Development of a sediment toxicity test for the South African coastal environment using the endemic amphipod, *Grandidierella lignorum* Barnard 1935 (Amphipoda: Aoridae)

By

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ABSTRACT

Contaminants introduced in solution to coastal waters eventually accumulate in sediment. Pollution by these contaminants is only evident when biological effects occur. Geochemical procedures lack the ability to identify biological effects of pollution. Biological methods (i.e. community structure analyses and/or bioassays) are currently the best available techniques for pollution assessment. Standardised and locally relevant protocols for pollution assessment are lacking in many developing countries, including South Africa. This study aims to develop a sediment toxicity testing protocol using an amphipod species endemic to South Africa, *Grandidierella lignorum*. Initial research focussed on establishing ranges of physico-chemical parameters (i.e. salinity, temperature, sediment grain size and organic matter content) within which sediment toxicity tests should be performed. The sensitivity of the amphipod was then determined by exposing the amphipod to cadmium, copper and zinc at various salinities. Lastly, the amphipod was exposed to effluents (to test the amphipod's sensitivity in water only tests) and whole sediment (to tests the amphipod's sensitivity to solid phase material). *G. lignorum* tolerates salinities between 0 and 56, but prefers salinities between 7 and 42. Preferred salinity range is modified by temperature, with salinity of 42 becoming less tolerable. Salinities between 7 and 35 are most preferred at 10-25°C. *G. lignorum* prefers fine- (27.48±12.13%), medium- (25.11±12.99%) and coarse-grained sand (21.45±8.02%). Sediment with low (≤2%) organic matter content is most preferable, regardless of sediment grain size or type of organic matter (protein-rich vs. carbohydrate-rich).

Cadmium toxicity decreased with increasing salinity (LC_{50}: 0.34 ± 0.17 mg l^{-1} (salinity of 7), 0.73 ± 0.05 mg l^{-1} (salinity of 21) and 1.08 ± 0.49 mg l^{-1} (salinity of 35)). Zinc toxicity increased with decreasing salinity (1.56 ± 0.33 mg l^{-1} at a salinity of 21 to 0.99 ± 0.13 mg l^{-1} at a salinity of 7) and with increasing salinity (from salinity of 21 to 0.82 ± 0.19 mg l^{-1} at a salinity of 35). Copper toxicity did not differ significantly with salinity and ranged between 0.72 ± 0.18 mg l^{-1} (salinity of 35) and 0.89 ± 0.24 mg l^{-1} (salinity of 21). Toxicity testing using *Grandidierella lignorum* should be performed in coarse- to fine-grained sediment at salinities of 7 - 35, at 10 – 25°C. Amphipods do not need to be fed during toxicity testing. A control chart using cadmium as a reference toxicant was established to determine the acceptability of toxicity results. Toxicity test results should be accepted when cadmium toxicity falls between 0.49 and 4.02 mg l^{-1}. The amphipod responded consistently to effluents and was able to discriminate polluted and unpolluted sediment in Durban Bay. Recommendations for refining the effluent and sediment toxicity test are suggested.

PREFACE

The experimental work described in this thesis was carried out in the School of Life Sciences, University of KwaZulu-Natal, Durban, from April 2011 to December 2013, under the supervision of Dr. Brent Newman (Council for Scientific and Industrial Research) and Dr. Ursula Scharler (University of KwaZulu-Natal). 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.

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

Signed: _____ Name: _____ Date: _____

As the candidate's co-supervisor I have approved this thesis for submission.

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DECLARATION: PLAGIARISM

I, Ntuthuko Fortune Masikane declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
   a. Their words have been re-written but the general information attributed to them has been referenced.
   b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed: .....

DECLARATION: Publications

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis are as follows:

Publication 1

Masikane, N. F., Newman, B. K., Scharler, U. M. Accepted. Salinity tolerance of the amphipod *Grandidierella lignorum* (Amphipoda: Aoridae). African Journal of Aquatic Science.

Publication 2

Masikane, N. F., Newman, B. K., Scharler, U. M. In preparation. Influence of grain size and organic content on sediment selection and survival in the laboratory by the amphipod *Grandidierella lignorum* (Amphipoda: Aoridae).

Publication 3

Masikane, N. F., Newman, B. K., Scharler, U. M. In preparation. Influence of salinity and temperature on growth, reproduction and fecundity in the amphipod *Grandidierella lignorum* (Amphipoda: Aoridae) in the laboratory.

Publication 4.

Masikane, N. F., Newman, B. K., Scharler, U. M. In preparation. Sensitivity of the amphipod *Grandidierella lignorum* to cadmium, copper and zinc in the laboratory.

Publication 5.

Masikane, N. F., Newman, B. K., Scharler, U. M. In preparation. Whole effluent and sediment toxicity testing using the amphipod *Grandidierella lignorum* (Amphipod: Aoridae).

Author Contribution

Publications 1-5. Concept and experimental design (B. Newman and N. Masikane). Data analysis and manuscript preparation (N. Masikane, B. Newman, U. Scharler).

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Dedication

This work is dedicated to my parents; Mr. Thulasizwe John Masikane and Mrs. Jabulile Monica Masikane. Not enough gratitude can be expressed for their unwavering support during my years of study.

"*Ecotoxicology is not a luxury research area, undertaken only in rich countries. It is relevant and important to all countries and all habitats, and it is vital if we are to protect our living heritage from the cocktail of chemicals present in all environments.*"

[1](Hermens et al. 2004).

1

[1] Hermens JL, Ankley GT, Sumpter JP (2004) Ecotoxicology - a multidisciplinary, problem-driven science. Environmental Science and Technology 38: 446A-447A.

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Chapter 1

General Introduction

Background

Standardised methods for sediment toxicity testing were developed in the 1990s (Burton et al. 1992). This coincides with the period when the output on marine pollution studies had declined fivefold in South Africa (O'Donoghue and Marshall 2003). This was despite increasing concern regarding the status of local estuaries due to anthropogenic impacts. More than 80% of the estuarine surface area in South Africa is in a poor state, and sediment contamination is recognised as one of the emerging pressures (Van Niekerk et al. 2013). Biological tools for assessing sediment contamination in the estuarine and marine environment in South Africa are currently under-developed. Sediment bioassays are commonly used tools for assessing sediment toxicity and the use of endemic species as test organisms should be promoted (Costa et al. 1998). There are no certified tests used on a routine basis to test the toxicity of water and sediment in marine or estuarine environments in South Africa (Slabbert et al. 1998; Wepener and Chapman 2012). There is, however, an urgent need to develop biological sediment assessment tools for South Africa, since some ecosystems (e.g. harbours) are showing ever increasing signs of contamination. For example, iron concentrations in sediment in Richards Bay harbour have almost doubled between the 1970s to 1990s (see Wepener and Vermeulen 2005). The development of a sediment toxicity test must include *inter alia* assessment of the test organism's sensitivity to non-contaminant factors (e.g. temperature, salinity and sediment parameters), sensitivity to common contaminants of sediment, and sensitivity to field-contaminated sediments. The collation of this information can then inform the protocol for assessing sediment toxicity (e.g. Costa et al. 1998).

Ecotoxicological studies in South Africa

Excellent reviews on marine pollution in South Africa have been published (e.g. O' Donoghue and Marshall 2003; Wepener and Degger 2012) and it is not the intention of this study to repeat this work, but it is recognised that a lot more work in toxicology studies across all science disciplines in South Africa is still required (Gulumian et al. 2005). In the marine sector, ecotoxicological research has been undertaken since the 1960s (O' Donoghue and Marshall 2003; Wepener and Chapman 2012). The analysis of 65 ecotoxicology papers referenced by O'Donoghue and Marshall (2003) showed that more than 50 species have been used as test organisms over the last six decades and these range from small-sized organisms, such as bacteria, to high trophic level organisms, such as fish (Table 1.1).

Table 1.1. Species that have been used in toxicological and/or ecotoxicological research in South Africa.

	Taxa	Reference
	Bacteria	
1	*Aeromonas hydrophila*	Thompson & Watling 1987
2	*Bacillus* sp.	Thompson & Watling 1987
3	*Enterobacter cloacae*	Thompson & Watling 1987
4	*Escherichia coli*	Thompson & Watling 1983, 1984, 1987
5	*Klebsiella oxycota*	Thompson & Watling 1987
6	*Pseudomonas* sp	Thompson & Watling 1987
	Phytoplankton	
7	*Chaetoceros calcitrans*	Hilmer & Bate 1983
8	*Pavlova lutheri* (*Diacronema lutheri*)	Hilmer & Bate 1983
9	*Phaeodactylum tricornutum*	Hilmer & Bate 1983
10	*Tetraselmis suecica*	Hilmer & Bate 1983
11	*Pseudoisochrysis paradoxa*	Hilmer & Bate 1983
	Algae	
12	*Cladophora* sp.	Hemens & Warwick 1972
	Grasses and Kelp	
13	*Zostera capensis* (roots)	Hemens & Warwick 1972
14	*Laminaria pallida*	Cook 1978
	Nemertea	
15	*Cerebratulus fuscus*	Brown 1974
	Isopoda	
16	*Eurydice longicornis*	Brown 1974
17	*Exosphaeroma truncatitelson*	Brown 1974
18	*Pontogeloides latipes* (*Excirolana latipes*)	Brown 1974
	Amphipoda	
19	*Grandidierella lignorum*	Connell & Airey 1979, 1982
20	*Grandidierella lutosa*	Connell & Airey 1979, 1982
	Mysida	
21	*Gastrosaccus psammodytes*	Brown 1974
	Prawns and shrimps	
22	*Callianassa kraussi* (Callichirus kraussi)	Jackson 1982, Jackson 1985, O' Donoghue & Marshall 2006, Thwala et al 2011
23	*Penaeus monodon*	Hemens & Warwick 1972
24	*Palaemon pacificus*	Hemens & Warwick 1972, Moldan & Chapman 1982, Moldan & Chapman 1983, Achituv & Cook 1984, Hennig 1986
25	*Penaeus indicus* (*Fenneropenaeus indicus*)	Hemens & Warwick 1972, Hemens et al 1975, McClurg 1984
26	*Upogebia africana*	Hill 1977

Table 1.1. *continued*

	Taxa	Reference
	Lobster	
27	*Jasus lalandii*	Cook 1978, Lipschitz 1982, Hennig 1986
	Brachyura (crabs)	
28	*Chiromantes eulimene*	Thwala et al. 2011
29	*Diogenes brevirostris*	Hennig 1986
30	*Sesarma catenata* (*Parasesarma catenatum*)	Malan 1986, Malan 1988
31	*Tylodiplax blephariskios*	Hemens & Warwick 1972, Hemens et al. 1975
	Mollusca (Limpets, oysters, abalone)	
32	*Crassostrea cucullata* (Saccostrea cucullata)	Watling 1981b, Watling 1982
33	*Crassostrea gigas*	Watling 1978, Watling 1981a, Watling 1981b, Watling 1982, Watling 1983a, Watling 1983b
34	*Crassostrea margaritacea* (Striostrea margaritacea)	Watling 1981a, Watling 1981b, Watling 1982, Watling 1983a
35	*Haliotis midae*	Shackleton et al. 2002, Stofberg et al. 2011
36	*Patella granularis* (*Scutellastra granularis*)	Hennig 1986
37	*Siphonaria capensis*	Marshall et al. 2004
	Bivalves	
38	*Aulacomya ater* (Aulacomya atra)	Cook 1978
39	*Choromytilus meridionalis*	Currie et al. 1974, Cook 1978,Watling 1981a, Watling 1983a, Hennig 1986
40	*Donax serra*	Watling & Watling 1983, Stenton-Dozey & Brown 1994
41	*Mactra lilacea*	Beckley 1981
42	*Mytilus galloprovincialis*	Mason 1988a, b
43	*Perna perna*	Hemens & Warwick 1972, Watling 1981a, Watling & Watling 1982, Watling 1983a, Hodgson & Hoebeke 1984, Gregory et al. 1999, Anandraj et al. 2002, Gregory et al. 2002, Vosloo et al. 2012
	Gastropoda	
44	*Bullia digitalis*	Brown 1964, Brown & Currie 1973, Brown et al. 1974, Cuthbert et al. 1976, Golombick & Brown 1980, Brown et al. 1982, Hennig 1986
45	*Bullia rhodostoma*	Watling & Watling 1983
46	*Bullia laevissima*	Brown 1964
47	*Nassarius kraussianus*	Marshall & Rajkumar 2003
	Echinodermata	
48	*Echinometra mathaei*	Connell et al. 1991
49	*Parechinus angulosus*	Greenwood & Brown 1974, Greenwood 1983, McGibbon & Moldan 1986, Wynberg et al. 1989
	Tunicates and Fish	
50	*Ambassis safgha*	Hemens & Warwick 1972
51	*Liza dumerili*	Mzimela et al. 2002
52	*Mugil cephalus*	Hemens & Warwick 1972, Hemens et al. 1975
53	*Pyura stolonifera*	Liebrich et al 1995
54	*Rhabdosargus holubi*	De Kock & Lord 1988
55	*Terapon jarbua*	Hemens & Warwick 1972
	Mixed taxa	
56	Meiofauna community	Gyedu-Ababio & Baird 2006

The majority of toxicity studies focused on the individual or cellular level of biological organisation, with only one study focusing on community level organisation (i.e. Gyedu-Ababio and Baird 2006). Toxicity tests can be performed at various levels of biological organisation (Chapman 1995; Van Straalen 2003) (e.g. from sub-cellular to species level). However, it should be noted that no single species is universally more sensitive to all types of contaminants, hence the need to use multiple species in sediment toxicity tests (Cheung et al. 1997; Macken et al. 2008; Kennedy et al. 2009).

Contaminants that have been studied for their toxicity since the 1960s in South Africa include oils (e.g. water soluble fraction, polycyclic aromatic hydrocarbons (PAHs)), metals and organic contaminants (Wepener and Degger 2012). Metals were the most studied class of contaminants (Figure 1.1); and cadmium, copper, zinc and lead were the most frequently used contaminants in laboratory experiments (Figure 1.2).

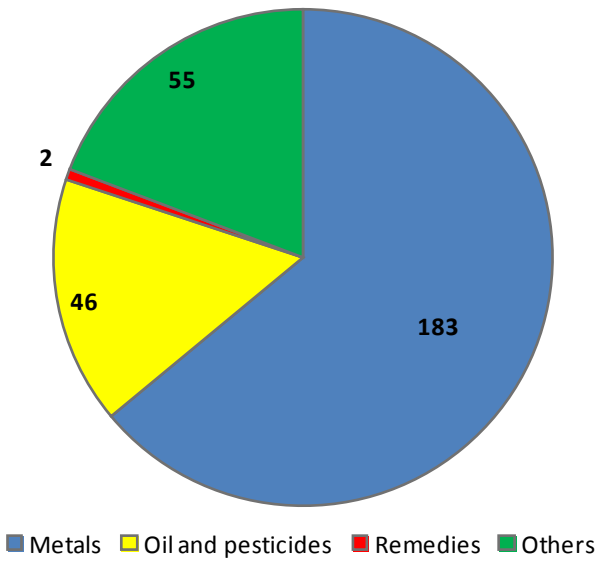

Figure 1.1. Contaminants of interest in toxicity and/or ecotoxicity studies in South Africa over the last six decades. Data represents the number of times test organisms were exposed to each contaminant class. Remedies refer to substances used to ameliorate contamination, such as oil dispersants and chelating agents (i.e. ethylenediaminetetraacetic acid (EDTA)).

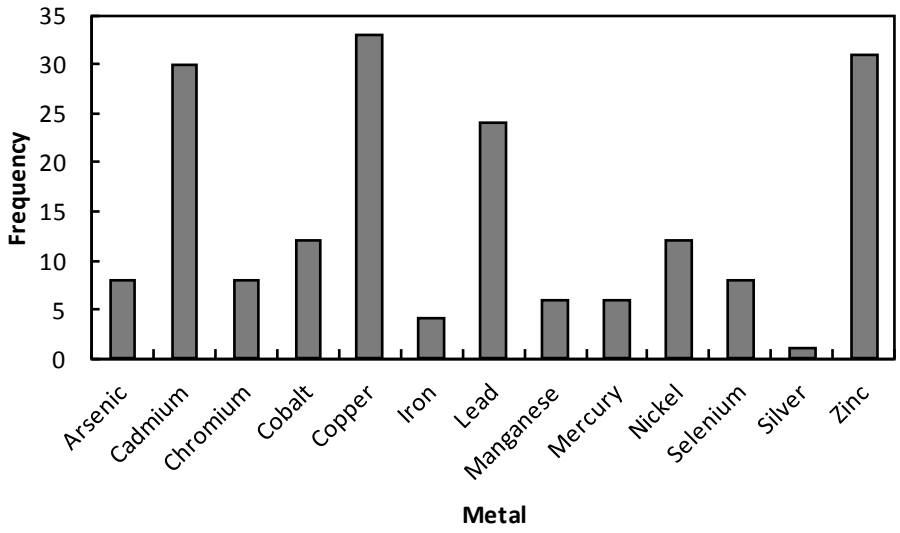

Figure 1.2. Frequency at which metals were used as contaminants of interest in historical ecotoxicity and/or toxicity studies in South Africa.

Characteristics of a good toxicity testing species

A good toxicity testing species should at least meet the following criteria, or possess the following features (Burton et al. 1992; EPA 1994; Chapman 1995; Costa et al. 1998; Chapman and Wang 2001; Chapman 2002; Peters et al. 2002; Bat 2005):

1. Must be endemic.
2. Should represent different taxa, different trophic levels and different exposure pathways (e.g. porewater, overlying water and sediment).
3. Must have an intimate relationship with the sediment (e.g. burrower, deposit feeder).
4. Should be sensitive to most contaminants of sediment.
5. Must be widely distributed in the natural environment.
6. Must be ecologically or economically relevant.
7. Must be available all year-round and easily collected.
8. Must be amenable to laboratory culture.
9. Must tolerate wide variations of natural parameters such as salinity, temperature and sediment particle size.
10. Availability of a toxicity database for the test species.
11. Should be abundant in the field.
12. Should be easy to identify.

***Grandidierella lignorum*: a potential toxicity test organism for South African coastal ecosystems**

Amphipods with a greater degree of contact with sediment through a burrowing and deposit feeding behaviour (i.e. infaunal amphipods) are ideal for sediment toxicity tests as these amphipods are exposed to whole sediment, interstitial water and overlying water to some degree (Burton et al. 1992). Tube burrowers are generally more exposed to overlying water than interstitial waters. Infaunal amphipods (i.e. free burrowers) in South Africa predominantly belong to the families Phoxocephalidae and Urothoidae (Griffiths 1976), but these are generally not numerically abundant in estuaries.

Grandidierella lignorum Barnard 1935, a gammaridean amphipod belonging to the family Aoridae (www.marinespecies.org), meets most of the requirements for a toxicity testing species. It is endemic to estuaries in South Africa (Griffiths 1976) and is distributed in all biogeographic regions. It has been collected in the cool temperate Great Berg River Estuary (Wooldridge and Deyzel 2009) and in warm temperate estuaries, including the Gamtoos, Swartkops, Sundays, Kariega, Keiskamma and East Kleinemonde River estuaries (Schlacher and Wooldridge 1996a; Teske and Wooldridge 2001; Wooldridge and Bezuidenhout 2008). It has also been collected in numerous estuaries in the subtropical region (Stow 2011). *G. lignorum* lives in tubes it constructs using detritus and sediment particles (Boltt 1969; Barnard et al. 1988). When constructing and/or maintaining burrows (including burrow extension), it feeds on microbes adhered to faecal pellets by browsing (Boltt 1969). It is, however, not limited to deposit feeding but is also a filter feeder (Schlacher and Wooldridge 1996b; Wooldridge and Bezuidenhout 2008). While *G. lignorum* feeds at the lower trophic level in the food web (i.e. a primary consumer; Wooldridge and Bezuidenhout 2008), it forms an important food source for juvenile fish such as *Rhabdosargus holubi* and *Lithognathus lithognathus* (Schlacher and Wooldridge 1996c), and is thus ecologically important. Its distribution within the estuary is not influenced by parameters such as sediment grain size and salinity (Teske and Wooldridge 2003). The amphipod does not seem to prefer any particular sediment grain size (Boltt 1969) and can tolerate a wide range of salinities (Boltt 1969; Thwala 2006) and temperature (Thwala 2006). It is sensitive to contaminants of sediment (Vivier 2010) and is amenable to laboratory culture (Connell and Airey 1979). Available toxicity data for *G. lignorum* (i.e. toxicity database) includes exposures to fluoride, cadmium and zinc (Connell and Airey 1979, 1982, Thwala 2006, Vivier 2010).The rich baseline information of this amphipod allows for its potential use as a toxicity testing organism for the South African coastal environment.

Aim

The primary aim of this study was to develop a sediment toxicity test for the estuarine and coastal environment of South Africa using the estuarine amphipod, *Grandidierella lignorum*. Specific aims are stated in chapters of this thesis that deal with different aspects of developing the toxicity test. The sediment toxicity test developed in this study is referred to as a second generation toxicity test by Chapman and Wang (2001). These tests are generally performed using euryhaline and/or stenohaline species for toxicity testing of estuarine sediments, but it is advised that the test salinity should match that measured in porewater. Sediment toxicity in estuarine ecosystems has been tested using marine or freshwater organisms, with test salinities being modified to suit the preferences of the test organism and consequently failing to recognise the influence this has on the bioavailability of sediment contaminants (Chapman and Wang 2001).

Thesis layout

This thesis is written up in a scientific publication format and the repetition of some information was thus unavoidable. The following two chapters aim to define test conditions of salinity, temperature, sediment grain size and organic matter content for *Grandidierella lignorum.* Results of this chapter would form the basis for the development of a chronic toxicity test for future investigations. The third chapter determines the sensitivity of the amphipod to common contaminants of sediment and establishes a quality control tool in a form of a control chart. Control charts aid in accepting or rejecting toxicity test results. The fourth chapter exposes the amphipod to liquid waste and contaminated sediment with the aim of determining the ability of the amphipod to discriminate toxicity in different exposure phases (i.e. water only and whole sediment toxicity). The last chapter synthesises the findings of this study and makes recommendations. An additional chapter in the appendix section investigates the influence of salinity and temperatures on the amphipod's growth, reproduction and fecundity.

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Chapter 2

Salinity tolerance of the amphipod *Grandidierella lignorum* (Amphipoda: Aoridae)

Abstract

The endemic amphipod *Grandidierella lignorum* is an organism potentially suited to the toxicity testing of coastal water and sediment in South Africa. The primary aim of this study was to define the range of salinity and temperature at which *Grandidierella lignorum* can be used for toxicity testing, to avoid potential confounding effects induced by these non-contaminant factors on test data interpretation. The data are also discussed in the context of the known ecology of this amphipod. Amphipods were exposed in the laboratory to salinities of 0 to 56 (increments of 7) for 96 hrs. Salinities were prepared using natural seawater and synthetic sea salts. *Grandidierella lignorum* tolerated all salinities, but showed highest survival at salinities of 7 to 42. Salinity tolerance was modified by temperature, with highest survival between 10 and 25^{o}C. These represent the range of conditions at which toxicity testing can be performed. Salinity tolerance did not differ significantly between natural and synthetic seawater. Synthetic sea salt can thus be used to manipulate the salinity of media for toxicity testing without acting as a confounding variable. Tests performed also highlight the importance of resting laboratory cultured *G. lignorum* between harvesting events.

Introduction

Salinity and temperature are important environmental factors that influence estuarine inhabitants (Kinne 1963, 1964). The survival of individuals and success of estuarine populations is partly dependent on the ability to tolerate the variable salinities that characterise this environment. Salinity tolerance defines the potential for penetrating the estuarine environment while temperature influences the range of salinity tolerated (Kinne 1963). Stenohaline species are restricted to the lower or upper reaches of estuaries depending on their origin (i.e. freshwater or marine) while euryhaline species can potentially be found along the length of an estuary. However, other environmental (e.g. sediment grain size) and biological (e.g. competition) factors may influence estuarine distribution (e.g. Barnes 1967, McLachlan and Grindley 1974, Teske and Wooldridge 2004, Rhodes-Ondi and Turner 2010). Consequently, many decapod crustaceans inhabit restricted regions of the estuarine environment, where salinity variation is within the range tolerated in the laboratory.

Amphipods are widely used for water and sediment toxicity testing (e.g. Woodworth et al. 1999, Bat 2005, Ré 2007). The estuarine amphipod *Grandidierella lignorum*, which is endemic to South Africa (Griffiths 1976), has been identified as an organism that can potentially be used for toxicity testing of

coastal water and sediment in South Africa. It meets most of the requirements for an organism to be considered suitable for this purpose (see Burton et al. 1992, EPA 1994, Bat 2005), including its wide geographical distribution (from Great Berg estuary in the west to Nhlabane estuary in the east; Vivier and Cyrus 1999, Wooldridge and Deyzel 2009), ease of collection, handling and maintenance in the laboratory (Connell and Airey 1979, Thwala 2006) and burrowing lifestyle (Boltt 1969a, Barnard et al. 1988). Although *G. lignorum* is widely tolerant of salinities <35 (Boltt 1969a), tolerance to salinities >35 has been poorly investigated. Thwala (2006) exposed *G. lignorum* to a salinity of 45, but the experiment was not replicated.

The primary aim of this study was to define the range of salinity and temperature at which *Grandidierella lignorum* can be used to test the toxicity of water and sediment to avoid the potential confounding effects induced by these non-contaminant factors on toxicity test data interpretation. The findings are discussed in this context and in the context of the known ecology of *G. lignorum*.

Natural seawater is the preferred medium for preparing laboratory culture water and for manipulating the salinity of water, testing for toxicity and for preparing overlying water for sediment toxicity testing. Seawater prepared using synthetic sea salts is commonly used for this purpose if natural seawater is not available (EPA 2001). However, synthetic seawater is known to affect the survival, growth and reproduction of some amphipods (e.g. Emery et al. 1997). An additional aim of this study was thus to determine whether *Grandidierella lignorum* shows differences in tolerance to salinities prepared using natural and synthetic seawater.

Materials and Methods

Maintenance of amphipods in the laboratory

Amphipods were collected from Intshambili River estuary on the subtropical northeast coast of South Africa (30°38’ S, 30°32’ E). In the laboratory the amphipods were maintained in four culture tanks (L x B x H: 53 x 33 x 15 cm) containing about 2 cm of medium- to fine-grained sediment and about 10 cm of UV sterilised, filtered (10 µm) natural seawater (salinity = 35). The culture water was aerated continuously. The amphipods were fed *ad libitum* three times a week on ground fish flakes (Tetramin®) and about 80% of the culture water was replaced weekly. The cultures were maintained at 22°C under a 12 hr light: 12 hr dark photoperiod in a temperature controlled environmental chamber. Under these conditions *Grandidierella lignorum* reproduced successfully, allowing the regular harvesting of individuals for experimental purposes.

