The use of the Toxicity Identification and Evaluation (TIE) protocol in the Port of Durban, South Africa

by

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As the candidate's supervisor I have approved this dissertation for submission.

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ABSTRACT

The Port of Durban, with its close proximity to industrial, urban and agricultural activities, receives a number of chemical pollutants that settle out and accumulate in sediments. Chemical analysis of these sediments has indicated elevated levels of chemicals that, according to sediment quality guidelines, might cause adverse biological effects. However, elevated concentrations alone do not necessarily imply that chemicals are present in bioavailable concentrations high enough to be harmful to organisms that come into contact with them. Thus, chemical tests alone cannot provide an accurate indication of the potential adverse biological effects of these chemicals.

In this regard, toxicity tests of sediment porewaters have been developed using sea urchin gametes to assist in determining the bioavailability of chemicals present in porewaters. Further, procedures such as Toxicity Identification and Evaluation (TIE), which involves the manipulation and/or treatment of toxic porewater, have also been developed to assist in the isolation and identification of chemicals causing porewater toxicity.

In this research, on a number of sampling occasions between July 2007 and July 2009, three replicate sediment samples were extracted from a site in the Port of Durban known to contain sediment with potentially toxic porewater. Results of initial toxicity tests, using the sea urchin fertilisation test indicated the presence of toxic porewater although, in some instances, porewater toxicity was highly variable between replicate samples. However, results from TIE procedures performed to reduce potentially toxic concentrations of metals, ammonia and organic compounds did not resolve the primary cause of porewater toxicity.

Further research indicated that chemicals including hydrogen sulphide, which can occur naturally in organically enriched sediments, may have been confounding factors that masked the potential toxicity of other chemicals present in the sediment samples. Consequently, a sampling strategy and modified TIE procedure have been recommended. The sampling strategy has been designed to assist with detecting and understanding any sample variability that may occur. The modified TIE procedure, which suggests initial procedures to determine and reduce/remove the possible confounding effects of potential naturally occurring compounds such as hydrogen

sulphide from the porewater, could be used in future to understand and evaluate the quality of contaminated sediments from similar environments.

PREFACE AND DECLARATIONS

The experimental work described in this dissertation was carried out in the School of Science and Agriculture, University of KwaZulu-Natal, Durban and at the Environmental Analytical Laboratories of the CSIR in Durban 2004 to 2010, under the supervision of Professor John Cooke and Doctor Andre Vosloo.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

I, Gary Angus Parsons declare that

1. The research reported in this dissertation, except where otherwise indicated, is my original work.
2. This dissertation has not been submitted for any degree or examination at any other university.
3. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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Chapter 1

Introduction

1.1 BACKGROUND

Estuaries are generally complex, dynamic environments due to the interaction between local physical, geological, chemical and biological features (Dauvin *et al.* 2006). In addition, given their position at the ocean-land interface, estuaries are also important sites for various human activities and developments, including industry, marine transportation, agriculture, fisheries, and tourism (Liu *et al.* 2003). In fact, many of the great cities of the world are situated on estuaries (Connell 2005) and the Port of Durban "flanked by two originally wood-clad ridges and connected by a channel with the Indian Ocean is the natural feature to which Durban as a settlement owes its existence" (Horwood 1969).

There are two predominant inputs into an estuary, namely, from the marine and terrestrial environments. From the marine environment there are inputs of sediments, organic matter and water (with a salinity of approximately 35). From the land, fresh water runoff from terrestrial sources flows into rivers and as with inputs from the marine environment, rivers also contain organic matter and sediment. At the point where fresh water from a river meets the saline conditions of the marine environment, the marine and terrestrial inputs interact and mix to establish an estuarine environment. This environment is neither characterised by freshwater or by saline water, but a combination of both which results in a unique environment that supports plant and animal life that has adapted to these specific conditions characterised by variable pH, salinity, nutrients and temperature (Hedges and Keil 1999; Dauvin *et al.* 2006).

Ports are important developments built to accommodate shipping trade, and are thus important contributors to the local and national economy (Marshall and Rajkumar 2003). The construction of a port in an estuarine area usually involves a great deal of estuary reshaping, dredging and destruction of natural habitat. In most cases an estuarine system is hardly recognizable after port construction is completed, as the

protection of the environment was, until relatively recently, regarded as secondary to trade concerns and construction requirements (Wooldridge *et al.* 1999).

The Port of Durban (Figure 1.1) has been constructed within the extensive estuarine system known as the Durban Bay and has a total land and water area of approximately 1854 hectares (http://www.npa.co.za/Ports/Durban/Overview, 23/08/2005). It is the busiest port on the African continent, with a ship traffic volume of over 4000 commercial vessels each year, and plays a pivotal role in the life of the city by contributing more than fifty billion rand to the local and national economy. It is a direct employer of about 6000 people as well as approximately 30000 people who are indirectly dependent upon the port and its activities. Durban's location is such that it is one of only a few cities in the world where the port is virtually adjacent to the central business district (http://www.npa.co.za/Ports/Durban/Overview, 23/08/2005).

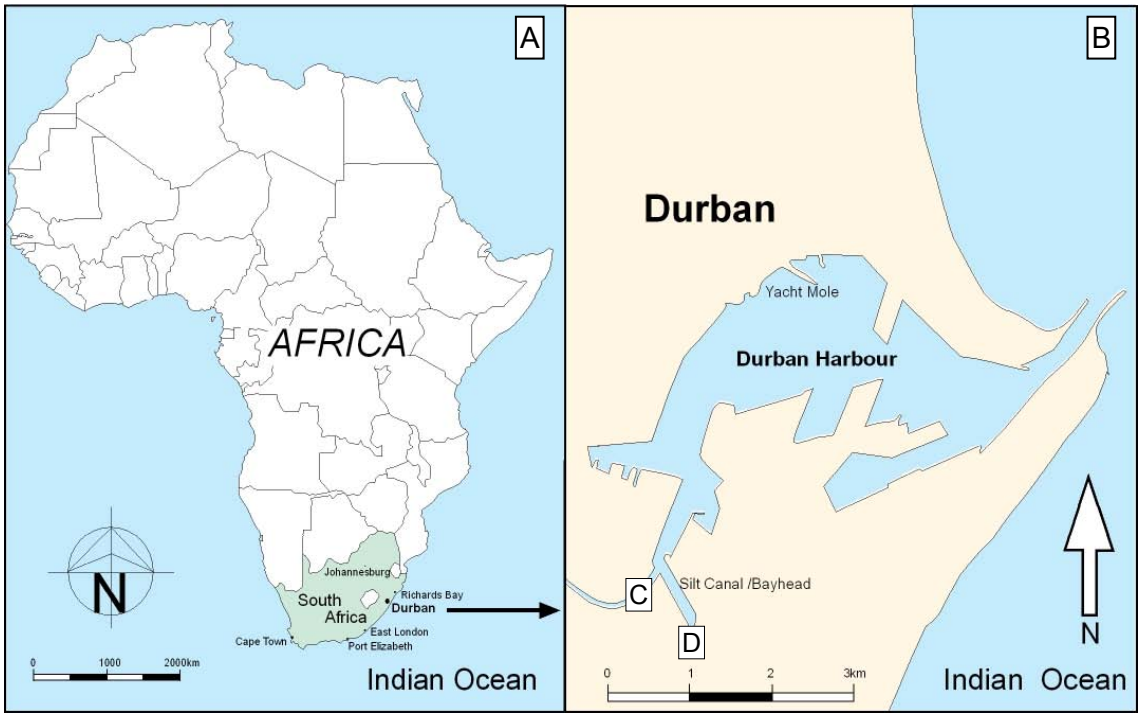

Figure 1.1 Location (A) and plan (B) of the Port of Durban with inputs from the Mhlatuzana and Umbilo Canals (C) and the Manzimnyama Canal (D)

Modification of the estuary to accommodate the Port of Durban has led to changes in the character and dynamics of the estuarine environment, causing a transformation from a natural setting to an almost completely modified one. Before construction began, Durban Bay was a sheltered lagoon comprising sand banks, multiple channels of various depths, extensive mangrove and swamp areas, and vegetated islands (Van Rooyen 2001). Safe entrance into the bay was restricted by a sandbar across the port entrance and there were no landing berths of any type at which ships could dock. Developments since the early 1900's when the sand bar was dredged (Van Rooyen 2001) have given rise to the Port of Durban, a busy and important economic asset to the region (Marshall and Rajkumar 2003). With these developments, the nature of the estuary has changed completely. In fact, the previously natural, pristine estuary had a relatively low salinity with limited intertidal exchange with the sea due to the sand bar and a number of freshwater inputs including the Mgeni River (Leuci 1998). Additional modifications since the 1930s, including construction of breakwaters at the port entrance to allow ships entry into the port, quays and jetties within the port for ships to dock, dredging of channels to allow safe ship passage within the port, removal of most of the natural vegetation, canalisation of inflowing rivers, and general urbanisation and industrialisation, have given rise to an environment that is far from natural (Van Rooyen 2001).

1.2 SOURCES OF ANTHROPOGENIC CONTAMINANTS INTO THE PORT OF DURBAN

The Port of Durban and its infrastructure are used in a number of ways, and these include shipping, coal and container transport, small craft areas, and dry dock ship repair and repainting facilities (Pillay 2003). Consequently, the Port of Durban, as a working harbour with local and international shipping traffic, and as an estuary with seawater, freshwater, and other external inputs, is susceptible to anthropogenic contamination of its environmental resources from a wide range of port and catchment based activities and marine sources (Walmsley *et al.* 1999). There are fresh water inputs into the port from three canalised rivers (the Umbilo, Manzimnyama and Mhlatuzana Rivers; see Figure 1.1) as well as from storm water outfalls that drain surface runoff from the surrounding city of Durban. In addition, water circulation in the port is controlled and restricted in order to ensure safe operating conditions for ships and other craft that use the port facilities (Walmsley *et al.* 1999). This will encourage

the settling out and accumulation in bottom sediments of contaminants entering these calmer waters of the port (Chapman and Mann 1999; Beiras *et al.* 2003; Kwok *et al.* 2005).

Thus, the modified estuarine conditions and the concentrated development within its catchment area have increased the likelihood of anthropogenic contaminants reaching the port environment (Walmsley *et al.* 1999; Wooldridge *et al.* 1999; Wakeman and Themelis 2001; Fang *et al.* 2005; Dauvin *et al.* 2006). Examples of point and diffuse sources of anthropogenic contamination include:

- Ballast water discharged by ships using the harbour (Niimi 2004), along with other discharges of contaminants such as fuels and heavy oils that may occur.

- The canalised rivers together with other canals and storm water drains that flow into the port from the surrounding urban and industrial areas. Local industrial effluent and effluent from other activities that occur along the banks of these canals and rivers, for example, domestic effluent and sewage inputs from informal settlements and agricultural runoff.

- Activities within the port, such as ship building and repair, may also introduce chemicals such as anti-fouling additives (e.g. tributyl tin) in ship paints and trace metals from welding or sand blasting processes.

1.3. CLASSIFICATION OF ANTHROPOGENIC CONTAMINANTS IN THE AQUATIC ENVIRONMENT

Anthropogenic contaminants released into the environment include nutrients, metals and organic chemicals. In coastal environments such as estuaries, these contaminants tend to precipitate out of the water column and become integrated into the bottom sediments (e.g. Beiras *et al.* 2003). In this way, sediment concentrations of these contaminants can sometimes be found at concentrations that are orders of magnitude higher than those detected in the water column. Thus, sediments can be sources of contaminants which may be present in a bioavailable form in sufficiently high concentrations, that they have the potential to induce a toxic response in organisms

living in or close to these sediments (e.g. Fent 2004). Specific contaminant groups are considered below.

Nutrients, particularly nitrates and phosphates, are generally not directly toxic but can cause environmental problems when present at high concentrations (Losso *et al.* 2004; Walker *et al.* 2006). Major sources of nutrients to surface waters include agricultural fertilisers and effluent. Excess fertiliser materials not used by plants for growth pass through or are washed from soil and can enrich adjacent water courses. The increase in nutrients in these water courses may result in the development of algal blooms, which can eventually lead to oxygen depletion and result in adverse biological effects such as fish kills (Hylland 2006). In the past, the Port of Durban has experienced algal blooms with resultant oxygen depletion and fish kills (Van Rooyen 2001).

Sources of metals released into the environment include the natural weathering process and activities such as mining and smelting (Liu *et al.* 2003). As sinks for these contaminants, many port environments have been shown to contain elevated metal concentrations with some being high enough to potentially cause environmental harm (Furness *et al.* 1990; Leuci 1998; Wakeman and Themelis 2001; Marshall and Rajkumar 2003). Additionally, metals are non-biodegradable (Walker *et al.* 2006; Newman and Watling 2007) and therefore can persist in the environment unlike some organic compounds that can be transformed over time to less harmful derivatives.

Some metals, such as calcium, sodium, iron, copper, zinc and selenium are essential for biological processes while some are non-essential, including cadmium, lead and mercury. Long term exposure to elevated metal concentrations may be toxic to an organism, especially if it does not have any biological defense to deal with the elevated concentrations (Hylland 2006). Connell (2005) concluded that metals such as mercury, cadmium and lead together with excessive quantities of metals such as copper were generally considered to be the most toxic metals to a wide range of organisms (e.g. algae, plants, vertebrates and mammals) as well as marine animals (e.g. polychaetes, molluscs, crustaceans and fish). The toxicity mechanism for metal ions appears to fall into three main categories: (1) by bonding to and blocking of essential biological functional groups of biomolecules including proteins and enzymes, (2) by displacing the essential metal ion in biomolecules and (3) by modifying the active chemical conformation of biomolecules (Walker *et al.* 2006).

Some metals in the non-ionic form are relatively non-toxic, for example, liquid mercury, lead and tin. However, the toxicity of these and other metals is greatly enhanced if they become bound to an organic ligand (e.g. tributyltin (TBT) and methylmercury) (Marshal and Rajkumar 2003; Fent 2004; Walker *et al.* 2006). This bonding forms lipid soluble organometallics that are capable of crossing cell membranes and thus accumulating within the cells of organisms (Connell 2005). As a result, these compounds have in the past been used as biocides (e.g. in cooling towers and antifouling agents in ship paints). They are very effective but their use has been banned in most parts of the world because they are non selective and very harmful to other animals in the environment. Thus, some organometallics have caused numerous environmental problems over the years such as the TBT induced imposex in *Nassarius kraussianus* detected in the Port of Durban (Marshall and Rajkumar 2003)).

Other organic chemicals include petroleum bi-products, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and dioxins, and pesticides. Some of these contaminants occur naturally (e.g. crude oil and natural gas) while others are manmade e.g. pesticides such as dichlorodiphenyltrichloroethane (DDT). Whatever their source, however, organic chemicals are xenobiotics which infers that they are compounds foreign to organisms (Walker *et al.* 2006). The behaviour of an organic compound in the environment will tend to depend on its molecular structure and polarity, including molecular properties such as size, shape, and functional groups (Ehlers and Loibner 2006; Hedges and Kiel 1999; Marshall and Rajkumar 2003).

Petroleum hydrocarbons (PHCs) include PAHs (Figure. 1.2) as well as other aliphatic and aromatic hydrocarbons such as paraffins and olefins. These compounds are mainly produced by petrochemical industries and are used for fuel, in the leather industry, as sources of raw materials for the detergent and the specialty chemicals industry, as plasticisers, solvents, and lube oil components. The toxicity of PHCs has been found to vary according to their chemical structure. As a general rule, however, aromatic hydrocarbons tend to be more toxic than the other forms (e.g. aliphatic) of hydrocarbons (Connell, 2005).

PAHs are among the contaminants most frequently detected in sediments. Sources of PAHs include agricultural and industrial combustion processes and petroleum containing wastes. PAHs are dispersed in the environment through run-off, industrial

and sewage discharges, and atmospheric input/fallout (Jorgensen *et al*. 2008). One reason that PAHs are a cause for concern is their apparent ability to cause mutagenic and carcinogenic activity within animals inducing, for example, chromosome aberrations and liver cancer in fish (Connell 2005; Jorgensen *et al*. 2008). However, the degree of effect and the mechanism of action of these organic contaminants on animals is complex and presently not well understood (Connell 2005).

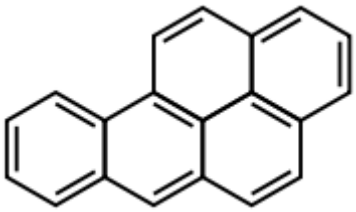

Figure 1.2 An example of a PAH structure (Benzo (a) pyrene)
(Source: Morrison and Boyd, 1987)

Polychlorinated biphenyls (PCBs, an example of the structure is shown in Figure 1.3) have in the past been used extensively for insulating and cooling electrical equipment, particularly in transformers, large capacitors, and fluorescent lamp ballasts because they are relatively chemically inert, non-flammable and have a low electrical conductivity. PCBs have in the past also used in paints, adhesives, sealants, pesticides, plasticisers, lubricants, inks and carbonless copy paper. Most of these former uses of PCBs are presently considered illegal or are strictly regulated (Ishikawa *et al*. 2007; Macken *et al*. 2008). However, PCBs can still be released into the environment from their illegal use, their inadvertent generation during certain production/recycling processes, and from storage and disposal facilities. Generally, the acute toxicities of PCBs are considered to be relatively low. PCBs have a tendency to produce chronic (long term low level exposure) toxic effects (Connell 2005).

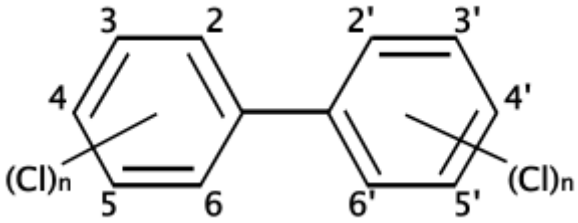

Figure 1.3 The basis of a PCB (Biphenyl) onto which chlorine atoms can bind. (Source: Morrison and Boyd, 1987)

Pesticides are organic chemicals used for the eradication of insects and other animals that interfere with human practices such as agriculture. Examples of pesticides in use, or those that have been used in the past include

- Endosulphan (active ingredient used as a broad band insecticide in many pesticide formulations for a variety of fruit and vegetables),
- Chlordane as a pesticide against ants and termites, DDT (Figure 1.4) effective but banned for most of its former uses although still used in some parts of the world for mosquito control and
- Aldrin (with its breakdown product Dieldrin, both of which are banned), used in the past to control locusts and mosquitoes, as a wood preservative, and for termite control.

