The use of zebrafish to assess water quality and remediation efforts.

by

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Biochemistry, School of Life Science of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg campus, South Africa. The research was financially supported by the National Research Foundation and the UKZN Institute for the Development and Dissemination of African Science (IDDAS).

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

Signed: Prof. RAYMOND HEWER

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ABSTRACT

Although wastewater effluents continue to be significant polluters of aquatic ecosystems in developing countries with limited water resources, little is known about the ecotoxicity induced by these effluents on fish throughout their early life stages. Several wastewater treatment plants (WWTPs) in South Africa (SA) do not adequately meet the minimal wastewater treatment requirements established by the country's Department of Water and Sanitation (DWS). Moreover, contaminants of emerging concern (CECs) originating from synthetic or natural sources, are widely distributed in aquatic environments of SA. This includes a broad range of natural and chemical compounds, such as aspirin (44243 ng/L), Fluoroquinolones (27100 ng/L), Atenolol (25900 ng/L), Nalidixic acid (25234 ng/L) and Ciprofloxacin (20514 ng/L). In addition to chemical compounds, endocrine disrupting chemicals, pharmaceuticals and personal care products are also distributed in the water systems. In the process of wastewater treatment, agents such as flocculants, coagulants, chemical precipitants (e.g., calcium hydroxide or sodium hydroxide) and chlorine disinfectants are utilized in wastewater treatment settings. However, research to understand the adverse effects that can be caused by these agents on aquatic organisms is still ongoing in SA. In order to bridge this knowledge gap, advanced techniques could be employed to help reveal adverse effects of wastewater as well as any shortcomings of current water remediation techniques. Using an appropriate aquatic model organism with highly conserved physiological pathways present in higher vertebrates (including humans), a rich behavioural repertoire, and occurrence in a variety of habitats would be a novel approach. To this effect, this study employed zebrafish with the aim to monitor six distinct wastewater samples from various regions of SA and to assess the effectiveness of currently used water remediation techniques such as chlorination. Two wastewater effluents, namely, Southern Works Final Effluents (SWFE) and Jacob's Incoming (JB) alerted potential toxicity during chemical characterization with sub-optimal pH (SWFE = 9.02 ± 0.16 and JB = 5.65 ± 0.02) and total alkalinity of zero (0 mg/L) detected for both effluents. The lethal toxicity of these effluents was seen by the elevation of mortality rate up to 77 ± 2.89 % and 100 ± 0.00 %, respectively for SWFE and JB at 40 %, with corresponding LC_{50} values of 17.77 % and 16.46 %. The zebrafish jaw and face, heart, brain, fins, notochord, somite and tail were significantly deformed ($p < 0.05$) post-exposure to these effluents, as revealed by morphological scores upon the analysis of the zebrafish's body structure. Moreover, there was a delay in development due to the aforementioned effluents, unsuccessful hatching, craniofacial abnormalities, pericardial and yolk sac oedema, notochord abnormality somite defects and spinal cord curvature. In addition, locomotor activity of zebrafish was inhibited following observation of distance travelled, frozen moments, acceleration rates, swimming trajectories and exploration rate. Surprisingly, safety of these wastewaters was restored by chemical precipitation revealing non-lethal pH ranges of 6.02 - 8.02 and 6.65 - 7.65 for SWFE and JB, reducing the mortality rate to non-significant levels ($p > 0.05$) compared to the control. Also, sodium bicarbonate ($NaHCO_3$) at 120 mg/L was found effective at supplementing the wastewater total alkalinity. In contrast, Amanzimtoti water before and after chlorination (TB and TA), Incoming Badulla (IB) and Chatsworth Incoming (CI) exhibited no consistent lethality effects on zebrafish and induced no apparent stress as demonstrated by insignificant expression ($p > 0.05$) of the stress protein: heat shock protein 70 (HSP70). However, the insignificant mortality

rate ($p > 0.05$) in the water tested before (TB) and after (TA) chlorination appeared to be the same (~25 %) indicating that chlorination is not enough at completely remediating wastewater. Our study is a pioneer in evaluating the ecotoxicological impact of wastewater effluents from localized regions of a developing country like South Africa in relation to the adjustment of water quality parameters for the neutralization of contaminants. To better understand emerging contaminants released as effluents in SA's water bodies and their interactions with aquatic organisms at the adult stage, more studies needs to be developed.

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ACRONYMS AND ABBREVIATIONS

2D- Two dimensional

AMRB - Antimicrobial-resistant bacteria

AMSB - Antimicrobial-susceptible bacteria

ARV - Antiretroviral

CECs - Contaminants of emerging concern

CF - Cystic fibrosis

China CDC - China Centers for Disease Control

CI - Chatsworth Incoming

COVID-19 - Coronavirus disease 2019

dM - Deformed muscles

DO - Dissolved oxygen

dpf - Days post-fertilization

DWAF- Department of Water Affairs and Forestry

DWS - Department of Water and Sanitation

ECs - Emerging contaminants

EDCs - Endocrine-disrupting chemicals

GC/MS - Gas Chromatographic-Mass Spectrophotometer

GMS - General morphology score

HAART- Highly Active Antiretroviral Therapy

HIV - Human immunodeficiency virus

hpf - Hours post-fertilization

HRP - Horse radish peroxidase

HSP70- Heat shock protein 70

IB – Incoming Badulla

JB - Jacobs Incoming

KZN - KwaZulu-Natal

MDRP - Multi-drug resistance

MIN – Minute

MPC - Maximum permitted concentration

NaHCO3 - Sodium bicarbonate

NCBI - National Center for Biotechnology Information

NDs - Nanodiamonds

NVP – Nevirapine

OCPs - Organochlorine pesticides

OECD - Organisation for Economic Co-operation and Development

PCPs - Personal care products

PPT - parts per thousand

QDs - Quantum dots

RBD - Relative band density

SA – South Africa

SARS-CoV-2- Severe acute respiratory syndrome coronavirus 2

SDS-PAGE - Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

SWFE – Southern Works Final Effluents

TA – Toti after chlorination

TB – Toti before chlorination

TBS- Tris-buffered saline

US- United States

USA- United States of America

UTI- Urinary tract infection

UV- Ultra-violet

WHO - World health organization

WWTP - Wastewater treatment plant

YS - Yolk sac

YSL - Yolk syncytial layer

ZET - Zebrafish embryotoxicity test

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Water pollution has been purported as a major contributor towards global mortality and morbidity, which is of a great concern, because water systems are the most important resources for life (Isiuku and Enyoh,2019). Many harmful substances are released into the aquatic systems, including toxic metals, microorganisms, agricultural and industrial chemicals (Alegbeleye *et al.*,2016;Isiuku and Enyoh,2019;Posthuma *et al.*,2020). Amongst these contaminants are emerging contaminants (ECs), which are not commonly monitored but may negatively impact living organisms and the ecosystem at large (Sauvé and Desrosiers,2014;Nawaz and Sengupta,2019). Even though harmful substances are released into the aquatic environment, there are limited studies that report the detrimental impacts of water contaminants detected in localized water systems in developing countries like South Africa (SA). Research conducted in developed countries such as Croatia revealed that wastewater can cause developmental malformations, impair hatching rate, induce neurotoxicity and edema of zebrafish while cumulatively increasing mortality in a concentration-dependant manner (Babić *et al.*,2017;Ribeiro *et al.*,2020). It is evident that water released into the environment as wastewater can exhibit some level of lethality towards aquatic species and may harm other living organisms. This includes humans who are at a high risk of experiencing adverse effects upon exposure to wastewater. In an attempt to monitor water systems using a model that shares similarities with living organisms such as humans, zebrafish have been utilized over years in water testing (Zhang *et al.*,2018;Shao *et al.*,2019;Ribeiro *et al.*,2020).

Despite mice being the most widely used research model worldwide, the utilization of zebrafish to conduct research has exponentially increased in science. Globally, there are over 1000 laboratories that use zebrafish as a model organism and over 100 zebrafish-related entries are entered per year on PubMed (Teame *et al.*,2019;Abdulrazaq *et al.*,2020). Zebrafish have become one of the most valuable aquatic animal models to advance knowledge in different research aspects. In biomedical experimentations, zebrafish are preferred due to the high degree of functional and sequence homology they share with mammals (Howe *et al.*,2013;Le Bras,2021). It is worth noting that despite the physiological differences, 84% of genes that are linked to human diseases have a counterpart in zebrafish (Howe *et al.*,2013;Can *et al.*,2020). Zebrafish possess all of the critical organs required for metabolism in humans. For these reasons, they are utilized to characterize human diseases (Rissone and Burgess,2018;Teame *et al.*,2019;Robea *et al.*,2020;Kurnia *et al.*,2021). In addition, reliable projections have revealed that using zebrafish allows for the most accurate and cost-effective high throughput screening that can be performed in a multi-well cell culture plate (Frieberg,2018;Hong *et al.*,2021). This is mainly due to the different advantages of zebrafish embryos, which include high fecundity, short generation time, ex utero development, and small embryo size (Feitsma and Cuppen,2008;Lawrence and Mason,2012;Bozkurt,2020). Embryos are preferred in toxicological experiments mainly due to their translucent nature that allows for direct observation of internal zebrafish organs and embryonic development a (Vacaru *et al.*,2014;Westhoff *et al.*,2020;Vauti *et al.*,2020). Zebrafish have been extensively used as a model organism in genetics as well. In this field, zebrafish were

employed to serve the purpose of genome editing (Sun *et al.*,2020b;Kim and Zhang,2020;Meshalkina *et al.*,2020), zebrafish mutant generation (Zhang *et al.*,2020b), studying human genes (Rosello *et al.*,2021) and for the discovery of new disease targets (Rubbini *et al.*,2020). In addition, this model organism has been used extensively in drug discovery and testing chemical compounds for a variety of diseases (Parng *et al.*,2002;Khedkar *et al.*,2018;Aspatwar *et al.*,2019), including accelerating the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) drug and vaccine testing (Fernandes *et al.*,2020;Kraus *et al.*,2020). Moreover, numerous studies have been developed for assessing water quality using zebrafish, due to the increasing rate of water pollution that poses serious threats to the ecosystem and human health (Frieberg,2018;Zhang *et al.*,2018;Shao *et al.*,2019).

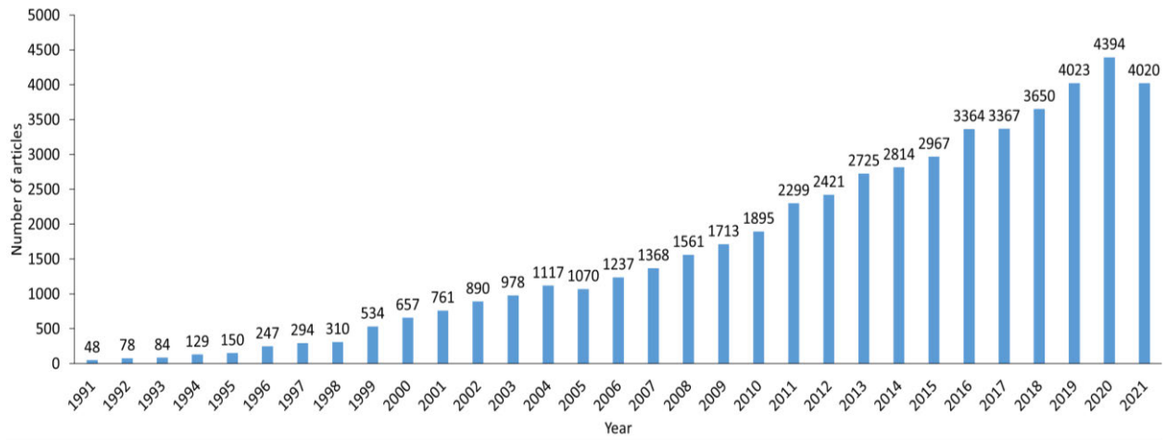
Although extensive research has been carried out using zebrafish to assess water quality, there is limited knowledge about the identification of ECs. Also, risks posed by ECs and methods to reduce them in localized water systems remains scarce in developing countries. This indicates that there is still a need for the advancement of knowledge to evaluate water quality in localized water environments. In addition to the aforementioned knowledge gaps, several wastewater treatment plants (WWTPs) poorly comply to the minimum wastewater treatment standards set by the national Department of Water and Sanitation (DWS) in SA (Kretzmann *et al.*,2021;DWS,2022). Only a few are in excellent or acceptable compliance to wastewater treatment standards. To monitor the effect of water remediation efforts, previous studies have tested water remediation agents such as flocculants and coagulants using zebrafish models (Faria *et al.*,2018a;Moura *et al.*,2019). However, the safety of water remediation methods used in developing countries is still not well understood. According to previous findings, chlorination does not ensure a complete removal of viruses such as SARS-CoV-2 (Zhang *et al.*,2020a) and other contaminants in water (Pignata *et al.*,2012;Zerva *et al.*,2021). This further emphasizes the need for the advancement of knowledge in water remediation methods and their safety, worldwide. Another concern is freshwater scarcity which is a major problem, globally. Approximately one-third of the drinking water is obtained from natural sources like dams, canals, rivers, and lakes (Jonnalagadda and Mhere,2001). In addition, SA is classified as a semi-arid country which suffers from freshwater shortage (Hove *et al.*,2019;Jury,2021). Due to water shortage, there has been an increasing use of partial or untreated wastewater for irrigation in agriculture since it is easily accessible and has high nutrient content (Adegoke *et al.*,2018). Irrigation with wastewater can affect the quality of crops produced by farmers, decrease crop yield, and compromise human and livestock health due to unmonitored contaminants that lead to waterborne diseases (Shuval,1991;Keraita *et al.*,2008;Alghobar and Suresha,2016;Elahi *et al.*,2017). Given the global burden of water pollution with ECs that are not well understood, freshwater scarcity and failure of WWTPs to comply with national standards in underdeveloped countries, there is a continuous drive to advance knowledge in monitoring and managing water resources. In this study, we aim to investigate adverse effects due to contaminants found in wastewater effluents, sampled downstream of different factories in SA. Also, we sought to assess remediation efforts such as chlorination at a molecular, cellular and tissue level of zebrafish. The current study is based on the hypothesis that contaminants introduced to localized water systems and methods of water remediation may exhibit toxic effects on the health of living organisms. This can give insight as to how humans, livestock, and the ecosystem can potentially be affected while contributing towards

the advancement of research on water systems. To the best of our knowledge, this study is unique in lethal toxicity of wastewater from localized areas in a country like South Africa in relation to the adjustment of water quality parameters for the neutralization of contaminants.

1.2 Zebrafish (*Danio rerio*) as a model organism

Background

Zebrafish are tropical freshwater fish native to habitats in South East Asia (Spence *et al.*,2008;Kalueff *et al.*,2014). They belong to the minnow family (Cyprinidae) of the Cypriniformes order. Their use as laboratory model organisms was first introduced during the 1960s by a molecular biologist called George Streisinger along with his colleagues at the University of Oregon in the United States of America (USA) (Eisen,2020). Ever since their discovery, zebrafish have gradually risen as an excellent animal model for biomedical and biological research. Studies on zebrafish have gained further momentum over the years due to two major accomplishments which are genome sequencing (Howe *et al.*,2013) as well as the establishment of genome-editing technology (Gaj *et al.*,2016). Furthermore, entries pertaining to zebrafish research that are entered each year on PubMed are illustrated in **Figure 1.1**. The data presented shows that there has been a significant increase in the number of zebrafish publications over the past three decades (1991 to 2021), with a total number of 45,847 entries throughout these years. This includes different article types such as clinical trial articles, meta-analysis, randomized controlled trials, books and documents, reviews and abstracts (NCBI,2021c). The article types mentioned above range from publications in the field of drug discovery (MacRae and Peterson,2015), cardiac toxicology (Heideman *et al.*,2005;Wilkinson *et al.*,2014) understanding diabetic complications (Jörgens *et al.*,2012), genotoxicity (Chakravarthy *et al.*,2014;Albadri *et al.*,2017), haematopoiesis (Jing and Zon,2011;Boatman *et al.*,2013;Gore *et al.*,2018) and neurological developmental disorders amongst other fields (Fontana *et al.*,2018;Pitchai *et al.*,2019;Abugable *et al.*,2019;De Abreu *et al.*,2020). According to results in **Figure 1.1**, from the year 1991 to 1993, the number of publications made per year were low, below 100, which shows that a few fields of research utilized zebrafish as a model organism. Thereafter, the number of publications increased significantly reaching above 600 publications in the 2000s, which is over 5-fold higher than the number of publications made in the early years (1991 to 1993). This indicates that around the beginning of the 2000s zebrafish were gaining a greater recognition in different fields of research. The increase continued reaching to over 1000 publications following the year 2003, which also escalated to a very high number overtime reaching 4020 publications by 2021 (NCBI,2021c). Although there are 1000s of publications made on PubMed each year according to current data, articles that are related to assessing wastewater and water contaminants using zebrafish comprise a ~0.2% (~70 articles) portion on PubMed (NCBI,2021b). Within the portion of 0.2%, only 7 articles have been published and entered onto PubMed regarding the effect of wastewater and water contaminants on zebrafish biological pathways (NCBI,2021a). This stresses the need to bridge the knowledge gap in research concerning the monitoring of water systems.



F**igure 1.1: Number of PubMed publications made on zebrafish per year.** The number of publications made per year on PubMed on zebrafish from 1991-2021. Adapted from literature (NCBI,2021c).

1.3 Studies conducted in South Africa using zebrafish

Zebrafish have been used in an array of scientific research fields in developing countries, including SA. Due to SA being a leading pesticide user in Sub-Saharan Africa, an investigation of the occurrence of pesticides in the surface waters of this country was carried out. Through the use of zebrafish, the sublethal effects of a pesticide called carbaryl were investigated (Mensah *et al.*,2012). Carbaryl (1-naphthyl methylcarbamate) is a broad-spectrum man-made pesticide which is a white crystalline solid that is commonly sold under the brand name Sevin (Krieger,2010). It was first registered in the Unites States as the third most used insecticide for controlling fire ants, aphids, ticks, fleas, spiders, and many other outdoor pests. When insects touch or eat carbaryl, it disrupts their nervous system by continuous stimulation of the nerves and this then results in the inability to contract breathing muscles, ultimately causing death to insect pests (Center,2003;Bond *et al.*,2016).

Although carbaryl is a broad-spectrum pesticide that is used to eradicate insect pests, it may drift into the aquatic systems (Relyea,2005), for such reasons the effect of this pesticide was investigated on the gonad differentiation and embryonic development of zebrafish (Mensah *et al.*,2012). After zebrafish embryos and larvae were exposed to carbaryl, adverse effects occurred. It was observed that the hatching rate in zebrafish embryos was reduced, gonad differentiation became impaired and acute morphological defects developed in newly hatched embryos. In another study conducted in SA, zebrafish larvae were proven as a useful tool to test acute behaviour-based toxicity of carbon- and metal-based nanomaterials. This study was conducted as nanoparticles can be toxic to organisms living in the aquatic systems, the environment, as well as humans (Zhu *et al.*,2006;Brand *et al.*,2020). Zebrafish were found to be highly susceptible to nanomaterials indicating higher acute toxicity for quantum dots (QDs), a metal-based nanomaterial associated with cadmium dissolution toxicity compared with nanodiamonds (NDs) that is a carbon-based nanomaterial. Erratic and hyperactive swimming coupled with a significant increase in locomotor activity was observed in 7 dpf zebrafish larvae exposed to quantum dots QDs, In contrast, behaviour remained substantially unaffected in zebrafish exposed to NDs (Brand *et al.*,2020).

In an attempt to assess the toxicology and dosage of *Sutherlandia frutescens* (*S. frutescens)* two extracts of *S. frutescens* were prepared (one in ethanol whilst the other was produced using water) and applied to developing zebrafish embryos in a previous study (Chen *et al.*,2018). *Sutherlandia frutescens* is one of the most promising medicinal plants indigenous to South Africa that is used in traditional medicine to treat diseases like diabetes, cancer and to boost the immune system (Chadwick *et al.*,2007;Gouws *et al.*,2021).The ethanol and aqueous extracts of this medicinal plant led to bleeding and pericardial cyst formation when applied at high concentrations to the zebrafish embryo culture. Chronic teratogenic toxicities, leading to yolk sac swelling, pericardial edema and other abnormal developmental characteristics, were also detected. The ethanol extracts of *S. frutescens* were more toxic to the larvae than the aqueous extracts, thus, confirming the preference for aqueous preparations of *S. frutescens* in traditional medicine (Chen *et al.*,2018).

1.4 Developmental stages of zebrafish

1.4.1 One cell stage

The zebrafish lifecycle starts with a single cell called the zygote, which forms at time zero of fertilization, with the chorion still intact on top of the membrane. At this initial step, the chorion lifts away and swells up from the fertilized egg. Cytoplasmic movements are also activated by fertilization at this stage, which are easily observed within about 10 minutes. The non-yolky cytoplasm begins to stream toward the animal pole, allowing for the formation of a blastodisc which is segregated from the clearer yolk vegetal cytoplasm (Kimmel *et al.*,1995;Kane,1998) (**Figure 1.2 A**). Commencing at the first mitosis, the non-yolk cytoplasm of the blastodisc duplicates in successive rounds forming blastomeres inside the blastodisc. The so-called blastomeres form from the 2-cell stage (approximately 30 min of development) and eventually reaches the 8-cell stage (**Figure 1.2 B**) and further develop to the 64-cell stage. Throughout this period the embryo is staged based on the number of cells formed.

1.4.2 Blastula period

The term blastula refers to the stage when the blastodisc containing blastomeres becomes ball-like in shape, with 128 cells early in the blastula stage until the onset of gastrulation. At the early blastula stage, cells continue to duplicate after every 15 minutes giving rise to about 1000 cells within 3 hours post-fertilization (hpf) in a synchronous manner as before. In later divisions, there is a loss of global synchrony, since cell cleavages have now become ‘metasynchronous’ due to mitoses not occurring at the same time. Cell cycle lengthening at the beginning of cycle 10 (3 hpf) marks the beginning of the midblastula transition. The midblastula is named after the shape of the embryo during this stage. During this period, the marginal cells undergo a collapse and release their cytoplasm as well as nuclei into the immediate neighbouring cytoplasm of the yolk. Thus arises a yolk syncytial layer (YSL) formed from marginal cells of the blastoderm. The YSL formation is temporarily accompanied by zygotic gene transcription. The late blastula stage begins, at this point both the blastodisc and YSL become thin and spreads on top of the yolk cell (**Figure 1.2 C**). Epiboly begins during this period. During epiboly, the blastoderm and YSL migrate towards the vegetal pole forming an inverted bowl over the yolk. At 50% epiboly stage, the

blastoderm reaches the equatorial region, and deep cell epiboly stops while other movements of gastrulation begin (**Figure 1.2 D**) (Kimmel *et al.*,1995;Bruce,2016).

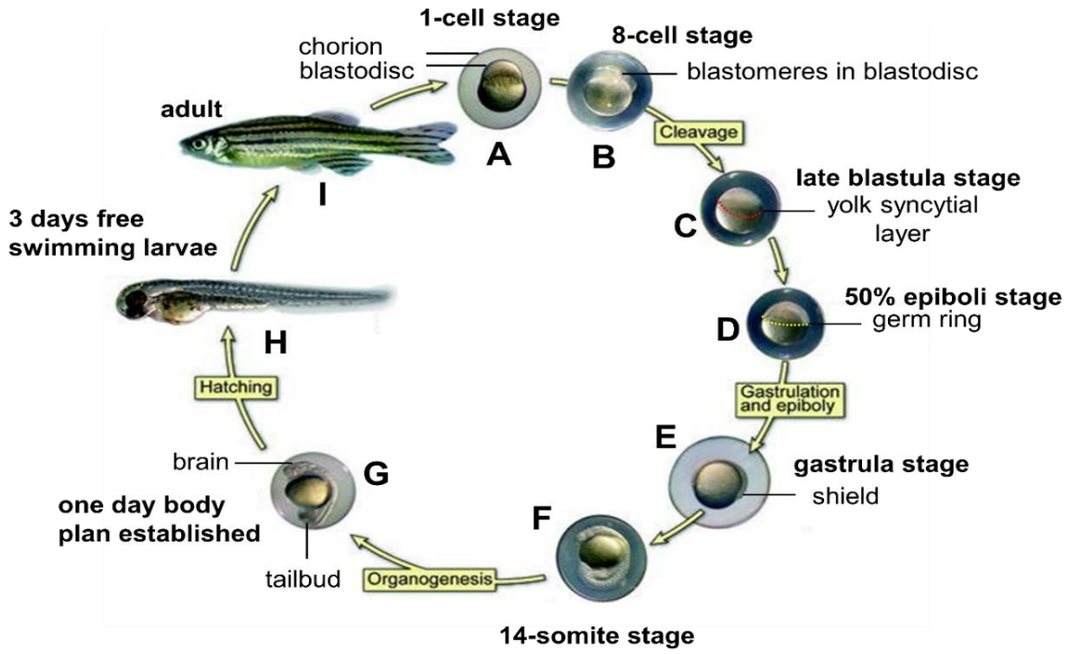


Figure 1.2: Zebrafish developmental from the embryonic to adult stage. The cycle starts at the 1-cell stage (A) with a fertilized egg. The embryos undergo quick development through the 8-cell stage (B), late blastula stage (C), 50 % epiboly (D), and gastrula stage (E) up to the 14-somite stage (F). The complete body plan of the zebrafish embryo becomes established at 24 hpf (G) and all organs are now present. The embryo then hatches outside the chorion and grows into a free-swimming larva by 3 dpf (H). After 3 months larva grows thus entering the adult stage (I). Taken from previous work (Willemsen *et al.*,2011).

1.4.3 Gastrulation and epiboly

Within minutes of reaching 50 % epiboly, a thickened marginal region called the germ ring becomes visible. The germ ring consists of an outer layer (epiblast) and an inner layer (hypoblast). The epiblast forms the embryonic ectoderm and the hypoblast forms the embryonic mesoderm and endoderm. The formation of the germ layers allows for bodily organs and tissues to develop in their respective locations. Each germ layer gives rise to specific tissue types. The gastrula period extends till about 10 hpf. Throughout this time percentage epiboly serves as the staging convention. Simultaneously with the germ-ring formation, convergence movements then give rise to a local proliferation of cells along the germ ring, termed the embryonic shield (**Figure 1.2 E**). Epiboly temporarily pauses during these events and continues after the shield forms. The blastoderm margin advances around the yolk cell thus covering it completely and somites begin to develop sequentially in the tail and trunk providing the most useful staging index (e.g., 14 somite stage at 16 hpf) (**Figure 1.2 F**). Approximately 3 somites develop per hour for the first six, and thereafter 2 per hour (Hanneman and Westerfield,1989;Shabnam *et al.*,2019).