Salinity tolerance

Amphipods were exposed to salinities between 0 and 56, at increments of 7. Salinities were prepared in two ways. First, salinities <35 were prepared by diluting UV-sterilised and filtered (10 µm) natural seawater (salinity = 35) with distilled water and salinities >35 by adding Instant Ocean® synthetic sea salt to natural seawater treated in the same manner described above. Distilled water was used for the 0 salinity treatment. Salinities prepared in this manner are referred to as natural seawater, despite the use of synthetic sea salt to prepare salinities >35. Second, salinities were prepared by adding Instant Ocean® synthetic sea salt to distilled water. Salinity was checked using an Atago (PAL-065) digital refractometer.

Amphipods were fed at least 6 hrs prior to experiments, but not during experiments. Amphipods were harvested by manually disturbing the sediment in culture tanks, which forced amphipods from the sediment. Juvenile amphipods (average length ± 1 SD: 2.59 ± 0.47 mm, *n* = 720; based on measurements made after tests) were isolated by passing the culture water through sequential nylon sieves with a mesh size of 1000 and 500 µm. Amphipods were then stepwise acclimated from salinity 35 to test salinities at a rate of ≤3 every 2 hrs. Apparently healthy (i.e. actively swimming) amphipods were then individually transferred to glass vials filled with 30 ml of water using a wide bore glass pipette.

Survival was monitored at 24 hr intervals for 96 hrs. The media was not renewed or aerated. Amphipods were considered dead when no activity was evident, including pleopodal beats or twitches after gentle mechanical stimulation with a glass rod. Experiments were repeated three times using amphipods from each laboratory culture, providing a total of 12 experiments with natural seawater and 12 experiments with synthetic seawater.

The tolerance of *Grandidierella lignorum* to instantaneous changes in salinity was also investigated, by directly exposing amphipods from salinity 35 to salinities between 0 and 56 (increments of 7). The experiment was repeated four times using amphipods from two cultures. Salinities were prepared in the same manner described above, using natural seawater.

Influence of temperature on salinity tolerance

The influence of temperature on salinity tolerance was investigated by exposing amphipods to salinities between 7 and 42 (increments of 7) at 10, 15, 20, 25 and 30°C. These temperatures encompass the range to which *Grandidierella lignorum* is likely to be exposed in the natural environment in the long-term. Salinities were prepared in the same manner described above, using natural seawater. A total of 24 amphipods were individually acclimated to experimental salinities at a rate of ≤3 per hr and then to experimental temperatures at a rate of ≤3°C per 2 hrs. Temperatures were maintained constant in an environmentally controlled chamber. Survival was monitored at 24 hr intervals for 96 hrs. The media was not renewed or aerated. Experiments were repeated three times for each salinity-temperature combination, using amphipods from all four cultures.

Statistical analysis

Statistical analyses were performed using SPSS software (version 21). A Model 1 three factor analysis of variance (ANOVA) was used to compare survival as a function of salinity, media type (i.e. synthetic vs. natural seawater) and laboratory culture. Survival for the instantaneous salinity exposure experiment was compared using one factor ANOVA. The combined influence of salinity and temperature on survival was evaluated using a Model 1 two factor ANOVA. A Tukey HSD *post hoc* test was used to identify treatments that differed significantly at $P = 0.05$.

Results

Salinity tolerance

Although *Grandidierella lignorum* survived for 96 hrs at all salinities prepared using natural and synthetic seawater, survival differed significantly between certain salinities (Figure 2.1 and Table 2.1). The highest survival (generally >80%) for each culture was at salinities between 7 and 42 (Figure 2.1).

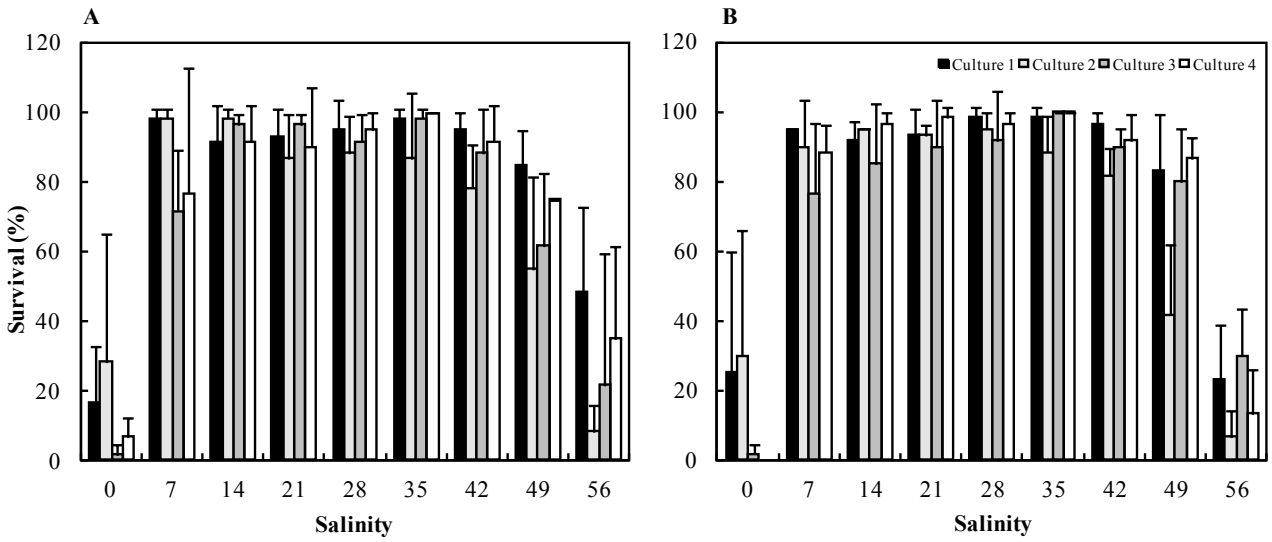

Figure 2.1. Mean survival (± standard deviation) of *Grandidierella lignorum* at various salinities prepared using natural (a) and synthetic seawater (b).

Table 2.1. Results of three factor ANOVA evaluating survival of the amphipod *Grandidierella lignorum* as a function of test salinities prepared using natural and synthetic seawater (i.e. media type) for amphipods taken from different laboratory cultures.

Source	*df*	*F*	*P*
Main Effects			
Test salinity	8	90.625	<0.0005
Media type	1	0.112	0.738
Amphipod culture	3	4.863	0.003
Interactions			
Salinity x media type	8	0.357	0.941
Salinity x amphipod culture	24	1.846	0.015
Media type x amphipod culture	3	0.386	0.763
Amphipod culture x media type x salinity	24	0.533	0.963

Survival at each salinity did not differ significantly between natural and synthetic seawater (F = 0,112, P = 0.738). Survival data for both types of media were thus pooled to compare salinity tolerance between cultures. Salinity tolerance differed significantly between cultures ($F_{(\text{salinity x amphipod culture})}$ = 1.846, $P_{(\text{salinity x amphipod culture})}$ = 0.015), due mainly to the significantly lower survival of amphipods from culture 2 at salinities between 35 and 49 compared to one or more other cultures (Table 2.2).

When amphipods were directly exposed to test salinities without acclimation the best survival (80%) was measured at salinities between 7 and 42 (Figure 2.2), which is the similar to the 'preferred' salinity range for amphipods that were stepwise acclimated.

Table 2.2. Survival of *Grandidierella lignorum* taken from four laboratory cultures at various salinities. Letters in superscript within a row represent the results of Tukey HSD *post hoc* tests. Survival was not significantly different in rows with no letters.

Salinity	Culture 1	Culture 2	Culture 3	Culture 4	*F*	*P*
	Mean±SD	Mean±SD	Mean±SD	Mean±SD		
0	20.83±24.78	29.17±32.62	1.67±2.58	3.33±5.16	2.862	0.063
7	96.62±2.62	94.17±9.70	74.17±17.15	82.50±24.24	2.676	0.075
14	91.67±7.53	96.67±2.58	90.83±12.81	94.17±7.36	0.582	0.634
21	93.33±6.83	90.00±8.94	93.33±9.31	94.17±12.01	0.563	0.646
28	96.67±6.06	91.67±8.16	91.67±10.33	95.83±3.76	0.547	0.656
35	98.33±2.58^{ab}	87.50±13.69^{a}	99.17±2.04^{b}	100.00±0.00^{b}	4.467	0.015
42	95.83±3.76^{a}	80.00±9.49^{b}	89.17±8.61^{ab}	91.67±8.16^{ab}	3.533	0.033
49	84.17±12.01^{a}	48.33±22.29^{b}	70.83±19.08^{ab}	80.83±7.36^{a}	5.408	0.007
56	35.83±22.89	7.50±6.89	25.83±25.58	24.17±22.00	1.965	0.152

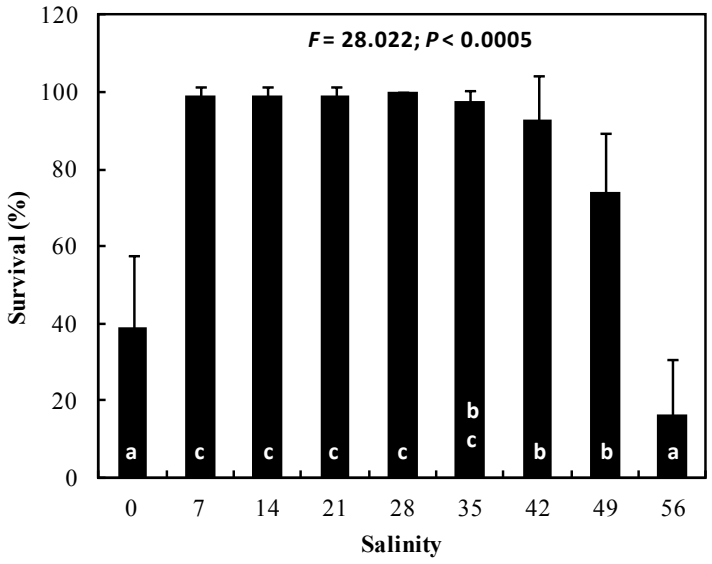

Figure 2.2. Mean survival (± standard deviation) of *Grandidierella lignorum* at various salinities following direct exposure from a salinity of 35. Similar letters in bars indicate that survival was not statistically significantly different while different letter indicate a statistically significant difference.

Influence of temperature on salinity tolerance

Temperature significantly influenced salinity tolerance ($F_{(salinity\ x\ temperature)}$ = 3.553, $P_{(salinity\ x\ temperature)}$ < 0.0005) (Figure 2.3a). These experiments were performed about two weeks after the salinity tolerance experiments. However, it was suspected that the amphipods may not have been sufficiently rested between experiments. Thus, the experiments were repeated after a further rest period of four to six weeks. The influence of temperature on salinity was again significant for amphipods rested for four to six weeks ($F_{(salinity\ x\ temperature)}$ = 9.181, $P_{(salinity\ x\ temperature)}$ < 0.0005) (Figure 2.3b). However, the influence of temperature on salinity tolerance of amphipods rested for a short period (i.e. two weeks) differed to that for amphipods rested for a long period (i.e. four to six weeks) (Figure 2.3a,b). For example, amphipods rested for two weeks tolerated (≥90% survival) a salinity range of 14 to 35 at 15°C, but a narrower range of 28 to 35 at 25°C (Figure 2.3a). Amphipods rested for four to six weeks, in contrast, tolerated (≥90% survival) a salinity range of 7 to 35 at 10 to 25°C. The influence of high temperature (i.e. 30°C) on survival became apparent at low (7) and high salinities (≥35) (Figure 2.3b).

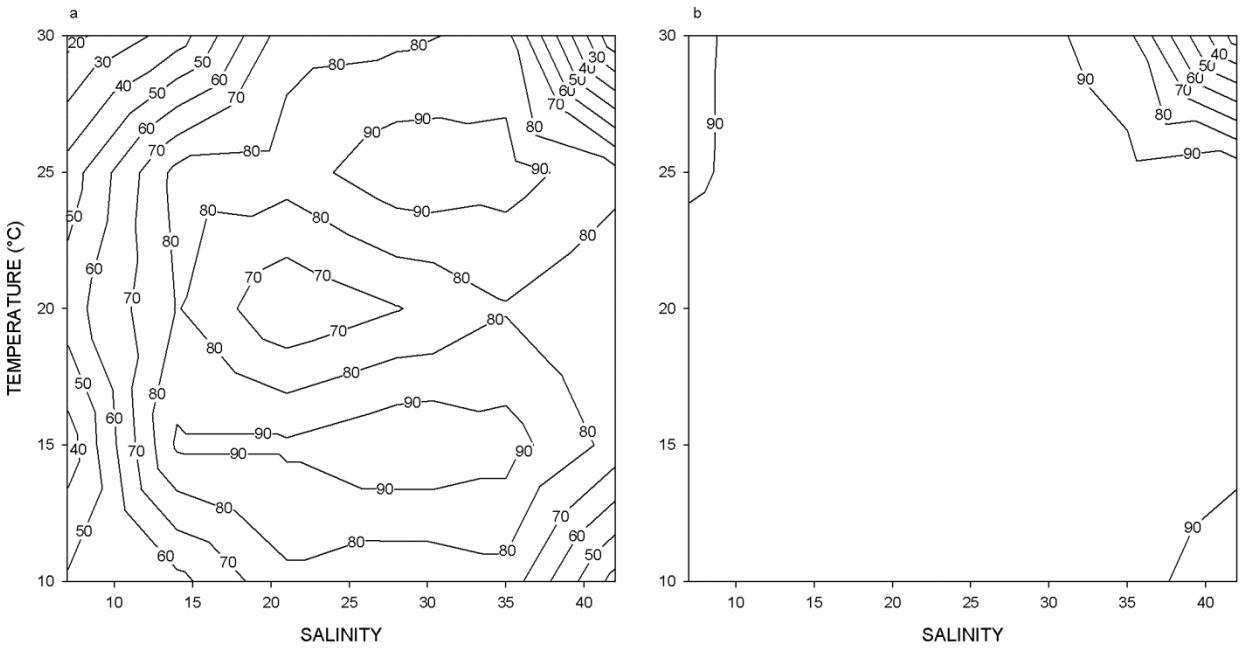

Figure 2.3. Interactive effect of salinity and temperature on survival of *Grandidierella lignorum* rested for two weeks (a) and four to six weeks (b) between experiments.

To determine the influence of resting period on survival a Student's *t*-test was used to compare survival between amphipods rested for two weeks and four to six weeks between experiments. Survival of amphipods rested for two weeks was generally significantly lower than for amphipods rested for four to six weeks (Figure 2.4). However, at certain combinations of salinity and temperature no significant difference was evident. This included salinities of 14 to 35 at 15°C, 28 to 42 at 25°C, and 35 to 42 at 30°C.

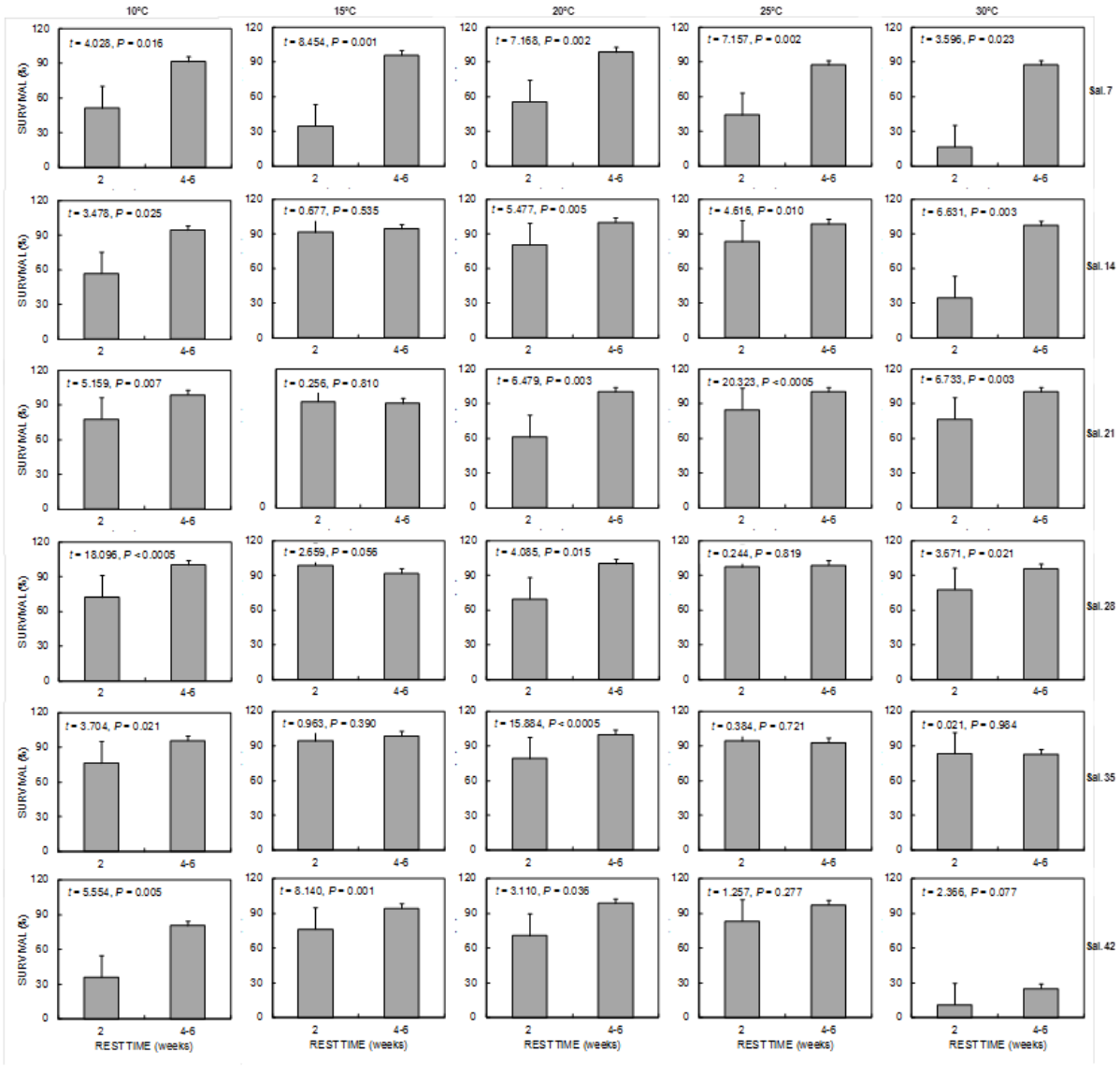

Figure 2.4. Mean survival (± standard deviation) of *Grandidierella lignorum* between resting periods when exposed to the combination of salinity and temperature. Sal. = salinity.

Discussion

Grandidierella lignorum is euryhaline, capable of tolerating salinities between 0 and 56 for 96 hrs in the laboratory. However, survival at salinities of 0 and 56 was, on average, low (<40%). The 'preferred' salinities are between 7 and 42, where survival usually exceeded 80%. The wide salinity tolerance of *G. lignorum* in the laboratory agrees with and to some extent explains its wide axial distribution in estuaries. For example, Schlacher and Wooldridge (1996) recorded *G. lignorum* at

salinities between 0.1 and 34 in the Gamtoos River estuary. *Grandidierella lignorum* is also able to tolerate wide, abrupt changes in salinity. Euryhalinity and tolerance of wide, abrupt decreases in salinity are important adaptations for organisms resident in South African estuaries. The majority of estuaries along this coastline are small, narrow and shallow. Along most of the coastline a significant proportion of the mean annual rainfall may fall over a period of a few days, resulting in a strong inflow of freshwater. This results in a wide, rapid decrease in salinity throughout the estuary, which may remain low for periods from a few days to a few weeks (e.g. McLachlan and Grindley 1974, Day 1981, Robertson 1984, Hanekom 1989, Henninger et al. 2008). Many estuaries also naturally close off from the sea and the salinity may decrease due to freshwater inflow. *Grandidierella lignorum* may thus need to withstand long periods of low salinity under flooding conditions or in closed estuaries. The water column in open and closed estuaries along the west, south and southeast coasts of South Africa may also become hypersaline when evaporative losses exceed freshwater inflow, although the salinity rarely exceeds ~42 (e.g. Hill 1981, Hodgson 1987, Whitfield and Bruton 1989, Teske and Wooldridge 2001, Harrison 2004). This is well within the salinity range tolerated by *G. lignorum* in the laboratory.

The salinity tolerance of *Grandidierella lignorum* is comparable to that for other gammarid amphipods, including *Corophium volutator, Leptocheirus plumulosus* and *Corophium multisetosum* (McLusky 1967, Emery et al. 1997, Ré et al. 2009). Some of these amphipods (e.g. *Corophium multisetosum*) can even reproduce successfully in freshwater (Cunha et al. 2000). *Grandidierella lignorum* does not appear to live permanently in freshwater apart from certain relict estuarine lakes in northern KwaZulu-Natal. However, this is a special case since the ionic composition of water in these lakes (e.g. elevated sodium ion concentrations) appears to facilitate survival of *G. lignorum* and a number of other organisms typically only found in South African estuaries (Allanson and van Wyk 1969, Boltt 1969a, 1969b, Reavell and Cyrus 1989).

The modifying influence of temperature on the salinity tolerance of estuarine organisms is well known and was apparent also for *Grandidierella lignorum*. Temperatures between 25 and 30 °C reduced survival at salinities of 7 and >35 for amphipods rested for four to six weeks between experiments. Thwala (2006) investigated the influence of temperature on the salinity tolerance of *Grandidierella bonnieroides* by exposing the amphipod to a salinity range of 0 to 55 and temperatures ranging from 10 to 30°C. The amphipods tolerated salinities of 0 to 45 and

temperatures of 10 to 26°C. Highest survival (≥80 %) was reported at a salinity range of 5 to 35, at 16 to 22°C. *Grandidierella lignorum* tolerates a similar range of salinity, but at a wider temperature range (10 to 25°C).

The implication of euryhalinity in *Grandidierella lignorum* in the context of toxicity testing is that this amphipod can be used to test the toxicity of water samples or sediment with porewater at salinities between 7 and 42 without salinity acting as a confounding factor in data interpretation. In cases where the salinity of samples requiring toxicity testing falls outside this range the salinity must be manipulated to at least 7 or 42, by dilution using natural seawater or addition of synthetic sea salts. Survival of *G. lignorum* was not adversely affected by exposure to experimental media prepared using synthetic sea salts, which will thus not act as a confounding factor during toxicity testing.

This study provides evidence that the frequent harvesting of *Grandidierella lignorum* from cultures in the laboratory may act as a confounding factor in toxicity tests. Amphipods rested for only two weeks between experiments showed a higher mortality when exposed to various salinity-temperature combinations than amphipods rested for four to six weeks. The reason is unknown but may reflect the energetic demands of burrow construction. Laboratory cultures of *G. lignorum* should thus be rested for at least four weeks but preferably six weeks between harvesting events.

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Chapter 3

Influence of grain size and organic content on sediment selection and survival in the laboratory by the amphipod *Grandidierella lignorum* (Amphipoda: Aoridae)

Abstract

Sediment grain size and organic content are important factors that influence the composition of benthic invertebrate communities. These factors also influence contaminant bioavailability, and thus have the potential to act as confounding factors during toxicity testing. Determining an amphipod's preference for sediment grain size and organic content is thus crucial for defining the conditions under which a sediment toxicity test should be performed. *Grandidierella lignorum* was offered a choice of six sediment grain size classes and varying organic content (0 - 8% dry weight) to determine their preference. Amphipods showed no significant preference for sediment grain size in two of three experiments, but consistently tended to avoid very coarse-grained and muddy sediment. Amphipods preferred sediment with low organic matter (<2%) and this was not influenced by the type of organic matter present (protein-rich vs. carbohydrate-rich organic matter) or sediment grain size. Survival of the amphipod in sediment devoid of organic matter over a 10 day period was significantly higher in the fine-grained (survival of 92%) and very fine-grained sediment (74%) but low in the mud fraction (30%). Thus, for toxicity tests on *G. lignorum*, sediment with a wide range of grain sizes but with low organic matter can be tested.

Introduction

Sediment is the major sink for particle reactive contaminants (including particulate and dissolved forms) anthropogenically introduced to aquatic ecosystems. With continued input and limited sediment redistribution (e.g. no scouring and/or flushing), contaminants can accumulate in sediment to such high concentrations that they adversely affect bottom-dwelling organisms through direct and indirect toxicity (Long and Chapman 1985; Chapman et al. 1987). Although chemical analyses can identify whether sediment is contaminated, the presence of contaminants in sediment does not mean they are adversely affecting bottom-dwelling organisms. For contaminants to cause an adverse ecological effect they must be in a bioavailable form, that is, in a form that can cross biological membranes. To identify whether contaminants in sediment are in a bioavailable form requires some form of biological assessment, either through the analysis of benthic invertebrate community structure and composition or through toxicity testing (Long and Chapman 1985; Long 2000; EPA 2001; Long et al. 2001; McPherson et al. 2008). A common form of sediment toxicity testing involves the exposure of test organisms to whole sediment under controlled conditions in the laboratory.

The grain size composition and organic content of sediment are important factors that influence the structure and composition of benthic invertebrate communities (Gray 1974, Snelgrove and Butman 1994, Van Tomme et al. 2013). It is generally accepted that muddy sediment supports deposit feeders while filter feeders usually flourish in sandy sediment (Biernbaum 1979; Snelgrove and Butman 1994). Since bottom-dwelling organisms select for, survive or grow best in certain types of sediment, the development of a sediment toxicity test must identify how these non-contaminant factors influence test organisms. If these factors are not considered they may induce responses in test organisms that are not a result of exposure to contaminants, confounding the interpretation of toxicity test results (Lacey et al. 1999; Postma et al. 2002).

Factors influencing an amphipod's selection for a particular substrate include *inter alia* sediment grain size, sediment thickness or depth, food availability, food quality, food condition (i.e. fresh vs. aged) and biotic interactions (Meadows 1964a,b, Boltt 1969, DeWitt 1987, DeWitt et al. 1988). Amphipods are known to select for a specific sediment composition. For example *Corophium volutator* selects for fine sand whereas *C. arenarium* selects for coarser sand (Meadows 1964b). Food availability (including biofilms coating sediment particles) plays an important role in sediment selection (Meadows 1964a). Doig and Liber (2010) also demonstrated that >75% of the amphipod *Hyalella azteca* remains burrowed in its preferable fine sand in the presence of organic matter, but burrowing is reduced by as much as 62% in the absence of sediment organic matter. The correlation between sediment grain size and organic matter content however makes it difficult to differentiate which variable is more important to benthic fauna (DeWitt 1988, Snelgrove and Butman 1994). Both variables are thus frequently investigated in toxicity studies, particularly because of their potential to confound toxicity test results.