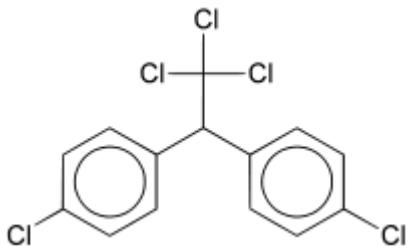

Figure 1.4 An example of a pesticide is Dichlorodiphenyltrichloroethane (DDT) (Source: Finar, 1967)

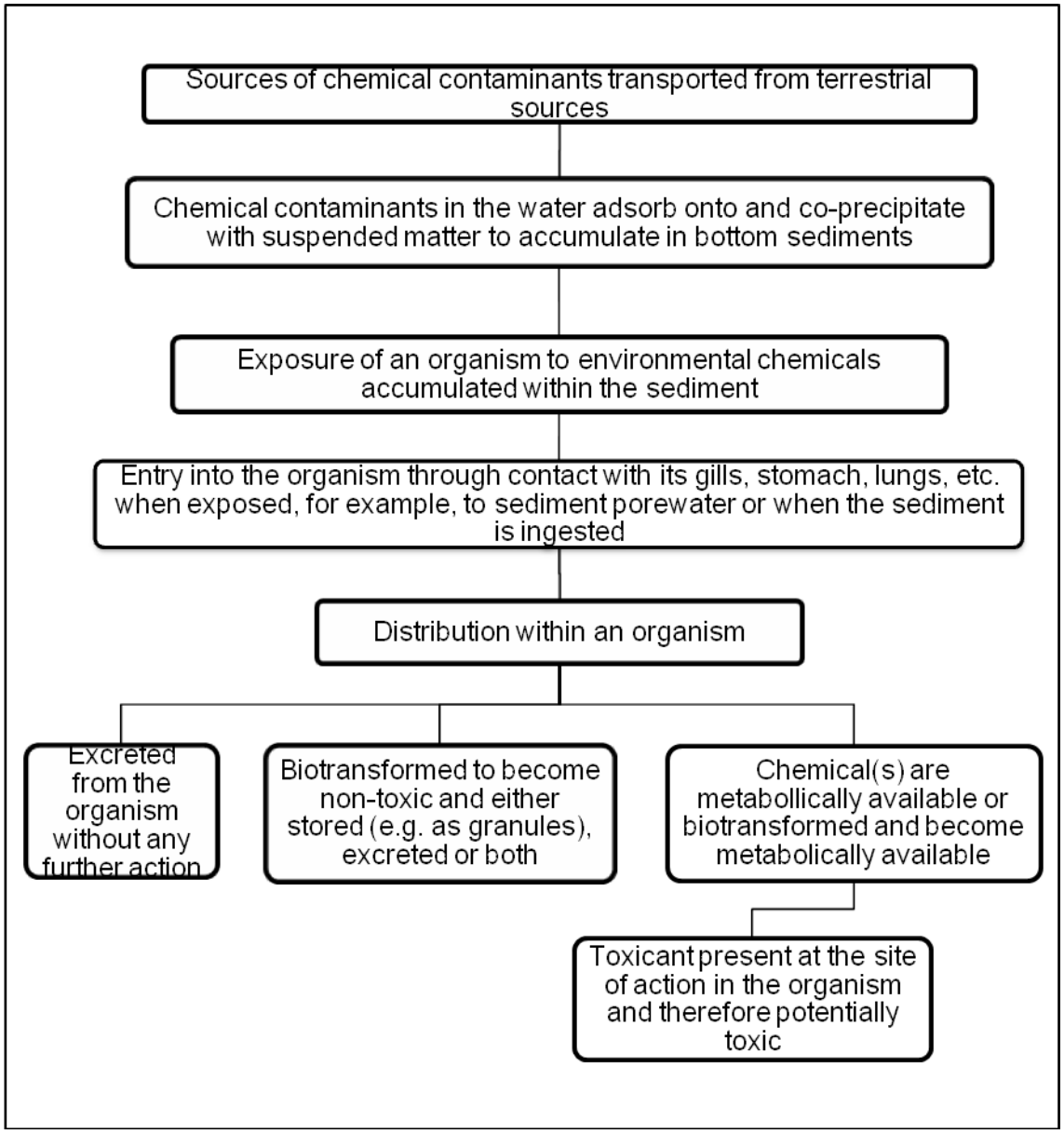

Figure 1.5 A generalised transfer processes of a bioavailable chemical from the environment and sediments to the site of potential toxic action within an organism (adapted from Rainbow 2002 and Connell 2005).

Polychlorinated hydrocarbon pesticides generally tend to be relatively resistant to attack by abiotic and biotic agents in the environment and, as a result, degradation proceeds at a slow rate with half lives sometimes lasting many years. Despite their relatively low aqueous solubility, members in this group of chemicals tend to be very toxic to aquatic non-target organisms such as fish. For example, 4, 4'-DDT has a LC50

value of between 0.40 to 89 µg/L for estuarine fish. While the mechanism of toxic action of these chemicals is unclear, they generally tend to act as neurotoxins (Connell 2005).

1.4 ORGANISMAL RESPONSE TO CHEMICAL CONTAMINANTS

Contaminants released both naturally and anthropogenically into the environment need to be present in a bioavailable form before they will exert any deleterious influence on plants and animals which are exposed to them (Fent 2004). For aquatic organisms, the predominant routes of exposure will tend to be contaminants dissolved in water, suspended particulate matter and other sources of food (Figure 1.5) (Eggleton and Thomas 2004; Fent 2004; Rainbow 2007; Macken *et al*. 2008). These other food sources, especially for deposit feeders such as polychaetes that tend to reside in enriched sediments, will include ingestion of sediment from which adsorbed nutrition can be extracted before the sediment passes out of their bodies (Jorgensen *et al.* 2008). The rate of uptake of these bioavailable contaminants will depend on the extent of organism exposure (e.g. acute or chronic exposure), low or high bioavailable contaminant concentrations (which will be influenced by factors including water and sediment physicochemical properties), exposure routes including contact with skin, gills or internal body surfaces, and mechanism of uptake including passive and/or facilitated diffusion (Eggleton and Thomas 2004; Fent 2004).

Once taken up by the organism, both essential and non-essential or xenobiotic compounds in a metabolically available form can potentially exert a toxic effect unless the organism can physiologically respond to prevent this. Most organisms possess mechanisms that are triggered on detection of toxic levels of chemicals, for example, the induction of enzymes from the Cytochrome P450 family in the case of organic compounds (Jorgensen *et al*. 2008). Biotransformation in this case may involve metabolising these compounds into more water soluble forms that can be excreted by the organism (Jorgensen *et al.* 2008). In the case of excess quantities of bioavailable metals, organisms often biotranform these into inert granules that can be stored in the body and, in some cases, excreted (Rainbow 2002).

The extent to which an organism can cope with potentially toxic contaminants will depend on the rate of uptake into the organism as well as the rate of biotransformation

into a less or non-toxic form and excretion. If the rate of uptake is greater than the rate of biotransformation and excretion then concentrations of the contaminant could reach levels that induce sub-lethal or lethal toxic effects. Conversely, if the rate of uptake is less than the rate of biotransformation and excretion then concentrations may not reach toxic levels or toxic levels could decrease to a point below a no-effects concentration.

The methods and rate of uptake, biotransformation, storage and excretion of contaminants will depend upon the individual organism. Studies have clearly shown that there are differences in these rates which can be both intra- and interspecific (e.g. Rainbow 2002; Jorgensen *et al.* 2008). This is the case because no fixed rule exists that defines the rate of uptake, method and rate of ingestion and biotransformation and/or storage and/or excretion between individuals and/or groups of organisms. Thus, each organism's response will be unique depending on the concentrations of bioavailable contaminants, the organism's contaminant coping mechanisms and the physicochemical conditions present at the time of exposure.

1.5 SEDIMENT QUALITY TESTING AND GUIDELINES

When contaminated waters enter into a sheltered water body such as an estuary, it generally tends to slow down allowing suspended matter and sediments that have been transported in them to settle and deposit onto bottom sediments. These deposits may contain co-precipitated chemical contaminants that accumulate on the bottom sediments to levels that could potentially cause adverse impact on the local flora and fauna (Chapman and Mann 1999; Ho *et al.* 2002; Beiras *et al.* 2003; Losso *et al.* 2004; Walker *et al.* 2006).

The Port of Durban and other ports in South Africa dredge sediment from shipping channels to ensure the safe passage of ships. However, because of the vicinity of the Port of Durban to the CBD, industry and other anthropogenic inputs, the sediments from many areas contain high levels of contaminants. Nevertheless, sediments need to be dredged and, if they meet the criteria set by the relevant government department, they are disposed of within the sea disposal site offshore of Port entrance. The South African government is a signatory to the London Convention of 1972 that regulates the international practice of dumping of dredged spoil at sea. This membership and

subsequent South African government policy and legislation require the monitoring of sediment quality before sediments are dredged and disposed. The purpose of this monitoring is to assist in ensuring that the dredged sediments do not cause a deleterious impact in the receiving environment.

In determining whether sediment quality is acceptable, the potential of chemical contaminants to cause adverse impact on the local flora and fauna needs to be assessed (Chapman and Mann 1999; Ho *et al.* 2002; Losso *et al.* 2004; Walker *et al.* 2006). Whether chemical contaminants present within sediments have any deleterious effect on the environment is an important question to answer in trying to understand sediment quality. In this respect, interpretive tools or guidelines have been developed to aid in the assessment of potential chemical ecological hazards and in some cases to also assist in the identification of the probable sources of contamination. Derived from different approaches, these tools or guidelines can provide useful information to environmental scientists and managers (Ehlers and Loibner 2006; Fang *et al.* 2005; Long *et al.* 1998; Newman and Watling 2007).

One interpretive tool for assessing chemical analytical results conducted on contaminated sediments are sediment quality guidelines (SQGs) (O'Connor and Paul 2000) or sediment quality values (SQVs) (Chapman and Mann 1999). SQGs/SQVs have predominantly been developed using two approaches, namely the empirical and the mechanistic (Vidal and Bay 2005). Empirical guidelines or values are derived from the statistical analysis of correlated sediment chemistry and toxicology data to identify chemical concentrations associated with various levels of biological effects. Mechanistic models, while also evaluating sediment chemistry and toxicology data, have been developed by incorporating additional factors governing contaminant equilibrium partitioning (e.g. sulphides and their binding of metals which is also dependent on additional factors such as pH and the dissolved oxygen concentration) that affect the bioavailability of adsorbed chemical contaminants (Vidal and Bay 2005). Whatever the method of derivation, SQGs/SQVs are currently widely used to help assess the chemical quality of sediments. The question to be answered, however, is whether the information gained from using SQGs/SQVs is sufficient to accurately determine the sediment quality and whether any potentially negative impacts on the surrounding flora and fauna could occur. O'Connor and Paul (2000) are critical and warn that chemical data by itself and comparison to SQGs/SQVs should not be used to

predict hazards except perhaps in cases of extreme contamination. Another precaution is that SQGs/SQVs are not universally applicable because natural background chemical concentrations differ from one area to another (Newman and Watling 2007). Therefore, the use of SQGs/SQVs should perhaps be limited to areas with similar background concentrations and/or specifically developed for the area of application.

Appropriately selected and applicable SQGs/SQVs can, however, be valuable first tools for interpreting whether contaminant concentrations could negatively impact surrounding organisms. Unfortunately, South Africa has not yet developed its own SQGs but has rather adopted SQGs, an example would be those developed by Long *et al.* (1995) to interpret contaminant concentrations. The assumption that these SQGs, which were developed for areas of the United States of America (Long *et al*. 1995), are applicable to sediments in the Port of Durban is clearly a risk. One reason for this is that natural geology will vary between different geographical regions. In addition, the potential impact of contaminants on flora and fauna might vary from region to region due to the differing sensitivities of the local plants and animals (Paquin *et al*. 2000; Eggleton and Thomas 2004). Therefore, local SQGs developed considering local sediment characteristics and flora and fauna sensitivities would provide more appropriate guideline values to assist with determining the potential negative impact of contaminants in the local sediments (Vidal and Bay 2005).

1.6 TOXICITY IDENTIFICATION AND EVALUATION PROTOCOL

From the above discussions it is clear that the chemical analysis of sediment samples alone will provide incomplete information regarding contaminant bioavailability. Further, comparing these chemical results to general SQGs/SQVs may yield useful but inappropriate information with regards to the biological effects of the chemical contaminants in a particular site. Due to these shortcomings, other methods of analysis have been developed to assist in establishing the magnitude and nature of any effect to an organism when it is exposed to one or more chemicals over time (Kwok *et al.* 2005; Walker *et al.* 2006). One such method is toxicity testing, which can be a valuable tool used to identify whether chemical contaminants in a sample could have a deleterious effect on biota in the environment (Beiras *et al.* 2003; Losso *et al.* 2004).

At the Council for Scientific and Industrial Research (CSIR) in Durban, South Africa, with the need for a simple, reliable, sensitive and rapid indication of toxic responses to chemical contaminants, an acute toxicity testing procedure was developed using the gametes of South African species of sea urchins (Airey 1989). Two species are used: *Echinometra mathaei* (a summer spawning species); and *Tripnuestes gratilla* (a winter spawning species). The toxicity tests are conducted by extracting and exposing sperm to contaminated water for a fixed period and then adding sea urchin eggs and allowing time for fertilisation of those eggs to occur. When the eggs are examined and compared to a reference sample consisting of uncontaminated water, if any statistically significant impedance of fertilisation occurs, the contaminated sample can be classified to have a degree of toxicity. Sea urchin toxicity tests similar to this are recognised worldwide as being pertinent tools in the determination of toxic effects of contaminated waters (Losso *et al.* 2004).

Additional procedures, developed in conjunction with toxicity testing include toxicity identification and evaluation (TIE) procedures. These procedures use toxicity tests with the separation of various treated fractions of the contaminated water to help understand whether sediments and their porewaters are toxic and if they are what the potential causes of the toxicity could be. Beginning in the late 1980s, the United States Environmental Protection Agency (USEPA) began developing TIE methods to identify toxic contaminants in complex samples (Kwok *et al.* 2005). The focus of TIE development was to expand on the number of methods that were available to identify the probable causes of toxicity. In essence, the TIE procedure involves the treatment or manipulation of toxic sediments and/or their porewater to aid in isolating the various groups of toxic chemical compounds present (Ho *et al.* 2002; Kwok *et al.* 2005; Macken *et al.* 2008).

At the beginning of the testing procedure and after each treatment, the resultant toxicity of the sample porewater is tested. If sediment or its porewater is not toxic at the beginning of the procedure, then no further testing would be required. However, if toxicity is detected, a series of further treatments and toxicity tests can be conducted to help isolate the most probable group or groups of chemical compounds causing the observed toxicities (Science Application International Corporation 2003; USEPA 2007). In this way, TIE procedures provide two essential pieces of information that chemical tests and comparison of the results to SQGs cannot. Firstly, they indicate whether

sediment porewater is toxic to the test organism which is usually chosen as an environmental indicator. Secondly, TIE procedures provide an indication of the potential causes of the toxicity. As this information is generally the most pertinent reason for initial sediment quality analysis, it is an important reason why TIE procedures could be valuable in making more informed decisions with regards to the assessment and handling of potentially contaminated sediment.

1.7 RATIONALE FOR THIS RESEARCH

Methods for TIE have been applied in many areas of the world to assess the degree of contamination in sediments and their porewaters (Ho *et al.* 2002; Science Application International Corporation 2003; Kwok *et al.* 2005; USEPA 2007; Macken *et al.* 2008). However, in the South African coastal environment, no local SQGs have been developed to assess chemical results obtained. In addition, protocol on toxicity testing of contaminated marine sediments and porewaters do not exist and where they are requested, are applied in a fairly haphazard manner. Background models for metals from some areas of the South African coast (e.g. south-eastern Cape coastline of South Africa) have been developed and can be used to assist in the interpretation of metal results obtained from analysis of sediments collected in these regions (Newman and Watling 2007). However, more research is required and additional tools need to be developed in South Africa to assist in the toxicity assessment of contaminated sediments and their porewaters.

In this context, the aim of this research is to investigate and apply these internationally recognised TIE procedures together with toxicity testing in the analysis of local toxic sediment porewaters and to assist in the determination of the potential chemical contaminants that may cause this toxicity.

1.8 OUTLINE OF THE DISSERTATION

In chapter 2, the experimental methods and materials used are detailed, including details of the sampling site within the Port of Durban, the extraction of porewater from sediment, the TIE procedure performed, the toxicity testing protocol, and the methods used to analyse the data.

Chapter 3 details the results of the experiments performed while chapter 4 discusses these. In this chapter an attempt is also made to integrate the findings of this research and arrive at recommendations with regards to the potential future use of toxicity tests and TIE procedures as sediment quality assessment tools for assisting in the management of contaminated sediments as well as suggesting potential future research ideas and strategies.

Chapter 2

Materials and methods

2.1. SAMPLING SITE SELECTION

Not all sediments that are chemically contaminated actually cause adverse biological effects. Whether toxic effects are manifested depends on the contaminants being in a bioavailable form (Fent 2004; Ehlers and Loibner 2006). Chemicals that have been rendered inactive by, for example, being tightly bound to sediment particles, are not available to cause toxic effects.

In order to determine whether bioavailable and toxic chemicals were present in the sediment, sea urchin fertilisation toxicity tests, described in greater detail later in this chapter, were conducted on porewaters from sediments that had previously been shown to have high concentrations of chemical contaminants (Leuci 1998; Parsons 2005). The results of these tests were used to select a sampling site for further investigation of toxic sediment porewaters by Toxicity Identification and Evaluation (TIE).

