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An Integrated Approach to the Toxicity Evaluation of Irish Marine Sediment - Chemical Assessment

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AN INTEGRATED APPROACH TO THE TOXICITY EVALUATION OF IRISH MARINE SEDIMENT- "CHEMICAL ASSESSMENT"

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Abstract

This project was a collaborative project between the Marine Institute (MI) in Galway and Radiation and Environmental Science Centre (RESC) in the Dublin Institute of Technology (DIT). In Ireland at present, sediment quality assessments are generally reliant on chemical analysis alone with limited bioassay techniques available to further characterise the sediment. Some causative agents of toxicity to biological organisms are below analytical detection limits. Integration of bioassay data with chemical analysis is essential in order to complete a full ecotoxicological assessment of the quality of the marine environment. This project describes the chemical analysis of marine sediment for persistent pollutants from selected locations around the coast of Ireland. A novel analytical technique is developed for extraction and quantification of organotins (OTCs) from sediment and for subsequent exposure onto two fish cell lines. Fish cell cultures are additionally exposed to a range of reference OTC chemicals. The method for organotin extraction is additionally utilised in a Toxicity Identification Evaluation study whereby a crude solvent extract is assayed on two biological organisms namely the Microtox® (employing the marine bacterium *Vibrio fischeri*) and the marine copepod *Tisbe battagliai* and chemically analysed. A further fractionation of the extract is then performed and further testing conducted on the organisms, therefore potentially pinpointing the source of toxicity. An *in-situ* study using caged *Nucella lapillus* and *Crassostrea gigas* to monitor TBT induced bioeffects in Irish harbours was also developed which was correlated with stable isotope ratios, condition indices and measurement of OTCs in the various biota tissues and sediment samples. This short term exposure method showed a rapid development of imposex in gastropod species and shell abnormalities in oysters at a TBT polluted location.

DECLARATION

I certify that this thesis which I now submit for examination for the award of PhD, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for an award in any other Institute or University.

The work reported on in this thesis conforms to the principles and requirements of the Institute's guidelines for ethics in research.

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Signature _____Date _____

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Abbreviations

AAS	-	Atomic Absorption Spectrometry
AB	-	Alamar Blue
AE	-	Assimilation Efficiencies
AMAP	-	Arctic Monitoring and Assessment Program
ANOVA	-	Analysis of Variance
AQC	-	Analytical Quality Control
ASE	-	Accelerated Solvent Extraction
BAC	-	Background Assessment Concentration
BC	-	Background Concentration
BDF	-	Bioassay Directed Fractionation
BRC	-	Background Reference Concentration
BT	-	Butyltin
CA	-	Cluster Analysis
CaBS	-	Capacity Building Scheme
CEMP	-	Coordinated Environmental Monitoring Programme
CG	-	Crassostrea gigas
CI	-	Condition Index
CP	-	Contracting Party
CRM	-	Certified Reference Material
DBT	-	Dibutyltin
DCM	-	Dichloromethane
DDT	-	Dichloro-Diphenyl-Trichloroethane
DIC	-	Dissolved Inorganic Carbon

DIT - Dublin Institute of Technology

DMSO - Dimethylsuphoxide

DNA - Deoxyribonucleic acid

EAC - Environmental Assessment Criteria

EC - Effective Concentration

ECD - Electron Capture Detection

EDA - Effects Directed Analysis

EOC - Elemental Organic Carbon

EQS - Environmental Quality Standard

ER - Effects Range

ER_L - Effects Range Low

ER_M - Effects Range Median

EROD - Ethoxyresorufin-O-Deethylation

FAA - Flame Atomic Absorption

FIMS - Flow Injection Mercury System

FID - Flame Ionisation Detection

FPD - Flame Photometric Detection

GC- - Gas – Chromatography

GC-DC-PFPD- Gas Chromatography Dual Channel Pulsed Flame Photometric Detection

GFAA - Graphite Furnace Atomic Absorption

GSI - Geological Survey of Ireland

HC - Hydrocarbon

HCB - Hexachlorobenzene

HELCOM - Helsinki Commission

HEPES	-	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	-	High Performance Liquid Chromatography
HSP	-	Heat sensitive Proteins
IAEA	-	International Atomic Energy Agency
IBR	-	Integrated Biomarker Index
ICES	-	International Council for the Exploration of the Sea
ICP-MS	-	Inductively Coupled Plasma Mass Spectrometry
ICP-OES	-	Inductively Coupled Plasma Optical Emission Spectroscopy
IFE	-	Institute for Energy Technology
IR	-	Integrated Response
ISQ	-	Irish Sediment Quality Guideline
JAMP	-	Joint Assessment and Monitoring Guidelines
K_{OC}	-	Partitioning Rate
LC	-	Lethal Concentration
LoD	-	Limit of Detection
LOEC	-	Lowest Observed Effect Concentration
LOI	-	Loss on Ignition
LoQ	-	Limit of Quantification
MAC	-	Maximum Allowable Concentration
MBT	-	Monobutyltin
ME	-	Mytilus edulis
MFD	-	Marine Framework Directive
MI	-	Marine Institute
MIP-AES	-	Microwave-Induced Plasma Atomic Emission Spectrometry
MRG	-	Metal Rich Granules

MS	-	Mass Spectrometry
MTLP	-	Metallothionein-like proteins
MT	-	Metallothionein
NL	-	Nucella lapillus
NOAA	-	National Oceanic and Atmospheric Administration
NOEC	-	No Observed Effect Concentration
NR	-	Neutral Red
OC	-	Organochlorine
OT	-	Organotin
OTC	-	Organotin Compound
OM	-	Organic Matter
OSPAR	-	Oslo and Paris Commision
PAH	-	Polycyclic Aromatic Hydrocarbons
PBS	-	Phosphate Buffered Saline
PCA	-	Principle Components Analysis
PCB	-	Polychlorinated Biphenyl
PEL	-	Probable Effect Level
PFPD	-	Pulsed – Flame – Photometric – Detection
PLHC – 1	-	Poeciliopsis lucida hepatocellular carcinoma (cell line)
PNEC	-	Predicted No Effect Concentration
POC	-	Particulate Organic Carbon
POM	-	Particulate Organic Matter
POP	-	Persistent Organic Pollutant
PSD	-	Particle Size Distribution
PVC	-	Poly vinyl chloride

QUASH	-	Quality Assurance of Sample Handling
QUASIMEME	-	Quality Assurance of Information for Marine Environmental Monitoring in Europe
RESC	-	Radiation and Environmental Science Centre
RPSI	-	Relative Penis Size Index
RSD	-	Relative Standard Deviation
RTG – 2	-	Rainbow Trout Gonad (cell line)
SEC	-	Size Exclusion Chromatography
SEM	-	Standard Error of the Mean
SI	-	Stable Isotope
SIM	-	Single Ion Monitoring
SQG	-	Sediment Quality Guideline
STEB	-	Sodium Tetraethylborate
TBT	-	Tributyltin
TBA	-	Tetrabutylammonium
TIE	-	Toxicity Identification Evaluation
TOC	-	Total Organic Content
TPT	-	Triphenyltin
UCM	-	Unresolved Compound Mixture
UK	-	United Kingdom
UNEP-MAP	-	United Nations Environment Program – Mediterranean Action Plan
US-EPA	-	United States Environment Protection Agency
USGS	-	United States Geological Survey
VDS	-	Vas Deferens Sequence

VDSI	-	Vas Deferens Sequence Index
WFD	-	Water Framework Directive
WGMS	-	Working Group on Marine Sediments
WHO	-	World Health Organisation
WKIMON	-	Workshop on Integrated Monitoring of contaminants and their effects in coastal and open-sea areas
WOE	-	Weight-of-Evidence
WP	-	Work Package

Scope of this thesis

The presence of anthropogenic pollutants throughout all compartments of the marine environment has been of national and international concern for a number of decades. A great number of regional and international contaminant monitoring programs currently exist with primarily goals to: identify pollutants of immediate concern and to assess temporal and spatial aspects of contamination, usually with the ultimate aim to reduce contaminant inputs and to minimise impacts of pollution on the marine environment.

Sediments play an important role for the distribution and dynamics of pollutants in the aquatic environment. They ultimately can act as a sink for anthropogenic pollutants. In Ireland, when an application for disposal of dredged material is made, a chemical assessment of sediment must be performed. At present, sediment quality assessments rely on chemical analysis alone with limited bioassay techniques available to further characterise the toxicity of the sediment. Some causative agents of toxicity are below analytical detection limits. Integration of bioassay data with chemical analysis is essential for full ecotoxicological assessment of the quality of the marine environment. Organisations such as the Oslo and Paris Commission (OSPAR) and International Council for the Exploration of the Sea (ICES) support integrated approaches for monitoring persistent pollutants and their effects in the marine environment and open-sea areas.

This was a three and a half year collaborative project between the Marine Institute (MI) in Galway and Radiation and Environmental Science Centre (RESC) in the Dublin Institute of Technology (DIT). The aim of this particular thesis was to implement the chemical assessment component and ultimately to integrate chemical results with biological data.

Chapter 1 introduces the need for an integrated approach and outlines the current monitoring programmes in place by organisations supporting integrated approaches such as OSPAR/ICES. Other legislative frameworks are described such as the Water Framework Directive under which there is a requirement for substantial monitoring of priority and relevant pollutants in transitional and coastal waters to achieve "good ecological status by 2015". The ecotoxicological significance of marine sediment monitoring is discussed in addition to the role of biomarkers and sediment toxicity bioassays. Pollutants relevant to this thesis are further discussed including their sources, chemical properties and ecotoxicological relevance. Toxicity Identification Evaluations (TIEs) are briefly overviewed and discussed. The evaluation of sediment quality using the United States Environmental Protection Agency (US-EPA), OSPAR and Irish Sediment Quality Guideline (ISQ) approaches including the development of effects range levels and derivation of background assessment criteria are described for use in subsequent chapters. This chapter includes an introduction to normalisation procedures which are utilized throughout this thesis. A sediment assessment approach known as the "Fullmonti" which is currently being developed at OSPAR is also described.

The selection of sites for determinations of concentrations of organic and inorganic contaminants in surficial sediments from three coastal locations around Ireland i.e. at Dublin Port and the inner Dublin Bay, Dunmore East and Omey Island is described in **Chapter 2** of this thesis. This analysis was completed to classify extent of contamination at sites selected for cell line assaying, imposex determinations, genotoxicity, and for the bioassay test battery employed. It was also completed to demonstrate some spatial aspects at the sites for example, to see if pollution levels differed upstream, but mainly to assess the

level of contamination from urban inputs/industrial activities etc. The chemical assessment of sites is performed by comparing dry weight and normalised concentrations of contaminants with OSPAR background assessment criteria, NOAA ER_L/ER_M levels and Irish Sediment Quality (ISQ) guideline upper and lower action levels. The data generated in this chapter shall contribute and be the basis of an integrated assessment. Integration of this data with a selected battery of bioassays and biomarkers is further addressed in later chapters.

The sources, physical and chemical properties, endocrine disrupting properties, analytical methods as well as legislation of organotins are further discussed in **Chapter 3**. This chapter describes the optimisation and validation for a method which extracts organotin compounds in their chloride form for subsequent exposure to biological organisms and for separation and quantification of organotin species in marine sediment. Validation of methodology is discussed including the following parameters: accuracy, precision, specificity, linear range, repeatability, reproducibility and limits of detection/quantification.

Chapter 4 reports on the toxicity evaluation of four organotin compounds in the rainbow trout gonad cell line (RTG-2) and toxicity of a solvent extract to both the RTG-2 cell line and the topminnow hepatocellular carcinoma cell line (PLHC-1) integrated with chemical analysis. The extraction of organotin compounds (OTCs) in their salt form from marine sediments for exposure to fish cell cultures is reported. Organotin compounds derivatisation followed by gas-chromatography-pulse-flame-photometric-detection (GC-PFPD) and quantitation is described. Prior to sediment extract exposure, the toxicities of four individual organotin compounds were established with the RTG-2 cells as this data was not previously available in the peer reviewed literature.

Chapter 5 reports on an *in-situ* study using transplanted *Nucella lapillus* and *Crassostrea gigas* species to monitor TBT-induced biological effects and the integration of the resulting data with chemical concentrations in the tissues of the biotic species and in sediment. The degree of imposex in the gastropod as measured by the vas deferens sequence index (VSDI) and Relative Penis Size Index (RPSI) and the extent of shell thickening in the oysters was investigated at t=0 and t=18 weeks. Stable isotope ratios of carbon $\delta^{13}C$ and nitrogen $\delta^{15}N$ are also reported in this study to provide information on predator/filter feeding activities and relative trophic status for the caged species. The application of cost effective caging techniques in potential organotin/TBT hotspot locations to complete integrated biological effects and chemical measurements in the absence of resident gastropod populations is discussed.

In **Chapter 6** metal uptake rates and rapid biotic accumulation of metal levels as demonstrated in the test species is discussed. This study is further described as a valid tool for bio-monitoring in metal impacted areas.

In **Chapter 7** of this thesis, a bioassay directed fractionation procedure is developed and described whereby organotins and other anthropogenic compounds are extracted from the bulk sediment, fractionated and analysed using a variety of analytical techniques, and these solvent extracts are exposed to two test organisms namely *Vibrio fischeri* and *Tisbe battagliai*. The analytical and biological procedures and results are further described including analytical methodology employed for the identification of toxic fractions. Mixture toxicity is discussed in terms of species sensitivity differences to both crude contaminant extracts and fractionated extracts. An insight into the contaminants present in

the sediment is provided and discussed and concluded to be a very useful technique for sediment quality assessments.

Chapter 8 of this thesis reports on the use of a scoring system developed with indices which allows for comparison of bioassay, biological effects, biotic and sediment chemistry data from a number of sites described in previous chapters. Generation of an integrated biomarker response "IBR" type index is described for use in future sediment quality assessments.

In summary this thesis reports on a wide range of integrated techniques developed to expand Ireland's capacity for integrated monitoring.

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CHAPTER 1-GENERAL INTRODUCTION

1.1 General Introduction

The marine environment receives inputs of hazardous substances from polluted rivers, direct discharges and atmospheric deposition (amongst others), becoming the ultimate repository for a variety of persistent chemicals. In environments where organisms are exposed to a cocktail of potentially harmful substances, increases in disease prevalence and potentially adverse effects on population growth, reproduction and survival may often result. Thus there is an increasing requirement for the availability of analytical (and biological assessment) methodologies that allow for the detection of anthropogenically-induced changes to individual compartments of our marine ecosystem.

Sufficiently sensitive, selective, and robust analytical and assessment "tool-kits" need to be in place in order for scientists and policy makers to advise on appropriate remedial responses and choose regulatory action to further protect the marine environment and its resident organisms. Difficulties can often arise in the development of techniques that allow for the 'health' of the marine environment and of its individual components to be assessed, however a number of such tool-kits are either routinely utilised or are under development in a variety of fora; these are discussed in greater detail below. Few data are available for integrated monitoring in Ireland. At present, assessment and monitoring of sediment quality in Ireland is predominantly reliant on chemical analysis, as limited biological techniques are available to further characterise the sediment.

1.1.1 Monitoring and assessment - the need for an "integrated" approach

Biological responses to chemical exposure may be observed when the causative substance is below current chemical analytical detection limits; e.g. the development of imposex in

gastropods due to tributyltin (TBT). In this case the development of appropriate tools ultimately has led to mandatory monitoring of TBT-specific biological effects under the Oslo Paris Commission (OSPAR) Coordinated Environmental Monitoring Programme (CEMP).

OSPAR has obligations to monitor the quality of the marine environment and its compartments (water, sediments, and biota), the activities and inputs that can affect that quality, and to assess impacts of contaminants on the marine environment as a basis for identifying priorities for action. OSPAR, together with the Helsinki Commission (HELCOM), has agreed on an ecosystem approach to managing the marine environment, and to understanding and assessing impact of human activities.

The original OSPAR Joint Assessment Monitoring Programme (JAMP) guidelines for monitoring contaminants in biota and sediment or biological effects do not provide guidance for the optimum approach to monitoring to support the "integrated" assessment of concentrations and effects of contaminants across the OSPAR Maritime Area. However, the guidelines do contain references to supporting measurements (chemical data, physical data, biological data) which aid the interpretation of monitoring data. Consequently, chemical and biological effects data have usually been collected, reported and assessed separately by individual Contracting Parties (CPs). Contracting parties are countries or states which are involved in the mandatory monitoring of contaminants/biological effects as agreed by OSPAR.

Integrated monitoring and assessment of contaminants and their effects will contribute more effectively to the integrated assessment of the full range of human impacts on the quality status of the marine environment as part of such an ecosystem approach.

1.1.1.1 The OSPAR Hazardous Substances Strategy

The objective of the OSPAR Hazardous Substances Strategy (OSPAR Commission, 2003) is to prevent pollution of the maritime area by continuously reducing discharges, emissions and losses of hazardous substances, with the ultimate aim of achieving concentrations in the marine environment near background values for naturally occurring substances and close to zero for man-made synthetic substances. The Hazardous Substances Strategy further declares that the Commission will implement this Strategy progressively by making every endeavour to move towards the target by the year 2020 (OSPAR Commission, 2003).

OSPAR has developed a Joint Assessment and Monitoring Programme (JAMP) providing the basis for the monitoring activities undertaken by Contracting Parties to assess progress towards achieving OSPAR objectives (Figure 1.1). In relation to hazardous substances, the JAMP seeks to address the following questions:

1) In marine environments, what are the concentrations and effects of hazardous substances on the OSPAR List of Chemicals for Priority Action ("priority chemicals")? 2) Are they at, or approaching, background levels for naturally occurring substances and close to zero for man made substances?

3) Are there problems emerging related to the presence of hazardous substances in the marine environment? In particular, are any unintended/unacceptable biological responses, or unintended/unacceptable levels of such responses, being caused by exposure to

hazardous substances? OSPAR uses the Coordinated Environmental Monitoring Programme (OSPAR, 2005) which requires temporal and spatial monitoring of quality assured data for a range of contaminants, and effects measurements, to answer the above questions.

OSPAR is involved in the ongoing development of criteria such as Background Concentrations (BCs), Background Assessment Concentrations (BACs), and Environmental Assessment Criteria (EACs) for contaminants in sediments, biota and sea-water OSPAR is also developing assessment criteria for TBT-specific biological effects; this effort will provide "tool-kits" by which the "health status" of the ecosystem can be assessed. A number of the OSPAR developed assessment criteria are relevant to this project and will be discussed throughout the thesis.

1.1.1.2 Legislative frameworks

It is widely accepted that chemical monitoring is no longer sufficient to assess pollution impacts in the marine environment. Both chemical and biological monitoring are recommended by OSPAR, the Workshop on Integrated Monitoring of Contaminants and their Effects in Coastal and Open-Sea Areas (WKIMON) and within the Water Framework Directive (WFD). The WFD (WFD2000/60/EC) has a requirement for monitoring of priority and relevant pollutants in transitional and coastal waters to achieve "good ecological and chemical status" by 2015. While compliance of priority substances with Environmental Quality Standards (EQS) will be the basis of assessing chemical status compliance, integrated monitoring will be an extremely important tool in assessing water quality, for instance in investigative monitoring, to ensure effective management decisions

are taken. Integrated chemical and biological effects monitoring will be an essential assessment approach within the Marine Framework Directive (MFD), and will build on the activities of the International Council for the Exploration of the Sea (ICES, 2004) and OSPAR.

To further support the OSPAR goals outlined above, integrated approaches to monitoring contaminants in the marine environment and the biological responses to the presence of hazardous substances are required. Such approaches provide greater interpretative power in assessments of the state of the OSPAR Maritime Area with respect to hazardous substances and an improved assessment of progress towards achieving the objectives of the OSPAR Hazardous Substances Strategy.

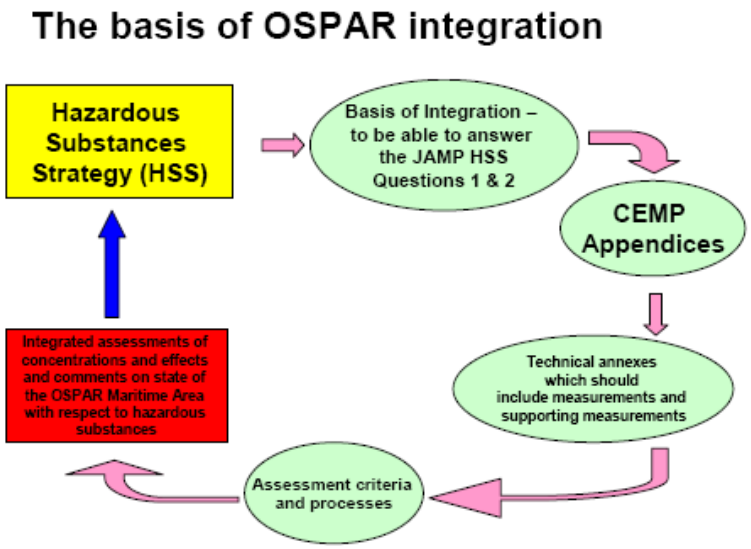

Figure 1.1: The basis of OSPAR integration (reproduced from WKIMON, 2007)

In recent years International HELCOM, OSPAR, the Arctic Monitoring and Assessment Program (AMAP) and the United Nations Environment Program Mediterranean Action Plan (UNEP-MAP) have increased their focus on the integration of both chemical

measurement and biological effects monitoring as an important element in linking contaminants and ecological responses and in assessing the overall quality of the marine environment in accordance with advice of ICES. The ICES WKIMON working groups' primary aims are to provide guidance on integrated chemical and biological effects monitoring within the OSPAR area, with special reference to the Coordinated Environmental Monitoring Programme (CEMP) issues and the list of OSPAR priority chemicals.

WKIMON initiatives, e.g. the integration of datasets, the development of analytical and biological effects capabilities, and the application of assessment criteria to analytical data form a major focus for this thesis and will be described in detail throughout.

1.1.2 "Integration approaches"

An integrated approach to monitoring is based on the simultaneous measurement of contaminant concentrations (in biota, sediments and/or water), biological effects (species/population/individual) parameters, and a range of physical and other water quality measurements (e.g. TOC and temperature) so as to permit data normalisation and ultimately enable assessment against criteria. The availability of enhanced datasets provides assessors with information on measurements of related concentrations and effects and on the environmental variables which influence the effects measurement.

Integration of chemical and biological effects measurements increases the interpretive value of the individual measurements and assists in the assessment of the significance (and potentially of "hot-spot" locations). Combining such surveys can additionally be more cost

effective as greater datasets are concurrently collected and can be directly compared using tools to provide integrated assessments.

Selected parameters/methodologies must be able to separate contaminant-related effects from other factors (e.g. natural variability). The methods used should ideally have an ability to predict effects on "ecosystem health". They should be sensitive to contaminants, i.e. provide "early warning".

In order to develop an integrated approach, measurements from a number of individual assessments must be simultaneously collected and then resulting datasets assessed together. A wide range of compartments (fish disease status, benthic community analysis, *in-vitro* bioassays, *in-vivo* biological effects studies, tissue and/or water/sediment contaminant levels etc.) may all provide information useful in describing the health status of an ecosystem. This thesis is primarily focused on the latter four compartments, each of whose significance is further discussed below and/or throughout the thesis.

1.1.2.1 Sediments and ecotoxicological significance

Sediment is an integral component of aquatic ecosystems, providing habitat, feeding, spawning, and rearing areas for many aquatic organisms. Depending on their physico-chemical properties as well as geochemical and hydrodynamic conditions, sediments can act as a contaminant source or sink. Dredging or other recirculation operations can disturb sediments and thus mobilise contaminants. Sediment can also serve as a reservoir for pollutants and therefore may become a potential source of pollutants to the water column, organisms, and ultimately human consumers of those organisms.

All pollutants can arise from a number of anthropogenic sources, including municipal and industrial discharges, urban and agricultural runoff, atmospheric deposition, port operations, sewage discharges and poor environmental management ultimately ending up in sediments in harbours and ports. Contaminated sediment can cause lethal and sub-lethal effects in benthic (sediment-dwelling) and other sediment-associated organisms. Sediments provide essential habitat for many freshwater, estuarine, and marine organisms. Furthermore, some sediment pollutants can bioaccumulate through the food chain and pose health risks to wildlife and human consumers even when sediment-dwelling organisms are not themselves impacted (US-EPA, 2007).

Sediments are particulate matter which can be transported by fluid flow and are eventually deposited on the bottom of seas, oceans and lakes over time. Sediments can act as an ultimate sink for recalcitrant pollutants which reside in the marine ecosystem. They play an important role for the distribution and dynamics of pollutants in the aquatic environment.

Chemicals in sediments can be responsible for ecotoxicological and adverse ecological effects (Ho et al. 2002). Since sediment is a heterogeneous medium, organisms living within the sediment can be affected by different degrees of contamination when in contact with different compartments. The various phases which are biologically tested include whole sediment, interstitial water, elutriate and organic extracted contaminants the latter being the 'worst case scenario'. In general, the chemical analysis for the suite of contaminants is conducted on the < 2 mm and < 0.063 mm fractions of sediment. The < 2 mm fraction is often deemed to be indicative of the bioavailable fraction of contaminants however some contaminants have an affinity for smaller particles and the investigation of the differences in concentrations of pollutants in both fractions would be a more informative approach.

Assessments of sediment quality commonly include analyses of anthropogenic contaminants, benthic community structure, physicochemical characteristics, and direct measures of whole sediment and pore water toxicity with a variety of approaches to overall assessment in use or under development, two assessment approaches are further described in section 1.4.

At present, in Ireland, limited techniques are available to undertake a complete bio-monitoring programme. Through this project, skills in both the chemical and biological analysis are developed, enabling measurement of the impact of various contaminants on the marine environment. As no single bioassay can detect all potential biological hazards, a test battery approach which incorporates a multi-trophic, multi-exposure phase assessment approach is used in this project in order to characterise Irish Marine sediments. In addition

selective and sensitive analytical chemistry methodology needs to be in place to determine environmental concentrations of a wide variety of contaminants.

1.1.2.2 A role for "bio-markers/bioanalysis"

The production and use of chemicals has historically led to contamination of the marine environment. Typically, monitoring and assessment of contaminant residues in the marine environment has focused on chemical concentration measurement of a limited number of contaminants (e.g. hydrocarbons, pesticides, heavy metals and various other pollutants etc.) with the specific aim of determining their spatial distribution and temporal trends. It is now recognised that chemical analysis is time consuming and expensive and therefore timelines and budgets limit the true chemical characterisation of the sediment. Also, chemical analysis of pollutants, provide information on ecosystem health and rarely address the actual impacts of contamination on ecosystems. Additionally, the interaction of complex factors, such as bioavailability and the combined effects of cocktails of chemicals are not adequately assessed by such approaches.

Any suite of monitoring techniques must span the range of ecological complexity from sub-organism level to populations and ecosystems. Measurement of biochemical markers or 'biomarkers' (biochemical and /or physiological changes in organisms exposed to contaminants) in individual organisms *in situ* can provide sensitive and specific early warning signs of biological stress in response to pollution. In contrast, measurements at a broader ecosystem scale may be insufficiently sensitive or unable to discern contributory cause-effect relationships.

"Bioanalyses" are tools that establish the ecological relevance of aquatic pollution, as they enable the effects of complex mixtures to be monitored. Bioanalyses can measure combined effects (additive, synergistic, antagonistic) and assist in identification of problems and risks relating to incidents. Bioanalyses can thus establish a relationship between pollutants and ecological effects. Additionally bioanalyses provide data for the precautionary principle approach of the WFD, which is monitoring of priority and relevant pollutants in transitional and coastal waters to achieve "good ecological and chemical status" by 2015, and also for other legislative requirements to be put into practice.

Few data exist in Ireland to complete an integrated chemical and biomonitoring programme in accordance with our OSPAR commitments. Selecting and improving the capacity to complete such a programme is the primary focus of this thesis. This project integrates sediment toxicity testing using a suite of standardised bioassays (Macken, 2007) with chemical analysis of sediment and biological samples for a suite of inorganic and organic contaminants. This project proposes the use of biomarkers using an *in situ* study with caged *Nucella lapillus* and *Crassostrea gigas* to monitor bio effects but also a multi-trophic range of bioassays covering the range of bio-complexity and which in addition, offer the potential by which specific contaminants can be identified. The ecotoxicological tools used by Macken (2007) in this project are outlined in Table 1.1.

Table 1.1 Summary of battery of bioassays implemented for the ecotoxicological evaluation of Irish marine sediment (Macken, [PhD thesis] 2007)

Trophic level	Species	Test	Duration	Endpoint	Sediment Assessed	Phase				Reference
						PW	AE	OS	WS	
Bacteria	*V. fischeri*	Microtox Acute test	5, 15, 30min	Reduction in bioluminescence		*	*	*		Stronkhorst et al., 2003
	V. fischeri	Microtox Basic SPT/SPT	5/20 min						*	Kwan and Dutka, 1992
Microalgae	*T. suecica*	Growth inbibition	72h			*	*			Walsh et al., 1985
Copepoda	*T. battagliai*	Acute Test	24h & 48 h	Mortality		*	*			Thomas et al., 2003
Amphipoda	*C. volutator*	Acute Test	10d	Mortality					*	Bat and Raffaeli, 1998
Cell culture	*RTG-2*	AB/NR	24 and 96h	Reduction in fluorescence		*	*			Davoren et al., 2005
	PLHC-1	AB/NR	24 and 96h	Reduction in fluorescence		*	*			Davoren et al, 2005

PW= Porewater, AE= Aqueous Elutriate, OS= Organic Solvent, WS= Whole Sediment, SPT= Solid Phase Test, AB= Alamar Blue, NR= Neutral Red

1.2 Pollutants relevant to this study

It is not the purpose of this thesis to conclusively review the physico-chemical and/or toxicological potential of the vast array of pollutants that may occur in the marine environment. "Key/priority" pollutants and those identified through WKIMON and OSPAR priority pollutants listings, provide the primary focus of this study. Summary reviews of contaminant groupings relevant to this study are presented below and are further described within individual chapters in the thesis as appropriate.

1.2.1 Toxic elements

Heavy metals exist naturally in sediment and some are essential elements for living organisms however mining, industrial and agricultural anthropogenic inputs can elevate concentrations above natural background levels (Saari et al 2007; Osán et al., 2007; Ghrefat and Yusuf 2006; Osher et al., 2006; Ip et al., 2004). Heavy metals in bottom sediments form a potential hazard to water quality and aquatic life (Förstner, 1979). Elements of concern include mercury, lead, cadmium, copper, zinc, arsenic, nickel and chromium. Ionic mercury has been found to have immunotoxic effects on the blue mussel (*Mytilus edulis*) (Duchemin et al., 2008); a toxic effect was also observed from methylmercury and mercury chloride at various concentrations exposed to Crassostrea gigas haemocytes (Gagnaire et al., 2004). Inorganic copper compounds have been found to elicit toxicity to rainbow trout gill cells (Bopp et al., 2008). A variety of elements have been found to be toxic to the bacteria *Vibrio fisheri* in the following order: copper > chromium (VI) > mercury > cadmium > zinc > chromium III > nickel > lead > arsenic (Hsieh et al., 2004). Dissolved metals in porewaters are more bioavailable and toxic than particulate metals (Atkinson et al., 2007).

1.2.2 Organochlorine compounds

Halogenated compounds such as polychlorinated biphenyls (PCBs) and organochlorine compounds (OCs) are persistent man made compounds and are of specific concern due to there toxicological and carcinogenic properties (Galanopoulou et al. 2005). PCBs consist of 209 congeners differing in the number and position of chlorine atoms on the two coupled phenyl rings. Their commercial use in dielectric fluids in capacitors and transformers, hydraulic fluids, lubricating and cutting oils, additives in paints, adhesives, sealants and plastics was based mainly on properties such as chemical stability, low flammability, and electrical insulating properties (El-Kady et al., 2007). Polychlorinated biphenyls have entered the marine environment by leakage, discharge, recycling, transboundary influx via major rivers and long-range atmospheric transport (Van Wezel et al., 2000). PCBs possess a low water solubility, a high *n*-octanol/water partition coefficient, and a high persistence (Geyer et al., 1984), particularly those which are highly chlorinated. Many toxic responses have been found with the co-planar dioxin-like PCBs as well as the non co-planar PCBs (Van Wezel et al., 2000). Within the 209 PCB congeners, the number and location of the chlorine atoms attached to the biphenyl molecule determine the potency and nature of toxicity of each PCB (Fadhel et al., 2002). Multiple *ortho*- substituted PCBs (non co-planar PCBs) have effects such as reproductive toxicity, promoter activity, neurotoxicity, effects on vitamin A metabolism and alterations in thyroid hormone levels (Van Wezel et al., 2000). The chlorinated pesticide, Dichloro-Diphenyl-Trichloroethane (DDT), was used as an insecticide and its use is now banned in Ireland however this pesticide is still in use in some countries. Environmental problems from the use of many halogenated chemicals such as DDT began to surface during the 1950s and 1960s (Carson, 1967; Pikkarainen,

2007). Although use of these compounds has been banned for many years, their resistance to degradation makes them prone to persist for years in marine sediment in Ireland and in other nations (Geyer et al., 1984).

1.2.3 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are another group of chemicals that can be present in marine sediment. They are hydrocarbons composed of two or more fused benzene rings and almost never occur alone and are usually present as complex mixtures having a wide range of toxicological effects on aquatic organisms. PAHs originate from three main anthropogenic sources: fossil fuels (petrogenic), burning of organic matter (pyrogenic) and conversion of natural organic precursors in the environment by rapid chemical/biological processes (biogenic) (Neff et al., 2005). These compounds can enter the marine environment from industrial sources, adsorb to sediments and persist (Grundy et al., 1996). Some PAHs and their derivatives effect Deoxyribonucleic Acid (DNA) by inducing mutation effects and have carcinogenic properties (Bihari et al., 2006), some PAHs have also been found to elicit toxicity in the green algae *Scenedesmus subspicatus* (Djomo et al., 2004). Many PAHs are acutely toxic to fish and other aquatic organisms in the presence of environmentally realistic intensities of solar ultraviolet radiation (Choi and Oris, 2003). However aliphatic hydrocarbons may also be a potential hazard to aquatic species, and it has been reported that low aromatic content oil can be more toxic than oil containing high concentrations of PAHs. Polycyclic aromatic hydrocarbons are not necessarily always the cause of major toxicity (Barron et al., 1999) from petroleum oil exposure.

PAHs are of interest to this study as the sampling strategy included sites within port areas (Dublin and Dunmore East) potentially subject to PAH pollution pressures.

1.2.4 Organotin compounds

Organotin compounds (OTCs) enter the marine environment through a number of sources such as biocides, fungicides, insecticides, polyvinylchloride (PVC) stabilisers, industrial catalysts and wood preservatives (Díez et al., 2005). Tributyltin (TBT) being one of the most toxic contaminants found in the marine environment is used as a paint additive to prevent biofouling (growth of aquatic organisms on ship hulls) TBT leaches from the paint resulting in pollution of harbours, ports and coastal areas (Fent, 1989). The degradation products of TBT, dibutyltin (DBT) and monobutyltin (MBT) are used as stabilisers in PVC production (Fent, 1989). In 1987, Ireland banned the use of all organotin containing compounds on vessels less than 25m being one of the first countries to do this (Minchin, 2003).

Organotin compounds are organic derivatives of tetravalent tin and may be represented by the general formula $R_pSnX_{(4-p)}$ where R is an alkyl or aryl group (e.g. methyl-, butyl-, ethyl-, phenyl-) and X is an anionic group such as a halide, oxide or hydroxide (Hoch, 2001). TBT exists in seawater as three species, hydroxide, chloride and carbonate (WHO, 1990). The nature of the anionic group, pH, temperature, and ionic strength influences the physico-chemical properties and more relevantly the solubility in water and non-polar solvents. In general, the solubility of OTCs in water increases as the number and length of the organic substitutes decrease. In aqueous solution, OTCs exist as cations below pH 4 or as neutral hydroxides above pH 5 (Weidenhaupt et al., 1997).

The clay fraction of sediment particulate matter plays a key role in the distribution and fate of OTCs in the marine environment. The absorption of OTCs to sediments occurs

primarily by reversible formation of complexes between the tin atom and carboxylate and phenolate ligands present in Particulate Organic Matter (POM). If undisturbed, the release of OTCs from deeper sediments is slow, however storms or dredging activities can increase the potential of these contaminants being distributed in the overlying water column (Berg et al., 2001). The presence of OTCs in sediment and overlying water makes OTCs available to multi-trophic organisms residing in the various compartments of the marine environment. Aquatic organisms accumulate OTCs through bioconcentration and biomagnification pathways (WHO, 1990). Bioconcentration is the accumulation of OTCs in an organism by absorption from the sediment or water phase irrespective of any intake with food, whereas biomagnification is the increase of the level of OTCs that occurs within an organism as the result of consuming other OTC contaminated species.

Degradation of OTCs occurs through successive losses of organic groups from the tin atom. For example, TBT is debutylated to its di- and mono- metabolites, DBT and MBT. Strong ultraviolet radiation has been shown to be the fastest route of degradation of OTCs in water; microorganisms such as bacteria or microalgae are also capable of degrading TBT. The kinetics of biodegradation depends on conditions such as temperature, dissolved oxygen, pH, the level of mineral elements, and the presence of easily biodegradable organic substances. Biodegradation also depends on the concentration of TBT being lower than the toxic threshold for the bacteria (WHO, 1990).

Table 1.2 Physical and chemical properties of organotin compounds

	TBT-Cl	DBT-Cl_2	MBT-Cl_3	TPT-Cl	DPT-Cl_2
CAS name	Tributyltin chloride	Dibutyltin chloride	Butyltin trichloride	Triphenyltin chloride	Diphenyltin dichloride
CAS number	1461-22-9	683-18-1	1118-46-3	639-58-7	1135-99-5
Molecular formula	$C_{12}H_{27}ClSn$	$C_8H_{18}Cl_2Sn$	$C_4H_9Cl_3Sn$	$C_{18}H_{15}ClSn$	$C_{12}H_{10}Cl_2Sn$
Molecular weight	325	303.8	282.2	385.5	343.8
Appearance	Liquid-clear	Solid-colourless	Liquid-clear	Solid faintly beige	Solid white
Boiling point (ºC)	140	135	93	240	333-337
Melting point (ºC)	-16	37	**	103	41-43
Relative density g/cm3 (20ºC)	1.2	1.37-1.4	1.693	**	**
Solubility in water (20ºC)	**	soluble	**	40 mg/litre *	**

*: pH not given

**: data not available

TBT has been linked to reductions in meat weight, increased shell thickness and distortion. In Pacific oysters (Minchin, 2003), TBT has been linked to imposex in the dogwhelk *Nucella lapillus* at concentration levels as low as 1ng/L with severe cases resulting in sterilisation of the organism (Bryan et al., 1986; Gibbs and Bryan, 1986; Bryan et al., 1987). TBT has also been found to be toxic to marine molluscs and copepods have been reported to show an LC_{50} from 0.6 to 2.2µg l^{-1} (WHO, 1990). OTCs are of interest in this study as the sampling strategy included Port based sites, potentially subject to OTCs pollution pressures.

1.3 Toxicity Identification Evaluation (TIE)

Toxicity Identification Evaluation (TIE) procedures attempt to identify the class of chemical which is responsible for toxicity by applying a combination of toxicity testing and chemical manipulations which narrows down and pinpoints the source(s) of toxicity.

Another type of TIE approach is bioassay directed fractionation (BDF) whereby a solvent extraction of the sediment is performed and transferred to a suitable medium for bioassay testing. Should this elicit toxicity, a further fractionation of the extract is performed to separate compounds/groups of compounds and each fraction is further tested on the organism thereby pinpointing the potential cause of toxicity.

TIEs and BDFs represent a worst case scenario of contaminant exposure to organisms and are not representative of the bioavailable fraction, however, they can be used to compare with exposure of other aquatic system compartments such as porewater and elutriates and have an important role to play in assessments of sediments. TIE/BDF approaches have an important role in this thesis and are further described in Chapter 8.

1.4 Evaluation of sediment quality using sediment assessment criteria

As previously discussed, a number of methodologies exist for the assessment of contaminant levels in sediments. Such criteria are important in monitoring toxic effects and ensuring appropriate guidelines are put in place for regulating dumping of dredged materials. A number of criteria exist but those relevant for this study are a) US-EPA, b) OSPAR Background Assessment Criteria and c) Irish Sediment Quality (ISQ) guidelines which are discussed below.

1.4.1 Summary of the US-EPA approach to effects level assessment criteria

The US-EPA has developed Effects Range Low and Effects Range Median (ER_L and ER_M respectively) concentrations for marine sediments which can be used to identify relatively non-contaminated/contaminated samples which pose a risk of limited toxicity/toxicity. This effective multi-factorial approach is routinely utilised for the assessment of the quality of coastal and estuarine environments. ER_M is defined as the median concentration (50th percentile) of a contaminant observed to have adverse biological effects in literature studies. A more protective indicator of contaminant concentrations is the ER_L criterion, which is the 10th percentile concentration of a contaminant, represented by studies demonstrating adverse biological effects in the literature. Ecological effects are not likely to occur at contaminant concentrations below the ER_L criterion (Long et al., 1998). ER_L – ER_M values (See Table 1.3) are based on the composition of sediments in which biological effects have been observed, although it should be noted a lack of clear causality may lead to false positives in the datasets.

The approach uses a multi-factorial assessment for estuarine quality covering water, sediment and aspects of biota. A "traffic light" style assessment is performed for each parameter in each estuary based on assessment criteria and then combines assessments across determinands, estuaries and regions to produce national scale assessments. The approach makes a consistent comparison of four primary indices of estuarine condition (water quality index, sediment quality index, benthic index, and fish tissue contaminants index).

The water quality index is made up of five component indicators: dissolved inorganic nitrogen, dissolved inorganic phosphorus, chlorophyll *a*, water clarity, and dissolved oxygen. The development of indices for water quality is based on rating criteria for each sampling site.

The sediment quality index is based on three component indicators of sediment condition: direct measures of sediment toxicity, sediment contaminant concentrations, and the sediment total organic carbon (TOC) concentration. Chemical characterisation is performed on the sediment through chemical analysis; sediment toxicity is evaluated by measuring the survival of the marine amphipod *Ampelisca abdita* following 10-day exposure to the sediments in the laboratory; and the sediment TOC concentration is measured on a dry-weight basis. Benthic community attributes are included in this assessment of estuarine condition as an independent variable rather than as an indicator of sediment quality. Once all three sediment quality component indicators (sediment toxicity, sediment contaminants, and sediment TOC) are measured for a given site, a sediment quality index rating is calculated for the site. The site is rated "good" if none of the component indicators are rated poor, and the sediment contaminants indicator is rated "good" when no ER_M values are exceeded, and less than five ER_L values are exceeded. The site is rated as "fair" if none of the component indicators are rated poor, and the sediment contaminants indicator is rated fair i.e. five or more ER_L values are exceeded; and finally the site is rated as "poor" if one or more of the component indicators are rated poor i.e. an ER_M value is exceeded for one or more contaminants.

Worms, clams, and crustaceans are creatures that inhabit the bottom of an aquatic environment and are collectively called benthic macro invertebrates, or benthos. Indices produced for the benthic community reflect changes in range and abundance of pollution-tolerant and pollution sensitive species. A high benthic index rating means that samples which are taken from an estuary's sediments contain a wide variety of species, a low proportion of pollution tolerant species, and a high proportion of pollution sensitive species. A low benthic index rating indicates that the benthic communities are less diverse than expected, are populated by more pollution-tolerant species than expected, and contain fewer pollution sensitive species than expected.

A marine organism can uptake contaminants in a variety of different pathways including direct uptake from contaminated water, consumption of contaminated sediment, or consumption of previously contaminated organisms. Pollutants can be bioaccumulated in organisms and can be biomagnified through the food chain through the consumption of these organisms resulting in elevated quantities in the higher trophic levels. Chemical analysis data is assessed with the use of guidance values for whole body fish contaminants. A good rating is given if all chemical concentrations fall below the guidance levels; a fair rating is given if at least one chemical contaminant's concentration falls within the range of the guidance criteria and a poor rating is given if at least one chemical contaminant concentration exceeds the maximum value in the range of the guidance criteria.

Overall condition for each region is calculated by summing the scores for the available regional indices and dividing by the number of available indices.

Table 1.3 ER_M and ER_L [Long et al., 1998] Guidance Values in Sediments (µg kg^{-1} dry sediment)

Analyte[b]	ERL	ERM	Metal[a]	ER_L	ER_M
Acenaphthene	16	500	Arsenic	8.2	70
Acenaphthylene	44	640	Cadmium	1.2	9.6
Anthracene	85.3	1,100	Chromium	81	370
Fluorene	19	540	Copper	34	270
2-Methyl naphthalene	70	670	Lead	46.7	218
Naphthalene	160	2,100	Mercury	0.15	0.71
Phenanthrene	240	1,500	Nickel	20.9	51.6
Benz(a)anthracene	261	1,600	Silver	1	3.7
Benzo(a)pyrene	430	1,600	Zinc	150	410
Chrysene	384	2,800			
Dibenzo(a,h)anthracene	63.4	260			
Fluoranthene	600	5,100			
Pyrene	665	2,600			
Low molecular-weight PAH	552	3,160			
High molecular-weight PAH	1,700	9,600			
Total PAHs	4,020	44,800			
4,4'-DDE	2.2	27			
Total DDT	1.6	46.1			
Total PCBs	22.7	180			

1.4.2 Summary of the OSPAR approach to effects level assessment criteria

OSPAR are currently developing Environmental Assessment Criteria (EACs) based on available ecotoxicological information, however these are currently not sufficiently refined to contribute usefully to assessments of OSPAR monitoring data. In the absence of clear OSPAR/ICES ecotoxicological assessment criteria, the US-EPA ER_L – ER_M values were compared to the contaminant data derived, (it should be noted that the ER_L – ER_M approach does not require the application of sediment normalisation criteria, as described in section 1.5, to correlate for differences in sediment composition between locations). OSPAR contracting parties often derive sediment ecotoxicological quality and/or contaminant temporal/spatial information using chemical monitoring data combined with statistical

comparison to established threshold/evaluation criteria, these techniques are further described.

OSPAR approaches to the assessment of monitoring data of contaminants in marine sediment and biota lies in the application of two types of assessment criteria: Background Concentrations (and associated Background Assessment Concentrations) and Environmental Assessment Criteria (formerly Ecotoxicological Assessment Criteria). The former are chemical expressions of quality, but are linked to the ultimate objective of the OSPAR hazardous substances strategy of achieving "concentrations in the marine environment near background values for naturally occurring substances and close to zero for man-made synthetic substances". The primary function of EACs is to identify potential areas of concern, to identify unintended/unacceptable biological responses, or unintended/unacceptable levels of such responses, being caused by exposure to hazardous substances. OSPAR EACs and the US-EPA ER_L – ER_M (Effects Range Low and Median respectively) system for the interpretation of the potential ecological significance of the contaminant concentrations in sediments will be utilised in subsequent chapters in this project.

In order to create comparability between data within and between stations, and to allow comparison with assessment criteria, it is necessary to complete a number of processes namely: a) select the appropriate basis for comparison purposes, b) derive appropriate "background" and statistical "background assessment concentrations" for data comparison purposes, c) complete appropriate contaminant normalisation techniques, each of which is further discussed below, d) complete a statistical assessment.

1.4.2.1 Selection of basis for contaminant comparisons

The choice of an appropriate basis primarily needs to meet several considerations: scientific validity, uniformity for groups of contaminants for particular matrix; and a minimum loss of data. Dry weight (dw) conversions are deemed to be the most appropriate basis for sediment determinations for both organic and metals analyses (OSPAR Commission, 2006).

1.4.2.2 Determination of Background Concentrations (BCs) for sediments

Background concentrations (BCs) are an established assessment criterion within OSPAR and can be defined as,

> *The concentration of a contaminant at a "pristine" or "remote" site based on contemporary or historical data.*

*(*OSPAR, 2004*)*

Background concentrations are needed to assess progress towards the OSPAR objective of achieving background/near background concentrations of contaminants, and in assessing the anthropogenic contribution to the observed concentrations of contaminants in the environment.

Background Reference Concentrations (BRCs) (now BCs) for contaminants in sea water, sediment and biota, and EACs for trace metals, PCBs, PAHs, TBT and some organochlorine pesticides, were originally adopted by OSPAR in 1997 as assessment tools for use in preparing the previous CEMP assessment and the Quality Status Report 2000 (OSPAR Commission, 2000).

In 2004, the ICES Working Group on Marine Sediments in Relation to Pollution (WGMS, 2004) and the Working Group on Statistical Aspects of Environmental Monitoring (ICES, 2004) constructed draft background concentrations for OSPAR Coordinated Environmental Monitoring Programme (CEMP) for metals (cadmium, mercury and lead) and PAHs in sediments. These BACs have been utilised in Chapter 2 of this thesis.

OSPAR calculated background concentrations by taking median concentrations of contaminants measured in core (pristine) samples in appropriate regions. Data sets potentially impaired by anoxic conditions or possibly anthropogenic contamination were excluded. Metal concentrations were normalised to 5.0 % aluminium (and lithium) [see section 1.5] and the BC was statistically derived. The BC for PCBs, PAHs, OTs and OCs was set at zero and precision of analytical measurements evaluated prior to normalisation to 2.5% organic content. Details of how BCs were calculated are reported in CEMP 2005 assessment report.

1.4.2.3 Use of BCs in the derivation of BACs

Statistical tests are required to determine whether concentrations of a contaminant, derived from monitoring data, comply with background concentrations as discussed above, i.e. ultimately assisting in achievement of the OSPAR policy objective for hazardous substances namely:

"achieving concentrations in the environment near background values for naturally occurring substances and close to zero for man-made synthetic substances."

(OSPAR, 2004)

The OSPAR method uses derived background concentrations and adopts a precautionary statistical approach to the comparison of monitoring data with the BCs. The method requires the establishment of a secondary concentration level, the Background Assessment Concentration (BAC). The BAC is a concentration near to the background whose value is contaminant specific and is dependent on the residual variation within data at the BC. The BC for xenobiotics (PAH, OCs and PCBs etc) has been set at zero, and in these cases the variance used to derive BACs was the variance at a low concentration that is small but detectable by common analytical methods.

The use of BACs is considered: a) statistically sound and based on a precautionary approach; b) having potential for wide applicability covering all contaminants, natural and man-made in all regions of the OSPAR Convention Area (providing BCs are available); c) applicable to sediment and biota, and also potentially to water, d) having application as a strategic management tool by countries wishing to assess the status of their marine environments allowing OSPAR to test its policy objectives.

1.4.2.4 Technical method for deriving BACs

BACs are used to make precautionary tests of whether observed concentrations are near background. The BAC is a concentration greater than the BC that quantifies what is meant by *near background* or *close to zero*. The test assumes that the mean concentration [c] is above background (i.e., [c] > BAC) unless there is statistical evidence to show that it is near background (i.e., [c] ≤ BAC). Formally, the null and alternative hypotheses are:

H_0: [c] > BAC (i.e., concentrations above background)

H_1: [c] ≤ BAC (i.e., concentrations near background)

and H_0 is rejected in favour of H_1 if the upper confidence limit on [c] is below the BAC.

BACs should be both low enough to reflect near background concentrations and high enough that we are likely to conclude that concentrations are near background when [c] = BC. In the absence of other objective means of setting the BAC, the precision of data can be used to set a provisional BAC. Specifically, the BAC can be set to give a 90 % probability of concluding that concentrations are near background when [c] = BC. Details on BAC construction are presented in the OSPAR CEMP report 2005.

1.4.3 Summary of Irish Sediment Quality Guidelines for sediment assessment

Irish guidelines have recently been published for the assessment of dredged material for disposal in Irish waters (Cronin et al., 2006). The purpose of these guidelines is to establish a comprehensive national framework for assessing the quality of dredged material and in particular, for assessing likely impacts arising from the dumping at sea of contaminated sediments. For metals, upper level guidance values were derived from samples collected from reference sites around the Irish coast whereas lower level values were derived using the 95 percentile of remaining background data. For OCs, PCBs and PAHs, 95 percentiles of background data were used to derive the lower level guidance values. In the absence of matching chemical and ecotoxicological data derived on Irish sediment samples, upper guidance values are based on available ecotoxicological datasets i.e. Effects Range Median/ Probable Effect Level (ER_M/PEL). Data for organics data in the < 2 mm sediment was not normalised for TOC content. The upper and lower level Irish quality guidance figures are presented in Table 1.4 below. For dredged material assessment, sediments can be classified into three categories: class one where contaminant concentrations are below the lower action level and no biological effects are likely, class

two where sediments are marginally contaminated and concentrations fall between the upper and lower action levels; and class three where sediments are heavily contaminated and very likely to induce toxicity in marine organisms. In the case of class 3 sediments, it is unlikely that dumping at sea will be permitted and alternative management and disposal of material would take place (Cronin et al., 2006).

Table 1.4 Proposed upper and lower level Irish Sediment Quality guidance levels

Parameter	Units (dw)	Proposed lower level	Proposed upper level[1]
Arsenic	mg kg^{-1}	9[2]	70
Cadmium	mg kg^{-1}	0.7	4.2
Chromium	mg kg^{-1}	120	370
Copper	mg kg^{-1}	40	110[3]
Lead	mg kg^{-1}	60	220
Mercury	mg kg^{-1}	0.2	0.7
Nickel	mg kg^{-1}	21[2]	60
Zinc	mg kg^{-1}	160	410
∑ TBT & DBT	mg kg^{-1}	0.1	0.5
γ- HCH (Lindane)	µg kg^{-1}	0.3	1
HCB	µg kg^{-1}	0.3	1[3]
PCB (101, 118, 138, 153, 180, 28, 52)[4]	µg kg^{-1}	1	180
PCB ∑ 7[5]	µg kg^{-1}	7	1260
PAH (∑ 16)	µg kg^{-1}	4000	

[1] ER_M (rounded up)
[2] ER_L (rounded up)- no background Irish data available
[3] Probable effects level (PEL) used as ER_M considered high
[4] PCB (individual congeners of ICES 7)
[5] PCB Σ ICES 7

1.5 Normalisation of sediment data

Contaminant fate and transport in marine sediments is primarily driven by the energy of the system at the test site. In high energy environments, sediments tend to be relatively coarse grained with low cohesion, contaminant binding capacity, and low depositional rates while in lower energy environments, greater deposition of fine grained sediments occurs resulting in higher adherence capacity for contaminants. As a consequence, contaminant concentrations tend to be generally more elevated in clay rich sediments compared to those from sandy regions, where granulometric dilution effects result in lower and more variable contaminant concentrations in the sediment, therefore spatial variance in pollutant levels may be related to variance in the systems ambient energy (Birch et al., 2001).

Assessments are often completed on site-specific contaminant background levels from sediments of different origins, textural, physicochemical, and compositional characteristics. Without correction for variable contaminant background levels and uptake capacity, such comparisons can be biased, even where only fine-grained sediments are analyzed, such background values have been reported to span two orders of magnitude for individual metals (Chapman et al., 1999).

Normalisation has been defined by Kersten and Smedes (2002) and others as a procedure to correct both background and contaminant concentrations for the influence of the natural variability in sediment granulometry and mineralogical composition mediated by the ambient energy of the aquatic system (Loring, 1991; Loring and Rantala, 1992; Daskalakis and O' Connor, 1995) with a primary aim being the capacity to differentiate between natural variability and anthropogenic inputs.

Several normalisation methods are commonly used including, sieving, (granulometric) or geochemical normalisation techniques. After normalisation of equally contaminated, pre-treated and analysed sediments with different grain-size distributions, the normalised contaminant concentrations should be similar and should show no correlation with the concentration of the normaliser itself. A number of normalisation approaches and their application in the determination of different contaminant types are further described below.

1.5.1 Granulometric normalisation

It is generally accepted that the clay portion of sediments tend to have the highest affinity/binding capacity for contaminants. Clay minerals naturally contain metals and in

general the natural contaminant burden increases with the clay content (< 0.002 mm) thus clay content is considered as a primary normaliser. Separation of the clay fractions is laborious, and contaminant information from larger mesh sizes (< 0.016 mm < 0.020mm, < 0.125 mm and < 0.150 mm) including the silt fraction is often utilised in international monitoring programmes. In principle the < 0.063 mm fraction separates silt from sand, with the silt fraction being primarily composed of quartz particles. Positive correlations to the clay content have been reported particularly where loses occurs in the watershed. Consequently, sieving has been utilised for primary normalisation of heavy metals in addition to organic contaminants (Klamer et al., 1990).

1.5.1.1 Sieving quality assurance

The EU project Quality Assurance of Sample Handling (QUASH) determined that wet sieving can reduce the between-laboratory variability in the sieving yield (sieving error) to lower than that of the analytical variability (analytical error), and of the compositional variability at individual sampling sites (field error) (Krumgalz et al., 1989). Agitation of sediments is recommended followed by homogenization of the sieved sample (www.quasimeme.marlab.ac.uk/QUASH/quash.htm). A further interlaboratory comparison exercise (Smedes, 1997) found there was a strong correlation between the clay content and the < 0.016 mm fraction, however the clay content in the < 0.063 mm fraction showed a range of a factor of four implying that sieving does not result in mineralogically homogeneous samples, thus grain-size separation alone does not necessarily reduce the differences between the composition of the sieved samples. It was concluded that sieving alone is insufficient as a final normalisation procedure (Smedes et al., 2000) especially in

areas of different mineralogical composition, where further geochemical normalisation is recommended.

1.5.2 Geochemical normalisation (metals)

In a two-component linear mixing model, with both a primary metal-bearing component and quartz, a geochemical normalisation model can be formulated. Such models require that elements, (e.g. aluminium) are present as they represent proxies for the clay portion of the sediment. Accordingly, silicon, representing a metal-devoid quartz grain content, exhibits a strong negative correlation to that of other elements.

Both Al and Si, may, in principle, be used as geochemical normalisers or ‘‘co-factors’’ for grain-size/compositional variation, for metals analysis. Even though site dependent aluminium contents of the clay fraction (< 2 µm) may vary between 2 % and 10 %, Al is primarily used as a normaliser in estuarine/coastal monitoring programmes (Windom et al., 1989; Hanson et al., 1993; Summers et al., 1996; Balls et al., 1997; Covelli & Fontolan, 1997; Weisberg et al., 2000). Additionally organic matter (OM) and Fe/Mn oxides can also bind contaminants but do not contain silicon or aluminium. Elements representing these components therefore exhibit a similar strong negative correlation to Si and a strong positive correlation to the clay fraction, and hence to the metal content (Helz and Sinex, 1986; Rule, 1986). Particular care is advised where historic pollution events and/or diagenetic processes may artificially or in the case of the latter, naturally enrich sediments (Gobeil et al., 1997) potentially introducing elements suitable for use as a normalising co-factor. Other elements including, lithium (Loring, 1990; Rowlatt & Lovell, 1990;

Miseroccchi et al., 2000), Sc, Cs, Rb and Y (less frequently) have also been utilised as normalising co-factors (Ackermann, 1980; Grant and Middleton, 1998; Prokisch et al., 2000) while care is advised where TOC is used as it is also a reactive component in early-diagenetic processes.

1.5.3 Geochemical normalisation (organic contaminants)

Organic contaminants such as polychlorinated biphenyls and polycyclic aromatic hydrocarbons have a strong affinity to organic matter (OM) due to their hydrophobic nature (Schwarzenbach et al., 1993). Consequently, environmental partitioning of organic contaminants has generally been predicted based on their partitioning rate into the bulk organic carbon in the sediment. There is no conclusive consensus as to which parameter best represents the OM content (total organic carbon, TOC; elemental organic carbon, EOC; particulate organic carbon (POC); loss on ignition (LOI); at different temperatures, etc.). A study of 23 European estuaries (Kersten and Smedes 2002) revealed that the EOC co-factor (equal to the TOC) seems to be preferable due to its much stronger correlation to OM. TOC was the method chosen in the course of this study.

1.5.4 Normalisation procedures

Kersten and Smedes (2002) recommended a two-tiered normalisation approach including both sieving and co-factor measurement as preferable to single normalisation techniques.

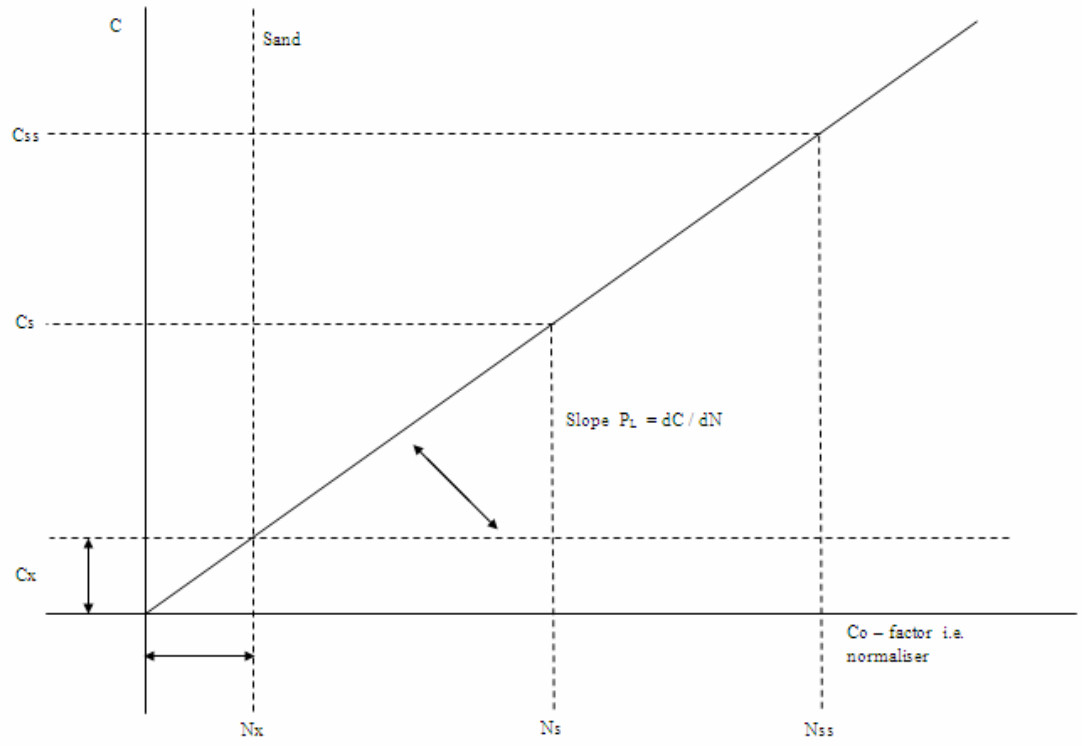

Figure 1.2: Regressions between different primary normalisers analyzed in sediments from 23 representative estuaries in Europe (reproduced from Kersten and Smedes 2002).

