The biomass, survival, reproductive and biomarker responses of Helix pomatia to soil contaminated with treated and untreated wastewater.

Semase Matseleng
University of the Free State - Qwaqwa Campus
Ozekeke Ogbeide (ozekeke.ogbeide@uniben.edu)
University of the Free State - Qwaqwa Campus https://orcid.org/0000-0003-3699-1515
Patricks' Otomo Voua
University of the Free State - Qwaqwa Campus

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Abstract

Wastewater treatment facilities in developing countries like South Africa are major sources of contaminants via effluent into the environment, which could portend high toxicity risks for non-target flora and fauna. To this end, a study was conducted to determine the ecotoxicological responses of selected organism to treated and untreated wastewater from the wastewater treatment plants in an industrial town. The snail *Helix pomatia* was exposed to OECD artificial soil spiked with untreated or treated wastewater at the following concentrations: 0, 25, 50, 75, 100%. The ecotoxicological responses of *Helix pomatia* to wastewater were determined by assessing the biomass, survival, reproduction and biomarker responses (Catalase – CAT and Acetylcholinesterase – AChE activities). The overall results showed significant effects on the survival, reproduction and biomass of *H. pomatia.* Similar results were observed for juvenile emergence. An EC_{50} of 5.751% for egg production and an EC_{50} of 6.233% for juvenile emergence were determined in the untreated wastewater. Such indices could not be computed for the treated wastewater, indicating a decreased in toxicity between the untreated and the treated samples. For both the AChE and CAT activities, there was no statistical difference between treated and untreated wastewater treatments. The results from this study highlight the toxic effects of untreated wastewater and indicate that treated wastewater (effluent) released from the wastewater treatment plant in Phuthaditjhaba remains suitable for invertebrate fauna such as *H. pomatia.*

1. Introduction

Growing anthropogenic activities in South Africa and the world over have resulted in the subsequent contamination of water bodies because of the introduction of different types of toxic pollutants (Oberholster and Ashton, 2008; Namugize et al., 2018). A major source of these pollutants is wastewater derived from various industrial, domestic and agricultural processes (Holt, 2000; Ma et al., 2009). Treated wastewater is used mainly for irrigation (both agricultural and landscape), seawater barriers, industrial and urban needs (Tchobanoglous et al., 2011).

Although wastewater treatment plants were established to rid raw sewage of pollutants, it has been reported that they 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; Rodriguez-Mozaz et al., 2015). Most treatment plants in municipalities within South Africa lack adequate facilities, sufficient financial resources and proper skills to manage sewage water and sludge (Mema, 2010). Pollution from wastewater treatment has significant consequences on river ecosystems (Bernhardt and Palmer, 2007; Grant et al., 2012; Moloi et al., 2020).

Exposure to wastewater is known to increase mortality, decrease survival and cause changes in biomarker responses in aquatic organisms. Effects on survival and mortality have been reported on the zebrafish *Danio rerio* (Gellert and Heinrichsdorff (2001); the Japanese rice fish *Oryzias latipes* and the freshwater shrimp *Macrobrachium nipponens* (Gerhardt et al. (2002); the ridge mussel *Amblema plicata* and the Asian clam *Corbicula fluminea* (Nobles and Zhang (2015); the amphipod crustacean *Gammarus* *gauthieri* (Haouache and Bouchelta (2016); the freshwater isopod *Asellus aquaticus* (Plahuta et al. (2017); the waterflea *Ceriodaphnia dubia* (Ziajahromi et al. (2017), and on the round goby *Neogobius melanostomus* (McCallum et al. (2017). Effects of reproductive output have been documented on the zebrafish *Danio rerio* (Babić et al. 2017); the waterlouse *Asellus aquaticus* (Plahuta et al. 2017); the roundworm *Caenorhabditis elegans* (Abbas et al. 2018); and on the amphipod crustaceans *Gammarus roeseli* and *Gammarus pulex* (Peschke et al. 2019).

Recent reports have suggested that effluent and sewage sludge from the wastewater treatment plant located in the eastern Free State region of South Africa have high concentrations of metals, with potential ecotoxic effects on organisms (Moloi et al., 2020). The study by Mosolloane et al. (2019) remains one of few in South Africa to include the ecotoxicological assessment of wastewater management wastes. Previous studies have only focused on the physicochemical, and microbial assessment of wastewater treatment plant (WWTP) by-products in several South African municipalities (Samie et al., 2009; Olujimi et al., 2012; Naidoo and Olaniran, 2014; Odjadjare and Olaniran, 2015; Adefisoye and Okoh, 2017; Edokpayi et al., 2017; Fagbayigbo et al., 2018; Salaudeen et al., 2018; Adeniji et al., 2019; Assress et al., 2020).

Despite mounting evidence for increased contamination of aquatic and terrestrial ecosystems by WWTPs effluent, the Drakensberg Afromontane region of South-Africa, remains an understudied region in terms of pollution monitoring or ecotoxicological studies. This is despite increased pollution threats from the urbanisation and industrialisation of the region, which is seeing an accelerated opening of waste generating industries (Liedtke, 2018). The obvious implications of these changes are a chemical overload of the wastewater treatment facilities, which are already struggling and a heightened likehood of envrionmental contamination.

This study seeks to address the gap in data from ecotoxicological studies by investigating the potential effects of treated and untreated wastewater on one of the model organisms most likely to endure the anticipated environmental pollution from WWPTs. Their wide distribution and ability to exhibit a quick response to exposure to various pollutants have conferred several terrestrial species of molluscs great potential for use in risk assessment of contaminated soil (Radwan et al., 2019). They have become greatly desirable biomonitors and sentinels in environmental pollution biomonitoring (Gomot de Vaufleury and Pihan, 2000; Mariet et al., 2017; Mleiki et al., 2017). In the matter of assessing ecotoxicological effects of soil pollutants, terrestrial mollusc are mostly used because of their unique life cycle that may interface the soil-plant-air systems, integrating several sources of contamination and the integration of the three routes of contamination: inhalation, cutaneous routes and ingestion (Regoli et al., 2006; De Vaufleury, 2015; Mariet et al., 2017). Other desirable qualities, suitable for ecotoxicity testing, include their high tolerance to many pollutants, and relatively high toxicant tissue accumulation.