2.2 SEDIMENT SAMPLING

2.2.1. Intra-site variability

During a pilot study to determine a suitable sampling site to conduct this research, a high level of variation in porewater toxicity was evident between replicate sediment samples (grabs) taken from the same site in the Silt Canal of the Port of Durban (S29°54'00.4", E31°0'22.0"; Figures 2.1 and 2.2)). The probability of high variation occurring within a small spatial range of a non-turbulent aquatic environment was assumed to be low and possibly indicated that sampling errors or changes occurring to the samples following collection might exist. In response, the sample collection and handling procedure described in section 2.2.2 below was designed to assist in identifying possible reasons for the observed variability.

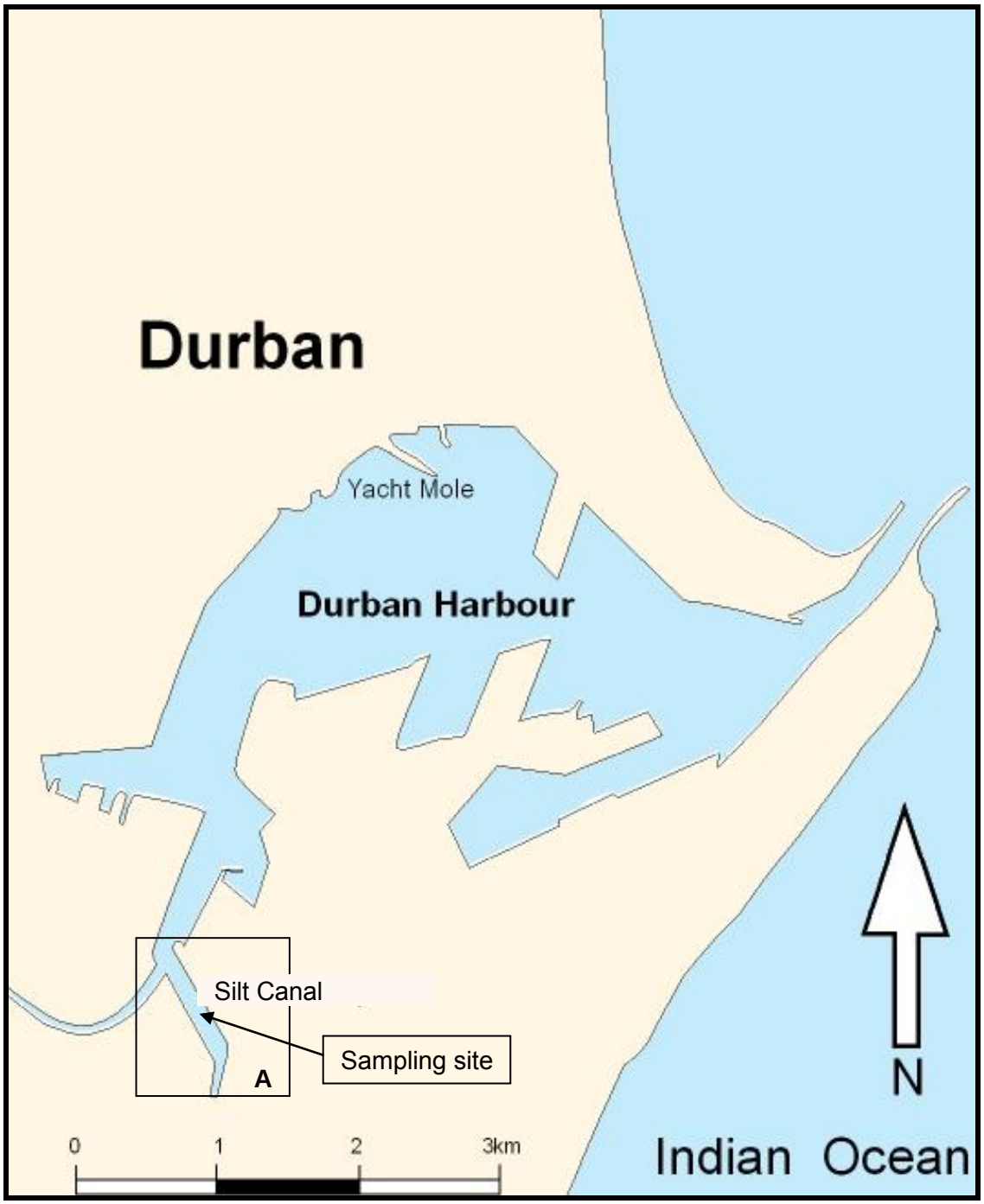

Figure 2.1 Location of sampling site within the Port of Durban. Area A is enlarged on the next page.

Figure 2.2 Location of the sediment sampling site in, and inputs from the Mhlatuzana, Umbilo and Manzimnyama canals into the Silt Canal in the Port of Durban (from Google Earth, 04/12/2009

2.2.2. Sample collection

Sediment was collected on a number of occasions (see Chapter 3 for collection dates) from a site in the Port of Durban (the Silt Canal (S29°54'00.4", E31°0'22.0"; Figures 2.1 and 2.2)). Three replicate sediment samples were collected on each sampling occasion, using a stainless steel Van Veen grab (surface area approximately 400 cm^2, Volume approximately 3100 cm^3).

The first sediment sample was transferred from the grab using a stainless steel spoon into three separate labeled 1 L Schott glass bottles. The containers were filled to the brim with sediment to exclude air and sealed to limit oxidation of the sediment and porewater. Each bottle was then covered in aluminium foil to minimise exposure to light, and held in a cool box until return to the laboratory. Two further sediment samples were taken in the same manner and similarly treated. Thus, a total of nine sediment sub-samples were collected. During sampling, appropriate observations and photographic records of the visual appearance of sediment samples as well as variations between grabs (e.g. depth of grabs) were recorded. Once back in the laboratory, samples were stored at approximately 4°C until further analyses.

2.3. EXTRACTION OF POREWATER

On the day that toxicity tests were conducted, sediment porewater was extracted from sediment by centrifuging. This was carried out by filling each of four clean centrifuge tubes with approximately 250 g of sediment and centrifuging for 20 minutes at 2000 revolutions per minute. Note that laboratory procedures for cleaning laboratory ware include washing with a phosphate free detergent, rinsing with tap water, soaking in dilute nitric acid (approximately 10%) and finally rinsing thoroughly with deionised water (with a resistivity of approximately 14 to 18 Ohm.cm).

To ensure a similar storage time for each grab sample before centrifugation, sub-samples were centrifuged using a staggered approach. To illustrate, the centrifuging process took approximately 30 minutes per sub-sample and centrifuging was conducted so that sample 1A was centrifuged after 30 minutes, sample 2A after 60 minutes, sample 3A after 90 minutes, sample 2B after 120 minutes and so forth. Table 2.1 demonstrates this sequence for all sub-samples of each grab and indicates that the

resulting total storage time before centrifugation for each sediment grab sample was similar when this approach was adopted. As such, the procedure was designed to account for any potential variability in toxicity between grab samples caused by the difference in storage time before centrifugation.

Table 2.1 Porewater extraction strategy

Grab number and replicate	A	B	C	Total time
Grab 1	(i) 30 minutes	(vi) 180 minutes	(viii) 240 minutes	**450 minutes**
Grab 2	(ii) 60 minutes	(iv) 120 minutes	(ix) 270 minutes	**450 minutes**
Grab 3	(iii) 90 minutes	(v) 150 minutes	(vii) 210 minutes	**450 minutes**

The porewater, which ranged in volume from about 30 to 60 mL per centrifuge tube, present as the supernatant, was removed from the centrifuge tubes using a clean glass pipette and transferred to a clean glass beaker. Following extraction, the salinity and pH of the porewater was measured using a model 340i WTW multimeter and TetraCon 325 salinity electrode and Metrohm 691 pH meter and glass pH electrode respectively. The remaining porewater was then decanted into 250 mL clean Schott glass bottles until TIE procedures were conducted. The total volume of porewater extracted from about 1 L of sediment was approximately 250 mL. To minimise any potential oxidation of porewater, the bottles were filled to the brim and capped tightly, leaving as little headspace as possible. Further, the bottles were wrapped with aluminium foil to minimise exposure to light. Porewaters were then stored in a refrigerator at approximately 4°C usually for no more than a few hours until toxicity tests were begun.

2.4. THE TIE PROCEDURE

The procedure developed by the USEPA (2007) and described in Figure 2.3 provides a pathway for evaluating the causes of porewater toxicity. The first step of the TIE procedure was to conduct an initial toxicity test on the porewater to determine whether it was toxic. Porewater that was not toxic requires no further testing. However, if toxicity was detected, the question to be answered by conducting the TIE procedures would

be: “What is the probable cause of toxicity in the sample” (USEPA 2007). Thus, once porewater toxicity was determined the treatments detailed in the TIE procedures (Figure 2.3) could be implemented, with their purpose being to assist in the investigation and characterisation of the most probable chemical groups or species responsible for the initially observed toxicity.

As indicated in Figure 2.3, the TIE procedure involves an initial or baseline toxicity test and thereafter three possible treatments. Treatments were performed in parallel.

- A baseline toxicity test to determine the toxicity of the initial porewater sample, which will serve as a comparison after other procedures.
- A solid phase extraction (SPE) procedure aimed at removing potentially toxic non polar organic compounds
- Graduated pH adjustments to determine the sensitivity of toxicity to pH.
- The additions of EDTA to porewater in order to complex bioavailable metals and thus reduce toxicity that may be caused by specific cationic metals.

Therefore, the TIE procedure follows a systematic pathway of removing groups of potential toxicants from the porewater. After each treatment, a sub-sample of treated porewater was collected and toxicity tests were conducted to determine whether there was any alteration in toxicity. The treatments for each component of the TIE procedure are given in more detail below and the results reported in Chapter 3 of this dissertation.

2.4.1 Toxicity testing procedure

Adult sea urchins were collected from Vetch’s Pier just north of the Port of Durban entrance channel and transported to the laboratory in a bucket of well aerated seawater. Depending on the season, either *Echinometra mathaei* (Oval Urchin, a summer spawner) or *Tripneustes gratilla* (Short spined urchin, a winter spawner) were collected. In the laboratory, sea urchins were kept in unfiltered natural seawater in flow through tanks connected to a protein skimmer at ambient temperature until required for toxicity tests.

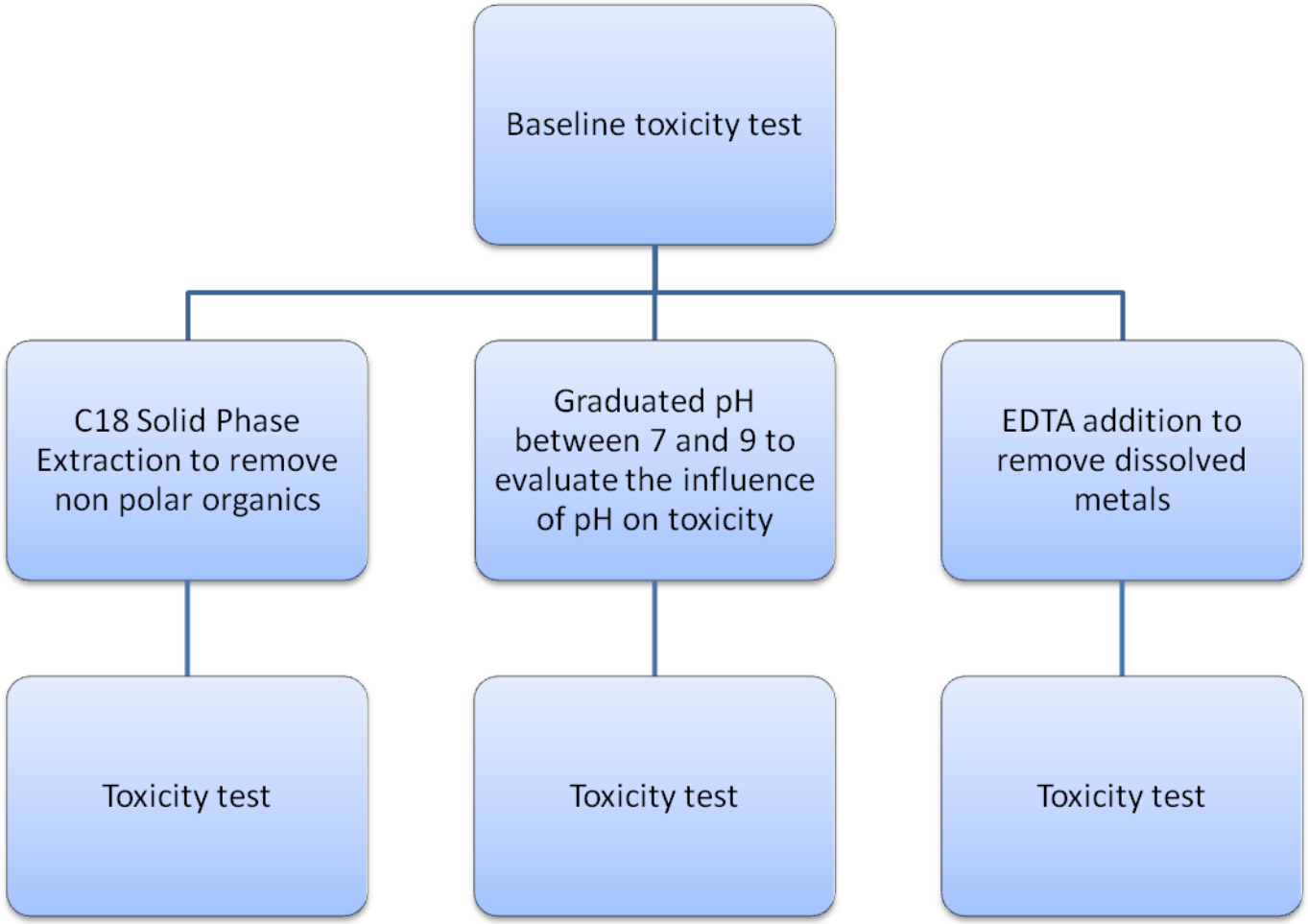

Figure 2.3 The TIE procedure (adapted from USEPA 2007).

All toxicity tests were conducted in a room with the temperature controlled at 22 ± 1ºC. In order to gather gametes for the toxicity testing procedure, approximately 1 mL of 0.5 mol/L KCl was injected through the peristomial membrane and into the perivisceral coelom of an urchin. Once spawning was induced in this manner the males became distinguishable from females, as sperm has a milky white appearance and eggs a golden brown colour. Eggs were collected by inverting the female urchins over beakers filled with seawater so that the genital pores were submerged. The eggs were allowed to settle at the bottom of the beaker. Sperm were collected dry in Pasteur pipettes. If induced spawning did not produce eggs or sperm that appeared plentiful and healthy, these gametes were excluded from the testing.

The quality of eggs and sperm was tested prior to experiments by adding diluted sperm from each male to eggs from each female in 20 mL of control seawater in test vials and allowing fertilisation to proceed for 10 minutes. Eggs were then examined under a microscope for the presence of a fertilisation membrane (Figure 2.4). Combinations of sperm and eggs that did not produce at least a 90% fertilisation success were rejected as healthy gametes were expected to have a higher fertilisation success rate.

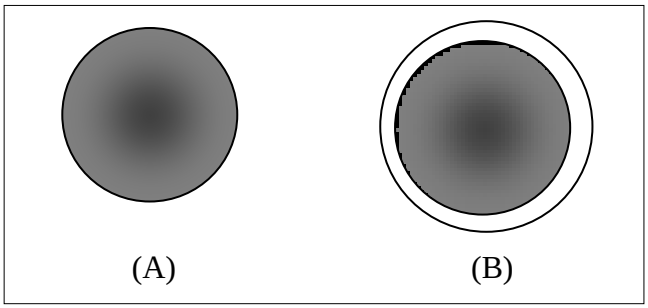

Figure 2.4 An illustration of the difference between an unfertilised egg (A) and a fertilised egg with an outer fertilisation membrane (B).

Sperm were activated by exposure to seawater. Aliquots of 100 µL of sperm suspension were then transferred either to control samples containing uncontaminated seawater or to the experimental porewater samples. A minimum of three replicates for each sample were tested and at least 10 mL of porewater was used per replicate. After ten minutes of sperm exposure, approximately 1 mL of diluted eggs were added and left for a further ten minutes, and then preserved by the addition of 100 µL of concentrated formalin (40% v/v) solution. To determine the fertilisation success in the

test solution, a sample of at least 100 eggs was microscopically examined for the presence of a fertilisation membrane (Figure 2.4). The number of fertilised and unfertilised eggs per a sample of 100 was then recorded and reported as a percentage of fertilised eggs.

2.4.2. Baseline untreated porewater toxicity

The TIE procedure began by performing a baseline toxicity test to determine if the porewater was toxic. This was conducted by sub-sampling at least three equal replicates of between 10 to 40 mL of the untreated porewater and conducting the sea urchin toxicity test on each. The exact volume of porewater used per replicate was dependent on the volume of porewater extracted, which was usually limited. An advantage of the sea urchin toxicity test was that it could be conducted in relatively small volumes of porewater unlike other tests such as amphipod toxicity tests. These sea urchin toxicity tests were conducted to provide a reference toxicity value, which was used for comparison with toxicity levels observed in subsequent treatments. Only those porewaters that inhibited fertilisation by 20% or more were considered for further testing. The 20% level was chosen as the threshold level of toxicity so that test samples could be statistically differentiated from false positive control levels that usually occur in the range of between 1 and 10%.

2.4.3 Removal of cationic metal concentrations

Ethylenediaminetetraacetic acid (EDTA), a strong chelating agent, was added to at least 30 mL of porewater. The USEPA TIE procedure (USEPA 2007) recommends a final EDTA concentration of 30 mg/L in the toxicity test solution. This was achieved by adding 50 µL of 18 g/L EDTA to 30 mL of test water (or multiples of this depending on the volume of test water treated). Upon mixing, EDTA could chelate certain cations if they were present to produce metal complexes which, although not removed from the solution before toxicity testing, were rendered non-bioavailable and hence non-toxic. In addition, toxicity tests were also conducted on control seawater that had the same concentration of EDTA added to discern that the EDTA did not itself cause toxicity.

EDTA has the ability to chelate certain cations of aluminium, barium, cadmium, cobalt, copper, iron, lead, manganese, nickel, strontium and zinc, but forms only weak

chelates with other metal cations such as silver and chromium. Thus, on comparison of the baseline toxicity results and toxicity results from the porewater treated with EDTA, any statistically significant reduction in the overall toxicity could signify the presence of EDTA complexed metal cations in toxic concentrations. It should be noted, however, that while addition of EDTA effectively reduces the toxicity of certain metals and metal mixtures, in some cases residual metal toxicity may still occur because the concentrations of metals were in excess of the complexing capacity of the volume of EDTA used.