The general model for normalisation based on a geochemical component mixing model from Kersten and Smedes (2002) is reproduced in Figure 1.2. C_X and N_X (pivot values for the co-factor and the contaminant respectively) which essentially are contents possibly present in the coarse material (e.g., Al in feldspar or EOC in charcoal grains) and have been estimated from samples without fines. The regression line between the contaminant and co-factor will originate from that point. Regressions of co-genetic data sets but with different contamination levels will have this point in common but tend to develop different slopes from this ‘‘turning point’’. In principle therefore, only one additional sample is required to estimate the slope for a co-genetic sample set if this turning point is known.

The slope for this sample with a contaminant content "CS" and a co-factor content "NS" can be expressed and contaminant concentrations normalised as follows:

Equation of the line: $$C_{SS} = (C_M - C_X)\frac{N_{SS} - N_X}{N_M - N_X} + C_X \quad (1)$$

where:

C_{SS} Normalised concentration

C_M Measured concentration of contaminant

C_X Pivot value for the contaminant

N_X The pivot value for the cofactor

N_M The measured concentration of the cofactor

N_{SS} Reference composition of the sediment as represented by cofactor content

The constants C_X and N_X (i.e. the pivot values for contaminant and cofactor) have been discussed in Annexes 8 and 9 to the 2002 report of the ICES Working Group on Marine Sediments in Relation to Pollution (WGMS). The report describes how to estimate pivot values for contaminants and co-factors.

Regression lines drawn for samples from different areas may thus be used to compare their degree of contamination. The steeper the gradient, the more contaminated an area is considered to be (Kersten & Kröncke, 1992; Fukue et al., 1999). Positive residuals (i.e. points above the regression line) are greater than would be predicted from the contaminant/co-factor relationship, and thus may represent hot-spot samples. An important prerequisite for the regression approach is that sufficient co-factor variability data are available.

1.6 Summary of integrated assessment approaches

Sediment pollution is a global problem. In order to identify substances which have an adverse effect on organisms, an approach integrating chemical and bioassay data in monitoring and assessment is necessary. A wide range of international organisations use sediment assessment techniques however, no universal decision making framework is used. Current methods used by ICES and OSPAR include the "FullMonti" approach and is further described.

1.6.1 FullMonti approach

The FullMonti is a weight-of-evidence approach which derives scores for contaminants, biological effects, and biology, and then combines these three scores into an overall assessment indicating the health status of the system. The score of biological effect is weighted from top to bottom with the most significant effects (i.e. reproduction, growth, behaviour, survival) weighted with a value of 10. Subcellular biomarkers are weighted with lower values, ethoxyresorufin-O-deethylation (EROD) induction for instance is assigned a value of 3. In addition, the response level is scored according to suggested intervals in the assessment criteria (green, amber and red having values of 1, 5 and 10 respectively). An overall score for biological effects is derived by mean value of total score multiplied with weighted values. For contaminants, the scores for each pollutant are integrated into single scores for sediment, shellfish and fish tissue, which again can be integrated into one overall score for contaminants. In the end a final integrated score can be extracted based on the overall scores for contaminants, biology and biological effects.

1.7 Project Description

The project was a collaborative project between the Marine Institute (MI) in Galway and Radiation and Environmental Science Centre entitled "An Integrated Approach to the Toxicity Evaluation of Irish Marine Sediment". Funding for this research was provided by the Technology Sector Research: Strand III: Core Research Strengths and from Dublin Institute of Technology's Capacity Building Scheme (CaBS) for Strategic Research.

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Two simultaneous projects were conducted, one dealing with ecotoxicological evaluation of sediment [Ailbhe Macken (RESC)] and the other dealing predominantly with the sampling and chemical analysis [Michelle Giltrap (MI)]. The aim of the project was to design and implement an integrated programme for ecotoxicological evaluation by integrating results of both bioassays and chemical analysis. The RESC component dealt predominantly with the optimisation of a multi-phase and multi-trophic battery of bioassays for ecotoxicological assessment. The MI component concurrently investigated the development of a sampling strategy and the chemical characterisation of the sediment.

The final aim was to integrate all results from bioassays and chemical analysis and performing an integrated assessment by using a weight-of-evidence approach currently being developed by OSPAR. The approach developed in this thesis complements other techniques such as benthic diversity monitoring, biomarker techniques and comprehensive chemical analysis that are part of a full integrated assessment approach.

The overall objective of this project is to design and implement an integrated programme for ecotoxicological evaluation of Irish sediments by correlating results from biotests and chemical analysis and link sediment contamination with ecotoxicity measurements by

examining the relationship between observed toxicity and key contaminants. This shall enable Ireland to contribute to the valuable work undertaken by organisations such as ICES and OSPAR who are currently tending towards integrated approaches. This current study wishes to report contaminant levels and ultimately to integrate this data with the ecotoxicological assessment of sediments at selected sites.

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2

CHAPTER 2 THE DISTRIBUTION OF PERSISTENT POLLUTANTS IN IRISH SEDIMENTS FROM THREE CASE STUDY SITES AROUND IRELAND

2.1 Introduction

Global industrial and agricultural processes, marine traffic, sewage discharges, industrial wastewater, and poor environmental management can all contribute to the widespread contamination of the marine environment. Marine sediments can act as an ultimate sink for recalcitrant pollutants. These pollutants tend to be persistent in the environment (Clark, 1992) and can reach levels that are toxic to resident marine organisms.

Heavy metals exist naturally in sediment and some are essential for living organisms however mining, industry and agricultural anthropogenic inputs can raise concentrations above natural background levels (Ghrefat and Yusuf, 2006; Osán et al., 2007; Saari et al., 2007). Elements such as mercury, lead, cadmium, copper, arsenic, nickel and chromium have previously been reported to elicit toxicity in aquatic species at various concentration levels (Spehar and Fiandt, 1986).

Organotin compounds enter the marine environment through a number of sources such as biocides, fungicides, insecticides, polyvinylchloride (PVC) stabilizers, industrial catalysts and wood preservatives (Díez et al., 2005). Tributyltin (TBT) being one of the most toxic contaminants found in the marine environment is used as a paint additive to prevent biofouling and its leaching from paints results in pollution of harbours, ports and coastal areas (Fent, 1989). Ireland was one of the first countries to ban the use of all organotin containing compounds on vessels < 25m in length in 1987 (Minchin, 2003). However, this compound may still enter the marine environment through other sources via wastewater since TBT is used as a biocide in preserving wood, textiles, paper and stonework. Its degradation products namely dibutyltin (DBT) and monobutyltin (MBT) are used as stabilizers in PVC production (Fent, 1989) these derivatives having in some cases been

found to be less toxic than their parent but reported to elicit toxicity in some aquatic species (Huang et al., 2004; Cooney, 1995; Bouchard et al., 1999). TBT has been linked to reductions in meat weight, increased shell thickness and distortion in Pacific oysters (Minchin, 2003) and has been shown to cause imposex, the imposition of male characteristics on the female gastropod, the dogwhelk *Nucella lapillus,* following exposure to concentration levels as low as 1 ng/l with severe cases resulting in sterilisation of the organism (Bryan et al., 1986; Gibbs and Bryan, 1986; Bryan et al., 1987). The degradation of TBT follows the pattern TBT < DBT < MBT (Hoch, 2001). In contrast to degradation of TBT in seawater which is highly variable depending on pH, temperature, turbidity and light and its half life ranging from days to weeks (Díaz et al., 2007), degradation of TBT in sediments is slow with a half life of between 1 and 5 years in oxic marine sediments (Hoch, 2001). Decreasing concentrations of TBT with depth of sediment core has also been reported, indicative of debutylation of TBT (Hwang et al., 1999).

Polycyclic aromatic hydrocarbons originate from pyrolytic processes, but they may also originate via petrogenic sources such as crude oils or refinery products, atmospheric deposition, industrial discharges and through oil spills (Webster et al., 2001) or via natural processes.

Halogenated compounds such as PCBs and OCs are persistent man made compounds and are of specific concern due to their toxicological and carcinogenic properties (Galanopoulou et al., 2005). PCBs consist of 209 congeners differing in the number and position of chlorine atoms on the two coupled phenyl rings. They were commercially used in dielectric fluids in capacitors and transformers, hydraulic fluids, lubricating and cutting oils, additives in paints, adhesives, sealants and plastics and their use was based mainly on

properties such as chemical stability, low flammability, and electrical insulating properties (El-Kady et al., 2007) and can enter the marine environment by leakage, recycling, transboundary influx via major rivers and long-range atmospheric transport (Van Wezel et al., 2000). Within the 209 PCB congeners, the number and location of the chlorine atoms attached to the biphenyl molecule determine the potency and nature of toxicity of each PCB (Fadhel et al., 2002).

Thousands of chlorinated compounds such as DDT, chlordanes, toxaphene, heptachlor etc. have historically been used (and many in some cases are still applied) as pesticide controls in agriculture. Although use of a number of these compounds has been banned for many years, they have been reported to persist in sediment (Doong et al., 2002). Some PAHs and their derivatives have been found to cause DNA inducing mutation effects and have carcinogenic properties (Bihari et al., 2006). PAH compounds have previously been shown to cause undesirable toxic effects in organisms (Hatch and Burton, 1999; Djomo et al., 2004). Further physico-chemical and toxicological information for pollutants of interest in this study are presented in chapter 1.

2.1.1 Sediment quality assessments

Sediments may act as an ultimate sink for certain pollutants, therefore a number of worldwide conventions (e.g., London Convention 1972 (LC) (www.Londonconvention.org); Oslo/Paris Convention (OSPAR) (www.ospar.org) and, the Helsinki and Barcelona Conventions have all produced guidelines for the chemical and ecotoxicological assessment of sediment quality. Different assessment methodologies are suggested ranging from physico-chemical and/or biological approaches, to the management

of different routes of disposal or use of dredged materials. Most conventions propose methods based on 'weight-of-evidence' (WOE) approaches which can consist of initial screening approaches (e.g. particle-size characteristics of the sediment), collation of local and historic information, (e.g., surrounding industries, pollution sources, and collectors) and in some cases biological screening tests, progressing where appropriate to more detailed assessments (e.g. chemical characterisation of the material). Where sediment quality assessment is not possible from initial and primary assessments, direct measurements of toxicity, comparison to appropriate assessment criteria and/or other case-specific studies as described throughout this thesis may be required.

2.1.1.1 Summary

OSPAR background assessment criteria (BACs) have been established for 10 parent PAHs, 8 metal compounds and a number of OCs in marine sediment as part of the OSPAR Coordinated Environmental Monitoring Programme (OSPAR, 2007). Data in this current study were additionally compared to Effects Range (ER) values established by the US National Oceanic and Atmospheric Administration (NOAA) as sediment quality guidelines. The ER-low (ER_L) value is defined as the lower ten percent of the effect concentration and the ER-Median (ER_M) as the median of effects concentration. The ER_L and ER_M are generated from biological effects data and pollutant modeling systems and in general predict a level of contamination of each pollutant where toxicity may occur. Adverse effects on organisms are rarely observed when concentrations fall below the ER_L value (Long et al., 1998).

Irish guideline upper and lower action levels have also been recently derived using Irish sediment data (Cronin et al., 2006). These guidelines were established in order to assess impacts of dumping at sea of contaminated sediments. Sediment concentrations in this study are compared to these upper and lower levels. The assessment methodologies utilised in this study are further described later.

Concentrations of heavy metals, organotin compounds (TBT and DBT), polyaromatic hydrocarbons (acenaphthene, acenaphthylene, anthracene, benzo[a]anthracene, benzo[a]pyrene, benzo[b]anthracene, benzo[b]fluoranthene, benzo[e]pyrene, benzo[ghi]perylene, benzo[j]fluoranthene, benzo[k]fluoranthene, chrysene, dibenzo[a,h]anthracene, dibenzothiophene and isomers C_1-C_3, fluoranthene, fluorine, indeno [1,2,3-cdpyrene, naphthalene and isomers C_1-C_3, perylene, phenanthrene and isomers C_1-C_3), hydrocarbons, polychlorinated biphenyls (PCB-28, -52, -101, -105, -118, -128, -138, -153, -156, -170, -180), and organochlorines (1,2,3-trichlorobenzene, 1,2,4-trichlorobenzene, 1,3,5-trichlorobenzene, aldrin, cis-chlordane, trans-chlordane, o, p'-DDE, p, p'-DDE, o, p'-DDT, p, p'-DDT, p,p'-TDE, dieldrin, endosulfan A, endosulfan B, endrin, hexachlorobutadiene, alpha-HCH, beta-HCH, delta-HCH, gamma-HCH, hexachlorobenzene, isodrin) were measured in sediment collected from selected locations around the Irish coast. Sites were selected as they were utilised in an integrated chemical analysis and biological effects project to assess the chemical and ecotoxicological quality of sediments at these locations. The data collected provide a "snapshot" of persistent pollutant levels in surficial sediments at selected sites and thus provide essential contaminant information to assist in completion of integrated ecotoxicological assessments. Data is compared to OSPAR BAC, NOAA ER_L/ER_M and Irish guideline upper and lower action levels for various contaminants.

The selection of sites was based on three different studies within the project. Table 2.1 below demonstrates which sites were chosen for each study. The Dublin Bay [inner port site] (DB1) located in the West Alexandra basin, the Bull Lagoon site (DB6) in the north of the bay and the Dunmore East site (DE1) in County Waterford were selected for full ecotoxicological and chemical evaluation. Ecotoxicological testing of the sediments are presented elsewhere (Macken et al., 2008 accepted manuscript). A caging study (reported in chapter 5) was also conducted using caged *Nucella lapillus* and *Crassostrea gigas* to assess bio-effects in Irish coastal waters. Three sites were selected for this study which were Dublin port site (DB6), Dunmore East site (DE2) and Omey Island (OI1) and are detailed below in Table 2.1. Pollutant data are described for metals, organotins, polycyclic aromatic hydrocarbons, hydrocarbons and organochlorine compounds at these ecotoxicological and caging study sites but in addition to this, other locations were selected in Dublin port and surrounds, from Omey Island on the West Coast of Ireland and from Dunmore East (see Table 2.1 and Figure 2.1), thus providing a means of assessing spatial contaminant influences within the test sites.

Table 2.1: Summary of sampling information and TOC values for sites selected for ecotoxicological, caging, and spatial monitoring study locations.

Location	Identification	Ecotoxicology	Caging study	Spatial Pollutants	MI Ref	Latitude	Longitude	TOC (%) <2mm	TOC (%) <0.063 mm
Alexandra Basin West	DB1	•		•	MSC/07/1002	6.2187	53.3485	3.70	3.11
Alexandra Basin West	DB2			•	MSC/07/1064	6.2163	53.3484	1.89	0.85
Alexandra Basin West	DB3			•	MSC/07/1065	6.2146	53.3464	1.07	1.74
Alexandra Basin East	DB4			•	MSC/07/1066	6.2114	53.3465	1.22	1.63
North Bank Lighthouse	DB5		•	•	MSC/06/1074	6.1700	53.3500	0.60	1.30
Bull Lagoon	DB6	•		•	MSC/05/1037	6.1300	53.3480	0.28	1.50
Dunmore East	DE1	•		•	MSC/06/1038	6.9921	52.1477	1.52	< 0.40
Dunmore East	DE2		•	•	MSC/07/1068	6.9922	52.1475	2.30	1.42
Dunmore East	DE3			•	MSC/07/1069	6.9920	52.1477	1.31	1.29
Omey Island	OI1		•	•	MSC/07/1070	10.170	53.5300	22.6	30.3

TOC : Total Organic Carbon
DB: Dublin Bay; DE- Dunmore East; OI- Omey Island

2.2 Materials and Methods

2.2.1 Site selection and sediment sampling

Dublin port is Ireland's premier port handling 50 % of all of Ireland's imports and exports. In 2006 throughput amounted to 29.3 million tonnes (Dublin Port Authority, 2006) and an estimated 8000 vessels pass through it annually (Buggy and Tobin, 2006). The relatively industrialised and urban surroundings make it a major potential source for pollution. Sampling sites in Dublin port and inner bay were selected around the west inner Alexandra Basin (DB1, DB2, DB3), east inner Alexandra Basin (DB4), and in the vicinity of the main marine traffic channel and at a fixed navigation mark under the North Bank lighthouse (DB5) (site used for caging study in chapter 5) at the entrance to the estuary which is sheltered by two breakwaters (the North and South Bull Wall) extending into Dublin Bay. Additionally a site located in the Bull Lagoon (DB6), to the North of Dublin port at the inner Dublin Bay was selected as it has fewer industrial influences/inputs than that of Dublin port itself. The site at the inner Alexandra Basin (DB1) and the Bull Lagoon site (DB6) were selected for full ecotoxicological evaluation which is described in detail by Macken et al. (2008).

Dunmore East harbour is in County Waterford in the South East of Ireland and is protected by a breakwater on an exposed coast. Elevated organotin levels have previously been reported in the inner harbour of this fishing port (Enterprise Ireland 2002) therefore three sites were selected in the inner Dunmore East harbour (DE1, DE2 and DE3) near the dry dock operation lift, the DE2 site was also selected for the caging study in chapter 5. The inner Dunmore East Harbour site (DE1) was selected for full ecotoxicological evaluation (Macken et al., 2008) including *in-vitro* cell line testing which is described in chapter 4 and

was also selected for a Toxicity Identification Evaluation (TIE) which is later described in chapter 8.

Omey Island (OI1) is an island off County Galway in the West of Ireland with no industrialised and very few urban surroundings. Omey Island was selected as a potentially low level contaminated site on the west coast of Ireland and is only accessible at low tide. This site was also selected as a control location for the caging study described in chapter 5. A map of the sampling locations can be seen in Figure 2.1 below. Sampling was performed spatially with multiple grabs combined. This coupled with dual normalisation where appropriate (< 0.063 mm and to TOC etc) is suitable for OSPAR reporting and is fit for purpose.

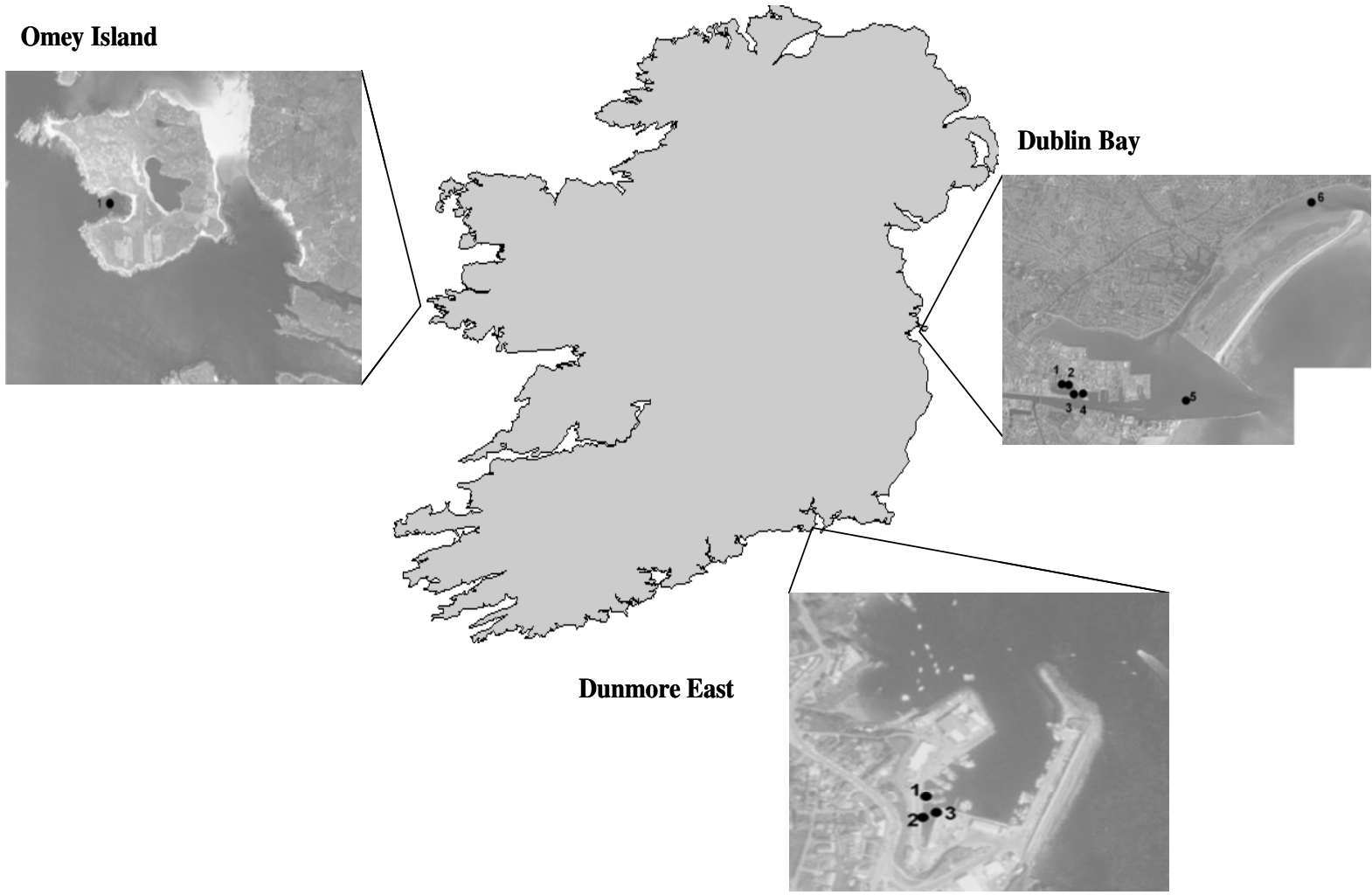

Figure 2.1: Map of sampling locations in the Dublin Bay, Dunmore East and Omey Island regions

The upper 0-5 cm of sediment was collected with a Van Veen Grab sampler at high tide in the Dublin Port area (Site Nos. 1-5) while all other locations were sampled under low tidal conditions. Clean sampling equipment was prepared including jars, spatulas and the grab sampler washed in hexane: acetone 1:1 (v/v) for organics analysis and acid washed by soaking in 30 % v/v nitric acid for metals analysis. Sediments were placed into solvent washed jars for organics analysis and acid washed jars for metals analysis. Prior to analysis the sediments were homogenised, wet sieved to the < 2mm and < 0.063 mm fractions, frozen to -20 ºC and subsequently freeze dried at -30 ºC.

Representative aliquots were then analysed under sub-contract by the Environment Agency (EA) laboratory, UK for metals, PCBs, OCs, PAHs and total organic carbon (TOC %). OTC analysis was performed at the Marine Institute (MI) and the National Environmental Research Institute (NERI) in Roskilde Denmark.

2.2.2 Analytical methodology

2.2.2.1 Metals analysis

The freeze dried samples for metals analysis were initially digested with a mixture of nitric, perchloric and hydrofluoric acids. The digest was subsequently evaporated to dryness and re-dissolved in hydrochloric acid, with metals measured using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

2.2.2.2 Polycyclic aromatic hydrocarbon analysis

Samples for PAH analysis were initially extracted with an Accelerated Solvent Extraction (ASE) system using a dichloromethane: acetone (1:1) solvent mixture. The extract was cleaned up with Gel Permeation chromatography (GPC) and Silica gel prior to analysis by GC/MS-SIM on a J & W Scientific, HP-5MS, 30m column.

2.2.2.3 Organochlorine analysis

For the analysis of PCBs and OCs, sediments were initially spiked with representative surrogate standards prior to extraction with a mix of Dichloromethane/Acetone using Accelerated Solvent Extraction (ASE). Solvent extracts were reduced in volume and interfering organic compounds of high molecular weight, elemental sulphur and mineral oils were removed using High Resolution Size Exclusion Chromatography (SEC/GPC) followed by further clean up on Florisil and Silica columns for the isolation of PCBs. The cleaned up extract was concentrated to low volume prior to injection onto a SGE HT-8 50m GC column with Mass Spectrometric detector operating in selective ion monitoring mode.

2.2.2.4 Organotin analysis

Organotin speciation was performed in the Marine Institute and is described briefly below (a detailed method is outlined in chapter 4). The sediment was digested with dilute HCl and shake extracted with dichloromethane and the organic layer centrifuged and collected. This was solvent exchanged to methanol for derivatisation with sodium tetraethylborate (10% w/v in methanol solution). The ethylated OT compounds were then back extracted into hexane and the organic layer concentrated. Sulphur was

removed using tetrabutylammonium (TBA) sulphite. The organotin speciation method was optimised to extract OT compounds in their chloride form for subsequent exposure of extracts to various biological organisms for ecotoxicological testing. No recovery correction standard was employed during the course of this study to ensure it did not effect the cell line toxicity as described in chapter 4. Finally, tetrapropyltin was added to the analytical extract (approx 1 ml hexane) as an instrumental internal standard prior to analysis by gas chromatography pulsed flame photometric detection (GC-PFPD).

The < 0.063 mm fraction of three sediment samples (DB1, DB3 and DB4) were analysed for OTCs at NERI, Roskilde in Denmark. The analytical method used for the organotin determination is previously described in Strand (2003) with the following modifications. In brief, the sediment fraction of less than 2 mm, was spiked with tri-*n*-propyltin as a recovery standard, hydrochloric acid added and treated with ultrasound, followed by pH adjustment (pH = 5 ± 0.5), in situ ethylation and extraction with pentane as the organic solvent. Solvent extract clean-up procedures were performed using silica gel in order to minimize GC column contamination. The quantification of organotin was performed using a modified Varian 3500 gas chromatograph and dual channel pulsed flame photometer detector (GC–DC-PFPD).

2.2.2.5 Total organic carbon analysis

Total organic carbon was determined in the < 2 mm and the < 0.063 mm freeze dried sample fractions by an ISO 17025 accredited flash combustion method using a Thermo flash Elemental Analyser as described below. Sediment samples were weighed, treated with sulphurous acid for removal of inorganic carbonates and heated to 900 ºC under a

constant flow of helium and introduction of oxygen. Flash combustion followed by quantitative combustion was completed. The individual components were separated and eluted in the order, N_2-CO_2-H_20 and measured using a thermal conductivity detector.

2.2.3 Quality Assurance of analysis

Quality control for all analyses was evaluated by the use of appropriate laboratory and/or certified reference materials with individual analytical batches. Organotin recoveries were determined utilising the butyl- and phenyltin certified freshwater reference material BCR646 run alongside analytical batches. Recoveries of TBT ranged from 63.3-68.1 % and DBT from 79.2-84.2 %. The HISS-1, MESS-3 and PACS-2 marine sediment reference materials were used to evaluate recoveries of metals and the NIST SRM 1944 CRM was used to evaluate PCB/OC and PAH recoveries. All certified reference materials were found to be within acceptable [Z] score limits. No recovery correction was applied for any of the data reported herein.

Quality control of total organic carbon measurements was ensured by analysing two QUASIMEME laboratory proficiency materials (QTM080MS and QOR090MS), which returned acceptable |Z| score limits (-0.6 and -0.2 respectively). Additional QC criteria including analysis of blanks, assessment of duplicate sample analysis and repeat analysis were also included on a per batch basis performed. All QA data was deemed acceptable with the exception of analysis of PCBs/OCs where limits of detection for analytes were higher than expected and therefore QA was not deemed acceptable for these analyses which is described later in this chapter.

2.3 Results and Discussion

In this study, OSPAR Background Concentrations (BCs) are used to assess the levels of 8 elements (arsenic, cadmium, chromium, copper, mercury, nickel, lead and zinc), 10 parent PAHs (anthracene, fluoranthene, phenanthrene, naphthalene, pyrene, benzo[a]anthracene, benzo[a]pyrene, benzo[ghi]perylene, chrysene, indeno1, 2, 3-cdpyrene), 7 PCBs (PCB-28, -52, -101, -118, -138, -153, -180) and 3 OCs (HCB, γ-HCH, o, p'-DDD, o, p'-DDE, p, p'-DDE, p, p'-DDT). In general, BCs for naturally occurring compounds such as PAHs are typically in the range of concentrations found in un-contaminated areas of the OSPAR maritime area. The concentration is described as 'near background' if the mean concentration detected is below the corresponding BAC. BACs are normalised to 2.5 % TOC for organic compounds and 5 % aluminium for metal compounds and are expressed in either mg kg^{-1} or μg kg^{-1} as previously described. The mean normalised concentration and an approximate upper 95 % confidence interval was generated [mean + ($\mathbf{t}_{d,f\ 0.95}$ x SE) where the SE is estimated at 15% to account for analytical error] (CEMP guidelines) to allow for comparison with BCs (Webster et al., 2007).

2.3.1 Data treatment and evaluation

Sediment contaminant data are discussed in a dry weight basis by normalising to 2.5 % TOC for organic contaminants and normalising to 5 % aluminium content for inorganic contaminants (in accordance with OSPAR BAC).

2.3.2 Physico-chemical characterisation of sediments

Hydrophobic persistent pollutants such as PAHs, PCBs/OCs, and OTCs accumulate in marine sediments to different degrees as a function of the sediment characteristics. Sediments with high organic carbon content and/or with smaller particle sizes tend to accumulate persistent organic pollutants (POPs) to a greater extent than coarse, sandy sediments. TOC values in sediment from this study ranged from 0.28 to 1.89 % (DB6 and DB2 respectively) in the < 2mm fraction and < 0.4 to 3.11 % (DE1 and DB1 respectively) in the < 0.063mm fraction.

The sampling location at Omey Island which had a very high TOC (30.3 %) is inter-tidal and may be subject to terrestrial peat/soil based influences and thus may not be truly representative of general marine sediments in the surrounding area. Therefore data at this site cannot be compared directly for all contaminant groups.

2.3.3 Assessment of metals in sediments

Table 2.3 displays the metal concentrations in various sites in both the < 2 mm and < 0.063 mm fractions (dry weight only). Normalised concentrations are presented graphically. All values are determined on a dry weight and normalised basis. The lowest levels of metals were found in the DB5 site in the < 0.063 mm fraction. The Omey Island site was determined to have the second lowest levels however based on TOC content, this site is unrepresentative of a marine sediment sample. From Figures 2.2 and 2.3 it can be seen that on a dry weight basis, the highest levels of metals were displayed in the < 2 mm fraction of the DB1 site with elevated levels of lead (265 mg kg^{-1}) and zinc (755 mg kg^{-1}) however,

when normalised to 5 % aluminium, the highest levels were shown in DB5 with elevated levels of copper, lead and zinc (2301, 2771 and 26780 mg kg^{-1} respectively). When data was normalised, all sites showed elevated levels of metals in both fractions.

The most elevated level of copper was determined at the Dunmore East site (DE2) in the < 2 mm and < 0.063 mm dry weight fractions (317 and 464 mg kg^{-1} respectively). These levels were similar to those of a previous report by Enterprise Ireland indicating high levels of copper (290 mg kg^{-1} of copper in the < 2mm fraction) at this site. Levels of zinc were also elevated at the DE sites, the highest level shown in DE2 (345 mg kg^{-1} dry weight).

On a dry weight basis, the metal concentrations in the < 2 mm and < 0.063 mm fractions of the sediment in the outer Dublin Bay area near the North Bank Lighthouse, DB5 (Figure 2.2) were found to be lower for all metals compared to the < 2mm fraction in the inner port (west Alexandra Basin DB1). On a dry weight basis, this inner port site (DB1) had the most elevated level of zinc (755 mg kg^{-1}). The enclosed environment of the Alexandra Basin has reduced marine flushing, increased sediment deposition and thus high residence of contaminants in surface sediment in the surrounding marine environment. The industrial impacts and shipping activity alone is a major source of pollution to this area. The export of lead and zinc ores contribute to the significant concentrations in the Alexandra Basin (Davoren et al, 2005). A history of heavy metal contamination has been previously shown to be a concern in North Dublin Bay (McBreen and Wilson, 2005).

Levels of lithium, arsenic, chromium, and nickel were similar in the dry weight < 2mm fraction of the Bull Lagoon (DB6) compared with the other Dublin Port sites. Mercury, cadmium, copper, lead and zinc values at the Bull Lagoon site were lower than the inner

Dublin Port site (DB1) (see Table 2.3). When these data were normalised to 5 % aluminium, the DB5 showed the highest levels of metal contamination. Anthropogenic influence in North Dublin Bay has been shown to reduce rapidly with distance from the port. Buggy and Tobin (2006) found similar levels of metals in Dublin Bay as those determined in this present study.

Table 2.4 presents metal concentrations normalised to 5 % aluminium as per Kersten and Smedes (2002) methodology discussed in chapter 1, section 1.5.4 of this thesis. Data is presented for the < 0.063 mm fraction in DB5 and DE2 for comparison with BAC data and OSPAR assessment criteria. Table 2.4 additionally presents comparison of < 2 mm dry weight fraction concentrations with ER_L/ER_M and Irish Sediment Quality Guideline (SQG) upper and lower levels in accordance with methodology set in chapter 1. It can be observed that concentrations of metals in the Dublin Port and Dunmore East exceed OSPAR BAC criteria where available.

The ER_L represents a chemical concentration below which the probability of toxicity is minimal (Long et al., 1998). In DB1, all metal concentrations with the exception of chromium were above both the ER_L and lower Irish SQG value. In DB5 however, copper, lead and zinc values exceeded both the ER_L and lower Irish SQG level. At the Bull Lagoon site (DB6) arsenic, nickel and lead were elevated above the ER_L and Irish lower level guideline level however zinc was not above the lower Irish SQG level. At DE1, copper and zinc were above ER_L and lower Irish SQG level. At DE2, copper, zinc, arsenic, nickel and lead concentrations were above ER_L and lower Irish SQG level. Concentrations for zinc and lead at the DB1 site, lead at the DB5 site, and copper at the DE2 site were above the ER_M and upper Irish SQG levels therefore adverse effects are potentially expected upon

exposure to contaminants at these sites. Concentrations of copper, zinc, manganese and vanadium were higher in DE2 compared to the DE1 site, which indicates that DE2 may be closer to the pollution source. This further stresses the importance of representative sampling even in locations of close proximity.

It was concluded as expected, that industrial areas showed more elevated levels of metals than areas which had little industrial/ urban influences, for example, the inner Alexandra Basin site (DB1) showed a more elevated level of zinc than the outer Dublin Port site at the navigation point of the Northbank lighthouse and to a lesser extent at Dublin Bay (DB6). Anthropogenic inputs and localised current flow which decreases the potential of marine flushing are potentially the causative factors in the elevated levels of metals in these areas. The potential for adverse effects on resident organisms, as evidenced by assessment criteria being exceeded, was demonstrated in sites DB1, DB5 and DE1.

Table 2.3: Concentrations of metals (dry weight mg kg^{-1}) in < 2mm and < 0.063mm (in parenthesis) fractions of sediments from selected Irish coastal locations. Aluminium concentrations are in g kg^{-1}.

	DP 1	DP 5	DP 6	DE 1	DE 2	OI 1*
Aluminium	19.2 (NA)	3.54 (14.7)	20.3(NA)	12.8 (NA)	26.9 (12.7)	15.4 (74.5)
Mercury	0.28 (NA)	< 0.05 (< 0.05)	0.08 (NA)	0.05 (NA)	nd (0.09)	nd (<0.05)
Lithium	33.7 (NA)	8.44 (28.8)	36.6 (NA)	29.4 (NA)	33.5 (28.3)	9.65 (4.55)
Arsenic	11.7 (NA)	2.62 (9.6)	15.4 (NA)	7.05 (NA)	12.4(11.5)	2.52 (1.68)
Cadmium	3.23 (NA)	0.19 (0.19)	0.37 (NA)	0.48 (NA)	0.38 (0.41)	0.42 (0.19)
Chromium	41.7 (NA)	12.5 (37.1)	55.5 (NA)	41.7 (NA)	51.0 (51.8)	31.3 (30.3)
Copper	78.8 (NA)	51.0 (32.1)	33.3 (NA)	76.8 (NA)	317 (464)	23.9 (15.1)
Lead	265 (NA)	62.2 (46.1)	68.2 (NA)	45.4 (NA)	53.4 (68.2)	18.0 (11.1)
Nickel	28.4 (NA)	10.9 (24.3)	35.6 (NA)	18.6 (NA)	21.1 (24.8)	10.3 (13.3)
Zinc	755 (NA)	590 (175)	154 (NA)	242 (NA)	345 (308)	13.2 (8.21)
Manganese	nd (nd)	nd (nd)	nd (nd)	nd (nd)	333	43.7 (nd)
Vanadium	nd (nd)	nd (nd)	nd (nd)	nd (nd)	46.1	35.8 (nd)

nd = not detected
NA = not analysed
* High TOC therefore data at this site used for comparison purposes only

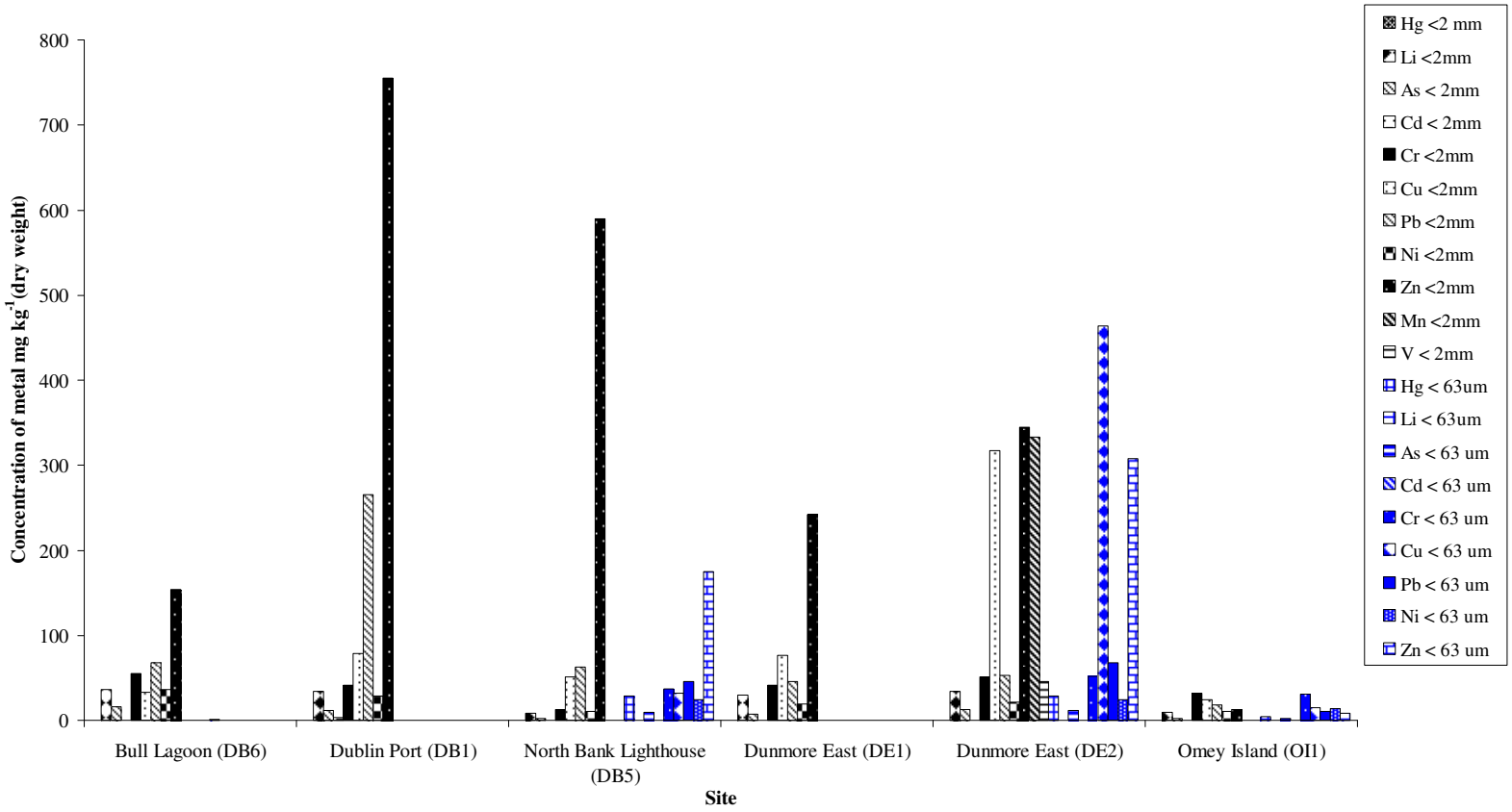

Figure 2.2: Concentrations of metals (dry weight mg kg^{-1}) in < 2 mm and < 0.063 mm fractions of sediments from selected Irish coastal locations.

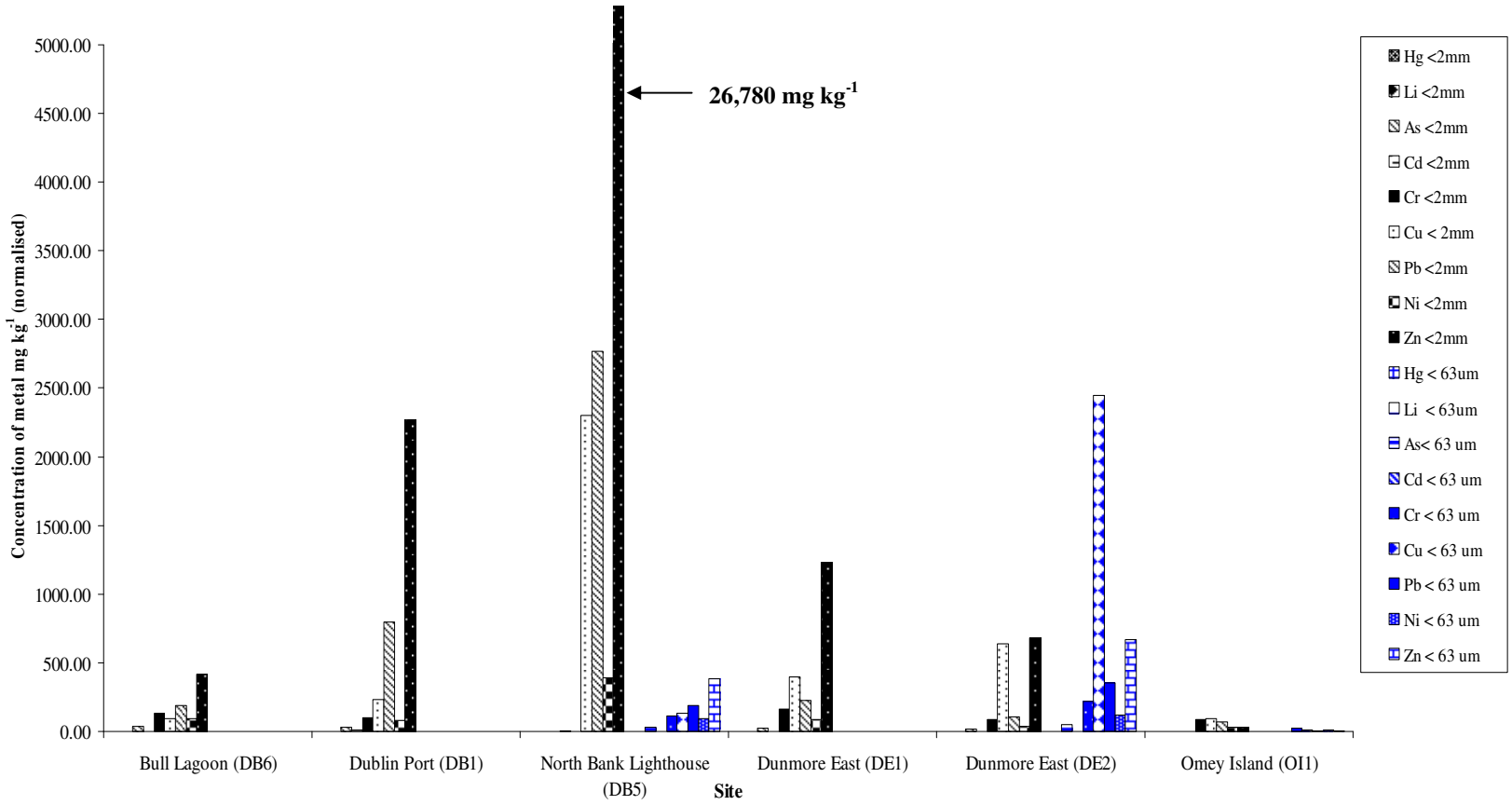

Figure 2.3: Concentrations of metals (mg kg^{-1}) normalised to 5 % aluminium in < 2 mm and < 0.063 mm fractions of sediments from selected Irish coastal locations.

Table 2.4: Concentrations of 8 elements (mg kg^{-1}) from various locations in the < 0.063 mm fraction normalised to 5 % aluminium compared to available BACs as derived by OSPAR CEMP 2004 and dry weight concentrations in the < 2 mm fraction compared to NOAA ER_L/ER_M values derived by Long et al., 1998 and Irish sediment quality guidelines (SQGs) upper and lower levels (Cronin et al., 2006) derived from whole sediment (< 2mm) data which are not normalised and are therefore compared to dry weight whole sediment concentrations. The concentrations in brackets are the < 0.063 mm normalised upper 95 % upper confidence limit and values above the BACs are in bold black, concentrations above ER_L and ER_M, bold blue and bold red respectively.

	Site	As	Cd	Cr	Cu	Hg	Ni	Pb	Zn
Provisional BAC		25	0.31	81	27	0.07	36	38	122
ER_L		8.2	1.2	81	34	0.15	20.9	46.7	150
ER_M		70	9.6	370	270	0.71	51.6	218	410
Irish SQG lower level		9	0.7	120	40	0.2	21	60	160
Irish SQG Upper level		70	4.2	370	110	0.7	60	220	410
c	DB1	11.7	3.23	41.7	78.8	0.28	28.4	265	755
a	DB5	**31.4**	**0.72**	**116**	**134**	**0.21**	**96.2**	**191**	**725**
b	DB5	**36.1**	**0.83**	**134**	**154**	**0.25**	**110**	**220**	**834**
c	DB5	2.62	0.19	12.5	**51.0**	<0.05	10.9	**62.2**	**590**
c	DB6	**15.4**	0.37	55.5	33.3	0.08	**35.6**	**68.2**	**154**
c	DE1	7.05	0.48	41.7	**76.8**	0.05	18.6	45.4	**242**
a	DE2	**47.9**	**2.0**	**218**	**2449**	**0.48**	**120**	**352**	**1594**
b	DE2	**55.1**	**2.4**	**250**	**2816**	**0.55**	**138**	**404**	**1833**
c	DE2	**12.4**	0.38	51.0	**317**	nd	**21.1**	**53.4**	**345**
a	OI1	2.14	0.13	24.3	10.2	0.03	9.55	7.94	8.14
b	OI1	2.46	0.15	27.9	11.7	0.04	11.0	9.13	9.36
c	OI1	2.52	0.42	31.3	23.9	nd	10.3	18.0	13.2

a- < 0.063 mm fraction concentration normalised to 5 % aluminium for BAC assessment
b- Upper confidence limit of < 0.063 mm fraction concentration normalised to 5 % aluminium for BAC
c- < 2 mm fraction dry weight concentration for comparison to SQGs and ER_L/ER_M data

2.3.4 Assessment of organotin compounds in sediments

The dry weight concentrations of the butyltin compounds TBT and DBT at the various sites are presented in Table 2.5. As observed in Figure 2.4, dry weight levels of organotin compounds (OTCs) were found to be highest at the DE2 in the inner harbour and lowest at DP5 at the outer Alexandra basin in the < 2mm fraction. The high TOC % values of 22.6 and 30.3% in the < 2mm and < 0.063mm fractions respectively of the Omey Island site could be due to terrestrial peat based influences. This site contained relatively elevated levels of OTCs (∑TBT and DBT 244 and 787 µg kg^{-1} in dry weight < 2 mm and < 0.063 mm fractions respectively) however this site cannot be used as a direct comparison to other marine sediments as it was more terrestrial based.

In general, the levels of OTCs were elevated (TBT was found to be in the range of 2125 – 22,707 µg kg^{-1} dry weight) in the three Dunmore East Harbour sites. The three sites at Dunmore East (DE1, DE2 and DE3) differed significantly in concentration demonstrating the importance of spatial sampling even within close proximity more significantly when monitoring TBT concentrations since a single paint fleck from a boat hull can elevate the concentration substantially. A study undertaken by Enterprise Ireland (consulting company) in 2002 determined similar levels of OTCs in spatial sediments from Dunmore East harbour. Levels of DBT were also found to be elevated compared to the levels outlined in the Enterprise Ireland report (Enterprise Ireland, 2002). Observed levels may potentially be related to the dry dock operations in close proximity to the sampling sites at Dunmore East. These observed levels could potentially threaten the surrounding marine ecosystem considering that TBT has been linked to imposex in 116 gastropod species at levels as low as 0.5 ng Sn/L (Birchenough et al., 2002, Santos et al., 2002). Elevated OTCs

pose an undesirable effect on surrounding ecosystems since strong correlations between concentrations of TBT in sediment and the marine mollusc *N. pernula* has been previously reported indicating transfer of this toxic substance into the marine food chain (Strand et al., 2003). It has been shown that levels of OTCs in harbours are higher than levels in estuarine and coastal sediments (Díaz et al., 2007). Chapter 5 describes a caging study utilising *Nucella lapillus* and *Crassostrea gigas* to monitor TBT induced bioeffects on organisms and demonstrates adverse effects at DE2 after an 18 week exposure.

Despite the concentration of TBT differing by an order of magnitude among Dunmore East samples (concentration of TBT at DE2 is 10.3 times higher than at DE1), TBT was found to be the dominating OTC species in the < 2 mm fraction of the three Dunmore East sites following the pattern as decribed by Hoch (2001). TBT domination was also evident for DB1 and DB2, however Dublin Bay locations DB3, DB4 and DB5 showed an equal or higher ratio of DBT than TBT indicating other inputs of DBT. On a dry weight basis DB1 in the west Alexandra Basin showed the most elevated OTC concentrations in the < 2mm fraction of all the Dublin Port sites analysed with a TBT level of 6621 µg kg^{-1}. A toxic effect on inhabiting gastropods has previously been reported by Wilson (2003). Also in this area, Buggy and Tobin (2006) showed levels of 0.1-1.5 µg kg^{-1} of TBT in sediments in the Tolka Estuary in Dublin, which is in the mid region of the Alexandra basin and the North Bull Island. These levels are low compared to the inner port and Bull Lagoon values in this study.

No comparison was made to OSPAR BACs as there is no available BAC for any of the OTCs however values are compared to NOAA ER_L/ER_Ms and Irish SQGs. The sediment quality guidelines have upper and lower action levels of 100 and 500 µg kg^{-1} for the sum of

TBT and DBT. Irish guideline values are based on information from the US-EPA comprising chemical and biological effects data for more than 1000 sediment samples using the Effects range low/ Effects range median approach however more accurate guidance values would be derived from Irish chemical and ecotoxicological data (Cronin et al., 2006). Levels of OTCs exceeded the Irish guideline values at all of the Dunmore East sites and at DB1 thus very strong evidence of adverse effects on marine organisms in particular at the Dunmore East site inducing imposex in the gastropod *Nucella lapillus* and shell thickening in the Pacific oyster *Crassostrea gigas* which is described later in chapter 5.

Overall, elevated levels of organotins were found in areas of high level boating activity. Dunmore East harbour (DE2) showed the highest concentrations of organotins whereas lowest levels were observed in the < 0.063 mm fraction of the inner Alexandra Basin (DB3). Despite the ban of TBT on boats less than ten metres in length, it is still present in harbours and ports at relatively elevated levels which may potentially pose a risk for the surrounding ecosystems.

Table 2.5 Concentrations of OTCs (dry weight µg kg $^{-1}$) in < 2 mm and < 0.063 mm (in parenthesis) fractions of sediments from selected Irish coastal locations.

	DB 1	DB 2	DB 3	DB 4	DB 5	DB 6	DE 1	DE 2	DE 3	OI 1
Tributyltin (TBT)	6621 (1934)	95.4 (75.5)	9.10 (16.9)	39.1 (18.5)	nd (109)	42.4 (NA)	2125 (NA)	22707 (6182)	11772 (NA)	192 (742)
Dibutyltin (DBT)	1362 (770)	72.4 (64.2)	53.1 (12.1)	39.3 (11.7)	36.2 (64.9)	75.9 (NA)	790 (NA)	7362 (965)	3555 (NA)	52.1 (45.6)

nd = not detected
NA = not analysed

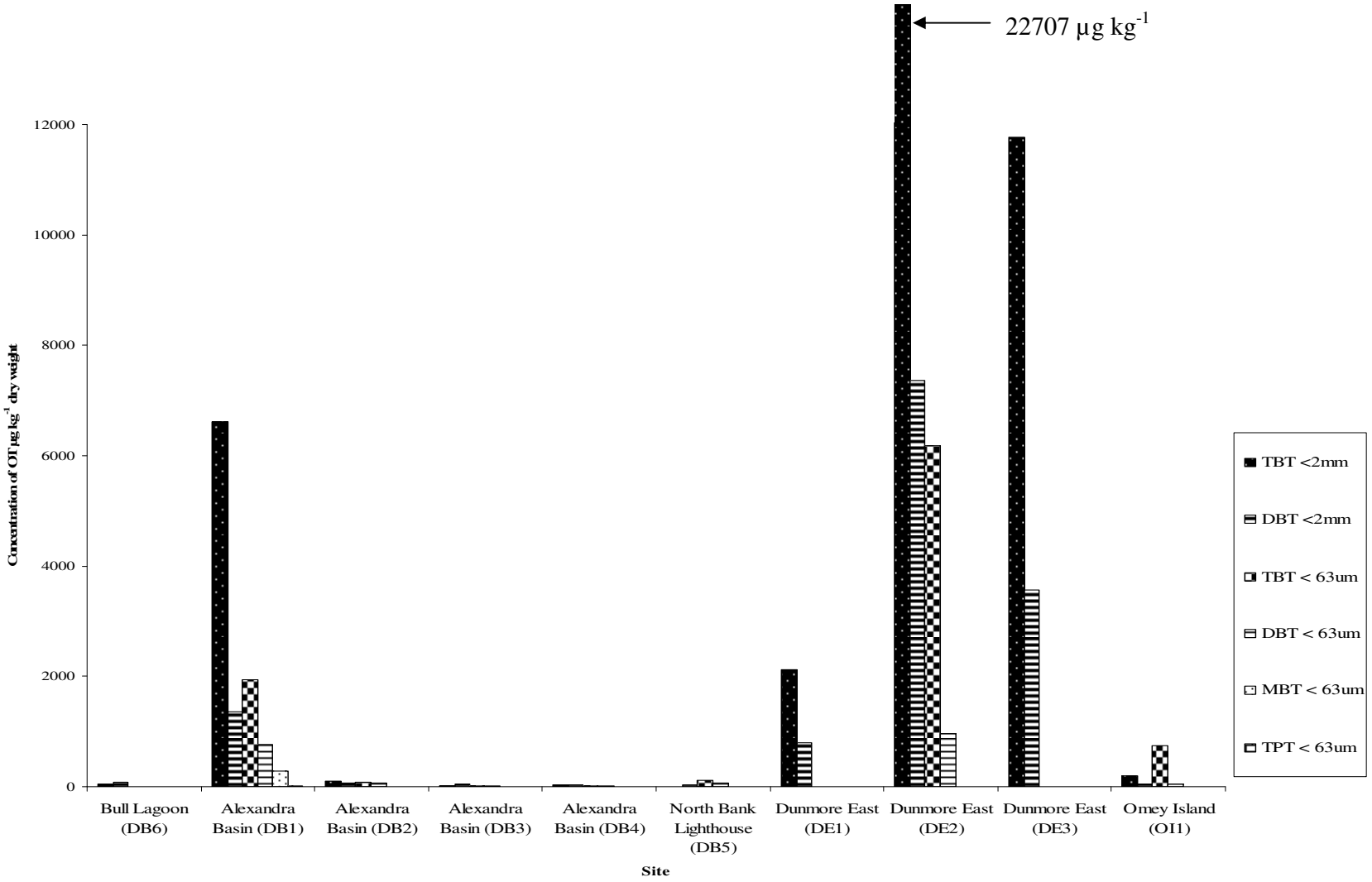

Figure 2.4: Concentrations of organotins (dry weight µg kg^{-1}) in < 2mm and < 0.063mm fractions of sediments from selected Irish coastal locations

2.3.5 Assessment of polycyclic aromatic hydrocarbons in sediments

Dry weight based oncentrations of PAHs detected in sediments are presented in Table 2.6. PAHs were detected in all samples analysed, the sum of all PAHs being 461– 12297.8 µg kg^{-1} dry weight in < 2mm fractions in Bull Lagoon (DB6) and Dunmore East (DE2) respectively and 3203- 9161 µg kg^{-1} dry weight in < 0.063 mm fractions at locations Dublin Bay (DB5) and Dunmore East (DE2) respectively. Higher PAHs levels were found in the < 0.063mm fraction indicating a correlation between PAHs and TOC content. The % TOC was found to be variable i.e. 0.28-1.89 % (DB6-DB2) in the < 2mm fractions and < 0.4-3.11 % (DB5- DB1) in the < 0.063 mm fractions. TOC values were highest at the Omey Island site (OI1) however this is due to this site being more terrestrial based than all other sites and cannot be used for comparison of data.

On a dry weight basis the highest concentrations of PAHs were found in the < 2mm fraction of the Dunmore East (DE2) site and the lowest concentrations found at the Bull Lagoon site (DB6) (12297 and 461 µg kg^{-1} respectively). Levels of PAHs at the Omey Island site in the < 0.063 mm fraction was determined to be 11993 µg kg^{-1} for the $\sum$ 21PAHs however, results for this site are not further discussed due to a high TOC % at this site. Elevated levels of $\sum$ 21PAH in the dry weight < 2mm fraction were also present in the inner Alexandra Basin (DB1) (6357 µg kg^{-1}). Concentrations decreased at the outer Alexandra Basin site at the North Bank lighthouse (DB5) (1270 µg kg^{-1}) and even lower levels observed at North Dublin Bay in the Bull Lagoon site (DB6) (461 µg kg^{-1}). However, normalisation of results to 2.5 % TOC demonstrated levels to be elevated to 5290 and 4117 µg kg^{-1} at DB5 and DB6 respectively. Potential geochemical differences could account for the normalisation differences however particle size distribution (PSD) data was

unavailable. Concentrations of hydrocarbons, PAHs and metals have been previously reported to be more elevated in the inner Dublin harbour and Tolka estuary but not elevated in the Bull Lagoon (Wilson, 2003). In general, on a 2.5 % TOC normalised basis, the lowest concentrations were found in the < 2 mm fraction of the DE1 site and the highest levels were found in the < 0.063 mm fraction at DE2 site, ∑ 21PAHs being 1992 and 13367 µg kg^{-1} for DE and OI respectively.

A wide range of PAH compounds were found in the < 2mm fractions of Dublin Port sites (DB1 and DB5) and the < 0.063 mm fraction of Dunmore East sites (DE1 and DE2) compared to the Bull Lagoon site (DB6). Alkylated isomers of dibenzothiophene, naphthalene and phenanthrene were measured in two sites in the < 0.063 mm of the Dublin Port site (DB5) and in both fractions of the Dunmore East site (DE2).

Levels of PAHs in all sites on a dry weight and 2.5 % normalised basis are presented in Figures 2.6 and 2.7 respectively. In general, the levels of PAH contamination at the sites analysed in this study were higher than literature values from Cork harbour which presented results of ∑ 21PAHs in the range of 170- 795 µg kg^{-1} dry weight in the < 0.063 mm fractions and 528 - 2878 µg kg^{-1} dry weight in the < 2mm fractions (Kilemade et al., 2004). The levels of the PAHs in this present study however were higher in the < 0.063 mm fraction than the < 2mm fraction which was not evident in data from Cork Harbour. Levels of PAHs in other areas such as the coastal areas in the Shetland and Orkney Islands have been previously reported to be in the range < LoD to 22619 µg kg^{-1} (Webster et al., 2001). These concentrations reported by Webster (2001) are higher than levels found at Dunmore East and Dublin Port locations despite the highly industrialised inputs into the areas.

2.3.5.1 Polycyclic aromatic hydrocarbon ratios in sediment

Petroleum contains lower molecular weight PAHs such as phenanthrene and anthracene. Due to its higher stability phenanthrene is higher relative to anthracene. PAHs of petrogenic origin are generally characterised by high phenanthrene: anthracene P/A ratios (>10) (Kilemade et al., 2004). Pyrogenic or petrogenic sources of the PAHs can be identified by evaluating compositions of the PAHs (Kilemade et al., 2004) and ratios of parent to alkyl-substituted PAH congeners. Ratios of the priority pollutants phenanthrene to anthracene (P/A) and fluoranthene to pyrene (FL/PY) are commonly used (Neff et al., 2005). Other ratios such as methylphenanthrene to phenanthrene (MP/P) and fluoranthene + pyrene to methylfluoranthene+ methylpyrene (FL+PY)/ (MFL+MPY), (Webster et al., 2001), chrysene to benzo[*a*]anthracene (Chry/BaA) and benzo[*a*]pryene to benzo[*ghi*]perylene (B(a)P/B[*ghi*]P) can be used to differentiate between sources.