Several studies have also utilised biomarker responses in land snails during risk assessment of contaminated sites. When exposed to contaminated soils, an increased generation of reactive oxygen species (ROS) in terrestrial snails, resulting in the overriding of antioxidant defences systems, has been reported (El-Shenawy et al., 2012; Radwan et al., 2019). Among the antioxidant defences, catalase (CAT), appear to be one of the most sensitive to the proliferation of ROS (El-Shenawy et al., 2012). CAT protects cells from damage by converting hydrogen peroxide (H_2O_2) into oxygen and water (Bhagat et al., 2016). A positive correlation between CAT activity in gastropods and exposure to contaminants has been reported by several studies including Chandran et al. (2005) and Wang et al. (2014) in the giant African snail (*Achatina fulica)*; Al-Daihan et al. (2010) in the fresh water snail *Biomphalaria arabica;* Ma et al. (2014) in tadpole snail (*Physa acuta*); Cabecinhas et al. (2015) in the flat top shell (*Gibbula umbilicalis*); Ali and Ali (2015) in the freshwater snail *Lymnea luteola*; and Khalil (2015) in the freshwater snail *Lanistes carinatus.*

Similarly, acetylcholinesterase activity (AChE) has also become a choice biomarker in ecotoxicological studies, as it is used as a sensitive biomarker of exposure to potential neurotoxic chemicals (Fairbrother et al., 1998). Several studies have utilized this biomarker in studies involving gastropods including Zawisza-Raszka et al. (2010) in the snail *Helix aspersa*; Mazzia et al. (2011) in the snail *Xeropicta derbentina*; and Banaee et al. (2019) in the snail *Galba truncatula*.

In the present study, we examined the responses of the land snail *Helix pomatia* exposed to concentrations of wastewater using standard life cycle parameters including survival, biomass, reproduction and biomarker responses. We hypothesize that the presence of pollutants in the wastewater from the WWTP could illicit adverse biological effects, which could lead to a decline in biodiversity necessary for ecosystem balance and functioning. The specific objectives of this study include determining the effects of treated and untreated wastewater on the survival, reproduction, biomass, catalase and acetylcholinesterase activity in *H. pomatia*.

2. Methods And Methods

2.1. Study area

Treated and untreated wastewater samples were collected from the wastewater treatment plant in the town of Phuthaditjhaba (28° 30' 28.3" S; 28° 49' 39.7" E). This town is located within the Drakensberg Afromontane region of Southern-Africa. The selected wastewater treatment plant receives raw wastewater from regional industries and residential areas in the surroundings of Phuthaditjhaba. The plant receives 6 to 6.8 million litres of wastewater per day (Mosolloane et al., 2019; Moloi et al., 2020).

2.2. Experimental organism

In this study, a surrogate mollusc; *Helix pomatia* was used under laboratory conditions to ascertain the potential effects of treated and untreated wastewater. The choice of the test organism was based on the fact that terrestrial gastropods are good bio-indicators of environmental status and they attain higher bioaccumulation for many toxicants (Regoli et al. (2006). Adult specimens of *H. pomatia* were purchased from a local breeder and maintained in OECD artificial soil (OECD 1984) to allow for stabilization. A temperature of 20^{o}C was maintained through during the period of acclimatization which lasted 5 days before bioassays could commence.

2.3. Collection of wastewater samples

The treated and untreated wastewater samples were collected in 25 L plastic jerry cans from the raw sewage inlet (28°30'28.4"S 28°49'44.1"E) and the treated effluent outlet situated on the banks of the Elands River (28°30'15.3"S 28°49'24.2"E) which is the receiving waterbody nearby

2.4. Preparation of Exposure Substrates

The OECD artificial soil was used as the exposure substrate and was prepared following OECD recommendations (OECD 1984), by mixing 10% of sphagnum peat (air-dried and sieved-2mm), 20% of kaolin clay and 70% of quartz sand (air-dried). The sampled wastewater samples were prepared into the following concentrations using distilled water. The following wastewater concentrations (treated and untreated) were obtained including 100% (pure wastewater), 75% (75% wastewater + 25% distilled water), 50% (50% wastewater + 50% distilled water), 25% (25% wastewater + 75% distilled water). Clean distilled water was used in the negative control. One liter (1L) of each solution of treated and untreated wastewater was used to spike 2000g of OECD soil to form a wastewater/OECD soil treatment in 25L black plastic storage boxes (330×220×345mm). This experimental setup was prepared based on bioassays for Helicidae described in De Vaufleury (2006), Gomot-de Vaufleury and Bispo (2000) and Gimbert et al. (2006).

2.5. Experimental procedure

2.5.1. Effects of wastewater on *Helix pomatia*

All snails before the exposure were washed and kept in 25 L black boxes (330×220×345mm) perforated at the top to allow for gaseous exchange for 5 days to acclimatize to the exposure substrate. A cohort of 10 adult *Helix pomatia* was exposed in 2000g of prepared wastewater/OECD soil treatment and clean distilled water/OECD soil controls. The exposures were carried out in triplicates for both treated and untreated wastewater samples for a period of 60 days based on a modified method described by Gimbert et al. (2006). The snails were fed once a week with 5 g of washed lettuce during the period of exposure. During the course of the experiment, snail faeces were removed regularly.

2.5.2. Survival and mortality

Survival and mortality of *H. pomatia* were determined using the method described by De Vaufleury (2006). Mortality was monitored every 24 hours. Unresponsive snails after external stimulation were counted as dead. The survival rates of snails in each exposure container was determined at the end of the exposure period.

2.5.3. Reproduction

Reproduction was estimated by counting the number of eggs produced during the experiments. Whenever present, the eggs were collected from each exposure container, put in 10-cm diameter Petri dishes and incubated at 20^oC until hatching. Upon hatching, the juveniles were counted and transferred to different breeding boxes with clean OECD artificial soil.

