

Imposex in marine gastropods from the Atlantic coast of South Africa

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DECLARATION

I declare that “Imposex in marine gastropods from the Atlantic coast of South Africa” to be my own work, that is has not been previously submitted for any degree or examination purposes at this or any other university, and that all the sources used or quoted, have been indicated and acknowledged.

Signed: Nicole Roos

Date: 29 April 2016

DEDICATION AND ACKNOWLEDGEMENTS

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ABSTRACT

Tributyltin (TBT) is an organotin compound used as an antifouling agent in paint that is applied to the hulls of vessels to prevent the formation of biofilms. This highly toxic antifouling compound leaches into the water and has subsequent negative effects on marine life, especially marine gastropods. One of the most serious effects of exposure to TBT is the growth of a penis/vas deferens in female gastropods; this phenomenon is referred to as imposex (imposed sex). Marine gastropods are therefore useful bioindicators as imposex is related to TBT exposure. Imposex may affect the reproduction of these organisms and thus affect coastal ecology. The consumption of TBT-contaminated foodstuffs may also pose a risk to human health. The use of antifouling paints containing organotin compounds such as TBT continues in developing countries, despite the fact that the International Maritime Organization (IMO) banned the global use of organotin compounds in antifouling paints applied to ships in 2008, by country Parties electing to do so.

This is the first report of an imposex-sediment survey used to evaluate TBT contamination along South Africa's Atlantic coastline. This was achieved by sampling caenogastropods from sites presumed impacted and non-impacted by TBT pollution and by using various biological indices: Percentage Imposex (% I), Relative Penis Length Index (RPLI), Relative Penis Size Index (RPSI), and Male:Female ratio (M:F). TBT and DBT (dibutyltin) concentrations were measured in sediments to provide additional information regarding TBT pollution as gastropods usually inhabit these sediments and imposex may be related to these concentrations.

This study showed that imposex is prevalent along the coastline and high concentrations of TBT have been measured, which is of major concern. Results have shown that TBT and DBT levels are related to boating activity, including imposex prevalence.

Research will make valuable information available to necessary parties so that regulations and protective measures may be enforced to protect our marine life.

Key words: TBT (Tributyltin); Imposex; Caenogastropods; Biomonitoring; RPLI; RPSI; M:F ratio; Organotin compounds (OTCs); Endocrine disruption; Marine sediments.

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LIST OF ACRONYMS

% I	Percentage Imposex
AF	Antifouling
AFS	Antifouling Systems
ANOVA	Analysis of Variance
ATSDR	Agency for Toxic Substances and Disease Registry
CDCH	Caudodorsal Cell Hormone
Cu ₂ O	Cuprous Oxide
CuSCN	Copper Thiocyanate
DBH	Dorsal Body Hormone
DBT	Dibutyltin
DDT	Dichloro Diphenyl Trichloroethane
DFA	Department of Foreign Affairs
DNA	Deoxyribonucleic acid
DS	Dumpton Syndrome
EDCs	Endocrine Disrupting Compounds
ELH	Egg-laying Hormone
EPA	Environmental Protection Agency
GPS	Global Positioning System
H ₀	Null Hypothesis
H _A	Alternative Hypothesis
IMO	International Maritime Organisation
ISI	Intersex Index
M:F	Male to Female Ratio
MBT	Monobutyltin

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MFPL	Mean Female Penis Length
MIPs	Molluscan Insulin-like Peptides
MMPL	Mean Male Penis Length
MPA	Marine Protected Areas
OTCs	Organotin Compounds
PAH	Polycyclic Aromatic Hydrocarbon (compound)
PCB	Polychlorinated Biphenyls
POPs	Persistent Organic Pollutants
PSA	Particle Size Analysis
RPLI	Relative Penis Length Index
RPSI	Relative Penis Size Index
RXR	Retinoid X Receptor
SAS®	Statistical Analysis System
Sn	Tin
TOC	Total Organic Carbon
TBT	Tributyltin
TBTCI	Tributyltin Chloride
TBTO	Tributyltin Oxide
TCMTB	Thio Cyano Methyl Thio Benzothiazole
TPT	Triphenyltin
VDSI	Vas Deferens Sequence Index
WCNP	West Coast National Park
WHOI	Wood Holes Oceanographic Institute
WHO	World Health Organisation
ZPT	Zinc Pyrithione

CHAPTER 1: INTRODUCTION

“For most of history man has had to fight nature to survive, in this century he is beginning to realise that, in order to survive, he must protect it “. – Jaques Yves Cousteau

South Africa has one of the most beautiful coastlines in the world. It stretches 3650 kilometres (Griffiths *et al.*, 2010) and consists of distinct ecosystems, including rocky, sandy, mixed shores, kelp beds, estuaries, and seagrass communities (Mead *et al.*, 2013). The coastline is exposed to two oceans, the warmer Indian Ocean to the east and the colder Atlantic Ocean to the west (Griffiths *et al.*, 2010); which is the contributing factor to the abundant marine life (Branch *et al.*, 2010).

South Africa's coastline is relatively pristine when compared to other countries (Branch *et al.*, 2010; Wepener and Degger, 2012) and it is well conserved, 23% of it being under formal protection (Griffiths *et al.*, 2010). The coastline provides many resources not only for subsistence, but also for the aesthetic value thereof. However, these resources are limited and they are being exploited (Branch *et al.*, 2010). South African seas might not be as polluted as many other areas of the world, but this does not mean that it may not end up in a similar situation. In South Africa, marine pollution is mainly concentrated in densely populated, coastal areas such as Durban, Cape Town and Port Elizabeth (Griffiths *et al.*, 2010; Mead *et al.*, 2013; Nel *et al.*, 2015). Human impacts are evident within each South African coastal ecosystem, but to varying degrees of intensity, depending on where the ecosystems are situated (Mead *et al.*, 2013).

South African marine ecosystems are under threat from many anthropogenic activities such as coastal development; runoff of non-point pollutants e.g. pesticides, fertilisers, livestock waste etc.; point-source pollution such as oil spills and antifoulants from ocean-going vessels; overfishing; estuarine and wetland degradation; invasion of non-native species; and climate change (Griffiths *et al.*, 2010; Mead *et al.*, 2013). It is also expected that climate change may act synergistically with these human effects, increasing the vulnerability of coastal ecosystems and their associated biota (Mead *et al.*, 2013). The consequences of the above activities have been to mankind's

disadvantage as it affects public health, marine resources, marine organisms, and transportation (Goldberg, 1995).

Marine organisms that survive in extremely polluted waters may accumulate high concentrations of pesticides, metals and metalloids, and other contaminants, and this may be dangerous to those who consume them, such as predators and humans (Sindermann, 2006). There are approximately 70 000 anthropogenic chemicals used every day (Dachs and Mejanelle, 2010; Sumpter, 2002) and the ocean has become a sink for most of these chemicals. It is in the ocean where they degrade at varying rates and extents, to produce more chemical by-products (Sumpter, 2002). Pollutants threaten all levels of biological organisation, from the molecular to ecosystem level (Fent, 2003) and this impacts species richness, composition, and food web structure, and degradation of ecosystem services, which in turn affects the economics of a country (Begon *et al.*, 2006; Dachs and Mejanelle, 2010; Miller and Spoolman, 2012). Diversity, productivity and stability determine ecosystem structure (Worm and Duffy, 2003). All organisms are linked and if one organism disappears, this will certainly cause disorder in nature (Worm and Duffy, 2003).

Antifoulant paints (AF) are protective coatings that contain one or more biocides to control the accumulation of organisms on surfaces (Boxall *et al.*, 2000; Chambers *et al.*, 2006). They have gained much attention due to their usage and persistence in the marine environment, but none so much as Tributyltin (Yerba *et al.*, 2004).

Tributyltin (TBT) is an antifouling agent that has been used extensively on marine vessels of all sizes since the 1960s (Smith *et al.*, 2008); its uncontrolled use in the 1970s and early 1980s had various harmful environmental consequences (Axiak *et al.*, 2003; Birchenough *et al.*, 2002; Evans and Nicholson, 2000). These included effects such as shell thickening in oysters, *Crassostrea gigas* (Alzieu *et al.*, 1986; Alzieu, 2000a; IMO, 2002), and imposex in dog-whelks, *Nucella lapillus* (Gibbs and Bryan, 1987; Gibbs *et al.*, 1997).

Marine gastropods are useful bioindicators of TBT pollution, because they develop imposex, which is the imposed development of male organs in female gastropods (Bryan *et al.*, 1987; Matthiessen and Gibbs, 1998). Imposex is directly related to TBT

exposure (Smith, 1981a; Smith, 1981b; Smith, 1981c), and thus to maritime (boating) activity. It affects the reproduction of these organisms and coastal ecology as TBT may have the potential to biomagnify along food webs (Strand and Jacobsen, 2005). The consumption of TBT-contaminated foodstuffs may also pose a risk to human health (Swennen *et al.*, 2009).

The causal relationship between TBT and effects such as imposex and shell thickening in oysters provided enough evidence for Europe to impose initial regulations on organotin-based antifoulants in the 1980s (Alzieu *et al.*, 1986; Alzieu *et al.*, 1989; Bauer *et al.*, 1995; Champ, 2000).

In an effort to reduce the effects on coastal ecology, France was the first country to regulate the use of organotin containing antifouling paints in 1982 on boats less than 25 m in length (Alzieu, 1998; Alzieu, 2000a; Champ, 2000; Evans, 1999; Fent, 2006; Sousa *et al.*, 2014). Numerous countries followed suit such as the UK, USA, Canada, Australia, Japan, Spain, Norway, Switzerland, etc. during the late 1980s and early 1990s (Alzieu, 1998; Champ, 2000; Fent, 2006; Fernandez and Pinheiro, 2007; Omae, 2006).

The International Maritime Organisation (IMO) controls the regulatory processes for the safety of shipping and environmental concerns related to shipping (Castro *et al.*, 2012b; IMO, 2014). In 2001, the Convention on Antifouling Systems (AFS) was adopted by the IMO and established to ban the usage of organotins in antifouling paints used on vessels and to inhibit the future application of other harmful compounds in antifouling systems (Champ, 2000; IMO, 2014; Sonak *et al.*, 2009). It called for a global ban of the application of organotin compounds as biocides in antifouling systems on ships by 1st of January 2003, and a complete ban by 1st January 2008 (Fent, 2006; IMO, 2002). On the 17th of September 2008, the Convention came into force and thereby prohibiting the usage and presence of TBT-based antifoulants on vessels on a global scale (IMO, 2014; Matthiessen, 2013).

South Africa became a full member of the IMO in February 1995 after having observer status from 1948 and is Party to many of the conventions or protocols of the IMO,

including the International Convention on the Control of Harmful Antifouling Systems on Ships (AFS Convention) (DFA, 2006).

Once regulations were enforced, TBT levels in coastal waters, sediments and tissues of molluscs were significantly reduced worldwide (Evans and Nicholson, 2000) along with its subsequent effects (Evans and Nicholson, 2000) and the recovery of bivalves and marine gastropods had started to occur (Alzieu *et al.*, 1986; Birchenough *et al.*, 2002; Blanck and Dahl, 1998; Bray *et al.*, 2011; De Wolf *et al.*, 2004; Dyrinda, 1992; Evans *et al.*, 1995; Evans and Nicholson, 2000; Evans *et al.*, 2000b; Fent, 2006; Law *et al.*, 2012; Rees *et al.*, 2001; Smith *et al.*, 2008).

Total prohibition cannot automatically ensure complete recovery from the damage caused by highly persistent and toxic compounds, such as TBT, once they have entered the environment (Kim *et al.*, 2014; Matthiessen, 2013). Its effects will still be observed in coastal environments for a number of years (Alzieu, 2000b; Fent, 2006; Gibbs, 2009; Titley-O'Neal *et al.*, 2011). TBT contamination and its effects in marine gastropods are still observed globally (Castro and Fillman, 2012; Castro *et al.*, 2012a; Choi *et al.*, 2013; Fent, 2006; Hoch, 2001; Sousa *et al.*, 2009; Tallmon, 2012). Recovery from imposex effects at sub-lethal levels can take up to 15 years (Hawkins *et al.*, 2002).

Many factors contribute to the presence of TBT in the environment such as the release from ships, desorption from sediments, shipyard activities, illegal use of TBT, use on foreign vessels, or from other sources other than those used for antifouling purposes (Sousa *et al.*, 2009; Ueno *et al.*, 2004). Although many countries have joined the IMO and banned organotin compounds, TBT contamination remains a persistent problem because there are other countries, especially developing countries, that have not become Party to the convention and they continue to produce and make use of TBT. Many countries continue producing and selling TBT-containing antifoulants due to lack of regulations on its production and use (Antizar-Ladislao, 2008; Bigatti *et al.*, 2009; Champ, 2000, Harino *et al.*, 2008; IMO, 2014; Kotrikla, 2009; Tallmon, 2012).

There is very limited information available regarding TBT compounds in the environment of South Africa. Imposex from South Africa is only reported by two

studies; one looking at imposex (Marshall and Rajkumar, 2003), and one investigating TBT concentrations in sediment (Okoro, *et al.*, 2016). In this study, I will investigate both imposex and TBT concentrations in sediment.

This project will assess the current situation regarding TBT pollution and its incidence in biota by using imposex in gastropods as a biomarker for its availability along the Atlantic coast of South Africa; this was achieved by collecting caenogastropods and sediment from sites along the coast from Cape Agulhas to Port Nolloth, to cover presumed TBT-polluted sites (harbours-high marine traffic) and including areas which are presumed pristine areas (MPAs-low marine traffic). Various indices: the Relative Penis Length Index (RPLI), Relative Penis Size Index (RPSI), Male:Female ratio (M:F), Imposex Percentage (% I) were used to quantify imposex and subsequently, the intensity of TBT pollution. The prevalence of imposex along the coastline will indicate the use of TBT and its associated impacts. This study will inform necessary parties, which will generate the need for concerted mitigation efforts.

Aim: The aim of this study was to determine the prevalence of imposex associated with TBT contamination along the Atlantic Coast of South Africa by using biological and chemical analyses. This will provide the necessary authorities with valuable information regarding the current presence of TBT, so that they can make informed decisions regarding the usage and regulation of antifouling paints along the coast.

To achieve the above, the following objectives were set:

- Study the presence and prevalence of imposex in intertidal caenogastropod species from a range of sites along the Atlantic shore of South Africa;
- Measure the presence and concentrations of TBT (and DBT) in sediments from the sites;
- Develop a broader understanding of the prevalence of TBT and imposex along the Atlantic coastline of South Africa;
- Identify gastropod taxa best suited for describing impacts for follow-up, and further study;
- Provide background levels against which future management practices may be evaluated.

CHAPTER 2: LITERATURE REVIEW

2.1 Fouling and antifouling

Fouling or biofouling, is the attachment of marine organisms to structures submerged in the marine environment (IMO, 2002; Yerba *et al.*, 2004) such as platforms, jetties, and ship hulls (Chambers *et al.*, 2006). A ship can collect about 150 kg/m² of fouling organisms during a six-month stay at sea (Radke *et al.*, 2008). The process of fouling consists of the adherence of various elements from molecules to various organisms to substrata (Almeida *et al.*, 2007; Cao *et al.*, 2011; Chambers *et al.*, 2006). Once the attachment of species to a substrate is prevented, fouling may be controlled (Chambers *et al.*, 2006).

Fouling is a major problem for the maritime industry (Cao *et al.*, 2011; Dafforn *et al.*, 2011). Its effects are recognised globally (Evans *et al.*, 2000c). Biofouling can clog systems in heat exchangers, block flow in pipelines; reduce the life span of offshore platforms and on ship hulls; fouling causes frictional drag due to roughness, which leads to fuel consumption, and an increase of the frequency of dry-docking operations. This leads to increased costs to naval and commercial shipping, nuclear, and thermal plants (Alzieu, 1998; Chambers *et al.*, 2006; Champ, 2000; Evans *et al.*, 2000c; Yerba *et al.*, 2004). Biofouling can also result in the transportation of harmful invasive species (Dafforn *et al.*, 2011).

Antifouling of boats and ships is not a new concept. Effects of marine biofouling have been battled with since man began navigating the seas (Fernandez and Pinheiro, 2007; WHOI, 1952). Due to the fouling problem, antifouling systems have been developed (Chambers *et al.*, 2006; Radke *et al.*, 2008). Anti-fouling systems are defined by the AFS convention as “a coating, paint, surface treatment, surface or device that is used on a ship to control or prevent attachment of unwanted organisms” (IMO, 2014). Effective antifouling systems are essential for the shipping industry and are of economic and environmental importance (Du *et al.*, 2014; Voulvoulis *et al.*, 2002a).

Most antifouling systems take the form of protective coatings (Boxall *et al.*, 2000; Chambers *et al.*, 2006; Fernandez and Pinheiro, 2007) that contain biocides which

slowly leach over time into the surrounding medium (Boxall *et al.*, 2000; Fernandez and Pinheiro, 2007). They are toxic to fouling organisms (Boxall *et al.*, 2000) and have harmful effects on non-target species (Alzieu, 2000a; Camillo *et al.*, 2004; Dafforn *et al.*, 2011; Terlizzi *et al.*, 2004).

2.2 Organotin compounds and TBT

2.2.1 Properties

Tin is a universal element (ATSDR, 2005; Omae, 2006). It combines with other chemicals to form various compounds. Industrially important tin compounds can be classified as inorganic (those without a tin-carbon bond) and organic (those with a tin-carbon bond) (ATSDR, 2005). Organotin compounds (OTCs) belong to the most important anthropogenic organometallic compounds and are characterised by the presence of one or more carbon-tin bonds (Fent, 2006; Murata *et al.*, 2008; Omae, 2006; Radke *et al.*, 2008).

Organotins are expressed by the formula R_nSnX_{4-n} where: R = carbon-bonded organic group (alkyl or aryl group, butyl, phenyl, octyl group); X=inorganic anion (O^- , OH^- , Cl^- , F^- , SH^- , etc); $n=1-4$ (n being four different types of organotin compounds, mono-, di-, tri-, and tetraorganotin) (Hoch, 2001; Kot and Namiesnik, 2000; Omae, 2003; Radke *et al.*, 2008).

The toxicity of organotin compounds differ as the effects of the substances on organisms depend on the nature, number, and type of organic groups (R) bonded to the Sn cation (EPA, 2003; Hoch, 2001; Kot and Namiesnik, 2000). Organotins that are the most toxic to organisms are those where $n=3$, for instance, the triorganotin compounds (R_3SnX) (EPA, 2003; Fent, 2006; Maguire *et al.*, 1982; Namiesnik and Szefer, 2010; Omae, 2003; Radke *et al.*, 2008).

Tributyltin compounds are classified according to the presence of covalent bonds between three carbon atoms and a tin atom (see figure 1). They have the formula $(n-C_4H_9)_3Sn-X$, where X is an anion (e.g. acetate, carbonate, chloride, fluoride, hydroxide, oxide, or sulphide) or a covalently linked group (EPA, 2003; Sarti *et al.*, 2011). The nature of X determines the physical-chemical properties, particularly the

relative solubility in water and non-polar solvents and the vapour pressure. Its toxicity is mostly influenced by the alkyl substitutes than the anionic substitutes. An increase in the n-alkyl chain length decreases toxicity (Antizar-Ladislao, 2008; Hoch, 2001; Maguire *et al.*, 1982).

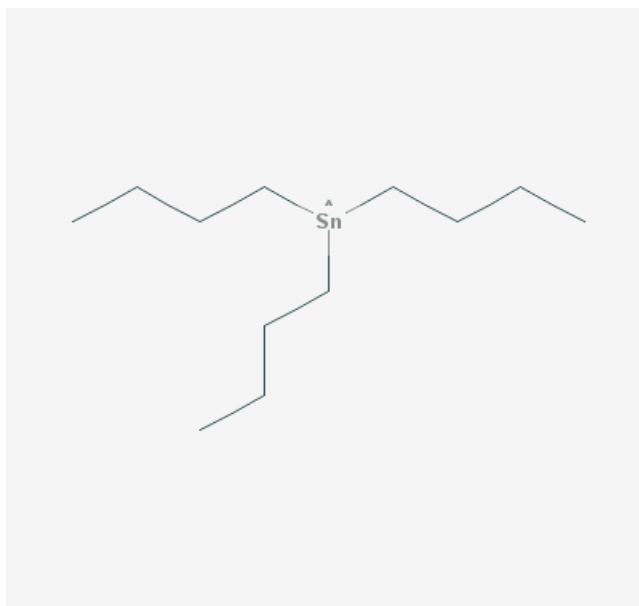


Figure 1: 2-Dimensional structure of Tributyltin (National Centre for Biotechnology Information, 2015)

Tributyltin oxide (TBTO) and tributyltin chloride (TBTCl) are usually used in laboratory experiments to investigate organotin toxicity (Antizar-Ladislao, 2008). Tributyltin compounds are expected to exist mainly as tributyltin hydroxide, tributyltin chloride, and tributyltin carbonate in the environment (WHO, 1999). Tributyltin is usually manufactured as TBT oxide, TBT fluoride, and TBT chloride and is degraded by debutylation to form dibutyltin (DBT), monobutyltin (MBT), and eventually inorganic tin (Sternberg *et al.*, 2010).

2.2.2 History of TBT

Tributyltin was first used as a mothproofing agent in the early 1920s (Dafforn *et al.*, 2011; Landmeyer *et al.*, 2004). The biocidal properties of TBT were later discovered in the 1950s by van der Kerk and Luijten at the Institute for Organic Chemistry in the Netherlands (Hoch, 2001; Omae, 2003; Omae, 2006).

Due to its biocidal activity, TBT was incorporated in antifouling coatings known as 'free-association paints' in the 1960s to replace toxic materials such as copper, lead, mercury, arsenic, DDT etc. (Abbott *et al.*, 2000; Chambers *et al.*, 2006; EPA, 2003; Evans *et al.*, 2000c; IMO, 2002; Omae, 2003; Qian *et al.*, 2010; WHOI, 1952; Yerba *et al.*, 2004). During the 1970s TBT was used in 'self-polishing copolymer' (SPC) formulations to prevent the unwanted growth of marine organisms on the hulls of commercial vessels (Almeida *et al.*, 2007; IMO, 2002). Self-polishing copolymer paints became very popular in the 1980s and were used on most of the world's commercial fleet (Evans *et al.*, 2000c; Goldberg *et al.*, 2004; Konstantinou and Albanis, 2004).

The extensive use and persistence of TBT compounds caused adverse effects on the aquatic environment and organisms, in particular, molluscs at nominal concentrations (Abbott *et al.*, 2000; Almeida *et al.*, 2007; Evans *et al.*, 2000b; Fent, 2006; IMO, 2002; Kot and Namiesnik, 2000; Omae, 2003).

2.2.3 Effects and toxicity

Tributyltin is a notorious, marine pollutant (Guðmundsdóttir *et al.*, 2011) due to its harmful effects on the marine environment (Yang *et al.*, 2006). Tributyltin is highly effective against the growth of various fouling organisms, but its effects have far-reaching consequences as it affects non-target organisms, including humans (Champ, 2000; EPA, 2003; Li and Collin, 2009).

Tributyltin is one of the most toxic biocides ever introduced into the aquatic environment (Alzieu *et al.*, 1989; Duft *et al.*, 2005; Fent, 2003; Fent, 2006; Hoch, 2001) due to characteristics such as high toxicity, persistence in the environment, high lipophilicity (leading to bioaccumulation) and low water solubility (ATSDR, 2005; De Mora *et al.*, 2003; Fent, 2003; Fent, 2006; Omae, 2006; Qian *et al.*, 2010; Radke *et al.*, 2008; Sekizawa *et al.*, 2003; Stewart and de Mora, 1990).

Tributyltin has had deleterious effects to the marine environment by affecting various marine organisms at different stages in their lifecycles via multiple pathways (Fent, 2003; Haggera *et al.*, 2005) at low concentrations (EPA, 2003). Effects caused by sublethal contamination are not as easily observed such as lethal contamination

(Fernandez and Pinheiro, 2007). These chronic effects may be observed in the form of reproductive disturbances and subsequent population declines (Stewart and de Mora, 1990). The EPA, 2003 has set the following criteria in order to protect organisms from chronic effects; levels of TBT should not exceed 0.072 µg/l in freshwater and 0.0074 µg/l in saltwater environments.

The harmful effects of TBT were observed as early as 1975 in the Pacific oyster, *Crassostrea gigas* that were farmed in Arachon Bay, France (Alzieu, 2000b; Evans and Nicholson 2000). The oysters were severely affected by a complete lack of reproduction and juvenile recruitment and the appearance of shell calcification of adult oysters lead to stunted growth (Alzieu, 1986; Alzieu, 1998; Alzieu, 2000a; Dyrinda, 1992). Molluscs are known to be the most sensitive to TBT exposure (Alzieu, 2000b), particularly gastropods and oysters (Ten Hallers-Tjabbes, 2003). This is mainly due to their slow rates of metabolism and excretion (WHO, 1999).

Many organisms are sensitive to TBT and exhibit various effects at wide ranges of concentrations (Alzieu, 2000a; WHO, 1999). The most notorious effect of TBT is imposex (Bigatti *et al.*, 2009; Gibbs and Bryan, 1987; Negri and Marshall, 2009). Effects such as imposex can be induced at sublethal concentrations 1 ng/l (Alzieu, 1998; Alzieu, 2000b; Gibbs *et al.*, 1987; Nias *et al.*, 1993; Sayer *et al.*, 2006).

Apart from causing shell deformations in oysters and imposex in gastropods, TBT is also lethal, and has produced developmental, behavioural, and reproductive toxic effects as well as various other kinds of physiological effects (Horiguchi *et al.*, 2012; IMO, 2002; Murata *et al.*, 2008) toward a wide range of organisms (Sousa *et al.*, 2014) such as bacteria (Martins *et al.*, 2005), bivalves (Hagger *et al.*, 2006), cnidarians (Negri and Heyward, 2001; Negri and Marshall, 2009; Watanabe *et al.*, 2007), echinoderms (Marin *et al.*, 2000), amphibians (Guo *et al.*, 2010), fish (Nakayama *et al.*, 2004; Sarria *et al.*, 2011; Zhang *et al.*, 2007; Zhang *et al.*, 2008; Zhang *et al.*, 2009; Zheng *et al.*, 2005), algae (Mercier *et al.*, 1997; Sidharthan *et al.*, 2002), and plants (Jensen *et al.*, 2004) etc.

Tributyltin compounds cause a number of cellular, biochemical and molecular effects (Fent, 2006). Tributyltin causes cytotoxicity (including disruption of cell membranes),

apoptosis, DNA damage and inhibition of critical enzyme activity and affects ion-pumping (Alzieu, 1998; Alzieu, 2000b; Gadd, 2000; Hagger *et al.*, 2006; Negri and Marshall, 2009; Omae, 2006).

Tributyltin and its metabolites have been found in various tissues of marine organisms (Antizar-Ladislao, 2008) such as squid (Yamada *et al.*, 1997), sharks and tuna (Kannan *et al.*, 1996; Ueno *et al.*, 2004), marine mammals e.g. dolphins, whales, seals (Tanabe *et al.*, 1998), sea otters (Kannan *et al.*, 1998; Murata *et al.*, 2008), dugongs (Harino *et al.*, 2007), and terrestrial organisms such as bats (Lilley *et al.*, 2013), racoons (Takahashi *et al.*, 1999), and birds (Guruge *et al.*, 1997). Their ubiquitous presence in higher trophic animals around the world (Guruge *et al.*, 1997) have raised concerns regarding the extent to which these organisms accumulate these compounds (Evans, 1999) as they cause negative effects, such as immune suppression (Choi *et al.*, 2013; Harino *et al.*, 2007; Kannan *et al.*, 1996; Kannan *et al.*, 1998).