Where data were available, ratios discussed above were determined and are presented in Table 2.6. The low (P/A), (FL/PY) and (Chrys/BaA) ratios in the < 2 mm sediments (Table 2.6) suggest pyrogenic sources. High (B(a)P/B[*ghi*]P) ratios i.e. > 0.8 are indicative of stationary source combustion emissions [e.g. power plants, gas stations etc.] (Kilemade et al., 2004).

From Table 2.6 it is observed that the Bull Lagoon site (DB6) , Dublin Port locations DB1 and DB5 and Dunmore East (DE1) all contained high B(a)P/B[*ghi*]P ratios indicating that stationary source combustion emissions may be potential sources of PAHs at these sites. To support this, it has also been previously established that by plotting the FL/PY ratio

against either P/A ratio or the MP/P ratio, a petrogenic or pyrogenic source can be predicted. In general, high FL/PY ratios and low P/A or MP/P ratios are characteristic of pyrogenic PAHs. (Webster et al., 2001) Two sites at Dublin Port (DB1 and DB5), one at Dublin Bay (DB6) and two Dunmore East sites (DE1 and DE2) were the only sites with concentrations of these compounds greater than the limit of quantification (> LoQ). A plot of the FL/PY ratio against the P/A ratio is presented in Figure 2.5 indicating PAHs from these sites are primarily from pyrogenic origin.

Comparison to OSPAR background assessment criteria for 10 PAHs (not exclusively priority PAHs) monitored in sediment in the < 0.063 mm fraction normalised to 2.5 % organic carbon ($\mu g\ kg^{-1}$) is presented in Table 2.7. These background concentrations are compared to normalised concentrations of PAHs from two sites (DB5 and DE2 with OI1 also included). It is shown that in the DB5 and DE2 sites, concentrations of all PAHs are above background levels. At the OI1 site however, all levels are near background level however, the high TOC % at this site deem it unrepresentative of a marine sediment sample**.** The figures in brackets are the 95 % upper confidence limit and values above the BACs are in bold.

The NOAA effects range low/ effects range medium data [ER_L/ER_M] and Irish guideline lower level ($\mu g\ kg^{-1}$ dry weight) are also presented in Table 2.7 for these 10 PAHs and sum 16 PAHs and compared to the dry weight concentrations in the < 2 mm fraction of all sites analysed. At the DB1 site, all PAHs with the exception of fluoranthene, pyrene and benzo-[ghi]-perylene exceeded the ER_L. At DB6 and DE2 only naphthalene exceeded the ER_L level however volatility of this compound makes it very difficult to measure accurately in freeze dried samples. All PAH levels in the < 2mm fraction were below the ER_L level at

the DE1 site. No concentration levels of PAHs exceeded the ER_M at any of the sites and therefore adverse effects, from PAH pollutants on aquatic species would potentially not be expected at these sites.

Irish SQGs for PAHs were derived from 95 percentile data of background values, from Marine Institute data, 2002-2003 (Cronin et al., 2006). These values were not normalised for organic carbon and the guidance lower level was based on the sum of 16 PAHs. From Table 2.7 it is shown that the lower level guidance value is set as 4000 $\mu g\ kg^{-1}$. This lower level Irish guideline value was exceeded at one site only (DB1) in the inner Alexandra Basin.

Table 2.6: Concentrations of PAHs (dry weight µg kg^{-1}) in both < 2 mm and < 0.063 mm fraction (in parenthesis) in sediments from various sites around the coast of Ireland

	DP 1	DP 5	DP 6	DE 1	DE 2	OI 1
Acenaphthene	75.1 NA)	30.0 (13.5)	<10.0 (NA)	<10.0 (NA)	<12.0 (13.8)	NA(<10.0)
Acenaphthylene	nd (NA)	0.00 (27.9)	nd (NA)	nd (NA)	31.8(14.1)	NA(<5.0)
Anthracene	168 (NA)	37.7 (35.9)	<10.0 (NA)	29.4 (NA)	82.8(79.1)	NA(<10.0)
Benzo[a]anthracene	382 (NA)	72.5 (86.1)	13.5 (NA)	63.6 (NA)	84.7(71.7)	NA(<6.0)
Benzo[a]pyrene	432 (NA)	72.0 (89.0)	14.1 (NA)	62.8 (NA)	70.8(70.4)	NA(<5.0)
Benzo[b]anthracene	306 (NA)	<10.0 (40.6)	<10.0 (NA)	<10.0(NA)	<10.0 (156)	NA(<10.0)
Benzo[b]fluoranthene	431 (NA)	69.7 (138)	17.5 (NA)	88.6 (NA)	136.0(152)	NA(<10.0)
Benzo[e]pyrene	495 (NA)	78.0 (109)	20.3 (NA)	<89.1(NA)	99.8(95.2)	NA(<10.0)
Benzo[ghi]perylene	374 (NA)	48.7 (112)	14.3 (NA)	64.1 (NA)	86.6(94.9)	NA(<10.0)
Benzo[j] fluoranthene	351 (NA)	48.3 (62.2)	11.7 (NA)	<54.7 (NA)	53.2(36.2)	NA(<10.0)
Benzo[k] fluoranthene	202 (NA)	38.5 (49.1)	<10.0 (NA)	<43.5 (NA)	41.2(43.3)	NA(<10.0)
Chrysene	435 (NA)	87.6 (139)	18.1 (NA)	129 (NA)	183(202)	NA(13.9)
Dibenzo[ah]anthracene	106 (NA)	11.0 (26.6)	<10.0 (NA)	15.2 (NA)	19.6(<10.0)	NA(<10.0)
Dibenzothiophene	nd (NA)	nd (22.8)	nd (NA)	nd (NA)	<14.0(23.6)	NA(<10.0)
DiBzThiphenes C1	nd (NA)	nd (61.5)	nd (NA)	nd (NA)	262(182)	NA(<10.0)
DiBzThiphenes C2	nd (NA)	nd (114)	nd (NA)	nd (NA)	1110(649)	NA(17.6)
DiBzThiphenes C3	nd (NA)	nd (97.0)	nd (NA)	nd (NA)	1480 (884)	NA(16.4)
Fluoranthene	561 (NA)	164 (164)	27.0 (NA)	117 (NA)	164(150)	NA (24.0)
Fluorene	173 (NA)	48.8 (29.3)	12.6 (NA)	30.0 (NA)	34.6(23.1)	NA(<10.0)
Indeno1,2,3-cdpyrene	472 (NA)	59.8 (102)	17.3 (NA)	91.3 (NA)	107.0(83.0)	NA(<5.0)
Naphthalene	192 (NA)	44.9 (143)	189 (NA)	< 43.1(NA)	239(24.2)	NA(<15.0)
Naphthalenes C1	nd (NA)	nd (117)	nd (NA)	nd (NA)	<10.0(22.4)	NA(<10.0)
Naphthalenes C2	nd (NA)	nd (185)	nd (NA)	nd (NA)	nd(149)	NA(13.0)
Naphthalenes C3	nd (NA)	nd (138)	nd (NA)	nd (NA)	595(580)	NA(19.0)
Perylene	166 (NA)	27.3 (35.9)	<10.0 (NA)	<51.6 (NA)	48.7(54.1)	NA(11,600)
Phenanthrene	419 (NA)	171 (137)	17.6 (NA)	65.4 (NA)	112(130)	NA(<10.0)
Phenanthrene C1	nd (NA)	nd (219)	nd (NA)	nd (NA)	945(873)	NA(19.2)
Phenanthrene C2	nd (NA)	nd (333)	nd (NA)	nd (NA)	2720(1,940)	NA(31.3)
Phenanthrene C3	nd (NA)	nd (221)	nd (NA)	nd (NA)	3310(2,140)	NA(46.0)
Pyrene	617 (NA)	150 (155)	28.1 (NA)	153 (NA)	235(215)	NA(16.8)
Sum 16 PAHS	5039 (NA)	1106 (1350)	409 (NA)	1006 (NA)	1640 (1381)	NA (171)
Sum all PAHs	6,165 (NA)	1,260 (3,203)	401 (NA)	909 (NA)	12297(9,151)	NA(11,817)
P/A Ratio	2.49 (NA)	4.54 (3.81)	1.76(NA)	2.22 (NA)	1.35(1.64)	(1)
FL/PY Ratio	0.91 (NA)	1.09 (1.05)	0.96 (NA)	0.77 (NA)	0.70(0.70)	(1.43)
Chry/B[a]A	1.14 (NA)	1.21(1.61)	1.34 (NA)	2.03 (NA)	2.16 (2.82)	(2.31)
B[a]P/B[ghi]P	1.16 (NA)	1.48 (0.79)	0.99 (NA)	0.98 (NA)	0.82(0.74)	(0.5)
TOC %	3.70 (3.11)	0.60 (1.30)	0.28 (1.50)	1.52 (<0.4)	2.30 (1.42)	22.6 (30.3)

nd: not detected
NA: not analysed
P/A: Phenanthrene: Anthracene; FL/PY: Fluoranthene: Pyrene; Chry/BaA: Chrysene/Benzo-[a]- anthracene; B(a) P/B[ghi]P: Benzo-[a]-pryene/Benzo[ghi]perylene
DiBzThiphenes C1,C2 and C3: Mono-, di and tri methyl-dibenzothiophene, Phenanthrene C1,C2 and C3: Mono-, di and tri methyl-phenanthrene, Naphthalenes C1, C2 and C3: Mono-, di and tri methyl-naphthalene

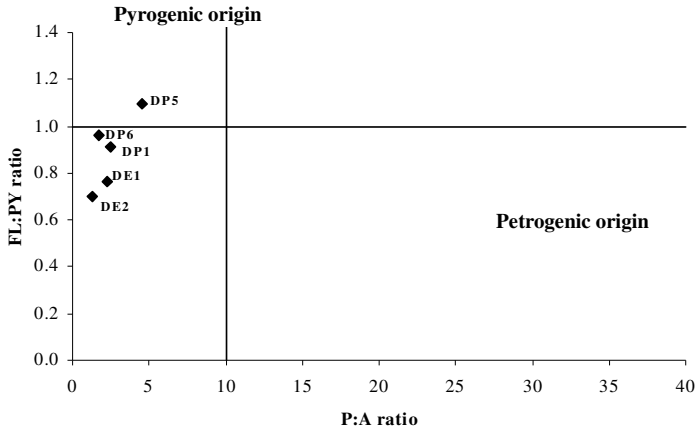

Figure 2.5: Ratios of PAHs P:A versus FL:PY for Dublin Port locations 1, 5 and site 6 (North Dublin Bay) and Dunmore East sites 1 and 2 for the < 2 mm fraction. Pyrogenic and petrogenic zones based on Webster et al. 2001.

Table 2.7: Provisional Background Assessment criteria (BACs) for 10 PAHs monitored in the < 0.063 mm fraction under the OSPAR CEMP (µg kg^{-1} dry weight) normalised to 2.5 % organic carbon. The figures in brackets are the 95 % upper confidence limit and concentrations above the BACs values are in bold. The NOAA ER_L/ER_M values and Irish guideline lower level are presented for these 10 PAHs and sum 16 PAHs respectively. Exceedences above ER_L, ER_M and Irish SQG lower level are in blue, red and green respectively.

Location	Site	*Anthracene*	*Fluoranthene*	*Phenanthrene*	*Naphthalene*	*pyrene*	*Benzo[a] anthracene*	*Benzo[a]pyrene*	*Benzo[ghi] perylene*	*Chrysene*	*Indeno123 cdPyrene*	*Sum 16 PAHs **
Provisional BAC		5	39	32	8	24	16	30	80	20	103	
ER_L		85.3	600	240	160	665	261	430		384	690	
ER_M		1100	5100	1500	2100	2600	1600	1600		2800	2600	
Irish SQG lower level												4000
a	**DB1**	**168**	**561**	**419**	**192**	617	**306**	**432**	374	**435**	472	**5039**
a	**DB5**	**69.3**	**315**	**264**	**275**	**298**	**166**	**171**	**215**	**267**	**196**	
b	**DB5**	**79.4**	**363**	**303**	**316**	**343**	**190**	**197**	**248**	**307**	**226**	
c	**DB5**	37.7	164	171	44.9	150	72.5	72.0	48.7	87.6	59.8	1106
a	**DB6**	<10.0	27.0	17.6	**189**	28.1	< 10.0	14.1	14.3	18.1	17.3	409
a	**DE1**	29.4	117	65.4	< 43.1	153	63.6	62.8	64.1	129	91.3	1006
a	**DE2**	**139**	**264**	**229**	**42.6**	**379**	**126**	**124**	**167**	**356**	**146**	
b	**DE2**	**160**	**304**	**263**	**49.0**	**435**	**145**	**143**	**192**	**409**	**168**	
c	**DE2**	82.8	164	112	**239**	235	84.7	70.8	86.6	183	107	1640
a	**OI1**	0.83	1.98	0.83	1.24	1.39	0.50	0.41	0.83	1.15	0.41	
b	*	0.95	2.28	0.95	1.42	1.59	0.57	0.47	0.95	1.32	0.47	
c	**OI1** *	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
	OI1 *											

a- < 0.063 mm fraction concentration normalised to 2.5 % TOC
b- Upper confidence limit of < 0.063 mm fraction concentration normalised to 2.5 % TOC
c- < 2 mm fraction dry weight concentration
*Note: Sum 16 PAHs include anthracene, acenaphthylene, fluoranthene, phenanthrene, pyrene, benzo-[a]-anthracene, benzo-[a]-pyrene, benzo-[ghi]-perylene, acenaphthene, chrysene, dibenzo-[ah]-anthracene, benzo-[k]-fluoranthene, benzo-[b]-fluoranthene, naphthalene, fluorene and indeno-[1, 2, 3-cd]-pyrene

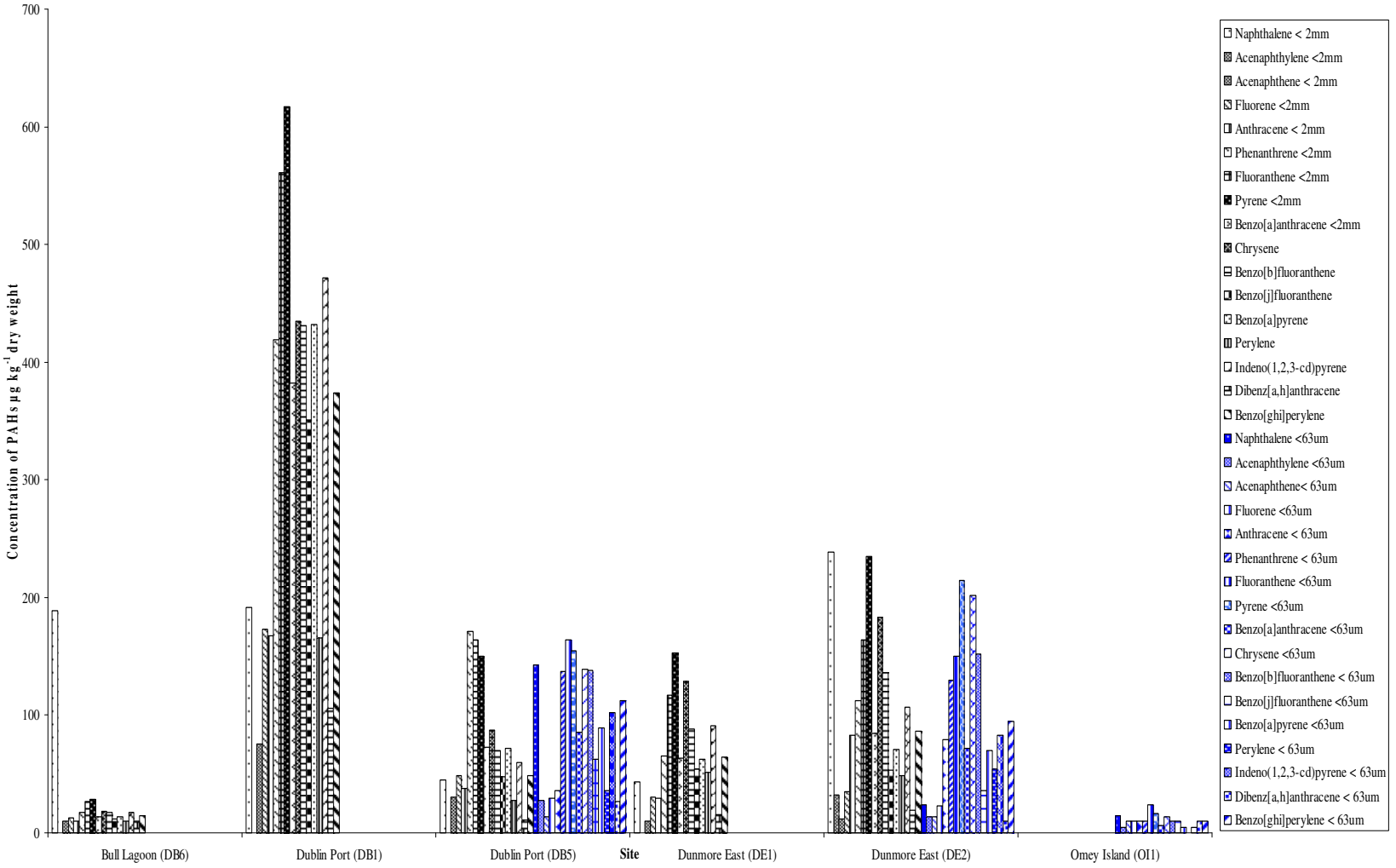

Figure 2.6: Dry weight concentrations of PAHs (µg kg^{-1}) in order of increasing molecular weight in < 2 mm and < 0.063 mm fractions of sediments from selected Irish coastal locations.

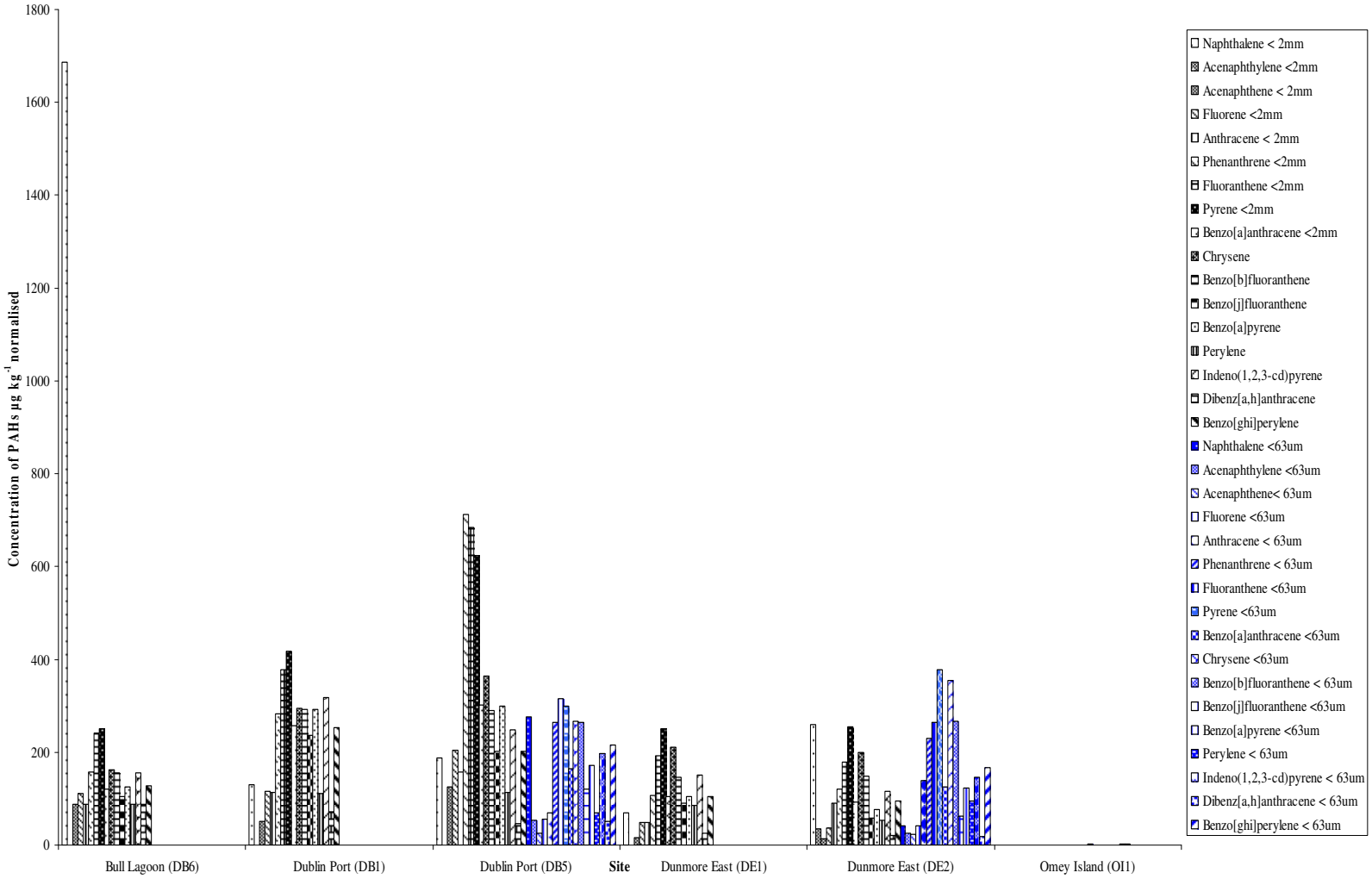

Figure 2.7: Concentrations of PAHs (µg kg^{-1}) in order of increasing molecular weight normalised to 2.5 % TOC in < 2 mm and < 0.063 mm fractions of sediments from selected Irish coastal location**s.**

2.3.6 Assessment of hydrocarbons in sediment

Gas Chromatography Flame Ionisation Detection (GC-FID) and Gas Chromatography Mass Spectrometry (GC-MS) have been found to be very useful tools in hydrocarbon profiling of sediment extracts and gives an indication of source identification (Webster et al., 2001). For sediments containing long-chain odd carbon alkanes (nC_{21}-nC_{33}) it is assumed that they have no petrogenic contamination (Webster et al., 2001). When crude oils degrade, the n-alkanes are lost initially and eventually all n-alkanes disappear and the so called "hump" appears when analysed with GC-FID. This "hump" is known as an unresolved complex mixture (UCM) and is composed of the more persistent compounds in the oil such as branched and cyclic compounds and is characteristic of petrogenic pollution.

Sediments from North Dublin Bay site at the Bull Lagoon (DB1), Dublin Port inner Alexandra Basin (DB1) and Dunmore East (DE1) sediment samples which were also used for ecotoxicological testing, were analysed for hydrocarbons with GC-FID in the < 2mm fraction. A number of reference oils and an n-alkane mix (C_{10}-C_{40}) were analysed with the samples. Figure 2.8 presents the various overlayed chromatograms of the various reference oils and oil products and an n-alkane mix analysed as reference standards.

The < 2mm Bull Lagoon (DB6) sample is not further discussed as there was a low level of hydrocarbon pollution evident. It can be seen in Figure 2.9 that the Dublin Port (DB1) location sample showed a profile of hydrocarbons, which showed similar characteristics to the petroleum standard hydrocarbon profile (Figure 2.8). In the Dunmore East sample (DE1) GC-FID chromatogram however (Figure 2.10) it can be observed that a number of long chain hydrocarbons C_{20}-C_{38} are present in the sample. These hydrocarbons have a

similar profile to that of the spilt/degraded crude oil which can be seen in Figure 2.8 however in this reference crude oil chromatogram there are no dominating alkanes and in the DE sample the C_{28}-C_{32} are the dominating n-alkanes. The dominating odd carbon n-alkanes C_{29} and C_{31} could potentially be an indication that these hydrocarbons originate from biogenic processes and there is no petrogenic influences at this site correlating well with the PAH results which have previously been discussed. Interestingly, the Bull Lagoon sediment (DB6) which was observed to have a low hydrocarbon profile which correlated with a low TOC value (0.28 %) at this site compared with the other two samples, DB1 and DE1 which were observed to have hydrocarbon profiles and contained higher TOC content (3.70 and 1.52 % respectively).

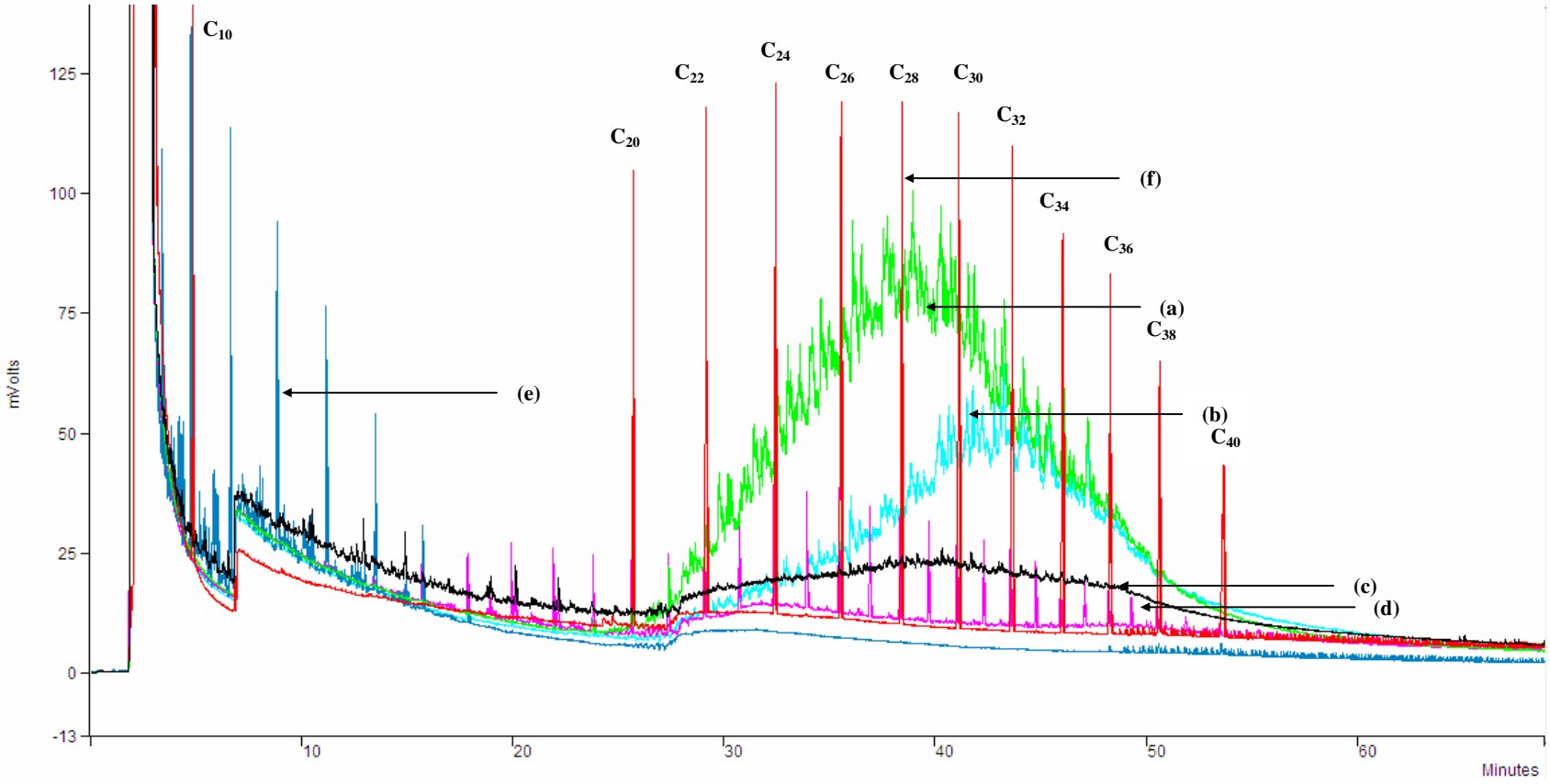

Figure 2.8: GC-FID overlayed chromatogram of (a) lubricating oil (green), (b) marine lubricating oil (light blue), (c) alba oil (black), (d) spilt/degraded crude oil (purple)(e) petroleum (dark blue) and (f) a long chain hydrocarbon mix C_{10}-C_{40} (red).

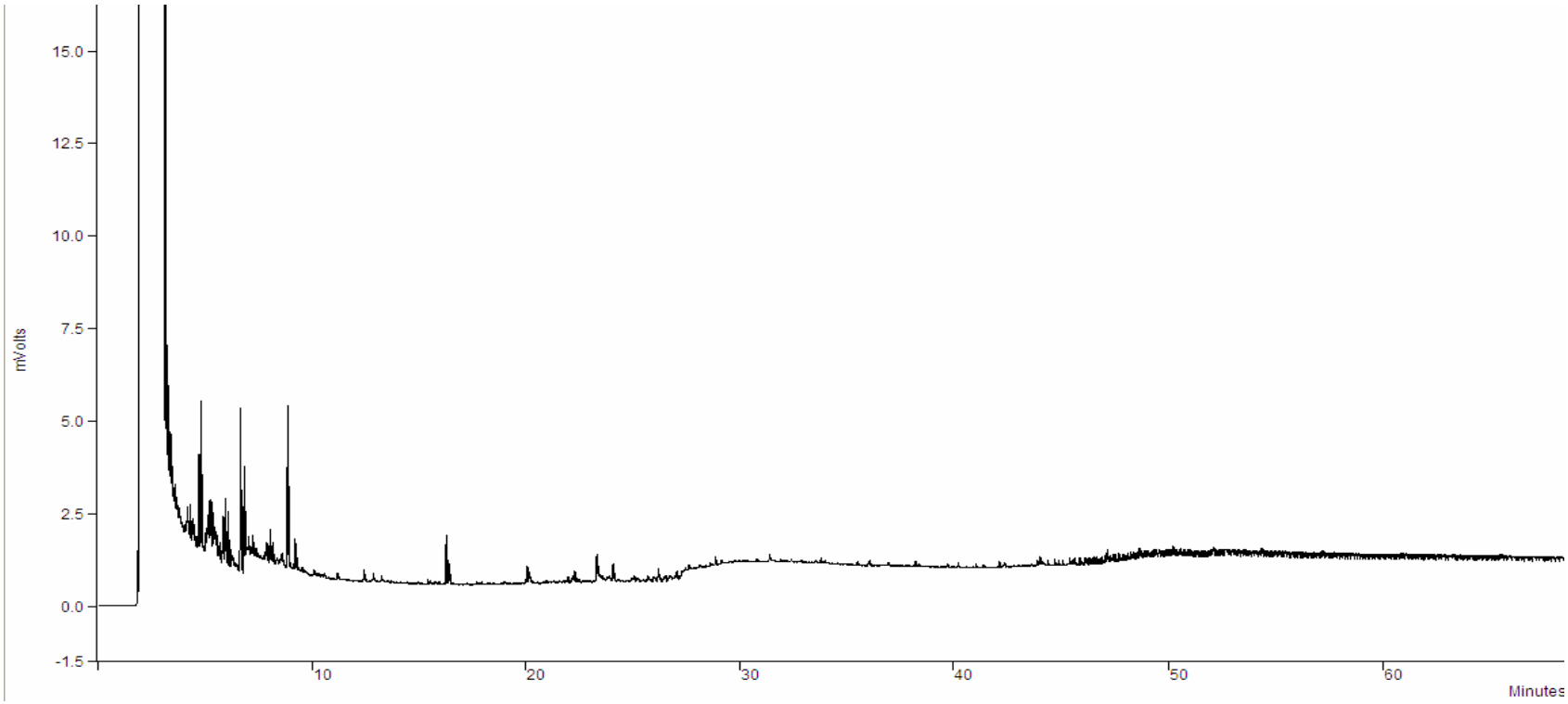

Figure 2.9: GC-FID chromatogram of the < 2 mm fraction of a Dublin Port (DB1) sediment sample**.**

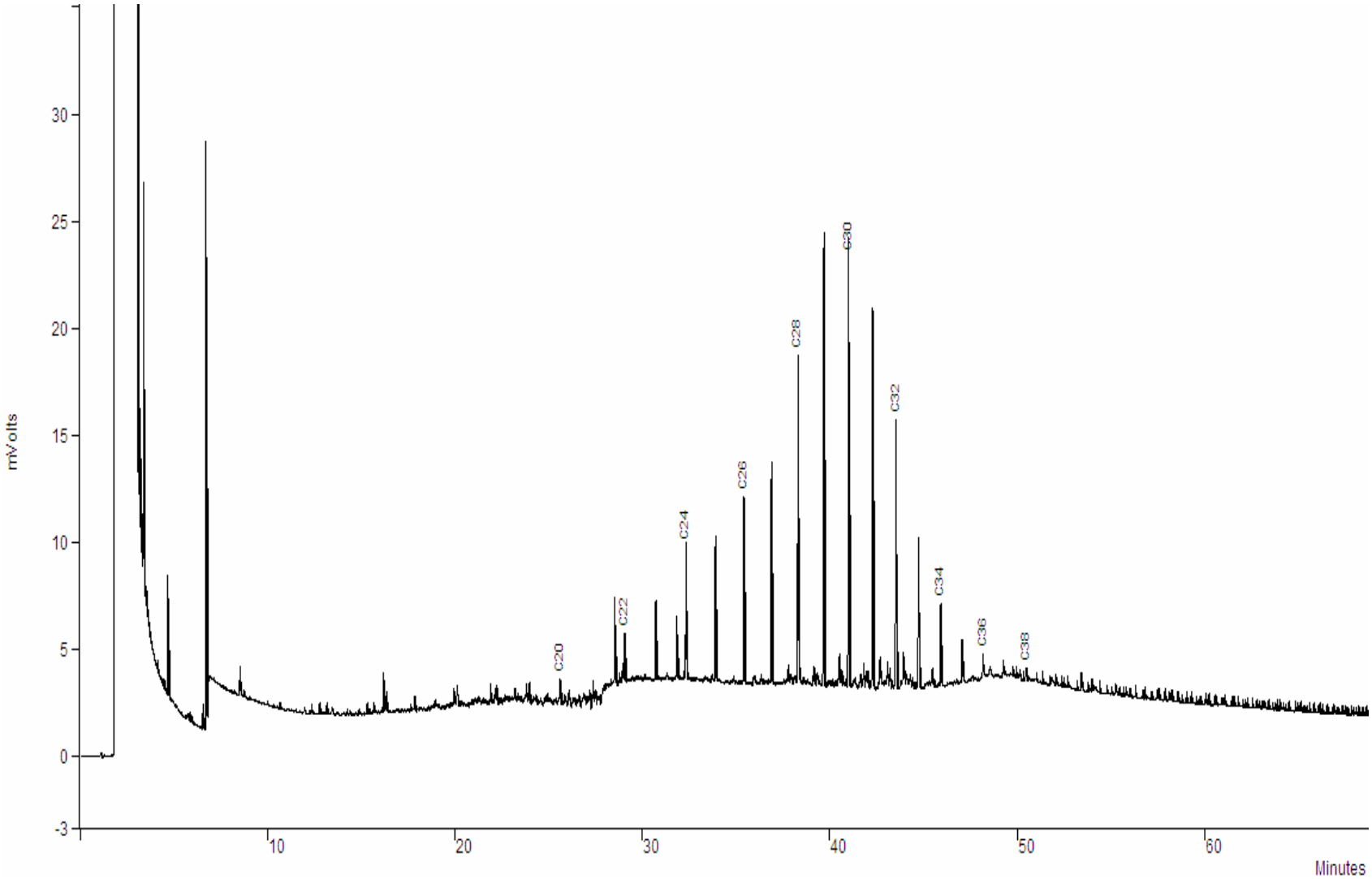

Figure 2.10: GC-FID chromatogram of the < 2 mm fraction of Dunmore East (DE1) sediment sample.

2.3.7 Assessment of organochlorine compounds in sediments

PCBs are persistent and hydrophobic pollutants due to their low rates of degradation, low water solubility, and high degree to which they partition to particles and organic carbon (Kilemade et al., 2004). Therefore sediments serve as a sink for these recalcitrant compounds. The sediments which were analysed in this study did not show elevated levels of PCBs and OCs while limits of quantification were relatively high (approximately 3 µg kg^{-1}dry weight) for each compound for these samples primarily as a result of small sample size and also as a result of small sample size and analytical interference however, potential for indoor background interference resulting in elevated LoDs for certain contaminants cannot be discounted.

Tables 2.8 and 2.9 display the concentrations of PCBs and OCs in both the < 0.063 and < 2 mm fractions in various sediments analysed for the dry weight determination. In some cases the detection limits were raised due to interference (e.g. for endosulfan B and aldrin in sites DB1 and DE 1). On a dry weight basis, the highest upperbound (i.e. where < LoQ the LoQ has been used) levels of PCBs were determined in the DE1 site (∑PCBs 36 µg kg^{-1}) and lowest levels were determined to be in the < 2 mm fraction of the DE2 site (∑PCBs 0.9 µg kg^{-1}). Where concentrations are < LoQ, normalization has been completed for indicative purposes only. On a 2.5 % TOC normalised basis however, the highest concentrations of PCBs were shown in the Bull Lagoon (DB6) (∑PCBs 295 µg kg^{-1}) in the < 2mm fraction and the lowest concentrations were found in the < 2 mm DE2 site (∑PCBs 1.00 µg kg^{-1}). The highest levels of OCs are in the < 2mm fraction of Dublin Port (DB1) (∑OCs 229 µg kg^{-1}) on a dry weight basis and in < 2mm fraction of the Bull Lagoon site (DB6) (∑OCs 589 µg kg^{-1}) on a normalised basis. The lowest values were shown in the

DE2 site on both a normalised and dry weight basis. Levels of PCBs and OCs at the Omey Island site were low in comparison to other sites but the high TOC % in this sample suggests that this is not marine sediment and concentrations cannot be compared to other sites data.

The limits of quantification in this study were found to be quite high compared to other studies in Ireland. Kilemade (2004) determined levels in Cork Harbour to be detected at or below the detection limit also however the detection limits ranged from nd to 0.2 µg kg^{-1} for PCBs and nd to 0.2 µg kg^{-1} for OCs so therefore a direct comparison cannot be made.

While the majority of sites showed levels > LoQ, a comparison to Background Assessment Criteria (BACs) for 7 PCBs and hexachlorobenzene (HCB) monitored in sediment under the OSPAR CEMP (µg kg^{-1}) normalised to 2.5 % organic carbon was completed on upperbound levels and is presented in Table 2.10. The figures in brackets are the 95 % upper confidence limit ($t_{0.95}$ ± SE @ 15%) and values above the BACs are in bold. These BACs are compared to < 0.063 mm normalised data in two sites (DE2 and OI1).

The DE2 site contains levels that exceed the background level with the exception of PCBs 153 and 180. The OI1 site however contains low levels which are below the background assessment criteria and therefore the levels of these contaminants are near background at this site. However this site was determined to have a high TOC % and therefore cannot be directly compared with other site data.

The ER_L /ER_M levels (µg kg^{-1} dry weight) are also presented for these 7 PCBs and 4 organochlorine pesticide compounds. In all sites, levels of PCBs do not exceed the ER_L or

ER_M level and therefore are unlikely sites for ecotoxicological concern with these specific contaminants. The limits of quantification for the PCB and OC compounds however are elevated and therefore a comparison cannot be made with ER_L /ER_M or Irish sediment quality guideline data.

It was concluded that as a result of the high LoQs (primarily due to small sample size) with the exception of the DE2 site that data for PCBs and OCs are of limited use for integrative purposes.

Table 2.8: Concentrations of PCBs (dry weight µg kg^{-1}) in both < 2 mm and < 0.063 mm fraction (in parenthesis) in sediments from various sites around the coast of Ireland

	DB 1	DB 5	DB 6	DE 1	DE 2	OI 1*
PCB-028	<3.60(NA)	< 3.00(NA)	<3.00(NA)	<3.00(NA)	<0.10(NA)	nd(nd)
PCB-052	<3.50(NA)	< 3.00(NA)	< 3.00(NA)	<3.00(NA)	<0.10(<0.20)	nd(<0.1)
PCB-101	4.00(NA)	< 3.00(NA)	< 3.00(NA)	3.90(NA)	<0.10(<0.30)	nd(<0.1)
PCB-105	<3.00(NA)	<3.00(NA)	<3.00(NA)	<3.00(NA)	nd(<0.10)	nd(<0.1)
PCB-118	3.40(NA)	<3.00(NA)	<3.00(NA)	3.40(NA)	<0.3(nd)	nd(nd)
PCB-128	<3.00(NA)	<3.00(NA)	<3.00(NA)	<3.00(NA)	nd(<0.10)	nd(<0.10)
PCB-138	4.30(NA)	< 3.00(NA)	< 3.00(NA)	4.70(NA)	<0.10(nd)	nd(nd)
PCB-153	<3.00(NA)	< 3.00(NA)	< 3.00(NA)	<3.00(NA)	<0.10(<0.30)	nd(<0.1)
PCB-156	<3.00(NA)	<3.00(NA)	<3.00(NA)	<3.00(NA)	nd(<0.20)	nd(<0.1)
PCB-170	<3.00(NA)	<3.00(NA)	<3.00(NA)	<3.00(NA)	nd(nd)	nd(nd)
PCB-180	<3.00(NA)	<3.00(NA)	<3.00(NA)	<3.00(NA)	<0.10(nd)	nd(nd)

nd: not detected
NA: Not analysed
* For reference only

Table 2.9: Concentrations of OCs (dry weight µg kg^{-1}) in < 2 mm fraction in sediments from various sites around the coast of Ireland

	DP 1	DP 5	DP 5	DE 1	DE 2	OI 1*
1,2,3-Trichlorobenzene	<3.00	<3.00	<3.00	<3.00	nd	NA
1,2,4-Trichlorobenzene	<4.80	<3.00	<3.00	<3.80	nd	NA
1,3,5-Trichlorobenzene	<3.00	<3.00	<3.00	<3.00	nd	NA
Aldrin	**	<3.00	<3.00	**	<1.0	NA
***cis*-Chlordane**	<3.00	<3.00	<3.00	<3.00	nd	NA
***Trans*-Chlordane**	<3.00	<3.00	<3.00	<3.00	nd	NA
o, p'-DDE	<3.00	<3.00	<3.00	<3.00	nd	NA
p, p'-DDE	<3.00	<3.00	<3.00	<3.00	<1.0	NA
o, p'-DDT	<14.5	<3.00	<3.00	<25.8	<1.0	NA
p, p'-DDT	<18.5	<3.00	<3.00	<22.5	<1.0	NA
p, p'-TDE	<5.30	<3.00	<3.00	<3.00	<1.0	NA
Dieldrin	<7.30	<3.00	<3.00	<3.00	<1.0	NA
Endosulfan A	<23.9	<3.00	<3.00	<3.00	nd	NA
Endosulfan B	<94.6	<4.6	< 3.00	**	nd	NA
Endrin	<12.9	<3.00	<3.00	<3.00	<1.0	NA
Hexachlorobutadiene	<3.00	<3.00	<3.00	<3.00	<1.0	NA
α-HCH	<3.30	<3.00	<3.00	<5.50	<1.0	NA
β-HCH	<3.00	<8.80	<3.0	<3.00	<1.0	NA
δ-HCH	<3.00	<3.00	<3.00	<3.00	<1.0	NA
γ-HCH	<11.2	<3.00	<3.00	<3.00	<1.0	NA
Hexachlorobenzene	<3.00	<3.00	<3.00	<3.00	<1.0	NA
Isodrin	<3.00	<3.00	<3.00	<3.00	<1.0	NA

nd: not detected
NA: Not analysed
* For reference only
** Interference raised for this sample

Table 2.10: Provisional Background Assessment criteria (BACs) for 7 PCBs monitored in sediment under the OSPAR CEMP (µg kg^{-1}) normalised to 2.5 % organic carbon. The figures in brackets are the 95 % upper confidence limit and values above the BACs are in bold. Comparisons to NOAA ER_L and ER_M levels are completed in (c) and Irish sediment quality guideline values are also presented for various organochlorine compounds.

	PCB 101	*PCB 118*	*PCB 138*	*PCB 153*	*PCB 180*	*PCB 28*	*PCB 52*	*HCB*	*γ-HCH*	*o, p' DDD*	*o, p' DDE*	*p, p' DDE*	*p, p' DDT*
BAC	0.14	0.17	0.15	0.19	0.10	0.22	0.12	0.16					
ER_L	22.7	22.7	22.7	22.7	22.7	22.7	22.7			1.58	2.2	2.2	1.6
ER_M	180	180	180	180	180	180	180			27	27	27	27
Irish SQG lower level	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.27	0.21				
Irish SQG Upper level	180	180	180	180	180	180	180	230	1				
DP 1													
c	4.00	3.40	4.30	< 3.00	< 3.00	< 3.60	< 3.50	< 3.00	< 3.00	nd	< 3.00	< 3.00	< 18.0
DP5													
c	< 3.00	< 3.00	< 3.00	< 3.00	< 3.00	< 3.00	< 3.00	< 3.00	< 8.80	nd	< 3.00	< 3.00	< 3.00
DP6													
c	< 3.00	< 3.00	< 3.00	< 3.00	< 3.00	< 3.00	< 3.00	< 3.00	< 3.00	nd	< 3.00	< 3.00	< 3.00
DE1													
c	3.90	3.40	4.70	< 3.00	< 3.00	< 3.00	< 3.00	< 3.00	< 3.00	nd	< 3.00	< 3.00	< 22.5
DE 2													
a	**0.18**	**0.18**	**0.18**	0.18	**0.18**	**0.35**	**0.53**						
b	**(0.20)**	**(0.20)**	**(0.20)**	**(0.20)**	**(0.20)**	**(0.40)**	**(0.61)**						
c	< 0.10	< 0.30	< 0.10	< 0.10	< 3.00	< 3.00	< 3.00	< 1.00	< 1.00	nd	nd	< 1.00	< 1.00
OI 1													
a	0.01	0.01	nd	nd	nd	0.01	0.01						
b	0.01	0.01				0.01	0.01						
c	nd	nd	nd	nd	nd	nd	nd	NA	NA	NA	NA	NA	NA

a- < 0.063 mm fraction concentration normalised to 2.5 % TOC for BAC comparison
b- Upper confidence limit of < 0.063 mm fraction concentration normalised to 2.5 % TOC for BAC comparison
c- < 2 mm fraction dry weight concentration for comparison to SQG and ER_L/ER_M criteria

2.4 Conclusion

Chemical analysis was performed on sediments from a range of selected locations around the coast of Ireland. Levels of metals, PAHs, and organotins were found to be elevated in the inner harbours as opposed to the sites with less significant industrial inputs. The most detrimental and toxic compound TBT was determined to be dominant in all sites and was found in elevated levels at Dunmore East Harbour. Ratio profiling of PAHs proved to be a useful tool in determining the source however this was questioned in some cases with oil fingerprinting techniques. Even with elevated LoQs, levels of OCs were relatively low in all locations in both < 2mm and in < 0.063 mm sediment fractions. Comparison of chemical data with OSPAR background assessment criteria, NOAA ER_L/ER_M levels and Irish guideline upper and lower action levels was useful in determining whether adverse effects could be expected at specific sites. Chemical analysis alone however, is not sufficient and very limited in providing information on ecosystem health and does not address the actual impacts of the contamination on ecosystems. The data generated in this chapter shall contribute and be the basis of an overall assessment and integration of this data with a selected battery of bioassays and biomarkers. Elevated pollution levels (especially for TBT at Dunmore East sites) and the potential for associated biological effects are further discussed in chapter 4 (cell line study), chapter 5 (nucella study) and overall in chapter 8 (integration of all data).

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3

CHAPTER 3 OPTIMISATION AND VALIDATION OF A METHOD FOR SPECIATION OF ORGANOTINS IN MARINE SEDIMENT

3.1 Introduction

3.1.1 Organotin usage patterns

Worldwide usage of organotin compounds (OTCs) in a variety of marine related applications has increased in the past 50 years. As a consequence of such applications, considerable quantities of organotins have entered the marine environment. Organotin compounds such as tributyltin (TBT) have been used in marine environments as effective antifouling agents in marine paints for shipping and for fish culture (Minchin, 2003). TBT has been shown to degrade to its di- and mono- metabolites (Tsunoi et al., 2002) however these degradation metabolites dibutyltin (DBT) and monobutyltin (MBT) have been used previously as stabilisers in polymers such as poly vinyl chloride (PVC) (Ikonomou et al., 2002) and can enter the marine environment from leaching of PVC pipes (Leroy et al., 1998).

3.1.2 Summary of biological effects of organotin compounds

In general, the higher substituted organotins have been found to be the most toxic to marine organisms. TBT being one of the most toxic compounds, has been found to cause chronic and acute poisoning in aquatic organisms for example, the development of the motile spores of a green macroalga was the stage most sensitive to TBT (5-day EC_{50}: 0.001 $\mu g\ L^{-1}$). There was also reduced growth of a marine angiosperm observed at TBT concentrations of 1 mg/kg in sediment but no effect at 0.1 mg/kg (World Health Organisation, 1990). *Nucella lapillus* are recognised internationally as a bioindicator of TBT contamination. Exposure of TBT to *Nucella lapillus* causes imposex in the dogwhelk, this condition manifesting itself as the imposition of male

characteristics on the female dogwhelk following exposure to concentration levels as low as 1ng L^{-1} with severe cases resulting in sterilisation of the organism (Bryan et al., 1986; Gibbs and Bryan, 1986; Bryan et al., 1987). TBT has also been linked to reductions in whole soft tissue weight, increased shell thickness and shell deformation in Pacific oysters (Alzieu et al., 1982). As organotin compounds are recognised pollutants of priority concern both biological and chemical monitoring of these compounds in Ireland is mandatory within the OSPAR and ICES (WKIMON) framework. Many countries have restricted the use of TBT but Ireland was one of the first countries to ban the use of all organotin containing compounds on vessels less than 25m in 1987 (Minchin, 2003).

3.1.3 Nomenclature and physico-chemical properties of OTCs

Organotin compounds are organic derivatives of tetravalent tin and may be represented by the general formula $R_pSnX_{(4-p)}$ where R is an alkyl or aryl group (e.g. methyl-, butyl-, ethyl-, phenyl-) and X is an anionic group such as a halide, oxide or hydroxide (Hoch, 2001). TBT exists in seawater as three species: hydroxide, chloride and carbonate which remain in equilibrium. At pH values less than 7.0 the predominant forms are the hydroxide and chloride and at pH 8 are the chlorides, hydroxides and carbonates and above pH 10 the hydroxide and carbonate predominate (World Health Organisation, 1990). In general OTCs existing as neutral ion pairs and complexes or as cations in the aquatic system have low water solubility (Rüdel, 2003) however, di and mono- butyltin have a higher solubility in water (Hoch, 2001). The log K_{ow} for TBT-Cl and TPT-Cl has been reported to be in the ranges 2.3-4.4 and 2.0-4.1 respectively (Rüdel, 2003), thus OTCs are strongly bound to the particulate phase and have been found to bind to oxides, hydroxides and organic material (Hoch, 2002). The bioavailability of organotin compounds is greatest at neutral and slightly alkaline pH values and is reduced in the

presence of dissolved organic carbon. Bioconcentration of TBT and TPT in marine aquatic systems has been shown to decrease with increasing concentrations of humic acids (Rüdel, 2003).

Butyltin compounds are strongly bound to particulate matter. Tributyltin is bound by hydrophobic forces whereas monobutyltin is bound by a high electrical charge (Smedes et al., 2000). The affinity of these compounds for the particulate phase makes them liable to enter the food chain through sediment dwelling organisms. Therefore it is essential that determination of the individual OTCs is completed and that sensitive and reproducible analytical methodologies are developed to quantitate these compounds. This study reports the optimisation and validation for speciation of OTCs in marine sediment. The relevance of sediments in terms of ecotoxicological potential is addressed in Chapter 2 of this thesis.

3.1.4 Analytical methods

Analytical methods for the speciation of OTCs in sediment samples can often be complex. Difficulties arise in setting up single methods that are capable of extracting and quantifying all OTCs in a matrix, this primarily being a result of the wide range of hydrophobicities of OTCs. This chapter concentrates on the validation of a method for the determination of OTCs within the OSPAR framework and concurrently a method suitable for the extraction of OTCs into a media suitable for ecotoxicological bioassay use.

3.1.4.1 Extraction

A wide variety of extraction procedures have been reported for sediment OTC speciation analyses including leaching with acid followed by shaking (Bravo et al., 2004), leaching with acid followed by sonication (Millán et al., 2000) and pressurised fluid extraction (Wasik et al., 2007). The most common method of extracting OTCs from the sediment particles is leaching with strong acids [e.g. hydrochloric acid (HCl)]. Using this method, a solvent may be added to enhance the extraction of tributyltin species (e.g. methanol). The OTCs are therefore present in a methanolic environment and ready for derivatisation. Other methods of extraction include leaching under acidic conditions with simultaneous extraction into an organic phase using various acids and solvents and *in situ* derivatisation with simultaneous extraction into an organic phase.

In this study, leaching with a high concentration of HCl was chosen followed by extraction to the organic phase using dichloromethane (DCM) which is immiscible with water. Subsequent solvent exchange to methanol was required for the derivatisation reaction. Extraction into DCM prevents the problems associated with high acid concentration used in derivatisation processes as discussed below. The method as described in this chapter allows for the extraction of the organotin salts in their chloride form for subsequent testing on a cell line which is discussed in Chapter 4 entitled Toxicity Evaluation of Irish Marine Sediment using two fish cell lines Integrated with Chemical Analysis (submitted manuscript 2008).

3.1.4.2 Derivatisation

Derivatisation reactions applied to organotin analysis are generally hydride generation with sodium borohydride ($NaBH_4$), ethylation with sodium tetraethylborate ($NaBEt_4$) and alkylation with Grignard reagents. In this study, ethylation with $NaBEt_4$ was chosen. Derivatisation with $NaBEt_4$ must take place in an aqueous/methanolic

environment and this reagent can be used directly in contact (*in-situ*) with the sample or after extraction into an organic extract (post extraction derivatisation). For *in-situ* derivatisation, a simultaneous derivatisation/extraction is performed which reduces the number of analytical steps compared to a post extraction derivatisation method and hence reduces potential sources of error. The optimum pH for carrying out this reaction has been proven to be 4-5 (Quevauviller et al., 2000). At lower pH values the conversion of $NaBEt_4$ to $NaBH_4$ was observed (Quevauviller et al., 2000) and the reagent decomposes faster as it reacts with protons (Smedes et al., 2000). The time of reaction proved to be the most important for the stripping efficiency of the organotin species (Quevauviller et al., 2000) as the derivatising reagent can degrade in approximately 60 secs. This reagent is not stable in the presence of strong acids and decomposes therefore the pH is crucial in organotin derivatisation. Ethylation with $NaBEt_4$ presents high yields of derivatisation in comparison to hydride generation.

Hydride generation is also suitable for aqueous samples however this type of reaction is hindered by interferences with humic acids, low yields, and poor reproducibility for sediments with high organic carbon content. Grignard reactions are performed on the organic extract rather than the aqueous phase. These reagents react violently with acids, water, alcohols, ketones and more steps are needed in the method creating potential sources of error such as contamination risks, decomposition and losses. Also, using the Grignard technique with sediments containing a high quantity of sulphur is hindered as elemental sulphur is co extracted and alkylated causing interferences on GC-MS and GC-FPD (Quevauviller et al., 2000).

3.1.4.3 Clean up of organotin compounds

Leaching followed by extraction to an organic phase will potentially co-extract a variety of other compounds from the sediment sample. Sulphur and sulphur containing compounds, oil, and many other anthropogenic and natural compounds can potentially be co-extracted. Also, by-products from ethylation with sodium tetraethylborate such as boroxin, diethylsulphide and diethyltrisulphide will be present in large quantities (Wasik et al., 2007). Various desulphurisation methods have been used previously including the use of deactivated copper, tetrabutylammonium sulphite (TBA) and mercury which eliminate elemental sulphur in the extract. TBA was utilised for sulphur removal during this project.

Silica (SiO_2), alumina (Al_2O_3) and florisil column clean ups have been used previously to remove interfering polar compounds for sediment extracts. In this study, alumina was chosen as a clean up reagent and was deactivated with 5% water (highly deactivated materials [> 5 % water] are not recommended, as OTCs can potentially degrade during elution). Hexane washed sodium sulphate has been previously used for removal of water from the organic extract.

There was no significant toxicity observed when method blank extracts (in suitable substrate media) were exposed to *Vibrio fisheri*, *Tisbe battagliai* or *Onchorynchus mykiss* RTG-2 cell line (see chapter 4). These method blanks incorporating the use of tetrabutylammonium sulphite for the removal of sulphur, alumina 5 % deactivated with water and sodium sulphate for removal of water were deemed to be suitable for proceding to the cell line study as outlined in Chapter 4.

3.1.4.4 Separation and Quantification of organotin compounds

Methodologies for separation and quantification of organotin species generally involve gas chromatography (GC) coupled to an element selective detector. Commonly used detectors include atomic absorption spectrometry (AAS), microwave-induced plasma atomic emission spectrometry (MIP-AES), inductively coupled plasma-optical emission spectrometry (ICP-OES), inductively coupled plasma- mass spectrometry (ICP-MS), mass spectrometry (MS), flame photometric detection (FPD) and more recently pulsed flame photometric detection (PFPD) (Bravo et al. 2004). In this study, GC coupled to PFPD was used for separation and quantification of OTC species. PFPD has been shown to have detection limits for organotin compounds 25 to 50 times lower than those obtained with conventional FPD (Bravo et al., 2004). The PFPD sensitivity is attributed to the reduction of flame background and chemical noise due to its filtration in time, dark current reduction due to its current gating, higher signal brightness due to lower combustible gas flow rate and smaller combustor volume as well as the use of broad band colour glass filters. The possible separation in time of the signal from unwanted hydrocarbon emission results in a significant improvement of the selectivity and therefore the PFPD is a specific detector with total intolerance against hydrocarbon compounds (http://www.tau.ac.il/chemistry/amirav/pfpd.shtml).

3.1.4.5 Quality assurance of methodology

Addition of a recovery standard is crucial for quality assurance as the extraction and derivatisation can easily be incomplete. Tripropyltin chloride has been used extensively as a recovery standard for OTC analysis but could not be used in this study as the method was optimised for cell culture exposures (as described in chapter 4) as this compound has been found to be toxic to cells at a very low concentration (Brüschweiler et al., 1995). The freshwater sediment certified reference material BCR646 was analysed with every batch to further ensure compliance with internal quality control

procedures and recoveries of analytes were based on a long term reproducibility study using this material. This was the only CRM available with certified values of the butyl- and phenyl-tin compounds and was used extensively in the literature with anlaysis of marine samples. Blanks were analysed with every batch to ensure no contamination was present.

In summary the purpose of this chapter was to develop a method for the extraction of OTCs (and co-extracts) from sediment into a form suitable for both the quantitation of OTC concentrations and for transfer of extract to media suitable for use in cell line assays and toxicity identification evaluation as described in chapters 4 and 6 respectively.

3.2 Materials and methods

3.2.1 Materials

Tributyltin chloride (CAS No. 1461-22-9), dibutyltin dichloride (CAS No. 683-18-1), monobutyltin trichloride (CAS No.1118-46-3), triphenyltin chloride (CAS No.639-58-7) and external standard tetrapropyltin were obtained from LGC Promochem (UK). Diphenyltin dichloride (CAS No.1135-99-5) was obtained from Sigma-Aldrich (Ireland). Stock solutions of ethylated standards and the external standard tetrapropyltin were prepared in hexane and stock solutions of the chlorides were prepared in methanol. The freshwater sediment certified reference material, BCR646 and OTC quantification external standard tetrapropyltin were obtained from LGC Promochem (UK). Hydrochloric acid (33%) was obtained from AGB, Ireland. The solvents, dichloromethane, propan-2-ol, hexane, acetone and methanol (all pestiscan grade) were obtained from Labscan, Ireland. Tetrabutylammonium hydrogen sulphate, sodium sulphite, sodium acetate, sodium hydroxide, sodium

sulphate and the derivatising agent sodium tetraethylborate 97% (STEB) were obtained from Sigma-Aldrich (Dublin, Ireland). The ethylated organotin standards in hexane were obtained from the Quality Assurance of Information for Marine Environmental Monitoring in Europe (QUASIMEME) project office (Wageningen, The Netherlands).

3.2.2 Gas chromatographic analysis-separation, detection and quantification

GC allows the separation of volatile and semi-volatile compounds through gas phase molecular interactions with a stationary phase. As compounds travel through the capillary column to the detector they are separated by differences in their boiling point, solubility, adsorption and molecular structure. Compounds are separated by their partition via sorption processes on a high boiling point liquid stationary phase bonded to the walls of a narrow bore capillary column. The GC is coupled to a PFPD detector for detection of analytes. The details of the GC conditions are detailed below. This method was based on an in-situ method by Strand et al. (2003) and modified to be a post extraction derivatisation of OTCs for cell culture exposure.

3.2.2.1 GC conditions employed for the analysis of organotins in sediment

For analysis of OTCs, a Varian Gas Chromatograph equipped with an 8400 autosampler and a pulsed flame photometric detector fitted with a BG12 filter was used. Separation of ethylated OTCs was performed using a ZB-1 capillary column 30m x 0.32mm (Phenomenex) coated with 100% dimethyl polysiloxane (PDMS 0.25µm thickness). Helium was used as carrier gas (flow rate: 2.0ml min^{-1}). The column was held at 70 ºC for 3 min and subsequently increased to 220 ºC at a rate of 5 ºC min^{-1} and held for 7 min. The injector was maintained at a temperature of 240 ºC in split/splitless mode. A high transmission band filter (390 nm ID) and interference filter (610 nm) was used to

observe the emission corresponding to the Sn-C and Sn-H molecular bonds. The signal acquisition was carried out with a gate delay of 3.0 ms and a gate width of 2.0 ms after each flame ignition. An air- hydrogen flame was used. The compressed air is divided into two flows: The first one "Air 1" is mixed with hydrogen and carries the effluent from column to the detector. The second one "Air 2" is added to the mixture in the detector to control the ignition rate of the pulsed flame. The flow rates of the three gases were as follows: Air 1 flow 22.0 ml min^{-1}, H_2 flow 25.0 ml min^{-1}, Air 2 flow 30.0 ml min^{-1}. The composition of the fuel gas mixture can influence the emission profile.

