance at several harbour locations: Delfzijl (location 1); Harlingen (location 8); Den Helder (location 14); IJmuiden (location 15); and Rijnmond (location 18). It is unclear from the observations alone whether this was caused by high levels of TBT or other stress factors (e.g. salinity, excessive sand transport, etc.). The WOE approach can help to indicate the likelihood of TBT causing this absence. High risk values (PAF > 20%) and maximum ISI levels have been predicted for Harlingen (location 8), Den Helder (location 14) and Rijnmond (location 18), so it is not unlikely that high TBT pollution caused the absence of periwinkles from these harbours.

Based on the evaluation of TBT effects on intersex development in the periwinkle *L. littorea*, and considering ecological health status as a criterion for decision-making, with reference to Table 3, LOEs were derived for *L. littorea*, representing the species used in the OSPAR monitoring guideline. The relationships between LOEs obtained from comparison of observed effects in the field aid the environmental assessment process. Table 3 brings together the objectives of the ecological health strategy for TBT substances and existing action by water managers.

**Table 3:** Combinations of LOE results and likely ecological health status in relation to TBT pollution.

<table>
<thead>
<tr>
<th>LOE – Chemistry</th>
<th>LOE – Toxicology</th>
<th>LOE – Ecology</th>
<th>Likely ecological health status in relation to TBT</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predicted risk; Predicted ISI</td>
<td>Predicted ISI</td>
<td>Observed ISI</td>
<td>Presence of gastropods at all locations</td>
<td>PAF &lt; 10%; ISI &lt; 0.3</td>
</tr>
<tr>
<td>PAF &lt; 10%; ISI &lt; 0.3</td>
<td>ISI &lt; 0.3</td>
<td>+</td>
<td>Good</td>
<td>No action</td>
</tr>
<tr>
<td>PAF &gt; 10%; ISI &gt; 0.3</td>
<td>ISI &gt; 0.3</td>
<td>+</td>
<td>Poor</td>
<td>Mitigating measures and monitoring of recovery</td>
</tr>
<tr>
<td>PAF &gt; 10%; ISI &gt; 0.3</td>
<td>ISI &gt; 0.3</td>
<td>-</td>
<td>Poor</td>
<td>Mitigating measures and monitoring of recovery</td>
</tr>
</tbody>
</table>

LOE = Integrated ecological risk assessment is based on multiple Lines Of Evidence
As gastropods are the most sensitive species to TBT, few of these species are likely to be present in a TBT-contaminated harbour environment. The consequences of the absence of this group for the functioning of the system should be evaluated. Probabilistic risk assessment based on chemical data might provide a generic ecosystem health indicator related to TBT exposure to improve our understanding of the impact of organotin pollution. The results of such an assessment should be validated in the field, in order to reveal the actual field status. Given the relationships presented, observations with ISI as a specific indicator could serve to validate the generic assessment. Inspection of location-specific conditions alongside generic risk prognosis improves the assessment of impacts related to TBT exposure. This study provides evidence of the poor ecological health status of some Dutch harbours as a result of TBT pollution and promotes the use of integrated approaches like the WOE to help practitioners improve ecological impact assessment.

Conclusion

In this study an integrated ecological risk assessment including three LOEs has been applied to assess the risk from TBT pollution in coastal and harbour environments. Only two measured ISI values for coastal environments fell within the predicted ISI ranges (North Sea and Western Scheldt), while the rest of the observations were below the calculated ranges. In harbours there is good correspondence between observed ISI levels and the predicted ISI ranges for each harbour. This study provides evidence of the poor health status of some Dutch harbours as a result of TBT pollution and promotes the use of integrated approaches like the WOE to help practitioners improve ecological impact assessment.

Acknowledgements

We would like to acknowledge the valuable contribution of the laboratory personnel at the Directorate-General of Public Works and Water Management’s (RWS) National Institute for Coastal and Marine Management (RIKZ) for the TBT analyses. The authors wish to express their gratitude to Johan Jol (IMARES) for his invaluable support in conducting the fieldwork. Ivonne Rietjens and Tinka Murk (WUR) are thanked for their critical review of a first version of the manuscript.

Supplementary data

Derivation of a representative $K_{oc}$ for Dutch waters

The measured exposure concentrations from the field survey are used as a base for a probabilistic risk assessment. In such an assessment the exposure is presented as an exposure distribution. In this study the exposure distribution presents the spatial variation in the concentrations measured in e.g. a specific harbour or for all harbours. The exposure distribution can be compared to the species sensitivity distribution (SSD) for TBT. The SSD represents the average sensitivity of species and the variation among species. As the effect data of TBT, on which the SSD is based, is derived from concentrations in water it was necessary to recalculate all exposure
concentrations to a concentration in the water column. For open waters TBT concentrations in suspended matter and in sediment are available. Both data sets are used to calculate the distribution of water concentrations as there was no significant difference in the distribution of water concentrations based on suspended matter or sediment data alone.

The concentration in the water column can be derived from the corresponding concentration in the sediment, assuming thermo-dynamic partitioning equilibrium. This can only be done for the locations were the fraction organic carbon was measured. For the harmonisation of exposure data to water concentrations the formulas 1 and 2 taken from the European Commission Technical Guidance Document on Risk Assessment (EC, 2003) were applied.

\[
P_{EC_{marine\ sediment}} = \left( \frac{K_{p_{susp-water}}}{Rho_{susp}} \right) \times P_{EC_{seawater}} \times 1000
\]

in which:

- \( PEC_{seawater} \) = concentration in seawater [mg*l-1]
- \( K_{p_{susp-water}} \) = suspended matter-water partitioning coefficient [m3*m-3]
- \( Rho_{susp} \) = bulk density of suspended matter [kg*m-3]
- \( PEC_{sediment} \) = predicted environmental concentration in sediment [mg*kg-1]

The solid-water partition coefficient (Kp) in each compartment (sediment/suspended matter) can be calculated from the Koc value, and the fraction of organic carbon in the compartment (foc)) by predictions outlined in equation 2.

\[
K_p = f_{oc} \times K_{oc}
\]

in which:

- \( K_{oc} \) = partition coefficient organic carbon-water [l*kg-1]
- \( f_{oc} \) = weight fraction of organic carbon in compartment [kg*kg-1]
- \( K_p \) = partitioning coefficient solid-water [l*kg-1]

In literature Koc values are reported from 3.0 – 6.2 (Lepper, 2002). The selection of the Koc highly determinates the final results of the risk assessment. Normally the lowest Koc value will be selected to calculate the worst-case water concentrations. In this study it was decided to assess the value of the Koc by comparing the fitting the distribution of calculated water concentrations to the distribution measured water concentrations. This will result in a Koc value representative for the Dutch situation. Figure SI-1 presents the result of this fitting procedure. The estimated Koc value that fitted best to the Dutch situation is 5.34. This value is within the range of the reported Koc values from literature (Lepper, 2002). The value of 5.34 is used to calculate water concentrations from all sediment and suspended matter TBT concentrations. Figure SI-1 presents the estimated exposure distribution for harbours together with the distribution of measured water concentrations.

The Species Sensitivity Distribution for TBT-Sn

The Species Sensitivity Distribution (SSD) applied in this study is derived from data published by Lepper (2002). The SSD describes the average sensitivity of species and the variation among species (Posthuma et al., 2002). The SSD for TBT is based on toxicity data for 29 marine
and freshwater species representing seven taxonomic groups (algae, annelida, crustacea, echinodermata, insecta, mollusca, pisces). An overview of the species and effect data (NOECs) is presented in Table SI-1. The SSD is presented in figure SI-2. From Table SI-1 it seems that the marine species are more sensitive to TBT-Sn than fresh water species. However, this observation can also be a result of the different composition of the freshwater and marine dataset (Wheeler et al, 2001). In order to include a maximum of taxonomic groups in the SSD the marine and freshwater data were pooled.

![Figure SI-1: Distribution of the measured and calculated (from sediment) water concentrations for Dutch harbours, using a Koc value of 5.34.](image1)

![Figure SI-2: Species Sensitivity Distribution for TBT-Sn based on the data for 29 marine and freshwater species presented by Lepper (2002).](image2)
Assessing the ecological impact of organotin pollution in Dutch marine and brackish waters

Table SI-1: Ecotoxicity data for TBT for marine and freshwater species taken from Lepper (2002). The data is used to construct the SSD for TBT as presented in Figure SI-2

<table>
<thead>
<tr>
<th>Species</th>
<th>Taxonomic group</th>
<th>fresh water / salt water</th>
<th>ng/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucella lapillus</td>
<td>Mollusca</td>
<td>sw</td>
<td>1</td>
</tr>
<tr>
<td>Phyllospora comosa</td>
<td>Algae</td>
<td>sw</td>
<td>1</td>
</tr>
<tr>
<td>Mercenaria mercenaria</td>
<td>Mollusca</td>
<td>sw</td>
<td>2</td>
</tr>
<tr>
<td>Buccinum undatum</td>
<td>Mollusca</td>
<td>sw</td>
<td>3</td>
</tr>
<tr>
<td>Saccostrea commercialis</td>
<td>Mollusca</td>
<td>sw</td>
<td>5</td>
</tr>
<tr>
<td>Nucella Lima</td>
<td>Mollusca</td>
<td>sw</td>
<td>6</td>
</tr>
<tr>
<td>Eurytemora affinis</td>
<td>Crustacea</td>
<td>sw</td>
<td>10</td>
</tr>
<tr>
<td>Ophioderma brevispina</td>
<td>Echinodermata</td>
<td>sw</td>
<td>10</td>
</tr>
<tr>
<td>Crassostrea gigas</td>
<td>Mollusca</td>
<td>sw</td>
<td>11</td>
</tr>
<tr>
<td>Mytilus edulis</td>
<td>Mollusca</td>
<td>sw</td>
<td>31</td>
</tr>
<tr>
<td>Salmo gairdneri</td>
<td>Pisces</td>
<td>fw</td>
<td>40</td>
</tr>
<tr>
<td>Dunaliella tertiolecta</td>
<td>Algae</td>
<td>sw</td>
<td>50</td>
</tr>
<tr>
<td>Neanthes arenaceodentata</td>
<td>Annelida</td>
<td>sw</td>
<td>50</td>
</tr>
<tr>
<td>Poecilia reticula</td>
<td>Pisces</td>
<td>fw</td>
<td>57</td>
</tr>
<tr>
<td>Pacaenonetes pugio</td>
<td>Crustacea</td>
<td>sw</td>
<td>57</td>
</tr>
<tr>
<td>Oncorhynchus mykiss</td>
<td>Pisces</td>
<td>fw</td>
<td>63</td>
</tr>
<tr>
<td>Acanthomysis sculpa</td>
<td>Crustacea</td>
<td>sw</td>
<td>90</td>
</tr>
<tr>
<td>Acartia tonsa</td>
<td>Crustacea</td>
<td>sw</td>
<td>100</td>
</tr>
<tr>
<td>Gasterosteus aculeatus</td>
<td>Pisces</td>
<td>sw</td>
<td>100</td>
</tr>
<tr>
<td>Daphnia magna</td>
<td>Crustacea</td>
<td>fw</td>
<td>160</td>
</tr>
<tr>
<td>Pimephales promelas</td>
<td>Pisces</td>
<td>fw</td>
<td>170</td>
</tr>
<tr>
<td>Gammarus oceanicus</td>
<td>Crustacea</td>
<td>sw</td>
<td>300</td>
</tr>
<tr>
<td>Lymnea stagnalis</td>
<td>Mollusca</td>
<td>fw</td>
<td>320</td>
</tr>
<tr>
<td>Cyprinodon variegatus</td>
<td>Pisces</td>
<td>sw</td>
<td>340</td>
</tr>
<tr>
<td>Phoxinus phoxinus</td>
<td>Pisces</td>
<td>fw</td>
<td>462</td>
</tr>
<tr>
<td>Hexagenia sp.</td>
<td>Insecta</td>
<td>fw</td>
<td>500</td>
</tr>
<tr>
<td>Arenicola cristata</td>
<td>Annelida</td>
<td>sw</td>
<td>2500</td>
</tr>
<tr>
<td>Selenastrum capricornutum</td>
<td>Algae</td>
<td>fw</td>
<td>4000</td>
</tr>
<tr>
<td>Chlorella pyrenoidosa</td>
<td>Algae</td>
<td>fw</td>
<td>18000</td>
</tr>
</tbody>
</table>
Chapter 6

Rational application of bioassays in hazard, risk and impact assessments of dredged sediments

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To be submitted in Marine Pollution Bulletin

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Abstract: Given the potential environmental consequences of dumped dredged harbour sediments it is vital to establish the potential risks from exposure before disposal at sea. Currently, European legislation for disposal of contaminated sediments at sea is based on chemical analysis standards for a limited number of well known contaminants for which maximum acceptable concentrations, action levels (ALs), have been set. The present paper addresses the issue of the applicability of in vitro and in vivo bioassays for hazard, risk and local impact assessment of dredged polluted sediments to be disposed of at sea. It discusses how and to what extent selected bioassays can fill in the gaps left open by chemical analysis and the way in which the bioassays may contribute to the present licensing system for disposal. Three different purposes for application were distinguished: the most basic application (A) is a rapid determination of the hazard (potential toxicity) of dredged sediments which is then compared to ALs in a licensing system. As with chemical analysis on whole sediment extracts, the bioavailability of the chemicals is not taken into account. As in vivo assays with whole sediment usually are sensitive to matrix effects which is not the case with in vitro bioassays with sediment extracts. A selection of specific in vitro bioassays can be suitable fast and standardized additions for the licensing system. When the outcome of (A) does not convincingly demonstrate whether the sediment is clean enough or too polluted, further bioanalysis can help the decision making process (B). More aspects of the mostly unknown complex chemical mixtures are taken into account, including the bioavailability and chronic toxicity focusing on ecologically relevant endpoints. The ecotoxicological pressure imposed by the dredged sediments can be quantified as the potentially affected fraction (PAF) based on chemical or biological levels of contaminants in sediment or biota. To validate the predicted risk, the actual impact of dumped harbour sediments on local ecosystems (C) can be determined using a dedicated set of in vitro and in vivo bioassays as well as bioindicators selected based on the information obtained from (A) and (B) and on the characteristics of the local ecosystem. Conversely, the local sediment impact assessment (C) can direct fine-tuning of the selection of chemical and bioassay analyses and for setting safe levels in the licensing system. It is concluded that in vitro and in vivo bioassays and biological are useful tools in the process of hazard, ecotoxicological risk and impact assessment of dredged harbour sediments, provided they are consciously chosen and quality criteria for assay performance are achieved.

Introduction

Sedimentation of suspended particles in delta areas such as those in the Netherlands is a natural process that provides the primary food source for filter-feeding macro invertebrates (Wood and Armitage, 1997). However, in harbours and waterways, frequent removal of sediment is required to prevent obstruction of important shipping activities. An average of 26 million cubic meters of sediment has to be dredged every year (Table 1) from eight major tidal harbours along the Dutch coast (Fig. 1). Annually, over 90 million tonnes are disposed of at sea within the OSPAR maritime area (OSPAR, 2005) and hundreds of millions of tonnes are disposed of worldwide (Lauwert et al., 2004; Witt et al., 2004; Bolam et al., 2006). World wide, harbour sediments are contaminated with persistent organic pollutants (POPs), including polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), tributyltin (TBT), mineral oil, and toxic metals like mercury, and several other sometimes unidentified chemicals which pose a hazard for the receiving marine systems (Stronkhorst and van Hattum, 2003). Given the potential environmental consequences of dumped dredged harbour sediments, it is vital to establish the potential risks before disposal at sea.
Therefore, minimum quality criteria are set for harbour sediments to be disposed of at sea, to ensure that the chemical impact on the receiving environments is zero or acceptably low (Stronkhorst et al., 2003b; Lauwert et al., 2006). Currently, legislation for disposal of contaminated sediments at sea is based on chemical analytical standards for a limited number of well known contaminants (OSPAR, 2004; Alvarez-Guerra et al., 2007b) for which maximum acceptable concentrations, Action Levels (ALs), have been set. This approach, however, focuses on local acute toxic effects which are unlikely to occur because of the major dilution of the chemicals in the water phase of marine and estuarine environments during disposal. Actually, at the disposal location, the physical covering of the local benthic ecosystem with meters of dredged sediment often poses a much greater acute threat than toxic chemicals (Stronkhorst et al., 2003a; Bolam et al, 2006). On the other hand, several of the sediment-associated, persistent, bioaccumulating and toxic (PBT) chemicals known to induce chronic sublethal effects are bound to the small sediment particles and transported to locations far away from the original dumping site (Sonneveldt and Laane, 2001). Given the potential chronic environmental consequences of these PBT chemicals and their bioaccumulation in the food chain, it is vital to improve the assessment of the full environmental risk from contaminants in disposed dredged material, including the risk for chronic effects at locations at a long distance away from the disposal site.

A number of shortcomings are associated with the current chemical analytical approach. No ALs exist for more recently identified contaminants found in sediments, including priority substances such as polybrominated diphenyl ethers (PBDEs), perfluorinated chemicals (PFCs such as perfluorooctane PFOS and perfluorooctanoic acid PFOA) and phthalates (EU, 2006). In addition, dredged sediments with concentrations of contaminants below the individual lower ALs are dumped in North Sea coastal waters, although potential additive toxicity of the identified and unidentified chemicals present is not known. On the other hand, if levels exceed the upper limit of ALs, the dredged material has to be stored in costly repositories, although exceedance of the ALs does not necessarily mean that upon disposal environmental damage will occur.

Table 1: Yearly volumes of dredged material for disposal at sea for different Dutch harbours and the % of dredged material that exceeds the action limit (AL) for disposal at sea.