1.4.4 Organogenesis

This long period encompasses the remainder of the first day of development, it is the period when the embryo begins the subdivision of the body plan. In this developmental stage, more somites develop from the anterior (near the head region) and sequentially towards the posterior (close to the tail of the embryo). Eighteen somites have formed at 18 hpf, and a total number of

approximately 30 pairs eventually forms. Among these, 13 are formed posterior to the anus, 10 above the yolk extension and 7 above the yolk cell (Shabnam *et al.*,2019). The embryo elongates, rudiments of primary organs become visible, the tail bud becomes more prominent, and the first body movements occur after the one-day body plan has been established (**Figure 1.2 G**).

1.4.5 Pharyngeal stage

The duration of this period is from 1 to 2 dpf. At this stage, the body axis starts to lengthen and the head lifts dorsally and straightens out. Furthermore, the notochord is well defined. There is the formation of the ventral and dorsal stripe. The nervous system is void at this point and it expands at the anterior. The brain is now made up of 5 different lobes, seven pharyngeal arch's, pectoral fins and the circulatory system. Also, the heart starts to beat, and the associated blood flow can be recognized as it circulates. Involuntary movements continue to occur and tactile sensitivity appears. In addition, melanogenesis begins, until the pigment extends over the entire body of the embryo.

1.4.6 Hatching stage

The hatching stage takes 2 to 3 dpf. In this period, most of the larva hatch outside their chorion (3 days free-swimming larva) and continue developing at the same rate as earlier (**Figure 1.2 H**). At this point the jaws and gills are still developing, however, morphogenesis of many of the organs is now complete and considerably slows down, with a well-defined gut including its associated organs. Pectoral fins have now lengthened with a flat blade. The mouth of the fish is wide open, protruding on the anterior just beyond the eyes. The cleithrum (first visible bone in the zebrafish) appears in the dorsal region. Zebrafish sub-intestinal veins are also prominent, ventrally to the gut. Melanin accumulates in the region overlying the rudiment of the swim bladder, darkening this area.

1.4.7 Early larval stage-adult stage

By day 3, prominent changes occur which includes continued anterior mouth protrusion and swim bladder inflation. Early larva starts to swim more actively while moving their pectoral fins, jaws, eyes as well as opercular flaps. Later on, the zebrafish enters the juvenile stage, with a full-body length, fins, and pigment. After 90 dpf the zebrafish reaches the adult stage, becomes fertile, and is now able to search for food (**Figure 1.2 I**) (Kimmel *et al.*,1995).

Table 1.1: Advantages and disadvantages of using zebrafish as a research model

Advantages	Disadvantages
1. Short generation time - Approximately 2 - 4 months and small size of embryos that allows for high throughput screening.	***1. Monitored daily*** - Zebrafish are fed on a daily basis, they require aged water and their tanks should always be clean and monitored, which can be time consuming.
2. Low housing cost - This allows for easy maintenance.	***2. Unpredictable reproductive cycle*** - Zebrafish can delay with embryo production, depending on their living conditions and age.
3. Year-round spawning - Spawning during the year enables continuous research.	***3. Genome duplication*** - Genome duplication, numerous genes are present as duplicate copies in zebrafish, which makes it challenging to determine functional roles since more than one copy encodes similar proteins such as transporters and receptors with similar biochemistry and pharmacological properties.
4. High fecundity - They produce about 300 - 600 embryos per female at a time.	***4. Behaviour*** - Have a limited behavioural repertoire and complex behavioural patterns mature over time, such as social behaviours (Kalueff *et al.*,2014).
5. Optical transparency - Allowing for imaging of internal organs during developmental stages.	***5. Environmental conditions*** - Greatly influenced by their environment and requires many environmental variables (e.g., temperature, lighting, population density, water quality, and nutrition must be kept optimal for accurate data interpretation.
6. External fertilization - Embryos can be accessed non-invasively.	***6. Body size*** - The small size makes functional studies of zebrafish hearts challenging (Huo *et al.*,2015).
7. Minimal parental care - Zebrafish are independent immediately after hatching. They do not rely on their parents for nutrition and learning basic survival skills.	
8. Similar to mammals - Share a high genetic similarity with mammals (e.g., humans) which allows for common molecular pathways to be studied.	
9. Permeability of embryos	

- The early embryo is permeable to test compounds and this makes zebrafish embryos suitable for drug testing (Ali *et al.*,2011).	

1.5 Zebrafish vs mouse as a model organism

In the early days of biomedical research, researchers developed mouse models to provide insight into the underlying mechanisms of many diseases, determining drug efficacy, and to predict the health responses of patients (Justice and Dhillon,2016). As years went by, newer studies have shown tremendous attention towards zebrafish for studying human pathology, drug screening, and other experimental procedures (Goldsmith and Jobin,2012;de Abreu and Kalueff,2021;Choi *et al.*,2021). The attributes of zebrafish in comparison to mice are shown in **Table 1.2** to highlight the reasons for the sudden replacement of mice by zebrafish in the research field. According to comparisons made in **Table 1.2**, zebrafish are preferred due to that they require a low space for housing and low costs of maintenance as opposed to mice. Zebrafish can be maintained in the same space in larger populations compared to mice since they have a smaller body size. During maintenance, they are fed about 4 % of their body weight 2 to 3 times a day and from one month onwards feeding them only once per day has no negative effects on their growth, health and reproduction (Teame *et al.*,2019;Aleström *et al.*,2020). In contrast, mice consume about 10 % of their body weight 15 to 20 times a day (Weiskirchen *et al.*,2020), which can be more costly and time-consuming. Once they reach their reproductive stage, zebrafish produce a large number of embryos that are fertilized externally with a life span of 3 years compared to mice. The production of many embryos makes them especially useful for high-throughput screening, meaning the duration of experiments and the quantity of the tested sample is reduced, the technical expertise required for its evaluation is less intensive than that of an equivalent study performed in mice. In addition, external fertilization and transparency of their embryos make them suitable for microscopy-based screens and fluorescence imaging from the onset of fertilization without carrying out any invasive procedures. In addition, the number of orthologous genes they possess per human gene can be more than one, with protein coding that are about 26 000. Similarly to mice, their genome has been fully sequenced and it is amenable to gene modification. This provides information that can be of medical value. Genetic mutations and variants that can lead to diseases or increase the risk of disease development can be identified.

Table 1.2: Attributes of the zebrafish as a research model organism in contrast to mice

Attribute	Zebrafish	Mouse
		
1) Husbandry:		
Space for housing	Low[a]	High[a]
Cost of maintenance	Low[b]	High[b]
Number of offspring per mating pair	50-300[c]	2-12[c]
Life span	3 years[c]	2 years[c]
Route of fertilization	External[c]	Internal[c]
Nature	Translucent at early stages[a]	Non-translucent[a]
Amenability to in vivo fluorescence imaging	High[c]	Low[c]
2) Genome:		
Number of orthologous genes per human gene	Often, more than one[c]	Usually one[c]
Number of protein coding genes	~26,000[c]	~23,000[c]
High-quality reference genome sequence available?	Yes[c]	Yes[c]
Amenable to targeted gene modification?	Yes[c]	Yes[c]

[a](Ackerman and Monk,2016) [b](Veinotte *et al.*,2014) [c](Kwon *et al.*,2019)

1.6 Studies that investigate water quality / contamination in South Africa

The South African constitution states that people have a right to a constant supply of safe and clean drinking water. To fulfill this right, it is essential to continuously monitor water at different stages of the water supply system (rivers, dams, and water cleaning facilities). Various studies have been conducted in SA for this purpose. The presence of bacterial pollutants at different sites in the Diep and Plankenburg rivers of the Western Cape were investigated and compared (Alegbeleye *et al.*,2016). In this study, nineteen isolates of bacteria were isolated from the surface water and sediment samples at the end of the survey. These were strains belonging to the genera *Raoultella, Bacillus, Pseudomonas, Klebsiella, Escherichia, Enterobacter, Exiguobacterium, Acinetobacter, Serratia, Aeromonas, Staphylococcus* and *Citrobacter*. Bacterial counts detected for both rivers were above 5 cfu / 100 mL, which is the maximum recommended total coliform limit for no risk according to the South African Department of Water Affairs and Forestry (DWAF,1996). Higher microbial load was obtained from sediment samples compared to surface water samples. Seasonal variation was also observed in terms of microbial counts, higher microbial counts were obtained during summer sampling time compared to winter sampling time. Members of the genera *Raoultella, Bacillus, Pseudomonas, Escherichia, Acinetobacter, Serratia, Aeromonas, Staphylococcus, Exiguobacterium, Citrobacter, Klebsiella* and *Enterobacter* were detected along the Plankenburg River while microorganisms belonging to genera *Escherichia, Bacillus, Pseudomonas, Acinetobacter, Aeromonas, Serratia,* and *Enterobacter* were isolated from the Diep River.

Amongst the detected species were harmful bacteria such as *Raoultella, Pseudomonas, Enterobacter, Serratia, Staphylococcus*, and *Citrobacter. Raoutella* is commonly found in the environment, particularly in water, soil, and fish. It has been found to cause a variety of infections, such as necrotizing fasciitis, cystitis, cholecystitis, pancreatitis, hepatic disease, and soft tissue infections (Kalaria *et al.*,2017). *Pseudomonas* can lead to urinary tract infection, blood stream infection, pneumonia, pharyngitis, bone and skin infections, ear, eye, and central nervous system infections. In addition, they colonize lungs of cystic fibrosis (CF) patients and contribute to chronic progressive pulmonary diseases and the high death rate in these patients (Golemi-Kotra,2008). On the contrary, *Enterobacter* species are responsible for causing many nosocomial infections, and less commonly community-acquired infections, including urinary tract infections (UTI), respiratory infections, soft tissue infections, osteomyelitis, and endocarditis, among many others (Ramirez and Giron,2020). *Serratia* can lead to pneumonia, urinary tract infection, bacteraemia, biliary tract infection, wound infection, meningitis, and endocarditis (Katib *et al.*,2020;Gadhiya *et al.*,2021). *Staphylococcus aureus* is a virulent pathogen that can cause a wide variety of diseases, ranging from moderately severe skin infections to fatal pneumonia and sepsis (Archer,1998;Cheung *et al.*,2021). *Citrobacter spp.* are opportunistic pathogens in humans that can lead to invasive disease, including infections of the urinary tract, respiratory tract, central nervous system, skin, and soft tissue (Archer,1998;Cheung *et al.*,2021). Due to the high microbial burden of both pathogenic and non-pathogenic bacteria in these rivers, it is evident that more advanced water quality models and techniques need to be established for comparison when

assessing microbial pollution and mechanisms of microbial transport, in both surrounding areas and within the river systems (Alegbeleye *et al.*,2016). In addition to microbes, organochlorine pesticides (OCPs) are other contaminants that affect water quality in SA, thus posing threats to the health of humans and the ecosystem (Zhao *et al.*,2013;Taiwo,2019). As a result, water systems need to be monitored for the presence of OCPs. To investigate the impact of OCPs on water systems, samples were collected in the Olifants River in a previous study (Moja *et al.*,2017). After purification of OCP extracts, gas chromatographic-mass spectrophotometry (GC/MS) was used for analysis. It was observed that the water found in the river catchment area were notably above the World Health Organization (WHO) drinking water quality maximum limits due to the OCPs detected. As a result, local authorities were urged to strengthen the evaluation and penalties against polluters of rivers (Moja *et al.*,2017).

The increasing amount of pharmaceutical products in the aquatic systems found in SA is another concern since they can potentially cause adverse effects on aquatic organisms. Furthermore, SA is the largest consumer of antiretroviral (ARV) drugs worldwide (Venter *et al.*,2017). For this reason, the potential impact of ARVs such as nevirapine (NVP) in SA surface water was assessed on the growth of Mozambique tilapia. After a chronic exposure at the laboratory, it was found that NVP detected in SA waters, did not detrimentally affect the early juvenile growth of the tilapia fish being tested (Nibamureke *et al.*,2019). However, further investigation is still needed to test NVP on all life stages of fish (such as zebrafish) to safeguard water systems. In SA, dams are one of the water resources we rely on for our water supply. Therefore, it is vital to maintain the quality and safety of water in these dams. To assess the quality of water in one of the dams in SA (Vaalkop dam) the pH, dissolved solids, conductivity, colour and turbidity, *Escherichia* (*E. coli)*, total coliforms, alkalinity, hardness, chlorophyll, precipitation potential, and organic carbon were monitored. All of these parameters were within acceptable limits except for conductivity and coliform counts (Nyende-Byakika,2018). Other studies that have been conducted in SA to monitor water quality involve the use of water samples collected from wastewater treatment facilities. In one of these studies, the magnitude of activation of immune/inflammatory cells (derived from the blood of healthy adult human volunteers, n=3) was determined post-exposure to water samples through monitoring the synthesis of pro-inflammatory cytokines. This technique gave an insight into pathogens' presence in the water, which can lead to the production of cytokines (Adebayo *et al.*,2014).

1.7 Deaths caused by unsafe water

Freshwater scarcity is a major problem worldwide. Approximately one-third of the drinking water is obtained from natural sources like dams, canals, rivers, and lakes (Jonnalagadda and Mhere,2001). Given the shortage of water issue in SA, contamination of available water systems is of a huge concern and it threatens the sustainability of the current water supply. According to the WHO, about 829 000 people die each year due to diarrhoea as a result of unsafe sanitation, hand hygiene, and drinking water (WHO,2019). Amongst deaths caused by different risk factors in Africa, air pollution followed by unsafe water contributes largely towards these deaths. There are over 712 479 as well as 542 857 deaths caused by air pollution and unsafe water respectively.

Contrary to air pollution and unsafe water, approximately 391 657 and 275 813 deaths are associated with unsafe sanitation and childhood malnutrition (Yiu,2019).

1.8 Contaminants of emerging concern (CECs)

The term contaminants of emerging concern (CECs) describe contaminants that have been detected in water bodies. These contaminants are usually not monitored but can cause human health or ecological problems. In addition, they are not regulated under current environmental laws. They are classified as “emerging” due to that they are newly identified chemicals that are naturally occurring or manufactured and are detected in different matrices at low levels (Sauvé and Desrosiers,2014;Nawaz and Sengupta,2019). Within CECs, various chemical and biological compounds, such as pharmaceuticals, hormones, and personal care products (PCPs) are included (Vasilachi *et al.*,2021).

1.8.1 Potential sources of contaminants of emerging concern

Contaminants of emerging concern can enter water systems after being discarded as waste through surface run-off, effluent discharge, or infiltration and seepage into the water table. As a result, these contaminants enter the public water supply system. They are released from urban, industrial, agricultural, and other anthropogenic activities (Abdulrazaq *et al.*,2020). Below are different sources and activities that contribute towards the disposal of CECs in the water. Industrial activities, domestic household, aquaculture, animal farming and agricultural services can lead to the spillage of CECs into water systems directly or indirectly according to illustrations in **Figure 1.3**.

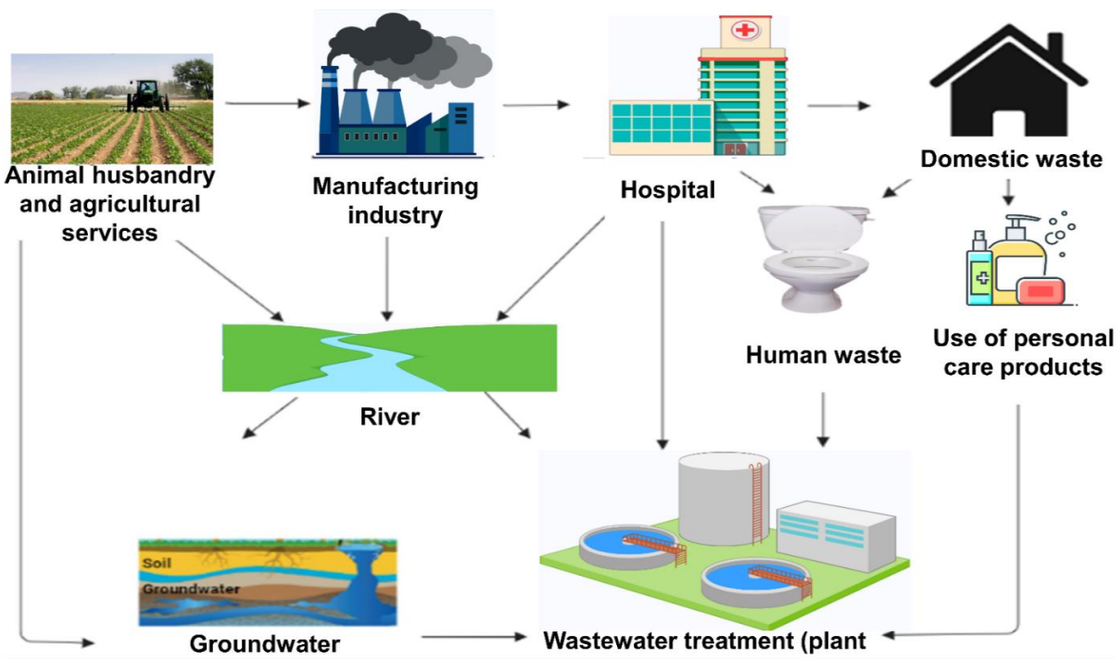


Figure 1.3: Origins of emerging pollutants and their routes in the environment. Sources and pathways for emerging contaminants to reach various receptors. Emerging pollutants can be discharged either directly, as sewage, drinking water, effluents and manure as well as surface run offs (prepared by author).

1.8.2 Prevalence of contaminants of emerging concern in South Africa

Figure 1.4 presents the prevalence of emerging contaminants (ECs) found in different water systems that are situated around SA (Wood *et al.*,2016;Rimayi *et al.*,2018a;Gani *et al.*,2021). There is a high number of contaminants in the East followed by the North zone as compared to other zones. In the East zone, the following compounds were more prevalent in this order: Aspirin (44243 ng/L)> Fluoroquinolones (27100 ng/L) >Atenolol (25900 ng/L) >Nalidixic acid (25234 ng/L) >Ciprofloxacin (20514 ng/L). Most of these compounds are reported from the Msunduzi River in Pietermaritzburg except for Atenolol, which was found high in Umgeni River, Durban. The high prevalence of these compounds in the Msunduzi river is highly associated with the location of the Msunduzi river. This river passes through the city and is therefore greatly impacted negatively by human activities which involve the disposal of pharmaceuticals in water systems (Agunbiade and Moodley,2014;Matongo *et al.*,2015).

In the North, the Hartbeespoort dam (situated within small towns) and Roodeplaat dam (located within the Roodeplaat Nature Reserve) was evaluated for the presence of contaminants (Marchand *et al.*,2012;Rimayi *et al.*,2018b;Rimayi *et al.*,2018a;Gani *et al.*,2020). These dams supply water for irrigation schemes, households (via municipalities), industrial activities and recreation, which increases the chances of contamination. Compounds that were highly detected along the north region in the Hartbeespoort and Roodeplaat dam include herbicides and ARVs in the following order: Terbuthylazine (1969 ng/L) > Nevirapine (1480 ng/L) > Atrazine (1237 ng/L) > Zidovudine (973 ng/L) > Stavudine (778 ng/L) (Rimayi *et al.*,2018a;Gani *et al.*,2020). According to literature, there are ARVs used in Highly Active Antiretroviral Therapy (HAART) such as nevirapine and zidovudine which are not effectively removed in wastewater treatment plants WWTPs in SA (Prasse *et al.*,2010). In addition, SA has been reported as the largest ARV consumer in the world (Venter *et al.*,2017). This explains the reason behind the increased concentrations of these compounds in surface waters found in provinces across SA.

Caffeine (158 ng/L) and the antiretroviral drug zidovudine (51.7 ng/L) were the only ECs detected along the Southern region. Although caffeine is a naturally occurring compound that is widely used as a stimulant in medicine and a flavour enhancer in beverages and snacks, the low detection reported in **Figure 1.4** indicates that there is not much spillage of caffeine onto the water systems found across SA (Gani *et al.*,2020;Völker *et al.*,2020). Similarly to caffeine, zidovudine had a low maximum concentration. This can be due to that in most provinces, the administration of zidovudine has been stopped in patients, since this drug can have severe side effects (Tadini *et al.*,1991;Demir and Laywell,2015;Edwards *et al.*,2021
). No data was found in literature with regards to ECs present in rivers located in the West zone, possibly due to that several tributaries (the Harts River, the Riet River, the Modder River, the Seekoei River) to the Vaal River that flows within the West zone, has not been investigated for ECs (Gani *et al.*,2020). However, in a recent study, diclofenac was the most dominant compound detected, with the highest concentration than the other pharmaceutical compounds that were investigated in edible fish from the Kalk Bay harbour in Cape Town along the West zone (Ojemaye and Petrik,2019;Ojemaye and Petrik,2021). This indicates that some level of ECs are present in

the water systems located in the West region, regardless of the knowledge gap concerning EC pollution in surface water sources along the West.

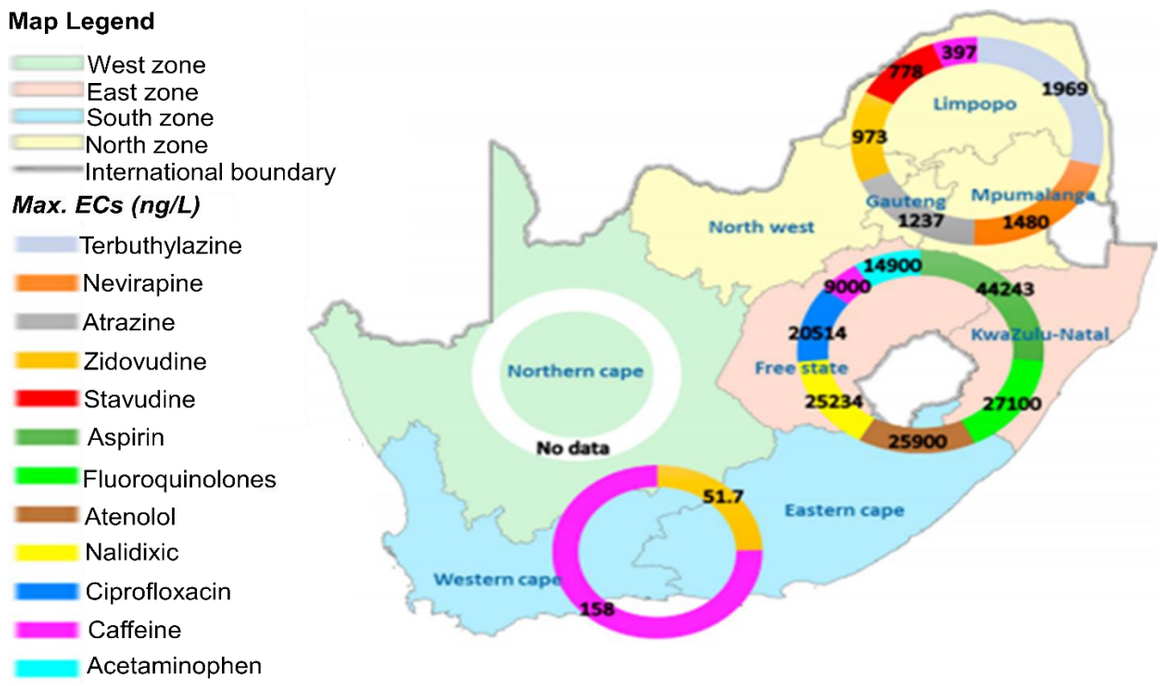


Figure 1.4: Map of different emerging contaminants in the freshwater of South Africa. Compounds of high concentration from the selected areas are indicated in their respective areas. Adapted from a previous study (Gani *et al.*,2020).

1.8.3. Examples of contaminants of emerging concern

a) Endocrine-disrupting chemicals (EDCs)

Endocrine-disrupting chemicals such as 17β-estradiol, estrone, estriol, and natural estrogens are ECs that are consistently discharged directly into disposal sludges, surface waters that have WWTP effluents, and in storm water runoff (Nazari and Suja,2016). The main concern about these chemicals is that among ECs, these compounds can result in adverse effects on human and animal health, directly affecting the endocrine system. According to literature, EDCs may be unsafe even at low doses (below ng/L) (Dorne *et al.*,2007;Brausch and Rand,2011;Loos *et al.*,2013). Low concentrations of EDCs could add to the endogenous hormone concentration in an organism's body, producing an effect that is much greater than would be predicted based on its ability to bind to the receptor in isolated systems (Gore *et al.*,2015). Endocrine disrupting chemicals are capable of blocking or imitating natural hormones that are responsible for the well-functioning of some organs (Vieira *et al.*,2020). The different ways in which EDCs affect the endocrine system inside the body are illustrated in **Figure 1.5**. Under normal circumstances hormones bind onto receptors resulting in the activation of a signal transduction mechanism that ultimately leads to a cellular response (**Figure 1.5 A**). Upon exposure, endocrine disruptors can mimic or partly mimic naturally occurring hormones in the body like estrogens (the female sex hormone), androgens (the male sex hormone), thus, potentially leading to overstimulation (**Figure 1.5 B**). In addition, EDCs can bind receptors within a cell and block the endogenous hormone from binding. The normal signal then fails, which then prevents the production of a cellular response (**Figure 1.5 C**) (Okoro *et al.*,2017).

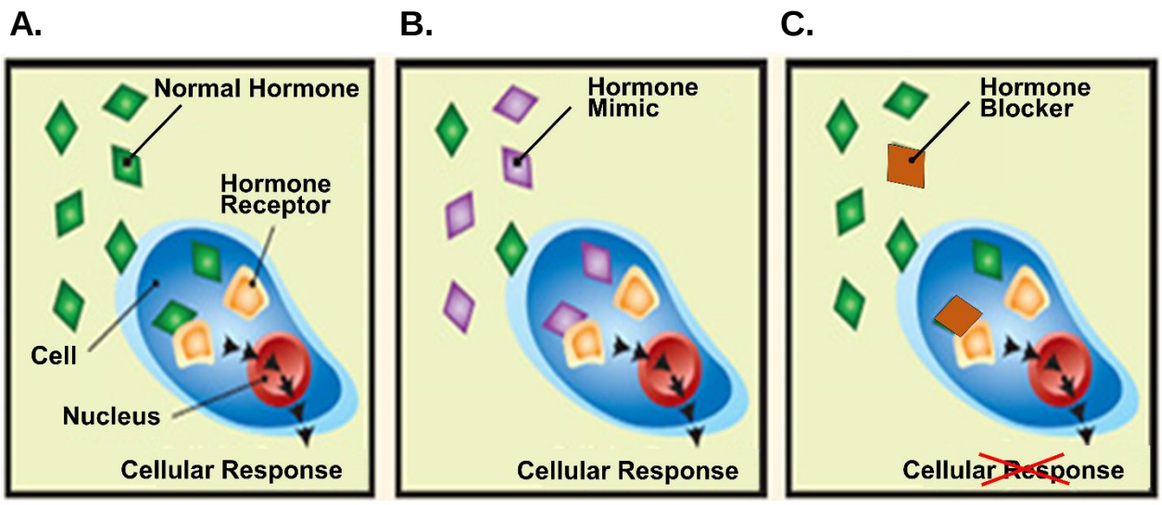


Figure 1.5: The effect of endocrine disrupting chemicals on the endocrine system. Endocrine system under normal conditions with no disruptors (**A**), endocrine disruptors mimics (**B**) or blocks (**C**) endogenous hormones. Normal hormone (green), hormone receptor (yellow), nucleus inside the cell (red), hormone mimic (purple), and hormone blocker (brown). Adapted from a previous study (Okoro *et al.*,2017).

b) Pharmaceuticals

Pharmaceuticals are a milestone in the development of human science. They have improved the quality of life, prolonged people's life spans, and cured many deadly diseases. However, this accomplishment of pharmaceuticals has now led to their emergence as rapidly spreading environmental pollutants. Pharmaceuticals can have negative and off-target effects on humans and aquatic organisms when deposited in aquatic systems. In addition, the efficiency of remediation can be <10% in the presence of pharmaceuticals such as atenolol, carbamazepine, acetylsalicylic acid, diclofenac, propranolol, mefenamic acid, clofibric acid atenolol, and lincomycin (Tauber,2003;Metcalfe *et al.*,2004;Richardson and Ternes,2018).