2.5.4. Biomass

The snails were weighed individually before the exposure and at the end of the exposure period, at day 60. Biomass gain or loss were estimated by subtracting the weights at day 0 from the weights at day 60. Positive values indicated weight gain while negative values indicated weight loss. After the experiments, they were washed with distilled water and preserved at -80°C in an ultra-low temperature freezer (Blizzard NU-998282) until biomarker experiments could be conducted.

2.6. Biomarker responses

2.6.1. Preparation of homogenates

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 rpm at 4°C for 10 minutes and the supernatants were used for biomarker and protein analysis. Both catalase and acetylcholinesterase procedures were performed on ice.

2.6.2. *Catalase* (CAT)

Catalase activity was measured according to the method described by Cohen et al. (1970). The reaction mixture consisted of 93 µl of 30% 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, using the Bio-Rad model 680 microplate reader, within 30–60 seconds of $KMnO_4$ addition. The following calculations were performed to determine CAT activity (in µmol H_2O_2 /min/mg protein) in the samples:

k = log (S0/S3) x 2.3 / t

Here k represents the first-order reaction rate constant, S0 equals to an average of standard absorbance reading, S3 equals to standard minus average absorbance of sample and t is the time taken to measure the reaction.

2.6.3. *Acetylcholinesterase* (AChE)

The acetylcholinesterase method was performed 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 a wavelength 412nm (405nm) in 1-minute intervals over 6 minutes. AChE activity was determined by calculating the average absorbance of the readings at each interval from 0 to 6 minutes. AChE activity was calculated as follows (absorbance /min/ mg protein) = (Abs/min) ÷ mg protein. Therefore, the inhibition percentage was computed by using the control's AChE activity as the standard activity.

2.6.4. Protein quantification

The protein concentration of each homogenate 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. 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) were added, mixed well and incubated at room temperature for 5 minutes. Thereafter, the absorbance was measured at 595 nm, using the 0 mg/mL BSA standard solution as a blank. The BSA standard solutions were used to plot a standard curve for estimating the concentrations of unknown protein samples.

2.7. 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). Data obtained for reproduction, survival and biomass was subjected to One-way ANOVA, with Bonferroni post-test using Sigma Plot version 13.0. The level of significance was set to be $p < 0.05$.

3. Results

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

Survival data showed that there was a significant decrease in the survival of *H. pomatia* exposed in all soil spiked with the untreated wastewater, compared to the respective control (Fig. 1; $P < 0.05$). In the soil spiked with treated wastewater, a similar significant decrease was only found in the snails exposed to the 25 and 50% treatments compared with the respective control ($P < 0.05$; Fig. 1). Comparing homologous concentrations of treated and untreated wastewater, showed significant differences in the 75 and 100% treatments, with lower survival occurring in the treatments spike with the untreated wastewater. For both exposure groups, no LC_{50} could be determined because mortality rates hovered around 50–55% in the worst-case scenario.

3.2. Effects of treated and untreated wastewater the reproduction of *Helix pomatia*

3.2.1. Cocoons production

The cocoon production of *H. pomatia* exposed to all the treatments spiked with the untreated wastewater was significantly reduced when compared to the respective control (Fig. 2; $P < 0.05$). Reproduction was totally inhibited in treatment spiked the 100% untreated wastewater. An EC_{50} = 5.751 % was calculated for *H.pomatia* exposed to untreated wastewater. Similarly, *H. pomatia* exposed to the soil spiked with the treated wastewater, also showed a significant decrease ($P < 0.05$), in reproduction in all treatments compared to the control group except for the 100% treatment which was similar to the control group (Fig. 2).

Comparing the homologous treatments showed no statistical differences except between the 100% treatments where reproduction did not occur in the untreated influent.

3.2.2. Juvenile emergence (hatching success)

The hatching success of *H. pomatia* was observed after 60 days of exposure. Results show that there was no statistical difference (Fig. 3; $P > 0.05$) between *H. pomatia* exposed in OECD soil spiked with the untreated wastewater (75%, 50%, 25%), and those in the control group. Apart from the highest concentration (100%), where there was a significant difference from the control (Fig. 3; $P < 0.05$). The total lack of reproduction in the pure untreated influent (100%) contributed in the generation of the relatively low EC_{50} of 6.233% for hatching success.

Similarly, there was no significant difference (Fig. 3; $P < 0.05$) in the number of juveniles that emerged for *H. pomatia* exposed in OECD soil spiked with the treated wastewater at 100%, 75%, 50%, and 25% concentration compared to the control group.

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 between the groups (Fig. 3; $P > 0.05$). In the highest concentrations, nevertheless, no hatchlings were recorded in the pure influent due to the lack of eggs laid in this treatment.

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

H. pomatia lost significant biomass in all treatments spiked with the untreated influent when compared to the respective control (Fig. 4, $P < 0.05$). In the treatments made with the treated effluent, biomass loss was only significant in the 25, 50 and 75% treatment, when compared to the respective control (Fig. 4, $P < 0.05$). The snails exposed to the control treatment and pure effluent (100% treated wastewater) showed no statistical differences in biomass. Comparing homologous treatments of treated and untreated wastewater showed no significant differences, except in the highest treatments where more biomass loss occurred in the 100% untreated wastewater (Fig. 4, $P < 0.05$).

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

3.4.1. Acetylcholinesterase (AChE) activity

In the soil spiked with the untreated wastewater, there was no statistical difference in Acetylcholinesterase (AChE) activity in earthworm between the treatment groups and the control group ($P > 0.05$, Fig. 5). Similarly, AChE activity in the soil spiked with the treated wastewater showed no statistical difference between the control and the treatments ($P > 0.05$).

3.4.2. Catalase (CAT) activity

As with AChE, CAT activity showed no statistical difference between the respective control and the treatments groups for treated and untreated wastewater ($P > 0.05$, Fig. 6). Similarly, no statistical differences in CAT activity between the untreated and treated treatments were found, although higher CAT activity seemed to occur in the treatments made using the untreated influent (Fig. 6).