Very little research has investigated the influence of sediment parameters to estuarine benthic fauna of South Africa (e.g. Boltt 1969) and these include field survey observations (e.g. Teske and Wooldridge 2003). This is despite the fact that sediment is more important than salinity in influencing the distribution of benthic fauna in South African estuaries, which is in contrast with the situation in the northern hemisphere (Teske and Wooldridge 2003). Field observations suggest that the distribution of amphipods such as *Grandidierella lignorum, G. lutosa* and *Corophium triaenonyx* is not influenced by salinity or sediment within South African estuaries (Teske and Wooldridge 2003). Laboratory experiments however, showed that *G. lignorum* prefers muddy sediment (Boltt 1969).

Grandidierella lignorum has a potential to be used as a sediment toxicity testing organism as it meets the requirements for toxicity test organisms. It is widely distributed and has been collected from cool temperate (e.g. Wooldridge and Deyzel 2009), warm temperate (e.g. Schlacher and Wooldridge 1996; Teske and Wooldridge 2001) and subtropical estuaries (Vivier and Cyrus 1999; Stow 2011). It is easy to culture and handle in the laboratory (Connell and Airey 1979, Thwala 2006) and it appears to be broadly tolerant to physico-chemical parameters of relevance in toxicity testing, such as salinity, temperature and sediment particle size (e. g. Boltt 1969; Thwala 2006). In a sediment selection test, Boltt (1969) did not remove organic matter content from sediment samples. It is therefore, unknown whether *G. lignorum* shows a preference for sediment of a specific grain size or can survive in all types of sediment. Similarly, whether organic content influences sediment selection is unknown. The primary aim of this study was thus to determine whether *G. lignorum* shows a preference for sediment of a specific grain size or organic content, for the purpose of defining the conditions under which toxicity testing of whole sediment should be performed and as an aid to determining potential toxic effects when these amphipods are exposed to sediment not conforming to that preferred. The implications of the findings are also discussed in the context of the known ecology of *G. lignorum*.

Materials and Methods

Maintenance of amphipods in the laboratory

Amphipods were collected from the Intshambili River estuary on the subtropical northeast coast of South Africa (30°38'S, 30°32'E). In the laboratory the amphipods were maintained in four culture tanks (L x B x H: 53 x 33 x 15 cm) containing about 2 cm of medium- to fine-grained sediment and about 10 cm of UV sterilised and filtered (10 µm) natural seawater (salinity = 35). The culture water was aerated continuously. The amphipods were fed *ad libitum* three times a week on crushed fish flakes (Tetramin®), and 80% of the culture water was replaced weekly. The cultures were maintained at 22°C under a 12 hr light: 12 hr dark photoperiod in a temperature controlled environmental chamber. Under these conditions *G. lignorum* reproduced successfully, allowing for the regular harvesting of individuals for experimental purposes.

Sediment grain size and organic content preference

Sediment was collected from the Mlalazi River estuary (28°56'S, 31°48'E) using a Van Veen grab. In the laboratory the sediment was dried in an oven at 56°C, where after organic matter in the sediment was destroyed by muffling at 600°C for 6 hrs. The sediment was then passed through a sieve stack to isolate six grain size classes according to the Udden-Wentworth scale (Wentworth 1926; Blair and McPherson 1999); namely very coarse-grained sand (VC, 1 - 2 mm), coarse-grained sand (Co, 0.5 - 1 mm), medium-grained sand (Me, 0.25 - 0.5 mm), fine-grained sand (Fn, 0.125 - 0.25 mm), very fine-grained sand (VF, 0.63 - 0.125 mm) and mud (Mu, <0.63 mm).

Sediment grain size preference experiments were based broadly on the procedures described by Meadows (1964a). Choice chambers and tanks for housing the chambers were constructed from perspex (Figure 3.1).

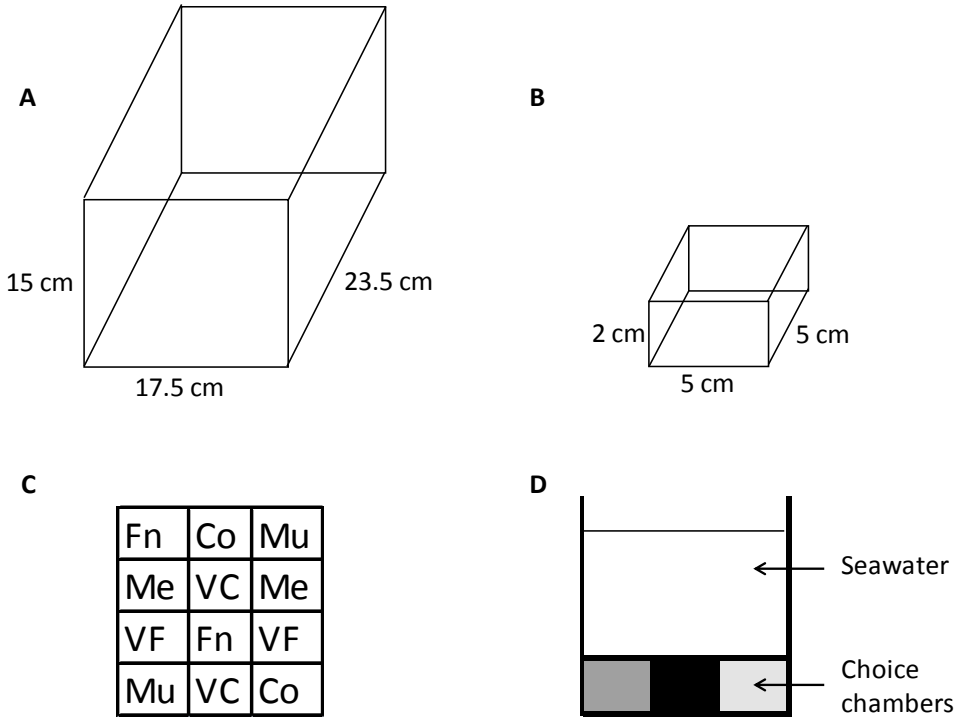

Figure 3.1. Experimental design for sediment grain size and organic content preference experiments. Represented are specifications for the experiment tank (a), specifications for choice chambers (b), example of randomised placement of choice chambers in an experimental tank (c), and the whole set up of the experiment (d).

Sediment of each grain size was offered in duplicates in each experimental tank (i.e. two choice chambers for every sediment grain size). Choice chambers (*n* = 12) with sediment of different grain sizes were randomly placed in the tanks (*n* = 3) (see Figure 3.1c). Sediment in choice chambers was filled to the brim so that amphipods can easily move between choice chambers. Filtered (10 µm) seawater was slowly introduced to the tanks by passing it through an air stone, to avoid sediment re-suspension. Juvenile amphipods (*n* = 50) were then introduced to the tanks. Amphipods were introduced just below the water surface and after introduction, amphipods were observed for a minute. Water in the tanks was gently aerated using air stones suspended some distance above the bottom, to avoid sediment re-suspension. The tanks were left for 24 hrs, where after the overlying water was siphoned off without disturbing the sediment and the number of amphipods in each choice chamber was counted. The experiment was repeated three times.

To determine whether *Grandidierella lignorum* selects for sediment based on the amount and type of organic matter present, amphipods were offered sediment devoid of or fortified with organic matter. Tetramin® fish flakes (rich in protein) and lucerne pellets (rich in carbohydrates) were ground using a coffee bean grinder, sieved through a 0.25 mm mesh screen, and an appropriate mass added to muffled medium-grained or fine-grained sand at 0, 0.5, 1.0, 2.0, 4.0 and 8.0% of dry weight. Concentrations of up to 4% organic content have been measured in estuaries (see Stow 2011) while concentrations >8% have been measured in harbours (Wepener and Vermeulen 2005, CSIR 2011). Medium- and fine-grained sand was chosen because they were identified as the preferred grain sizes from the above experiment. Sediment-organic matter mixtures were added to choice chambers that were then randomly placed in three experimental tanks, following the procedures described above. The experiment was repeated three times.

To determine whether *Grandidierella lignorum* is able to survive in various grain size classes of sediment devoid of organic matter for 10 days, which is the standard duration of an acute whole sediment toxicity test (e.g. EPA 1994), amphipods were exposed to muffled sediment in 1 L glass containers (bottom surface area: ~20 cm^2). The depth of sediment in the containers was about 2 cm. The containers were filled with ~700 ml of UV sterilised and filtered (10 µm) seawater. Each sediment grain size class offered for the grain size preference experiments was offered in three replicate containers. Test containers were covered with plastic petri dishes to limit excessive

evaporative loss and the water was continuously aerated by passing air through glass pipettes suspended some distance above the bottom, to avoid sediment re-suspension. Dissolved oxygen was measured prior the introduction of amphipods using an oxygen meter probe that was calibrated following manufacture specifications. After 24 hrs, 20 juvenile amphipods were introduced. The amphipods had been fed 12 hrs before transferred to the experimental containers. Amphipods were not fed during the experiment. Dissolved oxygen (mg l^{-1}) was maintained above 4 mg $l^{-1}$and salinity at 35 ± 1. Dissolved oxygen and salinity were monitored every two days and adjusted when necessary. Oxygen supply to test container was increased if dissolved oxygen fell below 4 mg l^{-1} and distilled water was added to test containers if salinity increased above 35.

Statistical analyses

Statistical analyses were performed using SPSS software (version 21). The proportion of amphipods retrieved from each sediment grain size was compared using a one factor analysis of variance (ANOVA) on ranked data, since assumptions of normality and equal variance were violated. This analysis was performed separately for the three replicate experiments to determine the reproducibility of results, and then repeated for pooled data. To determine if *G. lignorum* showed a preference for sediment containing different types and quantities of organic matter, a two factor ANOVA was performed on arcsine transformed data. This analysis was performed separately for the three replicate experiments to determine reproducibility of results, and repeated for pooled data for both medium-grained and fine-grained sediment. A three factor ANOVA was performed to determine if sediment grain size (medium- and fine-grained sand) influenced amphipods' selection for organic matter type and quantity. The survival of amphipods exposed to sediment devoid of organic matter during a 10 day period was determined by comparing mean survival using a one factor ANOVA. Significant differences (at P = 0.05) between treatments were identified by performing a Tukey HSD *post hoc* comparison test in all experiments.

Results

Although amphipods showed no significant selection for any grain size class in Experiment A and B, a higher proportion of amphipods were recovered from coarse-, medium- and fine-grained sediment (Figure 3.2a,b).In Experiment C, there was a significant selection for coarse-, medium-, fine and very-fine grained sediment (Figure 3.2c). Very coarse-grained sand and mud were thus the least selected grain size classes in all experiments. Analysis of pooled data (Figure 3.2d) showed that a significantly

higher proportion of amphipods were retrieved from fine- (27.48 ± 12.13 %), medium- (25.11 ± 12.99 %) and coarse-grained sand (21.45 ± 8.02 %) compared to other grain size classes. The proportion of amphipods retrieved from the remaining grain size classes was not significantly different, although the proportion retrieved from very fine-grained sand (15.03 ± 8.82%) was on average somewhat higher compared to coarse-grained sand (5.93 ± 5.94%) and mud (4.99 ± 4.87%) (Figure 3.2d).

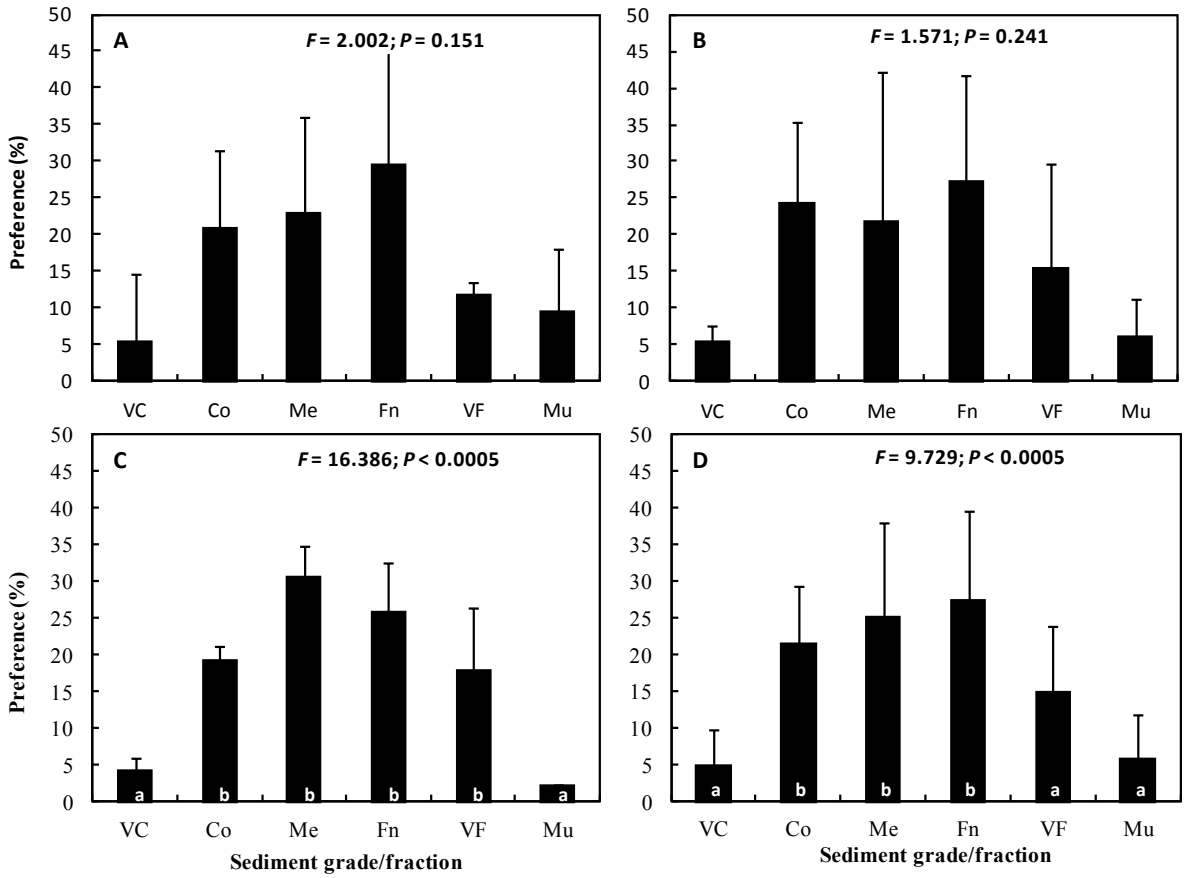

Figure 3.2. Proportion of *Grandidierella lignorum* (n = 3) retrieved from different sediment grain size classes. Graphs *a* - *c* present results of individual experiments while *d* presents pooled data. Similar letters represent no significant differences between compared sediment size classes while no letters represent no significant differences between all sediment size classes.

The proportion of amphipods recovered from sediment fortified with different amounts of organic matter was consistent between experiments. The data were thus pooled for each type of organic matter and sediment grain size class (Figure 3.3a,b). Amphipods preferentially selected for sediment

with no organic matter, with the proportion recovered generally decreasing with increasing organic matter content (see post hoc results within Figure 3.3a,b). The preference for sediment fortified with organic matter was not based on the type of organic matter (Fine-grained sand: $F_{interaction}$ = 0.623; $P_{interaction}$ = 0.683, Medium-grained sand: $F_{interaction}$ = 0.422; $P_{interaction}$ = 0.832), but based on organic matter concentration (Figure 3.3a,b). A three factor ANOVA confirmed the preference for sediment with no organic matter and that this was not influenced by the type of organic matter ($F_{(\text{concentration x organic matter})}$ = 0.572; $P_{(\text{concentration x organic matter})}$ = 0.722) or sediment grain size ($F_{(\text{concentration x sediment grain size})}$ = 0.463; $P_{(\text{concentration x sediment grain size})}$ = 0.803) (Figure 3.3c).

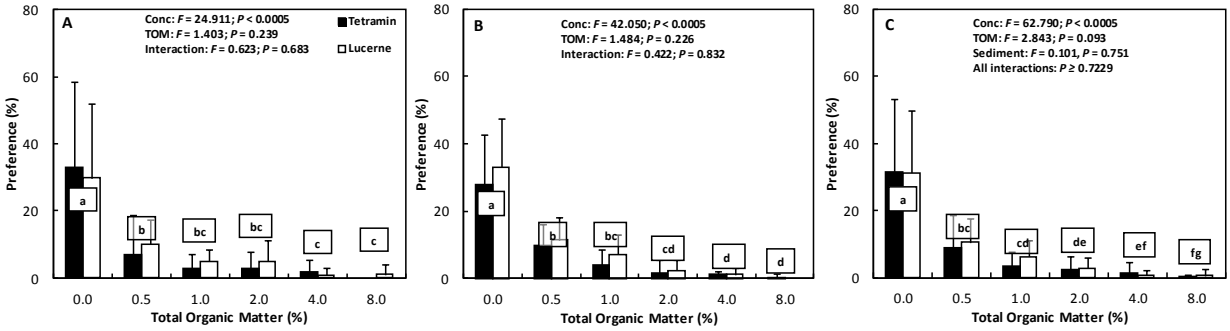

Figure 3.3. Proportion of *Grandidierella lignorum* (n = 3) retrieved from fine-grained (a) and medium-grained sand (b) fortified with two types of organic matter. The influence of sediment grain size on sediment selection based on the amount of organic content and type of organic matter type is shown in (c). Similar letters represents no significant difference in selection of sediment based on amount of organic content. Conc: organic matter content, TOM: type of organic matter, Sediment: medium- and fine-grained sediment.

Survival of *Grandidierella lignorum* exposed to sediment of various grain sizes devoid of organic matter for 10 days differed significantly (F = 8.871; P = 0.001) (Figure 3.4). The highest survival was recorded in fine-grained sand (91.67 ± 14.43 %) and the lowest in mud (29.59 ± 1.91 %).

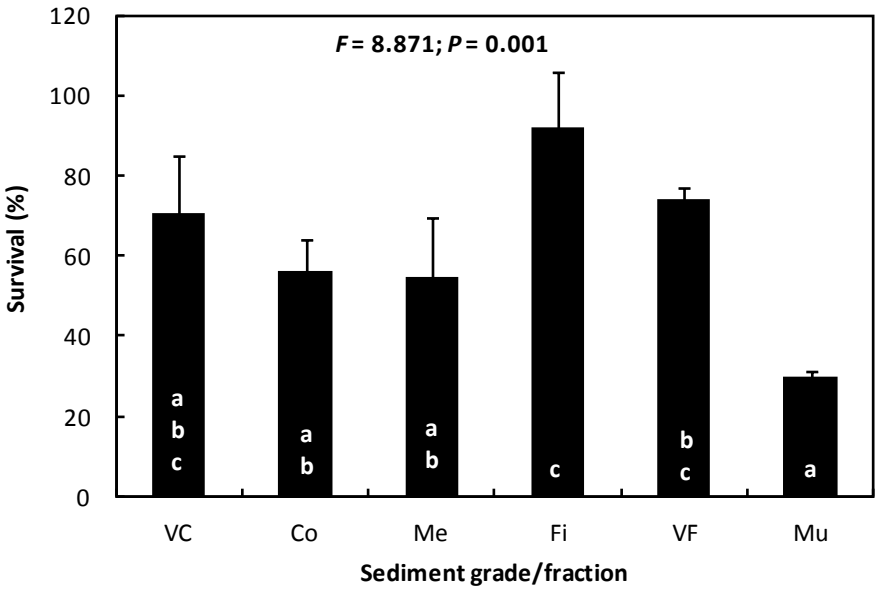

Figure 3.4. Mean survival ± SD (n = 3) of *Grandidierella lignorum* after 10 days exposure to different sediment grain size classes devoid of organic matter. Similar letters represent no significant difference in survival between sediment grain size classes.

Discussion

Grandidierella lignorum was retrieved from all sediment grain sizes offered, but showed a preference for coarse-, medium- and fine-grained sand. Based on these findings *G. lignorum* should theoretically be most abundant in the natural environment in sediment dominated by these grain sizes. However, the preference for sediment of a specific grain size class in the laboratory may not correspond to occurrence in the natural environment, where amphipods are highly unlikely to be exposed to sediment comprised of only one size class. Furthermore, other factors may influence where amphipods are found in the natural environment, including salinity tolerance and competition. Nevertheless, the experimental findings agree with the trend in abundance of *G. lignorum* in the field (L Vivier (University of Zululand) and F Mackay (Oceanographic Research Institute), personal communication).

An amphipod's preference for sediment of a particular grain size is probably partly related to the energetic costs associated with construction and maintenance of burrows or tubes (Meadows 1964a), and may explain the selection for some sediment grain size classes over others by *G. lignorum*. Constructing burrows in unfavourable sediment takes longer and requires more

maintenance, increasing the energetic cost (Doig and Liber 2010). If the energetic costs of burrow construction and maintenance are high then this may act as a confounding factor in whole sediment toxicity tests. If the preference for some sediment grain size classes over others by *G. lignorum* is related to the high energetic costs associated with burrow construction and maintenance, then this amphipod should not be used to test the toxicity of sediment dominated by very coarse-grained sand or mud. The general avoidance of mud may also be due to the clogging of gills.

Mud and/or some organic matter is required by *Grandidierella* species for the construction of burrows (Boltt 1969; Barnard et al. 1991). This is also true for *Corophium volutator*, which burrows even in unfavourable sediment if there is a small amount of mud available (Meadows 1964a). However, this does not tally with the findings of this study, which showed that amphipods not only burrowed in sediment devoid of mud but also devoid of organic matter. *G. lignorum* generally avoided mud in the experiments, yet it has been collected from mud dominated sediment in several South African estuaries (see Stow 2011). A possible explanation may be that the mud in experimental chambers was of a fluid nature and thus less viscous compared to the natural environment where it is mixed with variable amounts of other grain sizes and organic matter. Constructing and maintaining burrows in fluid mud may be energetically more expensive compared to other types of sediment. Mud devoid of organic matter (or with low organic content) is unstable and subject to resuspension and transportation or erosion (and is probably less viscous) (Uncles et al. 2006). This sediment would be unfavourable for burrow construction. Sediment organic matter encompasses microphytobenthic diatoms, whose production of the sticky mucopolysaccharides bind sediment particles together and in turn reduces sediment erosion (Sutherland et al. 1998; Uncles et al. 2003). This then increases the stability of muddy sediment, reducing the potential for erosion and thus supporting burrow construction. It was also observed during the experiments that mud particles clung to the setae of pereiopods of *G. lignorum* in the experiment when the amphipod landed on the muddy sediment. This would not only clog the gills but would also impair the ability of the amphipod to construct burrows, since amphipod silk used to glue sediment particles together is obtained from the pereiopods (Boltt 1969; Barnard et al. 1991). *G. lignorum* avoided muddy sediment in the selection experiment by swimming until it encountered favourable sediment grain sizes (*pers. obs.*). Survival of the amphipod in natural muddy sediment is thus expected to be higher than that recorded in the laboratory due to the presence of organic matter.

Grandidierella lignorum repeatedly preferentially selected for sediment with no organic matter in the laboratory. Preference decreased with increasing organic matter content, but was not affected by the type of organic matter. Amphipods select patches of sediment in the natural environment, using mechanical (i.e. grain size) or chemical cues (e.g. from food) (Meadows 1964a; Meadows 1964b; De Lange et al. 2005). The preferential selection of sediment devoid of organic matter by *G. lignorum* is interesting in that this amphipod has been collected from estuaries where the sediment organic matter content is as high as 4% (Stow 2011). Perhaps the condition of organic matter is particularly relevant for *G. lignorum.* Some amphipods prefer fresh organic matter (e.g. *Monoporeia affinis*) while others prefer aged organic matter (e.g. *Pontoporeia femorata*) (Byrén et al. 2006). Boltt (1969) observed *G. lignorum* re-ingesting faecal pellets during its burrow extension and maintenance.

In conclusion *Grandidierella lignorum* significantly selected for coarse-, medium- and fine-grained sediment over mud and very coarse-grained sediment. When exposed to the same sediment grain sizes, but over a 10 day period, which is a standard period for acute whole sediment toxicity test (EPA 1994), amphipod survival was still highest in favoured (e.g. fine-grained) sediment. When offered varying amounts of organic matter mixed in favourable sediment grain sizes, the amphipods consistently selected for sediment devoid of organic matter. This suggests that *G. lignorum* does not need to be fed during acute sediment toxicity tests. It is therefore suggested that sediment overly dominated by very coarse-grained sediment should not be tested by *G. lignorum*. The amount of mud in sediment composition that can be tested for toxicity requires further investigation. Mud is necessary for burrow construction in *Grandidierella* species (Boltt 1969, Barnard et al. 1991) but 100% mud is not favourable to *G. lignorum*.

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Chapter 4

Sensitivity of the amphipod *Grandidierella lignorum* (Amphipoda: Aoridae) to cadmium, copper and zinc in the laboratory

Abstract

One of the requirements for a toxicity test organism is that it should be sensitive to common contaminants of sediment. Metals were used in this study to determine the sensitivity of the amphipod *Grandidierella lignorum* under different test conditions (i.e. salinity). A quality control tool for assessing the sensitivity of the amphipod in toxicity tests (i.e. control chart) was also generated to aid in determining the acceptability of toxicity test results. Amphipods were exposed for 96 hrs to increasing concentrations of cadmium, copper and zinc at salinities of 7, 21 and 35. The toxicity of the metals (using LC_{50} as an indicator of toxicity) was compared between salinities. Cadmium toxicity decreased linearly with increasing salinity, from 0.34 ± 0.17 mg l^{-1} at a salinity of 7 to 1.08 ± 0.49 mg l^{-1} at a salinity of 35. Zinc toxicity increased with increasing and decreasing salinity, from 1.56 ± 0.33 mg l^{-1} at a salinity of 21 to 0.82 ± 0.19 mg l^{-1} at a salinity of 35 and 0.99 ± 0.13 mg l^{-1} at a salinity of 7. Copper toxicity, on the other hand, was not influenced by salinity. These responses were comparable to published data. A control chart was generated from a total of 20 LC_{50}'s, with upper and lower control limits measured as 0.49 mg Cd l^{-1} and 4.02 mg Cd l^{-1}, respectively.

Introduction

A reference toxicant is a standard chemical substance that is used to determine the sensitivity of the toxicity test population for a given toxicity assessment (Environment Canada 2005). Reference toxicity tests should be performed concurrently with toxicity tests (e.g. whole sediment, porewater, elutriate and effluent toxicity) to determine the status and/or sensitivity of the test population (EPA 1994; Environment Canada 2005). A result of a reference toxicity test, the LC_{50} (median concentration that causes death to 50% of the test population) is compared to a recent set of LC_{50}'s plotted as a control chart. An LC_{50} of a test population that is in poor health will often fall outside the control chart limits (Environment Canada 1990, Environment Canada 2005). Control charts can also be used to determine the precision of a laboratory that routinely performs toxicity tests and/or to compare precision between laboratories (i.e. inter-laboratory comparison) (Environment Canada 1990, 2005; Bay et al. 2003). A reference toxicant should *inter alia* be able to determine the sensitivity or health status of the test organism, be soluble and stable in solution, be readily available in pure form, be easily analysed in the laboratory, and have an established toxicity (Environment Canada 1990). Some of these characteristics are, however, difficult to satisfy.

