Development of an embryo-larval chronic toxicity test using the sea urchins *Tripneustes gratilla* and *Echinometra mathaei*

By

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ABSTRACT

Toxicity tests use organisms to assess the effects of the bioavailable fraction of toxicants in effluents and environmental media, such as water and sediment. Test organisms must be highly sensitive and ecologically relevant. Gametes of the sea urchins *Echinometra mathaei* and *Tripneustes gratilla* are used for acute toxicity testing purposes in South Africa through the fertilisation test (*e.g.* for testing effluents and seawater samples). However, this test has raised questions on the longer-term, sub-lethal effects of toxicants in effluents and other environmental media tested. The aim of this study was thus to develop a chronic toxicity test using the larvae of these sea urchins, to allow for more comprehensive toxicity testing. To achieve this aim, the influence of temperature on larval development to the 4-arm pluteus stage was first evaluated, to determine the duration of the toxicity test. Embryos of the sea urchins were exposed to three temperatures, namely 20, 23 and 26 °C, and development was documented at 24 h intervals until the 4-arm pluteus larva was attained. The optimum temperature for normal larval development for both species was 23 °C, with the 4-arm pluteus stage attained at 72 h for *E. mathaei* and 96 h for *Tripneustes gratilla*. The sensitivity of the sea urchin larvae to potential reference toxicants copper and zinc was evaluated. Reference toxicants are critical for quality assurance in toxicity testing. The EC_{50}s were used to construct control charts for each species. The control charts and coeffiecient of variation identifies the preferred reference toxicant, for *E. mathaei* as zinc and for *T. gratilla* as copper. The larval development test was then used to test the toxicity of seawater desalination brine, effluent from two wastewater treatment works and water collected in a marine environment that receives effluent from a pulp mill effluent concurrently with the fertilisation test. This was to compare the sensitivity of each test in detecting toxicity. The minimum acceptable toxicant dilution (MATD) was calculated from dose-response curves using the linear interpolation model and the index of difference was used a measure of sensitivity. The larval development test was generally less sensitive in detecting toxicity of seawater desalination brine and effluent compared to the fertilisation test, but the difference was negligible. In contrast, the larval development test was more sensitive in detecting toxicity of seawater samples collected from the receiving water of pulp mill effluent, but the results were highly variable. The fertilisation test would be the preferred choice for toxicity testing of seawater desalination brine, effluent and receiving water samples of pulp mill effluent as it is generally more sensitive, rapid and cost effective for sample analysis. However, this is not a definitive decision because the larval development test may be more sensitive in other applications using different types of effluent or water samples from receiving environments.

Keywords: Sea urchin, *Echinometra* mathaei, *Tripneustes* gratilla, acute toxicity, chronic toxicity, larval development, fertilisation, effluent, brine, seawater

PREFACE

The experimental work described in this thesis was carried out at the Council for Scientific and Industrial Research (CSIR) in Durban from November 2018 to December 2018, under the supervision of Dr. Brent K. Newman (CSIR) and Dr. Ntuthuko F. Masikane (University of Zululand). The study represents original work by the author and has 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

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I acknowledge that I have read and understood the University's policies and rules applicable to postgraduate research, and I certify that I have, to the best of my knowledge and belief, complied with their requirements.

I confirm that I had obtained an ethical clearance certificate for my research (UZREC 171110-030 PGM 2019/22) and that I have complied with the conditions set out in that certificate.

I further certify that this research dissertation is original, and that the material has not been published elsewhere, or submitted, either in whole or in part, for a degree at this or any other university

I declare that this research dissertation is the product of my own work and effort.

I have, to the best of my knowledge and belief, complied with the University's Plagiarism Policy and acknowledged all sources of information in line with normal academic conventions.

I have submitted earlier drafts of this document to a text matching software (*i.e.*Turnitin).

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Date:

DECLARATION: PUBLICATIONS

Details of contribution to publications that form part and/or include research presented in this dissertation are as follows:

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DEDICATION

This work is dedicated to my parents, Mr Rubendren Pillay and Mrs Eve Jessica Pillay. They have been there every step of the way in supporting me to the completion of this study. I can never thank them enough for being the foundation of my success.

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CHAPTER 1: General Introduction

The South African coastline is about 3650 km in length and is home to at least 12914 species of marine biota (Griffith *et al*., 2010). The richness and diversity of this coastal environment is a national asset in both economic and social terms (DEA, 2014). As a result of increased human activity, however, water quality along parts of the South African coastline has been compromised. For example, the densely populated KwaZulu-Natal coast and city hubs of Cape Town and Port Elizabeth produce high volumes of marine pollution (Griffith *et al*., 2010), mainly from industries, mining activities, sewage from wastewater treatment plants and excessive nutrients from fertilisers used for agricultural purposes (Griffin *et al*., 2019). Pollutants from these sources are directly or indirectly discharged into nearshore coastal areas.

The National Water Act (Act 36 of 1998) (RSA, 1998) governs the protection, use, development, conservation, management and control of South Africa's water resources (Singh & Nel, 2017). Therefore, any discharge to a water resource must comply with the standards prescribed under the National Water Act together with the minimum requirements governing effluent disposal (DWS, 2017). The Department of Environmental Affairs established that the disposal of effluent into coastal waters is a viable solution, provided that environmental sustainability and marine ecosystem integrity is maintained (DWS, 2017). Considering this the Integrated Coastal Management Act, (Act No. 24 of 28) was promulgated just over a decade ago to promote better management and control of South Africa's coastal and marine environment (Taljaard *et al.,* 2019). The Integrated Coastal Management Act stipulates that the discharge of effluents into coastal waters should be regulated through the issuance of Coastal Waters Discharge Permits.

Traditionally, the regulation and control of effluent discharge has been assessed by comparing the physico-chemical properties of an effluent to water quality guidelines or other targets. However, there are limitations to using only physico-chemical properties to assess complex effluents (Roux, 1994):

- The influence of physico-chemical properties (*e.g.* pH, temperature and dissolved organics) that affect chemical toxicity are not considered.

- Receiving water quality objectives based on single-substance toxicological data cannot account for the complex composition of effluents.
- The antagonistic, additive and synergistic effects of chemicals are not considered.
- Investigators cannot identify and/or quantify every chemical in an effluent.
- The toxicological effects of a chemical depending on its bioavailability, which cannot be identified through chemical analyses alone.

Although there are limitations, physico-chemical data along with biological data from toxicity tests can be used to gain more insight into the characteristics of an effluent and its potential effects on the environment (Palmer *et al*., 2004). Hence, this places importance on the use of toxicity tests to complement physico-chemical analyses in monitoring and assessing potentially harmful contaminants in water (Palmer *et al.,* 2004).

Ecotoxicology serves as the scientific basis for the ecological risk assessment of contaminants (Ashaur & Escher, 2010). Whole effluent toxicity (WET) testing is conducted to determine the aggregate toxic effect of an effluent on a test organism (USEPA, 2002). The main objective of toxicity testing is to estimate the 'safe' or 'no effect' concentration of an effluent, in other words the concentration that will allow the normal proliferation of biota in a receiving water (USEPA, 2002). The no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) can be estimated by comparing the responses of test organisms exposed to serial dilutions of a tested substance to the responses of organisms in the control which is done by hypothesis testing (USEPA, 2002). The NOEC (together with the LOEC) has received much criticism, for the following reasons. The NOEC which is selected from one of the tested concentrations, is dependent on the researcher (*i.e.* the concentrations used and their spacing), which is scientifically inappropriate (OECD, 1998). Therefore, test precision is compromised. It is less likely to detect differences from the control if there is greater experimental variability (OECD, 1998). Thus, poor quality data is favoured.

Considering this, it's still used as a standard measure to provide an acceptable level of aquatic ecosystem protection because it is both conceptually and computationally simplistic to understand and calculate using analysis of variance *i.e.* if there's no significant difference of the test concentration from the control then there's no effect (Davies-Coleman & Palmer, 2004; Griffith *et al.,* 2019; OECD, 1998).

Recently, the EC_x value of interest or an arbitrary percentage effect (for example 5 %, 10 % or 25 %) can be calculated from a dose-response curve and has been suggested as an alternative to the NOEC (Ishnard *et al*., 2001; Green *et al*., 2012 Kennedy *et al*., 2019). These arbitrary percentages can be either under or over conservative depending on the response or species (Green *et* al., 2012). In this study, the EC_x percentage was calculated using an equation that accounts for within-test variability which provides a more realistic effect of toxicity that is relevant to the test procedure. The EC_x value is highly model-dependent (Green, 2016). The use of the EC_x approach has received much apprehension as it requires 'good statistical modelling results from the application of knowledge (of the system being modelled), experience (with modelling systems of this type), and skill (based on technical understanding and tools available)' (Fox & Landis, 2016).

Toxicity tests are performed using seawater from an uncontaminated site that is preferably away from the receiving environment of the effluent that is being tested. This water serves as both the control and dilution water of an effluent that is being tested, or as the control for testing of receiving waters and thus provides a closer simulation of the chemical interactions that may occur when an effluent is discharged (ARC, 1992). Test organisms are exposed to various dilutions of effluent and incubated under controlled temperature and photoperiod conditions for a specific duration that is generally dependent on the endpoint being tested and the test organism. The test endpoint may consist of one of many variables (*e.g.* mortality, reduced fertilisation, development success) (USEPA, 1995).

The median effective concentration or median lethal concentration (EC_{50} or LC_{50}) is used to determine the concentration that results in 50% of the test organism's mortality, inhibited development or some other identifiable endpoint (Johnson & Finley, 1980). As part of routine monitoring, the tests need to follow standard protocols which include quality control procedures, such as reference toxicant testing (ARC, 1992; Environment Canada, 1999). The EC_{50} or LC_{50} values obtained from reference toxicant testing are used to plot a control chart. The control chart is used as a tool to document a test organism's performance under laboratory conditions and its ability to produce repeatable results (ARC, 1992; Environment Canada, 1999; USEPA, 2000).

Many international organisations, such as the American Standard Testing Methods (ASTM), United States Environmental Protection Agency (USEPA), Environment Canada (EC), and Australia and New Zealand Environment and Conservation Council and Agriculture and

Resource Management Council of Australia and New Zealand (ANZECC and ARMCANZ) have developed standard testing protocols using gametes and larvae of sea urchins belonging to the genera *Parencentrotus, Strongylocentrotus, Hemicentrotus, Pseudicentrotus, Anthocidaris,* and *Arbacia* amongst others (USEPA, 1995; ANZECC and ARMCANZ, 2000; EC, 2011; USEPA, 2012; ASTM, 2012; Beiras, 2018).The use of sea urchin gametes and larvae in acute and chronic toxicity tests can be described as follows.

Acute toxicity refers to a toxicant that can induce an immediate (short-term) effect on a test organism (ECETOC, 2004). The sea urchin fertilisation test is a cellular level acute test that measures the toxicity of test solutions to sperm fertility. Sperm are exposed to a test sample for a certain period prior to the addition of eggs, and the ability of the sperm to fertilise the eggs is determined. The endpoint is fertilisation success, which is indicated by a fertilisation membrane. The duration of sperm fertilisation tests varies depending on the species used and can be as short as 10 minutes to as long as 60 minutes (Environment Canada, 2011). These acute tests are preferred over chronic tests because of the relatively low cost and rapidity in producing results (ECETOC, 2004).

Chronic toxicity refers to the continuous exposure to a toxicant over a relatively long period (sub-lethal effects) (ECETOC, 2004). The sea urchin larval development test assesses the effect of a test sample, or dilution of a chemical or effluent on larval development and growth (Byrne *et al.*, 2003). These tests involve the exposure of fertilised eggs to test solutions for a period of 48 to 96 hours post fertilisation depending on the species and temperature of exposure (Anselmo, 2012; Pagano *et al*., 2017). The endpoint is the proportion of individuals that show normal larval development. This test is less commonly used because of cost and time issues. A chronic toxicity test is often conducted after an acute toxicity test has identified that the test sample provides cause for concern (ECETOC, 2004).

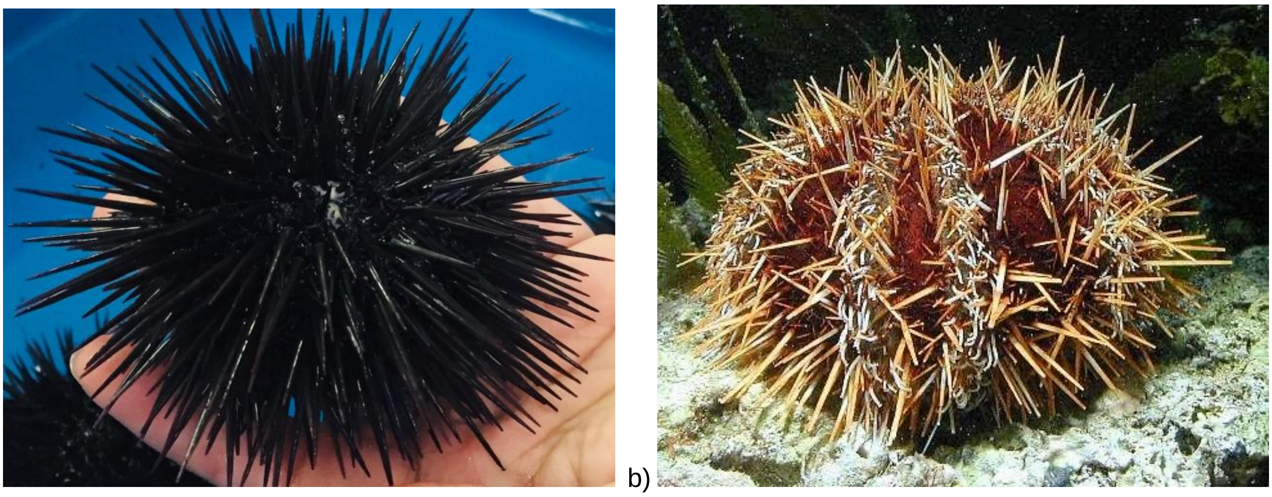
The chronic (larval development) test can be reflective of the biochemical or physiological effects of toxicity which may compromise the fitness and biological functions to survive or reproduce (Goh *et al*., 2014). Therefore, the acute (fertilisation success) test cannot determine the effect of a toxicant on organismal survival. In effect, endpoints of sea urchin acute and chronic toxicity tests provide an indication of the possible ecosystem effects and are used in the management of whole ecosystems and communities (Griffin *et al*., 2011). However, chronic (sublethal effects) such as retarded development can impair growth and reproduction by lowering fecundity (eggs per female) and increase susceptibility to predation

(USEPA, 1994). Hence, chronic toxicity tests can provide an ecologically significant measure of toxic effect (USEPA, 1994). Furthermore, this may impact recruitment and thus the success of a population. Sea urchins are keystone species and have been used in ecological risk assessments because the impact of their loss or addition has shown to have significant effects on community composition, structure and function (Maloney, 2019).

It is impractical to provide toxicity data for all potential species present in a given environment. Due to this limitation, test organisms are used as a representative of the effect of toxicity on major ecosystem components (Wilson, 2006). The ideal test organism should have the following characteristics (Buikerna *et al*., 1982; Masikane, 2014; Pagano *et al*., 2017)

- commercially and/or ecologically relevant,
- may cause cascading effects along the food chain,
- easily available and amenable to culture or maintenance in the laboratory,
- able to maintain genetic consistency,
- has enough background information (*i.e.* basic understanding of physiology, genetics, taxonomy and role in the natural environment),
- a wide geographical distribution to provide relevant data,
- highly sensitive and consistent in response to toxicants,
- tests are cost effective and can be conducted over short periods (*i.e.* hours to days)

The test species used in this study are the sea urchins *Echinometra mathaei* and *Tripneustes gratilla* (Figure 1.1) which fall within the phylum Echinodermata. These are a diverse group of marine deuterostomes in the animal kingdom (Mishra, 2013) that share an evolutionary link between vertebrates and invertebrates (Ruocco *et al*., 2017). *E. mathaei* belongs to the family Echinometridae and is commonly referred to as the burrowing urchin (Ghory *et al*., 2018, Brasseur *et al*., 2018). *T. gratilla* belongs to the family Toxopneustidae and is commonly referred to as the collector urchin (Tuang-tuang *et al*., 2018). Both species are primary consumers inhabiting temperate, tropical and subtropical regions (Vaïtilingon et al., 2005; Mishra, 2013). These urchins are extensively distributed from the Central Pacific through to the African Coast in the Indian Ocean, Western Pacific, Southern Islands, Australia and southern Japan (Toha *et al*., 2005, Mishra, 2013). The adults are economically highly valued, as their roe is harvested for human consumption (Biukerna *et al*., 1982; Balisco, 2015). Several countries, including Australia, Japan, the Philippines and South Africa have placed a high commercial value on *T. gratilla* (Darius *et al*., 2018). The presence of *T. gratilla* and *E. mathaei* in coral reef areas are indicative of their ecological role in maintaining the flow of matter and energy (Dalabajan *et al*., 2018, Keshavarz *et al*., 2017)**.** These urchins graze on seaweed or seagrass in coral reef areas and by an indirect effect of feeding the detachment of leaves forms part of the detritus (Lawrence, 2020). Another indirect effect of feeding is nutrient cycling where the production of faeces re-enters the community and becomes available to other consumers (Lawrence, 2020).



a) b)

Figure 1. 1: Images of sea urchins a) *Echinometra mathaei* and b) *Tripneustes gratilla.* (Source: http://atj.net.au/marineaquaria/Tripneustes_gratilla.html)

Sea urchin gametes and larvae have been used in developmental and cellular research since the mid-1800s (Canty, 2009). Numerous studies have used the gametes and larvae of *T. gratilla* and *E. mathaei* for testing the toxic effects of metals (Heslinga, 1976; Ghorani *et al*., 2012; Edullantes & Galapate, 2014; Tualla & Bitacura, 2016; Darius *et al*., 2018), effluents (Vazquez, 2013) and temperature (Rupp, 1973; Alsaffar & Lone, 2000; Brennand *et al*., 2010).

The larvae of *E. mathaei* (a summer spawner) and *T. gratilla* (a winter spawner) were selected for toxicity testing purposes in this study because sperm and eggs of one or other species are available throughout the year. The alternation between summer and winter spawning species allows year-round testing of effluents or other environmental matrices.

The larvae are also lecithotrophic (larval development is fuelled using nutrients from the egg). Static tests can thus be conducted without having to add food, which may be a confounding factor in any observed toxic effect (Shackleton *et al*., 2002; Whitehill, 2012). Lastly, the use of local species for testing purposes provides a greater ecological relevance in determining the effects of effluents on receiving waters**.**

Although the application of ecotoxicology is not yet widespread in South Africa, the sea urchin fertilisation test is routinely used to test the toxicity of effluents discharged into receiving waters along this coastline (Shackleton *et al*., 2002, CSIR, 2017). However, this test has raised questions on the longer-term, sub-lethal effects of effluent discharges. It is for this reason that this study expands on the acute sea urchin fertilisation test, by developing a chronic larval development toxicity test.

1.1 Aim

Ecotoxicology studies in estuarine and marine environments in South Africa are scarce, as are the tests available for toxicity testing. The development and standardisation of the sea urchin larval test would assist in understanding the chronic effects of environmental pollution along this coastline. The aim of this study was thus to develop chronic toxicity tests based on the larval development of the sea urchins *E. mathaei* and *T. gratilla.*

1.2 Objectives

1) Determine the optimal temperature for larval development and suitable test duration for *E. mathaei* and *T. gratilla.*

2) Develop control charts (consisting of reference toxicity test data using copper and zinc) to assess whether *E. mathaei* and *T. gratilla* perform consistently in toxicity tests. Using this information, determine which of the metals is the most effective reference toxicant for quality assurance purposes.

3) Apply and compare the sensitivity of the sea urchin fertilisation test and larval development test to real world situations, including the testing of effluent, brine from seawater desalination and the receiving waters of pulp mill effluent for the purpose of determining if the larval development test provides different information for decision-making compared to the fertilisation test, and should thus be used in preference to the fertilisation test.

1.3 Thesis outline

The chapters of this thesis are written in scientific publication format, and there is thus an unavoidable repetition of some information.

Chapter 2

This chapter aims to identify an optimum test temperature which is based on normal larval development of *E. mathaei* and *T. gratilla* to the 4-arm pluteus stage. Embryos of *E. mathaei* and *T. gratilla* are exposed to three temperatures and their development is documented over 24 h intervals. The influence of temperature on total larval length is also investigated in this chapter.

Chapter 3

The sensitivity of *E. mathaei* and *T. gratilla* to the reference toxicants copper and zinc is established. Control charts are constructed using the EC_{50} values obtained from each test and are then used as a tool to identify the most appropriate reference toxicant for each species.

Chapter 4

This chapter compares the results of the fertilisation and larval development test following exposure to various real-world samples *i.e.* seawater desalination brine, effluent and water samples from pulp mill effluent receiving water. The sensitivity of each test in detecting toxicity is compared to determine if the larval development test provides different information for the purpose of decision-making and should either replace or be used in conjunction with the fertilisation test.

Chapter 5

This chapter synthesises the results of the previous three chapters in achieving the overall aim of developing a chronic larval development toxicity test using sea urchins *E. mathaei* and *T. gratilla* and provides recommendations for future research.

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CHAPTER 2: The effects of temperature on larval development of sea urchins *Echinometra mathaei* and *Tripneustes gratilla*

2.1 Abstract

Temperature dictates the performance of biochemical and physiological processes, which ultimately affect organismal traits such as development and survival. This study aimed to determine the effect of temperature on the development rate and size of larvae of the sea urchins *Echinometra mathaei* and *Tripneustes gratilla* to the 4-arm pluteus stage, to identify an optimum temperature for chronic toxicity testing purposes. This was done by incubating fertilised eggs in water baths set at three temperatures, namely 20, 23 and 26 °C. Temperature selection was based on those likely to occur along the east coast of South Africa in the summer and winter seasons. The gametes and larvae were preserved at 24 h intervals to document the developmental stages to the 4-arm pluteus. Morphometric measurements were made by measuring the total larval length of the 4-arm pluteus. The 4-arm pluteus stage was attained at 23 °C and 26 °C for both species. However, development was accelerated at 26 °C, as the 4-arm pluteus was attained 24 h in advance when compared to 23 °C. The temperature identified to run the larval development test is 23 °C, with a duration of 72 h for *E. mathaei* and 96 h for *T. gratilla*. Total larval length was greater at 26 °C with a mean ± standard deviation of 246.41 ± 24.25 and 253.48 ± 30.72 compared to 214.23 ± 16.90 and 226.52 ± 31.6 at 23 °C, for *E. mathaei* and *T. gratilla.*

2.2 Introduction

The early development of sea urchins is highly sensitive to external stimuli and has been used for decades for the toxicity testing of pollutants for environmental monitoring purposes (Kobayashi, 1971; Byrne, 2011; Pagano *et al*., 2017). Some of the factors that affect the rate of larval development include temperature, salinity and food availability (McClanahan & Muthiga, 2013). According to numerous studies, temperature is the most important factor affecting larval development rate and this is species-specific for sea urchins (Rupp, 1973; Mita *et al.*, 1984; Rahman *et al.*, 2007; O' Connor *et al*., 2007; Azad, 2011; Parvez *et al.*, 2018; Tuang-Tuang *et al*., 2018). Since temperature dictates the performance of biochemical and physiological processes it may influence organismal traits, such as development and survival (O'Connor *et al.*, 2007). Hence, the metabolic rate influencing larval development is dependent on temperature (Byrne, 2011). Outside the optimal temperature range metabolic activity is affected, and this can result in slower development, developmental abnormalities or decreased survival (Rahman *et al.*, 2009; Azad, 2011).

