Ecotoxicological effects of treated and untreated wastewater on the life cycle of *Helix pomatia* (Pulmonata) exposed in artificial soil

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Declaration

This thesis presents my original work for the Master of Science degree hereby submitted at the University of the Free State. I declare that I have not previously, in any form, submitted it at any university of a degree.

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Abbreviations and Acronyms

BSA - Bovine serum albumin

OECD – Organisation for economic co-operation and development

ICP-OES – Inductively coupled plasma optical emission spectrometer

GC – Gas chromatography

GC/MS – Gas chromatography/mass spectrometry

AChE- Acetylcholinesterase

CAT- Catalase

MAP- Maluti-A-Phofung

HCL- Hydrochloric Acid

n.d –Not Determined

ROS- reactive oxygen species

Abstract

Water treatment plants, which are established to convert wastewater into reusable water of suitable quality are under gross neglect in South Africa. As such, most of these treatment plants do not function optimally. The present study was conducted to determine the ecotoxicological effects of treated and untreated wastewater from the Maluti-A-Phofung wastewater treatment plant in Phuthaditjhaba. To do so, Wastewater samples were collected from the treatment plant, while a total of 300 Individuals of *Helix pomatia* were purchased from a breeder in Phuthaditjhaba. Each treatment was comprised of 10 snails placed in 2000g of OECD soil spiked with different concentrations (0, 25, 50, 75 and 100%) of wastewater. The ecotoxicological effects of the wastewater were determined by assessing the biomass, survival reproduction (cocoon production and juvenile emergence), and biomarker responses (Catalase activity Acetylcholinesterase) of *H. pomatia.* The results showed that there was a significant decrease ($P<0.05$) in the survival of *H. pomatia* from the control for both treatments groups. Reproduction results showed that there was a significant reduction ($P<0.05$) in cocoon production between *H. pomatia* exposed in controls and all the treatments groups for untreated and treated effluent. Similar results were observed for juvenile emergence. The results also indicated that *H. pomatia* exposed to untreated and treated wastewater showed significant reductions in biomass compared to the control. The differences were between the control groups and 100% groups for both exposure substrates ($P < 0.05$). An EC_{10} of 0,425% and EC_{50} of 6.233% were calculated for juvenile emergence and EC_{10} of 5,751% and EC_{50} of 5.751% were calculated for egg production in untreated wastewater. After 60 days of exposure, biomarkers were conducted and the results showed there was no statistical difference in AChE activities for *H. pomatia* exposed in control soils and soils spiked with treated and untreated wastewater, despite the fact that the activity seemed higher in *H. pomatia* exposed to soils spiked with treated wastewater. Similar results were found in catalase activity for H. pomatia exposed in soils spiked with treated and untreated wastewater, despite the fact that higher CAT activity seems to occur in the untreated wastewater

Results from this study highlight the toxic effects of wastewater pollution in the Maluti-A-Phofung wastewater treatment plant. If the wastewater treatment plant

continues to improperly manage their wastewater with similar contents or more this could lead to a decline in biodiversity, and also contribute to the alteration of ecosystem balance.

Keywords: Acetylcholinesterase, Catalase, *Helix pomatia*, Reproduction, Survival, Wastewater.

Chapter 1
1. Introduction and Literature Review

1.1 General information on wastewater

Wastewater is defined as water that has been used in the home, domestic, agriculture or as part of industrial processes (Bianco *et al*. 2013). Wastewater treatment plants produce two by-products called wastewater and sewage sludge (Snyman 2007). Statistics provided by Snyman in 2007 indicate that approximately 89% of wastewater treatment plants in South-Africa treat less than 500 m^3/ day (< 0.5 ml/ day) and a further 11% treat between 500 and 2000 m^3/ day. Wastewater can cause harmful algal blooms, which are induced by an overabundance of nutrients in the water and this leads to disease and death of organisms (Leomanni *et al*. 2015). Treated wastewater is used mainly for irrigation (both agricultural and landscape), seawater barriers, industrial and urban needs (Tchobanoglous *et al*. 2011). In addition, recycled water can augment strained water resources.

The use of biosolids remains challenging particularly for defence urban use. In both water re-use and biosolids application to land, the primary challenge remains public perception (Escher *et al*. 2012).

One of the most common ways of controlling sewage induced pollution is through wastewater treatment. The main function of a wastewater treatment plant is to convert wastewater into an effluent that can either be reused or transferred into rivers. There are different stages of wastewater treatment, primary secondary and tertiary stage.

1.2 Stages of a wastewater treatment plant

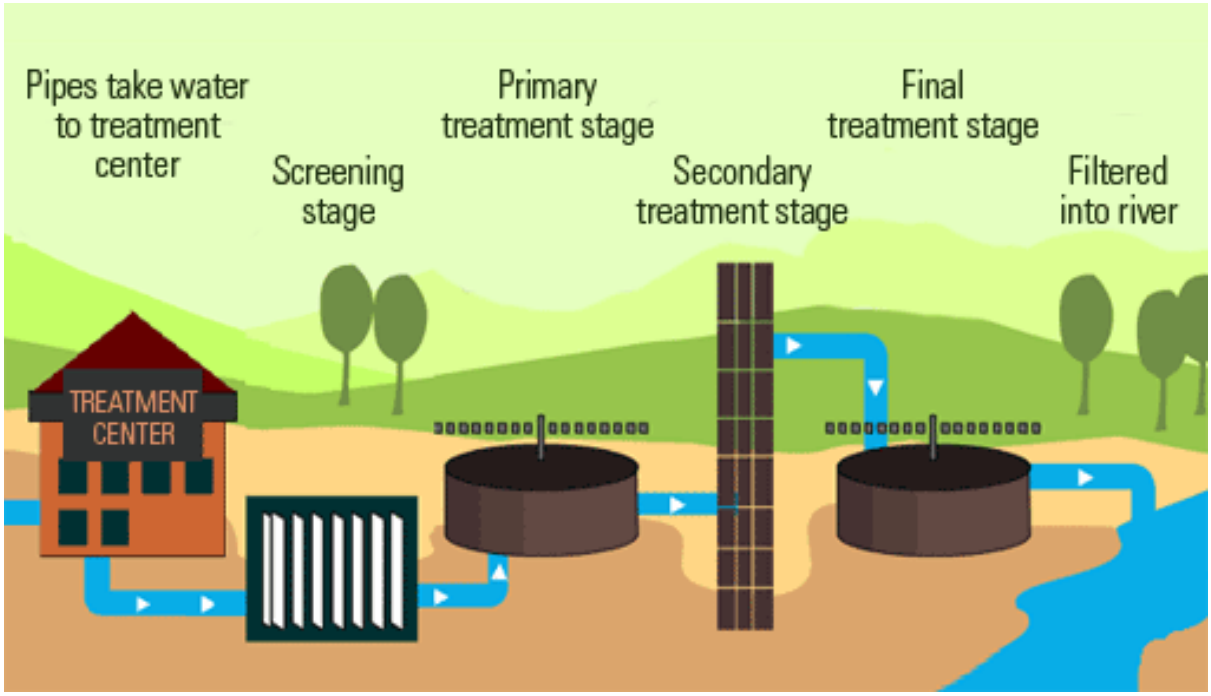

Figure 1: Different stages of a wastewater treatment plant

Source: http://eschooltoday.com/pollution/water-pollution/sewage-treatment-process

1.2.1 Preliminary treatment (screening)

The influent in sewage water passes through a bar screen to remove large particles such as plastics, cans, sticks etc that might damage the equipment. This is most commonly done with an automated mechanically raked bar screen in modern plants serving large populations, while in smaller or less modern plants, a manually cleaned screen may be used (Haandel *et al*. 2014) special equipment is also used to remove grit that gets washed into the sewer and Wastewater is placed in holding tanks and solids settle to the bottom where they are collected and lighter substances are scraped off the top.

1.2.2 Primary and secondary treatment

According to Younghee *et al*. (2017), after sewage has been screened, wastewater pumped from the centrifugal pumps goes to three digesters called primary circulators. Wastewater is broken down by aerobic bacteria incorporated into the wastewater treatment system then the rest of the water is moved to the secondary treatment. At this stage, dissolved and suspended biological matter is removed using micro-organisms in a controlled environment and water is put into larger tanks called

aeration lanes. Air is pumped into the water to encourage bacteria to break down the tiny bits of sludge that escaped the sludge scrapping process (Kirk *et al*. 2002).

1.2.3 Tertiary treatment

At this stage, three processes occur: aeration, settling and decanting.