Cadmium, copper and zinc are commonly used inorganic reference toxicants (Environment Canada 1990), and are widely used as the reference toxicant for toxicity tests where amphipods are used as the test organism (e.g. Hyne and Everett 1998; McGee et al. 1999; McNulty et al. 1999; Lee 2003; Prato and Biandolino 2005). The potential of these metals for use as reference toxicants was evaluated by experts in the United States of America (USA) and Canada (Environment Canada 1990). These metals represent essential and non-essential metals. Copper and zinc fulfill important roles in metabolic processes. For example, copper is a component of haemocyanin and plays a role in respiration of molluscs and crustaceans, while zinc is an integral part of many enzymes, including carbonic anhydrase (Bat 2005, Rainbow 2007, Rainbow and Luoma 2011). Cadmium has no known physiological role in crustaceans and is thus considered a non-essential metal (Rainbow 2007). Both essential and non-essential metals can be toxic (e.g. Grosell et al. 2007, Rainbow 2007).

The primary aim of this study was to determine the sensitivity of the amphipod *Grandidierella lignorum* to cadmium, copper and zinc. One of the characteristics of a good toxicity test organism is its sensitivity to common contaminants of sediment (see Burton et al. 1992; Peters et al. 2002; Bat 2005). The reference toxicants used in this study provide a means for determining the sensitivity for *G. lignorum*. Since salinity is known to influence the toxicity of metals to many aquatic organisms (McLusky et al. 1986), the influence of salinity on metal toxicity was also investigated. This is significant since the findings of Chapter 2 showed that *G. lignorum* can be used to test the toxicity of both estuarine and marine waters. For this purpose, reference toxicity tests were performed at salinities of 7, 21 and 35. This study contributes to the limited information on the interactive effects of salinity and metal toxicity to estuarine fauna of South Africa.

The use of a control chart has been highlighted above, yet this tool for quality assurance and quality control purposes is rarely used in South Africa, or it is simply not reported. Vivier (2010) compared reference toxicity test results with those for the amphipod *Leptocheirus plumulosus* provided by DeWitt et al. (1996). Such a comparison only highlighted the comparative sensitivity of *G. lignorum* to *L. plumulosus*. It did not determine if the sensitivity of *G. lignorum* was within an acceptable range to decide on the acceptbility of toxicity test results. This highlights the importance of a quality assurance and quality control tool for toxicity tests using *G. lignorum.* Therefore, a further aim of this study was to establish a quality control tool for *G. lignorum* in the form of a control chart for future use.

Materials and Methods

Concentrations of metals were prepared from stock solutions of 100 mg Zn l^{-1} ($ZnCl_2$, CP (Chemically Pure) Grade), 100 mg Cu l^{-1} ($CuCl_2 \cdot 2H_2O$, CP Grade) and 1000 mg Cd l^{-1} ($CdCl_2 \cdot H_2O$, AR (Analytical Reagent) Grade) in distilled water to prevent precipitation. Stock solutions were stored in clean, acid-washed Schott® bottles. Filtered (10 µm) and UV sterilised seawater was used to dilute appropriate volumes of stock solutions to prepare test media (salinity-metal concentration combinations). The range of metal concentrations used in experiments was determined from range finder tests (i.e. pilot studies). Based on the results, amphipods were exposed to nominal concentrations of 0 - 6.4 mg Cd l^{-1}, 0 - 4.8 mg Cu l^{-1} and 0 - 3.2 mg Zn l^{-1}. Actual exposure concentrations were not confirmed by chemical analysis of test media.

Reference toxicity tests were performed with juvenile amphipods (2 - 4 mm) that passed through 1 mm mesh but were retained by 0.5 mm mesh. The amphipods were fed on crushed fish flakes (Tetramin®) approximately 12 hrs prior to experiments. Test concentrations were prepared in 1 L glass containers 24 hrs prior to experiments. Toxicity tests were performed following standard procedures (i.e. Environment Canada 1990, Environment Canada 2005; EPA 1994). Briefly, amphipods were sieved from their cultures by disturbing culture sediment and 20 amphipods randomly selected and added to test solutions using a wide-bore glass pipette. Amphipods were acclimated to test salinities before they were exposed to metal concentrations. Test concentrations were offered in triplicate and glass containers were loosely covered to limit evaporative loss during the experiment. Survival was monitored at 24 hr intervals over a 96 hr period. Each toxicity test was repeated three times to determine the reproducibility of results. Toxicity tests were performed at salinities of 7, 21 and 35, to determine the influence of salinity on metal toxicity. Low salinities were prepared by diluting filtered (10 µm) and UV-sterilized seawater with deionised water. Experiments were performed at 22°C and at 12 hr light: 12 hr dark cycle, without aeration. This is comparable to methods used in previous studies (i.e. Thwala 2006; Vivier 2010), and test conditions (i.e. temperature and salinity) are within the tolerable range for *G. lignorum* (see Chapter 2). Results of toxicity tests were deemed acceptable when survival in the control media (0 mg l^{-1}) was ≥80%.

A control chart was created using one of the reference toxicants. Toxicity tests for generating the control chart were performed at a salinity of 35 and at 22°C. These conditions are the same as those at which amphipods were cultured in the laboratory. More importantly, environmental health

monitoring for South African coastal ecosystems is strongly focused on higher salinity systems, such as ports (e.g. Vermeulen and Wepener 1999; CSIR. 2011; Greenfield et al. 2011). Salinity of the nearshore environment of South Africa ranges between 34.7 and 35.4 (DWAF 1995). The control chart was generated from a total of 20 LC_{50}'s. Reference toxicity tests for generating the control chart were performed in triplicate except for the last two results. This means that three reference toxicity tests were performed simultaneously using amphipods from the same culture. The last two LC_{50}'s were obtained from monthly assessment of amphipod sensitivity.

To illustrate the usability of the control chart, three LC_{50}'s obtained by the use of 'stressed' amphipods in toxicity tests were plotted on a control chart. 'Stressed' amphipods in this study refer to cultured amphipods that had just been exposed to new sediment after a routine maintenance that required sediment change. These amphipods had been exposed for less than two weeks to the new sediment. Sediment change during culture maintenance involves vigorous sediment disturbance, sieving of amphipods, and then exposure to new sediment. This procedure is probably associated with a suite of stresses. The toxicity test using the stressed amphipods was repeated three weeks later to determine if the health status of the new population had improved. McNulty et al. (1999) also used sieving as a stress factor in their reference toxicity test study.

The toxicity of metals was summarised as LC_{50}'s calculated using US EPA Probit Analysis software. Where test conditions for the probit method were violated (e.g. data did not fit probit model), LC_{50}'s were estimated using the Trimmed Spearman Karber (TSK) method. The probit method is a parametric statistical test for estimating LC_{50} while TSK is a non-parametric test for estimating LC_{50} where data does not fit the probit model (EPA 1994). Toxicity of metals (LC_{50}'s) between different salinities was compared using one way ANOVA (SPSS version 21). LC_{50}'s used to generate a control chart were calculated using the probit method and followed Environment Canada (2005) guidelines. The variability of LC_{50}'s used to generate the control chart was determined by calculating the coefficient of variation (CV) (Environment Canada 1990, Environment Canada 2005) and CV > 30% indicated low consistency between toxicity tests (i.e. low reproducibility). It is generally recommended that the CV does not exceed 30%, however, this value is not based on empirical evidence (Environment Canada 1990). Toxicity of metals to *Grandidierella lignorum* was also compared to other amphipods (potential toxicity test species and standard toxicity test species) based on published literature.

Results

A total of four out of 27 LC_{50}'s violated conditions of the probit method and were consequently determined using the TSK method. Three of these LC_{50}'s were calculated at a salinity of 7 (one LC_{50} for each reference toxicant), and the other at a salinity of 35 (Figure 4.1). The LC_{50} for cadmium at a salinity of 7 ranged between 0.15 - 0.44 mg l^{-1} (Figure 4.1a). The LC_{50} for copper at a salinity of 7 ranged between 0.52 - 1.00 mg l^{-1} (Figure 4.1d), while that for zinc ranged between 0.85 - 1.11 mg l^{-1} (Figure 4.1g). At a salinity of 21, the LC_{50} ranged between 0.68 - 0.78 mg l^{-1} for cadmium (Figure 5.1b), 0.62 - 1.03 mg l^{-1} for copper (Figure 4.1e), and 1.18 - 1.80 mg l^{-1} for zinc (Figure 5.1h). At a salinity of 35 the LC_{50} ranged between 0.62 - 1.62 mg l^{-1} for cadmium (Figure 4.1c), 0.52 - 0.84 mg l^{-1} for copper (Figure 4.1f) and 0.61 - 0.97 mg l^{-1} for zinc (Figure 4.1i).

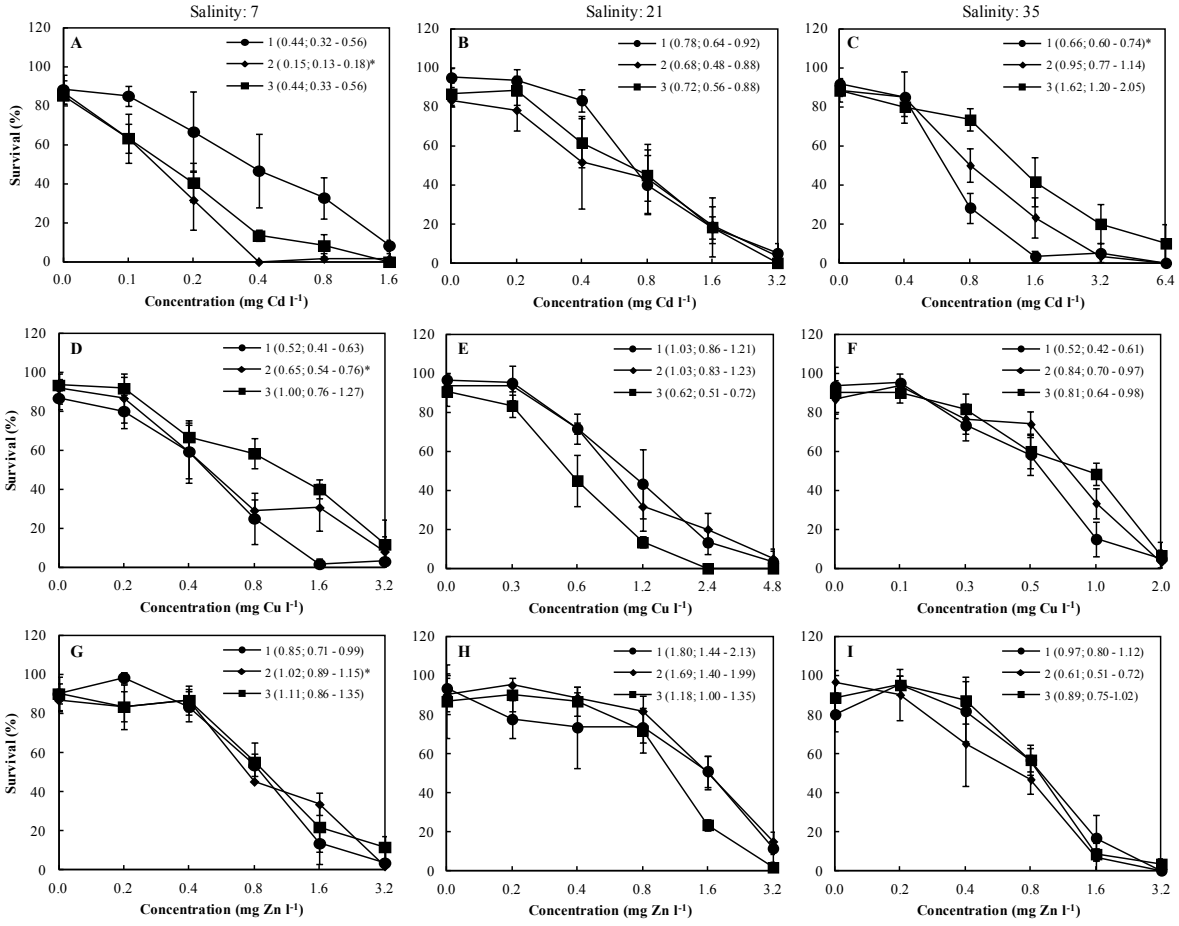

Figure 4.1. Survival of *Grandidierella lignorum* following exposure to dissolved cadmium, copper and zinc at various concentrations after 96 hrs of exposure at different salinities. Represented in each graph is the mean survival (n = 3) for all toxicity tests (represented by 1, 2, 3). Measurements represent mean LC_{50}'s and associated 95% confidence intervals. LC_{50}'s calculated using the Trimmed

Spearman Karber method are denoted by * - all other LC_{50}'s were calculated using the probit method.

Metal toxicities between replicate experiments sometimes varied considerably. For example, the toxicity of cadmium at a salinity of 7 did not differ significantly between experiments 1 and 3, but both differed significantly to experiment 2 (based on the comparison of confidence intervals) (Figure 4.1a). Copper toxicity between experiment 3 differed significantly to experiment 1, but not to experiment 2 (Figure 4.1d), while at salinity of 21 copper toxicity in experiment 3 differed significantly to that in experiments 1 and 2 (Figure 4.1e). The toxicity of cadmium at a salinity of 35 differed significantly between all three experiments (Figure 4.1c).

The influence of salinity on metal toxicity is shown in Figure 4.2. Cadmium toxicity decreased linearly (r^2 = 0.772, P = 0.007) with increasing salinity. Toxicity decreased from 0.34 ± 0.17 mg l^{-1} at a salinity of 7 to 1.08 ± 0.49 mg l^{-1} at a salinity of 35. The LC_{50} calculated for a salinity of 21 was 0.73 ± 0.05 mg l^{-1} (Figure 4.2a). Although cadmium toxicity decreased with increasing salinity, the difference between salinities was not statistically significant (Figure 4.2a). The LC_{50} for copper ranged between 0.72 ± 0.18 mg l^{-1} at a salinity of 35 and 0.89 ± 0.24 mg l^{-1} at a salinity of 21. The LC_{50} for copper at a salinity of 7 was 0.72 ± 0.25 mg l^{-1}. Copper toxicity did not differ significantly between salinities (Figure 4.2b). Zinc toxicity showed a different response to that of cadmium and copper. Toxicity increased either side of the salinity of 21, from 1.56 ± 0.33 mg l^{-1} at a salinity of 21 to 0.99 ± 0.13 mg l^{-1} at a salinity of 7 and 0.82 ± 0.19 mg l^{-1} at a salinity of 35 (Figure 4.2c). Zinc toxicity at a salinity of 7 was statistically similar to that at a salinity of 35, but toxicity at salinities of 21 and 35 was significantly different (Figure 4.2c). The order of toxicity at different salinities, based on absolute LC_{50}'s, was Cd>Cu>Zn at salinities ≤21, but Cu>Zn>Cd at a salinity of 35.

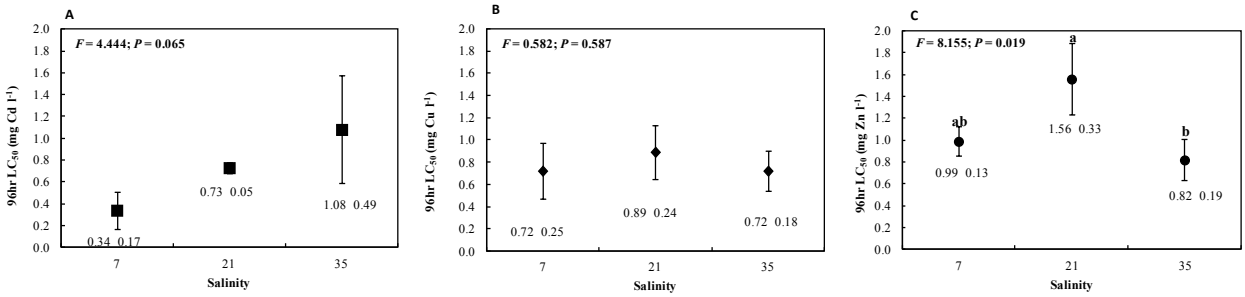

Figure 4.2. Influence of salinity on toxicity of cadmium (a), copper (b) and zinc (c) toxicity to the amphipod *Grandidierella lignorum*. Similar letters between data points represent no significant differences. Graphs are presented on a similar scale to facilitate comparison of toxicity

A control chart using cadmium as the reference toxicant at salinity of 35 and temperature of 22°C is shown in Figure 4.3. The mean LC_{50} was calculated as 1.40 ± 0.002 mg l^{-1}. The upper control limit was 4.02 mg l^{-1} and the lower limit was 0.49 mg l^{-1}. Data used for generating a control chart were normally distributed (Z = 1.071, P = 0.201) and the variability between LC_{50}'s used to generate the chart (i.e. coefficient of variation) was 35.76%. A total of four LC_{50}'s fell outside the control limits, namely Test 4 and Tests 13 - 15. The latter tests represent LC_{50}'s calculated using stressed amphipods (Figure 4.3).

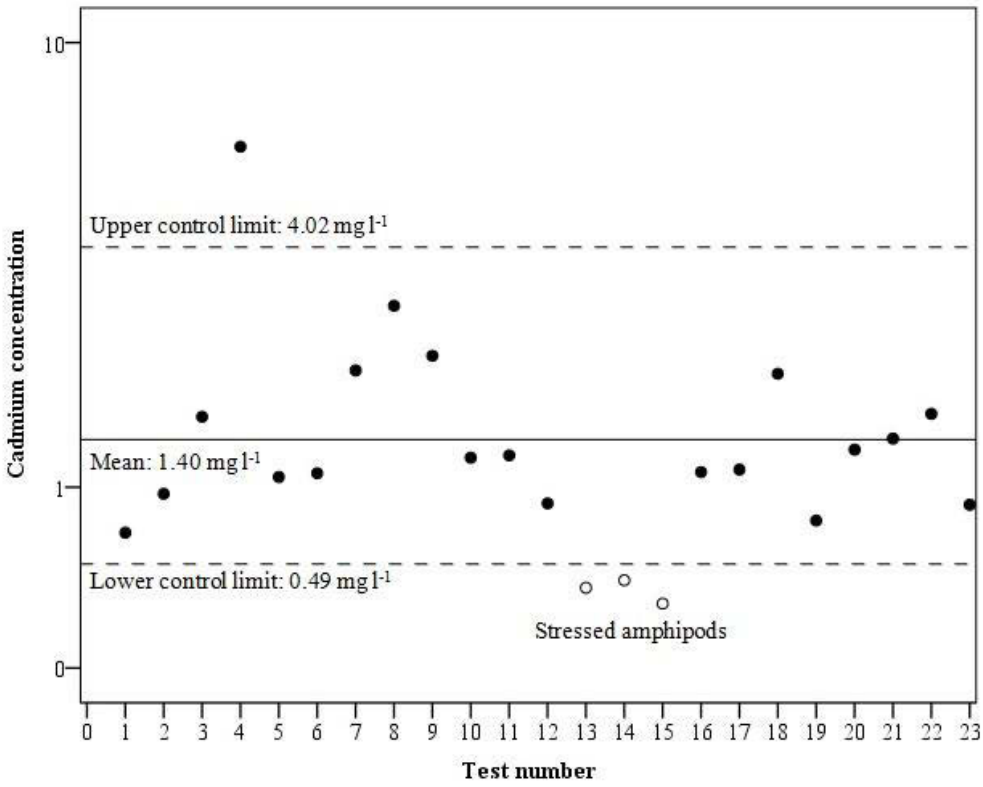

Figure 4.3. A cadmium reference toxicant control chart for *Grandidierella lignorum*. Open symbols (Test numbers 13 - 15) indicate LC_{50}'s for a 'stressed' population and the subsequent three LC_{50}'s (Test Numbers 16 - 18) for the stressed population after three weeks of rest.

Discussion

The LC_{50}'s calculated for *G. lignorum* in this study are at least two orders of magnitude higher (except for Papenkuils river estuary) than concentrations measured in overlying water of South African estuaries (e.g. Watling and Emmerson 1981, Watling and Watling 1982, Watling et al. 1985, Orr 2007, Vivier 2010). These L_{C50}'s are however, comparable to that of other amphipods under laboratory conditions (see Table 5.1). Sensitivity to cadmium at salinities above 30 compared favourably to that for *Grandidierella japonica*, *Gammarus aequicauda, Melita koreana, Mandibulophoxus mai* and *Corophium acheriscum*. At salinities below 8, it was comparable to that for *Leptocheirus plumulosus*, which is a standard toxicity testing organism in the United States of America. The toxicity of copper to *G. lignorum* at salinity of 35 was similar to that of *G. aequicauda*, while zinc toxicity was comparable to that of *Melita awa, M. matilda, M. plumulosa* and *Chaetocorophium* cf *lucasi*. *G. lignorum* is sensitive to dissolved metals under different test

conditions (i.e. varying salinity). Its sensitivity under these varying conditions is comparable to that for other amphipods used or proposed for use as toxicity testing organisms. Sensitivity to contaminants is one of the requirements for a toxicity test organism.

Table 4.1. Sensitivity of *Grandidierella lignorum* to reference toxicants compared to other amphipod species proposed or used as toxicity testing organisms. Ranges reported in parentheses represent 95% confidence limits.

Species	Salinity	Temperature (°C)	96hr Cd LC_{50} (mg l^{-1})	96hr Cu LC_{50} (mg l^{-1})	96hr Zn LC_{50} (mg l^{-1})	Reference
Corophium sp	5		-	0.009 (0.0049-0.0165)	-	Hyne & Everett 1998
Grandidierella lignorum	5	22	0.87	-	-	Thwala 2006
Grandidierella lignorum	7	22±1	0.34±0.17	0.72±0.25	0.99±0.13	This study
**Leptocheirus plumulosus*	8	25	0.25	-	-	McGee et al. 1999
Corophium sp	10		-	0.0285 (0.0083-0.098)	-	Hyne & Everett 1998
Corophium triaenonyx	20	25	1.6	-	-	Vivier 2010
**Eohaustorius aesturius*	20	15	-	33.3	-	Anderson et al. 2008
Grandidierella lignorum	20	25	1.1	-	-	Vivier 2010
Grandidierella lignorum	21	22±1	0.73±0.05	0.89±0.24	1.56±0.33	This study
Grandidierella lignorum	25	22	1.94	-	-	Thwala 2006
Chaetocorophium cf *lucasi*	30	21±1	-	-	1.13 (0.87-1.39)	King et al. 2006
Haustorioides indivisus	30	20	1.50	-	-	Lee et al. 2005
Haustorioides koreanus	30	20	2.77±2.44	-	-	Lee et al. 2005
Hyale crassicornis	30	21±1	-	>1	-	King et al. 2006
Hyale longicornis	30	21±1	-	>1	>0.5	King et al. 2006
Mandibulophoxus mai	30	20	1.12±0.36	-	-	Lee et al. 2005
Melita awa	30	21±1	-	0.12 (0.098-0.120)	0.71 (0.47-0.96)	King et al. 2006
Melita matilda	30	21±1	-	0.18 (0.15-0.21)	0.65 (0.09-1.45)	King et al. 2006
Melita plumulosa	30	21±1	-	0.12±0.019	0.64 (0.39-0.91)	King et al. 2006
Monocorophium acherusicum	30	20	1.37±0.38	-	-	Lee et al. 2005
**Grandidierella japonica*	35	19.5	1.17	-	-	Hong & Reish 1987
Grandidierella lignorum	35	22	1.78	-	-	Thwala 2006
Grandidierella lignorum	35	22±1	1.08±0.49	0.72±0.18	0.82±0.19	This study
Melita koreana	35	20	1.2-1.3	-	-	Lee 2003
Gammarus aequicauda	36	16±2	0.71 (0.44-1.14)	0.82 (0.53-1.28)	-	Prato & Biandolino 2005

*Standard toxicity test species

The use of juvenile *Grandidierella lignorum* in this study and use of adults by Thwala (2006) permits a comparison of cadmium toxicity between these life stages. As expected, juveniles were more sensitive (at least 1.65 times) than adults. Test conditions between the current study and that of Thwala (2006) were similar except that amphipods were exposed in a group of 20 individuals in the current study while Thwala (2006) exposed amphipods individually in glass pill vials. Nevertheless, both studies highlight the importance of salinity on cadmium toxicity to *G. lignorum*. The influence

of salinity on metal toxicity to malacostracans is well known, particularly for larger crustaceans such as crabs and mysids (e.g. Nugegoda and Rainbow 1989; Wright 1995; Wildgust and Jones 1998; Leornard et al. 2011; Thwala et al. 2011). The general trend is for increasing metal toxicity with decreasing salinity (see McLusky et al. 1986).

Grandidierella lignorum showed three distinct responses to metal toxicity. In the first instance toxicity increased with decreasing salinity. For example, cadmium toxicity increased linearly with decreasing salinity. The toxicity of cadmium to juvenile *G. lignorum* follows the general rule of increasing metal toxicity with decreasing salinity (McLusky et al. 1986), but is in contrast to that for the adult *G. lignorum*. Cadmium toxicity to adult male *G. lignorum* increased with decreasing salinity (from 1.94 mg l^{-1} at a salinity of 25 to 0.87 mg l^{-1} at a salinity of 5) and with increasing salinity (1.78 mg l^{-1} at salinity of 35) (Thwala 2006). The second response is that of increasing metal toxicity with decreasing and increasing salinity. For example, zinc toxicity increased from salinity of 21 (1.56 ± 0.33 mg l^{-1}) to salinity of 7 (0.99 ± 0.13.mg l^{-1}), and increased from salinity of 21 to salinity of 35 (0.82 ± 0.19 mg l^{-1}). A similar response has been recorded for the shrimp *Farfantepenaeus paulensis* (Barbieri and Doi 2011) and mysid *Neomysis integer* (Wildgust and Jones 1998). In the last response, metal toxicity was not influenced by salinity. In this instance, copper toxicity did not differ significantly between test salinities.

The toxicity of metals to aquatic organisms can be explained in terms of the interaction between physico-chemical properties of the test medium (i.e. water chemistry) and physiological processes (Rainbow 1995; Worms et al. 2006). Physiological processes include osmotic and ionic regulation. In a dilute medium, including less saline water where concentrations of metal ions (e.g. Cd^{2+}) are high (Rainbow 1995), the amphipod faces an osmotic influx of water. Since a constant cellular volume has to be maintained in order not to compromise the integrity of the cell, excess water gained through osmotic diffusion must be expelled to the extracellular environment (i.e. osmoregulation) and some of the dissolved essential salts (e.g. Na^{2+}, Ca^{2+}) may be lost during this process. These losses are not only caused by water chemistry, as essential salts such as calcium are also lost during moulting (Ahearn et al. 2004). To compensate for the losses the amphipod has to actively transport ions against a concentration gradient (i.e. from the dilute media into the body where concentrations are higher). This is energetically expensive and generally takes place across transporter proteins

embedded in the lipid bilayer of cells (Péqueux 1995; Henry 2001; Lucu and Towle 2003; Freire et al. 2008; Palmgren and Nissen 2011).