E. mathaei, the 'rock-boring' urchin, is oval shaped, purple in colour and has long and stout spines that taper to a sharp tip (Branch & Branch, 2010). *T. gratilla,* the 'collector' urchin, has short white spines that are roughly even in length with tube feet that stretches out to trap food and debris (Branch & Branch, 2010). Their diet consists mainly of algae and seaweed and are likely to be grazing in reef areas or rocky shores. *E. mathaei* and *T. gratilla* are dominantly subtropical and tropical urchins that are widely distributed througout the Indo-Pacific region, therefore *E. mathaei a*nd *T. gratilla* share a similar biogeographic distribution along with the temperature variations in these environments (Vaitilingon *et al.*, 2005; Tuang-Tuang *et al.,* 2018). Both species are thermotolerant as they inhabit areas with a wide temperature range (Rahman *et al.,* 2007; Rahman *et al*., 2009; Keshavars *et al.,* 2017).

Sea temperatures during spawning are more favourable for spawning to ensure fertilisation success (Sewell and Young, 1999). Thereafter, embryos and larvae are subjected to temperature fluctuations in the water column throughout their planktonic phase. Temperature effects on the early larval development of *E. mathaei* and *T. gratilla* were performed by Rahman *et al.* (2007) and Rahman *et al*. (2009) in Okinawa Island, Japan where spawning sea surface temperatures were 25 – 28 °C for *E. mathaei* and 22 – 26.5 °C for *T. gratilla*. Normal larval development was documented between 22 – 28 °C for *E. mathaei* and 22 – 29 °C for *T. gratilla* (Rahman *et al.,* 2007; Rahman *et al*., 2009). In Malaysia, *T. gratilla* and *Diadema setosum* are exposed to average annual seawater temperatures ranging from 27 – 31 °C and normal larval development to the 4-arm pluteus was observed at 22, 25 and 28 °C for *T. gratilla* and at 25 – 28 °C for *Diadema setosum* (Sarifundin *et al.,* 2016; Parvez *et al.,* 2017). This shows that temperatures required for normal larval development are strongly related to those occurring during spawning but can also occur outside this temperature range.

The development and growth of sea urchins consist of the planktonic, free-swimming larval stage in the water column which after days or weeks metamorphose and settles to become benthic juveniles and then adults (Hammond and Hoffman, 2010; Parvez *et al.,* 2017). Reproduction in sea urchins can happen either periodically or continuously throughout the year. Although sea urchins are dioecious, their sex can only be determined upon release of their gametes. External fertilisation occurs in the water column by broadcast spawning. Fertilised eggs are referred to as embryos, which develop synchronously by holoblastic cleavage until they become a blastula (Ghorani *et al*., 2012). During blastulation cells converge to the periphery while gradually forming the central cavity called the blastocoel (Ghorani *et al.*, 2013). The invagination of the vegetal plate forms the primitive gut or archenteron and is now referred to as the gastrula (Ghorani *et al.*, 2013). Throughout the process of gastrulation, mesodermal primary mesenchyme cells migrate and fuse to form a single syncytium (multinucleate cell resultant of multiple cell fusion of uninuclear cells) within which the calcium carbonate endoskeleton is secreted (Adomako-Ankomah & Ettensohn, 2013). The gastrula shifts to a pyramidal shape which is then called a prism larva. Thereafter, the formation of budding post-oral arms progresses into a 4-arm pluteus larva which has a distinctive tripartite digestive system and fully extended post-oral arms (USEPA, 1995; Annuziata *et al.*, 2013). These are composed of calcified structures which mainly function in feeding and swimming (Chan *et al*., 2015). This early embryonic-larval development is lecithotrophic, that is, development is sustained by the nutrients supplied by the egg. However, shortly after the 4-arm larval stage is reached feeding begins (Whitehill, 2012).

The aim of this study was to determine the effect of temperature on the early development of larvae of the sea urchins *E. mathaei* and *T. gratilla*, for the purpose of identifying an optimal temperature for chronic toxicity testing purposes.

2.3 Methods and materials

2.3.1 Collection and maintenance of urchins

Adult sea urchins were collected from Vetches Reef and seawater was collected from Vetch’s Beach (29º51’59.27”S, 31º 2’53.91”E) (Figure 2. 1) in Durban on the east coast of South Africa, during the spawning season for each species. *E. mathaei* (summer spawner) was collected from November 2018 to April 2019 and *T. gratilla* (winter spawner) from May 2019 to October 2019. The sea urchins were transported to the toxicology laboratory at the CSIR campus in Durban, in buckets filled with seawater from the source of collection to ensure minimal stress on the urchins. Upon arrival at the laboratory, sea urchins were gradually acclimated to the water temperature in the tanks. A maximum of 10 sea urchins were transferred to flow-through, aerated tanks (40 L) containing natural seawater at ambient temperature. The sea urchins were fed daily with fragments of *Ulva* spp algae.

Figure 2. 1: Aerial view of seawater collection point from Vetches Beach and sea urchin collection point at Vetches Reef.

2.3.2 Test procedure

A single male-female pair was used for each test. Three replicate tests were conducted. Seawater for testing purposes were stored in tightly sealed 25 L buckets. Prior to testing dissolved oxygen (7.5 ± 0.5 mg l^{-1}) and pH (8.1 ± 0.1) was checked using a YSI probe, and salinity (35 ± 1) was checked using a refractometer, in accordance with CSIR standard operating procedure for the sea urchin fertilisation test. Urchins were injected with 1 - 2 ml of 0.5M KCl through the peristomial membrane (Figure 2. 2) to induce spawning. Collection of gametes were done immediately after the release from urchins were visible.

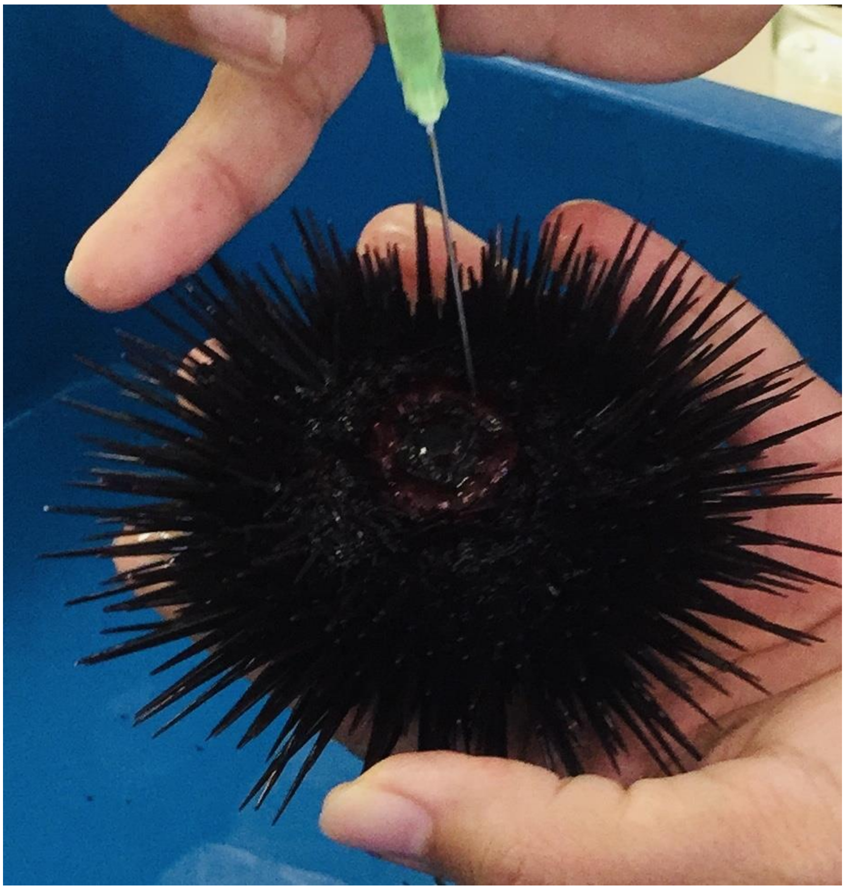


Figure 2. 2: *Echinometra mathaei* injected with 0.5M KCl through the peristomial membrane.

Females were inverted over a glass beaker filled with filtered seawater to collect the eggs. Sperm from the males were collected ‘dry’ using a Pasteur pipette to prevent activation and preserve its viability. Immature eggs and debris were removed by washing and decanting 3 - 4 times with filtered seawater. Egg quality and sperm motility were checked at 10× magnification using the Zeiss Axioskop 2 light microscope. Eggs that had a uniform shape and distinct nuclei were considered good quality and highly motile sperm were used. Immediately before the test, sperm were added to filtered seawater to activate the sperm. Sperm density was not calculated but a sperm dilution series was used to determine the appropriate volume of diluted sperm to yield 95 % fertilisation of 400 - 500 eggs per ml in the egg stock solution.

Fertilised eggs (400 - 500 per ml) were pipetted into glass vials (four replicates per time interval) filled with 20 ml of filtered seawater and then submerged in the tanks. Each vial was covered with a plastic lid to limit evaporation and prevent minimal changes in salinity. At 24 h intervals, the embryos and larvae were fixed with 40% formaldehyde. These intervals were selected to ensure that the main developmental stages could be observed and documented. The 4-arm pluteus was selected as the endpoint because larvae are lecithotrophic and are self-sustaining until this stage. The total test duration was 72 h for *E. mathaei* and 96 h for *T. gratilla*.

The effect of temperature on larval development was investigated at three temperatures, namely 20, 23 and 26 °C. These temperatures broadly encompass temperatures in the natural range of the sea urchins at the collection site. Seawater temperatures along the east coast of South Africa were recorded at a range of 22.3 – 27.8 °C in summer and 19.7 – 23.7 °C in winter (https://www.seatemperature.org/africa/south-africa/durban.). These temperatures are reflective of those experienced by *E. mathaei* and *T. gratilla* during their respective spawning seasons. This study included an expected annual minimum (20 °C), maximum (26 °C) and average (23 °C) seawater temperatures for both species although it might be outside of their range during their breeding season. Water baths equipped with heaters were set at 20, 23 and 26 °C (Figure 2. 3) and housed in a temperature-controlled room set to a temperature < 20 °C. The water temperature was monitored daily using a calibrated thermometer and remained constant throughout each experiment.

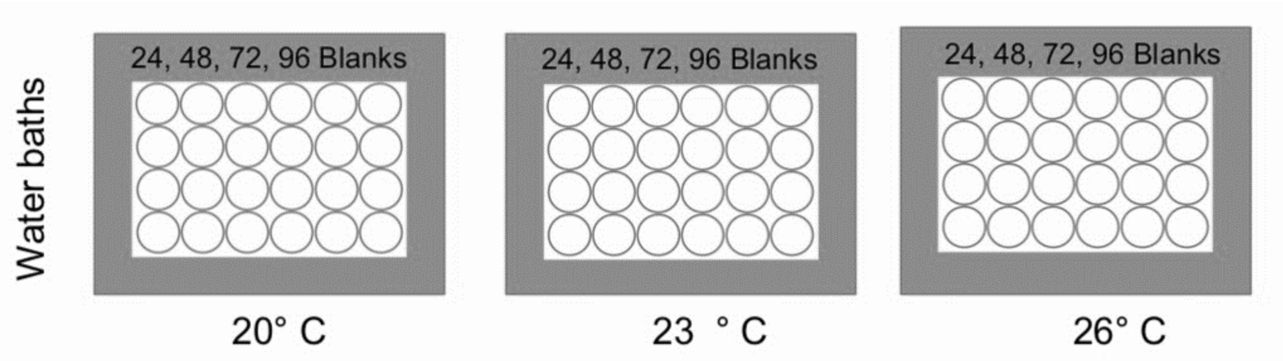


Figure 2. 3: Experimental test set-up of temperature exposures at 20, 23 and 26 °C. Each set of rows (n = 4) represents independent 24 h intervals of embryo-larval development.

2.3.3 Embryo-larval classification and measurements

Development stage(s) were assessed for 100 embryos observed under a light microscope at 10× magnification for each time interval and temperature. Following, Conway *et al*. (1984), USEPA (1995), Smith *et al*. (2008), Carbelleira *et al*. (2011), and Annunziata *et al.* (2013) embryos were scored as blastula, gastrula, prism, 2-arm and 4-arm pluteus larva (Figure

2.4) and abnormal. Normal pluteus larvae were used as the endpoint and classified based on the following characteristics: have a pyramid shape; fully differentiated gut and post-oral arm length greater than the total arm length (USEPA, 1995).

The predominance of abnormalities present in each replicate was classified as follows:

- Pathological hatched or pre-hatched - Single (*i.e.* fertilised eggs) or multicellular embryos with or without a fertilisation membrane, appears as dark masses of cells or dissociated blobs of cells.
- Necrosis - Cell death as a result of external interference that causes an uncontrolled release of the inflammatory cellular contents (Fink & Cookson, 2005).
- Inhibited - Embryos or larvae at the blastula, gastrula and prism stage, have no or undeveloped skeleton.
- Gut abnormalities - Normal overall appearance where guts are absent, undifferentiated, incorrectly shaped or positioned or projected outside the embryo or larvae (exogastulated)
- Retarded – Delayed development at the 2-arm pluteus stage or has an irregular shape
- Skeletal malformations - May have either bent, spread out or asymmetrical post-oral arms or separated or crossed tips at the apex of the larvae

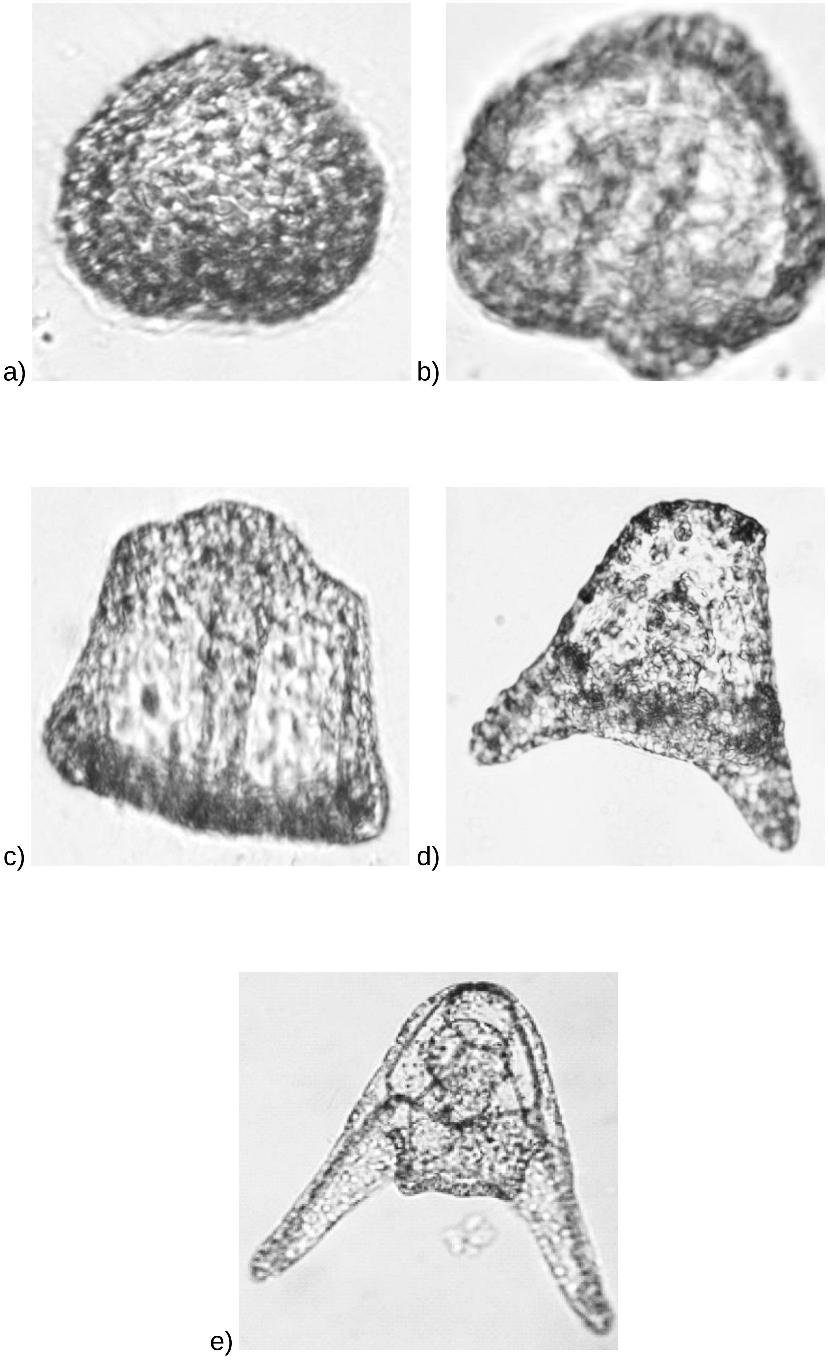


Figure 2. 4: Examples of the developmental stages of *Tripneustes gratilla* at a) blastula, b) gastrula, c) prism, d) 2-arm pluteus and e) 4-arm pluteus.

Ten 4-arm pluteus larvae per replicate (*n* = 4) were photographed using a Zeiss Axioskop 2 fitted with a camera. The total length of the larvae from the apex to the end of the post-oral arm (Figure 2.5) was then measured using ZEN imaging software.

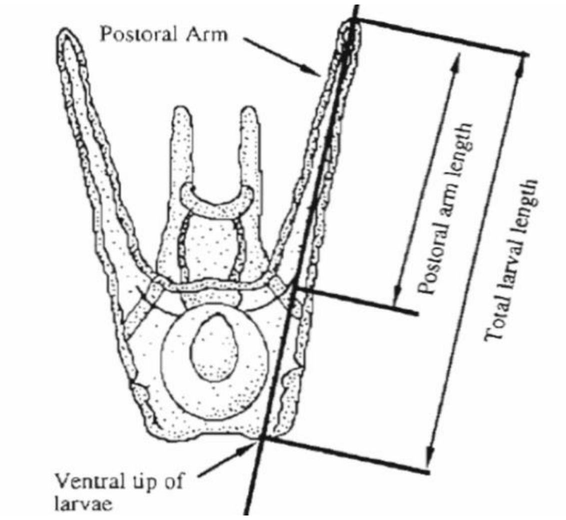


Figure 2. 5: Morphometric measurements of the total larval length using the 4-armed pluteus larvae (from Lamare & Barker, 1999).

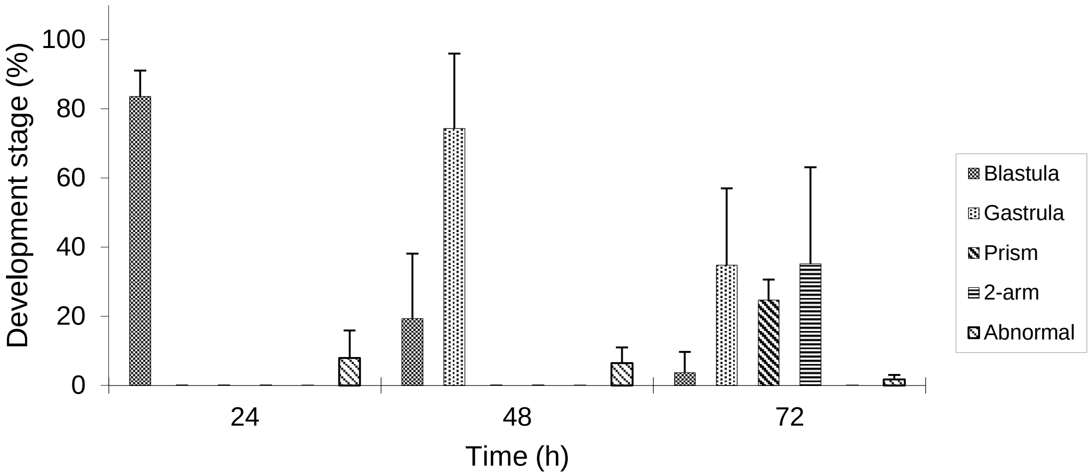
2.3.4 Statistical analyses

SigmaPlot 12.0 software was used to perform t-tests to compare the development success (proportions arcsine transformed) and morphometric measurements between species and temperatures. To test for normality and homogeneity of variance, Shapiro-Wilks and Levene's test were used. The Mann-Whitney U Rank sum test was used to compare data when the assumptions for normality and homogeneity of variance were violated. The significance level was $\alpha = 0.05$. The mean and standard deviation represent the data (*n* = 12) for each developmental stage at each time interval for the different temperatures. The acceptability criteria for each test was > 80 % normal larval development following USEPA (1995).