Several factors including physicochemical properties (e.g., hydrophobicity and biodegradability) of the targeted compounds, as well as operating conditions of WWTPs processes, can affect the removal efficiency of pharmaceuticals in wastewater. Highly resilient chemicals can be a challenge to remove effectively by WWTPs (Snyder,2008;Moslah *et al.*,2018). Antiretrovirals are one of the emerging class of pharmaceuticals, meaning, over time new ARV drugs that combine excellent potency with greater tolerability, convenience, and safety are being developed globally. This emphasizes how ARVs can become more of a threat to the health of the ecosystem if these drugs are continuously handled and disposed poorly.

c) Personal care products (PCPs)

According to previous studies, over the years there has been an increasing awareness about the unintentional presence of PCPs in the aquatic environment at concentrations that are capable of having toxicity effects to the aquatic organisms (Nikolaou *et al.*,2007;Kanama *et al.*,2018;Mhuka *et al.*,2020). Personal care products form a diverse class of common household products used for health, hygiene and beauty purposes. Examples of these products includes disinfectants, detergents, insect repellents, preservatives, ultra-violet (UV) filters, cosmetics and dental care products (Boxall *et al.*,2012;Montes-Grajales *et al.*,2017). Many personal care products and their metabolites are biologically active, meaning they can impact non-target aquatic organisms even though they are found at relatively low concentrations (Mimeault *et al.*,2005;Franzellitti *et*

al.,2013;Ford and Fong,2016). Human-use PCPs are generally excreted and emitted into the sewerage system following usage. In addition, water bodies are contaminated by PCPs mainly through the sewage effluents from wastewater treatment plants due to their incomplete or inefficient removal, when sewage effluent is used for irrigation or where sewage sludge is applied as a fertilizer to agricultural land (Mema,2010;Boxall *et al.*,2012;Blair *et al.*,2013).

One of the most widely used PCPs that poses a threat in water systems are pesticides. Pesticides detected in water were found to be above the maximum standards regulated by the European Health-Based Chemical Standards, which specifies 0.1 µg/L as an acceptable limit for any pesticide and 0.5 µg/l for total pesticides in water systems (Dalvie *et al.*,2003;Dolan *et al.*,2013;Elfikrie *et al.*,2020;Campanale *et al.*,2021). This was reported in different studies conducted in SA, Mexico and the United States (US) (Machete and Shadung,2019;Silva-Madera *et al.*,2021;Stackpoole *et al.*,2021), providing evidence that constant monitoring of water systems is of high importance. Additionally to the presence of pesticides in water systems, in literature acute exposure to pesticides is associated with dizziness, headache, skin allergies, burning of eyes, blurred vision, and swelling of body and muscle cramps (Kafle *et al.*,2021). While chronic exposure can potentially lead to leukaemia, neurological disorders , and cancer in both human and animal carcinogens (Alavanja *et al.*,2004;Parrón *et al.*,2011;Kumar *et al.*,2014;Hu *et al.*,2017;Portier,2020;Calaf *et al.*,2021;Karalexi *et al.*,2021). This highlights that non-monitored water systems are of a serious threat to the health of living organisms.

1.9. Wastewater treatment

1.9.1 Wastewater treatment plant processes

a) Preliminary treatment

Wastewater treatment plants play an important role in the water cycle and in pollutant removal through preliminary, primary, secondary, and tertiary treatments (**Figure 1.6**). In the first stage, coarse and fine solid material is removed mechanically. The wastewater passes through screens, which trap pieces of wood, rags, wire, etc. The extracted material is usually buried, but it may be burned (**Figure 1.6 A**).

b) Primary treatment

During primary treatment, sewage flows slowly through grit tanks, where particles of sand or grit settle out (sedimentation) allowing liquid to leave the primary sedimentation tanks (**Figure 1.6 B**). However, this liquid still contains fine solids and dissolved matter, so secondary treatment required.

c) Secondary treatment

Secondary treatment is used to convert dissolved and suspended pollutants into a form that can be removed, producing a relatively highly treated effluent. The liquid from primary treatment is poured over beds of broken stones, gravel, coke or plastic, which provide a large surface area for oxidation. Micro-organisms (mainly bacteria) living within the filter bed break down the organic matter in the liquid during the activated sludge processes, thus producing large particles that settle at the bottom of the aeration tanks by sedimentation **(Figure 1.6 C**).

d) Tertiary treatment

At this point, the quality of the water is improved further before it is released as effluent discharge to lakes, rivers, seas or other places. This treatment involves filtering the water for the removal of any inorganic substances such as nitrogen, phosphorus including bacteria or viruses that could be harmful to humans. Various types of tertiary treatment exist, e.g., nutrient stripping, disinfection by UV light or filter membranes (**Figure 1.6 D**) (Englande *et al.*,2015;Kesari *et al.*,2021).

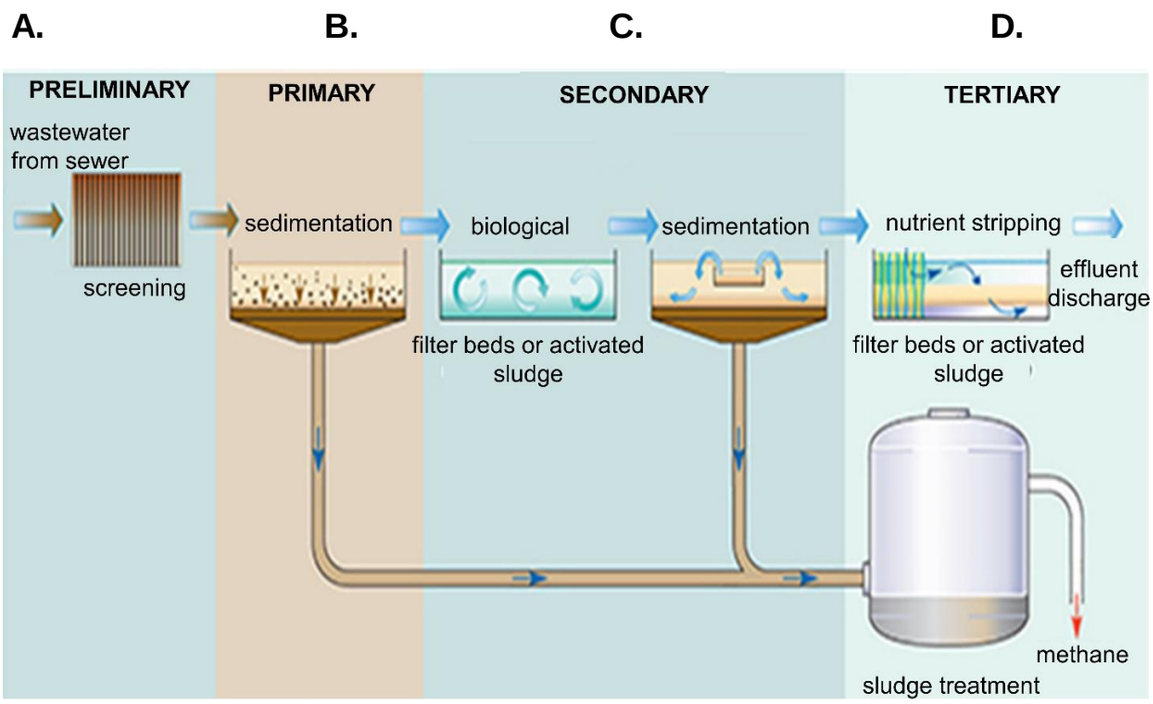


Figure 1.6: Flow diagram of the three main stages of a wastewater treatment plant (WWTP). Sewage passes through (**A**) preliminary, (**B**) primary, (**C**) secondary, and (**D**) tertiary treatment. Taken from literature (OpenLearn,2020).

1.9.2 The quality of effluent from wastewater treatment plants in South Africa

Figure 1.7 illustrates the national Department of Water and Sanitation (DWS) map showing compliance of WWTPs to minimum wastewater treatment standards in SA (DWS,2022). According to the DWS, more than 50 % of all SA's sewage treatment plants are failing as they do not fully comply with treatment standards, which is prominently marked by the pink regions. This indicates that numerous WWTPs spew billions of litres of sewage that are not properly treated or entirely untreated. The regulator under the DWS determined that 334 (39 %) of systems were identified to be at a critical score level. This compared to the 248 (29 %) of the systems in 2013 indicates that there has been a regress in the state of the wastewater systems. This decline is at both the treatment and sewer collection levels. The Green Drop audit process established that water services institutions with low levels of investment in infrastructure, and low capacity in respect of skilled personnel, were more likely to have wastewater systems in a critical state. In addition, according to the DWS 2022 report, lower performing municipalities generally have lower technical skills ratios, with several shortfalls highlighted. The most prominent risks were observed at treatment level, and pointed to works that exceeded their design capacity, dysfunctional processes, and equipment (especially disinfection), lack of flow monitoring, and effluent and

sludge non-compliance. The DWS is hopeful that the 2021 audits will set a baseline from where a positive trajectory for wastewater services and improved performance will follow. The average score across all provinces was 49.9 %, indicating more than half our raw sewage and industrial waste is not being treated to standards. Half of the 850 municipal treatment facilities in the report are failing and 334 (39 %) are in a critical state. Upon perception of the compliance scores reported in **Figure 1.7**, SA's wastewater systems are deteriorating over time.

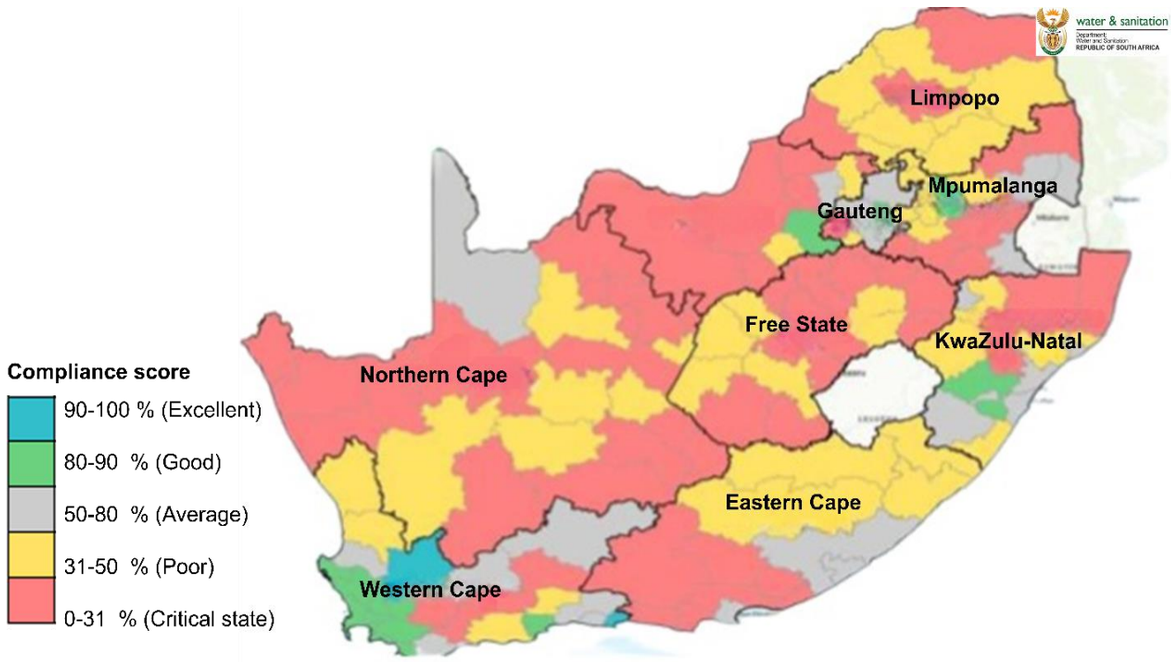


Figure 1.7: The national Department of Water and Sanitation (DWS) map depicting wastewater treatment plant (WWTP) scores of compliance to the national water treatment standards across South Africa. Weighted scores calculated by the Green Drop Wastewater Services Audit based on three indicators (amongst other factors) of effluent quality detected in WWTPs. These indicators are microbiological, chemical and physical state of the water. Compliance score percentages are assigned according to the number of standards the WWTP effluent meets on all three indicators and related factors, which are either excellent compliance (blue): 90-100 %, good compliance: 80-90 %, average compliance: 50% – 80 %, poor compliance: 31-50 % and critical sate where WWTPs meets 0-31 % of the standards pertaining to the three indicators and related factors, implying that it is on a critical state. Scores of 0-31 % also represent areas where there is no monitoring data submitted to the DWS. Adapted from literature (DWS,2022).

1.9.3 Wastewater treatment plants in a critical state in SA and way forward reported by the DWS

Upon review of the state of wastewater systems by the DWS on a province scale, compliance scores are as illustrated in **Figure 1.8** (DWS,2022). Limpopo has the highest number of its systems that are in a critical state, reported as 78 %, followed by the Northern Cape with about 76 % failing WWTPs, North West scored 69 %, Free State placed at 67 %, Mpumalanga with 43 %, Eastern Cape at 39 %, Gauteng sitting at 15 %, KwaZulu-Natal at 14 %, and Western Cape with a score of 11 %. According to the DWS, wastewater management is of concern across SA, as indicated by the above-mentioned scores highlighting the dismal state of wastewater management in the country. This poses a risk to both the environment and public health and calls for interventions.

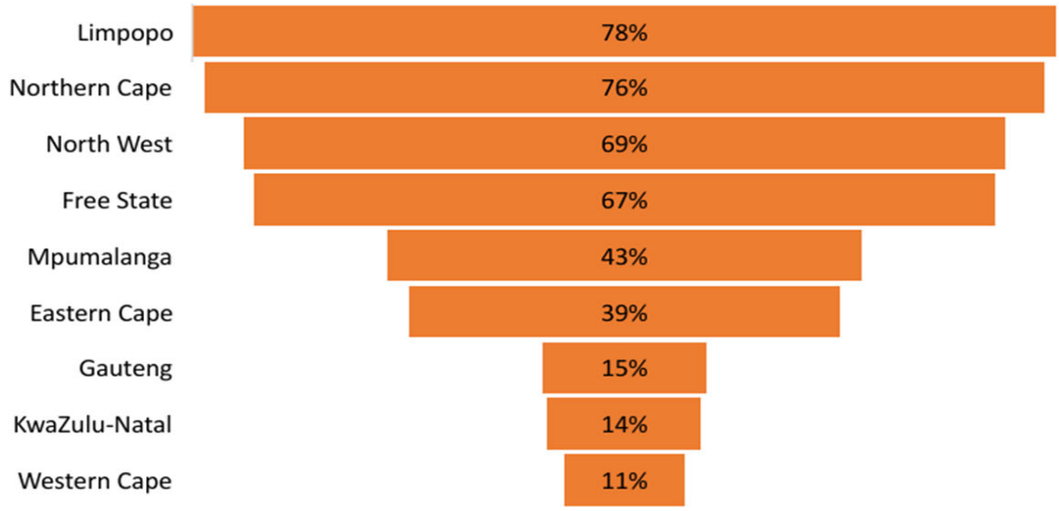


Figure 1.8: A funnel chart of WWTPs that are in a critical state in South Africa. Each bar represents a province along with the average compliance score of WWTPs in the respective province. The scores were allocated as per the DWS standards set for a water cleaning facility. The average score for each province represents WWTPs with excellent compliance (blue): 90-100 %, good compliance: 80-90 %, average compliance: 50% – 80 %, poor compliance: 31-50 % and those in a critical sate or where no data was submitted: 0-31 %. Adapted from the DWS Green Drop National report for 2022 (DWS,2022).

1.9.4 Way forward to improve WWTPs that are in a critical state in South Africa

According to the latest Green Drop Certification report (DWS,2022), it seems that infrastructure and skills shortages are at the heart of the problem. This means the auditors identified a trend, therefore, it follows that taking care of the skills shortage and infrastructure and asset neglect (such as pumps and motors), will not only improve Green Drops scores, but will do much to protect the health and wellbeing of the communities these municipalities serve. The development of a Water Services Improvement Programme will also be made a priority according to the DWS. In addition, to improve the conditions in WWTPs, the national government will ensure that grant funding allocated to the water sector will be allocated with the objective of restoring functionality of existing wastewater infrastructure according to the findings of the 2022 DWS report.

1.10 Analytical procedure to monitor adverse effects caused by water contaminants

The zebrafish embryotoxicity (ZET) test

The ZET test is a cheap, medium-throughput approach that allows for continuous monitoring of acute toxicity of chemicals on the embryonic developmental stages of zebrafish (OECD,2013). During the ZET test, fertilized eggs of zebrafish are exposed to a treatment sample for 96 h. Subsequently, lethality endpoints are classified in two ways to detect toxicity effects on zebrafish embryo development in comparison to a standard (negative control). Firstly, lethality is evaluated based on observations made every 24 h under the microscope (World Precision Instruments, Japan). These observations include coagulation of fertilized eggs, lack of tail detachment from the yolk sac, lack of somite formation, and absence of heartbeat. After the exposure time, acute toxicity is measured based on the outcomes in any of the four observations recorded, followed by the calculation of the LC_{50} according to the Organisation for Economic Co-operation and Development: OECD 236 (2013) guidelines (OECD,2013). Secondly, teratogenicity is assessed based on unusual eye development, lack of movement, edema, and lack of pigmentation (Chahardehi *et al.*,2020). In the ZET test, acute toxicity is monitored from the one-cell stage (0 hpf) until the pharyngeal stage (1 dpf) (Willemsen *et al.*,2011).

- *Indicators of lethality for the zebrafish embryotoxicity test*

Developmental alterations observed during the ZET test as stated in the OECD guidelines are summarised in **Figure 1.9** (OECD,2013). Gastrulation arrest which is often considered as a precursor of coagulation is shown (**Figure 1.9 A**). This is a state of an embryo where it is unable to change from its blastula stage with a single layer of cells to a gastrula stage containing multiple layers of cells. It may also be observed in embryos that are severely delaying to develop and thereafter be recorded as a sublethal endpoint (von Hellfeld *et al.*,2020). Coagulation can be observed at an early stage of embryonic development at 24 hpf (**Figure 1.9 B**) and only rarely, in later developmental stages (**Figure 1.9 C**). Lack of tail detachment is shown in increasing severity (**Figure 1.9 D-F**), where it occurs in various degrees which includes the development of a short tail than usual (**Figure 1.9 D**), an extremely short tail (**Figure 1.9 E**), and in a case where there is no tail development (**Figure 1.9 F**). The 24 h old embryo in presented in **Figure 1.9 G** and **Figure 1.9 I** depicts a zebrafish embryo that does not show any somite formation which becomes evident from 24 hpf. Panel H indicates the absence of somite due to deformed muscles (dM) that are situated behind the yolk sac (YS). Lack of heartbeat can be observed under the microscope (World Precision Instruments, Japan) by the non-convulsion of the heart that is situated below the mouth as illustrated in J (von Hellfeld *et al.*,2020).

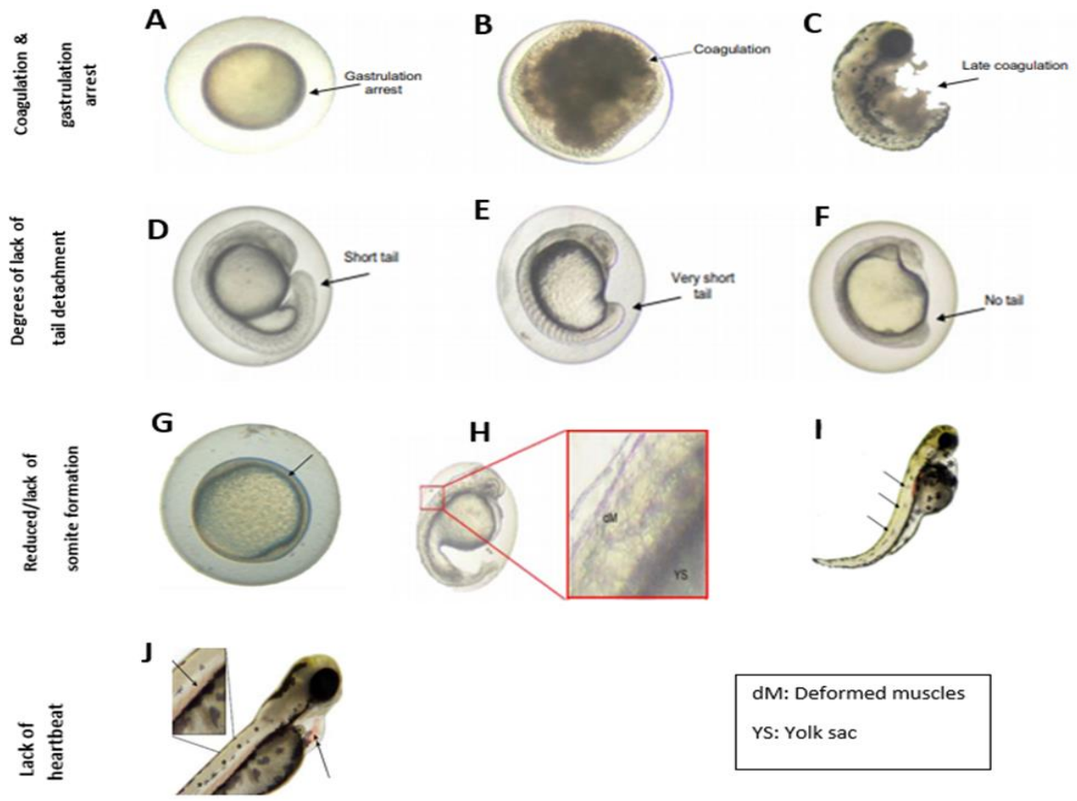


Figure 1.9: Overview of the four core lethality endpoints. The first row shows gastrulation arrest (A), coagulation of a fertilized zebrafish embryo (B) and late coagulation-coagulation at a later developmental stage (C). Second row depicts slightly reduced tail detachment of the zebrafish embryo (D), a barely detached tail (E) and complete non-detachment of the tail (F). In the third-row embryos showing no sign of somite formation (G), reduced somite formation (H) and larvae showing no somite formation (I) are represented. Lack of heartbeat is shown by non-convulsion of the zebrafish's heart (double arrow) and blood cells' immobility (J). Adapted from (OECD,2013).

1.11 Study rationale, aims, objectives and hypothesis

a) Study rationale

Globally, water pollution has been purported as a major cause of mortality and morbidity worldwide. A lot of harmful substances are released into the aquatic systems including toxic metals, microorganisms, or agricultural and industrial chemicals. These contaminants can lead to serious adverse effects on different living organisms and the ecosystem at large, which are highly dependent on clean water. In addition, SA is a water-scarce country, with the majority of WWTPs that are in a critical state, needing urgent intervention. Large amounts of CECs are spewed onto water systems posing a challenge pertaining to efficient removal by WWTPs. This prompts the need for the development of techniques to constantly monitor water systems and more advanced water remediation techniques. Also, there are limited studies that report on the lethal toxicity products used in WWTPs in developing countries. Moreover, wastewater contaminants on living organisms in relation to sub-optimal parameters detected in wastewater are of concern to be well understood. To gain insight into the underlying alterations that can occur due to water contaminants, a novel approach could be employed using zebrafish. In this study, monitoring the zebrafish's health after exposure to wastewater is aimed to uncover the potential effects of wastewater pollutants in SA in relation to sub-optimal water parameters and current remediation methods. This will in turn reveal the impact that could potentially be incurred by other aquatic and non-aquatic living organisms.

b) Aims, objectives and hypothesis

The observation of zebrafish in different developmental stages is an efficient method for determining the toxicity effect of different substances. In this study, this method was utilized along with assays that will test the effect of different wastewater effluents on the physiology of zebrafish. It has been hypothesized that contaminants introduced to localized water systems and methods of water remediation may exhibit toxicity effects on the health of zebrafish and their biological pathways. This study aims to investigate the health state of zebrafish and the occurrence of alterations due to contaminants in localized water systems and remediation efforts. The current study was carried out under the following objectives:

1. Assessing the mortality percentage post exposure and monitor the hatching rate to detect for lethal toxicity.
2. Determination of neurotoxicity in zebrafish by observation of spontaneous movements per minute.
3. Detection and quantification of the heat shock protein (HSP70), in an attempt to measure the expression of stress-related proteins.
4. Examination of the potential of contaminants to alter the zebrafish swimming patterns.
5. Assessing the efficiency of chlorination on water remediation.

CHAPTER 2: METHODS AND MATERIALS

2.1 Sampling of wastewater

The wastewater samples were provided by the eThekwini Metropolitan Municipality (KwaZulu Natal, South Africa), each of the samples were collected in June (winter), downstream different factories depicted by different symbols on the geographical map (**Figure 2.1**). Sampling sites were chosen based on effluent flow prediction. The first sample collection site was the Amanzimtoti River. Two water samples were collected from this site, one before chlorination (TB: Amanzimtoti before chlorination) and the other after chlorination (TA: Amanzimtoti after chlorination). Liquid gas chlorine that was 99.5% pure was used to disinfect the Amanzimtoti water in order to inactivate disease-causing pathogens, such as bacteria, viruses, and protozoans (Bailey *et al.*,2021). Incoming raw sewage was collected from the Badulla Line (IB: Incoming Badulla) and the river that flows through Chatsworth (CI: Chatsworth Incoming). In addition to the incoming raw sewage, other effluents were from the Southern Wastewater Treatment Works (SWFE: Southern Works Final Effluents). Industrial Jacob's sewage (JB: Jacob's Incoming) was also sampled. Samples (2 L from each site) were collected and stored at 28.5°C in 500 mL aliquots in Schott bottles (Merck, USA) before use.