Transporter proteins are generally referred to as P-type ATPases (Palmgren and Nissen 2011). The activity of ATPases increases in low salinity, thus facilitating the active transport of ions against concentration gradients (Brooks and Mills 2006). Essential ions would be transported across P_{2A}/P_{2B}-ATPases and P_{2C}-ATPases while metals would be transported across P_{1B}-ATPases (Palmgren and Nissen 2011). It is, however, suggested that transport sites (channels or proteins) are not highly selective (i.e. cannot discriminate between essential and non-essential metals), but transport ions of similar ionic radius and/or coordination geometry (Rainbow 1995; Worms et al. 2006). For example, Cd^{2+} and Ca^{2+} have similar ionic radii (109 pm and 114 pm, respectively) and thus compete for binding sites at the Ca^{2+}channel (Rainbow 1995). Therefore, trace metals can utilise several uptake routes into the cells (i.e. P_{2A}/P_{2B}-ATPases, P_{2C}-ATPases and P_{1B}-ATPases). When physiological processes like the production of metallothionein and glutathione (chelating agents) fail to cope with the influx of ions (essential and non-essential), particularly in a dilute medium, water chemistry becomes an important predictor of toxicity (Rainbow 1995). Chelating agents such as metallothionein bind the free metal ions in the cell, thus reducing their roles in metabolism, and also regulate the concentration of essential metals within the cell (Amiard et al. 2006).

The linear increase in cadmium toxicity with decreasing salinity suggests that water chemistry was more important in explaining toxicity to *Grandidierella lignorum* in this study. Metal toxicity occurs when metal uptake surpasses detoxification and excretion (Rainbow 2007. Crustaceans, including amphipods, cannot regulate cadmium in their bodies (Rainbow 2007). Furthermore, increased cadmium uptake at dilute media is known to damage amphipod gills, thus affecting their osmoregulatory capacity (see Issartel et al. 2010). Zinc toxicity at both low and high salinities suggests a strong interaction between physiology and water chemistry. Increased zinc toxicity at high salinity would be a result of active transportation from the dilute media, since the high concentration of chloride ions at high salinity would decrease zinc bioavailability (see Rainbow 1995). Increased zinc toxicity at low salinity would be best explained in terms of water chemistry. Essential metals such as zinc can be transported into cells regardless of concentrations in the dilute media and their uptake is largely regulated (Worms et al. 2006). Copper toxicity did not differ

significantly between salinities suggesting some form of regulation by *G. lignorum*. Accumulation and toxicity of copper to the crab *Hemigrapsus crenulatus* is also known to be independent of salinity (Lee et al. 2010), whilst salinity had a significant influence on copper toxicity to the killifish, *Fundulus heteroclitus* (Grosell et al. 2007). Copper toxicity in *F. heteroclitus* increased above and below salinity 10 (i.e. tolerance to copper was highest at salinity 10). The chemistry of the test media (or salinity), however, could not explain the toxicity of copper to *F. heteroclitus* since the most toxic forms of copper (i.e. Cu^{2+} and $CuOH^{+}$) were also abundant at the tolerable salinity (Grosell et al. 2007). It was concluded that copper toxicity is best explained by physiology, since it disrupts osmoregulation and acid-base balance in fish and invertebrates (Grosell et al. 2007). Other studies have also demonstrated that amphipods find it difficult to regulate cadmium compared to copper and zinc. For example, the amphipod *Orchestia gammarellus* detoxifies copper and zinc faster than it detoxifies cadmium, and these metals are temporarily stored in the ventral caeca pending excretion (Nassiri et al. 2000). Zinc and copper toxicities to *Grandidierella lignorum* may be overestimated in this study, since precipitation of these metals at higher salinities and higher concentrations was observed. This may influence the estimation of the LC_{50}'s and the hierarchical toxicity mentioned in the Results section. Copper is known to precipitate above 3 mg l^{-1} in estuarine and/or marine waters (McPherson and Chapman 2000).

Since zinc and copper precipitated at higher salinities and higher concentrations, cadmium was chosen as the reference toxicant for *Grandidierella lignorum*. Cadmium is a commonly used reference toxicant and has been adopted as such in South African toxicity studies (e.g. Vivier 2010). Very few published studies, however, report on control charts (e.g. Abessa and Sousa 2003). This limits comparison with the current study. Nevertheless, the control chart generated here for *G. lignorum* was useful in identifying stressed populations. Cadmium toxicity to stressed amphipods was very high and fell below the lower control limits. This suggests that cadmium toxicity interacted (additively or synergistically) with some stressor/s to increase cadmium toxicity. After resting the stressed amphipods for a further three weeks, cadmium toxicity was within the acceptable range and amphipods could then be used in a toxicity test. Variability between LC_{50}'s used to determine control limits in this study (CV: 35.76%) was slightly above recommendations of 30% (Environment Canada 1990, Environment Canadan2005), but this is not uncommon. The coefficient of variation between 18 LC_{50}'s used for the amphipod *Tiburonella viscana* was 33.6% (Abessa and Sousa 2003). Furthermore, 5% of the LC_{50}'s in a control chart will fall outside the control limits just by chance (Environment Canada 1990, 2005). Therefore, the LC_{50} measured in Test Number 4 is not a major

concern. Generally, when an LC_{50} falls outside the control limits, an investigation should ensue. Factors such as the preparation of stock solutions and environmental parameters in laboratory cultures are amongst the factors that should be investigated (Environment Canada 1990, 2005).

In conclusion, the sensitivity of *Grandidierella lignorum* to metals has been demonstrated and is comparable to that of other amphipods used or considered for use as toxicity test organisms. The role of salinity in metal toxicity was also evident for *G. lignorum*, and was metal-specific. Toxicity of the non-essential metal (i.e. cadmium) increased linearly with decreasing salinity while the amphipod showed the ability to regulate the toxicity of essential metals (zinc and copper). The influence of salinity on metal toxicity is variable between life stages, as was indicated for juvenile and adult *G. lignorum* in this study. *G. lignorum* can be used to test the toxicity of water from estuarine and marine ecosystems. This study has also generated the first control chart for *G. lignorum* and has demonstrated its usefulness in identifying stressed populations. Use of quality control tools, such as a control chart, should be a common practice and South African toxicity testing laboratories are encouraged to report on these results as they determine the acceptability of toxicity test results.

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Chapter 5

Whole effluent and sediment toxicity testing using the amphipod *Grandidierella lignorum* (Amphipoda: Aoridae)

Abstract

The usefulness of the amphipod *Grandidierella lignorum* as a toxicity testing organism was demonstrated through the toxicity testing of liquid waste (effluent) and sediment. Sea urchin fertilisation tests were used to complement data generated. Effluents collected in July and August from two wastewater treatment plants (Durban Central Works and Durban Southern Works) were assessed for toxicity. Effluent toxicity was generally higher for effluents collected in August than for effluents collected in July. The opposite was true for the sea urchin gametes. Additionally, sea urchin gametes were more sensitive than the amphipod, but the difference was variable. The sensitivity of *G. lignorum* to effluent toxicity was satisfactory and provides evidence of its suitability for effluent toxicity testing. The toxicity of 15 sediment samples collected in Durban Bay was assessed using a 10 day whole sediment toxicity test. Three sediment samples were toxic to *G. lignorum* while elutriates of four samples were identified as such using sea urchin gametes. Two of the samples toxic to sea urchin gametes were also toxic to *G. lignorum*. The most toxic sediment was situated off the inflows of rivers and in an area where vessel maintenance is undertaken. Recommendations for refining the sediment toxicity test are provided.

Introduction

Investigations on marine pollution in South Africa have declined drastically since the 1980s (O' Donoghue and Marshall 2003; Wepener and Degger 2012), yet the coastal environment is increasingly threatened and shows signs of degradation (Van Niekerk et al. 2013). The health status of estuaries has been assessed at a regional and country-wide scale using multiple lines of evidence (e.g. Cooper et al. 1994; Forbes and Demetriades 2009; Van Niekerk et al. 2013) and one of the emerging concerns is sediment contamination (see Van Niekerk et al. 2013). Sediment health should be assessed using multiple lines of evidence, such as in the Sediment Quality Triad (SQT) developed by Long and Chapman (1985). In this approach, data on sediment chemistry, macrofaunal community assemblage and sediment toxicity are integrated to summarise sediment quality. Tools for differentiating possible anthropogenic enrichment of metals from background concentrations have been developed and applied to selected coastal environments of South Africa (e.g. Newman and Watling 2007; Orr et al. 2008; Vivier 2010; CSIR. 2011), but chemical approaches do not infer toxicity as they only provide an estimate of possible biological effects of contaminants when compared to sediment quality guidelines (O'Connor and Paul 2000; Chapman 2007; Simpson and Batley 2007). Additionally, the chemical analyses of sediment may be costly and it is not possible to measure all potential chemicals in sediment that may be causing toxicity (Bay et al. 2005; Bay et al.

2011). Even if this were possible, many of the chemicals have no associated toxicity information, and unknown additive, synergistic and antagonistic effects. Bioassays (i.e. sediment toxicity tests) are increasingly being used to determine the toxicity of sediments and to identify areas of concern from a contamination perspective (e.g. Bay et al. 2005; Anderson et al. 2007; Bay et al. 2011; Greenstein et al. 2013). Unfortunately, standard (or certified) protocols for the toxicity testing of sediment in estuarine and marine ecosystems in South Africa have not been developed (Slabbert et al. 1998; Wepener and Chapman 2012). Organisms that have been used for sediment toxicity testing in South Africa include gastropods (e.g. *Bullia rhodostoma*, *B. digitalis*) and bivalves (e.g. *Donax serra*) (Watling and Watling 1983; Stenton-Dozey and Brown 1994), but these organisms are not recommended due to their biology and behaviour. They reproduce seasonally and are difficult to maintain and/or rear in the laboratory. These organisms can also isolate themselves from the contaminated environment by closing their valves or the operculum over an extended period of time (Brown 1982; Watling and Watling 1983; Stenton-Dozey and Brown 1994). Sea urchin gametes have also been used to screen toxicity for the marine environment (see Greenwood and Brown 1974; Brown and Greenwood 1978; Greenwood 1983; McGibbon and Moldan 1986; Wynberg et al. 1989; Connell et al. 1991), but these toxicity tests can only be used for dissolved contaminants in stenohaline marine conditions.

The lack of standardised sediment toxicity testing procedures in South Africa makes it difficult to determine, with some degree of certainty, sediment quality in coastal environments. A sediment toxicity testing protocol is thus urgently required. A regional survey of 58 estuaries of KwaZulu-Natal (a subtropical bioregion in the east coast of South Africa) in the mid 1990's revealed that the majority of estuaries were in a healthy state, with only 10% of the estuaries scoring <15.1 on a scale of 0 (poor) to 30 (good) (Cooper et al. 1994). This was based on the results of the Environmental Health Index that was developed by integrating three indices; the Biological Health Index, the Water Quality Index and the Aesthetic Health Index (see Cooper et al. 1994 for the components of each index). A subsequent small scale survey showed that more than 50% of the 16 estuaries in the eThekwini area of KwaZulu-Natal, including Durban Bay, are highly degraded (Forbes and Demetriades 2009). A country-wide assessment of nearly 300 estuaries has also revealed that <10% of the estuaries are in a poor state, but when the assessment is based on estuarine area rather than the number of estuaries, >80% of estuarine area or habitat is in a poor state (Van Niekerk et al. 2013). Lines of evidence used in these surveys, however, did not include sediment chemistry and toxicity bioassays, but sediment contamination has been identified as an emerging issue of concern

(see Van Niekerk et al. 2013). Since *Grandidierella lignorum* meets the requirements of a toxicity test organism (see Chapter 1) and has been shown to be sensitive to sediment contaminants (i.e. spiked sediment toxicity testing by Vivier (2010)), this amphipod can be used for screening field contaminated sediment and thus delineate polluted areas of an aquatic ecosystem. Amphipods are commonly used as sediment toxicity test organisms (Bat 2005). The influence of non-contaminant factors such as salinity, sediment grain size and sediment organic content to *Grandidierella lignorum* are addressed in previous chapters (Chapter 2 and Chapter 3). The aim of this study was to determine if *G. lignorum* can be used to screen polluted sediment. The use of this amphipod for a water only type toxicity test is also demonstrated, using effluent from wastewater treatment plants.

Materials and Methods

Whole effluent toxicity testing

Liquid waste (i.e. effluent) was collected from two wastewater treatment plants in Durban (Durban Central Works and Durban Southern Works). The Central Works plant handles domestic wastewater while the Southern Works plant handles a combination of industrial and domestic wastewater. Both plants discharge effluent offshore through deepwater outfalls. The experimental procedure outlined in EPA (2002) was used in a slightly modified form. Thus, since the aim of the study was to investigate the absolute effect of effluents on amphipod survival, environmental parameters such as dissolved oxygen, salinity and pH were not manipulated. Whole effluent toxicity (WET) tests were performed using effluent collected in July and August 2013. The collection of effluent and testing procedure was identical on both occasions except for the measurement of ammonia (see below). Effluent samples were collected in 10 L plastic containers that had been thoroughly washed in 10% hydrochloric acid. During collection it was ensured that there was no headspace in the sample containers. Effluent samples were transported to the laboratory (transport time: <2 hrs), where they were immediately prepared for toxicity testing.

The effluent was serially diluted with filtered (10 µm), UV sterilised natural seawater (salinity of 35). Effluent was diluted at a factor of 0.5 to produce test concentrations of 100, 50, 25, 12.5 and 6.25% effluent. Dilution water was used in control treatments and was diluted to a salinity similar to that of the lowest effluent dilution (i.e. 6.25% effluent). Effluent concentrations were prepared in clean 1 L glass containers and two replicates per effluent dilution were prepared for toxicity testing. Salinity, pH, dissolved oxygen (mg l^{-1}) and ammonia (mg l^{-1}) were measured in all test containers at the start

and end of the experiment. For the second test (August), however, ammonia was only measured in a single replicate container per effluent concentration. Twenty immature amphipods obtained from laboratory cultures were randomly selected and introduced to the effluent media. The experiment ran for 96 hrs and amphipods were not fed. Test media were also not renewed. Reference toxicity tests were performed in conjunction with the WET test to determine the sensitivity of the test population. Cadmium was used as the reference toxicant (see Chapter 4). WET and reference toxicity tests were performed at 22°C and 12hr light: 12 hr dark photoperiod. Test results were deemed acceptable when survival in the control treatment was ≥80 %. WET testing was also performed using the sea urchin (*Tripneustes gratilla*) gamete fertilisation test for comparative purposes. Urchins were induced to spawn by injecting approximately 1 - 2 ml of 0.5M KCL into the perivisceral coelom through the peristomial membrane and eggs were collected into glass beakers filled with seawater (salinity of 35). Sperm was collected and stored 'dry' using a glass pipette. Eggs were then introduced to four replicate samples of the effluents followed by the introduction of male gametes (sperm). Fertilisation was allowed to take place for 10 mins, after which the test was terminated by adding formaldehyde. Natural seawater was used as the control media (i.e. negative control). Fertilisation success was determined under a binocular microscope by counting the number of fertilised eggs (expressed as percent fertilised eggs). Successful fertilisation was identified by the presence of the fertilisation membrane (see McGibbon and Moldan 1986).

Whole sediment toxicity testing

Study site

The sediment samples tested for toxicity were collected in Durban Bay (29° 52' 9.50"S, 31° 3' 31.76"E, Figure 5.1). The Bay plays an important ecological role by providing habitat for aquatic fauna (Forbes et al. 1996). The intertidal mudflats and sandflats in the Bay provide habitat for macrozoobenthic fauna (Pillay 2002) and the pelagic environment provides a nursery function for juvenile fish (Forbes et al. 1996; Pillay et al. 2008). Intertidal sediment grain size has increased in coarseness over the years, resulting in an altered benthic community structure that currently supports high densities of sand prawns (Pillay et al. 2008). The Bay receives inflows from three rivers. The Amanzimnyama River discharges into the head of the Silt Canal, while the Umhlathuzana River joins the Umbilo River before entering the middle reach of the Silt Canal. The lower reaches of these rivers are canalised. The Bay also receives surface runoff via numerous stormwater drains. Sampling sites were positioned across the Bay to cover the majority of subtidal sediment habitats. Sites 1-3 were located within the Silt Canal, with Site 1 located at the inflow of the Amanzimnyama

River and site 2 off the inflow of the Umhlathuzana/Umbilo Rivers. Site 5 was located in Congella Basin. Sites 4, 6, 7 and 8 were located within the Maydon Wharf channel. Sites 10 and 11 were located in the Pier 2 Basin, while Sites 12 and 13 were located in Point Basin. Site 14 was located in the Entrance Channel. Site 15 was located in Island View Basin (Figure 5.1).

Figure 5.1. Site description (top) and sample site location (bottom) within Durban Bay. Control sediment was collected outside the Bay, from the sandy shoreline at Vetch's Point.

Sediment collection, transportation and storage

Composite sediment samples were collected for toxicity testing. These samples comprised three replicate sediment samples collected with a Petite Ponar grab at each site. The sediment samples were transferred to a glass bowl and homogenised using a high density polyethylene (HDPE) scoop, then transferred into plastic bags that were sealed in such a manner to limit air space. The sediment samples were temporarily stored in a cool dark place (storage time: <8 hrs from the collection of the first sediment sample) pending transport to the laboratory. In the laboratory the sediment was prepared immediately for toxicity testing. Sediment for grain size, organic matter and chemistry analyses was also collected by the Council for Scientific and Industrial Research (CSIR) from the same sites, at the same time that sediment samples for toxicity testing were collected. The chemical analyses procedures are not described in detail here since this did not form a component of this study. However, in summary the sediment samples were homogenised and 1 g subsamples digested (with the aid of a microwave) with concentrated nitric acid, perchloric acid and hydrogen peroxide in high-pressure vessels. The digested material was then diluted with deionised water and metal concentrations were quantified using Inductively Coupled Plasma Optical Emission Spectroscopy. Organic solvents (i.e. hexane and dichloromethane) were used to extract organic chemicals from sediment and interfering substances were removed from the extracts. Concentrations of organic chemicals were then analysed using Gas Chromatography Mass Spectrometry and/or high performance liquid chromatography (CSIR 2011, 2013).

Laboratory procedures

Sediment for toxicity testing was press sieved through a 2 mm mesh screen to remove larger fauna and debris (e.g. shells and litter) that may influence amphipod behaviour and/or survival during toxicity testing. Press sieved sediment was collected in a clean (acid washed) glass bowl and re-homogenised. Composite sediment from each site was then transferred to three replicate 1 L glass containers to a depth of 2 cm. The glass containers provide a bottom surface area of approximately 20 cm^2. Sandy beach sediment, also used in amphipod cultures, was used as control sediment in the absence of previously identified reference sediment. Filtered (10 µm mesh), UV sterilized seawater (salinity of 35) was gently introduced over test sediment to a total volume of approximately 900 ml. Containers were randomly placed in a controlled temperature room and continuously aerated (trickle flow aeration) through 1 ml glass pipettes for 24 hrs prior to the introduction of amphipods. Temperature in the controlled temperature room was maintained at 22°C and a 12hr light: 12hr dark photoperiod. This is similar to the conditions under which the amphipods were reared and also to

conditions used by other workers (Thwala 2006; Vivier 2010). On the day of amphipod exposure, selected physico-chemical parameters were measured before amphipods were introduced. Salinity was measured with a digital refractometer while dissolved oxygen and pH were measured with hand held probes. Ammonia was measured from two replicate containers using the cuvette system that utilises refraction and colorimetry. This method is, according to the manufacturer (HANNA Instruments), adapted from the ASTM D1426-92 and Nessler method.

Juvenile (2 – 4 mm) amphipods were isolated from cultures by sieving. Individuals retained by 0.5 mm mesh sieve were acclimated for 2 hrs in seawater before introduction to the sediment toxicity testing containers. Twenty actively swimming amphipods were randomly selected with the aid of a glass pipette and introduced to three replicate containers (i.e. 60 amphipods per sediment sample collected from each site). The sediment toxicity test was performed for a standard duration of 10 days without water change or feeding (EPA 1994). At the end of the test, salinity, pH, dissolved oxygen and ammonia was measured. Amphipods were then retrieved from sediment by sieving through 0.5 mm mesh sieve and the number of live amphipods counted. A reference toxicity test (also known as the positive control), using cadmium as a reference toxicant, was performed concurrently with the sediment toxicity test. The reference toxicity test was performed in a static water only set-up for 96 hrs in the same controlled temperature room, using amphipods from the same culture.

An elutriate toxicity test was also performed for the same sediment samples using gametes of the sea urchin *Tripneustes gratilla*. The elutriate was prepared by adding three parts sea water to one part sediment and vigorously shaken in a rotary shaker at 800 rev/min for 1 hr. The slurry was then centrifuged at 2000 rpm for 15 mins and the elutriate transferred to four replicate glass vials in which the fertilisation test was performed. The test followed the procedure described previously for WET testing.

Analyses

Sediment samples were analysed for *inter alia* 15 metals, polychlorinated biphenyls, tributyltin and polycyclic aromatic hydrocarbons by the CSIR. To determine if metals exceed background concentrations in the Bay, concentrations were superimposed on baseline models developed for the Bay. Examples of the models are provided by (Newman and Watling 2007). The baseline models

were also used to calculate enrichment factors for metals, which identify how many times a metal concentration exceeds or falls below the highest concentration predicted for uncontaminated sediment in the Bay. Enrichment Factor (EF) was calculated as, $EF = [M/N]_{sample}/[M/N]_{baseline}$, where $[M/N]_{sample}$ represents the ratio of the metal concentration to that of the normaliser (i.e. aluminium) in a sediment sample and $[M/N]_{baseline}$ represents the ratio of metal and normaliser of uncontaminated sediment (Newman and Watling 2007).

The Trimmed Spearman Karber method was used to estimate LC_{50}'s in WET tests. Toxicity of whole sediment and elutriates was assessed by comparing survival in test treatments to that in control treatments using a Student's *t*-test. Where survival was significantly lower than that of the control treatment at $P < 0.05$, the sediment or elutriate was regarded as toxic. The relationship between amphipod survival and the concentration of chemicals measured in the sediment was also assessed using the Spearman Correlation test.

Results

WET tests

Physico-chemical measurements for effluent toxicity tests using *Grandidierella lignorum* is presented in Figure 5.2 (Central Works effluent) and Figure 5.3 (Southern Works effluent). The pH was between 6.5 and 8 (a range of 6 – 9 is recommended for *Leptocheirus plumulosus*) for all toxicity tests. The trend for salinity, dissolved oxygen and ammonia was similar for all effluent toxicity tests. High salinity (range: 30 – 33) was measured in low dilutions (6.25% effluent) and decreased with increasing effluent concentration. Dissolved oxygen in the control treatments ranged between 4.2 and 6.2 mg l^{-1}, and decreased with increasing effluent concentration. Hypoxic or anoxic conditions were generally measured above 12.5 – 25% effluent. Ammonia concentrations in the control treatments ranged between 3.21 – 5.85 mg l^{-1} and concentrations increased with increasing effluent concentration. Ammonia concentration for undiluted effluent was higher than 50 mg l^{-1}, which is above the measurement capability of the ammonia colorimeter used in this study.

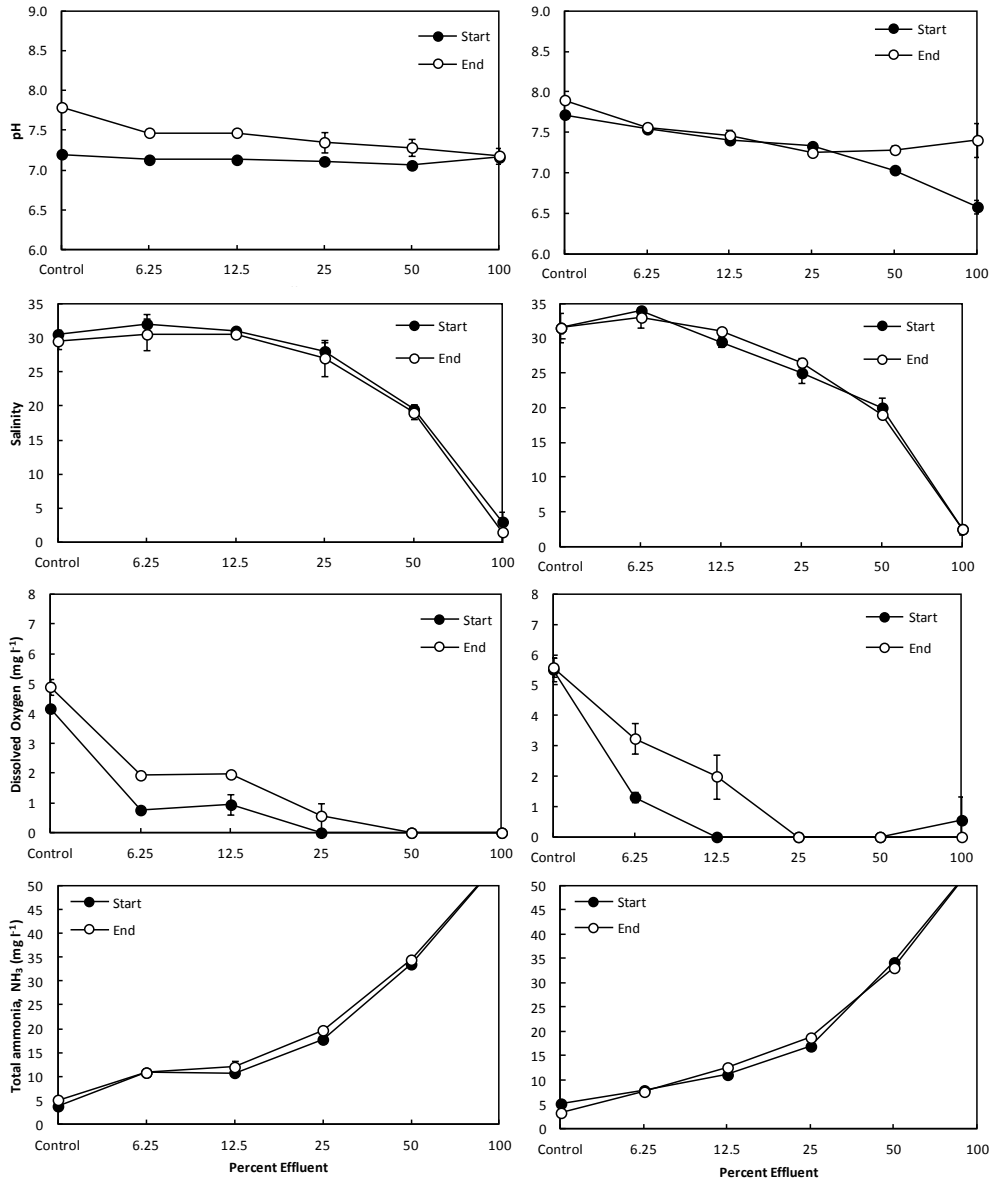

Figure 5.2. Measurements of physico-chemical parameters for WET tests of effluent collected from the Central Works wastewater treatment plant in July (left) and August (right). Data represents measurements made at the start and end of the toxicity tests.