2.4.4. Removal of Organics by solid phase extraction

This manipulation path of the TIE procedure involved eluting porewater through a Solid Phase Extraction (SPE) column to remove any potentially toxic organic compounds. Choosing an appropriate SPE column to remove as many of these compounds as possible meant that the column should contain an appropriate packing material to adsorb these from the porewater. The USEPA (2007) recommends use of a C_{18} sorbent column which tends to remove neutral non-polar compounds, including a number of hydrocarbons, polychlorinated biphenyls and pesticides. Thus by passing the porewater through the C_{18} column (Phenomenex STRATA C18-E solid phase extraction column, part number 8B-S001-HCH) and collecting and testing the elutriate for toxicity, the removal of any potentially toxic organic compounds could result in a decrease in toxicity. This was accomplished by activating the column with 5 mL each of acetonitrile, methanol and deionised water. Once the column was activated, a porewater sample was passed through it at a vacuum pump regulated column flow rate of approximately 1 mL/minute. Elutriate was collected in clean glass vials and then tested for toxicity. A new column was prepared for each replicate, and the eluted sample columns were stored at approximately 4°C for further analysis if required.

As in the earlier steps, any resulting statistically significant decrease or removal of toxicity from the porewater following this stage would indicate the presence of toxic organic chemicals in the porewater. If it was necessary to determine the identity of the organic compounds that were thought to cause the toxicity, this could be accomplished by extracting the adsorbed organic chemicals from the SPE column followed by analysis using Gas Chromatograph Mass Spectrometry (GC-MS).

2.4.5. Manipulation of ammonia and hydrogen sulphide by altering pH

The removal of ammonia by seaweed as recommended by the USEPA (2007) may not always be successful for a number of reasons, including insufficient exposure time and too high concentrations for adequate ammonia removal. This step, therefore, could fail to completely remove the ammonia and residual quantities may still cause toxicity.

An alternate/additional procedure could be conducted to facilitate a better understanding of potential toxicity caused by ammonia as well as hydrogen sulphide. This procedure involves the manipulation of the porewater pH at constant temperature. In water, ammonia exists in two forms, either as the toxic undissociated ammonia molecule (NH_3) or as the relatively non-toxic ammonium ion (NH_4^+).The manipulation of pH influences the ionic state between NH_4^+ and NH_3 as indicated by:

$$H_2O + NH_3 \leftrightarrow NH_4^+ + OH^-$$

Generally, as the pH of a solution decreases, the NH_4^+ concentration increases and the undissociated NH_3 concentration decreases.

Hydrogen sulphide, which was another chemical that could potentially cause toxicity in porewater, also exists in two forms, namely as the toxic undissociated hydrogen sulphide molecule (H_2S) and as a sulphide ion (S^{2-}) as indicated:

$$H_2S \leftrightarrow 2H^+ + S^{2-}$$

Generally, as the pH of a solution decreases, the undissociated H_2S concentration increases and the undissociated S^{2-} concentration decreases.

In this treatment of porewater to determine the potential toxicity of ammonia and/or hydrogen sulphide, the pH was adjusted to cause a change in either molecules concentration. Reducing the pH with 1 mol/L hydrochloric acid (HCl) will increase the concentration of H_2S but will decrease the concentration of NH_3. Alternatively, by increasing the pH with 1 mol/L sodium hydroxide (NaOH), the concentration of NH_3 increases while the concentration of H_2S will decrease. Toxicity tests were performed on the adjusted pH waters. If the high pH solution was determined to be more toxic,

then the assumption made was that the cause of toxicity was likely to be unionised NH_3. An increase in porewater toxicity that had been adjusted to a lower pH was assumed to indicate a toxic response probably due to unionised H_2S.

Before the manipulation of the pH as described, an experiment was conducted to determine the pH tolerance of sea urchin gametes. The results of this experiment were then used to determine to what extent the pH could be manipulated. This was accomplished by using contaminant free control seawater and adding various volumes of 1 mol/L hydrochloric acid and sodium hydroxide to adjust the pH from approximately 6.5 to 9.0. pH was measured just before the commencement of the urchin fertilisation tests using a standardised pH meter (Metrohm 691 pH meter) and glass pH electrode. The results of this experiment, presented in the Chapter 3, were then used to guide the pH manipulation process so that the pH tolerance level of the sea urchins gametes was not exceeded.

2.5 TOLERANCE TESTS

During the above experiments, it was determined that unionised hydrogen sulphide and ammonia could be produced naturally by the anaerobic biological decomposition of organic material. Thus, because toxic levels of naturally occurring chemical substances might cause the „*masking*' of the toxicity of other anthropogenic contaminants, further experiments to assist in determining what influence unionised hydrogen sulphide and ammonia have on the urchin fertilisation tests were conducted

Test solutions of total ammonia (NH_3 + NH_4) were prepared by dissolving analytical grade ammonium chloride (NH_4Cl, molar mass = 53.492 g/mol) in contaminant free seawater. Concentrations of total ammonia in the range of 0 to 60 mg/L were prepared at a pH of 8.1 ± 0.1. Ammonia concentrations were verified using a Bran and Luebbe AA3 Autoanalyser. These solutions were then tested for toxicity according to procedures described previously.

Test solutions of total sulphide (H_2S + HS^- + S^{2-}) were prepared by dissolving analytical grade sodium sulphide ($Na_2S.9H_2O$, molar mass = 240.784 g/mol) in contaminant free sea water. From this solution, working solutions in the range of 0 to 4 mg/L total sulphide (pH of 8.1 ± 0.1) were prepared for toxicity testing. As hydrous sodium

sulphide was not a primary standard, the concentrations of the working solutions were determined by colourimetric analysis using the Merck sulphide test kit. Generally, because of the volatility of H_2S it is prudent to determine its concentration at least at the start and end of the toxicity test. However, because of the relatively short toxicity testing time of 20 minutes, this determination was not conducted at the end of the experiment.

2.6. CHEMICAL ANALYSIS

The purpose of analysing for sediment and porewater chemical parameters was to gather information that could be used to better understand the toxicity results and/or any variability noticed in the toxicity results obtained. Additional analysis including dissolved nutrients such as ammonia, nitrate, nitrite and orthophosphate were determined by automated segmented flow colourimetric analysis using a Bran and Luebbe AA3 Autoanalyser. Sediment particle size and total organic material analysis were determined by wet and dry sieving techniques and hydrogen peroxide digestion respectively.

2.7. DATA ANALYSIS

The percentage of fertilised eggs was considered when evaluating the toxicity of seawater control samples, baseline porewater and subsequent TIE procedures. Statistics performed on this data included a test for normal distribution and equal variance (passed if $P>0.05$; this P value indicated the probability of being incorrect in concluding that the data was not normally distributed (using the Kolmogorov-Smirnov test for a normally distributed population) or of equal variance (by checking the variability of the group means)) followed by one way analysis of variance (ANOVA) using the Holm-Sidak test to compare multiple samples to a baseline or control. If the data was not normally distributed, statistical significance was determined by one way ANOVA on ranks by Dunn's method. Statistical significance for the one way ANOVA analyses was inferred when $P<0.05$, i.e. where the probability that the multiple comparison did not detect a significant difference was less than 5%. When comparing chemical data between samples, one way ANOVA was performed unless the data was not normally distributed. If the test for normal distribution failed (i.e. $P<0.05$), a Kruskal-Wallis one way ANOVA on ranks was performed. In addition, to compare the observed

toxicity with the physico-chemical parameters measured, a Pearson product moment correlation was conducted on the chemical and toxicity data. Statistical significance in the Pearson correlation analyses was also inferred when the probability of the correlation occurring by chance was less than 5% (i.e. when $P<0.05$). The smaller the P value reported was, the greater the probability that the variables were correlated. The statistical analyses described above were performed using SigmaStat 3.1 and SigmaPlot 9.0 (Systat Software Inc, San Jose, CA, USA). The effects concentration (EC) values for all chemicals were calculated using REGTOX-EV6.xls (Eric Vindimian http://eric.vindimian.9online.fr), a curve fitting macro developed for Microsoft® Excel.

Chapter 3

Results

3.1. TOXICITY TESTS: INITIAL RESULTS

Samples for these experiments and all other samples for this research were collected at a site in the Silt Canal located at latitude S29°54'00.4", and longitude E31°0'22.0" (Figure 2.2). Initial toxicity tests were conducted to determine the toxicity of the sediment porewater and thus suitability to conduct further TIE procedures. These results are given in Table 3.1.

Table 3.1 Porewater[$] toxicity (presented as mean ± S.D.) as measured by the fertilisation success of *Tripneustes gratilla* (Short-spined urchin). Each grab sample toxicity value was derived from the mean of four toxicity test replicates.

	% Fertilization
Control	98.5 ± 0.58 (n=1)
Grab 1	0 ± 0.00* (n=1)
Grab 2	97.5 ± 0.58 (n=1)
Grab 3	99.0 ± 0.82 (n=1)

[$] Sampled July 3rd, assayed July 18th, 2007

* Determined to be statistically significantly different (P>0.05) from the control sample using one way ANOVA followed by the Holm-Sidak test (normal distribution and equal variance tests both satisfied, i.e. P>0.05).

Porewater extracted on the 18th of July 2007 from the first sediment sample was toxic and prevented any fertilisation of sea urchin gametes from occurring (Grab 1, Table 3.1). However, these results also indicated an unexpected variability in toxicity even from sediments sampled from fundamentally the same area (Grabs 2 and 3, Table 3.1). Results presented in Table 3.2 from sediment sampled on the 6th of September 2007 and its porewater analysed on the 11th of September 2007 indicated similar between

sample variability, with the porewater from two sediment grab samples being toxic and the third being non toxic. This clearly indicated the need to conduct further investigations in an attempt to understand the potential causes of the observed grab sample variability.

3.2 VARIABILITY EXPERIMENTS

Sediment samples from the Port of Durban on the 6th of September 2007 were collected to investigate the variability in grab sample toxicity and what the potential causes were. The depth of the water column at this site at the time of sampling was approximately six meters and the sediment sampled was a soft and silty black mud (Figure 3.1). Once taken, the samples were kept cool and out of sunlight in a cooler box until they were brought back to the Laboratory and stored at approximately 4°C. On the 11th of September 2007, all the porewaters were extracted as per the sequence presented in Table 2.1. Once extracted, toxicity tests and various physico-chemical analyses were conducted on the same day. These results are recorded in Table 3.2. In addition to these tests, particle size and total organic material (TOM) analyses on the sediments were also conducted. The results of these analyses are presented in Table 3.3.

Figure 3.1 An example of the sediment sampled from this site in the Port of Durban.

The toxicity tests indicated that two of the three grab sample porewaters were toxic (Table 3.2). However, intra-grab variability in toxicity was not observed as indicated by the small standard deviations between replicate toxicity tests (Table 3.2). Apart from the statistically significant difference determined between the pH of Grab 1 and 2 (Table 3.2), the physical and chemical analyses conducted on the porewater did not immediately indicate any compelling reason why this variability in grab sample toxicity existed. In an attempt to determine the potential reasons for any inter-grab variability in toxicity, all toxicity and physico-chemical variables measured were subjected to correlation analysis using all the individual replicate data items in the data set. Pearson Product Moment Correlation was performed to establish whether any statistically significant correlations between the measured variables and toxicity existed. The correlation coefficients (r) and probabilities (P) are presented in Table 3.4 and the corresponding scatter plots are presented in Figure 3.2.

Table 3.2 Physico-chemical characteristics of porewater[$] from the Port of Durban and concomitant porewater toxicity (presented as mean ± S.D. n = number of grab sample replicates) as measured by the fertilisation success of *Tripneustes gratilla.*

	Toxicity Tests	**Total Ammonia (NH_3 + NH_4)**	**Unionised Ammonia**	**Ortho Phosphate**	**Nitrite**	**Nitrate**	**pH**	**Salinity**
	%Fertilisation	mg/L as N	mg/L as N	mg/L as P	ug/L as N	ug/L as N		
Control	97.7 ± 0.58 (n=1[#])	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
Grab 1	97.8 ± 0.44 (n=3[#])	39.2 ± 6.61 (n=3)	2.0 ± 0.31 (n=3)	12.7 ± 4.34 (n=3)	14.0 ± 9.54 (n=3)	52.3 ± 1.53 (n=3)	8.01 ± 0.02[¤] (n=3)	33.6 ± 0.29 (n=3)
Grab 2	0.11 ± 0.33* (n=3[#])	37.7 ± 0.69 (n=3)	2.3 ± 0.14 (n=3)	16.5 ± 9.87 (n=3)	8.3 ± 1.15 (n=3)	80.0 ± 46.7 (n=3)	8.10 ± 0.02[¤] (n=3)	33.8 ± 0.00 (n=3)
Grab 3	0.11 ± 0.33* (n=3[#])	36.9 ± 1.22 (n=3)	2.0 ± 0.07 (n=3)	17.3 ± 2.95 (n=3)	10.7 ± 2.89 (n=3)	58.0 ± 29.1 (n=3)	8.04 ± 0.03 (n=3)	33.9 ± 0.06 (n=3)

N.M. = not measured

[#] Each replicate was derived from three toxicity test replicates

[$] Sampled September 6th, assayed September 11th, 2007

* Determined to be statistically significantly different from the control sample using one way ANOVA on ranks by Dunn's method.

[¤] Determined to be statistically significantly different from each other by one way ANOVA (normal distribution and equal variance passed i.e. P>0.05). One way ANOVA analysis (normal distribution and equal variance all having P>0.05) comparing between grab variation for each of the other porewater physico-chemical variables indicated no statistically significant difference between the grab samples (i.e. P<0.05).

Table 3.3 Sediment particle size characteristics[$¤] expressed as a percentage and presented as mean ± S.D.

Size (mm)	<2.0	1.0-2.0	0.5-1.0	0.25-0.5	0.125-0.25	0.063-0.125	<0.063	
								%TOM[#]
Grab 1	0 ± 0.00 (n=3)	0 ± 0.00 (n=3)	0.05 ± 0.09 (n=3)	0.49 ± 0.31 (n=3)	2.07 ± 1.47 (n=3)	1.14 ± 0.73 (n=3)	96.2 ± 2.50 (n=3)	6.03 ± 1.12 (n=3)
Grab 2	0 ± 0.00 (n=3)	0 ± 0.00 (n=3)	0.10 ± 0.06 (n=3)	0.56 ± 0.28 (n=3)	1.60 ± 0.72 (n=3)	1.04 ± 0.44 (n=3)	96.7 ± 1.41 (n=3)	5.99 ± 0.79 (n=3)
Grab 3	0 ± 0.00 (n=3)	0 ± 0.00 (n=3)	0.03 ± 0.06 (n=3)	0.28 ± 0.34 (n=3)	1.20 ± 0.70 (n=3)	0.69 ± 0.16 (n=3)	97.8 ± 1.23 (n=3)	6.41 ± 1.16 (n=3)

[$] Sampled September 6th, 2007.

[#] %Total organic material

[¤] One way ANOVA analysis (normal distribution and equal variance passed, i.e. P>0.05) comparing the between grab variation within each sediment size class indicated no statistically significant difference between the sediment samples (P<0.05).

From these results, statistically significant correlations between nitrite and salinity (r = -0.856, P = 0.003), percentage fertilisation and pH (r = -0.695, P = 0.038) and between pH and unionised ammonia (r = 0.738, P = 0.028) were indicated (Table 3.4, Figure 3.2). Other potentially statistically significant correlations were detected between unionised ammonia and nitrite (r = -0.661, P = 0.052) and between percentage mud and percentage total organic material (0.638, 0.064). When considering the scatter plots (Figure 3.2), it can be seen that one of the reasons for the significant negative correlation observed between porewater nitrite concentrations and salinity was the datum point reflected in the lower right hand corner of Figure 3.2 (c) i.e. at high NO_2 and low salinity. Removing this result from the scatter plot changes the correlation coefficient to from -0.856 to 0.676. Therefore, while this result may be valid, without additional supporting data to confirm the association between nitrite and salinity it might be wrong at this stage to assume a strong negative correlation. With regards to the pH and unionised ammonia correlation, a review of the scatter plot indicated that this relationship was possible (Figure 3.2 (b)). This was not surprising as unionised ammonia was calculated using the pH and the total ammonia concentration, and this relationship is such that as pH increases, so does the concentration of unionised ammonia. The correlation between percentage fertilisation and pH was difficult to interpret (Figure 3.2 a) as toxicity was either no different from the control (Grab 1) or almost completely toxic (Grabs 2 and 3,Table 3.2). Thus, there were no results for pH between 1 and 97 percent fertilisation to verify this correlation. However, two data observations may support the correlation. The first was that a significant difference was determined between the higher pH of the toxic second grab sample compared to the lower pH of the non-toxic first grab sample (Table 3.2). The second was the higher mean pH of 8.07 for the toxic samples compared to the lower mean pH of 8.01 for the non toxic sample. Both these observations tend to indicate that as pH increased so the percentage fertilisation decreased (i.e. toxicity increases). However, because of the lack of data points along the length of the scatter plot, it would be difficult to conclusively state that the correlation between pH and percentage fertilisation was statistically or biologically significant from these data.

Table 3.4 Pearson Product Moment Correlation matrix (r = correlation coefficient, P = probability, n = 9) for toxicity and other measured physico-chemical variables for analyses conducted on the 11th of September 2007.