3.2.2.2 Stationary phase selection

The ZB-1 column or equivalent has been used for organotin separation in the literature (Bravo et al., 2004). This low polarity column is composed of 100 % dimethyl-polysiloxane and has a temperature limit of 370 ° C. The DB-5 column (5 % phenyl-95 % dimethylpolysiloxane) has been more extensively used in the literature (Jacobsen et al., 1997; Ikonomou et al., 2002; Wasik et al., 2007).

3.2.2.3 Optimisation of injection temperature

The optimisation of injection temperature was carried out using one standard (approximately 360 ng g^{-1}). A temperature range of 240 – 280 ºC was tested (range taken from literature) for optimal injection conditions. The optimum peak height for all ethylated OTCs was obtained with an injection temperature of 240 ºC.

3.2.2.4 Repeatability of injection type using an ethylated organotin mixture

The repeatability of the injection type was tested using standard on-column, split/splitless, and pressure pulse injection by analysis of an ethylated standard mixture (n=15). The ratio of peak heights tributyltin: tetrapropyltin was measured for each

injection type and relative standard deviations % RSD values were found to be 8.6, 5.5 and 4.3 % for on-column, split/splitless and pressure pulse respectively. Therefore pressure pulse was chosen as injection type for this method.

3.2.2.5 Repeatability of instrumental internal standard tetrapropyltin

The repeatability of the instrumental internal standard tetrapropyltin was tested by running a tetrapropyltin standard (concentration 595.9 ng g^{-1}) in hexane (n=14). Measurement of peak height for the 14 standard analyses showed a mean peak height of 4322 ± 222 and a % RSD of 5.1 %.

3.2.2.6 Optimisation of external standard

The concentration of external standard to be added to each sample as an injection correction standard was decided using the concentration range of organotins found in the literature and the GC calibration curve. Figure 3.1 below demonstrates the concentration of external standard versus peak height response. Three different concentrations (313.3, 595.9, 1164 ng g^{-1}) of tetrapropyltin were analysed (n=3). The highest concentration, 1164 ng g^{-1} did not show overloading but had greater error associated with it compared to the lower concentrations tested. The concentrations of butyltins found in various sediments in the Irish marine environment have been found to range from 0.01 ng g^{-1} (unpublished Irish data) to 2000 ng g^{-1} (Enterprise Ireland, 2002) therefore an external standard concentration of approximately 300 ng g^{-1} was chosen as an instrumental internal standard concentration.

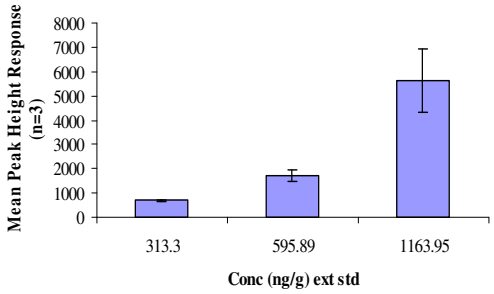

Figure 3.1: Concentration instrumental internal standard versus mean peak height response.

3.2.2.7 Optimisation of pH for derivatisation

The pH dependency on derivatisation yield of five organotin compounds was tested by derivatising an organotin chloride mix containing tributyltin chloride (TBT-Cl), dibutyltin dichloride (DBT-Cl_2), monobutyltin trichloride ($MBTCl_3$), triphenyltin chloride (TPT-Cl) and diphenyltin dichloride (DPT-Cl_2) in methanol using a pH range from pH 3-6. Analysis was completed on these samples in order to determine the optimal pH conditions.

3.2.2.8 Methodology for pH dependency study

Approximately 1ml of the organotin mixture was spiked onto 10 mls of 0.4M HCl in an acid washed glass centrifuge tube. After pH adjustment, (pH range 3-6) one ml of sodium tetraethylborate in methanol (10 % w/v) was added and the mixture was immediately vortexed for 5 mins. The derivatisation step was repeated on the extract in triplicate for maximum derivatisation efficiency. Thereafter 10 mls of hexane was added and the solution was vortexed for 5 mins. After settling for 2 mins, the hexane

layer (top) was removed. The extraction into hexane was also repeated in triplicate for maximum recovery. The three hexane layers were pooled and concentrated to approximately one ml, 70 mg of instrumental internal standard was added and extracts were analysed using GC-PFPD.

3.2.2.9 Results of pH dependency study

The % recovery for each analyte was calculated based on the spiking concentrations and the results for the pH dependency study are presented in Figure 3.2 which displays the % recovery for each of the various pH derivatisation yields for each analyte over the pH range (n=3).

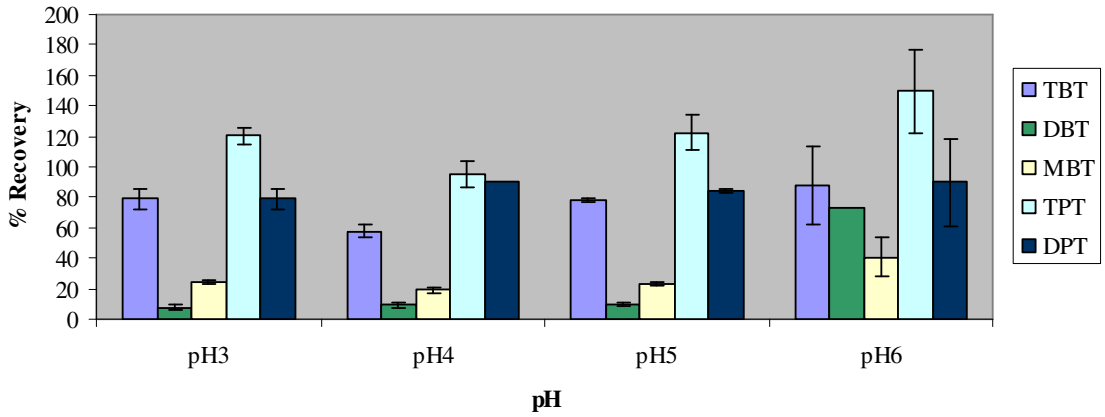

Figure 3.2: The percentage recovery of five organotin analytes over the pH range 3-6

The % recoveries for the organotin analytes proved to be highly variable. The optimum pH for TBT was between pH 5-6 with recoveries of 77.6 and 87.6 % which was similar to the literature value of pH5 ± 0.5 (Strand et al., 2003). There was greater recovery error associated with pH 6 (SD± 25.6%) for TBT demonstrating that pH 5 would be more appropriate as the optimum pH for this analyte. Low recoveries were obtained for DBT and MBT with the exception of pH 6 which demonstrated recoveries of 73.5 % (n=1) for DBT and 40.8 % for MBT (n=3).

Further testing and recoveries of these analytes from certified reference materials is later described. TPT demonstrated percentage recoveries in excess of 100 % potentially demonstrating contamination of either reagents used in the procedure or standards with triphenyltin. Blank extracts did not however show positive recoveries. DPT was shown to have optimum recovery of 90 % at pH 4. Figure 3.3 demonstrates that other organotins were detected such as monophenyltin trichloride (MPT) which was not present in the organotin mixture and therefore could potentially be a degradation product of TPT or DPT or could be present in one of the reagents or standards used.

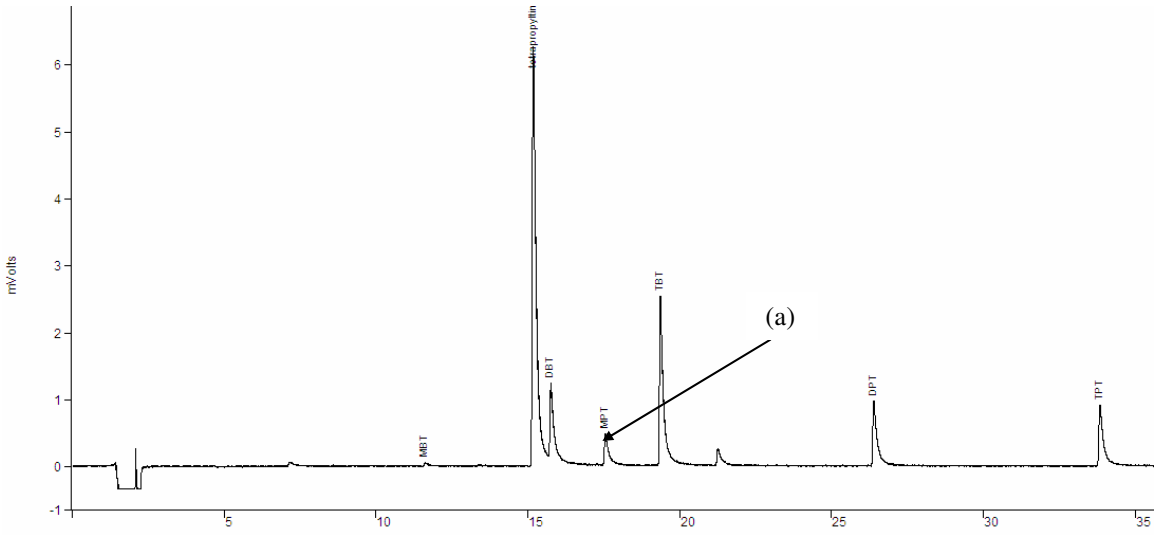

Figure 3.3: Chromatogram of derivatised OTCs showing presence of (a) MPT

3.2.3 Method for speciation of organotins in marine sediment

An adaption of an *in-situ* ethylation method (Strand et al., 2003) was employed for extraction of sediments for both quantification and cell line assay purposes with modifications detailed below.

Freeze dried sediment samples (0.5 g) were digested in a solution of HCl: H_20 (1:1 v/v) for 30 min in an ultrasonic bath. The mixture was then extracted with dichloromethane (DCM) for 30 min using a vortex mixer and the organic layer was decanted. The digestion procedure was completed on the sample in triplicate to maximise recovery. The DCM layer was firstly solvent exchanged to hexane and then subsequently to methanol, the pH was adjusted to 5.0 ± 0.5 with 10 % sodium acetate and 20 % sodium hydroxide in deionised water and ethylation of organotins completed using a 10 % w/v solution of sodium tetraethylborate (STEB) in methanol. The ethylated OTCs were then extracted into hexane and a clean up was performed on the organic extract with sodium sulphate and 5 % water deactivated alumina. Sulphur was removed from the extract using tetrabutylammonium sulphite which is described. To prepare the sulphite reagent, tetrabutylammonium hydrogen sulphate (3.39g) was dissolved in organic-free reagent water ($100cm^3$). This was extracted three times with three aliquots of hexane ($20cm^3$) to remove impurities. The hexane extracts were discarded and sodium sulphite (25g) was added to the water solution. The resulting solution, [tetrabutylammonium sulphite (TBA)] which is saturated with sodium sulphite was used for sulphur removal from the solvent extracts. The extract (1ml) was transferred quantitatively with hexane (1ml) to a 60ml turbo vap vial and TBA-sulphite (1ml) and propan-2-ol (2ml) was added and the bottle capped. This mixture was shaken for at least 1 min. Clear crystals (precipitated sodium sulphite) were indicative that sufficient sodium sulphite was present. If precipitated sodium sulphite disappeared, more crystalline sodium sulphite was added in approximately 0.100g portions until a solid residue remained after repeated shaking. Purified water (5ml) was added and shaken for at least 1 min and the solution was allowed to stand for 5-10 min. The hexane layer (top) was then transferred to a concentrator tube and was concentrated to approximately 1ml. The actual volume of the final extract was recorded. Tetrapropyltin was then added to the extract in the gas chromatography (GC) vial as an injection correction standard and the sample extract

was analysed using GC-PFPD. No procedural internal standard was employed during the course of this study as suitable compounds *e.g.* tripropyltin chloride have been previously found to be toxic to biological organisms at very low levels (Brüschweiler et al., 1995). The freshwater sediment certified reference material BCR646 was analysed with every batch to further ensure compliance with internal quality control procedures. A diagram outlining the method for speciation of organotins in marine sediment is presented in Figure 3.4 and is detailed below.

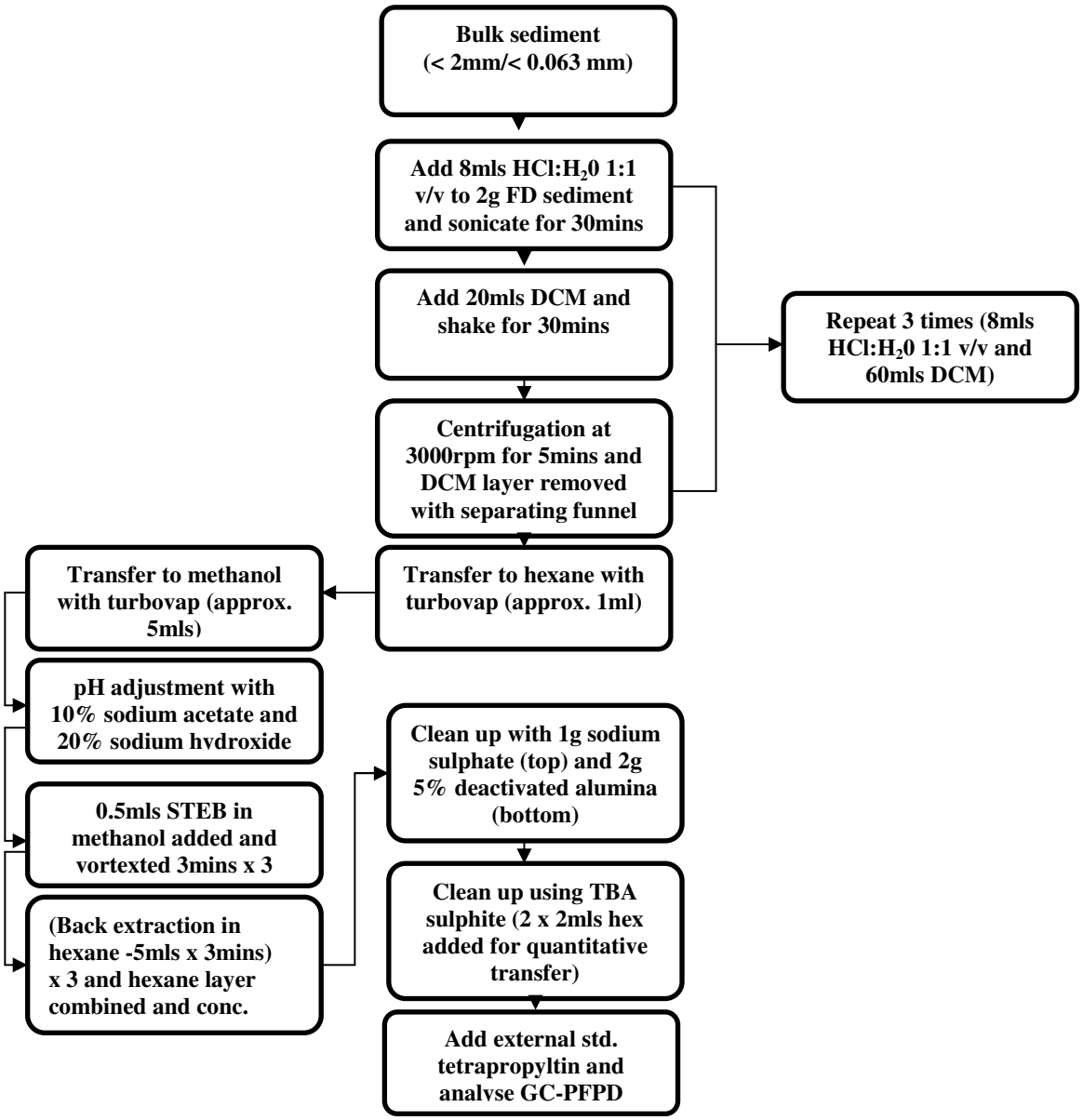

Figure 3.4: Method diagram for speciation of organotin compounds in marine sediment for analytical quantification.

3.3 Validation of Methodology

Method validation provides an assurance of reliability and is sometimes referred to as "the process of providing documented evidence that the method does what it is intended to do". Method validation was completed to ensure that an analytical methodology is accurate, precise, specific, reproducible, and linear over specified ranges ensuring that only the analyte will be analysed.

3.3.1 Accuracy

Accuracy is the measure of exactness of an analytical method (EURACHEM, 1998). It measures the closeness of agreement between the value accepted either as a conventional true value or a reference value and the result obtained from a particular method. Accuracy can be calculated by comparison of observed results from environmental samples to expected values.

Accuracy was determined with the freshwater certified reference material BCR646 which has certified values of tri-, di- and mono- butyltin and tri-, di- and mono-phenyltin. Certified values and percentage recoveries of the butyltins were determined in the sediment and results are presented in Table 3.1 below.

Table 3.1: Percentage recoveries of butyltins in the certified reference material BCR646 showing upper and lower control limits (UCL/LCL) as ± 2 SD mean % recovery.

Analyte	Certified value (uncertainty) µg kg^{-1}	Recovery (%) 1*	2*	3*	Mean	% RSD	UCL	LCL
TBT	480(80)	63.2	63.2	64	63.4	0.71	63.9	63.0
DBT	770(90)	69.2	71.4	74.4	71.7	3.65	74.3	69.0
MBT	610 (120)	29.1	30.8	29	29.6	3.28	30.6	28.7

* Replicate number

3.3.2 Precision

Precision can be defined as the measure of the amount of scatter of data points around the mean and can be determined by multiple analysis of a homogeneous sample. It is a measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation (% RSD). Precision of analysis (% RSD) was tested by analysis of one sample which contained the three analytes TBT, DBT and MBT at different concentrations and analysed 10 times. Precision of analysis was determined for the three analytes, TBT, DBT and MBT as 4.95, 9.31 and 9.81 % respectively.

3.3.3 Specificity

Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix (EURACHEM, 1998). It is a measure of the degree of interference from active ingredients, impurities or degradation products amongst others, ensuring that a peak response is due to a single component only i.e. that co-elution does not occur. Matrix

effects are sample dependent; therefore compounds that could cause interference in analyte peak identification will change depending on the sample type.

Identification of each sample analyte peak required that the relative retention time of the analyte to a specified internal standard be similar to the relative retention time observed with an analytical standard. Relative retention times were calculated by dividing the retention time (RT_2) of the external standard (ES) by the retention time (RT_1) recorded for the analyte peak.

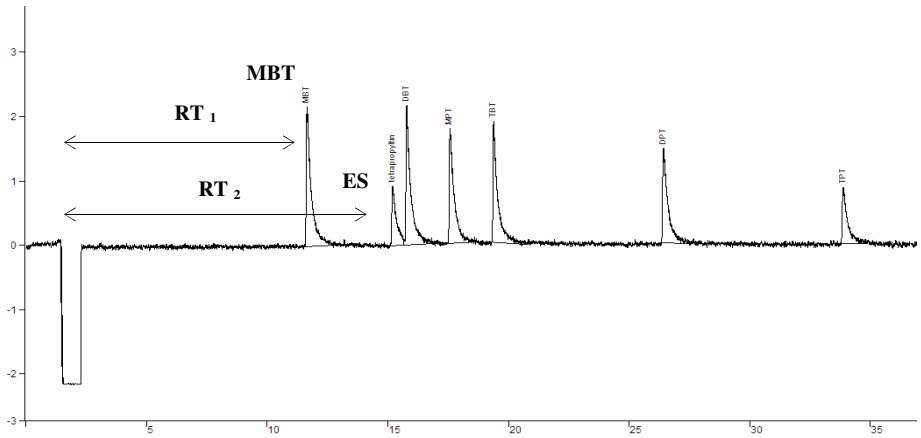

Figure 3.5: Chromatogram of ethylated butyl- and phenyl-tin compounds indicating relative retention times

Table 3.2: Retention and relative retention times of six ethylated organotin compounds.

Analyte	RT	RT_1	RT_2	RT_2/RT_1
MBT	11.6	12.9	9.3	0.72
DBT	15.8	12.9	13.5	1.05
MPT	17.6	12.9	15.3	1.19
TBT	19.4	12.9	17.1	1.33
DPT	26.4	12.9	24.1	1.87
TPT	33.9	12.9	31.6	2.45
TetPT	15.2			
Solvent quench	2.3			

RT- Retention Time
RT_2/RT_1-Relative retention time

Relative retention times (RT_2/RT_1) were calculated for MBT, DBT and TBT on a phenomenex ZB-1 column using tetrapropyltin as external standard.

3.3.4 Linear range

Linearity shows the ability of the method to produce results that are directly proportional to analyte concentration within a given range. Linearity is generally reported as the R^2 of the regression line. An acceptable linear range has an R^2 value range of 0.9995-1.0000. Range is the interval between the upper and lower levels of analyte (inclusive) that has been demonstrated to be determined with precision, accuracy and linearity using the method as written. Linearity statistics for calibration curves corrected for instrumental internal standard are outlined in Table 3.4 below.

Table 3.4: Linear range and regression statistics for TBT, DBT and MBT

Analyte	Points in regression	Range ($\mu g\ kg^{-1}$)	R^2
TBT	8	41.8-2496.5	0.9995
DBT	8	40.2-2406.7	0.9996
MBT	8	38.1-2278.8	0.9997

3.3.5 Determination of instrument LoD and LoQ

The LoD can be defined as the lowest concentration of an analyte in a sample that can be detected, but that is not quantified. It is a limit test that specifies whether or not an analyte is above or below a certain value within a certain degree of confidence. The sample LoD was expressed as the analyte concentration corresponding to a mean sample matrix blank response plus three standard deviations (+3s).

The LoQ can be defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. Validation of the LoQ for this method was expressed as the analyte concentration corresponding to a mean sample blank value +10s. The sample LoD and LoQ for the three OTCs are presented in Table 3.5 below.

Table 3.5: Limit of Detection (LoD) and Limit of Quantification (LoQ) determined for three OTCs (ng g^{-1})

	TBT	**DBT**	**MBT**
LoD ng g^{-1}	19.1	59.3	22.9
LoQ ng g^{-1}	40.1	106.3	51.7

The instrument LoD is determined as the concentration (ng g^{-1}) of the second lowest standard on the calibration curve for each given analyte. This concentration is then multiplied by the weight of each sample analysed to determine the ultimate "sample" LoD for each sample (approx. 0.5g). This means that there is an individual LoD for each analyte in each sample and will be used when reporting results. An example of the calculation LoD x sample wt = LoD g/sample) is presented in Table 3.6 and therefore, is expressed as an absolute weight of analyte (rather than concentration) which is the sample LoD.

Table 3.6: Sample limit of Detection (LoD) determined for three OTCs (ng)

MI Ref	MSC/07/1002	MSC/07/1064	MSC/07/1065	MSC/07/1068	MSC/07/1073
Weight (g)	0.5254	0.5339	0.5294	0.5312	0.5012
TBT LoD (ng)	44.3	45.0	44.6	44.8	42.2
DBT LoD (ng)	42.7	43.4	43.0	43.1	40.7
MBT LoD (ng)	40.4	41.1	40.7	40.8	38.5

The sample and instrument LoD for the three analytes (e.g. 19.1 and 44.3 ng g^{-1}) was elevated compared to other studies using the same detector. Strand et al. (2003) reported LoDs of 0.1 ng g^{-1} for all butyltins in sediment.

3.3.6 Reproducibility of method performance

Reproducibility is the closeness of agreement between individual results using the same method and test substance, but a different set of laboratory conditions such as analysis of CRMs on different days. The reproducibility of the method was tested by analysing the same certified reference material (CRM BCR646) in different batches on different days. Quality control (QC) charts were generated for the three OTCs and are presented in Figures 3.6, 3.7 and 3.8 below.

The mean concentration (μ) and standard deviation (s) was calculated from the results obtained from 17 batches of analyses. A mean result outside the μ ± 3s action limits would require investigation however no results were outside these action limits for all three OTCs on QC charts. If one mean result is outside the μ ± 2s this serves as a warning of possible loss of statistical control and indicates that a possible source of error might be present. If two mean results are outside the μ± 2s this shows a drift from statistical control. If there are eight successive results on the same side of the x-axis further investigation is needed and this is seen as a bias. However no negative or positive bias was observed for any of the three OTCs with the exception of the

monobutyltin control chart where the last point was out of range and removed from the chart.

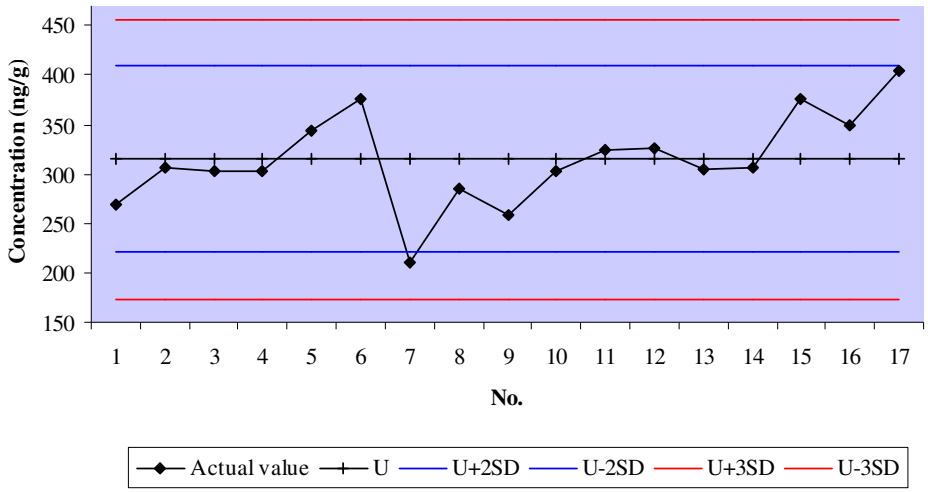

Figure 3.6: Control chart of tributyltin (TBT) in certified reference material BCR646

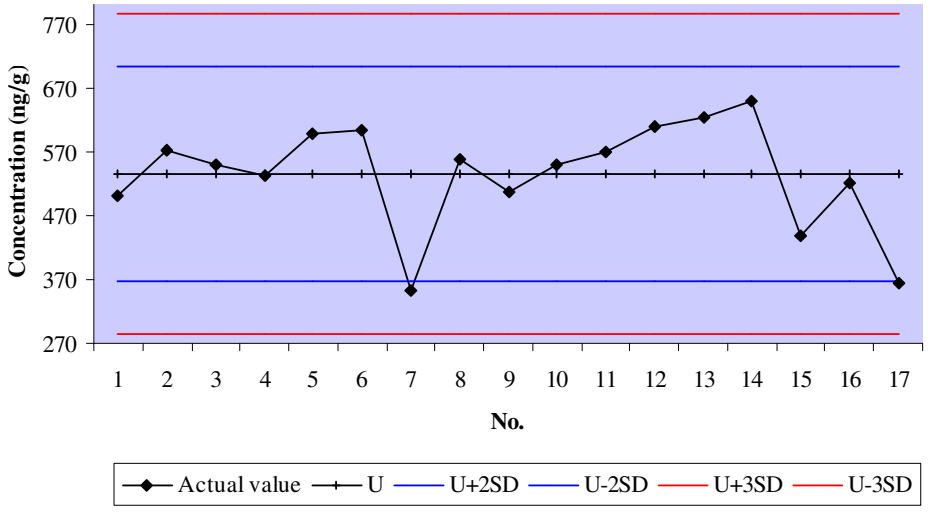

Figure 3.7: Control chart of dibutyltin (DBT) in certified reference material BCR646

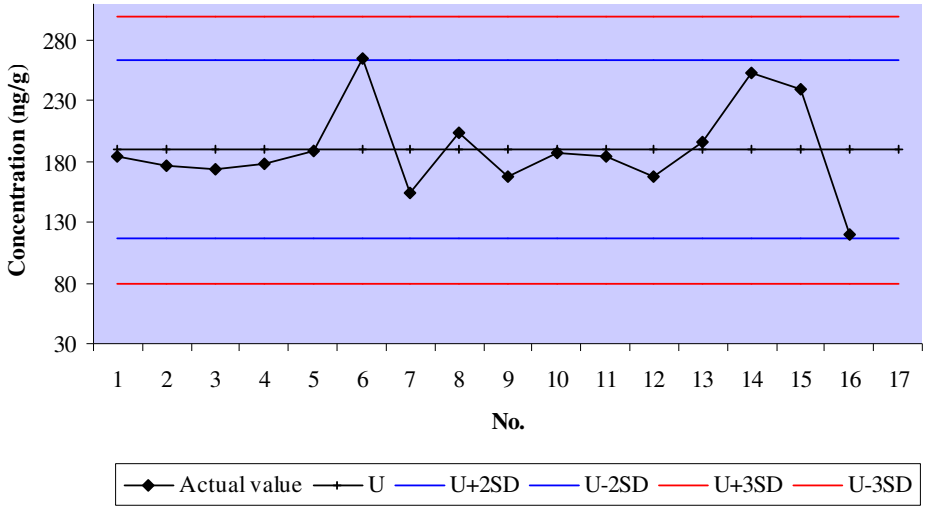

Figure 3.8: Control chart of monobutyltin (MBT) in certified reference material BCR646

3.3.7 Summary discussion on validation parameters

The ultimate aim of this study was to develop a method for the extraction of OTCs in their salt form for exposure to a cell line (chapter 4) and subsequent derivatisation for quantification using GC analysis. Procedural internal recovery standards such as tripropyltin have been found to be highly toxic to cell cultures therefore the described method did not incorporate the use of an internal recovery standard for cell line assay work which is essential for quality assurance as losses can potentially occur at the extraction and derivatisation stage. However, the quality of each batch was assured by running the CRM BCR646 within every batch to provide a % recovery concentration for each analyte.

Accuracy of the method was determined (n=17 extractions) with three concentrations of each compound in the CRM BCR646 and showed recoveries of 63.4, 71.7 and 29.6 %

for TBT, DBT and MBT respectively. The precision of analysis demonstrated good precision for TBT with a % RSD of 4.95 %. Elevated LoDs were determined for the three compounds (19.1, 59.3 and 22.9 ng g^{-1} for TBT, DBT and MBT respectively) in comparison to other studies using different methodology however for this particular project, all sites analysed demonstrated high quantities of butyltin compounds and low LoDs were not required. Overall the method showed good reproducibility and repeatability however detection limits would need to be improved for better sensitivity for future work.

3.4 Conclusion

An analytical method for extraction of OTCs in their chloride form and subsequent derivatisation and quantification was optimised and validated. Leaching with acid using ultrasound followed by extraction with DCM was demonstrated to be the most effective technique as opposed to pressurised fluid extraction which proved to be problematic due to the precipitation of complexing agents during the highly pressurised extraction. Sodium tetraethylborate was used as an effective derivatisation reagent however it was difficult to obtain commercially due to its explosive nature. Reproducibility of the method was demonstrated for TBT, DBT and MBT using control charts and recoveries of these three analytes were found to be 63.4, 71.7 and 29.6 % respectively. The low MBT recovery again demonstrates the difference in hydrophobicities of the three OTCs. The LoD/LoQ obtained for this study were high in comparison to other studies but did not affect the results for this present study as all samples were found to have elevated concentrations of OTCs. However further work is required to obtain lower LoD/LoQ values for future analysis.

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4

CHAPTER 4 TOXICITY EVALUATION OF ORGANOTIN COMPOUNDS IN IRISH MARINE SEDIMENT USING TWO FISH CELL LINES INTEGRATED WITH CHEMICAL ANALYSIS

Paper submitted to Toxicology in Vitro (09/05/2008)

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4.1 Introduction

Persistent organic pollutants (POPs) can pose a potential risk of causing adverse effects to organisms which reside in marine environments. Sediments can serve as an ultimate sink for these POPs and it is therefore important that the levels of such priority pollutants can be accurately determined and that sensitive and reproducible biological effects methodologies are developed to allow the long term effect of such contaminants on these organisms to be monitored. Presently, sediment quality in Ireland is predominantly reliant on chemical analysis alone and established procedures to characterise potential toxicity are limited. The integration of both chemical analysis and bioassay data, however, provide a more informative basis for in depth assessment of the sediment quality. Organisations such as the Oslo and Paris commission (OSPAR) and the International Council for the Exploration of the Sea (ICES) with responsibility for the provision of advice on marine ecosystems are tending towards integrated approaches (ICES, 2007).

Organotin compounds (OTCs) have been found to be of toxicological concern in organisms in the marine environment (Rüdel, 2003) therefore, sensitive biological indicators of organotin contamination need to be established to assess sediment toxicity. In general, it has been found that the greater the degree of substitution on the tin atom, the more toxic the compound (Forsyth and Casey, 2003). Tributyltin (TBT) and triphenyltin (TPT) enter the marine environment mainly from their use in antifouling paints used for shipping (Gomez-Ariza et al., 1994) and on fish cages (Minchin, 2003) with both compounds degrading to their di- and mono- metabolites in the marine environment. The TBT degradation metabolites dibutyltin (DBT) and monobutyltin (MBT) have been used as stabilisers in polymers such as poly vinyl chloride (PVC) (Ikonomou, 2002). Triphenyltin has also reportedly been used as an agricultural

fungicide and algicide (Gomez-Ariza et al., 1994) and its metabolites can also be found in the marine environment but their presence is due to degradation of triphenyltin only. Ireland was the first country to ban the use of all organotin containing compounds on vessels less than 25m in 1987 (Minchin, 2003).

In general the solubility of OTCs in water is low due to their hydrocarbon substituents. Organotins exist as neutral ion pairs and complexes or as cations in the aquatic system. The log K_{ow} for TBT-Cl and TPT-Cl has been reported to be in the ranges 2.3-4.4 and 2.02-4.08 respectively (Rüdel, 2003), these compounds are therefore tightly bound to the particle phase and have been found to bind to oxides and hydroxides and organic material (Hoch et al., 2002). Bioavailability of organotin compounds is greatest at neutral and slightly alkaline pH values and is reduced in the presence of dissolved organic carbon. Bioconcentration of TBT and TPT in marine aquatic systems has been shown to decrease with increasing concentrations of humic acids. Bioconcentration through the water phase has been shown to be significantly greater than bioaccumulation through the marine food chain (Rüdel, 2003).

Tributyltin has been found to cause limb deformities in crab species, affect arm regeneration in brittle stars and induce high larval mortality of the common mussel (Forsyth and Casey, 2003). Exposure to this compound has also been linked to reductions in tissue weight, increased shell thickness and distortion in Pacific oysters (Minchin, 2003). Tributyltin has been unequivocally linked to imposex in the dogwhelk *Nucella lapillus*, this condition manifesting itself as the imposition of male characteristics on the female dogwhelk following exposure to concentration levels as low as 1 ng/L with severe cases resulting in sterilisation of the organism (Bryan et al., 1986; Gibbs and Bryan, 1986; Bryan et al., 1987). *Nucella* species are recognised within international fora (e.g. OSPAR) as a suitable bioindicator of TBT contamination. As

OTCs are recognised pollutants of priority concern both biological and chemical monitoring of these compounds in Ireland is mandatory within the OSPAR framework.

Several analytical methods have been developed for the determination of OTCs. In general speciation of OTCs in sediment involve an acid digestion step, followed by extraction into an organic phase for subsequent derivatisation, clean up and chromatographic separation and detection. A wide range of extraction and derivatisation techniques have been reported and these are the most critical steps in the analysis (Quevauviller et al., 2000). Overall, Gas Chromatography is the most commonly used separation technique but HPLC can also be used when coupled to inductively coupled plasma mass spectrometry (Smedes et al., 2000). A wide range of very selective detection methods are available. The pulsed flame photometric detector (PFPD) offers very selective and sensitive detection of the OTCs and reduces interferences such as sulphur emitting species (Bravo et al., 2004, Bancon-Montigny et al., 2000). Current practices involving the production of solvent extracts intended for testing on biological organisms involve extraction of many anthropogenic compounds with a variety of solvents (Hollert et al., 2000; Brack et al., 2002; Houtman et al., 2004; Biselli et al., 2005; Brack et al., 2005).

The use of a single test species in toxicity testing may result in an inaccurate measurement of the potential toxicity of a particular marine pollutant. A single species test regimes cannot adequately address issues such as differences in species sensitivity, exposure routes and pollutant mode of action. A test battery approach employing numerous test species representing multiple trophic levels is therefore recommended (Matthiessen et al., 1998; Ahlf et al., 2002; Davoren et al., 2005b). *In vitro* cell cultures are becoming a frequent element of this test battery approach (Jos et al., 2003) as cell culture techniques are more practical both ergonomically and economically (and more

ethically responsible) than use of whole animal tests. Fish cell lines have also proven to be an inexpensive and sensitive bioanalytical tool for sediment quality assessment (Fent, 2003). Multiple endpoints for cytotoxicity testing have been shown to be a useful means of assessing the influence of different mechanisms of cell death for comparability of sensitivity (Weyermann et al., 2005).

The RTG-2 cell line derived from rainbow trout gonads *(Onchorhyncus mykiss)* and the PLHC-1 fish hepatoma cell line *(Poeciliopsis lucida)* are both established cell lines and have been widely used in a number of cytotoxicity studies for a number of contaminant classes (Fent 2001; Caminada et al., 2006; Davoren and Fogarty, 2006; Zurita et al., 2007a, 2007b). While the toxicity of OTCs has been established with the PLHC-1 cells, an extensive search of the literature revealed a lack of corresponding data for the RTG-2 cells. In addition, very few studies to date have investigated the use of fish cell lines as bioanalytical tools to evaluate the toxicity of environmental samples (e.g. solvent extracts).

This study reports on the extraction of OTCs in their salt form for subsequent exposure to cell cultures, in parallel with sediment extract derivatisation and quantification using GC-PFPD. The aims of this were to (1) to develop an analytical method suitable for the extraction and quantification of the organotin residues in sediment matrices with the additional requirement of being adaptable to enable transfer of extracted residues into an organic solvent for subsequent testing on the cell lines, (2) to determine the toxicity of five OTCs to the RTG-2 cell line using multiple endpoint measurements (3) to evaluate the selective sensitivities and different endpoints of this cell line for each of the OTCs used in this study, (4) to assess the sensitivity of both the RTG-2 and PLHC-1 cell lines to this solvent extract compared to the detection limits with the analytical technique, (5)

to assess the potential of employing both cell lines as screening tools of organotin contaminated sediments.

4.2 Materials and methods

4.2.1 Chemicals

Tributyltin chloride (CAS No. 1461-22-9), dibutyltin dichloride (CAS No. 683-18-1), monobutyltin trichloride (CAS No.1118-46-3), triphenyltin chloride (CAS No.639-58-7), were obtained from LGC Promochem (UK). Diphenyltin dichloride (CAS No.1135-99-5) was obtained from Sigma-Aldrich (Ireland). The organic solvent dimethylsulphoxide (DMSO) Sigma-Aldrich (Dublin, Ireland), was employed as an organotin solubilising agent for use in preparation of stock solutions for cytotoxicity assays. To ensure minimal background interference from solvents in bioassays the maximum allowable concentration (MAC) of DMSO did not exceed 1 % (v/v) in any of the cytotoxicity tests. Stock solutions of each chemical were prepared in the appropriate media. Cell culture media was purchased from Sigma-Aldrich (Dublin, Ireland) and all supplements and the trypsinisation solution were purchased from Biosciences (Dublin, Ireland). The freshwater sediment certified reference material, BCR646 and OTC quantification external standard tetrapropyltin were obtained from LGC Promochem (UK). Hydrochloric acid (33%) was obtained from AGB, Ireland. The solvents, dichloromethane, propan-2-ol, hexane, acetone and methanol (all pestiscan grade) were obtained from Labscan, Ireland. Tetrabutylammonium hydrogen sulphate, sodium sulphite, sodium acetate, sodium hydroxide and the derivatising agent sodium tetraethylborate 97% (STEB) were obtained from Sigma-Aldrich (Dublin, Ireland). The ethylated organotin standards in hexane were obtained from the Quality Assurance of Information for Marine Environmental Monitoring in Europe (QUASIMEME) project office (Wageningen, The Netherlands).

4.2.2 Site selection and sediment sampling

The sampling site at Dunmore East Harbour, Co Waterford 52º 08.843'N, 6º 59.480'W was selected on the basis of a previous report indicating elevated levels of TBT (up to 6.4 mg kg^{-1}) (Enterprise Ireland, 2002). Sediment was collected with a Van Veen Grab and the top 0-5 cm was removed for use as a test material. Samples were stored at -30 ºC. Sediment samples were subsequently thawed and wet sieved to the < 2mm fraction. Thereafter the < 2mm fraction was frozen at -30 ºC and freeze dried. The map of the sampling site can be seen in Figure 4.1 below.

Figure 4.1: Sampling site at Dunmore East harbour

4.2.3 Physical and chemical characterisation of the sediment

Total organic carbon was determined in the < 2mm fraction by Alcontrol Laboratories (Dublin, Ireland) using the combustion method, which conforms to ISO 17025. The accuracy of this method was ensured by analysing two QUASIMEME laboratory proficiency materials, which were found to be within acceptable |Z| score limits.

4.2.4 Analytical methodology for speciation of OTCs

An adaptation of an *in situ* ethylation method (Strand et al., 2003) was employed and resulting modifications are detailed below.

Freeze dried sediment samples (0.5g) were digested in a solution of HCl:H_2O (1:1 v/v) for 30 min in an ultrasonic bath. The mixture was then extracted with dichloromethane (DCM) for 30 min with shaking and the organic layer was removed. The procedure was repeated in triplicate to maximise recovery. The DCM layer was solvent exchanged to hexane and then subsequently transferred to methanol. The pH was adjusted to 5.0 ± 0.5 with 10 % sodium acetate and 20 % sodium hydroxide in deionised water and ethylation of organotins completed using a 10 % w/v solution of sodium tetraethylborate in methanol. The ethylated organotins were then back extracted into hexane and the organic extract was then cleaned up with sodium sulphate and alumina deactivated with 5% water and sulphur was removed from the extract using tetrabutylammonium sulphite. Tetrapropyltin was then added as an injection correction standard to the extract in the GC vial and the sample extract was analysed using a gas chromatograph-pulsed flame photometric detector (GC-PFPD). No internal recovery standard was employed during the course of this study as

suitable compounds *e.g.* tripropyltin chloride have been previously found to be toxic to biological organisms at very low levels (Brüschweiler et al., 1995).

The freshwater sediment certified reference material BCR646 was analysed with every batch to further ensure compliance with internal quality control procedures.

4.2.5 Cell culture

RTG-2 cells (Catalogue number 90102529) derived from rainbow trout gonads, were obtained from the European Collection of Cell Cultures (Salisbury, UK). The PLHC-1 cell line (CRL-2406) derived from a hepatocellular carcinoma in the topminnow were from the American Type Culture Collection and purchased from Promochem (UK). Both cell types were maintained in Dulbecco's Modified Medium Nutrient Mixture/ F-12 Ham (DMEM) supplemented with either 10 % (RTG-2) or 5% (PLHC-1) foetal calf serum (FCS) and 45 IU ml penicillin, 45 μg ml streptomycin. The RTG-2 medium was also supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid (HEPES) and 1% non-essential amino acids. Cultures were maintained in a refrigerated incubator (Leec, Nottingham, U.K.) at either 20°C (RTG-2) or 30°C (PLHC-1) under normoxic atmosphere.

4.2.6 Cytotoxicity testing

4.2.6.1 Organotin chemical exposure

Individual wells of a 96-well microplate (Nunc, Denmark) were seeded with 100 μl of cell suspension at a seeding density of 2 x 10^5 cells per ml for RTG-2 cells and 8 x 10^5 cells per

ml for PLHC-1 cells for 24 h exposure periods. RTG-2 cells were also exposed for 96 h and these plates were seeded at a density of 1.5 x 10^5 cells per ml, which was found to be optimal to achieve the desired confluence for this exposure period. Test chemicals were prepared in a reduced serum medium (5% FCS). Range finding tests were conducted on the five organotin compounds with the employed concentration range of 1 x 10^{-9} to 1 x 10^{-3} M with the exception of MBT (1 x 10^{-8} to 1 x 10^{-3} M). Definitive testing was then conducted on four of the organotin compounds (DBT, TBT, DPT and TPT) with the RTG-2 cell line. The PLHC-1 cell line was only exposed to TBT within the same definitive range as the RTG-2 cell line. Six replicate wells were used for each control, solvent control and test concentration per microplate.

4.2.6.2 Preparation of sediment extract for cell line testing

Approximately 26g of sediment was extracted in 2g aliquots and the extracts combined. To each 2g freeze dried sediment, a solution of HCl:H_2O 1:1 v/v (8 ml) was added and this was sonicated using an ultrasonic bath for 30 min. Twenty ml of dichloromethane (DCM) was then added and the mixture was shaken for 30 min using a multitube vortexer. The slurry was then centrifuged for 5 min and the DCM layer was removed. Further sonication and shaking were repeated twice and the DCM layers combined. The DCM layer was then concentrated and transferred to hexane under a N_2 stream using a turbovap concentrator. The hexane layer was evaporated to 1 ml in the turbovap and passed over a 1 g column of hexane washed sodium sulphate. A sulphur clean up was performed using tetrabutylammonium sulphite. The hexane layer was then transferred to 1 ml of hexane:acetone 9:1 v/v and ultimately transferred to 900 µl of DMSO. Both a method blank and sediment sample extract (Dunmore East) were prepared for cell line testing.

Stock solutions of sediment and blank extracts were prepared with a ratio of 1:24 dilution of DMSO to cell culture media. This stock solution was tested at dilutions 1:1 to 1:256 in media in six replicates per plate for a period of 24h (maximum concentration of DMSO was 2 % which has been shown to have no significant effect on the RTG-2 cells). A flowchart showing the analytical methodology and preparation of sediment extract for cell line testing is presented in Figure 4.2 below. Two different paths are followed using the same extraction. The analytical method is outlined in red and cell culture methodology is outlined in blue while the black outline indicates combined methodologies.

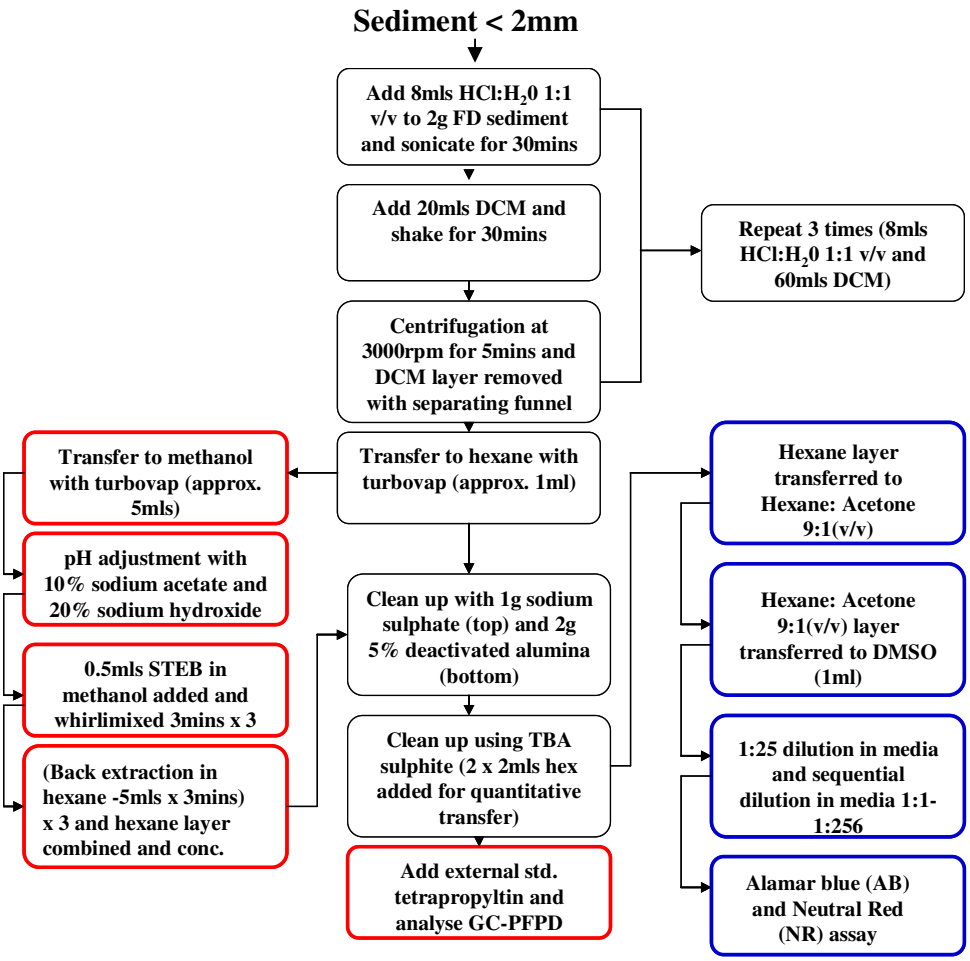


Figure 4.2: A schematic diagram of analytical methodology and cell line testing, the red outline indicating analytical methodology, blue outline indicating bioassay methodology and black indicating methodology used for both bioassay and analytical.

Following exposure of the cells to the individual OTCs or the sediment extract for the appropriate incubation period the test medium was removed; cell monolayers washed with phosphate buffered saline (PBS) and cytotoxicity assessed using the Alamar Blue and Neutral Red assays conducted subsequently on the same set of plates as previously described (Schirmer et al., 1998; Davoren and Fogarty, 2006).

4.2.7 Statistical analysis

Fluorescent units (AB and NR assays) was measured using a microplate reader (TECAN GENios, Grödig, Austria). Experiments were conducted in triplicate (three independent exposures). Cytotoxicity was expressed as the mean percentage inhibition relative to the unexposed control ± standard deviation (SD), and was calculated using the formula [100-((Mean Experimental data/Mean Control data) x 100)]. Control values were set at 0% cytotoxicity. Cytotoxicity data (where appropriate) was fitted to a sigmoidal curve and a four parameter logistic model was used to calculate the 50 % Effective Concentration (EC_{50}), which is defined as the concentration of test compound causing a 50% inhibition of cell viability compared to untreated controls. The EC_{50} values are reported at ± 95 % Confidence Intervals (± 95% CI). This analysis was performed using Xlfit3™ a curve fitting add-in for Microsoft® Excel (ID Business Solutions, UK). Statistical analyses were completed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison testing. The respective no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values were calculated for all data and statistical significance was accepted at $P \leq 0.05$ throughout.

4.3 Results

4.3.1 Chemical analysis of the sediment

The total organic carbon content of the < 2mm fraction of sediment was found to be 1.52 % for the Dunmore East site. The < 2mm fraction of the sediment was used for extractions as this fraction is most representative of the fraction available to organisms which reside in the sediment. The Gas Chromatography-Pulsed Flame Photometric Detector (GC-PFPD) chromatogram of the Dunmore East sediment sample extract (1:10 dilution in hexane) is presented in Figure 4.3 below. Three distinct butyltin peaks, tributyltin, dibutyltin and monobutyltin and a peak indicative of the external standard tetrapropyltin are detected. However two unknown peaks are also observed which could be by-products from ethylation with sodium tetraethylborate such as boroxin, diethylsulphide and diethyltrisulphide or ethylated sulphur species, all which have been found previously to be present in large quantities (Smedes et al., 2000).

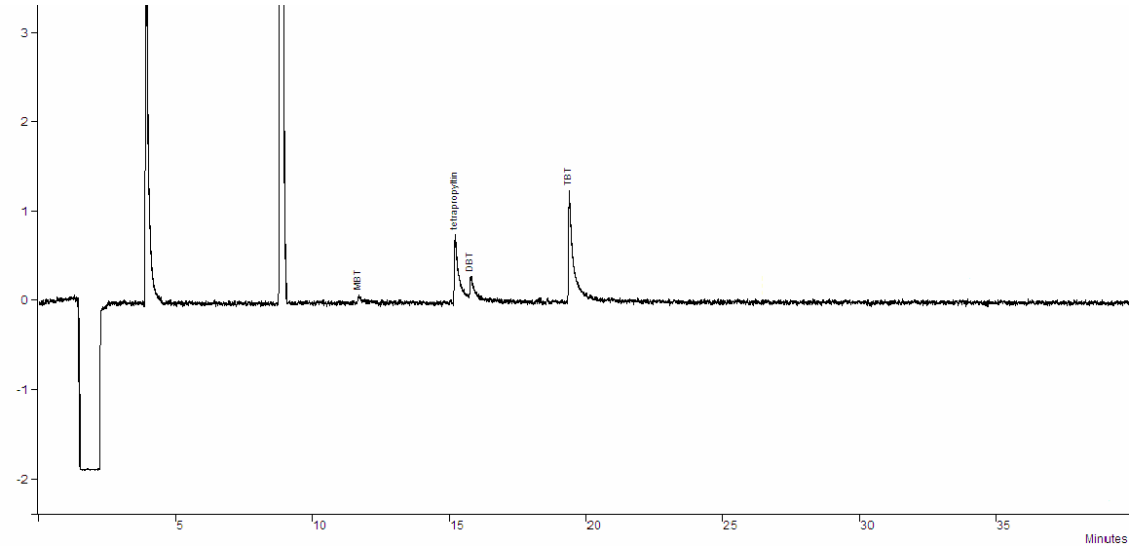


Figure 4.3: GC-PFPD chromatogram of the Dunmore East sediment sample extract (1 in 10 dilution in hexane). TBT, DBT and MBT and the injection correction standard peak, tetrapropyltin are highlighted.

4.3.1.1 Quality assurance of analysis

The concentrations of the various butyltin compounds and the percentage recoveries for the Dunmore East sample and the freshwater sediment certified reference material (CRM) BCR646 are shown in Table 4.1. The recoveries of OTCs in the CRM can be used to correct for concentrations of the various organotins in the environmental sample.

Table 4.1 Concentrations (µg kg^{-1} dry weight) and recoveries (%) of various butyltins in the Dunmore East sediment sample extract and certified values in the reference material BCR646.

Analyte	BCR646 Certified value and uncertainty	Conc OTCs in Dunmore East extract[1]	% Recovery ±SD of OTCs in BCR646 (n=3)
TBT ($Sn(C_4H_9)_3^+$)	480 (80)	2125	70.4±2.40
DBT ($Sn(C_4H_9)_2^{2+}$)	770 (90)	790	52.0±12.1
MBT ($Sn(C_4H_9)^{3+}$)	610 (120)	609	34.6±15.9

[1] Concentrations reported are not recovery corrected

4.3.2 Cytotoxicity tests with RTG-2 cells and organotin compounds

Cytotoxicity data for the RTG-2 cells following exposure to the four individual OTCs, TBT, DBT, TPT and DPT for 24 h and 96 h are presented in Tables 4.2 and 4.3 respectively. Cytotoxicity was found to be greatest for the higher substituted organotin compounds TBT and TPT. Range finding tests confirmed that MBT was significantly less toxic than the other compounds so that no further definitive testing was conducted. Concentration response cytotoxicity charts, as quantified with the AB and NR assays are presented for the four individual compounds TBT, DBT, TPT and DPT after 24 hours (Figures 4.4 to 4.7) and after 96 hours (Figures 4.8 to 4.11).

Table 4.2: Cytotoxic effects on RTG-2 cells after 24 hour exposure with four OTCs and an organotin mixture

Compound	**Exposure period and endpoint**	**EC_{50} (µM)[a]**	Fit Statistic **(r^2)**	**NOEC[b] (µM)**	**LOEC[c] (µM)**
TBT (0.1-10 µM)	24 h AB inhibition	1.03 (0.75-1.31)	0.981	0.1	0.5
	24 h NR inhibition	3.43 (-0.09-6.96)	0.800	0.1	0.5
DBT (0.1-100 µM)	24 h AB inhibition	6.27 (4.15-8.38)	0.986	1	5
	24 h NR inhibition	9.89 (5.94-13.85)	0.981	1	5
TPT (0.1-10 µM)	24 h AB inhibition	0.65 (0.003-1.31)	0.891	0.1	0.5
	24 h NR inhibition	1.57 (-0.37-3.52)	0.850	0.1	0.5
DPT (1-10 µM)	24 h AB inhibition	2.40 (1.78-3.02)	0.974	<1	1
	24 h NR inhibition	4.42 (3.50-5.33)	0.971	1	2

[a]Values represent EC_{50} (µM) with 95% confidence intervals in parentheses.
[b]NOEC, no observed effect concentration, the highest observed concentration at which no significant cytotoxic effect ($P \leq 0.05$) was detected.
[c]LOEC, lowest observed effect concentration, the lowest observed concentration at which a significant cytotoxic effect ($P \leq 0.05$) was detected.

Table 4.3: Cytotoxic effects on RTG-2 cells after 96 hour exposure with four OTCs.

Compound	Exposure period and endpoint	EC_{50} (µM)[a]	Fit Statistic (r^2)	NOEC[b] (µM)	LOEC[c] (µM)
TBT (0.1 -10 µM)	96 h AB inhibition	0.94 (0.75-1.12)	0.989	0.5	0.75
	96 h NR inhibition	2.22 (0.72-3.73)	0.891	0.5	0.75
DBT (0.1-100 µM)	96 h AB inhibition	2.38 (1.97-2.79)	0.999	0.5	1
	96 h NR inhibition	5.06 (2.43-7.68)	0.979	1	5
TPT (0.1 -10 µM)	96 h AB inhibition	0.24 (0.20-0.27)	0.997	0.1	0.5
	96 h NR inhibition	0.31 (0.2-.0.41)	0.988	0.1	0.5
DPT (1-10 µM)	96 h AB inhibition	0.82 (-0.58-2.22)	0.987	<1	1
	96 h NR inhibition	0.89 (0.09-1.68)	0.775	<1	1

[a]Values represent EC_{50} (µM) with 95% confidence intervals in parentheses.
[b]NOEC, no observed effect concentration, the highest observed concentration at which no significant cytotoxic effect ($P \leq 0.05$) was detected.
[c]LOEC, lowest observed effect concentration, the lowest observed concentration at which a significant cytotoxic effect ($P \leq 0.05$) was detected.

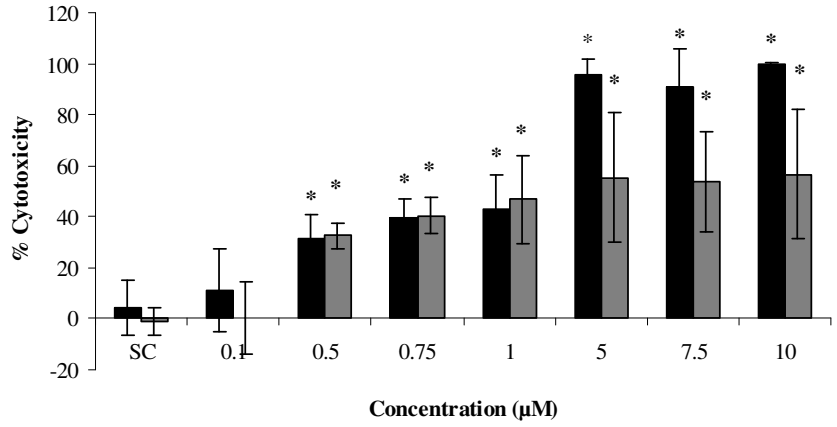


Figure 4.4: Cytotoxicity of TBT after 24 h exposure to RTG-2 cells determined by the AB assay (■) and NR assay (■). Data is expressed as a percentage of unexposed controls ± SD of three replicates for each exposure concentration. *Denotes a significant difference from the control ($P \leq 0.05$).

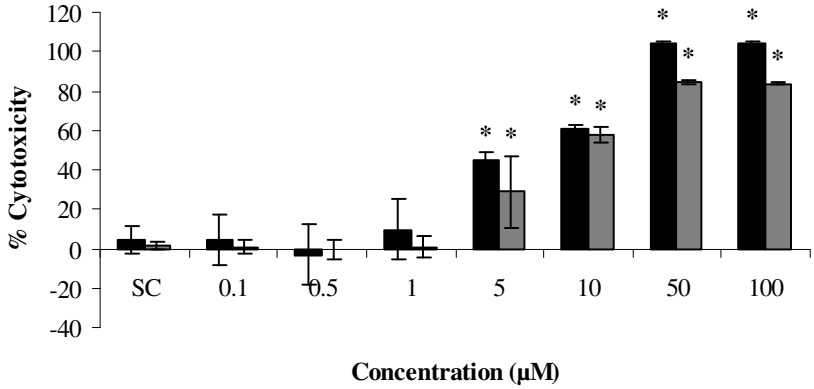


Figure 4.5: Cytotoxicity of DBT after 24 h exposure to RTG-2 cells determined by the AB assay (■) and NR assay (■). Data is expressed as a percentage of unexposed controls ± SD of three replicates for each exposure concentration. *Denotes a significant difference from the control ($P \leq 0.05$).

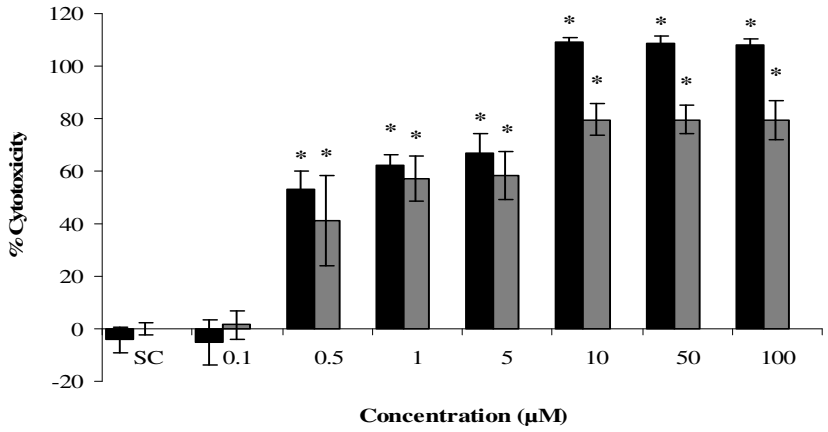


Figure 4.6: Cytotoxicity of TPT after 24 h exposure to RTG-2 cells determined by the AB assay (■) and NR assay (■). Data is expressed as a percentage of unexposed controls ± SD of three replicates for each exposure concentration. *Denotes a significant difference from the control ($P \leq 0.05$).