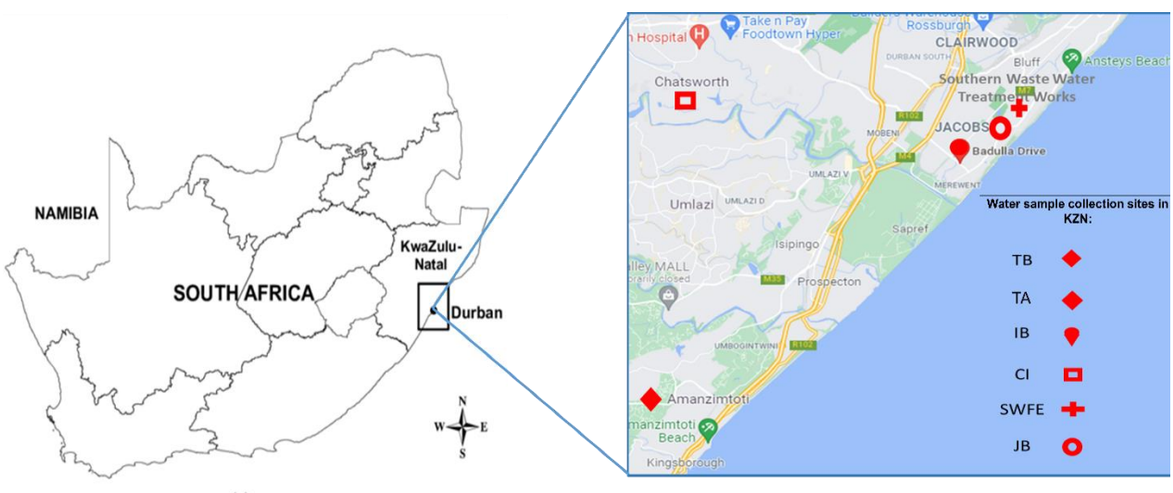


Figure 2.1: Geographical map of wastewater sampling sites in SA at KwaZulu-Natal (KZN). Effluent samples were collected downstream different factories on the regions assigned by different symbols. Wastewater symbols for TB: Amanzimtoti before chlorination, TA: Amanzimtoti after chlorination, Incoming badulla, CI: Chatsworth Incoming, SWFE: Southern Works Final Effluent and JB: Jacob's Incoming wastewater

2.2 Physicochemical characterization of wastewater

Wastewater/effluent samples were centrifuged (8600 *x* g, 15 min) using the MIKRO 200R (Labotec, South Africa) to remove physical components that may interfere with the sensitivity of the assays and to increase the reliability of the results (**Figure 2.2**). Physico-chemical parameters were assessed to detect for any deviations of the parameters from recommended standards. Parameters measured include pH, measured by the Orion Star A111 pH Benchtop Meter (Thermo Scientific, USA). Total chlorine, free chlorine, total alkalinity, hardness, nitrate and nitrite measurements were taken using a colorimetric kit (Vansful, China), while dissolved oxygen (DO) was measured using a portable pH/DO meter kit (Bante Instrument, China).

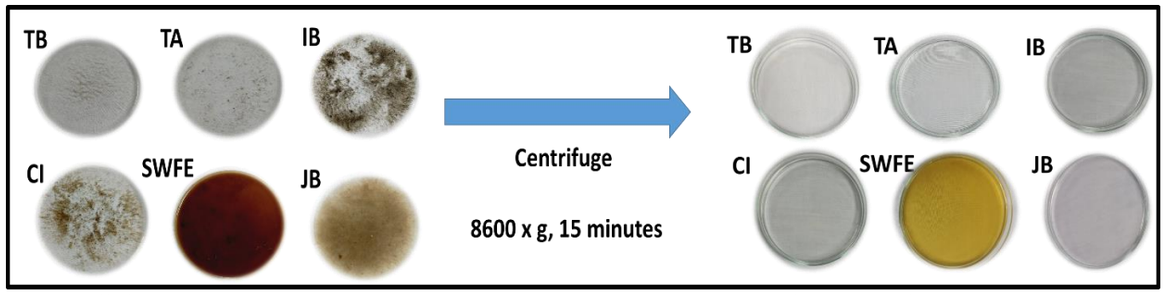


Figure 2.2: Wastewater before and after sediment removal. Sample TB: Amanzimtoti before chlorination, TA: Amanzimtoti after chlorination, IB: Incoming Badulla, CI: Chatsworth Incoming, SWFE: Southern Works Final Effluents and JB: Jacob's Incoming. Image captured using a digital camera.

2.3 Zebrafish maintenance and breeding

Adult wild-type zebrafish (*Danio rerio*) were sustained and bred in 10 L tanks under controlled water temperature (28 ± 5 °C), pH (7.0 ± 0.5), photoperiod (light: dark cycle 14:10 h) and dissolved oxygen (> 80 %). Fish were fed with TetraPro Energy Fish Flakes (Tetra®, Germany) twice a day, supplemented with rotifers (Ocean Nutrition, US) every second week. To obtain embryos, males and females in a 2:3 ratio were placed in the same tank in the afternoon prior to breeding. The breeding procedure was equipped by mesh lid containers which served as collection reservoirs of embryos released after spawning. Embryos were collected 2 - 3 hpf from each container, transferred to glass petri dishes and carefully rinsed with E3 medium (5 mM NaCI, 0.17 mM KCI, 0.33 mM $CaCI_2$, 0.33 mM $MgSO_4$, 5% Methylene Blue) before experiments. Viable fertilized embryos were selected under the microscope (World Precision Instruments, Japan) prior to exposure to the test samples. All experiments were carried out with ethics approval granted by the Animal Research Ethics Committee (AREC/029/019) at the University of KwaZulu-Natal on 04 August 2021 attached in **Appendix A**. Additionally, this study is in compliance with Section 20 of the animal disease act, 1984 (ACT NO 35 OF 1984) stated by the Department of Agriculture, Land Reform and Rural Development in SA (**Appendix B**).

2.4 Range-finding test

A range-finding test was conducted in line with recommendations of the zebrafish OECD guidelines (OECD,2013) in order to select the appropriate concentration range to use in the ZET. Briefly, five concentrations of the centrifuged effluent samples (spaced equally by a constant factor) were prepared in E3 medium ranging from 20 - 100 % of the effluent (20, 40, 60, 80 and 100 %). Ten embryos per concentration were exposed to 250 µL of each concentration in a 96-well plate (one embryo per well) and monitored for mortality over 144 hpf. The concentration range of 20 - 100 % was selected for wastewater TB, TA, IB and CI (20, 40, 60, 80 and 100 %). In contrast, 8 - 40 % was found suitable for SWFE and JB (8, 16, 24, 32 and 40 %) due to the 100 % fish mortality observed above the concentration of 40 % within 24 h of exposure to these samples. In addition, reducing the sample concentration of SWFE and JB allowed for the construction of a dose response curve where the acute toxicity of the effluents was calculated using the median lethal concentration (LC_{50} value).

2.5 Zebrafish embryotoxicity (ZET) test

Following the range-finding test, the ZET assay was performed as per the OECD guidelines (Ribeiro *et al.*, 2020, OECD, 2013). In short, effluent stock solutions were prepared in E3 medium 24 h prior to the experiments and stored at 28.5 °C. This was carried out 2-3 hpf in triplicates, with 20 embryos being exposed to each test concentration at a time, in 96-well plates (NEST Biotechnology Co., Ltd, China). The E3 medium was used as the negative control (0% concentration of effluent), followed by incubation at 28.5 °C for 144 h. Fish were fasted during the period 0-144 hpf while water was changed daily for all treatment groups. Visualization of embryo development was carried out under the microscope (World Precision Instruments, Japan) at 24, 48, 72, 96, 120 and 144 hpf. This procedure aimed at assessing the mortality rate (%), hatching rate (%) and frequency of morphological changes (%). Embryos without visible heartbeats, tail detachment, somite formation, and those showing signs of coagulation were considered dead (OECD, 2013, Ribeiro *et al.*, 2020). At the end of the exposure period, Graphpad Prism was used to construct a dose response curve in order to determine LC_{50} values.

2.6 Determining the effect of wastewater on each morphological endpoint of the zebrafish

The general morphology score (GMS) system was utilized (with amendments) to evaluate the zebrafish for any teratogenicity due to the wastewaters, according to the method of Hermsen and colleagues (Hermsen *et al.*,2011). At 144 hpf, triplicate viable zebrafish were assessed for each effluent concentration for any morphological deformities. A score was assigned based on the degree of development of different morphological traits: somites, notochord, tail, fins, heart, brain, facial structures, jaw and pharyngeal arches. Scores were assigned on a scale of 1 - 5, with 5 being allocated to correct morphology and a minimum score of 1 indicating a total lack of the structure under consideration.

2.7 Tolerance of zebrafish to various levels of pH in wastewater SWFE and JB

Due to high mortality and the sub-optimal pH detected in wastewater SWFE (9.02) and JB (pH 5.65), pH was neutralized using sodium hydroxide and hydrochloric acid (Merck, USA) in an attempt to remediate the water. A range of acidic to basic water concentrations were prepared for SWFE (pH 3.02, 4.02, 5.02, 6.02, 7,02, 8.02, 9.02, 10.02 and 11.02) and JB (pH 3.65, 4.65, 5.65, 6.65, 7.65, 8.65, 9.65, 10.65 and 11.65) using E3 medium. Tank water at adjusted pH (3.00, 4.00, 5.00, 6.00, 7.00, 8.00, 9.00, 10.00 and 11.00) was also tested to delineate if pH was the sole contributing water quality parameter towards the toxicity of SWFE and JB. Groups of twenty zebrafish embryos were exposed to each of the experimental concentrations at the various pH levels. All experiments were repeated three times, using different batches of embryos and zebrafish were inspected for the mortality percentage and malformations 144 hpf.

2.8 Tolerance of zebrafish to different total alkalinity in wastewater SWFE and JB

To investigate whether total alkalinity was an associated factor to toxic effects of wastewater, samples found with sub-optimal levels of total alkalinity were assessed to a further extent following the method of Furtado and co-workers (Furtado *et al.*,2011). In summary, SWFE and JB with 0 mg/L of total alkalinity detected during characterization, were supplemented with 120 mg/L of sodium bicarbonate ($NaHCO_3$) from Merck Chemicals (PTY) LTD, South Africa. This was in order

for alkalinity to be increased to fall within acceptable limits recommended for fish. Twenty embryos per wastewater concentratrion were exposed to the water prior alteration of total alkalinity (0 mg/L) and post adjustment (120 mg/L). Each experiment was performed with a different batch of embryos as all experiments were repeated three times. Zebrafish were inspected for the mortality percentage and evident body malformations 144 hpf. Tank water served as control, with conditions altered similarly to wastewater.

2.9 Neurotoxicity

Spontaneous movement rate per minute (n.min−1) was observed 24 hpf under the microscope (World Precision Instruments, Japan) to determine neurotoxicity induced by the water/effluent samples. Ten zebrafish per wastewater concentration were assessed for movement. In this study spontaneous movement was used as a suitable sublethal endpoint due to that altered locomotion is associated with the development of neurotoxic effects in literature (Selderslaghs *et al.*,2010;Ribeiro *et al.*,2020). Prior to the assessment of spontaneous movement, larvae were screened for teratogenic effects. Larvae which displayed malformations, although in low numbers were excluded for locomotor analysis.

2.10 Sample preparation

Zebrafish embryos collected from natural spawning were exposed to wastewater (TB, TA, CI and IB) exhibiting less toxicity at an organism and tissue level of the fish. Three days post-fertilisation, 25 free-swimming larvae in each wastewater were euthanized by submersion into an ice water bath for at least 20 minutes to ensure death. Thereafter, the fish were transferred to microcentrifuge tubes containing 1.5 mm Zirconium beads (BeadBug™, USA) and the lysis buffer (Pierce™ RIPA buffer, Thermo Scientific, USA) supplemented with 1× protease inhibitor cocktail (Melford, UK) and 1 U/mL DNase (Takara Bio, USA). Subsequently, the zebrafish larvae were subjected to lysis upon bead-beating using the BeadBug™ microtube homogenizer (Merck, Germany) for protein extraction. The positive control samples included zebrafish larvae heat shocked at 37 °C, for 1 h, while zebrafish grown under optimal conditions (E3 medium) were used as the negative control.

2.11 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Cell lysates (~25 zebrafish larvae per well) were separated on a 12.5 % reducing SDS-PAGE gel with the SeeBlue Plus2 Pre-stained Protein Standard molecular weight (Thermo Fisher Scientific, Massachusetts, USA) marker in accordance with the method of Laemmli (Laemmli,1970). Briefly, samples were loaded onto the gel with an equal volume of the reducing treatment buffer [125 mM Tris-HCI, 4 % (w/v) SDS, 20 % (v/v) glycerol, 10 % (v/v) 2-mercaptoethanol, pH 6.8] following incubation in boiling water, for 90 s. After cooling, samples were loaded onto the gels and subjected to run at 20 mA on a tank buffer [250 mM Tris-HCI, 192 mM glycine, 0.1 % (w/v) SDS, pH 8.3]. Gels were either stained with Coomassie blue [0.125 % (w/v) Coomassie blue R-250, 50 % (v/v) methanol, 10 % (v/v) acetic acid] overnight or unstained for analysis on the western blot. For the detection of the heat shock protein 70 (HSP70), proteins from an unstained 12.5 % SDS-PAGE gel were transferred to a nitrocellulose membrane (Pall Corporation, New York, USA) using

the Novex™ Semi-Dry Blotter (Thermo Scientific, USA). Thereafter, the membrane was blocked in 5 % (w/v) non-fat milk containing Tris buffered saline (TBS, 20 mM Tris–HCl, 200 mM NaCl, pH 7.4), for 1 h. The membrane was washed 3 x 5 min with TBS and incubated overnight with the primary antibody: chicken anti-HSP70 affinity purified IgY, produced in mouse (Cell Signalling Technology, Massachusetts, USA) made in 1 % (w/v) non-fat milk with TBS. Following incubation, the blot was washed as before (3 x 5 min) and incubated with the horse radish peroxidase (HRP)-conjugated goat anti-chicken IgY (IgG) rabbit anti-chicken IgY secondary antibody conjugated to horse-radish peroxidase secondary antibody (Jackson ImmunoResearch Laboratories, Inc., Pennsylvania, USA) diluted 1:10 000 in 5 % (w/v) non-fat milk, for 1 h. The blot was further washed, and the protein bands were detected with an enhanced chemiluminescent (ECL, Bio-Rad, USA) substrate kit. The SDS-PAGE gel and western blot images were captured using the G:BOX Chemi XR5 imaging system along with the GeneTools 1.8.0 software (Syngene, Cambridge, UK). To analyse the western blot the band intensity was quantified for each protein band using and normalised against the amido black total protein stained blot. Data was presented as mean relative band density (RBD). All band intensities were determined by applying the densitometry software called Image J, according to a previously developed method (Delport and Hewer,2022).

2.12 Behavioural analysis of zebrafish using Toxtrac

The Toxtrac software was used to track the locomotor behaviour of zebrafish larvae (144 hpf) following the method of (Klein *et al.*,2021) with minor modifications. Briefly, locomotor activity of four video replicates were recorded simultaneously per concentration of the wastewater. Videos of 1 – 2 minutes were taken, with three larve placed on a bright background per video (**Appendix E**). Captured videos were ran through the Toxtrac software ensuring an average of ≥95 % visibility ratethroughout every experiment. Numerous locomotor parameters were produced using ToxTrac (average speed, mobility rate, distance travelled, frozen events, acceleration, tracking trajectories and exploration rate). We chose the five latter ones as endpoints because they are commonly used as a parameters (Faria *et al.*,2018b;Li *et al.*,2018;Hussain *et al.*,2020;Yuan *et al.*,2021;Rao *et al.*,2022).

2.13 Statistical analysis

All statistical analyses were carried out using GraphPad Prism 9 XML Project software, including LC_{50} values that were calculated within 95 % confidence limits. Significance is represented with p-values showing a degree of statistical significance that varies. The different asterisks depict p-values less than 0.05 (*), 0.01 (**), 0.001 (***) or 0.0001 (****).

CHAPTER 3: RESULTS

3.1 Physicochemical analysis of wastewater samples

The physicochemical parameters of wastewater samples are presented in Table 3.1 along with the maximum permitted concentrations (MPCs) of these parameters as recommended in literature for aquatic species. Parameters taken into consideration were pH (MPC = 6.8 - 8.5), total chlorine (MPC = 0 mg/L), free chlorine (MPC = 0 mg/L), total alkalinity (MPC = 50 - 150 mg/L), hardness (MPC = 75 - 200 mg/L), nitrate (MPC ≤ 200 mg/L), nitrite (MPC = 0 mg/L) and dissolved oxygen (MPC ≥ 4 mg/L) (Lawrence *et al.*,2012;Tye *et al.*,2018;Aleström *et al.*,2020;Cueto-Escobedo *et al.*,2021;Del Vecchio *et al.*,2022). Notably, two effluent samples, namely SWFE and JB, presented indices that were not within the permissible concentrations stipulated for aquaculture. In these samples, pH was recorded as 9.02 ± 0.16 and 5.65 ± 0.02 for SWFE and JB respectively, while total alkalinity of 0 mg/L was detected in both samples. In contrast, the physicochemical properties detected in wastewater samples TB, TA, IB, CI and the control (E3 medium, 0 %) conformed with the recommended standards.

3.2 Multiple biomarkers of zebrafish

3.2.1 Mortality rate

Average mortality rate obtained due to sample TB, TA, IB and CI were below 50 % at all concentrations tested (20, 40, 60, 80 and 100%) (Table 3.2). Notably, a proportion of 25 % zebrafish larvae died in raw effluents (100 %) of TB and TA. Similarly, 33 % and 22 % zebrafish succumbed in the presence of undiluted IB and CI samples, respectively. In addition, the mortality percentages obtained following exposure to TB and TA, were statistically similar ($p > 0.05$) to those of the control sample (E3 medium, 0 %) at all concentrations, while the death rate at ≥ 80 % IB and CI varied statistically to the control group. Alarmingly, raw wastewater SWFE and JB brought about 100 % lethality within 24 hpf (results not shown). As per the resultant dose response curves, SWFE (**Figure 3.1 A**) and JB (**Figure 3.1 B**), led to a cumulative increase in average mortality upon increase in concentration that varied significantly ($p < 0.05$, **Appendix C**) from the control at concentrations ≥ 8 %. The highest concentration tested (40 %) for SWFE and JB elevated the death rate to 77 ± 2.89 % and 100 ± 0.00 %, respectively, with corresponding LC_{50} values of 17.77 % and 16.46 %.

Table 3.1: Physicochemical parameters of wastewater collected from KwaZulu-Natal in South Africa along with the maximum permitted concentrations (MPC) for zebrafish maintenance and breeding

Water parameters	MPC	Control (E3 medium)	TB	TA	CI	IB	SWFE	JB
pH	6.5 - 8.0[a]	7.01 ± 0.04	7.32 ± 0.03	7.42 ± 0.22	6.92 ± 0.45	6.81 ± 0.10	**9.02 ± 0.16**	**5.65 ± 0.02**
Total chlorine (mg/L)	0[b,d]	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Free chlorine (mg/L)	0[b,d]	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Total alkalinity (mg/L)	50 - 150[c]	53.33 ± 23.09	93.33 ± 23.09	106.67 ± 23.09	53.33 ± 23.09	93.33 ± 23.09	**0 ± 0.00**	**0 ± 0.00**
Hardness (mg/L)	75 – 200[a]	75.00 ± 43.30	166.67 ± 72.17	141.67 ± 101.04	141.67 ± 101.04	75.00 ± 43.30	166.67 ± 72.17	183.33 ± 115.47
Nitrate (mg/L)	≤ 200[c]	0 ± 0.00	75.00 ± 43.30	15.00 ± 8.66	150.00 ± 86.60	66.67 ± 28.87	0 ± 0.00	0 ± 0.00
Nitrite (mg/L)	0[c]	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Dissolved oxygen (mg/L)	≥ 4[c,e]	32.89 ± 1.35	10.93 ± 0.25	10.75 ± 2.11	23.34 ± 0.88	24.88 ± 1.22	8.99 ± 1.19	4.65 ± 0.33

[a]Del Vecchio et al., 2022, [b]Aleström *et al.*, 2020 [c]Lawrence and Mason, 2012 [d]Tye *et al.*, 2018 [e]Cueto-Escobedo et al., 2021. TB- Amanzimtoti before chlorination, TA- Amanzimtoti after chlorination, IB- Incoming Badulla, CI- Chatsworth Incoming, SWFE- Southern Works Final Effluents and JB- Jacob's Incoming. Bold : Values above MPC.

Table 3.2: Mortality rate recorded for zebrafish embryos during 144 h exposure to wastewater samples TB, TA, IB and CI

Type of wastewater	Mortality rate (%)					
	Wastewater concentration (%)					
	0	20	40	60	80	100
TB	6.67 ± 2.89	20.00 ± 8.66	13.33 ± 10.41	18.33 ± 7.64	16.67 ± 7.64	25.00 ± 15.00
TA	1.67 ± 2.89	15.00 ± 13.23	16.67 ± 7.64	21.67 ± 10.41	10.00 ± 5.00	25.00 ± 18.03
IB	5.00 ± 5.00	21.67 ± 20.21	25.00 ± 22.91	30.00 ± 17.32	28.33 ± 15.28	33.33 ± 5.77 *
CI	5.00 ± 5.00	13.33 ± 7.64	15.00 ± 10.00	18.33 ± 7.64	20.00 ± 10.00*	21.67 ± 7.64 **

TB: Amanzimtoti before chlorination, TA: Amanzimtoti after chlorination, IB: Incoming Badulla, CI: Chatsworth Incoming, SWFE: Southern Works Final Effluents and JB: Jacob's Incoming. Mortality is depicted as mean ± SD for triplicate experiments (20 embryos per concentration for each experiment). Asterisks depict p-value less than 0.05 (*), 0.01 (**), 0.001 (***) or 0.0001 (****), compared to the control group (E3 medium, 0%). Data analyzed by GraphPad Prism 9 XML Project software.

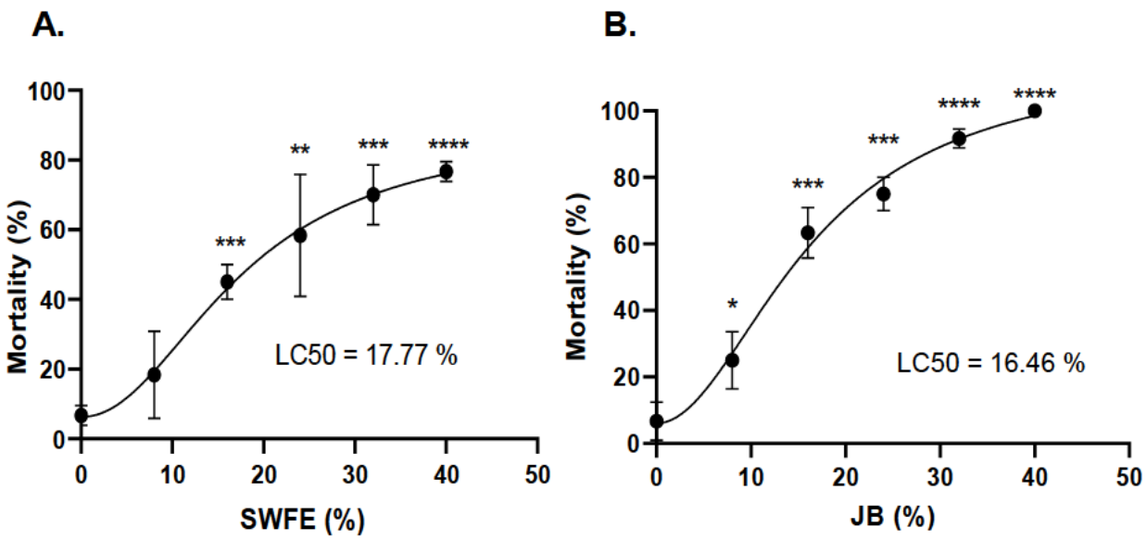


Figure 3.1: Mortality rate recorded for zebrafish embryos during 144 h of exposure to wastewater (A) SWFE and (A) JB. SWFE: Southern Works Final Effluents and JB: Jacob's Incoming data expressed in mean ± SD (n=3, 20 embryos per concentration for each experiment). Asterisks depict p-value less than 0.05 (*), 0.01 (**), 0.001 (***) or 0.0001 (****), comparing each concentration with the control (E3 medium, 0%). Data analyzed by GraphPad Prism 9 XML Project software, with LC_{50} percentages calculated within 95 % confidence limits.

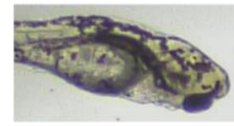
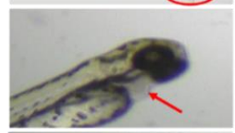
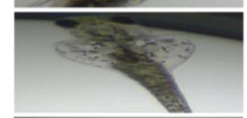
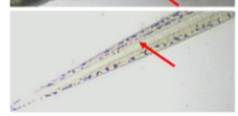
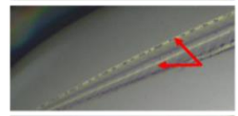
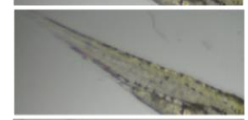
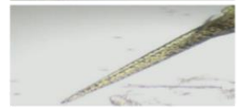
3.2.2 Analysis of morphological endpoints

The normal development of the zebrafish jaw and face, heart, brain, fins and pharyngeal arches, notochord, somite and tail are illustrated in **Table 3.3** along with abnormalities observed 144 hpf. In relation to the data set in **Table 3.3**, the development of zebrafish morphological structures are quantitatively demonstrated in **Figure 3.2** by score, with a score of 1 indicating the least developed structure and 5 representing a correctly developed structure. There was no observable teratogenicity due to TB, TA, IB and CI within the concentration range of 0 - 100 % (**Table 3.3**) and scores obtained for these effluents were statistically similar ($p > 0.05$) to those obtained for the control (E3 medium, 0 %) (**Figure 3.2 A-D**).

In contrast, exposure to wastewater SWFE and JB (at concentrations ≥ 32 %), significantly ($p < 0.05$) affected the development of eight out of nine body structures being assessed, compared to the control (**Table 3.3; Figure 3.2 E-F**). For the facial structure (shown by an open bracket), the eyes of the exposed fish were not in a suitable position and the otic capsule (ear) was not well-defined with the olfactory region appearing as underdeveloped (**Table 3.3 A**). In addition, the lower jaw was misshapen (encircled) and did not protrude as depicted for normal fish (**Table 3.3 A**). The previously described face and jaw was from an individual amongst fish exposed to 40 % SWFE with average scores of 3.7 and 2.3, assigned respectively (**Figure 3.2 E**). Furthermore, some of the fish displayed severe pericardial oedema following exposure to 40 % SWFE and 32 % JB with an enlarged and malformed heart (**Table 3.3 B**), with respective average scores of 2.3 and 3.3 in SWFE and JB (**Figure 3.2 E-F**). The brain was also affected, with some of the individuals exposed to 40 % SWFE presenting a frontal brain segment that was not well defined (shown by an arrow) with an average score of 4 (**Table 3.3 C; Figure 3.2 E**).