Unlike other pollutants, TBT accumulation does not necessarily depend on lipid content (Ueno *et al.*, 2004). Organotins have a higher affinity to proteins than to lipids and therefore accumulate at higher levels in the liver and kidneys than in most organs (Kannan *et al.*, 1996; Omae, 2003; Strand and Jacobsen, 2005; Tanabe, 1999; WHO, 1999; Ueno *et al.*, 2004). Tributyltin possesses both lipophilic and ionic properties; its lipophilic characteristic encourages its accumulation in lipids, and its ionic property enables TBT to bind to macromolecules such as glutathione, which is found in the liver and kidneys of mammals (Kannan *et al.*, 1996; Kannan and Falandysz, 1997).

Lipid-soluble substances such as TBT have a low solubility in aquatic environments and show a strong tendency for bioaccumulation (Stewart and de Mora, 1990) and potential trophic transfer in aquatic food webs, which may adversely affect human health and the environment (Antizar-Ladislao, 2008; Lilley *et al.*, 2013; Kannan and Falandysz, 1997; Radke *et al.*, 2008).

Little is known about species-specific processes of accumulation and ecotoxicological implications of organotins for food webs, as there are differences in the accumulation potential between species, even between species at the same trophic level. This may

be attributed to differences in exposure routes, metabolism and elimination of organotin compounds (Lilley *et al.*, 2013; Strand and Jacobsen, 2005; Tanabe *et al.*, 1998; Yang *et al.*, 2006).

Acute exposure to TBT in humans can cause irritation of the respiratory tract, eyes, and skin (ATSDR, 2005; WHO, 1999). Organotins have been detected in human tissues (Sousa *et al.*, 2014) such as blood and liver (Aluoch and Whalen, 2005; Antizar-Ladislao, 2008; Kannan and Falandysz, 1997; Kannan *et al.*, 1999; Takahashi *et al.*, 1999). The toxicology of organotin compounds to humans is not fully understood, but the organotin toxicity to animals may be extrapolated to humans (Cao *et al.*, 2009; WHO, 1999; Yang *et al.*, 2006).

The assessment of human health usually involves identifying acute and chronic toxicity of the chemical to substitute mammalian species such as rats and mice (LeBlanc and Bain, 1997). Various effects have been observed in rodents when exposed to TBT such as immune suppression, developmental effects, and reproductive toxicity (ATSDR, 2005; Chen *et al.*, 2008; Hoch, 2001; Kishta *et al.*, 2007; Sekizawa *et al.*, 2003; WHO, 1999).

Tributyltin may be responsible for weight gain in mammals, as an obesogen (Grün and Blumberg 2006; Iguchi *et al.*, 2007). TBT also disrupts the immune cell's function by inhibiting the activity of natural killer (NK) cells, which are used to reduce the immune system's ability to combat viral infection and tumours in animals and humans (Aluoch and Whalen, 2005; Thomas *et al.*, 2004; Whalen *et al.*, 1999).

2.2.4 Sources

The main source of TBT pollution is from antifouling paints used on ocean-going vessels (Alzieu, 2000b; Axiak *et al.*, 2000; Antizar-Ladislao, 2008; ATSDR, 2005; Chiavarini *et al.*, 2003; Du *et al.*, 2014; Fent, 2006; Laughlin *et al.*, 1988; Sousa *et al.*, 2009). Other sources include commercial harbours, marinas, ferry terminals, shipyards, dry dock and naval facilities, moorings, fishing harbours, and areas associated with high boating activity (Champ, 2000; Clark *et al.*, 1988; Evans and Nicholson 2000). In harbours, and areas mentioned previously, pollution is

concentrated, because there is normally reduced water exchange and dilution from outside (Sindermann, 2006). Antifouling paint particles shed from abandoned structures, grounded ships, and particles generated during the maintenance of boat's hulls, provide significant, localised sources of recent and historic use to the environment during the resuspension or dredging of sediment to the overlying water column (Radke *et al.*, 2008; Ruiz *et al.*, 2008; Smith *et al.*, 2006; Turner, 2010). Tributyltin concentrations can be expected to remain high in sediments from harbours, marinas dockyards etc., not only from a few vessels that are illegally coated with it, but from sources of TBT not derived from anti-fouling (AF) paints, such as preservative or disinfecting agents (Sousa *et al.*, 2009). Shipping lanes are expected to have higher TBT concentrations than more remote areas, due to frequent TBT input from shipping traffic (Dafforn *et al.*, 2011; Strand *et al.*, 2003).

Contaminated sites cause significant environmental hazards to aquatic ecosystems. They are important sources of pollution and may result in ecotoxicological effects. At severely contaminated sites, acute effects may occur, but the core problem lies in possible long-term chronic effects (Fent, 2003).

2.2.5 Routes of exposure

The movement of a chemical through various environmental matrixes to the organism is known as the exposure pathway and the way, in which the chemical moves from the exposure medium into the body, e.g. ingestion, inhalation or dermal contact is referred to as the route of exposure (Rodricks, 2007).

Aquatic organisms are directly exposed to contaminants in water and sediment (Sekizawa *et al.*, 2003). In molluscs, uptake from food is more important than the direct uptake from water (WHO, 1999). TBT accumulated by fishes is also mainly via their food (Lee *et al.*, 2006). Dolphins and higher trophic level predatory fish, such as sharks and tuna, may be exposed to organotins through their diet (Kannan *et al.*, 1996). After toxicants enter marine organisms via food, water, or sediment, they can be stored or eliminated, depending on the organism's metabolism (Schratzberger *et al.*, 2002). Organotins are bioconcentrated from water and bioaccumulated through aquatic food webs (Sekizawa *et al.*, 2003), but as previously mentioned, little is known

about the accumulation and toxic effects in higher trophic levels (Hoch, 2001). The effects of TBT contamination are not necessarily limited to organisms living in aquatic environments, but also to those that consume organisms from these areas (Cao *et al.*, 2009).

Humans are exposed to organotin compounds in multiple ways (Sousa *et al.*, 2014), but the main route of exposure for humans and other animals is the dietary intake of TBT compounds (Antizar-Ladislao, 2008; Newman, 2010; Nielsen and Strand, 2002; Sekizawa *et al.*, 2003; WHO, 1999). Tributyltin has been detected in foods such as dairy products, seafood, meat, farm products, and liquid foods (Aluoch and Whalen, 2005; Cao *et al.*, 2009). Organotins can also be introduced into food products through its consumption by livestock, especially poultry, through the ingestion of contaminated fish and gastropods (Cao *et al.*, 2009). Seafood is one of the main sources of protein and forms an important part of the diet for humans (Yang *et al.*, 2006). Seafood such as fish, mussels and crabs collected from the marine environment contain various concentrations of OTCs, thereby exposing humans and possibly affecting their health (ATSDR, 2005; Hoch, 2001; Kannan and Falandysz, 1997).

The determination of organotin concentrations in seafood may help us to understand levels of contamination in certain coastal areas and to predict the possible health threat of organotin compounds (Lee *et al.*, 2006). The safe daily limit for TBT exposure in humans is 0.25 µg/kg body mass (Sarti *et al.*, 2011). In recent times, tributyltin levels in seafood have decreased; therefore, human exposure to organotins from other sources has become significant (Sousa *et al.*, 2014). Now, humans are mainly exposed to organotins during their everyday lives through direct contact with treated products like PVC pipes, PVC products etc. (ATSDR, 2005; Cao *et al.*, 2009; Hoch, 2001) and by inhalation and ingestion of dust that contains organotins (ATSDR, 2005; Kannan *et al.*, 1999; Sousa *et al.*, 2014).

2.2.6 TBT in the environment

The distribution and persistence of TBT in the aquatic environment is complex (Evans, 1999). Local conditions modify the ultimate persistence and fate of TBT in the environment (Langston and Pope, 1995). The persistence of OTCs in the environment

depends on physical (e.g. sorption to suspended solids and sediment), chemical (e.g. chemical and photochemical degradation) and biological (e.g. uptake and biological degradation) removal mechanisms (Senda *et al.*, 2009; Hoch, 2001).

Due to their hydrophobicity and high affinity for particulate matter, pollutants such as TBT accumulate in sediments (Hoch, 2001; Omae, 2006; Sousa *et al.*, 2014). Sediments act as a sink for organotin compounds released into the aquatic environment, and may exert toxic effects long after anthropogenic inputs have stopped (Langston *et al.*, 2009; Kot and Namiesnik, 2000; Li and Collin, 2009; Stewart and de Mora, 1990). Sediments also serve as important means to assess pollution as they can be used to establish a historical record of physico-chemical changes in the marine environment (Radke *et al.*, 2008). In sediment, TBT can reach concentrations several orders of magnitude higher than in the overlying water column (Langston and Pope, 1995). Sorbtion is responsible for reducing the bioavailability and toxicity of biocides in the water column and is the main pathway for accumulation in sediments (Voulvoulis *et al.*, 2002b). Between 10% and 95% of TBT introduced into water is estimated to undergo sorbtion onto particulate matter (WHO, 1999). The half-lives of organotin compounds in seawater vary from days to months, because they are readily sorbed onto suspended particles or sorbed by bacteria and algae (Sayer *et al.*, 2006; Stewart and de Mora, 1990; WHO, 1999), but those in sediment, especially anaerobic sediments, have been several years (Langston *et al.*, 2009; Dowson *et al.*, 1996; Kannan *et al.*, 1998; McClellan *et al.*, 2006; Omae, 2003; Omae, 2006). Harbours, ports and estuaries with heavily sedimented bottoms are at risk of being contaminated by TBT for many years, because it decomposes much slower in anaerobic sediments (IMO, 2002).

A scenario for maximum persistence of TBT in the environment would include an anaerobic environment sheltered from ultraviolet light, and an abundance of organic matter of small particle size onto which the organotins could sorb. This is similar to the properties of the estuarine benthic environment near many of the marinas and, except for some possible action by ultraviolet light. This is also similar to the inter-tidal areas inhabited by gastropods (Smith, 1981c).

Organotins can be remobilised through desorption (Ruiz *et al.*, 2008) and by disturbances such as storms and dredging (Fent, 2006; Stewart and de Mora, 1990). Desorption of pollutants from the sediment into the water column can lead to the disturbance of the marine ecosystem's balance (Radke *et al.*, 2008).

One reason for the persistence of organotins in the environment is their lipophilic character (Hoch, 2001). The lipophilic nature of TBT facilitates its bioaccumulation by living organisms (Alzieu, 1998). Bioaccumulation occurs in most aquatic organisms (WHO, 1999). The accumulation of TBT by higher trophic aquatic organisms occurs either via direct uptake from the water or in combination with diet (Hoch, 2001).

Tributyltin compounds concentrate in the sea surface micro-layer and in sediments, which can be harmful to organisms that dwell there (Clark *et al.*, 1988; Maguire *et al.*, 1982; WHO, 1999). Bioavailability is a key factor responsible for ecotoxicological effects of contaminants (Fent, 2003). The bioavailable fraction of TBT is important for uptake, and eventually, for the concentration at target sites within the organisms. Only the bioavailable fraction induces ecotoxicological effects (Fent, 2003). The concentrations of TBT in the water and/or sediment cause a directly related response to sediment-dwelling gastropods (Axiak *et al.*, 2000; Bigatti and Penchaszadeh, 2005).

Unlike other persistent organic pollutants (POPS), TBT has low vapour pressures indicating that these compounds are less volatile, have low mobility, and as a result have highly localised characteristics (Kannan and Tanabe, 2009; Hoch, 2001; Ueno *et al.*, 2004). However, they are ubiquitous in the environment and have even been found in remote areas such as the Great Barrier Reef, and in the Antarctic and Arctic (Negri and Marshall, 2009; Strand and Asmund, 2003). This demonstrates the global occurrence of TBT (Fent, 2006). The widespread contamination of TBT may result from water currents and international shipping traffic (Kannan and Tanabe, 2009). The long-range atmospheric transport of TBT and its metabolites is unlikely to be the source for TBT in the remote oceanic environment (Guruge *et al.*, 1997).

The process of methylation may produce volatile organotin compounds in sediments and this may lead to remobilisation of organotins into the water column and possibly into the atmosphere (ATSDR, 2005). The same processes that circulate

organochlorine chemicals around the globe may also result in unexpected distributions of OTCs (Sekizawa *et al.*, 2003). Decisions regarding the management of TBT and TPT in the environment need to consider regional and global scale pollution (Sekizawa *et al.*, 2003).

In the environment, TBT degrades in a stepwise manner to less toxic derivatives, DBT and MBT, and eventually into harmless inorganic tin in the environment (Clark *et al.*, 1988; Fent, 2006; Langston *et al.*, 2009; Omae, 2006) by the progressive loss of organic groups from the Sn cation (Alzieu, 1998; Hoch, 2001). Organotins, such as TBT are degraded by sunlight (photolysis), chemicals (mineral acid, carboxylic acid, alkalimetals etc), and microorganisms including other organisms such as mussels, fish and mammals to lower concentrations (Alzieu, 1998; Hoch, 2001; IMO, 2002; Landmeyer *et al.*, 2004; Omae, 2003; Omae, 2006; Radke *et al.*, 2008; Stewart and de Mora, 1990).

The rate of degradation is expressed as the proportion of metabolites remaining in the sample as a function of time, and is also a function of microbial populations in the water column, and temperature (low in winter) (Langston *et al.*, 2009). Degradation also depends on environmental conditions such as pH, turbidity and light (Alzieu, 2000b; Radke *et al.*, 2008; WHO, 1999). Hydrolysis of organotin compounds can occur under conditions of extreme pH, but this rarely occurs under normal environmental conditions (WHO, 1999). The temperature of waters influences TBT degradation. Tributyltin persists in colder rather than warmer waters (Negri and Marshall, 2009) as cooler temperatures inhibit the growth of TBT-degrading microorganisms (Stewart and de Mora, 1990).

Photolysis seems to be the most rapid mode of degradation in water (Clark *et al.*, 1988), but is limited by the depth of water as it becomes less effective the deeper the light penetrates (Clark *et al.*, 1988; WHO, 1999). Biological degradation is the major breakdown pathway of organotins in aquatic and sedimentary environments (Clark *et al.*, 1988), but it is inhibited by the high concentrations of OTC and other conditions affecting the life span of the microorganisms (Hoch, 2001; Stewart and de Mora, 1990).

2.2.7 Other possible pollutants

The use of imposex as a biomarker for TBT pollution is based on the assumption that no other pollutant, or environmental stress, is able to induce such a biological response (Axiak *et al.*, 2003). Despite the widespread use of imposex as a biomarker of TBT contamination, many questions remain unanswered such as what other factors may induce imposex (Garaventa *et al.*, 2006a).

Exposure to TBT compounds is the most probable cause of imposex in gastropods, but others have questioned that TBT may not be the only cause (Bryan *et al.*, 1988; Evans *et al.*, 1995; Evans, 1999; Evans and Nicholson, 2000; Evans *et al.*, 2000a; Matthiessen and Gibbs, 1998; Nias *et al.*, 1993).

Some studies have suggested that there are other possible inducers of imposex (Lahbib *et al.*, 2010). Imposex has been observed in gastropods when exposed to TPT (Camillo *et al.*, 2004; Horiguchi *et al.*, 1997; Oehlmann *et al.*, 2007; Schulte-Oehlmann *et al.*, 2000; Shim *et al.*, 2000; Stange *et al.*, 2012), which was used as a cotoxicant along with TBT, in antifouling paint (Fent, 2006; Lee *et al.*, 2006).

Bryan *et al.*, (1988) compared the effectiveness of TBT chloride and five other organotin compounds in promoting the development of imposex in the dog whelk *N. lapillus* and found that in *N. Lapillus* imposex was not a specific response to TBT since a much similar response is caused by Tri-n-propyltin. Nias *et al.*, (1993) reported that other factors such as copper and environmental stress may also induce imposex. There is a possibility that compounds in the birds excreta had caused imposex (Evans *et al.*, 2000a). Imposex may be caused by four types of compounds: tin (Sn) and/or Sn derivatives, tin 'cocktails', steroids, and other contaminants (Titley-O'Neal *et al.*, 2011). Stress may also play a role in the development of imposex (Garaventa *et al.*, 2008). Garaventa *et al.* (2006a) indicated that imposex occurred in gastropods prior to the use of TBT.

However, available environmental evidence points to TBT as the causal agent for imposex development in marine organisms (Abbott *et al.*, 2000; Chiavarini *et al.*, 2003; Evans *et al.*, 2000a; Gibson and Wilson, 2003; Oehlmann *et al.*, 2007). Axiak *et al.*,

2003 mentioned that imposex should still be considered as an effective biomarker of TBT pollution. Biological measurements should nevertheless be confirmed by chemical tests of TBT in tissue, sediment and/or water samples (Evans and Nicholson, 2000).

2.3 Endocrine disruption

Since industrialisation and urbanisation, vast amounts of chemicals have been released into the environment (Colborn *et al.*, 1993; Namiesnik and Szefer, 2010). They are a major cause for concern, as some have been known to have sub-lethal, yet harmful effects in animals and humans in the form of endocrine disruption (Colborn *et al.*, 1993; Crisp *et al.*, 1998; Duft *et al.*, 2007; Schulte-Oehlmann *et al.*, 2006; Sumpter, 2002).

Potential human health issues can include breast cancer and endometriosis in women, testicular and prostate cancer in men, abnormal sexual development, reduced male fertility, alteration in pituitary and thyroid gland functions, immune suppression, and neurobehavioral effects (Crisp *et al.*, 1998).

Inconsistencies remain regarding our knowledge of potential mechanisms of action of endocrine disrupting chemicals (EDCs), despite the possible biological link between a number of environmental pollutants, reproductive abnormalities, and cancers. Many endocrine-disrupting chemicals exhibit multiple mechanisms of toxicity acting at different sites within the body (Hagger *et al.*, 2006).

The US Environmental Protection Agency (EPA) define EDCs as 'an exogenous agent that interferes with the synthesis, secretion, transport, binding action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behaviour' (EPA, 1997).

Endocrine disruptors possess a wide range of characteristics (Schulte-Oehlmann *et al.*, 2006). Endocrine disrupting chemicals that are the most cause for concern are highly toxic; have high bioaccumulation potential; are highly persistent; biologically active at extremely low concentrations, and have low vapour pressures, which

facilitate their widespread dispersal (Colborn *et al.*, 1993; Schulte-Oehlmann *et al.*, 2006; Tanabe, 2002).

A group of pollutants that have the above physical and chemical characteristics are known as POPs (Stockholm Convention, 2008).

Persistent (long-lived) substances remain in an organism or its environment for an extended period and this may cause chronic effects. Persistence means that the risk of damage to the environment as well as fauna and flora remain for a long time even if emissions have stopped, because the quantities that have already entered the environment may remain there for many years (Bernes and Naylor, 1998).

Persistent organic pollutants may be transported over far distances by rivers, oceans and air. Even in low concentrations, these substances impact ecosystems and affect human health on a global scale (Bouwman, 2004).

On the 17th of May 2004, a multi-government response managed by UNEP Chemicals became an international convention called the Stockholm Convention on Persistent Organic Pollutants. The aim of this convention is to safeguard human and ecosystem health from the effects of exposure to POPs. South Africa joined the convention in 2005 (Bouwman, 2004).

Persistent organic pollutants do not only affect local ecosystems nearby pollution sources, but also areas such as the Arctic and the Antarctic (Tanabe, 2002). Endocrine disruption is a global phenomenon (Schulte-Oehlmann *et al.*, 2006) and it should be observed on a global basis like that of global warming (Tanabe, 2002).

Most examples of endocrine disruption in individuals are those from aquatic habitats or those that feed on aquatic organisms (Sumpter, 2002) and this may be because EDCs are especially persistent in these habitats (Porte *et al.*, 2006).

Endocrine disrupting chemicals affect reproductive hormone-receptor systems (Depledge and Billinghamurst, 1999) and this may alter the sexual characteristics of organisms (Newman, 2010). The nuclear receptors of hormone systems are more

than likely to be targets of endocrine disruptors, because their ligands are fat-soluble and low-molecular-weight agents, as are the environmental pollutants (Nishikawa, 2006). The disruption of hormonal systems can affect sexual organ development, behaviour and fertility (Newman, 2010).

Most studies on EDCs have focused on their effects on reproduction of individuals as it seems to be mostly affected and it is often observable (Sumpter, 2002; Warring and Harris, 2005).

Some chemicals may act by directly binding to receptors, acting either as hormone-mimics (agonistic effect) where the substance attaches to the receptor to produce hormone-like action or they may inhibit hormone action (antagonistic effect) where the substance binds to the receptor but does not activate it (Crisp *et al.*, 1998; Oetken *et al.*, 2004; Schulte-Oehlmann *et al.*, 2006). For example, estrogenic (agonistic) chemicals mimic estrogen by binding to estrogen receptors. Antagonistic or anti-estrogenic substances block receptors, preventing normal binding of estradiol (Newman, 2010).

Endocrine disrupting chemicals may also act indirectly by affecting the synthesis or availability of endogenous hormones (Warring and Harris, 2005) by interfering with biochemical processes associated with the production, availability, or metabolism of hormones (Oehlmann and Schulte-Oehlmann, 2003). They may also cause the enlargement of organs or tissues and cause tumour promotion under certain circumstances (Crisp *et al.*, 1998).

It is expected that EDC's mode of action is the same throughout the major evolutionary phyla and it is probable that EDCs affect all phyla studied so far (Warring and Harris, 2005); however, there are differences in susceptibility to EDCs between phyla which need further attention (Taylor and Harrison, 1999).

In the animal kingdom, endocrine systems of different phyla have evolved and diverged over time (Oehlmann and Schulte-Oehlmann, 2003). Vertebrate (fish, amphibians, reptiles, birds, and mammals) endocrine systems indicate many similarities and therefore they respond in a similar way, with a similar degree of

sensitivity, to both steroidal hormones and EDCs. However, it is very doubtful that all invertebrates respond to endocrine-disrupting chemicals as vertebrates do, and it is unlikely that all invertebrates respond in the same way (Sumpter and Johnson, 2005).

Over the years, a number of studies have provided evidence on endocrine disruption in invertebrates (Depledge and Billingham, 1999; Janer and Porte, 2007). However, the knowledge available is still lacking (Duft *et al.*, 2007; Lafont and Mathieu, 2007) as more comprehensive information regarding the effects on and mechanisms of action of EDCs in invertebrates has only been obtained from a few cases (Oehlmann and Schulte-Oehlmann, 2003). T

The limited information regarding endocrine disruption in invertebrates is partially because their hormone systems are rather poorly understood in comparison with vertebrates (Matthiessen and Gibbs, 1998; Oehlmann and Schulte-Oehlmann, 2003; Oetken *et al.*, 2004). This is a shame as some of the best-documented examples for harmful effects in wildlife populations from exposure to endocrine-active substances come from studies performed on invertebrates (Oetken *et al.*, 2004).

2.3.1 TBT as an endocrine disruptor

Tributyltin's high toxicity, persistence and bioaccumulation properties (Fent, 2006) make it a notorious endocrine disruptor (De Mora *et al.*, 2003; Giusti *et al.*, 2013; Guo *et al.*, 2010; Strand and Asmund, 2003). The best example of endocrine disrupting effects of pollutants is the case of TBT and imposex (Abidli *et al.*, 2009; Matthiessen, 2008; Nishikawa, 2006). The case of TBT and its population-level impact on caenogastropods proves the overall vulnerability of these invertebrates to EDCs (Alzieu, 2000b; Bryan *et al.*, 1986; Oehlmann *et al.*, 2007).

It is essential that more studies are designed and implemented to expose the links between pollution effects on individuals, and ultimate impacts on populations (Matthiessen, 2000; Sumpter, 2002).

2.4 Gastropods

Molluscs are one of the major groups of the animal kingdom (Hickman *et al.*, 2008). The word Mollusca is derived from the Latin word 'mollis', meaning soft (Fretter and Graham, 1962). The phylum name is based on its characteristic soft body usually enclosed within a hard, calcareous shell (Russell-Hunter, 1983). The Mollusca form the second largest invertebrate phylum with about 110 000 living molluscan species known to science (De Moor and Day, 2002; Matthiesen, 2008; Russell-Hunter, 1983). Only the phylum Arthropoda has more species (De Moor and Day, 2002; Morton, 1960). There are more than twice as many species of molluscs as there are vertebrates (Russell-Hunter, 1983). In southern Africa, there are over 5000 molluscan species (Branch *et al.*, 2010).

Molluscs are highly successful in terms of numbers of individuals and species because they have one of the most successful patterns of animal construction (Russell-Hunter, 1983). They also represent a variety of lifestyles (Matthiessen, 2008; Morton, 1960). They are found in a great range of habitats: from the tropics to the polar seas, at high altitudes, the abysses of the ocean, coral reefs, mudflats, deserts and forests, rivers, lakes, and underground (Hickman *et al.*, 2008). They feed on every possible food source and some are even parasitic. They vary in size from microscopic organisms to the giant squid (Hickman *et al.*, 2008).

There are three structurally distinct regions of molluscs, which show different types of growth and function, namely: the head-foot region, which consists of some nerve concentrations, most of the sense organs and all locomotory organs; the visceral mass (hump) which contains the digestive, reproductive and excretive organs; and the pallium (mantle) which hangs from the visceral mass, enfolds it and secretes the shell (Kensley, 1973; Russell-Hunter, 1983).

The major classes of Mollusca are Gastropoda, Bivalvia and Cephalopoda (Russell-Hunter, 1983). The Gastropoda are the most numerous (Kensley, 1973). They are also the most successful group of molluscs and are adapted to many ecological habitats (Day, 1974; Kensley, 1973). Their success can be attributed to their hard, calcareous shell, which protects them from predators and desiccation (Day, 1974).

Gastropoda are divided into the archaeogastropods (e.g., limpets and topshells), mesogastropods (e.g., periwinkles), neogastropods (e.g., whelks), tectibranchs (sea slugs), saco-glossids (sea slugs), pleurobranchomorphs (sea slugs), nudibranchs (sea slugs), and pulmonates (Matthiessen, 2008). Caenogastropods comprise the majority of the Mesogastropoda and all of the Neogastropoda (Ponder *et al.*, 2008). Caenogastropoda is the dominant group of marine gastropods in terms of species numbers, diversity of habit and habitat and ecological importance (Ponder *et al.*, 2008).

Many Caenogastropods are well-known marine snails and include the Littorinidae (periwinkles), Cypraeidae (cowries), Cerithiidae (creepers), Calyptraeidae (slipper limpets), Tonnidae (tuns), Cassidae (helmet shells), Ranellidae (tritons), Strombidae (strombs), Naticidae (moonshells), Muricidae (rock shells, oyster drills, etc.), Volutidae (balers, etc.), Mitridae (miters), Buccinidae (whelks), Terebridae (augers), and Conidae (cones) (Ponder *et al.*, 2008). There are also well-known freshwater families such as the Viviparidae, Thiaridae, and Hydrobiidae and a few terrestrial groups, Cyclophoroidea (Ponder *et al.*, 2008).

Molluscan health and diversity are important for various ecological and commercial reasons (Ketata *et al.*, 2008; Matthiessen, 2008; Rittschof and McClellan-Green, 2005). Molluscs are food for many vertebrates such as echinoderms, fish, birds, and mammals (Oehlmann *et al.*, 2007) and the biomass of these species dominates the lower trophic levels serving as a trophic link in many ecosystems (Ketata *et al.*, 2008; Russell-Hunter, 1983).

Molluscs are ecosystem engineers as they have important roles in biogeochemical cycling (e.g. detritus processing in both aquatic and terrestrial environments) (Ketata *et al.*, 2008). They have started receiving more attention as ecotoxicological test organisms due to their high diversity and ecological importance (Duft *et al.*, 2007), including their extremely high sensitivity to pollutants (Matthiessen, 2008; Rittschof and McClellan-Green, 2005) and highly conserved control and regulatory pathways that are often similar to vertebrate systems (Rittschof and McClellan-Green, 2005).