<table>
<thead>
<tr>
<th>Harbour location</th>
<th>Number of harbour sections</th>
<th>Harbour area [m²]</th>
<th>Yearly average volume dredged [m³]</th>
<th>Yearly average volume dredged as dw (kg/dw)</th>
<th>% Exceeding AL</th>
<th>Samples analyzed (1999-2005)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delfzijl</td>
<td>11</td>
<td>2 074 729</td>
<td>1 863 175</td>
<td>1 453 276 500</td>
<td>1.9</td>
<td>66</td>
</tr>
<tr>
<td>Harlingen</td>
<td>21</td>
<td>195 220</td>
<td>1 477 919</td>
<td>1 152 776 820</td>
<td>1.8</td>
<td>48</td>
</tr>
<tr>
<td>Den Helder</td>
<td>15</td>
<td>506 495</td>
<td>955 671</td>
<td>745 423 380</td>
<td>0.7</td>
<td>42</td>
</tr>
<tr>
<td>IJmuiden</td>
<td>30</td>
<td>208 798</td>
<td>2 908 735</td>
<td>2 268 813 300</td>
<td>2.1</td>
<td>128</td>
</tr>
<tr>
<td>Scheveningen</td>
<td>9</td>
<td>191 322</td>
<td>290 000</td>
<td>226 200 000</td>
<td>1.2</td>
<td>33</td>
</tr>
<tr>
<td>Rotterdam</td>
<td>234</td>
<td>62 664 047</td>
<td>17 037 650</td>
<td>13 289 367 000</td>
<td>11.7</td>
<td>1030</td>
</tr>
<tr>
<td>Rijnmond</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vlissingen</td>
<td>11</td>
<td>4 451 785</td>
<td>772 156</td>
<td>602 281 680</td>
<td>0.4</td>
<td>15</td>
</tr>
<tr>
<td>Eemshaven</td>
<td>5</td>
<td>1 794 066</td>
<td>832 236</td>
<td>649 144 080</td>
<td>0.3</td>
<td>28</td>
</tr>
<tr>
<td>Total</td>
<td>331</td>
<td>72 086 462</td>
<td>26 137 542</td>
<td>20 387 282 760</td>
<td>-</td>
<td>1390</td>
</tr>
</tbody>
</table>
To prevent unexpected environmental damage but also unnecessary costs, policy- and decision-makers must be able to characterize the real environmental risks of the disposal of dredged sediments at sea. For this purpose, it was suggested to include bioassays in the decision making for disposal. Bioassays in this context are biological assays to determine the toxic potency of whole sediment or sediment extracts. The main added value expected from bioassays is the detection of yet unknown chemicals and mixture effects. Bioassays can be performed with whole animals (*in vivo*) or with isolated parts (*in vitro*) such as cells or enzymes. *In vivo* bioassays with full sediment are expected to take the bioavailability of the chemicals into account (Maas and Van den Heuvel-Greve, 2005). Further, the possible local impact of dumping dredged sediments can be monitored. In addition to application of bioassays to the local sediment, also local biological indicators can be used. These bioindicators are local species or groups of species whose population or health status can be used to determine environmental integrity. Such organisms can be monitored for specific changes (biochemical, physiological, or behavioural) that may indicate a problem within their ecosystem. Bioindicators can tell us about the cumulative effects of different pollutants in the ecosystem, which physical and chemical testing cannot (Simeonov and Hassanien, 2009).
In the recent past, in the Netherlands a preliminary application of three bioassays to obtain realistic environmental quality standards for sediments was performed (Staatscourant, 2004), but resulted in the conclusion that these bioassays provided only limited additional value. This outcome might however be explained by the fact that the selection of these bioassays was based merely on their coincidental availability instead of on rational choices (Hattum and Kruseman, 2005). Therefore, the present paper addresses the applicability of in vitro and in vivo bioassays as tools in the process of developing rational sediment ALs to decide whether dredged harbour sediments can safely be disposed of at sea. The starting point is not a judgment of the suitability of available bioassays, but the desire to increase their functionality for achieving specific goals complement the current chemical based ALs in environmental hazard and risk assessment. When bioassays prove to be a useful complement instead of merely ballast, it will be important to introduce them in an international setting. Currently, the ALs for allowing disposal of dredged material at sea differs between countries. As a result, the costs of keeping the harbours and their waterways to the right depth can differ greatly between countries, which may distort the international competition between harbours (Bruinsma and Jonkeren, 2006). Therefore, it is important that bioassays can be applied internationally in a comparable way.

Previous application of bioassays in sediment toxicity testing

Many countries have set their own ALs for determining whether dredged material may be disposed of at sea based on determination of a number of conventional contaminants (OSPAR, 2004; Alvarez-Guerra et al., 2007b). The ALs described in Sediment Quality Guidelines (SQG) are based on the concentrations of a number of conventional contaminants in sediments (Long et al. 2000, 2006; US EPA 2003a, 2003b, 2003c, 2005, 2008; Alvarez-Guerra et al., 2007b). In 2004, for the first time in the Netherlands bioassays were included in hazard assessment for the licensing of disposal of dredged sediments. In the so-called Chemical Toxicity Test (CTT) for dredged sediments (Table 2A), the ALs for three bioassays were included (Staatscourant, 2004; Schipper and Schout, 2004). The three bioassays that were sufficiently developed to be included were an in vitro reporter gene assay which detects dioxin-like compounds by dioxin-receptor mediated luciferase gene expression (DR-Luc or DR-CALUX-assay, Aarts et al., 1995; Stronkhorst et al., 2002), an in vivo toxicity assay for growth and survival of the marine amphipod Corophium volutator (ASTM, 1992), and an in vitro assay for bacteria, the Microtox Solid Phase (SP)\textsuperscript{*} assay (Stronkhorst et al., 2003b). A great deal of effort was put into the evaluation of quality criteria for these bioassays as their outcome could influence important and costly management decisions for dredged harbour sediments. As for chemical analysis, bioassay specifications must include Good Laboratory Practices (GLP) in terms of selectivity, reproducibility, and relevance for the PBTs in harbour sediments (Schipper et al., 2001). The main problem when applying the bioassays for general toxicity testing on the whole sediments was the occurrence of false positives induced by matrix factors present in the samples. As a result, toxicity indicated by these bioassays was often not related to chemical contamination. Also the toxic fraction could not be determined (Stronkhorst, 2003c; Schipper and Klamer, 2006).

In the analyses (Schipper and Stronkhorst, 1999) confounding matrix factors such as sulphur, ammonium or lack of oxygen caused no toxicity. Only the in vitro bioassay using clean organic sediment extracts yielded reproducible outcomes for the total dioxin-like toxic potencies that could be explained by (elaborate, costly) chemical analyses. As can be seen in Table 2, the final CTT hazard assessment for the eight harbour locations (Fig. 1) mainly indicated exceedance of the ALs for the metals Zn, Pb, Hg, Cu, the PCBs and PAHs, the organo-metallic compound...
**Table 2:** Action levels (AL) applied in the Chemical Toxicity Test (CTT) system to judge whether dredged material from Dutch harbours can be disposed at sea. Underlined values exceeds the AL threshold. The three bioassays in the marked Box are evaluated in Table 3. Loswal Noord is the Reference station where harbour sediments are dumped.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Wadden Sea area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Min</td>
</tr>
<tr>
<td><strong>Corophium volutator</strong></td>
<td>Mortality %</td>
<td>50</td>
</tr>
<tr>
<td><strong>Microtox SP</strong></td>
<td>Biolum (1/EC50)</td>
<td>100</td>
</tr>
<tr>
<td><strong>DR-CALUX</strong></td>
<td>ng TEQ/kg d.w.</td>
<td>50</td>
</tr>
<tr>
<td><strong>Tributyltin (TBT)</strong></td>
<td>μg Sn/kg d.w.</td>
<td>100-250</td>
</tr>
<tr>
<td><strong>Copper (Cu)</strong></td>
<td>mg/kg d.w.</td>
<td>60</td>
</tr>
<tr>
<td><strong>Arsenic (As)</strong></td>
<td>mg/kg d.w.</td>
<td>29</td>
</tr>
<tr>
<td><strong>Cadmium (Cd)</strong></td>
<td>mg/kg d.w.</td>
<td>4</td>
</tr>
<tr>
<td><strong>Mercury (Hg)</strong></td>
<td>mg/kg d.w.</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Chromium (Cr)</strong></td>
<td>mg/kg d.w.</td>
<td>120</td>
</tr>
<tr>
<td><strong>Zinc (Zn)</strong></td>
<td>mg/kg d.w.</td>
<td>365</td>
</tr>
<tr>
<td><strong>Nickel (Ni)</strong></td>
<td>mg/kg d.w.</td>
<td>45</td>
</tr>
<tr>
<td><strong>Lead (Pb)</strong></td>
<td>mg/kg d.w.</td>
<td>110</td>
</tr>
<tr>
<td><strong>Sum 10-PAH’s</strong></td>
<td>mg/kg d.w.</td>
<td>8</td>
</tr>
<tr>
<td><strong>Hexachlorobenzene</strong></td>
<td>μg/kg d.w.</td>
<td>20</td>
</tr>
<tr>
<td><strong>Som DDT/DDD/DDE</strong></td>
<td>μg/kg d.w.</td>
<td>20</td>
</tr>
<tr>
<td><strong>Mineral oil C10-40</strong></td>
<td>mg/kg d.w.</td>
<td>1250</td>
</tr>
<tr>
<td><strong>Sum 7-PCB’s</strong></td>
<td>μg/kg d.w.</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 2: continued.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>North Sea area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortality %</td>
<td></td>
<td>(°) Mean (°) Min Med Max n</td>
</tr>
<tr>
<td>Corophium volutator</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Microtox SP</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>DR-CALUX</td>
<td>ng TEQ/kg d.w.</td>
<td>50</td>
</tr>
<tr>
<td>Tributyltin (TBT)</td>
<td>μg Sn/kg d.w.</td>
<td>100-250</td>
</tr>
<tr>
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<td>60</td>
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</tr>
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<td>110</td>
</tr>
<tr>
<td>Sum 10- PHA’s</td>
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<td>mg/kg d.w.</td>
<td>20</td>
</tr>
<tr>
<td>Mineral oil C10-40</td>
<td>mg/kg d.w.</td>
<td>1250</td>
</tr>
<tr>
<td>Sum 7- PCB’s</td>
<td>mg/kg d.w.</td>
<td>100</td>
</tr>
</tbody>
</table>

nd = not determined; < dl = < detection limit; (°) Staatscourant, 2004; (°) Akerman et al. (2004); (°) Ariese et al., (1999);
Rational application of bioassays in hazard, risk and impact assessments of dredged sediments

TBT, and the dioxin-equivalents (TEQs) based on the *in vitro* bioassay DR-Luc response (Table 2, Fig. 2). Altogether, the added value of the two bioassays that were included in the CTT for dredged sediments and were performed with whole sediment, was not convincing. They apparently mainly added false positives due to sensitivity to matrix effects (Stronkhorst et al., 2003b).

It is important to note that tests for chronic effects were not included, nor were *in vivo* toxicity experiments performed with sediment extracts. In addition to the CTT example discussed above, *in vivo* and *in vitro* bioassays have been successfully applied for sediment quality assessment in combination with chemical analyses in several specific studies. Examples are the development of European otter (*Lutra lutra*)-based quality objectives for PCBs in sediment (Smit et al., 1996) and the Dutch national survey on estrogenic compounds (Vethaak et al. 2005; 2006). In USA, the “Green book” (US EPA/ACE, 1991) and Inland Testing Manual (US EPA, 1998) provide guidance for the testing and evaluation of the disposal of dredged material. However, because of experienced shortcomings in the applicability of the tools and models, further research is being conducted to increase the effectiveness of sediment management (Muñns et al., 2002; Chapman et al., 2002). Useful lessons can be learned from these studies applying bioassays and bioindicators, and we identified basic principles to consider when deciding whether to include bioassays for the hazard and risk assessment of dredged harbour sediments. This paper aims at presenting a comprehensive approach for the application of both *in vitro* and *in vivo* bioassays for hazard assessment, ecotoxicological risk assessment, and location-specific impact assessment for dredged harbour sediment disposal. We will also pay attention to quality requirements, extraction of the bioavailable fraction of sediment contaminants, sample preparation, and possibly relevant bio-activation of parent chemicals. We will illustrate our discussion with results obtained for dioxin-like compounds and TBT in sediments.

**Application of bioassays for hazard, risk and impact assessment of dredged harbour sediments**

When judging the applicability of bioassays in the management of dredged harbour sediment it is important to i) specify the goals of protecting of human and ecological health, ii) define the required information to base management decisions and iii) determine the quality of bioassay performance to be included. Bioassays could be applied for three specific purposes (Figure 3). The most basic purpose is the rapid determination of the hazard (potential toxicity) of dredged sediments as a worst-case estimation in a licensing system. The chemical levels or toxic potencies are compared with ALs defined in terms of SQGs developed to protect both human and ecological health (Box A, Figure 3). When the result of this analysis is not sufficiently conclusive, additional testing has to be performed (Box B, Figure 3) in order to enable an adequate risk assessment. This testing and assessment may take into account the bioavailability of the toxic chemicals in the dredged sediment and possible toxic effects on ecologically relevant endpoints and biomarker responses related to potentially long term sub-lethal effects on human and ecological health. A third testing strategy (Box C, Figure 3) might be required to determine the actual impact of dumped harbour sediments on local ecosystems and accumulation of persistent toxic chemicals in human and feral food chains. To this end, bioassays can be performed with sediment extracts and whole sediment in addition to chemical analysis. Also, *in situ* bioassays and local impact assessments should be performed on the relevant locations, including sampling in a gradient from the dumping site or over time following disposal. The characteristics of suitable *in vitro* and *in vivo* bioassays in combination with other analyses for the three purposes (Boxes
A, B and C, Figure 3) are discussed below, and examples will be given from already published or newly collected data for dioxin-like compounds and TBT.

**Figure 2:** Percentage of sediment samples from harbours and navigation channels along the Dutch coast that exceed the action levels (ALs) of the Chemical Toxicity Test (source: data from RWS RIKZ, period 2000-2005) (n=1276).

**Figure 3:** Application of *in vitro* and *in vivo* bioassays for: Box A) rapid screening for licensing of disposal of the dredged sediments; Box B) additional testing for further risk assessment when Box A was not conclusive; Box C) site-specific determination of the impact of dumping of dredged harbour sediments. Based on Boxes B and C, the sediment quality guidelines (SQGs) and action levels (ALs) for the licensing system (Box A), results can be used for fine-tuning the selection of chemical and biological analyses, and ALs. The symbols in the Figure; (X) an endpoint had a poor response; (V) indicates a good response (e.a. no exceedances); (?) shows a non-definitive finding.
Box A) Bioassays for hazard assessment of dredged harbour sediments

The aim of the hazard assessment of dredged harbour sediments is to establish whether chemicals are present at concentrations that are too toxic to be disposed of in the environment. Ideally, this should not only protect the local environment at the dumping site from acute toxic effects, but also prevent long-term effects at locations to which the fine sediment particles may travel. Currently, the decision whether to dispose of or store dredged sediment is based on the analysis of well-known key toxicants: metals, TBT, PCBs and PAHs (Table 2). Dredged material exceeding ALs for these chemicals are not eligible for disposal (DelValls et al., 2004). Not all relevant contaminants, however, are included in the analyses (Alvarez-Guerra et al., 2007a), in part because it is too costly to determine them all, but also because unidentified contaminants present in concentrations below the limit of detection cannot be measured. Examples are dioxins and dioxin-like compounds, several classes of halogenated flame retardants, and perfluorinated compounds (PFCs). In addition, by using the SQGs, mixture toxicity is not included in the current regulations and guidelines for the disposal of dredged material in marine and estuarine waters (Schipper and Schout, 2004; Alvarez-Guerra et al., 2007a). The question is to what extent bioanalysis with in vitro and in vivo bioassays can contribute to fill this gap in hazard assessment in a cost-effective way. These bioassays could be mechanism-specific; for example, detecting dioxin-like, estrogenic or androgenic compounds in in vitro reporter gene assays (Schipper et al., 2009) or non-specific, detecting acute toxicity with in vitro or in vivo bioassays for general toxicity.

In a large survey with nearly 1400 dredged sediment samples from locations in eight Dutch harbours (Table 2, Figure 1), the CTT test battery was applied, including two bioassays for general toxicity. The outcome of the in vivo bioassay with C. volutator and the in vitro Microtox Solid Phase (SP)® assay did not show a clear relationship with the toxic chemicals detected (table 2) (Schipper and Schout, 2004). As can be seen in Table 3, toxicity indicated by C. volutator is related in up to 29% of the cases to confounding matrix effects. Each confounder (e.g., ammonium) has an AL and if that AL is exceeded and toxicity was observed (Postma et al, 2002; Ringwood et al., 1996), it was considered a false positive. No relationship was found between the bioassay responses with whole sediment and the chemically indicated pollution gradient. The toxicity of the sediment matrix itself appeared to be more important for the responses than the chemical contaminants present in the samples, thus preventing detection of real toxic effects. For the C. volutator assay, this is in accordance with results from earlier studies, showing that confounding factors such as concentration of ammonium, pH, salinity and oxygen level in the sediment have a major impact on the toxic response. This seriously hampers the use of the bioassay in the toxicological hazard assessment of the dredged material (Kohn et al., 1994; Stronkhorst et al., 1996; Postma et al., 2002; Lahr et al., 2003; Van Hattum and Kruseman, 2005). In up to 84% of the applications of the Microtox Solid Phase (SP)® assay, the cellular response could be related to matrix effects such as ammonium or sulphide concentration, oxygen level or grain size (Table 3).

Although confounding factors could in theory induce multi-stress responses at local disposal sites, this is probably not relevant for the judgment of dredged sediment. This is because, after disposal, the matrix conditions will be different and not relevant anymore. In the CTT test, only the bioassay applied for testing the sediment extracts, namely the reporter gene assay for dioxin-like compounds (DR-Luc or DR-CALUX assay) yielded consistently useful results. This in vitro bioassay with transgenic H4IIE rat hepatoma cells responds with luciferase production upon activation of the arylhydrocarbon (Ah) receptor (Aarts et al., 1995). When matrix factors
Table 3: Examples of *in vitro* and *in vivo* bioassays for which standard operating procedures have been developed (Schipper and Stronkhorst 1999) and studied for their applicability in the licensing system for disposal of dredged sediment (own results) or were evaluated in other studies (e.g. ER-CALUX).