4. Discussions

This study presents more evidence that supports the usefulness of terrestrial mollusc in ecotoxicological assessment (Gomot de Vaufleury and Pihan, 2000; Regoli et al., 2006). In South Africa, aside from the release of improperly treated wastewater (Moloi et al., 2019), there is a worrying trend of wastewater spillage or leaks from raw sewage pipes, which constantly leads to the contamination of the terrestrial environment (Eales, 2011; Govender et al., 2011). Results from this study indicate that there could be a constant decline in keystone invertebrate species both from untreated wastewater spillages and from the release of treated wastewater.

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

The results shown in Fig. 1, suggests that in the OECD soil spiked with the treated wastewater, there was a significant reduction ($P < 0.05$) in the survival of *H. pomatia* for all 25 % and 50 % treatment groups. However, survival remained unaffected for 75 and 100% compared to the control. This indicates that as the concentration of treated wastewater increased the survival of *H. pomatia* remained unchanged. 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 Brönmark (1992) also reported very low mortality for pond snail, *Lymnaea stagnalis* exposed to DDT treatments when compared to the control.

On the contrary, the survival of *H. pomatia* exposed to OECD soil spiked with untreated wastewater was significantly reduced in all treatments (Fig. 1; $P < 0.05$) compared to the control. 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 Clarke et al. (2009), who reported a decrease in survival of the Ramshorn snail (*Planorbarius corneus*) exposed to sewage sludge compared with 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 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., 2014; Edokpayi et al., 2015; Moloi et al., 2019; Mosolloane et al., 2019; 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 Watton and Hawkes (1984) and Jobling et al. (2003).

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

4.2.1. Cocoon production

Reproductive parameters in gastropods including egg-laying, hatchability and embryo development have become important endpoints in ecotoxicological bioassay (De Vaufleury, 2015; Khangarot and Das, 2010). In the present study, there was a significant decline in cocoon production in *H. pomatia* exposed to soil spiked with untreated wastewater, with complete inhibition observed at the highest concentration (100%; Fig. 2) compared with the control. Also, there was a significant decrease in cocoon production in *H. pomatia* exposed to soil spiked with concentrations (25%, 50%, and 75%) of treated wastewater compared to the control. This implies that increasing concentration of untreated and treated wastewater reduced the ability of *H. pomatia* to reproduce, thus underscoring the toxicity of wastewater (treated and untreated) 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 especially estrogens. As stated earlier in this study, the observed toxicity of treated and untreated wastewater could be because of the presence of diverse contaminants (Moloi et al., 2019; Mosolloane et al., 2019). 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 routinely 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, De Castro-Catala et al. (2013) reported a reduction in eggs produced by the freshwater snail *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.

4.2.2. Juvenile emergence (hatching success)

Hatching success for *H. pomatia* in soil spiked with treated and untreated wastewater were not similar to results observed in cocoon production for *H. pomatia*. There was no significant reduction ($P < 0.05$; Fig. 3) in hatching success for *H. pomatia* exposed to the soil spiked with treated and untreated wastewater when compared to the control group. Only raw untreated wastewater showed ability to significantly reduce the hatching success of *H. pomatia*, indicating the toxicity of untreated wastewater to *H. pomatia's* life cycle.

While studies that have looked at the effect of wastewater on the hatchability of *H. pomatia* are scarce, 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 Schirling et al. (2006) 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*

Reduction in biomass observed in *H. pomatia* exposed to soils spiked with treated and untreated wastewater implies that increasing concentration of untreated wastewater had a negative impact on the wet weight of *H. pomatia*. This reduction could be linked to a decline in food consumption (plant and soil) by *H. pomatia* during the period of exposure because of an increase in toxicity of the substrate (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) reported a decrease in weight of *H. aspera* exposed to Chromium. Schuytema et al. (1994) also report growth inhibition and weight decrease in *H. aspera* exposed to a cocktail of pesticides. De Vaufleury and Pihan (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.

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

4.4.1. Acetylcholinesterase (AChE) activity

AChE is one of the most efficient enzymes of the nervous system that plays a role in neurotransmission in snails, and its activities have been used as a sensitive biomarker in biomonitoring programmes (Lionetto et al., 2011; Singh et al., 2011). In this study, results of AChE activity in *H. pomatia* exposed to soil spiked with the untreated and treated wastewater showed that there was no statistical difference between the control group and all treatments groups (Fig. 5). Even though 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) also reported no change in AChE activity after exposing a freshwater snail, *Lymnaea acuminate* to synthetic pyrethroid cypermethrin. Similarly, Crane et al. (1999) investigated the effects of the organophosphorus insecticide pirimiphos-methyl on the amphipod, *Gammarus pulex*. Results showed that AChE activity remained unaltered after the experiment. Also, Bonnard et al. (2009) reported no significant difference in AChE activities in the Bivalve, *Scrobicularla 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 and Taheri, 2019). In this study, the results showed, there was no statistical difference between CAT activities in *H. pomatia* exposed in the control soil and soils spiked with treated and untreated wastewater (Fig. 6). In each treatment concentration There was also no difference between CAT activities in *H. pomatia* exposed to treated and untreated wastewater. This result suggests that this test organism 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, Cochón et al. (2007) have also reported no significant change in catalase activity after exposing freshwater snail, *Biomphalaria glabrata* to paraquat. Also, Cabecinhas et al. (2015) 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 Vrankovic et al. (2012) who reported significant increases in CAT activities in *Holandriana holandrii* exposed to river water polluted with domestic and industrial effluents. Similarly, 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.

5. Conclusion

From the observed results, it is quite clear that treated and untreated wastewater from Phuthaditjhaba treatment plant had significant effects on the survival, reproduction and biomass of *H. pomatia* species, which could indicate potential harm to terrestrial invertebrates. However, treated and untreated wastewater had no significant effect on biomarker activities (CAT & AChE) in *H.pomatia*. Results from this study highlight the toxic effects of wastewater pollution in the Maluti-A-Phofung wastewater treatment plant. There is an urgent need for the monitoring of these treatment plants and the implementation of urgent mitigation efforts aimed at improving the quality of operations within these facilities.