Aeration- surface aerators mix the incoming effluent with the activated sludge, keeping it in suspension in the presence of supplied oxygen. Micro-organisms in the sludge use the organic material in the effluent as a food source for normal life functions and synthesis of new bacteria (Apha 1998).

Settling- aeration switch off and effluent becomes still. Imrary (2005) state that having no oxygen supply, micro-organisms use carbon in the organic matter as a food source, converting nitrates to nitrogen gas which is released to the atmosphere. During this phase, solids settle down from the liquid and that makes water clear in the upper part of the tank.

During the last stage of treatment, wastewater is passed through a settlement tank and the water is allowed to flow over a wall where it is filtered to remove remaining particles. The filtered water is then released into the river.

1.3 Effects of wastewater on river ecosystems

Pollution from wastewater treatment has significant consequences on river ecosystems (Bernhardt & Palmer, 2007; Grant *et al*. 2012). Rodriguez-Mozaz *et al*. (2015) reported that wastewater treatment plants do not remove all contaminants from sewage water, and this leads to a complex mixture of contaminants being released into freshwater ecosystems (Petrovic *et al*. 2002). Improperly treated wastewater can affect the turbidity of water in natural ecosystems which reduces the amount of light available to plants. It can also smother organisms in natural habitats, damage fish gills and the respiratory structures of other organisms. (Odum *et al*. 1979). Munawar *et al*. (1993) state that sewage may release warmer or cooler water into rivers. In humans, a variety of acute illness (Diarrhoea, cholera and hepatitis A) are associated with wastewater pollution (Cleuvers 2003).

1.4 Impacts of chemical pollutants in wastewater

Chemical pollutants are substances introduced into the environment as a result of man's activities and in quantities sufficient to produce undesirable effects (Michael 2003). Main sources of chemical pollutants in wastewater are human or animal waste, agricultural chemicals such as pesticides, herbicides, and fertilizers (Akpor *et al*. 2014). From previous studies, it has been shown that chemical pollutants have lots of effects on wastewater and aquatic organisms. Katja *et al. 2012* conducted a study on the effects of organic pollutants from wastewater treatment plants on aquatic invertebrate communities. The index spear $_{\text{pesticide}}$ was used to detect the effects of agricultural pesticide on the macro-invertebrate community, and the results showed that wastewater treatment plants are the main sources of oxygen depleting organic pollution, regardless of the technological improvements in wastewater management in several years.

Moreover, Akpor *et al. (2014)* investigated a study on pollutants in wastewater effluents: impacts and remediation processes. The major contaminants in wastewater are nutrients (Phosphorus, nitrogen), hydrocarbons and heavy metals. Excessive nutrients in wastewater may lead to harmful or unwanted microbes such as *pfisteria* which may cause respiratory irritations and gastrointestinal diseases to aquatic organisms (Morris 2001). According to Zhang *et al*. (2011) hydrocarbon pollutants in wastewater cause health and environmental risks, which are of great concern. When hydrocarbons are in contact with receiving water bodies could cause problems to fishery, marine habitats wildlife also a host of diseases and disorders to human and animals. Sources of heavy metals in wastewater are industrial, petroleum contamination and sewage disposal (Santos *et al*. 2005). High concentrations of heavy metals such as zinc, copper and iron in wastewater could affect the physiological state of aquatic organisms. Oyewale (2000) and Oladele *et al*. (2012) reported that a high presence of zinc in wastewater can cause an increase in water acidity which could affect the cultivation and yield of crops.

1.5 Organisms used to determine the effects of wastewater.

Municipal wastewater has been shown to contain harmful compounds which cause damage to the environment. For instance, Watton *et al*. (1984) studied the effects of sewage effluent on gastropod populations in experimental streams. Two gastropods, *Lymnaea perega* and *potamopyrgus jenkinsi* were exposed to treated effluent mixed with river water. The results showed abundance and biomass of *P. jenkinsi* were significantly lower in the presence of effluent, while reproduction and biomass of *L. perega* were significantly greater in the presence of effluent. Schulte-Oehlmann *et al.* (2000) tested the effects of Triphenyltin(Fungicide detected in wastewater) on mollusc, prosobranch snails. Snails were exposed to varying concentrations of trihenylthin (25, 150, 200 and 500ng) and there was a reduction in egg production until no reproduction was observed in the highest concentration (500ng). In addition, Joblin *et al*. (2003) compared the responses of molluscs to environmental estrogens and an estrogenic effluent. Individuals of prosobranch molluscs, *potamopyrgus antipodarium* were exposed to estrogenic chemicals(EE_2, bisphenol-A) and treated sewage effluent. The effects of EE_2 and effluent wastewater caused a decline in embryo production in P. antipodarum. Sanchez-Meza *et al*. (2007) also conducted a study on toxicity assessment of complex industrial wastewater using water fleas, *Daphnia pulex. D. pulex* was used to assess and compare toxicity between the effluent and influent wastewater from the activated sludge process. For each sample of wastewater, a duplicate bioassay was conducted using *D. pulex*. The results showed a significant decrease between influent and effluent wastewater. More recently Zounkora *et al*. (2014) investigated the effects of urban river pollution on the mud snail, *potamopyrgus antipodarum*. The snails were exposed for 8 weeks to water in the downstream of a wastewater treatment plant. Greater mortality and decreased embryo production of *P. antipodarum* were observed after the exposure.

1.6 Previous studies done on *Helix pomatia*

In ecotoxicology, the effects of pollutants on the relationship between the organism and its environment are studied. *Helix pomatia* are regarded as relevant organisms to be used in toxicity evaluation (Regoli *et al.* 2005). For example, Russel *et al*. (1981) assessed the ecological effects of municipal waste on forest land. *Helix pomatia* was exposed to varying concentrations of cadmium (0, 25,50,300, and 1000 ppm). Food consumption declined with each increasing cadmium concentration. For reproduction, there was no indication of the effects on 10ppm Cd. At 25ppm and above, reproduction declined. Gomot-de vaufleury (2000) has also investigated the use of different chemicals (Cu, Zn, Pb and pentachlorophenol) to test the effects of toxicity on the growth of *Helix pomatia.* Juvenile snails were exposed for 4 weeks to food contaminated with Cu, Zn, Pb and pentachlorophenol. Cu and Zn had sublethal effects on growth and pentachlorophenol inhibited growth. Coeurdassier *et al*. (2001) used *Helix pomatia* as a bioindicator of organophosphorus exposure. Snails were exposed to concentrations of organophosphorus pesticide dimethoate for 4 weeks. Growth parameters decreased with the increasing exposure to the pesticide. Moreover, Sverdrup *et al*. (2006) conducted a study on the effects and uptake of PAH in artificial soil. The results showed no effect on the mortality of *H. pomatia* during the period of exposure. Lastly, Gimbert *et al*. (2006) also investigated the chronic exposure to contaminated soil using terrestrial snail, *Helix pomatia.* Snails were exposed to cadmium contaminated soils for 84 days. The results showed no increase in biomass at all concentrations of cadmium.

1.7 Problem statement

The study will be addressing the effects of OECD soil spiked with treated and untreated wastewater on a terrestrial snail (*Helix pomatia*). This study may reveal cases of improper treatment of wastewater occurring in the MAP municipality. This will force other wastewater treatment plant to improve their function, as well as to check if they follow the standards of wastewater treatment (Kratz and kifferstein 2005). Even though animal testing alone cannot provide the final answer to the question “Is it safe to use water from Maluti-A-phofung wastewater treatment plant?” it may reveal problems that can be clarified using other methods. This might raise the need for funds from the government in helping to treat and save water. Proper treatment will reduce the chances of humans and animals getting diseases (Leomanni *et al*. 2015) unintentional killing of aquatic organisms as well as to prevent damage to the soil. The species that will be used to determine the ecotoxicological effects of OECD soil spiked with treated and untreated wastewater samples are the terrestrial snail of the family Helicidae: *Helix pomatia*. This species is commonly known as “garden snail”. Terrestrial snails of the family Helicidae are hermaphrodites, they can produce sperm and egg and mate as male, female, or both (Joris *et al*.1998). This species is herbivorous and consumes many types of plant matter. It finds its food in flowers, herbs and bark trees (Iglesias and Castillejo 1999).