<table>
<thead>
<tr>
<th>Bioassay</th>
<th>Detected effects</th>
<th>Endpoint</th>
<th>Levels of indication</th>
<th>Known active toxic compounds</th>
<th>Performance characteristics (sensitivity detection limit; variability; reproducibility)</th>
<th>Confounding factors</th>
<th>Percentage false positive data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corophium \textit{volutator}</td>
<td>general toxicity</td>
<td>mortality</td>
<td>individual</td>
<td>Zn, Cr, Cu, Cd, Hg, Ni, nonylphenol, TBT, oxides, propanone, ammonia, pH, salinity, oxygen, sulfur</td>
<td>45% ; 54 %; 11 – 136%</td>
<td>ammonium, pH, salinity, oxygen, sulfur</td>
<td>0 – 29%</td>
</tr>
<tr>
<td>Microtox SP</td>
<td>general toxicity</td>
<td>growth, metabolism</td>
<td>Cellular</td>
<td>see above, all directly toxic compounds</td>
<td>20% ; 36% ; 20 – 45%</td>
<td>ammonium, pH, O2, sulfide, grain size</td>
<td>6 – 84%</td>
</tr>
<tr>
<td>DR-Luc</td>
<td>dioxin like compounds</td>
<td>TCDD-equivalents (TEQ)</td>
<td>molecular, indicative of chronic physiological effects</td>
<td>2,3,7,8,-TCDD; PCDD’s; PCN’s; PCDF’s; PCB’s; PBDE’s; PBB’s</td>
<td>0.2 ng TEQ/kg, 30 – 68%</td>
<td>None, provided good clean-up</td>
<td>0%</td>
</tr>
<tr>
<td>ER-Luc</td>
<td>Estrogenic compounds</td>
<td>activation Estrogen receptor</td>
<td>molecular, indicative of chronic physiological effects</td>
<td>17β-estradiol, xenoestrogens (^{(1,2)})</td>
<td>6.8 ng EEQ/kg, 5 – 15%</td>
<td>Natural estrogenic compounds</td>
<td>nd</td>
</tr>
</tbody>
</table>

\(^{(1)}\) Legler et al., 1999; \(^{(2)}\) Murk et al, 2002; \(^{(3)}\) Legler et al. 2003

\(\text{nd} = \text{not determined}\)
are removed with the destructive methods for cleaning sediment extracts, \textit{in vitro} bioanalysis with the DR-Luc assay was shown to quantify dioxin-like compounds in a reproducible way (Murk et al., 1996, 1998; Stronkhorst et al., 2002; Besselink et al., 2004). It is important to note that without a thorough clean-up, this bioassay will also generate false positives as natural compounds and PAHs can transiently induce the DR-Luc assay (Hamers et al., 2000; Hurst et al., 2004, Houtman et al., 2004). In that case, the exposure period also will strongly influence the response. After 6 hours of exposure, mainly the PAHs induce luciferase production, after 48 hours only the persistent dioxin-like compounds. This occurs because most PAHs are easily degraded, and the exact time dependency for degradation is related to PAH load, composition and the biodegradation capacity of the cells (Hamers et al., 2000). As dioxin-like compounds induce a fundamentally different toxic profile than PAHs, their response should not be included in the DR-Luc bioassay response for dioxin-like compounds. PAHs already are included in the initial chemical ALs (Table 2).

The DR-Luc bioassay with the destructive clean-up proved to be useful for quantification of low and high levels of dioxin-like contaminants (further referred to as ‘dioxins’) in dredged material. As with chemical analysis on total sediment extracts, the bioavailability of the chemicals is not taken into account in the Box A hazard assessment. High concentrations of dioxins were present in dredged material from the transect of Rotterdam harbour (Table 2). The dioxin-equivalent values (TEQs) determined for these sediments ranged from of 0.1 to 689 ng TEQ/kg dw with a median value of 27 ng TEQ/kg dw. The AL for the CTT-test is 50 ng TEQ/kg dw based on i) the chemical SQG for dioxins (25 ng TEQ/kg dw) and ii) an assumed dilution factor for the dispersion of dredged material in coastal waters (Stronkhorst et al., 2002). Of the TEQs determined with bioanalysis, with full clean-up 40-50% could be attributed to dioxins, polychlorinated dibenzo-dioxins (PCDDs) and polychlorinated dibenzo-furans (PCDFs), and PCBs. (Stronkhorst et al., 2002; Klamer et al., 2005). As dioxins are key contaminants in several harbour sediments, chemical analysis of these compounds is more expensive and time-consuming, and not all compounds contributing to the total TEQ can be chemically analyzed because of their low levels or lack of chemical standards. It is concluded that for this group of compounds \textit{in vitro} bioanalysis can contribute significantly and in a meaningful way to the licensing system of dredged harbour sediments. Bioavailability of the compounds is not relevant for Box A assessment instead a worst-case approach is considered more appropriate here.

Comparable reporter gene bioassays could be applied for other endpoints, such as for estrogenic activity (Legler et al., 2002), androgenic activity (Blankvoort et al., 2005), or thyroid hormone activity (Freitas et al., 2009). However, an important difference between toxicological analysis of hormone-like activity with that of dioxin-like activity, is that a destructive clean-up of the sediment extract will result in losing hormones and hormone-like compounds whereas several naturally occurring hormone-like constituents may be present in the sediments (Houtman et al., 2004). This poses the risk of either false negatives if clean-up is too destructive, or false positive results when; for example, natural phyto-estrogens are not removed properly. As a result, unexplained estrogenic activity can be found in sediment samples from reference as well as harbour locations (Legler et al., 2003; Houtman et al., 2006; Schipper et al., 2009). This effect could be reduced by separating the non-polar fraction of the extract containing the well-known estrogenic compounds from the polar fraction containing the unidentified estrogenic compounds (Legler et al., 2003) that were able to induce estrogenic activity \textit{in vivo} (Legler et al., 2006a). In dredged harbour sediments, estrogenic compounds do not seem to be relevant contaminants from a risk management point of view. The levels of estrogenicity (EEQ) in harbour sediments are in the range of average river sediments namely 1.3 pmol EEQ/g dw (Houtman et al., 2004) to
38.4 pmol EEQ/g dw (Schipper et al., 2009). In addition, most estrogenic compounds are readily degradable, and during transport to sedimentation areas such as the Wadden Sea (Stronkhorst et al., 2002), extensive dilution and metabolism occur. Therefore, inclusion of bioassays for the estrogenic potency of harbour sediments may not be relevant for the licensing system for disposal of dredged sediments in marine environments.

Due to the extensive dilution upon disposal, bioassays for compounds that display toxicity only at relatively high concentrations, such as narcotics, would not be included in a Box A application, unless a local impact assessment at the dumping site (Box C, Figure 3) indicated otherwise.

As far as it is known, near industrialized areas, harbour sediments contain large amounts of trace metals and organometals (Table 2). The most important of these are addressed by the standard chemical analyses in the licensing system (Alvarez-Guerra et al., 2007a). It can not be dismissed that acutely toxic organic compounds are also present in the sediments. These compounds could be detected with in vitro or in vivo assays using organic sediment extracts, as developed for rain water extracts (Hamers et al., 2001) and later applied to sediment extracts as well (Houtman et al., 2004). Therefore, for testing in the licensing system (Figure 3, Box A), application of an in vitro assay for general toxicity with sediment extracts could be useful. This alternate approach could be taken instead of using the less successful in vitro Microtox SP® test with whole sediment initially applied within the CTT approach as described above. As the nature of the sample preparation will strongly affect the outcome of the bioassays for general toxicity, it is vital that this issue be taken into account. For all in vitro tests sulphur has to be removed from the extract (Murk et al., 1998; Houtman et al., 2004), for in vivo tests this is not really important (Gutleb et al., 2007a, b). For ethical and practical reasons in vitro tests may be preferred compared to in vivo tests. When the outcome of all tests neither convincingly indicates the sediment is sufficiently clean nor that it is too polluted to be disposed of at sea, additional testing including in vivo bioassays are needed for further risk assessment (Box B).

**Quality control when applying bioassays in a licensing system**

In a licensing system, not only rapid determination but also high standards of quality assurance and quality control (QA/QC) are needed to guarantee the quality of chemical and biological data on which the assessment is to be based (US EPA, 1995; Stronkhorst et al., 2004; Schipper and Klamer, 2006). In practice, misinterpretation and automatic disqualification of the dredged material must be avoided because of the potentially great economical consequences based on the outcome of the analyses (Alvarez-Guerra et al., 2007a). For the chemical analyses of traditional sediment toxic chemicals (Table 2) well developed Good Laboratory Practice (GLP) protocols exist. When in vitro bioassays are to be included in the licensing system, bioassay protocols and sample preparation methods must include GLP specifications in terms of selectivity, repeatability and reproducibility of the responses (ASTM, 1992; Schipper and Stronkhorst, 1999). With whole sediment bioassays false-positives occur too often because of high variability in the responses (Stronkhorst et al., 2003b) and the unpredictable interference of the sediment matrix with the bioassay responses. Therefore, extensive validation is required before whole sediment tests can be accepted for application in a licensing system. As bioavailability is not relevant for Box A hazard assessment of dredged material, in vitro or in vivo bioassays for general toxicity (i.e., non-dioxin) with sediment extracts from which matrix factors have been removed could be worth inclusion in the licensing system. Examples of in vitro bioassays for general toxicity are the microtiter method with bioluminescent bacteria (Hamers et al., 2001) or an assay with mammalian cells (e.g., measuring reduced cell proliferation with the resazurin method, Schriks et al.,...
Rational application of bioassays in hazard, risk and impact assessments of dredged sediments

The \textit{in vitro} bioassay for dioxins with cleaned sediment extracts (DR-CALUX) proved to comply with the QA/QC criteria needed to guarantee the reliability of data in an inter- and intra-laboratory study (Besselink et al., 2004). The chemical stability of dioxins makes it possible to apply destructive clean-up procedures which remove all matrix factors. Sample extraction and cleanup for other \textit{in vitro} bioassays for specific mechanisms of toxicity require further development to make sure that the chemicals of interest are not lost or unwanted chemicals included in the sediment extract to be tested. Table 4 summarizes possible bioassays that could be performed in addition to chemical analyses with the dredged sediment in a licensing system.

**Box B) Ecotoxicological risk assessment, using additional bioassays**

For specific situations, a full ecotoxicological risk assessment could be performed for disposal of dredged harbour sediments. For example, when there are indications for the need for additional toxicity testing or when the outcome of the basic testing (Box A) is not sufficiently

| **Table 4:** Summary of the uses of bioassays for hazard, risk and impact assessment of dredged harbour sediments to be disposed of at sea explained in Figure 3 (Boxes A, B and C). Chemical analyses are mentioned in italics. |

<table>
<thead>
<tr>
<th><strong>Box A</strong></th>
<th>Useful</th>
<th>Not useful for sediments to be disposed of</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Currently used for:</strong> licensing disposal harbour sediment rapid screening</td>
<td>\textit{In vitro} bioassay DR-Luc (purified extract) \textit{Chemical analyses (PAHs, PCBs, toxic metals, TBT) chemical anal.)}</td>
<td>\textit{In vivo} bioassays with cultured or wild species</td>
</tr>
<tr>
<td><strong>Possibly relevant to be added to data base</strong></td>
<td>\textit{In vitro} bioassay general toxicity (extract) \textit{In vitro} bioassay thyroid hormone disruption (extract) (incl. bioactivation)</td>
<td>\textit{In vitro} estrogenicity or androgenicity (extract) Chemicals that only are toxic in high concentration (narcotics, nanoparticles) Chemical analysis of lipophillic POPs in water</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Box B</strong></th>
<th>Useful</th>
<th>Not useful for sediments to be disposed of</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Currently Advanced risk assessment harbour sediment before disposal</strong></td>
<td>\textit{In vivo} bioassays with cultured species &amp; ecologically relevant endpoints: growth Development, survival, reproduction PAF or msPAF</td>
<td>\textit{In vivo} bioassays with wild species</td>
</tr>
<tr>
<td><strong>To be added in cases of indications for relevance and bioavailability</strong></td>
<td>\textit{In vivo} bioassays with relevant exposure conditions, routes and endpoints Prolonged ELS and metamorphosis \textit{In vivo} bioassays with sediment extracts \textit{In vitro} bioassays specific mechanisms Internal effect levels Specific chemicals (e.g. PFACs, HBCDs, PBDEs)</td>
<td>ELS not longer than free feeding Chemicals that only are toxic in high concentration (narcotics, nanoparticles) Chemical analysis of lipophillic POPs in water</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Box C</strong></th>
<th>Useful</th>
<th>Not useful local sediments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Currently Local sediments impact assessment</strong></td>
<td>Bioaccumulation and biomagnifications Population decline of local organisms \textit{In vivo} bioassays with local sediment \textit{In vitro} bioassays with local sediment (extract) Bioindicators Biomarkers Bioavailability of compounds</td>
<td>Black Box gene arrays Nanoparticles POPs in water Narcotic effects</td>
</tr>
<tr>
<td><strong>To be added in cases of indications for relevance</strong></td>
<td>Specific genomics biomarkers \textit{In vitro} bioassays with bioactivation \textit{In vivo} bioassays with local sediment extracts Local PAF or msPAF &amp; validation Body burden toxic compound Pharmaceuticals</td>
<td></td>
</tr>
</tbody>
</table>
conclusive. For this ecotoxicological risk assessment, more specific in vivo and in vitro bioassays could be included and additional chemical analysis might have to be performed. The selection of the additional in vitro and in vivo bioassays to be applied should be based on rational arguments and not just depend on the availability of the assays. The most direct approach to determine the ecotoxicological risk of the disposal of dredged sediment is by testing these sediments with in vivo bioassays using marine species. This can yield an indication of the toxic potency of the mostly unknown chemical mixture, and can be performed with the whole sediment or sediment extracts. The advantage of testing the whole sediment is that bioavailability of the chemicals is taken into account. The disadvantage is that matrix effects such as ammonium or sulphur toxicity may influence the outcomes, unless a way is applied to remove this source of toxicity (Ferretti et al., 2000; Burgess et al., 2003). When testing extracts, matrix effects can be prevented much more easily, but the effects of bioavailability are lost. A third option, that includes bioavailability and avoids matrix effects, is to extract only the bioavailable fraction of the organic compounds using solid phase extraction methods such as Tenax or HPCD (hydroxypropyl-β-cyclodextrin) and to test this extract in the bioassays (Puglisi et al., 2007; Weert et al., 2008). The endpoints studied in the additional bioassays should ideally be ecologically relevant (e.g. survival, development, reproduction) and the tests preferably performed with cultured instead of harvested marine species to reduce the often-occurring high biological variation (Stronkhorst et al., 2003b) and prevent false positives due to additional stress. The latter has been shown to occur for heart urchins (Echinocardium cordatum) in which the variability of the bioassay outcomes have been reduced by about 50% when using cultured instead of harvested urchins (Schipper et al., 2008b). Further, several yet not applied in vitro bioassays are available that could serve to improve characterization of the toxic chemicals present in dredged harbour sediment (Houtman et al, 2006; Hamers et al., 2009; Freitas et al., 2009).

In vitro bioassays for specific mechanisms of toxicity could be included in Box B, based on the expected chemicals of interest, but also to indicate the presence of yet unknown toxic chemicals. Ecotoxicological risk assessments for dredged sediment can be based on analysis of the chemicals of interest compared to (no) effect levels from the literature. Most toxicity data have been observed for fresh water organisms, and it is worthwhile to study whether this is mainly due to differences in bioavailability, or that internal effect concentrations are in the same order of magnitude for marine and fresh water species. Complementary to chemical analyses, the toxic potencies of the sediments can be quantified using validated in vitro bioassays and the results compared to (no) effect levels in these bioassays with standard chemicals and/or mixtures. Based on the concentrations of selected chemicals in the dredged sediments and the (no) effect levels of these chemicals for several species, a species sensitivity distribution (SSD) may be constructed (Posthuma et al., 2002). From this SSD, a potentially affected fraction (PAF) of species can be calculated (Aldenberg and Slob, 1993; Aldenberg et al, 2002). This has the advantage compared to the Predicted Environmental Concentration (PEC)/Predicted No Effect Concentration (NEC) ratio approach that not only ‘risk’ (PEC/NEC>1) or ‘no-risk’ (PEC/NEC<1) for the most sensitive species is indicated but the likelihood of effects is predicted based on statistical analysis of all species tested (Posthuma et al., 2002). As the PAF includes the variation in sensitivity among all species tested, it gives a more ecologically relevant indication of the risk of dumping dredged sediment. Ideally, a multi-substances PAF (msPAF) for the most important contaminants can be constructed based on the principle of effect addition (Smit et al., 2008).

Very suitable for Box B applications are mechanism-specific in vitro bioassays which have been linked to long term in vivo toxic effects (Murk et al., 1996; Foekema et al., 2008) and even to chronic population effects when the relationship with long term health effects have been
defined (Box C, Figure 3). Once the relationship with the chronic in vivo effects has been established (Box C), the results of the in vitro bioassays with sediments can be scaled relative to the responses of sediments known to induce adverse effects and with responses of reference locations. In this way, the ‘so what’ question for a given in vitro response can be answered. This has been performed for dioxin-like, estrogenic, thyroid hormone-like, and genotoxic potencies in Dutch sediments (Murk et al., 1998; Vethaak et al., 2005; Houtman et al. 2004; Hamers et al., 2009). Table 4 indicates relevant in vitro bioassays for additional risk assessment (Box B, Figure 3) with methods covering additional toxicological mechanisms or groups of chemicals (e.g. AR-Luc, ER-Luc, TR-Luc).