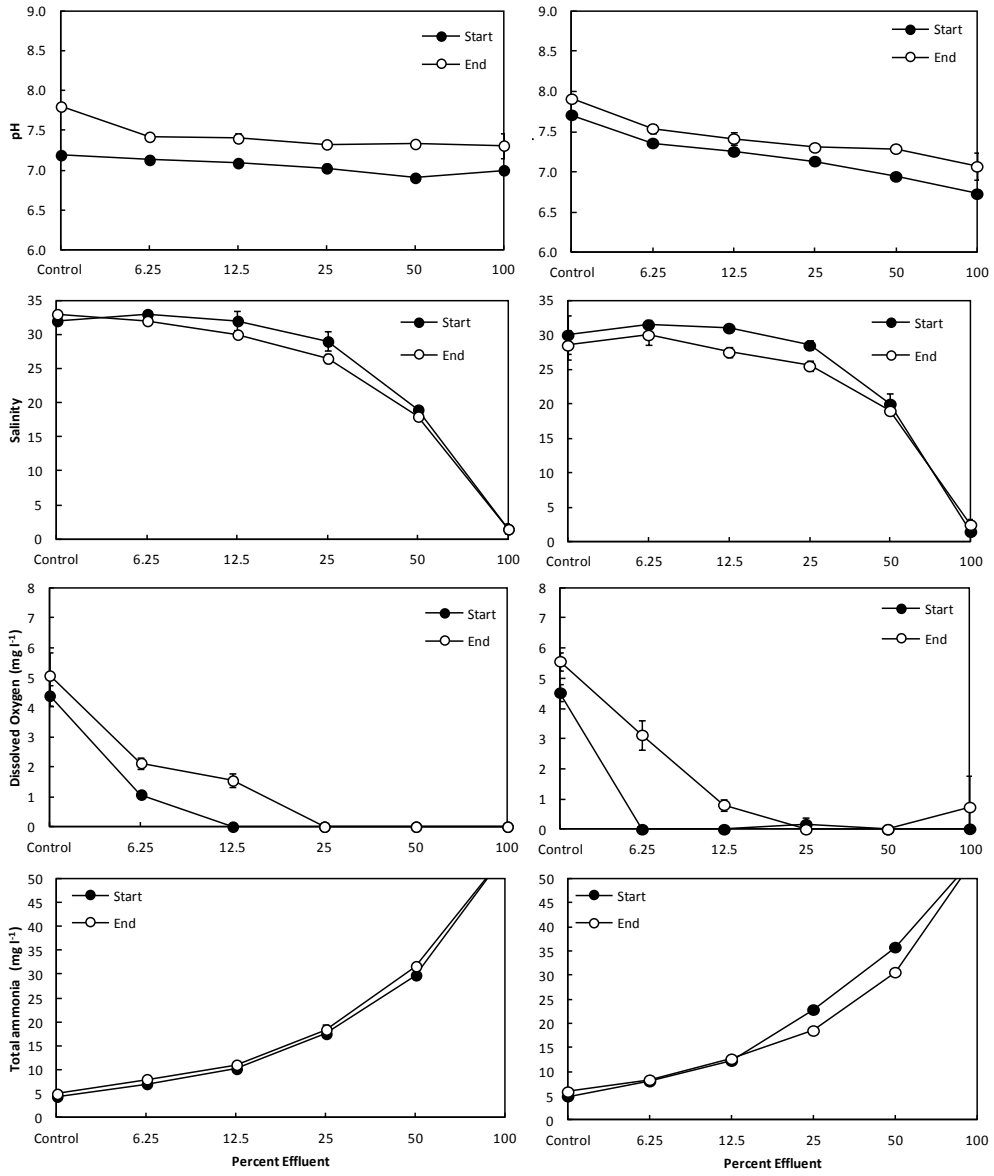

Figure 5.3. Measurements of physico-chemical parameters for WET tests of effluent collected from the Southern Works wastewater treatment plant in July (left) and August (right). Data represents measurements made at the start and end of the toxicity tests.

Cadmium toxicity (as LC_{50}) measured in the reference toxicity tests ranged between 1.41 mg l^{-1} in July and 0.86 mg l^{-1} in August. These concentrations are between the lower and upper control limits (0.49 – 4.02 mg.l^{-1}) and the effluent toxicity data was thus acceptable. Effluent collected at the Central Works wastewater treatment plant was less toxic to *Grandidierella lignorum* in July (LC_{50}: 15.85% effluent) than in August (LC_{50}: 7.41% effluent, Figure 5.4). On the contrary, inhibition of

fertilisation in the sea urchin *Tripneustes gratilla* was more pronounced for effluent collected in July than in August.

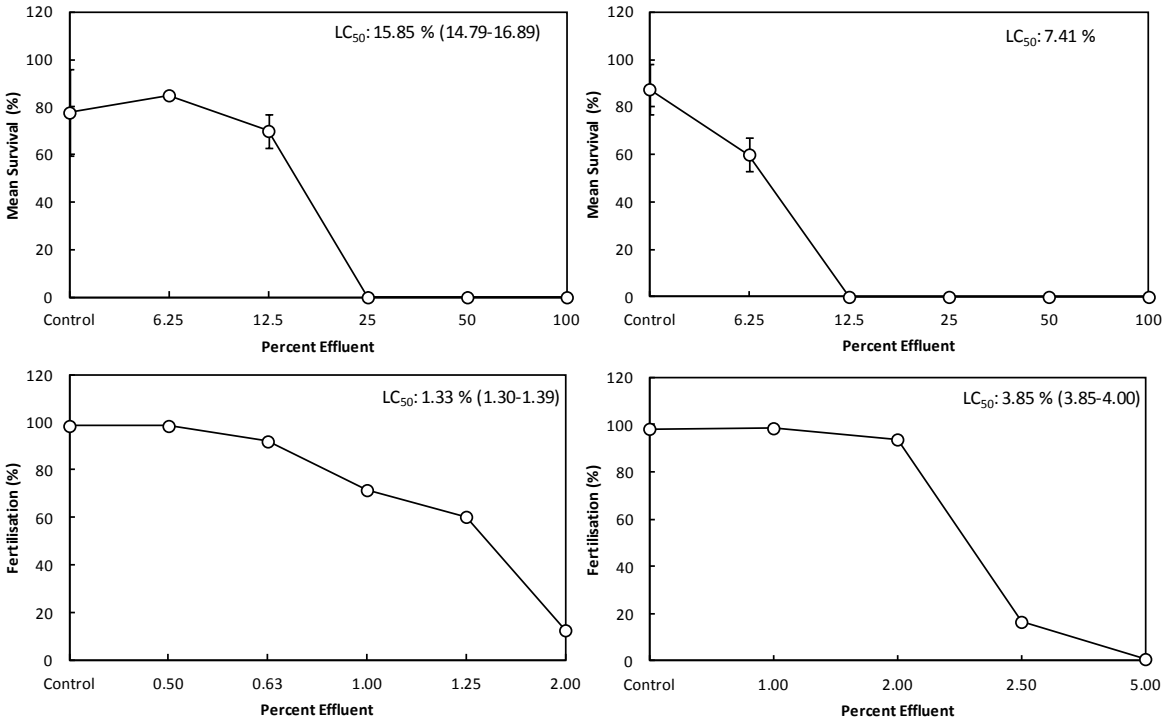

Figure 5.4. Toxicity of the Central Works effluent measured with amphipod *Grandidierella lignorum* (top) and sea urchin *Tripneustes gratilla* (bottom) in July (left) and August (right).

Effluent from the Southern Works wastewater treatment plant was also less toxic to the amphipod in July than in August while higher fertilisation inhibition was measured in effluent collected in July than in August (Figure 5.5). The sea urchin gametes consistently showed higher sensitivity to the effluent than the amphipod.

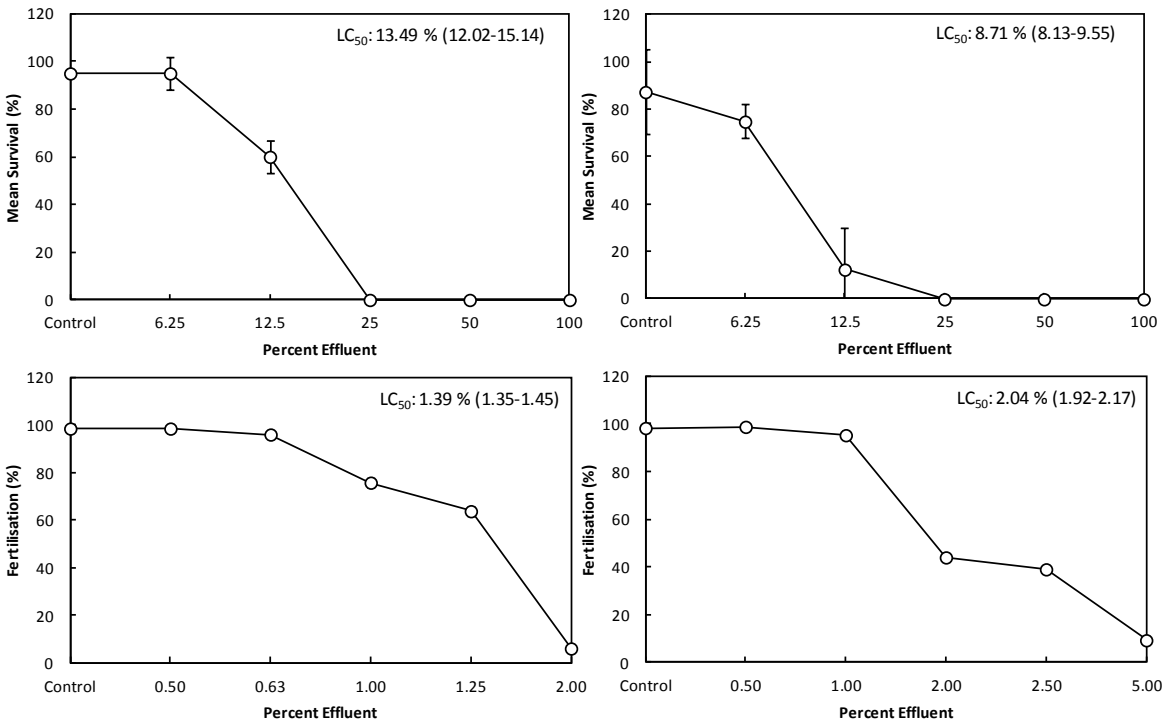

Figure 5.5. Toxicity of Southern Works effluent measured with *Grandidierella lignorum* (top) and *Tripneustes gratilla* (bottom) in July (left) and August (right).

Sediment toxicity testing

Sediment parameters

The sediment at the majority of sites was dominated by sand, but by mud at sites 1, 3, 4 and 6. The sediment at sites 1, 3 and 4 also had high organic content (Figure 5.6). The control sediment was dominated by sand (Figure 5.6). Medium- and fine-grained sand had the highest contribution (51% and 43%, respectively) to sediment composition of the control site. Control sediment also had a low organic content (i.e. 0.6%).

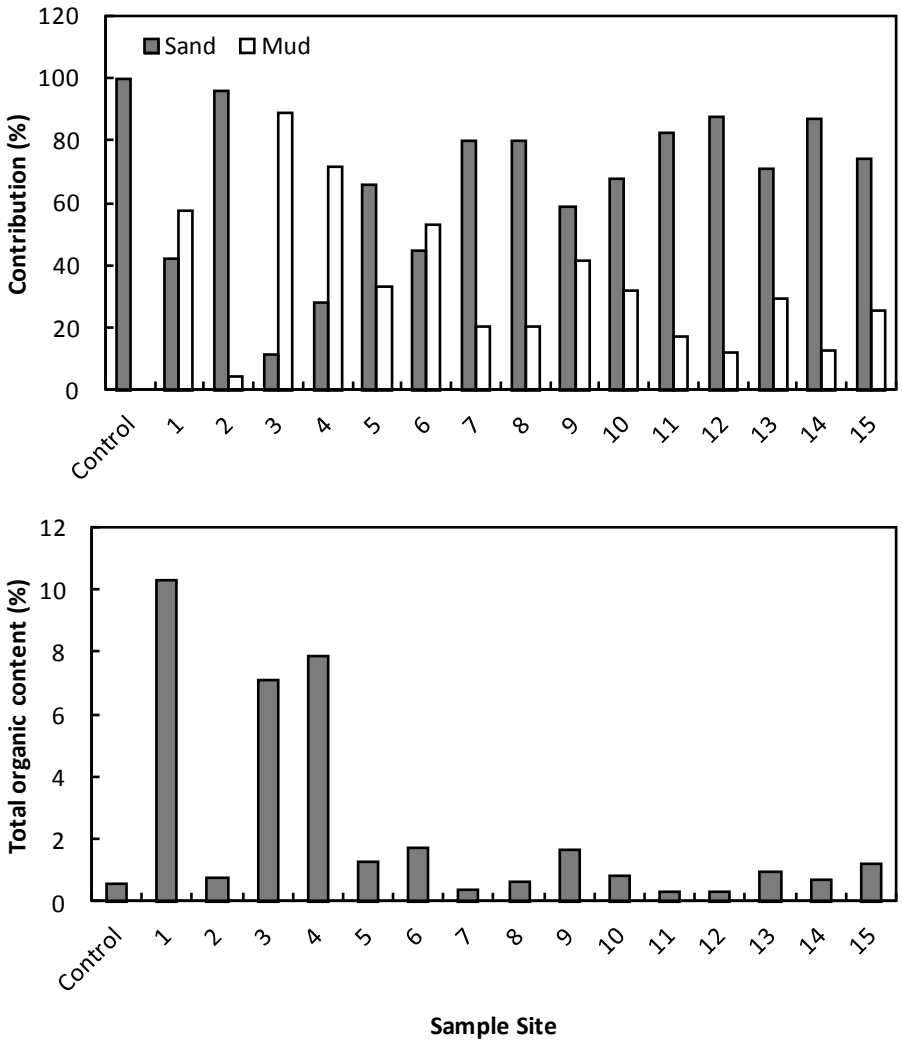

Figure 5.6. Grain size composition and total organic content of sediment collected in Durban Bay.

Metal enrichment or contamination was measured in eight sediment samples, collected at sites 1 - 6, 9 and 14 (Figure 5.7a). A total of 10 metals in sediment collected from site 1 (Silt Canal) exceeded background concentrations while at site 5 (Congella Basin) nine metals exceeded background concentrations. Although more metals exceeded background concentrations at site 1 compared to site 5, the highest metal concentrations were measured at site 5 (Figure 5.7b). Cadmium, copper and chromium were some of the metals present at highest concentrations in sediment in the Silt Canal and Congella Basin. High concentrations for other chemical compounds were either measured in Congella Basin sediment (e.g. tributyltin (TBT)) or from both Congella Basin and Silt Canal sediment (e.g. PCBs and PAHs, Figure 5.8a-c).

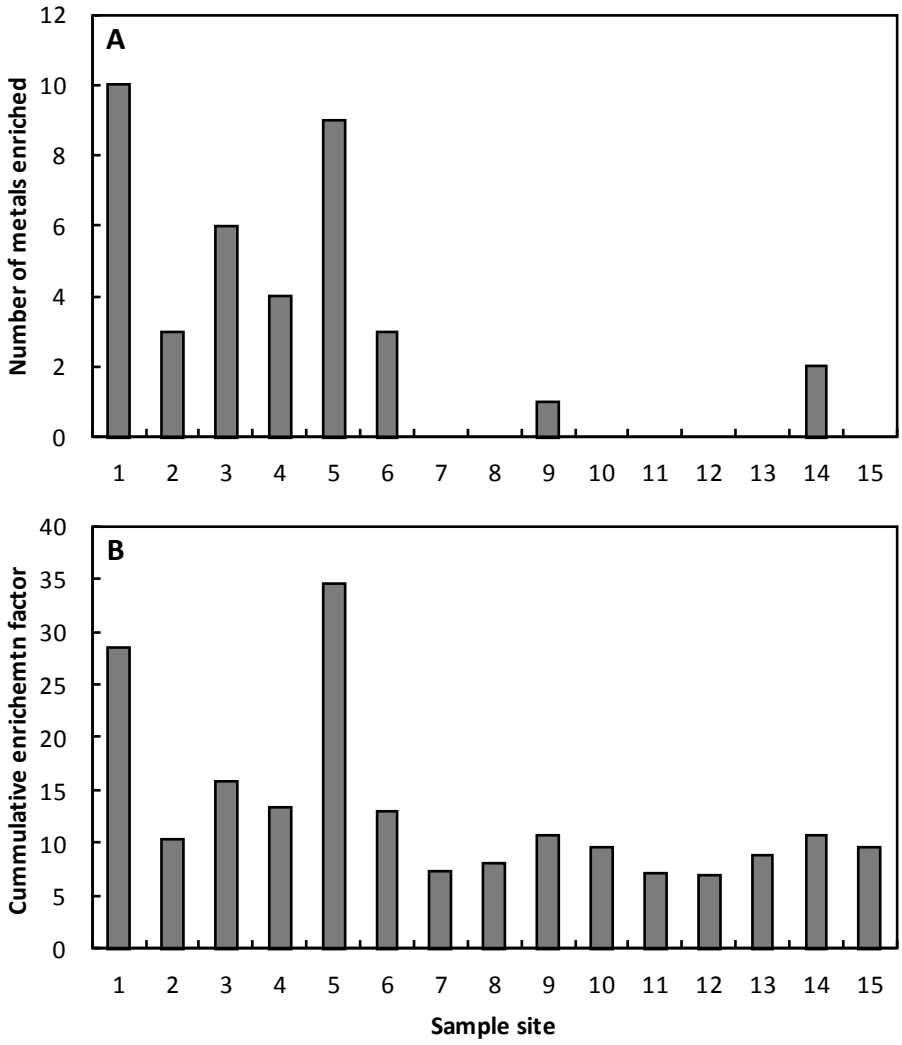

Figure 5.7. Number of metals in sediment that exceeded baseline concentrations (i.e. metal contamination) (a) in Durban Bay and their cumulative enrichment factor (b).

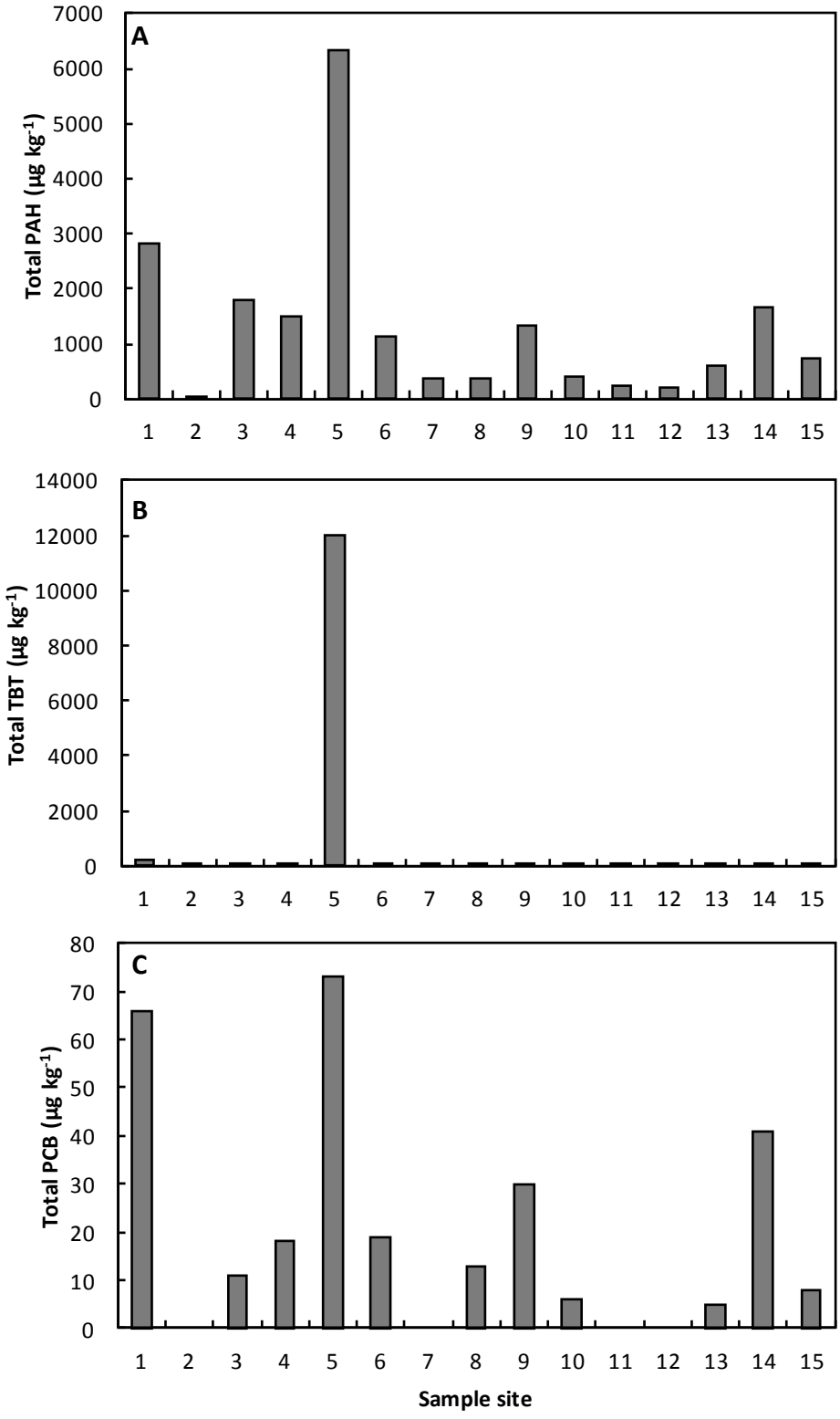

Figure 5.8. Organic chemical compound concentrations in sediment collected in Durban Bay.

Sediment toxicity test conditions

Physico-chemical conditions (e.g. pH, dissolved oxygen and salinity) for the sediment toxicity test were within acceptable limits (Figure 5.9). The pH was within the range of 6 – 9 while dissolved oxygen was >4 mg l^{-1}. Salinity in test containers was within the tolerable range for *Grandidierella lignorum*, generally around a salinity of 36. High ammonia concentration (25.15 ± 0.78 mg l^{-1}) were measured in sediment collected from site 1 (Silt Canal), otherwise, ammonia concentrations were below 5.5 mg l^{-1}. The tolerable ammonia concentration for *G. lignorum* is unknown.

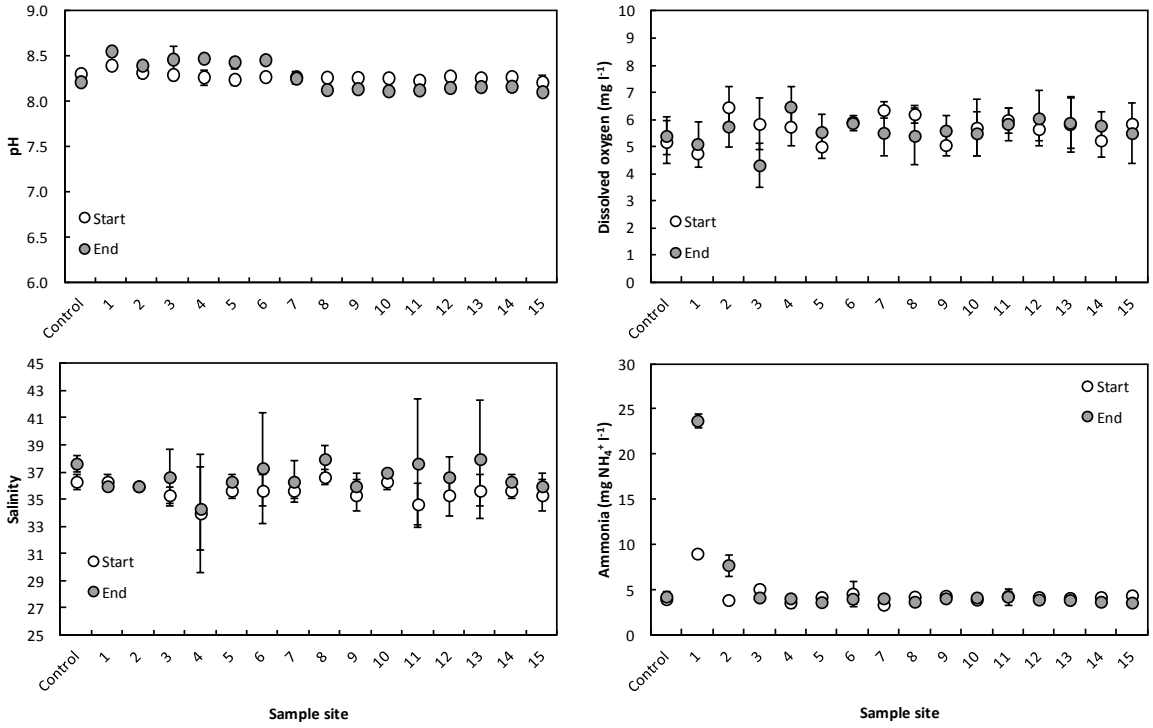

Figure 5.9. Physico-chemical conditions measured during the whole sediment toxicity test. Data represent measurements made at the start and end of the toxicity tests.

Sediment toxicity

Sediment samples collected from three sites were toxic to *Grandidierella lignorum* (Figure 5.10a). Two of these sites (sites 1 and 3) were situated in the Silt Canal and the other (site 5) was situated in Congella Basin. Sediment at site 1 was the most toxic, followed by sediment at sites 5 and 3, respectively. The elutriates of sediment collected from four sites significantly inhibited fertilisation in the sea urchin *Tripneustes gratilla*. These sediment samples were collected from site 1 in the Silt

Canal, site 4 in Maydon Wharf Channel, site 5 in Congella Basin, and site 9 (Figure 5.10b). Sediment at site 5 was the most toxic, followed by sediment from site 9.