Despite their ecological importance, caenogastropods have played a minor role in ecotoxicological research and laboratory testing of chemicals compared to other invertebrate groups and this is mainly due to the fact that their life cycles are relatively longer compared to other invertebrates and that the maintenance of healthy populations requires greater technical effort, especially for species with a planktonic larval phase (Oehlmann *et al.*, 2007). Caenogastropods are one of the most endangered taxonomic groups in aquatic ecosystems, because of their long life spans and sensitivity to organic chemicals (Oehlmann *et al.*, 2007).

2.4.1 The gastropod reproductive system

Gastropods make use of various reproductive strategies including gonochorism (separate sexes), protandritic hermaphroditism (male-to-female sex change), protygynitic hermaphroditism (female-to-male sex change), reversible hermaphroditism (successive changing between male and female), simultaneous hermaphroditism, and parthenogenesis (asexual reproduction) (Kensley, 1973; Moore, 2006; Oberdörster and Cheek, 2000; Sternberg *et al.*, 2010). The variety of reproductive strategies makes these species very useful candidates for EDC research (Oehlmann *et al.*, 2007).

Fertilisation in gastropods can either be external (Kensley, 1973), where eggs and sperm are released into the water, or internal, (Hickman *et al.*, 2008; Moore, 2006) within the female's oviduct (Kensley, 1973).

In individuals with external fertilisation, the egg divides by spiral cleavage and ultimately forms a trochophore, which develops into a free-swimming veliger larvae. Other gastropods produce egg capsules, which are attached to substrata from where a miniature gastropod, characteristic of the particular species, emerges (Fretter and Graham, 1962; Hickman *et al.*, 2008; Kensley, 1973).

Caenogastropods are gonochoristic with internal fertilisation, through copulation, and development is partially or completely in an egg capsule that is generally externally deposited (Ponder *et al.*, 2008). Along the female duct there are numerous glands which can be viewed from the right side. The glands are: the albumen gland (secretes

the albuminous fluid), followed by a mass of deep brown tubules known as the sperm ingesting gland (ingests spermatozoa), and then the egg capsule gland (secretes the bulk of the wall of the capsule in which the eggs are laid). The eggs are attached to substrata by a special gland in the foot known as the pedal gland. The vagina opens on the right anterior extremity of the mantle cavity, ventral to the anus. It is where the penis is inserted, the sperm is deposited, and from which the fully formed egg capsules pass to the exterior. The ovipositor carries the egg capsule out of the mantle cavity where it is released (Fretter and Graham, 1962; Giese and Pearse, 1977).

In most male gastropods, sperm are transferred to the oviduct by a penis, which is situated behind the right cephalic tentacle. The vas deferens opens at or near the tip of the penis. The vas deferens may be enlarged and glandular, forming a prostate (Fretter and Graham, 1962; Giese and Pearse, 1977).

According to Connell *et al.* (1999), reproduction is the most significant stage in the life cycle of an individual as it determines the survival of organisms. The reduction in the fitness of individuals because of reproductive impairment is perhaps the most useful of sublethal effects measured by ecotoxicologists. Reproduction remains one of the most frequently measured qualities in both field and laboratory studies of effects of contaminants (Newman, 2010).

2.4.2 The gastropod endocrine system

The endocrine system consists of chemical messengers (hormones) that are secreted to control various processes of a multi-cellular organism (Ketata *et al.*, 2008). This system allows organisms to react to numerous external (environmental) and internal (physiological) signals (Ketata *et al.*, 2008). All known invertebrates, including molluscs, rely on hormones for the control of many processes in the body, including development, growth behaviour and reproduction (Depledge and Billingham, 1999; Matthiessen, 2008; Pinder *et al.*, 1999; Oehlmann and Schulte-Oehlmann, 2003; Oetken *et al.*, 2004). The molluscan endocrine system is the most diverse hormonal system of the invertebrate phyla (Ketata *et al.*, 2008). In vertebrates, hormones are secreted by true glands while in invertebrates, such as gastropods, secretory structures are neurosecretory organs or cells (Oehlmann and Schulte-Oehlmann,

2003; Oetken *et al.*, 2004; Sternberg *et al.*, 2010). The neurosecretory organs or cells occur in the cerebral, pleural, pedal and abdominal ganglia, and they produce neuropeptides with hormonal action (Ketata *et al.*, 2008; Matthiessen, 2008).

Molluscan neurohormones involved in reproductive processes include APGW amide, the caudodorsal cell hormone (CDCH), the dorsal body hormone (DBH), the egg-laying hormone (ELH) and molluscan-insulin-like peptides (MIPs) (Ketata *et al.*, 2008; LeBlanc *et al.*, 2005; Sternberg *et al.*, 2010).

There has been great preservation in the development of the endocrine system and there are some close similarities between the vertebrate's and invertebrate's endocrine systems (Matthiessen, 2008). Vertebrate-type steroids have been identified in a number of species of molluscs. Vertebrate-type sex-steroids such as testosterone, estradiol-17b, and progesterone are considered to be present in the main classes of molluscs, i.e., cephalopods, gastropods, and bivalves (Lafont and Mathieu 2007). It has been mentioned that vertebrate-type hormones may have a functional role in and/or maintaining imposex (Oberdörster and McClellan-Green, 2002; Oehlmann and Schulte-Oehlmann, 2003; Oetken *et al.*, 2004) and may play a role in molluscan sex differentiation and reproduction (LeBlanc *et al.*, 2005).

Some argue that vertebrate-type hormones may not be involved (Nishikawa, 2006; Scott, 2013). Sufficient knowledge regarding the role of vertebrate steroids in the endocrine system of most invertebrates is still lacking (Oberdörster and Cheek, 2000; Pinder *et al.*, 1999). The endocrine system of molluscs appears to be dissimilar in many aspects to those of vertebrates and sex-steroids might not have the same importance in all mollusc species (Ketata *et al.*, 2008; Scott, 2013). Sex steroids in molluscs appear to be chemically but not mechanistically or functionally identical with those in vertebrates (Matthiessen, 2008). A particular hormone may induce distinctly different responses in invertebrates and vertebrates (Depledge and Billingham, 1999). There has been no substantial evidence that vertebrate sex steroids have endocrinological or reproductive roles in molluscs (Scott, 2013). Scott, 2013, insists that it has never been more than a hypothesis that TBT affects gastropod reproduction by interfering with the testosterone production in these organisms. Therefore, some other mechanisms must be in play.

There is a striking difference between vertebrates and invertebrates with respect to their nuclear receptor sets (Nishikawa, 2006). Hormones need to bind to a receptor in order to function, but the absence of certain hormone receptors in gastropods (Köhler *et al.*, 2007; Oberdörster *et al.*, 2005; Nishikawa, 2006; Sternberg and LeBlanc, 2006) indicate that certain hormones found in gastropods, if functional, must act via some other signalling pathway other than those in vertebrates (Köhler *et al.*, 2007; Sternberg *et al.*, 2010). Endocrine systems are very complex and they vary among species and hinder the extrapolation of the effects of xenobiotics from species to species, not to mention from phyla to phyla (Janer and Porte, 2007).

2.5 Imposex

Imposex is a global phenomenon that affects female gastropods (Matthiessen and Gibbs, 1998; Rato *et al.*, 2008), both freshwater and marine (Oehlmann *et al.*, 1996a). Imposex was first reported in 1970 (Matthiessen and Gibbs, 1998) by Blaber, who noticed a penis-like outgrowth on female gastropods (Gibbs and Bryan, 1996). This condition was later termed by Smith (1971) as imposex, the superimposition of male sexual characteristics onto normal, dioecious, female gastropods.

Only a decade later, Smith, 1981a; Smith, 1981b; Smith, 1981c, discovered a relationship between imposex and areas with high boating activity, such as harbours and marinas where the compound TBT was frequently used in antifouling paints on marine vessels. Bryan *et al.*, 1986; Bryan *et al.*, 1987; Gibbs and Bryan, 1996; Gibbs and Bryan, 1987; Gibbs *et al.*, 1987, confirmed Smith's findings by using the dog whelk, *Nucella lapillus*, as a bioindicator of TBT pollution. They revealed that imposex intensity correlated to the amount of TBT bioaccumulated in *N. lapillus* and that imposex could be induced by TBT when populations of *N. lapillus* were transplanted from 'clean' sites to areas heavily polluted by TBT, e.g. harbours.

Molluscs, such as gastropods, are particularly sensitive to TBT pollution. This is mainly because they inhabit sediments where TBT is known to accumulate (e.g. harbours and marinas) (Morcillo and Porte, 1999) and they bioaccumulate TBT due to their feeding habits coupled with their inability to metabolise and excrete these compounds (de Lima *et al.*, 2006; Oehlmann *et al.*, 2007; Sternberg *et al.*, 2010).

Imposex has been recorded in approximately 200 species of gastropods (Horiguchi *et al.*, 2012). It has been documented globally, along the coasts of the United States of America (Smith, 1981c), Caribbean (Strand *et al.*, 2009), Mediterranean (Axiak *et al.*, 1995; Chiavarini *et al.*, 2003; Garaventa *et al.*, 2006b; Rato *et al.*, 2008; Sousa *et al.*, 2005); Brazil (Camillo *et al.*, 2004; Castro *et al.*, 2012a; Toste *et al.*, 2011), Chile (Gooding *et al.*, 1999) Argentina (Averbuj and Penchaszadeh, 2010; Bigatti *et al.*, 2009, Bigatti and Penchaszadeh, 2005; Penchaszadeh *et al.*, 2001), Greenland (Strand and Asmund, 2003), Iceland (Guðmundsdóttir *et al.*, 2011), United Kingdom (Bryan *et al.*, 1986; Gibbs *et al.*, 1987; Minchin *et al.*, 1997; Oliveira *et al.*, 2009), Netherlands (Sumpter, 2002), Germany (Schulte-Oehlmann *et al.*, 1997), India (Vishwakiran *et al.*, 2006), China (Cao *et al.*, 2009; Huang *et al.*, 2008), Thailand (Bech, 2002; Swennen *et al.*, 2009), Japan (Horiguchi *et al.*, 1995; Tan, 1997), Australia (Gibson and Wilson, 2003; Nias *et al.*, 1993; Rees *et al.*, 2001), New Zealand (Stewart *et al.*, 1992), Tunisia (Abidli *et al.*, 2009), South Africa (Marshall and Rajukumar, 2003) etc.

Few cases have been reported for sub-Saharan regions. Marshall and Rajukumar, (2003) who discovered imposex in *Nassarius kraussianus*, have carried out only one study in South Africa. Okoro *et al.* (2016) reported concentrations of TBT in Cape Town Harbour, but did not look at imposex. More data are needed regarding imposex in Africa, the Middle East, and Latin America (Titley-O'Neal *et al.*, 2011). The global occurrence of imposex in a large number of gastropod species and associated population declines in coastal areas emphasises the hazards and risks associated with these antifouling paints (Chiavarini *et al.*, 2003; Fent, 2006).

The main gastropods species used worldwide as sensitive bioindicators of exposure to organotin contamination are from the Muricidae family (Castro and Fillman, 2012), especially those belonging to the genus *Nucella* (Gooding *et al.*, 1999). Other families that are affected include Buccinidae, Nassariidae, Conidae etc. (Horiguchi *et al.*, 2012; Oetken *et al.*, 2004).

TBT accumulation and the effect of imposex vary according to the species of gastropod (Barreiro *et al.*, 1999; Evans and Nicholson 2000; Gibbs, 1996; Ketata *et al.*, 2008; Matthiessen and Gibbs, 1998). The difference in TBT accumulation may be

explained by their diverse ecology (Schulte-Oehlmann *et al.*, 1998) and diet, which affects the TBT body burden of gastropods in direct relation to the trophic level they consume through the food web (Oehlmann *et al.*, 1998a). Predatory gastropods accumulate more TBT, followed by scavengers, herbivores, and lastly freshwater species (Schulte-Oehlmann *et al.*, 1998). The reason predators accumulate more organotins may be due to biomagnification (Schulte-Oehlmann *et al.*, 1998).

TBT accumulation differs according to their sex; females may accumulate more TBT than males because they degrade TBT slower, have higher food consumption (accumulate TBT via food web), and they have a higher lipid content (Schulte-Oehlmann *et al.*, 1995; Stroben *et al.*, 1992).

The extent to which gastropods are affected by imposex depends on their reproductive strategy. Some species develop directly from egg to snail, while others have a mobile larval stage (Sternberg *et al.*, 2010). Unlike gastropods that develop directly, gastropods with a free-swimming larval stage, known as veliger larvae (Hickman *et al.*, 2008), are less susceptible to population decline and local extinction, because they are capable of extensive dispersion and can therefore colonise areas over a wide range (Bryan *et al.*, 1987; Sousa *et al.*, 2009; Sternberg and LeBlanc, 2006; Stewart *et al.*, 1992).

Reproduction in some populations is affected by imposex to the point of extinction, while in others it is not affected at all (Axiak *et al.*, 2003; Matthiessen and Gibbs, 1998; Swennen and Horpet, 2008). There are several species that do not display a response to TBT exposure (Titley-O'Neal *et al.*, 2011). Imposex has been discovered in species before TBT introduction and for some it may occur naturally (Garaventa *et al.*, 2006a; Titley-O'Neal *et al.*, 2011). This 'pseudo-imposex', not induced by TBT, occurs in female gastropods from the Volutidae family (Swennen and Horpet, 2008).

Female gastropods affected by imposex are characterised by the presence of normal female sexual anatomy (albumen gland, ingesting gland etc.) and supplementary male accessory sex organs such as the sperm duct, seminal vesicle, external sperm groove and penis (Lima-Verde *et al.*, 2010; Matthiessen 2013; Oberdörster and McClellan-Green, 2002; Oehlmann *et al.*, 1996b; Sternberg *et al.*, 2010). They start to develop a

penis and vas deferens similar to that of the male of the same species (Axiak *et al.*, 2003; Gibbs and Bryan, 1987; Oehlmann and Schulte-Oehlmann, 2003; Shi *et al.*, 2005). First, it starts as a formation of a bud penis followed by the development of the vas deferens instead of the penis (Gibbs and Bryan, 1987; Lahbib *et al.*, 2010) behind the right tentacle (Lima-Verde *et al.*, 2010). TBT causes a reduction of female glands, e.g. albumen, ingestion and capsule glands (Oehlmann *et al.*, 1996a).

In extreme cases where there is considerable contamination (>6-8 ng/l TBT) (Gibbs and Bryan, 1987), imposex obstructs the oviduct in *N. lapillus* with the imposed male tissue and prevents the release of egg capsules (Bryan *et al.*, 1986; Bryan *et al.*, 1987; Gibbs *et al.*, 1987; Gibbs and Bryan, 1986; Gibbs *et al.*, 1988). The subsequent accumulation of unlaidd capsules may rupture the capsule gland (Evans *et al.*, 2000a).

In *O. erinacea* and *U. cinerea*, the edges of the developing oviduct fail to fuse so that a gutter-like structure appears in the adult female; this inhibits the formation of egg capsules because material normally secreted to manufacture egg capsules would spill into the pallial cavity therefore disrupting the transfer of packages of eggs to the exterior through the vulva (Gibbs *et al.*, 1990; Gibbs, 1996). Both effects interfere with sexual development (Matthiessen and Gibbs, 1998). This may have severe consequences for gastropod populations causing sterility, female mortality, reduced fecundity and ultimately population decline, and, in worst cases, local extinction (Evans *et al.*, 2000a; Gibbs *et al.*, 1986; Ketata *et al.*, 2008; Oehlmann *et al.*, 1996b. Oehlmann *et al.*, 1998a; Schulte-Oehlmann *et al.*, 1997; Shim *et al.*, 2000; Stroben *et al.*, 1992).

Certain populations of females are capable of recolonizing areas highly polluted by TBT as they may develop a condition, known as Dumpton Syndrome (DS) (Gibbs, 2005). DS is a phenomenon that occurs in female populations of *N. lapillus*. Females affected by DS are characterised by a reduction in the effect of imposex (penis development may be lacking and vas deferens formation may be weak) (Barreiro *et al.*, 1999; Gibbs, 2005). It is a recessive condition that causes the underdevelopment of the reproductive tract in males of *N. lapillus* and in its extreme for can lead to a lack of a penis (aphally) and an incompletely developed vas deferens.

Male gastropods may also suffer from the consequences of TBT pollution. In some cases, when imposex levels are extremely high, the male penis may become deformed and mating may be prevented (Bryan *et al.*, 1987). It is disadvantageous to male *N. lapillus* gastropods because it can affect them to an extent where they are incapable of breeding (Gibbs, 2005). However, it does benefit female *N. lapillus* populations, because it increases their survival rate by reducing the sterilising effect of imposex by limiting the development of the vas deferens. In DS affected females, the vas deferens is less inclined to develop thick enough to interfere with normal egg laying (Gibbs, 2005). It has been suggested that natural selection has increased the frequency of DS due to the fitness advantage it represents for females in those areas (Barreiro *et al.*, 1999).

Imposex is irreversible (Axiak *et al.*, 2003; Bryan *et al.*, 1986; Gibbs *et al.*, 1987; Gibbs, 2008; Matthiessen and Gibbs, 1998; Matthiessen, 2013); therefore, the occurrence of a penis in females may be difficult to distinguish whether it is from previous or recent exposures (Axiak *et al.*, 2003; Titley-O'Neal *et al.*, 2011).

Populations with high a degree of imposex are characterised by a low percentage of females (male-biased) and an absence of juveniles and egg capsules (Bryan *et al.*, 1986; Bryan *et al.*, 1987). An indication that TBT pollution is decreasing would be when affected populations become recolonized by younger, imposex-free individuals (Axiak *et al.*, 2003; Matthiessen, 2013; Sousa *et al.*, 2005). Other indications include a decrease in imposex severity, increased fecundity, and the presence of juveniles and/or females (Titley-O'Neal *et al.*, 2011). Recolonisation and recovery will only take place over the long term if the remaining uses of TBT are effectively managed and the development and use of less environmentally harmful alternatives occurs (Matthiessen and Gibbs, 1998).

Intersex, as compared to imposex, is the disturbance of the congruity between gonad and genital tract (Bauer *et al.*, 1995). It occurs in the female periwinkle *Littorina littorea* when exposed to TBT. Littorinds that are affected by intersex are characterised by the development of male features on the female organs or female sex organs are replaced by male formations (Oehlmann and Schulte-Oehlmann, 2003). No superimposition of male characters occurs, but the organs of the oviduct are modified

towards a male morphological structure such as a non-functional prostate gland (De Wolf *et al.*, 2004; Strand and Asmund, 2003). Intersex can reduce fecundity due to sterility; however, populations are unlikely to become extinct owing to free-swimming veliger larvae of the species (Oehlmann *et al.*, 1996a). Intersex index (ISI) calculated as the average intersex stage of a population and is used to measure intersex intensity (Oetken *et al.*, 2004).

Dogwhelks are especially sensitive to TBT (Minchin *et al.*, 1997), for example, in *N. lapillus*, imposex can be induced at extremely low levels (1 ng/l) (Bryan *et al.*, 1986). Concentrations of more than 2-4 ng/l Sn are capable of inhibiting breeding activity which causes sterility, subsequent population decline, and ultimately local extinction (Chiavarini *et al.*, 2003; Gibbs, 1996; Gibbs and Bryan, 1987). The periwinkle, *L. littorea*, are less sensitive than other species and can be used to evaluate TBT pollution where other, more sensitive, gastropods are absent by looking at intersex incidence (Schulte-Oehlmann *et al.*, 1998; Minchin *et al.*, 1997). Imposex is used to monitor areas with low to moderate TBT pollution (Oehlmann *et al.*, 1998a). Intersex can be used to monitor TBT pollution where imposex-affected species, such as dogwhelks are absent, especially in heavily polluted areas, because *L. littorea* are more tolerant to high TBT concentrations (Bauer *et al.*, 1995; Bauer *et al.*, 1997; Minchin *et al.*, 1997; Morton, 2009; Oehlmann *et al.*, 1998a; Oehlmann and Schulte-Oehlmann, 2003; Oetken *et al.*, 2004).

Imposex is a specific response to organotin pollution (Oehlmann and Schulte-Oehlmann, 2003; Oetken *et al.*, 2004; Santos *et al.*, 2005; Strand *et al.*, 2009) especially TBT (Barroso *et al.*, 2002; Ketata *et al.*, 2008; LeBlanc *et al.*, 2005) and to a certain extent, TPT pollution (Barroso *et al.*, 2002; Horiguchi *et al.*, 1997; Rato *et al.*, 2008). Imposex intensity is related to TBT bioavailability in the environment (Axiak *et al.*, 1995; Bigatti *et al.*, 2009; Matthiessen and Gibbs, 1998; Penchaszadeh *et al.*, 2001; Ten Hallers-Tjabbes *et al.*, 2003). Populations that inhabit areas with high boating and shipping activity show the highest degree of imposex (Bryan *et al.*, 1986; Smith *et al.*, 2006; Sousa *et al.*, 2005), but imposex prevalence decreases as distance increases from these 'hot spots' of pollution and become null in areas without boating traffic (Bigatti *et al.*, 2009; Evans and Nicholson, 2000; Li and Collin, 2009; Minchin *et al.*, 1997). The coincidence between high degrees of imposex and proximity to

harbours and marinas indicates that the pollutant is almost certainly associated with shipping or boating activity (Bigatti *et al.*, 2009; Bryan *et al.*, 1986).

2.5.1 Imposex indices

A number of indices are used to quantify imposex (Gibbs and Bryan, 1996). The main indices used are: Percentage Imposex (% I), Mean Male Penis Length (mMPL), Mean Female Penis Length (mFPL), Male to Female ratio (M:F), Relative Penis Length Index (RPLI), Relative Penis Size Index (RPSI), Vas Deferens Sequence Index (VDS/VDSI), etc. (Titley-O'Neal *et al.*, 2011).

Percentage imposex, the proportion of females with imposex in a population, is the most popular and simplest index available (Gibbs and Bryan, 1996). Before TBT regulations, there was no differentiation between highly polluted sites, because most populations of females had imposex and therefore the index was not as effective, but since TBT levels have declined, it has become useful (Titley-O'Neal *et al.*, 2011).

Sites heavily polluted by TBT have fewer females due to imposex, and therefore consist mostly of males. The M:F is used to describe whether imposex affected populations diverge from the normal 1:1 ratio of male to female gastropod populations (Titley-O'Neal *et al.*, 2011).

Indices related to penis length measures are mFPL, mMPL RPLI and RPSI. The mFPL and mMPL are required to calculate RPSI and RPLI, but they may be used as individual indices to measure imposex severity (Titley-O'Neal *et al.*, 2011). The sizes of female penises within any one population are usually found to be remarkably consistent and the mean lengths provide a useful parameter (Gibbs and Bryan, 1996). The intensity of imposex may be used to assess TBT pollution (Schulte-Oehlmann *et al.*, 1995). Both the RPSI and RPLI are used for this purpose (Gibbs *et al.*, 1987; Titley-O'Neal *et al.*, 2011).

Relative penis length index is calculated as the $\text{mFPL/mMPL} \times 100$. Length is the most useful parameter of penis size to measure; however, it does not take mass into consideration (Bryan *et al.*, 1986; Gibbs *et al.*, 1987).

Body size, including penis size can differ significantly between populations (Gibbs *et al.*, 1987). Variability is reduced by calculating the RPS value based on a comparison of female and male penis size in individuals from the same population. Relative penis size index is the $(mFPL)^3 / (mMPL)^3 \times 100$ for the population (Bryan *et al.*, 1986; Gibbs *et al.*, 1987; Gibbs, 1996). The RPS index for any population is defined as the mean bulk of the female penis and is expressed as percentage of the mean bulk of the male penis. The bulk of a penis is calculated as the cube of its length. The rationale for this is that length is a convenient measure or surrogate of tissue mass (Tan, 1999). An RPS value of 50% indicates that the mean penis size of the female is half the bulk of that of the male (Bryan *et al.*, 1986; Gibbs *et al.*, 1987).

When the degree of imposex exceeds 70%, male penises become extremely deformed, such as penises that are three times their normal size, and bifurcation of the penis (Bryan *et al.*, 1986). In cases like these, mean male penis lengths may be greater in such populations, and therefore the highest degrees of imposex may be underestimated (Bryan *et al.*, 1986). In cases where the occurrence of a penis is always 100% in females and the RPSI does not vary, it can be assumed that the population has pseudo-imposex where female penises occur naturally (Swennen and Horpet, 2008). For comparisons among populations, the RPSI has demonstrated that it is a reliable measure (Gibbs *et al.*, 1987) and a simple and effective way of estimating the level of imposex (Gibbs and Bryan, 1996) except where contamination is severe (Gibbs *et al.*, 1987). Accurate assessment of penis size is a necessary step towards determining the severity of imposex (Tan, 1999).

The RPSI does not identify the presence of sterilised females and therefore a VDSI was developed (Gibbs *et al.*, 1987). Structural changes in the development the vas deferens are assessed using the VDSI (Gibbs and Bryan, 1996). The vas deferens sequence index denoted in the literature as VDS/VDSI is the mean imposex stage of a population (Titley-O'Neal *et al.*, 2011). This is based on the division of imposex into six stages ranging from early development to the final stage of sterilisation; from stage 0 (normal) to stage 6 (grossly affected) (Bryan *et al.*, 1988; Gibbs *et al.*, 1987; Oehlmann *et al.*, 1996a) and therefore VDS provides a more sensitive method of categorising the intensity or expression of imposex (Gibbs *et al.*, 1987). Many have modified the VDSI (Oehlmann *et al.*, 1991; Shi *et al.*, 2005) as each species has their

own penis/imposed penis characteristics. What applies to one species may not necessarily apply to another (Tan, 1999). It allows the interspecific comparison of the TBT sensitivity of different species and informs about the reproductive capability of a population (Oehlmann *et al.*, 1996a).

2.5.2 Pathway of TBT-induced imposex

Morphological characteristics of imposex have to begin with changes in biochemical levels in gastropods (Shi *et al.*, 2005). Tributyltin's mode of action at environmental concentrations causes imposex in female caenogastropods via endocrine disruption, but proving the biochemical mode of action for a given compound remains difficult (Matthiessen, 2008; Oehlmann *et al.*, 2007; Omae, 2003).

The link between imposex and TBT exposure has been well established (Gibbs, 1996) and various studies have indicated that TBT has various negative impacts on female gastropod populations, but the underlying mechanism of imposex is still unknown due to a lack of knowledge regarding their endocrinology (Cajaraville *et al.*, 2000; Evans *et al.*, 2000a; Janer and Porte, 2007; LeBlanc *et al.*, 2005; Lima *et al.*, 2011; Morcillo and Porte, 1999; Oberdörster *et al.*, 2005; Schulte-Oehlmann *et al.*, 2000; Sternberg *et al.*, 2010).