Also, it is especially important when designing in vivo bioassays, to choose the right exposure routes, life stages and observation periods for chemicals known to induce chronic effects. For example, exposure of very young sole (Solea solea) larvae to dioxin-like PCB126 resulted in toxic effects at field relevant levels only when the observation period was prolonged (Foekema et al., 2008). When the assay was terminated at the start of the free feeding stage, according to common practice, few adverse effects were observed. The same response has been demonstrated with tadpoles (Gutleb et al., 2007b). Also, imposex in gastropods was initially difficult to demonstrate upon TBT exposure, because the animals initially were exposed as adults or juveniles instead of in the egg stage which is the most sensitive life stage for this effect (Mensink et al., 1996). Not only the relevant life stage, but also the exposure conditions for the animals should be taken into account, temperature could greatly influence both the bioavailability and the toxicity of chemicals. For example, paraffin with a melting point at 15°C is not toxic below that temperature (Huwer et al., 1997), while the sensitivity of Spisula subtruncata to sediment associated benzo-a-pyrene is much greater below 5ºC than above this temperature (Foekema et al., unpublished results).

Results obtained using additional bioassays in the more advanced ecological risk assessments (Box B) could eventually lead to the decision to include some new chemical analyses or bioassays in the rapid screening (Box A). This could improve detection of toxic chemicals that may currently be overlooked but appear to be relevant for dredged harbour sediments. It is important to stress that the new chemical analyses or bioassays to be included in the licensing system do not need to be identical to the assays that brought forward the yet unidentified ecotoxicological issue in the additional testing procedures (Box B). Tests to be added for a certain endpoint need to be standardised, inexpensive, quick, high throughput, and validated for that specific endpoint. Such tests may still need to be developed for inclusion in the licensing system.

**Box C) Contribution of bioassays and bioindicators to local impact assessment**

The impact of dumped dredged sediments on the receiving ecosystem also depends on local conditions and existing contamination. So, although the hazard and risk assessment of the dredged sediments can be the same (i.e., Figure 3, Boxes A+B), the local impact assessment is site-specific. Therefore, management of sediments and dredged material will involve application of chemical analyses and bioassays for the licensing of disposal (Figure 3, Box A) and ecotoxicological risk assessment to identify effects not (yet) included in the standard testing (Figure 3, Box B). In addition, the assessment will also include bioassays and bioindicators to evaluate the original local sediment quality and changes over time or in a gradient from the dumping site after disposal (Figure 3, Box C). The endpoints to test for in this last objective will depend partially on the results obtained from Boxes A and B. It is logical to apply the bioassays that gave positive responses with the dredged sediment to the receiving sediments as well. For example, in a study...
with disposed harbour sediments known to contain dioxins, the changes in dioxin-equivalents in sediment extracts were studied after dumping over time and in an gradient. In addition, in locally collected starfish (*Asterias rubens*), the cytochrome P450 activity was determined (as EROD activity) (Stronkhorst et al., 2003a). Similarly, when increased TBT-levels are expected, the focus will be on indicators for imposex in gastropoda, in addition to the chemical analyses of TBT in the sediment and in gastropods (Schipper et al., 2008a). Table 4 summarizes possible bioassays and indicators that could be performed in addition to the analyses conducted with the dredged sediment before disposal, in order to obtain a good sediment toxicity profile (Figure 3, Box B). Further, bioassays and indicators can provide an adequate estimate of the original local sediment quality and changes over time or in a gradient from the dumping site after disposal (Figure 3, Box C).

In impact assessment studies (Figure 3, Box C), the causal relationship between exposure and effects in situations with multiple stressors that can impair the local ecosystem can be revealed by applying biomarkers. The exposure should ideally include internal dosage levels to be able to link to internal effect levels in laboratory bioassays with sediments under very different conditions. The exposure, both in the sediment and in the animals can be determined using chemical analyses, but also by bioanalysis applying *in vitro* bioassays for specific endpoints for quantification of toxic potencies as has been done with e.g. European otter (Murk et al., 1998), flounder and bream (Legler et al., 2006b), and sole (Foekema et al., 2008). Based on the outcome of the impact assessments from better studied locations, the value of bioassays for risk assessment can be validated by combining all available information (obtained in Boxes A, B and C type studies). The combination of mechanistic *in vitro* and *in vivo* bioassays with ecologically relevant endpoints in benthic fauna and bioaccumulation in biota (mammals, fish or snails) can help to answer the ‘so what’ question that ideally should be answered when presenting bioassay results. The assessment of the potentially affected fraction (PAF) of species by the toxic chemicals after dumping of the dredged material, based on SSDs constructed from *in vivo* bioassays with several species (Box B), can be validated as well as based on actual impact assessment studies. Of course, it is important to focus on the right endpoints in the ecosystem, that can be mechanistically related to the bioassays and toxic compounds analyzed. When biomarkers have been determined in the Box B bioassays as well, SSDs for biomarkers and *in vivo* SSDs can be linked enabling the prediction of the *in vivo* effects based on the biomarker responses in local organisms (Smit et al., 2009). This can provide practical tools for responsible authorities to obtain information about the ecotoxicological status of their local environment including the sediment. This is discussed further below for dioxins and TBT.

Furthermore, it is important to realize that after disposal of the dredged sediments along the coastal North Sea, most of the persistent contaminants are transported northward by the tidal current along the coast to locations far away from the original dumping site (Stronkhorst, 2003a). Here they become available for bioaccumulation and even biomagnifications both in the feral as well as the human food chain (Reichenberg and Mayer, 2006; Leonards et al., 2008). Therefore, effects at the sedimentation sites should be taken into account as well, especially for the risk assessment of PBT chemicals (see below). Results obtained from the ecotoxicological impact assessment (Box C) could eventually lead to the decision to include some additional chemical analyses or bioassays in the rapid screening (Box A) or advanced risk assessment (Box B). This would improve detection of toxic chemicals that may currently be overlooked but appear to be relevant for dredged harbour sediments, or be used to update the current chemical standards in SQG and ALs for already regulated contaminants.

In the next sections, the usefulness of the application of bioassays and bio-indicators for hazard, risk and impact assessment is illustrated based on experiences with dioxins and TBT.
**Example application of bioassay and biomarker analyses for TBT in Boxes A, B and C**

TBT is a very persistent compound with high direct toxicity as well as chronic endocrine toxicity, especially in gastropods (Mensink et al., 1996). It has been applied as a biocide in ship hull paints to prevent fouling. Currently, dredged material is tested for the presence of TBT, and when the sediment exceeds the AL for TBT, it is not eligible for disposal (Figure 3, Box A). In dredged material from most Dutch harbours, the TBT concentrations do not exceed the AL of 250 µg Sn/kg dw, but in some sections of the harbours these levels are far exceeded (Table 2). Along the estuarine and coastal North Sea, moderate to low TBT concentrations are present, so if sediment exceeding the AL were disposed of there the polluted dredged harbour sediments would possible increase the level of pollution. An exception is the Western Scheldt shipping route, where high concentrations are found. For rapid determination in the licensing system, when considering TBT, the chemical analysis is reliable and specific enough, and there is no additional role for bioassays to determine the hazard of the dredged material.

To assess the possible ecotoxicological risk of TBT present in sediments to a further extent (Figure 3, Box B), bioassays can play an additional role by taking bioavailability into account under relevant exposure conditions. It was mentioned above that in laboratory experiments, imposex only is induced in whelk that are exposed as eggs immediately after fertilization (Mensink et al., 1996). For TBT, an ecologically relevant endpoint is intersex in *L. littorea* and other gastropods, which can be included in a SSD (Schipper et al., 2008a). From this SSD, and based on the analyzed levels of TBT in the dredged sediment, the PAF can be estimated. This has been performed for the sediment levels analyzed for this study (Table 2) and by applying the SSD from Schipper et al., (2008a). The calculated PAF of species (Table 5) indicates risk in most harbours, and no-risk in the North Sea and Wadden Sea.

![Figure 4: Population development of *Nucella lapillus* in the period 1945-2007 along the Dutch coast, expressed relative to the average number of organisms present in the period 1900-1950 (set at 100%) (source: own data, RWS/Stichting Anemoon).](image)

In a local impact assessment (Box C), the predicted intersex (expressed as PAF) is compared with the actual occurring intersex (Schipper et al. 2008a). In the local sediments of the harbours, there is a good correlation between the predicted risk and the observed Intersex Index (ISI). No toxic effects were observed at the disposal sites for dredged sediments: Loswal Noord (Table 2) and in the North Sea (Stronkhorst et al., 2003a). However, along the Dutch coast, the
impact of TBT on gastropods is still evident and results show imposex (Table 5) in *N. lapillus* at stations kilometres away from TBT-treated shipping lines. The TBT-induced effects in sampled gastropods were inversely related to the distance from the disposal site (Santos et al., 2004) and correlated with the TBT levels in the suspended matter in the North Sea. This suggests that even when the dumped sediments do not exceed the ALs, an impact can be observed in the receiving environment when applying suitable effect biomarkers. It was revealed that effects of the low TBT levels can be predicted from the bioassay-based ecotoxicological risk assessment and that Box C effects on bio-indicators, such as observed Vas Deferens Sequence Index (VDSI) or ISI of the gastropods (Table 5) and ecological effects such gastropod population decline (Fig 4), could be related to the chemical sediment quality (Box A analysis) and the PAF (Box B analysis).

Table 5: Example application of process in Box B to evaluate the risk of TBT in Dutch sediments and biota. Ecotoxicological risk determined as potentially affected fraction (PAF %) based on the sediment levels interpolated in the SSD for TBT (from Schipper et al., 2008a), in the field observed intersex index (ISI) values in periwinkle *L. littorea* and *Vas Deferens Sequence Index* (VDSI) in dogwhelk *N. lapillus* as biomarkers for TBT-exposure (unpublished own results). Based on a combination of the criteria ISI<0.3, VDSI>1 and TBT-PAF<10%, the risk status of the sediments is classified (last column).

<table>
<thead>
<tr>
<th>Location</th>
<th>Mean [TBT] in sediment (ug/kg dw)</th>
<th>[TBT] in biota (ug/kg dw) in <em>L. littorea</em> (1) or Mytilus edulis (2)</th>
<th>Observed ISI in <em>L. littorea</em> (min-max)</th>
<th>Observed VDSI in <em>N. lapillus</em> (min-max)</th>
<th>TBT PAF (%) (1)</th>
<th>Risk status in relation to TBT pollution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ems-Dollard</td>
<td>31</td>
<td>nd</td>
<td>0.0</td>
<td>#</td>
<td>5.1</td>
<td>Good</td>
</tr>
<tr>
<td>Wadden Sea</td>
<td>28</td>
<td>92 (1)</td>
<td>0.0</td>
<td>#</td>
<td>4.8</td>
<td>Good</td>
</tr>
<tr>
<td>North Sea</td>
<td>40</td>
<td>134 (2)</td>
<td>0.0-0.1</td>
<td>1.0-2.5</td>
<td>6.2</td>
<td>Good</td>
</tr>
<tr>
<td>Western Scheldt</td>
<td>136</td>
<td>509 (2)</td>
<td>0.0-0.2</td>
<td>S</td>
<td>15.3</td>
<td>Poor</td>
</tr>
<tr>
<td>Eastern Scheldt</td>
<td>35</td>
<td>59 (2)</td>
<td>0.0</td>
<td>1.0-6.0</td>
<td>4.2</td>
<td>Good</td>
</tr>
<tr>
<td>Delfzijl</td>
<td>17.3</td>
<td>150 (1)</td>
<td>0.1</td>
<td>#</td>
<td>13.4</td>
<td>Probably poor</td>
</tr>
<tr>
<td>Eemshaven</td>
<td>25.7</td>
<td>67-328 (1)</td>
<td>0.04-0.1</td>
<td>#</td>
<td>3.5</td>
<td>Good</td>
</tr>
<tr>
<td>Harlingen</td>
<td>30.0</td>
<td>71-102 (1)</td>
<td>*</td>
<td>#</td>
<td>26.9</td>
<td>Poor</td>
</tr>
<tr>
<td>Den Helder</td>
<td>17.3</td>
<td>975-1446 (1)</td>
<td>2.9-3.0</td>
<td>#</td>
<td>22.6</td>
<td>Poor</td>
</tr>
<tr>
<td>IJmuiden</td>
<td>22.2</td>
<td>51-996 (1)</td>
<td>1.2-1.4</td>
<td>#</td>
<td>10.2</td>
<td>Poor</td>
</tr>
<tr>
<td>Schouveningen</td>
<td>728.0</td>
<td>134-338 (1)</td>
<td>0.5</td>
<td>S</td>
<td>23.7</td>
<td>Poor</td>
</tr>
<tr>
<td>Rotterdam</td>
<td>41.5</td>
<td>34-741 (1)</td>
<td>0.7</td>
<td>S</td>
<td>21.4</td>
<td>Poor</td>
</tr>
<tr>
<td>Vlissingen</td>
<td>24.2</td>
<td>465-4062 (2)</td>
<td>0.5-2.6</td>
<td>S</td>
<td>18.7</td>
<td>Poor</td>
</tr>
<tr>
<td>Terneuzen</td>
<td>6.6</td>
<td>nd</td>
<td>nd</td>
<td>S</td>
<td>11.8</td>
<td>Poor</td>
</tr>
</tbody>
</table>

nd=not determined
* =no periwinkles observed in habitat
# =no dogwhelk habitat, species never observed before 1950
$ =no dogwhelk reproduction observed
(1) Schipper et al. (2008a); field collected periwinkles are from one location for an exposure period of approximately 2-3 year (min-max)
(2) Belfroid and Van der Hoeven (2006); reference mussels transplanted from one location for an exposure period of six weeks (mean(sd))

Since 1990, the use of TBT antifouling paint on ships less than 25 m in length is prohibited. In 1998, several of the EU member states proposed an international ban of TBT and since 2008 world wide all organotin compounds in antifouling paints are prohibited (Gipperth, 2009). As a result, the TBT-levels in coastal waters and in sediments are steadily decreasing (Ruiz et al., 2008; Choi et al., 2009). In the North Sea, the ecotoxicological risk of TBT from disposal of dredged harbour sediment now is reduced to a PAF<6.2% (Table 5). In the Eastern Scheldt,
biomonitoring of the abundance of the gastropod *N. lapillus* reveals a steady recovery of the populations since 1997 which is not yet occurring in the Western Scheldt (Fig 4).

Exposure modeling has indicated that TBT from dredged material contributes a very low amount to the total TBT levels in the North Sea sediment. The bulk is directly related to ship activity (about 93%) with minor contributions from river input (Van Hattum et al., 2002; Van Gils and Friocourt, 2008). TBT levels in the Dutch Wadden Sea; however, where sedimentation of the smallest particles takes place (Stronkhorst et al., 2003a) were included in the fate modeling. It is advisable to study the TBT impact over time in the Wadden Sea applying the same set of analyses. This could indicate that the ALs for TBT in disposed sediments need to be adjusted and is a good illustration of how Box C analysis might ultimately affect Box A analysis for the licensing system of dredged harbour material.

**Example of applications of bioassay and biomarker analysis for dioxins in Boxes A, B and C**

Dioxins are very persistent organic pollutants that are present in harbours as by-products of incomplete combustion and industrial processes (Besselink et al., 2004). Dioxins in sediments are not acutely toxic, but are known to induce developmental, reproductive and immune toxicity after chronic exposure at low levels. In the licensing system for qualifying dredged material for

<table>
<thead>
<tr>
<th>Location</th>
<th>Years of sampling</th>
<th>ng TEQ/kg dw (min-max)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>North Sea</td>
<td>1996</td>
<td>2-10</td>
<td>Stronkhorst et al. (2003a)</td>
</tr>
<tr>
<td>Western Scheldt estuary outflow</td>
<td>2000</td>
<td>15</td>
<td>Klamer et al. (2005)</td>
</tr>
<tr>
<td>Suspended matter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Western Scheldt</td>
<td>2003</td>
<td>20-40</td>
<td>Åkerman et al. (2004)</td>
</tr>
<tr>
<td>Harbours</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delfzijl</td>
<td>1999-2005</td>
<td>2 - 65</td>
<td>own results</td>
</tr>
<tr>
<td>Harlingen</td>
<td>1999-2005</td>
<td>1 - 47</td>
<td>own results</td>
</tr>
<tr>
<td>Den Helder</td>
<td>1999-2005</td>
<td>1 - 53</td>
<td>own results</td>
</tr>
<tr>
<td>IJmuiden</td>
<td>1999-2005</td>
<td>1 – 61</td>
<td>own results</td>
</tr>
<tr>
<td>Scheveningen</td>
<td>1999-2005</td>
<td>8 – 192</td>
<td>own results</td>
</tr>
<tr>
<td>Rotterdam Rijnmond</td>
<td>1999-2005</td>
<td>0 – 689</td>
<td>own results</td>
</tr>
<tr>
<td>Vlissingen</td>
<td>1999-2005</td>
<td>0 - 23</td>
<td>own results</td>
</tr>
<tr>
<td>Eemshaven</td>
<td>1999-2005</td>
<td>1 – 16</td>
<td>own results</td>
</tr>
</tbody>
</table>
disposal at sea, in vitro bioanalysis using the DR-Luc bioassay has been shown to be reliable, relatively inexpensive and fast (Stronkhorst et al., 2002, Besselink et al., 2004). Applying this bioanalysis or bioassay, reveals elevated concentrations of dioxins in dredged material from all Rotterdam harbour transects and the Western Scheldt, but low levels in sediments from other Dutch harbours and the Wadden Sea (Table 6).