Parameter	r and P	Total Ammonia ($NH_3 + NH_4$)	Unionised Ammonia (NH_3)	O-PO_4	NO_2	NO_3	pH	Salinity	%mud	%TOM[#]
% Fertilisation	r	0.268	-0.433	-0.352	0.402	-0.274	**-0.695**	-0.554	-0.294	-0.092
	P	0.485	0.244	0.353	0.283	0.475	**0.038**	0.122	0.443	0.814
Total Ammonia ($NH_3 + NH_4$)	r		0.492	-0.431	-0.334	-0.185	-0.223	0.199	0.472	0.504
	P		0.179	0.247	0.380	0.634	0.563	0.607	0.199	0.166
Unionised Ammonia (NH_3)	r			-0.069	**-0.661**	0.304	**0.738**	-0.554	0.411	0.432
	P			0.861	**0.052**	0.426	**0.023**	0.122	0.272	0.245
Ortho-Phosphate (O-PO_4)	r				0.048	0.540	0.260	-0.024	-0.320	-0.267
	P				0.902	0.133	0.499	0.952	0.401	0.487
Nitrite (NO_2)	r					0.036	-0.489	**-0.856**	-0.048	-0.203
	P					0.926	0.182	**0.003**	0.902	0.600
Nitrate (NO_3)	r						0.501	0.140	-0.117	0.055
	P						0.170	0.719	0.764	0.889
pH	r							0.474	0.096	0.102
	P							0.197	0.806	0.793
Salinity	r								0.284	0.415
	P								0.458	0.266
%mud (<0.063 µm)	r									**0.638**
	P									**0.064**

[#] %Total organic material

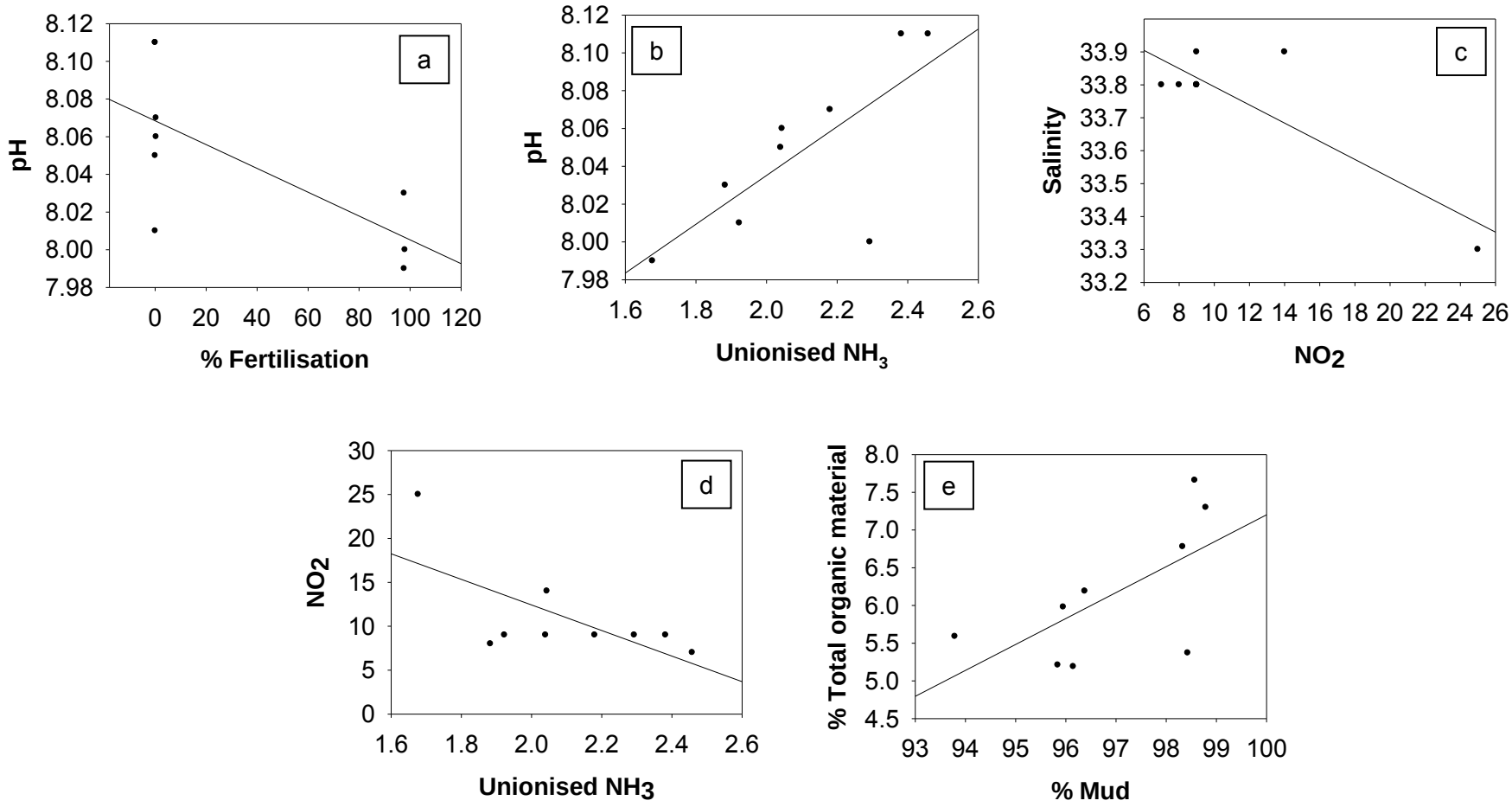

Figure 3.2 Scatter plots indicating the statistically significant correlations between variables measured in Tables 3.2 and 3.3

3.3 TIE PROCEDURES: INITIAL RESULTS

On the 22nd of April 2008 further toxicity tests were conducted on sediment porewater and all porewater samples were determined to be toxic (Table 3.5). These toxic porewaters were then subjected to TIE procedures in an attempt to investigate the potential causes of toxicity (Table 3.6) using *E. mathei* gametes.

Table 3.5 Porewater[$] toxicity (presented as mean ± S.D.) as measured by the fertilisation success of *Echinometra mathaei* (Oval urchin). Each of the grab sample replicate toxicity values was derived from the mean of three toxicity test replicates.

	Toxicity Tests
	(% Fertilisation)
Control	98.3 ± 0.58 (n=1)
Grab 1	0.33 ± 0.71* (n=3)
Grab 2	0.56 ± 0.73* (n=3)
Grab 3	0.33 ± 0.71* (n=3)

[$] Sampled April 15th, assayed April 22nd, 2008

* Determined to be statistically significantly different from the control sample using one way ANOVA followed by the Holm-Sidak test (normal distribution and equal variance tests both satisfied, i.e. P>0.05).

In Table 3.6 the control and control after treatment results (+ EDTA, at pH = 7.3 and post C18 column) indicate that the TIE procedures performed did not themselves affect the toxicity of the sample (percent fertilisations ranged from 98.0 ± 1 to 98.3 ± 0.58 (Table 3.6) and Kruskal-Wallis one way ANOVA on ranks indicated no significant difference (P<0.05) between them). The results and subsequent statistical analysis using one way ANOVA on ranks by Dunn's method (for this and the following procedures with P<0.05) also indicate that the addition of EDTA, a procedure designed to remove potential metal toxicants, did not result in a reduction in toxicity. Further, the TIE procedure that attempted to remove any toxic non-polar organic pollutants by passing the porewater through a C18 column was not successful in reducing toxicity either. In addition, adjusting the porewater pH from approximately 8 to 7.3, conducted

within the tolerance range of these sea urchin gamete fertilisation tests and designed to change the concentrations of unionised NH_3 and H_2S, was also not successful in reducing toxicity. Thus, these TIE results indicate that toxicity was not reduced during the procedures applied. It is important to note that not all the recommended procedures were followed and the reasons for this are explained in the discussion. The procedures omitted were aeration to remove volatile substances and increasing the pH to reduce the concentration of unionised H_2S. These omissions may indicate that unionised H_2S was the possible cause of toxicity in this instance.

Table 3.6 Porewater[$] toxicity (presented as mean ± S.D.) as measured by the fertilisation success of *Echinometra mathaei* before and after chemical manipulation as specified by the USEPA TIE procedures. Each of the replicate sample fraction toxicity values was derived from the mean of three toxicity test replicates. Porewater had a pH of 8.05 ± 0.02 and a salinity of 34.1 ± 0.1.

	Toxicity Tests
	(% Fertilisation)
Control	98.0 ± 1.00 (n=1)
Control + EDTA	98.3 ± 0.58 (n=1)
Control at pH = 7.3	98.0 ± 0.00 (n=1)
Control post C18 column	98.3 ± 0.58 (n=1)
Grab 1 Baseline	0.22 ± 0.44 (n=3)
Grab 1 + EDTA	0.33 ± 0.71 (n=3)
Grab 1 post C18 column	0.11 ± 0.33 (n=3)
Grab 1 to pH 7.3	0.11 ± 0.33 (n=3)
Grab 2 Baseline	1.78 ± 1.86 (n=3)
Grab 2 + EDTA	0.0 ± 0.00 (n=3)
Grab 2 post C18 column	0.22 ± 0.44 (n=3)
Grab 2 to pH 7.3	1.33 ± 1.80 (n=3)
Grab 3 Baseline	0.89 ± 1.17 (n=3)
Grab 3 + EDTA	0.11 ± 0.33 (n=3)
Grab 3 post C18 column	0.0 ± 0.00 (n=3)
Grab 3 to pH 7.3	0.0 ± 0.00 (n=3)

[$] Sampled 15th of April, assayed on the 25th of April 2008

3.4 TOLERANCE EXPERIMENTS

Considering that H_2S was suspected to be a possible cause of primary toxicity in the above investigation and as adjusting the pH was one of the procedures recommended for varying these concentrations, further pH and unionised NH_3 and H_2S tolerance experiments were conducted. In order to establish the tolerance of sea urchin gametes to a range of hydrogen ion, hydrogen sulphide and ammonia concentrations, sea urchin toxicity tests were conducted using solutions of known concentrations. The response of the urchin gametes, expressed as percentage fertilisation of eggs, was determined across a range of concentrations and the results plotted as effect concentration (EC) graphs. Hydrogen ion concentrations are presented as the negative log of the hydrogen ion concentration (pH).

Table 3.7 The effect of pH on the fertilisation success of *Echinometra mathaei*. Seawater pH was adjusted using 1 mol/L HCl or NaOH. Toxicity values at each pH were derived from the mean of three toxicity test replicates.

pH	Toxicity Tests (% Fertilisation)
7.19	97.7 ± 0.58
7.34	97.7 ± 0.58
7.51	98.0 ± 0.00
7.64	97.3 ± 0.58
7.89	98.3 ± 0.58
7.99	98.0 ± 0.00
8.20	98.3 ± 0.58
8.30	98.3 ± 0.58
8.41	97.7 ± 0.58
8.50	97.7 ± 0.58
8.61	23.7 ± 1.53
8.82	1.33 ± 0.58
8.97	0.00 ± 0.58

The pH tolerance tests were performed on both urchin species, once in March 2008 using the summer spawning Oval urchins (*Echinometra mathaei*) (Table 3.7), and once in July 2009 using the winter spawning Short-spined urchin (*Tripneustes gratilla*) (Table 3.8). *Echinometra mathaei* gametes were exposed to natural seawater pH solutions ranging from 7.19 to 8.97, and at all pHs within this range except those greater than 8.50, fertilisation was not impeded (Table 3.7). *Tripneustes gratilla* gametes were exposed to natural seawater pH solutions ranging from 6.51 to 8.82 and within this range, fertilisation was reduced at pH values of less than 8 (Table 3.8).

Table 3.8 The effect of pH on the fertilisation success of *Tripneustes gratilla*. Seawater pH was adjusted using 1 mol/L HCl or NaOH. Toxicity values at each pH were derived from the mean of three toxicity test replicates.

pH	Toxicity Tests (% Fertilisation)
6.51	0.00 ± 0.00
6.76	0.00 ± 0.00
7.02	0.00 ± 0.00
7.23	0.00 ± 0.00
7.57	0.00 ± 0.00
7.75	3.67 ± 0.58
7.98	54.0 ± 2.00
8.08	92.7 ± 0.58
8.17	99.7 ± 0.58
8.23	99.7 ± 0.58
8.27	99.0 ± 1.73
8.35	99.3 ± 0.58
8.42	99.7 ± 0.58
8.49	99.7 ± 0.58
8.60	99.3 ± 0.58
8.82	97.7 ± 0.58

With the above data on the tolerance of the different urchin species to pH, further tolerance tests using *T. gratilla* gametes were conducted using differing concentrations

of total NH_3 in natural seawater (Table 3.9). The pH of the seawater used was 8.15 ± 0.05 and at this pH the concentration of unionised NH_3 was calculated (approximately 7% at 22 ± 1°C). Effect concentration graphs were plotted and various effective concentrations were calculated (Figure 3.3; Table 3.10).

Table 3.9 The effect of ammonia on the fertilisation success of *Tripneustes gratilla*. Each toxicity value was derived from three toxicity test replicates.

		Toxicity Tests
	Unionised NH_3 concentration	**(% Fertilisation)**
Control	0 mg/L	98.7 ± 0.58
5 mg/L Total NH_3	0.35 mg/L	98.3 ± 0.58
10 mg/L Total NH_3	0.70 mg/L	98.0 ± 0.00
20 mg/L Total NH_3	1.40 mg/L	98.7 ± 0.58
30 mg/L Total NH_3	2.10 mg/L	98.3 ± 0.58
40 mg/L Total NH_3	2.80 mg/L	95.7 ± 0.58
50 mg/L Total NH_3	3.50 mg/L	24.3 ± 1.53
60 mg/L Total NH_3	4.20 mg/L	4.00 ± 1.00

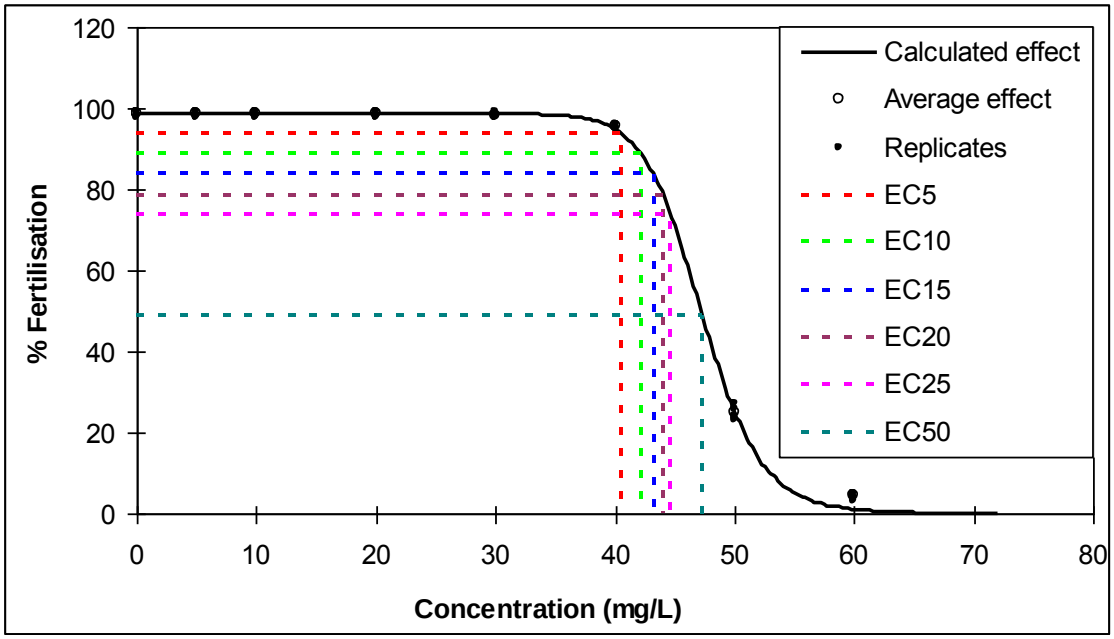

Figure 3.3 Effect concentrations (EC) determined from the total ammonia tolerance toxicity test for the species *T. gratilla*.

Table 3.10 Effect concentrations (EC) for total and unionised NH_3 determined from the total ammonia tolerance toxicity test for *Tripnuestes gratilla.*

Effects concentration	Total ammonia concentration (mg/L)	Unionised NH_3 concentration (mg/L)
EC5	40.6	2.84
EC10	42.2	2.95
EC15	43.2	3.02
EC20	44.0	3.08
EC25	44.7	3.13
EC50	47.3	3.31

Total sulphide tolerance tests were performed using a range of concentrations of total sulphide in natural seawater (Table 3.11). Solutions were prepared from sodium sulphide and concentrations were determined before the toxicity tests began to be approximately 95% of their calculated values. The pH of the seawater used was 8.15 ± 0.05 and at this pH the concentration of unionised H_2S was calculated (approximately 7% at 22 ± 1°C). Effect concentration graphs were plotted and various effect concentrations were calculated (Figure 3.4; Table 3.12).

Table 3.11 The effect of sulphide on the fertilisation success of *Tripneustes gratilla.* Each toxicity value was derived from three toxicity test replicates.

		Toxicity Tests
	Unionised H_2S concentration (µg/L)	(% Fertilisation)
Control	0	99.0 ± 1.00
950 µg/L Total S	66.5	37.7 ± 3.79
1900 µg/L Total S	133	1.67 ± 0.58
2850 µg/L Total S	200	0.00 ± 0.00
3800 µg/L Total S	266	0.00 ± 0.00

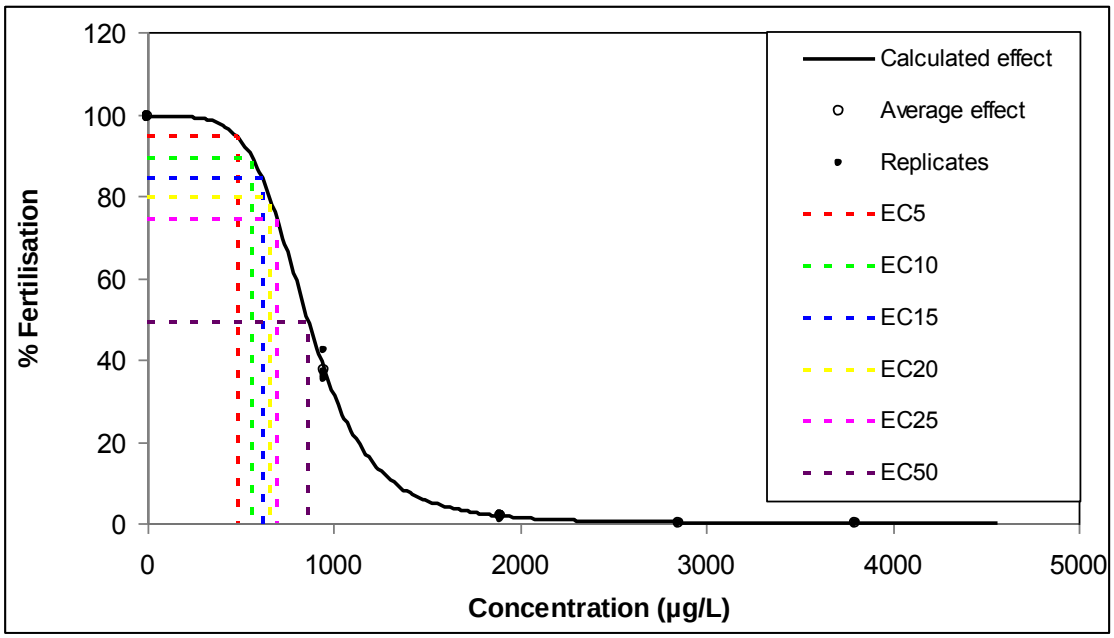

Figure 3.4 Effect concentrations (EC) determined from the total sulphide tolerance toxicity test *Tripnuestes gratilla*.