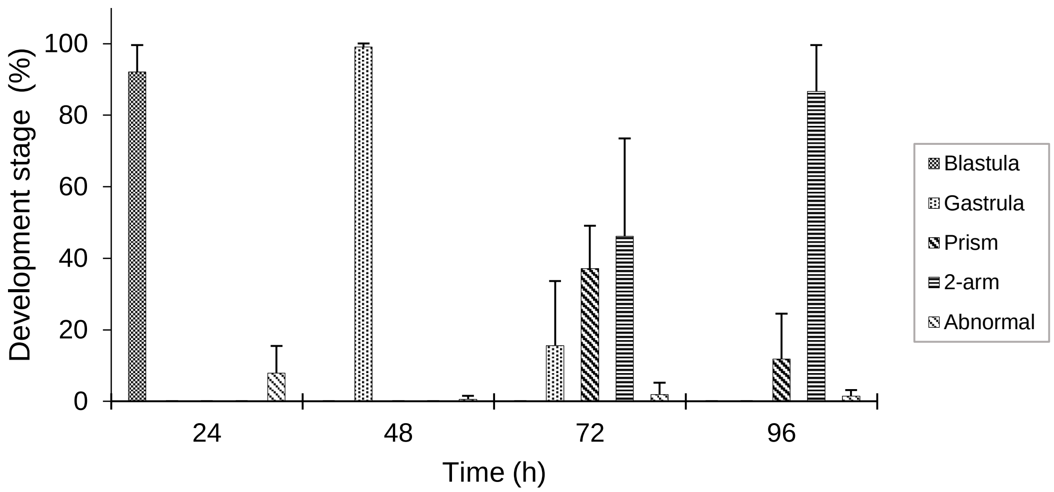
2.4 Results

Development at 20 °C

E. mathaei and *T. gratilla* followed a similar sequence of development, reaching the blastula stage at 24 h and the gastrula stage at 48 h (Figure 2.6). However, the development to 2-arm pluteus was achieved at 72 h for *E. mathaei* and at 96 h for *T. gratilla.* The 4-arm pluteus was not attained in both species. For *E. mathaei* and *T. gratilla*, the highest proportion of abnormalities, such as necrosis of the blastula, was recorded at 24 h (Table 1 and 2).



a)

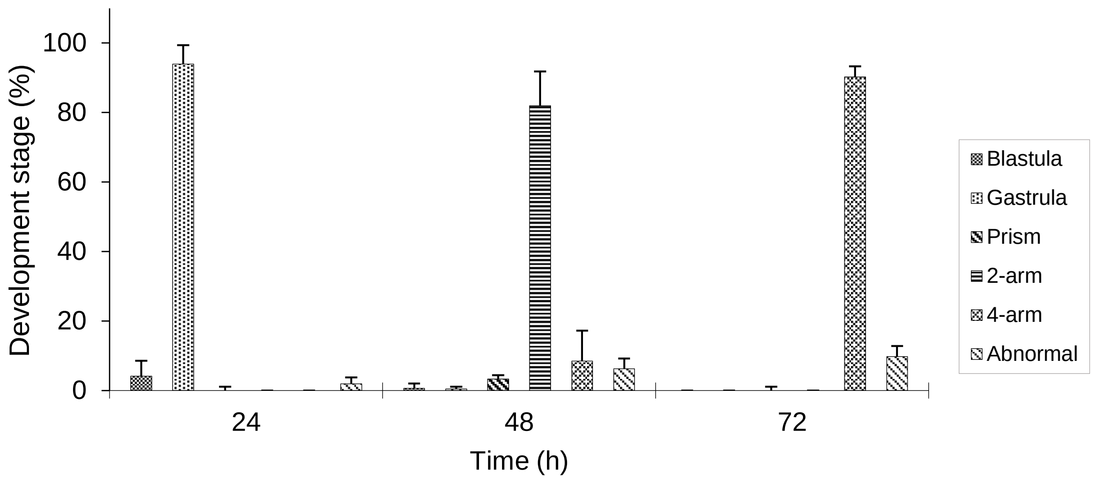


b)

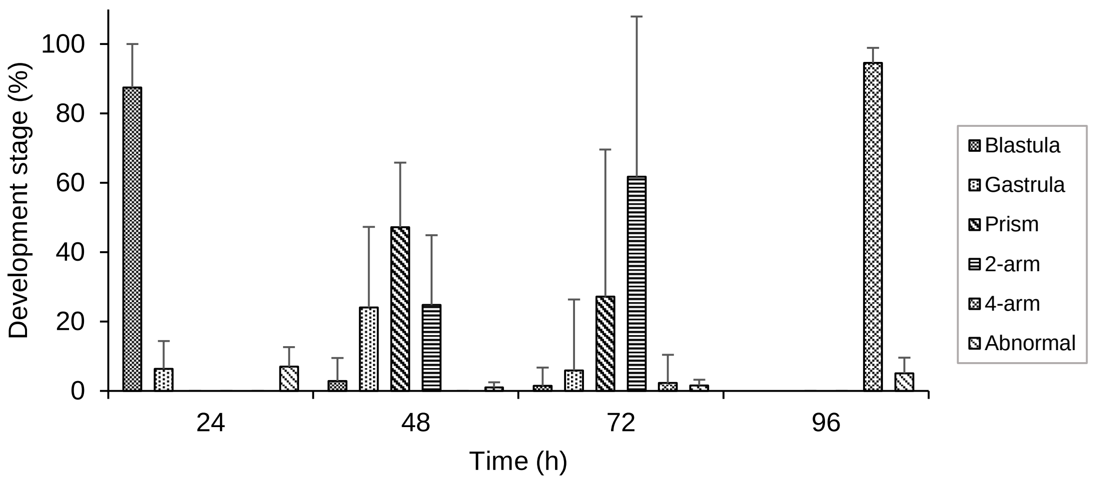
Figure 2. 6: The mean (± standard deviation; *n* = 12) proportion of various development stages of a) *Echinometra mathaei* and b) *Tripneustes gratilla* at different time intervals at 20 °C.

Development at 23 °C

E. mathaei embryos progressed to the gastrula stage at 24 h, whereas in *T. gratilla* they were in the blastula stage (Figure 2.7). In *E. mathaei,* the 2-arm pluteus was reached at 48 h and the 4-arm pluteus at 72 h. *T. gratilla* developed asynchronously (embryos and larvae were at different developmental stages) at 48 and 72 h. However, at 96 h development to the 4-arm pluteus was synchronous. Necrosis of the blastula and exogastrulation was observed at 24 h in *E. mathaei* and *T. gratilla*. In *E. mathaei,* there was a higher proportion of abnormal larvae at 72 h in comparison to *T. gratilla* at 96 h (Table 2.1 and 2.2). Examples of abnormalities at 72 h for *E. mathaei* and at 96 h for *T. gratilla* included; embryos and larvae inhibited at blastula, gastrula and prism stage, and retarded plutei and skeletal malformations.



a)

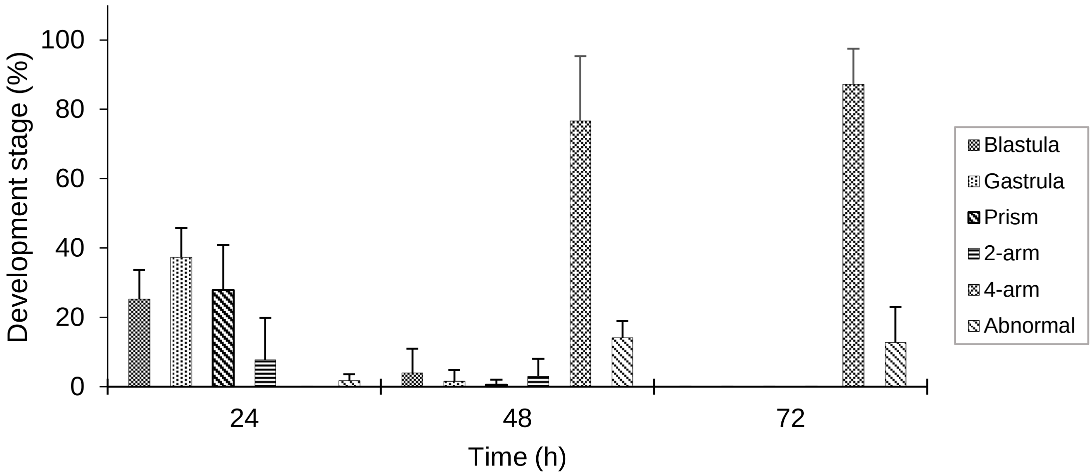


b)

Figure 2. 7: The mean (± standard deviation, *n* = 12) proportion of various development stages of a) *Echinometra mathaei* and b) *Tripneustes gratilla* at different time intervals at 23 °C.

Development at 26 °C

Although there was asynchronous development at 24 h for *E. mathaei*, most embryos and larvae progressed to the 4-arm pluteus at 48 h (Figure 2.8). In *T. gratilla*, the main development stage at 24 h was the gastrula, which progressed to the 2-arm pluteus at 48 h and 4-arm pluteus at 72 h (Figure 2.8). The highest proportion of abnormalities were recorded at 48 and 72 for *E. mathaei* (Table 2.1) whereas the proportion of abnormalities were < 5 % for *T. gratilla* at all time intervals (Table 2.2). In *E. mathaei,* retarded development and skeletal malformations were observed at 48 and 72 h whereas inhibited (blastula and gastrula) and retarded development was observed at 96 h for *T. gratilla.*



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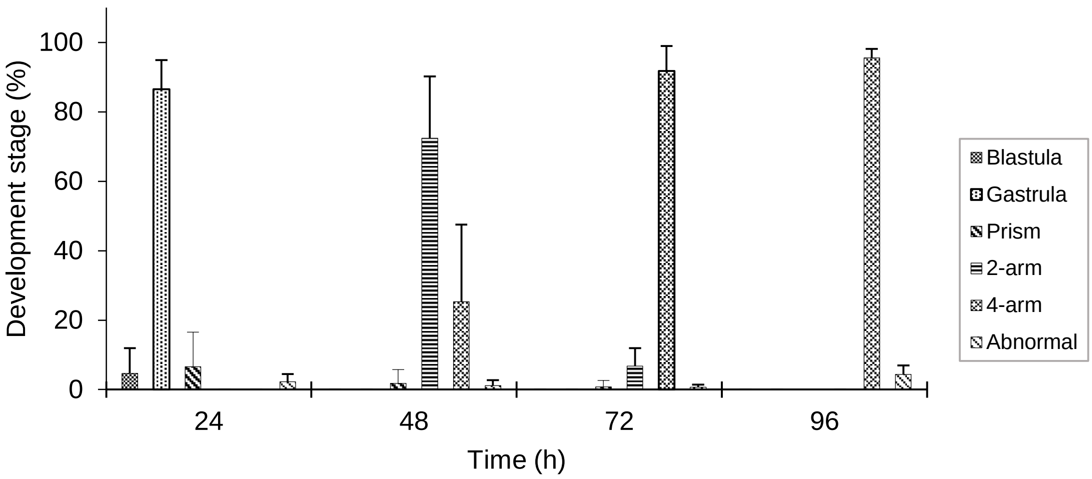


b)

Figure 2. 8: The mean (± standard deviation; *n* = 12) proportion of various development stages of a) *Echinometra mathaei* and b) *Tripneustes gratilla* at different time intervals at 26 °C.

Temperature and larval developmental rate

The mean proportion of larvae at different development stages for each temperature at 24 h intervals are represented in Table 2.1 for *E. mathaei* and Table 2.2 for *T. gratilla.*

Table 2. 1: The mean proportion of various development stages (*n* = 12) of *Echinometra mathaei* at different time intervals for each temperature.

Temperature (°C)	**Time (h)**	**Mean (%) in developmental stages**					
		Blastula	Gastrula	Prism	2-arm pluteus	4-arm pluteus	Abnormal
20	24	83.58					16.83
	48	19.33	74.25				6.42
	72	3.67	34.75	24.67	35.17		1.83
23	24	4.17	93.92				1.92
	48	0.67	0.42	3.33	81.92	8.50	6.25
	72					90.25	9.75
26	24	25.25	37.33	27.83	7.75		1.75
	48	4.00	1.58	0.58	3	76.67	14.17
	72					87.25	12.75

Table 2. 2: The mean proportion of various development stages (n = 12) of *Tripneustes gratilla* at different time intervals for each temperature.

Temperature (°C)	**Time (h)**	**Mean (%) in developmental stages**					
		Blastula	Gastrula	Prism	2-arm pluteus	4-arm pluteus	Abnormal
20	24	92.08					7.92
	48		99.08				0.58
	72		15.58	37.08	46.17		1.92
	96			11.83	86.67		1.50
23	24	87.50	6.33				7.00
	48	2.83	24.08	47.25	24.83		1.00
	72	1.50	5.92	27.17	61.75	2.33	1.58
	96					94.58	5.08
26	24	4.67	86.50	6.58			2.25
	48			1.83	72.42	25.33	1.17
	72			0.75	6.75	91.75	0.67
	96					95.58	4.42

Developmental success

There was no significant difference in development success to the 4-arm pluteus between 23 °C and 26 °C for *E. mathaei* ($U = 70.00$, $p = 0.931$) and *T. gratilla* ($t = -0.257$, $p = 0.799$) (Figure 2.9).

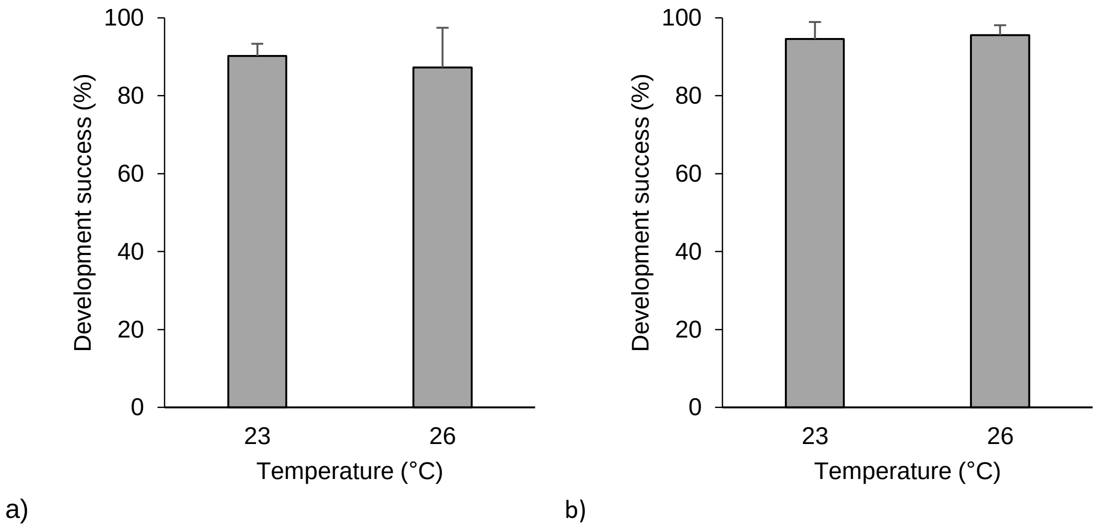


Figure 2. 9: Comparison of the development success (mean ± standard deviation) to the 4-arm pluteus stage at 23 °C and 26 °C for a) *Echinometra mathaei* and b) *Tripneustes gratilla.*

There was a significant difference in the developmental success between *E. mathaei* and *T. gratilla* at 23 °C (t = 2.882, p = 0.009) and 26 °C (U = 36.00, p = 0.039) (Figure 2.10).

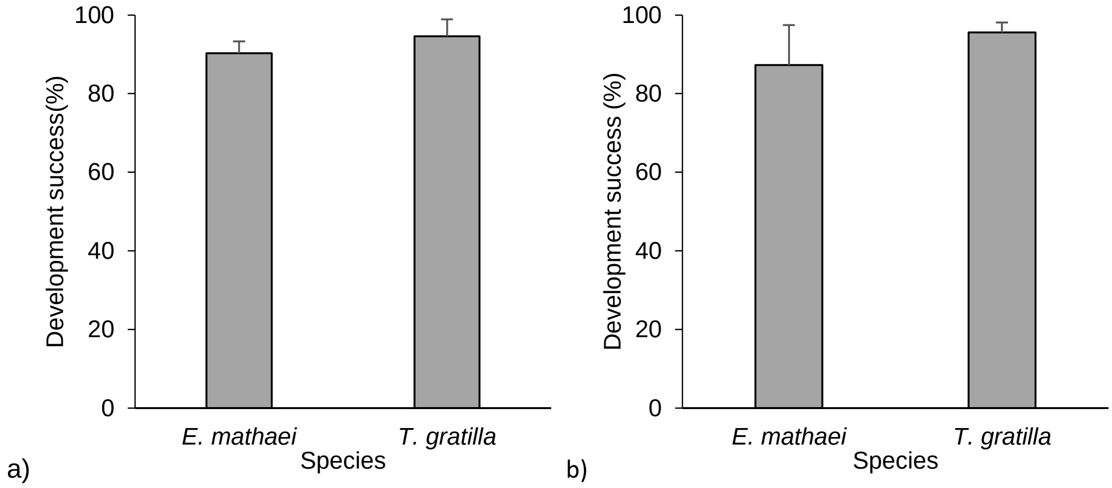
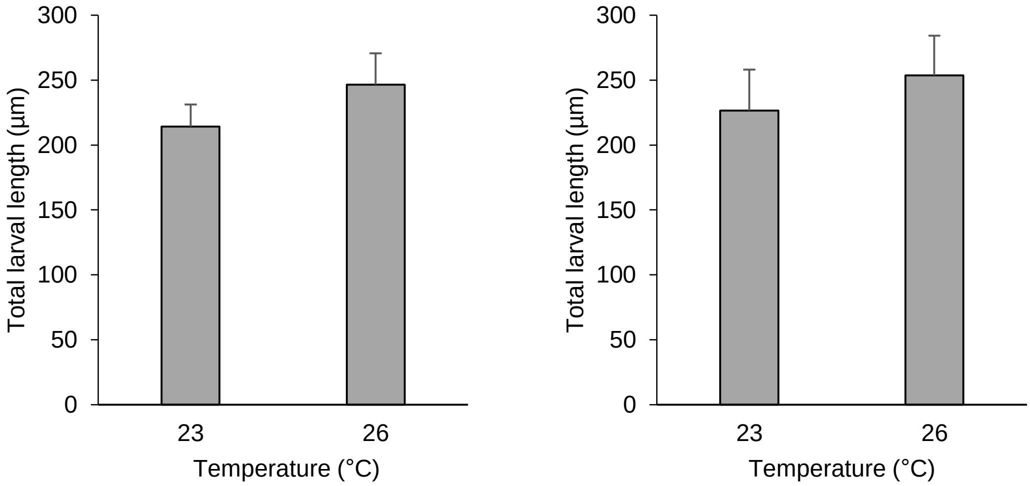


Figure 2. 10: Inter-species comparison of development success (mean ± standard deviation) to the 4-arm pluteus at a) 23 °C and b) 26 °C.

Temperature and total larval length

In *E. mathaei*, the total length of the pluteus was significantly larger at 26 °C (median = 250.19 µm) than at 23 °C (median = 216.18 µm) (U = 2190.00, $p < 0.001$) (Figure 2.9). In *T. gratilla*, the total length of the pluteus was also significantly larger at 26 °C (median = 245.85 216.79 µm) than at 23 °C (median =216.79 µm) (U = 3900.50, $p < 0.001$) (Figure 2.11).

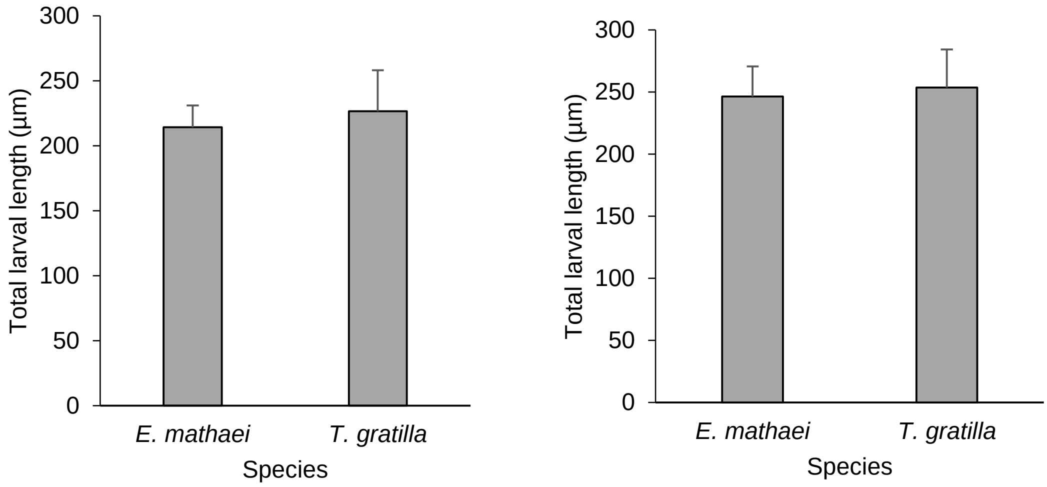
The mean ± standard deviation for *E. mathaei* was 214.23 ± 16.90 and 246.41 ± 24.25 and for *T. gratilla* 226.52 ± 31 and 253.48 ± 30.72 at 23 °C and 26 °C (Figure 2.11).



a) b)

Figure 2. 11: Comparison of the total length (mean ± standard deviation) of the 4-arm pluteus at 23 °C and° 26 °C for a) *Echinometra mathaei* and b)*Tripneustes gratilla.*

Comparison of the total larval length between *E. mathaei* and *T. gratilla* showed no significant differences at 23 °C (U = 6221.00, p = 0.055) and 26 °C (U = 6723.00, p = 0.376) (Figure 2.12).



a) b)

Figure 2. 12: Inter-species comparison of the total length (mean ± standard deviation)of the 4-arm pluteus at a) 23 °C and b) 26 °C

2.5 Discussion

The rate of the development to the 4-arm pluteus larval stage in *E. mathaei* and *T. gratilla* was temperature dependent. Temperature plays an integral role in the metabolic rate of ectotherms, which ultimately influences body temperature and thus respiration (Carey *et al*., 2016). In marine ectotherms, increased temperature acts as a catalyst in accelerating the metabolic rate until the physiological limit is attained, provided that other factors (*e.g.* pH, salinity and dissolved oxygen) are within the acceptable range (Carey *et al.*, 2016). Normal development of larvae in *E. mathaei* and *T. gratilla* occurred at 23 and 26 °C, but there was a delay in development at 20 °C as the 4-arm pluteus stage was not attained. Parvez *et al*., (2018) reported that embryos of *T. gratilla* incubated at 19 °C attained the 2-arm stage, but later exhibited abnormalities and mortality. At a temperature of 26 °C, development was accelerated for both species, where the 4-arm pluteus was reached 24 h earlier in comparison to 23 °C. Similarly, Rahman *et al.* (2009) and Parvez *et al.* (2018) showed the range for normal development of *T. gratilla* to be between 22-29 °C, and outside this range abnormality was prominent in embryonic development. Rahman *et al.* (2007) demonstrated that at 25 °C and 28 °C embryos of *E. mathaei* reached the 4-arm pluteus at 48 h with all larvae exhibiting normal development. Alsaffar & Lone (2000) showed that the optimal temperature for spawning of *E. mathaei* in Japanese waters is between 22-30 °C. *E. mathaei* and *T. gratilla* show development success at a wide temperature range, however the critical lower and upper thermal limit was found to be 16 °C and 34 °C (Rahman *et al.*, 2007; Parvez *et al.*, 2018).