As dioxins normally do not induce acute toxicity, in vivo bioassays used for additional testing (Box B) need to be carefully selected. Well known ecologically relevant adverse effects of dioxins include immune system toxicity (Ross et al., 1995), reduced fertility (Reijnders 1986, Leonards et al., 1995), and early life stage effects (Murk et al., 1996; Gutleb et al., 2007a; Foekema et al., 2008). Early life stage effects would be missed if these assays are performed as usual, including exposure and observation until the free feeding stage of the larvae. Only with prolonged observation, even without further exposure, do developmental effects become evident (Gutleb et al., 2007b; Foekema et al., 2008). In vivo effects of dioxins that could only be detected with chronic in vivo bioassays with vertebrates, can be predicted based on quantification of the internal dioxin-like toxic potencies determined using in vitro bioanalysis of internal TEQs. Using the water concentration for exposure assessment is not suitable for dioxins. Aquatic organisms can be exposed to dioxins directly from the (pore) water via their gills or skin, but the highest internal levels are reached in predators exposed via the food chain. As an example, the internal NOEC of a number of vertebrate animals is presented in Table 7. As for TBT, the toxicity of dioxins for several species can be combined into a SSD based on these NOECs (Figure 5). To be able to predict the PAF based on sediment levels, the internal NOECs (ng TEQ/g lw) can be calculated back to levels in their food (ng TEQ/g lw) and from that back to levels in the sediment (ng TEQ/g organic carbon (OC)). The bioaccumulation factors vary depending on the species and sexes, and, for fish eating mammals we used a BAF of 100 based on Traas et al. (2001) and for fish eating birds a BAF of 20 (based on Bowerman et al., 1995; Leonards et al., 2008) (ng TEQ/g lw/.ng TEQ/g lw). As a sediment to fish bioconcentration factor (ng TEQ/g lw/ ng TEQ/g OC) we chose to use four (Traas et al., 2001). Based on these factors, the internal NOECs can be translated back into sediment levels (Table 7) and from these data an SSD constructed as well (Figure 6). From this SSD, safe sediment TEQ-levels can be determined which provide a basis to define appropriate ALs for the licensing system. The current AL is 50 ng TEQ/kg-sediment which, without dilution, would imply a PAF of 96%. A possible known dilution after disposal could be taken into account. The average organic carbon (OC) content, of dredged harbour sediments is 5% (Stronkhorst et al., 2003a), which could be applied in case the TEQ is only determined based on sediment dry weight. The ranges of TEQs in dredged sediments from rivers in the Dutch coastal zone were 12-70 ng TEQ/kg dw, on average 24 ng TEQ/kg dw (Table 8). The Box B analysis using bioassays and sediment TEQ levels, reveals a PAF of 87-96% (an average of 91%). Of the TEQ-input in the North Sea, more than 90% is directly originating from rivers and indirectly via material dredged from waterways and harbours and only 14 of the total 188 g TEQ/year is originating from atmospheric deposition (Table 8).

To determine the actual impact of dioxins in dumped harbour sediments on local ecosystems (Box C analysis), biomonitoring should be performed, by taking samples in a gradient from the dumping site or over time. It is important, to take into account that most of the TEQs in the dumped dredged sediment will not settle, but will be carried, bound to small particles, with the current to the Wadden Sea (Stronkhorst et al., 2003a). TEQ-levels in Wadden Sea sediments vary from 13 to 31 ng TEQ/kg dw implying a PAF of 74 to 99%, respectively (Figure 6). The internal TEQ-levels recently determined with the DR-Luc assay in livers of stranded or drowned dead
Table 7: Example application of process in Box B to evaluate the risk of dioxins in Dutch sediments. No observed effect (NOEC) concentrations for chronic toxicity of dioxins in vertebrates (immune, reproductive and developmental toxicity) expressed as internal concentration (ng TEQ/g lw). The sediment to fish bioconcentration factor is set at 4 (ng TEQ/g Organic Carbon to ng/g lipid weight in fish) based on Traas et al. (2001). Based on a species-specific biomagnification factor (BMF) from fish to animal (ng TEQ/g lw) the internal NOEC is extrapolated to a NOEC in sediment. These data are used to construct the SSDs in Figures 5 and 6.

<table>
<thead>
<tr>
<th>Species</th>
<th>Endpoint</th>
<th>NOEC biota$^1$ (ng TEQ/g lw)</th>
<th>BMF$^2$ factor</th>
<th>BSAF$^3$ (BCF*BMF)$^3$</th>
<th>NOEC sediment (ng/kg OC)</th>
<th>NOEC $^5$ sediment (ng/kg dw)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common tern</td>
<td>Fledging success</td>
<td>4</td>
<td>20</td>
<td>80</td>
<td>50</td>
<td>2.5</td>
<td>Murk et al., 1994a, 1996</td>
</tr>
<tr>
<td>Forster’s tern</td>
<td>Prolonged incubation</td>
<td>5</td>
<td>20</td>
<td>80</td>
<td>62.5</td>
<td>3.125</td>
<td>Kubiak et al., 1989</td>
</tr>
<tr>
<td>Cormorant</td>
<td>Fledging success</td>
<td>20</td>
<td>20</td>
<td>80</td>
<td>250</td>
<td>12.5</td>
<td>Van den Berg et al., 1994</td>
</tr>
<tr>
<td>Bald eagle</td>
<td>Development</td>
<td>0.2</td>
<td>20</td>
<td>80</td>
<td>2.5</td>
<td>0.125</td>
<td>Bowerman et al., 1995</td>
</tr>
<tr>
<td>European otter</td>
<td>Retinoid biomarker</td>
<td>2</td>
<td>100</td>
<td>400</td>
<td>0.35</td>
<td>0.0175</td>
<td>Murk et al., 1998</td>
</tr>
<tr>
<td>Mink</td>
<td>Litter size</td>
<td>1</td>
<td>100</td>
<td>400</td>
<td>5</td>
<td>0.25</td>
<td>Leonards et al., 1995</td>
</tr>
<tr>
<td>Mink</td>
<td>Kit survival</td>
<td>6</td>
<td>100</td>
<td>400</td>
<td>15</td>
<td>0.75</td>
<td>Leonards et al., 1995</td>
</tr>
<tr>
<td>Harbour seal</td>
<td>Immune/Reproductive</td>
<td>0.06</td>
<td>30</td>
<td>120</td>
<td>0.15</td>
<td>0.0075</td>
<td>Ross et al., 1995; Reijnders et al., 1996</td>
</tr>
<tr>
<td>Fish larvae</td>
<td>Development</td>
<td>0.6</td>
<td>3</td>
<td>12</td>
<td>150</td>
<td>7.5</td>
<td>Foekema et al., 2008</td>
</tr>
</tbody>
</table>

$^1$ BMF: Bio magnification Factor
$^2$ BSAF: Biota/Sediment Accumulation Factor
$^3$ BCF: Bio concentration Factor sediment-to-fish has accumulation factor 4
$^4$ NOEC biota to construct SSD in Figure 5
$^5$ NOEC sediment to construct SSD (based on 5% Total Organic Carbon) in Figure 6
Table 8: Calculated annual input of TEQ in the Dutch coastal zone from the rivers, atmospheric emission and disposal of dredged material (period 1999-2005). For the TEQ-levels in sediment, the ecotoxicological risk is estimated as % Potentially Affected Fraction of species (PAF) and calculated based on the SSD for chronic dioxin-toxicity (Figure 6).

<table>
<thead>
<tr>
<th>Reference</th>
<th>Year</th>
<th>Sediment load (kiloton/yr)</th>
<th>mean [TEQ] (ng/kg dw) sediment</th>
<th>PAF (%) Median (Min-Max)</th>
<th>Calculated annual load TEQ (g/year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>North Sea area</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>North Sea Goeree</td>
<td>4</td>
<td>2005</td>
<td>na</td>
<td>18</td>
<td>90 (71-98)</td>
</tr>
<tr>
<td>North Sea Noordwijk</td>
<td>4</td>
<td>2005</td>
<td>na</td>
<td>20</td>
<td>91 (72-89)</td>
</tr>
<tr>
<td>Wadden Sea</td>
<td>4</td>
<td>2005</td>
<td>na</td>
<td>24</td>
<td>92 (74-99)</td>
</tr>
<tr>
<td>Rivers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhine (part of New Waterway)</td>
<td>5</td>
<td>2000</td>
<td>nd</td>
<td>19</td>
<td>90 (71-98)</td>
</tr>
<tr>
<td>Meuse (part of New Waterway)</td>
<td>5</td>
<td>2000</td>
<td>nd</td>
<td>37</td>
<td>94 (77-99)</td>
</tr>
<tr>
<td>Western Scheldt Terneuzen</td>
<td>4</td>
<td>2005</td>
<td>219</td>
<td>15</td>
<td>89 (69-98)</td>
</tr>
<tr>
<td>Ems-Dollard</td>
<td>This study</td>
<td>2005</td>
<td>61</td>
<td>18</td>
<td>90 (71-98)</td>
</tr>
<tr>
<td>Lake IJssel</td>
<td>This study</td>
<td>2005</td>
<td>227</td>
<td>21</td>
<td>91 (72-98)</td>
</tr>
<tr>
<td>Channel Gent-Terneuzen</td>
<td>This study</td>
<td>2005</td>
<td>3</td>
<td>10</td>
<td>86 (65-97)</td>
</tr>
<tr>
<td>New Waterway</td>
<td>This study</td>
<td>2001-2004</td>
<td>407</td>
<td>47</td>
<td>95 (79-99)</td>
</tr>
<tr>
<td>North Sea Channel</td>
<td>This study</td>
<td>2005</td>
<td>23</td>
<td>17</td>
<td>90 (70-98)</td>
</tr>
<tr>
<td>Haringvliet sea-locks</td>
<td>This study</td>
<td>2005</td>
<td>80</td>
<td>12</td>
<td>87 (67-97)</td>
</tr>
<tr>
<td>Seine</td>
<td>2003</td>
<td>751</td>
<td>nd</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>Elbe</td>
<td>8</td>
<td>2004</td>
<td>776</td>
<td>40</td>
<td>94 (78-99)</td>
</tr>
<tr>
<td>Weser</td>
<td>2000</td>
<td>299</td>
<td>nd</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>Yser</td>
<td>6</td>
<td>2004</td>
<td>18</td>
<td>19</td>
<td>90 (71-98)</td>
</tr>
<tr>
<td>UK-Scotland (Firth of Forth)</td>
<td>7</td>
<td>2005</td>
<td>153</td>
<td>17</td>
<td>87 (67-97)</td>
</tr>
<tr>
<td>UK-Midlands (Mersey)</td>
<td>7</td>
<td>2005</td>
<td>292</td>
<td>17</td>
<td>87 (67-99)</td>
</tr>
<tr>
<td>UK-South England (Southampton Water)</td>
<td>7</td>
<td>2005</td>
<td>19</td>
<td>70</td>
<td>97 (81-100)</td>
</tr>
<tr>
<td>Disposal site Loswal Noord</td>
<td>This study</td>
<td>1999-2005</td>
<td>312</td>
<td>33</td>
<td>94 (76-99)</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atmospheric deposition North Sea</td>
<td>nd</td>
<td>2007</td>
<td>nd</td>
<td>nd</td>
<td>Na</td>
</tr>
<tr>
<td>Total input North Sea Dutch Coastal zone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^1based on estimated TEQ-level of 40 ng TEQ/kg dw; ^2Source MWTL; ^31.0 ng TEQ / m²/year (Hovmand et al., 2007); ^4Åkerman et al., 2004; ^5Vethaak et al., 2002; ^6Sanctonum et al., 2007; ^7Hurst et al., 2004; ^8Oehme, 1998; ^9Calculation with ETX 2.0 model; nd = not determined; na = applicable
Harbour seals (*P. vitulina*) are in the range of 100-633 pg TEQ/g lw in the Dutch Wadden Sea (n=13), 144-565 pg TEQ/g lw in the Dutch Delta (n=6), and 106-355 pg TEQ/g lw in the Norwegian Sea (n=5) in 2008 (own unpublished data).

**Figure 5:** Species sensitivity distribution (SSD) for the NOEC of specific dioxin-induced effects in vertebrate species (common and Foster’s tern, cormorant, bald eagle, European otter, mink, harbour seal and sole) expressed as internal TEQ (μg TEQ/kg lipid weight).

**Figure 6:** Species sensitivity distribution (SSD) for the NOEC of specific dioxin-induced effects in vertebrate species translated into sediment levels (mg/kg dry weight) by applying estimated bioconcentration and bioaccumulation factors (explanation in text).

Interpolation in the internal NOEC-SSD confirmed that the current concentrations still are above safe levels (Figure 5). In the study of Ross et al. (2002), harbour seals with 209 ng TEQ/g lw were reported to reveal significantly more immunotoxic effects than the reference seals containing 62 pg TEQ/g lw. It would be useful to study specific dioxin-related biomarker responses
in the seals containing the highest levels of dioxins. A study with European otters killed either by traffic or in fishing nets, shows how population success can be related to individual animal health (Leonards et al., subm.), to biomarkers and internal TEQs (Murk et al., 1998), to TEQs in their prey (fish), and finally, to the TEQs in the local sediment (Traas et al., 2001). Based on such studies, as we demonstrated in our examples, internal NOECs can be related to sediment-based SSDs for ecotoxicological risk assessment of bioaccumulating chemicals inducing chronic toxicity. Especially with bioaccumulating chemicals for which exposure can occur via diverse routes, it is very important to determine the internal effects levels in in vivo studies. This allows comparison with field situations, as has successfully been shown with the internal effect levels for early development of sole larvae (with prolonged observation period) (Foekema et al., 2008). This revealed that current TEQ levels in European fish from more polluted locations are above the NOEC for larval survival. Dioxins are an example of persistent and accumulating chemicals for which the current in vivo bioassays for effects assessment are likely to underestimate the chronic risks. The example given demonstrates that with more dedicated bioassays and approach, the latest SSD and PAF approaches can be applied.

Conclusions and recommendations

When selected carefully bioassays have been shown to be useful tools in the performance of hazard, ecotoxicological risk and impact assessment of dredged harbour sediments. Here, three different purposes for bioassay application were distinguished (Figure 3). The most basic application (Box A) is a rapid determination of the hazard (potential toxicity) of dredged sediments which is then compared to ALs in a licensing system. In this process, the worst-case situation is considered and bioavailability of compounds in the dredged material is not taken into account as after dumping the new physical-chemical conditions will greatly alter the bioavailability. The tests have to be fast, inexpensive and standardized, and bioassays sensitive for matrix effects are not suitable. The ALs have to be derived from hazard assessment (Box A) and validated by (eco)toxicological risk assessment (Box B) and by impact assessment (Box C). Some in vitro assays that are specific for groups of compounds or toxicological endpoints can be used to supplement chemical analyses (Table 4). For example, the DR-Luc assay currently is included as an inexpensive and cost-effective alternative for dioxin-like compounds. In vitro assays for PBDEs and related compounds that induce thyroid hormone disruption could be useful additions. The organic sediment extract prepared for the in vitro bioassay could also be tested for cytotoxicity to give an alert for the presence of otherwise undetected toxic (organic) compounds and unknown PBT chemicals. When bioassays are conducted on extracts, instead of whole sediment, matrix effects which have been a problem in several studies using in vivo species are avoided. Finally, the in vitro bioassays should be standardized and validated to provide reproducible and trustable results.

In addition to application of bioassays in the licensing system, bioassays could also be applied for ecotoxicological risk assessment of the dredged sediment (Box B, Figure 3). In this application the bioavailability of the toxic chemicals in the dredged sediment would be included as well as chronic toxicity focusing on ecologically relevant endpoints. This is especially relevant in cases where the outcome of the first assessment (Box A) is not conclusive. Under these circumstances, further bioanalysis can help the decision making process by taking into account more aspects of the mostly unknown complex chemical mixtures present and, when considered relevant, the bioavailability of the chemicals. These bioassays should comply with quality
Rational application of bioassays in hazard, risk and impact assessments of dredged sediments

standards, but in addition exposure route, test conditions, and endpoints, should be based on rational considerations. However, when the outcome of all tests performed in the framework (Box A) and additional in vitro testing (Box B) still do not demonstrate convincingly whether the sediment is clean enough or too contaminated, additional testing is suggested. In such cases, an in vivo bioassay could be performed. As outlined in the present paper, it is recommend to use cultured species for in vivo bioassays as this strongly reduces the variability in the response. In addition, it is important to include relevant exposure routes in the in vivo bioassays; for example, maternal transfer of PBTs from the mother to the offspring. The introduction of the PAF method to assess the ecotoxicological pressure of dredged material is well applicable for contaminated sediments. For acutely toxic chemicals for which direct exposure is relevant, SSDs can be made with NOECs expressed as exposure concentration in the water. For bioaccumulating chemicals, such as dioxins, internal exposure is a more relevant parameter for defining the SSD of chronic effects. Using established food chain bioaccumulation factors and sediment to fish bioconcentration factors, the NOEC can be expressed in terms of sediment concentration. The concentrations of pollutants in the sediment or organisms, quantified either with chemical analysis or in vitro bioassays, can be interpolated in the SSDs to assess the PAF of species.

To determine the actual impact of dumped harbour sediments on local ecosystems, both in vitro and in vivo bioassays, as well as biomonitoring should be performed (Box C, Figure 3). When applying the proposed bioassay approach in risk management of dredged contaminated sediments, the most relevant in vitro and in vivo bioassays and bioindicators are selected for location specific impact assessment. In situations with strong dilution after disposal of dredged sediment, readily biodegradable compounds and chemicals with toxic effects at relatively high concentrations such as narcotics, may not be expected to be relevant for the impact assessment (Table 4). This means that impact assessment of dredged material in marine systems should involve an understanding of the ecotoxicological fate of chemicals, and include monitoring of effects with mechanistic in vitro bioassays and dedicated biomarkers to support management decisions. The implications of sediment contamination on the population development of local marine organisms can be monitored in combination with relevant bioindicators and, if possible, linked to in vitro bioanalysis of toxic potencies in the sediment, food chain and, in organisms. The predicted ecotoxicological risk, preferably expressed as PAF based on SSDs for ecological relevant endpoints, can thus be validated as well. This combination of in vitro and in vivo bioassays and bioindicators are valuable tools that complement chemical analysis in the assessment of the possible impact of disposal dredged material and sediments at specific locations in the marine environment.