1.8 Aims and objectives

The general objective of this study is to investigate the effects of OECD soil spiked with treated and untreated wastewater on life cycle parameters of *Helix pomatia*

1.8.1 Specific objectives

1. To assess effects OECD soil spiked with treated and untreated wastewater on the survival of *helix pomatia*
2. To assess the effects of OECD soil spiked with treated and untreated wastewater on the reproduction of *Helix pomatia*
3. To assess the effects of OECD soil spiked with treated and untreated wastewater on the biomass of *Helix pomatia*
4. To determine the biomarker (Catalyse and acetylcholinesterase) response of *Helix pomatia* after exposure to treated and untreated wastewater

1.9 Hypothesis of the study are:

Survival and reproduction of *Helix pomatia* is expected to decrease with the increasing concentrations of OECD soil spiked with untreated wastewater samples

Survival and reproduction of *Helix pomatia* are expected to increase with the increasing concentrations of OECD soil spiked with treated wastewater samples.

Biomass of *Helix pomatia* is expected to decrease with the increasing concentrations of OECD soil spiked with untreated wastewater

Biomarker response will not be the same in treated and untreated wastewater samples

Chapter 2

2. Materials and Methods

2.1 Study area

Treated and untreated wastewater samples were collected from the wastewater treatment plant in the town of Phuthaditjhaba (28º 30′ 28.3″ S; 28º 49′ 39.7″ E). This sampling site is located within the Drakensberg Afromontane region of Southern-Africa. The annual temperature of this area ranges from 35°C in mid-summer to -5°C in mid-winter (Department of water affairs 2011). Phuthaditjhaba is part of the Maluti-a-Phofung municipality, which hosts more than 300 factories in different industrial sectors such as clothing, furniture, agriculture, aluminium and glass products (Liedtke 2018: Mosolloane *et al.* 2019). The selected wastewater treatment plant receives raw wastewater from these industries and from the residential areas of Phuthaditjhaba, and neighbouring areas such as Bluegumbosch and Clubview. The plant receives 6 to 6.8ML volume of wastewater per day. The plant functions 24 hours a day, and discharges its treated effluent in a nearby holding pond and thereafter into the Elands River.

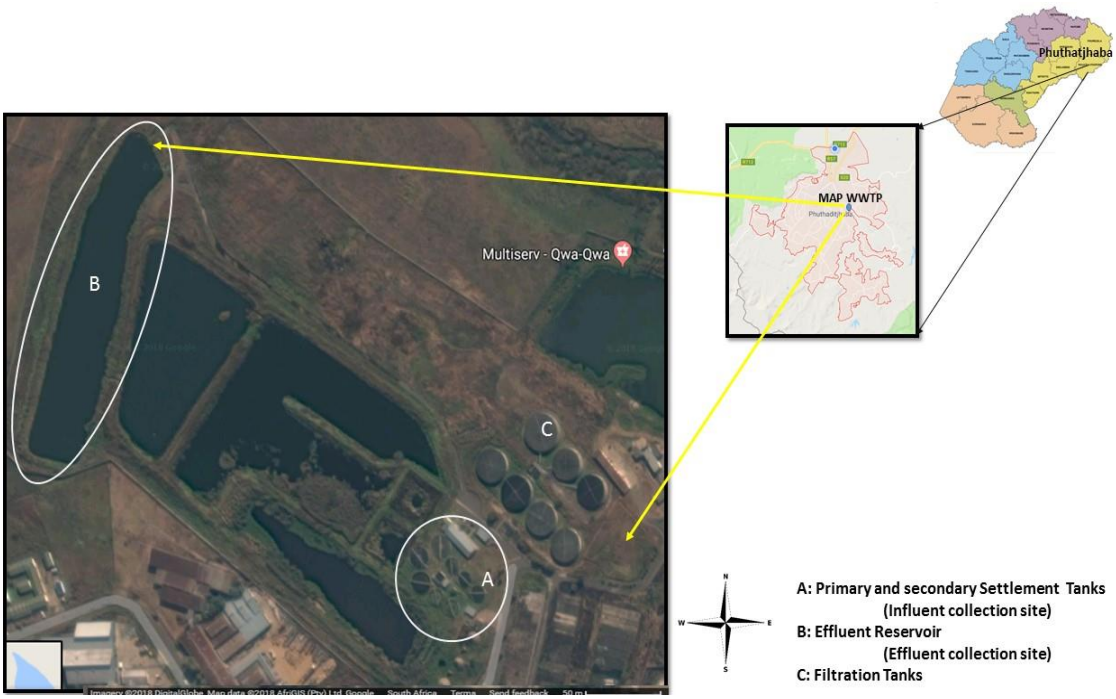

Figure 2: Demonstration of MAP wastewater treatment plant.

2.2 Collection of samples

2.2.1 Collection of samples for ecotoxicological assay

Treated and untreated wastewater samples were collected in 25 L plastic jerry cans from the raw sewage inlet and the treated effluent outlet.

2.3 Test species

The organisms used in this study are *Helix pomatia*. Their shell size is approximately 34-49mm(Korabek *et al.* 2015; Neubert 2014). The choice of the test organism was based on the fact that terrestrial gastropods are good bio-indicators of environmental status. (*Regoli et al.* 2005). They are in constant contact with the soil and/ or the vegetation, have a strong impact on nutrient cycling and are primary consumers. Furthermore, terrestrial snails are easy to handle and culture in the lab and field conditions (Elder & Collin.1991). Lastly, they are sentinel organisms, which, can attain higher bioaccumulation for many toxicants.

Table 1: the taxonomic classification of *Helix pomatia*

Kingdom	Animalia
Phylum	Mollusca
Class	Gastropoda
Order	Stylommatophora
Family	Helicidae
Genus	*Helix*
Species	*Helix pomatia*

2.3.1 The biology of *Helix pomatia*

The *Helix pomatia* is an air-breathing land snail that has a creamy to light brown shell with to five light brown bands, and are round or conical. A mature shell can range from 34-49mm height and about 35-50mm wide (Korabek *et al.* 2015; Neubert 2014). *H.pomatia* has a single lung and a muscular foot that help with locomotion. The foot contracts to produce movements and glands within it, release mucus that reduces friction with the underneath surface, reducing the risk of damaging their skin. Reproduction, the first cocoons are released 48 hours after copulation. Each cocoon has an incubation period of 7-14 days and hatchling reach maturity (sexually mature adult) within 45-59 days (Pollard 1975).

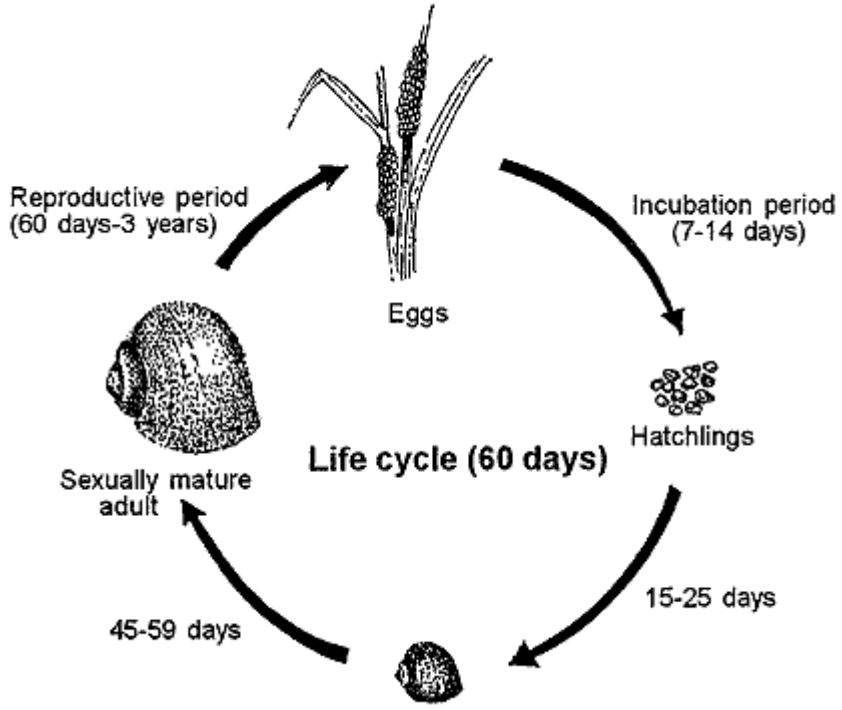

Figure 3: The life-cycle of *H.pomatia*. The first cocoons are released 48 hours after copulation. Each cocoon has an incubation period of 7-14 days and hatchling reach maturity (sexually mature adult) within 45-59 days (Pollard 1975).

Source:
https://za.pinterest.com/pin/855402522955124464/?d=t&mt=signupOrPersonalizedLogin

2.4 Snail breeding and exposure

2.4.1 Snail breeding

Individuals *Helix pomatia* were purchased from a breeder in Riverside situated in Free-State (South- Africa). Snails were bred indoors and outdoors.