In this study, larvae of both sea urchin species have shown normal development outside their natural spawning temperature range *i.e.* development to the 2-arm pluteus at 72 h for *E. mathaei* at 20 °C and to the 4-arm pluteus at 72 h and 96 h for *T. gratilla* at 26 °C. This could be strongly attributed to the thermal acclimatisation of female urchins during oogenesis (*i.e.* the formation of oocytes) (Visconti *et al.*, 2017). The duration which is required to grow and differentiate a population of oocytes is species-dependent but can range from one to three months prior to spawning (Song *et al*., 2006). Therefore, oogenesis would likely occur during the warmer summer months in *T. gratilla* and in the cooler winter months for *E. mathaei,* thus influencing embryo-larval development at the upper and lower thermal limits used in this study. Thus, the environmental history of the maternal parent plays a major role on the stress tolerance of the progeny (Visconti *et al.*, 2017) rather than the temperature acclimatisation of the adults during the breeding season. This is more likely to play a role in the thermal regimes required for the normal larval development of *E. mathaei* and *T. gratilla.* In addition, larval development is completely independent of the temperature acclimatisation of their parents during the breeding season because larvae are planktonic and adults are benthic organisms (Sherman, 2015). Therefore, conditions for normal larval development will differ from those of the adults.

A major requirement of a toxicity test is its sensitivity, which could increase with longer exposure periods, depending on the species being used. However, practicality is also necessary (Pérez *et al*., 2016). In other words, the test should not be of such a length that it becomes both onerous to perform and is cost inefficient. On the other hand, the test should not be too short in that the sensitivity of the test organism is comprised, thus yielding inaccurate results. Considering this, 23 °C was identified as the optimum temperature at which to run the chronic toxicity test for both species. However, there's a notable difference in the developmental rate of the 4-arm pluteus, *i.e.* 72 h for *E. mathaei* and 96 h for *T. gratilla* at 23 °C. Therefore, the larval developmental rate of *E. mathaei* and *T. gratilla* at 23 °C is species-specific. This temperature also provides greater ecological relevance as it coincides with the mean annual temperature of ~ 22 °C along the east coast of South Africa (Smit *et al.*, 2013). Furthermore, the development rate in *E. mathaei* and *T. gratilla* to the 4-arm pluteus at 23 °C falls in the expected range of 48 – 96 h required for chronic toxicity testing purposes using sea urchin larvae (Anselmo, 2011). Another reason for selecting 23 °C is the synchronous development of the 4-arm pluteus with few abnormalities observed. The occurrence of anomalies was likely due to natural circumstances as variability in species fitness is expected. Regardless, embryos of *E. mathaei* and *T. gratilla* were robust in the acclimation and adaptation to each temperature.

Total length was significantly longer at 26 °C in comparison to 23 °C for both species but there was no significant difference in total length between species at each temperature. Increased larval length is favourable for feeding, swimming and avoiding predation which ultimately affects the survival of larvae (Brennand *et al.*, 2010). Similarly, the total larval length of the 4-arm pluteus in *T. gratilla* and *Diadema setosum* was significantly larger at 25 °C than at 22 °C (Sarifundin *et al.*, 2016; Parvez *et al.*, 2018).

In conclusion, the normal larval development of *E. mathaei* and *T. gratilla* occurs at 23 °C and 26 °C. At 20 °C the 4-arm pluteus stage was not attained whereas development to the 4-arm pluteus was accelerated at 26 °C compared to 23 °C. For this study, 23 °C was selected as the optimal temperature for normal larval development to the 4-arm pluteus with test duration of 72 h for *E. mathaei* and 96 h for *T. gratilla*.

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CHAPTER 3: Copper and zinc as reference toxicants for the larval development test using sea urchins *Echinometra mathaei* and *Tripneustes gratilla*

3.1 Abstract

The sensitivity of *Echinometra mathaei* and *Tripneustes gratilla* to potential reference toxicants copper (Cu) and zinc (Zn) was evaluated to determine which was the most appropriate to use for the larval development test. Cu and Zn are essential metals, which are commonly used as reference toxicants in toxicity tests based on sea urchin larval development. Embryos were exposed to incremental concentrations of Cu and Zn for 72 h in the case of *E. mathaei* and 96 h in the case of *Tripneustes gratilla*. EC_{50} values from each test were used to construct a control chart as a quality assurance tool in accepting toxicity test results. *E. mathaei* showed a stable sensitivity response to Zn, having an acceptable coefficient of variation CV (%) of 19.92, whereas its response to Cu was highly variable with a CV (%) of 42.05. Hence, Zn would be a preferred reference toxicant to use for *E. mathaei*. The sensitivity response of *T. gratilla* to Cu and Zn was stable, having an acceptable CV (%) of 19.58 and 26.85 respectively, but Cu is the preferred reference toxicant due to a narrower CV (%). In comparison to other sea urchin species, *E. mathaei* and *T. gratilla* were more sensitive to Cu and Zn, thus satisfying the requirement of being highly sensitive as a toxicity test organism.

3.2 Introduction

E. mathaei (family Echinometridae) and *T. gratilla* (family Toxopneustidae) are closely related, belonging to the order Echinoida (Thomas, 1994; Matsuoka & Inamori, 1999). They are both commercially and ecologically important species, as their gonads (‘roe’) are harvested for human consumption and for their role in maintaining the flow of matter and energy in coral reefs (Biukerna *et al*., 1982; Balisco, 2015; Keshavarz *et al*., 2017; Dalabajan *et al*., 2018). Sea urchins are commonly used as test species in toxicity testing due to their ease of maintenance and spawning in the laboratory, and the high sensitivity of their gametes and larvae to toxicants (USEPA 2002; ASTM 2012; Pagano *et al*., 2017). The sea urchin larval development test has been widely used for assessing the sub-lethal effects of toxicants.

In toxicity tests, a negative control group (*i.e.* absent of the treatment) is used to assess the health of test organisms, by their performance (*e.g.* survival, development or reproduction) in comparison to other treatments in the test (USEPA, 1995). In the sea urchin embryo-larval development test, the control treatment provides the basis of the biological quality of the test organism, as it is imperative that only good quality gametes are used (Beiras *et al*., 2012). A reference toxicant serves as a positive control in toxicity testing to provide an overall measurement of the repeatability or reproducibility of the toxicity test method within a single laboratory or between laboratories over time (Environment Canada, 1999; USEPA, 2000). As a part of quality assurance, the results of reference toxicant tests are useful in identifying possible sources of variability, for example test organism health, difference among batches of organisms, changes in dilution water or food, and laboratory technician performance (de Vlaming *et al*., 2009).

The sensitivity of test organisms needs to be verified to establish the precision (repeatability) of results from toxicity tests. This is expressed by the median effective concentration (EC_{50}) or median lethal concentration (LC_{50}), which is the concentration that results in 50% of the test organism's mortality, abnormal development or some other identifiable endpoint (Johnson & Finley, 1980). The EC_{50} values are plotted on a control chart which is used to compare the most recent test to historical test performance as a quality assurance measure, to identify whether they fall within an acceptable range of variability (ARC, 1992; Environment Canada, 1999; USEPA, 2000). A minimum of five EC_{50} or LC_{50} values are adequate in establishing a control chart (Flemming *et al*., 2004). The data for the control chart is re-calculated with each successive test result. Therefore, the control chart functions as a tool in accepting the results obtained for each toxicity test using either the same or different batches of test organisms, under standard laboratory conditions (Lombardi *et al*., 2018).

South Africa's coastline is subjected to metal input from various sources such as natural weathering processes and anthropogenic activities like the discharge of domestic and industrial effluents and mining activities (Vetrimurugan *et al.,* 2017). The entry of Cu in the marine environment is through its use in pesticides for agricultural purposes and antifouling paints on ship hulls, buoys and underwater surfaces, whereas major contributors of Zn are smelting, ore processing and mine drainage (DEA, 2018). Marine organisms are highly susceptible to Cu and Zn in their natural environment and it is thus necessary to investigate the sensitivity of test species to these metals. Cu and Zn are suitable reference toxicants from an ecological perspective as sea urchin embryos and larvae are most likely to be exposed to these metals in their natural environment. For toxicity testing purposes, Cu and Zn are safe to handle and have shown to produce consistent results (EC, 1990; Lombardi *et al*., 2018). Sea urchin larval development is highly sensitive to essential metals Cu and Zn and are thus commonly used as reference toxicants in toxicity testing (Environment Canada, 1990; Cesar *et al*., 2002; Carballeira *et al*., 2011 and 2012; Edullantes and Galapate, 2014; Sartori *et* al., 2016; Rouchon & Phillips, 2017). The effects of cadmium, lead, mercury and nickel on sea urchin larval development have been investigated and can also be used as reference toxicants (Fernández and Beiras, 2001; Tualla and Bitacura, 2016) but these are generally carcinogenic.

The regulation concerning the acceptable levels of metals in South African waters is based on the South African Water Quality Guidelines for Coastal Waters (DWAF, 1995). Target values are set at 5 µg/L for total Cu and 25 µg/L for total Zn (DWAF, 1995) which is indicative that Cu is more toxic to marine primary consumers compared to Zn. These trace metals are homeostatically regulated in tissues (Fu et al., 2016), but bioaccumulation that occurs through exposure to excess concentrations results in abnormal development or even mortality (Gharred *et al*., 2015). It is suggested that Cu is the preferred reference toxicant for acute and chronic toxicity using sea urchins (USEPA, 1995). The usual toxic effects caused by Cu are through altered enzyme function which causes oxidative stress, disrupting ion regulation thus interfering with acid/base balance (Bielmyer *et al*., 2005) and restricting metabolic activity as a secondary effect of oxygen uptake inhibition (De Polo & Scrimshaw, 2012). Zinc as a priority pollutant has received little concern but is still prevalent in coastal waters (Rouchon and Phillips, 2017). The effect of Zn toxicity on embryos produces abnormalities as a result of the disruption to ribosomal RNA synthesis and the inhibition of endodermal and mesenchyme development (Tualla & Bitacura, 2016).

The aim of this study was to determine which of Cu or Zn is the most effective reference toxicant for chronic toxicity testing using larvae of the sea urchins *E. mathaei* and *T. gratilla*. This is the first study to determine the sensitivity and establish control charts using Cu and Zn as reference toxicants for the chronic larval development test using *E. mathaei* and *T. gratilla*.

3.3 Methods and materials

3.3.1 Test chemicals and seawater

Experiments were conducted at the toxicology laboratory at the CSIR in Durban in a temperature-controlled room. Cu and Zn were of analytical grade and purchased from Merck. Stock solutions of copper (1000 µg/L) and zinc (1000 µg/L) were prepared using ultrapure deionized water and stored at room temperature. Reference toxicant solutions were made up of filtered (20 µm) and UV-sterilised seawater with a salinity of 35 ± 1, dissolved oxygen of 7.5 ± 0.5 mg l^{-1} and pH of 8.1 ± 0.1, to ensure that any observable anomalies of sea urchin larvae, were mainly attributed to either Cu or Zn toxicity or as a result of unavoidable natural circumstances. The physico-chemical variables were in accordance with the CSIR standard operating procedure for the sea urchin fertilisation test. Preliminary trials were performed to determine the definitive test concentrations. Cu concentrations were 2.5, 5, 10, 15, 20 µg/L for *T. gratilla* and 5, 10, 15, 20, 30, 40 µg/L for *E. mathaei*. Zn concentrations for both species were 10, 20, 40, 80, 160 µg/L. These concentrations are nominal as the actual concentrations were not measured. Therefore, metal loss could have occurred as result of metal precipitation and thus leading to a reduced actual metal concentration than the one reported. It has been reported that nominal and dissolved concentrations for copper and zinc in seawater are agreeable (Rouchon and Phillips, 2017).

3.3.2 Toxicity test procedure

Static non-renewal tests were conducted using a minimum of five test concentrations and a negative control of filtered seawater. Urchins were injected with 1 - 2 ml of 0.5M KCl through the peristomial membrane. Females were inverted over a glass beaker filled with filtered seawater to collect the eggs. Sperm from the males were collected 'dry' using a Pasteur pipette to prevent activation and preserve its viability. Immature eggs and debris were removed by washing and decanting 3 - 4 times with filtered seawater. Egg quality and sperm motility were checked at 10× magnification using the Zeiss Axioskop 2 light microscope. Eggs that had a uniform shape and distinct nuclei were considered good quality and highly motile sperm were used. Immediately before the test, sperm were added to filtered seawater to activate the sperm. Sperm density was not calculated but a sperm dilution series was used to determine the appropriate volume of diluted sperm to yield 95 % fertilisation of 400-500 eggs per ml in the egg stock solution.

Fertilised eggs (400 – 500 per ml) were pipetted into glass vials (four replicates per row) containing serial concentrations (as stated above for each species) of reference toxicants, Cu and Zn and a negative control of filtered seawater. Larvae were preserved at 72 h for *E. mathaei* and 96 h for *T. gratilla* post-fertilisation by adding 100 µl of formalin to the test vials. Endpoints were the proportion of normal larvae (n = 100) and the predominance of abnormalities present in each replicate was classified as follows:

- Pathological hatched or pre-hatched - Single (*i.e.* fertilised eggs) or multicellular embryos with or without a fertilisation membrane, appears as dark masses of cells or dissociated blobs of cells.
- Necrosis - Cell death as a result of external interference that causes an uncontrolled release of the inflammatory cellular contents (Fink & Cookson, 2005).
- Inhibited - Embryos or larvae at the blastula, gastrula and prism stage, have no or undeveloped skeleton.
- Gut abnormalities - Normal overall appearance where guts are absent, undifferentiated, incorrectly shaped or positioned or projected outside the embryo or larvae (exogastulated)
- Retarded – Delayed development at the 2-arm pluteus stage or has an irregular shape

- Skeletal malformations - May have either bent, spread out or asymmetrical post-oral arms or separated or crossed tips at the apex of the larvae

Larvae were photographed at 10× magnification using a Zeiss Axioskop 2 fitted with a camera.

3.3.3 Statistics

Since the proportion of normal larval development was the endpoint, the EC_{50} was the index used to determine the chronic toxic effect of the two reference toxicants on each species. The EC_{50} was calculated by the linear interpolation model using CETIS software. Control charts were constructed for *E. mathaei* and *T. gratilla*, using the EC_{50} values obtained from each test performed with Cu and Zn and was plotted against the range limited by the cumulative mean, upper and lower confidence. The coefficient of variation (CV, %) is used as a measurement of variability of EC_{50}'s used to generate the control chart (Environment Canada, 1990). The value of a CV > 30% demonstrates low consistency between toxicity tests *(i.e.* low repeatability), but this is subjective as it is not based on empirical evidence (Environment Canada, 1990).

3.4 Results

In *E. mathaei*, the main abnormalities observed were a combination of inhibited, and retarded development and skeletal malformations at the lowest concentrations (5 µg/L and 10 µg/L), retarded development and skeletal malformations in the intermediate concentration (15 µg/L), and retarded and inhibited development in the highest concentrations (20 µg/L and 30 µg/L) of Cu (Figure 3.1). For Zn, the main abnormalities observed were a combination of inhibited and retarded development and skeletal malformations in the lowest concentrations (10 µg/L and 20 µg/L), retarded development and skeletal malformations in the intermediate concentration (40 µg/L), and inhibited development and skeletal malformations in highest concentrations (80 µg/L - 160 µg/L) (Figure 3.1)

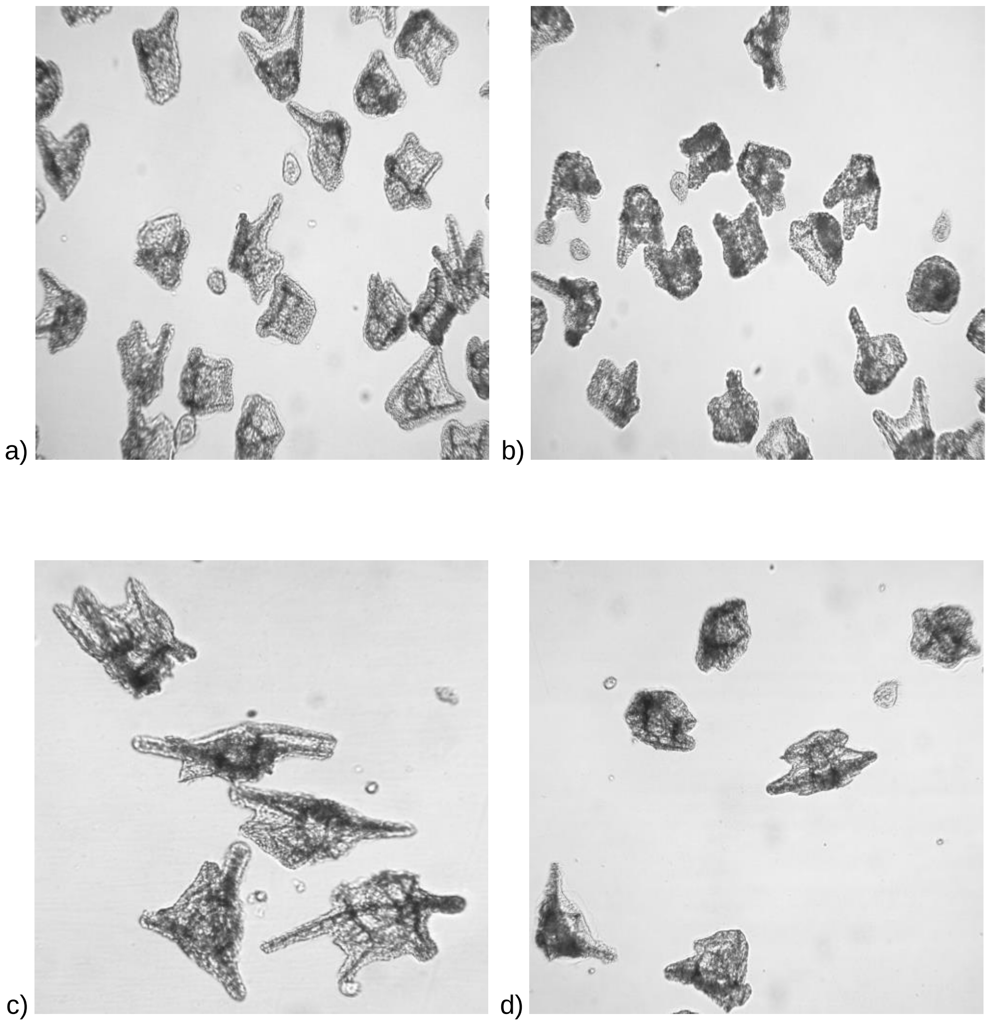


Figure 3. 1: Observed abnormalities of *E. mathaei*: a and b) retarded development at 20 µg/L and 30 µg/L concentrations of copper; c) severe skeletal malformations at 80 µg/L and d) larvae with skeletal malformations at 160 µg/L concentrations of zinc. (10x)

In *T. gratilla*, the main abnormalities observed in the lowest concentrations (2.5 µg/L and 5 µg/L) and the intermediate concentration (10 µg/L) were inhibited and retarded development, and development was inhibited at the highest Cu concentrations of 15 µg/L and 20 µg/L (Figure 3.2). For Zn, the main abnormalities observed in the lowest (10 µg/L - 20 µg/L) and intermediate (40 µg/L) concentrations were inhibited, and retarded development and a combination of inhibited, and retarded development and severe skeletal malformations in the highest concentrations of 80 µg/L - 160 µg/L (Figure 3.2).