Several hypotheses regarding the underlying mechanism of imposex induction have been proposed:

- Tributyltin induces imposex through an increased androgen level by inhibiting the activity of the P450 aromatase enzyme, which catalyses the aromatisation of androgens to estrogens (Bettin *et al.*, 1996);
- Tributyltin causes the accumulation of androgens in tissues as it decreases the elimination of testosterone as sulphate conjugates (Ronis and Mason, 1996);
- Tributyltin leads to elevated levels of free testosterone due to the inhibition of esterification of testosterone to fatty acid esters (Gooding *et al.*, 2003; LeBlanc *et al.*, 2005);

- Tributyltin may act as a neuro-toxicant by abnormally releasing APGWamide, which is a common peptide found in many species of molluscs, and is often associated with sex organ growth or reproductive behaviour (Oberdörster and McClellan-Green, 2002; Oberdörster *et al.*, 2005);
- Tributyltin may affect reproductive processes through DNA damage pathways (Hagger *et al.*, 2006);
- The most promising mechanism of TBT induced imposex may be through the involvement of the retinoid X receptor (RXR) signalling pathway (Lima *et al.*, 2011; Nishikawa *et al.*, 2004; Nishikawa *et al.*, 2006; Stange *et al.*, 2012; Sternberg *et al.*, 2010). TBT may act as an agonist of the RXR (Horiguchi *et al.*, 2008; Nishikawa *et al.*, 2004; Nishikawa *et al.*, 2006). TBT has been revealed as a high affinity ligand for the RXR, and the RXR ligand 9-cis retinoic acid causes imposex. TBT may cause imposex through the inappropriate activation of this signalling pathway (Sternberg *et al.*, 2010). TBT and TPT exert their actions via their interaction with the RXR (and not via androgens) (Scott, 2013).

Some authors mention that imposex may not only occur via a single pathway, but through multiple pathways that occur simultaneously (Matthiessen, 2008; Santos *et al.*, 2005; Stange *et al.*, 2012).

2.6 Biomonitoring and gastropods

Ecosystems consist of communities made up of individuals of numerous species. Observing the responses of these individuals and species will provide an indication of the effect of changes in the ecosystems where they live (Fernandez and Pinheiro, 2007; Thain *et al.*, 2008). Biological-effect methods can reveal connections between contaminants and ecological responses and are therefore important components in environmental monitoring programmes (Thain *et al.*, 2008). However, in order for these methods to be useful to make important decisions regarding the contaminants, it is necessary to determine the cause and effect relationship between the presence of the toxin and the response (Thain *et al.*, 2008).

Over the past decade, an increasing number of techniques (e.g. bioassays, biomarkers, and diseases) to measure the biological effects of contaminants have been incorporated into national and international monitoring activities (Thain *et al.*, 2008). A frequently used approach to evaluate ecological impacts of contaminated sites is field monitoring. This includes structural and functional measurements of local biota, and sometimes in situ (caged) studies (Fent, 2003).

Biological monitoring is the use of organisms to monitor contamination and to imply the possible effects on biota or sources of toxicants to humans (Dallas and Day, 2004; Rand, 1995). The application of biomarkers use biochemical, physiological, morphological or histological changes in individuals that are used during biomonitoring to imply presence of significant contamination or as early warning systems for future effects (Depledge and Billingham, 1999; Huggett *et al.*, 1992; Newman, 2010).

A fundamental concept used in biomonitoring is the biological indicator or indicator species (Rand, 1995). The condition of an ecosystem is evaluated by using biological indicators (Dallas and Day, 2004; Fernandez and Pinheiro, 2007; Huggett *et al.*, 1992; Jamil, 2001). Indicator species are valuable as they provide early warning signals of harm to the environment (Jamil, 2001). This allows the initiation of interventions and bioremediation strategies before irreversible environmental damage of ecological consequences occurs (Cajaraville *et al.*, 2000). Organisms that are affected at trace levels are the most effective i.e. high sensitivity (Terlizzi *et al.*, 2004). Other important characteristics for the selection of potential indicators are their abundance and distribution (Bech, 2002).

Molluscs are good indicators of contamination in aquatic and marine environmental monitoring and toxicology (Rittschoff and McClellan-Green, 2005). They are frequently used in biological monitoring of heavy metals and organic products of anthropogenic sources (Jamil, 2001) such as TBT. Reasons for this may be because of their relatively territorial/sedentary habit coupled with other criteria such as longevity, abundance, ease of collection and size, high bioconcentration factors and biological sensitivity to organotins (Langston and Pope, 2009; Jamil, 2001).

Molluscs are bioaccumulators as they accumulate contaminants through ingestion of food and other metal containing substances through direct sorbtion of dissolved constituents (Jamil, 2001). Bioaccumulation of organotins in molluscs is determined by their feeding habits, which differs between carnivores, suspension feeders and deposit feeders, according to the bioavailability and concentration in food and sediments as well as in water (Langston and Pope, 2009). The sensitivity of molluscs to the effects of TBT is partly because of TBT's bioaccumulation potential (Langston and Pope, 2009).

Marine gastropods are the most sensitive animals to environmental contamination due to organotin compounds (Abidli *et al.*, 2009). Gastropods that are affected by imposex may be used as sensitive bioindicators of TBT contamination (Lahbib *et al.*, 2010; Minchin *et al.*, 1995) as the distribution of imposex is related to boating activity (Oehlmann *et al.*, 1996a; Smith, 1981a, 1981b). Smith (1981c) first showed the relationship between imposex and organotins, mainly TBT, in England.

Various gastropod species inhabit the same sampling site (sympatric) which allows a direct comparison of their sensitivity to TBT by observing their development of imposex (Oehlmann *et al.*, 1996a). The development and effect of imposex differs significantly among species (Stewart *et al.*, 1992). Differences in habitat, diet and physiology are possible factors that may influence the varying response between species (Tan, 1999). The three closely related gastropod families that are mostly used to monitor TBT contamination worldwide are the Muricidae, Buccinidae and Nassariidae (Morton, 2009; Titley-O'Neal *et al.*, 2011). Muricidae are the most accessible (Gibbs *et al.*, 1997) and exhibit greatest TBT sensitivity (Gibbs *et al.*, 1997). The top four genera are: *Thais* spp., *Nassarius* spp., *Conus* spp., *Nucella* spp., (Titley-O'Neal *et al.*, 2011).

Imposex is used as a valuable bioassay approach to monitor organotin pollution (Lima-Verde *et al.*, 2010; Shim *et al.*, 2000; Sousa *et al.*, 2009) and to evaluate the associated ecological impact of endocrine disruption on the health and fertility of humans and wildlife (Axiak *et al.*, 2003; Oehlmann *et al.*, 1996b; Rato *et al.*, 2008). Imposex is therefore used extensively throughout the world along various coastlines (Evans *et al.*, 2000a; Oehlmann *et al.*, 1996a; Oehlmann *et al.*, 1998a; Rato *et al.*,

2008). It is assumed that no other pollutant or environmental stress is able to induce such a biological response (Axiak *et al.*, 2003; Terlizzi *et al.*, 2004).

The monitoring of imposex is important to establish the geographic distribution and temporal trends based on the bioavailabilities of organotins such as TBT in the marine environment (Smith, 1981a; Sousa *et al.*, 2009) in order to assess the effectiveness of the regulations and the continued impact of these pollutants from large vessels on marine biota (Alzieu, 2000b; Langston and Pope, 2009; Evans *et al.*, 2000a; Garaventa *et al.*, 2006a; Garaventa *et al.*, 2008; Oehlmann *et al.*, 1998b).

The use of organisms as a source of information on the environment provides valuable data on the health status of aquatic ecosystems, with numerous advantages over chemical analysis (Fent, 2006; Namiesnik and Szefer, 2010). Chemical analysis of environmental samples does not provide any indication of the disastrous effects of contaminants on the biota (Bech *et al.*, 2002; Cajaraville *et al.*, 2000). It is expensive, time consuming, and the sensitivity of the analytical instruments when measuring very low concentrations may be ineffective, especially for substances that may be present at low concentrations like TBT (Axiak *et al.*, 1995; Cao *et al.*, 2009; Dallas and Day, 2004; Duft *et al.*, 2007; Evans *et al.*, 1995). It is also possible that the snails have experienced TBT exposure, developed imposex, but at the time of sampling, that the TBT concentrations have dropped to below detectable concentrations. Due to the limitations of methods based on chemical determination of organotin concentrations, specific biological responses to TBT, such as imposex, are used as tools for biomonitoring purposes (Axiak *et al.*, 1995; Cao *et al.*, 2009; Evans and Nicholson 2000; Jamil, 2001). There is a disadvantage when using imposex to determine TBT pollution. Imposex in adult species with a long life span is indicative of long term exposure to TBT (Gibbs and Bryan, 1987). Since imposex is irreversible (Gibbs *et al.*, 1997), one cannot determine whether it was from past or present exposure to TBT that induced the condition (Bryan *et al.*, 1987).

Biomonitoring studies alone do not have substantial power to identify causative agents. Similarly, chemical analysis of pollutants in ecosystems alone cannot provide evidence for toxicological consequences in biota (Fent, 2003). Biological monitoring and chemical analysis are complementary (Dallas and Day, 2004). An integrated

approach considering environmental, chemical, toxicological and ecological concepts is needed for the understanding of ecotoxicological effects in contaminated ecosystems (Fent, 2003). Analysis of body burden of TBT can enhance the interpretation of the results and understanding of the time scale involved with the development of imposex (Bech *et al.*, 2002). Progress in the last decade has mainly been made in the global assessment of the organotin pollution using both chemical analysis and biomonitoring using imposex as a biomarker (Fent, 2006).

According to Wepener and Degger (2012), there is lack of a pollution-monitoring programme for South African oceans. The analysis of metal and organic pollutants is limited in South African ecotoxicology because of the high costs involved and the lack of accredited laboratories to perform the analysis (Wepener and Degger, 2012). There is insufficient information available regarding TBT contamination and its effects in the southern hemisphere (Marshall and Rajkumar, 2003), especially in Africa (Titley-O'Neal *et al.*, 2011). It is important to use imposex as a tool to monitor TBT along the coastline so that efficient and effective environmental management can take place.

2.7 Alternatives to TBT as antifoulants

The ban on TBT in antifouling systems has transformed the maritime paint industry (Almeida *et al.*, 2007; Yerba *et al.*, 2004) as it has been the main motivation in developing and replacing harmful organotin antifouling coatings with, hopefully, non-toxic alternatives (Chambers *et al.*, 2006; Qian *et al.*, 2010), which provide the same economic benefits as organotin compounds, while protecting the environment (Omae, 2003; Yerba *et al.*, 2004). There are a wide range of alternatives that are commercially available (Gipperth, 2009). More than 20 chemicals have been proposed as alternatives (Takahashi, 2009). They claim to be effective and environmentally friendly (Yerba *et al.*, 2004), yet there is a lack of knowledge regarding the environmental and economic impacts of these alternatives (Dafforn *et al.*, 2011; Gipperth, 2009; Konstantinou and Albanis, 2004; Qian *et al.*, 2010; Yerba *et al.*, 2004).

Available antifoulants fall into two basic types, those with tin-free biocides and those without biocides (Radke *et al.*, 2008). Biocides are chemicals used to eradicate living organisms (Dallas and Day, 2004). Most effective modern antifouling coatings contain

biocides (Champ, 2000; Evans *et al.*, 2000c) that are released from antifouling paint (Lambert *et al.*, 2006). The most common alternative to TBT-based antifoulants is copper (Dafforn *et al.*, 2011; Gipperth, 2009; Negri and Heyward, 2001; Omae, 2003; Stewart *et al.*, 2014). Copper compounds such as cuprous oxide (Cu_2O), copper thiocyanate (CuSCN) or metallic copper are usually used (Turner, 2010; Voulvoulis *et al.*, 2002a).

Copper has bactericidal, molluscicidal, and fungicidal properties and exhibits antifouling activities against organisms such as barnacles, tube worms and the majority of algal species (Omae, 2003). There are concerns over the release of copper into the marine environment and its associated toxicity (Evans, 1999; Gipperth, 2009; Stewart *et al.*, 2014). Restrictions on release rates have been introduced in some countries (Dafforn *et al.*, 2011; Fernandez and Pinheiro, 2007). Despite doubts concerning the impact of high copper concentrations in the marine environment, some claim that the use of copper-based coatings as an alternative to TBT would actually benefit the environment as it is less harmful (Abbott *et al.*, 2000) due to the low release rates of the paint formulations (Almeida *et al.*, 2007). Dafforn *et al.* (2011) advises caution in regulating and restricting the use of copper in order to allow enough time for the development of a sustainable replacement.

Certain species of algae and diatoms are resistant to copper and therefore copper is incorporated with additional biocides into antifouling paints (Omae, 2003; Thomas *et al.*, 2001; Turner, 2010; Voulvoulis *et al.*, 2002a). These biocides are referred to as booster biocides (Boxall *et al.*, 2000; Omae, 2003) as they enhance the efficacy of the formulation by inhibiting primary growth of copper-resistant fouling organisms such as algal slimes (Chambers *et al.*, 2006; Thomas *et al.*, 2001; Voulvoulis *et al.*, 2002a). They are mostly used as agrochemicals, pesticides, herbicides, fungicides or bactericides (Omae, 2003).

Copper compounds and booster biocides such as Irgarol 1051, Chlorothalonil, Dichlofluanid and Diuron, were introduced as alternatives to organotin compounds in antifouling products (DeLorenzo *et al.*, 2009; Kim *et al.*, 2014; Konstantinou and Albanis, 2004; Omae, 2003; Voulvoulis *et al.*, 2000; Voulvoulis *et al.*, 2002b). Irgarol 1051, diuron, Sea-Nine 211, dichlofluanid, chlorothalonil, zinc pyrithione,

TCMSpyridine, TCMTB, Kathon 5287, and zineb are the most commonly used booster biocides (Boxall *et al.*, 2000; Castro *et al.*, 2012b; Konstantinou and Albanis, 2004; Omae, 2003; Voulvoulis *et al.*, 2002a).

The most popular of these booster biocides are Irgarol 1051 and diuron (Konstantinou and Albanis, 2004) as they are more cost-effective (Chambers *et al.*, 2006; Omae, 2003). They are designed to inhibit algal photosynthesis (Evans *et al.*, 2000c; Fernandez and Pinheiro, 2007; Lambert *et al.*, 2006). Measurable concentrations of these biocides have been observed in aquatic environments, especially in areas such as marinas where there is high yachting activity (Omae, 2003; Thomas *et al.*, 2001; Voulvoulis *et al.*, 2000). Irgarol, in the marine environment, has been reported from a number of countries including Europe, USA, Japan, Australia, and Bermuda (Konstantinou and Albanis, 2004). There have been much doubt regarding its environmental effect (Yerba *et al.*, 2004) as it is highly toxic to autotrophic aquatic species such as cyanobacteria, algae, macrophytes, and symbiotic dinoflagellates in corals (Bao *et al.*, 2011; Voulvoulis *et al.*, 2000) which could subsequently impact higher trophic levels (De Lorenzo *et al.*, 2009). Diuron is one of the major herbicides used for general weed control (Voulvoulis *et al.*, 2000) and it may also affect algae (Lambert *et al.*, 2006). Irgarol and diuron are not environmentally acceptable alternatives due to their toxicity and persistence (Chambers *et al.*, 2006; Zhou, 2008).

Other booster biocides such as chlorothalonil and dichlofluanid are fungicides used in agriculture, with a wide range of actions against a number of organisms (Voulvoulis *et al.*, 2000). Thio cyano-methyl thio-benzothiazole (TCMTB), and TCMSpyridine exhibit environmental performance characteristics similar to TBT and should therefore be monitored before their use is permitted (Voulvoulis *et al.*, 2002a). Other biocides seem to be less hazardous to the environment such as ZPT, Zineb (Voulvoulis *et al.*, 2002a) and Sea-Nine (Yerba *et al.*, 2004).

Booster biocides have not been given much attention as compounds of environmental concern due to their relatively recent introduction, limited usage, and supposed lower toxicity in comparison to TBT (Voulvoulis *et al.*, 2000). There is limited monitoring data and little information on their fate, behaviour, toxicity and persistence in the marine environment which makes accurate risk assessments difficult (Lambert *et al.*, 2006;

Voulvoulis *et al.*, 2000; Voulvoulis *et al.*, 2002a; Voulvoulis *et al.*, 2002b; Yerba *et al.*, 2004). Therefore, any decisions on policies regulating these antifoulants cannot be fully informed (Voulvoulis *et al.*, 2002a) and their use should only be allowed once further studies have provided more information on their toxicity and persistence in aquatic environments (Dafforn *et al.*, 2011; Harino *et al.*, 2007; Voulvoulis *et al.*, 2002a). It is likely that these biocides will be used more frequently in antifouling products and the environmental occurrence in the future might become considerable (Harino *et al.*, 2007; Voulvoulis *et al.*, 2000). It is of concern that some of these biocides could be even more toxic than TBT to certain organisms (Bao *et al.*, 2011; Evans *et al.*, 2000c).

The environmental impacts of biocides and subsequent restrictions have been a driving force behind the development of more environmentally friendly antifouling alternatives, with specific focus on foul-release coatings and natural products (Dafforn *et al.*, 2011; Yerba *et al.*, 2004). Foul-release coatings interfere with normal adhesion of fouling organisms and natural products are isolated from organisms, which have antifouling properties (Clare, 1998). The development of antifoulants that are environmentally sound, such as natural products and foul release coatings, are supported to protect the marine environments (Omae, 2003; IMO, 2002).

Natural products are obtained from various organisms including marine microorganisms such as bacteria (Burgess *et al.*, 2003); marine plants (Omae, 2003); marine algae (Hellio *et al.*, 2001); marine invertebrates such as sponges, corals, sea urchins, tunicates etc. (Chambers *et al.*, 2006); terrestrial plants such as wasabi, green tea, bark of the wild cherry tree, oak leaves (Omae, 2003), chestnut, mimosa and quebracho (Stupak *et al.*, 2003); and marine animals such as dolphins, whales, bony and cartilaginous fish (Almeida *et al.*, 2007; Omae, 2003). Natural products should be explored as antifouling agents, because they are obtained from marine resources, they are present in natural environment, and they are biodegradable (Qian *et al.*, 2010). It is expected that environmentally friendly, natural product antifoulants may actually be utilised in the near future (Clare, 1998; Omae, 2003).

Foul-release coatings are promising eco-friendly alternatives that control fouling by interfering with the settlement and attachment mechanisms of biofouling organisms

(Omae, 2003; Yerba *et al.*, 2004), but they are only effective at high ship's speeds (Yerba *et al.*, 2004). Other biocide-free antifouling applications being investigated are electro-conductive coatings, microfiber coatings, ultrasonic waves, laser irradiation, 'smart' antifoulants that act when organisms attach themselves, and mechanical cleaning which is one of the oldest methods to control biofouling (Almeida *et al.*, 2007; Chambers *et al.*, 2006; IMO, 2002; Omae, 2003; Yerba *et al.*, 2004).

Available TBT alternatives have their pros and cons. The ultimate goal would be the complete removal of toxic antifoulants (Dafforn *et al.*, 2011) and their replacement with biocide-free antifoulants that protect ships while preserving the marine environment (Almeida *et al.*, 2007; Alzieu, 1998; Burgess *et al.*, 2003; Chambers *et al.*, 2006; Champ, 2000).

CHAPTER 3: MATERIALS AND METHODS

3.1 Species selection

Gastropods are known to develop a condition known as imposex when they are exposed to organotin compounds, particularly TBT (Bigatti *et al.*, 2009; Gibbs and Bryan, 1987; Negri and Marshall, 2009).

Caenogastropods used in this study were chosen according to the following criteria: those that might be affected by imposex; those that were easily accessible; those that had separate sexes (gonochoristic); those with a high abundance; and those that were easily identifiable.

3.2 Site selection

Sites selected for this study are from areas of high marine traffic that are presumed polluted, and areas of low marine traffic, supposedly free of organotin pollution. High impact sites were deliberately chosen to be near or within a marina or harbour, where contamination from antifouling agents would expect to be greatest. Low impact sites were isolated areas, chosen as reference sites, usually at the open coast or Marine Protected Areas (MPAs) where contamination by antifouling agents is expected to be minimal. Site selection was aimed to describe gradients of TBT contamination around likely hotspots, from inside the harbours towards less polluted sites along the coast, over an extensive area of the Atlantic coast of the South Africa.

3.3 Site Descriptions

This study area comprised the Atlantic coast of South Africa, from Cape Agulhas to Port Nolloth (see Figure 2 for position of sites on map).

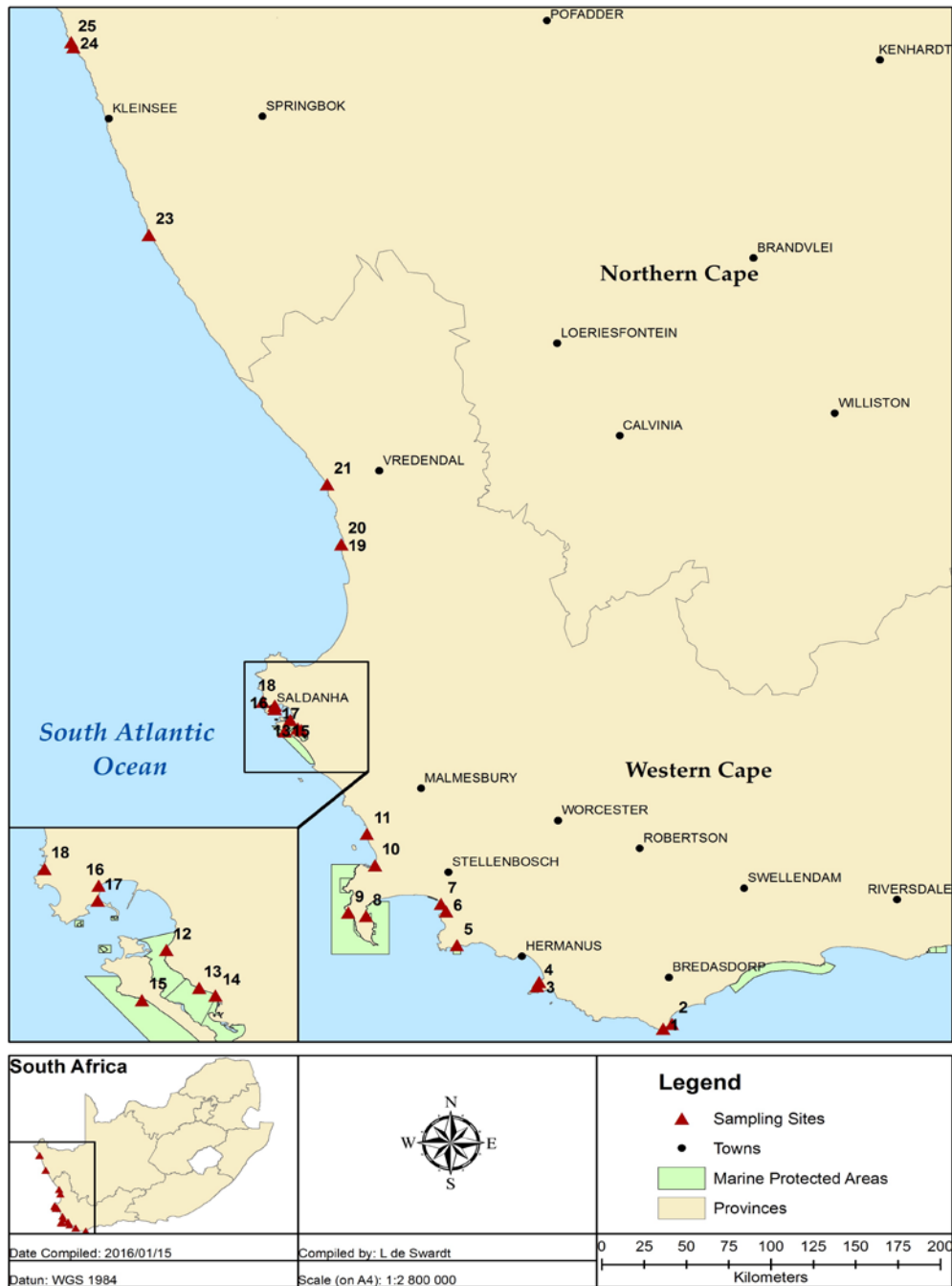


Figure 2: Map of site locations along the Atlantic coastline of South Africa

At each site, geographical co-ordinates were determined with a mobile GPS (Garmin) and observations were noted e.g. substrate, nature of shoreline, type of activity etc.

Table 1: Locations and basic characteristics of the sampling sites along the Atlantic coast of South Africa

SITE	COORDINATES	SAMPLING DATE	BOATING ACTIVITY	SITE INFORMATION	POPULATION BREEDING CAPACITY
1. Cape Agulhas	S34°49'42.9" E20°00'41.9"	2013/03/22	Low boating activity	Rocky shore; Pristine area, bordering on the Agulhas National Park; High diversity	No juveniles; egg capsules present
2. Struisbaai Harbour	S34°48'01.9" E20°03'31.4"	2013/03/22	Intermediate boating activity	Small harbour	No juveniles or egg capsules present
3. Gansbaai	S34°33'54.2" E19°21'09.6"	2013/03/23	Low boating activity	Rocky shore near harbour	No juveniles; egg capsules present
4. Gansbaai Harbour	S34°35'15.72" E19°20'32.78"	2014/03/30	High boating activity	Rocky shore; Fishing port; Aquaculture (Fishing pen); Harbour	No juveniles or egg capsules present
5. Betty's Bay	S34°21'25.56" E18°54'57.95"	2013/03/24	Low boating activity	Sandy beach and rocky shore	No juveniles or egg capsules present
6. Gordon's Bay Harbour	S34°9'54.71" E18°51'34.83"	2013/03/25	High boating activity	Rocky area; Fishing boats; moorings	No juveniles or egg capsules present
7. Strand	S34°07'23.0" E18°49'52.9"	2013/03/25	Low boating activity	Sandy beach with rock pools	No juveniles or egg capsules present
8. Simon's Town Yacht Club	S34°11'31.3" E18°26'01.1"	2013/03/29	High boating activity	Naval; Moorings; Shipyard; Marina; Harbour; Blue flag status	No juveniles or egg capsules present
9. Soetwater	S34°10'23.1" E18°20'19.0"	2013/03/30	Low boating activity	Pristine area; Rock pools; rough seas	No juveniles or egg capsules present
10. Lagoon Beach	S33°54'21.4 E18°28.51.4	2014/04/01	High boating activity	Sandy beach close to CPT Harbour. Various boating activities	Juveniles present; no egg capsules present
11. Melkbosstrand	S33°43'41.9" E18°26'20.4"	2013/04/01	Low boating activity	Sandy beach and rock pools	Juveniles present; no egg capsules present
12. Langebaan Zone A (WCNP)	S33°05'05.5" E18°01'51.3"	2013/03/28	Low boating activity	Beach and rocky shore; Bordering on West Coast NP; small yacht club in close proximity	No juveniles present; egg capsules present

CHAPTER 3: MATERIALS AND METHODS

SITE	COORDINATES	SAMPLING DATE	BOATING ACTIVITY	SITE INFORMATION	POPULATION BREEDING CAPACITY
13. Langebaan Zone B (WCNP)	S33°7'59.40" E18°4'13.20"	2014/04/05	Low boating activity	Rocky Shore; clean area, WCNP, little human influence	No juveniles or egg capsules present
14. Langebaan Zone C (WCNP)	S33°8'34.78" E18°5'23.77"	2014/04/05	Low boating activity	Rock on sandy beach; Clean Area, Lagoon, WCNP, No human influence	No juveniles or egg capsules present
15. Tsarsbank (WCNP)	S33°8'55.27" E18°0'5.71"	2014/04/02	Low boating activity	Rocky shore; Marine Protected Area (MPA); Open coast, rough seas	No juveniles or egg capsules present
16. Saldanha Bay	S33°00'09.2" E17°56'58.1"	2013/03/27	High boating activity	Sandy beach; Close to iron ore harbour and commercial harbour	No juveniles or egg capsules present
17. Saldanha Bay Harbour	S33° 1'18.16" E17°56'54.06"	2014/04/02	High boating activity	Rocky shore; Various boating activities (Shipyard, Fishing port, Naval etc.)	No juveniles or egg capsules present
18. Jacobsbaai	S32°58'51.6" E17°53'03.7"	2013/03/26	Low boating activity	Pristine area; rocky shore/beach	No juveniles present; egg capsules present
19. Lambert's Bay Harbour	S32°05'33.6" E18°18'07.9"	2013/04/03	Intermediate boating activity	Harbour; Shipyard; Fishing boats	Juveniles present; no egg capsules present
20. Bird Island (Lambert's Bay)	S32°05'33.6" E18°18'07.9"	2013/04/03	Low boating activity	Close to harbour; Separated by a wall; Open shore; Rocky shore; CapeNature Reserve	No juveniles or egg capsules present
21. Strandfontein	S31°45'14.7" E18°13'37.3"	2013/04/04	Low boating activity	Pristine area; Blue Flag beach	Juveniles and egg capsules present
22. Hondeklipbaai Harbour	S30°19'02.3" E17°16'27.2"	2013/04/05	Low boating activity	Rocky shore; small harbour	No juveniles or egg capsules present

SITE	COORDINATES	SAMPLING DATE	BOATING ACTIVITY	SITE INFORMATION	POPULATION BREEDING CAPACITY
23. Hondeklipbaai	S30°20'46.0" E17°16'50.3"	2013/04/05	Low boating activity	Pristine area; Aristea Shipwreck	Juveniles present; no egg capsules present
24. Port Nolloth (McDougall Bay)	S29°17'06.7" E16°52'45.6"	2013/04/06	Low boating activity	Clean area; Beach and rocky shore	No juveniles or egg capsules present
25. Port Nolloth Harbour	S29°15'26.1" E16°52'03.7"	2013/04/06	Low boating activity	Small fishing boats; rocky shore	No juveniles or egg capsules present

The sampling sites were classified according to the criteria used by Ten Hallers-Tjabbes *et al.*, (2003):

High boating activity: >10 ships

Intermediate boating activity: 5 – 10 ships

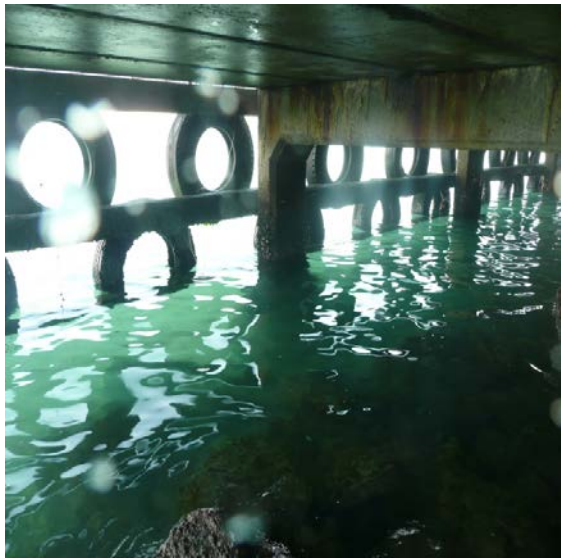
Low boating activity: <5 ships

Ports and Ships, (2015) was also referred to for maritime movements within the chosen harbours.