Exposure to 32 % JB also resulted in incorrect development of the pectoral fins (**Table 3.3 D; Figure 3.2 F**). Pectoral fins were not clearly visible and were forced to spread out sideways without lying flat on either sides of the body due to yolk sac oedema, thus preventing the fish from swimming upright and efficiently. The lack of well-defined pectoral fins resulted to a score of 2.7 being assigned post-exposure to 32 % JB (**Figure 3.2 F**). Despite the inefficiency of pectoral fins, pharyngeal arches developed well in all of the water samples with an average score of ≥ 4 being allocated for all wastewater samples (**Table 3.3 D; Figure 3.2**). The notochord and somite showed a severe lack of definition with average scores of 2.3 and 1.7, assigned correspondingly, in 32% JB (**Table 3.3 E-F; Figure 3.2 F**). According to illustrations in **Table 3.3**, the notochord was wider than normal, extending from the region at the front of the swim bladder to the tip of the tail while being indistinguishable from the somites along the tail due to poor development. Upon observation, somites appeared to be unclear, they were not visibly distinct from the notochord (**Table 3.3 E-F**). Clear lines that are outlined outwards from the centre of the tail during normal development were not well-defined following exposure. Another abnormality observed was the spinal cord curvature and a tail that had kinks from the frontal region of the swim bladder to the tip of the zebrafish tail at 40 % SWFE and JB at 32 %, scoring 3 and 3.7 (**Table 3.3 G; Figure 3.2 E-F**). Zebrafish exposed to 40 % JB all incurred death due to coagulation (**Figure 3.2 F**).

Table 3.3: A fully developed zebrafish, *Danio rerio* at six days post-fertilization (dpf) in comparison to abnormal zebrafish larvae exposed to Southern Works Final Effluent (SWFE) and Jacob's Incoming (JB) wastewater samples

Morphology	Normal development	Abnormalities	Wastewater and concentration
A. Jaw and Face			40% SWFE
B. Heart			40% of SWFE and 32% JB
C. Brain			40% SWFE
D. Fins and pharyngeal arches			32% JB
E. Notochord			32% JB
F. Somite			32% JB
G. Tail			40% SWFE and 32% JB

SWFE: Southern Works Final Effluents and JB: Jacob's Incoming

3.2.3 Tolerance of zebrafish to different pH in wastewater SWFE, JB and tank water

a) Mortality due to pH

There was an increase in mortality at the extremely acidic and basic regions seen during zebrafish exposure in wastewater SWFE and JB following pH adjustment (**Figure 3.3**). This was observed 144 hours post-exposure. At concentrations ≥ 8 % of SWFE, 100 % of the fish died at the lowest pH tested (pH 3.02) while a mortality rate of ≥ 85 % was observed at the highest pH tested (pH 11.02). In contrast, mortality was notably lowered (less than 12 %) at pH values between 6.02 and 8.02, with no statistical differences ($p > 0.05$) in any of the concentrations when compared to the control group. Comparing the effect of pH, mortality reported at pH 6.02 - 8.02 varied statistically ($p < 0.05$) in all concentrations compared to mortality at the acidic region (pH < 6.02) (**Figure 3.3 A; Appendix D.1**). A similar trend was observed under basic pH conditions (> 8.02), where the number of fish that died differed significantly ($p < 0.05$) to the fish recorded within pH 6.02 - 8.02 for all concentrations ≥ 8 %.

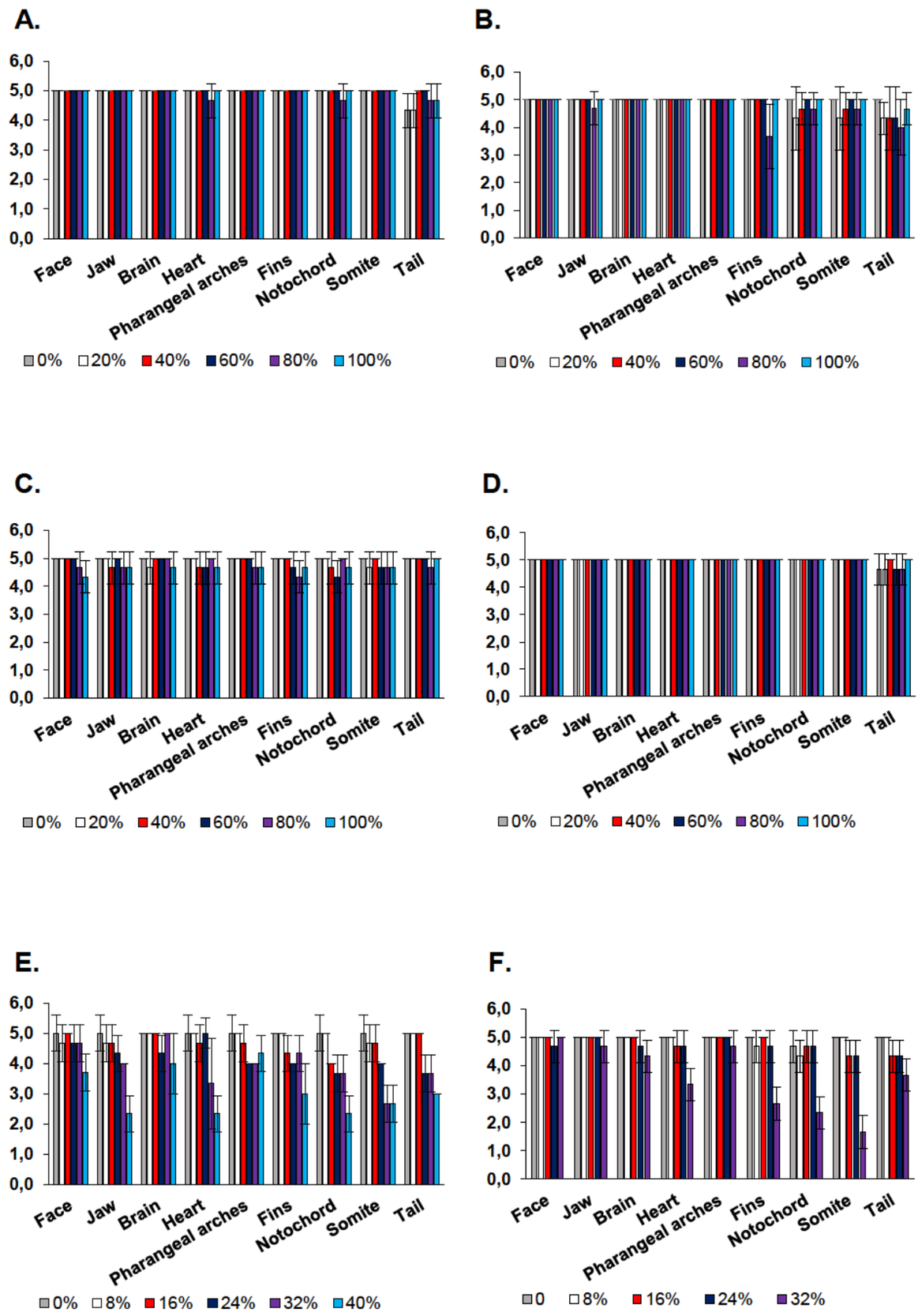


Figure 3.2: The morphological score indicating zebrafish embryo development after exposure to wastewater. A scoring of correct development of the face, brain, jaw, heart, pharyngeal arches, fins, notochord, somite and tail was determined after six days. Each morphological trait was scored out of five with a score of 5 indicating a fully developed structure at six dpf. Error bars indicate the standard deviation of three replicates for sample (A) TB- Amanzimtoti before chlorination, (B) TA- Amanzimtoti after chlorination, (C) IB- Incoming Badulla, (D) CI- Chatsworth Incoming, (E) SWFE- Southern Works Final Effluents and (F) JB- Jacob's Incoming.

Comparably to SWFE, wastewater JB led to 100 % mortality at highly acidic pH, specifically at pH 3.65 (≥ 32 % JB), pH 4.65 and 5.65 (40 % JB) (**Figure 3.3 B; Appendix D.2**). This was also observed at pH 11.65, where no fish survived at any concentration of JB. In addition to the high mortality observed, there was no statistically significant change in mortality at the range of pH 6.65 - 7.65 compared to the control group (E3 medium, 0 %) after the addition of wastewater JB (concentrations 8 - 40 %). Nonetheless, acute acidity (< 6.65) significantly increased ($p < 0.05$) mortality at JB concentrations ≥16 % compared to non-lethal pH of 6.65 - 7.65 (**Appendix D.2**). Similarly, basic environments of JB showed variation statistically compared to pH 6.65 - 7.65 ($p < 0.05$). Upon exposure to various pH of tank water diluted in E3 medium, there was no significant change ($p > 0.05$) in mortality due to the adjustment of pH within the range 3.00 - 8.00 (**Figure 3.3 C; Appendix D.3**). Also, the mortality rate observed at this range was not statistically different ($p > 0.05$) to the control (E3 medium, 0 %) for all concentrations (**Appendix D.3**). However, an increase of pH to ≥ 9.0 led to an increase in mortality percentage, which was statistically significant ($p < 0.05$) from about 24 % tank water. Notably, pH 10.0 (40 % tank water) and 11.0 (8 - 40 % tank water) resulted in 100 % death of the fish following exposure.

b) Malformations due to pH adjustment

Malformations induced by the lower and upper limits of pH in effluent SWFE and JB on zebrafish are shown in **Figure 3.4** and **Figure 3.5**. Prolonged exposure of zebrafish to SWFE and JB at highly acidic and basic pH conditions caused numerous morphological abnormalities in zebrafish, such as pericardial and yolk sac edema, spinal cord curvature, underdevelopment of facial features, unsuccessful hatching and hypopigmentation. These malformations were evident at a concentration as low as 8 % in effluent SWFE and 24 % in JB at the lowest pH tested, which was 3.02 and 3.65 respectively for SWFE and JB. In contrast, there was only one abnormality due to tank water at 40 % concentration (pH 3.00) even after the exposure of the fish to various pH levels.

3.2.4 Tolerance of zebrafish to different total alkalinity in wastewater SWFE, JB and tank water

Following effluent remediation by the increase of total alkalinity from 0 (sub-optimal) to 120 mg/L (optimal), there was a reduction in the mortality rate of fish exposed to SWFE (**Figure 3.6 A**) and JB (**Figure 3.6 B**), while the rate remained the same in tank water used as a control (**Figure 3.3 C**). Comparing the mortality rate at 0 vs 120 mg/L total alkalinity, there was a significant reduction ($p < 0.05$) in all concentrations ≥ 16 %, with a resultant maximum mean mortality of 25 % at the highest concentration tested for SWFE (40 %) post alkalinity increase (**Figure 3.6 A; Appendix D.4**). Also, as an outcome of the change in alkalinity, SWFE at concentrations of 8 - 32 % induced only 10 % mortality - which was insignificant ($p > 0.05$) compared to the control (E3 medium, 0%) (**Appendix D.4**). Similarly, upon the increase of alkalinity to 120 mg/L, a significant decrease in mortality ($p < 0.05$) was observed for wastewater JB at concentrations ≥ 16 % when compared to the death rate at 0 mg/L alkalinity (**Figure 3.6 B; Appendix D.4**). This led to a maximum of 63 % death in the presence of 120 mg/L alkalinity at the 40 % concentration from 100 % death at 0 mg/L (**Figure 3.6 B**). Contrary to SWFE and JB, mortality observed for tank water was not statistically different ($p > 0.05$) following the comparison of 0 mg/L and 120 mg/L (**Figure 3.6 C; Appendix D.4**). Also, the control group (E3 medium, 0 %) showed no significant variation

compared to tank water (for all concentrations of 8-40 %) under both 0 mg/L and 120 mg/L of total alkalinity.

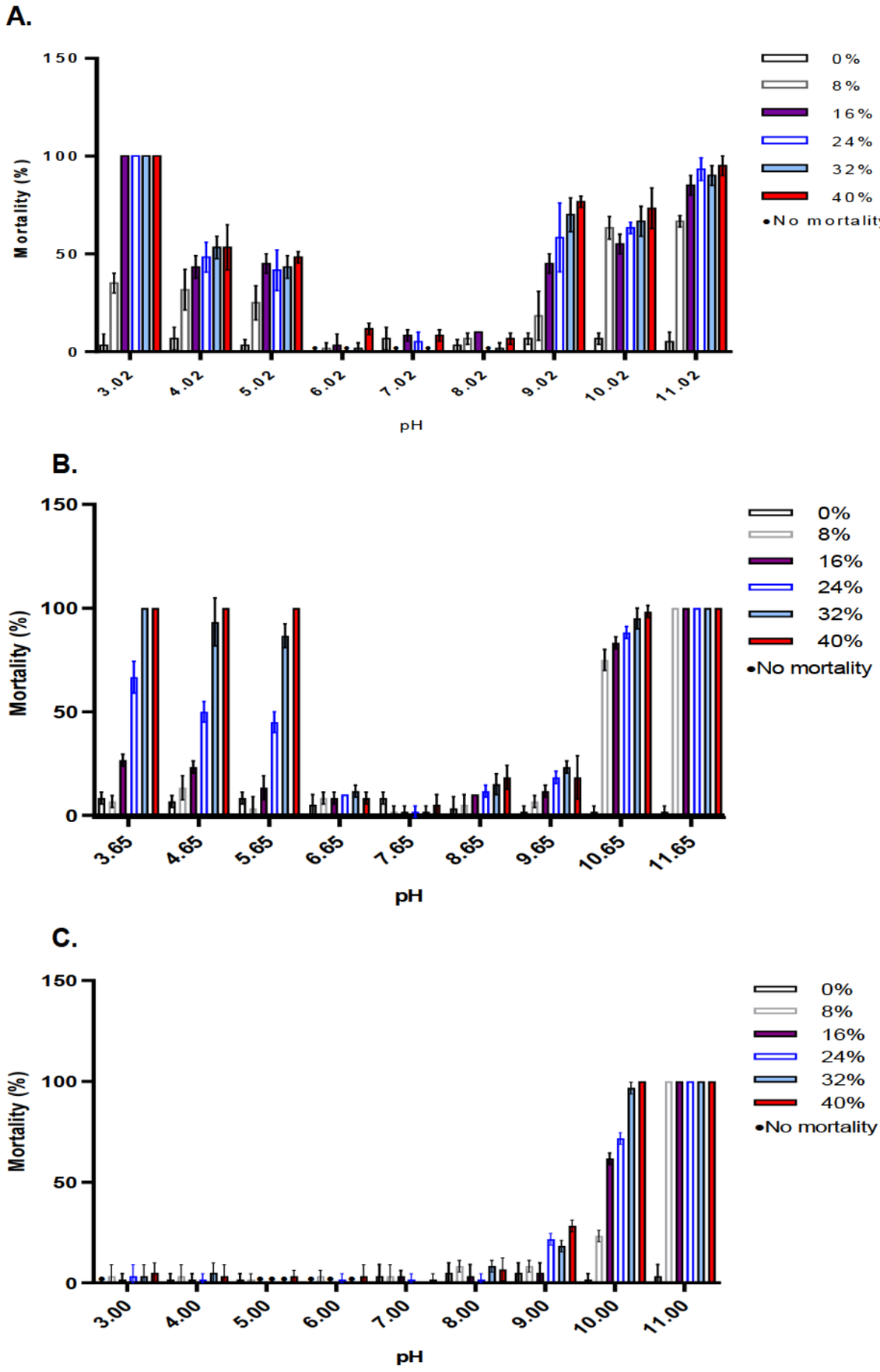


Figure 3.3: Mortality rate of zebrafish exposed to different pH levels of (A) SWFE, (B) JB and (C) tank water. Values are presented as mean ± SE (20 embryos per concentration for each experiment). Zero mortality is denoted as, no mortality: ●. Data constructed and analyzed by GraphPad Prism 9 XML Project software.

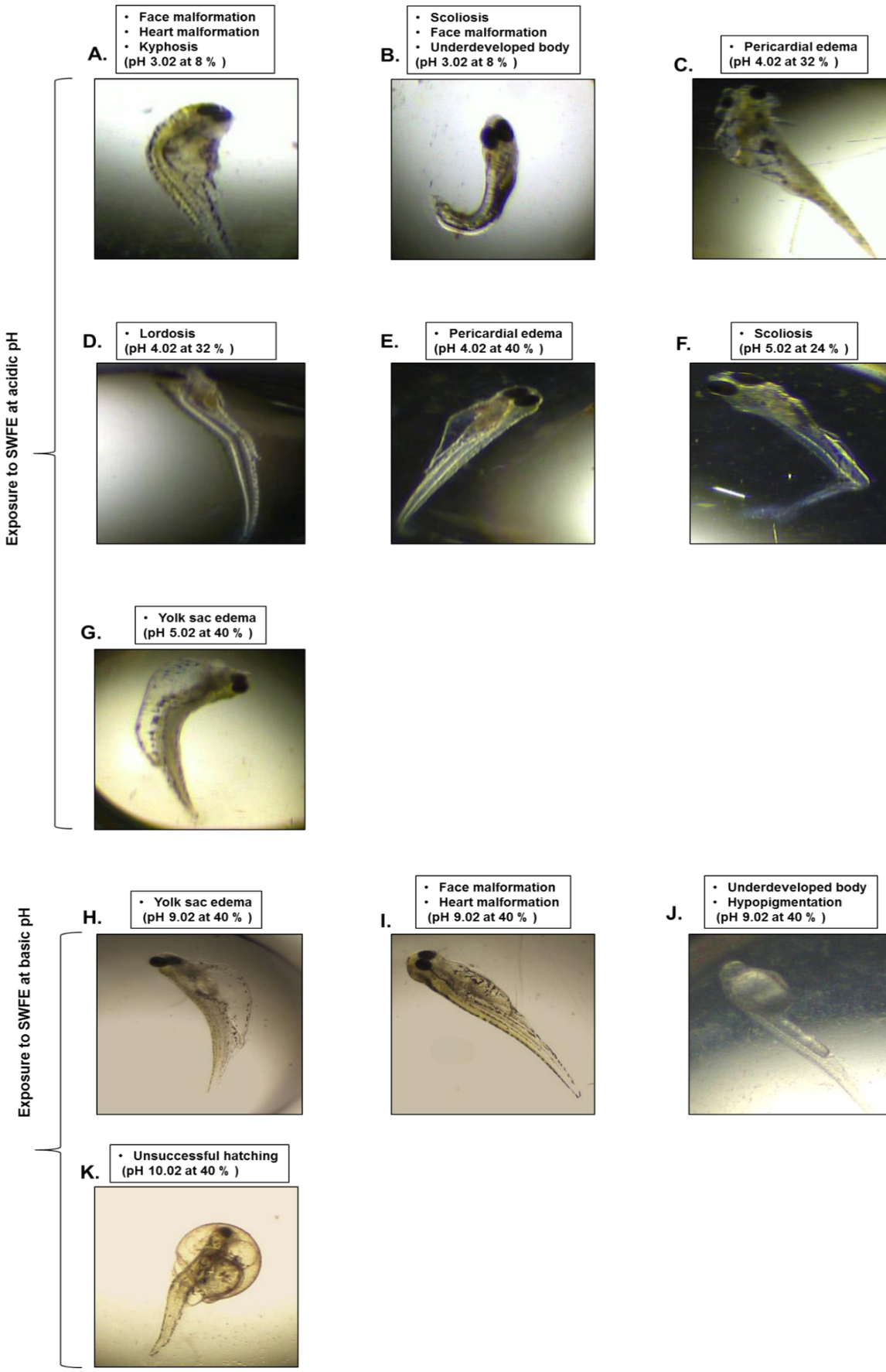


Figure 3.4 Malformations of zebrafish (144 hpf) due to change in pH of SWFE. The pH tested was 3.02 - 11.02. Edema: Swelling caused by excess fluid trapped in body tissues. Lordosis: Inward curvature of the spine. Scoliosis: Sideways curvature of the spine. Kyphosis: excessive outward curvature of the spine, causing hunching of the back.

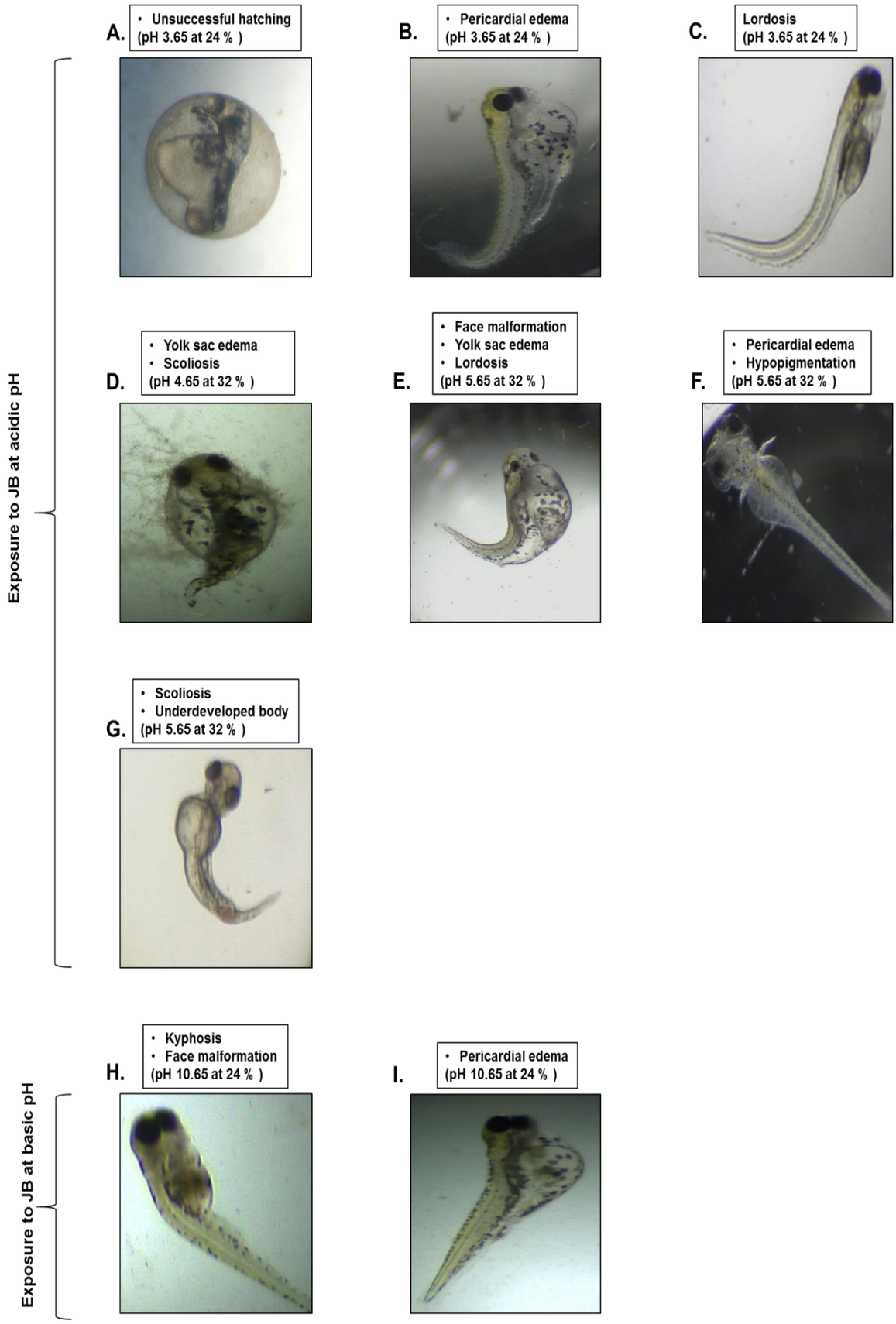


Figure 3.5: Malformations of zebrafish (144 hpf) due to change in pH of JB. The pH tested was 3.65 - 11.65. Edema: Swelling caused by excess fluid trapped in body tissues. Lordosis: Inward curvature of the spine. Scoliosis: Sideways curvature of the spine. Kyphosis: excessive outward curvature of the spine, causing hunching of the back.

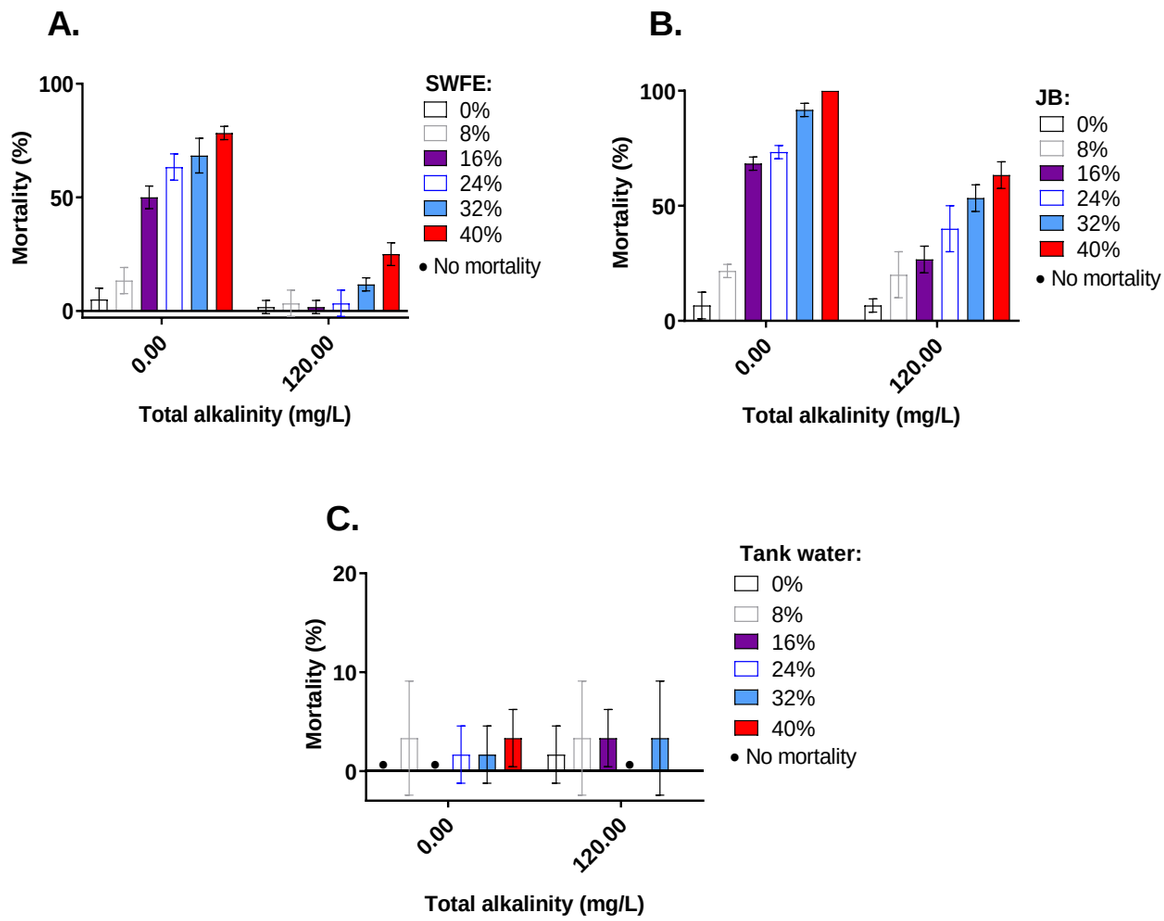


Figure 3.6: The effect of wastewater on zebrafish embryos at adjusted total alkalinity of (A) SWFE, (B) JB and (C) tank water. Alkalinity tested at 0 mg/L (sub-optimal) and 120 mg/L (optimal). The mortality rate of zebrafish embryos (144 hpf) is presented as mean ± SE for triplicate sets of data (20 embryos exposed per concentration for each experiment). Zero mortality is denoted as, no mortality: ●.