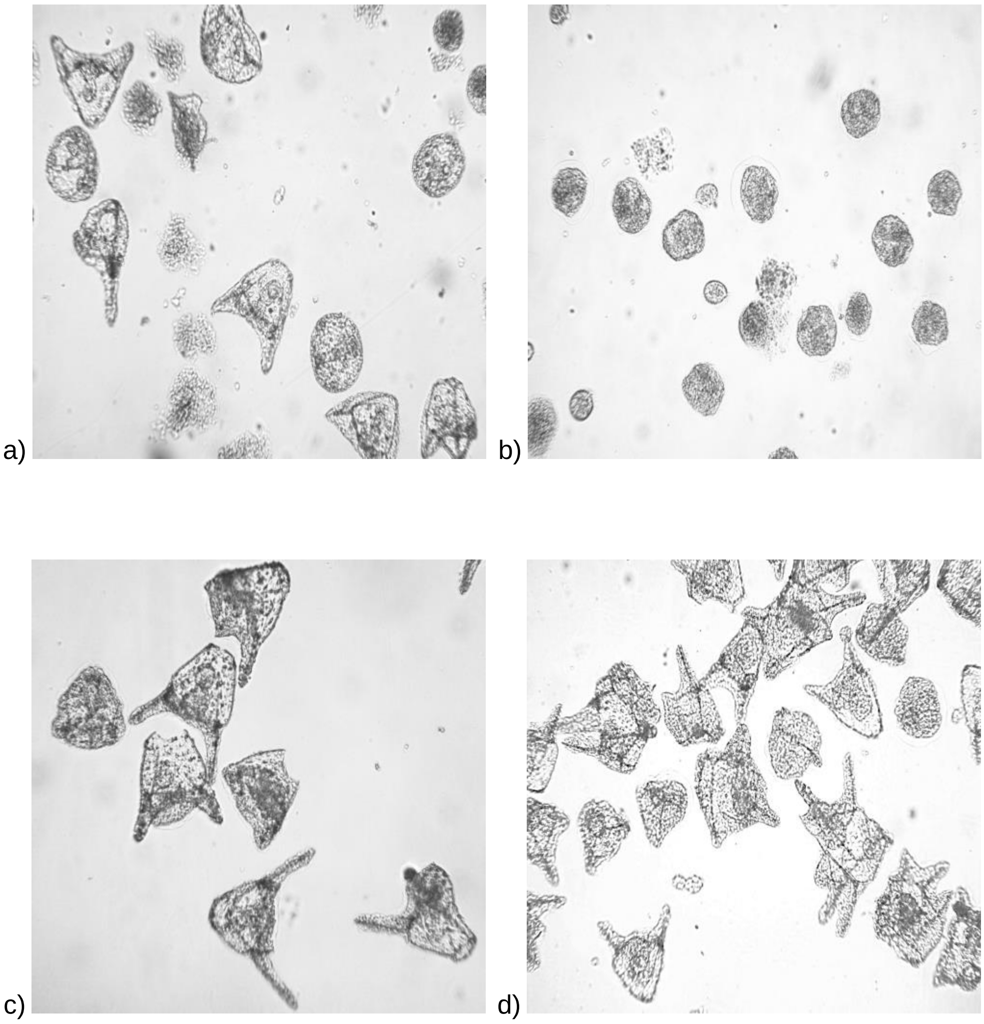
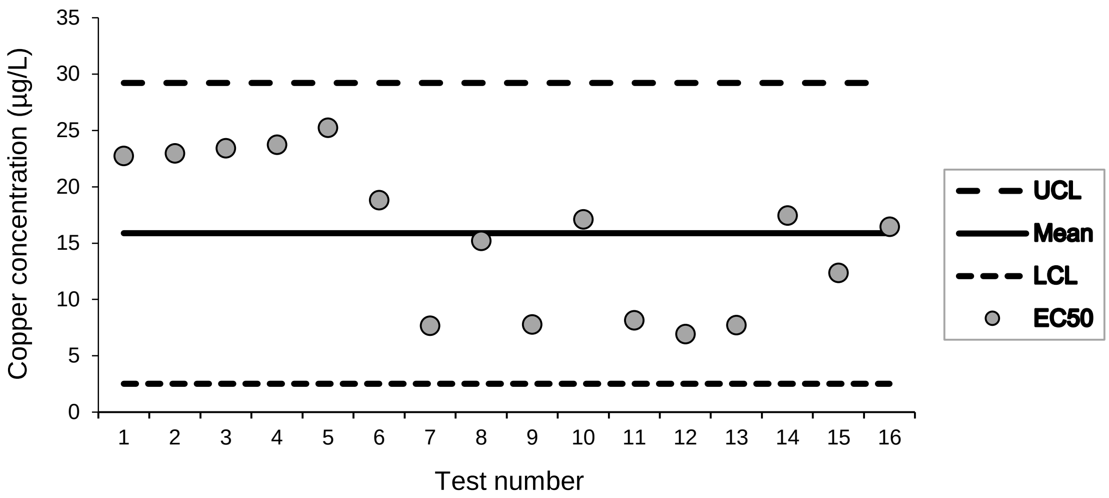
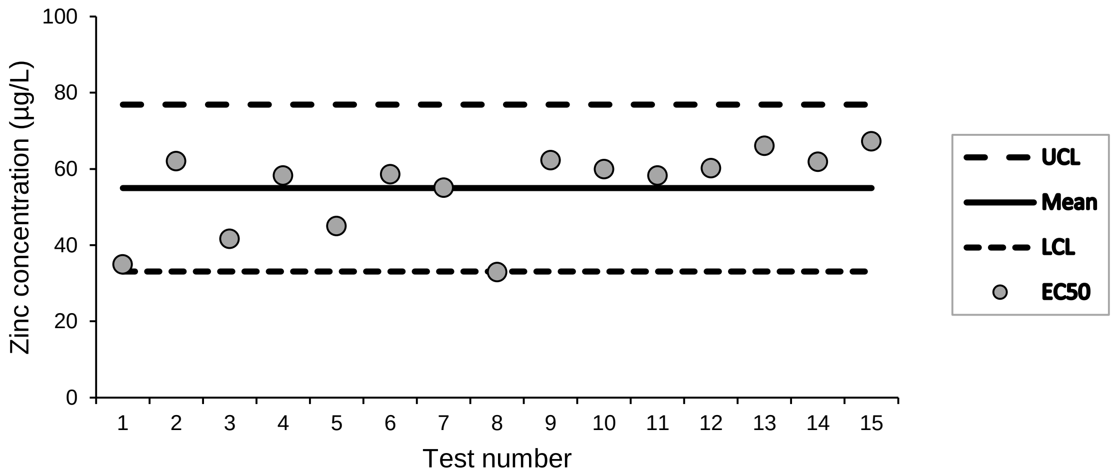


Figure 3. 2: Observed abnormalities of T. gratilla larvae: a) retarded at the 2-arm pluteus and inhibited (gastrula) at 15 µg/L, b) inhibited development (gastrula and prism) at 20 µg/L of copper; c) retarded development with skeletal malformations at 80 µg/L and d) retarded development with severe skeletal malformations at 160 µg/L concentrations of zinc. (10×)



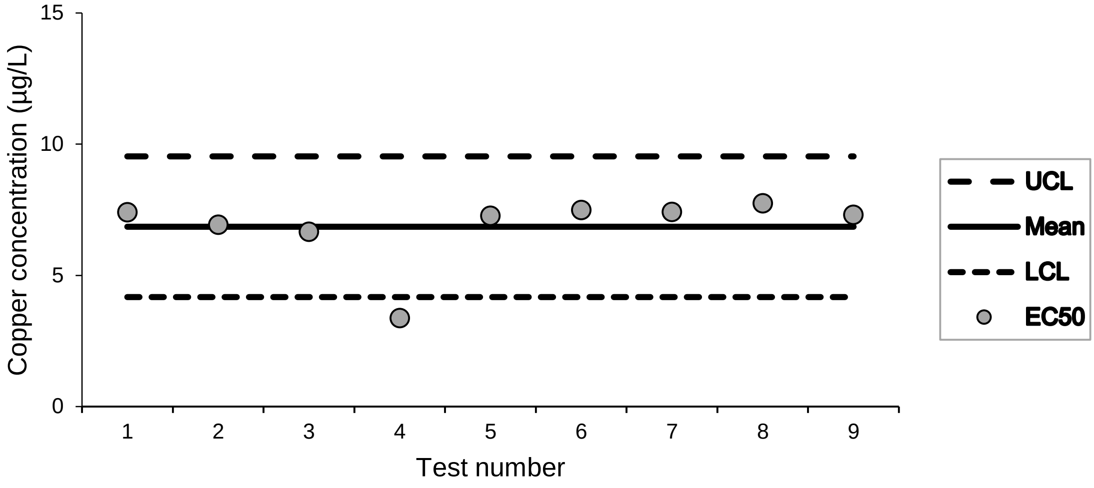
a)



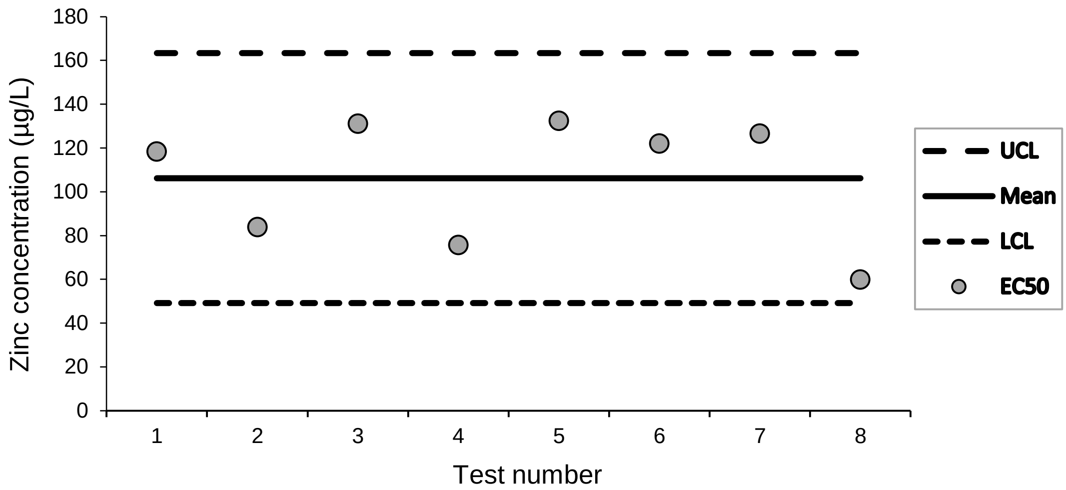
b)

Figure 3. 3: Control charts composed of EC_{50} values for *Echinometra mathaei* using a) copper and b) zinc reference toxicants. The upper confidence limit (UCL) and lower confidence limit (LCL) is equal to the mean ± two standard deviations.

The sensitivity of *E. mathaei* and *T. gratilla* to Cu and Zn is summarised in Table 3.6. *E. mathaei* showed a stable sensitivity response to Zn (Figure 3.3), with an acceptable CV (%) of 19.92, but its response to Cu (Figure 3.3) showed higher variability with a CV (%) of 42.05. *T. gratilla* showed a stable sensitivity response to Cu and Zn (Figure 3.4), having an acceptable CV (%) of 19.56 and 26.85 respectively.



a)



b)

Figure 3. 4: Control charts composed of EC_{50} values for *Tripneustes gratilla* using a) copper and b) zinc reference toxicants. The upper confidence limit (UCL) and lower confidence limit (LCL) is equal to the mean ± two standard deviations.

An overview of the EC_{50} and confidence limits calculated based on the data for each test using Cu and Zn are represented in Tables 3.1 and 3.2 for *E. mathaei* and Tables 3.3 and 3.4 for *T. gratilla*.

Table 3. 1: EC_{50} values and confidence intervals for *E. mathaei* in tests (n = 16) using Cu as the reference toxicant.

Test number	EC_{50} (µg/L)	Confidence Interval (95%)	
		Lower	Upper
1	22.74	21.11	24.04
2	22.98	21.56	23.97
3	23.43	22.5	24.19
4	23.73	21.21	25.26
5	25.26	24.82	25.82
6	18.83	18.14	19.67
7	7.69	7.15	8.24
8	15.71	6.72	15.84
9	7.80	7.10	8.02
10	17.13	16.64	17.59
11	8.15	7.74	8.57
12	6.94	6.47	7.29
13	7.75	7.66	7.85
14	17.47	17.11	17.83
15	12.36	11.09	13.43
16	16.47	16.11	16.85

Table 3. 2: EC_{50} values and confidence intervals for *E. mathaei* in tests (n = 15) using Zn as the reference toxicant.

Test number	EC_{50} (µg/L)	Confidence Interval (95%)	
		Lower	Upper
1	34.94	31.04	38.66
2	62.11	60.66	63.17
3	41.67	35.85	51.40
4	58.30	56.82	59.62
5	45.00	35.85	52.36
6	58.60	57.88	59.27
7	55.10	53.47	56.60
8	32.95	30.78	35.44
9	62.36	60.65	64.23
10	59.94	58.74	60.95
11	58.28	56.92	59.59
12	60.25	59.12	61.12
13	66.12	64.02	68.37
14	61.93	59.83	64.17
15	67.25	64.13	70.00

Table 3. 3: EC_{50} values and confidence intervals for *T. gratilla* in tests (n = 9) using Cu as the reference toxicant.

Test number	EC_{50} (µg/L)	Confidence Interval (95%)	
		Lower	Upper
1	7.41	7.30	7.51
2	6.93	6.62	7.22
3	6.66	6.11	7.07
4	3.37	2.93	3.79
5	7.27	6.63	7.80
6	7.49	7.03	7.98
7	7.42	7.33	7.50
8	7.75	7.59	7.90
9	7.31	7.03	7.57

Table 3. 4: EC_{50} values and confidence intervals for *T. gratilla* in tests (n = 8) using Zn as the reference toxicants.

Test number	EC_{50} (µg/L)	Confidence Interval (95%)	
		Lower	Upper
1	118.40	116.30	119.90
2	83.88	76.57	91.11
3	131.10	128.50	133.30
4	75.75	70.65	81.36
5	132.40	129.40	135.70
6	122.00	118.80	124.80
7	126.60	121.60	130.60
8	59.83	56.93	62.78

Table 3. 5: Summary of test results for *E. mathaei* and *T. gratilla* using Cu and Zn as reference toxicants. The upper confidence limit (UCL) and lower confidence limit (LCL) is equal to the mean ± two standard deviations and the coefficient of variation (CV, %) is equal to the standard deviation divided by the mean and multiplied by 100 to give a percentage value.

Species	Toxicant	Mean	Standard deviation	Lower	Upper	CV (%)
E. mathaei	Cu	15.87	6.67	2.52	29.22	42.05
	Zn	54.99	10.95	33.09	76.89	19.92
T. gratilla	Cu	6.85	1.34	4.17	9.53	19.56
	Zn	106.25	28.53	49.20	163.30	26.85

3.5 Discussion

The present study evaluated the methodology of a chronic larval development toxicity test using sea urchins *E. mathaei a*nd *T. gratilla*, by testing their performance to potential reference toxicants Cu and Zn. Larval development in *E. mathaei* and *T. gratilla* showed greater sensitivity to Cu at lower concentrations in comparison to Zn (Table 3.5). The effect of Cu and Zn on larval development is concentration dependent in which abnormalities are more evident at higher concentrations.

Kobayashi and Okamura (2005) reported normal development of sea urchin *Anthocidarus crassispina* larvae at concentrations less than 3.8 µg/L of Cu, but concentrations greater than 7.7 µg/L resulted in the formation of abnormalities such as retardation, like the results for *T. gratilla* in this study. Inhibited and retarded development of larvae at above threshold concentrations of Cu could be caused by altered enzyme function thus causing oxidative stress and subsequently decreasing metabolic rate (Bielmyer *et al*., 2005; De Polo & Scrimshaw, 2012).

The results in this study are also corroborated by those of Kobayashi and Okamura (2004, 2005), which showed that Zn at low concentrations (7.2 µg/L) had no inhibitory effect on larvae, while an intermediate concentration of 58 µg/L results in embryos inhibited at the gastrula stage and abnormal exogastrulae and high concentrations (120 - 480 µg/L) severely inhibited development at the blastula stage. In the present study, skeletal malformations such as bent arms and crossed tips at the apex were prominent at high concentrations of Zn. This is probably indicative of Zn playing a role in restricting the development of the endoderm, including its mesenchyme derivatives, thus causing skeletal malformations of the pluteus larvae (Kobayashi, 1990).

The narrow range of confidence intervals generated for each EC_{50} value are indicative of high precision of the methodology for each test using *E. mathaei* and *T. gratilla.* The CV (%) of 42.05 calculated for *E. mathaei* exposed to Cu was slightly higher than the recommended acceptable CV (%) of 30, but this is not uncommon (Environment Canada, 1990). This was also observed by Soares and Junior (2016), where Cu was shown to be highly toxic to larval development of the sea urchin *Lytechinus variegatus* with greater variability of results CV (%) = 34.41. Soares and Junior (2016) accounted for this variability due to the range between EC_{50} values irrespective of the number of tests, whereas Dermeche *et al*., (2012) attributed variability in results to poor quality brood stock, producing poor quality gametes that were more sensitive to Cu.

Control charts were constructed using EC_{50} values from the reference toxicant tests and this was also used to assess whether variation is consistent with a stable process (common cause variation) or inconsistent with a stable process (special cause variation) (Marshall & Mohammed, 2007). Contreras-León *et al*. (2013) state that a special cause variation can happen when either two or more consecutive values fall beyond the limits or when seven consecutive points fall on the same side (above or below) the midline. A special cause of variation was identified in the present study, where a pattern of seven consecutive EC_{50} values were above the mean for *E. mathaei* exposed to Zn. In some instances, organisms were exposed to a new batch of prepared test solutions, which could have resulted in a special cause of variation. In most cases when a special cause of variation occurs, an investigation is carried out and test procedures are reviewed (Masikane, 2013)

The sensitivity of *E. mathaei* and *T. gratilla* to Cu and Zn were compared to other sea urchin species at exposure durations between 48 to 96 h, based on published literature. Many studies have focused on species from the *Strongylocentrotus* and *Paracentrotus* genera, but the sensitivity to other genera may differ (Rouchon and Phillips, 2017). An inter-species comparison in this study shows that *T. gratilla* is more sensitive to Cu than *E. mathaei* whereas *E. mathaei* is more sensitive to zinc than *T. gratilla.* Considering the EC_{50} or LC_{50} range for other species exposed to Cu (5.4 - 389.0 µg/L) and Zn (10 - 446.7 µg/L), *E. mathaei* and *T. gratilla* exhibit generally a higher sensitivity to Cu and Zn (Table 3.6 and 3.7).

The differences in intra and inter-species sensitivity to Cu and Zn in sea urchins can be attributed to differences in accumulation rates, internal binding mechanisms, detoxification mechanisms and paternal history of exposure that is inherited by the offspring (Purbonegoro and Hindarti, 2019). An indication of surplus metal intake is the increase in metallothionein production and changes in lysosomal activity which aid in the process of homeostasis of cellular metals and function in detoxifying excess metals (Phillips e*t al.,* 2003). These processes may influence the differential sensitivity of each species to different toxicants.

Table 3. 6: Comparison of EC_{50} values for *E. mathaei* and *T. gratilla* to other sea urchin species exposed to copper for duration between 48 to 96 h.

Species	Test duration	EC_{50} or LC_{50} (µg/L)	Reference
Evechinus chloroticus	72 h	5.4	Rouchon & Phillips 2017
Strongylocentrotus purpuratus	48-96 h	6.3	King & Riddle, 2001
Tripneustes gratilla	**96 h**	**7.9**	**This study**
Heliocidaris tuberculata	72 h	8.0	Doyle *et al*., 2003
Heliocidaris tuberculata	96 h	9.4	King & Riddle, 2001
Hemicentrotus pulcherrimus	48 h	10-20	King & Riddle, 2001
Centrostephanus rodgersii	96 h	11.8	King & Riddle, 2001
Arbacia punctulata	48-96 h	14.0	King & Riddle, 2001
Strongylocentrotus purpuratus	96 h	15.3	Phillips *et al*., 2003
Echinometra mathaei	**72 h**	**15.9**	**This study**
Diadema savigni	48 h	19.0	Rosen *et a*l., 2015
Strongylocentrotus droebachiensis	48-96 h	21.0	King & Riddle, 2001
Paracentrotus lividus	48 h	<32.0	King & Riddle, 2001
Paracentrotus lividus	48 h	32.9	Lorenzo *et al*., 2002
Diadema setosum	48 h	43.0	Ramachandren *et al*., 1997
Paracentrotus lividus	48 h	46.0	Manzo *et al*., 2008
Paracentrotus lividus	72 h	62.0	Novelli *et al*., 2003
Paracentrotus lividus	48 h	66.8	Fernández & Beiras 2001
Paracentrotus lividus	48 h	85.0	Manzo 2004
Paracentrotus lividus	72 h	389.0	Dermeche *et al*., 2012

Table 3. 7: Comparison of EC_{50} values for *E. mathaei* and *T. gratilla* to other sea urchin species exposed to zinc for duration between 48 to 96 h.

Species	Test duration	EC_{50} or LC_{50} (µg/L)	Reference
Hemicentrotus pulcherrimus	48 h	10-20	King & Riddle, 2001
Diadema setosum	48 h	10-20	King & Riddle, 2001
Arbacia lixula	96 h	10-100	King & Riddle, 2001
Strongylocentrotus purpuratus	48-96 h	23.0	King & Riddle, 2001
Strongylocentrotus droebachiensis	48-96 h	27.0	King & Riddle, 2001
Evechinus chloroticus	72 h	27.7	Rouchon, & Phillips, 2017
Paracentrotus lividus	48 h	<33.0	King & Riddle, 2001
Paracentrotus lividus	72 h	49.0	Novelli *et al*., 2003
Echinometra mathaei	**72 h**	**55.0**	**This study**
Strongylocentrotus purpuratus	96 h	96.9	Phillips *et al*., 2003
Strongylocentrotus purpuratus	96 h	97.2	Phillips *et al*., 1998
Tripneustes gratilla	**96 h**	**106.3**	**This study**
Strongylocentrotus purpuratus	72 h	151.0	Nadella *et al*., 2013
Heliocidaris tuberculata	96 h	160.0	Doyle *et al*., 2003
Arbacia punctulata	48-96 h	205.0	King & Riddle, 2001
Heliocidaris tuberculata	96 h	280.0	King & Riddle, 2001
Centrostephanus rodgersii	96 h	289.0	King & Riddle, 2001
Paracentrotus lividus	72 h	446.7	Dermeche *et al*., 2012

In conclusion, Cu and Zn could be used as reference toxicants for *E. mathaei.* and *T. gratilla* based on the CV (%) of the EC_{50}. However, on closer inspection of the control charts, Cu would be the preferred reference toxicant for *T. gratilla* as the EC_{50} values obtained show more consistency. The preferred reference toxicant would be Zn for *E. mathaei* due to its stability in sensitivity response and narrow CV (%) in comparison to Cu, which had a CV (%) > 30. *E. mathaei* and *T. gratilla* are highly sensitive to Cu and Zn, qualifying their use in chronic larval development toxicity testing.

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CHAPTER 4: Practical application and comparison of the larval development and fertilisation test using sea urchins *Echinometra mathaei* and *Tripneustes gratilla*

4.1 Abstract

The current study focused on a comparative assessment of the sensitivity of the acute sea urchin fertilisation test and chronic larval development test to the toxicity of seawater desalination brine, effluent from two wastewater treatment works and seawater collected from a marine environment that receives pulp mill effluent. Sea urchin gametes and larvae were exposed to serial dilutions of seawater desalination brine and effluent. The 'low effect' EC_x value was derived using the linear interpolation model and was then used to calculate the minimum acceptable toxicant dilution (MATD) which is the minimum number of dilutions required to render an effluent non-toxic. The MATD was calculated for seawater desalination brine and effluent and the index of difference was used as a measure of sensitivity. The larval development test was generally less sensitive in detecting toxicity compared to the fertilsation test, but the difference was negligible. The fertilisation test required a higher number of dilutions to render samples of seawater desalination brine (50 to 75% of the time) and effluent (83 to 100% of the time) non-toxic. On the other hand, the larval development test was more sensitive in detecting toxicity of seawater samples from receiving waters of pulp mill effluent, but the results were highly variable. The fertilisation test would be the preferred choice in toxicity testing as it was more sensitive in detecting toxicity of seawater desalination brine, effluent from both wastewater treatment works. Although the larval development test was more sensitive in detecting toxicity, the results produced by the fertilisation test had less variability and thus would be preferable in toxicity testing of receiving water samples. Furthermore, the fertilisation test is rapid and cost effective for sample analysis and adequate for monitoring and decision-making purposes. However, this is not a definitive decision as the larval development test may be used in other applications of different types of effluent and receiving water systems to further investigate if there are differences in sensitivity compared to the fertilisation test.

4.2 Introduction

The use of the ocean, although controversial, is a feasible approach to effluent disposal provided this is done appropriately and strict conditions are placed on the quality of effluent discharged (CSIR, 2017). This method of effluent disposal is implemented in many countries (USEPA, 1989; ANZECC 2000). Contaminants in effluents pose a risk to marine biota if they are present at toxic concentrations. The assessment of effluent toxicity using the whole effluent toxicity (WET) method is suitable for testing complex mixtures since biological effects are a more realistic assessment of the bioavailable fraction (*i.e.* the fraction of the toxicant that can be assimilated by an organism) (USEPA, 1995; ANZECC, 2000). WET testing integrates interactions among complex mixtures of contaminants in an effluent and is intended to measure total effects on biological organisms, regardless of the effluents physico-chemical properties. It is thus understood that the WET test set-up is designed to simulate worst-case conditions that could occur in the receiving waters (USEPA, 1993; USEPA, 1994; ANZECC, 2000). WET tests can also be used to test the toxicity of water samples collected from the marine environment.