Table 3.12 Effect concentrations (EC) for total sulphide and unionised H_2S determined from the total sulphide tolerance test for *Tripnuestes gratilla*.

Effects concentration	Total sulphide concentration (µg/L)	Unionised H_2S concentration (µg/L)
EC5	485	34.0
EC10	561	39.3
EC15	614	43.0
EC20	657	46.0
EC25	695	48.7
EC50	862	60.3

These tolerance tests indicate that the EC5 and EC50 values for unionised NH_3 were 2.84 and 3.31 mg/L and for unionised H_2S were 34.0 and 60.3 µg/L respectively. They indicate that the *Tripneustes gratilla* gametes were approximately 50 to 100 times (on a concentration basis) more sensitive to unionised H_2S than to unionised NH_3.

Chapter 4

Discussion

4.1. THE DIRECT EFFECT OF SEAWATER pH ON SEA URCHIN GAMETE FERTILISATION

The results of the porewater toxicity test performed during September 2007 indicated two of three of the porewaters tested were toxic (Table 3.2). TIE procedures were not performed on these porewaters but additional chemical tests were conducted to assist with the evaluation of any potential causes of toxicity. Correlation analysis of the toxicity and chemical data indicated that pH was inversely correlated to percentage fertilisation and positively correlated to undissociated or unionised NH_3 (Table 3.5). In addition to the correlation between pH and percentage fertilisation, one of the recommended TIE procedures involved pH manipulation to adjust the bioavailable concentrations of pH dependent chemicals. Therefore, urchin tolerance experiments were conducted to investigate the possible direct and/or indirect effects that adjusting the pH might exert on percentage fertilisation. The tolerance ranges for fertilisation in each urchin species were determined in natural seawater (Tables 3.7 and 3.8) and are presented in Figure 4.1.

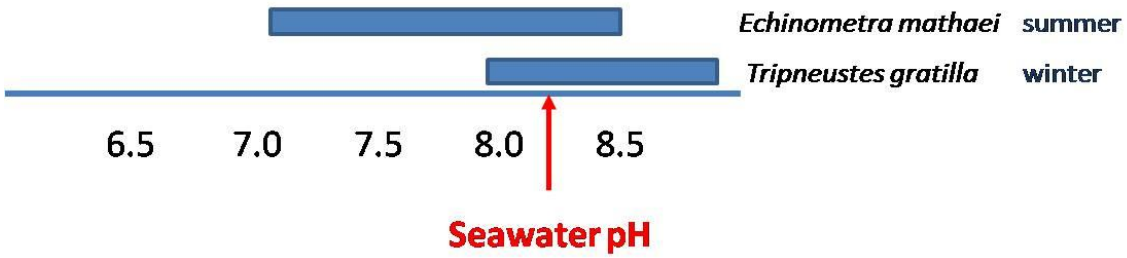

Figure 4.1 The pH threshold limits for fertilization in two species of sea urchins (summer spawning *E. mathaei* and winter spawning *T. gratilla*) used to conduct toxicity tests

These tolerance ranges indicate that gametes of *E. mathaei* had a broader range of pH tolerance when compared to *T. gratilla*. In addition, *E. mathaei* gametes were tolerant

of lower pH levels (fertilisation was not inhibited at the lowest pH of 7.19) compared to *T. gratilla,* which seemed to be sensitive to pH experimental values of 8 and lower (for *T. gratilla*, the EC50 value at the lower end of the pH scale was calculated to be 7.94). For *E. mathaei*, the results obtained in this research are similar to the results determined by Kurihara and Shirayama (2004) who determined that pH values above 7.1 did not significantly affect *E. mathaei* gamete fertilisation. Their research did not investigate the tolerance range of these gametes to pH levels higher than normal seawater.

The intolerance of *T. gratilla* to lower pH values might negatively impact the use of *T. gratilla* gametes in local toxicity tests as porewater pH values determined during this research tended to be lower than those of the natural seawater (i.e. porewater pH was 8.05 ± 0.06 compared to natural seawater with a pH of 8.15 ± 0.05) and thus closer to the *T. gratilla* lower tolerance threshold. However, in the toxicity tests where *T. gratilla* gametes were used the pH of the porewater did not appear to be the primary cause of any observed toxicity. Thus the lowest porewater pH tested was 8.01 ± 0.02 but the fertilisation of gametes for this sample were not significantly inhibited from the control sample (Table 3.2). In addition, the correlation between percentage fertilisation and pH was negative, indicating that the percentage fertilisation increased as pH decreased (i.e. porewater became less toxic as the pH decreased). Thus, for the research experiments involving *T. gratilla* it appears that the lower pH threshold of *T. gratilla* did not influence the results of toxicity testing of the porewater even though the porewater pH was close to the experimentally determined tolerance threshold for this species.

To better understand the possible effect of seawater pH on sea urchin gametes, various literature were reviewed. Seawater pH is primarily regulated by the concentration of dissolved carbon dioxide (CO_2) and the ionic equilibrium that results between hydrogen ions (H^+) and various inorganic carbon species such as CO_2, carbonic acid (H_2CO_3), bicarbonate ions (HCO_3^-) and carbonate ions (CO_3^{2-}) (Zhang 2000). The equilibrium between dissolved CO_2 and these ions has in the past meant that natural seawater has had a pH in the range 8.0 to 8.3 (Ocean Acidification fact sheet, Plymouth Marine Laboratory 2007) However, the pH of seawater has been forecast to decrease by approximately 0.3 to 0.5 pH units by the end of the 21st century if current CO_2 emission levels continue (IPCC 2007, Dashfield *et al.* 2008) due to the oceans acting as a major sink for CO_2 (Clark *et al.* 2009). The increased absorption of

CO_2 into seawater from the atmosphere reduces the pH because of its reaction in water as that increases the hydrogen ion concentration as follows:

$$CO_2 + H_2O \leftrightarrow H^+ + HCO_3^- \text{ (K = approximately } 10^{-6}\text{; Boyd 2000)}$$

As a result of the potential decrease in seawater pH, studies have begun to investigate whether these forecast changes will cause any adverse impacts on marine animals and particularly the more susceptible invertebrates including sea urchins (e.g. Kurihara *et al.* 2004, Shirayama and Thornton 2005, IPCC 2007, Saraswat *et al.* 2007, Dashfield *et al.* 2008, Fabry *et al.* 2008 and Hendriks *et al.* 2010). As a result, a number of the more sensitive life stages of sea urchin development have been investigated for hypercapnia (i.e. an increase in the concentration of carbon dioxide) and pH tolerance, including fertilisation and larval growth (e.g. Miles *et al.* 2007, Havenhand *et al.* 2008, Byrne *et al.* 2009 and Clark *et al.* 2009). A number of studies have investigated the effect of pH change on *E. mathaei* gamete fertilisation, embryogenesis and larval development (Kurihara *et al*. 2004, Kurihara and Shirayama 2004 and Shirayama and Thornton 2005). However, only one paper (Clark *et al.* 2009) and one presentation (http://stanford.sea.edu/research/Rizk_FINAL.pdf) were found to contain information pertaining to the development of *T. gratilla* larvae under reduced seawater pH conditions. Unfortunately, in this study the pH of the seawater was only adjusted once gamete fertilisation had occurred in natural seawater (i.e. the pH was not adjusted from an ambient of 8.2 (Clark *et al.* 2009)). Thus, no studies could be found to confirm the pH tolerance of *T. gratilla* gametes as determined in this research i.e. on fertilization.

When examining how the pH of seawater could influence sea urchin fertilisation (either directly because of the change in hydrogen ion concentrations and/or indirectly through the change in the bioavailable concentrations of pH dependent chemicals), the different stages preceding and including the development of a sea urchin egg fertilisation membrane (which distinguishes fertilized from unfertilised eggs) needs to be explained. These are briefly summarised later in this discussion.

Before this, however, it is necessary to consider the steps involved in the toxicity testing procedure used in this research. Initially, once sperm had been collected from the male urchin, they were added and exposed to the constituents of natural seawater (control samples) or porewater for 10 minutes before the addition of eggs. Therefore,

chemicals in a sample that might adversely impact the sperm could also affect their ability to fertilise the urchin eggs. The eggs, on the other hand, were only added 10 minutes after sperm exposure, where they could either be fertilised depending on the fertilising ability of the sperm (exposed for > 10 minutes) or egg (exposed for ≤ 10 minutes). In this way, the toxicity tests performed could be considered primarily spermiotoxicity tests (as the sperm were exposed for longer to potentially adverse sample constituents), although the shorter exposure time of eggs to the sample before fertilisation may also impact the eggs ability to be fertilised.

Considering the gamete fertilisation process, the first step that occurs in the natural environment is the release of sperm from the sexually mature male urchin. According to Gilbert (2006), sperm present in the male testes are initially kept immobile or immotile by a low internal pH of approximately 7.2. This low pH is maintained by a high concentration of carbon dioxide in the sea urchin gonad (Gilbert 2006). However, once urchin sperm are released into the seawater, their internal pH rises to approximately 7.6, resulting in the activation of the enzyme dynein ATPase. When ATP stored by the sperm is hydrolyzed by this enzyme, the energy released from this process becomes available to the flagella causing the sperm to become motile and thereby inducing the sperm to swim (Gilbert 2006).

Once the sperm are active, the second step in the fertilisation process involves the release of urchin specific chemicals from the jelly layer surrounding mature eggs (e.g. peptide molecules such as resact (Gilbert 2006) to attract sperm of the same species. The sperm then swim along this chemical concentration gradient in the water until they reach an egg. In addition to providing sperm with directional information, these peptides are also thought to further activate the sperm by increasing their rate of mitochondrial respiration and thus their mobility, increasing the probability of contact with an egg (Gilbert 2006).

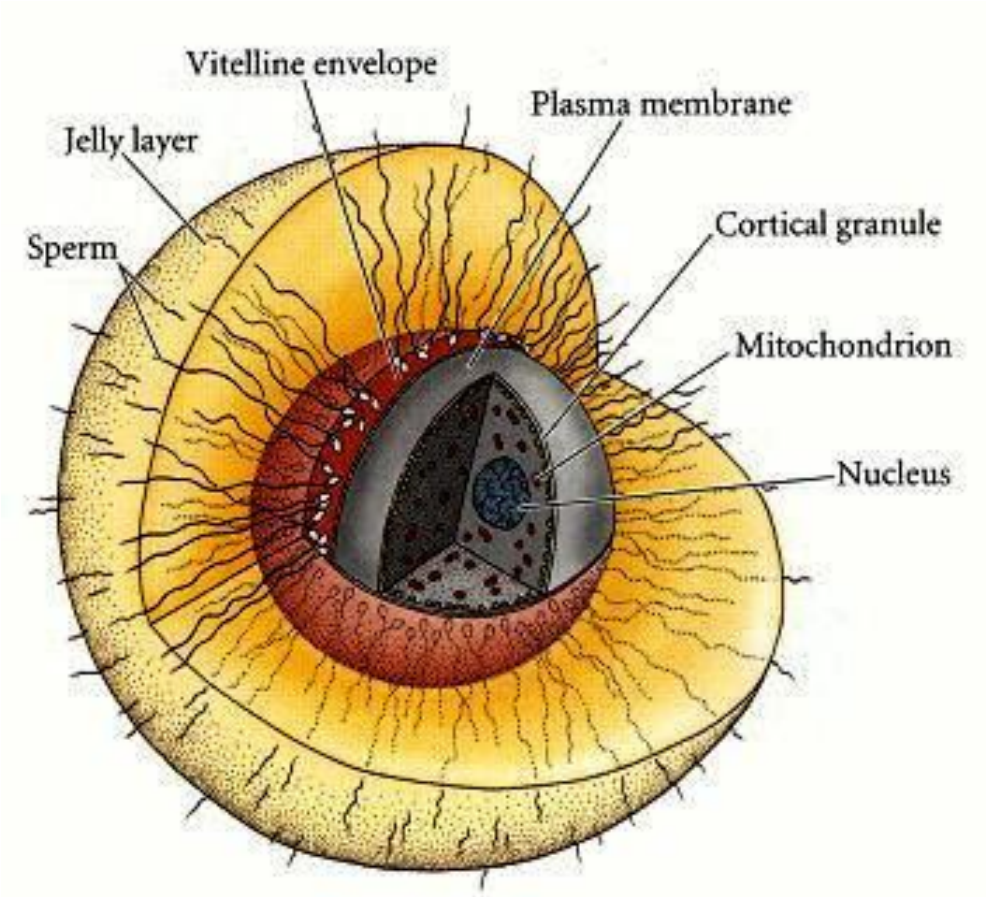

Figure 4.2 Structure of the sea urchin egg at fertilisation. Sources: http://www.ncbi.nlm.nih.gov (02/02/2010) and Gilbert (2006).

The third step, once sperm have been activated and have swum close to an egg, is contact with the egg jelly (Figures 4.2 and 4.3). Contact with this jelly initiates what is known as the acrosome reaction which consists of two main components, namely the fusion of the acrosomal vesicle to the sperm membrane which causes the release of the contents of the acrosomal vesicle followed by the extension of the acrosomal process where the actin globules are polymerised into filaments (i.e. longer strands rather than globules) (Gilbert 2006). A complex sugar in the jelly, which is also urchin species specific, initiates this reaction and during its course, a number of processes occur, including the generation of sperm membrane proteins. These proteins enable the transfer of sodium and calcium ions into the sperm membrane while releasing hydrogen ions and therefore it is assumed that this could raise the internal pH of the sperm even further. These and other processes facilitate the fusion of the acrosomal membrane with the sperm cell membrane while releasing enzymes that allow sperm to

lyse a pathway through the egg jelly until it comes into contact with the vitelline envelope of the egg (Gilbert 2006; Figure 4.3).

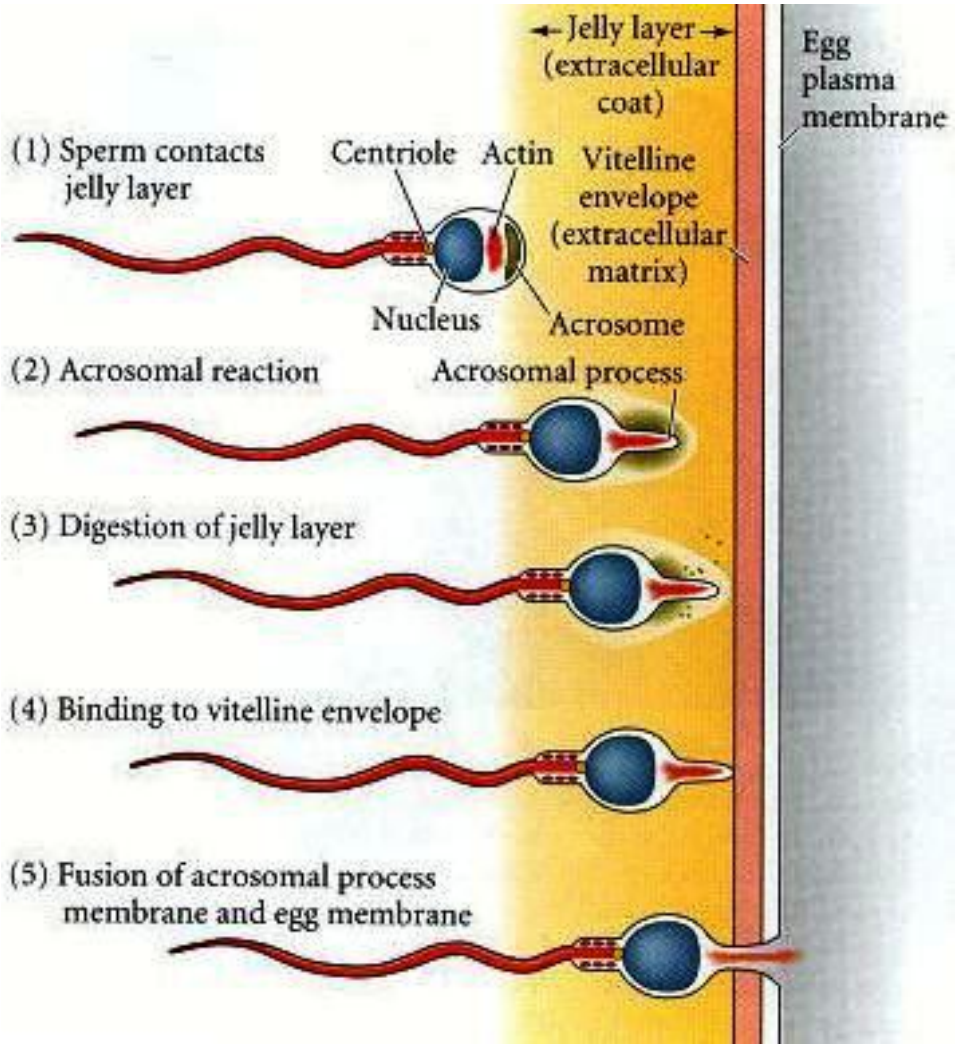

Figure 4.3 Summary of events leading to the fusion of sea urchin egg and plasma membranes. Sources: www.ncbi.nlm.nih.gov and Gilbert (2006).