Declarations

Consent to participate

NOT APPLICABLE. This wasn't required because we did not work with human subjects

Consent to Publish

NOT APPLICABLE. This wasn't required because we did not work with human subjects.

CONTRIBUTION	AUTHOR
Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND	Semase Matseleng Ogbeide Ozekeke Voua Otomo Patricks
Drafting the work or revising it critically for important intellectual content; AND	Ogbeide Ozekeke Voua Otomo Patricks
Final approval of the version to be published; AND	Voua Otomo Patricks
Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved	Ogbeide Ozekeke

- We confirm that the manuscript has been read and approved by all named authors.
- We confirm that the order of authors listed in the manuscript has been approved by all named authors.

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Competing Interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript

Research Ethics:

Ethical Approval: UFS-AED2016/0067. This project was part of a larger Project Title: Ecotoxicological and bacteriological assessment of water resources in the Afromontane region of the eastern Free State. Hence the Interfaculty Animal Ethics Committee approved the above project. UFS-AED2016/0067.

Availability of data and materials

Data used in this research will be made available by the authors upon request.

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Figures

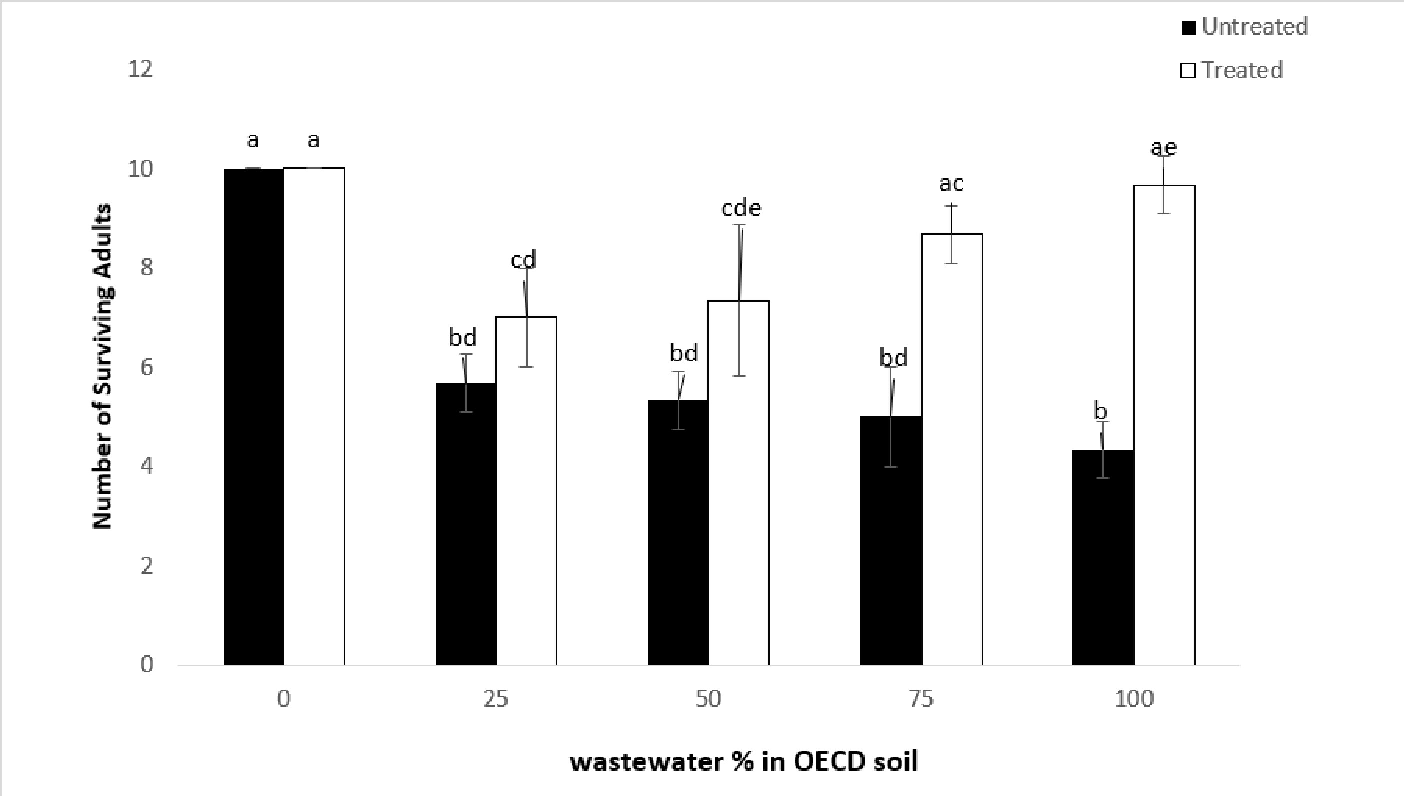

Figure 1

Survival of Helix pomatia adults after 60 days of exposure to artificial OECD soil spiked with treated and untreated wastewater from the Phuthaditjhaba wastewater treatment plant. n =30 snails per treatment. Error bars represent standard deviation. Different letters above the bars represent statistical differences between the treatments ($P \leq 0.05$).