Table 2: Photographs of sampling sites along the Atlantic coastline of South Africa



SITE 2: Struisbaai Harbour



SITE 3: Gansbaai



SITE 4: Gansbaai Harbour



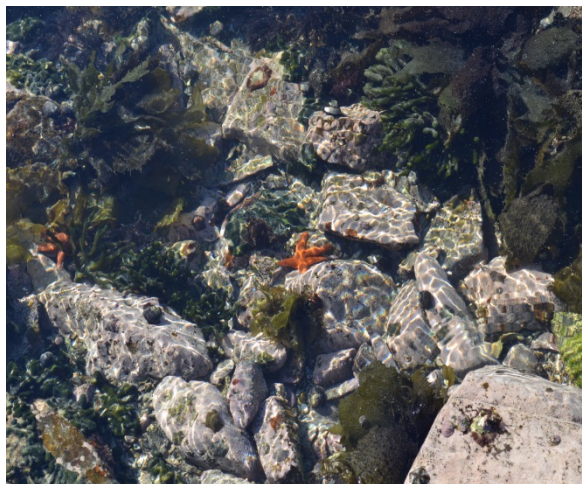
SITE 5: Betty's Bay



SITE 6: Gordon's Bay Harbour



SITE 7: Strand



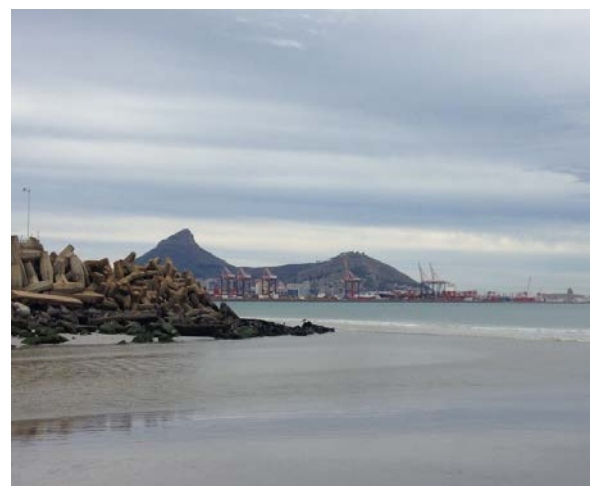
SITE 8: Simon's Town Yacht Club



SITE 9: Soetwater



SITE 10: Cape Town Harbour (Lagoon Beach)



SITE 10: Cape Town Harbour (Lagoon Beach)



SITE 11: Melkbosstrand



SITE 12: West Coast National Park (WCNP) Zone A



SITE 13: West Coast National Park (WCNP) Zone B



SITE 14: West Coast National Park (WCNP) Zone C



SITE 15: Tsarsbank



SITE 16: Saldanha Bay



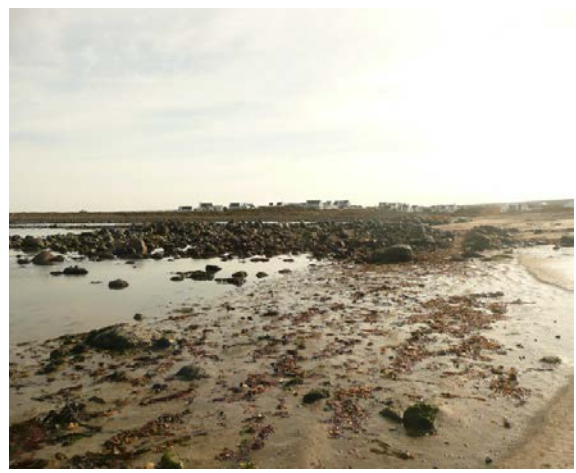
SITE 17: Saldanha Bay Harbour



SITE 17: Saldanha Bay Harbour



SITE 18: Jacobsbaai



SITE 19: Lambert's Bay Harbour



SITE 20: Bird Island (Lambert's Bay)



SITE 21: Strandfontein



SITE 22: Hondeklipbaai Harbour



SITE 23: Hondeklipbaai



SITE 24: McDougall Bay



SITE 25: Port Nolloth Harbour



3.4 Gastropod description

Table 3: Classification of gastropods used in this study (Branch et al., 2010; Bouchet and Rocroi, 2005; Day, 1974; Ponder et al., 2008).

KINGDOM	PHYLUM	CLASS	SUBCLASS	ORDER	FAMILY	GENUS	SPECIES	COMMON NAME
Animalia					Littorinidae	<i>Afrolittorina</i>	<i>knysnaensis</i>	Southern periwinkle
	Mollusca				Nassaridae	<i>Bullia</i>	<i>digitalis</i>	Finger plough shell
		Gastropoda				<i>Bullia</i>	<i>laevissima</i>	Fat plough shell
			Caenogastropoda			<i>Bullia</i>	<i>papyracea</i>	Papery burnupena
				Neogastropoda		<i>Bullia</i>	<i>rhodostoma</i>	Smooth plough shell
					Buccinidae	<i>Burnupena</i>	<i>catarrachta</i>	Flame-patterned burnupena
						<i>Burnupena</i>	<i>cincta</i>	Ridged burnupena
						<i>Burnupena</i>	<i>lagenaria</i>	Variable burnupena
						<i>Burnupena</i>	<i>rotunda</i>	Rotund burnupena
					Clavatulidae	<i>Clionella</i>	<i>sinuata</i>	Ribbed turrid
					Muricidae	<i>Nucella</i>	<i>cingulata</i>	Girdled dogwhelk
						<i>Nucella</i>	<i>dubia</i>	Common dogwhelk
					Muricidae	<i>Thais</i>	<i>capensis</i>	Knobbly dogwhelk

Table 4: Photographs of caenogastropods sampled along the Atlantic coastline

<i>Afrolittorina knysnaensis</i>	
	
<i>Bullia digitalis</i>	
	
<i>Bullia rhodostoma</i>	
	

Bullia laevissima



Burnupena papyracea



Burnupena catarrachta



Burnupena cincta



Burnupena lagenaria



Burnupena rotunda



Clionella sinuata



Nucella cingulata



Nucella dubia



Thais capensis**3.5 Gastropod Collection**

All collections were done with appropriate permits and permissions. Sampling was done during March-April 2013 and March-April 2014. The first collection was more extensive than the second was. The second collection was scheduled to achieve objectives that were not met during the first collection, due to permit restrictions, weather, time constraints, and the availability of gastropods. Both collections were scheduled during the same season (March/April) to reduce the influence of seasonality on results.

Sampling was scheduled and coordinated to sample comparable sites along the Atlantic coast of South Africa. Gastropods were collected from sites along the coast between Cape Agulhas and Port Nolloth, to cover presumed TBT-polluted sites (high marine traffic) and areas, which were presumed TBT-free (low marine traffic).

Gastropods were collected from the intertidal or sub-tidal zone by baited traps, or manually during low tide/mid-tide levels. A sample size with a maximum of 30 and minimum of 20 individuals of each species of marine gastropod were collected. This ensured that a proportion of females were obtained for statistical analysis.

The samples had to be transported from the Atlantic Coast to the laboratory at the North-West University in Potchefstroom, which was a +/- 1000 km journey. Logistical constraints made it necessary to freeze samples. Gastropod samples were placed in plastic bags and stored at -20°C in a portable freezer. The North-West University's

laboratory facilities were used to take necessary measurements and to perform imposex analysis.

3.6 Sediment Collection

TBT is deposited in marine sediments where it can remain bioavailable for many years (Strand and Asmund, 2003). It is necessary to collect sediment samples, because imposex development is related to levels of OTCs in gastropods and the sediments they inhabit (Ten Hallers-Tjabbes *et al.*, 2003).

All sampling apparatus and containers were washed and then rinsed with acetone and hexane before they were used. Sediment was collected from three different points within the site where the gastropods were sampled with a shovel from the top few centimetres (5 cm) where organotins are known to accumulate (Clark *et al.*, 1988; Maguire *et al.*, 1982). The sediment samples were placed in plastic bottles and then wrapped in aluminium foil (to protect against sunlight) and stored at -20°C in a portable freezer where they were transported to the North-West University in Potchefstroom until shipment to the laboratory for chemical analysis of TBT and DBT.

3.7 Measurements

Prior to imposex analysis, all the sampled gastropods were thawed and counted to determine the number of males and females. Snails were sexed, dissected and measured (shell length, total weight with and without the shell and penis size in males and imposexed females), using the same protocols and comparable instruments.

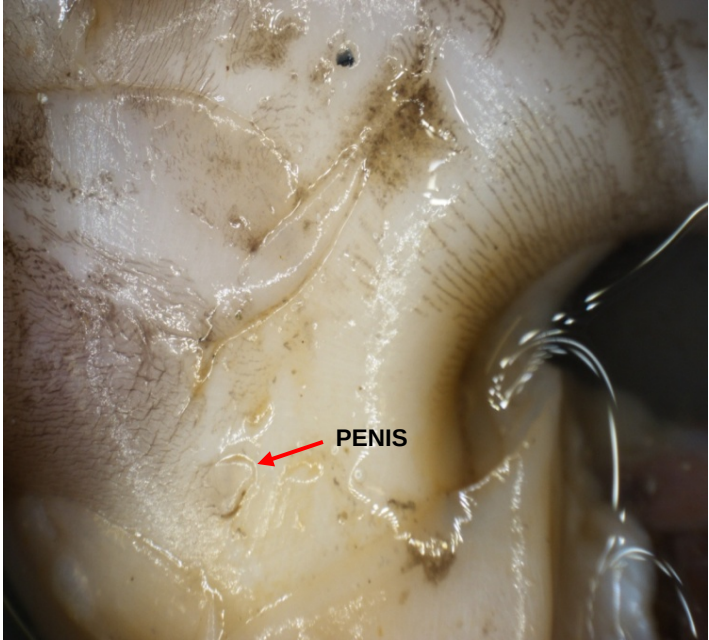
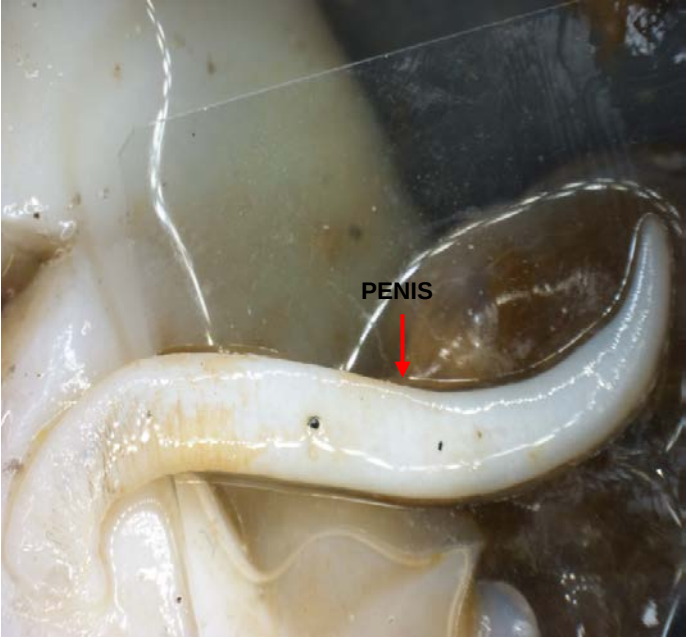
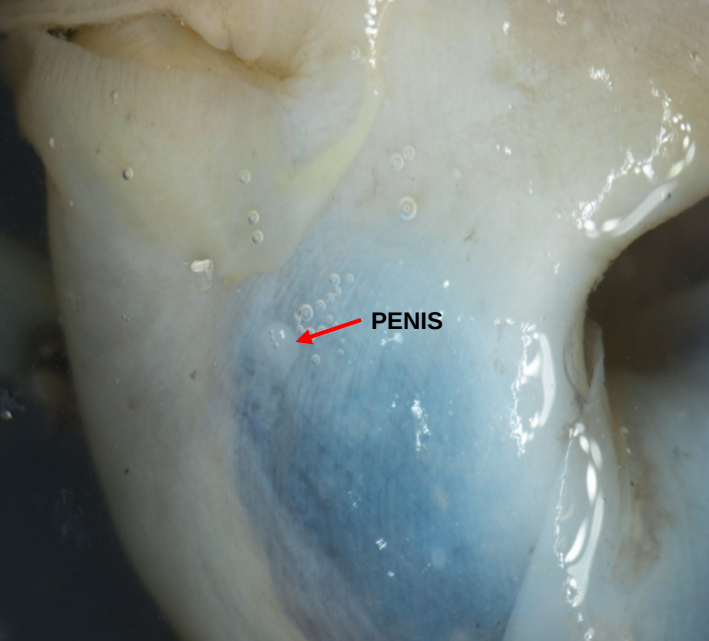
Mature adults were used rather than juveniles because their sex is easily distinguishable (Gibbs *et al.*, 1987).

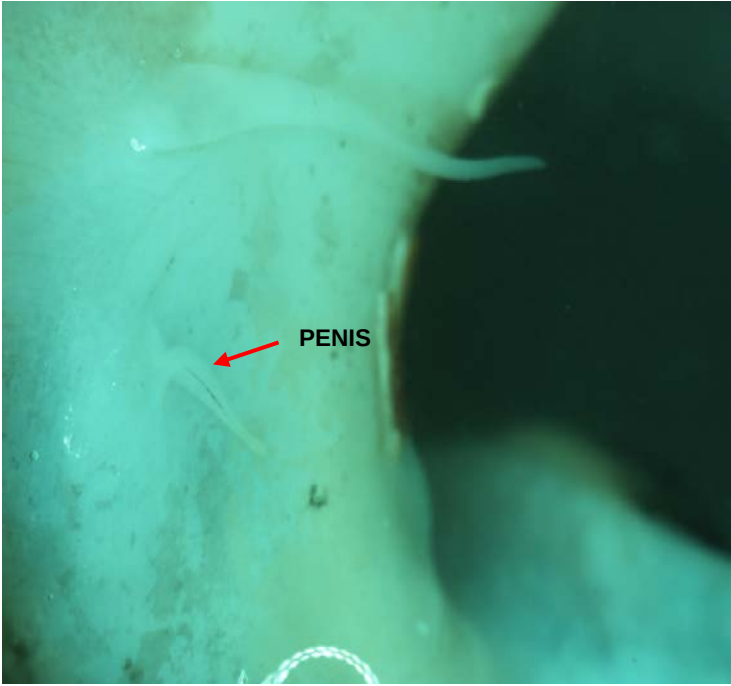
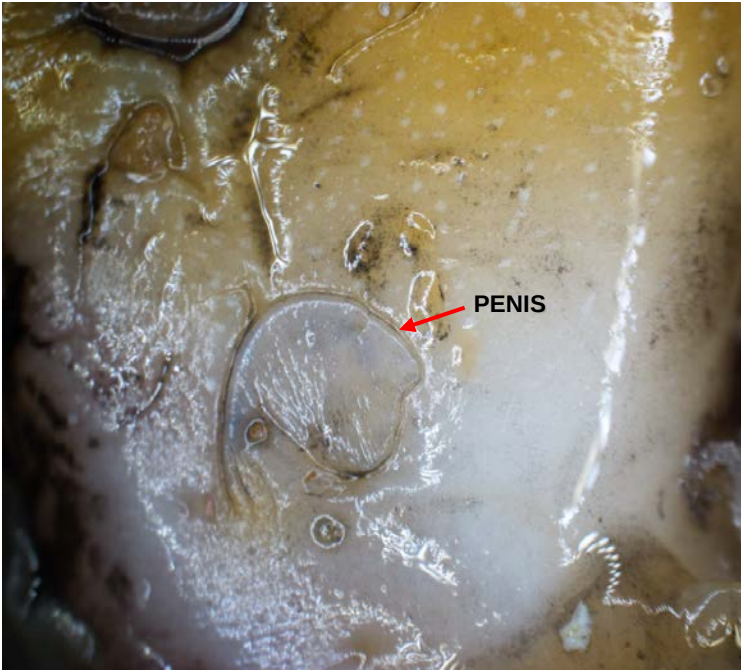
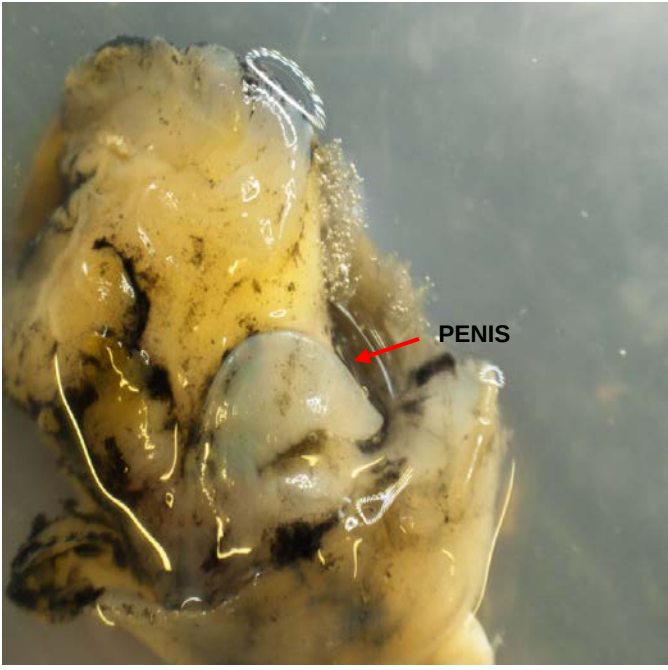


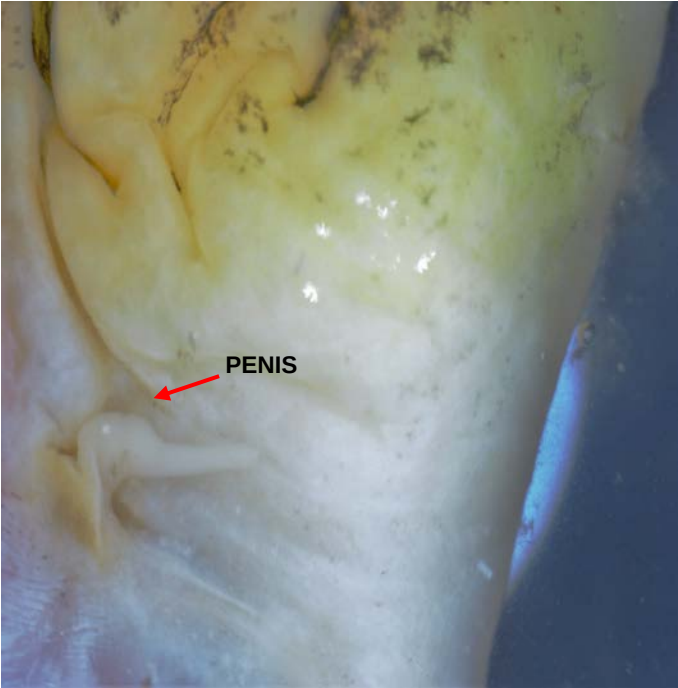
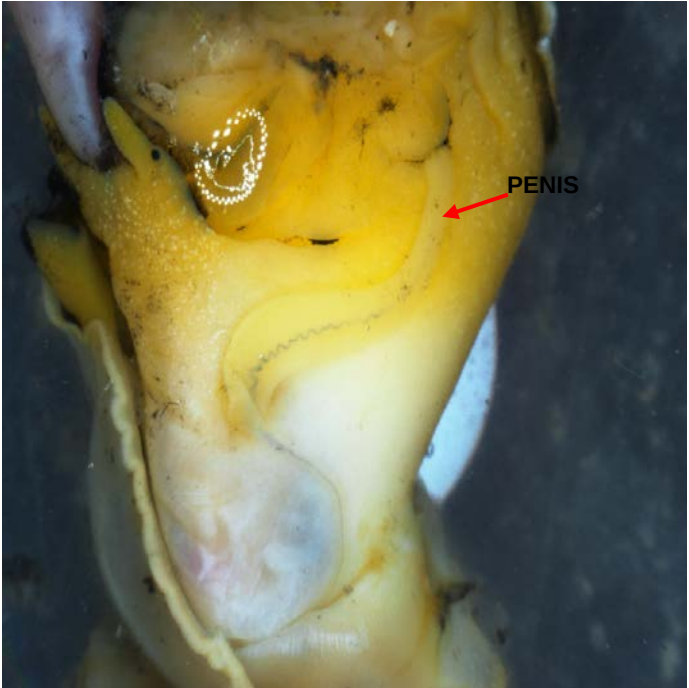
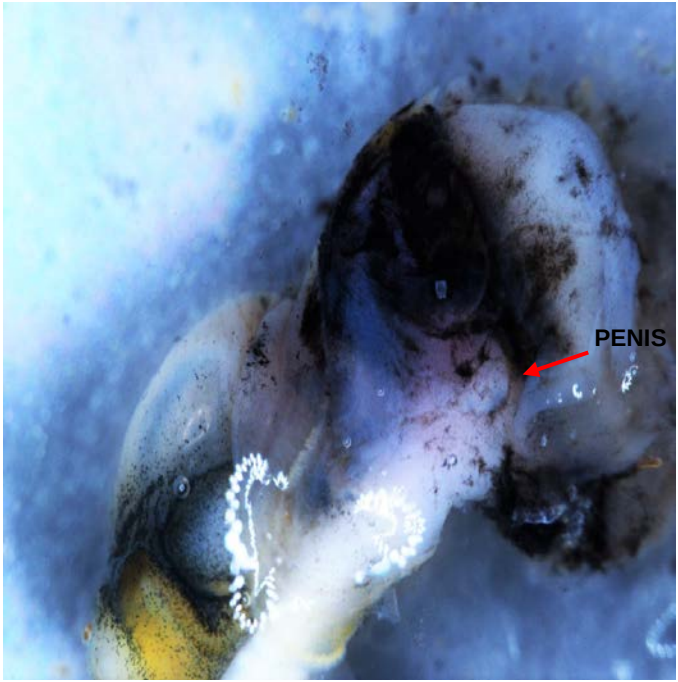
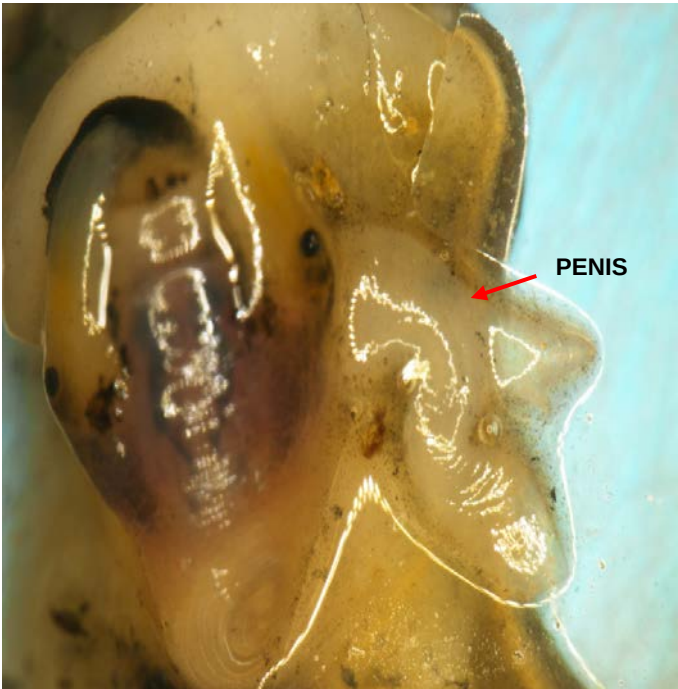
Figure 3: Gonad of female gastropod *Burnupena lagenaria* (indicated by arrow)

Females that had male sexual organs were classified as imposed sexed (Table 5). In certain instances, some females often had a penis the same size of males, so gastropods could not be sexed by observing the penis behind the right tentacle. The sexes of all collected gastropods were determined based on the presence or absence of sexual accessory glands (albumen, capsule, and pedal glands) along with the colour of the gonads (characteristic of each sex) (see figure 3). A feature in females that appears to be distinctive and easily recognisable is the sperm-ingesting gland. The gland is reddish/brown and is situated immediately posterior to the egg capsule gland (Gibbs *et al.*, 1987; Gibbs and Bryan, 1987; Gibbs, 1996). If the sex was not easily identified, sex was verified by examining gametes from a smear of the gonad.

Table 5: Photographs of imposex affected species and male gastropods of the same species

<i>Bullia laevissima</i>	
Imposexed Female	Male
	
<i>Bullia rhodostoma</i>	
Imposexed Female	Male
	

<i>Bullia digitalis</i>	
Imposexed Female	Male
	
<i>Burnupena cincta</i>	
Imposexed Female	Male
	

<i>Burnupena lagenaria</i>	
Imposexed Female	Male
	
<i>Afrolittorina knysnaensis</i>	
Imposexed Female	Male
	

Gastropods that were infected by trematode parasites were discarded. Gastropods are hosts of numerous helminth parasites. Some gastropods are of economic importance, because they act as hosts to parasites that are harmful to humans and various domestic animals (Fretter and Graham, 1962). Caenogastropods are known to be infected by various trematodes as their way of life allows plenty of opportunity for infection (Averbuj and Cremonte, 2010; Fretter and Graham, 1962).