The fourth step in the fertilisation procedure, which takes place just before the sperm binds with the egg, occurs when sperm have made their way through the jelly and make contact with the vitelline envelope. This contact causes the release of an urchin species specific acrosomal protein called bindin that facilitates the initial binding of the sperm to the egg (Figure 4.3). Prior to fertilisation, (i.e. in an unfertilised state), the internal potential of the egg is approximately -70 mV. However, within approximately

one second of fertilisation occurring, the internal potential changes to about 20 mV (i.e. a change from a negative to a positive internal voltage) (Gilbert 2006). This net change in potential upon egg fertilisation is caused by the influx of positively charged sodium ions from the surrounding seawater and prevents other sperm also fertilising the egg as they require an egg to have an internal negative potential (the reasons for this are not yet known although it has been speculated that it is because sperm most likely have some type of voltage sensitive component (Gilbert 2006)). This then is one of the ways in which polyspermy is prevented and is known as the fast block to polyspermy. This positive internal potential of the egg lasts for approximately 1 minute after fertilisation occurs (Gilbert 2006).

Another mechanism for preventing polyspermy in sea urchins, and one that results in the formation of the egg fertilisation envelope (which is used to microscopically identify fertilised eggs), is known as the slow block to polyspermy. Basically, the fertilisation envelope results from the fusion of components of the cortical granules with the vitelline envelope which becomes elevated from the egg cell membrane by hydrated mucopolysaccharides. This process begins approximately twenty seconds after fertilisation and lasts for about forty seconds, removing any sperm that may still be attached to the vitelline envelope (Gilbert 2006).

These steps in the fertilisation process have been highlighted as they provide some understanding of what is required from sea urchin male and female gametes before successful fertilisation can occur between them. It is apparent that all steps are vital as, without one of them, fertilisation might not occur, which in this research, would indicate toxicity. Considering the steps, a first observation is that sperm are kept immobile at low pH induced by high CO_2 concentrations in the male urchin gonad. This could indicate that one of the first hindrances to fertilisation might be the inactivation of sperm once they are transferred into a sample having a lower pH than natural seawater. Thus the internal pH of the sperm might not increase sufficiently or may even decrease causing the sperm to remain or become immobile. In addition, this mechanism by which urchins store their sperm also indicates that the sperm have been exposed to naturally high CO_2 and low pH conditions. Therefore, conditions of hypercapnia and the related reduction in pH are not foreign to sperm and the lack of fertilisation under these conditions may indicate that the observed toxicity is due in some part to sperm inactivation and thus reduced or complete immobility.

Perhaps the best way to determine this would be to microscopically observe the sperm upon exposure to the sample water with reduced pH values. Experiments like this were not performed during this research but other studies have investigated the effect of lowered pH conditions on sperm mobility and motility. For example, Havenhand *et al*. (2008) investigated the behaviour of *Heliocidaris erythrogramma* gametes to CO_2 induced acidification of natural seawater with a pH of 0.4 units below ambient (ambient pH was 8.1 while the pH was 7.7 under test conditions). The research of Havenhand *et al*. (2008) found that statistically significant reductions in sperm swimming speed (11.7% reduction) and motility (16.3% reduction) occurred as a result of the decrease in pH which consequentially resulted in an 11.3% reduction in gamete fertilisation success. Thus, they concluded that sperm swimming speeds and motility, influenced by pH, were key determinants governing fertilisation success. However, other research including that conducted by Byrne *et al*. (2009) has shown that the pH at which gametes are negatively impacted varies from experiment to experiment and from species to species. They found that fertilisation and 20 hour development of the same urchin species *H. erythrogramma* appeared not to be affected by a decrease in pH from ambient seawater (a pH range of 8.25 ± 0.02 (ambient) to 7.67 ± 0.02). This conclusion contrasted that reached in the research conducted by Havenhand *et al*. (2008) and Byrne *et al*. (2009) suggested that a reason for this could include the different experimental fertilisation conditions. The experimental conditions of Havenhand *et al*. (2008) involved exposure of both sperm and eggs to acidified seawater (pH of 7.7) for 30 minutes while Byrne *et al*. (2009) exposed only the eggs to acidified seawaters for 20 minutes while just exposing sperm to experimental seawater for a few seconds prior to adding them to the eggs. This raises the question of whether experimental conditions might also affect sea urchin fertilisation.

While the research of Byrne *et al*. (2009) and Havenhand *et al*. (2008) only adjusted the pH of test conditions to 7.7 and found contrasting fertilisation results, other research conducted to determine the relative sensitivity of urchin species to decreasing pH have indicated that reduced pH resulted in the reduction or prevention of fertilisation. For example, Kurihara and Shirayama (2004) investigated the potential effects of decreasing seawater pH due to increasing levels of CO_2 on the early development of the two sea urchin species *Hemicentrotus pulcherrimus* and *Echinometra mathaei*. Their research indicated that *Echinometra mathaei* fertilisation

was statistically significantly different from a control sample at pH levels of 7.1 and lower while for *Hemicentrotus pulcherrimus,* a significant difference was also observed at a at a slightly lower pH of 6.8. Byrne *et al* (2009) also presented similar pH data for four species of urchins including those investigated by Kurihara and Shirayama (2004) (i.e. *Strongylocentrotus purpuratus, Hemicentrotus pulcherrimus, Arbacia punctulata and Echinometra mathei*). These pH values ranged from 6.8 to 7.4.

However, other research indicates different results altogether. Consider, for example, research conducted by Pagano *et al*. (1985) who investigated how seawater with differing pH values would affect the sperm of the sea urchin species *Paracentrous lividus*. In one experiment, sperm were exposed to control and pH adjusted seawater until they were inactivated while in a second experiment, sperm were exposed to control and pH adjusted seawater for 60 minutes and then used for developmental experiments. Results from the first experiment indicated that sperm remained viable for about 6 hours in control natural seawater while in seawater with pH values adjusted to between 6 and 8, fertilisation capacity was actually prolonged (e.g. in seawater at a pH of 6.35, approximately 60% of eggs were fertilised after 13 hours of sperm exposure). An acid induced decrease in fertilisation was only observed when the pH was reduced to about 5 (Pagano *et al*. 1985). Results from their second experiment showed that sperm first exposed to seawater with pH values lower than 8 for 60 minutes resulted in fertilised eggs with mitotic and developmental abnormalities. In contrast, pH increases above normal values induced only a loss of fertilising capacity and/or death of sperm and appeared to be ineffective in inducing developmental and/or mitotic abnormalities in fertilised eggs.

Therefore, the fertilising capacity of *Paracentrous lividus* sperm in these experiments appeared to be sensitive to increased seawater pH while being able to tolerate considerable decreases in the pH of seawater. It is important to note, however, that only sperm were subjected to varied seawater pH values (5 to 9) while eggs were maintained and fertilisation and development occurred at a natural seawater pH (8.0 to 8.2).

Thus, the results of this research and the studies conducted by others including Pagano *et al.* (1985), Kurihara and Shirayama (2004), Havenhand *et al*. (2008) and Byrne *et al.* (2009) indicate that gamete tolerance and fertilisation success as well as

the success of subsequent life stage development of urchins will be affected by the pH of seawater. In these studies, however, it was also shown that sea urchin response will tend to vary and be dependent on the species specific sensitivity to seawater acidification, which gametes are exposed to the experimental conditions (i.e. eggs, sperm or both) and the nature of these conditions under which exposure occurs.

Considering these dependencies, it might be prudent when conducting future sea urchin fertilisation research, to attempt to closely simulate the conditions that would occur in the natural environment. For instance, in the natural environment both the urchin eggs and sperm are likely to be exposed to pH altered seawater before, during and after fertilisation. However, if studies only involve the exposure of sperm or eggs, it would then be prudent to only compare these findings with studies having similar experimental conditions. For example, a study might investigate and compare the fertilisation success of different urchin species by first only exposing sperm or eggs to seawater of decreasing pH before continuing with fertilisation and development under natural seawater pH conditions. Thus, a host of other experimental conditions as indicated by the studies mentioned in this research are also possible. In this way, confusion when comparing studies because of experimental differences could be minimised and not confound between study comparison.

4.2. THE INDIRECT EFFECT OF SEAWATER pH ON SEA URCHIN GAMETE FERTILISATION

Apart from the direct effect that a varying concentration of hydrogen ions (i.e. pH) may exert on sea urchin gametes, there are also indirect effects of pH changes. For example, metals (such as cadmium, copper and lead) and their complexes (e.g. metal sulphides) as well as other chemicals such as hydrogen sulphide (H_2S) and ammonia (NH_3) in water will experience a change in their degree of dissociation depending on the pH. The degree of chemical dissociation and therefore the concentrations of the undissociated and dissociated ions may influence the chemicals bioavailability and consequently the toxicity of the water being tested.

Results from toxicity tests conducted during September 2007 indicated that a positive correlation existed between pH and undissociated ammonia (Table 3.4). This is in agreement with literature as, according to authors such as Boyd (2000), the percentage

of undissociated NH_3 to total measurable ammonia (i.e. both undissociated (NH_3) and dissociated (NH_4^+) ammonia resulting from the chemical equilibrium in water: $NH_3 + H_20 \leftrightarrow NH_4^+ + OH^-$; the equilibrium is maintained by the prevailing physico-chemical conditions) will increase with increasing pH (Figure 4.4). To illustrate, in water at 20ºC and at a pH of 8, the percentage undissociated NH_3 relative to total ammonia is approximately 3.8% whereas at a pH of 8.4 and 8.6 it is 9% and 13.6% respectively (Boyd 2000). The USEPA (2007) also indicate that ammonia is approximately three times more toxic at a pH of 8 than it is at a pH of 6. Further, Boyd (2000) shows that the percentage of undissociated ammonia will increase with temperature, for example, at pH of 8 and temperature of 20ºC the percentage of undissociated ammonia is 3.8% compared to 7.5% at 30ºC.

When considering the chemical forms of total ammonia, undissociated ammonia is reported to be the more toxic form (Boyd 2000; USEPA 2007; Losso *et al*. 2009). Thus, any toxicity attributable to undissociated NH_3 would be expected to increase as the pH increased. Considering this positive correlation between pH and undissociated ammonia and the negative correlation indicated by this research between pH and percentage fertilisation (i.e. as pH increased, fertilisation decreased, Table 3.4), it might be feasible to surmise that the increase in porewater toxicity could have been due to the increase in the concentration of undissociated ammonia caused by an increase in pH. This relationship of increasing toxicity with increasing pH and thus increasing undissociated ammonia has been shown before (e.g. Arizzi Novelli *et al*. 2003; Stronkhorst *et al*. 2003; USEPA 2007). However, in this research when the correlation between the percentage fertilisation and undissociated ammonia was investigated, this relationship was not determined to be strong (Table 3.2). Further, no significant correlation was found between percentage fertilisation and the other measured physicochemical parameters. Therefore, these investigations tend to suggest that the measured physicochemical parameters were not the most likely causes of the observed decreases in fertilisation. For this reason, it is surmised that an unmeasured parameter was the primary cause of the sediment porewater toxicity.

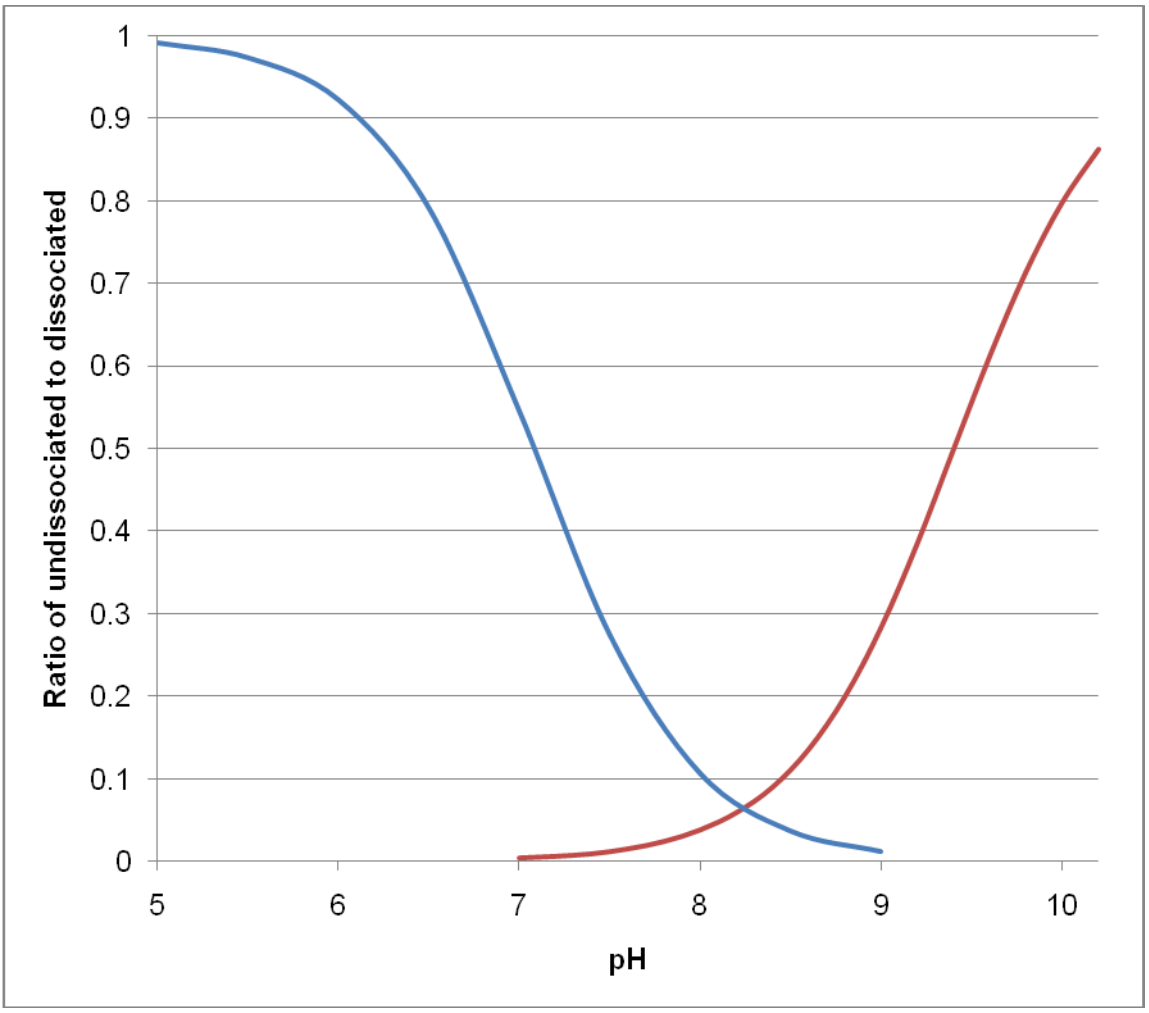

Figure 4.4 The relationship between pH to the proportions of undissociated and dissociated hydrogen sulphide (blue line) and ammonia (red line) in water at 20°C (Boyd, 2000).

In other toxicity tests conducted during April 2008 using gametes of *E. mathaei* sea urchins, results indicated that all the porewaters were toxic (Table 3.5). In an attempt to determine the potential causes of this toxicity, TIE procedures and additional toxicity tests were then performed. Unfortunately, the results of the TIE procedures conducted were inconclusive in that they did not identify any specific potential chemical cause of the toxicity (Table 3.6). Therefore, because of the TIE procedures performed, it was considered unlikely that either bioavailable metals, organic chemicals or undissociated ammonia were the primary causes of toxicity.

A possible cause of this toxicity, not investigated by the TIE procedures performed, might have been undissociated H_2S. Undissociated H_2S, like undissociated NH_3, is considered more toxic than the other chemical forms (HS^- and S^{2-}) and may cause porewater toxicity as well as preventing the detection of other toxicants in porewater if present in sufficient concentrations (Knezovich *et al*. 1996; Wang and Chapman 1999, USEPA 2007). When comparing the toxicity of H_2S to undissociated NH_3, adjusting the pH downwards will result in an increase in the proportion of undissociated H_2S compared to its dissociated ions (i.e. the opposite of what occurs with undissociated ammonia, Figure 4.4). For example, at 20ºC and at a pH of 7.5, the percent undissociated H_2S relative to total sulphide (i.e. the sum of H_2S, HS^- and S^{2-}) is approximately 27.5% whereas at a pH of 8 and 8.5 it is only 10.7% and 3.7% respectively (Boyd 2000). Therefore, decreasing the pH to 7.3 in the TIE procedures conducted during April 2008 would likely have resulted in a decrease in the concentration and thus toxicity potentially attributable to undissociated NH_3 as discussed above. However, it could have resulted in an increase in the concentration of undissociated H_2S. Thus, the potential net result of this pH adjustment depending on the initial concentration of H_2S could have been the increase in the porewater toxicity. As the porewaters remained completely toxic, this could imply that undissociated H_2S was a potential reason why no reduction in toxicity was observed.

No procedures (e.g. aeration or adjusting the pH upwards) were conducted during this research to reduce or remove H_2S (or any other oxidisable and/or volatile chemicals) from the porewater. The primary reason for this was the limited quantity of porewater available to conduct them. Limited volumes of porewater are not unique to this research and can be a restraint in porewater TIE studies (e.g. USEPA 2007; Macken *et al*. 2008), depending on the quantity of sediment sampled. A possible solution to this may be to collect larger quantities of sediment from which sufficient porewater can be obtained, for example, by increasing the size of the grab sampler or by pooling grab samples (Stronkhorst *et al*. 2003; Volpi Ghirardini *et al*. 2005). However, collecting larger samples can be problematic because of the lack of large grab samplers and/or a boat to manage such equipment. Pooling samples may also limit the ability of a study to determine whether sample variability exists and if so, to investigate/understand its potential causes.

Another reason why no procedures were conducted to reduce or remove H_2S from the porewater was the assumption that centrifuging would remove some, if not all, of the H_2S in the sample because of its volatile nature and the porewater's exposure to air during rigorous centrifugation. In addition, when pH tolerance experiments were performed to determine urchin gamete tolerance of changes in pH, it was noted that *Echinometra mathaei* gametes were relatively insensitive to low pH levels but much more sensitive to increasing pH levels above natural seawater (Figure 4.4, EC50 = pH 8.56). Thus, during experimentation it was deemed impractical and unnecessary to increase the pH of the sample above what is usual (i.e. ambient). Instead, only a procedure that involved decreasing the pH of the porewater was performed. In hindsight, when considering that a relatively small upward adjustment of the pH may have resulted in an exponential decrease in the undissociated H_2S concentration, a small change in pH between ambient and say pH 8.50 could have significantly reduced the undissociated H_2S concentration. Thus, the direct influence of H_2S on toxicity was not determined by the TIE procedures followed here, although this and other research into sediment porewater toxicity indicates that it might well be a contributor of toxicity.