Snails were bred in an open space (Garden) where vegetables such as spinach, carrots and lettuce are planted. Plants are watered in the morning and afternoon. Usually, in winter snails hibernate under the soil and come out in the morning. The garden was covered with a white net to prevent the snails from escaping. During the mating process, they fertilize each other, and they both lay around 20 eggs about 3 to 6 days after the copulation. Eggs and baby snails were monitored until they mature.

2.4.1.2 Indoor breeding

Snails were bred in a hatching box. They took soil from the garden and baked it in an oven to kill unwanted bacteria. Snails and baked soil were placed in a hatching box at the depth of 7cm. snails were fed lettuce, spinach and carrots three times a week and the feeding was changed after every two days to avoid mosquitoes and unwanted bacteria. Snails were given water in the morning and afternoon.

2.5 Preparation of the artificial soil

The artificial soil used in this experiment was prepared following the OECD guideline (OECD 2004). The soil was prepared by mixing the following:

- 10% sphagnum peat, air-dried and finely ground
- 20% kaolin clay
- 70% air-dried quartz sand

The OECD artificial soil contents were mixed a week before the experiment starts, and used as an exposure medium in this study.

2.6 Toxicity test

Prior to the exposure, snails were collected and brought to the laboratory where they were placed in 25L Black storage boxes (330×220×345mm) for 5 days to acclimatize. The lids of the containers were punctured to allow air exchange. The snails were fed lettuce and were sprayed with distilled water in order to maintain a moist environment. The individuals used for the test were aged 6 months and 12 months.

Exposure was done for a period of 60 days. Groups of 10 snails were placed into thirty plastic buckets each bucket was filled with 2000g of OECD soil spiked with treated and untreated wastewater; distilled water was added to reach 50% of the water holding capacity. The buckets were covered with a white net to prevent snails from escaping. The soils were then left to stabilise for three days prior to the exposure. The snails were fed 5 g of lettuce three times a week and the uneaten food was removed every two days. Mortalities were recorded every 24 hours according to the procedures by (Gimbert *et al*. (2006). During the exposure, eggs were collected from buckets filled with damp OECD soil. The eggs were then incubated in Petri-dishes at 20^{o}C until hatching. After hatching the juveniles were left to rest for 1 or two weeks and they were feeding on the calcareous shell of the egg. Laboratory observations suggest that quite frequently they will eat unhatched eggs and occasionally other young snails, leaving the empty snail shells. (Gomet 1998; Pollard 1975).

2.6.1 Maximum of 10 Snails placed in 2000g of OECD soil spiked with a 1L volume of water samples were exposed to varying concentrations of wastewater.

The following concentrations were prepared using distilled water:

100% (1000ml of sample+ 0% distilled water+2000g OECD soil)

75% (750ml of sample + 250ml of distilled water+2000g OECD soil)

50% (500ml of sample + 500ml of distilled water+2000g OECD soil)

25% (250ml of sample + 750ml of distilled water+2000g OECD soil)

0% sample+ 100% distilled water + 2000g OECD soil (control)

2.7 Determination of Biomarker responses

2.7.1 Preparation of homogenates

After 60 days of exposure, the snails were weighed, washed with distilled water, de-shelled, frozen and preserved at -80°C in an ultra-low temperature freezer (Blizzard NU-998282) until biomarker experiments were conducted. The samples were prepared by homogenizing snail tissue in a Tris/Sucrose buffer (pH 7.4) (1:5) for acetylcholinesterase and phosphate buffer (pH 7.4) for catalase. After homogenizing, the samples were centrifuged at 10 000 g at 4°C for 10 minutes and the supernatants were used for catalase and protein analysis. For acetylcholinesterase and protein analysis the homogenates were centrifuged at 9500 g at 4°C for 10 minutes. Both Catalase and Acetylcholinesterase procedures were performed on ice. Samples from two randomly selected snails of each treatment were analysed per microtiter plate in two replicates.

2.7.2 Protein quantification

The protein concentration was quantified using the Bradford assay (Bradford, 1976)). Protein standard solutions were prepared in duplicates using a 5 mg/mL bovine serum albumin (BSA) stock solution as indicated in Appendix 1-Table 1. Protein extracts were also prepared in duplicates in 2 mL plastic cuvettes by adding 10 µL of a protein sample, 10 µL of 0.1 M HCl and 80 µL of distilled water. In both the standard solutions and protein samples, 900 µL of a 1:4 diluted Bio-Rad Protein Assay Dye Reagent Concentrate (BIO-RAD, Hercules, California, USA) was added, mixed well and incubated at room temperature for 5 minutes. Thereafter, the absorbance was measured at 595 nm on a spectrophotometer, using the 0 mg/mL BSA standard 0solution as a blank. The BSA standard solutions were used to plot a standard curve for estimating the concentrations of unknown protein samples.

Table 2: The preparation of BSA standard solutions for protein quantification.

Concentration (μg)	BSA standard solution (μL)	Extraction buffer (μL)	0.1 M HCl (μL)	Distilled water (μL)
*0	0	10	10	80
5	1	9	10	80
10	2	8	10	80
20	4	6	10	80
40	8	2	10	80
50	10	-	10	80

2.7.3 Estimation of Catalase (CAT) activities

Catalase activity was measured according to the method described by Cohen *et al.* (1970). The reaction mixture consisted of 93 µl of 30% of hydrogen peroxide H_2O_2 solution with 10 µl of each sample homogenates. The mixture was left to rest for 3 minutes at room temperature before the addition of 19 µl of H_2SO_4 which was followed by 130 µl of $KMnO_4$ solution. Thereafter, absorbance reading was performed at 492 nm and the readings were taken within 30-60 seconds Using Bio-Rad model 680 microplate reader machine.

Specific activity (units/ mg protein) = (min^{-1} x dilution factor x 1cm light path)/ (43.6 x mg protein/ ml reaction mixture).

2.7.4 Estimation of Acetylcholinesterase (AChE) activities

Acetylcholinesterase method was done according to the method described by Ellman *et al.* (1961)

Initially, 210 µl of Potassium phosphate buffer, 10 µl of s-Acetylthiocholine iodide and 10 µl of Ellman's reagent were added into microplate wells and mixed thoroughly. The mixture was incubated for 5 minutes at 37°C. The samples were added, mixed and absorbance reading was done immediately. Absorbance reading was done at 412nm (405nm) in 1-minute intervals over a 6-minute period. AchE activity was determined by calculating the average absorbance of the readings at each interval from 0 to 6 minutes. The linear graph for each sample was drawn and expressed as the change in absorbance over time. Ache activity was calculated as follows (absorbance /min/ mg protein) = (Abs/min) ÷ mg protein.

2.8 Statistical analysis

Microsoft Excel was used to record the data and calculate the mean reproduction and survival used to construct the graphs. The effective concentrations (EC_{10} and EC_{50}) were calculated using the statistical package ToxRat professional version 2.10.05 (Toxicity Response Analysis and Testing; ToxRat solutions GmBH, Alsdorf, Germany). One-Way ANOVA was used to compare the results of reproduction, survival and biomass because the data were normally distributed and the equality of variance was verified. This analysis was performed using SigmaPlot version 13.0.

Chapter 3

3. Results

3.1 Effects of treated and untreated wastewater on survival, reproduction and Biomass of *H. pomatia.*

3.1.1 Effects of treated and untreated wastewater on the survival of *H. pomatia.*

In the OECD soil spiked with the treated wastewater, there was a statistical difference ($P<0.05$) between the number of *H. pomatia.* which survived in the control and the number of adult snails which survived in the 25% and 50% treatments ($P<0.05$), where the number of surviving adults was significantly lower in these two treatments when compared to the control (Figure 4).

In the OECD soil spiked with untreated wastewater, there were statistical differences ($P <0.05$) between the control and all the treatments. Survival was significantly lower in all the treatments spiked with the untreated wastewater when compared to the control ($P<0.05$; Figure 4).

Furthermore, when comparing homologous concentrations figure 1 shows that there was a statistical difference ($P<0.05$) between the survival of *H. pomatia e*xposed to soils spiked with untreated and treated wastewater for 75% and 100% treatment groups. An LC_{50} could not be determined due to insufficient mortality in this soil substrate.