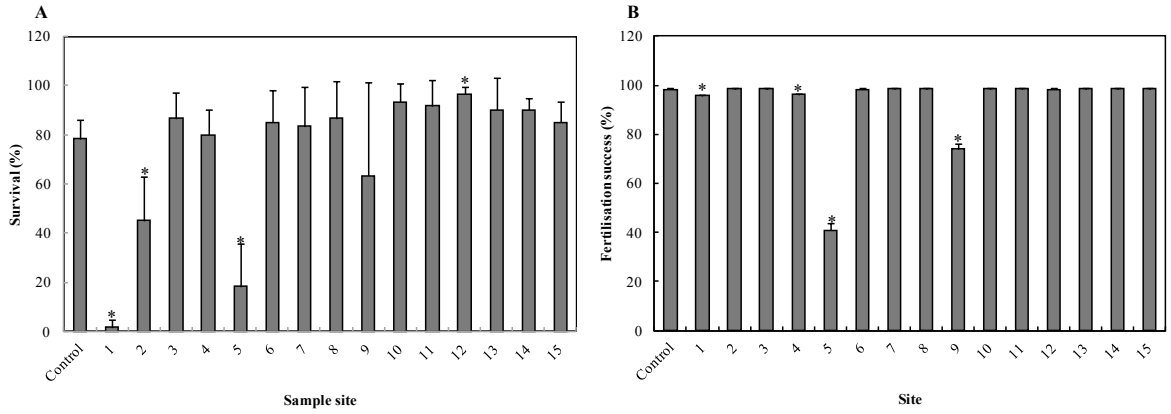

Figure 5.10. Mena (± standard deviation) survival of the amphipod *Grandidierella lignorum* following exposure to sediment collected from Durban Bay (A) and fertilisation success of *Tripneustes gratilla* gametes (B) following exposure to sediment elutriates collected at the same sites. Data denoted with an * represent survival significantly different to control treatment (i.e. toxicity).

There was a significant and negative relationship between the survival of the amphipod *Grandidierella lignorum* and the concentration of metals and organic compounds measured in the sediment collected from Durban Bay (Figure 5.11). The highest correlations were with copper (Spearman Correlation coefficient (ρ) = 0.738, *P* = 0.001) and cadmium (ρ = -0.727, *P* = 0.001) (Figure 5.11d,f). Only arsenic, beryllium, manganese and mercury showed no significant correlation with amphipod survival (Figure 5.11a,c,h,i).

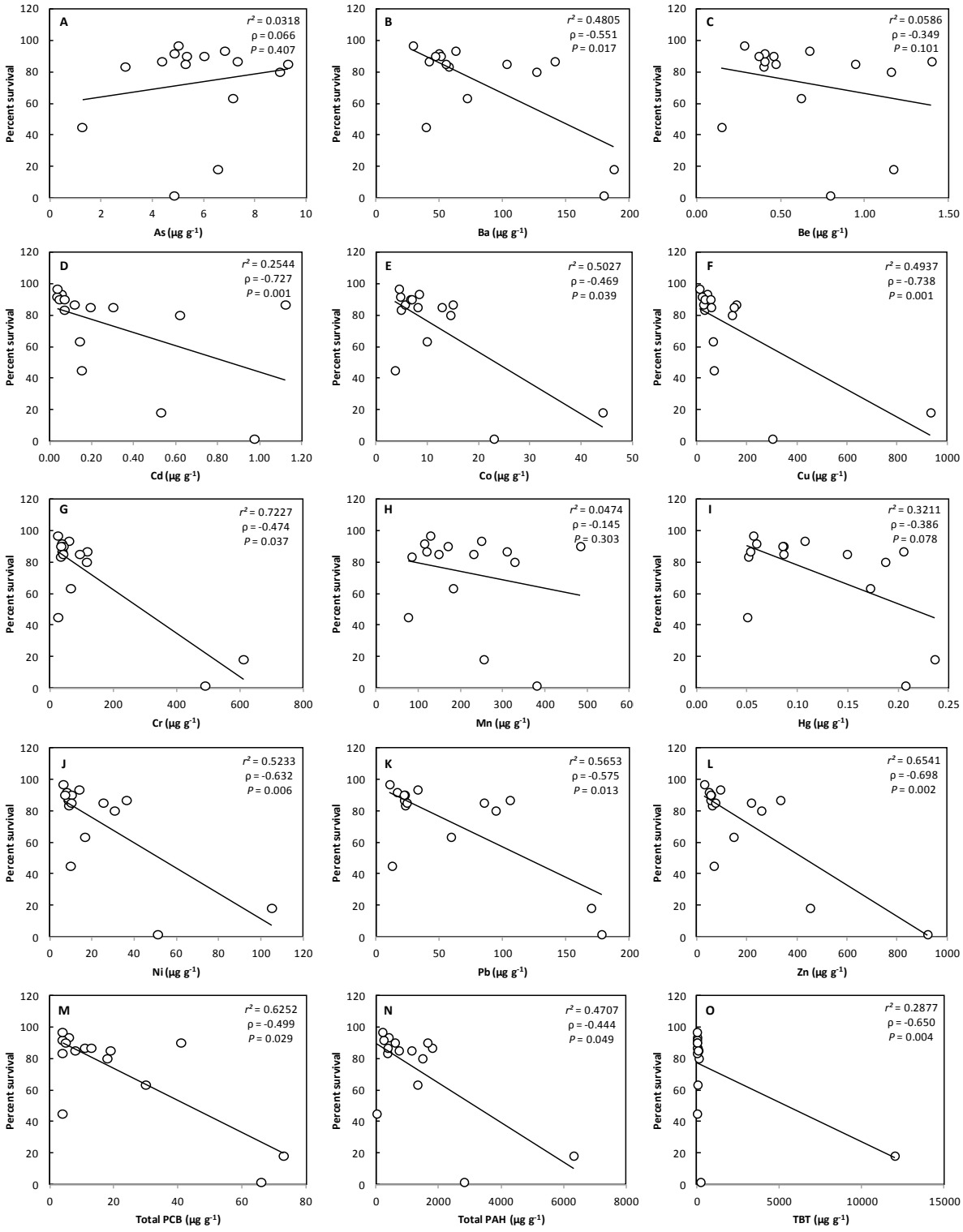

Figure 5. 11. Spearman correlation between mean survival (± SD) of *G. lignorum* and chemical concentrationmeasured in the sediment collected from Durban harbour.

Discussion

The aim of this study was to determine if the amphipod *Grandidierella lignorum* can be used to assess the toxicity of liquid waste (i.e. effluent) and sediment. The amphipod responded to all effluents tested, but when compared to the sea urchin was less sensitive. This was expected since early life history stages of aquatic organisms are known to be more sensitive than later life history stages (e.g. Gopalakrishnan et al. 2008). Similar findings have been shown by Cesar et al. (2004) and Ré et al. (2007). The differences in sensitivity between *G. lignorum* and *Tripneustes gratilla* were, however, variable. For example, effluent toxicity (July effluent from Central Works) to the sea urchin was approximately 12 times higher than that measured for the *G. lignorum*, but was only twice as toxic in August. More data is needed to correlate sea urchin and *G. lignorum* sensitivity. Interestingly, the sensitivity of *G. lignorum* to effluents seems to be comparable to the sensitivity of other amphipods exposed to effluents (e.g. *Allorchestes compressa* (Woodworth et al. 1999), *Gammarus chevreuxi* and *Corophium multisetosum* (Ré et al. 2007)). *Grandidierella lignorum* may thus prove to be useful for effluent toxicity testing, especially for receiving waters of estuaries where sea urchins cannot be used. Fertilisation of sea urchin gametes is sensitive to salinity (Carballeira et al. 2011). Effluent toxicity testing of receiving estuarine waters is urgently needed since effluent discharge is recognised as one of the emerging threats to estuaries of South Africa (Van Niekerk et al. 2013) and already has a significant impact in their ecological functioning. For example, effluent discharged into the Mhlanga River estuary dominates baseline flow during dry periods and increases breaching events (Lawrie et al. 2010).

Contaminants introduced to the overlying water (including contaminants from effluents) eventually sink to the bottom where they can negatively impact on resident fauna. Currently, whole sediment toxicity tests are currently not incorporated into coastal monitoring programmes in South Africa (e.g. Clark et al. 2010; CSIR 2012). This is due to the lack of standardised and/or certified protocols (Slabbert et al. 1998; Wepener and Chapman 2012). Previous investigations have resorted to correlating sediment metal concentration and metal body burden of test organisms to identify toxic sediments, but these procedures have been unsuccessful (Vermeulen and Wepener 1999) even though bioavailable fractions of metals were considered present in the sediment (Wepener and Vermeulen 2005). Metal toxicity does not always depend on the amount accumulated by the organism but depends on the metabolic bioavailability of the accumulated metals (Rainbow and Luoma 2011). A recommendation that bioassays be used to identify toxic sediments (Vermeulen and Wepener 1999) is described for *Grandidierella lignorum* in this study. Toxicity testing using this

amphipod revealed that sediment collected in some parts of Durban Bay is toxic. These sediments were also identified through chemical analysis as the most (and often highly) contaminated by metals, TBT, PCB's and PAH's. These high concentrations had a significant and negative correlation with amphipod survival. This suggests that *G. lignorum* is sufficiently sensitive to the presence of contaminants to be used for screening toxic sediment. Further support is provided by studies (CSIR, 2011) on the macrozoobenthic community at these same sites. Macrofaunal assemblages in Durban Bay basically separated into three assemblages. The first assemblage was less diverse and dominated by polychaetes and comprised of macrofauna collected from sites 1 – 6. This is the most contaminated part of the Bay. The least impacted macrozoobenthic community was at sites 7 – 10, where the taxonomic diversity was highest. Sediment at these sites was not toxic to *G. lignorum*.

Even though *Grandidierella lignorum* responded to toxic sediments in the Bay, the acceptability criterion of ≥80% survival in the control treatment was violated (survival: 78 ± 7.6%). A possible reason is that the grain size composition and organic content of the reference sediment was not suitable, although this sediment is successfully used for culturing amphipods in the laboratory. This sediment is, however, supplemented with a small amount of mud in laboratory cultures, but no mud was added to the control sediment. Whether this influenced survival is uncertain, but possible. It is thus recommended that suitable reference sediment should be identified for use in further studies. Grain size composition for sediment collected in the Bay was not expected to have acted as a confounding factor, even though *G. lignorum* prefers certain types of sediment (see Chapter 3). This conclusion is supported by high survival in sediment of different grain size composition of the sediment tested for toxicity (see Figures 5.6. and 5.10 a).

Sediment toxicity is not limited to a single mode of exposure since adsorbed contaminants can remobilise into the surrounding water during activities such as dredging and bioturbation (Durán et al. 2012). Therefore, tests that focus on other modes of toxicity (i.e. porewater, elutriate, sediment-water interface) using species from different trophic levels is recommended for a holistic toxicological assessment of sediment (Cheung et al. 1997; Liß and Ahlf 1997; Nendza 2002; Macken et al. 2008). The elutriate toxicity test was used to compliment results of whole sediment toxicity test. Elutriate toxicity tests are used to determine whether chemicals are released from sediment through resuspension, such as may occur during dredging or dredged material disposal (Macken et al. 2008), and may be used as a surrogate test where whole sediment toxicity testing is not possible

provided both tests have a comparative sensitivity (Haring et al. 2010). Toxicity of elutriates was assessed using the sea urchin fertilisation test. This test revealed that elutriates of sediment from four sites in Durban Bay were toxic. The sediment at two of the sites (sites 1 and 5) was also toxic to *Grandidierella lignorum*. However, the magnitude of toxicity was usually lower compared to the sediment toxicity test and the elutriate test also revealed toxicity at sites where no toxicity was identified using the amphipod. It must be noted that elutriate toxicity test may underestimate or over estimate sediment toxicity as elutriate preparation may reduce the concentrations of certain contaminants (e.g. ammonia) but increase concentrations of other contaminants that may not have been in a bioavailable form in the sediment because of their binding to sediment or organic matter (Ré et al. 2007).

This study has shown that *Grandidierella lignorum* is suitable for use in determining the toxicity of effluent and sediment. However, there are still several issues that require attention in this regard. These include an understanding of the sensitivity of this amphipod to ammonia, and the identification of a suitable reference sediment. Together with ammonia, hydrogen sulphide is a naturally occurring sediment contaminant and its influence on the amphipod needs to be quantified (e.g. Phillips et al. 1997). Sulphide influences adsorption and/or desorption of sediment contaminants such as metals (Durán et al. 2012).

While the dose-response relationship of *G. lignorum* and sediment metals has been demonstrated (Vivier 2010), the relationship to organic compounds is unknown. A toxicity test of sediment spiked with organic compounds is thus recommended. Future sediment toxicity tests will also benefit from toxicity identification evaluation (TIE) procedures that identify chemical substances responsible for toxicity.

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Chapter 6

Synthesis and recommendations for future studies

Synthesis

The estuarine amphipod *Grandidierella lignorum* has been recommended as a toxicity test organism for South African coastal environments (Vivier 2010). This species meets most of the requirements for a good toxicity test organism (see Chapter 1). The primary aim of this study was to develop a sediment toxicity test for estuarine and coastal environment test using this amphipod. To develop a sediment toxicity test using *Grandidierella lignorum*, it was necessary to define toxicity test conditions for some of the natural parameters such as salinity, temperature, sediment grain size and sediment organic matter. These are known to sometimes act as confounding factors for laboratory based toxicity tests (Postma et al. 2002), since macrofaunal community structure in the natural environment is also influenced by these parameters (Gray 1974; Snelgrove and Butman 1994; Lacey et al. 1999). Failure to understand the influence of these natural parameters and failure to account for their potential influence on toxicity test organisms may lead to erroneous interpretation of toxicity data, with implications where mitigation activities (which are generally costly) have to be taken.

Anecdotal evidence (e.g. Boltt 1969; Thwala 2006) suggests that *Grandidierella lignorum* is euryhaline, tolerating salinities between 0 – 45. This study has shown that salinity tolerance for *G. lignorum* is in fact wider than previously reported. *G. lignorum* tolerates salinities between 0 – 56, but prefers salinities between 7 – 42, which is further modified by temperature. *G. lignorum* prefers (survival >80%) salinities between 7 – 35 at 10 – 25°C. The constitution (or make) of seawater (i.e. natural or synthetic) does not influence salinity tolerance. This is particularly relevant for situations where natural seawater is not readily available. Some toxicity testing laboratories in South Africa are located inland, where natural seawater is obviously not readily accessible (Slabbert et al. 1998). *Grandidierella lignorum* can thus be used to test for samples with salinities of 7 – 35.

Sediment is one of the other parameters known to confound toxicity test data when it is not accounted for. In the natural environment sediment parameters such as grain size and organic matter are principal factors affecting macrofauna distribution (Gray 1974; Snelgrove and Butman 1994). The influence of sediment grain size and organic matter should thus be accounted for in a sediment toxicity test before toxicity is wrongfully attributed to contaminants when in fact the organism is responding to unfavourable conditions. For example, constructing burrows in unfavourable sediment is time consuming and energetically costly. These sublethal effects may

accelerate responses of interest (e.g. mortality or emergence from burrows) when amphipods are exposed to contaminated sediment that is of an unfavourable grain size. Boltt (1969) showed that *G. lignorum* prefers muddy sediment with high organic content. However, this study did not separately address the influence of sediment grain size from that of organic matter. The current study addressed this information gap, in the context of defining sediment toxicity test conditions. It is acknowledged that in the natural environment it is nearly impractical to dissociate the importance of sediment organic matter from sediment grain size in structuring benthic communities (Snelgrove and Butman 1994). Experiments on sediment grain size selection by *G. lignorum* showed that the amphipod does not show a statistically significant preference for a particular sediment grain size. This was based on low reproducibility of results (i.e. two test out of three showed no significant preference), but the analysis on pooled data of three experiments showed that the amphipod prefers fine-, medium- and coarse-grained sediment in that particular order. The preference of fine- and medium-grained sediment corresponds with areas of high abundance in estuaries. The sediment preference of *G. lignorum* was further evaluated over an extended period, similar to that of a standard acute sediment toxicity test (i.e. 10 days) and highest survival was recorded in fine- and very fine-grained sediment.

To determine the importance of organic matter, a source of protein-rich organic matter (fish flakes) and a source of carbohydrate-rich matter (lucerne) were fortified in fine-grained and medium-grained sediment at various concentrations. These types of organic matter were simultaneously offered to the amphipod, which consistently selected sediment without organic matter in both sediment grain sizes. Preference was not influenced by the type of organic matter or sediment grain size. Sediment with varying sediment grain sizes over a 10 day period can thus be tested without feeding the amphipods.

Natural parameters (e.g. salinity, temperature, sediment grain size) are not the only important factors for potential toxicity test organisms, but the sensitivity of these organisms must be determined. The sensitivity of *Grandidierella lignorum* was evaluated by exposing the amphipod to three metals that are commonly used as reference toxicants (i.e. cadmium, copper and zinc). These toxicants were dissolved in salinities of 7, 21 and 35. *Grandidierella lignorum* showed three responses to reference toxicants. Cadmium toxicity decreased linearly with increasing toxicity (1). Zinc toxicity increased with increasing and decreasing salinity (2). Copper toxicity was constant

between salinities (3). The sensitivity of *G. lignorum* was comparable to that of other amphipods used as standard toxicity test organisms. A control chart using cadmium as a reference toxicant was established and the initial lower and upper control limits were 0.49 mg l^{-1} and 4.02 mg l^{-1}, respectively. The importance of the control chart was demonstrated using stressed amphipods.

Lastly, the amphipod was used to test the toxicity of effluent and sediment. The amphipod was used in conjunction with the sea urchin fertilisation test. Results showed that the amphipod was sensitive to effluent toxicity and contaminated sediment. As far as sediment toxicity is concerned, three sediment samples were toxic to the amphipod whilst four sediment samples were toxic to the sea urchin. Two sediment samples that were toxic to the sea urchin were also toxic to the amphipod. The most toxic sediments were collected from the Silt Canal and Congella Basin where most of the sediment contamination by metals and organic compounds were also recorded. *G. lignorum* is sensitive to contaminated sediment and should be used in monitoring studies.

Conclusion and Recommendations

Conclusion

This study successfully developed a sediment toxicity test by defining test conditions for salinity, temperature, sediment grain size and organic matter content. Results from these experiments were used to generate provisional toxicity testing conditions for *Grandidierella lignorum* (Table 6. 1) and should be extended with the availability of data in the future. Additionally, the sensitivity of the amphipod was evaluated by exposing the amphipods to metal contaminants that were prepared in varying salinity. The amphipod responded satisfactorily to metal toxicants, taking into account the test conditions (i.e. salinity). The amphipod was then exposed to samples collected from the field and it was successful in screening sediment toxicity in Durban Bay.

Table 6. 1. Provisional test conditions for sediment toxicity assessment using *Grandidierella lignorum*.

	Parameter	Test condition
1	Test type	Whole sediment, static, non renewal
2	Test sediment grain size	100% fine-grained sediment, 88% mud
3	Test salinity	7 - 35 ± 5, low variation should however be maintained
4	Test ammonia	Not established
5	Sulfide	Not established
6	Temperature	10 - 25 °C
7	Photoperiod	12hr L: 12hr D
8	Test container	1 L glass container
9	Sediment volume	2 cm depth
10	Sediment preparation	Press sieve through 2mm where necessary
11	Overlying water	>600 ml
12	Test end point	Survival
13	Monitored physicochemical parameters	Salinity, pH, dissolved oxygen and ammonia
14	Test duration	10 d
15	Acceptability criteria	80% survival in the control treatment

Recommendations

Physico-chemical variables

Grandidierella lignorum does not select for a particular sediment grain size but high survival (92%) over a 10 d period was recorded in fine-grained sediment. Sediment is naturally present as a matrix of grain sizes and therefore, future investigations need to determine the maximum proportion of mud and sand in a sediment matrix that is not expected to negatively influence sediment toxicity test results. For example, the amphipod *Leptocheirus plumulosus* can be used to test the toxicity of sediment comprising up to 90% mud (>5% silt, and <85% clay (EPA 2001). With regard to natural chemical contaminants of sediment, the influence of ammonia and hydrogen sulphide to *G. lignorum* was not evaluated. These compounds occur naturally in sediment and can significantly confound toxicity test results (Moore et al. 1997; Phillips et al. 1997; Ferretti et al. 2000; McDonald 2005). Moore et al. (1997) have shown that the probability of statistical approaches to define dredged sediment as toxic increases by as much as 18% due to the influence of ammonia. The concentration of both compounds changes progressively over the duration of a toxicity test between the sediment and the overlying water (i.e. decrease or increase) (Phillips et al. 1997) and younger toxicity test amphipods (e.g. *Leptocheirus plumulosus*) are generally more sensitive than adults. The implications are that chronic toxicity tests initiated with younger organisms are likely to yield results of high sediment toxicity compared to tests initiated with adults (Moore et al. 1997). Therefore,

determining the toxicity of chemicals such as ammonia for *G. lignorum* will assist in determining the concentration at which sediment samples should be purged. The procedures for removing ammonia have been published by *inter alia* EPA (1994) and Ferretti et al. (2000).

Toxicity tests

It is recommended that for WET tests, effluent dilutions be modified to include more dilutions and greater than 50% effluent should be excluded in future studies. Complete mortality has been measured at ≥25% effluent. This will increase data points and consequently, the precision of statistical methods for estimating LC_{50}'s. The use of *Grandidierella lignorum* in assessing effluent toxicity of receiving waters is also encouraged, especially for estuarine conditions.

Lastly, whole sediment toxicity tests should be complimented with sediment chemistry and benthic community assemblage data in a weight of evidence approach to assess sediment health. But, whole sediment toxicity testing should not be limited to the solid phase test. Elutriates, porewater and sediment-water interface must also be tested to provide a holistic discrimination of the sediment health status. Sea urchins (*Tripneustes gratilla* and *Echinometra mathaei*) are already used routinely by the Council for Scientific and Industrial Research and can be developed to test toxicity at the sediment-water interface. The development of a chronic toxicity test using *G. lignorum* is recommended for future investigations. This however, requires the refinement of methods for evaluating growth and reproduction of the amphipod in the laboratory.

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Appendix

The research presented below is included as an appendix to this thesis based on the fact that the major focus of the research was on the development of acute toxicity tests. However, a start was made on investigating whether *Grandidierella lignorum* is also suitable for use in chronic sediment toxicity tests, wherein growth and reproduction are appropriate test endpoints.

Influence of salinity on growth, reproduction and fecundity of the amphipod *Grandidierella lignorum* (Amphipoda: Aoridae) in the laboratory

Abstract

Salinity and temperature do not only influence the distribution of fauna in estuaries but also their growth and reproduction. Growth and reproduction are critical endpoints for chronic toxicity tests. Their response to environmental factors (e.g. as salinity and temperature) should be investigated or understood in candidate toxicity test species. This assists in differentiating between growth that is influenced by environmental parameters and growth influenced by contaminants. Growth and reproduction of *Grandidierella lignorum* in three salinities (7, 21 and 35) was investigated at two temperatures (10°C and 22°C). Amphipods grew at a rate of 0.07 – 0.13 mm d^{-1} at 22°C and reached maturity after 21 to 28 days (size range of 2.30 ± 0.44 - 4.55 ± 1.51 mm). The brood size of the young females producing for the first time comprised 5 ± 2 eggs. This increased to 9 ± 3 eggs in the second brood, which was produced by Day 42. The number of eggs produced by females (i.e. brood size (BS)) increased in an allometric pattern with the increasing size of the females (FS; $Log_{10}BS = 3.191Log_{10}FS - 0.074$). Amphipods reared at 10°C grew so slowly (0.01 - 0.05 mm d^{-1}) that no amphipods had reached maturity after 42 days of exposure.

Introduction

Grandidierella lignorum is one of the most dominant macrozoobenthic amphipods in South African estuaries and provides a link between primary producers and secondary consumers. It burrows in surface sediment and filters suspended organic matter. It also browses for microbes adhered to deposited particles (Boltt 1969, Schlacher and Wooldridge 1996a; Wooldridge and Bezuidenhout 2008). While *G. lignorum* feeds at the lower trophic level in the food web (i.e. a primary consumer; Wooldridge and Bezuidenhout 2008), it forms an important food source for juvenile fish such as *Rhabdosargus holubi* and *Lithognathus lithognathus* (Schlacher and Wooldridge 1996b), and is thus ecologically important. It is widely distributed along the South African coast and has been collected in estuaries from all three biogeographic regions (e.g. Schlacher and Wooldridge 1996c; Teske and Wooldridge 2001; Wooldridge and Bezuidenhout 2008; Wooldridge and Deyzel 2009; Stow 2011). *G. lignorum* has a wide salinity tolerance (see Chapter 2) and may be distributed along the length of estuaries (see Schlacher and Wooldridge 1996c). The preferred salinity zone for *G. lignorum* in estuaries has not been identified but Teske and Wooldridge (2003) have shown that its distribution is not influenced by salinity and/or sediment grain size.

The interactive effects of environmental parameters on the biology of *G. lignorum* have however, been poorly studied. Thwala (2006) studied the influence of salinity on cadmium toxicity. The limited information on the biology of this amphipod thus hinders the development of a sediment toxicity test. Information on natural parameters, such as salinity and temperature tolerance, were addressed in Chapter 2, to define test conditions for an acute (typically 10 day long) sediment toxicity test. Information about reproduction and growth forms the basis for developing a chronic (typically ≥28 day long) sediment toxicity test. What is currently known for *G. lignorum* is that it completes its lifecycle within 30 days in the laboratory (Connell and Airey 1979), but its growth rate has not been quantified or reported. Several studies have shown that fecundity, reproduction and growth in crustaceans is influenced by salinity and temperature; and the importance of both factors is species-specific (Kumlu et al. 2001; Maranhão and Marques 2003; Ruscoe et al. 2004; Tsoi et al. 2005). The aim of this study was, therefore, to describe growth and reproduction of *G. lignorum* at different salinities and at two temperatures in the laboratory. Understanding the influence of environmental parameters on growth, embryonic development and fecundity is important in the development of a toxicity test since these investigations provide the ability to distinguish between natural variability and variability induced by chemical contaminants (Fockedey et al. 2005). Results of this study will contribute to the development of a chronic toxicity test.

Materials and Methods

Experiments

Grandidierella lignorum can tolerate salinities ranging from 0 and 56 but prefers salinities between 7 and 35 at 10 to 25°C (Chapter 2). Experiments on growth, reproduction and fecundity were thus performed at three salinities (7, 21 and 35) representing the preferred range and at two temperatures (10 and 22°C). A temperature of 10°C was the lowest that did not negatively influence salinity tolerance whilst 22°C was selected in favour of 25°C since amphipods were cultured at this temperature. Results for 22°C can thus be extrapolated to culture conditions. Amphipods used in experiments were reared in the laboratory at a salinity of 35 and at 12hr light: 12hr dark photoperiod. Gravid females (n = 200) were removed from cultures and acclimated to test salinities at a salinity change of ≤3 per 2 hrs (Tsoi et al. 2005). Once target salinities were reached, 50 gravid females were transferred to three smaller culture tanks (L x B x H: 23 cm x 17.5 cm x 15 cm) containing 2 cm of medium- to fine-grained sediment and 10 cm of overlying water of the target salinity. Salinity of 7 and 21 were prepared by diluting filtered (10 µm), UV sterilised seawater (salinity = 35) with distilled water. The cultures were then acclimated to test temperatures at ≤3°C

change every 12 hrs. Once the appropriate experimental temperature was reached, gravid females were acclimated in these conditions for a further three days after which neonates (≤7 day old) released were isolated from the respective cultures, by sieving. A total of 20 neonates were then selected under a dissecting microscope and introduced into 1 L glass containers containing water of a corresponding test salinity (*n* = 18 per salinity). Each container comprised 2 cm of medium- to fine-grained sediment and ~700 ml overlying water. Water in the containers was continuously aerated (trickle flow) with the aid of 1 ml glass pipettes and amphipods were fed 2 mg of ground TetraMin® fish flakes every two days. This is similar to the feeding scheme for *Leptocheirus plumulosus* (EPA 2001; McGee et al. 2004). Dissolved oxygen was monitored periodically (1 to 2 days) during the experiment and maintained above 4 mg l^{-1}, which is recommended for toxicity testing using the amphipod *Leptocheirus plumulosus* (EPA 2001). Salinities were maintained within ±1 of the target salinities. The dissolved oxygen meter was calibrated (according to manufacture specifications) before use while the digital refractometer was calibrated using distilled water.