WET testing is often used to assess the concentration of an effluent that is non-toxic to a test organism (Andersen *et al*., 1998). The "low effect" EC_x-value (concentration that corresponds to x % effect), which is determined using the point estimate approach, has been recommended by the international scientific community as an alternative to the No Observed Effect Concentration (NOEC) which uses hypothesis testing (Andersen *et al*., 1998, Diamond *et al*., 2012). The NOEC concept has been criticised based on the inexpedient method by which it is calculated, and the validity of the statistical approach used (Warne and van Dam, 2008). For this study, the "low effect" EC_x value was used to determine the minimum acceptable toxicant dilution (MATD), which represents the number of times the effluent must be diluted with clean seawater to render it non-toxic (CSIR, 2017).

Acute toxicity usually occurs or develops rapidly after an exposure to a toxicant whereas repeated or prolonged exposure of a toxicant may result in the occurrence of chronic toxicity (WHO, 1978). The two main tests using sea urchins are the acute fertilisation test and the chronic larval development test. The sea urchin fertilisation test assesses the ability of sperm to fertilise the egg after a specific period of exposure (10 to 60 minutes) to an effluent, or to dilutions of an effluent, whereas the sea urchin larval development assesses the ability of the larvae to develop normally during an exposure period (typically 48 to 96 hours depending on the species) to an effluent, or to dilutions of an effluent. The main difference between the tests is related to the duration of exposure (Woodworth *et al*., 1999). Early life stages of marine, invertebrates (*e.g.* their gametes and larvae) are most sensitive and vulnerable to environmental perturbations, thus constituting their use in the toxicity testing of effluents (Kobayashi 1971; Nacci *et al*., 1986).

The fertilisation test is advantageous in that it allows for rapid testing: it can be completed, including the analysis of results, within a day and the exposure of sperm to volatile compounds provides more accurate results of the transient toxicity through the measurement of fertilisation success (Phillips *et al*., 1998). However, the fertilisation test only provides information on lethal effects whereas the chronic larval development test evaluates sub-lethal effects (*e.g.* inhibited development or skeletal malformations). Despite the larval development test being short (a few days), the test is presumed to be ecologically relevant because sub-lethal effects may impact on recruitment to adult populations and even result in mortality, thus affecting the survival of a population (Tellis *et al*., 2014; Rouchon & Phillips, 2017)

The CSIR routinely uses gametes of the sea urchins *Echinometra mathaei* and *Tripneustes gratilla* to test the toxicity of effluents using the acute fertilisation test. The reason for using two species is due to seasonality in spawning where *E. mathaei* is a summer spawner and *T. gratilla* is a winter spawner. This allows for testing throughout the year. The fertilisation test is known to present certain limitations and it is uncertain if it should be replaced by or be performed in conjunction with a larval development test to better understand the toxicity of effluents and hence the implications of their discharge into the marine environment. The purpose of this study was thus to determine if these tests provide similar information on effluent and seawater toxicity, and if not to recommend if one test should be used in preference to the other. For this purpose, the sea urchin fertilisation and larval development tests were used to simultaneously test the toxicity of brine derived from seawater desalination, domestic and industrial effluents, and water samples collected in an area of the marine environment where effluent from a pulp mill is discharged.

4.3 Materials and methods

4.3.1 Sample collection and handling

4.3.1.1. Brine: Samples of brine and seawater were collected at or near two desalination plants in the City of Cape Town and couriered on ice to the CSIR Toxicology Laboratory in Durban. The brine was collected at a point immediately prior to entering outfalls discharging brine to the sea. Seawater (salinity of 35) collected relatively near the desalination plants was used for brine dilution purposes. Upon receipt the samples were logged in and then stored at 4 °C until testing, which was usually the next day but no later than four days after receipt. Samples of brine from seawater desalination Plant A (34º 5’10.44’’S, 18º33’22.84’’E) and Plant B (34º 4’18.30’’S, 18º41’18.31’’E) (Figure 4.1) were tested in December 2018 and February 2019 using *E. mathaei,* and in July and August 2019 using *T. gratilla.* A total of eight tests were performed, four tests for each seawater desalination plant.

a)

b)

Figure 4. 1: Aerial view of sample collection points, showing the position of a) desalination Plant A and b) desalination Plant B.

4.3.1.2. Effluent: Final effluent, that is, immediately before it enters the outfall, was collected from the Central Works (29º52'36.41'S, 31º 3'37.06'') and Southern Works (29º57'36.65''S, 30º58'4.14''E) wastewater treatment plants in Durban (Figure 4.2). The effluent was collected on a monthly basis between April to September 2019. The samples were held on ice until return to the CSIR Toxicology Laboratory in Durban. Upon receipt the samples were logged in and then stored at 4 °C until testing, which was usually the next day but no later than four days after receipt. Seawater (salinity of 35) collected at Vetches Beach (29º51'59.27"S, 31º 2'53.91"E) (Figure 4.3) in Durban was filtered (20 µm) and UV-sterilised and used for effluent dilution to allow definitive testing. Effluent samples collected from both wastewater treatment plants were tested in April and May using *E. mathaei,* and in June, July, August and September using *T. gratilla.* A total of 12 tests were performed, six tests for each wastewater treatment plant.

Figure 4. 2: Aerial view of Central Works and Southern Works outfalls in Durban. (CSIR, 2017)

Figure 4. 3: Aerial view of seawater collection point from Vetch's Beach.

4.3.3.3. Receiving waters: The toxicity of seawater samples collected in March and April 2019 near the outfall of Sappi Saiccor pulp mill discharge was tested. The seawater samples were collected at various distances to the south-southwest and north-northeast of the outfall diffuser section (Figure 4.4). Sites SA, SB and SC, and NA, NB and NC were situated about 40 m to the southwest and northeast of the diffuser section respectively. Sites S1 - S3 and N1 - N3, which were identified as reference sites, were situated between 500 – 2000 m to the south-southwest and north-northeast of the outfall diffuser section. Water samples were collected at the top and middle of the water column using a remotely triggered Niskin bottle. On retrieval the bottle was bled of a small amount of sample, closed, and inverted a few times to ensure the sample was well-mixed. Aliquots of sample were then transferred to sterile high-density polyethylene containers. Samples were collected at the surface and middle of the water column because the effluent discharged through the outfall is buoyant and ascends due to differences in density of effluent and surrounding seawater. In other words, the effluent is most likely to disperse and dilute closer to the top of the water column after discharge. The dilution water was collected at a reference site up-current and inshore of the outfall diffuser to ensure that it was absent of effluent.

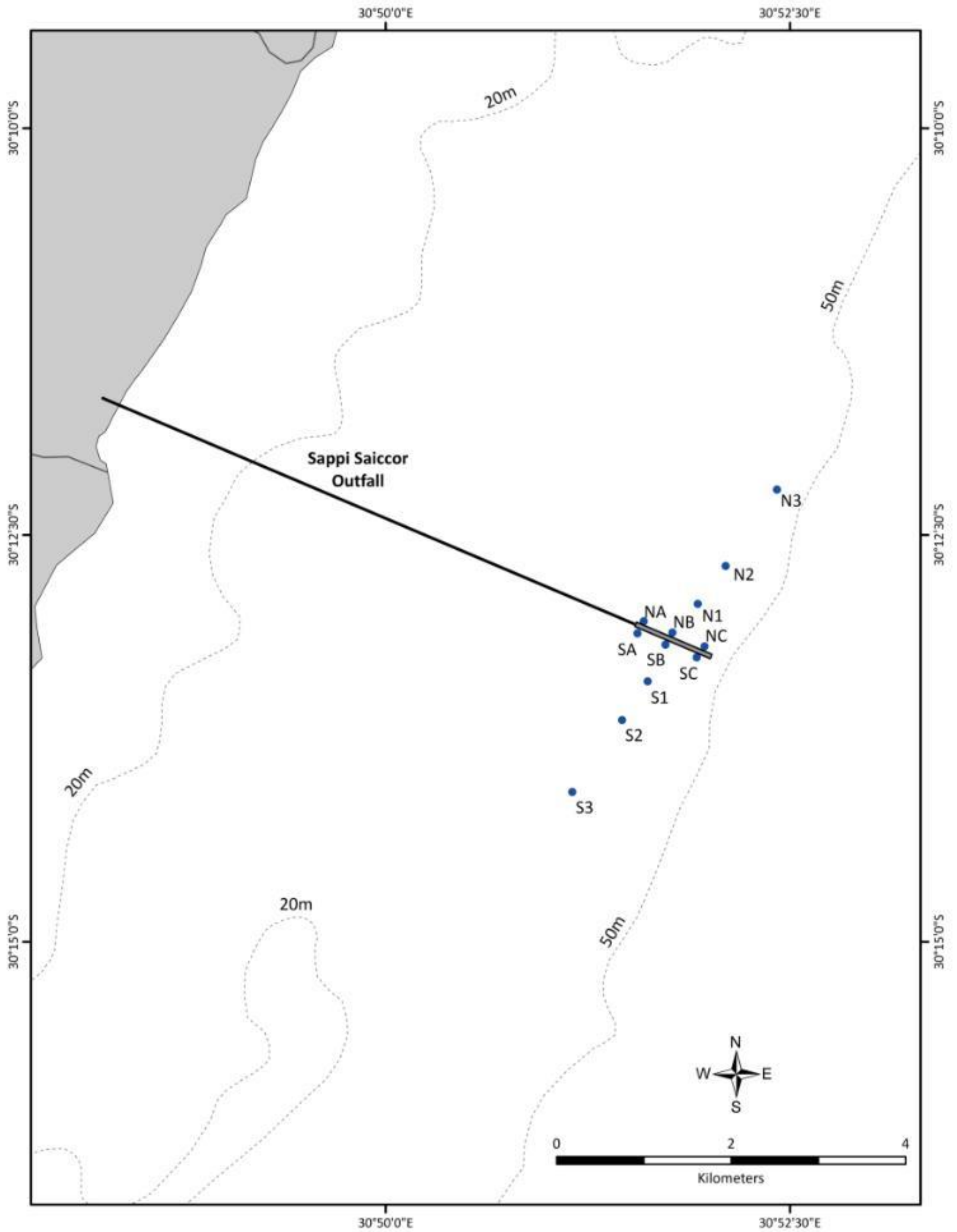

Figure 4. 4: Map illustrating the sites where water samples were collected for the Sappi Saiccor outfall monitoring programme (CSIR, 2018).

4.3.2 Sample preparation

4.3.2.1. Brine: Seawater collected near desalination Plants A and B was used as the control and dilution water. Seven concentrations of the brine were prepared using a 1:2 dilution series (v/v) with the receiving waters. The concentration series was 1.6%, 3.1%, 6.3%, 12.5%, 25%, 50% and 100%. Salinity was measured using an Atago refractometer. A 20 ml aliquot of seawater collected near the desalination plants (control) and brine concentration series was dispensed into glass vials.

4.3.2.2. Effluent: The effluent was serially diluted with filtered seawater to produce seven dilutions (5, 10, 20, 50, 100, 200, 500), by adding test samples to 20 ml of filtered seawater in glass vials at concentrations ranging from 0.002% to 0.2% v/v of wastewater. The lowest dilution of 0.2% was used to avoid a salinity adjustment which may be a confounding factor (sea urchin gametes are stenohaline and are unable to tolerate low salinities).

4.3.3.3. Receiving waters: A 20 ml aliquot of filtered seawater, dilution seawater and undiluted receiving water samples were dispensed into glass vials.

4.3.3 Sperm fertilisation and larval development toxicity tests

The sea urchin fertilisation and larval development tests were based on USEPA (1995) methods. Adult sea urchins were collected from Vetches Reef in Durban and maintained at ambient temperature in natural seawater in large recirculating tanks in the laboratory. Urchins were injected with 1 - 2 ml of 0.5M KCl through the peristomial membrane. Females were inverted over a glass beaker filled with filtered seawater to collect the eggs. Sperm from the males were collected ‘dry’ using a Pasteur pipette to prevent activation and preserve its viability. Immature eggs and debris were removed by washing and decanting 3 - 4 times with filtered seawater. Egg quality and sperm motility were checked at 10× magnification using the Zeiss Axioskop 2 light microscope. Eggs that had a uniform shape and distinct nuclei were considered good quality and highly motile sperm were used. Immediately before the test, sperm were added to filtered seawater to activate the sperm.

Sperm density was not calculated but a sperm dilution series was used to determine the appropriate volume of diluted sperm to yield 95 % fertilisation of 400-500 eggs per ml in the egg stock solution.

For the fertilisation test, sperm were exposed to a sample for 10 minutes, followed by the addition of viable eggs for a further 10 minutes. Fertilised eggs were identified by an elevated fertilisation membrane. For the larval development test, 1 ml of fertilised eggs (embryos) from a stock solution was added to each control and treatment. The exposure period of embryos was 72 h for *E. mathaei* and 96 h for *T. gratilla* at a temperature of 23 °C. The control and each treatment comprised four replicates. Each test was terminated with 40 µl of formaldehyde.

4.3.4 Toxicity assessment

Evaluation of fertilisation success and normal 4-arm plutei were performed on 100 individuals from each replicate (400 individuals per treatment). Fertilisation success was identified by the presence of an elevated fertilisation membrane. Normal 4-arm pluteus larvae were used as the endpoint and classified based on the following characteristics: have a pyramid shape, a fully differentiated gut and post-oral arm length greater than the total arm length (USEPA, 1995). The predominance of abnormalities present in each replicate was classified as follows:

- Pathological hatched or pre-hatched - Single (*i.e.* fertilised eggs) or multicellular embryos with or without a fertilisation membrane, appears as dark masses of cells or dissociated blobs of cells.
- Necrosis - Cell death as a result of external interference that causes an uncontrolled release of the inflammatory cellular contents (Fink & Cookson, 2005).
- Inhibited - Embryos or larvae at the blastula, gastrula and prism stage, have no or undeveloped skeleton.
- Gut abnormalities - Normal overall appearance where guts are absent, undifferentiated, incorrectly shaped or positioned or projected outside the embryo or larvae (exogastulated)

- Retarded - Delayed development at the 2-arm pluteus stage or has an irregular shape
- Skeletal malformations - May have either bent, spread out or asymmetrical post-oral arms or separated or crossed tips at the apex of the larvae

4.3.5 Quality assurance

A reference toxicant test using zinc for *E. mathaei* and copper for *T. gratilla* was performed concurrently to ensure that each species was responding as expected to a known toxicant. Test acceptability was achieved when (i) fertilisation in the control treatment was >90% and normal larval development in the control treatment was >80%; and (ii) the EC_{50} of the fertilisation success and larval development from the reference toxicant test was within the expected response range (see Figures 3.3b and 3.4a in Chapter 3).

4.3.6 Statistical analyses

4.3.6.1. Brine and effluent: Comparisons between treatments and the control was done using analysis of variance (ANOVA) and the MATD for the seawater desalination brine and effluent was determined by the linear interpolation method, using CETIS software. The MATD was derived from the "low effect" EC_x value, which was calculated as:

$$'Low\ effect\ ECx' = 100 - (CA - MSD)$$

Where: CA= control average and MSD= minimum significant difference.

The MSD was calculated from the percent minimum significant difference (PMSD) as follows:

$$MSD = (PMSD \times CA) \div 100$$

The MSD represents the difference that can be distinguished between the response of the individuals in the control and the response of the individuals in the treatment. This provides an indication of within-test variability and test method sensitivity (USEPA, 2000).

The index of the difference was used to determine whether the larval development test was more sensitive than the fertilisation test and calculated as:

$$Index\ of\ difference = \frac{MATD\ (LD)}{MATD\ (F)}$$

Where: MATD (LD) = minimum acceptable toxicant dilution of larval development success and MATD (F) = minimum acceptable toxicant dilution of fertilisation success. A value greater than one indicates that larval development is more sensitive, and a value less than one indicates that larval development is less sensitive. A value of one indicates that the sensitivity of larval development is equivalent to fertilisation. The index difference was used as a comparative measure of the sensitivities of the acute and chronic tests.

4.3.6.2. Receiving waters: Data expressed as a percentage was converted to proportions and then arcsine transformed before analysis. Sigmaplot 12.0 software was used to run a t-test comparing the larval development success and fertilisation success for each sample. ANOVA was used to identify significant differences between seawater samples and the filtered seawater and dilution water. Non-parametric tests were conducted using Kruskal-Wallis One Way on Ranks test when normality was violated and Dunnett's multiple comparisons test was used to identify seawater samples that were significantly different to the dilution water. In all cases the significance level was set at $\alpha = 0.05$.

4.4 Results

Although the test acceptability criterion for the control was set at > 80% for normal larval development, each control had > 90% normal larval development in this study.

The larval development test was more sensitive to the brine sample collected in July, and less sensitive to samples collected in December, February and August for seawater desalination Plant A (Figure 4.5). For seawater desalination Plant B, the larval development test was more sensitive for samples collected in December and August and was less sensitive to samples collected in February and July (Figure 4.5).

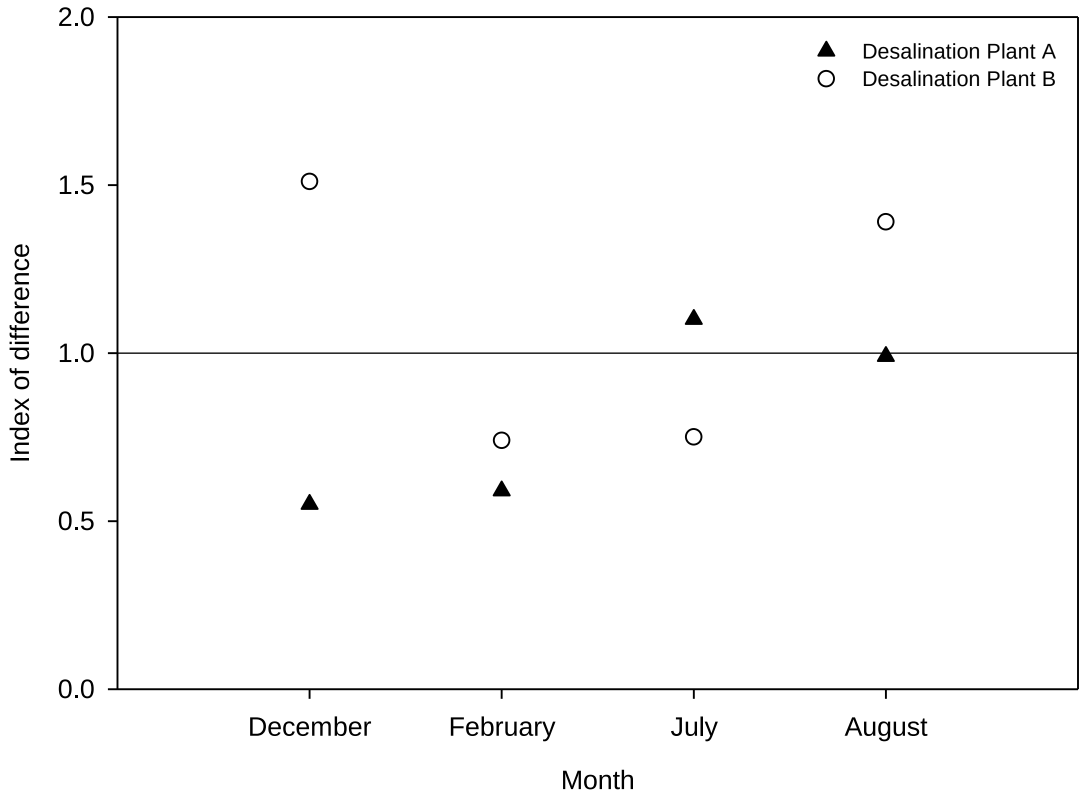

Figure 4. 5: Index of difference of the MATD for the larval development and fertilisation success tests using sea urchins *E. mathaei* (December and February) and *T. gratilla* (July and August) for seawater desalination Plant A and Plant B.

Table 4. 1: Minimum acceptable toxicant dilution (MATD) for larval development (MATD LD) and fertilisation success (MATD F), and the index of difference (IOD) for seawater desalination Plant A and B for each month.

Month/ Species	Plant A			Plant B		
	MATD (LD)	MATD (F)	IOD	MATD (LD)	MATD (F)	IOD
December *E. mathaei*	3.42	6.23	0.55	5.87	3.88	1.51
February *E. mathaei*	3.63	6.12	0.59	5.21	7.09	0.74
July *T. gratilla*	5.81	5.26	1.10	3.99	5.32	0.75
August *T. gratilla*	5.95	6.01	0.99	5.51	3.97	1.39

The MATD range for seawater desalination Plant A was 5.26 to 6.23 for the fertilisation test and 3.42 to 5.95 for the larval development test (Figure 4.7). The MATD range for seawater desalination Plant B was 3.88 to 7.09 for the fertilisation test and 3.99 to 5.87 for the larval development test (Figure 4.7). The difference in toxicity for the larval development test was 0.55 to 1.10 times that of the fertilisation test for seawater desalination Plant A and 0.74 to 1.51 times that of the fertilisation test or seawater desalination Plant B (Table 4.1).