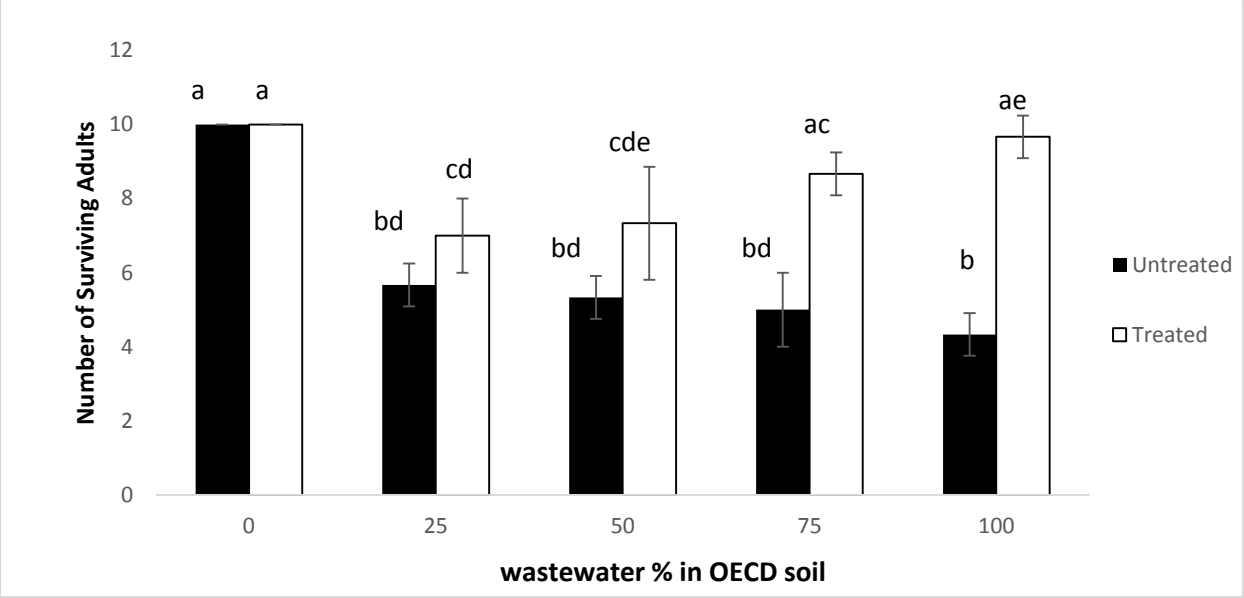

Figure 4: Comparison of the number of *H. pomatia* adults who survived after 60 days of exposure to OECD soil spiked with treated and untreated wastewater. n=30 per wastewater treatment. Error bars represent standard deviation. Different letters on the bars represent a significant difference between treatments ($P \leq 0.05$).

3.1.2 Effects of treated and untreated wastewater on the reproduction of *H. pomatia.*

3.1.2.1 Cocoons production

In figure 2, the results showed that in the OECD soil spiked with the untreated wastewater, there was a significant decrease in cocoon production from the control and all the treatments groups ($P<0.05$), especially at 100%, where it was completely inhibited. An EC50= 5.751% was calculated.

For *H. pomatia* exposed to OECD soil spiked with the treated wastewater, there was also a statistical difference ($P<0.05$) in cocoon production between the control and all the treatments except for 100% treatment group ($P>0.05$; Figure 5).

The comparison of the homologous treatments showed that there was no statistical difference ($P<0.05$) in cocoon production between H. *pomatia* exposed to OECD soil spiked with treated wastewater and OECD soil spiked with untreated wastewater for all the treatments except for the 100% treatments where there was a complete inhibition of cocoons in the untreated influent ($P<0.05$; Figure 5).

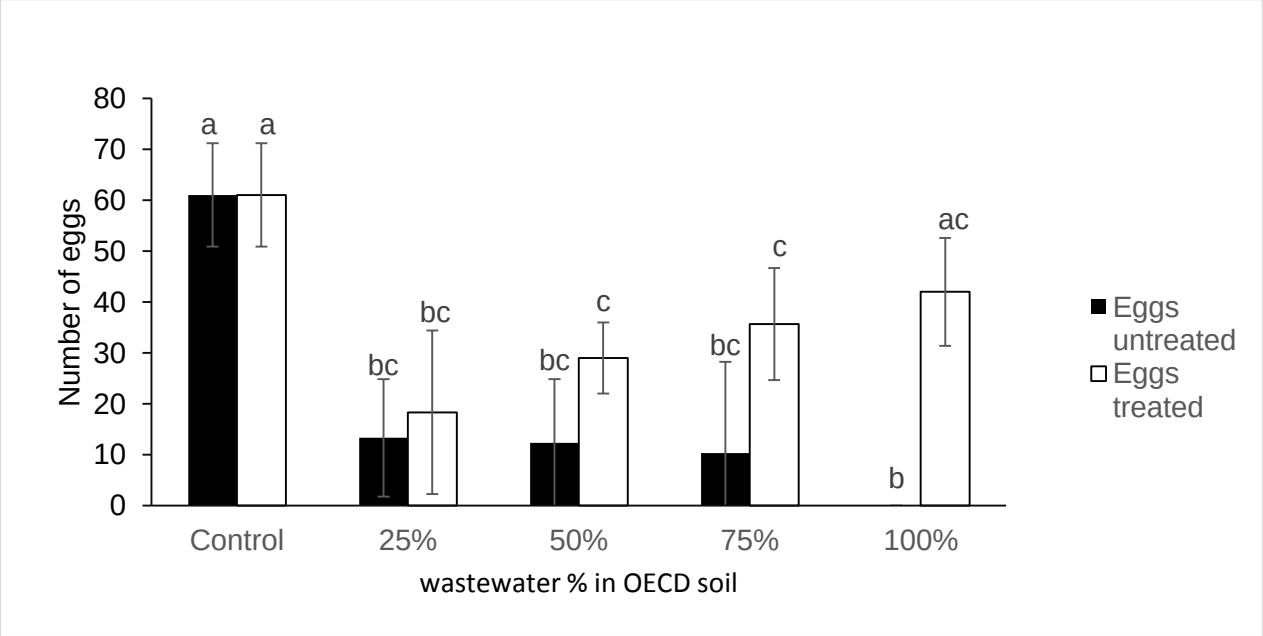

Figure 5: Comparison of the number of *Helix pomatia* cocoons reproduced after 60 days of adult exposure in OECD soil spiked with treated and untreated wastewater. n=30per wastewater concentration. Error bars represent standard deviation. Different

letters represent statistical significance between the control and the treatment. P≤0.05

3.1.2.2 Juvenile emergence (hatching success)

Juvenile emergence or hatchability for *H. pomatia* in OECD soil spiked with the treated wastewater showed that there was a statistical difference (P<0.05) between the control and all treatments apart from 100%, where there was no statistical difference with the control (Figure 6).

In the OECD soil spiked with the untreated wastewater, juvenile emergence or hatchability for *H. pomatia* showed that there was a significant reduction (P<0.05) between the control and all treatment groups, with complete inhibition of juvenile emergence observed at the highest concentration (100%). EC_{50}=6.233% was calculated.

The comparison of the juvenile numbers between the H. *pomatia* exposed in OECD soil spiked with the treated wastewater and OECD soil spiked with the untreated wastewater indicated that there was no statistical difference (P>0.05), except in the 100% treatments where significantly more juveniles hatched after exposure to the treated effluent (Figure 6).

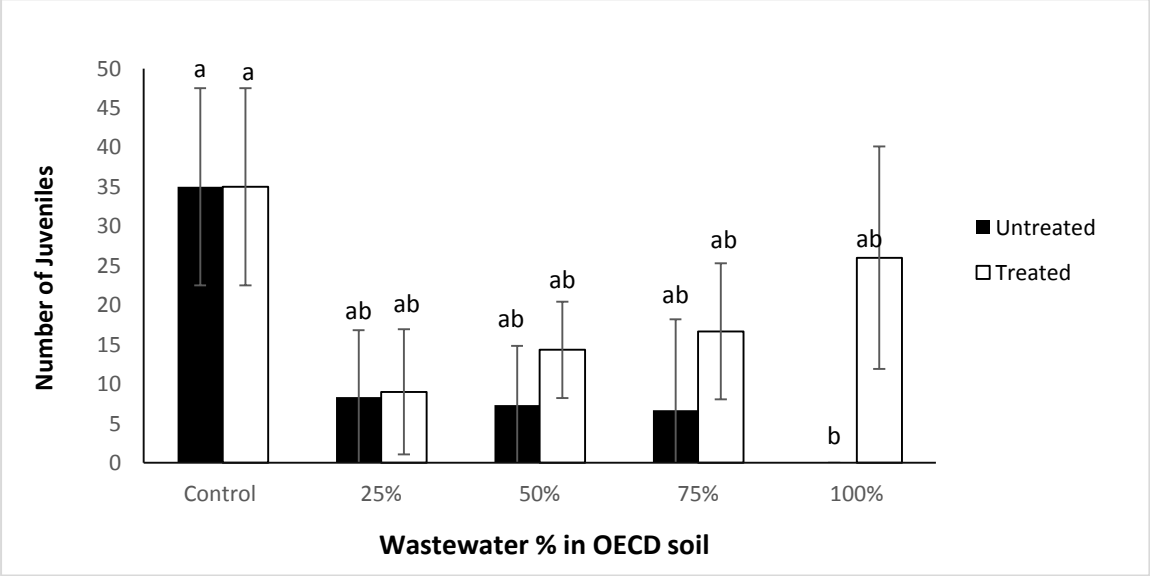

Figure 6: Comparison of the number of *Helix pomatia* juveniles reproduced after 60 days of exposure in OECD soil spiked with treated and untreated wastewater. n=30 per wastewater concentration. Error bars represent standard deviation. Different letters represent statistical significance between the control and the treatment. P≤0.05