In this proposed approach, results obtained using additional bioassays in more advanced ecological risk assessment (Box B) and impact assessment (Box C) could eventually lead to the decision to include some additional chemical analyses or bioassays in the rapid screening (Box A) to allow improved detection of toxic chemicals that may currently be overlooked but appeared to be relevant for dredged harbour sediments. Also, the SQG and ALs may be updated based on the new information. It is important to stress that the new chemical analyses or bioassays to be included in the licensing system need not necessarily be identical to the tests that brought forward the yet unidentified ecotoxicological consequences. Tests to be added for a certain endpoint need to be standardised, inexpensive, fast, high throughput and validated bioassays. Such tests may still need to be developed for inclusion in the licensing system.
Acknowledgements

We would like to acknowledge the valuable contribution in terms of financial support made by the Directorate-General of Public Works and Water Management (Rijkswaterstaat, RWS). The authors wish to thank Gmelig Meyling (Stichting ANEMOON), Klaas Kaag (IMARES) and Johan Jol (IMARES) for their invaluable support in conducting the fieldwork. This paper benefited greatly from the input of colleagues at RWS including Hans Klamer, Martine van den Heuvel-Greve (Deltares), and Jos van Gils (Deltares). We are grateful to Pim Leonards (IVM) for the analysis of the seal samples.
Chapter 7

Summary and conclusions

Sediment risk management

Sedimentation of suspended particles in delta areas like those in the Netherlands necessitates dredging of waterways and harbours to prevent obstruction of important shipping routes. Hundreds of millions of tonnes of sediment are removed worldwide each year. Dredged material is subsequently disposed of, either at sea or stored at high costs. Dredged material disposal is regulated in accordance to different environmental conventions and agreements on marine pollution prevention. In Europe, disposal of dredged material is based on three conventions: London Convention (1972), Oslo-Paris Convention (OSPAR, 1992), and Helsinki Convention (HELCOM, 1992). The purposes of these conventions are to limit environmental pollution resulting from disposal of dredged material in the marine environment of the North East Atlantic (North Sea) and the Baltic Sea area. Countries have set dredged material guidelines into force for disposal of dredged material into the sea (Alvarez-Guerra et al., 2007a). The Sediment Quality Guideline (SQG) is based on the levels of a number of conventional contaminants (Long et al. 2000, 2006; MacDonald et al., 2000; Alvarez-Guerra et al., 2007b). Many countries have based their action levels for determining whether dredged material may be disposed of at sea, on determination of a number of classical contaminants in the sediment. The potential toxicity of as yet unknown compounds is not included in the current licensing systems for considering disposal of dredged material in the marine environment. Given the complexity of contaminated sediments, integrated assessment methods with in vitro and in vivo bioassays and local bioindicators in addition to chemical analyses are needed to detect the biological effect of contaminants (Chapman and Mann, 1999).

This thesis focuses on the applicability of in vitro and in vivo bioassays and bioindicators as tools for evaluating the effects of complex chemical sediment contaminants in the process of deciding whether dredged harbour sediments can be safely disposed of at sea. To this end three objectives have been defined:

1. Analyze the main factors determining success and failure when applying bioassays for hazard, risk and impact assessments of dredged sediments.
2. Develop a rationale for the inclusion of bioassays in hazard, risk and impact assessments of dredged sediments.
3. Advise a rational choice of bioassays and biomarkers that will provide added value to the risk management process when considering the disposal of dredged sediments.
Combining the information obtained in these newly defined bioassays with existing chemical analyses may facilitate definition of not only the hazard but also the risk posed by the dredged sediment.

**Assessment of the biological effects**

**Chapter 2** presents results from a retrospective analysis to explore the applicability of fish biomarkers and sediment bioassays along contaminated salinity transects (Schipper et al., 2009a). In the Netherlands, the aquatic environment is dominated by the estuaries of several major European rivers that are the receiving waters for major industrial areas. The principal objective of the study was to test and compare available biological effect monitoring techniques in order to select the most appropriate, robust and reliable methods for further use in monitoring programmes for complex estuarine environments. The addition to monitoring programmes of *in vitro* and *in vivo* bioassays specifically suited to the assessment of this activity will provide useful information on the presence of these types of pollutants in water systems. The results obtained in such studies allow the gross toxicity of the sediments and bioavailability of certain classes of toxicants to be assessed. Our study focused on biomarker responses in European flounder (*Platichthys flesus*) as the sentinel fish species.

Sediment, water and European flounder samples were collected at two locations within the two transects at Port of Rotterdam and Port of Amsterdam. The sex, length, weight, somatic weight, liver weight and gonad weight of the European flounder, *P. flesus* were recorded and tissue samples were sub-divided for chemical and biomarker analysis. The biomarkers chosen in *P. flesus* tissue were cytochrome P450-1A content (CYP1A), metallothioneins (MT), glutathione-S-transferase activity (GST), superoxide dismutase activity (SOD), catalase activity (CAT) and several other biomarkers for oxidative stress, vitellogenin (VTG) in plasma in male fish, 1-OH-pyrene in bile, acetyl cholinesterase activity (AChE) in brain and muscle, RNA/DNA ratio in tissue, and liver histopathology. Sediment bioassays included *in vivo* tests with invertebrates species such as the amphipod *Corophium volutator*, the burrowing heart urchin *Echinocardium cordatum* and the polychaete *Arenicola marina*. Other bioassays were the Microtox Solid Phase® bioluminescence inhibition assay, the *in vitro* reporter gene assays DR-Luc and ER-Luc for dioxin-like and estrogenic effects respectively, and the recombinant Yeast Estrogen Screen (YES) assay.

Chemical analysis showed that body burden in benthic fish at least partly fingerprints the chemical characteristics of the sediment from the locations where they were sampled, though it did not always show a clear correlation between sediment contamination and contaminants in flounder. For several of the biomarkers, there was a distinct and significant difference between the average values at the saltwater and freshwater reference sites. The results of the biomarker responses and the sediment chemistry of the flounder and the sediments were correlated, though differences were apparent between types of specific and a-specific biomarkers. Most of the *in vivo* bioassays conducted with the sediments from the locations in the study barely demonstrated any differences between sites. The bacterial Microtox tests and the other *in vitro* bioassays with sediment extracts clearly indicated differences between the sediments from different locations. A strong effect was also found at Oranjesluis and Buitenhuizen with the 6h results (indicating PAHs) and 48h results (indicating dioxin-like compounds) of the DR-Luc. The greatest estrogenic activity (EEQ>10 pmol/g dry weight) was found in sediments from the interregional locations at Port of Rotterdam, Noordwijk and IJmuiden Harbour.

The results obtained, demonstrate that the assessment of the biological effects of marine and estuarine contamination could be an important element of programmes that aim to assess the
quality of the environment (Schipper et al., 2009a), since the bioassays and biomarkers applied, in contrast to mere chemical analysis, address bioavailability and can demonstrate links between contaminants, and biomarkers for organism health. The phase I biotransformation enzyme CYP1A in flounder was hardly correlated to sediment PCB and PAH levels, phase II enzyme GST was not different in fish from sites with different degree of pollution while in addition the antioxidant enzymes SOD, CAT, KMBA, MDA and WOP did not differ between the freshwater and saltwater reference sites. Therefore, it these biomarkers don’t seem to be suitable for monitoring the toxicological impact of contaminated sediment. The most suitable and successful biomarkers in the study presented in chapter 2 were those based on the induction of hepatic metallothionein content (indicating exposure to organotins) and concentration of 1-OH pyrene in bile (an indicator of exposure to PAHs). These specific biomarkers gave more straightforward results than gross indices and biomarkers of general damage or condition that can be influenced by many different stressors. In this study the mechanism-based in vitro reporter gene assays DR-Luc and ER-Luc applied to sediment extracts were much more distinctive than in vivo bioassays with macro invertebrates exposed to whole sediment using survival as an endpoint. Overall it has been concluded that the most suitable and successful biomarkers and bioassays in the study were those based on fairly specific action mechanisms and types of biological effect that can be applied to cleaned sediment extracts thus excluding matrix effects.

In chapter 3 one of these specific mechanism-based in vitro toxicity tests was studied in more detail.

Specific mechanism-based in vitro toxicity testing

Chapter 3 describes the promises and reproducibility of a specific mechanism-based in vitro bioassay applied to specifically prepared extracts from disturbing matrix effects and possibly interfering compounds have been removed (Besselink et al., 2004). The DR-Luc (dioxin responsive–luciferase reporter gene) bioassay was taken as the example and the bioassay performance criteria were determined for the analysis of dioxin-like compounds in sediment samples for the purposes of licensing of disposal of dredged harbour sediments at sea. The bioassay responses to the mixture of dioxin-like compounds could systematically be correlated to levels of dioxin-like contaminants in the sediments, and indicated the presence of congeners with a similar mode of action that were yet unknown or present in too low concentrations to be quantified by chemical analysis. Standard operating procedures for bioassays must include good laboratory practice in terms of selectivity, reproducibility, and the representativeness of exposure involving specific mechanisms (Schipper et al., 2001). For use in marine and estuarine environments, sediment bioassays should preferably have a strong response to contaminants, high tolerance to salinity, organic content and sediment types, and be based on relevant endpoints. However, when cleaned extracts are being used the sediment conditions are not relevant anymore because disturbing factors can be removed. For quantitative toxicological studies an intra- and interlaboratory validation study or ring test should be performed. In Chapter 3 the intra- and interlaboratory repeatability and reproducibility and the limit of detection and quantification of the DR-CALUX bioassay were determined by analyzing cleaned sediment extracts and dimethyl sulfoxide (DMSO) blanks. Six international laboratories were selected to participate. They performed the analyses according to the protocols supplied. Each of these laboratories analyzed blind samples in each of three phases of the study. Calibration curves were produced with a validated 2,3,7,8-TCCDD-
standard for quantification of the responses as 2,3,7,8-TCDD-equivalents (TEQs) in calibration curves.

All compounds showing an affinity towards the Ah receptor are detected by the DR-Luc bioassay. The average interlaboratory repeatability ranged from 14.6% for the analysis of pure compounds to 26.1% for the analysis of the whole sediment matrix.

It is concluded that the determination of dioxin-like activity in cleaned sediment extracts using the DR-Luc bioassay has a consistent reproducibility, and can be used as a reliable and robust tool in the hazard assessment of dredged materials in a licensing system. Its advantage over chemical analysis is that it also detects the effects of combined exposure as well as the effect of unidentified congeners or chemicals present in concentrations below the limit of detection of chemical analysis with a similar mode of action. In addition the use of cleaned sediment extracts circumvents the problem of disturbing sediment matrix effects.

The bioassay thus evaluated in chapter 3 is an example of an in vitro bioassay, whereas in the next chapter an in vivo bioassay specifically for exotoxicological testing in marine environments was developed.

Sediment assessment with cultivated organisms for use in in vivo toxicity testing

Echinoderms (Chapter 4) are a useful model for ecotoxicological testing in the context of hazard or risk assessment in the marine environment. Heart urchins naturally live in sandy, silt-rich areas of the North Sea with a relatively high level of organic material and can be kept in the laboratory for extended periods. However, field collection of E. cordatum is difficult in coastal zones because of heterogeneous population densities. Furthermore, urchins collected from the field may suffer acute trauma during collection, which is likely to influence their sensitivity to chemical contaminants in the bioassays and introduce experimental variability. The mortality results from in vivo field-collected heart urchin bioassays showed major variability in the coefficient of variation for samples from harbour sediments. The objective of the study described in chapter 4 was to develop and optimize the cultivation process for raising adult E. cordatum under laboratory conditions and to validate the use of these cultured heart urchins for sediment toxicity testing by comparing their responses with field E. cordatum in sediment toxicity studies (Schipper et al., 2008a). The study describes a culture method for the heart urchin E. cordatum under controlled laboratory conditions, providing organisms with low biological variation. Spawning and fertilization were successful in the spring, when a combination of induction with KCl and a low pre-temperature period was applied. With our optimized growth protocol, both larvae and juveniles have a growth rate comparable to E. cordatum in the wild. The sensitivity of field and cultured heart urchins to tributyltin (TBT) spiked sediment was tested using heart urchins of different weight classes. The in vivo bioassay with TBT spiked sediments revealed 14d LC50 values of 1,242 and 964 μg Sn/kg dw respectively in cultured and field-collected E. cordatum. The performance of field and cultured E. cordatum was also studied in sediment toxicity tests with polluted sediment, using mortality as the endpoint. The results revealed that cultured E. cordatum (maximum CV=9.6%) give less statistical variation in sediment toxicity in the polluted sediments than field heart urchins (maximum CV=18.9%). Although no externally visible signs of damage were noted in either field or cultured E. cordatum, in field E. cordatum evidence of cellular damage caused by infections and/or other stressors was detected. The TBT spiked sediment toxicity test also suggested higher sensitivity in field urchins as compared to cultured
urchins. The presence of increased melanin pigmentation, as detected in the field urchins, is often associated with ‘stress’, including pathogen insult, resulting in cell damage and recycling involving the production of pigments as part of the host defence mechanism. This may also explain the lower variability in the toxicity tests with cultured organisms.

It can be concluded that cultured heart urchins are less sensitive to TBT than field-collected *E. cordatum*. Furthermore, in whole sediment *in vivo* bioassays, the survival of cultured sea urchins was higher or at least similar to that of field-collected *E. cordatum*. The increased sensitivity of field urchins compared to cultured urchins in various toxicity tests may be due to multiple environmental stressors reducing their overall performance. On the whole, it was demonstrated that use of cultured *E. cordatum* provides a significant improvement in urchin-based bioassays for marine environmental toxicity testing, resulting in a more homogeneous, vital population and experimental data with lower variability. In risk assessment of dredged material, bioassays with cultured *E. cordatum* give less standard deviation in acute sediment toxicity tests using contaminated estuarine and marine sediments than field-collected animals. In addition the life-history of cultured heart urchins is known, and the animals are free of infection and ecological or collection trauma.

In the next chapter the studies were extended to perform an ecological risk assessment using all lines of evidence including chemical analysis, *in vivo* bioassays in the laboratory and in situ ecological biomarkers.

Assessing the ecological impact of organotin pollution, combining risk prognosis and field monitoring using the gastropod intersex bioassay

Chapter 5 describes the results of an integrated assessment using information from the toxicity determined in an *in vivo* bioassay, the bioindication obtained in the field and chemistry analysis of sediment levels tributyltin (TBT) (Schipper et al., 2008b). The objective of this study was to evaluate the ecotoxicological risk for observed TBT levels in local sediments predicted based on toxicological effect levels for 29 marine and freshwater species representing seven taxonomic groups that were combined in an species sensitivity distribution (SSD). TBT-based antifouling paints were commonly used on the hulls of boats to prevent corrosion and fouling. The biocide TBT is very persistent and accumulates in sediments. Especially snails have been shown to suffer endocrine-disrupting effects even at low exposure concentrations resulting in penis development in females. Therefore in addition to the abundance of the gastropod indicator species periwinkle *L. littorea* also the intersex index (ISI) was determined as biomarker for TBT-specific effects. At 42 Dutch coastal harbour locations and open water areas, biota, sediment, and suspended particulate matter were sampled for chemical, ecological and biomarker analysis. The ecological risk expressed as the potentially affected fraction of species (PAF) was calculated by interpolation of the TBT-levels in the SSD. The PAF for open coastal waters ranged from 4.2% to 15.3% and for harbours from 3.5% to 26.9%. Significant intersex levels were observed in waters where the risk was calculated above 10% PAF. Given the good correspondence between observed ISI occurrence in periwinkles (*L. littorea*) and the PAF predicted in the TBT-levels in the harbour sediments, the ISI in *L. littorea* could be used as a bioindicator for the local ecotoxicological health status. The study demonstrated a generally poor ecotoxicological health status of some Dutch harbours and the absence of *L. littorea* from some harbours could be explained by high TBT concentrations.
Rational application of bioassays for hazard, risk and impact assessments of dredged sediments

Chapter 6 addresses the issue of the applicability of *in vitro* and *in vivo* bioassays for hazard, risk and local impact assessment of dredged polluted sediments to be disposed of at sea. It discusses how and to what extent selected bioassays can fill in the gaps left open by chemical analysis and the way in which the bioassays may contribute to the present licensing system for disposal (Schipper et al., 2009b).

Annually, over 90 million tonnes of polluted dredged material are disposed of at sea within the OSPAR maritime area and hundreds of millions of tonnes are disposed of worldwide. Given the potential environmental consequences of dumped dredged harbour sediments it is vital to establish the potential risks from exposure before disposal at sea. Currently, the legislation of dredged material is based on chemical analysis of a limited number of well known contaminants only for which action levels (AL) have been set. However, a chemical approach ignores the presence of i) unidentified chemicals, ii) mixture effects, iii) the bioavailability of the chemicals and iv) effects on ecologically relevant endpoints. No ALs exist for more recent contaminants found in sediments, including potentially harmful priority substances such as polybrominated diphenyl ethers (PBDEs), perfluorinated chemicals like perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), toxic congeners of polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated naphthalenes (PCNs), and phthalates (EU, 2006). To be able to take into account the presence of unknown compounds, mixture effects, the availability of the compounds and effects on ecologically relevant endpoints, it has been suggested to include the results of bioassays as well in the licensing process for sediment disposal.

*In vitro* and *in vivo* bioassays have been shown to be useful tools in the process of hazard, ecotoxicological risk and impact assessment of dredged harbour sediments, provided they are consciously chosen. Three different purposes for application were distinguished (Fig. 1) for which the considerations differ.