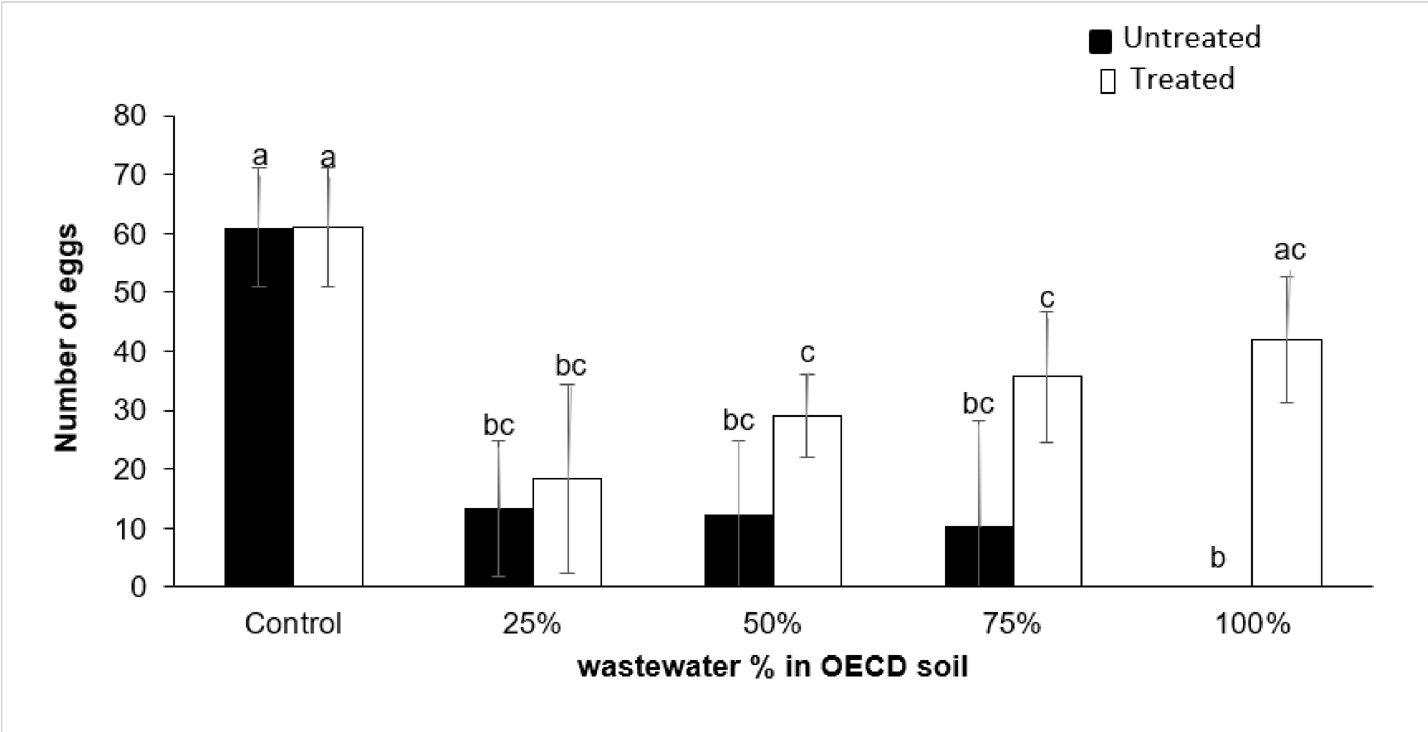

Figure 2

Survival of Helix pomatia adults after 60 days of exposure to artificial OECD soil spiked with treated and untreated wastewater from the Phuthaditjhaba wastewater treatment plant. n =30 snails per treatment. Error bars represent standard deviation. Different letters above the bars represent statistical differences between the treatments (P ≤ 0.05).

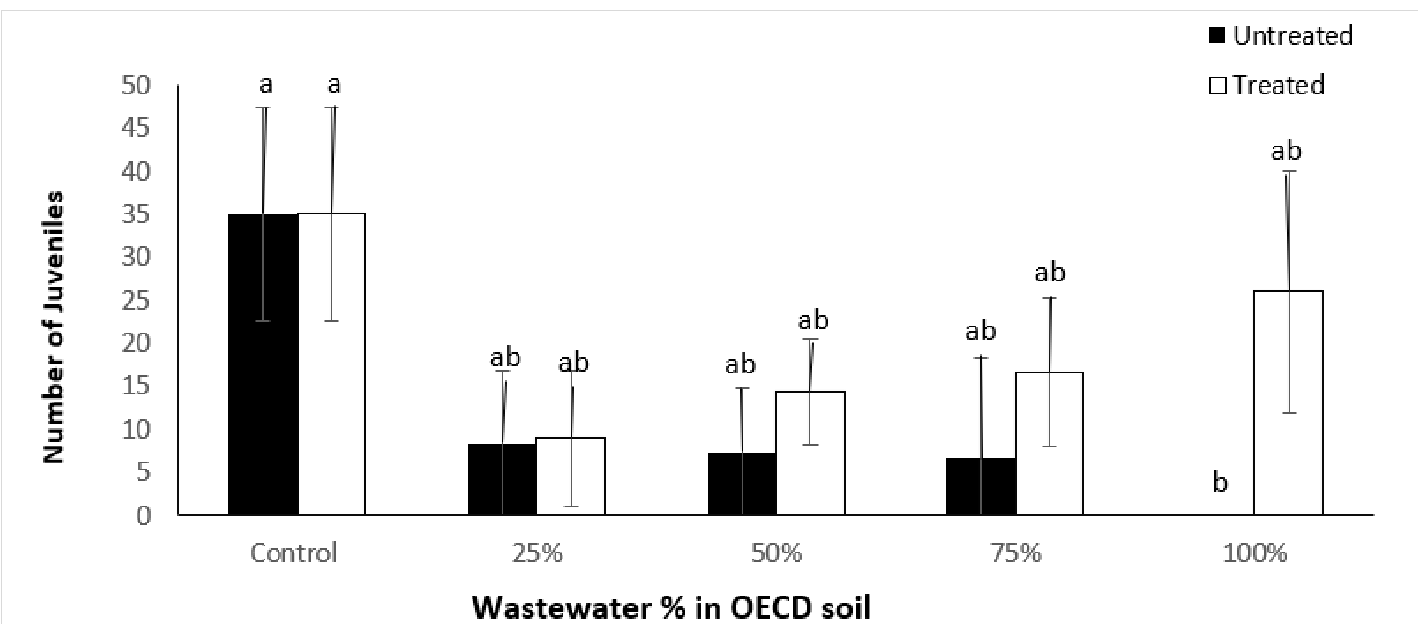

Figure 3

Hatching of Helix pomatia eggs after 60 days of exposure to artificial OECD soil spiked with treated and untreated wastewater from the Phuthaditjhaba wastewater treatment plant. n =30 snails per treatment. Error bars represent standard deviation. Different letters above the bars represent statistical differences between the treatments (P ≤ 0.05).