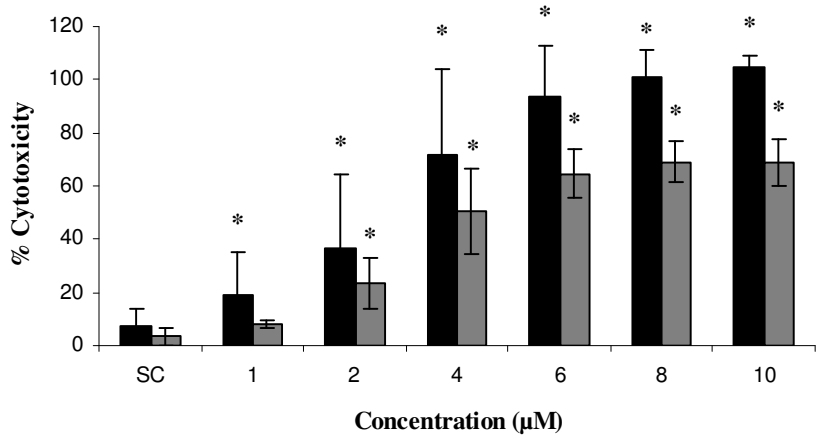


Figure 4.7: Cytotoxicity of DPT after 24 h exposure to RTG-2 cells determined by the AB assay (■) and NR assay (■). Data is expressed as a percentage of unexposed controls ± SD of three replicates for each exposure concentration. *Denotes a significant difference from the control ($P \leq 0.05$).

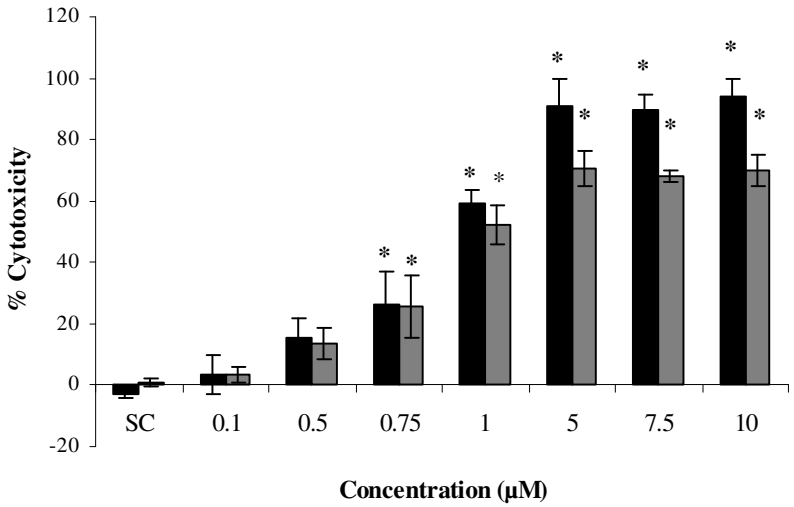


Figure 4.8: Cytotoxicity of TBT after 96 h exposure to RTG-2 cells determined by the AB assay (■) and NR assay (■). Data is expressed as a percentage of unexposed controls ± SD of three replicates for each exposure concentration. *Denotes a significant difference from the control ($P \leq 0.05$).

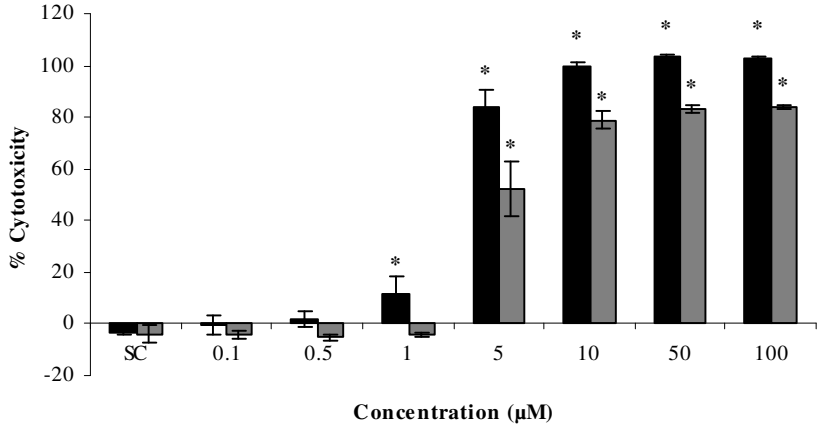


Figure 4.9: Cytotoxicity of DBT after 96 h exposure to RTG-2 cells determined by the AB assay (■) and NR assay (■). Data is expressed as a percentage of unexposed controls ± SD of three replicates for each exposure concentration. *Denotes a significant difference from the control ($P \leq 0.05$).

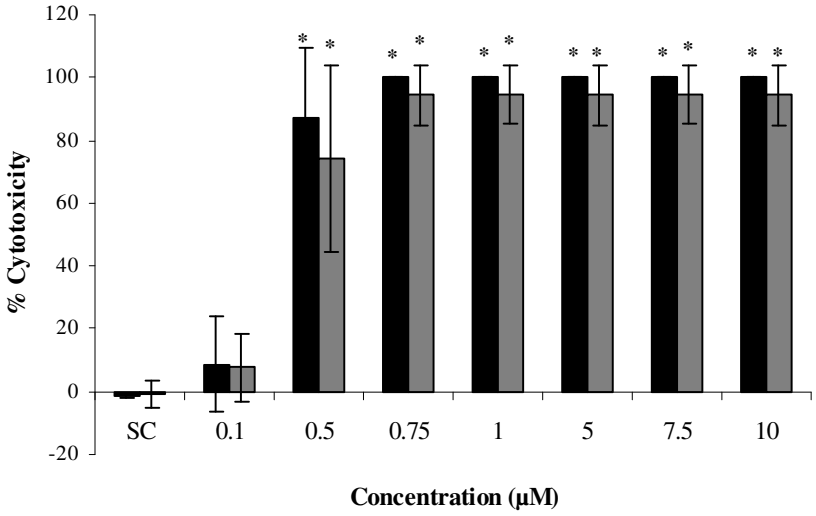


Figure 4.10: Cytotoxicity of TPT after 96 h exposure to RTG-2 cells determined by the AB assay (■) and NR assay (■). Data is expressed as a percentage of unexposed controls ± SD of three replicates for each exposure concentration. *Denotes a significant difference from the control ($P \leq 0.05$).

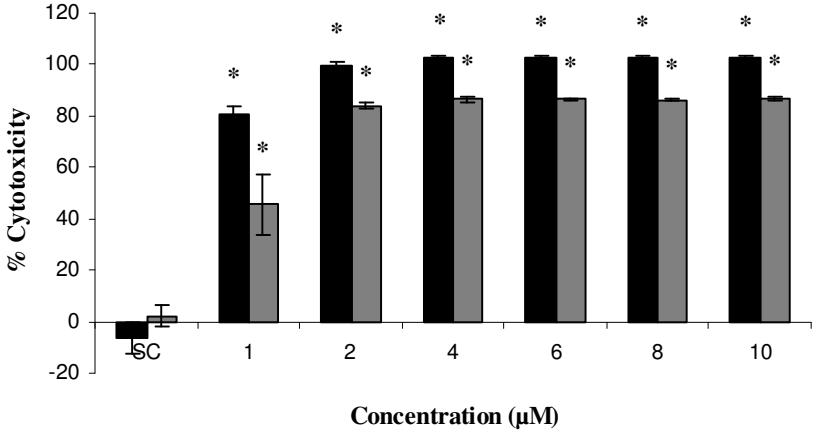


Figure 4.11: Cytotoxicity of TPT after 96 h exposure to RTG-2 cells determined by the AB assay (■) and NR assay (■). Data is expressed as a percentage of unexposed controls ± SD of three replicates for each exposure concentration. *Denotes a significant difference from the control ($P \leq 0.05$).

4.3.3 Cytotoxicity tests with PLHC-1 cells and TBT

Cytotoxicity data for the PLHC-1 cells following exposure to TBT for 24 h is presented in Table 4.4. The cytotoxicity chart for PLHC-1 cells as quantified with the AB and NR assays is presented for TBT after 24 h in Figure 4.12. Other individual OTCs were not exposed to PLHC-1 cells as similar work has been previously reported by Brüschweiler et al., (1995). A comparison of these PLHC-1 results with those determined with the RTG-2 cells in this study is presented in Table 4.5.

Table 4.4: Cytotoxic effects on PLHC-1 cells after 24 hour exposure with TBT chloride

Compound	Exposure period and endpoint	EC_{50} (µM)[a]	Fit Statistic (r^2)	NOEC[b] (µM)	LOEC[c] (µM)
TBT (0.1-10µM)	24 h AB inhibition	0.99 (0.74-1.25)	0.988	0.5	0.75
	24 h NR inhibition	1.01 (0.13-1.88)	0.844	0.1	0.5

[a]Values represent EC_{50} (µM)with 95% confidence intervals in parentheses.
[b]NOEC, no observed effect concentration, the highest observed concentration at which no significant cytotoxic effect ($P \leq 0.05$) was detected.
[c]LOEC, lowest observed effect concentration, the lowest observed concentration at which a significant cytotoxic effect ($P \leq 0.05$) was detected.

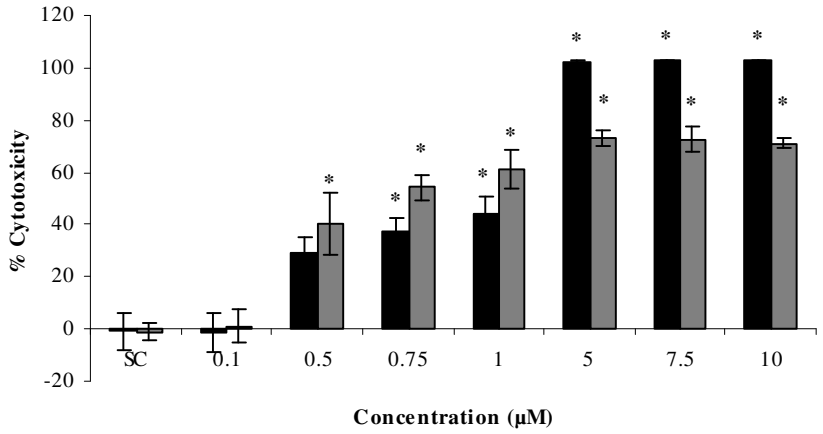


Figure 4.12: Cytotoxicity of tributyltin chloride to PLHC-1 cells determined by AB assay (■) and NR assay (■) following 24 h exposure. Data is expressed as a percentage of unexposed controls ± SD of three replicates for each exposure concentration. *Denotes a significant difference from the control ($P \leq 0.05$).

4.3.4 Cytotoxicity tests with RTG-2 and PLHC-1 cells with the organic extract and method blank

Cytotoxicity data for the RTG-2 and PLHC-1 cells following exposure to the Dunmore East organic extract are presented in Table 4.6. Cytotoxicity charts for RTG-2 and PLHC-1 cells as quantified with both AB and NR assays are presented for the Dunmore East extract and blank after 24 h exposure in figures 4.13 and 4.14 respectively.

Table 4.5: Comparison of cytotoxic effects on PLHC-1 cells and RTG-2 cells for four individual OTCs.

Compound	Exposure period And endpoint	PLHC-1 EC_{50} [a] (µM)	RTG-2 EC_{50} [b] (µM)
TBT-Cl	24 h AB inhibition	0.99[b]	1.03
	24 h NR inhibition	0.11 (1.01[b])	3.43
$DBT-Cl_2$	24 h AB inhibition	-	6.27
	24 h NR inhibition	20	9.89
TPT-Cl	24 h AB inhibition	-	0.65
	24 h NR inhibition	0.17	1.57
$DPT-Cl_2$	24 h AB inhibition	-	2.40
	24 h NR inhibition	15	4.42

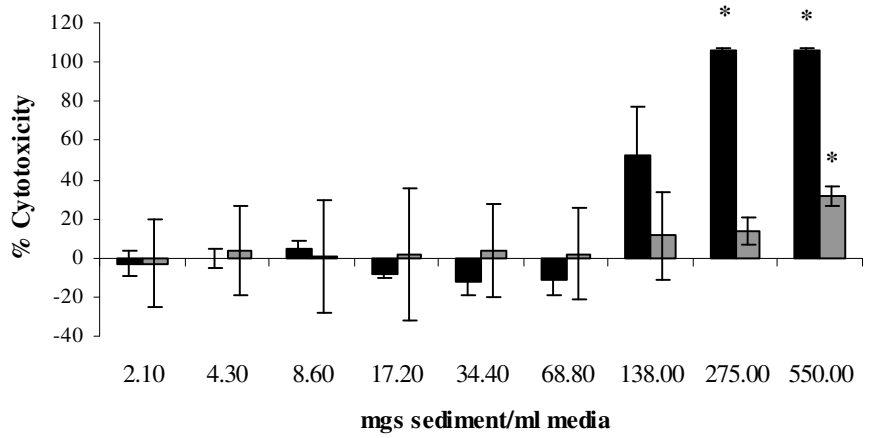
[a]Values obtained from Brüschweiler et al., 1995.

[b]Values determined in this study

Table 4.6: Cytotoxic effects on RTG-2 and PLHC-1 cells after 24 h exposure with the Dunmore East extract

Cell line	Exposure period and endpoint	Dunmore East extract EC_{50} (No. mgs sediment/ml media) ±SD (n = 3)	r^2
RTG-2	AB 24H	138.15 (±10.06)	0.983
	NR 24H	73.19 (± 24.85)	0.980
PLHC-1	AB 24H	66.91 (±16.61)	0.995
	NR 24H	63.97 (±17.93)	0.981

(a)



(b)

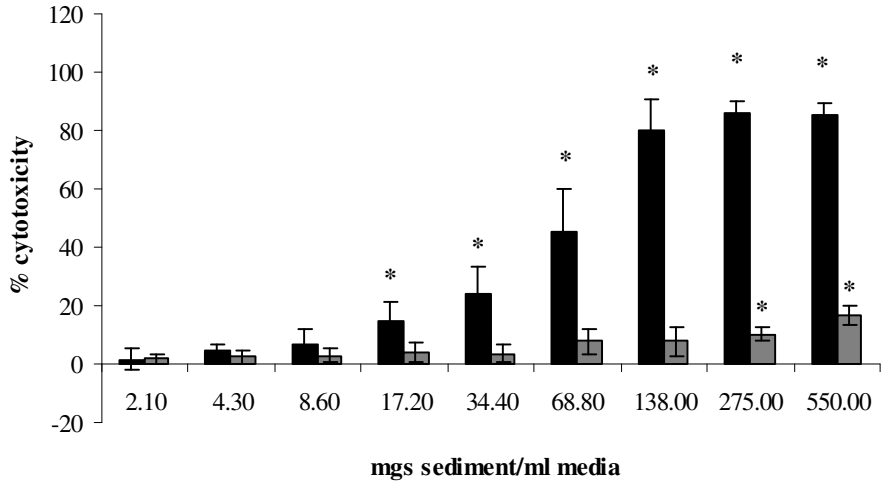
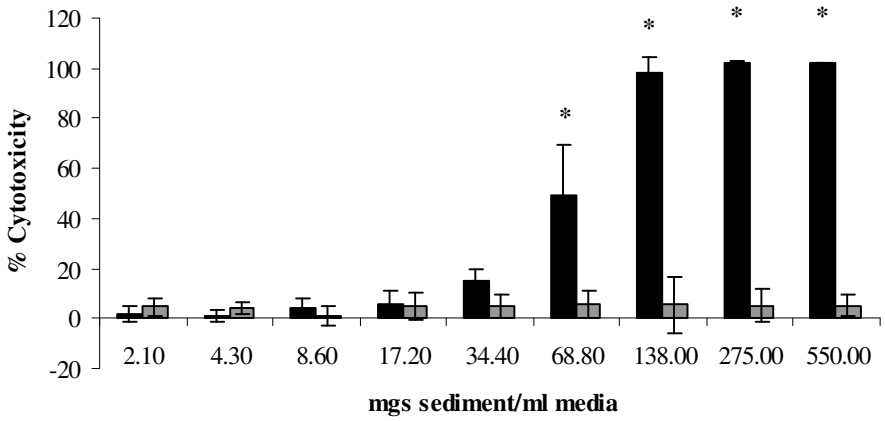


Figure 4.13: Cytotoxicity of the Dunmore East extract (■) and blank extract (■) to RTG-2 cells determined by AB assay (a) and NR assay (b) following 24 h exposure. Data is expressed as a percentage of unexposed controls ± SD of three replicates for each exposure concentration. *Denotes a significant difference from the control ($P \leq 0.05$).

(a)



(b)

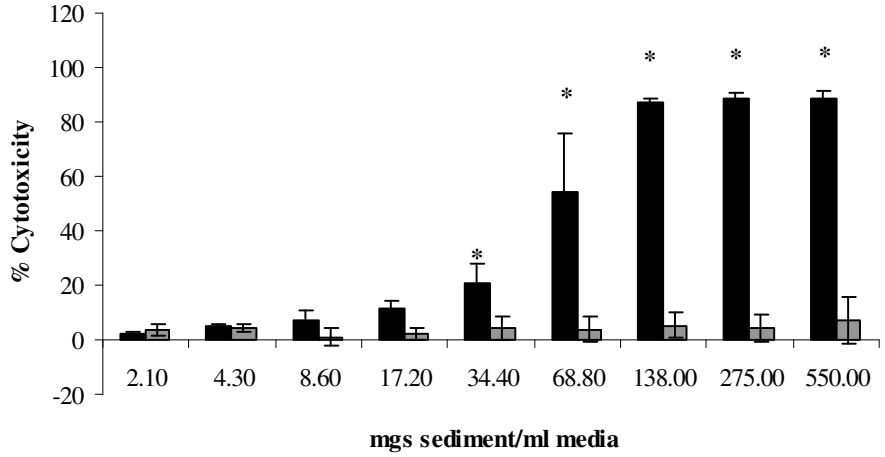


Figure 4.14: Cytotoxicity of the Dunmore East extract (■) and blank extract (■) to PLHC-1 cells determined by AB assay (a) and NR assay (b) following 24 h exposure. Data is expressed as a percentage of unexposed controls ± SD of three replicates for each exposure concentration. *Denotes a significant difference from the control ($P \leq 0.05$).

4.4 Discussion

Current methods of preparing extracts for exposure to biological organisms include extraction by a range of solvents and analytical techniques such as accelerated solvent extraction (ASE), soxhlet extraction, and ultra-turrax cold extraction (Hollert et al., 2000; Brack et al., 2002; Houtman et al., 2004; Biselli et al., 2005; Brack et al., 2005). Such techniques generally have focused on the extraction of contaminants such as estrogens, polychlorinated biphenyls, polyaromatic hydrocarbons and other hydrocarbon containing compounds but are not ideal for the extraction of organotins. Organotin compounds require an acid leaching step as well as a derivatisation step after extraction with organic solvents to convert the compounds to their volatile form for quantitation purposes with GC.

A GC-PFPD chromatogram of the detected butyltin species is shown in Figure 4.3. This study shows higher TBT recoveries (70.4 %) than DBT and MBT, (52.0 % and 34.6 % respectively) which were calculated from the mean recovery of the CRM BCR646 from three batches of samples (Table 4.1). The lower recoveries of these compounds have been previously documented (Smedes et al., 2000, Ikonomou et al., 2002). Extraction techniques such as soxhlet extraction were previously reported to have good TBT recoveries but low DBT and MBT recoveries (Smedes et al., 2000). The solvent extracts were found to elicit cytotoxicity, while, by comparison, the current method can be used successfully as a tool in assessing toxicity of OTCs in environmental samples. Accelerated solvent extraction, from our experience is unsuitable for the production of sediment extracts suitable for OTC toxicity testing due to the acidity of the extracts and use of complexing agents which themselves may be toxic to the cells. The method described in this study provides a mechanism whereby OTCs (and other co-extracted contaminants) can be analytically

quantified and are readily transferable to DMSO for use in exposure experiments in their salt form.

The analytical method displayed good reproducible recoveries for TBT but the recoveries for DBT and MBT were low in the CRM. Many other laboratories use tripropyltin chloride as an internal standard which would correct the actual recovery in each sample however this compound was previously found to be very toxic to PLHC-1 cells with an NR_{50} of 1 x 10^{-7}M (Brüschweiler et al., 1995). Therefore the concentration of each butyltin analyte is presented inTable 4.1(not recovery corrected with CRM recovery values) (Table 4.1).

Data presented in Table 4.2 demonstrate that the alamar blue assay was found to be the most sensitive for each of the individual OTC exposures after 24 h. Of the four individual compounds tested, TPT was found to be the most toxic after 24 h exposure with an AB_{50} value of 0.65 µM. After the 96 h exposure period (Table 4.3) increased cytotoxicity was observed for the RTG-2 cells for each of the individual OTCs for both of the endpoints studied as evidenced by the lower EC_{50} values. A parallel study conducted in our lab with *Vibrio fischeri* and *Tisbe battagliai* demonstrated a similar increase in toxicity over time (unpublished data). To the best of our knowledge, no toxicity data with the RTG-2 cell line and OTCs was available for comparative purposes in the peer reviewed literature

The inhibition of cytochrome P4501A by organotins in PLHC-1 cells has also been investigated (Brüschweiler et al., 1996). This cell line has also been used to study the cytotoxicity of pharmaceuticals along with the RTG-2 cell line and has been demonstrated to be more sensitive than the rainbow trout cell line for this class of compounds (Caminada et al., 2006). Cytotoxicity tests with the PLHC-1 cells after 24h exposure with TBT show

that there are comparable sensitivities between the AB and the NR assays (Table 4.4 and Figure 4.12). PLHC-1 cells have been previously employed to ascertain the toxicity of numerous OTCs (Brüschweiler et al., 1995). For this reason, only TBT was tested on the PLHC-1 cells for comparability purposes. An NR_{50} value for TBT was determined to be 1.0 µM which is one order of magnitude higher than the 0.11µM that was determined by Brüschweiler (1995). It must be highlighted that there was a wide confidence interval associated with our EC_{50} value for TBT (0.13-1.88 µM).

The RTG-2 cells were found to be more sensitive than the PLHC-1 cells to the di-substituted organotins, DBT and DPT but the PLHC-1 cells were found to be more sensitive for the higher substituted TBT and TPT using the NR assay. In this study the alamar blue assay was found to be more sensitive than the NR assay for all OTCs tested.

Sediment extracts are not intended to serve as an indicator of bioavailability of the OTCs to aquatic organisms, however, since these extracts can contain anthropogenic compound classes that are otherwise very tightly bound to particulate phases (i.e. a 'worst case scenario'), they do provide a good representation of the quality of the sediment in the overall assessment. Table 4.6 demonstrates that the NR assay was found to be the most sensitive for both cell lines when exposed to the environmental sample extracts. The PLHC-1 cells were found to be slightly more sensitive (NR_{50}, 63.97 mg sediment/ml media) to the Dunmore East extract than the RTG-2 cell line (NR_{50}, 73.19 mg sediment/ml media). It should be noted that the blank extract did elicit significant toxicity with the RTG-2 cells at the top concentration with both assay endpoints (AB assay at 550 mgs sediment per ml and NR assay at 275 and 550 mgs sediment per ml), however, the

sediment extract at the same concentrations was demonstrated to induce considerably more toxicity. The blank extract had no adverse effect on the PLHC-1 cells with either endpoint.

In Figure 4.13 (a) hormesis was demonstrated in the AB assay results upon exposure of the RTG-2 cells to both the Dunmore East extract and method blank using the AB assay. Hormesis can be described as a stimulatory response of the cells to low, non cytotoxic concentrations of chemicals or mixture of chemicals in the sample extracts therefore leading to an increase over controls (Calabrese and Baldwin, 2003). Hormesis was only observed for the AB assay for the RTG-2 cell line and not observed with the NR assay. This hormetic effect has been reported previously for RTG-2 cells on exposure to zinc salts and PLHC-1 cells on exposure to cadmium (Ni Shúilleabháin et al., 2004; Caminada et al., 2006). Hormesis has been associated with an increase in cell proliferation (Ni Shúilleabháin et al., 2004) therefore hormetic effects would be expected with the AB assay (as evidenced in this present study) on the RTG-2 cell line since the AB assay is generally used to assess cell proliferation.

The repeatability of the cytotoxic responses to pollutants is related to differences in metabolic activity of exposed cells and also to the type of toxic mode of action of chemicals or mixture of chemicals. The RTG-2 cell line has been found to retain a basic cytochrome P450-dependent monooxygenase activity whereas other cell lines have been found to lose their metabolic activity (Fent, 2001). In addition some of the chemicals in the anthropogenic cocktail can be light sensitive and temperature sensitive, repeatability can also be related to the storage of samples (De Lange et al., 2007).

A total of 26g of sediment was extracted for the purposes of the cytotoxicity study, this quantity being based on the TBT content in the <2mm fraction (2.125 mg/kg) and the demonstrated sensitivity of the RTG-2 cell line to TBT with the AB assay (1.03×10^{-6} mol/Litre) which when expressed as TBT equivalents equates to 369 ng TBT/ml. The actual EC_{50} obtained for the RTG-2 cells following exposure to the extract was 138 mg sediment/ml media with the AB assay (Table 4.6). Therefore the amount of available TBT equivalents in the extract was calculated to be 294 ng TBT/ml (2125/1000*138).

In summary the established sensitivity (EC_{50}) of the RTG-2 cells to TBT was calculated as being 369 ng/ml while the sediment extract was found to be slightly more toxic to the cells (EC_{50}, 294 ng TBT/ml). While we have demonstrated that the extraction technique is very effective in extracting TBT from marine sediments and that RTG-2 cells are sensitive to TBT, the greater toxicity exhibited by the extract suggests that the toxic effects observed are not due to TBT alone. We have shown that three OTCs were present in the extract (TBT, DBT and MBT), each of which may contribute to toxicity to differing degrees therefore increased sediment toxicity may be as a consequence of additive and/or synergistic effects between these OTCs and other anthropogenic compounds co-extracted by the described method. Further research into such mixture effects is therefore warranted. The identification of these compounds and their associated cytotoxicity was beyond the scope of the present study but is currently being investigated by means of a Toxicological Identification Evaluation (TIE) study.

4.5 Conclusion

This paper describes an optimised analytical method for the extraction and quantification of OTCs from a sediment extract, which were readily transferable to DMSO for use in cell exposure experiments in their chloride form. The method displayed good reproducible recoveries for TBT but the recoveries for DBT and MBT were relatively low. The method extracted butyltin compounds in their salt form for testing on both cell lines and demonstrated toxicity with approximately 26g of sediment, thus demonstrating that this technique can be used as a tool for sediment assessment. The toxicity of four individual OT compounds was also established with the RTG-2 cells. The AB assay was found to be the most sensitive for each of the individual OTC exposures after 24 h and TPT was found to be the most toxic compound tested. After the 96 h exposure period, increased cytotoxicity was observed for the RTG-2 cells for each of the individual OTCs for both of the endpoints studied as evidenced by the lower EC_{50} values. Comparing results from this study to previously published data, the RTG-2 cells were found to be more sensitive than the PLHC-1 cells to the di-substituted organotins (DBT and DPT) while the PLHC-1 cells were found to be more sensitive for the higher substituted TBT and TPT using the NR assay. The NR assay was found to be the most sensitive assay for both cell lines when exposed to the environmental sample extracts and the PLHC-1 cells were found to be slightly more sensitive to the Dunmore East extract than the RTG-2 cell line. In conclusion we have described a novel technique, integrating specific cytotoxicity tests with analytical chemistry that has not previously been performed for OTCs in sediment extracts.

4.6 References

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5

CHAPTER 5 AN IN-SITU STUDY USING CAGED *NUCELLA LAPILLUS* AND *CRASSOSTREA GIGAS* TO MONITOR TBT BIOEFFECTS IN IRISH COASTAL WATERS

5.1 Introduction

In recent years there has been increase in concern regarding the potential effects that pollutants may have on the health status of marine organisms. A number of adverse physiological and/or morphological changes have been reported in a variety of species as a result of exposure to anthropogenic substances. Organotins are one such group of compounds that are recognised as being of toxicological concern to resident marine organisms (Rüdel, 2003; Hagger et al., 2005; Macken et al., 2008). Tributyltin (TBT) and triphenyltin (TPT) enter the marine environment mainly from their use in antifouling paints used for shipping (Gomez-Ariza et al., 1994) and on fish cages (Minchin, 2003) with both compounds degrading to their di- and mono- metabolites in the marine environment. The TBT degradation metabolites dibutyltin (DBT) and monobutyltin (MBT) have been used as stabilisers in polymers such as poly vinyl chloride (PVC) (Ikonomou, 2002). Triphenyltin has also reportedly been used as an agricultural fungicide and algicide (Gomez-Ariza et al., 1994) and its metabolites can also be found in the marine environment, however, their presence is due to degradation of triphenyltin only.

TBT is known to be toxic to marine species at low water concentrations (Seinen, 1981, Beaumont and Budd, 1984; Hall, 1988), with the irreversible condition of imposex i.e. the imposition of male genital organs (penis and vas deferens) on female gastropods strongly associated with organotin exposure (Gibbs, 1987; Oehlmann et al., 1991). Reproductive failure has been reported to occur in severely affected organisms (Gibbs and Bryan, 1986; Gibbs et al., 1988; Horiguchi, 2000; Bryan et al., 1987) with severe imposex reported to lead to both sterility in females and detrimental reproductive effects on individuals and populations. Organotin related biological effects are commonly measured in a number of

gastropod species including, *Nucella lapillus* (dog whelk) and the *Littorina littorea* (periwinkle) for coastline monitoring of TBT-induced imposex and *Buccinum undatum* (common whelk) and *Neptunea antiqua* (red whelk) for the measurement of offshore effects (Bryan et al., 1988., Bailey et al., 1988; Gibbs et al., 1986; Harding et al., 1992, 1997 and 1998).

During the 1980s several Pacific oyster culture operations in Europe were adversely impacted with high levels of TBT reported in tissues. A cause and effect relationship was established between TBT contamination and reductions in meat weight and increased shell thickness in Pacific oysters (Alzieu et al., 1982) while Waldock et al., (1995) utilized the ratio of shell length to shell thickness (length/thickness) as an index of contamination. Increased shell thickening is not considered as being an organotin specific effect, however, shell thickening in *Crassostrea gigas* has been associated with trace levels of TBT in the water column (Lawler and Aldrich, 1987) and Medaković et al., 2006 have reported environmentally induced levels of tin in the chambers of oyster shells. In Ireland, grossly distorted oysters were reported from Cork Harbour and Baltimore, Co. Cork in 1985 (Minchin et al., 1987).

Ireland was one of the first countries to ban the use of all organotin containing compounds on vessels less than 25m in 1987 (Minchin, 2003). In 1989 the European Union (EU) adopted a ban on the application of tributlytin (TBT) containing antifouling paints to small vessels and subsequently in 1999 the International Maritime Organisation (IMO) (2004) adopted a resolution that called for global prohibition on the application of organotin compounds (OTCs) in anti-fouling paints on vessels by 1 January 2003 with a complete prohibition by 1 January 2008. This has now been implemented in the EU by Council

Directive 2002/62/EC. Ireland is additionally a contracting party to the Oslo Paris Commission Coordinated Environment Monitoring Programme (OSPAR CEMP) and as such has commitments on the provision of TBT specific biological effects data to OSPAR under the CEMP.

The use of cages to transplant dogwhelks has been previously reported (Harding et al., 1992; Quintela et al., 2000; Smith et al., 2006) and these species have been demonstrated to provide valuable biological effects information over short time periods in areas with high levels of TBT contamination. Additional studies have reported the use of marked and recaptured dogwhelks (Bryan et al., 1986, 1987; Foale, 1993; Bech et al., 2002). Recapturing procedures, whilst valuable techniques, can prove problematic as accessibility of food, predation and submergence/emergence times are generally uncontrollable variables (Smith et al., 2006). It has also been reported that the marking can result in animal stress thus reducing the chance of survival of the animal (Quintela et al., 2000). The use of the caging techniques in TBT hotspots can overcome a number of these problems. Using these particular technoques, specimens do not require marking and therefore this would possibly reduce stress on the individuals. Also, gastropods will have accessibility to food and potential threats of external predation can be reduced.

The measurement of stable isotopes (SI) of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) in marine species has previously been employed to provide a quantitative, continuous variable for studying relative trophic status, dietary preferences and the biomagnification of contaminants within complex food webs (De Niro and Epstein, 1978; Minigawa and Wada, 1984; Peterson and Fry, 1987; Rounick and Winterbourn, 1986; Hobson, 1999; Fisk et al.,

2001; Rüüs et al., 2002) and as such SI techniques were primarily employed during this study to provide information on nutrient assimilation within selected test species.

This study provides information on the application of caging methodologies for the simultaneous determination of a number of organotin specific biological effects measurements in two indicator species (Pacific oyster and dogwhelk). It additionally integrates biological effects information with derived tissue concentrations in the species. The potential for the utilisation of stable isotope techniques to trace organism acclimatisation, transplanting success and/or nutrient assimilation within the test system is further discussed. The present study additionally compares quantified organotin measurements to relevant sediment quality and biota assessment criteria and discusses the applicability of caging techniques for use in environmental monitoring assessments.

5.2 Materials and methods

5.2.1 Selection of test species

This study focused on the transplanting of indicator species collected from Omey Island: (*Nucella lapillus)*, Galway Bay: (*Crassostrea gigas* and *Mytilus edulis*) to two test sites (Dublin Port and Dunmore East) in addition to setting up of a control test system at Omey Island itself. Levels of imposex in *Nucella lapillus* have been shown to be very low or absent (Minchin et al., 1987), which when coupled with low levels of marine traffic and the absence of industrialised influences lead to this site being selected as the reference/control location. Dogwhelks for use in the study were obtained from littoral to sublittoral areas, no parasitism of the snails was found at this site. Oysters and mussels for all experiments were obtained from the southern shores of Galway Bay.

5.2.2 Site descriptions and collection of indicator species

At least 40 specimens of *N. lapillus* per site were collected at low tide from Omey Island (see Figure 5.1) in April 2007 which is a semi-exposed shore of bedrock and boulders located on the west coast of Ireland. Largest specimens and smaller juveniles were excluded, smaller juveniles were excluded as their presence is a valuable for continued reproductive activity and removal. *Nucella lapillus* species were subsequently transported to the laboratory in damp cool conditions and analysed for imposex and other biological effects within 48 hours.

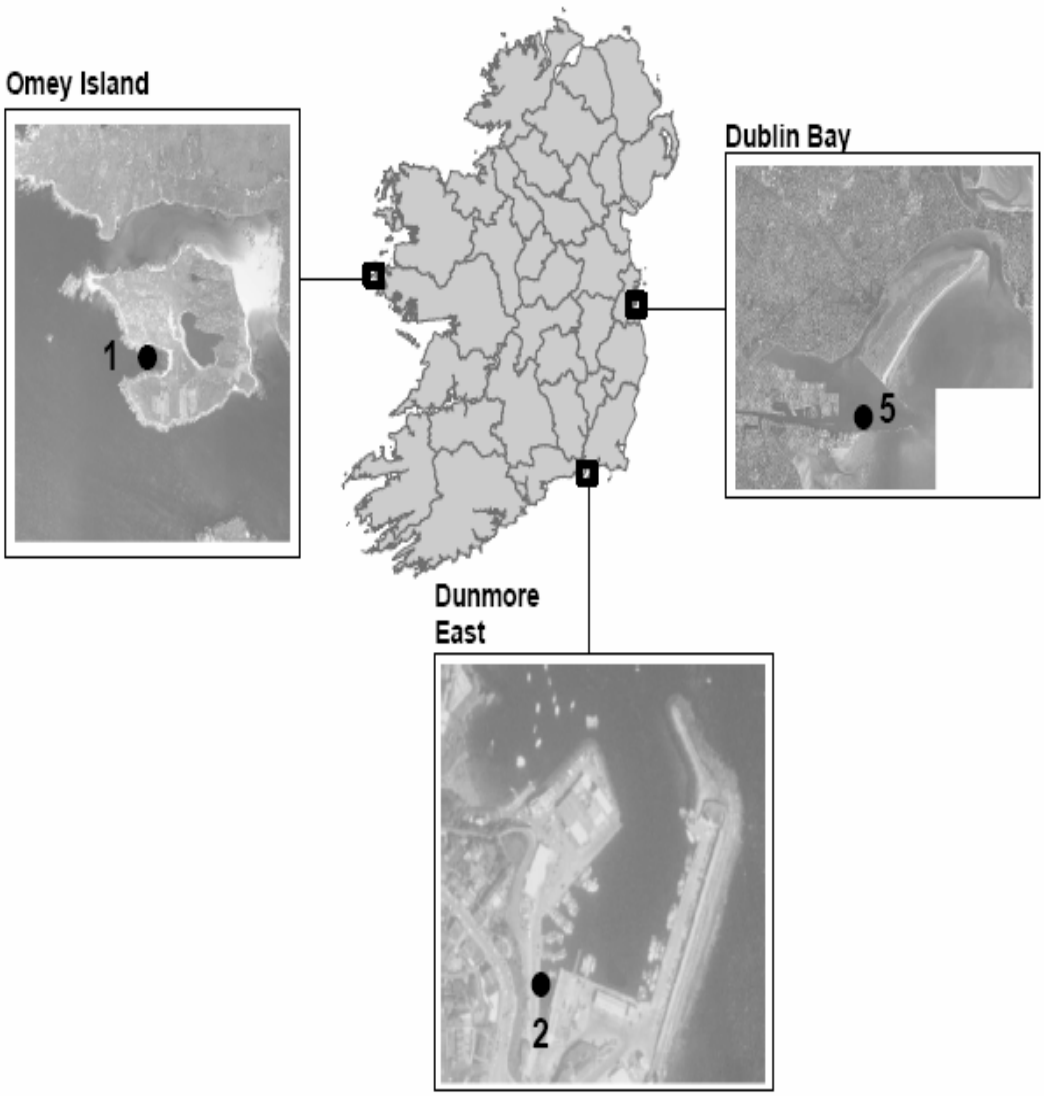

Figure 5.1: Map of transplant locations at Omey Island, Dunmore East and Dublin Bay

Dublin is the main shipping port on the east coast of Ireland. The chosen experimental site is near a fixed navigation mark at the entrance to the estuary sheltered by two breakwaters (the North and South Bull Wall) extending into Dublin Bay. The breakwater to the north becomes progressively flooded over from its extremity during a rising tide. Dunmore East is a fishing port with boat-lifting facilities on the south coast of Ireland. This harbour is protected by a breakwater on an exposed coast. Elevated organotin levels have previously been reported in the inner harbour at Dunmore East (Enterprise Ireland, 2002). The chosen experimental site was located underneath the harbour and the cages were secured to boulders beneath. Test site locations (coordinates) are detailed in Table 5.8 and locations are presented in Figure 5.1.

5.2.3 Caging study methodology

Under the OSPAR-CEMP, collection of periodic biological effects monitoring data with suitable gastropod species is mandatory for contracting parties. While a number of gastropod species are available in Irish waters, *N. lapillus* is an internationally recognised sensitive bioindicator of TBT contamination in addition to its relative abundance at the selected control location and its sensitivity it was chosen for this study. The additional inclusion of *C. gigas* to the test system, added another valuable potential bioindicator to the study.

A total of 7-8 dogwhelks and approximately 1kg of blue mussels (*Mytilus edulis*) were placed together in rigid plastic Northwest Trays® (51cm by 51cm and 53mm deep with a bar mesh of 5mm). Dogwhelks when presented with mussels have been shown to preferentially select these over other bivalves (Minchin, 1989). At each site, 10-12 trays containing both dogwhelks and oysters and one tray containing pacific oysters alone were

stacked and were secured to fixed or temporary moorings at each of the test sites (see Figure 5.2 below). During the experimental period of 18 weeks, trays were permanently submerged at both Omey Island and at Dublin Port while the tray stack was exposed at low tide at the Dunmore East test site. Complete submersion was not found to have an impact on survival of *mytilus galloprovincialis* in cages in previous studies (Quintela et al., 2000).

Figure 5.2: An open tray at t=0 with *N. lapillus* and *M. edulis* (a) and one set of closed trays ready to be transplanted (b) is also displayed.Transplant sites at the North Bank Lighthouse in Dublin Bay (c) where cages were secured and suspended to either side of the lighthouse onto ladders, Dunmore East (d) where cages were secured to boulders underneath the harbour on the opposite side of the synchro-lift and Omey Island (e) where a cage was attached to a buoy in the bay.

5.2.4 Measurement of a species condition index

In order to assess whether transplantation adversely affected test species, a simple surrogate condition index (CI) was derived by calculating the individual mean whole-body tissue dry weight for each test species at t=zero weeks and at t=18 weeks. This average weight was further divided by the mean organism length (mm) to derive a proxy indicator of condition that reduced the inherent variability associated with differences in growth of individual locations.

5.2.5 Determination of the Vas Deferens Sequence Index (VDSI) and relative penis size indices (RPSI)

The development of imposex in *N. lapillus* may be divided into seven stages, depending upon the developmental state of both the penis and vas deferens in the female (Gibbs *et al.*, 1987). Stage 0 is identified where no signs of imposex are observed. Stage 1 can be identified when the vas deferens begins at the site of the vulva with Stage 2 also showing a small penis behind the right eye tentacle. As imposex progresses, the vas deferens starts to develop from the penis (Stage 3) and will become continuous (Stage 4). Eventually, vas deferens tissue may proliferate over the opening of the vulva (Stage 5), rendering the female incapable of breeding since she can no longer release egg capsules. The trapped egg capsules form a solid mass within the capsule gland. In this final Stage (Stage 6), the capsule gland may eventually rupture, causing premature death of the female. Calculation of the mean Vas Deferens Sequence (VDS) for a group of females provides the Vas Deferens Sequence Index (VDSI) that may be used to compare the reproductive competency of different populations. The VDS was determined for each female and the mean VDS calculated to provide an estimate of the VDSI of the population. The

determination of imposex incidence was calculated from the VDSI, the VDSI calculated as (Sum of imposex stages of all females sampled)/number of females.

A second measure is the *relative penis size index* (RPSI), the ratio (female penis length)^3 / (male penis length)^3 x 100. This index provides a more sensitive indication of relative concentrations of TBT and is useful in areas close to release points, especially where VDSI levels are high.

5.2.6 OSPAR Assessment criteria for VDSI

Assessment criteria have been derived by OSPAR for Vas Deferens Sequence Index (VDSI) in *Nucella lapillus*. The assessment classes range from A to F and enable integration of biological effects and chemical data and comparison of sensitivity between other species.

TBT simulates an androgenic hormone in *Nucella lapillus* and in many other neogastropod snails, causing the formation of male organs superimposed upon the normal female reproductive tract. In severe conditions the vas deferens seals the vagina causing obstruction to the release of egg capsules. This condition not only renders the female snail sterile but can also result in mortality (Bryan et al., 1987). Six stages of male organ superimposition are presented in Table 5.1, ranging from 1(A), indicative of the least biological effect to 6 (F), the worst case likely to be observed. The spire-height of dogwhelks was measured from the shell apex to the most ventral part of the siphonal canal at the start and completion of field exposure.

Table 5.1 Oslo and Paris Commission biological effects assessment criteria for imposex in *N. lapillus*, based on VDSI (OSPAR, 2004).

Assessment Class	*N. lapillus* VDSI	Effects and impacts
A	VDSI = <0.3	The level of imposex in the more sensitive gastropod species is close to zero (0~30% of females have imposex) indicating exposure to TBT concentrations close to zero, which is the objective in the OSPAR strategy of hazardous substances.
B	VDSI = 0.3 - <2.0	The level of imposex in the more sensitive gastropod species (~30 ~100 % of the females have imposex) indicates exposure to TBT concentrations below the EAC derived for TBT e.g. adverse effects in the more sensitive taxa of the ecosystem caused by long-term exposure to TBT are predicted to be unlikely to occur.
C	VDSI = 2.0 - <4.0	The level of imposex in the more sensitive gastropod species indicates exposure to TBT concentrations higher than the EAC derived for TBT e.g. there is a risk of adverse effects, such as reduced growth and recruitment, in the more sensitive taxa of the ecosystem caused by long-term exposure to TBT
D	VDSI = 4.0 - 5.0	The reproductive capacity in the populations of the more sensitive gastropod species, such as *N. lapillus*, is affected as a result of the presence of sterile females, but some reproductively capable females remain e.g. there is evidence of adverse effects, which can be directly associated with the exposure to TBT.
E	VDSI = > 5.0	Populations of the more sensitive gastropod species, such as *N. lapillus*, are unable to reproduce. The majority, if not all females within the population have been sterilized.
F	VDSI = negative	The populations of the more sensitive gastropod species, such as *N. lapillus* and *Ocinebrina aciculata*, are absent/expired.

5.2.7 Measurement of biological effects in Nucella lapillus

Dogwhelk shells were crushed in a vice and the living snail was removed and examined for imposex features (VDSI). The length of the female penis, if present, and male penis was measured to 0.1mm following the procedure recommended by OSPAR (1998). Vas deferens sequence index (VDSI) and relative penis size index (RPSI) measurements follow

a standard methodology originally developed by Gibbs et al. (1987) and adopted by OSPAR. The method has a general uniformity of impact on dogwhelks in Europe (Oehlmann et al., 1998).

5.2.8 Measurement of biological effects in Crassostrea gigas

In this study the practice of measuring maximum length of the flat shell and thickness at the position of the adductor muscle was adopted. A great deal of biological variation can exist within any sampled population and consequently a large sample size (> 50 individuals) was investigated to derive a meaningful index.

Investigations into the presence/absence of a number of other biological features in *C. gigas* suspected to be linked to TBT and/or pollutant effects was also completed including;

1) the formation of an adductor pit in the upper (flat) shell and the occurrence of a gel between shell lamellae of the thickened flat valve,
2) shell thickness; the length of the upper flat shell and the thickness of this shell provides an index of shell-thickness as described by Waldock et al. (1995). At its most severe a low index is obtained when organisms are exposed to high levels of TBT contamination.
3) The presence of a brittle, thick and chambered flat shell (producing shell plates referred to here as lamellae) often containing a clear gel in its chambers.
4) the occurrence of a prolonged and dorsally extending spur to the cupped shell. These features are generally associated with shell distortion of highly contaminated Pacific oysters (Alzieu et al., 1982; Minchin et al., 1987)

5.2.9 Organotin analysis in biota

Following the measurement of imposex and shell thickening in *N lapillus* and *C. gigas* respectively, the biologically examined whole body tissue was collected from individuals, pooled, homogenised and stored at < -20^{o}C prior to the determination of TBT, DBT, MBT and TPhT in representative tissue aliquots. Organotin analysis in biota was carried out at the National Environmental Research Institute, Roskilde in Denmark using a method developed by Strand et al., 2003 and is summarised. To 2g (wet weight) of tissue, a methanolic solution of tripropyltin chloride was added as internal standard. The tissue was then digested with 1M HCl in an ultrasonic bath followed by treatment with an Ultraturrax© homogenisator. The pH was then adjusted to approximately 5 with a sodium acetate and sodium hydroxide solution before *in- situ* derivatisation with 1ml 10% sodium tetraethylborate in methanol and subsequent extraction with 10ml pentane. The derivatisation and extraction procedure was repeated 3 times for maximum recovery and the extracts were pooled, before being dried with sodium sulphate. The extracts were then gently evaporated to a volume of 100µl before butyltin and phenyltins are speciated and quantified with a gas chromatograph and a Pulsed Flame Photometric Detector (GC-PFPD). Final data were normalised to tissue dry weight content determined at 105ºC.

5.2.10 Stable isotope analysis in biota

Tieszen et al. (1983) reports that the presence of lipid in biotic tissue samples can affect isotopic ratios, therefore for the purposes of this study, lipid free tissue extracts were prepared on all tissue samples according to the method of Smedes (QUASH, 1998, 1999) prior to stable isotope analysis. Lipid free tissues were freeze-dried and approximately 1

mg of sample was transferred to a 9 x 15 mm tin capsule before combustion in the presence of O_2 and Cr_2O_3 at 1700°C in a Carlo Erba NCS 2500 element analyser. Reduction of NO_x to N_2 was then performed in a Cu oven at 650°C. Water was removed in a $KMnO_4$ chemical trap before separation of N_2 and CO_2 on a 3 m Poraplot Q GC column prior to on-line detection of $\delta^{15}N$ and $\delta^{13}C$ using a Micromass Optima Isotope Ratio Mass Spectrometer. Differences in stable isotope abundances are expressed by (δ) notation as the deviation from standards in parts per

thousand by

$$\delta X = [(R_{sample}/R_{standard})-1] \times 1000$$

where X relates to ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$. $R_{standard}$ for ^{13}C and ^{15}N relate to Pee Dee Belemnite standard and atmospheric N_2, values respectively.

5.2.11 Sediment collection and organotin analysis

Sediment was collected with a Van Veen Grab sampler at high tide in the Dublin Port area while the Dunmore East and Omey Island locations were sampled under low tidal conditions. The upper 0-5cm of sediment were removed for analysis using an appropriately washed spatula for organics analysis and placed into solvent washed jars for OTC analysis. Prior to analysis the sediments were homogenised, wet sieved to the < 2mm and < 0.063 mm fractions, frozen to -20 ºC and subsequently freeze dried at -30 ºC.

Determination of OTC levels in sediments was conducted in the Marine Institute laboratories in Galway, Ireland. In brief, the sediment was digested with dilute HCl and

extracted with dichloromethane and the organic layer centrifuged and removed. This was solvent exchanged to methanol prior to derivatisation with sodium tetraethylborate (10% w/v in methanol solution). The ethylated OTCs were then extracted into hexane and the this layer was pooled and concentrated to approximately 1 ml. Sulphur was removed using tetrabutylammonium (TBA) sulphite. The organotin speciation method was optimised to extract OTCs in their chloride form for subsequent exposure of extracts to various biological organisms for ecotoxicological testing. Finally, tetrapropyltin was added to the analytical extract (approx 1 ml hexane) as an instrumental internal standard prior to analysis by gas chromatography pulsed flame photometric detection (GC-PFPD).

5.2.12 Determination of total organic carbon in sediments

Normalisation to Total Organic Carbon (TOC) in sediments provides a means to compare OTC results from differing locations. TOC was analysed under sub-contract at the Environment Agency laboratories in Llanelli in Wales. Sediments were wet sieved to < 2 mm and < 0.063 mm fractions and freeze dried. Total organic carbon was determined in the < 2mm and the < 0.063mm fractions by an ISO 17025 accredited flash combustion method using a Thermo flash Elemental Analyser as described below. Sediment samples were weighed, treated with sulphurous acid for removal of inorganic carbonates and heated to 900 ºC under a constant flow of helium and introduction of oxygen. Individual components were separated and eluted in the order N_2-CO_2-H_2O and were measured using a thermal conductivity detector.

5.2.13 Quality assurance of determinations

A full analytical quality control program was completed for all analyses, details are presented below.

5.2.13.1 Quality assurance of biological measurements

During the past fifteen years Analytical Quality Control (AQC) procedures have been developed through QUASIMEME for the measurement of imposex with regular intercalibration studies being conducted Europe wide. OSPAR guidelines for TBT-specific biological effects monitoring are now in place. Bioeffects measurements were made by Marine Organism Investigations who received satisfactory results from two such intercalibration exercises in the QUASIMEME programme.

5.2.13.2 Quality assurance of sediment analysis

Quality control of organotin measurements for all sediment analyses was evaluated by the use of the certified reference material BCR646. Analyses of blanks showed no evidence of organotins. Organotin recoveries were determined utilising the butyl- and phenyltin certified freshwater reference material BCR646. Recoveries of TBT ranged from 63.3-68.1 % and DBT from 79.2-84.2 %.

Total organic carbon measurements were quality assured by analysing two QUASIMEME laboratory proficiency materials (QTM080MS and QOR090MS), which returned acceptable |Z| score limits (-0.6 and -0.2 respectively).

5.2.13.3 Quality assurance of biota analysis

Organotin QA was completed utilising the butyl- and phenyltin certified freshwater sediment reference material BCR646. Recoveries of TBT ranged from 63.3-68.1 % and 79.2-84.2 % for DBT. No recovery correction was completed on presented data.

Within the analytical batch, the certified reference material (CRM477) was analysed to quality assure analytical procedures. Quantification limits of 0.5ng Sn/g wet weight (ww) for butyltins (TBT, DBT and MBT) and 1ng Sn/g ww for phenyltins were determined, while repeatabilities were found to be within < 15 %. Organotins were absent from procedural blanks.

Stable isotope analysis was completed in the Institute for Energy Technology (IFE) in Kjeller, Norway. Accuracy and precision of $\delta^{15}N$ and $\delta^{13}C$ analyses was successfully evaluated against International Atomic Energy Agency (IAEA-N-1, IAEA-N-2) and US Geological Survey (USGS-24) standards in addition to an internal IFE trout standard material.

5.3 Results and discussion

5.3.1 Biological and morphological assessment

On completion of the 18 week study, tray fouling with tunicates and barnacles was evident in Dublin, and dogwhelks had become heavily fouled with barnacles (see Figure 5.3 below). In the Dunmore East and Omey Island sites, trays and dogwhelks showed little fouling (Figure 5.3). Most of the juvenile dogwhelks matured during the eighteen week study at all test sites and had a high survival rate. Some unexplained dogwhelks mortalities were observed, Dunmore East (6 %), Dublin Port (5 %) and Omey Island (1 %), dogwhelks have been known to feed on each other under crowded conditions, however no such feeding was evident in this study.

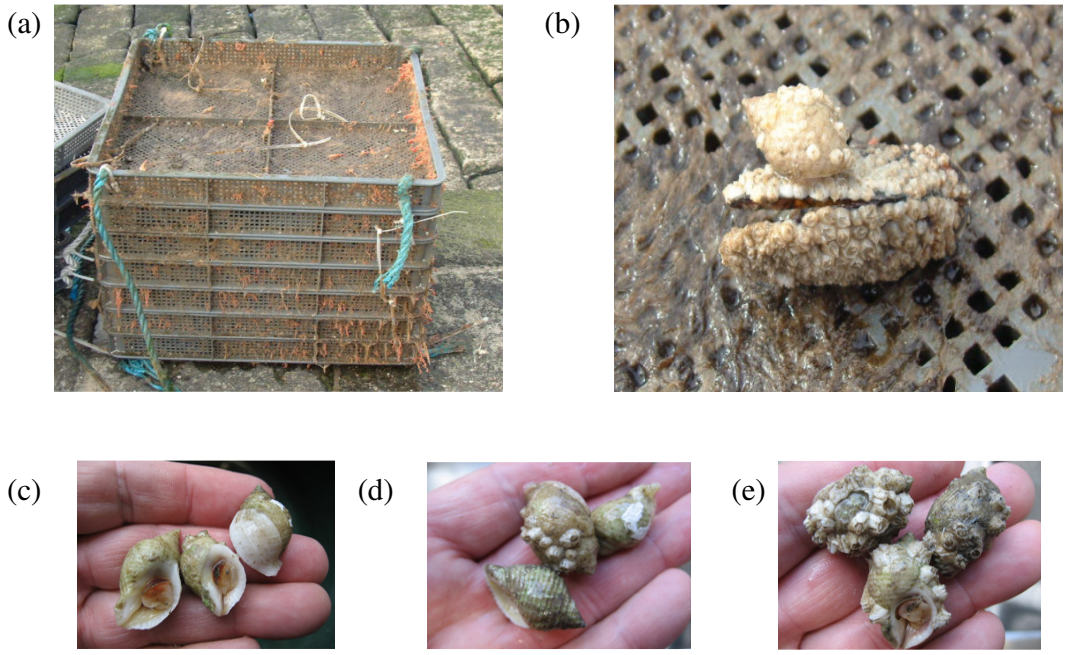

Figure 5.3: Photographs of fouling at the Dublin Port site after 18 weeks (a) fouling on one of the cages including ciona, barnacles, and tubeworms at the Dublin Port site (b) barnacles on the dogwhelk/mussel at Dublin Port (c) fouling on dogwhelks from Omey Island (d) fouling on dogwhelks from Dunmore East where shells were more brittle than other two sites (e) fouling on dogwhelks from Dublin Port which had a high degree of barnacles, some growing to the lip of the dogwhelks.

Mussels present in cages exhibited signs of dogwhelk predation as was further evidenced by the presence of a number of dogwhelk drill holes on collected shells (see Figure 5.4 below). Barnacles were present on the Dublin dogwhelks, some of which were vacant which may indicate feeding upon by dogwhelks. Pacific oysters transplanted to Dublin port had some barnacle and tubeworm fouling. Shell growth was found to be greatest at Dublin and lowest at Omey. Morphological features of Pacific oysters at the end of the

experimental period are displayed in Table 5.2 below. The condition of oyster flesh from Dunmore East and Omey Island was poor relative to Dublin port. No predation of oysters by dogwhelks was evident at any location.

Figure 5.4: Two drilled holes in the mussel shell demonstrating predation of dogwhelk on mussel.

Table 5.2: Morphological features of Pacific oysters at end of experimental period. Mean flat shell length and thickness (mm), and number of individuals with shell lamellae, gel between lamellae and a distinct adductor pit.

Location	Shell length	Shell thickness	Shell index	Shell lamellae	Gel between lamellae	Distinct adductor pit
Omey Island	31.0	1.40	22.1	4	0	0
Dublin Bay	49.2	2.12	25.4	18	1	0
Dunmore East	35.1	4.37	9.20	46	39	37

5.3.2 Condition indices of test species

Transplanted oysters at the Dublin Bay site grew at a much greater rate than those at the other two sites (see Table 5.3 below) and their relative condition factor (0.0059) was much greater than observed at the other two test sites. Dublin Bay can experience much greater nutrient loadings than at either of the locations. It was evident that oysters had no problem acclimatising to the environment in Dublin Bay compared to the mussels given the difference in stable isotope ratio (see Table 5.3). However, there are no native oysters to

compare the stable isotope signal with. Growth of both the mussels and dogwhelks at the test sites was less evident. The dry weight condition index of mussels transplanted to Dunmore East and to Dublin Bay was however lower than that observed from the control site. Results suggest that test species must be transplanted in a timely manner at test sites and that undue stress on animals must be avoided. It should additionally be noted that the mussels, oysters and dogwhelks used in the transplantation experiment originated from saline waters on the west coast of Ireland and that transplantation to variable salinity estuarine waters (e.g. Dublin Bay) with a higher nutrient loading may additionally place undue stress on experimental animals thus appropriate species selection must be carefully considered prior to such caging studies.

Table 5.3 Concentrations (µg kg^{-1} wet weight) of organotin compounds in the tissues of *Nucella lapillus* (NL), *Mytilus edulis* (ME) and *Crassostrea gigas* (CG) and the carbon and nitrogen ratios and condition index for associated samples

Location	Species	TBT	DBT	MBT	TPT	$\delta^{13}C$	$\delta^{15}N$	C:N	ML	DWCI
NewQuay	NL	<0.5	<0.5	1	<1	-16.6	11.8	3.85		
	ME	0.8	1.3	0.5	<1	-18	9.3	3.46		
	CG	<0.5	<0.5	<0.5	<2	-20.1	8.6	4.61		
Omey Island*	NL	<0.5	<0.5	<0.5	<1	-19.5	9.9	3.6	25.3	0.0028
Omey Island	NL	<0.5	<0.5	2.1	<1	-17	10.3	4.24	27.3	0.0071
	ME	<0.5	<0.5	<0.5	<1	-20.5	9.1	3.95	45.2	0.1470
	CG	5.3	1.6	<0.5	<1	-17.9	9	4.26	31.0	0.0016
Dunmore East	NL	113	71	11	<1	-17.3	9.4	4	27.8	0.0053
	ME	94	41	6.1	<1	-18.8	7.6	3.74	52.1	0.0056
	CG	116	18	5.9	<1	-18.1	9	3.71	35.1	0.0022
Dublin Bay	NL	1.8	1.3	<0.5	<1	-16.9	10.8	3.49	26.3	0.0063
	ME	1.1	0.7	<0.5	<1	-18.2	7.8	3.86	52.6	0.0106
	CG	11	0.8	<0.5	<1	-20.6	9.8	3.72	49.2	0.0059

*Control site at Omey Island, specimens were sampled at beginning of experimental period, i.e. T=0

ML= Mean length (mm)

DWCI = Dry weight condition index (dry weight (g) per unit length (mm) of species)

5.3.3 Occurrence of imposex in caged Nucella lapillus

Imposex was not detected in *Nucella lapillus* from the Omey Island control site throughout the 18 week experiment while dogwhelks at the Dublin site showed a VDSI (stage 1) for 5 (25 %) individuals and no other level of imposex was present at this site (Table 5.4). At Dunmore East however, Stage 4 VDSI was demonstrated in 14 individuals (58.3 %) with 100 % of the individuals displaying some level of imposex at this site (Table 5.4 below).

Table 5.4 Imposex of dogwhelks sourced from Omey Island at T=0 weeks and T=18 weeks.

VDSI	T=0 Omey	T=18 Omey	T=18 Dublin	T=18 Dunmore East
0	15	22	15	0
1	0	0	5	2
2	0	0	0	4
3	0	0	0	4
4	0	0	0	14
5	0	0	0	0
6	0	0	0	0
RPSI	0	0	0.0003	2.375
Number (%) imposex	0 (0%)	0 (0%)	5 (25%)	24 (100%)
Mean shell height		27.3	26.3	27.8

The RPSI index has been confirmed to be a more sensitive index of TBT pollution than the VDSI (Barrosa and Moreira, 2002). RPSI values for the three sites are outlined in Table 5.4. Dunmore East was found to have an RPSI of 2.375 which was much greater than that observed at the Dublin site (RPSI= 0.0003). Dogwhelks in Dunmore East showed a much stronger degree of imposex than observed in Dublin or Omey, while no sterile dogwhelks were detected, at Dunmore East it was the only site where distinct penises could be found on females (Table 5.4).