3.2.5 Hatching rate

The percentages of embryos hatched during zebrafish development (0 - 144 hpf) are indicated in **Figure 3.7**. During the early stages of development, no embryos hatched from 0-24 hpf in wastewater effluents tested (**Figure 3.7 A-F**). An exponential increase occurred in sample TB, TA, CI and IB (**Figure 3.7 A-D**) 24 to 48 hpf, in all of the concentrations tested (0 – 100 %). In contrast, hatching remained at low levels in SWFE and JB with the highest effluent concentration reducing hatching success to an average of ≤ 15 % at 48 hpf (**Figure 3.7 E-F**). After 72 hpf and beyond, hatching started to remain constant in all concentrations tested for TA, TB, IB and CI (**Figure 3.7 A-D**). Also, at the end of the experiment (144 hpf) wastewater TB, TA, IB and CI (20 – 100 %) exhibited no significant effect on the hatching percentage ($p > 0.05$) in comparison to the control (E3 medium, 0 %) (**Figure 3.7 A-D**). However, this trend was not consistent in concentrations tested for SWFE and JB. Instead proportions of hatched embryos were significantly reduced ($p < 0.05$) as opposed to control embryos upon the addition of effluent SWFE and JB at concentrations above 8 % (144 hpf). This occurred in a dose-dependent manner with the highest dosage (40 %) drastically lowering hatching success to a minimum of 20.00 ± 5.00 % and 6.67 ± 7.63 %, respectively for SWFE and JB at 144 hpf.

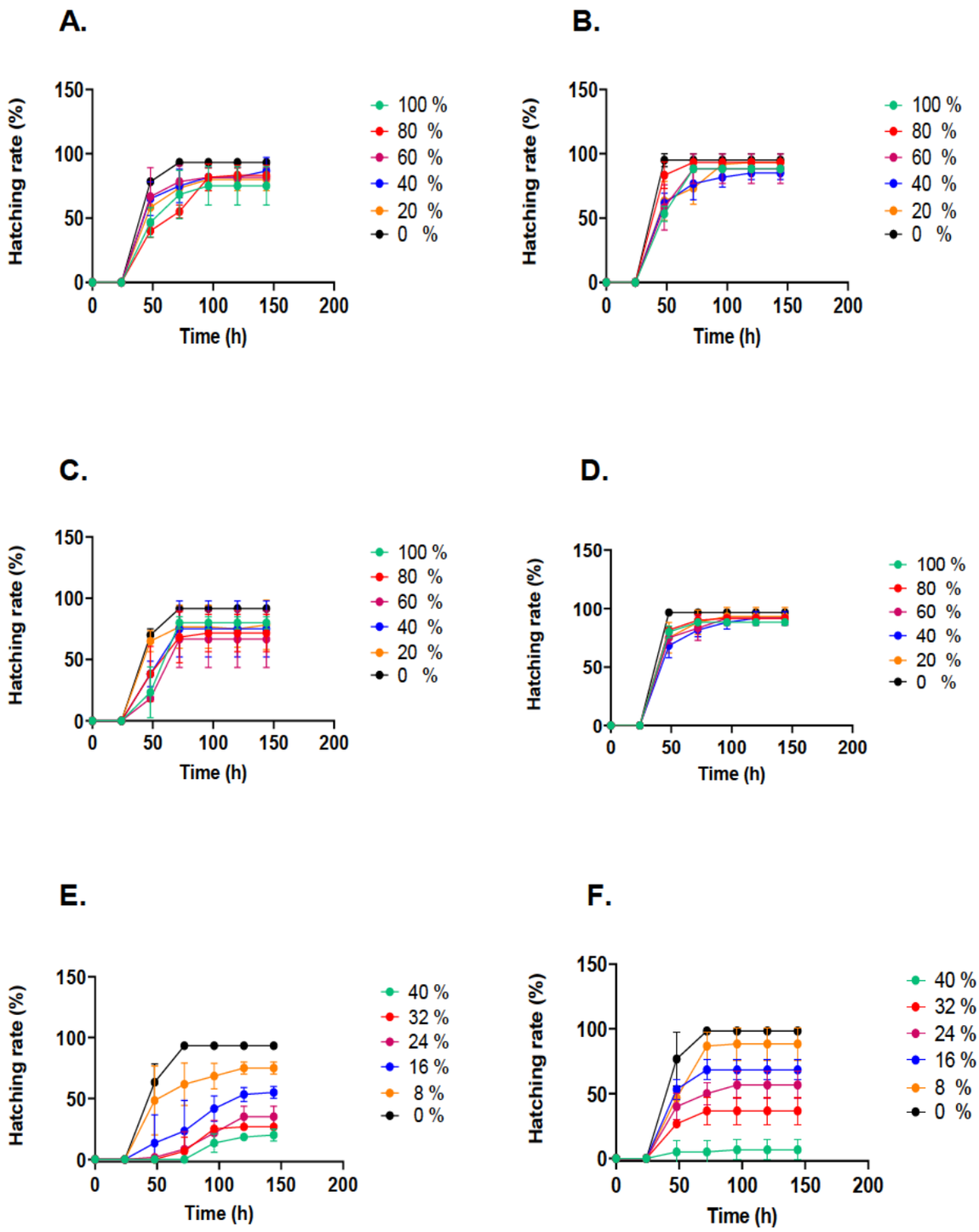


Figure 3.7 Hatching rate recorded for zebrafish embryos during 144 h exposure to wastewater samples. Sample (A) TB: Amanzimtoti before chlorination, (B) TA: Amanzimtoti after chlorination, (C) IB: Incoming Badulla, (D) CI: Chatsworth Incoming, (E) SWFE: Southern Works Final Effluents and (F) JB: Jacob's Incoming. Error bars depict triplicate experiments (20 embryos per concentration in each experiment). Data plotted using GraphPad Prism 9 XML Project software. Embryos were exposed to TB, TA, IB and CI at concentrations of 100, 80, 60, 40 and 20 % while SWFE and JB were tested at 40, 32, 24, 16 and 8 %.

3.3. Neurotoxicity

No neurotoxic effects occurred due to wastewater TB, TA and CI in all of the concentrations tested (20 - 100 %), as shown by the average spontaneous movements obtained. Spontaneous movements due to these effluents were statistically similar ($p > 0.05$) to those of the control zebrafish, which had a range of 0 - 4 mov.min^{-1} (**Figure 3.8**). Similarly, low concentrations of wastewater SWFE (8 %) and JB (≤ 16 %) induced no significant spontaneous movements ($p > 0.05$), in relation to embryos reared under optimal conditions. Notably, zero movement (0 mov.min-1) was recorded at higher concentrations of SWFE (≥ 16 %) and JB (≥ 24 %). This was, however, a reflection of delayed development observed under acute exposure to SWFE and JB, rather than wastewater impact on spontaneous movement. In contrast to the above-mentioned effluent samples, wastewater IB notably impacted spontaneous movements at effluent proportions ≥ 60 %, significantly increasing ($p < 0.05$) the average spontaneous movement to a maximum of about > 7.00 mov.min-1.

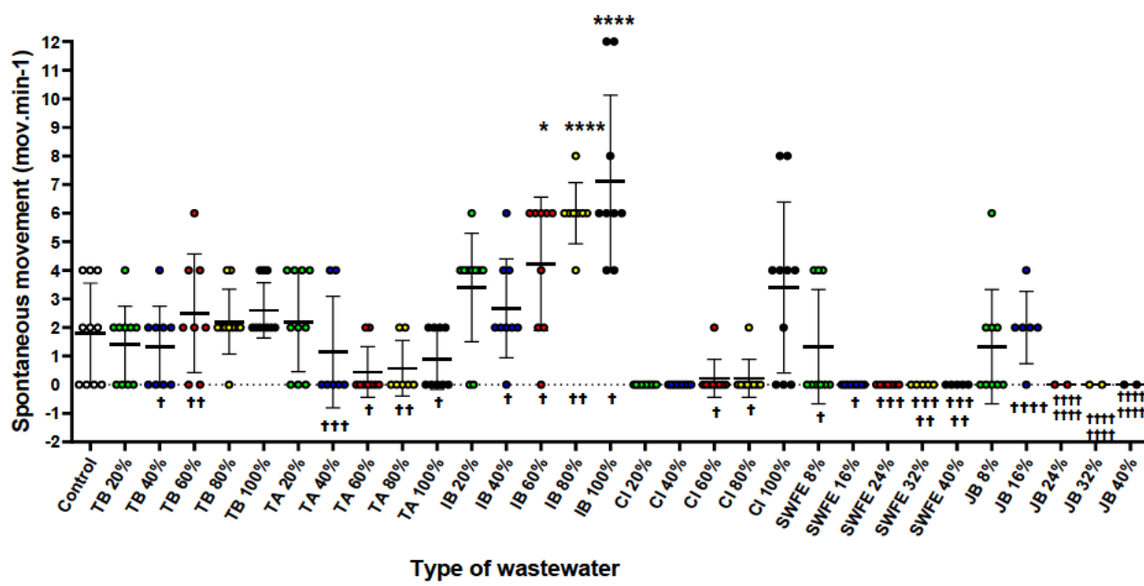


Figure 3.8 Effect of wastewater on zebrafish (24 hpf) spontaneous movement (mov. min−1) at the embryonic stage. Ten embryos were exposed per concentration of TB: Amanzimtoti before chlorination, TA: Amanzimtoti after chlorination, IB: Incoming Badulla, CI: Chatsworth Incoming, SWFE: Southern Works Final Effluents and JB: Jacob's Incoming. Control embryos were raised in E3 medium. Dead embryos were denoted as †. Data expressed as mean ± SD with asterisks representing p-value less than 0.05 (*), 0.01 (**), 0.001 (***) or 0.0001 (****). To analyze data GraphPad Prism 9 XML Project software was utilized.

3.4 Locomotor assay

Zebrafish larvae locomotor activity was quantified by deriving four swimming behavioural indicators, reported as; average distance travelled (**Figure 3.9**), frozen events (**Figure 3.10**), average acceleration (**Figure 3.11**), exploration rate and swimming trajectories (**Table 3.4**), during exposure to effluents. The distance travelled ranged from 989.88 to 104.23 mm following 144 h exposure to TB, TA, IB and CI, which was not significantly different ($p > 0.05$) to the range of 299.12 to 856.00 mm obtained for the control zebrafish (**Figure 3.9**). Similarly, zebrafish exposed to the above-stated effluents showed no statistical variation ($p > 0.05$) in the number of freezing events in relation to control counts (4 - 9 freezing events) (**Figure 3.10)**. Average acceleration rate also did not vary in a significant manner after treatment with TB, TA, IB and CI (**Figure 3.11**). In addition, projected moving trajectories revealed a similar trend in the coverage of surfaces of the 24-well plates for the control environment, TB, TA, IB and CI-exposed zebrafish (**Table 3.4**). This was further illustrated by the exploration rate ranging from 49.40 to 21.97 % for TB, TA, IB and CI, which remained within statistically similar levels with the non-exposed fish ($p > 0.05$).

Conversely, the average distance travelled by zebrafish larvae decreased significantly below the control range of 299.12 - 856.00 mm upon exposure to ≥ 32 % SWFE and ≥ 24 % JB effluents, reaching a minimum of 49.61 mm in SWFE and 48.00 mm in JB. Freezing counts were significantly upregulated ($p < 0.05$) in groups treated with SWFE and JB, upon concentration increase. A significant difference was also observed in average acceleration, where ≥ 32 % SWFE and ≥ 24 % JB reduced acceleration to minimum levels of 2.10 and 0.21 mm/s^2, respectively. Reduced movement of larvae in groups treated with SWFE and JB can also be seen in representative locomotor trajectories and exploration rate plots (**Table 3.4**), where the percentage of areas explored by zebrafish significantly decreased ($p < 0.05$) in a dose-dependent manner. Moreover, exposure to these wastewaters resulted to a loss in balance of the zebrafish larvae, causing the fish to lay down in one position, only moving its tail after several minutes of being still, or forcing the fish to settle to the bottom of the well-plate lying laterally on its body.

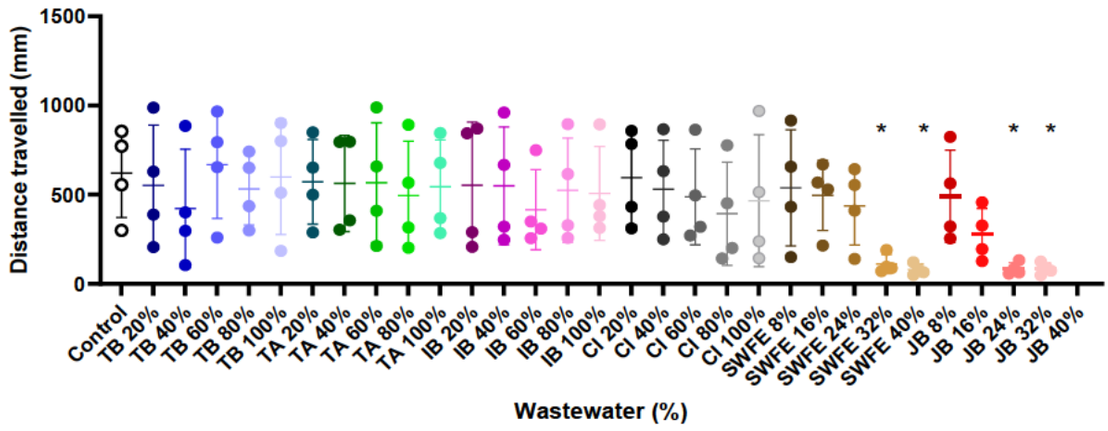


Figure 3.9: Average distances of zebrafish larvae (144 hpf) exposed to wastewater. Wastewaters tested to generate average distance (mm) using 1- 2 minute video recordings are TB: Amanzimtoti before chlorination (blue), TA: Amanzimtoti after chlorination (green), IB: Incoming Badulla (pink), CI: Chatsworth Incoming (black), SWFE: Southern Works Final Effluents (brown) and JB: Jacob's Incoming (red). Data expressed as mean ± SD with asterisks (*p < 0.05, **p < 0.01, ***p < 0.001 ****p < 0.0001) showing significant differences between the control and exposed groups (n=4, three fish per experiment). Distance travelled generated by Toxtrac software and analyzed using the GraphPad Prism 9 XML Project software.

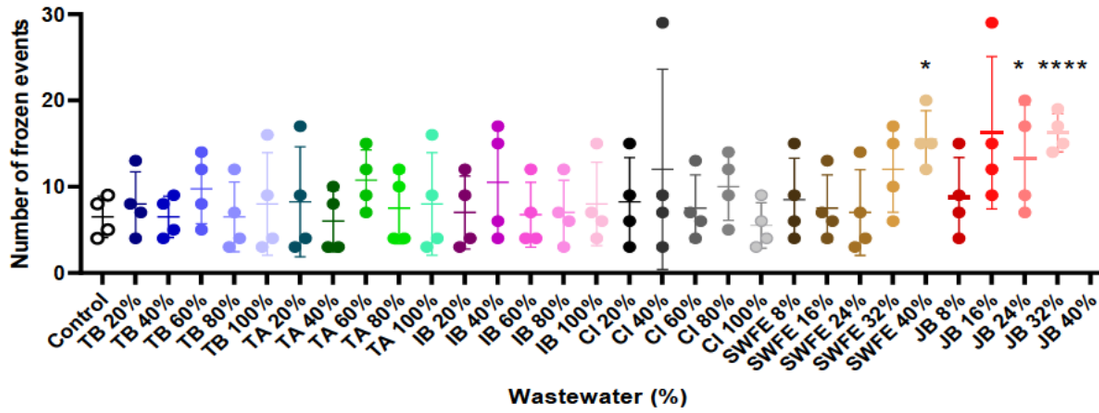


Figure 3.10: The number of frozen events of zebrafish larvae (144 hpf) in wastewater. The number of frozen events were recorded when zebrafish did not move more than 3 sec and/or 5 mm. Wastewaters tested were TB: Amanzimtoti before chlorination (blue), TA: Amanzimtoti after chlorination (green), IB: Incoming Badulla (pink), CI: Chatsworth Incoming (black), SWFE: Southern Works Final Effluents (brown) and JB: Jacob's Incoming (red). Data expressed as mean ± SD with asterisks (*p < 0.05, **p < 0.01, ***p < 0.001 ****p < 0.0001) showing significant differences between the control and exposed groups (n = 4, three fish per experiment. Frozen events generated by Toxtrac software and analyzed using the GraphPad Prism 9 XML Project software.

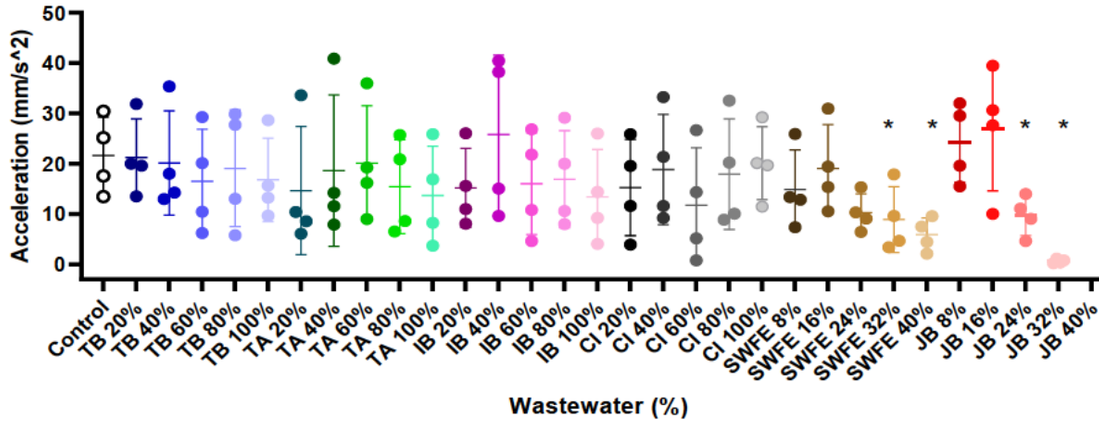
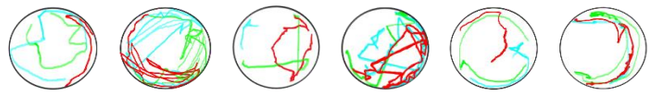
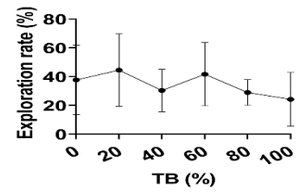
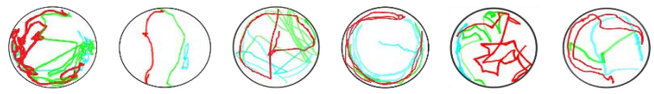
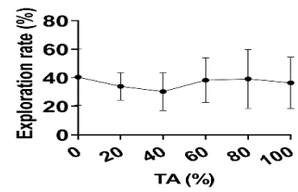
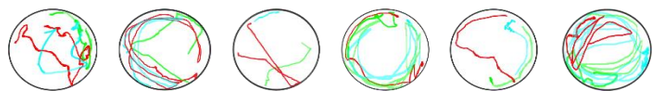
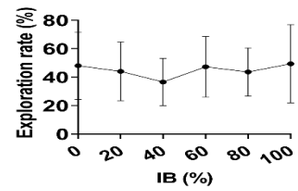
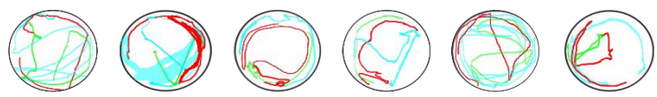
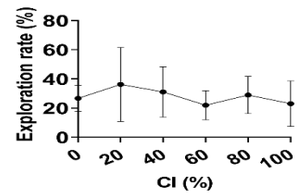
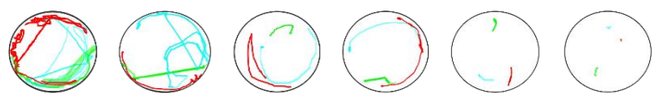
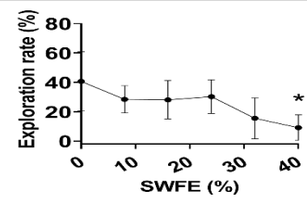
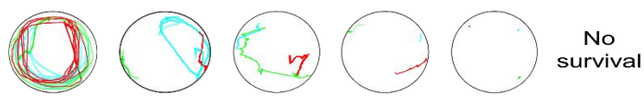
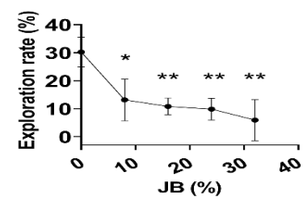


Figure 3.11: Average acceleration rate of zebrafish larvae (144 hpf) exposed to wastewater. Acceleration rate (mm/s^2) was recorded during exposure to TB: Amanzimtoti before chlorination (blue), TA: Amanzimtoti after chlorination (green), IB: Incoming Badulla (pink), CI: Chatsworth Incoming (black), SWFE: Southern Works Final Effluents (brown) and JB: Jacob's Incoming (red). Data expressed as mean ± SD with asterisks (*p < 0.05, **p < 0.01, ***p < 0.001 ****p < 0.0001) showing significant differences between the control and exposed groups (n = 4, three fish per experiment). Frozen events generated by Toxtrac software and analyzed using the GraphPad Prism 9 XML Project software.

Table 3.4: Movement trajectories of zebrafish larvae (144 hpf) exposed to wastewater with respect to the exploration rate

Wastewater	Zebrafish movement trajectories						Exploration rate (%)
Concentration (%)	0	20	40	60	80	100	
TB							
TA							
IB							
CI							
Concentration (%)	0	8	16	24	32	40	
SWFE							
JB							No survival 

Red, green and blue lines in the track visualization plots indicates the path followed by 3 independent zebrafish larvae (colour unique for each fish) during the 1 – 2 minute video recording. Experiments performed four times (n = 4), with one trajectory plot chosen to represent each concentration. Average exploration rate depicts the percentage of areas explored during swimming in TB: Amanzimtoti before chlorination, TA: Amanzimtoti after chlorination, IB: Incoming Badulla, CI: Chatsworth Incoming, SWFE: Southern Works Final Effluents and JB: Jacob's Incoming. Asterisks signify p-value less than 0.05 (*), 0.01 (**), 0.001 (***) or 0.0001 (****) comparing all treatments with control the (E3 medium, 0%). Trajectories and exploration rate generated by Toxtrac software and analyzed by the GraphPad Prism 9 XML Project software.

3.5 Expression of the heat shock protein 70 (HSP70)

To determine the physiological stress induced by wastewater that signalled low lethal toxicity (TB, TA, IB and CI) during characterization and previous assays, the expression of HSP70 was examined (**Figure 3.9**). Observation of stress protein expression that was instigated by these samples was done side by side in relation to the heat shocked (positive control) zebrafish larvae and fish reared under optimal conditions in E3 medium (negative control) (**Figure 3.9 A**). While analysing the expression of HSP70 in response to the different conditions, two bands were detected (**Figure 3.9 A**) – the first at approxiamately 70 kDa and the second at approximately 45 kDa. As expected, heat shocked zebrafish larvae expressed higher levels of HSP70, with statistically significant variation ($p < 0.05$) compared to the negative control group, for both bands (**Figure 3.9 A-C**). Conversely, protein levels of HSP70 in effuent TB, TA, IB and CI were expressed in a mannner that was not significant ($p > 0.05$) when compared to the detection observed in embryos kept in E3 medium, ie., negative control (**Figure 3.9 A-C**).

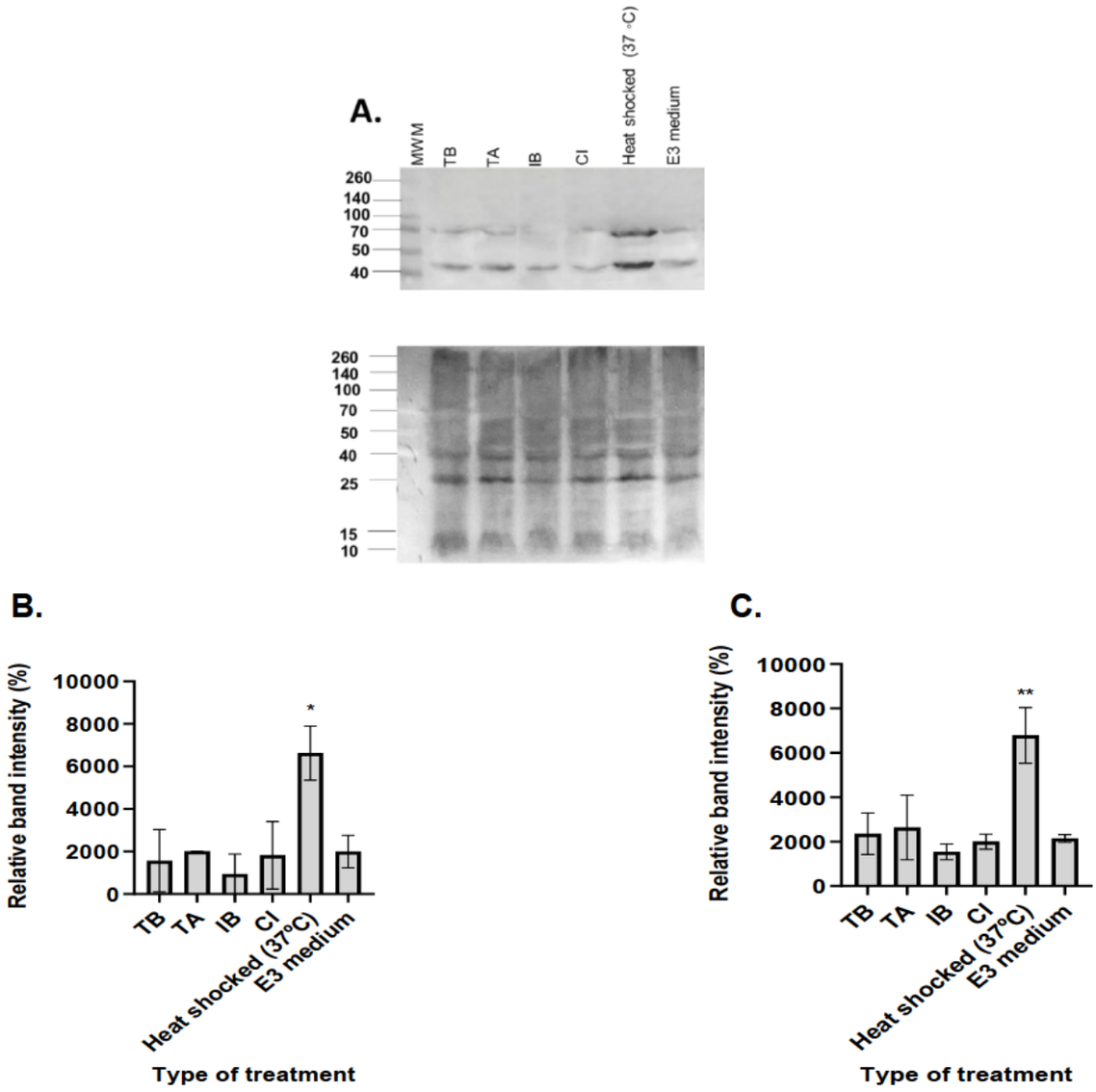


Figure 3.12: The detection of the heat shock protein 70 (HSP70) in relation to the protein concentration. (**A**) Western blot analysis of zebrafish (72 hpf) cell lysate with anti-HSP70 antibody in different wastewater samples (at 100 % concentration) as compared to the amido black total protein stain (TPS). MWM: Molecular weight marker, TB: Amanzimtoti before chlorination, B: TA: Amanzimtoti after chlorination, IB: Incoming Badulla and CI: Chatsworth Incoming. Positive control includes heat shocked zebrafish (37 °C, 1 h) while fish grown in E3 medium were the negative control. The corresponding relative band intensity for the (**B**) 70 kDa and (**C**) 45 kDa form of HSP is depicted as mean ± SD (n = 3). All band intensities were determined by applying the densitometry software (Image J).