The most basic application (Box A) is a rapid determination of the hazard (potential toxicity) of dredged sediments which is then compared to ALs in a licensing system. As with chemical analysis on total sediment extracts, the bioavailability of the chemicals is not into account in this box A hazard assessment. The bioavailability of the compounds will change greatly after disposal of the sediments, therefore a worst-case approach is considered most appropriate here. Because this system has to be fast and standardized, and *in vivo* assays with whole sediment usually are sensitive to matrix effects they are less suitable for Box A analyses. An example of successful application of an *in vitro* bioassay is the DR-Luc assay for dioxin-like compounds performed with sediment extracts after destructive sample clean-up. In general, the current chemical analyses supplemented with some mechanism- or compound specific *in vitro* assays with sediment extracts are useful (table 1). It is important to consider additions to the base set of analyses based on the outcome of Boxes A and C results revealing (groups of) compounds that are very relevant for dredged sediment but will be missed by the current chemical analyses and bioassays. For example *in vitro* assays for brominated flame retardants and related compounds that induce thyroid hormone disruption could be useful additions. To get an alert of the presence of otherwise un-assessed toxic (organic) compounds and unknown PBT chemicals the organic sediment extract can be tested for general cytotoxicity in an *in vitro* bioassay to help decide for disposal of dredged material under legislation. These *in vitro* assays are fast, with the extract preparation disturbing matrix effects can be prevented, and the assays should be selected to fill
Summary and conclusions

Figure 1. Application of in vitro and in vivo bioassays for: Box A) fast screening for licensing of disposal of the dredged sediments; Box B) additional testing for further risk assessment in case A was not conclusive; Box C) site specific determination of the impact of dumping of dredged harbour sediments. Based on boxes B and C, the sediment quality guidelines (SQGs), and action levels (ALs) for the licensing system (box A), results can be used for fine-tuning the selection of chemical and biological analyses and ALs.

the harbour-relevant main gaps left open by the chemical analyses. The in vitro bioassays should be standardized and validated to provide reproducible and trustable results.

When the outcome of all tests performed in the framework of the legislation (Box A) do not convincingly demonstrate whether the sediment is clean enough or too polluted, further bioanalysis can help the decision making process. In this second step (Box B, figure 1) more aspects of the mostly unknown complex chemical mixtures are taken into account and, when considered relevant, the bioavailability of the chemicals can be included. Given the strong dilution after disposal at sea, readily biodegradable compounds and chemicals or nanoparticles with toxic effects at relatively high concentrations only, such as for example compounds with narcotic effects, are not relevant for the impact assessment in the marine situation (Table 1). Bioassays for ecotoxicological risk assessment of the dredged sediment include the bioavailability of the toxic chemicals in the dredged sediment and chronic toxicity focusing on ecologically relevant endpoints. These tests can be in vitro bioassays with the biologically relevant extracts of the bioavailable fraction of the contaminants, or in vivo bioassays with whole sediment, performed preferably with invertebrate species. As outlined in chapter 4 it is recommend to use cultured species instead of animals collected from the field for in vivo bioassays as this strongly reduces the standard variation in the response. In addition it is important to include relevant exposure routes in the in vivo bioassays, for example maternal transfer of PBTs from the mother animal to the offspring. The introduction of the PAF method to assess the ecotoxicological pressure of dredged material is well applicable for polluted sediments. For acute toxic chemicals for which
**Summary and conclusions**

**Table 1:** Summary of usefulness of bioassays for hazard, risk and impact assessment of dredged harbour sediments to be disposed of at sea explained in figure 1 (Boxes A, B and C). Chemical analyses are mentioned *in italics.*

<table>
<thead>
<tr>
<th>Box A</th>
<th>Useful</th>
<th>Not useful for sediments to be disposed of</th>
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| Currently used for: licensing disposal harbour sediment rapid screening | *In vitro* bioassay DR-Luc (purified extract)  
*Chemical analyses (PAHs, PCBs, toxic metals, TBT)*  
*chemical anal.* | *In vivo* bioassays with cultured or wild species |
| Possibly relevant to be added to data base | *In vitro* bioassay general toxicity (extract)  
*in vitro* bioassay thyroid hormone disruption (extract)  
(incl. bioactivation) | *In vitro* estrogenticity or androgenicity (extract)  
*Chemicals that only are toxic in high concentration (narcotics, nanoparticles)*  
*Chemical analysis of lipophilic POPs in water* |

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<th>Box B</th>
<th>Useful</th>
<th>Not useful for sediments to be disposed of</th>
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| Currently Advanced risk assessment harbour sediment before disposal | *In vivo* bioassays with cultured species  
& ecologically relevant endpoints: growth  
Development, survival, reproduction  
PAF or msPAF | *In vivo* bioassays with wild species |
| To be added in cases of indications for relevance and bioavailability | *In vivo* bioassays with relevant exposure conditions, routes and endpoints  
Prolonged ELS and metamorphosis  
*In vivo* bioassays with sediment extracts  
*In vitro* bioassays specific mechanisms  
*Internal effect levels*  
*Specific chemicals (e.g. PFACs, HBCDs, PBDEs)* | *ELS not longer than free feeding*  
*Chemicals that only are toxic in high concentration (narcotics, nanoparticles)*  
*Chemical analysis of lipophilic POPs in water* |

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<tr>
<th>Box C</th>
<th>Useful</th>
<th>Not useful local sediments</th>
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| Currently Local sediments impact assessment | Bioaccumulation and biomagnifications  
Population decline of local organisms  
*In vivo* bioassays with local sediment  
*In vitro* bioassays with local sediment (extract)  
Bioindicators  
Biomarkers  
*Bioavailability of compounds* | Black Box gene arrays  
*Nanoparticles*  
*POPs in water*  
*Narcotic effects* |
| To be added in cases of indications for relevance | Specific genomics biomarkers  
*In vitro* bioassays with bioactivation  
*In vitro* bioassays with local sediment extracts  
Local PAF or msPAF & validation  
*Body burden toxic compound*  
*Pharmaceuticals* |

Direct exposure is relevant, SSDs can be made with NOECs expressed as exposure concentration in the water. For bioaccumulating chemicals such as dioxins internal exposure is a more relevant parameter for defining the SSD for chronic effects. Using established food chain bioaccumulation and sediment to fish bioconcentration factors the NOEC can be extrapolated to concentration/kg of sediment. The concentrations of pollutants in sediment or organisms, either quantified with chemical analysis or with *in vitro* bioassays, can be interpolated in the SSDs to assess the potentially affected fraction (PAF).

To determine the actual impact of dumped harbour sediments on local ecosystems, in addition to bioassays also biomonitoring should be performed on this location, testing for example samples collected in a gradient from the dumping site or over time. The application of *in vitro* bioassays for local impact assessment needs to be tailor-made for special cases to study the impact over time and to further develop and fine-tune the selection and performance of the bioassays to be applied to the dredged sediment. When applying the proposed bioassay approach in risk management of dredged contaminated sediments, the most relevant *in vitro, in vivo* bioassays...
and bioindicators are selected for location specific impact assessment. This means that impact assessment of dredged material should involve an understanding of the ecotoxicological fate of chemicals, and include monitoring of effects with mechanistic in vitro bioassays and dedicated biomarkers to support management decisions. The implications of contamination for population development of local marine organisms can be monitored in combination with relevant bioindicators and if possible linking these outcomes to in vitro bioanalysis of toxic potencies in the sediment, food chain and in the animals themselves. The predicted ecotoxicological risk, for example expressed as PAF based on SSDs for ecological relevant endpoints, can thus be validated as well. This combination of in vitro and in vivo bioassays and bioindicators are valuable tools that complement chemical analysis in the assessment of the possible impact of disposal dredged material and sediments at specific locations in the marine environment.

Results obtained upon using additional bioassays in more advanced ecological risk assessment (Box B) and impact assessment (Box C) could eventually lead to the decision to include some additional chemical analysis or bioassay in the routine screening (Box A) to allow improved detection of toxic chemicals that may currently be overlooked but appeared to be relevant for dredged harbour sediments. Also the SQG and ALs may be updated based on the new information. It is important to stress that the new chemical analysis or bioassays to be included in the licensing system need not necessarily be identical to the tests that brought forward the yet unnoticed ecotoxicological consequences. Tests to be added for a certain endpoint need to be standardised, cheap, fast, high throughput and validated (bio)assays for that specific endpoint. Such test may still need to be developed for inclusion in the licensing system.

Conclusions

The objective of the thesis “Assessment of effects of chemical contaminants in dredged material on marine ecosystems and human health” addresses the issue of the applicability of in vitro and in vivo bioassays as tools in the process of deciding whether dredged harbour sediments can be disposed of at sea. To aid selection of the most appropriate, robust and reliable bioassay methods for assessment programmes, the predictability and uncertainty in the responses of bioassays in hazard, risk and local impact assessment for management purposes have been explored. When are bioassays useful, and when are they merely demerits and a burden? A conceptual framework is proposed for selecting bioassays to evaluate contaminated dredged material. This framework incorporates rationally chosen chemical and toxicological assessment tools depending on the purpose to achieve a cost-effective strategy to protect marine ecosystems health.

Overall the following conclusions have been drawn concerning the contribution bioassays can make to hazard, risk and impact assessment in disposal of dredged polluted sediments:

1. Bioassays using cleaned sediment extracts are much more distinctive than bioassays with whole sediment for decision making in a licensing system. This is mainly due to the interference of matrix factors with the assessed endpoint when applying whole sediment instead of cleaned extracts.

2. Mechanism-specific in vitro bioassays, e.g. for dioxin-like or estrogenic activity, can fill in specific gaps left open by chemical analysis. They may detect unknown compounds, compounds that are present in levels below the limit of detection for chemical analysis, quantify mixture effects or quantify the presence of certain classes of compounds in a more cost effective way. Although
not specific, a bioassay for general toxicity (*in vitro* or *in vivo*) can be usefully applied for sediment extracts as well, to indicate unexpected toxic compounds.

3. For hazard assessment in a licensing system chemical analysis on total sediment extracts or bioanalysis in order to quantify the bioavailability of the chemicals does not need to be included because the bioavailability will change greatly after disposal of the sediments.

4. When the outcome of the hazard assessment of dredged harbour sediment does not convincingly demonstrate whether the sediment is either clean enough or too polluted, further bioanalysis can help the decision making process. In this further testing more aspects of the mostly unknown complex chemical mixtures should be taken into account, including the bioavailability and chronic toxicity as well as the effects of the whole mixture including as yet not chemically analysed compounds. The tests to be used should focus more on ecologically relevant endpoints, and rationally chosen experimental conditions and exposure routes.

5. Aqua-cultured species are more suitable for use in *in vivo* bioassays than animals collected in the field because they produce less standard deviation in the tests. This can be due to additional stress of collecting the animals and keeping them under stressful conditions, in addition to the unknown and variable life histories, contamination and health status of the wild animals.

6. Bioindicators of effect in the field such as intersex index (ISI) and population decline in gastropods are useful to reveal causal relationships between the exposure to toxic compounds and population effects on local marine organisms. When these effects correspond to the effects predicted based on bioassays in the laboratory, for example expressed as PAF of species, these field effects in gastropods, can be used as a bioindicator for the local ecotoxicological health status.

7. To validate the predicted risk, the actual impact of dumped harbour sediments on local ecosystems can be determined using a dedicated set of *in vitro* and *in vivo* bioassays as well as bioindicators selected based on the information obtained from the hazard and risk assessment and on the characteristics of the local ecosystem.

8. Local sediment impact assessment can direct the fine-tuning of the selection of chemical and bioassay analyses and the setting of safe levels in the licensing system. This could eventually lead to the decision to include some additional chemical analysis or bioassay in the routine screening.

9. Bioassays should comply with high quality standards, and especially when applying *in vivo* bioassays for further risk assessment also exposure route and duration, test conditions and endpoints should be based on rational considerations as well as valid protocols.

10. Rationally chosen *in vitro* and *in vivo* bioassays can provide valuable contributions to the hazard, risk and impact assessment of dredged harbour sediments under consideration for disposal in the environment.
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Nederlandse samenvatting

Om te voorkomen dat scheepvaartroutes dichtsliben, moeten waterwegen en havens in deltagebieden regelmatig worden gebaggerd. Wereldwijd worden op jaarbasis honderden miljoenen tonnen baggerspecie en sediment verwijderd. Nederland verspreidt jaarlijks meer dan 25 miljoen kubieke meter gebaggerd sediment in de Noordzee. Hierbij is het van belang te kunnen bepalen wat de risico’s zijn voor het ecosysteem. Worden relevante ecologische eindpunten, zoals reproductie van organismen, verstoord? In hoeverre is blootstelling aan chemische stoffen potentieel schadelijk voor organismen? Van het merendeel van de ruim 100.000 gesynthetiseerde chemische stoffen die in het Noordzeemilieu worden aangetroffen is maar weinig bekend over hun toxiciteit.

Het in sediment identificeren van chemische stoffen die een potentieel risico vormen voor het ecosysteem of voor de menselijke gezondheid, is dan ook het onderwerp van veel studies.

Havensedimenten en baggerspecie zijn vaak verontreinigd met persistente organische verbindingen, zoals polychloorbifenylen (PCB’s), polycyclische aromatische koolwaterstof-relmers (PAK’s), tributyltin (TBT), en ook met metalen en minerale olie. In het Nederlandse kustgebied zijn de afgelopen decennia allerlei biologische effecten van chemische stoffen waargenomen. Zo veroorzaakt TBT geslachtsafwijkingen bij zeeslakken. Relatief nieuwe chemische stoffen met potentieel schadelijke effecten zijn de zogenaamde prioritaire stoffen. Hieronder vallen onder meer broomhoudende vlamvertragers, polybroomdifeny lethers (PBDE’s), perfluorinated chemicalien (PFOS/PFOA), toxische congeneren polychloor dibenzofuranen dioxinen (PCDD’s), polychloor terfenyl furanen (PCDF’s), gepolychloreerde naftalenen (PCN’s) en ftalaten. Voor al deze stoffen geldt dat zij niet standaard worden gemeten in monitoringprogramma’s. Dit terwijl kennis over deze chemische stoffen nodig is om de ecologische gevolgen van het verspreiden van baggerspecie op zee te kunnen beoordelen.

Veel landen hebben milieunormen ingevoerd om vast te stellen of gebaggerd sediment op zee kan worden verspreid. Hieraan kleven nadelen. De bestaande normen zijn uitsluitend gebaseerd op het meten van een aantal klassieke chemische stoffen in sediment. In de huidige risicobeoordelingsystemen wordt niets gedaan om de toxiciteit vast te stellen van nog onbekende verbindingen. Gezien de complexe samenstelling van verontreinigd sediment, en de potentiële aanwezigheid van stoffencombinaties die schadelijk zijn voor zeeorganismen, dient de nadruk bij het sedimentonderzoek te liggen op het meten van sedimentgeassocieerde chemische stoffen, en de toxische verbindingen. Gezien de complexe samenstelling van verontreinigd sediment, en de potentiële aanwezigheid van stoffencombinaties die schadelijk zijn voor zeeorganismen, dient de nadruk bij het sedimentonderzoek te liggen op het meten van sedimentgeassocieerde chemische stoffen, en de toxische verbindingen. Gezien de complexe samenstelling van verontreinigd sediment, en de potentiële aanwezigheid van stoffencombinaties die schadelijk zijn voor zeeorganismen, dient de nadruk bij het sedimentonderzoek te liggen op het meten van sedimentgeassocieerde chemische stoffen, en de potentiële aanwezigheid van stoffencombinaties die schadelijk zijn voor zeeorganismen, dient de nadruk bij het sedimentonderzoek te liggen op het meten van sedimentgeassocieerde chemische stoffen, en de potentiële aanwezigheid van stoffencombinaties die schadelijk zijn voor zeeorganismen, dient de nadruk bij het sedimentonderzoek te liggen op het meten van sedimentgeassocieerde chemische stoffen, en de potentiële aanwezigheid van stoffencombinaties die schadelijk zijn voor zeeorganismen, dient de nadruk bij het sedimentonderzoek te liggen op het meten van sedimentgeassocieerde chemische stoffen, en de potentiële aanwezigheid van stoffencombinaties die schadelijk zijn voor zeeorganismen, dient de nadruk bij het sedimentonderzoek te liggen op het meten van sedimentgeassocieerde chemische stoffen, en de potentiële aanwezigheid van stoffencombinaties die schadelijk zijn voor zeeorganismen, dient de nadruk bij het sedimentonderzoek te liggen op het meten van sedimentgeassocieerde chemische stoffen, en de potentiële aanwezigheid van stoffencombinaties die schadelijk zijn voor zeeorganismen, dient de nadruk bij het sedimentonderzoek te liggen op het meten van sedimentgeassocieerde chemische stoffen, en de potentiële aanwezigheid van stoffencombinaties die schadelijk zijn voor zeeorganismen, dient de nadruk bij het sedimentonderzoek te liggen op het meten van sedimentgeassocieerde chemische stoffen, en de potentiële aanwezigheid van stoffencombinaties die schadelijk zijn voor zeeorganismen, dient de nadruk bij het sedimentonderzoek te liggen op het meten van sedimentgeassocieerde chemische stoffen, en de potentiële aanwezigheid van stoffencombinaties die schadelijk zijn voor zeeorganismen, dient de nadruk bij het sedimentonderzoek te liggen op het meten van sedimentgeassocieerde chemische stoffen, en de potentiële aanwezigheid van stoffencombinaties die schadelijk zijn voor zeeorganismen, dient de nadruk bij het sedimentonderzoek te liggen op het meten van sedimentgeassocieerde chemische stoffen, en de potentiële aanwezigheid van stoffencombinaties die schadelijk zijn voor zeeorganismen, dient de nadruk bij het sedimentonderzoek te liggen op het meten van sedimentgeassocieerde chemische stoffen, en de potentiële aanwezigheid van stoffencombinaties die schadelijk zijn voor zeeorganismen, dient de nadruk bij het sedimentonderzoek te liggen op het meten van sedimentgeassocieerde chemische stoffen, en de potentiële aanwezigheid van stoffencombinaties die schadelijk zijn vo
het mogelijk om de risico’s die verbonden zijn aan de verspreiding van baggerspecie op zee te minimaliseren.