The rapid development of imposex in the caged animals at Dunmore East reflects TBT contamination at this site. High levels of imposex (4.21 to 5.00) have previously been determined in animals collected on the more coastal and east facing side of this breakwater (see Table 5.5 below) with dogwhelks absent at the end of the breakwater and in the inner harbour in the vicinity of the present test sites. It is anticipated that the transplanted dogwhelks within Dunmore East harbour would have greater imposex levels given a longer exposure period. Low level TBT related bioeffects were determined in the Dublin site. No

imposex was noted at the control site Omey Island (see Table 5.4). Quintela et al. (2000) detected VDSI in *N. lapillus* species at stages 4-5 after five months of exposure in caging studies and Smith et al. (2006) demonstrated VDSI stage 5 for four out of thirteen females after six months of exposure.

Table 5.5 Historic mean VDSI (RPSI) values from studies adjacent to current experimental sites (Minchin, 2005).

Year sampled	Omey Island	Dunmore East [1]	Dublin[2]
1996	-	-	4.00 (14.00)
1997	-	4.60 (54.41)	-
1999	0.00 (0.00)	5.00 (78.16)	4.25 (31.25)
2005	-	4.21 (27.48)	4.00 (14.47)

[1]=Outer breakwater of harbour

[2]=North Bull Wall

Although sterile snails have historically been found in Dublin bay, TBT levels were not sufficient to result in the extinction of dogwhelks on either of the two breakwaters leading into the port (Table 5.5). Indications from a survey of Irish port regions (Minchin, 2005) show that there has been a general decline in TBT contamination in Irish waters, except for a number of fishing ports.

5.3.4 Shell thickening in Crassostrea gigas

Shell thickening has been reported as an initial indicator of TBT contamination in cultured Pacific oysters which developed distorted shells (Minchin et al., 1987) and can result in the shell gape becoming restricted (Alzieu et al, 1982).

Morphological features of Pacific oysters at the end of the experimental period and shell thickness indices are presented in Tables 5.2 and 5.6 respectively. The growth of oysters

was greatest in Dublin and five individuals had matured during the period. Growth of oysters was least at Omey Island. Shell thickening indices of 25.4 and 22.1 in Dublin and Omey Island respectively were determined. Shell thickening was most prevalent in Dublin Port. Oysters at Dunmore East demonstrated shell thickening of 9.2, lamellae and the presence of a gel in most shells, a gel was noted in one individual from Dublin. Shell lamella was noted at all sites however in only one individual from the control site (Omey Island). No shells had an elongated spur to the cupped shell at any site. Shell thickening effects have previously been demonstrated in Cork Harbour and thereafter at other sheltered Irish harbours (Minchin et al., 1987). Experimental studies involving the use of trays were undertaken in a bay in the north of Ireland and oysters demonstrated thickening in 70 days (Minchin et al., 1987) providing a shell index ranging from 5.9 to 7.7. Shell thickening and inner white lamellae and gel can be observed in oysters from Dunmore East in Figure 5.5.

(a)

(b)

(c)

Figure 5.5: Photographs of *Crassostrea gigas* after 18 weeks (a) demonstrating the shell thickening at Dublin Port (b) shell thickening indicated by thick white lamella on *Crassostrea gigas* from Dunmore East (c) *Crassostrea gigas* from Omey Island showing no degree of shell thickening with much smaller shells.

Table 5.6: Results of the shell thickness index for Pacific oysters

Shell Index	T=0 Galway Bay	T=18 Omey	T=18 Dublin	T=18 Dunmore East
0	0	0	0	2
5	0	0	0	34
10	0	2	6	10
15	8	15	6	2
20	9	12	14	2
25	10	12	9	0
30	12	6	9	0
35	8	1	5	0
40	2	1	0	0
45	0	1	0	0
50	1	0	0	0

5.3.5 Assessment of stable isotope ratio information

In order to investigate nutrient assimilation, feeding relationships, and the relative trophic status of indicator species, stable isotopes of both Carbon and Nitrogen were analysed in all indicator species at both the start (T=0) and end of the experimental period (T=18).

5.3.6 Assessment of $\delta^{13}C$ data

At the Dublin Bay test site $\delta^{13}C$ isotopes were most isotopically enriched in *N. lapillus* (NL) followed by *M. edulis* (ME) with *C. gigas* (CG) being the least enriched of the test species. At both the Omey Island and Dunmore East test sites the order was NL < CG < ME. Differences in the $\delta^{13}C$ profiles of test organisms suggest that dietary assimilation

and/or availability and utilisation of nutrients and especially in the case of oysters differed between locations. As previously discussed, much greater growth rate was detected in oysters transplanted to Dublin Bay compared to those transplanted to other sites. The $\delta^{13}C$ ratio observed in whole body oyster tissue was less enriched (-20.6‰) in the Dublin individuals than observed at the other sites (-18.1‰ and -17.9‰) at Dunmore East and Omey Island respectively) thus supporting the hypothesis of a greater influence of increased nutrient loadings and/or freshwater influences on the feeding regime of transplanted oysters in Dublin Bay. It should be noted that higher particulate matter content in the water column may affect the $\delta^{13}C$ values of organisms at the base of the oyster food chain and thus be reflected in the $\delta^{13}C$ ratio of the primary consumer themselves. Barnacles which were found to be vacant in Dublin may have potentially fed on these organisms, this however cannot be concluded.

Whole-body nutrient turnover rates have previously been reported to be of the order of 30-60 days (Riera and Richard, 1997) for *C. gigas*, therefore as oysters in this study have potentially been exposed to increased nutrient loadings in Dublin Bay, the observed isotopic differences between oysters in Dublin Bay and the other two locations would be expected.

As reviewed by Fukumori et al. 2008, $\delta^{13}C$ values of organisms in a marine trophic system have been shown to be influenced by phytoplankton growth rate (Laws et al., 1995), the occurrence of phytoplankton blooms (Nakatsuka et al., 1992; Gervais and Riebesell, 2001), primary productivity (Laws et al., 1995; Schell, 2000), and CO_2 concentration (Burkhardt et al., 1999; Tortell et al., 2000). Piola et al (2006) report that changes in oyster $\delta^{13}C$ values can be as a result of the consumption of phytoplankton with differing $\delta^{13}C$ isotope values

reflecting the source of dissolved inorganic carbon (DIC) used for photosynthesis. DIC values typically change along an estuarine gradient, from a depleted signature (more negative) where freshwater influences are evident to an enriched signature at the marine end, and phytoplankton $\delta^{13}C$ values have been shown to reflect this change (Deegan and Garritt, 1997). While no Particulate Organic Matter (POM) isotopic data are available from each of the sampling sites nutrient loadings at the Dublin Bay site would be expected to influence the isotopic ratios derived in whole body tissues.

Primary consumers of phytoplankton, such as oysters, are also likely to integrate this varied estuarine $\delta^{13}C$ signature. Oyster tissues from Dublin Bay show a relatively depleted $\delta^{13}C$ signature compared to the other sites. The strong positive correlation (R^2=0.60) observed between $\delta^{13}C$ ratios and the surrogate condition index of both oysters and mussels may indicate the potential influence of localised sewage-derived nutrients on oysters at this site.

5.3.7 Assessment of $\delta^{15}N$ data

Greatest $\delta^{15}N$ enrichment (9.4 to 10.8 ‰ at Dunmore East and Dublin Bay respectively) was found in *N. lapillus*, thus indicative of this organism's higher relative trophic status compared to the other test species. Enrichment in oysters was found to be greatest at the Dublin Bay site (9.8‰) however little difference was observed between $\delta^{15}N$ ratios in oysters between the sites (range 9.0 to 9.8‰). The $\delta^{15}N$ isotopic ratio determined in mussels was found to be most enriched at the reference site at Omey Island (9.1‰) compared to those at Dunmore east (7.6‰) and Dublin Bay (7.8‰). McKinney et al. (2001) reported a large variation of $\delta^{15}N$ values of ribbed mussel in coastal salt marshes

and suggested that $\delta^{15}N$ of the mussel is influenced by nitrogen derived from human activities, thus locally increased nutrient loadings (e.g. in Dublin bay) would be expected to give rise to changes in the overall $\delta^{15}N$ values derived.

SI data confirm that *N. lapillus* have the highest relative trophic status of the three test species and that derived isotopic ratios may be influenced by site specific nutrient inputs in all species. Overall strong correlations between $\delta^{13}C$ ratios and the surrogate dry weight condition of oysters and mussels were observed and relevance of SI tissue measurements in the description of nutrient assimilation pathways was demonstrated.

Figure 5.6 presents a plot of $\delta^{15}N$ versus $\delta^{13}C$ data for each of the three test species at the three selected locations. The highest values represent the highest trophic level when data is plotted. It is demonstrated that the *N. lapillus* species had the highest trophic status of all three species at Omey Island and Dublin Bay sites however, *C. gigas* was at the same trophic level at Dunmore East. The mussel *M. edulis* was the most predated species at the Dunmore East and Dublin Bay sites but showed the same trophic status at Omey Island to *C. gigas* species at Dublin Bay and Dunmore East.

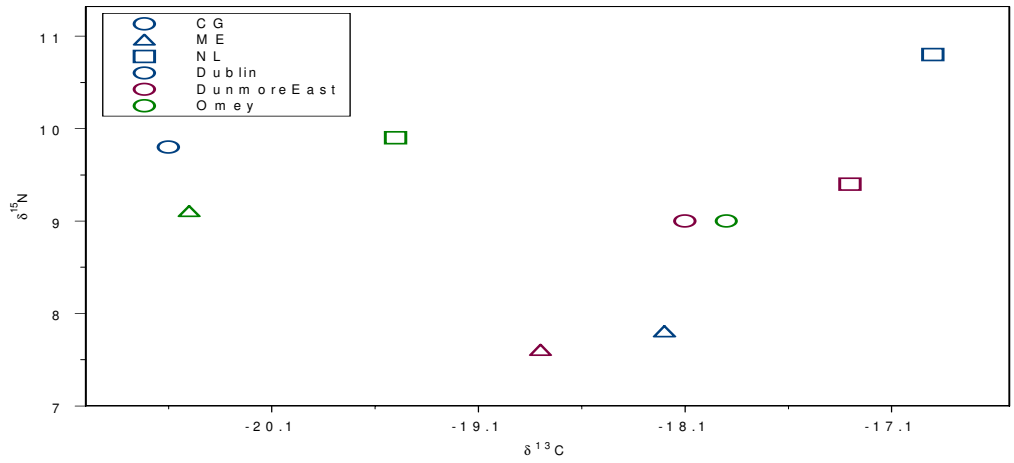

Figure 5.6: Stable isotope profiling of test species (ME=*Mytilus edulis*, NL= *Nucella lapillus* and CG= *Crassostrea gigas*) at test locations.

5.3.8 Organotin concentrations in transplanted animals and sediments

The concentrations of organotins in biota and sediment with the corresponding TOC and moisture contents are presented in Tables 5.3 and 5.7 respectively. On a dry weight basis, highest levels of OTCs were determined in the < 2mm fraction of the Dunmore East sediment (22707 µg kg^{-1} dry weight) and low levels were detected in the Dublin Port and Omey sediments (not detected and 192 µg kg^{-1} dry weight respectively). Low level TBT contamination is evident for Dublin sediments despite the high amount of shipping entering the port and passing in close proximity to the experimental site. Dublin Bay is further subjected to a significant level of water exchange and the sediment contains both low total organic carbon and a low level of clay.

Table 5.7 Concentrations of OTCs in sediment expressed as dry weight and normalised to total organic content µg kg $^{-1}$ in bold text, in < 2mm and < 0.063 mm (in parenthesis) fractions of sediments from adjacent to the caging study test sites.

	TOC	TBT (dw)	TBT (Normalised)	DBT (dw)	DBT (Normalised)
Omey Island	22.6 (30.3)	192 (742)	**21.2 (61.2)**	52.1 (45.6)	**5.76 (3.76)**
Dunmore East	2.30 (1.42)	22707 (6182)	**24681 (10884)**	7362 (965)	**8003 (1699)**
Dublin Bay	0.60 (1.30)	nd (109.2)	**nd (210)**	36.2(64.9)	**151 (125)**

nd=not detected

Normalisation to 2.5 % total organic carbon as per the methodology of OSPAR and Kersten and Smedes (2002), resulted in highest OTC levels being demonstrated in the < 2 mm sediment fraction from Dunmore East (24681 µg kg^{-1} dry weight) while lower levels were shown in the Dublin Bay and Omey Island samples (not detected and 21.2 µg kg^{-1} dry weight respectively). The Omey Island sediment sampling site while in close proximity to the cage study location may however be subject to terrestrial peat based influences and may not be truly representative of marine sediments in the surrounding area. TOC values ranged from 0.60 to 22.6 % in the < 2 mm fraction and 1.30 to 30.3 % in the < 0.063 mm fraction of the three sites (Table 5.8). TBT was the dominant species found in the Dunmore East site this being indicative of TBT inputs into Dunmore East harbour. TBT was not detected in the < 2mm fraction of the Dublin Port site therefore normalisation was not completed. It should be noted that in Dunmore East experimental trays were located close to the area where fishing craft have historically been maintained. Sediments at this location are subjected to disturbance from boat activity and may have resulted in plumes reaching the trays.

Table 5.8 Summary of sampling locations and physico-chemical information for caging study

	Identification	Latitude	Longitude	TOC (%) < 2 mm	TOC (%) < 0.063 mm
North Bank Lighthouse	DB5	6.17	53.35	0.6	1.3
Dunmore East	DE2	6.9922	52.1475	2.3	1.42
Omey Island	OI1	10.17	53.53	22.6	30.3

5.3.9 Organotin concentrations in tissues of test species

The *C. gigas* species accumulated the highest levels of TBT at each of the three test sites. Dry weight concentrations of the three OTCs determined were greater in *N. lapillus* compared to *M. edulis*. Of the three sites analysed, all three species from Omey Island were found to contain the lowest levels of butyltins (BTs). After the 18 week experimental period, it was found that *N. lapillus* had accumulated low levels of MBT compared to the T=0 control. TBT and/or DBT were not accumulated at the same rate at this location. Wet weight concentrations of butyltins in *M. edulis* from Omey were below the detection limit however *C. gigas* did accumulate some TBT and DBT. Highest levels of butyltins were accumulated in all species present in Dunmore East and distribution followed the pattern TBT > DBT > MBT. As previously discussed sediments at this location are subjected to disturbance from boat activity and this may result in test organisms being exposed to sediment plumes with elevated OTCs. At the Dublin Port site levels of TBT and DBT in

the three species were above the quantitation limits and again the distribution followed the pattern TBT > DBT > MBT, this being similar to the pattern in Dunmore East.

5.3.10 Comparison of mussel tissue results to assessment criteria

Ecotoxicological Assessment Criteria (EACs) for TBT were adopted as assessment tools by OSPAR in 1997. The EACs were intended to be used to identify potential areas of concern and to indicate which substances could be considered as a priority. Table 5.9 displays the current EACs for sediment, water and biota in relation to the various stages of imposex in *N. lapillus*. The quantities of TBT in each of the matrices needed to produce these biological effects are also presented. For Dunmore East, VDSI of 3.25 was demonstrated which, is represented by assessment class C in OSPAR assessment criteria. The concentration of TBT in sediment needed to produce such an effect ranged from 2 to < 50 µg kg^{-1} dry weight, however TBT in sediment at this site was at an elevated level of 22707 µg kg^{-1}. This value was elevated compared to the EAC of 0.01 µg kg^{-1} dry weight.

Table 5.9: OSPAR classification of various VDSI stages (A-F) in *Nucella lapillus* species relating to Ecotoxciological Assessment Criteria and concentrations of TBT (µg kg^{-1}) in water, mussels and sediment.

Nucella lapillus	Assessment class	A	B	C	D	E	F
	VDSI	< 0.3	0.3 - <2.0	2.0 - < 4.0	4.0 - 5.0	>5.0	
TBT Water	(ng TBT/l)	<0.025	0.025-0.25	0.25-5	5-7.5	7.5-37.5	>37.5
TBT mussel	(µg TBT/kg dw)	< 3	Mar-30	30 - <600	600-<900	900-4200	>4200
TBT sediment	(µg TBT/ kg dw)	n.d.	< 2	2 - <50	50-<200	200-500	>500
EAC water	(ng TBT/l)		0.1				
EAC Mussel	(µg TBT/ kg dw)		12				
EAC sediment	(µg TBT/ kg dw)	0.01					

5.4 Conclusions

The employment of caging technologies allows for the introduction of indicator species into locations where they may not be present or where they may have previously been affected by elevated contaminant levels. This provides an efficient mechanism whereby integrated biological and/or chemical effects measurements can be determined. It should be noted that prior to these transplantation studies the disease free status of the test organisms must be ensured and undesirable organisms are not introduced to the test site. The reported data are valuable as few studies are available that report measured OTC concentrations in biota with biological effects measurements in multiple caged indicator species. Biological indicators and measured biota and sediment concentrations indicate: 1) elevated levels of contamination of TBT at the fishing port of Dunmore East, 2) low contaminant levels were found at the test site in outer Dublin port and at the control site in Omey Island, 3) no TBT specific biological effects were noted at Omey Island, 4) imposex data at the Dunmore East location suggest exposure of *N. lapillus* to TBT concentrations higher than the Ecotoxicological Assessment Criteria (EAC) for TBT. Stable isotope data confirm that *N. lapillus* had the greatest relative trophic status of the three test species and that dietary assimilation of nutrients may be influenced by site localised nutrient inputs in all species. The relevance of SI tissue measurements in the description of nutrient assimilation pathways was demonstrated. This study has highlighted the value in using imposex for measuring the effects of organotins in the marine environment; and in light of legislative constraints of the use of TBT, the caging techniques described provide a means for biological effects monitoring to take place where resident gastropod populations are absent.

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6

CHAPTER 6 UTILISING CAGING TECHNIQUES TO INVESTIGATE METAL UPTAKE RATES IN *NUCELLA LAPILLUS*, *MYTILUS EDULIS* AND *CRASSOSTREA GIGAS*

6.1 Introduction

The pollution of marine environments by metal contaminants has been of increasing concern for a number of decades, however many gaps still remain in our understanding of the mechanisms underlying bioaccumulation and toxicity of metals in aquatic organisms. Ongoing studies on the mechanism of metal accumulation are required to further provide information for environmental risk assessment of metals in aquatic environments and to provide valuable seafood safety data, which may ultimately assist in consumer protection.

Goldberg (1975) first proposed the mussel-watch concept, this having since being adopted by a number of International metal monitoring programmes (such as OSPAR (OSPAR 2005) wide (including Ireland Mussel-watch programmes). These programmes are primarily based on a quantitative bio-indicator concept, using the ability of marine bivalves (usually mussels/oysters) to concentrate and accumulate contaminants in their tissues.

Utilisation of such sentinel species offers a "time-integrated" response to contaminant levels in the relevant environmental compartment and is considered much more efficient than direct/spot measurements in the water (Goldberg, 1975; De Kock and Van het Groenewoud, 1985; Claisse et al., 1992; Boisson et al., 2003; Andral et al., 2004). Interpretation difficulties can result as a consequence of contaminant dynamics, environmental factors (e.g. temperature, trophic conditions, contamination level, salinity, pH, redox potential, dissolved organic carbon, temperature, and food availability) (Bjerregaard and Depledge, 1994; Sunda and Huntsman, 1998) and (associated) interactions with the physiology (size, sex, sexual maturity, reproduction stages, and seasonal growth cycles) of the test species (Cossa, 1989; Rainbow et al., 1990; Bjerregaard and Depledge 1994; Wang and Eckmann, 1994; Wright, 1995; Lee and Luoma, 1998; Lee

et al., 1998). A better understanding of the mechanism of metals accumulation/elimination is fundamental to ensuring that test species are appropriately selected in order to deliver experimental, environmental and or food safety related goals.

6.1.1 Accumulation/elimination and toxicity of metals in marine organisms

Significant differences can exist in the abilities of marine species to accumulate metals. Silver, cadmium, zinc, copper, and mercury are generally accumulated to much higher concentrations in oysters as compared to mussels whilst scallops (*Pecten maximus*) have been reported to accumulate metals such as cadmium in the digestive gland and kidneys to a greater degree than other bivalves (Belcheva et al., 2006; Metian et al., 2007).

Oysters are known to accumulate high concentrations of zinc in the form of detoxified granules while mussels excrete a portion of accumulated zinc in granules from the kidney. Thus oysters are strong accumulators of zinc whereas mussels are weak net accumulators or partial regulators of zinc (Rainbow, 1992). Such information is fundamental when implementing monitoring programs where zinc levels are to be determined.

Mussels have been reported to accumulate relatively low cadmium and zinc concentrations while oysters have been shown to contain relatively high cadmium and zinc concentrations compared to other marine species. It has additionally been reported that even in unpolluted waters, neogastropods such as *Nucella (Thais) lapillus*, *Murex brandaris* and *Buccinum undatum* can accumulate cadmium at high concentrations (Bouquegneau and Martoja, 1982).

Toxicity of metals to marine organisms does not solely depend on total accumulated tissue concentrations but is related to a threshold concentration of internal metabolically available metal (Rainbow, 2007). Toxicity ensues when the rate of metal uptake from all sources exceeds the combined rates of detoxification and excretion (if present) of the metal concerned.

When metals first enter the body of crustaceans (after uptake from solution through permeable ectodermal surfaces or across the endoderm of the gut) they will initially be metabolically available, i.e. will have the potential to bind to molecules in the receiving cell or elsewhere in the body after internal transport via the haemolymph (Rainbow, 2007). Essential metals (e.g. zinc, copper) are then available to bind to sites where they can play an essential role in metabolic processes, or, if present in excess may cause toxic effects. Excesses of essential metal (and all non-essential metals) must be detoxified, e.g. bound in a storage organ within the body of the animal. Such bound forms in a "detoxified store" may be temporary in which they may be excreted (Rainbow, 1998; Rainbow, 2002).

Detoxification can also occur in the soluble phase. Certain trace metals e.g. zinc, copper, cadmium, silver and mercury, are associated with, and induce, metallothioneins, low molecular weight cytosolic proteins involved in the cellular regulation and detoxification of these metals (Roesijadi, 1993; Amiard et al., 2006). The presence of sulphur in cysteine residues in these proteins provides the high metal affinity of the molecule, sequestering metals in the cytoplasm and reducing their metabolic availability. Once the uptake rate of a trace metal is lower than the combined rates of detoxification and excretion, then the metal will not accumulate in the metabolically available component and toxicity will not ensue (Rainbow, 2002; Marsden and Rainbow, 2004). Conversely if the uptake rate exceeds that

of excretion/detoxification then the level of metabolically available metal will exceed a threshold and toxic metal can then bind to sites where they can interfere with normal metabolic functioning.

Differences in tissue metal concentrations can therefore be described as a function of the interaction of the physicochemical characteristics of the contaminant, environmental factors, physiology of the test species, and inter-specific differences in the biokinetics of uptake and elimination. Summary factors influencing metal accumulation in a number of marine species relevant to this work are further discussed below.

6.1.2 Factors influencing metals uptake

There have been numerous studies on factors controlling metal uptake in marine species. It is not the purpose of this chapter to fully report on all of these studies, however a number are summarised below.

6.1.2.1 Physiological factors influencing metals uptake

Wang and Rainbow (2006) reported that differences can exist in cadmium assimilation efficiencies (AE) among bivalve species feeding on a common laboratory food source. Predatory gastropods can also assimilate dietary cadmium at close to 100 % efficiency (Wang and Ke, 2002).

Major differences exist in the uptake rate constants of dissolved metals among the different species of bivalves with scallops and oysters having the highest uptake rate constants for

different metals as a result of high pumping rates. Differences in metal "efflux" rates are also important in determining inter-specific differences in accumulated metal concentrations in bivalves. The storage of accumulated metal in a detoxified form is required for a low efflux rate, particularly where the detoxification form is other than soluble metallothionein (Ng et al., 2007).

Differences in growth rate in accounting for the difference in metal accumulation among different bivalve species have not yet been explored. Growth rate is used in the biokinetic equation to calibrate growth dilution (analogous to metal efflux). The importance of the growth rate of the bivalves in controlling metal concentrations needs to be studied, especially when examining differences in metal concentrations in bivalves from different locations. Wang and Fisher (1997b) suggested that the growth rate is needed to predict the metal concentration and allometry of metal accumulation in the mussel *Mytilus edulis*, especially in the case of smaller mussels.

Biokinetic parameters (e.g., dietary assimilation, dissolved uptake and efflux rates) are therefore important in determining interspecies differences, while other parameters (such as feeding rate, growth rate) can be of less importance in metal accumulation. Since many species living in the same geochemical environment display contrasting metal concentrations, geochemical parameters (metal concentrations in water and food) may be less important in explaining the interspecies difference in metal body concentrations Wang and Rainbow (2005).

6.1.2.2 Subcellular fractionation of metals

It is now established that aquatic invertebrates possess diverse strategies in the handling and storage of accumulated metals; thus great differences in tissue metal concentrations across different phyla can exist (Rainbow, 1998; Rainbow, 2002; Wang and Rainbow, 2005).

Wang and Rainbow (2005) discuss the importance of the sub-cellular distribution of metals in bivalves and suggest that metals are generally fractionated into 5 operationally defined subcellular pools, consisting of 1) metal-rich granules (MRG), 2) cellular debris (mainly cellular membrane fragments), 3) organelles (metals bound with mitochondria, lysosomes, endoplasmic reticulum), 4) heat-sensitive proteins (HSP, including enzymes), and 5) heat-resistant proteins (generally considered to be metallothioneins or more correctly metallothionein-like proteins (MTLP) (Wallace et al., 2003). Different metals have contrasting associations with different subcellular pools, depending on species, exposure history, and other conditions.

Blackmore and Wang (2003a) have reviewed whether differences in the measured concentrations in biomonitors reflect local bioavailabilities or reflect inter-site differences in the organism's physiology and biochemistry, suggesting that biomonitoring data from different areas and even utilising different mussel species may be directly comparable thus supporting the concept of biomonitoring/"Mussel-Watch" programs. Such conclusions confirm that with appropriately selected test species and methodologies (e.g. caging techniques in areas where species may be absent) that caging of sentinel species can provide valuable information on (metal) pollutant levels in the marine environment.

6.1.2.3 Factors influencing metal uptake

Environmental factors can however be critical in influencing metal accumulation in organisms living in different environments and are discussed below.

6.1.2.4 Effect of salinity

Water salinity variations can directly affect the speciation potential of metals in the water column and thus both the metal uptake rate from solution and potentially the physiology of organisms. Factors leading to an increase in the local proportion of free ion concentration may increase metal bioavailability and thus increase uptake and toxicity, although such relationships especially in field situations can be complex (Pan and Wang, 2004; Chuang and Wang, 2006).

Blackmore and Wang (2003b) compared the biokinetics of metals in the green mussel *P. viridis* from two sites of contrasting salinity. Concentrations of metals (cadmium, chromium, selenium and zinc) were 1.2–6.4 times greater in mussels collected from the low salinity site compared to those from the high salinity site. The authors suggest that Cr (VI) and Se (IV), which are present as anions in solution are not affected by chloride complexation, thus behave similarly to cadmium and zinc in that their uptake from the dissolved phase increased with decreased salinity and dissolved speciation was not the only factor to affect metal uptake from solution by the mussels. Such studies demonstrate that changes in salinity can cause eco-physiological changes in particular organisms and subsequently cause an inter-populational difference in dissolved metal uptake rates.

A previous history of environmental exposure of a population to metals itself may result in changes within that populations metal handling physiology. Prior metal exposure may induce specific metal detoxification processes or physiological and biochemical changes that can subsequently affect the uptake of metals (Wang and Rainbow, 2005).

Shi and Wang (2004a) compared the cadmium biokinetics in clams *R. philippinarum* from a previously contaminated site and a "clean" site and found that the contaminated clam population had a higher metallothionein (MT) concentration compared to the uncontaminated population. No significant difference in the dissolved uptake rate constants, efflux rate constants and the clearance rates of the two were observed between the populations and the contaminated clam population had significantly higher cadmium and zinc AEs compared to the uncontaminated population. The authors suggest that AE differences may account for the higher cadmium and zinc tissue concentrations in the clams from the contaminated site and may suggest the presence of a "positive feedback" mechanism. Others studies (e.g. Rainbow et al., 1999) on amphipods (*Orchestia gammarellus*) and crabs (*C. maenas* and *Pachygrapsus marmoratus*) did not suggest such positive feedback.

Mussels rapidly accumulate heavy metals and can carry out depuration when animals are transplanted to clean locations or when environmental concentrations of contaminants decline (Okazaki and Panietz, 1981). It has been reported that despite depuration, detoxification and the potential for metals isolation, that heavy metals negatively affect growth of mussels and other bivalves (Rainbow, 1995; Manley et al., 1984; Din and Ahamad, 1995).

6.1.3 Use of caging studies

The translocation of sentinel species from a reference site to the study areas has been demonstrated as a useful strategy for the assessment of water quality in coastal and estuarine environments, either through bioaccumulation and/or biomarker response (Regoli and Orlando, 1994; Regoli, 2000; Da Ros et al., 2002; Nasci et al., 2002; Riveros et al., 2002; Riveros et al., 2003; Smolders et al., 2002; Romeo et al., 2003; Bodin et al., 2004; Bolognesi et al., 2004; Regoli et al., 2004). The use of cages to transplant dogwhelks has also been previously reported (Harding et al., 1992; Quintela et al., 2000; Smith et al., 2006).

Caging of marine species facilitate the investigation in areas where native organisms are absent, reduce the influence of genetic/population differences, of seasonal variability or adaptive phenomena. The use of caged organisms provides a time-integrated assessment of environmental quality over a translocation period, and reveals the early biological effects induced by accumulated pollutants. As discussed previously in chapter 5, caging of *Nucella lapillus* and *Crassostrea gigas* species demonstrated a high level of imposex (3.25 VDSI and 2.375 RPSI) and a high degree of shell thickening (shell index: 9.20) after only an 18 week period in a TBT contaminated site.

6.1.4 Stable isotopes in describing nutrient assimilation and metal uptake

Wang (2002) reports that aquatic invertebrates will take up trace metals into the body from solution through permeable body surfaces and from the gut. It is becoming increasingly appreciated that uptake of trace metals from the diet may be the major source of metals for

many aquatic invertebrates. The measurement of stable isotopes (SI) of nitrogen and carbon in marine species has previously been employed to provide a quantitative, continuous variable for studying relative trophic status, dietary preferences and the biomagnification of contaminants within complex food webs (De Niro and Epstein, 1978; Minigawa and Wada, 1984; Peterson and Fry, 1987; Rounick and Winterbourn, 1986; Fisk et al., 2001; Ruus et al., 2002; Hobson, 1999). The potential for the application of SI techniques to caging studies for metals uptake is evaluated.

This chapter proposes to investigate:

1) the potential application of caging techniques for the monitoring of metals uptake in three marine species (mussels, oysters and dogwhelks)
2) levels of metals in filter-feeding mussels and oysters and in the predatory dogwhelk (at T=0 and T=18 weeks) transplanted to 3 Irish coastal locations.
3) Similarities/differences in the metals accumulation pattern in the filter-feeding organisms and in the gastropods.
4) the potential application of stable isotope methodologies to track nutrient assimilation in these species.
5) a potential role for stable isotopes in modelling metals uptake in test species.
6) the potential for application of caging studies to support biomarker/ecotoxicological studies.

6.2 Materials and methods

6.2.1 Caging study methodology

The caging study was completed as reported in chapter 5 (section 5.2.3) of this thesis. Test species (oysters, mussels and dogwhelk) at T=0 and T=18 weeks were collected for biological effects measurement as described previously in chapter 5 (section 5.2.2). Whole

tissues remaining after biological effects measurements were completed were pooled on a species basis and analysed for a variety of metals as described below.

6.2.2 Biota metals analysis

A total of 23 metals were analysed in freeze dried tissues from each of the test species. The methodology for metals analysis is briefly described. Metal concentrations were quantified using a 7500cs ICP-MS (Agilent, Santa Claire, United States) with a Babington nebuliser connected to a cooled spray chamber (5 °C), introducing the mist into an Ar-plasma operating at 1500W with 15 L Ar per minute. Standard mass-overlap correction from US-EPA method 6020 was used to correct the signal before the calibration was calculated, and drift was corrected by using rhodium, iridium and indium as internal standards. Zinc was determined using acetylene-air flame atomic absorption on a PerkinElmer 5100PC (Perkin Elmer Corporation, Massachusets, United States); cadmium was determined by graphite furnace of the same instrument, using palladium-magnesium as modifier in a platform furnace and finally mercury was determined using cold vapor atomic absorption spectrometry on a Perkin Elmer flow injection mercury system (FIMS) 400 (Strand et al., 2005). All atomic absorption methods were based on external standard curves.

6.2.3 Stable isotope analysis

The methodology for stable isotopes analysis in tissues is described previously in chapter 5 (section 5.2.10).

6.2.4 Quality control of data

A full quality control programme was completed for both stable isotope analysis (see chapter 5, section 5.2.14.3) and metals as discussed below.

Quality assurance for metals was validated by the use of certified reference material (CRM) NIST 2976. With the exception of strontium and uranium, all where within 30 % of the target value for the metals, aluminium, potassium, iron, nickel, cobalt, copper and lead fell within 15 % (on ICP-MS, RSD of 3-12 % for 5 NIST digestions) zinc (Flame Atomic Absorption), cadmium (Graphite Furnace Atomic Absorption) and mercury (Cold Vapour) where all within 15 % (RSD < 5%). The RSD on replicate digestions was typically 2-5 % for AAS and 3-12 % for ICP-MS.

6.2.5 Data assessment

Concentrations of 23 metals (µg kg^{-1} dry weight) and for stable isotopes (‰) in the test species are presented in Table 6.2. For the purposes of further assessment concentration and stable isotope data were subdivided into five classifications, as follows;

1) Mussels- *Mytilus edulis* only (filter feeding).
2) Pacific Oysters- *Crassostrea gigas* only (filter feeding).
3) Dogwhelk- *Nucella lapillus* only (predatory gastropod).
4) Mussels and oysters combined (as indicators of filter feeders).
5) Data from all three test species.

Correlations were subsequently completed with S-Plus software between 23 metal concentrations (µg kg^{-1} dry weight), $\delta^{13}C$ and $\delta^{15}N$ for each of the above classifications allowing for the identification of strong positive and/or negative correlations between metals, between species (individual and combinations) and between locations.

Strong positive/negative correlations can indicate that parameters are closely associated, and in the case of metals that they may have similar assimilation and/or elimination mechanisms. In the case of filter feeding organisms (oysters and mussels) strong correlations in opposite directions may indicate that mechanisms for the assimilation/elimination of metals may differ within species. Strong correlations between stable isotope parameters and individual metals may indicate dietary related assimilation of metals.

For further illustrative purposes graphs (Figures 6.1 to 6.6) detailing increases/decreases in metal concentrations in each of the three test species relative to their appropriate t=0 reference sample were completed. Subtracting the log (x+1) metal concentration in the t=18 week samples from that of the t=0 week reference sample has the effect of "normalising" datasets to allow for visualisation of uptake/elimination of metals on both a location (Omey Island, Dublin Bay and Dunmore East) basis and on a test species basis.

Cluster Analysis (Pielou, 1984) was performed as Bray-Curtis with Single Linkage and plotted as % similarity. Principle Components Analysis (Jeffers, 1978; Pielou, 1984) was based upon a correlation matrix to calculate only necessary Eigen values for three axis. Both analyses were performed in Biodiversity Pro 2 (McAleece et al., 1997).

6.3 Results and Discussion

6.3.1 Overall observations on metals concentrations

Cadmium levels were found to be more elevated in both resident dogwhelks (t=0) and in dogwhelks transplanted to each of the test locations than in either tranplanted mussels or oysters (see Figures 6.1 to 6.6). Levels of cadmium are raised in the initial (t=0) sample and are still elevated in (t=18 weeks) at Omey island. Cadmium levels at Omey Island would be expected to be low therefore in the absence of additional supporting data it is unclear whether results suggest that the dogwhelk, *Nucella lapillus,* has a greater capacity to accumulate cadmium in their tissues or may or have a lower capacity to eliminate the metal than the other species. Exposure to cadmium is responsible for metallothionein induction in both *N. lapillus* and *T. clavigera*, and metal-rich granules (MRG) have also been shown to be involved in cadmium storage in detoxified form (Leung and Furness, 2001; Cheung et al., 2006). Correlations between cadmium levels in *Nucella lapillus* and $\delta^{13}C$ (r=-0.60) and $\delta^{15}N$ (r=-0.65) suggested dietary influences in cadmium uptake in the species, however this should be further investigated (as discussed in detail below) due to the small sample number (n=4) available.

Levels of zinc were more elevated in oysters than observed in either mussels or in the dogwhelks. Zinc levels in this thesis were also found to be more elevated for each of the test species located in Dunmore East. The capacity within the oysters to accumulate higher concentrations of zinc and (copper) in oysters is primarily related to the oysters' high pumping rate. Such haemocytes are typically present in all oysters (Roesijadi, 1996). George et al., (1978) report that based on observations of copper in the oyster *Ostrea edulis* relative to mussels, may be attributable to bonding to sulfur within hemocytes. This

mechanism may be responsible for the strong correlation between silver and copper concentrations as reported by Daskalakis (1996).

Levels of lithium were found to be lower in NL than in either CG or ME. Manganese levels were lower in CG and NL than in ME. Levels of iron were lower in NL transplanted to the three test sites than in other species (exception t=0 reference ME). Nickel was lower in NL transplanted to the three test sites than in other transplanted species. Cobalt was found to be more elevated in ME than in the other test species.

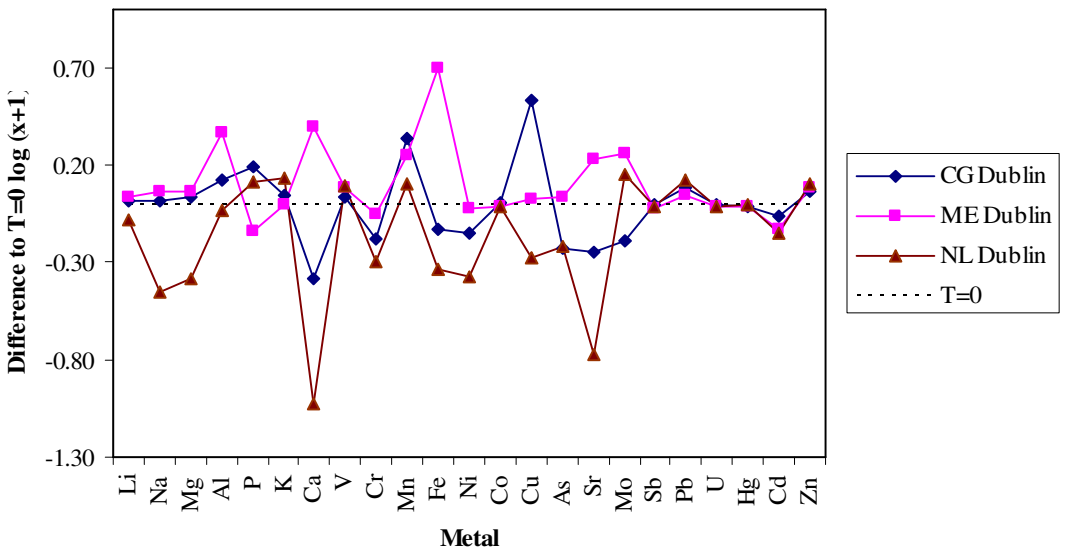

Figure 6.1: Levels of metals in *Crassostrea gigas*, *Mytilus edulis* and *Nucella lapillus* transplanted to Dublin, relative to the appropriate (t=0) reference. Units expressed as Log(x + 1) of t=18 week sample minus Log(x + 1) of t=0 reference.

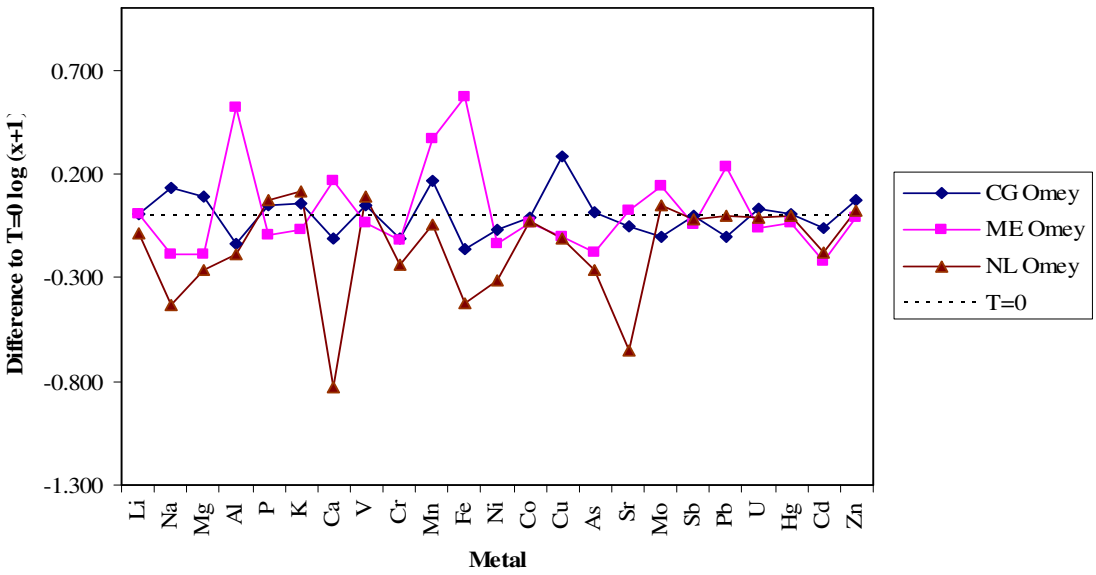

Figure 6.2: Levels of metals in *Crassostrea gigas*, *Mytilus edulis* and *Nucella lapillus* transplanted to Omey Island, relative to the appropriate (t=0) reference. Units are expressed as Log(x + 1) of t=18 week sample minus Log(x + 1) of t=0 reference.

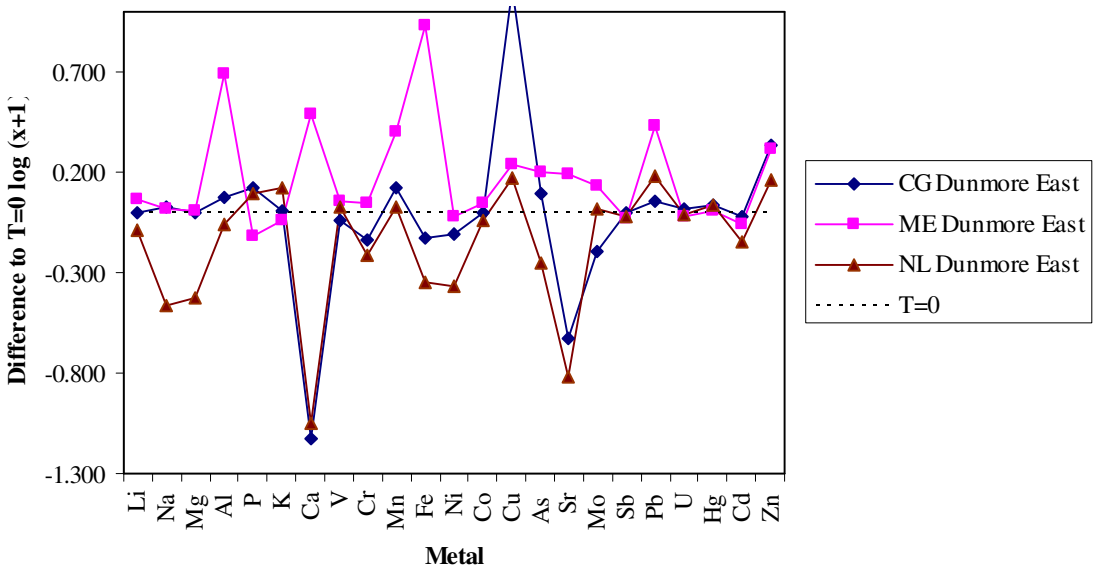

Figure 6.3: Levels of metals in *Crassostrea gigas*, *Mytilus edulis* and *Nucella lapillus* transplanted to Dunmore East, relative to the appropriate (t=0) reference. Units expressed as Log(x + 1) of t=18 week sample minus Log(x + 1) of t=0 reference.

Copper levels were more elevated in each of the test species transplanted to both Dublin and Dunmore East indicating increased concentrations of these metals at these two more industrialised locations. Similarly lead (with the exception of mussels in Omey) was more elevated in Dunmore East and Dublin than in other sites. Uranium levels were found to be lowest in dogwhelks at all sites and in mussels transplanted to Omey Island. Mercury was raised in each of the species transplanted to Dunmore East compared to at other sites.

Arsenic levels were higher in NL (range 38.4 to 71.2 µg kg^{-1} dry weight) than in either mussels or oysters at other test locations while antimony concentrations were found to be lower in dogwhelks than in mussels or oysters.

The above findings with respect to biotic accumulation of elevated metals levels at test sites in both Dunmore East and Dublin suggest rapid accumulation of bioavailable metals from the surrounding environment and provide a valid tool for biomonitoring metal impacted areas (Regoli and Orlando, 1994; Regoli et al., 2004).

6.3.2 Metal concentrations in Mytilus edulis between locations

In general, dry weight metal concentrations in transplanted and T=0 mussels were broadly similar in mussels between locations. Higher levels of aluminium, iron, lead, cadmium, copper, and zinc (201, 261, 4.42, 1.43, 14.1 and 251µg kg^{-1} respectively) in mussels transplanted to Dunmore East were found to be higher than in those transplanted to other locations.

Aluminium, calcium, manganese, iron, strontium, molybdenum and lead were found in greater levels in mussels transplanted to all three locations than in the reference (t=0) mussels. Calcium levels in mussels from Omey Island were much higher than in those at other locations, shell fragments if present in a sample would potentially result in such elevated calcium levels.

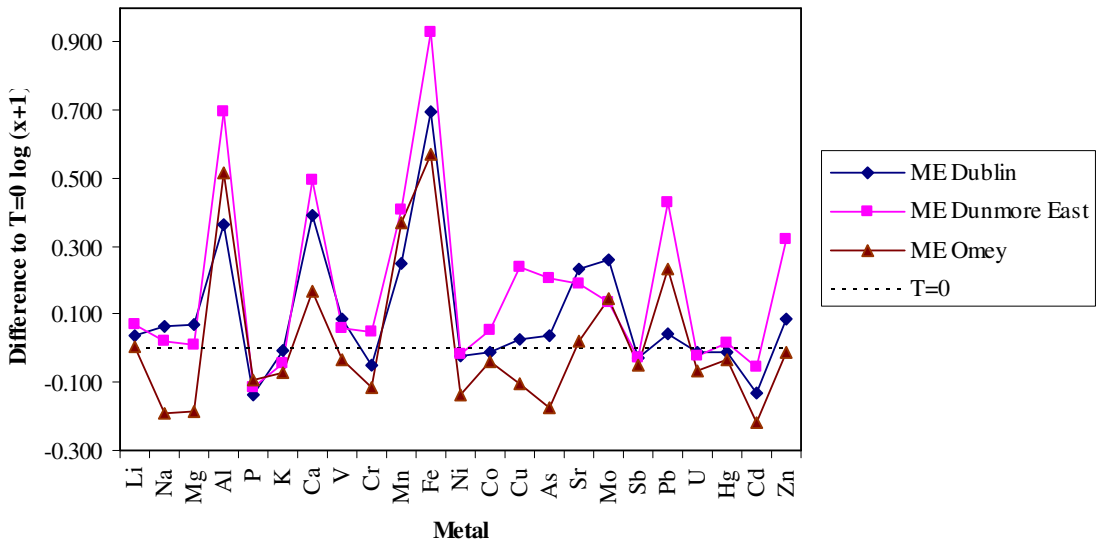

Figure 6.4: Levels of metals in *Mytilus edulis* relative to the Galway (t=0) reference. For graphical purposes units are expressed as Log(x + 1) of t=18 week sample minus Log(x + 1) of Galway t=0 reference.

6.3.3 Metal concentrations in Crassostrea gigas between locations

Concentration levels for the majority of metals were broadly similar in oysters transplanted to the test locations. Copper, lead and zinc levels in Dunmore East and copper and lead in Dublin Bay (DB5) transplants were higher than those observed in either the (t=0) or Omey Island samples indicative of the nature of activities in these locations. Both molybdenum and strontium were found to be lower in both Dublin Bay and Dunmore East transplanted samples than at the other two sites.

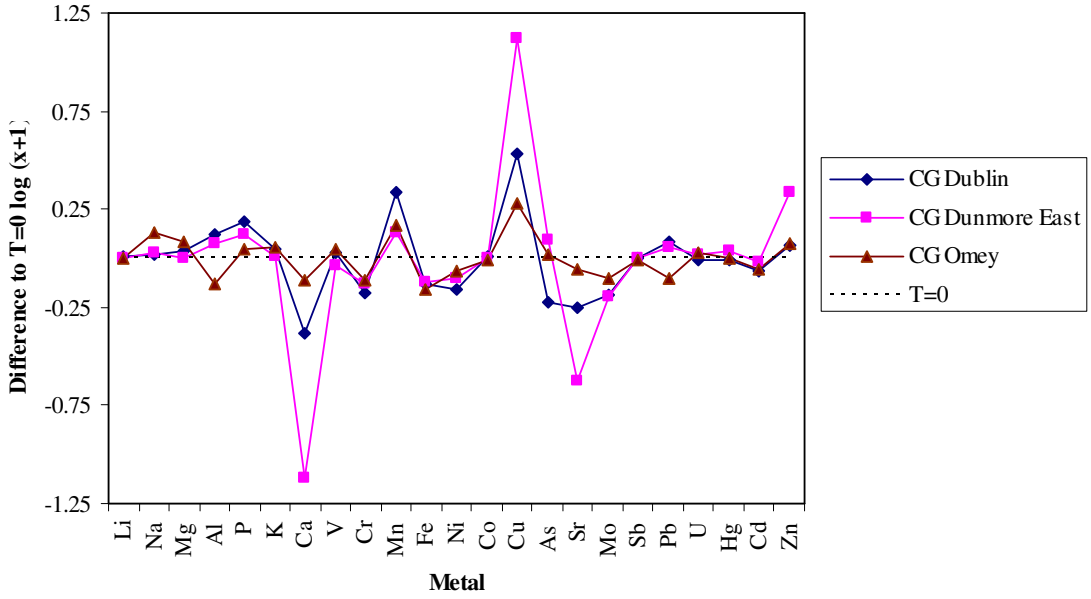

Figure 6.5: Levels of metals in *Crassostrea gigas* relative to the Galway (t=0) reference. For graphical purposes units are expressed as Log(x + 1) of t=18 week sample minus Log(x + 1) of Galway t=0 reference.

6.3.4 Metal concentrations in Nucella lapillus between locations

Stable isotope ($\delta^{15}N$) data suggest that *Nucella lapillus* occupy a higher trophic level than either *Mytilus edulis* or *Crassostrea gigas* and thus accumulation of metals may potentially result as a consequence of its predatory nature. Chromium, nickel, arsenic, strontium and iron levels in (t=0) dogwhelks were higher than those determined at the same location at T=18 weeks and in Dunmore East and Dublin after 18 weeks.

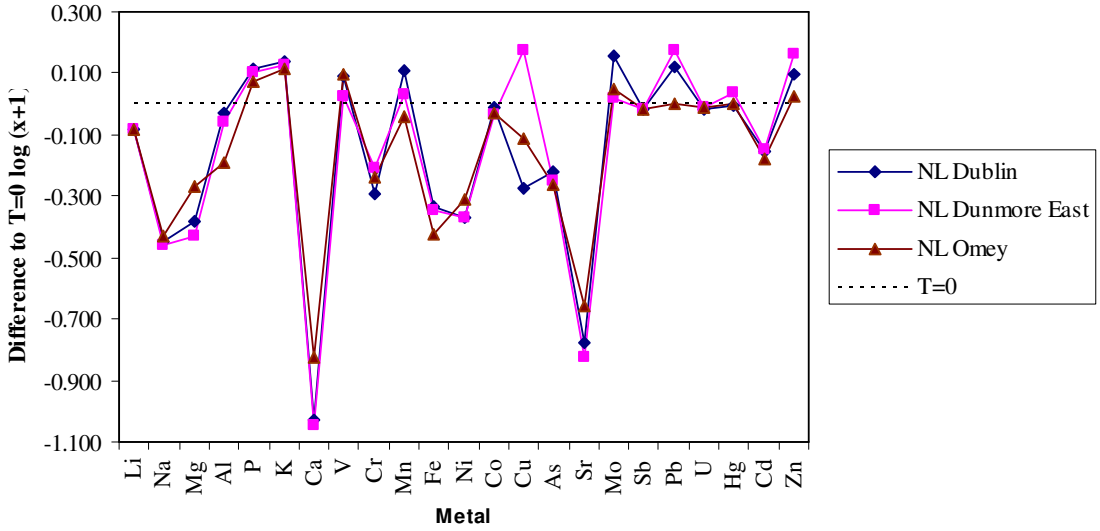

Figure 6.6: Levels of metals in *Nucella lapillus* relative to the Omey Island (t=0) reference. For graphical purposes units are expressed as Log (x + 1) of t=18 week sample minus Log (x + 1) of Omey Island t=0 reference.

Levels of lithium were found to be lower in dogwhelks than in either oysters or mussels with strong correlations between lithium and $\delta^{13}C$ (r=0.81) and $\delta^{15}N$ (r=0.82) indicating dietary assimilation.

Arsenic levels were found to be higher in dogwhelks (range 38.4 to 71.2 µg kg dry weight) than in either mussels or oysters at other test locations with strong correlations to both $\delta^{13}C$ (r=0.81) and $\delta^{15}N$ (r=0.82).

Raised imposex indices (see chapter 5) were determined in dogwhelks from Dunmore East and while elevated organotin levels have been found at this location, it may be of significance that a number of other metals are elevated in dogwhelks transplanted to Dunmore East.

6.3.5 Metal and stable isotopes correlations in classification groups

As previously discussed, stable isotopes provide a mechanism whereby dietary assimilation of nutrients can be tracked and where assimilation of contaminants can be modelled. A full discussion on the use of stable isotopes to trace nutrient assimilation in these caging studies is previously described in chapter 5 (sections 5.3.6 and 5.3.7), while the application of stable isotopes in tracing metals assimilation/elimination is further discussed below.

A substantial dataset containing concentration data from a total of 23 metals, 2 stable isotope and 5 biota classifications was available and a large correlations dataset was established. However the discriminatory power in such correlations was reduced based on the low number of samples (3 sites with 1 replicate each). Additionally due to the complex/dynamic nature of test sites it was deemed inappropriate to utilise SI techniques to identify potential dietary/trophic level correlations with this dataset. With additional discriminatory power in the form of an increased number of samples and establishment of the level of potential analytical errors associated with singlicate sampling SI techniques may prove to be valuable in further elucidating the influence of diet/trophic status in trace metal assimilation/elimination. It should however be noted that the use of stable isotopes to track nutrient flow within a food web system has been demonstrated in sections 5.3.6 and 5.3.7 of this thesis.

6.3.6 Principle Components Analysis and Cluster Analysis

In both Principle Components Analysis (PCA) (Figure 6.7) and Cluster Analysis (CA) (Figure 6.8) samples were grouped primarily by species; *C. gigas, M. edulis* and *N. lapillus*.

Samples of *N. lapillus* clustered closest together and separated furthest from other samples. Signals of site were present in the analysis. For all 3 species, samples showed the same site ordering along Axis 1 of the PCA within their respective clusters. Samples collected at the site of origin at t=0, showed the lowest score along the axis, followed in all three cases, by Omey Island, Dublin Port and finally Dunmore East.

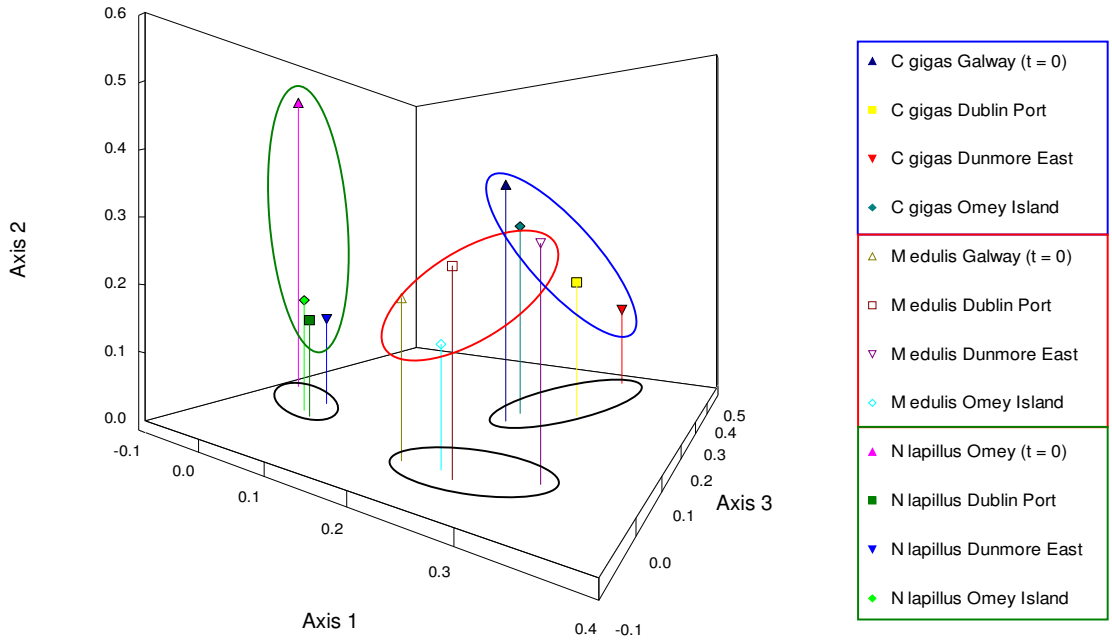

Figure 6.7: Principle Components Analysis Axis 1, 2 and 3 with clusters by species indicated.

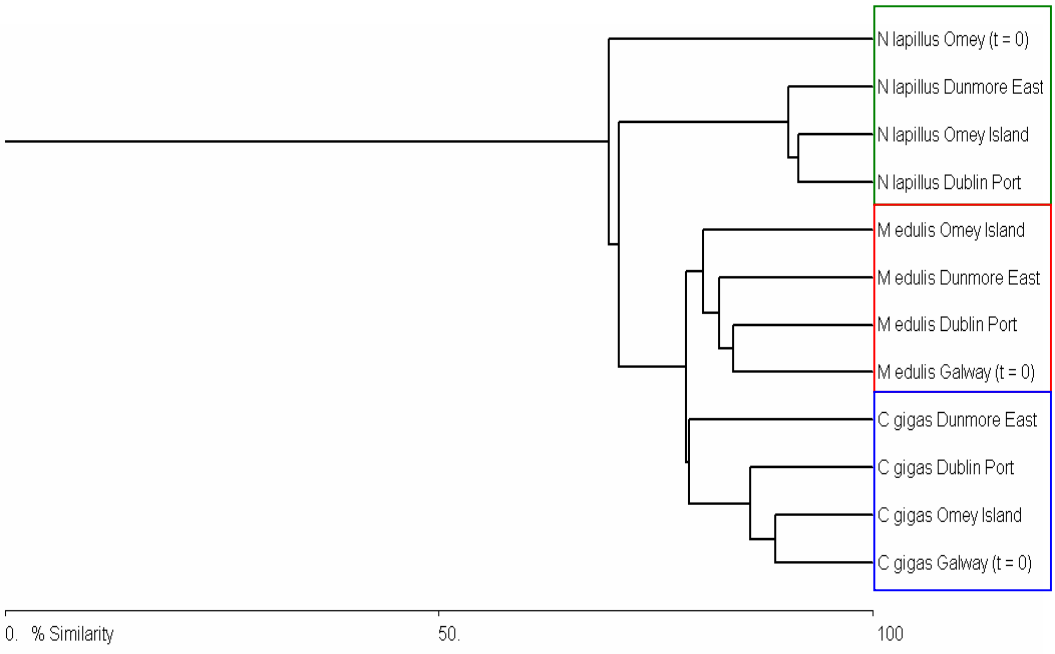

Figure 6.8: Bray-Curtis Cluster Analysis (single Linkage) with clusters indicated.

Results suggest that metabolism/ absorption and the accumulation of metals in tissues is species specific. Clearly, tissue loadings of chemicals from different species are not comparable. Within species however, results indicate that there is a site influence upon tissue concentrations (for example, at the Dunmore East site, turbulence from boating activities could effect sediment exposure) however this cannot be concluded from this study.

6.3.7 Statistics used for metals results

Significant differences (as derived by ANOVA) in dry weight metal concentrations between species were determined for lithium, aluminium, potassium, vanadium,

manganese, iron, arsenic, antimony, uranium, cadmium and zinc. The nature of these differences was further examined by the use of a Scheffe Post Hoc Test (See Table 6.1).

Table 6.1: Summary ANOVA statistics for transplantation experiment including F test statistic (F Critical = 4.25 2 and 6 df), probability statistic P and nature of difference between species as derived from Post Hoc Scheffe tests.

Metal	Sig Diff	F test (F crit. 4.25. 9,2 df)	Probability	ME - CG	NL - CG	NL - ME
li	✓	11.05	0.0038	0.85	0.006	0.014
Na		1.5				
Mg		0.92				
al	✓	5.78	0.024	0.99	0.046	0.049
P		2.807				
K	✓	12.6	0.0025	0.99	0.006	0.005
Ca		1.88				
V	✓	6.2	0.0203	0.625	0.103	0.02
Cr		0.02				
Mn	✓	13.998	0.0017	0.0035	0.0056	0.94
Fe	✓	4.405	0.046	0.4	0.046	0.347
Ni		0.16				
Co	✓	7.38	0.0127	0.046	0.82	0.018
Cu		2.03				
As	✓	15.899	0.001	0.812	0.004	0.002
Sr		0.808				
Mo		3.91				
Sb	✓	16	0.0011	0.33	0.01	0.0013
pb		2.29				
U	✓	5.42	0.0285	0.689	0.033	0.12
Hg		1.91				
Cd	✓	82.26	0.0001	0.999	0.00000562	0.0000055
Zn	✓	16.64	0.0009	0.0021	0.0029	0.97

Significant differences in levels of manganese, cobalt and particularly for zinc were reported between mussels and oysters. Differences in the levels of lithium, aluminium, potassium and antimony (lower in dogwhelks than in either mussels or oysters) arsenic and

cadmium (elevated in dogwhelks compared to mussels or oysters) were determined. These results are presented in graphs (Figures 6.9-6.14).