Parasitic trematode larvae settle in blood spaces associated with the gonad or digestive gland where they obstruct the flow of nutrients from the digestive gland to the gonad. The parasite may destroy the gonad and then pass to the digestive gland with devastating consequences (Fretter and Graham, 1962). The infected areas become a pale yellow. In certain species the larva can be observed as white threads, or in some, the gland may be a bright orange when infected (Fretter and Graham, 1962).

Trematode parasites control neuroendocrine pathways in host molluscs resulting in the reduction of sexual differentiation and function (Sternberg *et al.*, 2010). The parasite may affect the gonad by causing partial or complete castration (Averbuj and Cremonte, 2010). Parasitised adult snails showed a reduction of penis size in comparison with healthy males (Averbuj and Cremonte, 2010). In females, the ovary is destroyed and this may make it difficult to determine the sex of the infected individual. Some parasites may destroy the gonad by completely devouring it (Fretter and Graham, 1962).

In Figure 4, the following procedure was developed so that the examination and measurements were standardised:

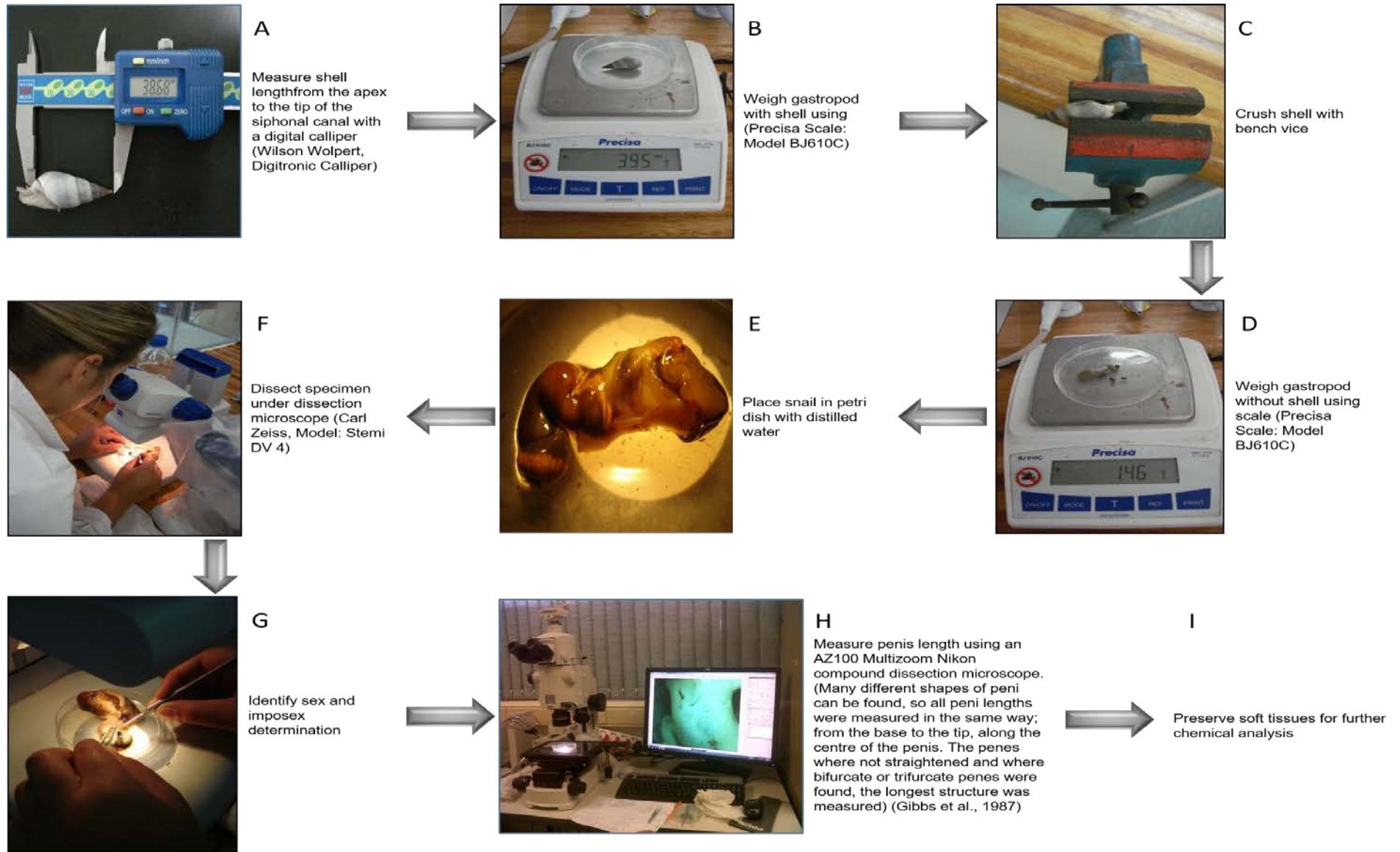


Figure 4: Procedure used for measurements and dissections in the laboratory

3.8 Imposex analysis

In Argentina, practical training was received at the institution, LARBIM-IBIOMAR-CONICET (Puerto Madryn), on sample collection procedures, sample preparation, examination and scoring of imposex). In females where a measurable penial outgrowth was absent, a value of zero was assigned to it and it was included in the calculation of the mean (Gibbs et al., 1987).

Once the necessary measurements were completed, the imposex parameters were calculated e.g. RPLI, RPSI, sex ratio etc.

Imposex can be adequately expressed as the percentage of females bearing a penis (Gibbs and Bryan, 1987). Percentages of imposex-affected females were calculated as the number of females with penis and/or vas deferens with respect to all females sampled of each species (Bigatti et al, 2009). Percentage imposex is calculated accordingly:

$$\% \text{ Imposex} = \frac{\text{Imposex Females}}{\text{Total Females}} * 100$$

Imposex might advance to the stage where all females are penis-bearing and this has therefore little meaning. Since the female penis enlarges with imposex development, its length can be compared to that of the male in the same population. Indices such as RPLI and RPSI are used in this instance. RPLI is calculated as follows:

$$RPLI = \frac{mFPL}{mMPL} * 100$$

where

mFPL= Mean Female Penis Length

mMPL=Mean Male Penis Length

Length does convey a true impression of the difference in size between a penis of 2 mm length and one of 4 mm, but the weight (volume) of the penis is related to the cube of its length and the RPS (Gibbs and Bryan, 1987). An RPS value of 50%

indicates that the mean penis size of the female is half the bulk of that of the male (Gibbs and Bryan, 1987). RPSI is calculated as follows:

$$RPSI = \frac{mFPL^3}{mMPL^3} * 100$$

where

mFPL = Mean Female Penis Length

mMPL = Mean Male Penis Length

Both the RPSI and RPLI measure imposex severity. RPLI is related to length while RPSI is related to weight. The larger the value of these indices, the more serious the imposex effect is at a particular site. The mFPL and mMPL are used to calculate the RPLI and RPSI.

3.9 Chemical analysis

Organotins vary remarkably in their physical, chemical and biological properties. Therefore, Sn has a larger number of its organometallic derivatives in commercial use than many other elements. Methods that are sensitive enough for an accurate and simultaneous determination of OTCs at extremely low concentrations (low ng/l Sn) in different environmental samples are necessary (Hoch, 2001).

The RPS Group in the United Kingdom who has EU accreditation performed analysis for TBT and DBT concentrations in sediment samples. TBT and DBT were analysed using GC-MS after extraction and derivatisation. The exact parameters for extraction, derivatisation and analyses are proprietary to the laboratory, and not available. Values are expressed as µg/kg dm (dry mass).

3.10 Statistical analysis

The data from the dissections and microscopic measurements were captured on a sampling sheet where it was entered into Microsoft® Excel 2010. All statistical analysis (non-parametric Kruskal-Wallis, ANOVA, level of significance at $p < 0.05$, etc.) was performed using SAS® 9.4. Maps were drawn using Microsoft® Power Maps 2013.

CHAPTER 4: RESULTS

4.1 TBT/DBT concentrations in sediments

Sediment samples were collected from all sites ($n = 25$). Six sites had high boating activity, while two had medium boating activity, and the remaining seventeen had low boating activity (See Table 7). Concentrations of DBT and TBT measured in sediment samples were below the limit of detection in 21 of the 25 sites. The limit of detection for DBT was $<5 \mu\text{g/kg}$ and for TBT $<2 \mu\text{g/kg}$ dry mass (dm).

Concentrations in sediment samples from Gordon's Bay Harbour, Simon's Town Yacht Club, Saldanha Bay Harbour, and Lambert's Bay Harbour were well outside the dynamic range of the standard method and required dilution to bring the results within range. [See note following] Concentrations of TBT in sediment ranged from $495 \mu\text{g/kg dm}$ to $20\,000 \mu\text{g/kg dm}$. DBT levels ranged from $315 \mu\text{g/kg dm}$ to $3\,740 \mu\text{g/kg dm}$. DBT and TBT concentrations are shown in Table 7. The levels of TBT and DBT were the highest in Gordon's Bay, followed by Simon's Town Yacht Club (False Bay), Saldanha Bay Harbour, and lastly, Lambert's Bay Harbour.

NOTE: These samples were diluted to bring the concentrations into range; however, the high responses of these compounds remained above the calibration range. The results were obtained from the diluted extracts by extrapolation of the calibration plot and therefore precision and accuracy may have been affected.

Gordon's Bay, Simon's Town Yacht Club and Saldanha Bay Harbour had high boating activity. Lambert's Bay had intermediate boating activity. Sites with high boating activity, such as Gansbaai Harbour, Lagoon Beach and Saldanha Bay, had TBT/DBT concentrations below the limit of detection. Struisbaai had intermediate boating activity; however, concentrations were also below the limit of detection.

Table 6: DBT and TBT concentrations ($\mu\text{g/kg}$) dm in sediment from the sites along the Atlantic Coastline.

SITE	BOATING ACTIVITY	DBT CONCENTRATION ($\mu\text{gDBT/kg}$)	TBT CONCENTRATION ($\mu\text{gTBTCl/kg}$)
1 Cape Agulhas	Low	< 5	< 2
2 Struisbaai	Medium	< 5	< 2
3 Gansbaai	Low	< 5	< 2
4 Gansbaai Harbour	High	< 5	< 2
5 Betty's Bay	Low	< 5	< 2
6 Gordon's Bay Harbour	High	591	20000
7 Strand	Low	< 5	< 2
8 Simon's Town Yacht Club	High	610	7930
9 Soetwater	Low	< 5	< 2
10 Lagoon Beach	High	< 5	< 2
11 Melkbosstrand	Low	< 5	< 2
12 Langebaan Zone A (WCNP)	Low	< 5	< 2
13 Langebaan Zone B (WCNP)	Low	< 5	< 2
14 Langebaan Zone C (WCNP)	Low	< 5	< 2
15 Tsarsbank	Low	< 5	< 2
16 Saldanha Bay	High	< 5	< 2
17 Saldanha Bay Harbour	High	3740	14400
18 Jacobsbaai	Low	< 5	< 2
19 Lambert's Bay Harbour	Medium	415	495
20 Bird Island	Low	< 5	< 2
21 Strandfontein	Low	< 5	< 2
22 Hondeklipbaai Harbour	Low	< 5	< 2
23 Hondeklipbaai	Low	< 5	< 2
24 McDougall Bay (Port Nolloth)	Low	< 5	< 2
25 Port Nolloth Harbour	Low	< 5	< 2

4.1.1 TBT/DBT inputs

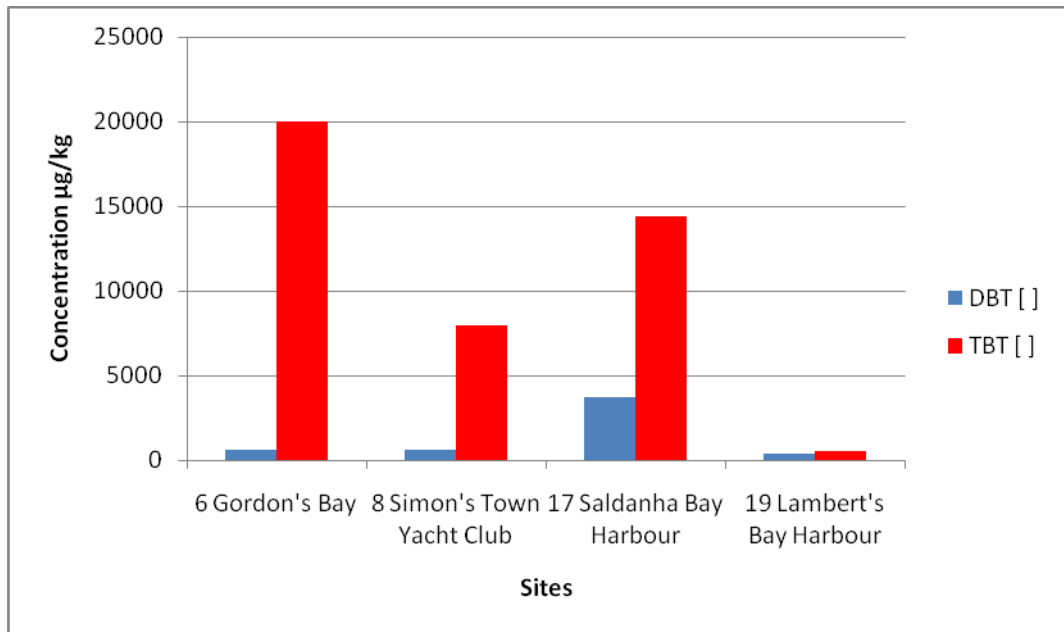


Figure 5: Organotin concentrations measured at sites with TBT and DBT above the limit of detection.

In Figure 5, it can be seen that TBT concentrations were much higher than DBT levels for Gordon's Bay, Simon's Town and Saldanha Bay. In Lambert's Bay, TBT concentrations were almost equal to those of DBT.

4.1.2 Boating Activity vs. DBT (µg/kg)

Hypotheses were tested to determine whether there was a relationship between perceived boating activity and levels of DBT.

The null-and alternative hypotheses were constructed as follows:

H₀: There is no relationship between the perceived boating activity and the measured levels of DBT.

H_A: A relationship exists between the perceived boating activity and the measured levels of DBT.

The Kruskal-Wallis test was used. The Kruskal-Wallis test is the non-parametric equivalent to the one-way ANOVA. Assumptions for the Kruskal-Wallis test are that within each sample the observations are independent and identically distributed and the samples are independent of one another. ANOVA test for the basis of 'Analysis of Variance', and is appropriate to test the means of different factor levels of a population or sample. The Kruskal-Wallis test was used, because no assumptions were made regarding the underlying distribution of the variables that were measured (thus non-parametric).

Table 7: Boating activity vs. DBT ($\mu\text{g/kg}$)

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable DBT $\mu\text{g/kg}$ Classified by Variable Boating activity					
Boating activity	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1. Low	36	810.00	882.0	20.134125	22.500
2. Medium	2	67.50	49.0	9.291478	33.750
3. High	10	298.50	245.0	18.883508	29.850
Average scores were used for ties.					

Kruskal-Wallis Test	
Chi-Square	13.3507
DF	2
Pr > Chi-Square	0.0013

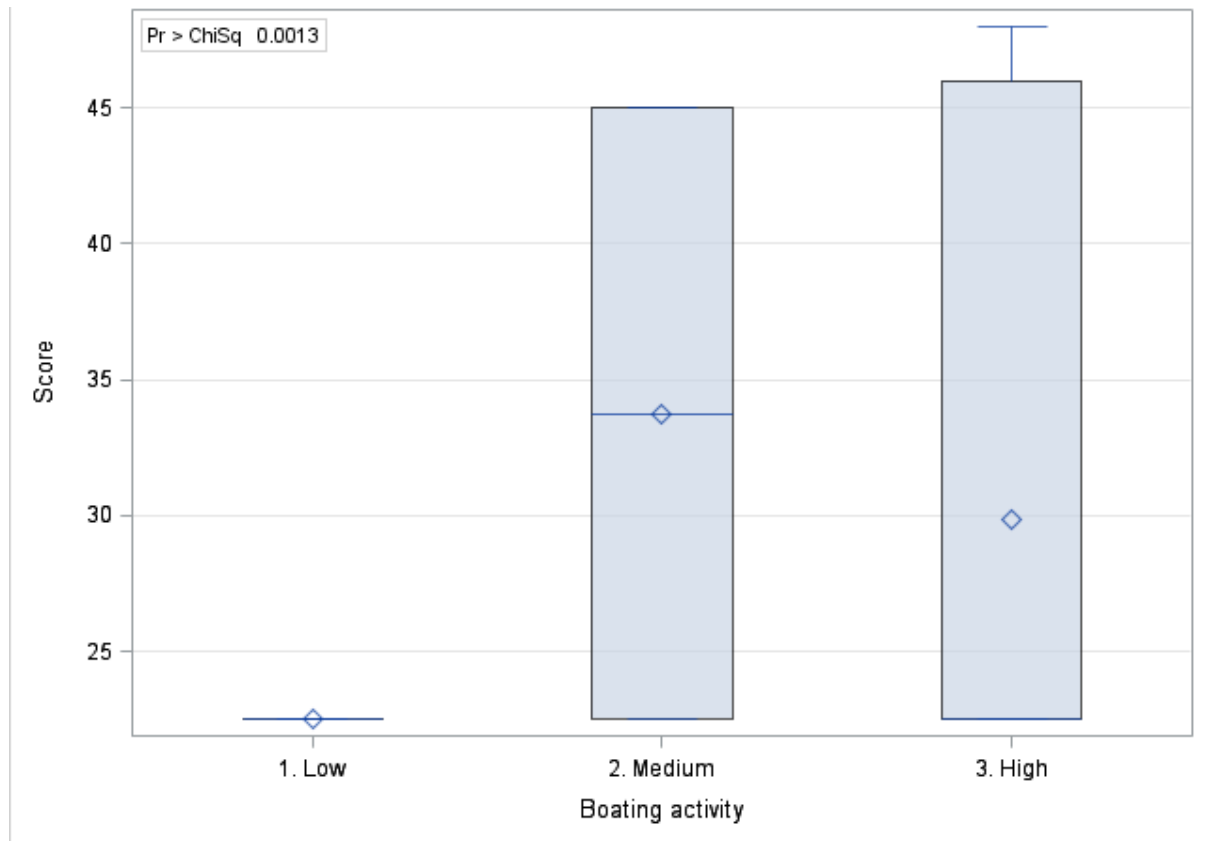


Figure 6: Wilcoxon box plot comparing the rank scores of Boating Activity vs. DBT ($\mu\text{g/kg}$)

Table 7 and Figure 6 show the output of the SAS® non-parametric one-way procedure. The Wilcoxon scores listed the expected value of the rank scores of DBT within each level of boating activity. As observed, for low levels of boating activity, the scores were less than the expected values under the null-hypothesis (H_0). The opposite is true for medium and high boating activity levels. This provides an idea of the directionality of the influence of boating activity on the levels of DBT; as boating activity increases, the levels of DBT also increase. This directionality is illustrated by Wilcoxon box plots in Figure 6. This illustrates the rank scores of DBT and its measured standard deviation. It can clearly be seen that low levels of boating activity did not produce such high ranking scores as medium and high boating activity.

In order to test this relationship properly, the results of the Kruskal-Wallis test should be referred to. According to the output in Table 7, the p-value ($\text{Pr} > \text{Chi-Square}$) was less than 0.05 (0.0013), which means that the null hypothesis is discarded, and the alternative hypothesis is assumed; that boating activity has a causal relationship with the levels of DBT.

4.1.3 Boating Activity vs. TBT ($\mu\text{g/kg}$)

The same results as for the DBT comparison were obtained (see Figure 7 and Table 8). All the sites that had measurable readings of DBT also had measurable levels of TBT. This means that the same conclusions can be drawn as for DBT hypothesis; in that there is a relationship between the perceived boating activities and TBT.

The output of the SAS® non-parametric one-way procedure can be observed in Table 8 and Figure 7.

Table 8: Boating activity vs. TBT ($\mu\text{g/kg}$)

The NPAR1WAY Procedure					
Wilcoxon Scores (Rank Sums) for Variable TBT $\mu\text{g/kg}$ Classified by Variable Boating activity					
Boating activity	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1. Low	36	810.00	882.0	20.134125	22.500
2. Medium	2	67.50	49.0	9.291478	33.750
3. High	10	298.50	245.0	18.883508	29.850
Average scores were used for ties.					

Kruskal-Wallis Test	
Chi-Square	13.3507
DF	2
Pr > Chi-Square	0.0013

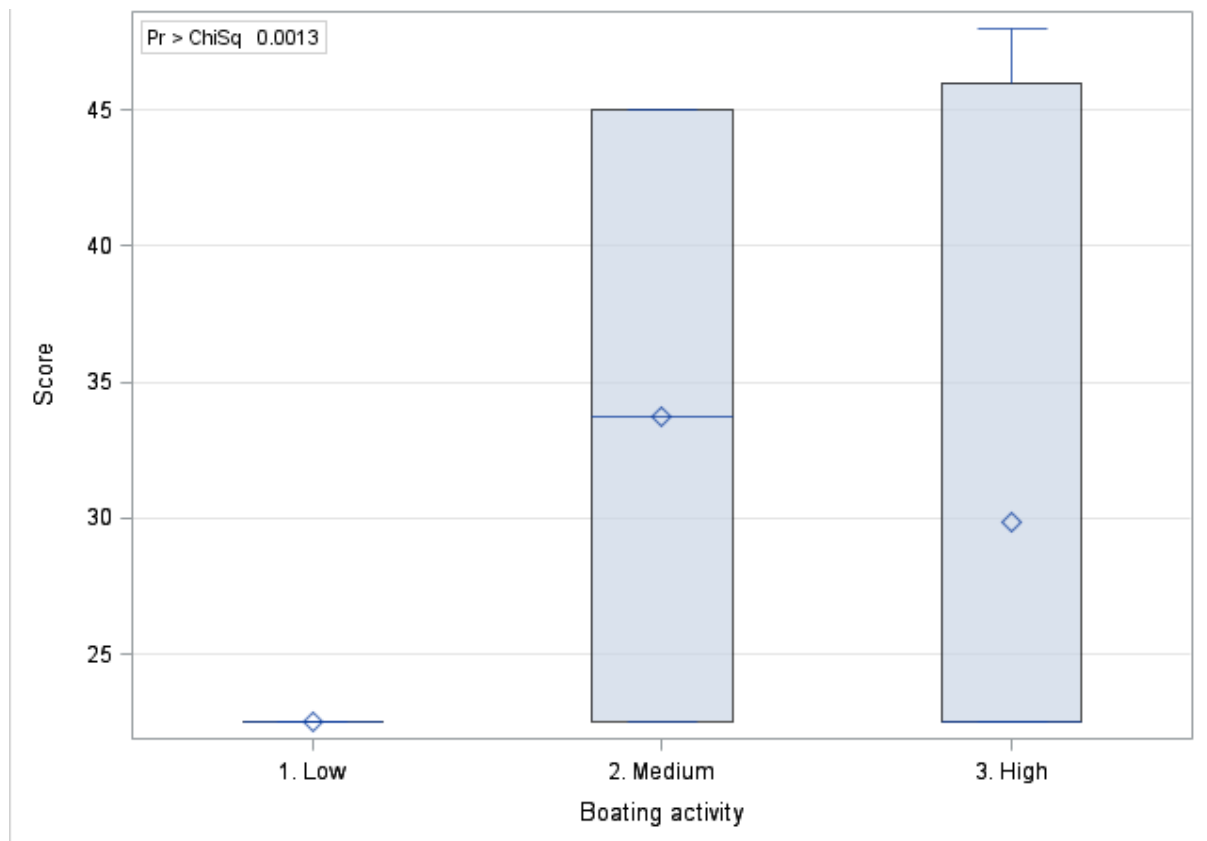


Figure 7: Wilcoxon box plot comparing the rank scores of Boating Activity vs. TBT ($\mu\text{g/kg}$)

4.2. Boating activity vs. imposex

Hypotheses were tested to determine whether a relationship existed between the observed levels of imposex and boating activity.

The null-and alternative hypotheses were constructed as follows:

H0: Mean levels of imposex do not differ for different levels of boating activity.

HA: Mean levels of imposex differ for different levels of boating activity.

Again, the SAS® non-parametric one-way procedure was used in order to determine if the null-hypothesis would be rejected or not.

Table 9: Boating activity vs. Imposex

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for variable Imposex Classified by Variable Boating activity					
Boating activity	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1. Low	36	789.0	882.0	28.599769	21.916667
2. Medium	2	40.0	49.0	13.198196	20.000000
3. High	10	347.0	245.0	26.823315	34.700000
Average scores were used for ties.					

Kruskal-Wallis Test	
Chi-Square	14.5368
DF	2
Pr > Chi-Square	0.0007

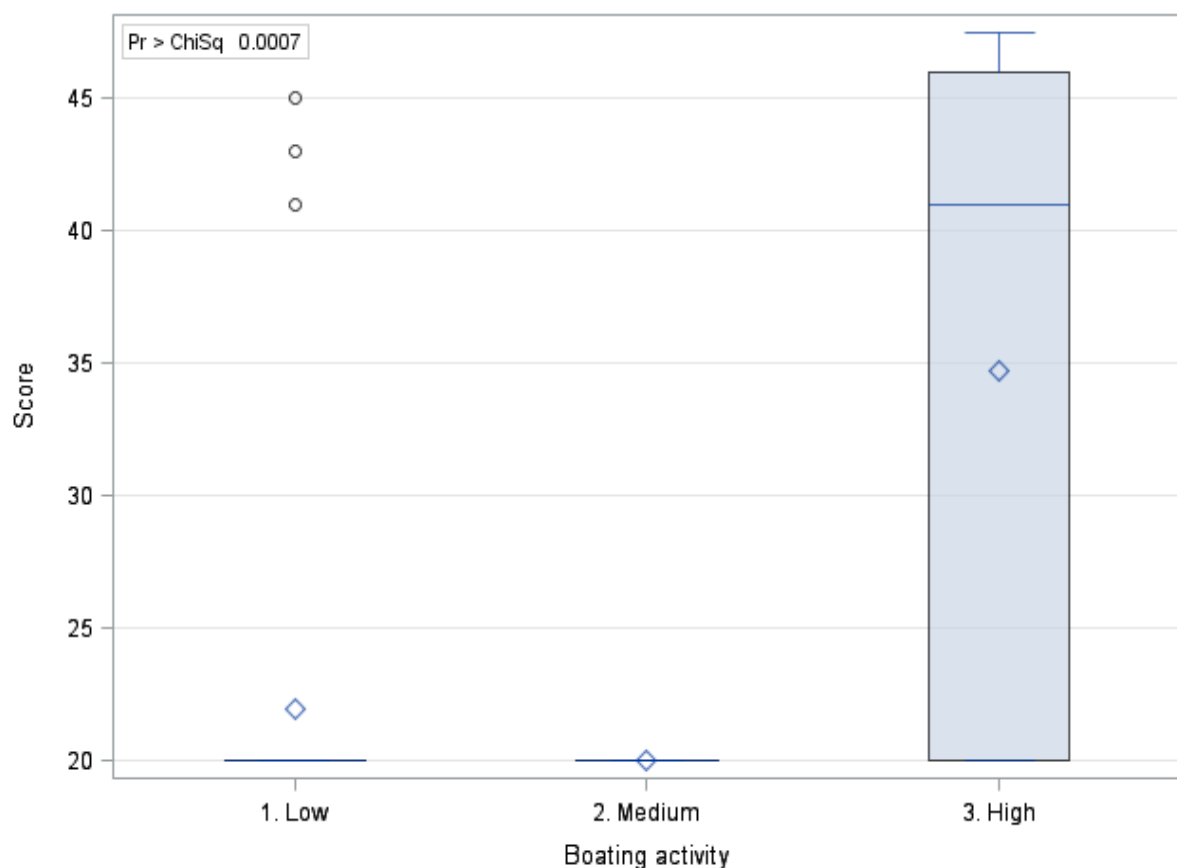


Figure 8: Wilcoxon box plot comparing the rank scores of Boating Activity vs. Imposex %

As boating activity increased, the measured levels of imposex also increased (Table 9). This directionality is further illustrated in Figure 8 whereby the Wilcoxon rank scores increased as boating activity increased.