In research conducted in similar environments as this area of the Port of Durban (soft silty mud with canalized river inputs), H_2S has been determined to be a primary cause of toxicity (e.g. Knezovich *et al*. 1996). These environments that receive anthropogenic organic material inputs may contain naturally high concentrations of H_2S and NH_3. Apart from any direct anthropogenic inputs of H_2S and NH_3 (e.g. from effluent spills from industries along the banks of the canals and storm water runoff draining surface contamination from surrounding urban and industrial areas into the canals), the natural anaerobic decomposition of organic matter by bacteria can also significantly contribute to overall concentrations of H_2S and NH_3 (Knezovich *et al*. 1996; Philips *et al.* 1997; Wang and Chapman 1999; Arizzi Novelli *et al.* 2003; Losso *et al.* 2007). Thus, whatever the source, concentrations of H_2S and NH_3 may be high enough in sediments and their porewaters to cause a toxic response and therefore potentially prevent the detection of toxicity due to other anthropogenic contaminants (e.g. bioavailable metals and organic chemicals). This potential masking effect observed in other TIE studies conducted in similar environments has lead to researchers describing toxicity attributable to NH_3 and H_2S, and especially high naturally occurring concentrations, as „*confounding*' chemicals in toxicity testing procedures (e.g. Arizzi Novelli *et al.* 2003; Losso *et al.* 2007).

Thus, as it is not always possible to collect sufficient porewater and because of the potential toxicity of undissociated H_2S and NH_3 in sediments from these types of environments, it is a major recommendation of this research that chemical analyses are conducted before any TIE procedures to determine whether undissociated NH_3 and/or H_2S could potentially be predominant causes of toxicity. If NH_3 and H_2S are present at potentially toxic concentrations, then procedures which first attempt to reduce or remove these concentrations before continuing with additional TIE procedures can be applied. This recommendation is illustrated in Figure 4.5.

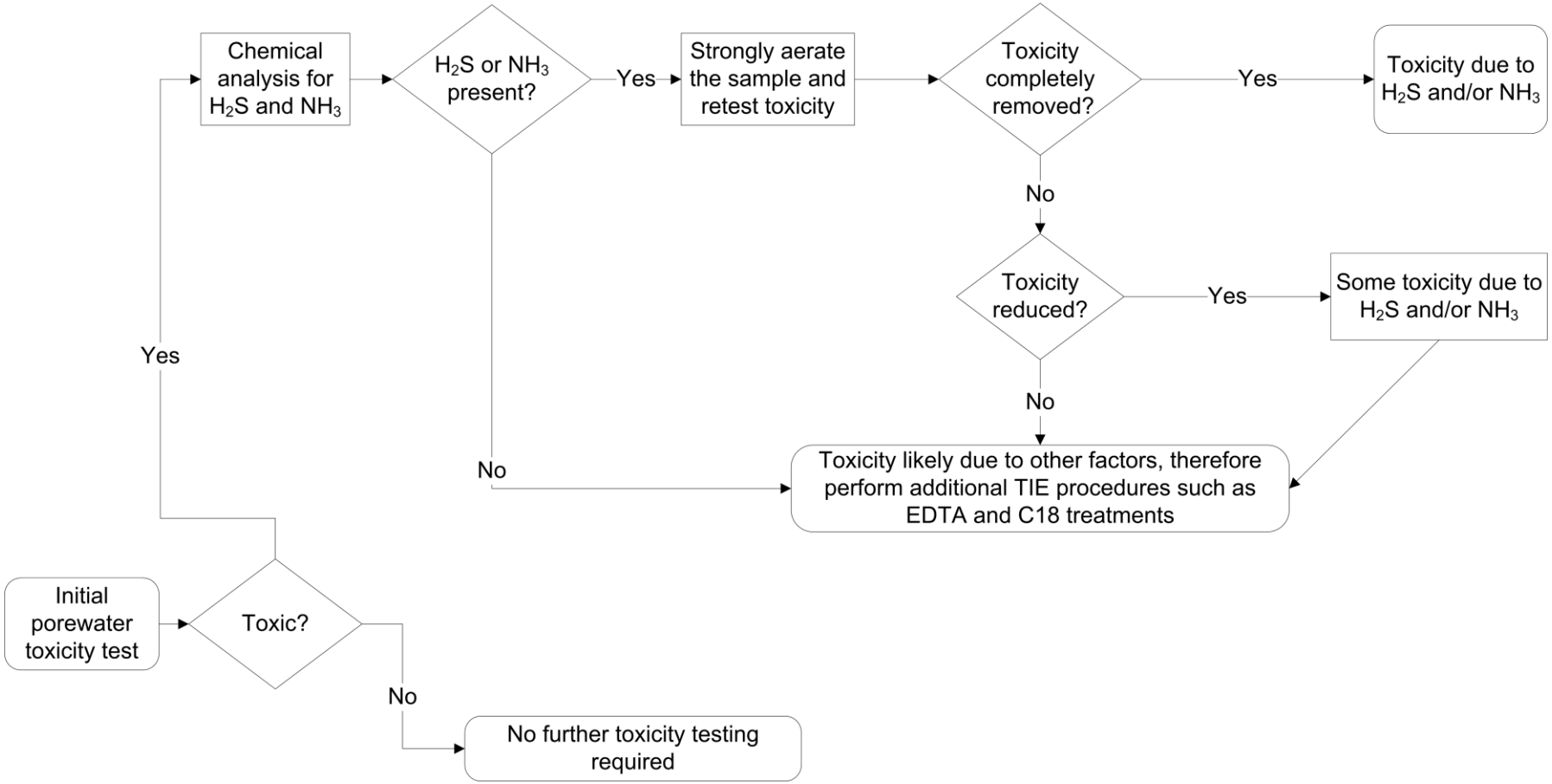

Figure 4.5 A recommended testing procedure when analyzing sediment porewater from environments where NH_3 and H_2S are suspected to be available at potentially toxic concentrations.

4.3. THE POTENTIAL EFFECT OF SAMPLE VARIABILITY ON TIE

In order to determine inter-grab variability and what the possible causes of it were, three grab samples of sediment were taken during each sampling event. Further, in order to determine whether any intra-grab variability existed, each grab sample was split into three equal fractions and all were tested separately for toxicity. In some cases, the results of these tests indicated significant inter-grab variability (e.g. sediment porewater toxicity tests conducted in July and September 2007). However, in all cases, intra-grab variability was low.

Sediments are known to accumulate environmental contaminants in the aquatic environment over time and have been successfully used in environmental contaminant assessments (Birch *et al*. 2001). However, the inter-grab sediment porewater toxicity variability noticed during this research raised some concerns about whether these toxicity results were an accurate indicator of contaminant status as it was initially assumed that sediment sampled from fundamentally the same site would exhibit similar porewater toxicities. Nevertheless, environmental sample variability is not unusual (e.g. Morrisey *et al.* 1994, Mackey and Mackay 1996 and Hewitt *et al*. 2007) and other researchers have indicated that there are three predominant causes of variability in environmental data (Pettersen *et al.* 1999 and Birch *et al*. 2001). These are spatial, sampling and analytical variability which together contribute to total variability.

Spatial variability refers to any variation in sediment composition including variation in physiochemical parameters such as sediment particle size distribution and metal and other contaminant concentrations. Consequently, spatial variability will tend to indicate the degree of sediment physiochemical heterogeneity within a defined area. This variability may be the result of factors including the concentrations and input rates of contaminants into the system over time, the various currents that distribute the contaminant load into and within an area, and the physical properties of the sea floor (Pettersen *et al.* 1999). A visual inspection of all sediment samples taken from this area of the Port of Durban indicated that the sediment consisted predominantly of grey/black mud. This was confirmed by particle size analysis of some samples which indicated that more than 90% of the sediment particles had a diameter of less than 63 µm. Therefore, as particle size appeared similar for all sediments, it was not suspected to be the main cause of any observed variability (this was also confirmed by correlation

analysis, see Table 3.4). Nevertheless, muddy sediments do have a greater propensity to adsorb chemicals compared to sandy sediments because of their larger total surface area and chemical properties. As such, changes in sediment particle sizes and other environmental characteristics could potentially regulate the bioavailability of chemicals between sediment and porewater, and represent another potential source of spatial variability.

Another potential source of variability, sampling variability, results from the physical sampling procedure employed. For instance, it is possible and likely that the same precise sampling position will be missed when collecting additional replicate samples because a sampling vessel is unable to perform fine manoeuvres into the exact same position every time even when using modern positioning techniques like GPS (whose accuracies can range up to ±10 m depending on the GPS specifications as indicated in the Garmin GPSMAP 76CSx Owner's Manual). In addition, even if a sampling vessel were initially able to manoeuvre into the same position on the water surface above the sampling area, collection of sediment from the same position on the seabed is not guaranteed because of vessel drift potentially caused by its own momentum, as well as the prevailing wind, currents and waves. Besides sampling vessel drift, a remotely-operated sampling device such as a grab sampler can drift horizontally out of position as it travels through the water column to the seabed. In murky and/or deep water, when the sampling device cannot be seen to strike the seabed, there is no way of ensuring that one is actually near to or in the same position previously sampled. This will most likely be the case when sampling seabed sediment, except perhaps when one is extracting samples from a shallow, calm, non-turbid water body. Further, if an area of sediment has previously been sampled by a destructive sampling technique such as a grab sampler, it is impossible to resample the same sediment again because it was unique and is no longer there. Sampling equipment striking the seabed may also disturb the sediments causing mixing and the potential change in the sediment physicochemical parameters. Thus, unless a sampling area is perfectly homogenous (i.e. no spatial variation exists), any subsequent sample retrieved from a specific site cannot be exactly the same as a previous sample.

Analytical variability implies variability introduced when preparing and analysing a sample. Examples of the potential sources of analytical variability include procedures occurring after the sample has been collected such as sample handling and storage,

preparation techniques and analytical methods (e.g. instrumental variability). Procedures used in this research for sample handling, storage, porewater extraction and toxicity testing were designed to investigate and minimise variability when preparing and analysing samples. For example, each grab sample was split into three equal fractions to determine any intra-grab variability and the porewater from these was extracted in a staggered manner to determine whether the time of extraction introduced any analytical variability. The resultant low intra-grab toxicity variability determined for these porewaters provides evidence that the procedures followed for sample preparation and analysis, including the extraction regime adopted, consistently contributed little toward total variability.

This evidence tends to eliminate analytical variability as a likely reason for the inter-grab variability observed. This fact has also been borne out by other researchers such as Pettersen *et al*. (1999) who found that the largest sources of variability were introduced by sampling procedure and spatial differences from the repeated samplings at each of three different sites. Pettersen *et al.* (1999) indicate that in their research, the contribution of analytical variability was generally less than 5% relative standard deviation (RSD) for a range of organic contaminants analysed, compared to sampling (ranged from 10 to 55% RSD) and spatial variability (which ranged from 5 to 30% RSD). The RSD calculated similarly for selected chemical parameters in this research (i.e. dissolved nutrients, Table 3.2) showed a similar trend with sampling and spatial variability ranging between 9 to 51% and 3 to 26% respectively. The laboratory analytical variability on the other hand, was also generally less than 5% RSD.

Other researchers, such as Morrisey *et al*. (1994) have investigated metal concentration variability on spatial scales from between two meters to approximately four kilometres. Their principal finding was that metal concentration variation occurred within what might appear to be a homogeneous area of sediment. They referred to this phenomenon as „*patchiness*' which appeared unrelated to any obvious environmental heterogeneity, highlighting the need to test for variation at different spatial scales when sampling. When samples are collected by remote methods, such as grab samplers, even obvious environmental patchiness may not be detected before the samples are taken and brought up to the water surface.

Another potential contributor to spatial variability when performing toxicity and other biological response tests, apart from those mentioned above, will be factors that govern the bioavailability of chemicals adsorbed onto sediment. Fine grained muddy sediment, with its propensity to adsorb chemicals because of a large total surface area, can act as a sink for contaminants. However, if environmental conditions change, they can also become a source of these same contaminants as they may be desorbed from the sediments. Thus, factors governing the adsorption and desorption of contaminants as well as the prevailing physicochemical conditions will affect their bioavailability. Some of these factors include sediment particle size, pH, salinity and the specific mechanisms by which contaminants adsorb onto sediments. The roles of particle size and pH have already been discussed.

Organic pollutants including polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) are adsorbed onto sediment by co-adsorbed matter such as organic material, soot carbon and lipids (Pettersen *et al.* 1999). Therefore, the amounts of these materials in sediments will influence how strongly and to what concentrations these organic contaminants will also adsorb onto sediments. Consequently, this will regulate the bioavailability and concentrations of organic contaminants available to organisms that live in the vicinity of organically enriched sediment. In addition, other contaminants including metals have their own mechanisms for binding to and accumulating in sediments that will likewise regulate their bioavailability (e.g. metal sulphide complexes tend to regulate metal bioavailability in anaerobic sediments, Mackey and Mackay (1996)).

Independent of the mechanisms that regulate contaminant adsorption onto, accumulation and desorption from sediments, are factors that influence the biological uptake, regulation and response of exposed organisms to bioavailable contaminants. Each organism's response will be unique and will vary depending on the concentrations of bioavailable contaminants, the stage in the lifecycle of the organism (e.g. egg or larvae, juvenile or adult), the organism's contaminant coping mechanisms and toxicodynamics, and the physicochemical conditions present at the time of exposure (Rainbow, 2002). Depending on these factors and the organisms present, the contaminants may exert a negative impact if present at metabolically available concentrations that the organism cannot cope with. Thus, toxicological variability may

also be introduced due to the variability in the response of an organism to the metabolically available contaminant concentrations.

Consequently, considering the potential sources of variability discussed above, it may not be unreasonable to experience the levels of variation encountered during this and other research. Thus, it is essential to design sampling strategies that attempt to understand a sampling area's variability in order to better interpret the relationships or patterns being sort (Hewitt *et al.* 2007). To this end, it is a further recommendation of this research to adopt a sampling strategy like that illustrated in Figure 4.6. In this sampling strategy, the overall toxicity of a sampling site can be determined together with those of the individual replicates.

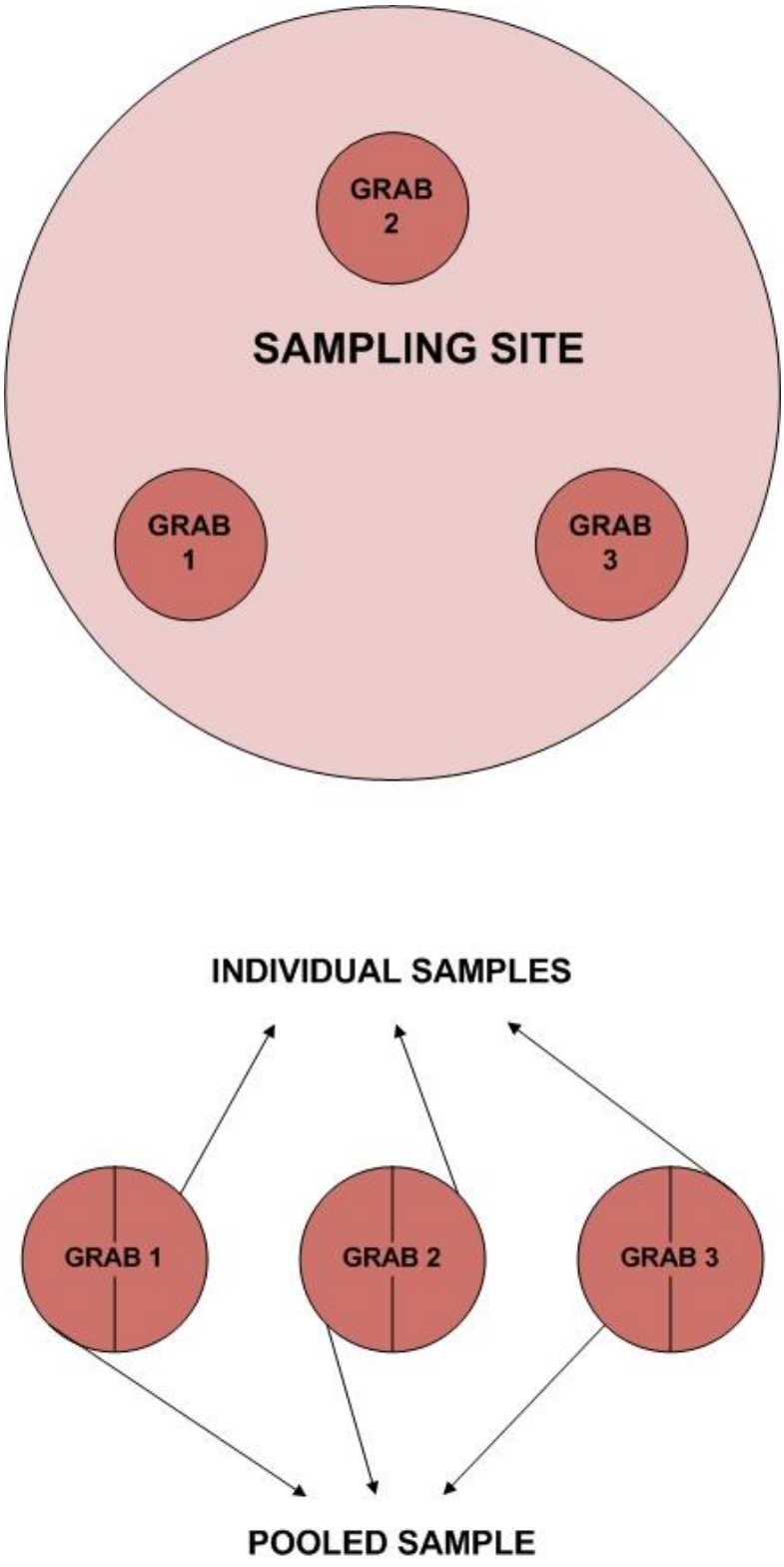

Figure 4.6 A recommended sampling strategy illustrating sample handling that will allow for the analysis of both individual samples and a pooled sample.

Chapter 5

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