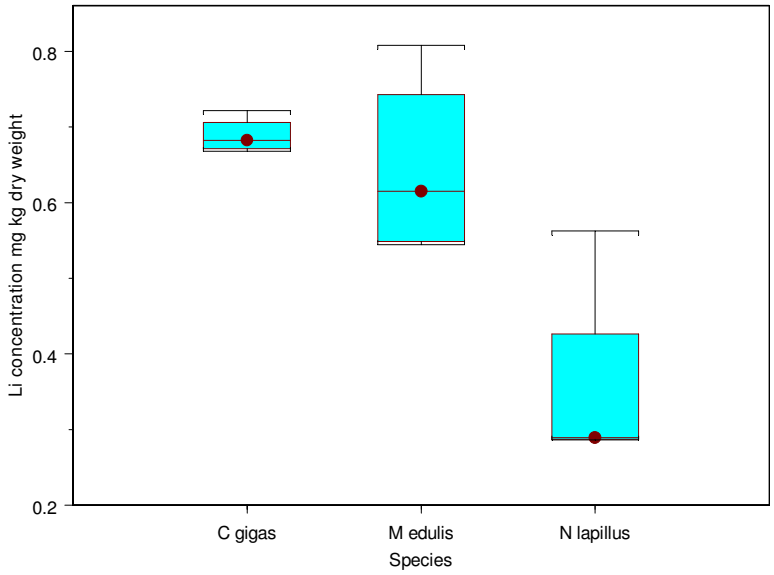

Figure 6.9: Concentrations of lithium (Li) in the caged species, *Crassostrea gigas*, *Mytilus edulis* and *Nucella lapillus* species after 18 weeks exposure.

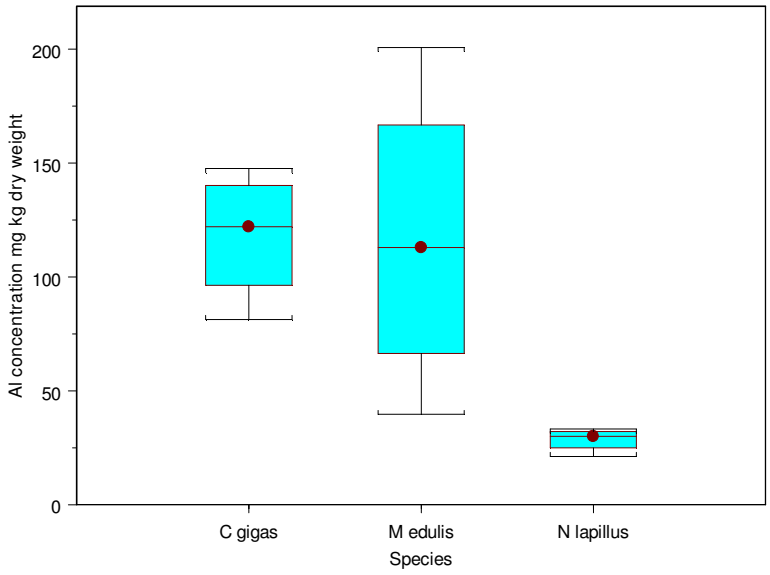

Figure 6.10: Concentrations of aluminium (Al) in the caged species, *Crassostrea gigas*, *Mytilus edulis* and *Nucella lapillus* species after 18 weeks exposure.

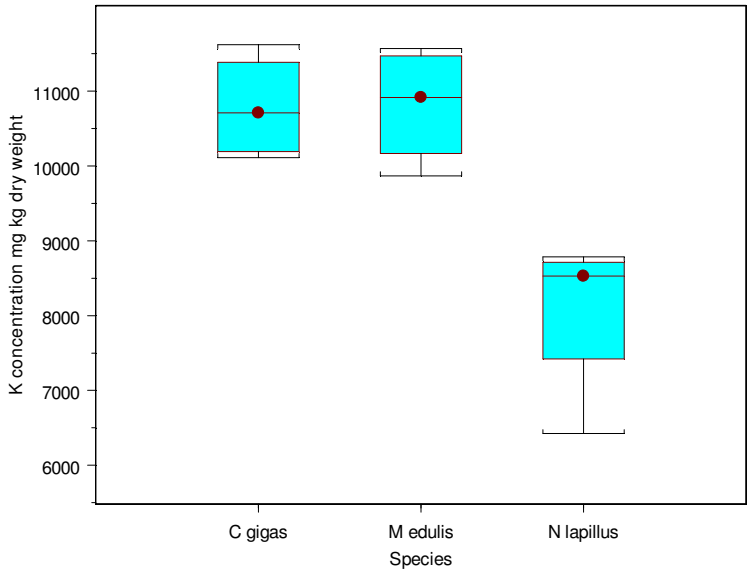

Figure 6.11: Concentrations of potassium (K) in the caged species, *Crassostrea gigas*, *Mytilus edulis* and *Nucella lapillus* species after 18 weeks exposure.

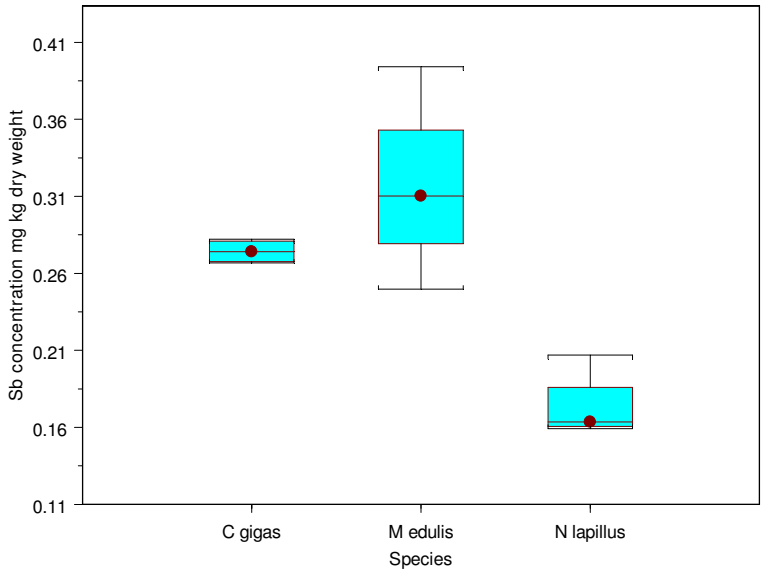

Figure 6.12: Concentrations of antimony (Sb) in the caged species, *Crassostrea gigas*, *Mytilus edulis* and *Nucella lapillus* species after 18 weeks exposure.

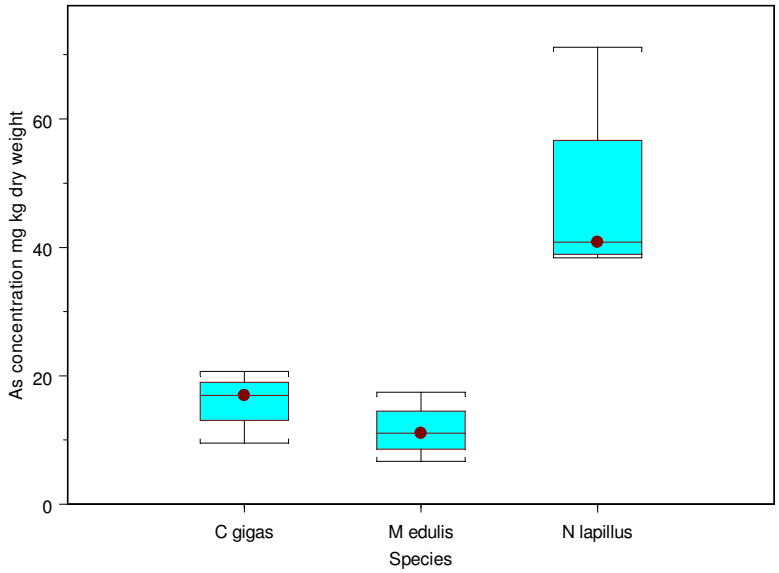

Figure 6.13: Concentrations of arsenic (As) in the caged species, *Crassostrea gigas*, *Mytilus edulis* and *Nucella lapillus* species after 18 weeks exposure.

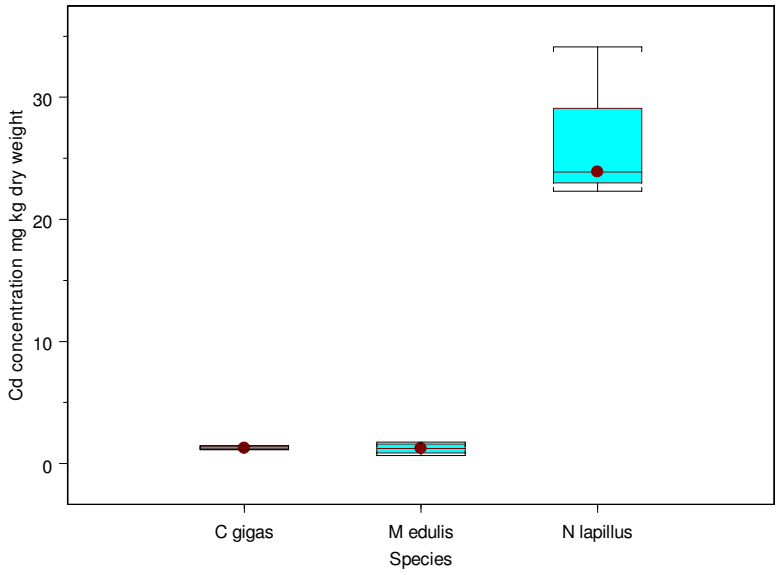

Figure 6.14: Concentrations of cadmium (Cd) in the caged species, *Crassostrea gigas*, *Mytilus edulis* and *Nucella lapillus* species after 18 weeks exposure.

6.4 Conclusions

Rapid biotic accumulation of metals was demonstrated in the test species, especially in both Dunmore East and Dublin suggesting the caging study as described is a valid tool for biomonitoring metal impacted areas. The use of SI techniques to model metal uptake in this study was statistically limited by sample size. Application of the SI technique to describe nutrient assimilation in the test species has been demonstrated. It has been demonstrated that the cage study is suitable for the deployment of indicator species to potential "hotspot" locations or where resident species may be absent. Further to this, the metals and stable isotopes datasets derived during this study are of significant value in supporting other biological effects (i.e. imposex in the dogwhelk *Nucella lapillus* and shell thickening in *Crassostrea gigas*) described throughout this thesis.

Table 6.2: Levels of metals (mg kg^{-1} dry weight) and stable isotope ratios $\delta^{13}C$ and $\delta^{15}N$ ($^{0}/_{00}$) in Crassostrea gigas (CG), Mytilus edulis (ME), Nucella lapillus (NL) at t=0 and t=18 weeks in Dublin, Dunmore East and Omey Island.

Location	Galway (t=0 wks)	Dublin	Dunmore East	Omey	Galway (t=0 wks)	Dublin	Dunmore East	Omey	Omey (t=0 wks)	Dublin	Dunmore East	Omey
Species	**CG**	**CG**	**CG**	**CG**	**ME**	**ME**	**ME**	**ME**	**NL**	**NL**	**NL**	**NL**
Dry Wt %	24.4	21.2	21.8	22.1	21.9	20.3	21.1	24.79	30.1	37.2	36.4	36.4
d13C	-20.1	-20.6	-18.1	-17.9	-18.0	-18.2	-18.8	-20.5	-19.5	-16.9	-17.3	-17.0
d15N	8.60	9.80	9.00	9.00	9.30	7.80	7.60	9.10	9.90	10.8	9.40	10.3
Li ICP	0.67	0.72	0.67	0.69	0.54	0.68	0.81	0.55	0.56	0.29	0.29	0.29
Na ICP	11,033	11,435	11,826	14,859	15,117	17,495	15,945	9,728	18,501	6,576	6,415	6,839
Mg ICP	2,132	2,310	2,133	2,611	2,696	3,153	2,746	1,757	5,541	2,305	2,068	2,992
Al ICP	111	148	133	81.3	39.7	93.3	201	133	33.3	31.0	28.9	21.2
P ICP	4,761	7,320	6,301	5,357	6,944	5,050	5,318	5,595	3,871	5,037	4,873	4,592
K ICP	10,110	11,150	10,266	11,618	11,569	11,370	10,460	9,866	6,424	8,784	8,641	8,416
Ca ICP	33,549	13,910	2,525	25,827	1,263	3,119	3,948	1,856	35,042	3,296	3,151	5,226
V ICP	1.05	1.21	0.88	1.29	1.11	1.59	1.41	0.95	0.52	0.88	0.61	0.88
Cr ICP	1.70	0.79	0.99	1.09	1.24	0.98	1.51	0.71	2.26	0.65	1.00	0.88
Mn ICP	21.3	47.1	29.0	31.8	4.64	9.02	13.4	12.1	10.6	13.8	11.4	9.5
Fe ICP	254	188	191	175	29.8	152	261	113	124	56.6	55.5	46.0
Ni ICP	1.58	0.81	1.03	1.21	1.56	1.43	1.47	0.88	2.57	0.51	0.53	0.75

Co ICP	0.49	0.52	0.48	0.46	0.67	0.62	0.88	0.54	0.51	0.47	0.39	0.42
Cu ICP	20.6	72.7	282	40.3	7.70	8.29	14.1	5.82	30.6	15.9	46.5	23.3
As ICP	16.6	9.5	20.7	17.3	10.5	11.6	17.4	6.68	71.2	42.2	39.4	38.4
Sr ICP	84.2	47.0	19.2	73.8	18.9	33.0	29.8	19.9	147	23.7	21.2	31.7
Mo ICP	1.85	0.84	0.83	1.27	1.00	2.64	1.73	1.80	0.56	1.23	0.63	0.76
Sb ICP	0.28	0.28	0.27	0.27	0.39	0.31	0.31	0.25	0.21	0.16	0.17	0.16
Pb ICP	1.20	1.65	1.51	0.74	1.01	1.22	4.42	2.45	0.54	1.05	1.32	0.54
U ICP	0.46	0.44	0.51	0.56	0.54	0.49	0.46	0.32	0.38	0.32	0.35	0.34
Hg - FIMS	0.13	0.10	0.23	0.13	0.15	0.12	0.18	0.06	0.18	0.16	0.28	0.18
Cd-GFAA	1.49	1.14	1.37	1.17	1.77	1.05	1.43	0.67	34.1	23.7	24.1	22.3
Zn-FAA	673	786	1,466	791	120	148	251	118	163	204	238	172

ICP: Inductively Coupled Plasma, GFAA: Graphite Furnace Atomic Absorption, FAA: Flame Atomic Absorption, FIMS: Flow Injection Mercury System, CG: Crassostrea gigas, ME: Mytilus Edulis, NL: Nucella lapillus

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7

CHAPTER 7 BIOASSAY-DIRECTED FRACTIONATION OF SOLVENT EXTRACTS OF MARINE SEDIMENTS FROM THE EAST COAST OF IRELAND

7.1 Introduction

Bioassay-directed fractionation (BDF) and Effects-directed analysis (EDA) are two valuable techniques that aim to establish a causal link between chemical substances and biological effects in environmental samples by combining chemical and biological analysis. Thousands of compounds can potentially exist in marine coastal sediments and usually different contaminants present their own mode of action on marine organisms. In ecotoxicological studies with marine species, effects of single substances are usually assessed. This scenario is not realistic of the true environmental exposure as sediment samples from individual locations can contain a mixture of contaminants. Ecotoxicological based assessments of contaminants in mixtures is difficult to quantify due to the potential for contaminant interactions (e.g. additive, antagonistic or synergistic effects). When dealing with marine species there are many substances for which no toxicity data currently exists. The measurement of biological effects on organisms and identification of the causative agent using chemical analysis is therefore essential for the risk assessment of sediments.

In BDF/EDA, the cause of toxicity is narrowed down by using a fractionation procedure which separates the complex mixture of contaminants into various groups. This procedure has been used previously for fractionation of groups of compounds such as polycyclic aromatic hydrocarbons (Grote et al. 2005), halogenated aromatic hydrocarbons such as coplanar polychlorinated biphenyls, polychlorinated naphthalenes, polychlorinated dibenzo-*p*-dioxins and dibenzofurans (Brack et al., 2005) alkylphenols, nitroaromatics, synthethic musks, organophosphorous compounds and brominated organic compounds (Biselli et al., 2004). Limited bioassay directed fractionation data is available for contaminant groups such as organotin compounds (OTCs). This is because a derivatisation

step is required in the quantification procedure and their extraction into suitable forms for bioassay testing may be problematic. The production of crude solvent extracts to test other compounds on biological organisms has previously been reported to incorporate mainly the use of soxhlet extraction using dichloromethane [DCM] (Brack et al., 2002; Brack et al., 2005; Grote et al., 2005), soxhlet extraction over 24 h with solvents of increasing polarity (Hollert et al., 2000) and a sequential extraction incorporating a range of solvents with increasing polarity using accelerated solvent extraction (ASE) (Biselli et al., 2004; Houtman et al., 2004). A direct analysis can therefore be performed with these extracts for quantification of many organic compounds but an additional derivatisation step is needed for organotin quantitation.

7.1.1 Bioassays used for bioassay directed fractionation

The primary consumer, *Tisbe battagliai* and the decomposer, *Vibrio fischeri* were selected for ecotoxicological testing of solvent extracts of samples. These two assays have previously been shown to be sensitive to organotin compounds (Macken et al., 2008).

Microbial tests such as the Microtox® Acute Test, which tests the acute toxicity of environmental samples and pure compounds based on the natural bioluminescence of *Vibrio fischeri* species, are suited for sediment toxicity testing primarily because they represent principal cell functions. The uptake of pollutants is more direct than with higher organisms where the main route of exposure may be ingestion of sediment (Liß and Ahlf, 1997). In the presence of certain pollutants, the natural bioluminescence is reduced and the toxicity is expressed as the sample concentration that produces a 10 or 50 % reduction of

the initial luminescence (EC_{10}/EC_{50}). This test has been observed to show differences in sensitivities to various organic and inorganic contaminants (Macken et al., 2008).

Copepods are the most abundant metazoans in the aquatic environment. Ecologically, copepods are an important trophic level, living on phytoplankton and serving as food for fish larvae. The marine copepod *Tisbe battagliai* has previously been observed to be sensitive to a range of complex mixtures and single substances (Hutchinson et al., 1994; Kirby et al., 1998; Macken et al., 2008) and requires low sample volumes (< 20 ml) for testing.

7.1.2 Aims of the study

The aims of this present study were to (a) prepare a crude contaminant extract using a method which has previously been reported to extract OTCs and other anthropogenic compounds (see chapter 4) for three sites in Ireland namely Bull Lagoon, Dunmore East and Alexandra Basin for assaying with *Tisbe battagliai* and the Microtox® system, (b) use chemical analysis to characterise these extracts (c) where toxicity is evident, further fractionate the extract/s with solvents of increasing polarity, (d) perform further bioassays on these fractions with *Tisbe battagliai* and the Microtox® system in tandem with additional chemical characterisation and ultimately (e) assess whether bioassay directed fractionation is a useful tool for fractionation of a contaminated sediment extract.

7.2 Materials and Methods

7.2.1 Sediment sampling and sample extraction

Sediments were collected in Dunmore East (DE1), Dublin Port west Alexandra Basin (DB1) and the Bull Lagoon (BL) as per 2.2.1 in chapter 2 of this thesis and is briefly described. Sediment was sampled from three sites on the east coast of Ireland (Bull Lagoon [latitude 6.1300, longitude 53.3480] Dunmore East [latitude 6.9921, longitude 52.1477] and Alexandra Basin [latitude 6.2187, longitude 53.3485]). Sediment was collected with a Van Veen Grab sampler and the top 0-5 cm was removed for use as a test material. Samples were stored at -30 ºC. Thereafter, the < 2mm fraction was frozen at -30 ºC and freeze dried. Sediment samples were subsequently thawed and wet sieved to the < 2mm fraction. A map of the sampling locations is shown in Figure 7.1.

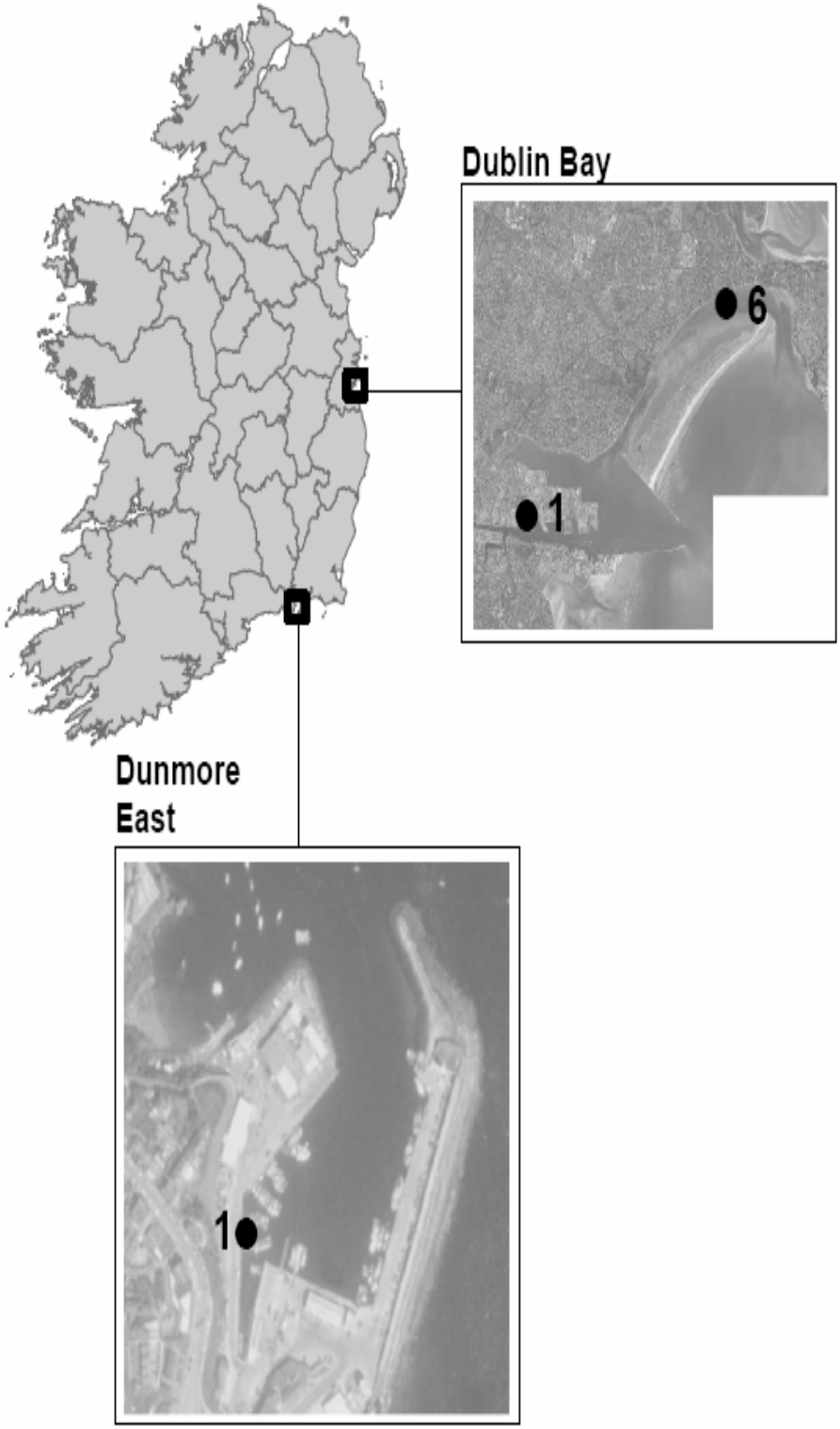


Figure 7.1: Map of sampling locations for the bioassay directed fractionation study.

7.2.2 Preparation of crude organic extract for assaying

A crude contaminant extract was prepared for each of the three test sites. Three separate extractions were prepared, (1) for the Microtox® assay, (2) for the *Tisbe battagliai* acute tests and (3) the final extract for the chemical analysis. A crude extract was also prepared for assaying with the RTG-2 and PLHC-1 cell lines which was previously described in section 4.2.6.2 of chapter 4 in this thesis.

For the *T. battagliai* test approximately 6 g sediment was extracted in total in 2 g fractions (see Figure 7.2) and the extracts combined. To each 2 g of freeze dried sediment, a solution of HCl: H_2O 1:1 v/v (8 ml) was added and this was sonicated using an ultrasonic bath for 30 min. Dichloromethane (20 ml) was then added and the mixture was shaken for 30 min using a multitube vortexer. The slurry was then centrifuged at 3000 rpm for 5 min and the DCM layer was removed. Sonication and shaking were repeated twice for maximum recoveries and the DCM layers combined. The DCM layer was then concentrated and transferred to hexane under a N_2 stream using a turbovap concentrator. The hexane layer was concentrated down to 1 ml with a turbovap concentrator and excess water was removed with 1 g of hexane washed sodium sulphate. Sulphur was removed from the extract using tetrabutylammonium sulphite as previously described. The hexane layer was then transferred to hexane:acetone 9:1 v/v (1 ml) and ultimately transferred to 0.25 ml of DMSO. A blank extract and a sample extract were prepared for each site.

For the Microtox® test, 1 g of sediment was extracted. Sediment weights used for extract preparation were based on previous chemical and bioassay data which determined each species' sensitivity to tributyltin (Macken et al., 2008).

Working solutions for the bioassay procedures were prepared by conducting a 1:100 dilution for the Microtox test® (with Microtox® diluent) and a 1:1000 dilution for the *T. battagliai* test (with natural seawater) which took the Maximum Allowable Concentration (MAC) of DMSO; 1 % for the Microtox® assay and 0.1 % for the *T. battagliai* assay, into account respectively. These MACs have previously been reported by Macken (2008) to show no discernible toxicity in both assays. The total crude extracts were then assayed with both species in at least triplicate in three independent experiments. The Dunmore East extract was also assayed on two cell lines namely RTG-2 and PLHC-1 (submitted manuscript) which is detailed in chapter 4.

7.2.3 Preparation of extract for chemical analysis

The analytical method was based on a method by Strand et al. (2003) with the following modifications. The hexane layer was transferred to methanol, the pH was adjusted to 5.0 ± 0.5 with 10 % sodium acetate and 20 % sodium hydroxide in deionised water and ethylation of OTCs completed using a 10 % w/v solution of sodium tetra ethylborate (STEB) in methanol. The ethylated OTCs were then back extracted into hexane and the organic extract was cleaned up with sodium sulphate and 5% water deactivated alumina with sulphur being removed from the extract using tetrabutylammonium sulphite. Tetrapropyltin was then added to the extract in the gas chromatography (GC) vial as an injection correction standard and the sample extract was analysed using GC-PFPD. No internal standard was employed during the course of this study as suitable compounds *e.g.* tripropyltin chloride have been previously found to be toxic to biological organisms at very low levels (Brüschweiler et al., 1995). The freshwater sediment certified reference material BCR646 was analysed with every batch to further ensure compliance with internal quality

control procedures. A diagram outlining the production of the crude organic extract for bioassay testing and chemical analysis is presented in Figure 7.2.

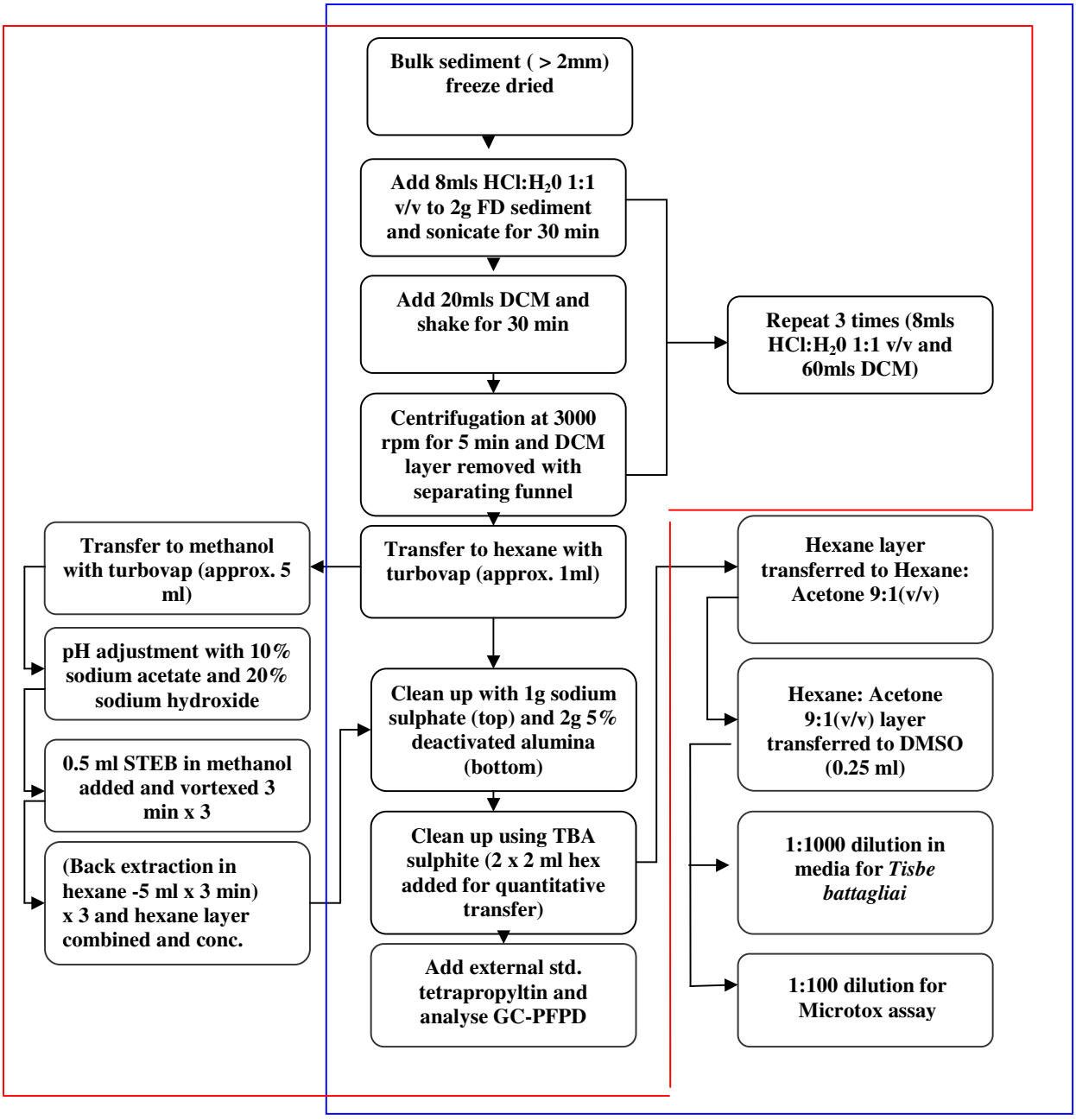


Figure 7.2: Diagram outlining the production of a crude organic extract for bioassay testing and chemical analysis. Colours distinguish between analytical and bioassay methodology blue indicating bioassay testing and red indicating analytical methodology.

7.2.4 Fractionation for bioassay testing and chemical analysis

On the basis of overall toxicity profiles observed on all assays including the RTG-2, PLHC-1 and OTCs concentration levels, further fractionation was conducted on the Dunmore East sediment extract. This sediment extract was fractionated according to Figure 7.3. The total crude solvent extract in DMSO (500 µl) which was remaining from the cell line assays (see chapter 4) was mixed with 3 g of 5 % deactivated alumina with water and placed in a thistle column (0.5 g sodium sulphate was placed at the bottom of the column). The extract was then eluted with solvents of increasing polarity into five primary fractions (fraction 1, 18 ml of *n*-hexane; fraction 2; 67.5 ml *n*-hexane/dichloromethane [95:5 v/v] fraction 3, 67.5 ml of dichloromethane, fraction 4, 70 ml of methanol/acetic acid [99:1 v/v]; fraction 5, 70 ml methanol). The elution of compounds with this procedure has previously been reported by Grote (2005).

Each of the fractions (1-5) was divided into three separate aliquots the first of which was used for tests with *T. battagliai*, the second in the Microtox® assay and the third fraction was used for chemical analysis (Figure 7.3). Calculation of quantities of sediment to be extracted for each bioassay was based on EC_{50} values of TBT-Cl obtained by Macken (2008) and extraction efficiencies of the various butyltin compounds from the OTC analytical method (see chapter 3). The solvent extracts for bioassay testing were solvent exchanged to DMSO (250 µl) from the original solvent prior to assaying. Fractionation of the Dunmore East total crude extract was not conducted for assaying with the RTG-2 and PLHC-1 cell lines as both the *T. battagliai*, and Microtox® assays were determined to be more sensitive to TBT-Cl than both cell lines as discussed later in this chapter.

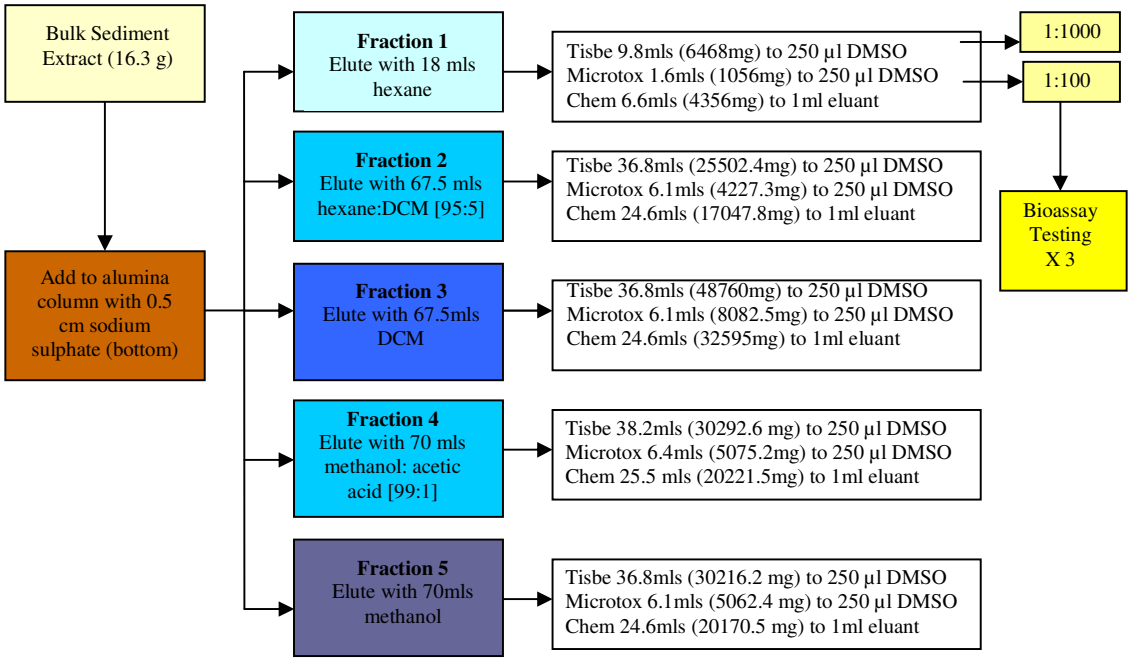


Figure 7.3: Fractionation procedure for the separation of organic compounds with increasing polarity fraction 1 (least polar) to fraction 5 (most polar).

7.2.5 Chemical analysis

Each of the five chemical analysis fractions were further divided into two aliquots. One half of each extract was transferred to methanol from the original solvent for OTC analysis. The pH of each methanolic extract was adjusted to 5.0 ± 0.5 with 10 % sodium acetate and 20 % sodium hydroxide in deionised water and ethylation of organotins completed using a 10 % w/v solution of sodium tetraethylborate in methanol. The ethylated organotins were then back extracted into hexane using a multitube vortexer and each fraction was concentrated down in hexane using a turbovap concentrator. This extract was analysed for organotins using a gas chromatograph coupled with a Pulsed Flame Photometric Detector (GC-PFPD). The other half of each of the fractions 1 – 3 in their original solvent were analysed with Gas Chromatography-Electron Capture Detection (GC-ECD) for analysis of chlorinated compounds such as polychlorinated biphenyls (PCBs) and organochlorine

compounds (OCs), GC coupled with a Flame Ionisation Detector (GC-FID) for the detection of hydrocarbons and oil residues and GC coupled with a Mass Spectrometer (GC-MS) using selected ion monitoring (SIM) for polycyclic aromatic hydrocarbon (PAH) identification were also conducted. The various instrument parameters used for analysis of the fractions are outlined in Table 7.1 below. This analysis was conducted in-house at the Marine Institute, Galway, Ireland. The last two fractions (fractions 4 and 5) are methanolic and therefore could not be analysed using gas chromatography techniques and were to be analysed using High Performance Liquid Chromatography (HPLC) however due to time limitations and the fact that the blank extracts demonstrated high toxicity to the Microtox® assay, this analysis was not performed and currently out of the scope of this thesis. Figure 7.4 summarises the chemical analysis conducted on the three solvent extracts.

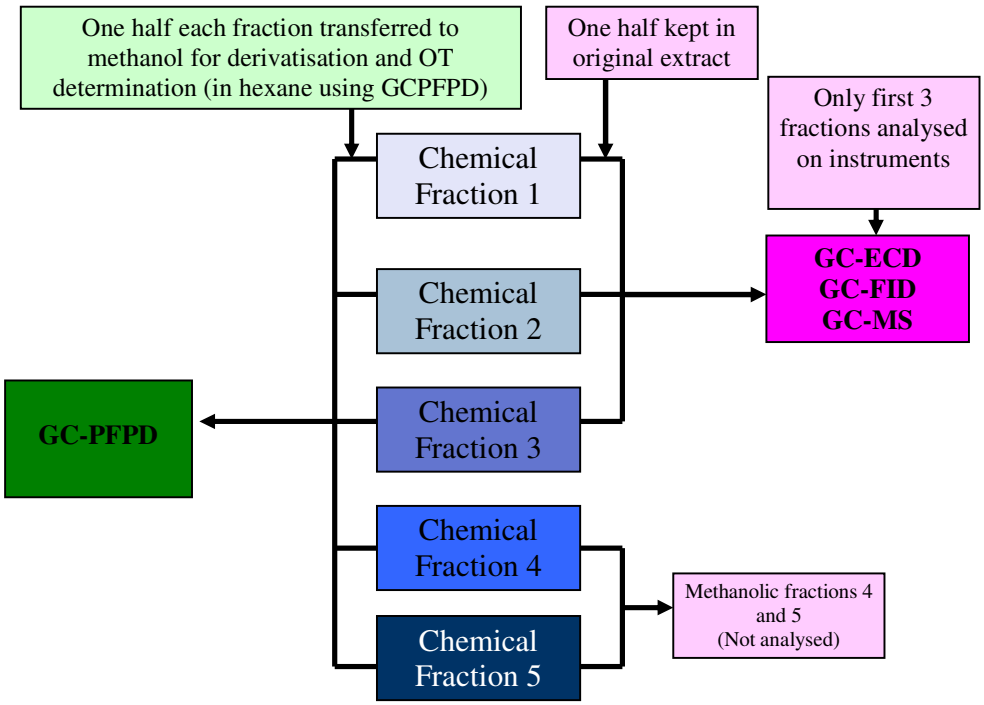


Figure 7.4: Schematic diagram of chemical analysis of solvent extract fractions (1 – 5).

Table 7.1: Instrument parameters for analysis of crude and fractionated contaminant extracts

Instrument		GC-FID	GC-ECD (Front)	GC-ECD (Middle)	GC-PFPD	GC-MS (PAH)
Analytical separation	Column	CPSIL5CB Column	HT8	CPSIL19CB	ZB-1	DB5-MS
	Length (m)	25	50	60	30	60
	Internal diameter (mm)	0.32	0.22	0.25	0.32	0.32
	Film thickness (µm)	0.12	0.25	0.25	0.25	0.25
	Carrier Gas	Helium	Hydrogen	Hydrogen	Helium	Helium
	Make up Gas	Hydrogen/Air	Nitrogen	Nitrogen	Hydrogen/Air	
	Column flow (ml/min)	1.0		1.3	2.0	
Injection parameters	Injection mode	User defined			Pressure pulse	Std on column
	Temperature (°C)	300	240	240	240	300
	Temperature hold time (min)	5	9	9		52
	Liner type	double gooseneck	single gooseneck	single gooseneck		single gooseneck
	Injection volume (µl)				1	1
Detector parameters	Detector temperature (°C)	300	280	280	300	
	Make up flow (ml/min)	25	25	25	22	
	Hydrogen flow (ml/min)	30			25	
	Source temperature (°C)					
	Air flow (ml/min)	325			30	
Temperature programmes	Initial temperature (°C)	60	80	80	70	50
	Initial time (mins)	1	1	1	3	1
	Ramp 1 (°C)	5	45	45	5	20
	Final temperature (°C)	300	180	180	220	100
	Hold time (mins)	20	0	0	7	0
	Ramp 2 (°C)		2	2		10
	Final temperature (°C)		235	235		220
	Hold time (mins)		0	0		0
	Ramp 3 (°C)		5	5		3
	Final temperature (°C)		275	275		280
	Hold time (mins)		20	20		0
	Ramp 4 (°C)					2.5
	Final temperature (°C)					320
	Hold time (mins)					
	Total time (mins)	69.0	58.7	58.7	40.0	53.5
External standard		N/A	PCB 112	PCB 112	Tetrapropyltin	N/A

7.2.6 Biological assays

For the identification of active toxicants in the solvent extracts, two bioassays were employed, the Microtox® acute assay and the acute mortality assay with *T. battagliai*. All extracts were tested in a geometric dilution series (according to Macken et al. 2008) with a final DMSO concentration of no greater than 1 % for the Microtox® and 0.1 % for the *T. battagliai* assay. For the blank assaying of the fractionated extracts only Microtox® blank data is described however future work will include blank assaying with *Tisbe battagliai* for future manuscript submission. All experiments were conducted in triplicate (Microtox®) or quadruplicate (*T. battalgiai*) and all testing was conducted in three independent experiments.

7.2.6.1 Microtox® assay

Lyophilised *Vibrio fischeri* bacteria (NRRL B-11177) and all Microtox® reagents were obtained from SDI Europe, Hampshire, UK. Phenol was used as a reference chemical and a basic test for phenol was run for every fresh vial of bacteria to ensure the validity of all tests. Each of the three solvent extracts was diluted 1:100 with Microtox® diluent in order to achieve ≤ 1 % DMSO at all concentrations. Sample blanks which underwent the same extraction process as the samples were also tested in parallel with each sample.

The 90 % test for aqueous extracts (Azur Environmental Ltd, 1998) was selected as it is the method validated by our laboratory for the assessment of other environmental samples (e.g. porewater and elutriate samples). Percentage inhibition after 5, 15 and 30 min was recorded for each extract and respective EC_{10} and EC_{50} values were calculated.

7.2.6.2 Tisbe battagliai assay

Toxicity testing using *T. battagliai* was performed according to the International Standard ISO/DIS 14669 (1997), with slight modifications. Each of the five solvent extracts were diluted 1:1000 with natural seawater in order to achieve ≤ 0.1 % DMSO at all concentrations. Organisms were exposed to a range of solvent extract concentrations (20, 40, 60, 80 and 100 %) using natural seawater as a diluent. The same concentration range was prepared for each fraction (1 -5). Survival was recorded after 24 h and 48 h and the lethal concentration at which 10 % and 50 % of the experimental population died (LC_{10} and LC_{50}) was calculated for each timepoint. A positive control using the reference chemical potassium dichromate was run alongside tests in order to verify the sensitivity of the copepods, results of which have previously been reported in the PhD thesis (Macken, 2007) and negative controls containing only natural seawater were also run concurrently.

7.3 Statistical analysis

All data are expressed as arithmetic mean ± standard error of the mean (SEM). The acute toxicity data for the Microtox® assays was analysed using MicrotoxOmni® software (SDI Europe, Hampshire, UK). Toxicity data were fitted to a sigmoidal curve and the Hill model was used to calculate Effective Concentration (EC) and Lethal Concentration (LC) values. This analysis was performed using REGTOX-EV6.xls (Èric Vindimian http://eric.vindimian.9online.fr/), a curve fitting macro for Microsoft® Excel. Statistical analyses were carried out using a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. These analyses were performed using MINITAB® release 14 (MINITAB Inc. PA, USA). Statistical significance was accepted at $p \leq 0.05$. Percentage

inhibition data generated by the MicrotoxOmni® software were Arcsin transformed prior to statistical analysis to improve normality and homogeneity of variances and reduce the influence of outliers.

7.4 Results

7.4.1 Results for Microtox® testing of crude extracts from three locations

The reduction in bioluminescence and corresponding EC_{10} and EC_{50} results for the total crude extract for each of the three sites, Bull Lagoon, Dublin Port and Dunmore East with the Microtox® assay is presented in Table 7.2 below. These extracts were assayed with the 90 % test for aqueous extracts and the Microtox® system. The 1:100 dilution of the stock solvent extract was used as the top concentration (90 % solvent extract). The dilution of the extract was conducted to eliminate toxicity arising from the DMSO carrier solvent. The concentrations of solvent extract (%) ranged from 0.35- 90.0 % for each site. Solvent extract blanks were prepared in a similar manner (1:100) and were found to have no significant toxicity from the extraction process or the carrier solvent. The Bull Lagoon extract was found to be the least toxic extract while the extract from the Alexandra Basin in Dublin Port was found to be the most toxic. The order of decreasing toxicity for each of the three sites was found to be Dublin Port > Dunmore East > Bull Lagoon demonstrating EC_{10} values at 30 min of 1.08, 11.6 and 26.9 % solvent extract respectively.

Table 7.2 Toxicity of solvent extracts from three Irish marine sediment sites to Microtox®

Site	Exposure time (min)	Maximum reduction in bioluminescence (%)	EC_{10}[a]	EC_{50}[b]
Bull Lagoon (DB6)	5	26.9	34.4 (3.05-54.6)	> 100 %
	15	28.1	29.3 (2.97-56.9)	> 100 %
	30	28.3	26.9 (2.89-48.0)	> 100 %
Dunmore East (DE1)	5	48.9	11.1 (6.47-38.0)	> 100 %
	15	49.1	9.98 (4.03-29.9)	> 100 %
	30	44.8	11.6 (3.53-36.3)	> 100 %
Alexandra Basin (DB1)	5	52.9	1.78 (0.86-4.48)	> 100 %
	15	51.8	2.60 (1.29-7.19)	> 100 %
	30	48.6	1.08 (0.40-3.07)	> 100 %

[a] EC_{10} values and corresponding 95 % confidence intervals in parentheses
[b] EC_{50} values (no confidence intervals presented)

7.4.2 Results for Tisbe battagliai assaying with crude extracts

The crude organic solvents from each of the three locations were assayed with *T. battagliai* results of which are further described. The crude solvent extract blank was not found to elicit any significant toxic effect to these organisms. The Bull Lagoon extract was found to be the least toxic with no significant toxicity being observed after 48 h. The Dunmore East was the next most toxic extract with 24 h and 48 h EC_{10} values of 65.9 (95% CI=44.0–78.8) % and 19.1 (4.2–34.7) % extract respectively. The Dublin Port solvent extract was the most toxic with EC_{10} values of 46.7 (23.5–70.0) % and 13.0 (5.2–22.9) % after 24 h and 48 h exposure respectively. The Dublin port extract was the only extract from which EC_{50} values could accurately be derived. Calculated values for this site were 86.3 (74.7–110.6) % and 46.6 (37.1–57.1) % of solvent extract after 24 h and 48 h respectively.

7.4.3 Chemical analysis results for each of the three locations

Chemical analysis results including metals and organics for the < 2 mm fraction of the sediment samples from the three locations are presented in Table 7.3. It should be noted that these concentrations are an overall representation of the chemicals present in sediment samples from the three locations however cannot be deemed representative of the chemicals that may be present in the bioassay solvent extract. The presence of various organic contaminants contained in the crude contaminant and blank extracts is presented in Table 7.4 and Figure 7.5. The extraction used in this study involved leaching with acid and therefore the solvent extract could potentially contain metals extracted from the sediment but metals were not quantified in the solvent extract in this study.

Figure 7.5 presents chromatograms obtained for the solvent extract and blank samples from each of the instruments including GC-PFPD which is organotin specific, GC-ECD which is extremely sensitive to chlorinated compounds and GC-FID which is very useful for hydrocarbon profiling. For GC-PFPD and GC-ECD, injection correction standards were introduced prior to analysis and for GCFID, a number of reference oils and an n-alkane mix (C_{10}-C_{40}) were analysed with the samples.

It is demonstrated from previous chemical results (see Table 7.3) and the organotin specific GC-PFPD chromatogram, that the Dunmore East total crude extract contained an elevated level of organotin contamination (in particular TBT which is one of the most toxic OTCs) but also had another distinct unknown tin peak present in the chromatogram. The Dunmore East extract also contained various chlorinated compounds and showed a distinct hydrocarbon profile containing long chain hydrocarbons C_{20}-C_{40} (see Table 7.4 and Figure 7.5). The Dublin Port extract was observed to have a variety of contaminants present

including the presence of OTCs, some chlorinated compounds and demonstrated a "petroleum like" profile with GC-FID analysis. The Bull Lagoon was observed to be the least contaminated showing some chlorinated compounds which could potentially be due to method contamination as these compounds were also observed in the blank extract (see Table 7.4) and demonstrated to have the lowest level of TBT however there was a distinct unknown tin peak present which was approximately 180 times greater than the injection correction standard peak tetrapropyltin which was at an approximate concentration of 300 ng g^{-1}.

It was concluded that the fractionation procedure would be conducted on the Dunmore East extract on the basis that this extract contained the most elevated level of the toxic compound TBT in addition to having significant levels of other pollutants present. The quantity of sediment to be extracted would be calculated from results obtained from a model compound study (Macken et al., 2007). The EC_{50} obtained from the model compound study for TBT was used to determine the number of grams of sediment to be extracted for both the Microtox® and *Tisbe battagliai* tests.

Table 7.3: Concentrations of metals and organics of the < 2 mm sediment fraction for the crude solvent extracts of the three sampling locations and Irish Sediment Quality Guideline upper and lower levels

Contaminant	Bull Lagoon	Dunmore East	Alexandra Basin	Proposed lower level[a]	Proposed upper level[a]
Metals (mg kg $^{-1}$ dry weight)					
Mercury	0.08	< 0.05	0.28	0.2	0.7
Aluminium	20300	12800	19200	-	-
Lithium	36.6	29.4	33.7	-	-
Arsenic	15.4	7.05	11.7	9.0	70.0
Cadmium	0.37	0.48	3.23	0.7	4.2
Chromium	55.5	41.7	41.7	120	370
Copper	35.3	76.8	78.8	40	110
Lead	68.2	45.4	265.0	60	220
Nickel	35.6	18.6	28.4	21	60
Zinc	154	242	755	160	410
Organic contaminants (µg kg $^{-1}$ dry weight)					
$\sum$ 16 - PAHs[b]	409	1006[*]	5039[†]	4000.0	-
$\sum$ 11 - PCBs[c]	33.0	36.0	36.8[†]	7.0	1260
$\sum$ 22 - OCs[d]	66.0	527[*]	279.3[†]	-	-
TBT	42.4	2125	6621		
DBT	75.9	790	1362	-	-
$\sum$ TBT &DBT	118	2915	7984	100	500
TOC (%)	0.28	1.52	3.7		

[a] Proposed Irish Sediment Quality guidance figures from the Marine Institute of Ireland (Cronin et al., 2006).
[b] ΣPAHs = Acenaphthene, acenaphthalene, anthracene, B[a]anthracene, B[a]pyrene, B[b]fluoranthene, B[ghi]perylene, B[k]fluoranthene[*], chrysene, DiB[ah]anthracene, fluoranthene, fluorene, indeno123cdPyrene, naphthalene[*†], phenanthrene, pyrene.
[c] Σ = Congeners 28[†], 52[†], 101, 106, 118, 128, 138, 153, 156, 170, 180.
[d] Σ = 1,2,3-Trichlorobenzene, 1,2,4-Trichlorobenzene[*†], 1,3,5-Trichlorobenzene, aldrin[*†], dieldrin[†], endrin[†], isodrin, cis-, trans-chlordane, o,p′-DDE, DDT[*†], p,p′-DDE, DDT[*†], TDE[†], Endosulphan A[†] and B[*†], HCB, HCBD, α-[*†], β-, γ-, δ-HCH[†].
[*] Raised LOD due to ion interference in Dunmore East sample.
[†] Raised LOD due to ion interference in Alexandra Basin sample.

Table 7.4: Chemicals observed to be present in crude extracts of BL, DP and DE and fractionated DE extract. Blank extract chemicals are also presented.

Site		Crude BL	Crude DP	Crude DE	Crude Blank	FR1 DE	FR2 DE	FR3 DE	FR1-3 BLANK
PCBs									
	PCB28		✓			✓			
	PCB 52	✓		✓	✓	✓	✓		
	PCB138	✓	✓	✓		✓			
	PCB 101		✓	✓	✓	✓	✓		
	PCB 118		✓	✓		✓			
	PCB 153		✓	✓		✓			
	PCB 105		✓	✓	✓	✓	✓		
	PCB156		✓	✓		✓			
	PCB180		✓	✓		✓			
OCs									
	a-HCH	✓	✓	✓	✓	✓	✓		
	b-HCH		✓						
	HCB	✓	✓	✓	✓	✓			
	lindane	✓	✓		✓				
	pp-ddd	✓	✓	✓	✓	✓			
	pp-dde	✓	✓	✓	✓	✓			
	pp-ddt		✓	✓		✓			
	dieldrin	✓	✓	✓	✓	✓	✓		
	trans -nonachlor	✓	✓	✓		✓			
HCs									
		Low level	Petroleum profile	C20-C40	Low level	C20-C40	Low level	C27-C40	low level
OTs									
	TBT	✓	✓	✓		✓	✓	✓	
	DBT		✓	✓		✓			
	MBT		✓	✓					
	Unknown peaks	Unknown OT peak*	Unknown peak**	Unknown OT peak*					
PAHs	Naphthalene					✓	✓		
	Acenaphthene					✓			
	Fluorene					✓			
	Phenanthrene					✓			
	Pyrene					✓			
	Fluoranthene					✓			

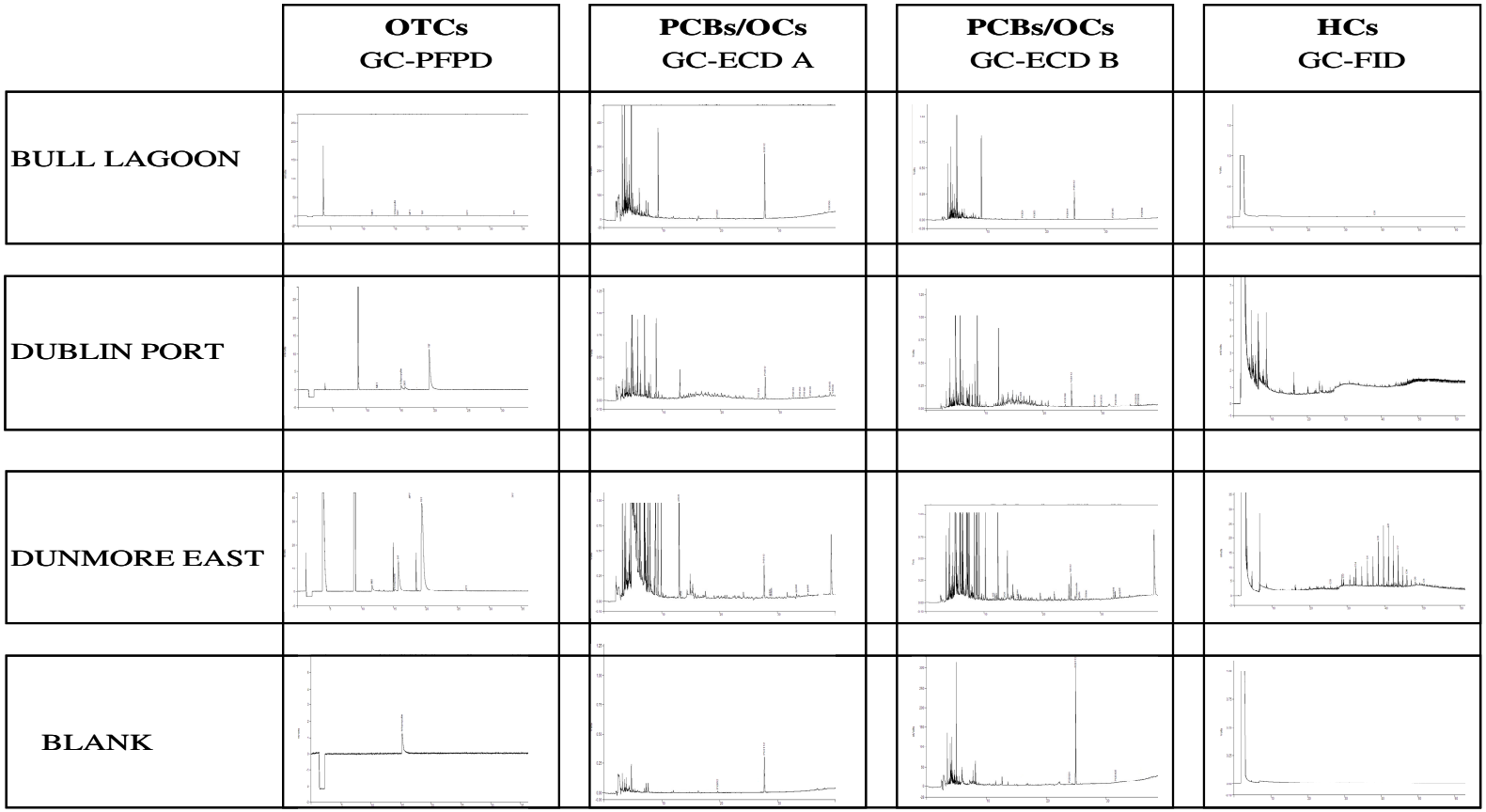


Figure 7.5: Chromatograms obtained using various instrumental techniques for each of the three crude extracts and method blank. Note: extracts were analysed on a dual column (A and B) with GC-ECD.

7.4.4 Results for Microtox® testing and Dunmore East fractionated extracts

Results of the Microtox® assay for all five Dunmore East fractions are presented in Table 7.5 and Figure 7.6. No significant toxicity was observed for any of the blank fractions prepared alongside each of the test fractions (1-3). The blanks extracts produced for Fraction 4 and 5 were both observed to cause significant toxicity in both bioassays therefore the toxicity elicited by the actual fractions cannot be attributed solely to the contaminants extracted in these fractions, therefore the results and discussion will focus primarily on fractions 1-3. Therefore, fractions 4 and 5 are not further discussed. Fraction 1 was found to be the most toxic with an EC_{10} of 0.15 % and an EC_{50} of 16.8 % solvent extract after 30 min, while fractions 2 and 3 are not significantly toxic at any concentration. Employing calculated ecotoxicity values (EC_{10} and EC_{50}) a sensitivity ranking order for all fractions to the Microtox® system is as follows: Fraction 1 > Fraction 3 ≥ Fraction 2. The toxicity of Fraction 1 is observed to increase with time which correlates with a previous study where TBT-Cl was tested using the Microtox® assay in a model compound study (Macken et al., 2007).

Table 7.5: Results of the Microtox® assay and five organic solvent fractions from Dunmore East sample.