There was significant relationship between imposex levels and boating activity as the p-value was 0.007. Therefore, the null hypothesis was rejected.

Given that the statistical tests imply that the mean levels of imposex for the different boating activities are in fact different, it can be seen from the one-way graph and from the Wilcoxon scores (Figure 8) that the directionality of the influence is ascending as boating activity increased.

4.2.1 Imposex Prevalence and Severity

Caenogastropods were sampled from the 25 sites. Imposex parameters were M:F ratio, % I, RPLI and RPSI, and are shown in Table 10. Thirteen species of caenogastropods, comprising of 1389 individuals, from the various sites along the Atlantic coastline were analysed.

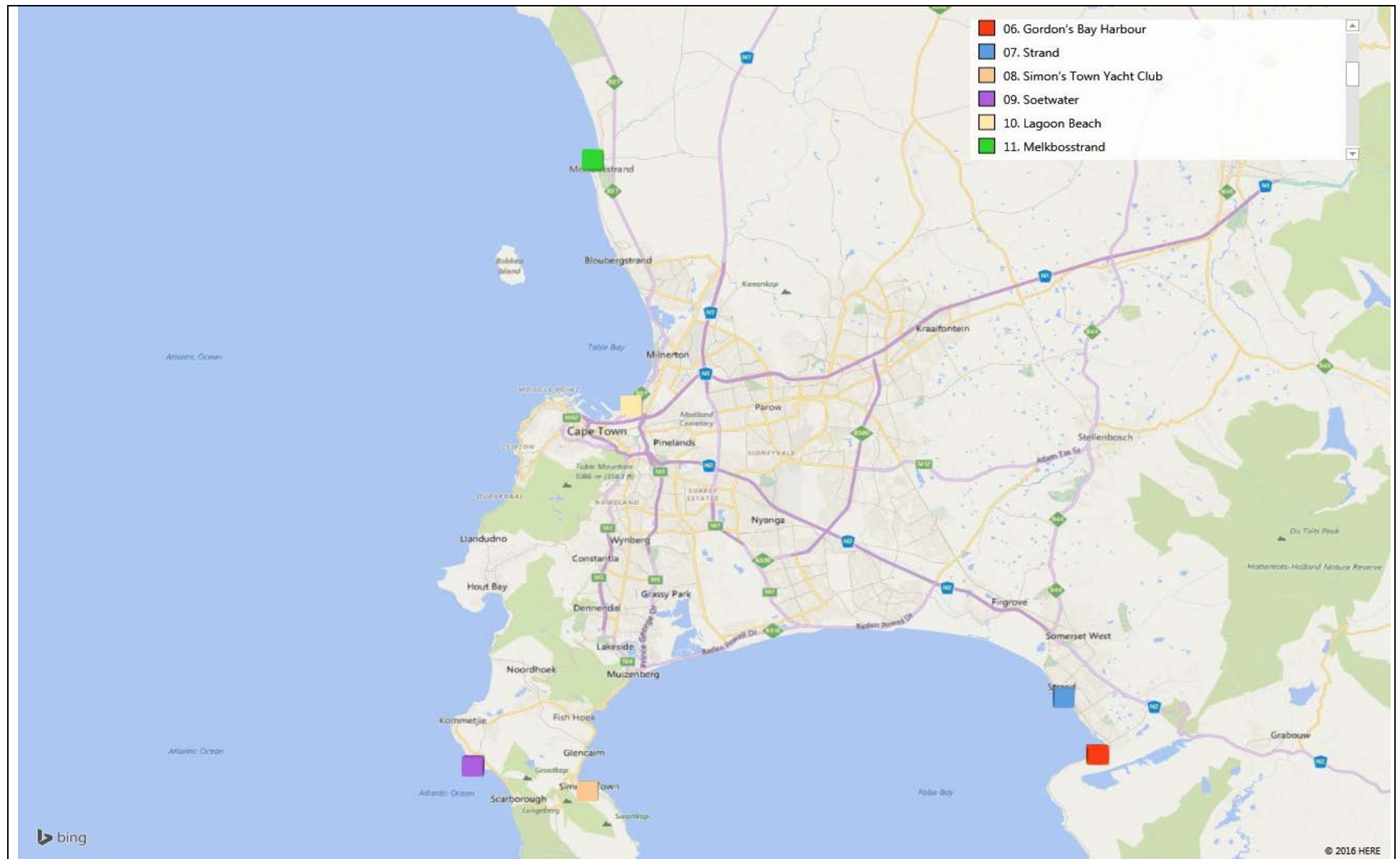
In Table 10, imposex was observed at eight of the twenty-five sites. Six species displayed imposex, namely: *Afrolittorina knysnaensis*, *Bullia digitalis*, *Bullia rhodostoma*, *Bullia laevissima*, *Burnupena cincta*, and *Burnupena lagenaria*.

Table 10: Maps of sites, boating activities, species, male to female ratio, % I, RPLI, RPSI, DBT and TBT concentrations, and the number of each species sampled from Cape Agulhas to Port Nolloth.

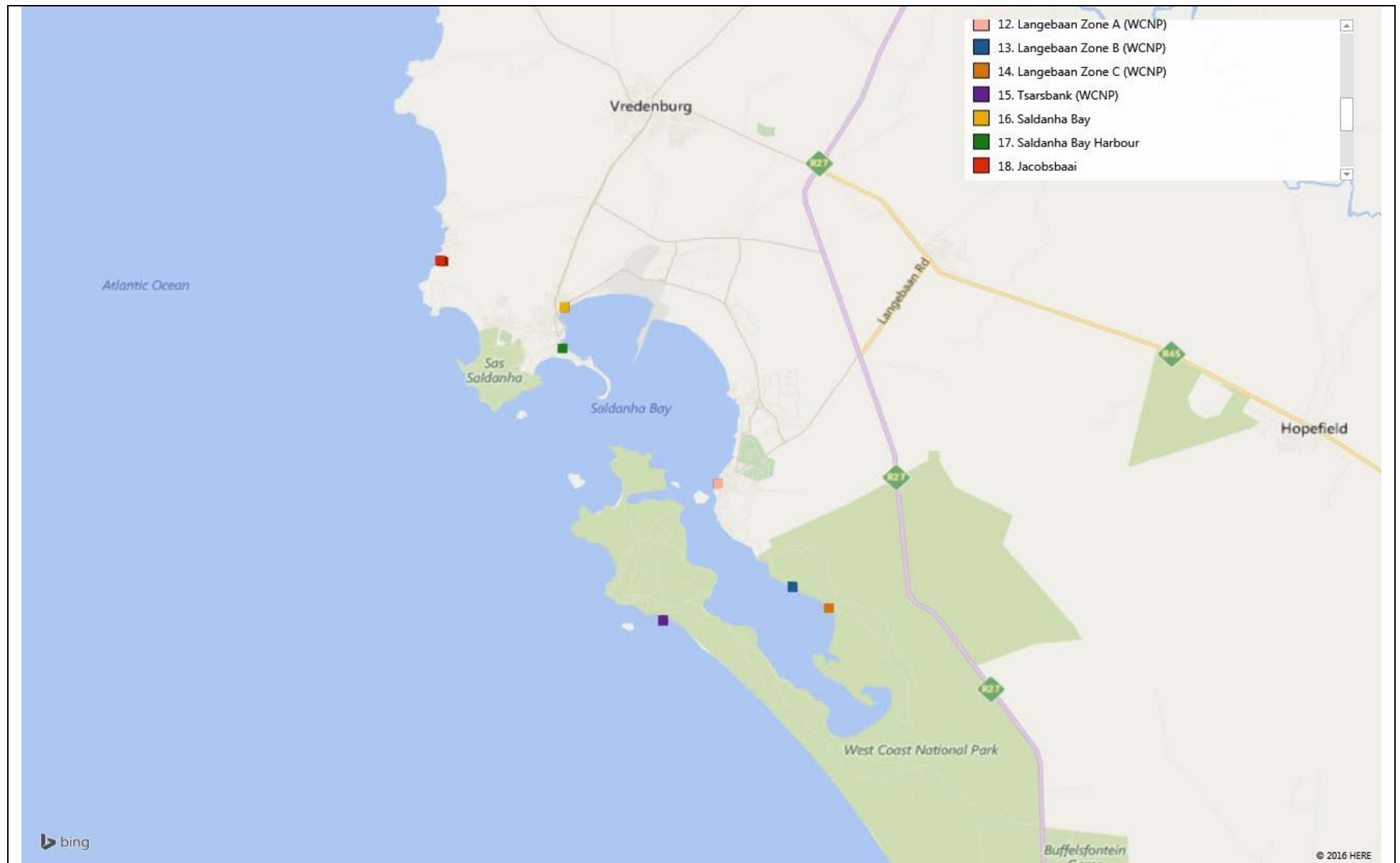
SITE	BOATING ACTIVITY	SPECIES	M:F RATIO	% IMPOSEX	RPLI	RPSI	DBT (µg/kg)	TBT (µg/kg)	N
1. Cape Agulhas	Low	<i>Afrolittorina knysnaensis</i>	6:24	0	0	0	< 5	< 2	30
		<i>Burnupena lagenaria</i>	16:14	14.3	9.8	0.09	< 5	< 2	30
2. Stuisbaai Harbour	Medium	<i>Thais capensis</i>	8:22	0	0	0	< 5	< 2	30
3. Gansbaai	Low	<i>Burnupena lagenaria</i>	10:20	0	0	0	< 5	< 2	30
4. Gansbaai Harbour	High	<i>Burnupena cincta</i>	12:12	91.7	9.4	0.08	< 5	< 2	24
		<i>Burnupena lagenaria</i>	23:7	0	0	0	< 5	< 2	30
		<i>Clionella sinuata</i>	1:29	0	0	0	< 5	< 2	30
5. Betty's Bay	Low	<i>Bullia rhodostoma</i>	13:17	52.9	1.9	0.0006	< 5	< 2	30
		<i>Burnupena cincta</i>	11:15	0	0	0	< 5	< 2	26



SITE	BOATING ACTIVITY	SPECIES	M:F RATIO	% IMPOSEX	RPLI	RPSI	DBT (µg/kg)	TBT (µg/kg)	N
6. Gordon's Bay Harbour	High	<i>Burnupena cincta</i>	13:10	100	80.4	51.9	591	20000	23
7. Strand	Low	<i>Burnupena catarrachta</i>	9:21	0	0	0	< 5	< 2	30
8. Simon's Town Yacht Club	High	<i>Afrolittorina knysnaensis</i>	16:14	0	0	0	610	7930	30
9. Soetwater	Low	<i>Afrolittorina knysnaensis</i>	14:16	0	0	0	< 5	< 2	30
		<i>Burnupena catarrachta</i>	4:26	0	0	0	< 5	< 2	30
10. Lagoon beach	High	<i>Bullia digitalis</i>	1:29	6.9	13.6	0.25	< 5	< 2	30
		<i>Burnupena rotunda</i>	8:12	0	0	0	< 5	< 2	20
11. Melkbosstrand	Low	<i>Bullia digitalis</i>	2:27	0	0	0	< 5	< 2	29
		<i>Burnupena catarrachta</i>	6:24	0	0	0	< 5	< 2	30



SITE	BOATING ACTIVITY	SPECIES	M:F RATIO	% IMPOSEX	RPLI	RPSI	DBT (µg/kg)	TBT (µg/kg)	N
12. Langebaan Zone A (WCNP)	Low	<i>Bullia laevissima</i>	8:19	36.8	2.8	0.002	< 5	< 2	27
		<i>Bullia rhodostoma</i>	0:30	0	0	0	< 5	< 2	30
		<i>Burnupena cincta</i>	13:17	0	0	0	< 5	< 2	30
		<i>Burnupena lagenaria</i>	12:18	0	0	0	< 5	< 2	30
		<i>Burnupena papyracea</i>	8:22	0	0	0	< 5	< 2	30
13. Langebaan Zone B (WCNP)	Low	<i>Afrolittorina knysnaensis</i>	11:19	0	0	0	< 5	< 2	30
		<i>Nucella dubia</i>	9:12	0	0	0	< 5	< 2	21
14. Langebaan Zone C (WCNP)	Low	<i>Burnupena cincta</i>	13:8	0	0	0	< 5	< 2	21
15. Tsarsbank (WCNP)	Low	<i>Afrolittorina knysnaensis</i>	5:25	0	0	0	< 5	< 2	30
		<i>Burnupena catarrachta</i>	12:18	0	0	0	< 5	< 2	30
16. Saldanha Bay	High	<i>Bullia digitalis</i>	6:24	100	13.2	0.23	< 5	< 2	30
		<i>Burnupena Cincta</i>	11:19	31.6	0	0	< 5	< 2	30
17. Saldanha Bay Harbour	High	<i>Afrolittorina knysnaensis</i>	12:18	50	48.3	11.2	3740	14400	30
18. Jacobsbaai	Low	<i>Afrolittorina knysnaensis</i>	9:21	0	0	0	< 5	< 2	30
		<i>Burnupena catarrachta</i>	11:19	0	0	0	< 5	< 2	30
		<i>Clionella sinuata</i>	0:30	0	0	0	< 5	< 2	30



SITE	BOATING ACTIVITY	SPECIES	M:F RATIO	% IMPOSEX	RPLI	RPSI	DBT (µg/kg)	TBT (µg/kg)	N
19. Lambert's Bay Harbour	Medium	<i>Burnupena catarrachta</i>	13:17	0	0	0	415	495	30
20. Bird Island (Lambert's Bay)	Low	<i>Burnupena catarrachta</i>	16:14	0	0	0	< 5	< 2	30
21. Strandfontein	Low	<i>Bullia digitalis</i>	8:22	0	0	0	< 5	< 2	30
		<i>Burnupena lagenaria</i>	7:23	0	0	0	< 5	< 2	30
		<i>Burnupena rotunda</i>	10:20	0	0	0	< 5	< 2	30
		<i>Nucella cingulata</i>	12:18	0	0	0	< 5	< 2	30
22. Hondeklipbaai Harbour	Low	<i>Burnupena catarrachta</i>	6:22	0	0	0	< 5	< 2	28
23. Hondeklipbaai	Low	<i>Afrolittorina knysnaensis</i>	11:19	0	0	0	< 5	< 2	30
		<i>Burnupena catarrachta</i>	13:17	0	0	0	< 5	< 2	30
24. Port Nolloth (McDougali Bay)	Low	<i>Burnupena catarrachta</i>	7:23	0	0	0	< 5	< 2	30
		<i>Clionella sinuata</i>	3:27	0	0	0	< 5	< 2	30
25. Port Nolloth Harbour	Low	<i>Afrolittorina knysnaensis</i>	14:16	0	0	0	< 5	< 2	30
		<i>Burnupena catarrachta</i>	13:17	0	0	0	< 5	< 2	30
		<i>Burnupena rotunda</i>	10:20	0	0	0	< 5	< 2	30



Sites with high boating activity, and high imposex prevalence were Gansbaai Harbour, Gordon's Bay Harbour, Lagoon Beach, Saldanha Bay, and Saldanha Bay Harbour.

Simon's Town Yacht Club had high boating activity, but no imposex was observed.

Lambert's Bay and Struisbaai Harbour had intermediate boating activity and no imposex.

Sites with low boating activity, and imposex prevalence were Cape Agulhas, Betty's Bay and Langebaan Zone A (WCNP).

The remaining sites (Gansbaai, Strand, Soetwater, Melkbosstrand, Langebaan Zone B and C, Tsarsbank, Jacobsbaai, Bird Island, Strandfontein, Hondeklipbaai Harbour, Hondeklipbaai, Port Nolloth (Mcdougall Bay) and Port Nolloth) were all pristine sites, with low boating activity and no imposex.

4.2.2 Boating Activity vs. RPLI

The next test was performed to determine whether or not boating activity also influenced the RPLI. This only entailed the sites where there were observed cases of imposex in females. The null-and alternative hypotheses were constructed as follows:

H₀: Mean levels of RPLI are not different for different levels of boating activity.

H_A: Mean levels are different.

Table 11: Boating activity vs. RPLI

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable RPLI Classified by Variable Boating activity					
Boating activity	N	Sum of Scores	Expected Under H ₀	Std Dev Under H ₀	Mean Score
1. Low	3	7.0	13.50	3.354102	2.333333
3. High	5	29.0	22.50	3.354102	5.800000

Wilcoxon Two-Sample Test	
Statistic	7.0000
Normal Approximation	
Z	-1.7889
One-Sided Pr < Z	0.0368
Two-Sided Pr > Z	0.0736
t Approximation	
One-Sided Pr < Z	0.0584
Two-Sided Pr > Z	0.1168
Z includes a continuity correction of 0.5.	

Kruskal-Wallis Test	
Chi-Square	3.7556
DF	1
Pr > Chi-Square	0.0526

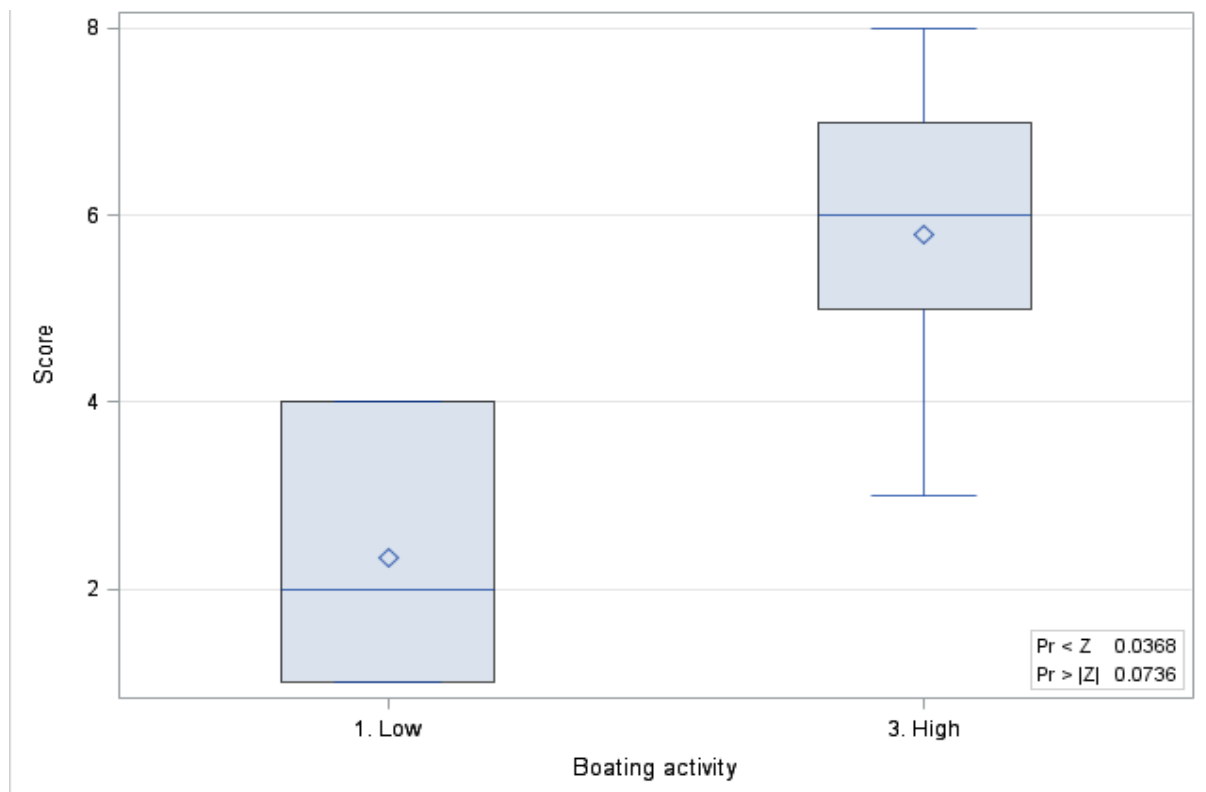


Figure 9: Wilcoxon box plot comparing the rank scores of Boating Activity vs. RPLI

As mentioned earlier, there was very little exposure in sites with medium boating activity (only two), that actually needed to be grouped with another level. This meant that only two levels were used to compare the relationship by using the Wilcoxon Two-Sample test. The results of this test are included in Table 11, whereby all the p-values of the test were low, but not always less than 0.05.

The test statistic for the Kruskal-Wallis test was 0.0526, which pointed to some uncertainty, which may be due to the fact that the sample size had to be reduced to only include the sites with observed imposex in females. Because of this sample reduction, the precision of measurement could be reduced and the null-hypothesis should be rejected, with caution.

This test showed that the higher the levels of boating activity, the higher the RPLI for imposexed females (Figure 9). The same conclusions were reached when looking at RPSI as they are related.

4.2.3 Boating activity vs. Male:Female Ratio (Male%)

The influence of boating activity on the male to female ratio (actual male percentage) of the sample was tested. The hypotheses were constructed as follows:

H0: Mean levels of the male proportion do not differ given different levels of boating activity.

HA: Mean levels differ given different levels of boating activity.

Table 12: Boating activity vs. Male:Female ratio (M%)

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable Male Classified by Variable Boating activity					
Boating activity	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
1. Low	36	840.50	882.0	41.918995	23.347222
2. Medium	2	53.00	49.0	19.344740	26.500000
3. High	10	282.50	245.0	39.315227	28.250000
Average scores were used for ties.					

Kruskal-Wallis Test	
Chi-Square	1.0063
DF	2
Pr > Chi-Square	0.6046

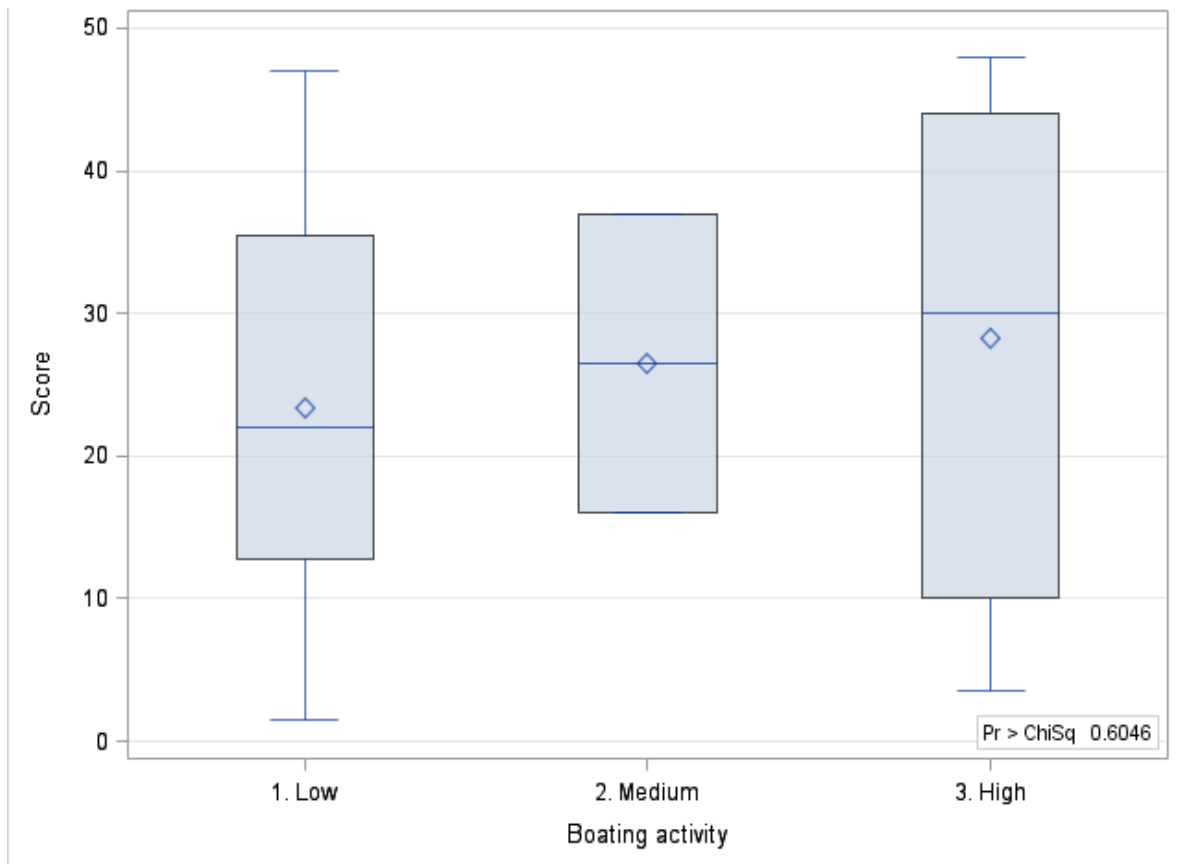


Figure 10: Wilcoxon box plot comparing the rank scores of Boating Activity vs. Male %

According to Figure 10, there was directional influence between the boating activity and the proportion of males in the sample. However, the p-value in Table 12 was 0.6. The null-hypothesis could not be rejected – the mean levels were not statistically different as boating activity increased.

4.3 Sensitivity of gastropods

Table 13: Sensitivity of gastropods

SPECIES	% I RANGE		RPLI RANGE		RPSI RANGE	
	LOW BOATING	HIGH BOATING	LOW BOATING	HIGH BOATING	LOW BOATING	HIGH BOATING
<i>Afrolittorina knysnaensis</i>		0-50		0-48.27		0-11.24
<i>Bullia digitalis</i>		6.9-100		13.19-13.55		0.23-0.25
<i>Bullia laevissima</i>	0-36.84		0-2.8		0-0.002	
<i>Bullia rhodostoma</i>	0-52.94		0-1.86		0-0.0006	
<i>Burnupena cincta</i>		31.58-100		9.42-80.37		0.08-51.92
<i>Burnupena lagenaria</i>	0-14.29		0-9.81		0-0.09	

The sensitivity of gastropods was inferred from the % I, RPSI, RPLI in relation to high and low boating activity among the various sites (Table 13) as imposex is correlated with boating activity.

Species that demonstrated a higher sensitivity towards TBT pollution were *B. laevissima* and *B. rhodostoma* that displayed relatively high % I and high RPLI and RPSI values at low boating sites. *Afrolittorina knysnaensis* that displayed low % I and RPLSI and RPLI values at high boating sites were presumed less sensitive.

CHAPTER 5: DISCUSSION

Imposex and associated TBT pollution in South Africa has only been reported by Marshall and Rajkumar in 2003, indicating that imposex was prevalent in gastropod, *N. kraussianus* populations in harbours along the Indian Ocean of South Africa. This also indicated that TBT was bioavailable in South African harbours.

In the present study, the presence of TBT was inferred from imposex prevalence in caenogastropods and TBT/DBT concentrations in sediment sampled from sites with varying degrees of boating activity, along the Atlantic coastline.

5.1 TBT and DBT concentrations in sediments

Sediments accumulate contaminants and serve as sources of pollution to the ecosystems that are associated with them (Burton Jr, 2002). Due to their hydrophobicity and high affinity for particulate matter, pollutants such as TBT accumulate in sediments (Hoch, 2001; Omae, 2006; Sousa *et al.*, 2014). TBT and DBT concentrations were measured in sediment from all sites (N=25) as imposex is known to be associated with TBT levels found in sediments (Penchaszadeh *et al.*, 2001). TBT and its breakdown product, DBT, were recorded in sediment. Another study by Okoro *et al* (2016) showed that TBT was also present in seawater and sediment in Cape Town Harbour. This indicates that TBT is bioavailable in South Africa, despite being banned by the IMO since 2008 (IMO, 2014).