3.1.3 Effects of treated and untreated wastewater on the Biomass of *H. pomatia.*

The effect of treated and untreated wastewater on the biomass of adult *Helix pomatia* was assessed after 60 days of exposure in the OECD soil. For *H. pomatia* exposed to soils spiked with treated wastewater, there was a statistical difference ($P<0.05$) in the biomass of between the control (pure OECD soil) and 25, 50, 75% treatment groups apart from 100% treatment group (Figure 7). There was also a significant difference ($P<0.05$) in biomass for exposed *H. pomatia* between control soils (pure OECD soil) and soils spiked with 25, 50, 75 and 100% untreated wastewater (Figure 7).

Overall, there was a statistical difference ($P<0.05$) in biomass changes between *H. pomatia* exposed in soils spiked with treated wastewater and soil spiked with the untreated wastewater. However, a further statistical test showed that the difference was between the control group and 100% group for both exposure substrate ($P>0.05$, Figure 7).

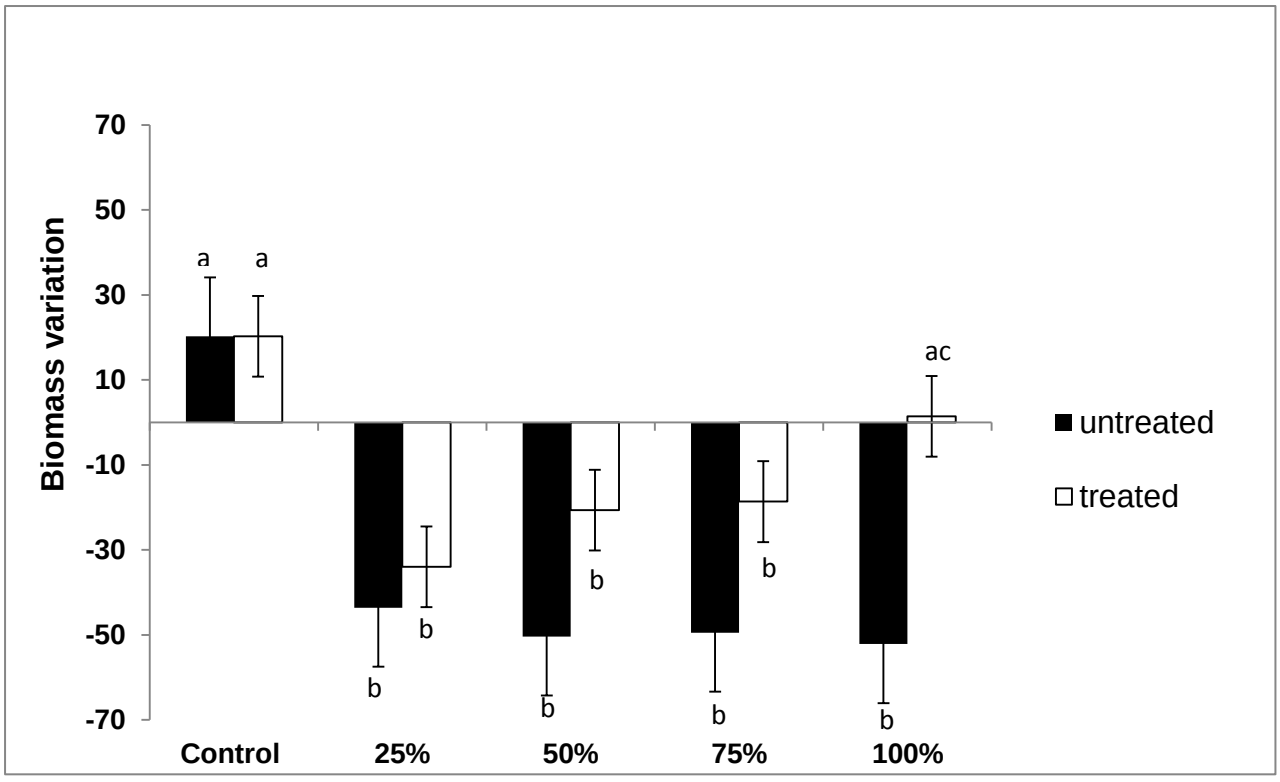

Figure 7: Change in biomass of *H. pomatia* after 60 days of exposure in treated and untreated wastewater. n= 30 per wastewater concentration Different letters above bars indicate a statistical difference between the groups. Error bars represent standard deviation. P≤0.05

3.2 Summary of ECX and LCX

When comparing the EC_X and LC_X values of OECD soil spiked with treated and untreated wastewater, LC50s could not be determined due to insufficient mortality in the soil substrates. Similarly, insufficient reduction in reproduction in OECD soil spiked with untreated wastewater meant that an EC50 could not be determined. EC10 and EC50 values are reported for OECD soil spiked with treated wastewater (Table 3)

Table 3: Effective concentrations on reproduction of *H.pomatia* exposed to OECD soil with treated and untreated wastewater for a period of 60 days.

	Effective concentration	OECD soil with treated wastewater	OECD soil with untreated wastewater
Reproduction(Juveniles)	EC10 EC50	n.d n.d	0,425 % 6,233%
Reproduction (Cocoons)	EC10 EC50	n.d n.d	0,380% 5,751%

n.d: Not determined

3.3 Biomarker responses in *H. pomatia* exposed to treated and untreated wastewater

3.3.1 Acetylcholinesterase (AChE) activity

In the OECD soil spiked with the treated wastewater, there was no statistical difference between all treatments and the control (P>0.05, Figure 8). Similarly, AChE activity in the OECD soil spiked with the untreated wastewater showed no statistical difference between the control and the untreated wastewater groups (P>0.05). Likewise, there was no statistical difference in AChE activity between homologous treatments spiked with the treated and untreated wastewater, this despite the fact that activity seemed to be higher in the treated wastewater (Figure 8).

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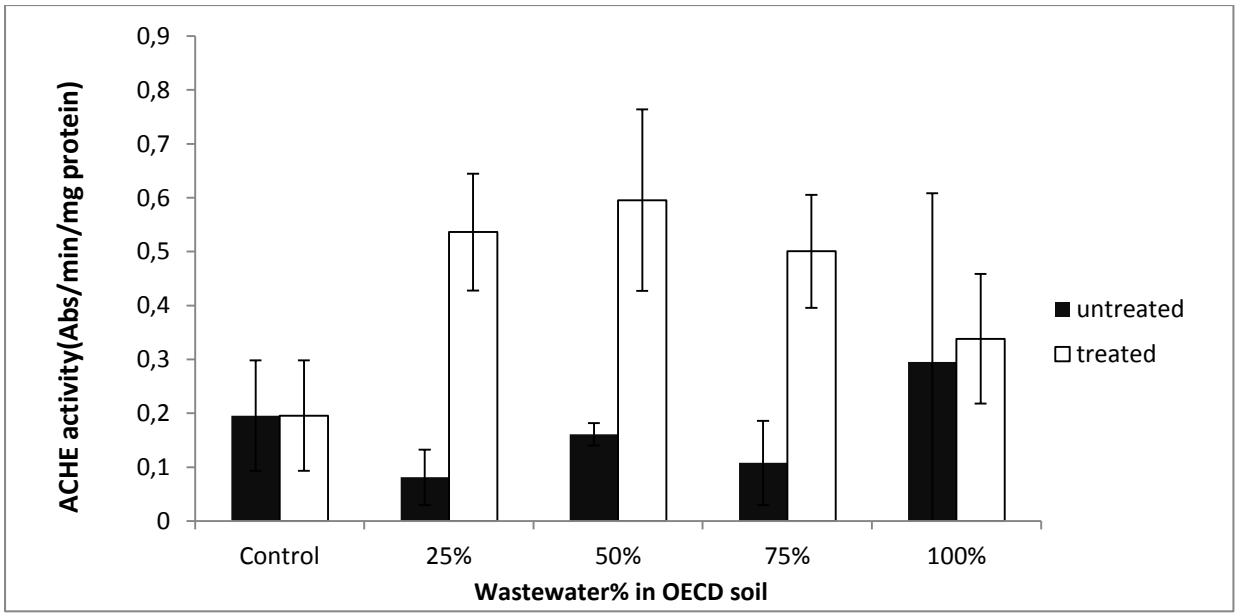