Experiments were performed in controlled temperature chambers. Amphipods were harvested every seven days from three containers per test salinity by sieving through 0.25 mm mesh screen and stored in glass pill vials at 7°C for ~12 hrs, to relax the animals before they were stored in formalin. When amphipods are immediately fixed in formalin they tend to curl up and measurements of young individuals become difficult (*pers. obs.*). Amphipod size was measured under a microscope equipped with a camera and imaging software. Body length was measured from the rostrum to the tip of the first uropod, since the base of the telson was difficult to identify with accuracy. The cephalon length was measured from the rostrum to the posterior end of the cephalon. All measurements were made along the dorsal side of individuals. Growth rate was estimated from body measurements taken on the first day of measurement (e.g. Day 7) and the last day (e.g. Day 35).

Statistical analysis

Estimates of body size per replicate container per salinity were based on ≥5 amphipods. There were instances where the number of amphipods retrieved was below five individuals and in some containers amphipods were not recovered at all. Comparison of age between replicate containers at the same salinity could not be performed due to small sample sizes for some replicates. This analysis would have assisted in determining the homogeneity of the size/age classes before comparison with

other salinities is performed. Data were thus pooled for all replicates and growth was only compared between salinities.

The relationship between body length and cephalon length was explored using a simple linear regression on log transformed data. Sizes of amphipods between salinities were compared by the independent sample *t*-test (the choice for *t*-test instead of the analysis of variance is discussed below). Fecundity was estimated from the number of eggs produced per female (i.e. brood size) and is an indication of successful reproduction. Only females with brood pouches that had not breached were used for this exercise. Due to the small number of gravid females recovered, this data was supplemented by analysing brood sizes of females removed from laboratory cultures. The relationship between brood size and female size was investigated using a regression analysis based on log transformed data.

Results and Discussion

Dissolved oxygen

Dissolved oxygen was generally above 4 mg l^{-1} in all experiments except on three occasions for amphipods reared in salinity 7 and on one occasion for amphipods reared in salinity 35 (Figure 1). This situation was short-lived, did not persist for more than 12 hrs and was not lethal since dead amphipods were not recovered. These low oxygen levels resulted from the failure of oxygen supply due to technical difficulties. While the disrupted oxygen supply did not result in the death of the amphipods (i.e. lethal effects), sublethal effects may have occurred and their effect on growth could not be quantified and/or was not evident in subsequent observations.

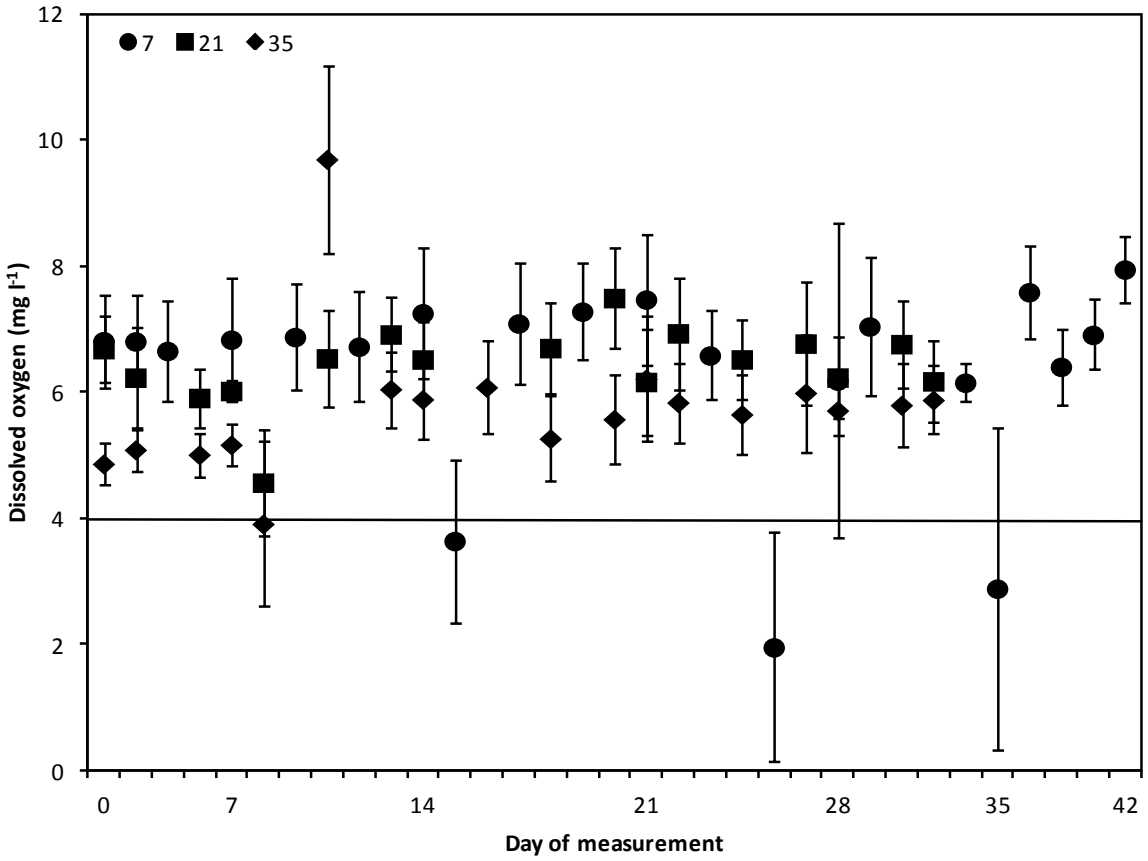

Figure 1. Dissolved oxygen (mean ± SD, n = 3 to 18) over the duration of the study. The dashed line represents the lower limit of dissolved oxygen considered acceptable in the experiments (EPA 2001).

Body and cephalon growth relationship

Cephalon length can be used to estimate the body length in situations where body length measurements prove to be difficult (i.e. measuring live organisms) (Delgado et al. 2011). Body size in *Grandidierella lignorum* is positively related to cephalon size (r^2 = 0.901, P < 0.0005, n = 270) (Figure 2) and growth of the body regions is allometric (slope: 1.128, t = 49.337, P < 0.0005), defined by the equation:

$$Log_{10}BL = 1.128 \cdot Log_{10}CL - 0.0141$$

where BL is the body length (mm) and CL the cephalon length (mm).

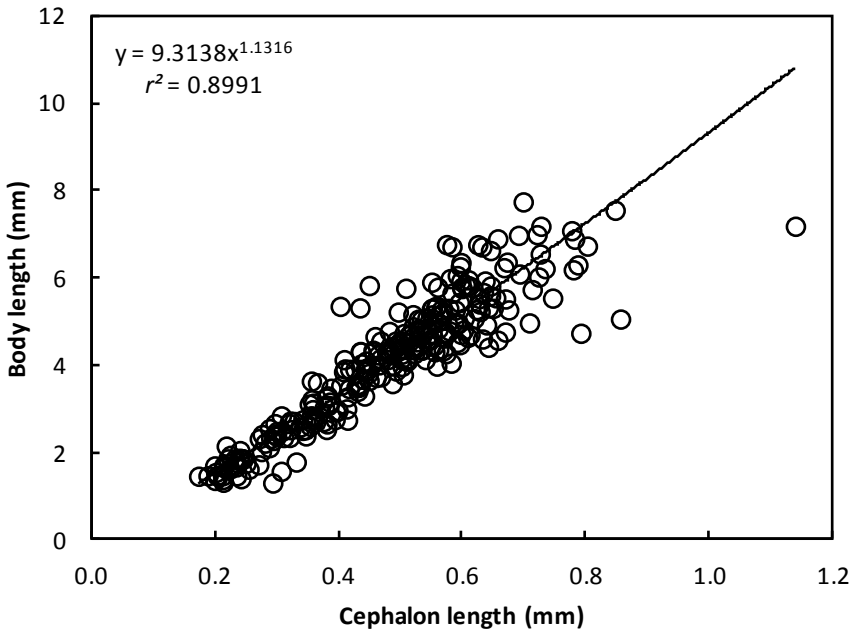

Figure 2. Relationship between body length and cephalon length (n = 270) in Grandidierella lignorum.

Growth

Growth of amphipods at 22°C was measurable in all salinities, but stunted growth was observed at a salinity of 21 (mean growth ± SD: 0.03 ± 0.02 mm d^{-1}, *n* = 81). Growth was high at salinities of 7 (0.11 ± 0.03 mm.d^{-1}; *n* = 251) and 35 (0.13 ± 0.13 mm.d^{-1}; *n* = 140), Figure 3a). Similarly, *Gammarus aequicauda* grows at an estimated rate of 0.12 mm d^{-1} when reared at a salinity of 9 and 0.14 mm.d^{-1} when reared at a salinity of 34 (Delgado et al. 2011). *G. aequicauda* is also a potential toxicity test organism in Europe (Prato and Biandolino 2005; Prato et al. 2009). *G. lignorum* seems to grow faster than the amphipod *Hyale crassicornis,* which grows at an estimated rate of 0.06 mm d^{-1} at salinity 10 (20°C) to 0.08 mm d^{-1} at salinity 30 (20°C) (Tsoi et al. 2005). Growth of *Grandidierella lignorum* at 10°C was reduced considerably, ranging between 0.01 ± 0.03 mm d^{-1} at a salinity of 7 and 0.05 ± 0.07 mm d^{-1} at a salinity of 35 (Figure 3b). At a salinity of 21, amphipods grew at 0.03 ± 0.02 mm d^{-1}, which is similar to that measured in the same salinity at 22°C. More importantly, the amphipods did not survive the duration of the experiment at 10°C (except for amphipods reared at salinity of 21) and no amphipods reached maturity. Further reference to growth and reproduction is thus restricted to data for 22°C.

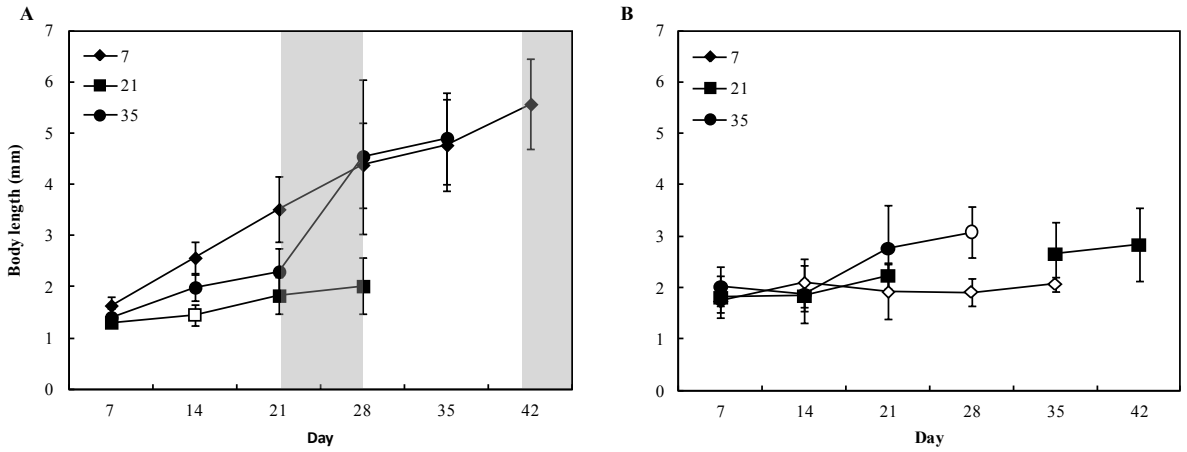

Figure 3. Mean body length (± SD) of the amphipod Grandidierella lignorum at salinities 7, 21 and 35 at 22°C (A), n = 81 to 244 (data for filled symbols) and n = 2 (data for open symbols) and at 10°C (B), (n = 30 to 70 (data for filled symbols), n = 1-5 (data for open symbols)). The grey area represents the estimated time at which neonates were produced. Open symbols in graphs represent data obtained from <5 amphipods.

Juvenile *Grandidierella lignorum*, defined by the lack of distinguishable primary sexual characteristics (i.e. oostegites), were generally ≤14 d old and ranged between 1.99 ± 0.27 to 2.57 ± 0.32 mm. This is larger than for *G. japonica* collected from the wild, which ranges between ~0.8 - 1.1 mm (Greenstein and Tiefenthaler 1995). Sex was generally distinguishable at Day 21, when immature (i.e. non-gravid females) adults were observed. Immature adults ranged between 2.30 ± 0.44 and 3.51 ± 0.63 mm. Distinction between the sexes was based on secondary and/or primary sexual characteristics, such as gnathopods for males (secondary sexual characteristic) and oostegites for females (primary sexual characteristic). Genital papillae in males (a primary sexual characteristic) could not be used as a diagnostic feature as they were difficult to identify due to their small size. Oostegites on the other hand were easily identifiable and lacked setae in immature females (*pers. obs.*). *G. lignorum* possesses a 'primitive' type of oostegites that are characteristically broad (*pers. obs.*). Broad oostegites are common in the family Aoridae, whereas families such as Corophiidae and Pontoporeidae possess both narrow and broad oostegites (Steele 1991). The sole purpose of distinguishing sexes was to determine if males and females attain sexual maturity at the same time.

Males and females attained sexual maturity within the same time period (21 to 28 days). Amphipods grew to a size of 4.77 ± 0.89 mm (*n* = 47) after 35 days of exposure to salinity of 7 and 4.91 ± 1.02 mm (*n* = 13) in salinity of 35. Amphipod size did not differ significantly between amphipods reared in salinity of 7 and 35 (*t* = 0.483, *P* = 0.631). Mean size of amphipods reared in salinity of 21 was not included in the analysis due to the slow (stunted) growth rate. These amphipods were approximately four times smaller than amphipods measured at a salinity of 7 and 35. Reasons for this slow growth are unknown but salinity and food availability are ruled out as factors. Dissolved oxygen was also maintained above 4 mg l^{-1} and is not suspected to have negatively influenced the experiment. In fact, dissolved oxygen at salinity of 21 was high (6.43 ± 0.62 mg l^{-1}) and did not differ significantly to that measured at salinity of 7 (6.34 ± 1.55 mg l^{-1}) (*P* = 0.573) during the study. Dissolved oxygen at salinity 35 (5.76 ± 1.13 mg l^{-1}) was significantly lower than that measured in salinity of 7 (*P* < 0.0005) and 21 (*P* = 0.004).

Fecundity

Mean body size for adults was measured at 4.38 ± 0.83 mm in Day 28. Brood pouches of some amphipods were already breached and neonates were observed, particularly at salinity of 35. This suggests that sexual maturity is reached between 21 and 28 days, corresponding to a size range of 2.30 ± 0.44 mm to 4.55 ± 1.51 mm (Figure 3). The initial brood size of females reared at a salinity of 7 was 5 ± 2 eggs per female (range: 3 - 7, *n* = 4). The second brood was produced at Day 42, when females produced 9 ± 3 eggs per individual (range: 5 – 13 eggs, *n* = 11). Brood pouches of amphipods reared at salinity of 35 were breached. As discussed previously, fecundity data were supplemented by data obtained from the laboratory cultures to determine the relationship between brood size and female size. The lowest number of eggs (*n* = 3) was produced by a female of 3.52 mm and the largest number of eggs (*n* = 35) by a female of 6.55 mm (Figure 4.4). Brood size was positively correlated with female size (r^2 = 0.796, *P* < 0.0005, *n* = 20) and defined by the allometric relationship:

$$\text{Log}_{10}\text{BS} = 3.191\text{Log}_{10}\text{FS} - 0.074 \ (t = 8.369, P < 0.0005)$$

where BS represent brood size (number of eggs per female) and FS represents female body size (mm).

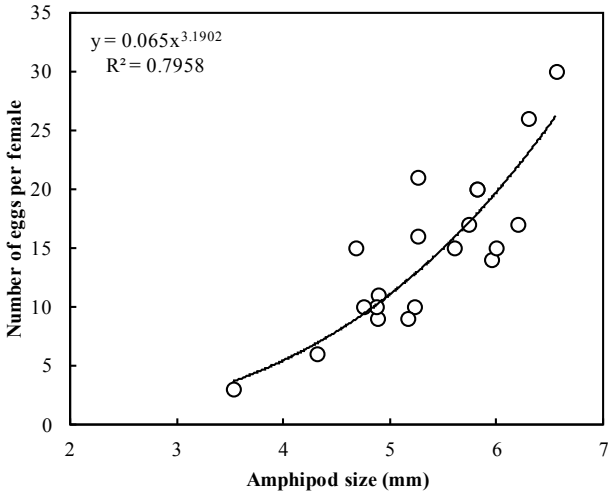

Figure 4. Relationship between brood size and female size (n = 20) in Grandidierella lignorum at 22°C.

The findings are supported by the observations of Connell and Airey (1979). According to Connell and Airey (1979), *Grandidierella lignorum* in their cultures reached sexual maturity at 3.4 mm and young females produced 1 - 2 eggs from their first brood. This number of eggs increased to 5 - 8 and 14 - 16 eggs per female in the second and third brood, respectively. Older females produced as many as 45 eggs per brood, which is similar to the brood size of the amphipod *Onisimus litoralis* that produces 42 ± 15 neonates per brood (Nygård et al. 2010). The brood size of 45 eggs in *G. lignorum* is generally smaller compared to that for *Corophium volutator* and *Echinogammarus marinus. C. volutator* produces an average of 96 neonates (Peters and Ahlf 2005) and *E. marinus* can produce as much as 67 neonates per brood (Maranhão and Marques 2003).

Microcosm study

Stunted growth of amphipods at a salinity of 21 at 22°C could not be explained, but could be the result of numerous factors including ammonia build up from feeding. The feeding regime and quantity of food offered to *Grandidierella lignorum* was adopted from that recommended for *Leptocheirus plumulosus* (EPA 2001; McGee et al. 2004). This may be inappropriate for *G. lignorum.* Ammonia was, however, not monitored in the previous study for logistical reasons. The study was then repeated with some modifications, so that ammonia could be monitored and is reported in this

section. In this experiment, neonates were not reared in discrete containers but were left to grow in the presence of adult females (i.e. parental care) in bigger culture containers (L x B x H: 23 cm x 17.5 cm x 15 cm). Adult amphipods were only removed when the first juveniles were removed for size measurements. Survival of some juvenile amphipods may be improved in the presence of parent amphipods (see Thiel 1998). This microcosm study was only performed at 22°C, but over 60 days and ≥5 amphipods were removed for body measurement at 10-day intervals. Dissolved oxygen and ammonia were monitored over 30 days. The amphipod population at the salinity of 21 incidentally crashed by Day 21 and this coincided with an increase in ammonia concentration (as NH_4^+) from 4.4 mg l^{-1} (Day 18, dissolved O_2= 6.45 mg l^{-1}) to 7 mg l^{-1} (Day 20, dissolved O_2= 6.03 mg l^{-1}). This is equivalent to 6.61 mg NH_3 l^{-1} (i.e. 7 mg NH_4^+ l^{-1} multiplied by 0.944, as per manufacturer (HANNA Instruments) instructions). This concentration is almost twice the LC_{50} of unionised ammonia (3.35 mg l^{-1}) in *Grandidierella japonica* (Kohn et al. 1994). Dissolved oxygen never decreased below 4 mg l^{-1} in this experiment (Figure 4.5b). A new culture at salinity of 21 was re-established for the experiment.

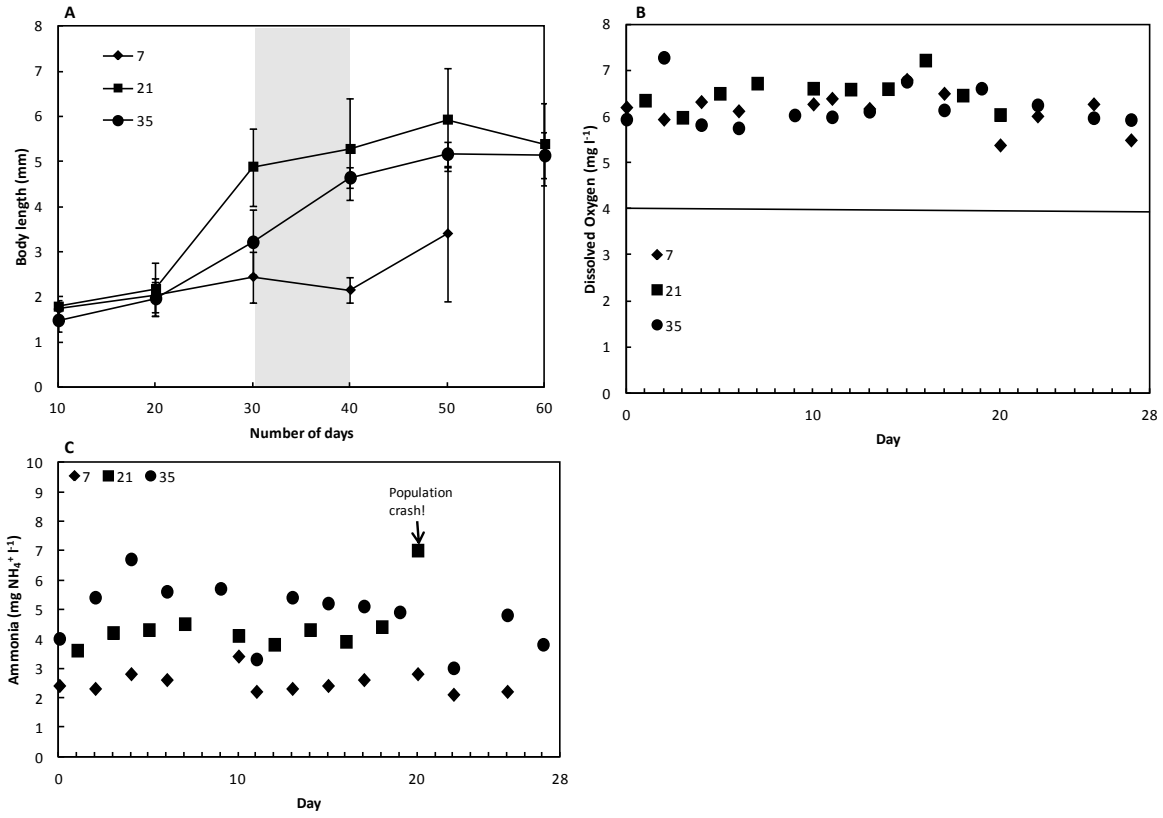

Figure 5. Growth (N = 33 to 58) of Grandidierella lignorum at three salinities and 22°C in a microcosm set-up.

Amphipods reared at salinity of 7 grew at an average of 0.04 ± 0.06 mm d^{-1} and the population did not survive for more than 50 days (Figure 5a). This slow growth cannot be explained since ammonia concentration measured over a 30 day period was low (2.51 mg NH_4^+ l^{-1}) and salinity was maintained at 7 ± 1. Synergistic effects between parameters cannot be excluded. Dataset for salinity 7 was excluded from further analysis (i.e. comparison of amphipod sizes between salinities). Amphipods reared in salinities of 21 and 35 grew at the same rate (salinity of 21: 0.07 ± 0.12 $mm.d^{-1}$, salinity of 35: 0.07 ± 0.06 $mm.d^{-1}$) and amphipods grew as large as 5.93 ± 1.14 mm (salinity of 21, Day 50). This similar to the mean amphipod length (6.28 ± 0.84 mm, data not presented) measured for adult amphipods in the culture tanks. The size of 60 day old amphipods at a salinity of 21 (5.39 ± 0.91 mm) did not differ significantly to that for amphipods reared at a salinity of 35 (5.13 ± 0.51 mm, t = 0.583, P = 0.569). Growth of amphipods beyond Day 50 had stabilised (Figure 5), but amphipods may continue to reproduce.

Conclusion

Growth and reproduction in *Grandidierella lignorum* differed significantly between 10°C and 22°C but did not differ significantly between salinities(35 vs. 7, t = 0.483, P = 0.631, discrete containers; 35 vs. 21, t = 0.583, P = 0.569, microcosm). Higher temperature supported a higher growth rate, while amphipods did not reach maturity at low temperature. Normal growth rate for neonates ranged between 0.07 - 0.13 $mm.d^{-1}$ and maturity was reached within 21 – 28 days at 22°C. Males and females matured within the same time period. The life cycle (from egg to mature adults) is thus completed within 30 days (also observed by Connell and Airey 1979) but the life span of the amphipod is >60 days in the laboratory.

Several challenges were experienced during this study. The pilot study failed when neonates were reared in either medium- or fine-grained sediment. When reared in a mixture of medium- and fine-grained sediment, neonates showed variable responses. They showed stunted growth at a salinity of 21, while growth did not appear to be impacted at a salinity of 7 and 35. Dissolved oxygen, food availability and salinity were excluded as important factors affecting neonate growth in medium- to fine-grained sediment. The possible effect of ammonia was further investigated in a microcosm study, where neonates were allowed to coexist with adult females for the first 10 days of their lives.

Some amphipod species require parental care in their early stages of development (see Thiel 1998). The microcosm study revealed that ammonia may have a significant role in amphipod growth. The population of amphipods reared at a salinity of 21 crashed when ammonia increased to 6.61 mg NH_3 l^{-1}(LC_{50} for *G. lignorum* is 3.35 mg NH_3 l^{-1}, Kohn et al. 1994). Ammonia toxicity at different salinities (see example in Chapter 4) should thus be investigated further. Stunted growth was also observed in the microcosm experiment, but at the different salinity (salinity 7). Amphipods reared at a salinity of 7 grew slower than those reared at 21 and 35, yet the ammonia concentration was low (2.51 ± mg NH_4^+ l^{-1}; range: 2.1 to 3.4 mg NH_4^+ l^{-1}). Further investigations on factors influencing the growth of *G. lignorum* should include sediment grain size composition, food quality, food quantity, feeding regime and ammonia. Besides challenges that befell this study, some conclusions can be made. For example, chronic toxicity tests using *G. lignorum* where growth and reproduction are endpoints should be 28 days long, since the amphipod completes its life cycle within 28 days. Further investigations are, however, necessary to determine whether chronic sediment toxicity tests should be initiated with neonates or immature individuals. Other estimations of growth such as weight should also be evaluated as a possible endpoint in chronic toxicity tests.

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