Extremely high concentrations of TBT and DBT were present in sediment from four sites, with the remainder being below the limit of detection ($<5 \mu\text{gDBT/kg dm}$ and $<2 \mu\text{gTBT/kg dm}$). Gordon's Bay Harbour had the highest recorded concentration of TBT and DBT, followed by Simon's Town Yacht Club (False Bay), Saldanha Bay Harbour and Lambert's Bay.

These heavily polluted sites contain diverse maritime facilities including shipyards, a naval base, fishing vessel docks and leisure-boat marinas; consequently, TBT input had been considerable. A small contribution may have arisen from overseas vessels from countries where there was no ban on TBT paint use.

Hydrodynamics and coastal morphology may play a role in the concentrations of TBT in the environment (Chiavarini *et al.*, 2003). The high degree of enclosure, slow water flushing rates of these harbours (resulting in reduced dilution of released TBT), and high marine traffic made these sites ideal environments for TBT accumulation.

The type of sediment may also influence the amount of TBT in the environment (Bigatti *et al.*, 2009). Fine-grained silts and clays, characteristic of benthic environments found in harbours, are known to sorb pollutants relatively efficiently (Namiesnik and Szefer, 2010). The type of sediments and amount of organic matter from the sites were not analysed, however it would be useful for future studies.

Lambert's Bay and Struisbaai were the only two sites with intermediate boating activity, but only Lambert's Bay had concentrations above the limit of detection. Lambert's Bay had a few fishing and mining boats berthed within the harbour. This harbour was semi-enclosed where little dilution may have occurred, which may have lead to the accumulation of OTCs in sediment.

Sites where there was high boating activity, such as Gansbaai Harbour, Lagoon Beach, and Saldanha Bay, where TBT and DBT levels were expected to be high, were below the limit of detection. This may be attributed to the hydrodynamics of these sites. The sites were open to the prevailing oceanic currents where pollution is usually dispersed and diluted. Other factors may also have played a role in the bioavailability of TBT at these sites, such as type of substrata and the amount of organic matter in the environment (Bigatti *et al.*, 2009).

Most of the sites where TBT and DBT levels were below the limit of detection were associated with low boating activity and were considered relatively unaffected when compared to the other sites. These sites were chosen as they were isolated, situated far from any obvious sources of pollution. The great hydrodynamic effectiveness of these sites may have reduced the accumulation of TBT and DBT in the sediments.

Results have shown that TBT pollution seems to be less prevalent than expected when compared to TBT concentrations in sediments from other countries (Antizar-Ladislao, 2008; Dafforn *et al.*, 2011; Kan-Atireklap *et al.*, 1997; Senthilkumar *et al.*,

1999). Results, in this study, have shown that TBT and DBT concentrations in sediments were localised, with higher concentrations being associated with high boating activity (Figure 6 and 7; Table 8 and 9).

5.1.1 TBT/DBT inputs

Tributyltin in the environment degrades in a stepwise manner to less toxic derivatives - DBT and MBT, and eventually into inorganic tin in the environment (Clark *et al.*, 1988; Fent, 2006; Langston *et al.*, 2009; Omae, 2006). The rate of degradation is the proportion of metabolites in the sample, and is dependent on time, microbial populations, and temperature (Langston *et al.*, 2009). A study performed by Diez *et al.*, 2002 determined the inputs of TBT into the environment by observing its rate of degradation. The concentration of metabolites in relation to the parent compound may be used as an indication of recent or past usage.

As shown in Figure 5, DBT and TBT concentrations were almost equal in Lambert's Bay Harbour suggesting that TBT had started to breakdown. TBT concentrations at this harbour may not have been from recent inputs. In contrast, TBT levels were considerably higher than DBT in Gordon's Bay Harbour, Saldanha Bay Harbour and Simon's Town Yacht Club, which indicates continued usage of TBT.

5.2 Imposex prevalence and severity

Imposex intensity is related to TBT bioavailability in the environment (Axiak *et al.*, 1995; Bigatti *et al.*, 2009; Matthiessen and Gibbs, 1998; Penchaszadeh *et al.*, 2001; Ten Hallers-Tjabbes *et al.*, 2003). Populations that inhabit areas with high boating and shipping activity show the highest degree of imposex (Bryan *et al.*, 1986; Smith *et al.*, 2006; Sousa *et al.*, 2005), but levels decrease as distance increases from these 'hotspots' of pollution and become null in areas without boating traffic (Bigatti *et al.*, 2009; Evans and Nicholson, 2000; Li and Collin, 2009; Minchin *et al.*, 1997). The coincidence between high degrees of imposex and proximity to harbours and marinas indicates that the pollutant is almost certainly associated with shipping or boating activity (Bigatti *et al.*, 2009; Bryan *et al.*, 1986).

Percentage imposex was used to determine the amount of penis-bearing females at a site and the RPSI and RPLI were used to measure the intensity of imposex (Gibbs *et al.*, 1987; Titley-O'Neal *et al.*, 2011). The higher these values were, the greater the intensity of imposex and therefore TBT pollution.

The present study found that % I, RPLI and RPSI were associated with boating activity - % I, RPLI and RPSI increased with increased boating activity - refer to Figure 8 and 9, including Table 9 and 11. TBT and DBT concentrations also increased with increasing level of boating activity. Imposex was present in eight sites - Cape Agulhas, Gansbaai Harbour, Betty's Bay, Gordon's Bay Harbour, Lagoon Beach, Langebaan Zone A (WCNP), Saldanha Bay, and Saldanha Bay Harbour. It confirms that imposex detected in female snails sampled from 25 localities on the Atlantic coast of South Africa is related to TBT pollution.

Six species displayed imposex, namely: *A. knysnaensis*, *B. digitalis*, *B. rhodostoma*, *B. laevissima*, *B. cincta*, and *B. lagenaria*. However, this does not suggest that the other species would not have developed the imposex effect. The other gastropod species were possibly less sensitive; or TBT pollution may have been absent from the sites they were sampled from.

Gansbaai Harbour, Gordon's Bay Harbour, Lagoon Beach, Saldanha Bay and Saldanha Bay Harbour were areas of high boating activity where gastropods displayed imposex. Gansbaai Harbour is a fishing port. Structures such as fishing pens were also present within the harbour. Tributyltin has been known to be painted on these structures (Champ, 2000). Species, *B. cincta*, *B. lagenaria* and *C. sinuata*, were collected from this site; only *B. cincta* had imposex. There was a high percentage (91.7%) of females affected by imposex. The RPLI was 9.42 and the RPSI was 0.08. These values were low, indicating low TBT pollution, which was confirmed by the low levels of TBT and DBT in sediment sampled from this site.

Burnupena cincta showed 100% of imposex in Gordon's Bay Harbour population. The RPLI and RPSI values were the highest at this site with an RPLI value of 80.4, and an RPSI value of 51.9. The RPLI and RPSI were indicative of advanced imposed penis development. The female population's penis development is almost the same as for

the male population. The high levels of imposex and high RPLI and RPSI values in *B. cincta* may be from the continuous input of TBT from the various maritime activities that occurred at this site. TBT pollution was evidently severe at this site.

Lagoon Beach is situated in close proximity to Cape Town Harbour. Cape Town Harbour is strategically positioned, almost at the southern tip of the African continent, as it is situated on one of the world's busiest trade routes. The port serves cargoes moving between Europe and the western hemisphere, and the Middle East and Australia (Transnet, 2010). During the 2011/2012 financial year, Cape Town handled 2,775 vessels (Ports and Ships, 2015). Gastropods collected from this site were *B. digitalis* and *B. rotunda*. Only, *B. digitalis* displayed imposex, with a low % I (6.9%), and low RPSI and RPLI values. OTC levels were below the limit of detection at this site, which may explain the low % I, RPLI, and RPSI.

At a high boating activity site, Saldanha Bay, both *B. digitalis* and *B. cincta* displayed imposex. *B. digitalis* displayed 100% imposex with reasonably low RPLI and RPSI values, while *B. cincta* displayed 31.6% imposex. Zero values were obtained for RPLI and RPSI for *B. cincta*, although imposex was evident, penis development was only in the early stages. The intensity of imposex was more severe in *B. digitalis* than *B. cincta*. *Burnupena cincta* did not form a penis, only developing a so-called 'bud penis' (beginning stage of imposex), which may indicate recent TBT exposure.

Fifty percent of *A. knysnaensis* females were affected by imposex in Saldanha Bay Harbour. The RPLI was 48.3 indicating that the penis length was almost half of the male population's. The RPSI was relatively low, at 11.2. These females were sampled from rocks where there is supposedly less exposure to TBT pollution and littorinids are known for their lowered sensitivity towards TBT pollution (Oehlmann *et al.*, 1998a). For this species to display imposex to such an extent demonstrates the high levels of TBT pollution at this site. Saldanha Bay is South Africa's largest and deepest natural port and as a result has gone through extensive harbour development (Clark *et al.*, 2012; Transnet, 2010). The presence of a shipyard, harbours, fishing ports, marinas, ore terminal, oil terminal, together with some ship mooring areas and dredging are considered potential sources of TBT pollution here.

Gastropods sampled from Gansbaai Harbour, Lagoon Beach, and Saldanha Bay displayed imposex, although, TBT and DBT concentrations were below the limit of detection. However, imposex may be induced below limits of detection (Bryan *et al.*, 1987). In the absence of other possible causes, these sites were therefore presumed polluted by TBT, because of imposex prevalence at these sites. Once chemical contamination concentration reaches a point at which it causes adverse effects (i.e. imposex) to biota, it is considered polluted (Burton Jr, 2002). In addition, the amount of chemical identified analytically is also not necessarily equivalent to the amount that is bioavailable (ATSDR, 2005). The presence of imposex at these sites may be due to previous exposure as imposex is irreversible (Matthiessen and Gibbs, 1998) and concentrations are likely to change over time because of tides and sediment dynamics, or even be a transient, once off, event.

Cape Agulhas, Betty's Bay and Langebaan Zone A (WCNP) were sites with low boating activity and were chosen because of their relatively pristine nature. Undetectable levels of TBT and DBT were also recorded, yet imposex was prevalent at these sites. As mentioned before, imposex may be induced at levels below the limit of detection, which may explain its presence at these sites.

Afrolittorina knysnaensis and *B. lagenaria* were sampled from Cape Agulhas. Imposex was only observed in *B. lagenaria*. A low percentage (14.3%) of females was affected by imposex, including low RPLI and RPSI values. The low % I, RPLI and RPSI values correlated with the undetectable levels of TBT pollution.

Betty's Bay was assumed to be an area free of TBT pollution as it was a pristine area, with no obvious sources of pollution. The site was also exposed to the open ocean where prevailing currents may remove pollutants. Two gastropod species, *B. rhodostoma* and *B. cincta* were collected from this site. Only one species, *B. rhodostoma* displayed imposex. 52.9% of female gastropods sampled from Betty's Bay developed imposex, yet the RPLI and RPSI values were very low. The low RPLI and RPSI levels recorded indicated lowered TBT pollution. This was confirmed by chemical analysis as TBT levels were undetectable in sediments.

Langebaan Zone A (WCNP) is situated in the West Coast National Park. Five gastropod species were sampled from this site. Only *B. laevissima* exhibited imposex. 36.8% of the female population were affected by imposex. Low RPLI and RPSI values were recorded, indicating low TBT pollution. These gastropods are known to live sub-tidally (Branch *et al.*, 2010). They were washed upon the shore, along with marine debris. The presence of imposex in these gastropods may have been due to exposure to TBT from an area other than where they were sampled from.

Imposex may be found in relatively pristine areas (Terlizzi *et al.*, 2004). Areas presumed to be the most pristine natural habitats, such as the Great Barrier Reef (GBR) and the Antarctic are impacted by TBT pollution (Negri and Marshall, 2009). External sources may have played a role in the distribution of TBT to these relatively pristine areas. Many scattered small wharves and vessel traffic (shipping lanes) may also be sources leading to the wide distribution of imposex to the remote places from harbours and shipyards (Shim *et al.*, 2000). South Africa is located along a primary shipping route between Europe, the Americas, and Asia (Marshall and Rajkumar, 2003). Tributyltin pollution in South Africa may not only be from obvious maritime activity, but could also be from its presence on wooden structures, as TBT is used for other industrial activities such as wood preservation (Burger *et al.*, 2008; Graham, 2008).

Imposex may have severe consequences for gastropod populations causing sterility, female mortality, reduced fecundity and ultimately population decline and in worst cases, local extinction (Evans *et al.*, 2000a; Gibbs *et al.*, 1987; Ketata *et al.*, 2008; Oehlmann *et al.*, 1996b. Oehlmann *et al.*, 1998a; Schulte-Oehlmann *et al.*, 1997; Shim *et al.*, 2000; Stroben *et al.*, 1992).

5.2.1 Sex ratio

According to Azuma *et al.* 2014, anthropogenic pollution may cause sex-biased asymmetry in population genetics. TBT-induced imposex leads to female proportional decline (Evans *et al.*, 2000a; Gibbs *et al.*, 1986; Ketata *et al.*, 2008; Oehlmann *et al.*, 1996b. Oehlmann *et al.*, 1998a; Schulte-Oehlmann *et al.*, 1997; Shim *et al.*, 2000;

Stroben *et al.*, 1992) as it reduces the female- to-male sex ratio, which may eventually lead to the local extinction of species (Shim *et al.*, 2000).

In Figure 10 and Table 12, boating activity in this study did not have an influence on the M:F ratio. The sex ratios for most species seem to be female-biased from the majority of sites, even those affected by imposex. Imposex may have not advanced to the stage where it may have caused female mortality. At sites where populations were male-biased, the sex ratio may have been influenced by factors other than imposex, such as feeding patches, reproductive migrations, parasitism etc.

5.3 Species sensitivity

Gastropods are known for their sensitivity towards pollutants and as a result have been used in numerous toxicological studies (Matthiessen, 2008; Oehlmann *et al.*, 2007). Gastropod's degree of sensitivity towards TBT pollution may vary between species; even between populations inhabiting different areas, therefore, imposex development and the effect of imposex may also vary significantly (Gibbs, 1996; Stewart *et al.*, 1992). Many factors play a role in the varying sensitivities exhibited by gastropod species. These factors include bioaccumulation potential (Langston and Pope, 2009), diet, physiology, habitat, etc (Tan, 1999). The extent to which female gastropods are affected by imposex may also depend on their reproductive strategy (Sternberg *et al.*, 2010). Reproduction in some populations is affected by imposex to the point of extinction, while in others it is not affected at all (Axiak *et al.*, 2003; Matthiessen and Gibbs, 1998; Swennen and Horpet, 2008).

Various gastropod species inhabit the same sampling site; therefore, a direct comparison of their sensitivity to TBT pollution may be made between them according to their development of imposex (Oehlmann *et al.*, 1996a). Species that demonstrated a higher sensitivity towards TBT pollution were those that displayed high % I and high RPLI and RPSI values at low boating sites, while those that only displayed low % I and low RPLI and RPSI values at high boating sites, were presumed less sensitive (Table 13). In this study, six species of gastropods developed imposex (see Table 13) – *B. laevissima*, *B. rhodostoma*, *B. digitalis*, *B. cincta*, *B. lagenaria* and *A. knysnaensis*, to varying degrees.

Gastropods from the genus, *Bullia*, exhibited the highest sensitivity towards TBT pollution, followed by *Burnupena* species, and lastly the Littorinids. *Bullia laevissima* were the most sensitive when compared to the other species of the same genus, followed by *B. rhodostoma* and *B. digitalis*. *Bullia laevissima* and *B. rhodostoma* both developed imposex at low boating activity sites. However, both species were sampled from Langebaan Zone A and only *B. laevissima* developed imposex. *Bullia digitalis* exhibited imposex at high boating activity sites, indicating that they were less sensitive when compared to the two other species. *Bullia* inhabit sandy areas, where TBT is known to accumulate. They are scavengers and may accumulate TBT through biomagnification. Species of the genus, *Bullia* have direct development within an egg capsule (Branch *et al.*, 2010), and as a result their spawn are not dispersed to cleaner sites and they are therefore more susceptible to TBT pollution and localised extinction.

Gastropod females from the genus, *Burnupena* were relatively sensitive to TBT pollution. Both, *B. cincta* and *B. lagenaria* developed imposex. It was difficult to ascertain which of the two species were most sensitive. Both appeared to be similarly sensitive, however, more detailed studies are needed to determine which is more sensitive.

Afrolittorina knysnaensis were the least sensitive as they displayed an average percentage of imposex at Saldanha Bay Harbour. *Afrolittorina knysnaensis* were the only gastropods sampled from Simon's Town yacht club, which had high boating activity and high concentrations of TBT and DBT, yet no imposex was observed. Littorinids are known for their lower sensitivity towards TBT pollution and are used in studies as biological indicators where more sensitive species are absent (Oehlmann *et al.*, 2007) or at sites with high TBT concentrations, where all highly sensitive species show no differences (e.g. all have 100% imposex or high RPLI/RPSI values) (Bigatti *et al.* 2009). *Afrolittorina knysnaensis*' lowered sensitivity to TBT pollution may be attributed to their diet as they are herbivorous. They also inhabit areas less exposed to TBT pollution and their developmental modality (free-swimming veliger larvae) enables them to recolonise areas that are TBT-free (Oehlmann *et al.*, 2007).

Bigatti *et al* (2009) reported that species which best reflected the degree of imposex were those inhabiting sandy/muddy or mixed bottoms and species inhabiting intertidal

hard bottoms, were not good indicators of TBT contamination. Similar results were found in this study. *Afrolittorina knysnaensis*, inhabited rocky areas, and were possibly less exposed to TBT pollution, which usually accumulates in sandy/muddy or mixed bottoms where species such as *B. laevissima*, *B. rhodostoma*, *B. digitalis*, *B. cincta* and *B. lagenaria* were sampled.

Even the most polluted of areas are not without species as certain species can tolerate such conditions. Species differ greatly in their ability to tolerate pollutants (Begon *et al.*, 2006). Littorinids can therefore be used as indicators in areas where there is a high TBT pollution as they are less sensitive, whereas whelks and plough shells should be used at areas where there are lower levels of TBT pollution as they are more sensitive.

CHAPTER 6: CONCLUSION

The main purpose of this study was to determine the presence of TBT contamination by using imposex as a biomarker for TBT pollution and by measuring concentrations of TBT in sediment from sites along the Atlantic coast of South Africa.

The above was achieved by determining the presence and prevalence of imposex in caenogastropods along the Atlantic coastline from sites with varying degrees of boating activity (e.g. those presumed impacted and non-impacted by TBT). Imposex was prevalent and therefore indicating TBT pollution. Tributyltin has been banned by the IMO since 2008 (IMO, 2014) and South Africa is a party to many of the conventions or protocols that fall under the IMO (DFA, 2006).

Imposex was detected at various boating sites. Imposex prevalence and intensity was related to boating activity, similar to results found by other studies (Bigatti *et al.*, 2009, Bryan *et al.*, 1987; Gibbs and Bryan, 1987; Smith, 1981a; Smith, 1981b; Smith, 1981c) Imposex was highest at high boating activity sites and low to null at intermediate and low boating activity sites.

Concentrations of TBT were determined in sediment by means of chemical analysis to provide supplementary information regarding the bioavailability of TBT in the environment. TBT and DBT were present. Concentrations in sediment of TBT and the breakdown product DBT were extremely high, far beyond the dynamic range of quantification of the method at four sites. The highest levels were recorded in Gordon's Bay Harbour followed by Simon's Town Yacht Club (False Bay), Saldanha Bay Harbour, and Lambert's Bay. The remaining sites had levels below the limit of detection.

A broader understanding regarding the usage of TBT along the Atlantic coast was developed. Tributyltin was found at harbours that were semi-enclosed by physical barriers, where little wave movement may not have compensated for the high amount of TBT input from various maritime activity. The sites where TBT levels were high were related to high boating activity, except for Lambert's Bay, which had intermediate boating activity. Only a few sites had high levels of TBT measured in the sediment,

however, these concentrations were extremely high, which is a major cause for concern. The imposex effect was recorded at eight sites; five being high boating activity sites and the rest being low boating activity sites. Imposex recorded at low boating activity sites may have been a result of various external sources of TBT.

Taxa best suited for describing TBT impacts were those inhabiting sediments where TBT was known to accumulate and those with a higher sensitivity. It was found that the genus *Bullia* were good indicators as imposex was induced in these species at low concentrations. Species belonging to the genus *Burnupena* were also potential biological indicators. In areas of high pollution where these gastropods may be absent (or if they exist, they all have 100% and too large RPLI/RPSI, which makes it difficult to compare), Littorinids, such as *A. knysnaensis* may be more suitable to determine TBT pollution as they displayed a lower sensitivity as they only developed imposex at high levels of boating activity. All of these species are commonly found and easy to collect in the studied sites. These results can be utilised for future biomonitoring studies in order to infer TBT contamination prior to performing expensive biochemical analysis to determine the bioavailability of a compound.

Gastropods probably represent the best-documented and population-relevant case of endocrine disruption in wildlife with the cause-effect relationship between imposex and antifouling coatings containing organotin compounds (Depledge and Billingham, 1999; Lafont and Mathieu, 2007; Matthiessen and Gibbs, 1998; Porte *et al.*, 2006; Sousa *et al.*, 2014; Sumpter, 2002; Sumpter and Johnson, 2005). It is crucial to develop more studies on invertebrates and endocrine disruptive effects as there is limited knowledge regarding the endocrinology of invertebrates (Porte *et al.*, 2006) and effects of EDCs on wildlife invertebrate species might have far-reaching adverse consequences for biodiversity and the sustainability of natural ecosystems (Depledge and Billingham, 1999). Invertebrates play important ecological roles. They are key components of all ecosystems (Depledge and Billingham, 1999; Oehlmann and Schulte-Oehlmann, 2003) and comprise about 95% of all terrestrial and aquatic animal species (Warring and Harris, 2005) which make them an important part of the global biodiversity (Oetken *et al.*, 2004).

The widespread usage of TBT and organotin compounds, their persistence in the environment, high toxicity towards non-target organisms together with their tendency to accumulate in marine organisms, have created economic costs associated with their severe consequences to aquatic life (Chiavarini, *et al* 2003; Mercier *et al.*, 1997). Tributyltin has had significant economic impacts in important breeding waters due to contamination and this has jeopardised the sustainable development of some marine activities (Alzieu, 1998). Seafood contaminated by TBT can cause a risk to the local population and tourism industry (Swennen *et al.*, 2009) as there are potential human health implications associated with the consumption of these marine organisms. It is of concern as imposex was recorded in gastropods where mussels were farmed in Saldanha Bay. This may affect the livelihoods of communities living along the coasts. TBT has become more than a local problem, but a global one (Fent, 2006; Champ, 2000; Dyrinda *et al.*, 1992). It is not only confined to inshore areas, but open oceans. What is the benefit if one country restricts the use of TBT while others are still using and abusing it? Countries that have banned the use of TBT may still be impacted by it when vessels from countries where TBT is not regulated enter their oceans. It is not only a problem for developed countries, but also one for developing countries, as these countries do not have the infrastructure capable of dealing with TBT contamination and its subsequent effects (Champ, 2000; Abbott *et al.*, 2000; Castro *et al.*, 2012a). Developed countries need to lead by example in order to prevent further contamination of organotins in the coastal waters of developing countries (Tanabe, 1999). The only option to eliminate TBT is the rapid ratification of the AFS Convention (IMO, 2014) by other countries.

In order to control TBT contamination not only does the application of TBT-based antifoulants need to be prohibited, but dry-dock practices need to be regulated (Axiak, *et al.*, 2000; Turner, 2010). Both production restrictions and dry-dock practices need to be audited regularly. So, also should the paints on ships that visit our waters. Although, being painted with TBT in itself is not illegal, preference should be given to shipping companies that use acceptable anti-fouling practices.

The situation regarding TBT contamination and associated imposex prevalence along the Atlantic coast reported in this study must be taken seriously by the relevant

authorities in order to develop regulations and policies regarding TBT usage and its removal, and implement them.

6.1 Recommendations and shortcomings

6.1.1 Fieldwork

- This was a single assessment to determine the presence of TBT levels in sediment and prevalence of imposex along the Atlantic coastline. It is necessary that the various sites should be monitored at regular intervals. The effects of seasonality need to be reduced by sampling at the same time. TBT concentrations are known vary between seasons (Okoro *et al.*, 2016).
- An extensive assessment of the entire coast of South Africa would be more informative. It would be interesting to see the difference between imposex effect and TBT pollution between two oceans that surround South Africa; the Atlantic and Indian Ocean.
- South Africa lacks criteria regarding TBT in the environment. Continued research of organotin compounds such as TBT remains necessary due to the lack of toxicity data generated.
- Male gastropods may also suffer from the consequences of TBT pollution. In some cases, when imposex levels are extremely high, the male penis may become deformed and mating may be prevented (Bryan *et al.*, 1987). This study was mostly concerned with the detrimental effects imposex has on females. It is necessary to determine possible harmful effects in male populations.
- Same species from various sites should be used for direct comparison as physiology and sensitivities differ among species. The result in different responses for each of the studied species must be studied in further detail.
- Transplanting healthy individuals to presumed contaminated sites may also be a useful indication of potential pollution. Imposex development provides limited

information on exposure period; adult snails living in uncontaminated conditions have been shown to display imposex features derived from TBT exposure during their juvenile stages (Marshall and Rajkumar, 2003).

- Other sediment quality parameters should be measured e.g. organic content, particle size, particulate organic carbon and trace metals. TBT in water should also be measured as certain gastropods are less exposed to TBT in sediment.
- Monitoring and observing oceans require an international effort and broad cooperation. This work will aid maritime management, provide information to public, and make them more aware of the current situation our oceans are facing.
- Other coastal and marine organisms may also be sensitive to TBT and DBT, and may need to be examined for possible impacts.
- Human consumption from contaminated molluscs and other seafood should be investigated.
- The presence/absence of juvenile snails and egg capsules should also be noted to establish if there were any reproductive side effects associated with imposex. The presence of juveniles or egg capsules in the field can be a valuable indication of continued reproductive activity in population.

6.1.2 Laboratory work

- The measurements of the male and female penises must be done with precision. There are various sizes and shapes of penises, therefore the measurement requirements must be standardised. It is recommended that the measurements be taken from the base of the penis along the centre, to the tip of the penis. It is crucial that methods should be standardised in order to maintain consistency between regions and to allow comparison.
- Penis size may be affected by thawing and freezing. If possible, measurements should be taken immediately. However, in this case, where penis measurements

were taken with absolute precision (with AZ100 Compound Microscope); there would not be great variability.

- A control group should be compared under laboratory conditions to compare results to as sites that are supposedly free from TBT pollution; e.g low boating activity sites (control sites) are not necessarily free from contamination. In addition, all chemicals are toxic under some conditions of exposure. It is important to determine what those conditions are.
- In addition to the indices used in this study, others may be utilised to quantify imposex such as the VDSI (Gibbs *et al.*, 1987).
- TBT levels should be measured in organisms from higher trophic levels to determine possible food web implications.
- The country lacks the means and capacity at all levels to address the ecotoxicological problems associated with EDC pollution (Burger *et al.*, 2008; Wepener and Degger, 2012). Capacity building at personal as well as institutional level is necessary for the success of any further projects in this field.
- The type of sediments from the sites were not analysed, however it would be useful for future studies as the type of substrata may play a major role in the bioavailability of TBT in the environment and therefore exposure to marine gastropods (Bigatti *et al.*, 2009).

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