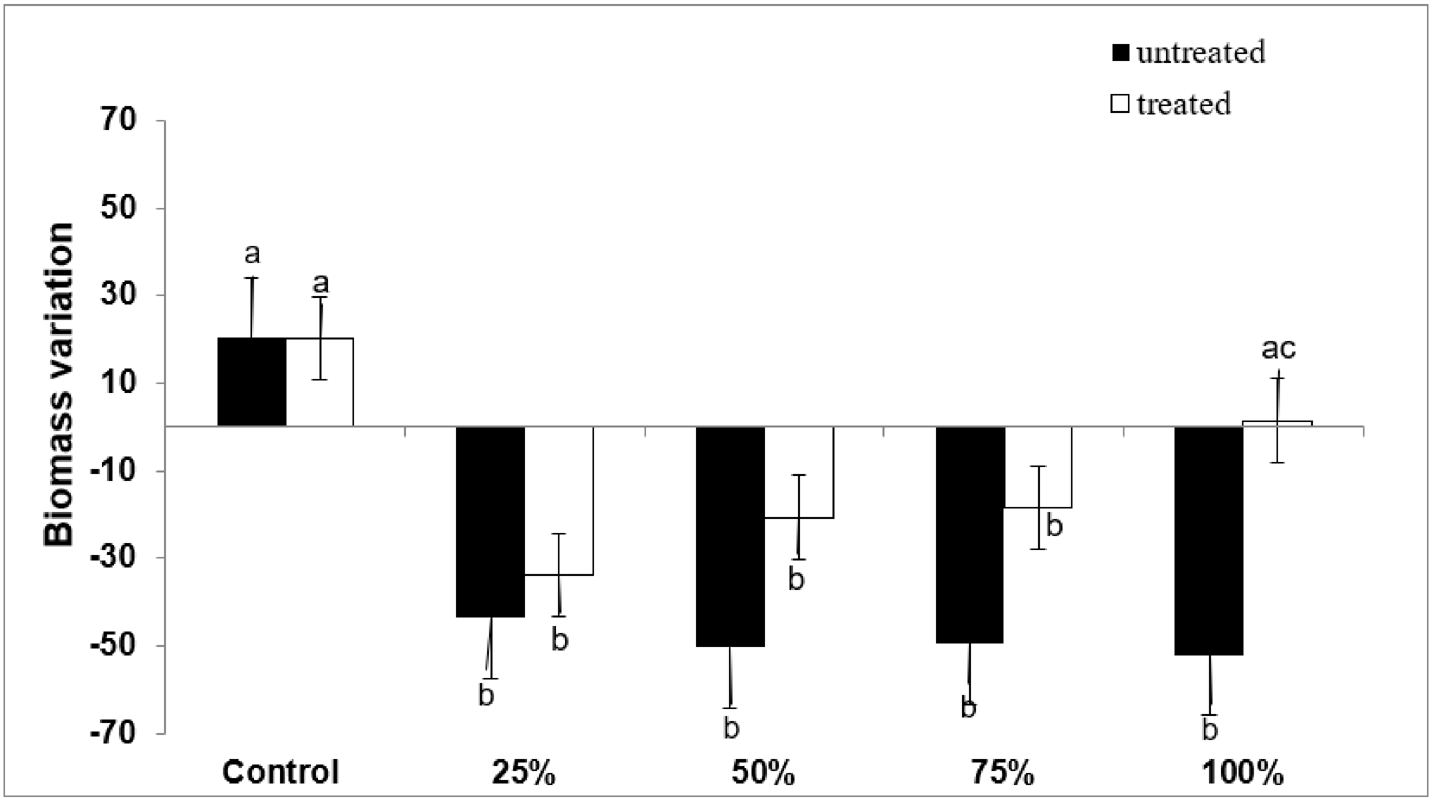

Figure 4

Biomass change of Helix pomatia after 60 days of exposure to artificial OECD soil spiked with treated and untreated wastewater from the Phuthaditjhaba wastewater treatment plant. n =30 snails per treatment. Error bars represent standard deviation. Different letters above the bars represent statistical differences between the treatments (P ≤ 0.05).