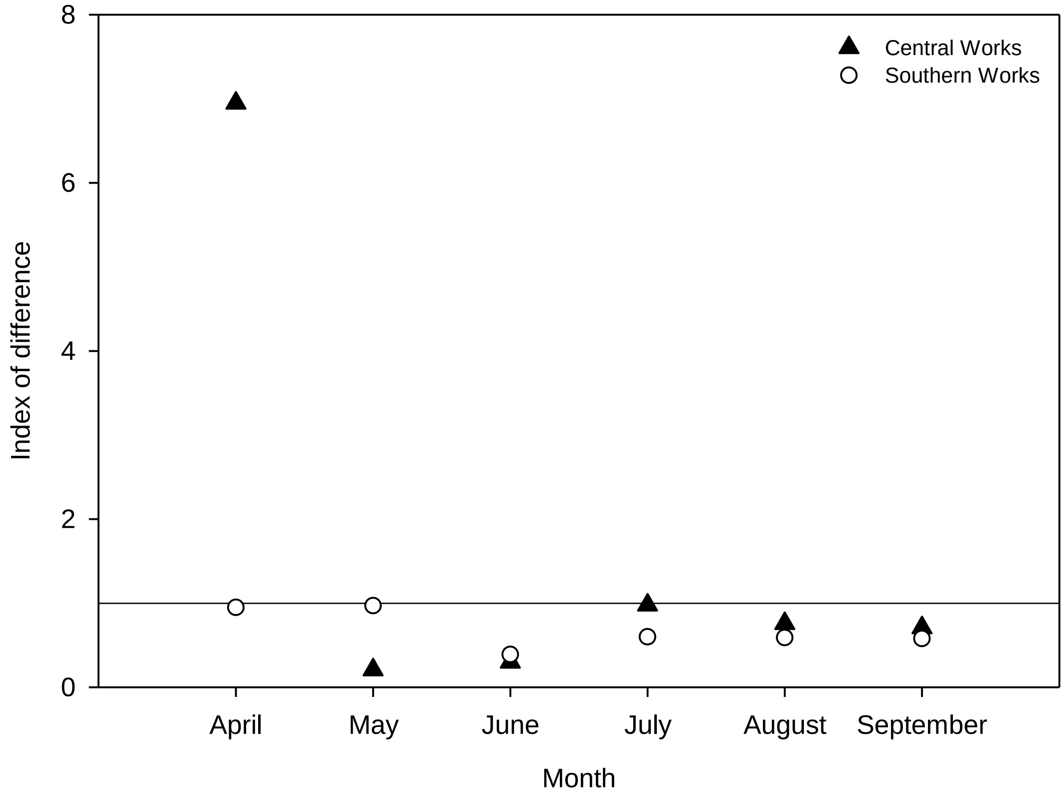

Figure 4. 6: Index of difference of the MATD for the larval development and fertilisation success tests using sea urchins *E. mathaei* (April and May) and *T. gratilla* (June, July, August and September) for Central Works and Southern Works effluent.

The MATD range for Central Works effluent was 17.33 to 153.85 for the fertilisation test and 9.56 to 120.48 for the larval development test (Figure 4.8). The MATD range for Southern Works effluent was 68.03 to 333.33 for the fertilisation test and 65.79 to 181.82 for the larval development test (Figure 4.8). The difference in toxicity for the larval development test was 0.21 to 6.95 times that of the fertilisation test for Central Works effluent and was 0.39 to 0.95 times that of the fertilisation test for Southern Works effluent (Figure 4.6, Table 4.2).

Table 4. 2: Minimum acceptable toxicant dilution (MATD) for larval development (MATD LD) and fertilisation success (MATD F), and index of difference (IOD) for Central Works and Southern Works effluent for each month.

Month/ Species	Central Works			Southern Works		
	MATD (LD)	MATD (F)	IOD	MATD (LD)	MATD (F)	IOD
April *E. mathaei*	120.48	17.33	6.95	78.74	82.64	0.95
May *E. mathaei*	9.56	46.17	0.21	65.79	68.03	0.97
June *T. gratilla*	46.17	153.85	0.30	131.58	333.33	0.39
July *T. gratilla*	18.73	19.05	0.98	181.82	303.03	0.60
August *T. gratilla*	70.42	92.59	0.76	123.46	208.33	0.59
September *T. gratilla*	49.26	69.44	0.71	96.15	166.67	0.58

The mean ± standard deviation of fertilisation and larval development were plotted against the dilutions for seawater desalination brine and effluents (Figures 4.7 and 4.8).The graphs show a similar series of converging dose-response curves and a pattern of ‘all or nothing’, which shows no significant effect at one effluent dilution to a complete effect at the next effluent dilution, and a stimulatory response at high dilutions and detrimental effect at low dilutions (USEPA, 2000). This stimulatory response pattern is typically found with sub-lethal endpoints such as growth, reproduction and larval development (USEPA, 2000). In such cases, larval development may increase relative to the control at high dilutions and decrease relative to the control at low dilutions. This also seems to be the case for fertilisation success.

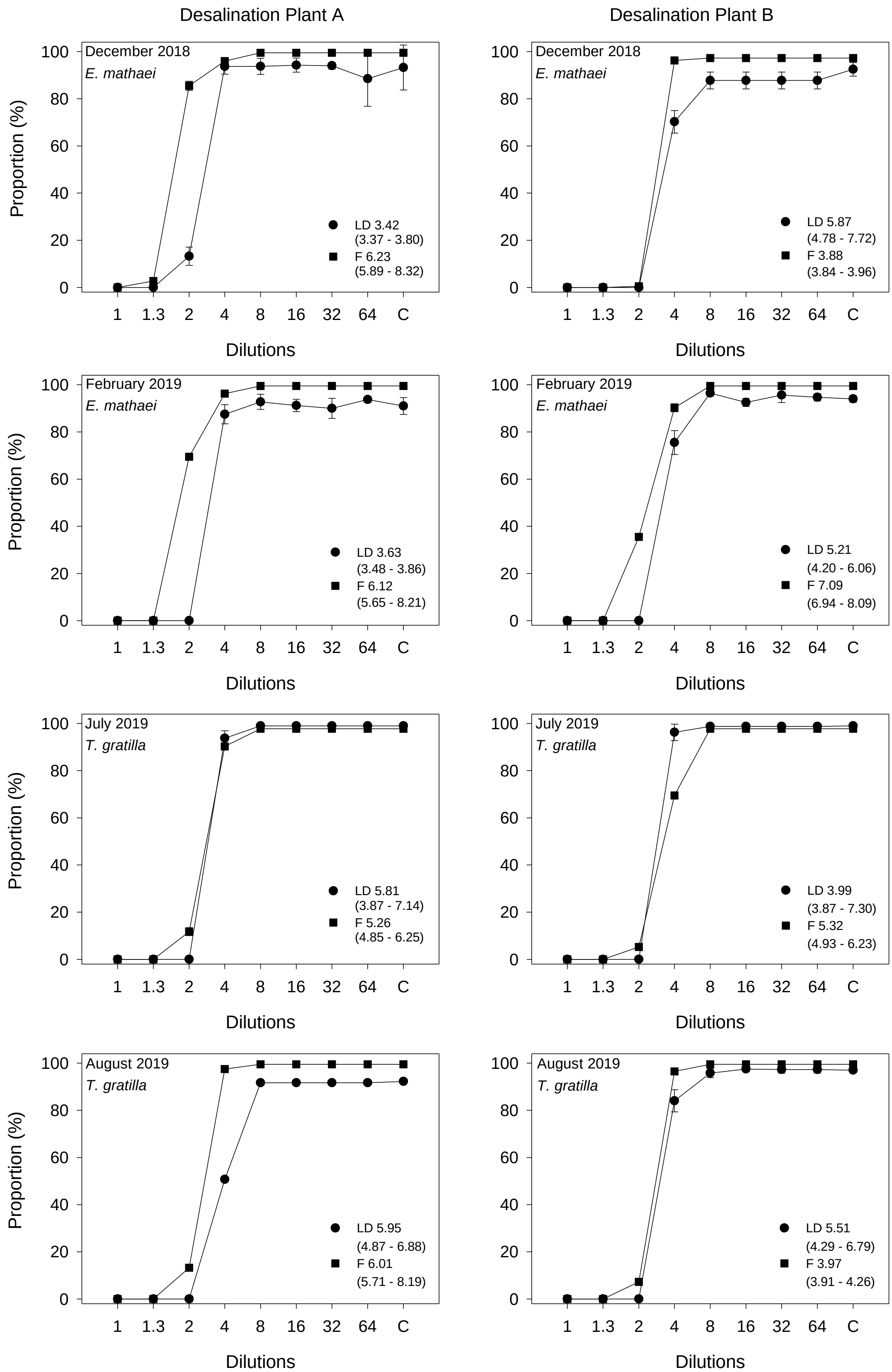

Figure 4. 7: The minimum acceptable toxicant dilution (MATD) and confidence interval for the larval development (LD) and fertilisation (F) tests using sea urchins *Echinometra mathaei* and *Tripneustes gratilla*. Mean ± standard deviation of four replicates. C = Control.

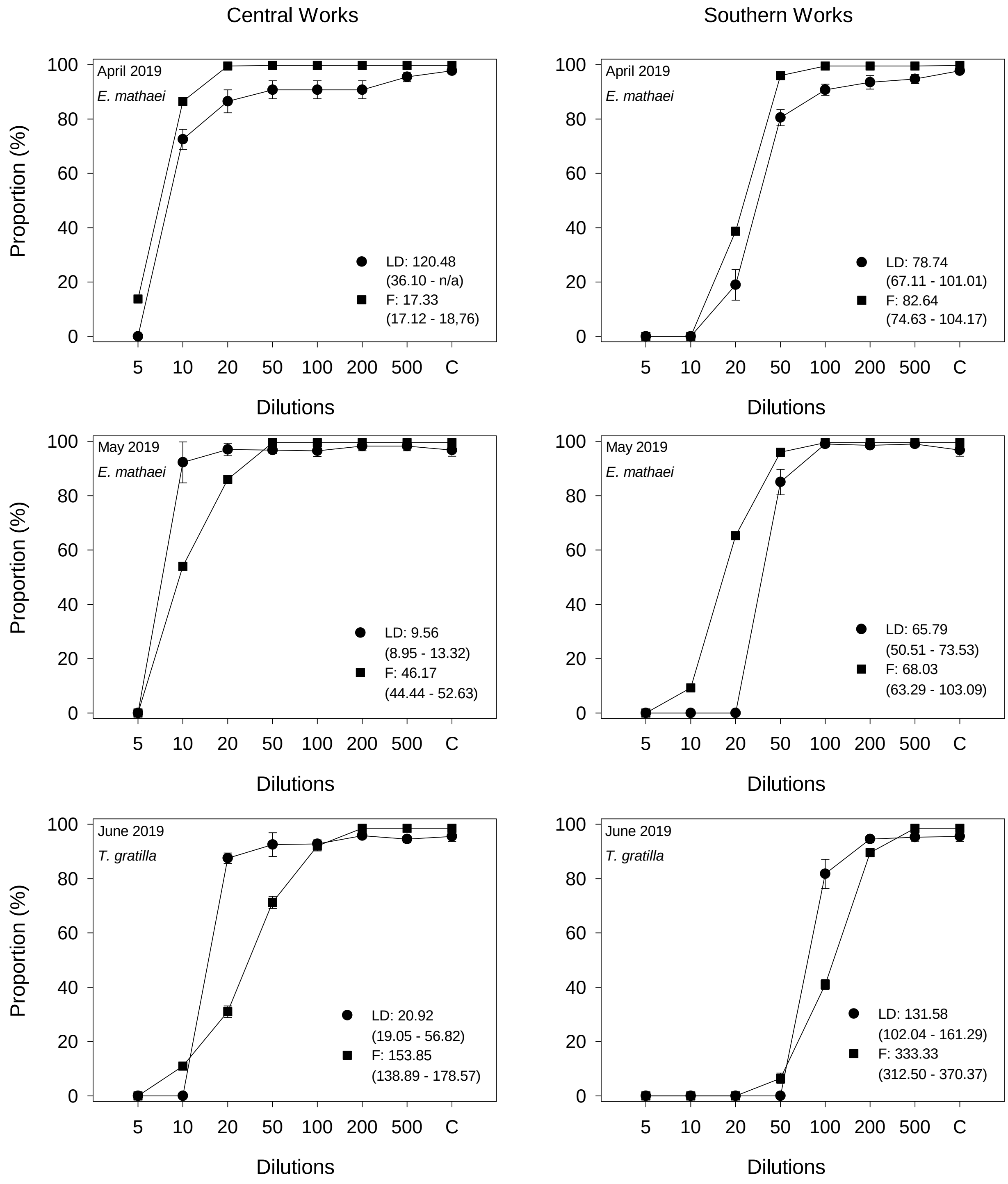

Figure 4. 8: The minimum acceptable toxicant dilution (MATD) and confidence intervals for the larval development (LD) and fertilisation success (F) using sea urchins Echinometra mathaei and Tripneustes gratilla. Mean ± Standard deviation of four replicates. C = Control.

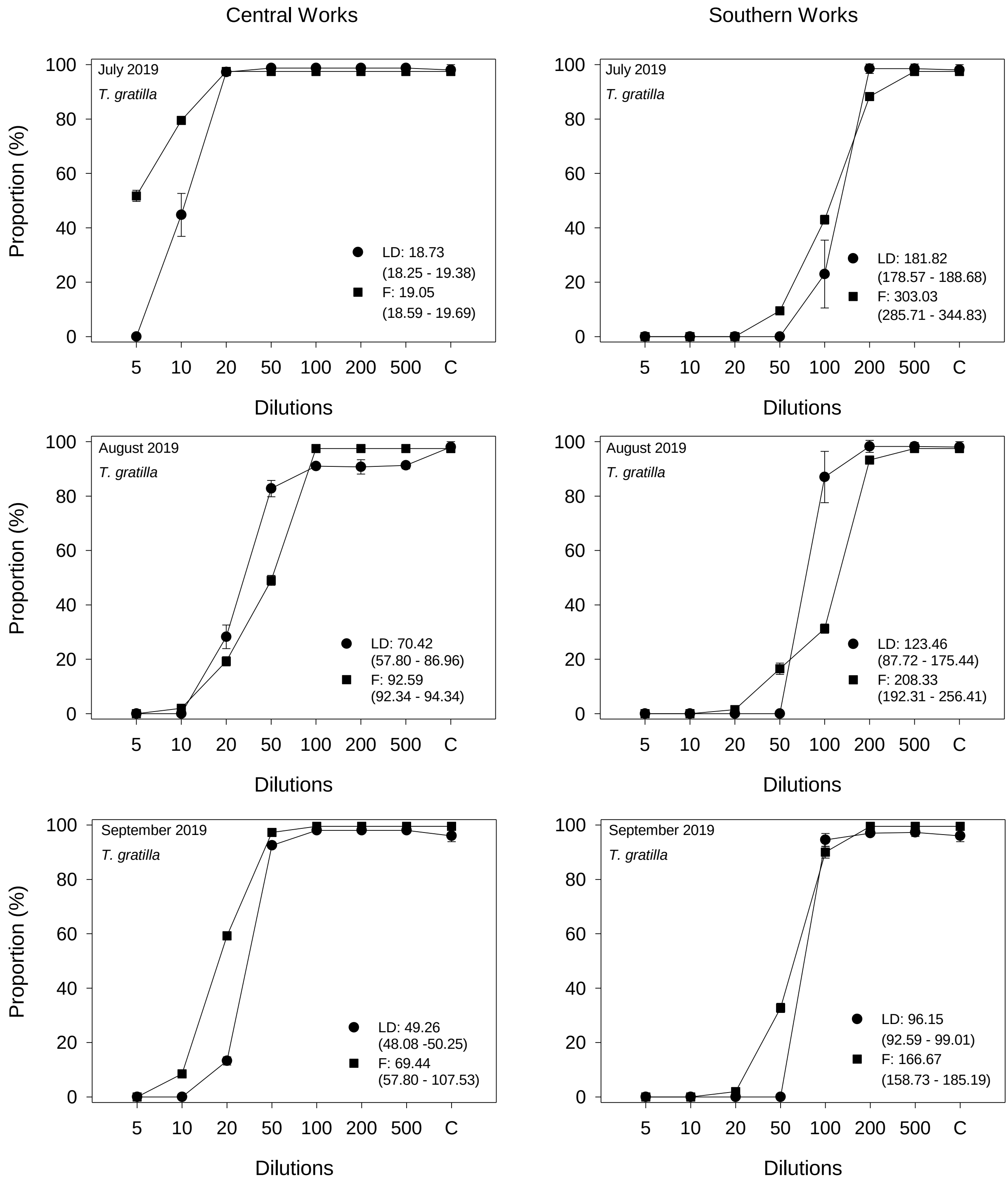

Figure 4. 8 continued. The minimum acceptable toxicant dilution (MATD) and confidence intervals for the larval development (LD) and fertilisation success (F) using sea urchins *Echinometra mathaei* and *Tripneustes gratilla*. Mean ± Standard deviation of four replicates. C = Control.

A comparison of the larval development and fertilisation success for seawater samples collected near the Sappi Saiccor outfall showed statistically significant differences for 18 of the seawater samples in Survey 1 (Table 4.3) and for 22 of the samples in Survey 2 (Figure 4.9 and Table 4.4). The mean values for larval development success were consistently lower than the fertilisation success apart from one sample (N2-T) in Survey 2. This indicates that the larval development test was more sensitive to toxicity.

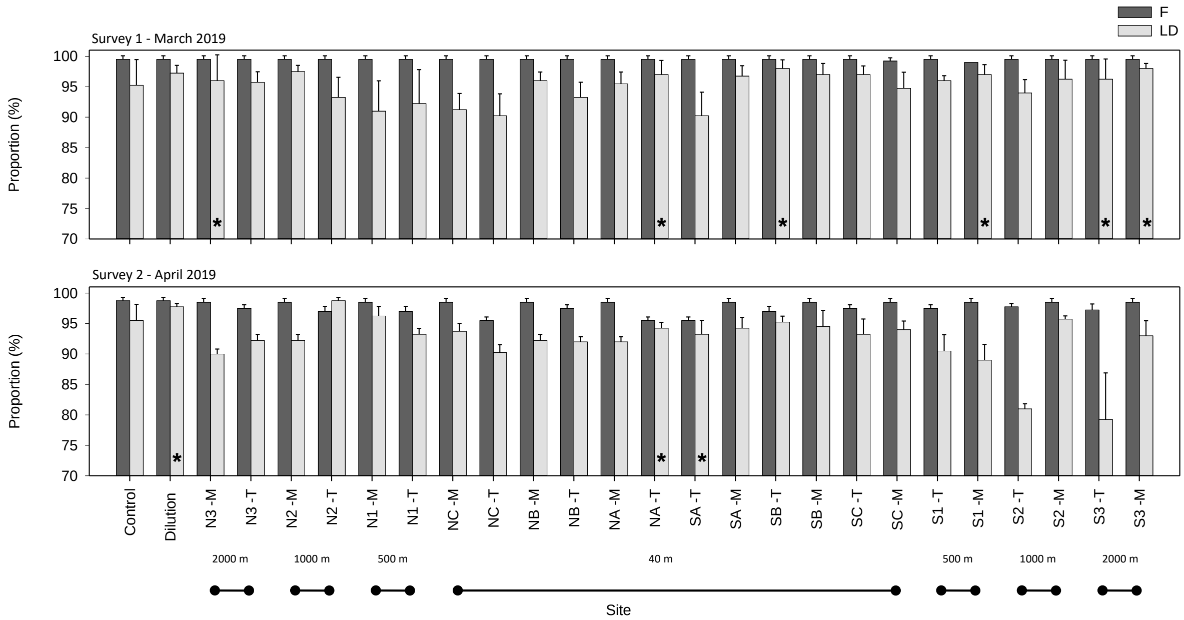

Figure 4. 9: Comparison of the average and (± standard deviation) fertilisation and larval development success of sea urchin *Echinometra mathaei* exposed to control and dilution water, and seawater samples collected in water quality surveys done in March and April for the Sappi Saiccor outfall monitoring programme. Sites were sampled at 40 m, 500 m, 1000 m and 2000 m from the outfall diffuser. T = surface water sample and M = mid-water water sample. Asterisks (*) denote no significant differences between the results of fertilisation success and larval development.

Table 4. 3: Average (± standard deviation) for fertilisation success and larval development at different sites for survey one of the Sappi Saiccor outfall monition programme. P-value < 0.05 indicates significant differences.

Sites	Fertilisation (%)	Larval development (%)	Statistic	P-value
Control	99.5 ± 0.6	95.3 ± 4.2	t = -2.98	0.025
Dilution	99.5 ± 0.6	97.3 ± 1.3	t = -3.10	0.021
N3 M	99.5 ± 0.6	96.0 ± 4.2	t = -2.18	0.072
N3 T	99.5 ± 0.6	95.8± 1.7	t = -4.18	0.006
N2 M	99.5 ± 0.6	97.5 ± 1.0	t = -3.28	0.017
N2 T	99.5 ± 0.6	93.3 ± 3.3	t = -4.67	0.004
N1 M	99.5 ± 0.6	91.0 ± 5.0	t = -4.92	0.003
N1 T	99.5 ± 0.6	92.3 ± 5.6	t = -4.01	0.007
NC M	99.5 ± 0.6	91.3 ± 2.6	U = 0.00	0.029
NC T	99.5 ± 0.6	90.3 ± 3.6	t = -6.50	0.001
NB M	99.5 ± 0.6	96.0 ± 1.4	t = -4.24	0.005
NB T	99.5 ± 0.6	93.3 ± 2.5	t = -5.49	0.002
NA M	99.5 ± 0.6	95.5 ± 1.9	t = -4.17	0.006
NA T	99.5 ± 0.6	97.0 ± 2.3	U = 2.00	0.114
SA T	99.5 ± 0.6	90.3 ± 3.9	U = 0.00	0.029
SA M	99.5 ± 0.6	96.8 ± 1.7	t = -3.16	0.020
SB T	99.5 ± 0.6	98.0 ± 1.4	t = -2.29	0.062
SB M	99.5 ± 0.6	97.0 ± 1.8	t = -2.89	0.028
SC T	99.5 ± 0.6	97.0 ± 1.4	t = -3.17	0.019
SC M	99.3 ± 0.5	94.8 ± 2.6	t = -4.03	0.007
S1 T	99.5 ± 0.6	96.0 ± 0.8	U = 0.00	0.029
S1 M	99.0 ± 0.0	97.0 ± 1.6	U = 2.00	0.114
S2 T	99.5 ± 0.5	94.0 ± 2.1	t = -5.35	0.002
S2 M	99.5 ± 0.6	96.3 ± 3.1	t = -2.67	0.037
S3 T	99.5 ± 0.6	96.3 ± 3.3	t = -1.73	0.135
S3 M	99.5 ± 0.6	98.0 ± 0.8	U = 1.00	0.057

Table 4. 4: Average and (± standard deviation) for fertilisation success and larval development at the different sites for survey two of the Sappi Saiccor outfall monitoring programme. P-value < 0.05 indicates significant differences.