CHAPTER 4: DISCUSSION

The global escalating inadequate management and disposal of urban, industrial and agricultural wastewater leads to an enormous strain to the limited available water resources worldwide, especiallly in developing countries such as SA. Also, to put into perspective, wastewater treatment facilities in SA are in a deteriorating state with lack of efficiency at removing the increasing ECs in water. As a consequence, it is critical to constantly monitor water quality including methods of remedition to remain well-informed of the environmental and health risks associated with these matters of concern. To accomplish this, advanced techniques could be employed to help reveal adverse effects of wastewater as well as any shortcomings of current water remedition techniques. A novel approach would be to use a suitable aquatic model organism that occurs in a wide range of habitats, with highly conserved physiological pathways that are found in higher vertebrates (including humans), along with a robust behavioral repertoire. By virtue of the above-stated issues, this study used zebrafish to monitor six different wastewater samples from different regions of SA to allow for investigation of the potential environmental and health defects due to wastewater and efficiency of water remedition methods.

4.1 Physico-chemical parameters of wastewater

Wastewater physicochemical properties were characterized for each of the six samples tested (TB, TA, IB, CI, SWFE and JB) in order to gain baseline information about the water quality. Most of the evaluated parameters presented indices within the permissible limits, indicating the minimum suitability for aquatic life (Lawrence *et al.*,2012;Tye *et al.*,2018;Aleström *et al.*,2020;Cueto-Escobedo *et al.*,2021;Del Vecchio *et al.*,2022). Notably, total chlorine (0 mg/L), free chlorine (0 mg/L), hardness (75-200 mg/L), nitrate (≤ 200 mg/L), nitrite (0 mg/L) and dissolved oxygen (≥ 4 mg/L) were at optimal levels. These outcomes were similar to values reported for effluents from WWTPs and hospital wastewater (Neri-Cruz *et al.*,2015;Ribeiro *et al.*,2020), where values did not exceed the prescribed guidelines for discharge of effluents into receiving waterbodies, appearing adequate for support of aquatic species survival. Although several of the currently investigated effluent water properties suggested the safety of the water for aquatic life, constant monitoring of wastewater effluents including all water systems at large, remains ideal. This is because parameters may vary from time to time primarily due to seasonal variation, weathering of rocks in water systems, depositions due to wind, leaching from soil, storm water run-off and aquatic biological processes (Singh and Tiwari,2019;Makuwa *et al.*,2022). Additionally, anthropogenic activities such as mining, agriculture, use of fertilizers, manures and pesticides, animal husbandry activities and pollution due to industrial effluents are amongst the principal factors that can lead to deviation of these physicochemical values from the optimal range in water systems. In addition, Mendonç*a et al*., concluded that physicochemical evaluation of samples does not always correlate with the observable effect induced on tested organisms. In their study, samples that alerted no potential hazard during chemical characterization surprisingly presented effects in tested organisms (Mendonça *et al.*,2009). Apart from the above-mentioned parameters, pH and total alkalinity indices reported for wastewater SWFE (pH = 9.02 ± 0.16 and

0 mg/L total alkalinity) and JB (pH = 5.65 ± 0.02 and 0 mg/L total alkalinity), did not conform to the permissible range. Similar values have been reported for wastewater effluents released into water resources in a number of countries across the world (Santos *et al.*,2008;Singh *et al.*,2012;Ribeiro *et al.*,2020;Butu *et al.*,2022) including developing countries, specifically, SA (Agoro *et al.*,2018;Olabode *et al.*,2020;Phungela *et al.*,2022), suggesting the inefficiency of the treatment works at producing effluents within the water quality guidelines. These observations signalled lack of safety and poor quality of wastewater SWFE and JB for aquatic life.

4.2 Lack of chlorine disinfection efficiency

In an effort to assess the effectiveness of chlorine disinfection as a widely used water remediation technique, the Amanzimtoti River, was treated with 99.5 % pure liquid gas chlorine. Briefly, chlorination of water is a highly recommended water disinfection method that is applied for the inactivation of waterborne pathogens including bacteria and viruses because of its easy deployment, broad sterilization, cost-effectiveness, and high efficiency (Lordache and Woinaroschy,2020). In previous studies this procedure has been found effective for the eradication of bacteria and viruses in water (Azuma and Hayashi,2021;Sun *et al.*,2021). However, during current investigations, chlorine disinfection with liquid gas chlorine was demonstrated as not highly efficient at disinfecting wastewater from the Amanzimtoti River. Surprisingly, the mortality rate of zebrafish embryos subjected to the chlorinated effluent (Amanzimtoti water after chlorination: TA) was ~25 %, showing no variation to the death rate obtained for embryos raised in non-chlorinated water (Amanzimtoti water before chlorination: TB). According to this data set, it can be deduced that contaminants in the Amanzimtoti wastewater were not completely eradicated by chlorination since the same number of fish died due to wastewater TB and TA. A possible explanation for the lack of efficiency demonstrated through our data and in previous work can be the ability of chlorination disinfection to promote microbial disinfectant resistance (Tong *et al.*,2021;Adefisoye and Olaniran,2022), thus promoting proliferation of microorganisms. In literature, the growth of microorganisms such as *E. coli* and *Zoogloea* bacteria have been shown to have the ability to multiply in chlorinated effluent (Pignata *et al.*,2012;Zerva *et al.*,2021). Perhaps pathogenic bacteria may have increased in numbers in the effluent even after chlorination of the wastewater, thus inducing harmful effects on zebrafish.

In accordance to our outcomes, Ponce-Palomera *et al.*, also demonstrated the inability of chlorination at sufficiently removing lethal wastewater substances, this was proven by the appearance of body malformations in zebrafish exposed to wastewater treated with chlorine gas (Ponce-Palomera *et al.*,2022). In their study, teratogenic effects characterized by edema of the pericardium, spinal curvature, incomplete eye formation and absence of the otolith resulted post chlorine disinfection. This clearly indicated the shortcomings of utilizing chlorine gas for the removal of water contaminants. Similar observations were made in other published work using sodium hypochlorite instead of chlorine gas (Da Costa *et al.*,2014;Affek *et al.*,2021), reporting the lack of chlorination at neutralizing or removing contaminants in wastewater, which further emphasizes that the use of the most commonly applied disinfectants in wastewater treatment should be reviewed.

Additionally, the lack of chlorination efficiency observed in this study was supported by Zhang *et al.* who revealed for the first time that water disinfection by chlorination does not secure a complete removal of viruses such as SARS-CoV-2 in medical wastewater (Zhang *et al.*,2020a). In their study, effluents and influents samples from the septic tank of a COVID-19 hospital in China were disinfected with 800 mg/L of sodium hypochlorite for 1.5 h with free chlorine of >6.5 mg/L. which was in accordance with the maximum guideline concentration of the World Health Organization suggested to be ≥ 0.5 mg/L for atleast 30 minutes contact time for chlorine as a residual disinfectant, and the China Centers for Disease Control and Prevention limits which recommends free chlorine above 6.5 mg/L after 1.5 h contact (WHO,2017;Chu *et al.*,2020;Wang *et al.*,2020;Clayton *et al.*,2021). However, following disinfection, free chlorine declined to non-detectable levels and the SARS-CoV-2 viral RNA was surprisingly present in the effluents. This indicated that even the recommended dosages of chlorine may not be effective at complete water remediation, as observed in the Amanzimtoti water after the application of the chlorine disinfectant. To overcome this challenge, an overdose of this disinfectant was found highly effective in documented findings (Zhang *et al.*,2020a). However, the major bottleneck faced by this approach pertains to high levels of disinfection by-products that may form as a result of the application of chlorine at increased concentrations (Richardson *et al.*,2007;Zhang *et al.*,2020a). These by-products include trichloromethane, tribromomethane, bromodichloromethane, and dibromochloromethane which can be harmful and carcinogenic (Li and Mitch,2018;Evlampidou *et al.*,2020;Mazhar *et al.*,2020). Based on earlier findings and observations made in the present study, water treatment guidelines recommended by the World Health Organization and China CDC need to be re-evaluated. Development of alternative strategies should be considered for the improvement of water remediation and reduction of disinfection by-products.

4.3 High mortality due to SWFE and JB

In addition to the inefficacy of chlorination to completely remediate effluents collected from the Amanzimtoti River, wastewater SWFE and JB revealed its toxic nature by significantly elevating the levels of mortality ($p < 0.05$) in a concentration-dependant pattern. The alarmingly high levels of mortality were observed alongside LC_{50} values of 17.77 % and 16.46 %, respective to SWFE and JB, with raw effluent prompting 100 % mortality (24 hpf). These observations together with the physicochemical parameters obtained for SWFE and JB (indicating sub-optimal indices in pH and total alkalinity) evidently signalled the threat posed by continuous discharge of wastewater effluents containing complex mixtures of contaminants from various sources. This brings into attention the importance of further analysis of these samples in future studies. Comparing the two samples, JB was slightly more toxic, taking into account the LC_{50} values obtained. In addition, zebrafish died most frequently due to coagulation and less commonly by lack of tail detachment, absence of heartbeat or lack of somite formation during the embryotoxicity test. These observations were generally in accord with various published work, where undiluted, raw and/or untreated hospital wastewater, mining effluent and receiving water, tannery emissions, as well as partially treated WWTP effluents caused high acute toxicity leading to 100 % death of the fish (Affandi *et al.*,2019;Ribeiro *et al.*,2020;Ashraf *et al.*,2020;Wittlerova *et al.*,2020a). Similar to our

findings, death increased proportionally to the effluent concentration in the aforementioned studies. The cumulative increase apparent post exposure to wastewater such as SWFE and JB can be credited to the complex mixture of a broad spectrum of foreign substances present in wastewaters, which are prevalent in varying nature and quantities, depending on the type of industry that serves as a source of origin. Moreover, numerous studies in various countries, including SA, have reported affluent and effluent wastewater as rich in, but not limited to, toxic heavy metals (Agoro *et al.*,2020;Iloms *et al.*,2020), carcinogenic and immunosuppressing industrial chemicals (Waheed *et al.*,2021;Kishor *et al.*,2021), oils and grease (Cirne *et al.*,2016;Wei *et al.*,2020) as well as disease-causing pathogens such as bacteria (Xie *et al.*,2022) and viruses (Rector *et al.*,2022). Therefore, the concerning high fish mortality can be a consequence of these contaminants. Not to disregard the accumulating synthetic and naturally occurring ECs which are not commonly monitored in water systems such as pharmaceuticals, pesticides, antibiotics, endocrine disrupting chemicals and UV filters (Kumar *et al.*,2022). In addition, the above-stated wastewater contaminants, particularly ECs, have been reported to pose a challenge for current WWTPs to effectively remediate water, resulting to their detection even after water treatment, at concentrations close to, or exceeding safe limits known to induce no effect (Tran *et al.*,2018). Taking into consideration the adverse effects and prevalence of wastewater contaminants, discharging of waste into water bodies linked to the inefficiency of WWTPs to completely remediate water before release into natural resources can pose harmful effects to the environment and health of aquatic and non-aquatic organisms. The major concern of wastewater discharge onto freshwater courses is the impact they have on public health, since scientists have identified microorganisms that can cause ill health, including bacteria, fungi, viruses, protozoa, protozoan parasites and parasitic worms (Alberts *et al.*,2002;Chahal *et al.*,2016;Barrow *et al.*,2020). This could subsequently result in the transmission of waterborne diseases of various magnitude to organisms that consume wastewater-contaminated water such as the wildlife animals, farmers' livestock and humans that use it for domestic and irrigation purposes amongst others. In addition, since humans share a high degree of similarity with zebrafish, they are amongst the living organisms at a high at risk of experiencing similar adverse effects upon chronic exposure.

In contrast to SWFE and JB, four wastewater effluents had a mortality rate below 50 %, with a lethal toxicity that was exhibited only at ≥ 80% in only IB and CI. There were no malformations observed due to all four of these effluents, suggesting that zebrafish could withstand or adapt in the presence of contaminants in the above-stated effluents. It is evident that the aforementioned effluents contained minor concentrations of pollutants that were less hazardous since zebrafish were able to resist any detrimental effects. There were no occurrences of edema (excessive accumulation of fluid inside the body cavity or tissues), underdevelopment, spinal cord curvature nor pigmentation reduction (Lent-Schochet and Jialal,2019). Concurrently, as observed previously, physicochemical parameters of these water samples indicated no potential threat for aquatic species, with indices that met the permissible water quality standards.

4.4 Malformations induced by SWFE and JB

Wastewater TB, TA, IB and CI had no significant effect on the early development of zebrafish during 6 days of exposure even at high concentrations (0 - 100 %). These results were similar to observations made for wastewater effluent samples in a previous study where wastewater did not cause any malformations in the developing zebrafish (Frieberg,2018), indicating that these samples had low levels of contaminants to give rise to adverse effects. In contrast, at concentrations above and equal to 24 %, wastewater SWFE and JB induced morphological changes such as craniofacial abnormalities, pericardial and yolk sac oedema, notochord and somite defects as well as scoliosis. In short, scoliosis refers to the by sideways curvature of the spine/tail (Thalengala *et al.*,2021). These findings are concordant with previously published work where zebrafish exposed to wastewater effluent samples collected from WWTPs in Brazil and hospitals, resulted in malformation of the head, yolk sac, tail, pericardial oedema, spinal cord curvature and pigmentation changes (Ribeiro *et al.*,2020;Wittlerova *et al.*,2020b). Similarly, Babić and colleagues reported that exposure of zebrafish to sewage effluents results in brain and eye retardation, malformation of the notochord and impaired muscle organization at 48 hpf (Babić *et al.*,2017). Based on these recent findings and our results, it is evident that wastewater makes up a complex mixture of highly toxic components at even low concentrations as observed for SWFE and JB. Literature also reveals that there is a broad range of synthetic and naturally occurring chemicals regularly detected in aquatic ecosystems as well as wastewater. This includes considerable amounts of pharmaceuticals, heavy metals, emerging contaminants such as endocrine disruptors, antibiotics, pesticides and other such pollutants (Peteffi *et al.*,2018;Patel *et al.*,2019;Kinuthia *et al.*,2020;Baralla *et al.*,2021;Cooper *et al.*,2021;Syafrudin *et al.*,2021). Amongst these, endocrine disruptors (e.g., bisphenol A) and herbicides (e.g., atrazine) were reported to cause craniofacial deformities in zebrafish embryos when tested at 0.0038 µM and ≥ 4 µM, respectively, which are concentrations that can be present in water bodies (Walker *et al.*,2018;Huang *et al.*,2020;Huang *et al.*,2021). Furthermore, Yuqiong and colleagues reported fungicides that are widely used for plant diseases in agricultural industries as a cause of abnormal length and width of zebrafish jaws (Wu *et al.*,2020). Given the fact that these contaminants can be detected in the aquatic environment, it is apparent that the aforementioned abnormalities observed may have been induced by their presence following exposure to wastewater. Similar to our results, abnormal eye development was also observed in a previous study under the influence of pharmaceuticals, which was indicated by the increase in thickness of the retina in zebrafish at environmentally relevant concentrations (≤ 50 µg/L) (Van de Perre *et al.*,2022). In addition, pericardial and yolk sac oedema, notochord and somite defects as well as scoliosis were reported to be caused by pollutants such as heavy metals synthetic compounds and antibiotics (von Hellfeld *et al.*,2020). This highlights the need for more efficient removal of pollutants from the aquatic environment since pollutants in different industries can be dispersed into localized water environments, potentially forming a considerable portion of wastewater that was seen to cause deformities in zebrafish in the present study.

4.5 Change in pH reduces toxicity of wastewater SWFE and JB

The acidification and alkalinisation of water systems as a result of climate change and other anthropogenic practices has become one of the serious environmental concerns globally (Oberholster *et al.*,2017;Van Niekerk *et al.*,2022;Jiang *et al.*,2022). Acid rain and chemical waste pollution can cause the decrease of local fish population by shifting the natural water pH to acidic. On the other hand, it may combine with the effects of ocean acidification to produce even more extreme events, resulting in an even greater impact on the biota pH. While several species have enough physiological plasticity to cope with acidification, many may not be able to cope with the two extremes of acidity and alkalinity in the marine and estuarine environment. In the current study, pH as one of the critical physicochemical parameters in water was assessed in all samples. Two of the wastewaters displayed sub-optimal pH, which were namely, SWFE and JB, with values that were outside the permissible limit (6.8 – 8.5), specifically pH 9.02 in effluent SWFE and 5.65 in JB. As demonstrated earlier, these samples had the highest proportion of mortality compared to the rest of the samples.

To gain an insight of the cause-effect relationship, between the sub-optimal pH of these effluents and zebrafish, a broad range of pH (including the recommended limits) was investigated for both samples. In an attempt to decipher the sub-optimal indices, it can be said that, generally sewage and industrial effluent contain a wide variety of foreign substances such as, but not limited to, pathogens and a broad mixture of hazardous chemicals and compounds that can be toxic and harmful to aquatic environments and the overall public health (El-Lathy *et al.*,2009;Ahmed *et al.*,2021;Zhang *et al.*,2021;Duan *et al.*,2022;Brunelle *et al.*,2022). The introduction of these contaminants to wastewater may be a result of uncontrolled industrial and domestic waste disposal, agricultural activities, surface run-offs or storm-water (Gothandam *et al.*,2020;Ahmed *et al.*,2021), thus causing a change in pH. Due to the high concentration of a variety of pollutants that may cause sub-optimal conditions for aquatic life and a stray in pH from the acceptable limits in wastewaters. In an effort to remediate effluent SWFE and JB, as well as to find the optimum pH to effectively neutralize contaminants in the wastewater, pH was adjusted to fall within the permissible pH limits of 6.8 - 8.5 while considering values in the lower and upper regions of this range. A very low mortality rate, statistically similar to the control group ($p > 0.05$) was obtained within the pH 6.02 - 8.02 and 6.65 - 7.65, respectively for SWFE and JB, indicating effective neutralization of contaminants in SWFE and JB. In addition, there were no body malformations on the zebrafish within this non-lethal range. Both of the non-lethal pH limits obtained for SWFE and JB were in accordance with literature, since zebrafish typically inhabit water environments with pH varying from 6 to 10 (Engeszer *et al.*,2007;Arunachalam *et al.*,2013;Aleström *et al.*,2020).

On the basis of these considerations, it is apparent that zebrafish were able to better withstand the toxicity of contaminants in wastewater at pH 6.02 - 8.02 and 6.65 - 7.65, respectively for SWFE and JB. This can be due to the circumstance that the obtained non-lethal limits were similar to the recommended pH in literature for aquatic species' survival. Therefore, within this non-deadly range of the effluent, zebrafish were in a healthy state with fully developed body structures, displaying no discernible malformations, thus exhibiting resistance to pollutants in the water with

a reduced mortality rate. Secondly, this notable resistance of zebrafish to SWFE and JB acquired through pH change, can be attributed to contaminants that were neutralized in the wastewater. This postulation can be supported by previous reports suggesting chemical pH adjustment with sodium hydroxide or calcium hydroxide as one of the conventional processes that significantly neutralizes industrial wastewater by removing heavy metals (Shah *et al.*,2021;Qasem *et al.*,2021). This process is generally referred to as chemical precipitation (Saleh *et al.*,2022). Different chemical precipitation techniques includes hydroxide precipitation, sulfide precipitation, chelating precipitation. In the current study, hydroxide precipitation was used. During this heavy metal removal technique dissolved contaminants such as metals were converted into insoluble particles upon the addition of the precipitant chemical, sodium hydroxide. Reduction in mortality after the addition of sodium hydroxide under our investigations can be explained by that sodium hydroxide reacted with heavy metal ions to form an insoluble precipitate of metal hydroxides thus allowing for removal of these metals, as explained in literature (Dahman *et al.*,2017;Saravanan and Kumar,2021). In support of our findings, the work of Balintova and Petrilakova reveals to a greater extent, how application of this technique can lead to the removal of considerable contents of heavy metals such as iron, copper, zinc, aluminium and manganese in an acid mine drainage solution (Balintova and Petrilakova,2011). In their study, neutralizing the acid mine drainage by increasing pH up to 8.2 with sodium hydroxide eradicated about 99.9 % of aluminium, 96.6 % of iron, 93.3 % of zinc, 92.3 % of copper, and 15.9 % of manganese in the solution. As additional evidence, a study conducted by Saloua *et al.,* demonstrated good efficiency of the uptake of copper, cadmium, iron, cobalt and zinc from industrial wastewater by chemical precipitation at an optimum pH of between 6.0 and 10.0 (Saloua *et al.*,2020). Taking into consideration the pH observed for neutralization of SWFE and JB (6.02 - 8.02 and 6.65 - 7.65, respectively), which led to a reduction in the mortality rate in the current investigation, this range is comparable to the optimum pH (6 - 10) reported for wastewater heavy metal removal in literature. Therefore, the remarkably drastic decrease in mortality in SWFE and JB under non-lethal pH can be postulated to be an outcome of the efficient removal of contaminants such as heavy metals upon change in solubility by pH adjustment.

Although chemical precipitation has been demonstrated to remove a considerable portion of contaminants in wastewater, despite the adjustment of pH, the efficiency of this method is dependent on several variables. These include metal ion concentration, type of metal present in solution, nature of the precipitant used and constituents which can compete or form soluble complexes with the target metal species, thus inhibiting precipitation (Dahman,2017;Pohl,2020;Serrano *et al.*,2021). Also, it is noteworthy that each dissolved metal has a distinct pH value at which the optimum hydroxide precipitation occurs (Abdullah *et al.*,1999;Zainuddin *et al.*,2019). More precisely, an ideal pH required for minimizing the solubility of one metal for its eradication may relatively increase the solubility of another. This variation in optimum pH for precipitation poses a challenge for remediation of wastewaters from industrial processes such as wastewater SWFE and JB since they contain several metals. Due to the above-mentioned limitation, further water remediation steps are of a necessity to follow during wastewater treatment after performing chemical precipitation.

4.6 High acidity and alkalinity increases toxicity of contaminants in wastewater

To further demonstrate the potential toxicity of wastewater SWFE and JB, acute acidity and alkalinity, outside the non-lethal range of pH was also assessed. Highly acidic (pH < 6.02) and basic (pH > 8.02) conditions of wastewater SWFE yielded a significant effect ($p < 0.05$) on zebrafish as the effluent concentration was increased, compared to the non-toxic range, with pH 3.02 exhibiting the highest toxicity since there was 100 % mortality at higher concentrations of the effluent. It was evident that continuous drop or rise in pH exerted a significant impact on the toxicity of contaminants in the effluent since this led to a decline in the number of zebrafish exposed during 144 hpf. With regards to the effect of JB, outcomes were markedly similar to those of effluent SWFE. It was noted that under the most acidic (pH < 6.65) and basic (pH > 7.65) JB environment the death rate significantly escalated ($p < 0.05$) in a concentration dependant manner, in contrast to the non-deadly pH levels. This further highlighted the endangerment of aquatic and non-aquatic species exposed to wastewater, since sample JB was illustrated to prompt high mortality at sub-optimal pH, like SWFE, with a noticeable 100% death at higher concentrations, specifically at pH 3.65, pH 4.65 and 5.65 and 11.65. These observations were in accord with the work of Affandi and co-workers, who reported the effect of tin mining effluent and receiving water at different pH using zebrafish as a model organism (Affandi *et al.*,2019). In their pioneering study, high acidity (pH < 6.19) at lethal concentrations of the mine effluent were demonstrated to be the fundamental cause of 100 % fish mortality. According to literature, exposure of aquatic species to pH as low as 2.0 and as high as 12.0 can cause fish to turn opaque and succumb within 2 hours of exposure (Zahangir *et al.*,2015a). This was further corroborated in an in vivo study of Zahangir *et al*., where changes in the level of pH were demonstrated to induce chronic physical and internal stress in aquatic organisms (Zahangir *et al.*,2015b), which potentially leads to death. As observed in the present study and previous work, deviation of pH from the recommended limits in wastewaters harms aquatic species.