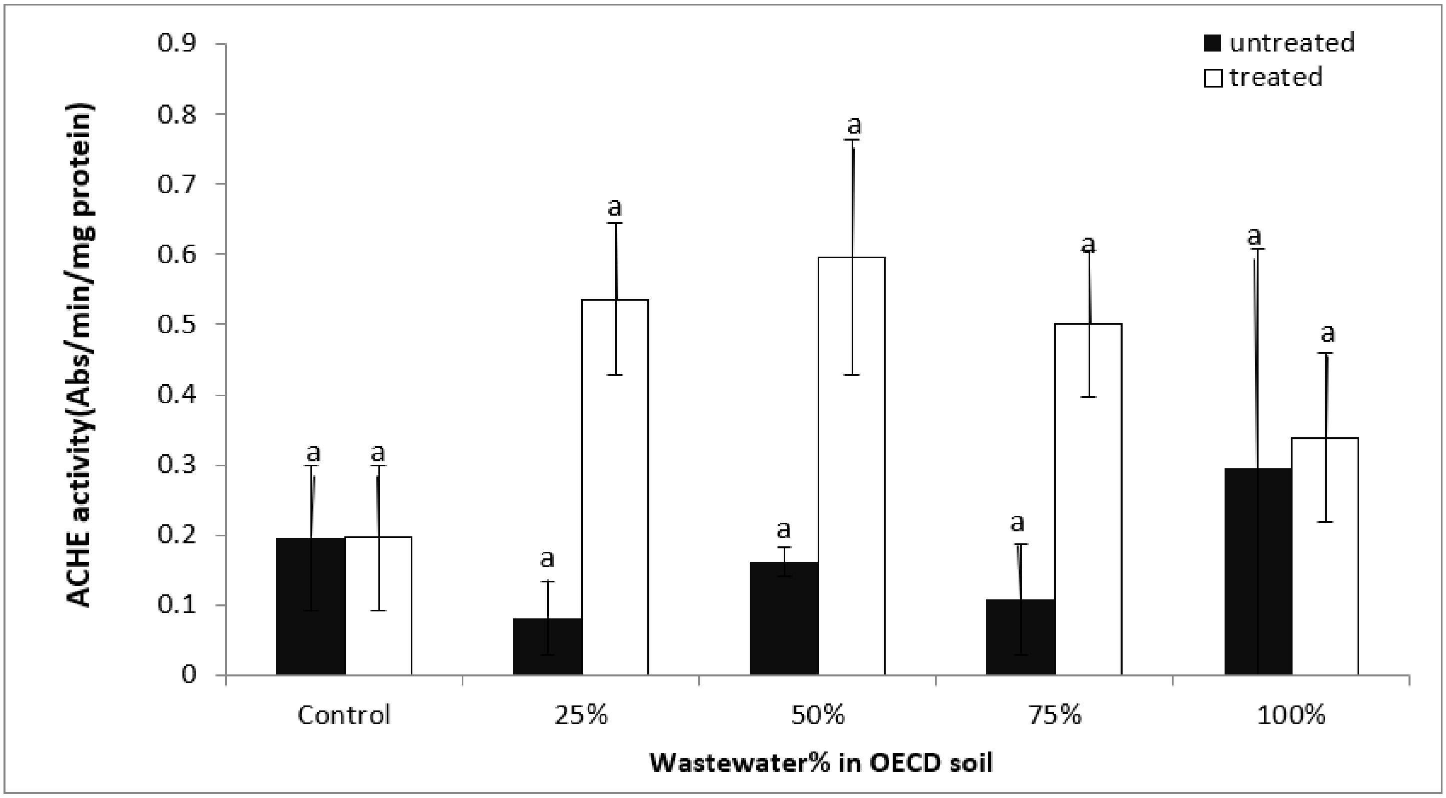

Figure 5

Acetylcholinesterase activity of H. pomatia after 60 days of exposure to artificial OECD soil spiked with treated and untreated wastewater from the Phuthaditjhaba wastewater treatment plant. Error bars represent standard deviation. Different letters above the bars represent statistical differences between the treatments ($P \leq 0.05$).

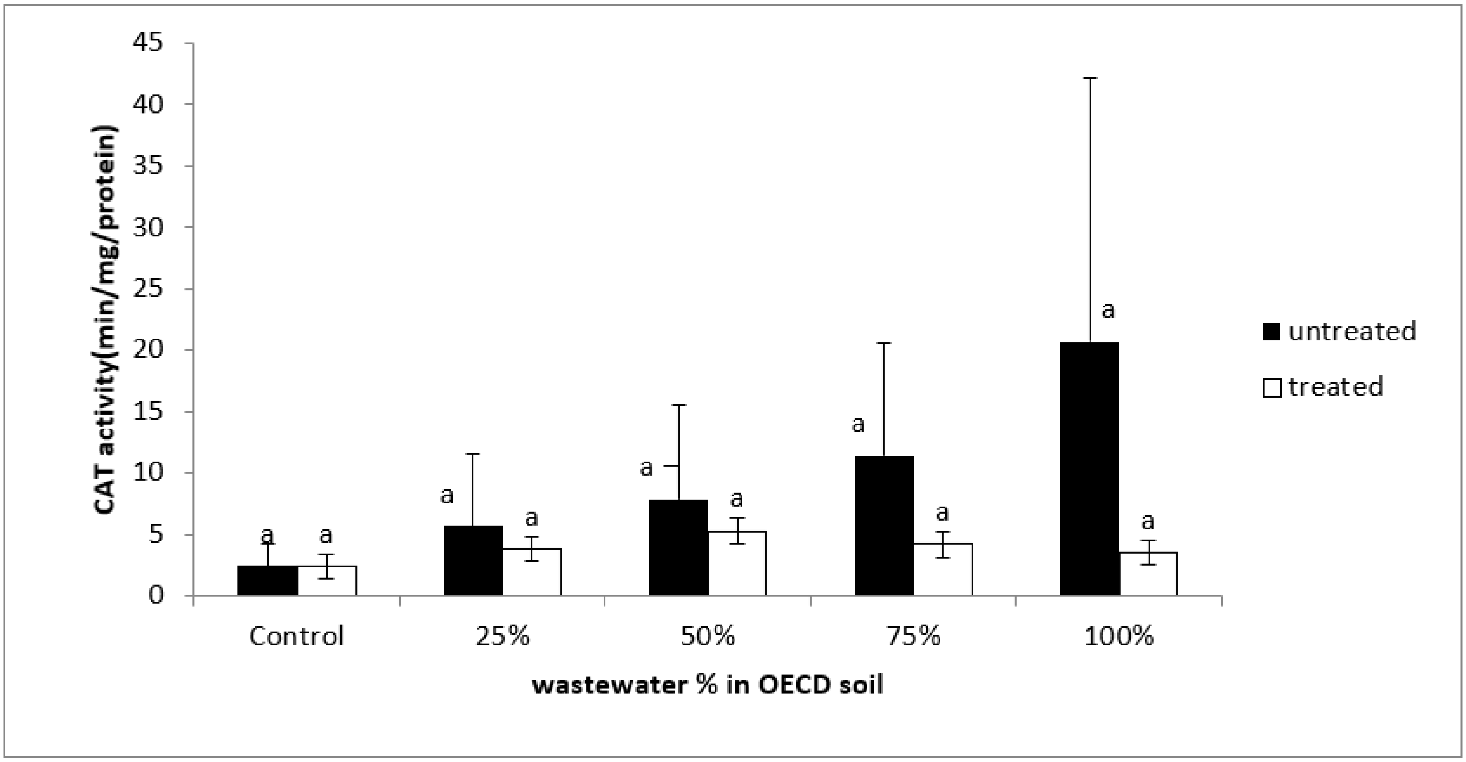

Figure 6

Catalase (CAT) activity of Helix pomatia after 60 days of exposure to artificial OECD soil spiked with treated and untreated wastewater from the Phuthaditjhaba wastewater treatment plant. Error bars represent standard deviation. Different letters above the bars represent statistical differences between the treatments ($P \leq 0.05$).