Figure 8: ACHE activity and inhibition of *Helix pomatia* after 60 days of exposure to OECD soil spiked with treated and untreated wastewater. Different letters indicate a statistical difference between treatments. Error bars represent standard deviations P≤0.05

3.3.2 Catalase (CAT) activity

As for the CAT activity in *H. pomatia* exposed to OECD soil spiked with the treated wastewater, the results showed no statistical difference between the control and all the treatments groups (P>0.05, Figure 9).

Similar results were observed in CAT activity in *H. pomatia* exposed to OECD soil spiked with the untreated wastewater.

A comparison of CAT activities in *H. pomatia* for homologous treatments, showed no significant difference (P<0.05) between treated and untreated wastewater for all treatment groups (Figure 9).

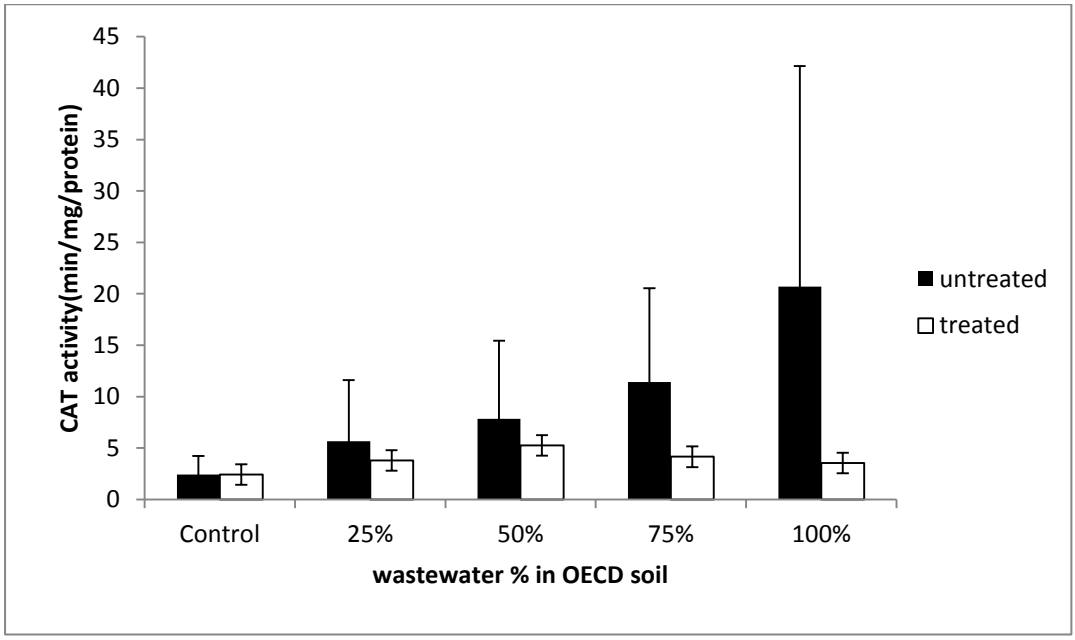

Figure 9: Catalase (CAT) activity of *Helix pomatia* after 60 days of exposure to OECD soil spiked with treated and untreated wastewater. Different letters indicate a statistical difference between treatments. Error bars represent standard deviation P≤0.05

Chapter 4

4. Discussion

4.1 Effects of treated and untreated wastewater on the survival of *Helix pomatia*

The results showing the effects of treated and untreated wastewater on the survival of *H. pomatia* are presented in Figure 1. In the OECD soil spiked with the treated wastewater, there were statistical differences ($P<0.05$) between the number of *H. pomatia* which survived in the control and the number all treatment groups apart from 75 and 100% where survival was least affected. Here, as the concentration of treated wastewater increased the survival of *H. pomatia* also increased. Results from this study corroborate reports from Gust *et al. (*2010), who reported no mortality in the adult freshwater snail, *Potamopyrgus antipodarum* exposed to wastewater treatment plant effluent discharges in France. Similarly, Sverdrup *et al*. (2006) in a study on the effects and uptake of PAH in artificial soil reported that there was no effect on the mortality of *H. aspera* during the period of exposure at a maximum concentration (2,800 mg/kg). Woin and Bronmark (1992) also reported very low mortality for pond snail, *Lymnaea stagnalis* exposed to DDT treatments when compared to the control.

On the contrary, for the survival of *H. pomatia* exposed to OECD soil spiked with untreated wastewater, there were statistical differences ($P<0.05$; Figure 1) observed between the control and all the treatments. This implies that the survival of *H. pomatia* decreased as the concentration of untreated wastewater increased. Increased mortality observed here could be due to the composition of pollutants in the untreated wastewater (de Vaufleury *et al*. 2006; Zounkova *et al*. 2014). This is similar to results by Clark *et al*. (2009), who reported a decrease in survival of Ramshorn Snail (*Planorbarius corneus)* exposed to sewage sludge compared those exposed to river water. Similarly, de Vaufleury *et al*. (2006) reported mortality for *H. aspera* exposed to sewage sludge diluted in a natural soil or in the artificial ISO

substrate. Furthermore, Zounkova *et al*. (2014) also reported mortality for mud snail (*Potamopyrgus antipodarum)* exposed for 8 weeks in cages permeable to sediment and water in the downstream of a WWTP.

Overall, *H. pomatia* showed more survival in soil spiked with treated wastewater compared to soil spiked with untreated wastewater. This could be attributed to the fact that untreated wastewater contains a cocktail of pollutants sourced from both municipal, domestic and agricultural districts (Akpor *et al*. 2014, Dhokpande *et al*. 2013, Edokpayi *et al*. 2015, Vandevivere *et al*. 1998). Most of these pollutants tend to be eliminated during the treatment process. Several studies have linked toxic effects in invertebrates to exposure to wastewater including Joblin *et al.* (2003) and Watton and Hawkes (1984).

4.2 Effects of treated and untreated wastewater on the reproduction of *Helix pomatia*

4.2.1 Cocoon production

Gastropods have become effective organisms that can be used for ecotoxicological bioassays. Therefore, reproductive parameters including egg-laying, hatchability and embryo development have become important endpoints in such bioassay (De Vaufleury, 2014; Das and Khangarot, 2010). In this study, there was a significant decline in cocoon production for *H. pomatia* exposed to soil spiked with untreated wastewater, with complete inhibition observed at the highest concentration (100%; Figure 2). Also, there was a significant decrease in cocoon production *for H. pomatia in the control group and those* exposed to soil spiked with concentrations (25%< 50%<75%) of treated wastewater. However, it was observed at the highest concentration (100%), there was no significant difference with egg production in the control (Figure 2). This implies that increasing concentration of untreated and treated wastewater reduced the ability *of H. pomatia* to reproduce, thus underscoring the toxicity of wastewater from the Phuthaditjhaba WWTP. Similar effects on egg production by treated and untreated wastewater observed in this study could indicate the inability of the WWTP of effectively remove substances that could inhibit reproduction. As stated earlier in this study, the observed toxicity of untreated and untreated wastewater could be because of the presence of diverse contaminants

(Mosolloane *et al.* 2019; Moloi *et al*. 2019). Moloi et *al.* (2019), in their study of metals in influents and effluents from Phuthaditjhaba WWTP, observed no significant difference between effluents and influents. Results in this study are similar to results by Schulte- Oehlmann *et al.* (2000) who reported a decrease in egg production of Prosobranch Snails exposed to varying concentrations of Triphenyltin (a broad-spectrum fungicide that could be detected in wastewater). Here there was a dose-dependent (75, 150, 250 and 500 ng TPT- Sn/L) reduction in egg production until no reproduction was observed for the highest concentration. Similarly, Castro-Català *et al*. (2013) reported a reduction in eggs produced by *Physella acuta* exposed to endocrine-disrupting compounds (including pesticides, pharmaceuticals which are constituent of wastewater) in situ in three Iberian basins. Cœurdassier *et al.* (2005) also reported a decrease in the number of eggs produced by *Lymnaea palustris* exposed to increasing concentrations (20, 30, 40 and 80%) of industrial effluent containing high levels of metals particularly Cr, Zn and Fe.