Sites	Fertilisation (%)	Larval development (%)	Statistic	P-value
Control	98.8 ± 0.5	95.5 ± 2.6	t = -2,90	0,027
Dilution	98.8 ± 0.5	97.8 ± 0.5	U = 1,50	0,057
N3 M	98.5 ± 0.6	90.0 ± 0.8	U = 0,00	0,029
N3 T	97.5 ± 0.6	92.3 ± 1.0	U = 0,00	0,029
N2 M	98.5 ± 0.6	92.3 ± 1.0	U = 0,00	0.029
N2 T	97.0 ± 0.8	98.8 ± 0.5	t = 3,89	0,008
N1 M	98.5 ± 0.6	96.3 ± 1.5	t = -2,97	0,025
N1 T	97.0 ± 0.8	93.3 ± 1.0	t = -5,81	0,001
NC M	98.5 ± 0.6	93.3 ± 1.3	t = -7,47	0,000
NC T	95.5 ± 0.6	90.3 ± 1.3	t = -8,08	0,000
NB M	98.5 ± 0.6	92.3 ± 1.0	U = 0,00	0,029
NB T	97.5 ± 0.6	92.0 ± 0.8	t = -10,73	0,000
NA M	98.5 ± 0.6	92.0 ± 0.8	t = -11,61	<0,001
NA T	95.5 ± 0.6	94.3 ± 1.0	t = -2,29	0,062
SA T	95.5 ± 0.6	93.3 ± 2.2	t = -1,95	1,000
SA M	98.5 ± 0.6	94.3 ± 1.7	t = -5,49	0,002
SB T	97.0 ± 0.8	95.3 ± 1.0	t = -2,80	0,031
SB M	98.5 ± 0.6	94.5 ± 2.6	t = -3,59	0,012
SC T	97.5 ± 0.6	93.3 ± 2.5	t = -3,83	0,008
SC M	98.5 ± 0.6	94.0 ± 1.4	U = 0,00	0,029
S1 T	97.5 ± 0.6	90.5 ± 2.6	t = -6,07	0,000
S1 M	98.5 ± 0.6	89.0 ± 2.6	t = -9,03	0,000
S2 T	97.8 ± 0.5	81.0 ± 0.8	t = -31,24	0,000
S2 M	98.5 ± 0.6	95.8 ± 0.5	U = 0,00	0,029
S3 T	97.3 ± 1.0	79.3 ± 7.6	t = -6,65	<0,001
S3 M	98.5 ± 0.6	93.0 ± 2.4	t = -5,26	0,002

In Survey 1, fertilisation success in the dilution water was 99.5% and was not significantly different to the other seawater samples which had a fertilisation success, greater than or equal to 99.0%. In contrast, larval development success in the dilution water was 95.3% and was significantly different to seawater samples NC-T and SA-T, which had a larval development success of 90.3% (Figure 4.10 and Table 4.3). Toxicity detected by the larval development test was within the range of 40 m from the outfall diffuser.

In Survey 2, fertilisation success in the dilution water was 98.8% and was significantly different to seawater samples NC-T, NA-T and SA-T which had 95.5% fertilisation success (Table 4.4). Larval development success in the dilution water was 97.8% and was significantly different to seawater samples N3-M, NC-T, S1-T, S1-M, S2-T, S3-T which had 90.0%, 90.3%, 90.5%, 89.0%, 81.0% and 79.3% larval development success (Figure 4.10, Table 4.4). Toxicity was detected in seawater sample NC-T for both tests. The fertilisation test detected toxicity within 40 m of the outfall diffuser and the larval development test detected toxicity towards the south-southwest of the outfall diffuser

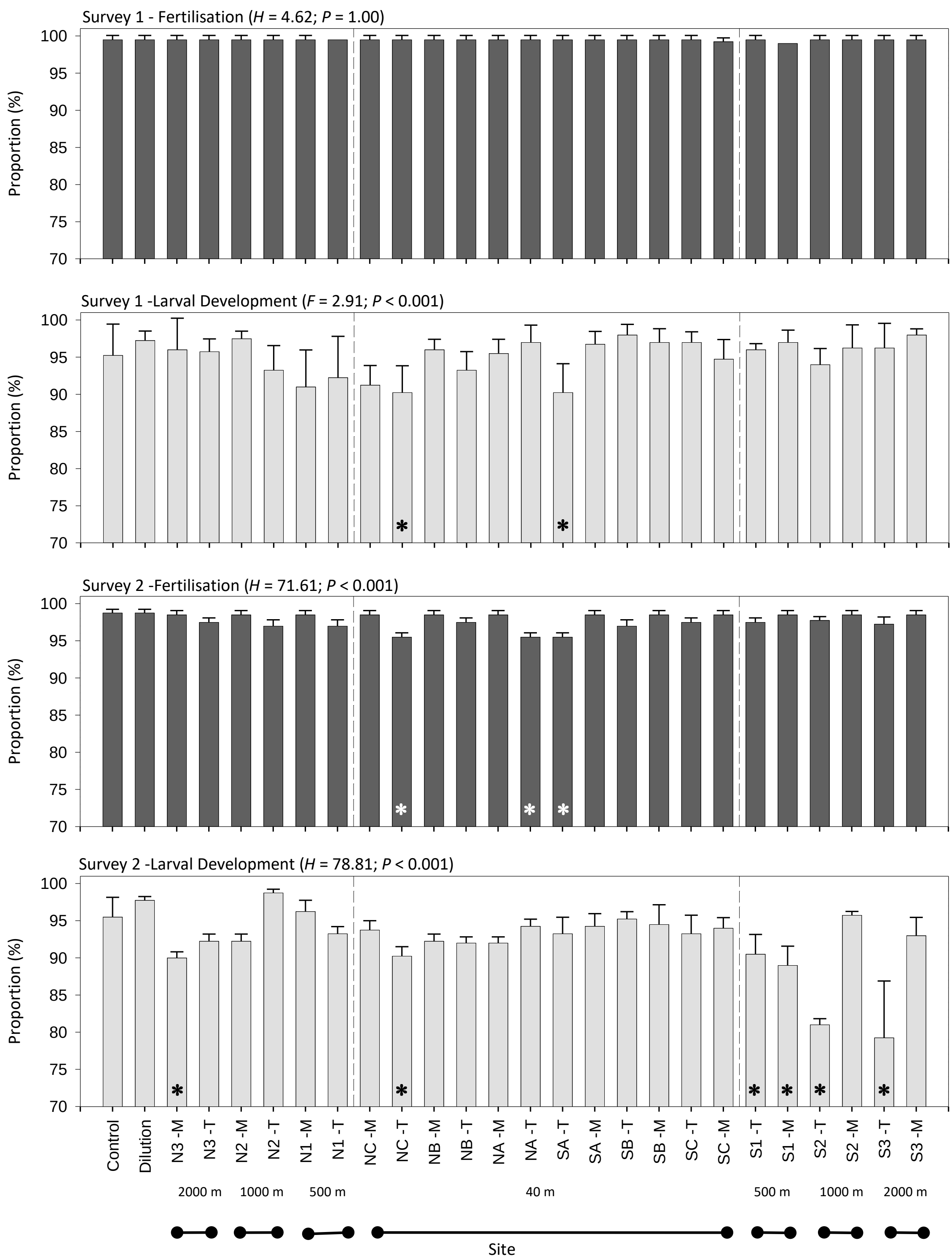

Figure 4. 10: Average (± standard deviation) fertilisation success and larval development of sea urchin *Echinometra mathaei* exposed control and seawater samples collected in water quality surveys done in March and April for the Sappi Saiccor outfall monitoring programme. Sites were sampled at 40 m, 500 m, 1000 m and 2000 m from the outfall diffuser. T = surface water sample and M = mid-water water sample. Asterisk (*) denote significant differences between the site and dilution water sample/

4.5 Discussion

The present study was conducted to determine whether there were similarities or differences in the sensitivity of the acute fertilisation test and chronic larval development test in detecting toxicity of seawater desalination brine, effluent from two wastewater treatment works, and seawater samples from the receiving water of pulp mill effluent. The main difference in toxicity testing of effluent and receiving water samples is that the objective is to quantify the toxicity of an effluent whereas with receiving water the objective is to determine whether a sample is toxic.

The fertilisation and larval development tests showed a similar dose-response to seawater desalination brine and effluents, but the fertilisation test exhibited a slightly higher sensitivity. This was contrary to the expectation that the chronic larval development test would be more sensitive. The fertilisation test required a higher number of dilutions to render the samples of seawater desalination brine (50 to 75% of the time) and effluent (83 to 100% of the time) non-toxic. Dinnel and Stober (1987) and Bay *et al*., (1993) also found the sperm of *Strongylocentrotus purpuratus* to be highly sensitive to effluent. However, the difference in toxicity detection of larval development success to fertilisation success was negligible. There was, however, one exception for the Central Works effluent sample tested in April, where the difference in toxicity detection of larval development success to fertilisation success was about seven times greater. In this instance the larval development test demonstrated its ability in being more sensitive in detecting toxicity than the fertilisation test.

The minimum initial dilution is the amount of dilution that occurs near an outfall diffuser section due to discharge momentum and buoyancy effects. Effluent discharges are usually designed to result in rapid initial dilution that is regulated by a mixing zone approach, wherein water quality criteria should be met at the boundary of the mixing zone as a precautionary measure to protect ecosystem and human health (Jenkins *et al.,* 2012). The recommended area of the mixing zone by Jenkins *et al.* (2012) is 100 m from the outfall structure covering all directions in the water column. Currently, there are no regulations on the minimum initial dilution for seawater desalination plants in South Africa. Desalination plants produce large amounts of hypersaline effluents, referred to as brine, which is then discharged into the sea (Quintino *et al*., 2008; Riera *et al*., 2011). To ensure that brine discharges are not harmful to marine organisms in coastal receiving waters, it is necessary that desalination plants monitor effluent toxicity (Riera *et al*., 2011; Ahmed & Anwar, 2012). Toxicity of discharged effluent from seawater desalination plants seems to be mainly attributed to the hyper salinity of the brine (Fernández-Torquemada *et al*., 2013). However**,** the toxicity of the brine may differ between desalination plants based on the types of chemicals used for source water conditioning (Voutchkov, 2011).

In the present study, the number of dilutions required to render the seawater desalination brine samples non-toxic was less than eight for the fertilisation and larval development test. This provides an indication that seawater desalination brine poses little harm on the receiving environment as this number of dilutions should be achieved within a short distance of the discharge point. Seawater desalination is a new system of providing fresh water for human consumption, thus there is limited research on the toxic effects of its waste on the marine environment. While there is currently no available literature on comparative studies on the effects of seawater desalination brine on sea urchin fertilisation and larval development success, Hobbs *et al*. (2008) showed that the sea urchin larval development test was the most sensitive to seawater desalination brine in comparison to other chronic tests using marine organisms.

A minimum initial dilution of 229 and 261 for Central Works and Southern Works wastewater treatment works outfalls respectively was estimated using numerical modelling (CSIR, 2017). Effluents from Central Works (domestic) and Southern Works (domestic and industrial) wastewater treatment works required dilutions up to 153.85 and 333.33 for the fertilisation test and 120.48 and 181.82 for the larval development test respectively. The MATD for all samples tested using the fertilisation and larval development test were within the initial dilution apart from two samples (June and July) of Southern Works effluent for the fertilisation test and one sample (August) of Central Works effluent for the larval development test. The statistical procedure used extrapolated the MATD for the larval development test to be 833.33 for the Central Works effluent sample (August) which exceeded the highest dilution of 500 that was used in the test. In this instance, USEPA (2000) suggests repeating the test with a new sample batch and additional dilutions, but this was not done in the present study. A suggested alternate approach to calculate a more realistic MATD for this specific case was to average the larval development success in the three effluent dilutions where larval development success reached a plateau, and to then substitute this for the control. The recalculated MATD using the substituted control as presented in this study as it more realistic and closer to the MATD for the fertilisation test.

The dispersion of pulp mill effluent is influenced by the hydro-dynamic characteristics (*e.g.* areas swept by strong currents or affected by wave action) of the receiving environment (Raventos *et al*., 2006; CSIR, 2018). Site-specific studies of the effects of the effluent on receiving water are typically conducted using an upstream or downstream approach which focuses on the sites where toxicity could be expected to occur (La Point & Waller, 2009). There was no consistent trend in the sensitivity of the fertilisation and larval development tests in detecting toxicity of seawater samples collected from the receiving waters of pulp mill effluent for each survey. The larval development test detected toxicity in seawater sample NC-T for Survey 1 and Survey 2 whereas the fertilisation test only detected toxicity in this sample for Survey 2. For seawater sample SA-T, toxicity was detected by the larval development test in Survey 1 and by the fertilisation test in Survey 2. This shows that both tests can be used in long-term monitoring of the effect of pulp mill effluent on the receiving waters and to document if specific seawater samples show repetitive toxicity.

The larval development test detected a higher prevalence of toxicity south-southwest of the pulp mill outfall diffuser in Survey 2. However, it is highly unlikely that these seawater samples were toxic due to effluent discharge as they were collected 500 to 2000 m away from the outfall diffuser section and there was little evidence for toxicity in samples collected nearer the diffuser section. Although the larval development test was more sensitive in detecting toxicity, the variability was much greater in comparison to the fertilisation test. Possible reasons for greater variability observed in the larval development test could be attributed to contamination that may have occurred during the sample collection, test procedure, or as a result of unknown environmental variables or specific toxicants in each sample (Landis & Hughes, 1993; Cavanagh *et al*., 1998). The fertilisation test showed lower variability, thus indicative of higher test precision and accuracy of results (Cavanagh *et al*., 1998).

In conclusion, sea urchin gametes and larvae are highly sensitive to toxicants, but there is uncertainty whether other organisms may experience the same effect of toxicity (CSIR, 2018). Chapman (1995) suggests that the only way to overcome this uncertainty is to generate data that compares the sensitivities of multi-organism tests. Considering this limitation, the early life stages of sea urchins serve as a conservative measure of the potential toxic effects of toxicants on marine receiving waters (CSIR, 2018). In the present study, the sea urchin fertilisation and larval development tests produced consistent dose-response results for seawater desalination brine and effluent. The sensitivity of both tests was comparable, although the larval development test was less sensitive in detecting toxicity of seawater desalination brine and effluent. The larval development test was more sensitive in detecting toxicity of receiving seawater samples, but the variation of the results was greater than that for the fertilisation test. The selection of a toxicity test usually considers the following factors; rapidity, sensitivity, acceptability, statistical robustness, reproducibility, standardised protocol, use in monitoring and decision-making purposes, cost effectiveness and the level of representation as a biological proxy (Altenburger *et al*., 2019). This considered, the fertilisation test would be the preferred choice in toxicity testing as it was more sensitive in detecting toxicity of seawater desalination brine and effluent. Although the larval development test was more sensitive in detecting toxicity, the results produced by the fertilisation test had less variability and thus would be preferable in toxicity testing of receiving water samples. Furthermore, the fertilisation test is rapid and cost effective for sample analysis and adequate for monitoring and decision-making purposes. However, this is not a definitive decision as the larval development test may be used in other applications of different types of effluent and receiving water systems to further investigate if there are differences in sensitivity compared to the fertilisation test.

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CHAPTER 5: Conclusions and recommendations

5.1 Toxicity test development

The primary aim of this study was to develop a chronic larval development toxicity test using the sea urchins *Echinometra mathaei* (summer spawner) and *Tripneustes gratilla* (winter spawner). The reason for using two species is to ensure an annual supply of gametes due to seasonal spawning variability. These species are widely distributed (Toha *et al*., 2005, Mishra, 2013) but also inhabit the east coast of South Africa and are thus more likely to be adapted to local conditions. Falkenburg & Styan (2015) state that the use of local species in whole effluent toxicity (WET) testing provides a better indication of the ecological effect of the effluent. These sea urchins are easily sourced and maintained in the laboratory. Furthermore, these species are highly sensitive to toxicants and play a major role in maintaining ecosystem integrity (Dalabajan *et al*., 2018, Keshavarz *et al*., 2017).

Although the methods followed for the larval development test were adapted from USEPA (1995), the test temperature which ultimately determines test duration, needed to be defined for each species. Both species were exposed to three temperatures (20, 23 and 26 °C) within their ecological range. The optimal test temperature for both species was 23 °C, at which the endpoint being the normal 4-arm pluteus was attained at 72 h for *E. mathaei* and 96 h for *T. gratilla* (Chapter 2). The results of this study corroborate with others who investigated the effects of temperature on larval development of *E. mathaei* and *T. gratilla* and found that the temperatures for normal development to be between 22 to 29 °C (Rahman *et al.*, 2009; Parvez *et al.*, 2018). Generally, the exposure duration and test conditions of toxicity tests attempt to simulate those that may be experienced by organisms in the natural environment (Chapman, 1995). It was also established that the total larval length of *E. mathaei* and *T. gratilla* was greater at 26 °C than at 23 °C.

5.2 Toxicity test sensitivity and reproducibility

The sensitivity of *E. mathaei* and *T. gratilla* larvae and the reproducibility of tests was tested using copper (Cu) and zinc (Zn). Control charts were constructed using the EC_{50} from each test for *E. mathaei* and *T. gratilla*, to document their response to Cu and Zn. The preferred reference toxicant is Cu for *T. gratilla* and Zn for *E. mathaei*. This is based on analysis of the control charts and coefficient of variation (CV, %) produced for each species. *E. mathaei* and *T. gratilla* were shown to be highly sensitive to Cu and Zn, which supports their use as toxicity test species. The use of the control chart in the present study has demonstrated its role in determining the preferred reference toxicant for *E. mathaei* and *T. gratilla* (Chapter 3).

Table 5. 1: Toxicity test conditions for the larval development of *E. mathaei* and *T. gratilla*.

Parameter	Test condition
1. Test type	Static, non-renewal
2. Test salinity	35 ± 1
3. Temperature	23 ± 1 °C
4. Photoperiod	12L: 12D
5. Test container	30 ml glass vial
6. Test volume	20 ml
7. Density	400 – 500 eggs per ml
8. Test endpoint	Normal larvae
9. Test duration	72 h (*E. mathaei*) and 96 h *(T. gratilla*)
10. Acceptability criteria	> 80% normal larvae in the control
11. No. of replicates	4
12. Dissolved oxygen (DO)	7.5 ± 0.5 mg l^{-1}
13.pH	8.1 ± 0.1

5.3 Toxicity test application

The larval development test using *E. mathaei* and *T. gratilla* was applied in the toxicity testing of seawater desalination brine, effluent from two wastewater treatment works and receiving water samples of pulp mill effluent, to demonstrate the environmental relevance of this test. For each application, larval development success was compared to the fertilisation success, to determine if there were differences in the sensitivity of each test in detecting toxicity. Both tests produced a comparable response in detecting toxicity of seawater desalination brine and effluent, which can be seen from the dose-response curves constructed (refer to Chapter 4). The larval development test was less sensitive in detecting toxicity of the seawater desalination brine and effluent, but the difference was negligible for all but one sample. In contrast, the larval development test was more sensitive in detecting toxicity of seawater samples of receiving waters of pulp mill effluent, but the variability of results was higher than that of the fertilisation test. Factors considered in the selection of a toxicity test include rapidity, sensitivity, acceptability, statistical robustness, reproducibility, standardised protocol, use in monitoring and decision-making purposes, cost effectiveness and the level of representation as a biological proxy (Altenburger *et al*., 2019). From the reference toxicant tests performed, CV values were < 20 % using Zn for *E. mathaei* and Cu for *T. gratilla.* This is well within the suggested standards required to determine the sensitivity of each species and overall credibility of the sea urchin larval development test. The larval development test compared to the fertilisation test has produced acceptable and statistically robust results. In terms of representation of a biological proxy, the larval development test would be more ecologically relevant in determining the chronic effects of toxicity whereas the fertilisation test can only provide information on the immediate effects of toxicity. Considering the aspects of sensitivity, rapidity and cost-effectiveness, the fertilisation test would be a suitable test mainly because it was more sensitive in detecting toxicity, sample analysis can be completed in a day and is low cost to conduct. The larval development test is a chronic test and would require a few days to produce results and is more costly to conduct in terms of test duration. Although the fertilisation test is preferred in detecting toxicity, the sea urchin larval development test should be considered a valuable comparative tool in assessing the chronic effects of effluent and receiving water toxicity in the marine environment.

5.4 Conclusion

The present study successfully developed a larval development chronic toxicity test using sea urchins *E. mathaei* and *T. gratilla* by defining the optimal test temperature which ultimately determined the test duration for each species. Results from this study were used to generate toxicity test conditions for each species (Table 5.1). The sensitivity and reproducibility of the larval development test was evaluated by the CV's and establishing control charts for each sea urchin species exposed to Cu and Zn. Their response to reference toxicants Cu and Zn were acceptable based on the control charts produced. The larval development test was applied in toxicity testing of seawater desalination brine, effluent from two wastewater works and seawater samples of receiving waters of pulp mill effluent and compared to the fertilisation test to determine if there were similarities or differences in the sensitivity of detecting toxicity. Although the fertilisation test was more suitable for use in toxicity testing, the chronic larval development test could be applied to other toxicants or different types of effluents and other receiving systems to further investigate and compare the difference in sensitivity to the fertilisation test.

5.5 Recommendations and future work

1. Percentage normal larvae is considered a standard endpoint, but Beiras (2018) suggests that larval length may provide a more sensitive and observer-independent response whereby a reduced number of individuals need to be analysed. In this study, the effect of temperature on larval length was investigated and significant differences were found in size, depending on temperature exposure. Future studies should investigate the effect of toxicity on larval length and a method as to how to classify the level of toxicity based on the length of larvae.

2. *E. mathaei* and *T. gratilla* attained the 4-arm pluteus larval stage at 26 °C at a shorter duration of 48 h and 72 h. Comparative studies should be conducted at 23 °C and 26 °C to

determine whether an increase in temperature and shorter test duration influences the sensitivity of the larval development test in detecting toxicity.

3. This study adopted its larval classification criteria from USEPA (1995) with slight modifications. It is highly recommended that a standardised larval identification be published to eliminate bias and to provide comparability between results of other studies.

4. Criteria should be established on what should be regarded as statistically significant differences versus biologically significant differences, especially for receiving water samples. Understanding biologically significant differences may assist in determining the actual effects of the effluent on the receiving environment.

5. Preliminary studies on the toxicity testing of sediment elutriate and sediment-water interface samples were conducted. Further studies should be conducted on refining the experimental design of the sediment-water interface test using the larval development test.

5.6 Limitations

1. Dissolved oxygen and pH were not measured daily due to a lack of instrumentation for this purpose, but these variables were measured before the start of the test. The physico-chemical variables were checked in accordance to the CSIR standard operating procedure for the sea urchin fertilisation test. It is unlikely that there were significant changes in the DO and pH as the proportion of normal larval development to the 4-arm pluteus in all tests were > 90 %.

2. The production of eggs and sperm are limiting factors in the number of experiments or samples that can be performed or tested. This can be problematic mainly during the transition between seasons.

3. Nominal concentrations of reference toxicants were used therefore unexplained variability could have occurred as a result of metal concentrations in solution not being at the actual concentration reported.

5.6 References

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