Dit proefschrift ‘Assessment of effects of chemical contaminants in dredged material on marine ecosystems health’, onderzoekt de toepasbaarheid van *in vitro* en *in vivo* bioassays en bio-indicatoren als mogelijke beoordelingsinstrument voor het vaststellen van de schadelijke effecten op zeeorganismen van complexe chemische verbindingen in sediment. Voor dit onderzoek zijn drie doelstellingen gedefinieerd:

1. het analyseren van de belangrijkste succes- en faalfactoren bij toepassing van bioassays om de schadelijkheid van baggerspecie en sedimenten te beoordelen;
2. het beargumenteren waarom bioassays van belang zijn in sedimentbeheer voor de ‘hazard’ en ecologische risicobeoordeling bij verspreiding op zee;
3. het formuleren van randvoorwaarden voor de toepassing van bioassays en bio-indicatoren die een toegevoegde waarde bieden in het sedimentbeheer bij de beoordeling van de schadelijkheid van deze sedimenten.

Hoofdstuk 2 geeft een overzicht van risicobeoordeling met behulp van biologische risicobeoordelingsmethodes die kunnen worden ingezet om de effecten van verontreiniging in het mariene en estuariene zeemilieu vast te stellen. De uitgevoerde studie naar de aanwezigheid van schadelijke stoffen in sediment op zeevissen toont aan dat er een relatie is tussen het voorkomen van chemische verontreiniging en de gezondheid van zeeorganismen. Het meten van biomarkers in de Europese bot (*Platichthys flesus*) geeft inzicht in de relatie tussen de concentraties van chemische stoffen in sediment en de gezondheid van vissen die hieraan zijn blootgesteld. De fase I en II biotransformatie-enzymen in vissen bleken in deze studie niet sterk te correleren met verontreinigingsniveaus.

Van de beproefde biomarkers zijn de succesvolste: metallothioneins in vissenlever en 1-OH pyreen in gal. Deze biomarkers reageren op door specifieke groepen van verbindingen die worden veroorzaakt door het in sediment aanwezige organotin (metallothioneins) en PAK’s (1-OH pyreen). In dit onderzoek zijn ook biologische effectmetingen uitgevoerd in sedimentextracten. Van de gebruikt methodes bleken de *in vitro*-assays DR-CALUX en ER-CALUX sterker onderscheidend te zijn dan de *in vivo*-sedimenttesten. De conclusie is de toepassing van biomarkers en bioassays op basis van het type biologisch effect van blootstelling of met een werkingsmechanisme reagerend op een specifieke stof, de succesvolle toxiciteitstesten zijn en daarom als indicator kunnen fungeren.

Hoofdstuk 3 beschrijft de voordelen van de *in vitro* DR-CALUX bioassay. Deze assay houdt rekening met de biologische beschikbaarheid en de potentiële bioaccumulatie en biomagnificatie in sedimentextracten van onbekende chemische samenstelling. De sedimentbeoordeling met de DR-CALUX bioassays is indirect verbonden met de ecologische risicobeoordeling. Het doel van dit onderzoek was het identificeren van de betrouwbaarheid en de optimale toepasbaarheid van de DR-CALUX *in vitro* bioassay voor de analyse van onder meer PAK’s in sedimentextracten. Dit met het oog op het gebruik van deze bioassay voor de beoordeling van baggerspecie. Met de DR-CALUX bioassays worden alle verbindingen met een gevoeligheid voor de Ah-receptor gedetecteerd. Om te bepalen of de DR-CALUX bioassay geschikt is bij het testen van baggerspe-ciemonsters, werd interlaboratoriumonderzoek uitgevoerd met gebruik van gestandaardiseerde protocollen en kwaliteitscriteria. De resultaten uit de DR-CALUX bioassay tonen aan dat deze
methode betrouwbaar en goed te reproduceren is voor het bepalen van dioxine en dioxineachtige activiteit in sediment. De conclusie luidt dan ook dat de DR-CALUX in-vitro bioassays robuust genoeg zijn om te worden toegepast voor de beoordeling van baggerspecie als onderdeel van wet- en regelgeving.

In hoofdstuk 4 wordt beschreven hoe een stekelhuidige, de zeeklit Echinocardium cordatum, een model vormt – als in vivo-bioassay – voor de risicobeoordeling van verontreinigd marien sediment. De zeeklit leeft in zandige gebieden van de Noordzee en kan in het laboratorium voor een langere periode worden bewaard. Het verzamelen van de E. cordatum uit de Nederlandse zeebodem is echter lastig, omdat de organismen een dichtopeengepakt op de bodem leven. Het doel van dit onderzoek: het ontwikkelen van een zeeklitweek waarmee, onder laboratoriumomstandigheden, toxiciteitstesten kunnen worden gedaan met volwassen E. cordatum. Een vergelijking van in vivo bioassays met gekweekte zeeklitten met in vivo assays met in het veld verzamelde organismen, laat zien dat het sterftecijfer bij assays met in het veld verzamelde zeeklitten sterker fluctueert. In vergelijking met uit de zeebodem verzamelde exemplaren, vertonen gekweekte zeeklitten in de in vivo bioassays een verminderde gevoeligheid voor stoffen als TBT. Bovendien zijn de overlevingskansen van gekweekte zeeklitten hoger of ten minste vergelijkbaar met die van in het veld verzamelde E. cordatum. De toegenomen gevoeligheid en verminderde vitaliteit van uit de zeebodem verzamelde zeeklitten wordt mogelijk veroorzaakt door meerdere milieustressoren. Het onderzoek toont aan dat gekweekte E. cordatum veel geschikter zijn voor in vivo bioassays. In de risicobeoordeling van baggerspecie geven gekweekte E. cordatum bij acute blootstelling aan verontreinigd sediment een kleinere standaarddeviatie en is nauwelijks sprake van multistress. Het kweken van de zeeklit E. cordatum maakt onderzoek mogelijk met organismen die een bekende levensgeschiedenis hebben, die geen infecties hebben door parasieten en niet lijden onder ecologische invloeden.

Hoofdstuk 5 beschrijft de resultaten van een geïntegreerde beoordeling met behulp van drie lijnen: toxisch, biologisch en chemisch. In het Engels zijn deze bekend als de ‘Lines of Evidence’, ofwel LOEs. Het doel van deze studie: het evalueren van de geïntegreerde ecologische risicobeoordeling op basis van LOEs, specifiek voor als instrument voor een betere beoordeling van de risico’s van TBT in de Nederlandse havens en open kustwateren. TBT-houdende, aangroeiwerende verven werden tot voor kort gebruikt op scheepsrompen, om aangroei van zeeorganismen te voorkomen. TBT is een zeer giftige chemische stof die hormoonontregeling veroorzaakt bij organismen, waaronder geslachtsverandering bij vrouwtjeszeeslakken (intersex en imposex). In de LOEs-studie waren de lijnen voornamelijk gericht op het meten van: (1) chemische concentraties TBT, (2) ecotoxicologische indicatoren en de resultaten van ecotox- icologische laboratoriumexperimenten en (3) het biologische effect intersex met de bioindicator alikruik L. littorea.

Alle drie de LOEs geven aanwijzingen over de ecologische gezondheidsstatus van de zeeslak, in relatie tot TBT-bvervuild. De onderzoeksresultaten laten zien dat hoge intersexindexniveaus (niveaus tot een maximum van 3,35) zich alleen voordoen als het voorspelde risiconiveau hoger is dan 20%. Intersex werd waargenomen in kustwateren waar het risico is berekend met een PAF-waarde van meer dan 10%. Deze resultaten wijzen erop dat ofwel de intersexindex (ISI) ISI-metingen in L. littorea niet gevoelig genoeg zijn voor de beoordeling van de gezondheid van het ecosysteem, ofwel dat het risicoprognose te conservatief is. De alikruiken zijn waarschijnlijk voornamelijk blootgesteld aan TBT die in het water is opgelost en is geadsorbeerd aan zwevend stof dat in het water circuleert. De ISI kan als een specifieke indicator fungeren voor het beleid,
om de ecologische gezondheidsstatus vast te stellen. Dit onderzoek levert het bewijs dat de ecologische toestand van een aantal Nederlandse havens slecht is, ten gevolge van TBT-vervuiling. En pleit voor het gebruik van geïntegreerde benaderingen zoals de Weight of Evidence (WOE) waarmee ecologische effecten beter beoordeeld kunnen worden.

In hoofdstuk 6 wordt een strategisch concept voorgesteld waarin in vitro, in vivo bioassays en bio-indicatoren worden toegepast voor het beoordelen van complexe chemische mengsels in baggerspecie en sedimenten. Dit om te kunnen vaststellen of deze baggerspecie en sedimenten – zonder risico’s voor zeeorganismen – op zee kunnen worden verspreid.

Voor de risicobeoordeling zijn de meest robuuste en betrouwbare bioassay-methoden onderzocht, op de toepassingsmogelijkheden alsook de voorspelbaarheid van de bioassays in sedimentbeheer. Wanneer zijn bioassays een waardevolle toevoeging? Wanneer zijn ze nutteloos? In het strategische conceptvoorstel kunnen bioassays worden ingezet op drie niveaus van toenemende complexiteit. Het meest elementaire niveau is een snelle risicobeoordeling van sediment voor het vaststellen de potentiële toxiciteit (Box A). Deze kan worden uitgevoerd met in vitro bioassays met een toxicologische werkingmekanisme voor specifieke chemische verbindingen. Op een hoger niveau van complexiteit (Box B) wordt de risicobeoordeling uitgevoerd inclusief de ecologisch relevante eindpunten en de biologische beschikbaarheid van de toxiche verbindingen. Dit om veranderingen in het ecosysteem te kunnen signaleren. Tot slot, de beoordeling van de ecotoxicologische kwaliteit van sediment en het mogelijke ecologische risico bij de verspreiding van dit sediment in het mariene ecosysteem (Box C).

Voor Box A wordt de toxische potentie van de baggerspecie vastgesteld met chemische analyses en indien gewenst met een of meer geselecteerde in vitro assays. Deze in vitro zijn snelle analyses die de belangrijkste hiaten in chemische analyses kunnen opvullen. Het vooraf bewerken van sedimentextracten voorkomt storende matrixeffecten. Voorbeelden van geschikte in-vitro bioassays zijn assays die dioxineachtige of estrogene activiteit detecteren of schildklierhormoonverstoring. In vivo bioassays in Box B zijn vooral bedoeld als aanvulling, voor het geval de uitkomst van de A-beoordeling onvoldoende duidelijk is. Bioassays in Box C zijn voor het vast stellen van ecologische risicobeoordelingen op maat. Ze zijn bedoeld om speciale situaties te onderzoeken, om biologische effecten in het ecosysteem te signaleren en, daarnaast, om de uitkomsten van Box A- en Box B-bioassays te verifiëren.

Vooral voor de in vitro-bioassays in Box A zijn standaardisatie en validatie de analysemethode van groot belang. Dit om betrouwbare en reproduceerbare resultaten te krijgen die vergelijkbaar zijn met chemische analyses. Bioassays met ecologisch relevante eindpunten en geselecteerde biomarkers moeten voldoen aan de kwaliteitsnormen, en daarnaast worden ingezet op grond van rationele argumenten.

De zeven hoofdstukken laten zien dat een rationelere benadering voor het opnemen van bioassays in de ecologische risicobeoordeling wenselijk is. Een wetenschappelijke verklaring bij het vaststellen van toxiciteit is nodig. Dit om onjuiste interpretaties te voorkomen die leiden tot het automatisch afkeuren van baggerspecie.

Bij toepassing van in vivo bioassays kunnen verstorende factoren, die multistress veroorza-ken bij organismen een mogelijke oorzaak zijn van gemeten toxische effecten.

Voor het in het zeemilieu vaststellen van een potentiële toxiciteit met sedimentbioassays is het gebruik van gekweekte zeeorganismen daarom de voorkeur boven het werken met organismen die zijn verzameld in het veld.
Het verdient aanbeveling om bij beoordelingsprocedures in wet- en regelgeving op te nemen dat *in vivo* bioassays gebruikt dienen te worden als waarschuwing- of signaleringssysteem. *De in vitro* assays DR-CALUX en ER-CALUX die zijn toegepast op sedimentextracten waren meer onderscheiden dan de *in vivo* bioassays. De toepassing van het voorgestelde conceptkader met bioassays, biomarkers en bio-indicatoren op drie complexiteitsniveaus biedt toegevoegde waarde bij de risicobeoordeling van sediment- en baggerspecie. Voor de risicobeoordeling in Box B, kunnen *in vivo* bioassays worden gebruikt, met name als de uitkomst van het assay in Box A onvoldoende duidelijk is. Voor de beoordeling van de ecotoxicologische kwaliteit van sediment en de mogelijke ecologische gevolgen bij de verspreiding van baggerspecie op zee is de toepassing van bioassays en bioindicatoren op Box C waardevol voor het voorspellen van mogelijke biologische effecten. De geïntegreerde ecologische risicobeoordeling levert, door het combineren van de resultaten uit de verschillende 3 LOEs, waardevol inzicht om de risico’s van TBT in de havens en open kustwateren beter te beoordelen. Gezien de voorbeelden die reeds beschikbaar zijn, luidt de conclusie dat zowel *in vitro* als *in vivo* bioassays een waardevolle bijdrage leveren aan risicobeoordelingen bij het sedimentbeheer van baggerspecie.
Curriculum vitae


Van 2001-2007 werkte Cor bij RWS als projectleider waterbodems bij de afdeling Onderzoek en Strategie van het Rijks Instituut van Kust en Zee (RIKZ). In de periode 2000-2006 heeft hij ecotoxicologisch onderzoek verricht naar de effecten en risico’s van bagsprespieverontreiniging bij de verspreiding hiervan in het mariene ecosysteem. Dit werk richtte zich op de mogelijkheden van beleidmatige implementatie van biologische assays voor een waterbodembeoordelingssysteem te gebruiken voor wet- en regelgeving. Dit onderzoek heeft de basis gevormd voor het voor u liggende proefschrift, dat onder begeleiding van prof. dr. A.J. Murk en prof. dr. I.M.C.M. Rietjens bij de sectie Toxicologie van Wageningen Universiteit is gerealiseerd. Sinds 2008 is Cor werkzaam als senior adviseur ecotoxicologie bij Deltares, unit Zee en Kustsystemen, afdeling Waterkwaliteit.
List of Publications

Publications related to the thesis


List of Publications


Presentations related to the subject of this thesis


Schipper C.A., and Mathijs G.D. Smit, Intersex in Littorina littorea as a measure of ecological risk from TBT exposure in Dutch marine coastal waters. SETAC North America annual meeting 2007, Milwaukee, USA.


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Dankwoord


Het feit dat dit proefschrift er ligt, is mede te danken aan de vele personen die een belangrijke bijdrage hebben geleverd aan mijn onderzoek. Ik wil mij eerst richten tot mijn promotoren Tinka Murk en Ivonne Rietjens. Beste Tinka, ik ontmoette je al in de periode negentig terwijl je zelf promotie onderzoek aan de WUR verrichtte. Jij bent de persoon geweest die mij attent deed op het vele beschikbare onderzoeksmateriaal en de wetenschappelijke ideeën een aanzet konden zijn tot a) het publiceren van meerdere manuscripten in wetenschappelijke tidschriften en b) de uiteindelijke bundeling ervan in een proefschrift. Je vertrouwen en de deskundigheid die je uitstraalde, maakte de uiteindelijke beslissing om te promoveren een logische stap voorwaarts. Grote waardering heb ik voor je aanhoudende persoonlijke betrokkenheid, van start tot afronding, gedurende het vijf jaar durende werk aan het proefschrift. De integere wijze waarop je direct of indirect feedback gaf heb ik als enorm stimulerend ervaren en is cruciaal geweest voor de totstandkoming van dit proefschrift. Beste Ivonne, ook jij stond aan de wieg van mijn onderzoek en bood gedurende het traject kritische en stimulerende begeleiding. Ik wil je daarvoor hartelijk bedanken evenals voor je, vlotte beoordeling met suggesties van de manuscripten en het vertrouwen dat je mij gaf.

Mijn inspirerende collega Martine van den Heuvel-Greve, lotgenoot op het RIKZ-veldstation Jacobahaven, op RIKZ-Arnestein, op RIKZ-Poelendaele en bij Deltares Delft, jou wil ik hartelijk danken voor de wijze waarop je de interessantste onderzoeksresultaten met mij besprak. Ik leerde van de temperamentvolle wijze waarop jij onderzoek uitvoert. Vele relativerende momenten herinner ik mij ook. Gesprekken over ecotoxicologische experimenten op het veldstation en over waarnemingen in het veld: de bruinvis die werd gesignaleerd in Jacobahaven, de manatee in Florida. Ook het gezellig kletsen over je kinderen na het weekend. Dick Vethaak wil ik hartelijk bedanken voor zijn inzet en zijn adviserende rol bij vele onderzoeks- en beleidsprojecten. Jij was het die mij in staat stelde onderzoek te verrichten op het RWS-veldstation. We hebben vele jaren met enige regelmaat samengewerkt, wat ook zichtbaar is in de vorm van geza-
Dankwoord

...
Lieve Thea, jij als mijn allergrootste ‘tijger’ zag ik soms met verwondering naar mij kijken wanneer ik weer eens vol enthousiasme mijn ‘ecotox’ verhalen over jou uitstortte. Het is vast niet altijd eenvoudig geweest om het wervelende en energetische – in één persoon vertegenwoordigd als projectleider, promovendus en sportliefhebber – goed te managen. Gelukkig bood je mij de ruimte en ondersteuning om te schakelen tussen verre reizen, proefschriftactiviteiten, sporten en inspanning/ontspanning. Zo gaf het vanuit Middelburg altijd weer een gevoel van thuiskomen in Den Haag. In de schaarse tijd die we naast al mijn activiteiten overhielden, hebben we kans gezien er mooie kleurrijke jaren van te maken. Ik ben er zeker van dat er nog vele kleurrijke zullen volgen.
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