In this study, overall results indicate that there was no difference in egg production between *H. pomatia* exposed to soil spiked with treated and untreated water.

4.2.2 Juvenile emergence (hatching success)

Hatching success for *H. pomatia* in OECD soil spiked with treated and untreated wastewater were similar to results observed in cocoon production for *H. pomatia*. There was a significant reduction in hatching success when compared to the control for *H. pomatia* exposed to OECD spiked with treated and untreated wastewater ($P<0.05$; Figure 3). Wastewater has been reported to contain endocrine disruptors and estrogenic compounds that affect the fecundity of invertebrates including snails (Mauricio *et al*. 2007; Schirling *et al*. 2006; De Vaufleury, 2014). There are no studies that have looked that the effect of wastewater on the hatchability of *H. pomatia*. However, results in this study are similar to results obtained by Gomot (1997), who reported that concentrations (25 and 100 µg/L) of Cd (a constituent of wastewater) decreased the hatching success of *Lymnaea stagnalis*. Similarly, Liu *et al.* (2013) also reported a significant reduction in hatching rate at 25-µg/L Cd treatment for freshwater snail, *Radix auricularia* eggs. Our results also conform with findings by who reported delayed hatching in isolated apple snail eggs exposed to 250 µg/L cadmium.

4.3 Effects of treated and untreated wastewater on the Biomass of *Helix pomatia*

Results from this study show that *H. pomatia* exposed to soils spiked with treated wastewater showed significant reduction ($P<0.05$) in the biomass between the control group (pure OECD soil) and 25, 50, 75% treatment groups apart from 100% treatment group. This implies that treated wastewater samples showed toxicity to *H. pomatia* after 60 days of exposure. Similarly, there was also a significant difference ($P<0.05$) in biomass for between *H. pomatia* exposed in control soils (pure OECD soil) and soils spiked with 25, 50, 75 and 100% untreated wastewater (Figure 4). This implies that increasing concentration of untreated wastewater had a negative impact on the biomass of *H. pomatia*. The reduction of biomass could be linked to a decline in food consumption (plant and soil) by *H. pomatia* (Wlostowski *et al*. 2016). The result from this study is in alignment with several studies that have reported a significant reduction in the biomass of invertebrates including gastropods because of exposure to contaminants. Cœurdassier *et al.* (2000) report a decrease in weight of *H. aspera* exposed to Chromium. Schuytema, *et al* (1994) also reports growth inhibition and weight decrease in *H. aspera* exposed to a cocktail of pesticides. De Vaufleury *et al* (2000) reported a reduction in biomass of *H. aspera* exposed to Cu, Zn Pb and Pentachlorophenol. More recently, Das and Khangarot (2011) also reported a dose-dependent growth (weight) inhibition in freshwater snail *Lymnaea luteola* exposed to Copper.

Based on results from this study, effluent released from Maluti-A-phofung wastewater treatment plant improved the biomass of *H. pomatia*, therefore, indicating its suitability to organisms within the environmental discharge

From on the results of this study, the first hypothesis can be accepted: survival and reproduction of *Helix pomatia* decreased with the increasing concentrations of OECD soil spiked with untreated wastewater samples. The second

hypothesis can also be accepted: survival and reproduction of *Helix pomatia* increased with the increasing concentrations of OECD soil spiked with treated wastewater samples although this was only statistically significant at the highest concentration. The third hypothesis can be accepted: Biomass of *Helix pomatia* decreased with the increasing concentrations of OECD soil spiked with untreated wastewater.

4.4 Biomarker responses in *H. pomatia* exposed to treated and untreated wastewater

4.4.1 Acetylcholinesterase (AChE) activity

AChE is one of the versatile enzymes of the nervous system that plays a role in neurotransmission, in snails and its activities have been used as a sensitive biomarker for biomonitoring pollution (Tripathi and Srivastava 2008; Singh *et al*. 2011; Singh *et al*. 2014). In this study, results of AChE activity in *H. pomatia* exposed to OECD soil spiked with the untreated and treated wastewater showed that there was no statistical difference between the control and all treatments groups (Figure 5). Despite the fact that the activity seemed to be higher in treated wastewater. Results in this study are similar to those reported by Singh and Agarwal (1987) who reported no significant changes in AChE activity after exposing a freshwater snail, *Lymnaea acumata* to synthetic pyrethroid permethrin. Singh and Agarwal (1991) reported no change in AChE activity after exposing a freshwater snail, *Lymnaea acuminate* to synthetic pyrethroid cypermethrin. Similarly, Crane *et al*. (1997) investigated the effects of the organophosphorus insecticide pirimiphos-methyl on the amphipod, *Gammarus pulex L*. Results showed that AChE activity remained unaltered after the experiment. In addition, Bonnard *et al*. (2009) reported no significant difference in AChE activities in the Bivalve, *ScrobicularIa plana* after copper exposure.

Results of this study do not agree with the findings of Banaee *et al*. (2019) who reported reduced AChE activities in the freshwater snail, *Galba truncatura* as a result of exposure to dimethoate alone and in combination with cadmium. Ma *et al*. (2014) also reported reduced AChE activity in the freshwater snail, *Physa acuta* in response to the toxicity of abamectin. Lastly, AChE inhibition in freshwater snail (*Galba*

truncatura) as a result of exposure to municipal sewage in Iran has also been reported by Banaee and Taheri, (2019).

4.4.2 Catalase (CAT) Activity

Catalase enzyme has become a valuable biomarker of oxidative stress in invertebrates exposed to toxicants. It plays a vital role in the breakdown of hydrogen peroxide to water and oxygen thereby acting as a defensive response against the overproduction of ROS induced by the presence pollutants (Banaee *et al*. 2014; Banaee *et al*. 2019). In this study, the results showed that there was no statistical difference between CAT activities in *H. pomatia* exposed in the control soil and all concentrations of treated and untreated wastewater (Figure 6). There was also no difference between CAT activities in *H. pomatia* exposed to treated and untreated wastewater. This result suggests that the animal was not under oxidative stress during the exposure period. Results observed in this present study agree with results from studies that have reported no change in catalase activities after exposure to contaminants. Bainy *et al*. (2000), reported that there was no significant effect on CAT activities for gastropod *Perna perna exposed* to sites contaminated in Santa Catarina Island, Brazil. Similarly, Cochon *et al*. (2007) have also reported no significant change in catalase activity after exposing freshwater snail, *Biomphalaria glabrata* to paraquat. In addition, Cabecinhas *et al*. (2014) have also observed no significant change in catalase activity in *Gibbula umbilicalis* after exposure to mercury chloride.

However, the results in this study do not agree with the findings of Vranković *et al*. (2012) who reported significant increases in CAT activities in *Holandriana holandrii* exposed to river water polluted with domestic and industrial effluents. Similarily, Bianchi *et al*. 2014 report elevated CAT levels in the gastropod *Diplodon chilensis* in response to sewage pollution.

This implies that CAT activities alone as an oxidative stress biomarker in *H. pomatia* in this study is not suitable to access stress caused by pollutants in wastewater.

Based on the biomarker results of this study, the fourth hypothesis cannot be accepted: Biomarker responses were the same in treated and untreated wastewater samples.

5 Conclusion

From the observed results, it is quite clear that treated and untreated wastewater from Phuthaditjaba treatment plant had significant effects on the survival of *Helix pomatia species*. In contrast, the same wastewater has the potential to reduce the reproduction of *H. pomatia* species despite the fact that reproduction seemed to be higher in OECD soil spiked with treated wastewater. Treated and untreated wastewater from Phuthaditjhaba treatment plant has the potential to cause harmful effects on terrestrial organisms. *H. pomatia*. These findings provide evidence on wastewaters potential ecological risk on the soil. Even though it did not cause any reduction/inhibition of AChE and CAT activity. Results from this study highlight the toxic effects of wastewater pollution in the Maluti-A-Phofung wastewater treatment plant. If the wastewater treatment plant continues to improperly manage their wastewater with similar contents or more this could lead to a decline in biodiversity, and also contribute to the alteration of ecosystem balance.

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