Fraction	Time (min)	EC_{10}[a] (%)	EC_{50}[a] (%)	NOEC[b] (%)	LOEC[c] (%)
1	5	0.15 (0.04 – 0.38)	46.7 (31.8 – 89.3)	2.81	5.62
	15	0.10 (0.04 – 0.38)	21.6 (15.8 – 29.9)	0.35	0.70
	30	0.15 (0.03 – 0.60)	16.8 (11.4 – 29.5)	2.81	5.62
2	5	NST[d]	NST	> 100	> 100
	15	NST	NST	> 100	> 100
	30	NST	NST	> 100	> 100
3	5	NST	NST	> 100	> 100
	15	NST	NST	> 100	> 100
	30	NST	NST	> 100	> 100
4	5	0.71 (0.35 – 1.88)	2.28 (1005 – 4.36)	1.41	2.81
	15	0.92 (0.48 – 2.38)	2.05 (1.97 – 4.02)	1.41	2.81
	30	1.55 (0.46 – 4.81)	3.48 (2.67 – 8.70)	2.81	5.62
5	5	0.77 (0.28 – 1.17)	10.1 (6.73 – 13.3)	2.81	5.62
	15	1.47 (0.61 – 2.81)	10.3 (7.01 – 13.3)	5.62	11.3
	30	2.10 (0.76 – 3.57)	10.3 (6.48 – 12.5)	5.62	11.3

[a] EC_{50} values and corresponding 95 % confidence intervals in parentheses
[b]NOEC, no observed effect concentration, the highest observed concentration at which no significant effect ($p \leq 0.05$) was detected
[c]LOEC, lowest observed effect concentration, the lowest concentration of the tested concentration at which a significant ($p \leq 0.05$) effect was detected.
[d]NST, No Significant Toxicity

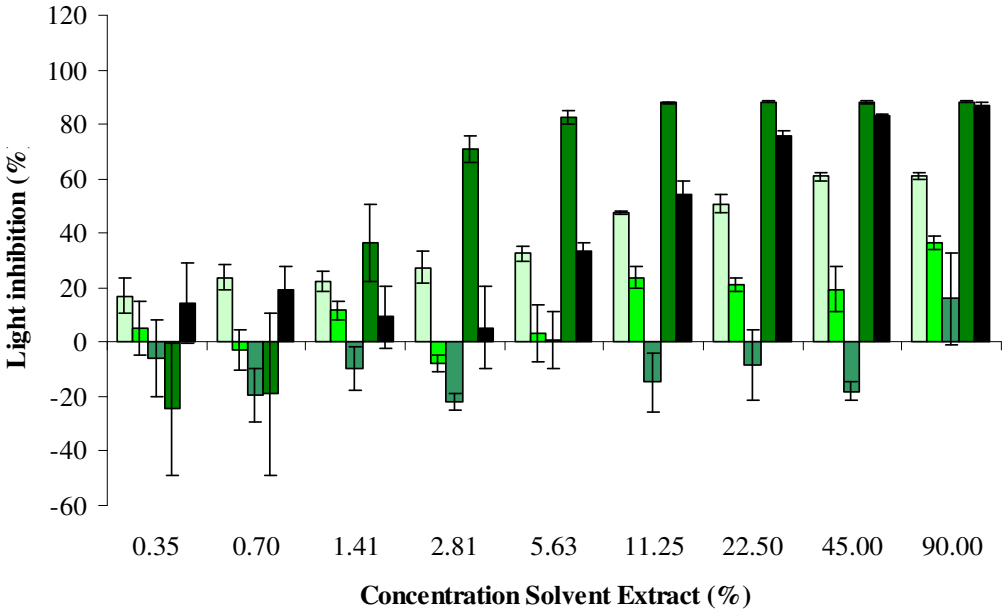


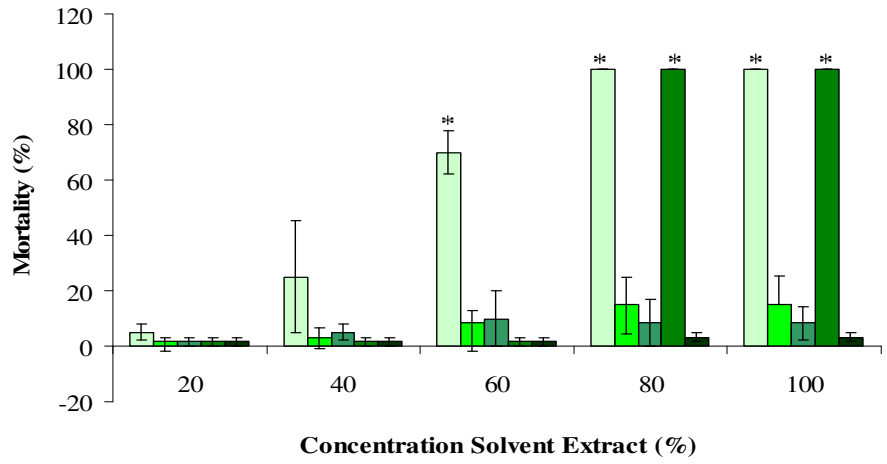
Figure 7.6: Microtox® assay results showing percentage light inhibition from 5 solvent extract fractions; Fraction 1 [hexane (100 %) exchanged to DMSO] (□), Fraction 2 [hexane:DCM (95:5 %) exchanged to DMSO] (■), Fraction 3 [DCM (100 %) exchanged to DMSO] (■), Fraction 4 [methanol: acetic acid (99:1 %) exchanged to DMSO] (■) and Fraction 5 [methanol (100 %) exchanged to DMSO] (■) from Dunmore East after 15 mins exposure. Data are expressed as a percentage of unexposed controls ± SEM of three independent experiments. * denotes significance from the control ($p \leq 0.05$).

7.4.5 Results for T. battagliai testing and Dunmore East fractionated extracts

The results of the *T. battagliai* assay and five solvent fractions are presented in Figure 7.7 and Table 7.6. As with the Microtox® test no significant toxicity was determined with any of the test blanks prepared alongside each fraction (1-3). From the results presented, fraction 1 was significantly toxic to *T. battagliai* at all concentrations above 60 % for 24 and 48 h. Fraction 4 was only significantly toxic at the top two concentrations after 24 and

48 h, while fractions 2, 3 and 5 were not observed to be significantly toxic at any concentration for the time intervals assayed. The toxicity of fractions 2 and 3 appeared to increase with time. However, this was not significant. The toxicity of the fractions to *T. battagliai*, in decreasing order was as follows: Fraction 1 > Fraction 4 ≥ Fraction 2 ≥ Fraction 3 ≥ Fraction 5.

(a)



(b)

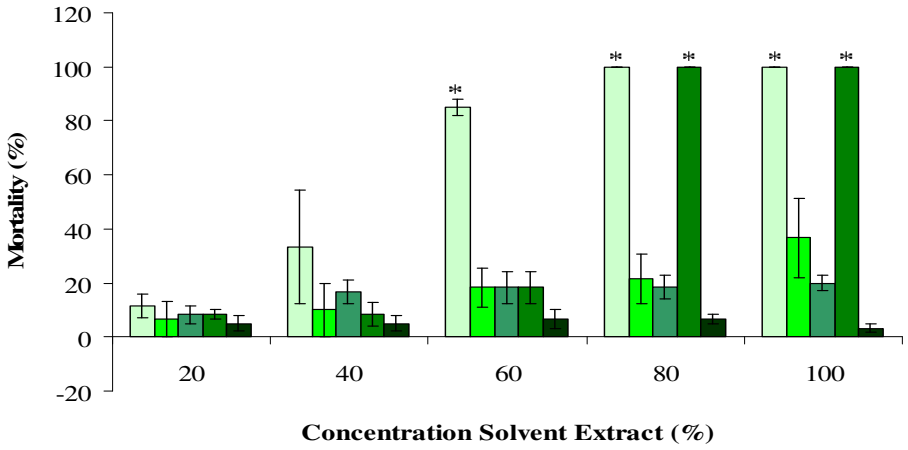


Figure 7.7: Percentage mortality of *T. battagliai* exposed to 5 organic sediment solvent extracts; Fraction 1 [hexane (100 %) exchanged to DMSO] (□), Fraction 2 [hexane:DCM (95:5 %) exchanged to DMSO] (□), Fraction 3 [DCM (100 %) exchanged to DMSO] (□), Fraction 4 [methanol: acetic acid (99:1 %) exchanged to DMSO] (□) and Fraction 5 [methanol (100 %) exchanged to DMSO] (□) from Dunmore East after (a) 24, and (b) 48 h exposure. Data are expressed as a percentage of unexposed controls ± SEM of three independent experiments. * denotes significance from the control ($p \leq 0.05$).

Table 7.6: Ecotoxicity results of the *Tisbe battagliai* assay and five solvent fractions from an original Dunmore East total solvent extract.

Fraction	Time (h)	EC_{10}[a] (%)	EC_{50}[a] (%)	NOEC[b] (%)	LOEC[c] (%)
1	24	33.3 (24.4 – 41.2)	49.6 (42.2 – 53.9)	40	60
	48	30.9 (20.1 – 38.2)	44.7 (37.1 – 48.0)	40	60
2	24	68.1 (20.6 – 115)	NA[d]	> 100	> 100
	48	39.6 (7.76 – 64.6)	NA	> 100	> 100
3	24	NA	NA	> 100	> 100
	48	17.8 (0.19 – 35.1)	NA	> 100	> 100
4	24	62.2 (60.7 – 63.4)	64.9 (63.7 – 66.1)	60	80
	48	58.84 (57.4 – 59.1)	62.5 (61.6 – 62.7)	60	80
5	24	NA	NA	> 100	> 100
	48	NA	NA	> 100	> 100

[a] EC_{50} values and corresponding 95 % confidence intervals in parentheses
[b]NOEC, no observed effect concentration, the highest observed concentration at which no significant effect ($p \leq 0.05$) was detected
[c]LOEC, lowest observed effect concentration, the lowest concentration of the tested concentration at which a significant ($p \leq 0.05$) effect was detected.
[d]NA = Not Applicable.

According to reported EC_{50} values for the fractions and the two assays, the Microtox® system was more sensitive. For example, fraction 1 was observed to be toxic in both assays, however, the 24 h EC_{50} value reported for *T. battagliai* (49.6 %) was greater than the 30 min EC_{50} value reported for the Microtox® system (16.8 %).

7.4.6 Chemical analysis results of fractionated extracts

According to the chemical analysis results for the first three fractions (Figure 7.8 and Table 7.4), fraction 1 contained approximately 100 times more TBT than fraction 2 and approximately 28 times more TBT than fraction 3. Fraction 1 also contained detectable levels of DBT with the ratio being 36:1 TBT: DBT found in the derivatised extract using

GC-PFPD. Trace levels of various polychlorinated biphenyls (PCBs) and organochlorines (OCs) were found in the underivatised extract fraction when analysed using a dual column GC-ECD system (see Table 7.4). A hydrocarbon profile was also detected which showed similar characteristics to that of a spilt crude oil containing hydrocarbons C_{20}-C_{40} in a "hump" like profile. Fraction 2 contained trace amounts of DBT and some TBT however to a much lesser extent than fraction 1. Some PCBs and OC compounds were observed and a very low level hydrocarbon profile. In fraction 3 a quantity of TBT in addition to an unknown OTC peak was observed which was previously demonstrated in the total crude contaminant Dunmore East extract chromatogram (see Figure 7.8). No chlorinated compounds were detected in fraction 3 however a hydrocarbon profile was detected containing C_{27}-C_{40} hydrocarbons in a long chain hydrocarbon profile which differentiated from the hydrocarbon profile found in fraction 1. Blanks for each of the fractions (1-3) showed little by the way of contaminant levels (see Table 7.4).

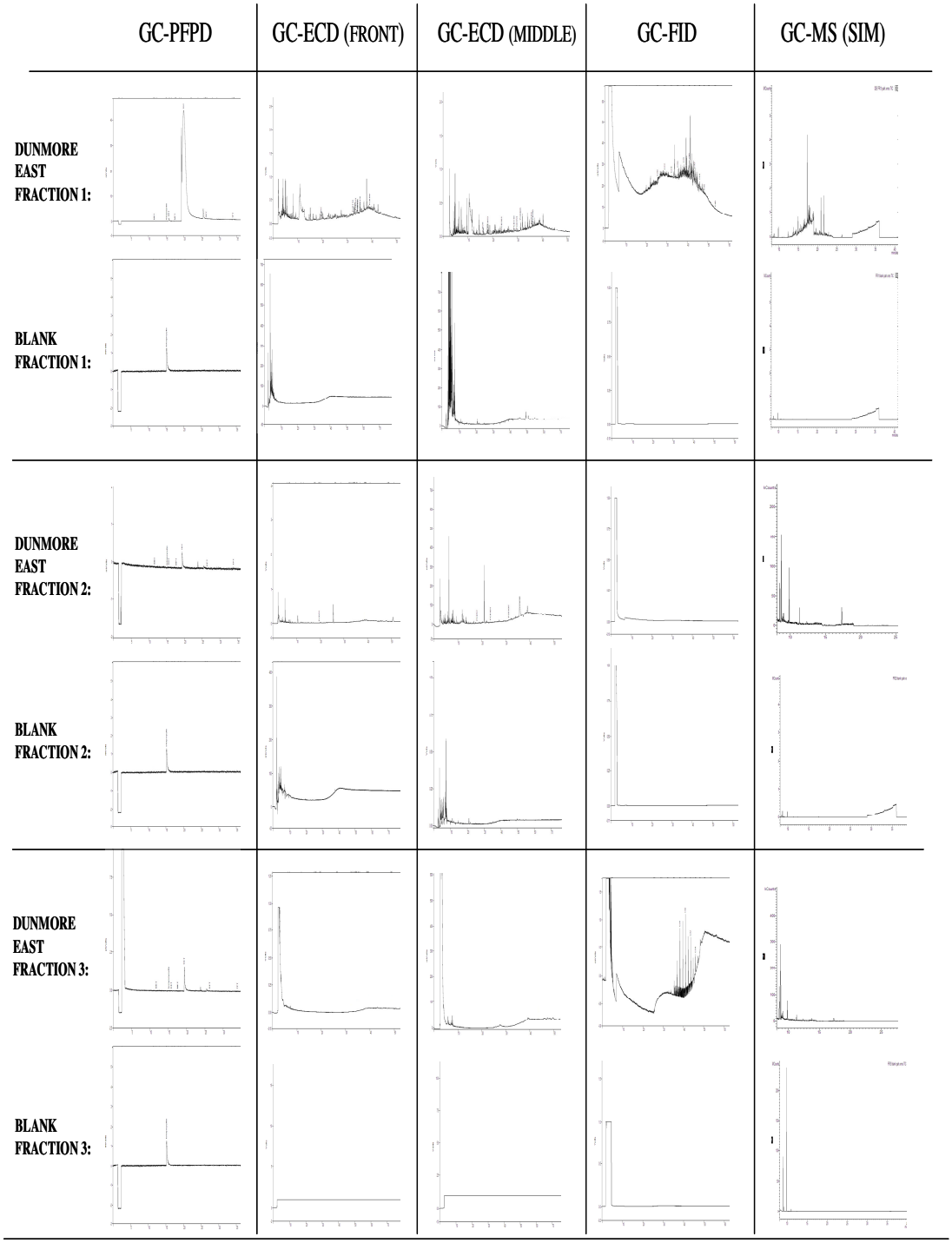


Figure 7.8: Chromatograms of the three fractions obtained from the fractionated Dunmore East sample and blanks.

7.5 Discussion

Bull Lagoon (DB6), according to the Microtox® crude solvent extract results, was the least toxic site. This result correlates with the chemical analysis of the bulk sediment (> 2 mm) as DB6 had the lowest total concentration for all organic contaminants (PAHs, PCBs, OCs and OTCs) (see Table 7.3). The chemical analysis results of the solvent extract (see Table 7.4 and Figure 7.5) also supports the low toxicity whereby only slight levels of chlorinated compounds were detected and a low level hydrocarbon profile was observed. However an unknown OTC was observed using GC-PFPD analysis. The toxicity of this extract was observed to increase with time suggestive of delayed response exposure to metals in the extract (Azur Environment Ltd., 1998). The levels of aluminium, lithium, arsenic, chromium and nickel were higher than for any other sites but were within the proposed Irish sediment quality guideline levels. Low level of organotins was detected at this site relative to the other sites (see Table 7.3).

The Dublin Port site (inner West Alexandra Basin) was the most toxic crude extract and toxicity was observed to decrease with time (Table 7.2) with the Microtox® assay indicative of organic contaminants such as PAHs where the sum of 19 PAHs at this site (5755 µg kg^{-1}) was found to exceed the proposed lower level Irish Sediment Guideline value (4000 µg kg^{-1}). The chemical analysis of this extract was observed to include many chlorinated compounds in addition to a "petroleum like" hydrocarbon profile using GCFID analysis. Macken et al. (2008) observed a similar time-dependent decrease in light levels with *V. fischeri* following exposure to the PAHs benzo[a]pyrene and fluoranthene. However, no definitive conclusions on the causative agents can be made as synergistic, antagonistic or additive effects of these contaminants are unknown.

Macken et al. (2008) determined that the Microtox® and *Tisbe battagliai* assays were sensitive to the chemical TBT-Cl with EC_{50}'s of 0.06 µM after 30 min for the Microtox® assay and 0.07 µM after 48 h with the *Tisbe battagliai* assay in comparison to a study conducted in this thesis (chapter 4, submitted manuscript) with RTG-2 and PLHC-1 cell lines where the EC_{50}'s for these cell lines and the compound TBT-Cl were determined to be 0.94 µM after 96 h exposure with the RTG-2 cell line and 0.99 µM after 24 h exposure with the PLHC-1 cell line. Therefore it was concluded that the Microtox® and *Tisbe battagliai* assays would be used for testing the fractionated extracts.

Despite the fact that the Dunmore East sediment was not shown to be as toxic as the Dublin Port, the crude extract was observed to contain many chlorinated compounds also and showed elevated levels of OTCs compared to the Dublin Port extract. A hydrocarbon profile was also evident and it can be observed that a number of long chain hydrocarbons C_{20}-C_{38} are present in the sample. These hydrocarbons have a similar profile to that of the spilt/degraded crude oil which can be seen in Figure 7.5. In the DE1 sample dominating n-alkanes are observed from C_{28}-C_{32}. The dominating odd carbon n-alkanes C_{29} and C_{31} could potentially be an indication that these hydrocarbons originate from biogenic processes. A similar study has previously investigated the use of hydrocarbon profiles to investigate point sources of PAHs (Webster et al., 2001).

The most toxic crude solvent extract to *T. battagliai* was the Dublin Port extract and the least toxic was from Bull Lagoon. For all three sites toxicity was observed to increase with time. Results from previous studies with *T. battagliai* and organic contaminants (Macken et al., 2008) showed an increase in toxicity after exposure to organotins (tributyltin, triphenyltin), PAHs (benzo[a] pyrene, fluoranthene) and a PCB (PCB-153). As the

analysed solvent extract was a crude contaminant extract this observation is not surprising. This is in contrast to the Microtox® assay, where an increase in toxicity was only observed after exposure to the organotins.

For the crude contaminant solvent extract it was shown that *T. battagliai* was more sensitive than the Microtox® assay. In contrast the results of the bioassays on the fractionated extracts differ in relation to species sensitivity. According to these results *T. battagliai* is less sensitive to fraction 1 than the Microtox® assay. Fractions 2 and 3 did not elicit a toxic effect in either assay. In a model compound study conducted by Macken et al. (2008), it has been observed that Microtox® is less sensitive than *T. battagliai* to a variety of organic contaminants. Interestingly it was also shown in chapter 4 (submitted manuscript) of this thesis that the PLHC-1 cell line was more sensitive than the RTG-2 cell line to the Dunmore East crude extract with EC_{50}'s of 64.0 and 73.2 mg sediment/ml media demonstrated for PLHC-1 and RTG-2 cell lines respectively after 24 h exposure with the neutral red (NR) assay. Therefore it can be concluded that for the sediment investigated here that the effects of mixture toxicity of organic extracts and also potentially co-extraction of metals may potentially play an important role. However, without full chemical analysis of the fractions no definite conclusions can be made.

Kammann et al. (2005) concluded in their integrated study that despite sophisticated fractionation and intensive chemical analysis, that major toxicant identification can be difficult and much of the observed effects of their study remained without explanation. They also concluded that the toxicity of their extracts was most probably from a combination of effects in the mixtures. Brack (2003) also stated that the identification of a compound in a fraction does not prove that the compound has caused the effect. Therefore

it is important to conduct confirmatory assaying at environmentally relevant levels of the compound to investigate if it could potentially be responsible for the observed effect. Although the importance of a confirmation step has been advocated throughout the literature (Brack, 2003) few studies focus on the problem of confirming the results of BDF procedures (Grote et al., 2005).

Fraction 1, which contained the majority of TBT was more toxic in the Microtox® assay than to *T. battagliai.* From previous work (Macken et al., 2008) it was observed that both assays were comparably sensitive to the organotin, 48 h EC_{50} of 0.068 (0.056 – 0.078) µmol l^{-1} for *T. battalgiai* and a 15 min EC_{50} value of 0.083 (0.078 – 0.132) µmol l^{-1} in the Microtox® assay. This further confirms that mixture toxicity could potentially be the cause of the differentiation in species sensitivity. From Figure 7.8 it is observed that Fraction 1 was found to contain a variety of compound groups in particular TBT, and showed overloading of the column with this compound indicating that this compound was the most dominant species overall. A hydrocarbon profile was demonstrated in a GC-FID chromatogram containing long chain alkanes (nC_{20}-nC_{40}) in fraction 1. These are present in this fraction in the form of a "hump". A "hump" such as this one observed is generally characterised as an unresolved complex mixture (UCM) and is generally composed of the more persistent compounds in an oil such as branched and cyclic compounds and is characteristic of petrogenic pollution. This hump is a result of when crude oils degrade and the n-alkanes are lost initially and eventually all n-alkanes disappear and the so called "hump" appears when analysed with GC-FID. This "hump" present in fraction 1 could therefore be a characteristic of petrogenic pollution and could potentially contribute to the overall toxicity observed from this extract.

Fraction 2 contained trace amounts of DBT and some TBT however to a much lesser extent than fraction 1. There were also some PCBs and OC compounds observed and a very low level hydrocarbon profile. In fraction 3 a quantity of TBT similar to that of fraction 2 was observed and there was no chlorinated compounds detected however a hydrocarbon profile was detected containing nC_{27}-C_{40} hydrocarbons in a long chain hydrocarbon profile which differentiated from the hydrocarbon profile found in fraction 1. This could potentially be the cause of fraction 3 being slightly more toxic than fraction 2 when assayed using Microtox®.

Overall, the order of toxicity of the total crude extract to the three sites was Dublin Port > Dunmore East > Bull Lagoon. For the fractionated Dunmore East extracts, the Microtox® assay was more sensitive than *Tisbe battagliai* to fraction 1 however, no significant toxicity was observed for fractions 2 and 3 with this assay. The order of toxicity of the fractionated Dunmore East extracts (1-3) to *Tisbe battagliai* were fraction 1 > fraction 3 > fraction 2. This correlated with the chemical analysis results where the order of decreasing quantities of OTCs was observed in this order also. Fraction 3 was also observed to have an unknown OTC peak in the chromatogram which could have potentially contributed to the toxicity of this extract to *Tisbe battagliai*. Fractions 1 and 3 also contained hydrocarbon profiles whereas fraction 2 did not. The potential for effects from co-extracted metals cannot be discounted. Chapter 2 reports elevated metal concentrations in Dublin Port and Dunmore East samples and these may have potentially be extracted into the bioassay solvent extracts however metals are not quantified in the solvent extracts obtained in this study and no definite conclusions can be made.

While BDF techniques described herein were inconclusive, their application is important in screening sediment for culprit compounds which could potentially be toxic to organisms. In comparison with other authors (e.g. Kammann and Brack), BDF in combination with chemical analysis can be a useful technique in deriving information related to the potential toxic nature of sediments. To the best of our knowledge, this project is the first to utilise an extraction method capable of extracting OTCs in a form suitable for ecotoxicological testing.

7.6 Conclusion

Methodology was developed for bioassay directed fractionation whereby OTCs and other anthropogenic compounds were extracted from the bulk sediment, fractionated and analysed using a variety of analytical techniques. Instrumental analysis led to the characterisation of the solvent extracts for a range of organic contaminants. The bioassay directed fractionation approach applied in this study led to the identification of the most toxic fraction isolated from the Dunmore East sediment. Bioassay-directed fractionation was successfully used to characterise compounds with a variety of polarities as contributing to the measured activity.

The need to use chemical and biological investigations in tandem to identify possible effects is further highlighted by this study as is the importance of using a battery of bioassays for any environmental assessment. When dealing with an unknown mixture (such as marine sediment) there is no way to predict the sensitivity of the assay being employed. As seen from this study a species that is considered highly sensitive (*T. battalgiai*) may in fact be less sensitive than expected when considering antagonistic, synergistic or additive effects that may be acting in a sample containing a mixture of compounds (both anthropogenic and biogenic).

The BDF methodology used during this study is suitable for the identification of toxic fractions with a demonstrated hazard in the marine environment. The benthic harpacticoid copepod, *T. battalgiai,* and the Microtox® system are particularly suitable for this sort of fractionation study as *T. battagliai* possesses a short life cycle, both assays require low sample volumes and there is minimum space, equipment and time required to conduct the assays (Hutchinson and Williams, 1998; Macken et al., 2008).

7.7 References

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8

CHAPTER 8 DETERMINATION OF AN "INTEGRATION RATIO"

8.1 Introduction

Contamination of sediment has been defined as a condition in which substances would not normally be found or where they occur above background levels (Chapman and Anderson, 2005). Sediment contamination is a global problem, however as yet there is no universal decision- making approach in place for the assessment of sediment quality.

This thesis has previously discussed the assessment methodologies used by ICES and OSPAR (background concentrations [BCs], background assessment concentrations [BACs] and environmental assessment criteria [EACs]) and the FullMonti approach which is under development in the UK and within ICES and the US-EPA assessment approach. These in addition to a further approach as proposed by Beliaeff and Burgeot (2002) are summarised below.

8.1.1 The "FullMonti" index

As previously discussed this is primarily a weight of evidence approach currently under development (primarily within the UK) for sediment assessment and its concepts are under review by international organisations such as ICES and OSPAR. This approach derives "scores" for a variety of compartments for contaminants, biological effects and biology and can combine these three measurements into an overall score which indicates the health status of the system.

The score of effect level is weighted from top to bottom with the most significant effects (i.e. reproduction, growth, survival) weighted with a value of 10. In addition, the response level is scored according to suggested intervals in the assessment criteria (green, amber and

red having values of 1, 5 and 10 respectively). An overall score for biological effects is derived by mean value of total score multiplied with weighted values. For contaminants, the scores for each pollutant are integrated into single scores for sediment, shellfish and fish tissue, which again can be integrated into one overall score for contaminants. In the end a final integrated score can be extracted based on the overall scores for contaminants, biology and biological effects.

These individual and an overall "final" score allows scientists and environmental managers to "visualize" how derived indices compare on a site specific basis. The "Fullmonti" index may in future years provide a mechanism whereby the derived indices may support legislative limits such as outlined within the Water Framework directive.

8.1.2 US-EPA assessment approaches

The US-EPA approach uses a multi-factorial assessment approach for estuarine quality including water, sediment and aspects of biota. A "traffic light" style assessment is performed for each parameter in each estuary based on assessment criteria and then combines assessments across determinands, estuaries and regions to produce national scale assessments. The approach makes use of four primary indices of estuarine condition (water quality index, sediment quality index, benthic index, and fish tissue contaminants index) in a standardised format.

The US-EPA water quality index is made up of five component indicators: dissolved inorganic nitrogen, dissolved inorganic phosphorus, chlorophyll *a*, water clarity, and dissolved oxygen. The water quality index rating is calculated for the site based on these

five component indicators. The development of indices for water quality is based on rating criteria for each sampling site.

The sediment quality index is based on three component indicators of sediment condition: direct measures of sediment toxicity, sediment contaminant concentrations, and the sediment total organic content (TOC) concentration. Chemical characterisation is performed on the sediment with chemical analysis, sediment toxicity is evaluated by measuring the survival of the marine amphipod *Ampelisca abdita* following a 10-day exposure to the sediments in the laboratory and the sediment TOC concentration is measured on a dry-weight basis. Benthic community attributes are included in this assessment of estuarine condition as an independent variable rather than as an indicator of sediment quality.

An overall condition for each region can be calculated by summing the scores for the available regional indices and dividing by the number of available indices and a final assessment on overall site status is deduced based on the number of indices found to fall within the acceptance criteria.

To conduct an assessment with either the FullMonti or US-EPA approach, a full suite of parameter data is needed. Such data would include water analysis for a suite of chemicals and benthic diversity indices measurement data, however the testing of these parameters is currently out of the scope of this thesis, therefore a subset of these parameters will be included.

8.1.3 Generation of an integrated biomarker response "IBR" type index

A further approach to integrating datasets is that as proposed by Beliaeff and Burgeot (2002) and as adopted by Damiens et al., (2007) in which a battery of biomarkers in transplanted mussels was measured and an integrated biomarker response (IBR) was calculated. A method for combining all the measured biomarkers responses into one general "stress index" was applied for each biomarker experiment.

For each biomarker: (1) calculation of mean and SD for each station was performed, (2) standardisation of data for each station: $x'_i = (x'_i - \text{mean } x)/s$, where x'_i is the standardised value of the biomarker, x_i is the mean value of a biomarker from each station, mean x is the mean of the biomarker calculated for all the stations, and s is the standard deviation calculated for the station-specific values of each biomarker. The result being variance = 1, mean = 0, (3) using standardised data, Z was computed as $+x'_i$ in the case of an activation and $-x'_i$ in the case of an inhibition, then the minimum value for all stations for each biomarker was obtained and added to Z. Finally the score B was computed as B = Z + lminl where $B \geq 0$ and lminl is the absolute value.

For all the biomarkers corresponding IBR values were therefore calculated by multiplication of the obtained value of each biomarker (Bi) with the value of the next biomarker, arranged as a set, dividing each calculation by 2 and summing-up of all values i.e. $IBR = \{[(B_1 \times B_2)/2]+[(B_2 \times B_3)/2]+\ldots[(B_{n-1} \times Bn)/2] +[(Bn \times B1)/2]\}$.

An overall indication of stress for each site can therefore be represented on the star plot which would give an indication of the quality of sediment at a particular site.

8.1.4 The Environmental Assessment Criteria concept

As previously discussed OSPAR/ICES have developed "provisional" Environmental assessment criteria (EACs) shown in Tables 8.2 and 8.3 as a basis to allow individual OSPAR contracting parties to assess the degree of toxicity of marine sediments within their region. EACs have been estimated to represent the contaminant concentration in the environment below which it can be assumed that no chronic effects will occur in marine species, including in the most sensitive species. The EACs have been modelled on predicted no effect concentrations (PNECs) as the concentration below which unacceptable effects will most likely not occur based upon the ecotoxicological data to the extent that is available for each of the contaminants concerned.

The methodology for deriving the EACs, which has been brought into line with the methodology for deriving quality standards under the Water Framework Directive involves the use of precautionary assessment factors where there is a limited availability of data. Because of limited toxicity data large assessment factors have been used in many cases to calculate EACs for water on which the EACs for sediments and biota are based. This means that many of the values are derived are precautionary and close to "background values" particularly for non-synthetic organic compounds. EACs have not been derived for some substances and matrices because of the limited quantity of ecotoxicological data for marine species. In some cases the EACs (shown in Tables 8.2 and 8.3) have been derived according to secondary poisoning as a tool for a better understanding of possible mechanisms and effects in food chains.

Due to these caveats, the EACs **should only be applied with caution for any current assessments**, **and should not be used as a trigger for source directed action without further evaluation**. The relationship between environmental assessment criteria (EACs) and background concentrations (BCs) and background assessment concentrations (BACs) is currently being considered as part of the process of developing integrated assessments of the status of hazardous substances in the OSPAR Maritime Area.

Furthermore, the environmental assessment criteria, set out below, do not:

(a) take into account specific long-term biological effects such as carcinogenicity, genotoxicity and reproductive disruption due to hormone imbalances, or;

(b) consider toxic effects resulting from combinations of substances.

The provisional EACs as described herein do however provide a basis to allow for the comparison of analytically derived sediment and mussel concentration data to contaminant levels that are currently deemed to present a toxicological risk to resident marine organisms.

The indices and EAC concept as described above all provide mechanisms that allow for the visualisation of the overall "health" of a particular site. Each of these indexing systems attempt to incorporate elements of differences in toxicity potential (e.g. in the form of weighting factors) of various chemicals and have a number of merits. The complex nature of the development of a complete indexing system (e.g. accounting for chemical toxicity, additive/synergistic effects, differences in sediment make-up and contaminant bioavailability etc.) was outside of the scope of this current work.

The primary focus of this thesis was on the development of Ireland's capacity to complete both biomarker and supporting chemical analysis tools for the assessment of the toxicity of marine sediments and to investigate the generation of an indexing system that incorporates elements of the biological and chemical work packages completed and thus forms the basis of this chapter.

This chapter describes an adaptation of the IBR concept of summing the indices derived from ranked analytical (based on the extent to which analytically derived and appropriately normalised data exceed the EAC) and biomarker data into a series of biomarker/contaminant integrated response (IR) indices. Plotting of the derived indices followed by the generation of site specific star plots allow for the visualisation of the degree of contamination/toxicity relative to each other at the selected locations.

8.2 Methods

8.2.1 Sample collection and data generation

Details on the collection of samples and the generation of analytical information relevant to IR generation are discussed throughout this thesis. Additionally, the work of Macken (2007) who completed a number of bioassay measurements on sediment porewater and sediment extracts is utilised for the generation of bioassay scores and site specific IRs

8.2.2 Generation of an IR index

For the generation of an index for sediments and biota, a ranking system (based on comparison to the EAC) has been developed. The system only allows for the comparison of toxicity between the test sites examined during the course of this work. The generation of the index requires a number of stepwise procedures to be completed.

8.2.2.1 Sediment and blue mussel data index generation

1) Sediment and mussel contaminant data must be converted to appropriately "normalised" data for comparison to the OSPAR/ICES EACs. Sediment data must be converted to 1 % organic carbon for metals assessment and for other contaminants (PCBs, TBT and PAH) to 2.5% organic carbon.

2) Calculate the extent to which analytical data exceeds the relevant EAC by dividing the normalised contaminant value by the appropriate EAC.

3) Identification of the site/sample that exceeded the EAC to the greatest degree.

4) Assign a score of 10 to the highest value and pro-rata assign scores to other sites.

5) Complete this process for all contaminants where EACs are available.

8.2.3 Biomarker/Biological effects

A score was completed for each parameter including the sediment toxicity bioassays (Macken et al., 2008) which included the PLHC-1 and RTG-2 cell line assays (see chapter

4) and from RPSI indices generated from the *in-situ* caging experiment (chapter 5). Two systems were employed as follows;

1. In bioassays where an EC_{10}/EC_{50} value of 100 % represented the lowest degree of toxicity, a score of "0" was assigned to this sample.

2. Scores were thus assigned to these assays by 10-[EC_{10}/10].

3. In mortality related bioassays where 100% mortality indicated the greatest degree of toxicity, a score of "10" would be assigned to such a sample, thus in these assays then the score would be calculated by EC_{10}/10.

8.2.4 Comparison of tissue and sediment data to EACs

For the contaminant data in tissue and sediment, EACs are used and the score for each contaminant in each site is calculated by how much it exceeded the EAC. Therefore only scores are derived for contaminants for which EACs are available.

Once comparison to the individual EACs was completed the sample/site which exceeded the EAC to the greatest degree was designated a score of "10", with other site/sample scores assigned a pro-rata score relative to the highest one.

Finally a score is created for each contaminant/effect and an overall score for the sample/site was obtained by using a method described by Damiens et al., (2007) as follows $[(B_1 \times B_2)/2]+[(B_2 \times B_3)/2]+\ldots[(B_{n-1} \times Bn)/2] +[(Bn \times B_1)/2]\}$. Star plots are then derived from data generated for each site.

8.3 Results and discussion

The development of indices, as described in this chapter provides a weight of evidence approach for the identification of potentially toxic chemical parameters in Irish marine sediments. Indices were generated for;

1) Bioassay data as generated by Macken (2007) in part fulfillment of this current project and for cell line assays as reported in chapter 4 of this thesis.
2) Metals contaminants in *M. edulis* as transplanted and reported in chapter 5 of this thesis.
3) Sediment chemistry analysis as reported in chapter 2 of this thesis.
4) Biological effects measurements (RPSI and shell thickening) in *Nucella lapillus* and *Crassostrea gigas* as described in chapter 5 of this thesis
5) Overall indices were calculated from a combination of 1-4 above.

8.3.1 Indices generated from bioassay component

As evidenced by Table 8.1, the overall scores derived from the bioassay component (a combination of data from Macken and this present thesis) of this project were relatively similar for the three selected sites. The Dunmore East site exhibited a greater toxicity in the case of *V. fischeri* in the porewater component compared to that observed at the other locations. The potential nature of this toxicity will be further discussed.

This thesis primarily reports on the development of techniques directed towards OTCs and as such the development of cell line assay techniques as described in chapter 4 primarily concentrated on the Dunmore East site only.

Table 8.1: Scores generated for individual bioassay measurements as reported by Macken (1-5) and as described in chapter 4 of this thesis (6-7).

	Sample/site "Score"		
Bioassay/organism	**DB1**	**DE1**	**DB6**
***V. fischeri* (1) PW (*)**	1.32	7.30	NA
***V. fischeri* (1) AE (*)**	NA	7.07	NA
***V. fischeri* (1) SE (*)**	9.89	8.84	7.31
***V. fischeri* (2) WS (*)**	5.34	9.16	NA
***T. suecica* (3) PW (*)**	5.92	6.64	NA
***T. suecica* (3) AE (*)**	NA	NA	NA
***T. battagliai*(48 h) (4) PW (*)**	9.13	8.29	8.24
***T. battagliai*(48 h) (4) SE (*)**	8.70	8.09	NA
***C. volutator* (5) (*)**	6.00	5.00	NA
RTG-2 (6) SE (Chapter 4)	NA	2.68	NA
PLHC-1 (7) SE (Chapter 4)	NA	3.60	NA
Overall site score	146	234	30.1
Score/n parameters	20.8	23.4	15.1

(*) Macken PhD Thesis, PW = Porewater, AE=Aqueous Elutriate; WS=Whole sediment, SE= Solvent extract.

DB1: Dublin Bay site 1; DE1: Dunmore East site 1; DB6: Dublin Bay site 6

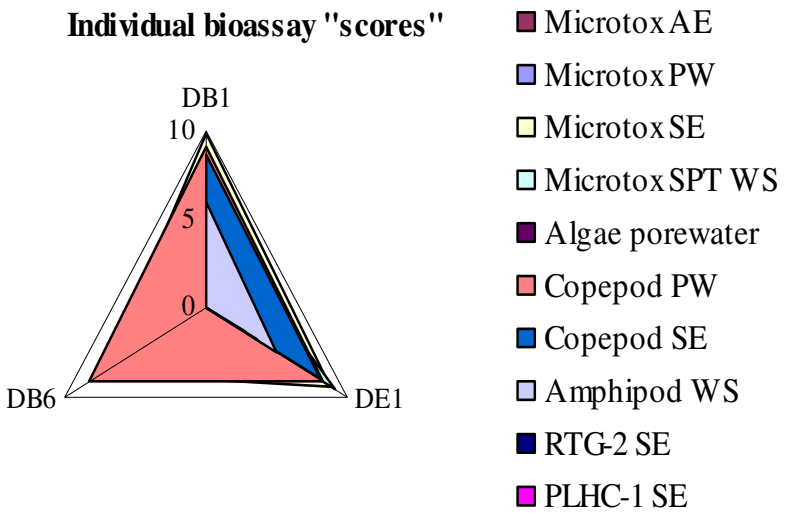

Figure 8.1: Star plot of site specific individual "scores" calculated for various bioassays as completed during this thesis and as reported in Macken [PhD thesis] (2007).

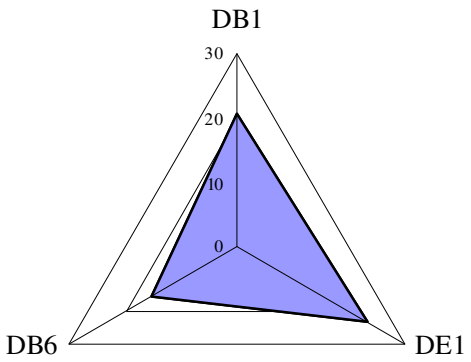

Figure 8.2: Star plot of site specific overall "IR" as calculated for various bioassays as completed during this thesis and as reported in Macken et al.,(2008).

While data for the cell line assay components are only available for the Dunmore East site, their removal from the overall index has a relatively small influence on the derived index at the site (overall 234 as against 209 upon removal) and 23.4 to 20.9 for the averaged index. This relatively minor decrease in index (~10%) supports the hypothesis that where multiple parameters are measured that the influence of the addition of additional stand alone test results (such as the cell line results described) do not dramatically alter derived indices.

8.3.2 Biotic component (metals and TBT)

Metals were measured in mussels at test sites as described in chapter 6. Following the 18 week testing regime, levels of lead (Pb), cadmium (Cd), mercury (Hg) and tributyltin (TBT) (only parameters where EACs have been generated) were determined in transplanted mussels (see Table 8.2 and Figure 8.3). Wet weight levels were converted to dry weight prior to comparison to the EAC as per assessment criteria requirements.

Table 8.2: Dry weight normalised metals and TBT values and derived site scores.

		Normalised Result				Sample "score"			
Biota M. edulis	**EAC mg/kg**	**OI1 (t=0)**	**DB5 (t=18)**	**DE2 (t=18)**	**OI1 (t=18)**	**OI1**	**DB5**	**DE2**	**OI1 (t=18)**
Pb	8.50	0.12	0.14	0.52	0.29	2.29	2.76	10.0	5.54
Hg	0.01	15.0	12.0	18.0	6.00	8.33	6.67	10.0	3.33
Cd	0.28	6.32	3.75	5.11	2.39	10.0	5.95	8.11	3.80
TBT	12.0	0.37	0.51	43.0	0.20	0.09	0.12	9.99	0.05
Overall site score						51.9	29.6	181	15.8
Score/n parameters						10.4	5.9	36.2	3.16

The average "score" suggests that the Dunmore East site (36.2) contains the greatest levels of the above contaminants compared to the other locations. As previously discussed Dunmore East exhibits elevated TBT contamination compared to the other locations and thus forms a substantial portion of the index at this location (approx. 50 %).

The impact of TBT contamination at this location is discussed throughout this thesis. The IR generated for DE2 suggests that TBT contamination plays an important role in describing the overall contaminant profile at the selected test site within Dunmore East, but also that further work may be merited to conclusively evaluate the relative degree of contamination at this location compared to at the other test sites.

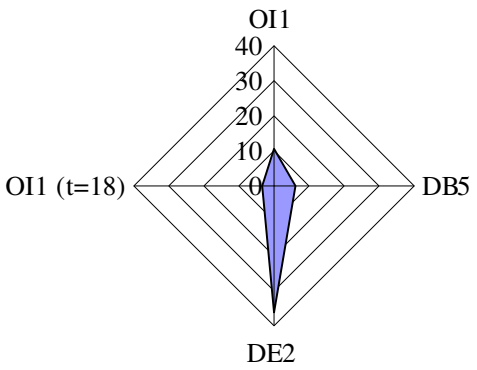

Figure 8.3: Star plot of site specific overall "IR" as calculated for various metals in *M. edulis* as completed during this thesis.

8.3.3 Sediment component (metals and organic compounds)

As per the biotic compartment, sediments data required normalisation prior to index generation. Normalised metals and organic contaminant values in sediments and derived site scores are presented in Table 8.3 and Figure 8.4. As per the metals and bioassay components the average index derived for Dunmore East (DE2) (34.7) was slightly greater than that determined for the Dublin Bay (DB5) site. The DE2 site provided the highest index for all of the metals measured in addition to anthracene, benzo[a]anthracene and pyrene. The Dublin Bay site (DB5) was shown to have a higher content of naphthalene and phenanthrene in addition to Benzo [ghi] perylene, Benzo [k] fluoranthene and Indeno[1,2,3-cd] pyrene than the other locations.

Table 8.3: Normalised metals and organic contaminant values and derived site scores

		Normalised result			Sample "score"		
Sediment parameter	EAC (ug/kg)	OI1 (t=0)	DB5 (t=18)	DE2 (t=18)	OI1	DB5	DE2
Mercury	0.22	0.01	0.17	0.29	0.26	6.00	10.0
Cadmium	0.06	0.59	2.59	5.01	1.17	5.17	10.0
Lead	2.20	1.05	16.3	22.1	0.47	7.39	10.0
TBT	0.01	6120	21000	1088451	0.06	0.19	10.0
PCB 153	40.0	0.0002	NA	0.009	0.23	NA	10.0
Naphthalene	43.0	0.03	6.40	0.99	0.05	10.0	1.55
Phenanthrene	1250	0.001	0.21	0.18	0.03	10.0	8.69
Anthracene	78.0	0.01	0.89	1.79	0.06	4.96	10.0
Fluoranthene	250	0.01	1.26	1.06	0.06	10.0	8.37
Pyrene	350	0.00	0.85	1.08	0.04	7.87	10.0
Benz[a]anthracene	1.50	0.55	52.1	183	0.03	2.84	10.0
Benzo[a]pyrene	625	0.00	0.27	0.20	0.02	10.0	7.24
Benzo [ghi] perylene	2.10	0.39	103	79.6	0.04	10.0	7.76
Benzo [k] fluoranthene	3.50	0.24	27.0	21.8	0.09	10.0	8.07
Indeno[1,2,3-cd] pyrene	1.50	0.28	131	97.4	0.02	10.0	7.45
Overall site score					0.46	380	555
Score/n parameters					0.03	23.7	34.7

The indexing system identifies (and is supported by hydrocarbon analysis described in chapter 2 and 7) that sources of PAHs at the two locations may differ to an extent, with as the DB5 site contains predominantly low aromatic PAHs as against the higher aromatic compounds which predominate at the DE2. Significantly TBT was again found to be elevated at DE2 compared to the other sites.

By generating an IR with PAH data alone, the DE2 and DB5 sites would be similar (19.3 and 19.6 respectively). This suggests that contaminants other than PAHs provide the majority of difference to the overall IR at this site, significantly TBT was again found to be elevated at DE2 compared to other sites.

It should be noted that as a high TOC content was determined in the Omey Island sample, the results for this site should be treated with caution and are included in this assessment primarily for reference only purposes.

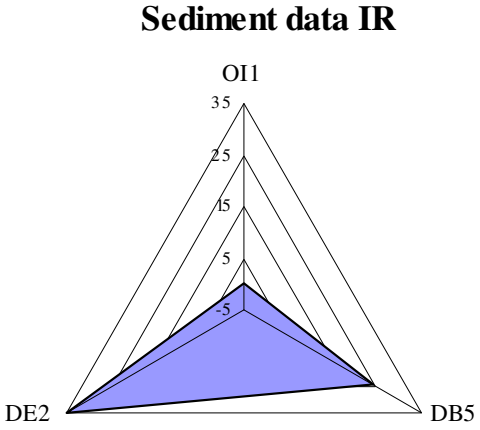

Figure 8.4: Star plot of site specific overall "IR" as calculated for various sediment parameters as completed during this thesis.

8.3.4 Biological effect indices

Derived values and calculated site scores for shell thickening and RPSI values at individual locations are presented in Table 8.4 and Figure 8.5. Data on shell thickening and RPSI have previously been reported in chapter 5. The DE2 site (score 22.7) was again found to show the most elevated indice. As the biological effects of shell thickening and imposex have been linked to TBT contamination and elevated "scores" for TBT have been observed

at Dunmore East in mussels and in sediments (and further supported by bioassay directed fractionation analysis in chapter 7) it is not unreasonable to assume that the effects discussed in oysters and in dogwhelks may primarily be as a result of OTC contamination at this location.

Table 8.4: Derived values and calculated site scores for shell thickening and RPSI at individual locations.

	Result				Sample "score"			
Biological effects	**OI1 (t=0)**	**DB5 (t=18)**	**DE2 (t=18)**	**OI1 (t=18)**	**OI1 (t=0)**	**DB5 (t=18)**	**DE2 (t=18)**	**OI1 (t=18)**
***C. gigas* (Shell thickening)**	NA	25.4	9.20	22.1	NA	7.46	9.08	7.79
***N. lapillus* (RPSI)**	NA	0.0003	2.38	NA	NA	NA	10	NA
Overall site score					NA	7.46	45.4	7.79
Score/n parameters					NA	7.46	22.7	7.79

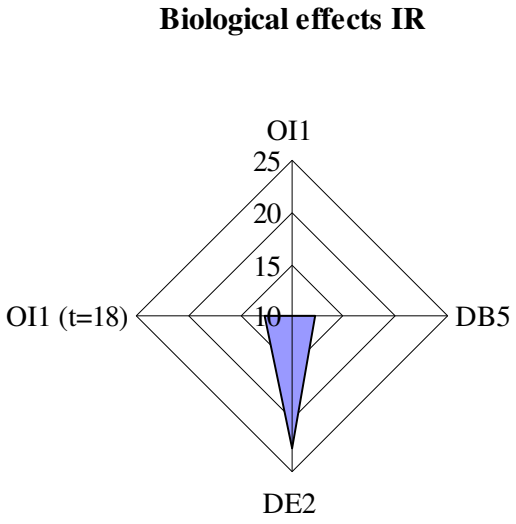

Figure 8.5: Star plot of site specific overall "IR" as calculated for various biological effects parameters (shell thickening and RPSI) as completed during this thesis.

8.3.5 Overall compartment based IR at test sites

As consistently discussed throughout this chapter the Dunmore East site have been found to exhibit the highest scores and compartment IRs with respect to the other locations tested. It is therefore not surprising that the mean compartment IR is much higher at this site (477) than at the other locations (see Table 8.5 and Figure 8.6).

Table 8.5: Derived compartment based IRs at individual locations.

Compartment	**OI1**	**DB5**	**DE2**	**OI1**
Biota	10.4	5.90	36.2	3.20
Sediment	0.03	23.73	34.7	
Biological effects		7.46	22.7	7.79
Total IR	0.15	181	1432	12.3
Mean IR (IR/n compartments)	0.08	60.2	477	6.15

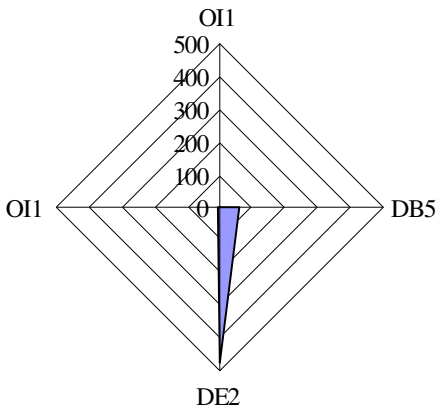

Figure 8.6: Star plot of site specific mean compartment "IRs" as calculated during this thesis.

8.4 Overall conclusions

This chapter generated an index system that allowed for the comparison of bioassay, biological effects and biotic and sediment chemistry data from a number of sites. As such, this index provides a mechanism which allows individual parameters to be compared with respect to each other and between locations. The system described does not include a mechanism for toxicity and/or synergistic/additive effects weightings which would ultimately provide further useful information on the potential for toxicity effects at individual sites. Additionally future IR generation should attempt to modify the scoring system so that the degree to which a parameter exceeds the assessment criteria (e.g. EAC) becomes standardised, thus allowing for long term (temporal) assessments to be completed independent of comparison to a "reference" location as was completed in this present chapter.

Beliaeff and Burgeot (2002) stated that the selection of an appropriate battery of biomarkers can avoid false–negative responses obtained with a single biomarker and allow information to be summarised in the form of a multivariate data set. According to Broeg and Lehtonen (2005), due to its mathematical basis, the IBR becomes more robust when the number of biomarkers increases. The number of biomarkers included in the calculation of the IBR plays an important role affecting the ‘‘relative weight’’ of each biomarker in the final index value. When the set of biomarkers is relatively large, e.g. 6–8, the weight of one factor is markedly reduced compared to cases when 3–4 biomarkers are used. The removal of individual parameters (e.g. cell-lines PLHC-1 and RTG-2 data which were only available for Dunmore East alone) from the bioassay IR resulted in only a small difference in the derived IR compared to when these parameters were included. Further validation, intercalibration and temporal monitoring (to assess trends and variability) of the biomarkers/bioeffects techniques used to generate the IR would further improve the robustness of the technique.

The primary objective of this chapter was to provide a visualisation mechanism whereby biological and chemical data can be scored relative to (provisional) assessment criteria and evaluated in an “integrated” manner. As such this index achieves this goal.

8.5 References

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9

CHAPTER 9 GENERAL CONCLUSIONS

9.1 Conclusions

This thesis formed part of a three and a half year collaborative project involving the Radiation and Environmental Science Centre (RESC) at DIT and the Marine Institute in Galway. The project was divided into three work packages. Work package 1 (WP1) mostly involved the optimisation and validation of a battery of bioassays for sediment toxicity testing and took place at the RESC [Macken PhD thesis 2007], work package 2 (WP2) which is the work described in this thesis, primarily involved the development of chemical assessment methodologies (analytical, bioassay, caging studies and assessment techniques) for a suite of organic and inorganic contaminants in marine sediment and took place at the Marine Institute in Galway. Work package 3 (WP3) involved the final integration of all data obtained from both PhDs and is discussed in chapter 8 of this thesis. The ultimate aim of this collaborative project was to design and implement an integrated programme for ecotoxicological testing and ultimately to correlate these results with chemical analysis data.

At the beginning of the project, a number of spatial sediment locations were chosen on the basis of being potentially contaminated/clean for optimisation of bioassays for sediment toxicity testing. A number of sites within Dublin Port and in the greater Dublin bay area were selected primarily on the basis of this region being Ireland's busiest passenger ferry port and its overall industrialisation which may potentially subject it to a greater degree of contamination. Two additional sites were selected in the Dublin Bay area, one directly under the North Bank Lighthouse (to primarily assess the potential for imposex effects in the main shipping channel area and to evaluate the potential influence of sewerage effluent into the Bay) and one in the North Bull Lagoon in Dublin Bay as the reference sediment for whole sediment bioassays. Dunmore East in Co. Waterford was selected on the basis of a

previous report where organotin and metal levels were elevated. The final selected location was Omey Island on the West coast of Ireland which was expected based on its location to be a pristine site. This site is unexposed to industrialised and urban inputs and species of the dogwhelk *Nucella lapillus* were collected here which were previously observed to show no signs of imposex.

A battery of bioassays was optimised by both Macken and Giltrap (this thesis) in WP1 and WP2 of this project including a variety of trophic levels from bacteria to fish cell lines. The battery of bioassays utilised throughout this project are shown in Table 1.1 in chapter 1 of this thesis. This battery was used for the ecotoxicological assessment of sediment by assaying with porewater, aqueous elutriate, whole sediment and solvent extracts.

A full chemical assessment of sediments at all sites was performed and is described in chapter 2 of this thesis. Concentration data are described for, heavy metals, organotin compounds (OTCs), polycyclic aromatic hydrocarbons (PAHs), hydrocarbons (HCs) and organochlorine compounds (OCs) at selected locations. Levels of metals were observed to be elevated at both Dublin and Dunmore East harbours, TBT and PAHs were observed in highest elevated levels at Dunmore East harbour however elevated levels were also determined in the Alexandra basin in Dublin Port. Overall levels of OCs were relatively low in all locations.

Pollutant levels were further assessed with respect to OSPAR BAC and US National Oceanic and Atmospheric Administration (NOAA) Effects Range Low (ER_L) and Effects Range Median (ER_M) criteria and Irish Sediment Quality Guidelines. It was demonstrated that Omey Island was near background for all contaminants where assessment criteria are

available however high TOC values suggest that terrestrial influences maybe present at the sampling site and thus this sample is unrepresentative of true marine sediments. Concentrations for zinc and lead in the inner Alexandra basin, lead at the North Bull Lagoon and copper at Dunmore East harbour were above the ER_M and upper Irish guideline levels therefore adverse effects on organisms which reside at these sites could potentially be affected by these specific contaminants/groups of contaminants. Concentration levels of PAHs did not exceed the ER_M at any of the sites and therefore adverse effects from PAHs alone on aquatic species would potentially not be expected at these sites. This finding is additionally evaluated in chapter 7 of this thesis where the potential influence of PAHs to the overall toxicity profile seems to be lower than that determined for other compounds (*e.g.* TBT). Therefore it can be concluded that toxicity of porewater from Dunmore East and Dublin port with the battery of bioassays could have potentially been induced by metals, organotin compounds or mixtures of contaminants.

A method for the extraction of organotin (OT) compounds in their salt form from marine sediments for assaying with biological organisms and two cell lines (PLHC-1 and RTG-2) was established and is reported in chapter 3 of this thesis. This method was used for the production of solvent extracts for assaying on biological organisms and was also successfully used for quantifying OTCs. Solvent extracts were assayed and toxicity was observed with the Dunmore East and Alexandra basin extracts, the Alexandra basin extract being the most toxic to both the Microtox® test and *T. battagliai* assay. In chapter 4 of this thesis it was shown that just 73.2 and 64.0 mg sediment /ml of media elicited 50 % cytotoxicity in the RTG-2 and PLHC-1 cell lines respectively however the cell lines utilised in this study were observed to be less sensitive than other organisms used in the battery in WP1 (Macken PhD thesis 2007). The data produced by both Macken and Giltrap have

been further evaluated in chapter 8 of this thesis in the form of an "integrated ratio" and will be further discussed below.

In chapter 4 of this thesis four organotin compounds namely TBT, DBT, TPT and DPT were assayed with the RTG-2 cell line to assess cytotoxic effects. The EC_{50}s calculated for these compounds after 96 h exposure using the alamar blue (AB) assay ranked the toxicity as TPT > TBT > DPT > DBT again demonstrating differences in sensitivities of various contaminants. The RTG-2 cell line was found to be the least sensitive species for exposure to organotin compounds.

In chapter 5, an in-situ study using caged *Nucella lapillus* and *Crassostrea gigas* was used to monitor TBT induced bioeffects in three sites namely Omey Island, Dunmore East and Dublin Port. TBT has unequivocally been linked to the imposex effect and associated with shell thickening and reduced meat yields in the Pacific oyster *Crassotrea gigas*. The degree of imposex in the gastropod as measured by the Vas Deferens Sequence Index (VSDI) and Relative Penis Size Index (RPSI) and the extent of shell thickening in the oysters was investigated at t=0 and t=18 weeks. After 18 weeks the Dunmore East site showed the highest level of imposex (3.25 VDSI and 2.375 RPSI) of all three sites. Concentrations of organotins in whole-body tissues of each test species were also measured at t=0 and t=18 weeks and the highest accumulation of all organotins was observed in all test species at the Dunmore East site compared to the other two test locations which may be associated with elevated sediment concentrations observed at this location.

Stable isotope ratios of carbon $\delta^{13}C$ and nitrogen $\delta^{15}N$ were used in this study to provide information on food sources for the caged species which assisted in studying relative

trophic status, dietary preferences and the biomagnification of contaminants within complex food webs. Greatest $\delta^{15}N$ enrichment (at Dunmore East and Dublin Bay respectively) was found in *N. lapillus*, thus indicative of this organism having higher relative trophic status compared to the other two test species. The employment of caging technologies allows for the introduction of indicator species into locations where they may not be present or where they may have previously been affected by elevated contaminant levels thus providing an efficient mechanism whereby integrated biological and/or chemical effects measurements can be determined. Imposex data at the Dunmore East location suggest exposure of *N. lapillus* to TBT concentrations higher than the Ecotoxicological Assessment Criteria (EAC) derived for TBT. This *in-situ* study proved to be a very effective technique for future integrated assessments as bio-effects were induced in a short period of time compared to other *in-situ* studies and bio-effect data correlated well with chemical measurements in biota and sediment

In chapter 6 which also involved the caging study, levels of metals were determined to investigate metal uptake rates in the species *Nucella lapillus*, *Mytilus edulis* and *Crassostrea gigas*. Rapid biotic accumulation of metal levels was demonstrated in the test species, especially in both Dunmore East and Dublin suggesting the caging study as described can be considered a valid tool for bio-monitoring in metal impacted areas.

In chapter 7 of this thesis, methodology was developed for bioassay directed fractionation whereby OTCs and other anthropogenic compounds were extracted from the bulk sediment, fractionated and analysed using a variety of analytical techniques. Instrumental analysis led to the characterisation of the solvent extracts for a range of organic contaminants. The bioassay directed fractionation approach applied in this study led to the identification of the

most toxic fraction isolated from the Dunmore East sediment. Bioassay-directed fractionation was successfully used to characterise compounds with a variety of polarities and hydrophobicities as contributing to the measured activity. The difference in species sensitivity to both crude contaminant extracts and fractionated extracts proved that mixture toxicity plays an important role in profiling sediment toxicity and thus highlights the need for a battery of bioassay tests for sediment quality assessments. Bioassay directed fractionation is no doubt a worst case scenario for sediment contamination but provided a useful insight into the contaminants present in the sediment and proved very useful for sediment quality assessments.

Chapter 8 of this thesis generated an index system that allowed for the comparison of bioassay, biological effects and biotic and sediment chemistry data from a number of sites. As such this index provides a mechanism which allows individual parameters to be compared with respect to each other and between locations. The system described did not include a mechanism for toxicity and/or synergistic/additive effects weightings which would ultimately provide further useful information on the potential for toxicity effects at individual sites. Additionally future IR generation should attempt to modify the scoring system so that the degree to which a parameter exceeds the assessment criteria (e.g. EAC) becomes standardised, thus allowing for long term (temporal) assessments to be completed independent of comparison to a "reference" location as was completed in this present chapter.

The relative robustness of the technique was demonstrated by the removal of individual parameters (e.g. cell-line PLHC-1 and RTG-2 data which were only available for Dunmore

East alone) from the bioassay IR and this resulted in only a small difference in the derived IR compared to when these parameters were included. Further validation, intercalibration and temporal monitoring (to assess trends and variability) of the biomarkers/bioeffects techniques used to generate the IR would further improve the robustness of the technique. The primary objective of this chapter was to provide a visualisation mechanism whereby biological and chemical data can be scored relative to (provisional) assessment criteria and evaluated in an "integrated" manner. As such the index presented in this thesis achieved this goal.

The extent of work completed in this thesis (and supported by Macken's PhD thesis) has further developed the capacity within DIT and the Marine Institute (and Ireland) to monitor pollutant levels and measure their potential toxic effects in the Irish marine environment. The variety of techniques developed and the important information generated for a number of Irish coastal sites greatly adds to the currently available data.

This PhD has described the chemical assessment component of a project entitled "An Integrated Approach to the Toxicity Evaluation of Irish Marine Sediments". It has highlighted the importance of coupling chemical analysis data with a multi-trophic, multi-phase battery of bioassays for integrated assessments of marine sediment. Current international approaches are utilising scoring systems with weight of evidence approaches similar to the approach used in chapter 8 of this thesis. The use of scoring systems in chapter 8 highlights the importance of using a triad approach including chemistry, benthic indices and sediment toxicity for sediment assessments. An integrated programme for ecotoxicological assessment of marine sediment was implemented and results of bioassays and chemical analysis were correlated.

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Giltrap, M., Macken, A., Foley, B., McGovern, E., McHugh, B., Davoren, M. 2007. Toxicity evaluation of organotin compounds in Irish marine sediment using two fish cell lines integrated with chemical analysis. Submitted to Toxicology in Vitro, May 2008

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Giltrap, M., Macken, A., Davoren, M., Foley, B., McGovern, E., Strand, J., Minchin, D., McHugh, B. An in-situ study using caged *Nucella lapillus* and *Crassostrea gigas* to monitor TBT induced bio-effects at three Irish coastal sites. Poster presented at SETAC North America 28th Annual Meeting, Milwaukee, Wisconsin, USA (Nov 2007).