In addition to the high mortality at sub-optimal pH, zebrafish displayed physical deformities following incubation under acidic and alkaline pH outside the non-lethal range, namely, edema of the pericardium and yolk sac, spinal cord curvature, craniofacial malformations, unsuccessful hatching and pigmentation changes. These results suggest that aquatic species can be highly sensitive to abrupt changes in pH in water systems since sudden pH adjustment was demonstrated to be capable of exerting considerable negative biological effects on the tested aquatic zebrafish. Moreover, this data emphasises that pH outside of the optimal range worsens the harmfulness of water contaminants. Occurrence of malformations within the lethal limits furthermore served as an indication that, although extreme pH conditions may not immediately result in acute mortality, chronic exposure of the few fish that survived in these conditions may lead to stress and subsequent health defects. This was also showcased in the previous work of Dos Santos *et al.,* where in agreement with our findings, they demonstrated that malformations manifested due to extreme levels of pH (Dos Santos *et al.*,2020). In their work, malformations were characterized by deformities in the yolk-sac, lordosis-type curving of the spine, decrease in total body length, as well as a decrease in both the depth and length of the head or absence of the head. Several earlier studies also revealed that exposure to decreasing acid concentrations

and increasing alkaline concentrations generally led to a decline in hatching rates, as observed in our current investigations (Zahangir *et al.*,2015a;Lin *et al.*,2019;Ismail and Aripin,2020). Additionally, at pH 3.0 adult zebrafish appeared pale in colour after approximately 1 h of exposure following observations made by Zahangir and colleagues, which was also found in our tested fish (Zahangir *et al.*,2015b). Despite the outlined malformations, other studies revealed that non-optimal water pH can immunosuppress aquatic organisms as far as causing breakdown of gill structure, suffocating the fish by mucus accumulation (pH 2.0 – 3.5), denaturation of cellular membranes (pH 9 - 14) and decreasing body length (pH 4.0) (Kwong *et al.*,2014;Liu *et al.*,2018). In marine fishes, acidification was found to a greater extent reported to impair sensory and brain functions (Simpson *et al.*,2011;Ferrari *et al.*,2012;Munday *et al.*,2014;Zahangir *et al.*,2015b). Our data and literature findings coherently show beyond doubt that pH fluctuations can result into decreased immune system function, which can pre-dispose the fish to various bacterial, parasitic and viral infections.

To help elucidate the enhanced lethality and health defects observed in the present study and previous work due to extreme effluent pH, a number of studies report on the relationship between pH and contaminants that may be found in wastewater (Roberts and Palmeiro,2008;Peng *et al.*,2021;Saalidong *et al.*,2022). According to literature, in addition to eradicating a part of wastewater contaminants by chemical precipitation, pH is capable of modifying the chemical state of numerous pollutants to a toxic form, as well as to interfere with their transport and bioavailability (Başak and Alagha,2010;Wang *et al.*,2016;Hong *et al.*,2020;Parvathy *et al.*,2022;Ohoro *et al.*,2022). This has been demonstrated in a number of studies, where contaminants such as ammonia have been reported to occur in an un-ionized toxic form that can kill aquatic species, under high pH environments (Levit,2010;Francis-Floyd *et al.*,2022). In addition, it has been reported that for every pH increase of one unit, the amount of toxic unionized ammonia increases about 10 times (Durborow *et al.*,1997). In contrast, compounds such as phenols have been revealed to increase in toxicity with decreasing pH (Saarikoski and Viluksela,1981). Moreover, the toxicity of ECs such as ionisable organic pollutants that include pharmaceuticals (antibiotic antimycin and antidepressant fluoxetine), pesticides (dichlorophenol) and personal care product ingredients (triclosan) can also be influenced by the variability in pH in aquatic systems (Marking,1975;Holcombe *et al.*,1980;Boström and Berglund,2015;Sun *et al.*,2020a;Ohoro *et al.*,2022). Zinc, cadmium, iron, manganese, copper and aluminium generally increase in concentration under low pH, accumulating into potentially more toxic forms (Spry and Wiener,1991;Johansson *et al.*,1995;De La Torre *et al.*,2010), as their precipitation occurs at higher pH levels (Singh and Rawat,1985;Balintova and Petrilakova,2011;Saloua *et al.*,2020). Therefore, theoretically, the high mortality proportion and body deformities evident under lethal pH in SWFE and JB can be attributed to the interference of pH with contaminants. In addition to heavy metals, compounds and ECs the elevated levels of toxicity in SWFE and JB may also be linked to harmful algae overgrowth, which thrives and multiplies in water with high pH (7.00 – 9.00) (Price and Farag,2013), or fungal growth that grows over a wide pH optimum in water (Ali *et al.*,2017). Moreover, their health can be compromised by not only the aforementioned contaminants but also microbial waterborne diseases caused by acid and/or alkaline tolerant pathogens. These includes, but not limited to, bacteria such as *Vibrio cholerae*, which is a

pathogenic agent of cholera that can grow optimally at pH 9.00 – 10.00 (Cabral,2010). The emerging metal tolerant pathogen: *Shigella sonnei* associated with shigellosis (bloody diarrhoea), predominantly found in sewage and wastewaters with the capability to tolerate metals dissolved at low pH (Silambarasan and Jayanthi,2010;Cabral,2010;Shad and Shad,2021). Also, agents of food-borne illnesses present in wastewater over a broad range of pH (4.05 –9.50) such as *Salmonellae* bacteria may be of a concern (Cabral,2010;Liu *et al.*,2018;Santiago *et al.*,2018). Upon consideration of the above-described pollutants that can be prevalent in wastewaters, along with the alerting harmful effect exerted by SWFE and JB, exposure under sub-optimal conditions highly endangers aquatic and non-aquatic living orgasms.

4.7 Change in pH of normal tank water provides contrasting findings to wastewater observations

In order to further demonstrate whether pH was a major contributing factor towards the neutralization of wastewater contaminants, various levels of pH were tested in tank water. Interestingly, there was no significant impact on mortality ($p > 0.05$) due to acidic pH, with only one fish developing edema post-exposure to the highest tank water concentration (40 %, diluted in E3 medium). Contrary to acidic environments, basic pH significantly elevated mortality percentage ($p < 0.05$), as expected. The resistance exhibited by zebrafish against acidic tank water contrasted outcomes obtained for SWFE and JB, which conveyed the detrimental effect of highly acidic and alkaline wastewater. Nonetheless, in support of these outcomes, diverse adaptation mechanisms of fish natively living in acidic environments have been reported (Kwong *et al.*,2014).

In literature, zebrafish physiological responses that ultimately contribute to ionic and acid–base homeostasis during exposure to acidic environments, includes the activation of the sodium / hydrogen exchanger (NHE) and H+-ATPase for acid secretion and Na+ uptake, cortisol-mediated regulation of transcellular and paracellular Na+ movements, as well as ionocyte proliferation (Goss *et al.*,1992;Claiborne *et al.*,2002;Gilmour and Perry,2009;Zimmer and Perry,2022). Furthermore, Zahangir *et al*., revealed that zebrafish can tolerate sub-optimal pH environments by developing biochemical and physiological adaptations to cope with these constraints (Zahangir *et al.*,2015b). The above proposed explanation from previous work corroborates our research outcomes and highlights that, pH alone was not the primary reason for the toxicity of SWFE and JB. Based on the survival of fish under acidic tank water, it is apparent that pH was not the only factor of concern but rather this parameter exacerbated the harmful effects of the foreign substances present in the tested effluents. Overall, these findings coincided with the earlier mentioned theory about the relationship between pH and water components (Roberts and Palmeiro,2008;Peng *et al.*,2021;Saalidong *et al.*,2022). Given the susceptibility issue of aquatic species to extreme levels of pH in contaminated water, it is evident that continuous uncontrolled waste disposal in conjunction with abrupt pH changes caused by natural or indirect anthropogenic processes, should be recognized as a matter of great concern.

4.8 Increase in toxicity of wastewater SWFE and JB due to sub-optimal total alkalinity

Upon water characterization, total alkalinity was another parameter that presented sub-optimal indices for SWFE and JB, which was detected as 0 mg/L for both samples. As a consequence, $NaHCO_3$ was used to supplement the wastewater alkalinity up to 120 mg/L, which is within the ideal range. This step was performed for the same reason as pH, which is to determine whether total alkalinity significantly contributes towards the lethality caused by contaminants in SWFE and JB. Following the evaluation of the effect of alkalinity at 0 vs 120 mg/L, there was a significant decrease in the zebrafish mortality rate ($p < 0.05$) at ≥ 16 % of SWFE and JB. This was postulated to be a sign of water contaminant neutralization / remediation.

A maximum mean mortality of 25 % was obtained in SWFE at 40 % which was 2.5 fold lower than the mortality obtained in the same concentration for JB. The observed variability revealed that contaminants in wastewater JB are more potent or in higher concentrations in comparison to SWFE pollutants, since a lower restoration occurred in water JB. Change in pH did not have a significant impact on zebrafish exposed to tank water. The high survival under sub-optimal total alkalinity suggest that zebrafish are able to withstand a broad range of total alkalinity. Similarly, previous studies demonstrated the ability of embryos to acclimatize under varying salt concentrations (Sawant *et al.*,2001). In their work, the survival and hatching of zebrafish reared at salinities of up to 2.00 parts per thousand (ppt) displayed were similar to the rate of fish raised at 0.30 ppt. In addition, salinity tolerance of the zebrafish embryos improved with advancing developmental stages. Although these findings reflect the ability of the fish to adapt to varying habitats, this can negatively impact egg production and survivability (Boisen *et al.*,2003).

According to Furtado *et al*., hydrated sodium bicarbonate appeared to be highly effective in retaining good water quality during rearing of shrimps (Furtado *et al.*,2011). According to their findings, water where alkalinity was maintained above 100 mg/L using $NaHCO_3$ led to improved shrimp growth. The improved survival of embryos under optimized total alkalinity appeared to differ significantly ($p < 0.05$) from the control group of shrimps which were raised without the correction of total alkalinity during the 60 day experiment. A lower growth performance occurred at the control environment, thus verifying the effectiveness of $NaHCO_3$ as an alkalizing chemical compound at supplementing total alkalinity. Similar observations were made in other work, highlighting the utilization of $NaHCO_3$ as an applicable and appropriate approach to allow for favourable water conditions leading to better physiological health, higher growth and net yield of aquatic species (Zhang *et al.*,2017;Martins *et al.*,2017). To a greater extent, other numerous earlier studies reveal the effect of different concentrations of $NaHCO_3$ on aquatic species such as the rainbow trout, pallid sturgeon, white sucker and fathead minnow amongst other fish (Farag and Harper,2012;Farag and Harper,2014;Harper *et al.*,2014). Our observations and the mentioned literature findings concordantly suggest that a drastic decrease in alkalinity below optimal levels as observed in SWFE and JB, can significantly impair the quality of water found in our aquatic systems.

Despite the apparent advantages of $NaHCO_3$ dosing to improve the life of aquatic species and quality of water under optimal conditions, the methodology is not without caveats. One of the

major concerns is variation in the tolerance of salts by different aquatic organisms, since some aquatic organisms evolved from marine water with the ability to tolerate high alkalinity, and also that, some fish have adapted periodic or seasonal changes in salt concentrations (Wurts,1998;Nielsen *et al.*,2003;Weber-Scannell and Duffy,2007). A few studies have been published that fall short of clearly illustrating the effects of $NaHCO_3$ in relation to the actual water components including toxic contaminants present in water systems. Much more augmented research should be conducted in this field to properly define potential adverse effects of $NaHCO_3$, mainly related to the interaction of pathogens and ionic composition with this alkalizing compound. Additionally, the effects of this method taking into account age within life stages of aquatic species is limited. Therefore, multiple approaches are still needed for the establishment of water-quality criteria research pertaining to supplementation of total alkalinity in water using $NaHCO_3$.

4.9 Reduction in hatching percentage due to SWFE and JB

Wastewater TB, TA, IB and CI had no significant impact ($p > 0.05$) on the proportion of hatched zebrafish embryos reared in over a period of 144 hpf. In these wastewater effluents, hatching occurred predominantly between 60 and 96 hpf as observed in the control, showing no variation in hatching success in comparison to untreated embryos 144 hpf ($p > 0.05$). This was a reflection of the high survival rate in these effluents, and therefore, it can be speculated that zebrafish hatched at a high rate due to being able to withstand the toxicity of contaminants in JB, thus escaping from the chorion to become free swimming larvae (Willemsen *et al.*,2011). This speculation is however controversial since other previous work report on the ability of ionic stress and chemicals such as tributyltin and ethanol, which can be found in aquatic environments, to prompt early hatching in previous studies (Liang *et al.*,2017;Ord,2019;Pinheiro-da-Silva and Luchiari,2021). This was explained as another important stress response that functions to enable zebrafish embryos to escape unfavourable conditions. As opposed to the above-stated effluents, SWFE and JB impaired the hatching rate of zebrafish in a dose-dependent manner with the highest concentration (40 %) impairing fish the most (SWFE = 20.00 ± 5.00 % and JB = 6.67 ± 7.63 %) at 144 hpf ($p < 0.05$), as a result of elevated levels of mortality and delayed development at the highest dosage of these effluents. These outcomes were in line with published work, reporting on the reduction in the hatching rate of zebrafish embryos exposed to wastewater released in water environments, negatively affecting the hatching success upon concentration increase (Ribeiro *et al.*,2020;Gauthier and Vijayan,2020;Golovko *et al.*,2021). Our outcomes suggested that wastewater effluents (such as SWFE and JB) contain contaminants that may be capable of highly interfering with the developmental stages of aquatic species such as zebrafish, thus inhibiting the hatching process. This raises a concern regarding spillage of these wastewaters into water resources. Moreover, literature reveals that the impairment of hatching in aquatic species can be a result of an interaction between heavy metals present in water bodies, with metalloproteases such as chorionase, which is an enzyme responsible for chorion (eggshell) disintegration during hatching of zebrafish (Hagenmaier,1974;Dave and Xiu,1991). Therefore, the inhibition of the hatching process due to SWFE and JB can perhaps be perceived as a metal-induced disturbance in the fish, leading to a decrease in chorionase efficiency. Another reason for the apparent decrease in hatching could be disturbances that occur during transcription and

translation which are caused by heavy metals therefore leading to a reduction in the synthesis of proteins such as chorionase (Kapur and Yadav,1982), as revealed in zebrafish embryos exposed to Cr, Mo and Zn (Gouva *et al.*,2020) as well as Ni and Cd (Aldavood *et al.*,2020). Also, it is worth considering that although the zebrafish embryo chorion acts as a protective barrier, it has pores with a diameter that is 0.50 - 0.70 µm wide for the uptake of nutrients, oxygen and excretion transport (Hamm *et al.*,2019). These pores can be permeable to other toxic compounds during exposure, which can accumulate in different body regions including the head that has glands responsible for producing chorionase, thus consequently decreasing chorion's production (Pelka *et al.*,2017;Pitt *et al.*,2018;d'Amora *et al.*,2021). In addition, observations made in the current study revealed that development was impeded in zebrafish exposed to wastewater SWFE and JB at respective concentrations of 8 % and 16 %, thus decelerating hatching rate. At the aforementioned concentrations, zebrafish were held at the embryonic gastrulation stage with no signs of tail development to facilitate escape of zebrafish from the chorion, thus hindering the hatching process. These findings were generally concordant with previous work where embryos exposed to 5 %, 10 %, and 50 % municipal wastewater effluent experienced delays in hatching due to hindrance of the somitogenesis stage (Gauthier and Vijayan,2020). In addition, Kime and colleagues also demonstrated that EDCs such as 17-a ethinylestradiol detected in surface waters and wastewater may cause a delay in development at concentrations as low as 0.005 ng/L (Kime and Nash,1999;Mita *et al.*,2017). Based on this information, it is apparent that hatching time may differ based on the general physiological stress and the total chemical exposure burden.

4.10 Neurotoxic effects induced by IB and growth retardation caused by SWFE and JB

Wastewater IB significantly increased the spontaneous movement rate of zebrafish at concentrations ≥ 60 %, as early as 24 hpf. This indicated an induction of neurotoxic effects in zebrafish, since altered locomotion is linked to neurotoxicity in literature (Selderslaghs *et al.*,2010;Ribeiro *et al.*,2020). Also, previous studies report that hyperactive spontaneous movement may result from the interaction of a substance with the nervous system (Ogungbemi *et al.*,2019). Therefore, upregulated spontaneous movement due to chronic exposure to IB, generally signalled the presence of harmful contaminants capable of interfering with the nervous system. However, further chemical characterization steps can be taken pertaining to this effluent sample, to help explain that postulation. An example of foreign substances detected in aquatic systems, that may interact with the nervous system are organophosphate pesticides, which were reported to increase spontaneous movement by stimulating nerve cells through the inhibition of acetylcholinesterase (Watson *et al.*,2014). Acetylcholinesterase is an enzyme that catalyses hydrolysis of the neurotransmitter at the synapse thus terminating neurotransmission across the synaptic gap (Rienda *et al.*,2021). Inhibition of this enzyme leads to an accumulation of acetylcholine in the synapse resulting in over-stimulation of the nervous system, which can result in hyperactivity in zebrafish (Watson *et al.*,2014;Greathouse *et al.*,2022). Recent studies also revealed that increase in spontaneous movement can be induced by changes in the gene expression of neural proteins, which includes proteins that are expressed by neurons located in the spinal cord and hindbrain (Wang *et al.*,2021). In addition, adding chemicals such as

polyacrylamide polymer and ferric chloride in the process of sewage treatment were reported to induce increased locomotion in zebrafish (Ribeiro *et al.*,2020). Previous studies also revealed that contaminants of emerging concern such as anti-depressants (amongst others) can cause altered locomotion (Suryanto *et al.*,2021). No movements were detected for SWFE and JB at ≥ 16 % and ≥ 24 %, respectively. Spontaneous movements were likely not detected due to the developmental retardation caused by these effluents. At 24 hpf, zebrafish were held at the gastrulation stage with no defined tail, which becomes prominent during the successional organogenesis stage to allow for movement (Willemsen *et al.*,2011). This indicated the potential threat of wastewater contaminants on stunting growth of aquatic organisms and other organisms that may be in contact with such wastewater.

4.11 SWFE and JB alter zebrafish swimming behaviour

Based on the herein recorded results, there were no differences in behavioural endpoints due to TB, TA, IB and CI, following the assessment of total distance, frozen events, acceleration rate and exploration rate 144 hpf. In addition to physicochemical parameter analysis, low mortality, successful hatching and lack of body malformations, these outcomes suggested that the level of pollutants in TB, TA, IB and CI generally seems to be insufficient to inhibit behavioral activity and/or the overall zebrafish health. Unexpectedly, this data was not in correspondence with observations made for spontaneous movements, which had previously revealed the ability of wastewater IB to induce neurotoxic effects as early as 24 hpf. Based on these observations, arguably, it can be postulated that zebrafish possess the ability to circumvent some neurotoxic effects over prolonged exposure, since no abnormal swimming behaviour was observed 144 h post-exposure to IB. In support of these findings, previous work reported on the resistance of zebrafish to wastewater effluents, showing normal travelling distance during behavioural investigations (Frieberg,2018). Although no persistent or large scale effects appeared pertaining to swimming behaviour in TB, TA, IB and CI, more sensitive assays are important to consider, in order to help elucidate the potential toxicity of these samples to aquatic species. This can be done beyond observation of mortality, morphology deformation and locomotion analysis.

Contrastingly, higher proportions of SWFE (≥ 32 %) and JB (≥ 24 %) significantly altered the locomotor activity of zebrafish 144 hpf, suggesting the presence of contaminants that may adversely impact the fitness and survival of aquatic species. This illustrated the risk that may be posed in recipient water resources polluted with wastewater effluents such as SWFE and JB. In addition, exposure to a lower concentrations of SWFE and JB led to less behavioural pattern impairment, whereas zebrafish larvae exposed to higher concentrations of these effluents experienced more prominent behavioural changes. This signalled that wastewater effluents concentrations might be positively correlated with developmental disruption of aquatic organisms. Also, there was a loss in balance in SWFE and JB-treated fish, causing the zebrafish to lay laterally on the bottom surface of the well-plate, further revealing the degree to which untreated sewage can damage aquatic organisms. In accordance to these outcomes, the ecotoxicity of untreated tannery, hospital and whole tin mining effluent have been reported to cause loss in fish

motility, decrease in total distance travelled and an alerting increase in frozen time (Affandi *et al.*,2019;Chagas *et al.*,2019;Rosales-Pérez *et al.*,2022). These behavioural alterations were attributed to the presence of various substances explained to interfere with locomotion in aquatic species, such as heavy metals, nanoplastics, CECs such as anti-depressants (e.g., diazepam), pesticides (e.g., fenvalerate and tebuconazole), endocrine-disrupting chemicals (e.g., bisphenol A) (Ogungbemi *et al.*,2019;Chen *et al.*,2021;Rao *et al.*,2022;Yao *et al.*,2022;Feng *et al.*,2022). More studies are required to determine the exact isolates in SWFE and JB which affect larvae locomotion.

4.12 Identification of an additional isoform of HSP70

The expression of HSP70 due to wastewater that yielded low sublethal effects in previous assays, was quantified to assess the level of stress induced by these effluents at a proteomic level. Interestingly, the detection of HSP70 was concurrently expressed with an unanticipated 40-50 kDa band, which was also statistically comparable ($p > 0.05$) to the negative control group. Our results accompany previous investigations that describes the detection of an HSP putative pseudogene expressed alongside with HSP70, under environmental stress (Bernabò *et al.*,2020). In short, the novel pseudo HSP70 gene encoding a putative long noncoding RNA was detected in an aquatic organism suggesting the existence of a new and unexpected mechanism to cope with extreme environmental changes in aquatic species. Moreover, Dutta *et al*., demonstrated the ability of antibodies raised against HSP70 to recognize multimeric aggregates of HSP70 (5 distinct protein bands), indicating multimeric forms of this protein (Dutta *et al.*,2013). Although we recognized a 40-50 kDa transcript using HSP70 antibodies in the current study, to understand and prove if this gene is an additional isoform of HSP70 or an uncharacterized gene, further analysis by sequencing and characterization to a greater extent is of a necessity.

4.13 Wastewater TB, TA, IB and CI induced no evident stress

During the analysis of the western blot, high levels of HSP70 were apparent within 1 h of exposure to 37 °C, as predicted for the positive control, suggesting high levels of stress induction in zebrafish. Markedly, the western blot analysis revealed that there was no statistical variation between HSP70 expressed under optimal conditions (E3 medium) and in the presence of raw effluents (100 %) of TB, TA, IB and CI. This data suggested that there was no proteotoxic effects or apparent stress incurred by zebrafish during 144 h of exposure to TB, TA, IB and CI. Moreover, similar obervations were made by Vinczeet *et al*., who demonstrated that samples collected from the polluted Neckar River water and sediment during Autumn in 2011 induced no stress reponse in zebrafish post exposure (Vincze *et al.*,2014). These findings emphasized the high tolerance of aquatic species such as zebrafish to pollutants present in rivers or introduced as a form of wastewater (due to natural or anthropogenic activities) into water systems. Moreover, our western blot outcomes correlated with the data obtained during characterization of wastewater by physicochemical parameters. Contradictory previous work demonstrated that there can be a

significant up-regulation in HSP70 indicating stress response in aquatic species exposed water resources such as polluted lakes (Wang *et al.*,2007), rivers containing emerging contaminants (Vincze *et al.*,2014) and industrial effluents (Janz *et al.*,1997). Inconsistency in the current data compared to these previous findings can be attributed to the use of different aquatic species, which may respond differently to environmental stress. Moreover, water resources such as lakes and rivers generally contain different types of contaminants, that can be detected in low or high concentrations as an influence of pollutant-producing natural and human activities taking place in that geographical area (Paulse *et al.*,2012;Khatri and Tyagi,2015;Gani *et al.*,2021;Wilkinson *et al.*,2022). Therefore, the induction of stress indicated by the expression of HSP70, can possibly vary following exposure to these water resources, depending on the composition of that particular water environment.

Moreover, it is noteworthy that heat shock proteins such as HSP70 can be both stress-inducible and constitutively expressed (Place *et al.*,2004), even though they were originally discovered as proteins expressed under environmental stress (Lindquist,1986;Ritossa,1996). This could explain the basal expression of HSP70 observed in TA, TB, IB, CI and E3 medium-exposed zebrafish upon densitometry analysis. The HSP70 gene can be expressed under non-stress conditions with multiple housekeeping chaperone functions which are essential for cellular machinery involved in protein homeostasis, pertaining to protein folding, intracellular translocation of newly synthesized proteins and/or suppressing apoptosis (Radons,2016;Genest *et al.*,2019;Albakova *et al.*,2020). In addition, it has been reported to be expressed during discrete periods in the development of several embryonic tissues including normal embryonic lens formation under optimal growth temperatures in zebrafish (Blechinger *et al.*,2002;Krone *et al.*,2003;Evans *et al.*,2005).

4.14 Conclusion

The present study is original in assessing the ecotoxicological impact of wastewater effluents from localized regions of a developing country like South Africa - further reporting on remediation of wastewater by chemical precipitation. The use of zebrafish embryos in biomonitoring programs is advised given the findings, which show that it is a superior experimental vertebrate for evaluating effluent toxicity. Two of the tested wastewater samples particularly induced lethal toxicity in zebrafish embryos and larvae, resulting in elevated mortality levels, body malformations, delay in hatching and the overall development, signs of neurotoxicity and impaired locomotion. The current study provided evidence of the potential environmental risk that wastewaters offer to aquatic life. More investigation is needed to better understand emerging pollutants discharged as effluents in South Africa's water bodies and their interactions with aquatic organism at the adult stage.

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APPENDIX A

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

04 August 2021

Prof Carola Niesler (215036364)
School of Life Sciences
Pietermaritzburg Campus

Dear Prof Niesler,

Protocol reference number: AREC/029/019
Project title: Establishment of zebrafish models for the study of human disease and toxicology.
Full Approval – Renewal Application

With regards to your renewal application received on 03 August 2021. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 03 August 2022.

Please note: the study renewal in 2022 must be uploaded to the RIG online system as a new application.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

Dr Sanil D Singh, BVSc, MS, PhD
Chair: Animal Research Ethics Committee

/kr

Animal Research Ethics Committee
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Pietermaritzburg
Westville

Figure A.1: Ethics approval (AREC/029/019) granted for the project entitled: "Establishment of Zebrafish models for the study of human disease and toxicology" on 04 August 2021

APPENDIX B

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Ms Marna Laing • Tel: +27 12 319 7442 • Fax: +27 12 319 7470 • E-mail: MarnaL@dalrrd.gov.za
Reference: 12/11/1/5 (2183NC)

Responsible person: Prof Carola U Niesler
Institution: University of KwaZulu-Natal, Carbis Road, Scottsville Pietermaritzburg 3201.
Email: Niesler@ukzn.ac.za

Dear Prof Carola Niesler,

PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Title of research project / study: "Establishment of Zebrafish models for the study of human disease and toxicology."

1. Based on the information provided in your application, the Director of Animal Health has no objection to this study. The study may continue if statement 1.1 to 1.4 hereunder (as applicable) are, and remain, accurate. **Should the scope of your research project change in any way you are required to inform the Section 20 Secretariat and may not proceed with any activities until written permission to do so have been granted by the National Director: Animal Health.**

   1.1. No work will be done with controlled and notifiable animal diseases (list can be obtained / requested from this office), which includes any animal diseases which do not occur in South Africa;
   1.2. No imported material of animal origin or imported animal pathogens will be utilized in the study;
   1.3. No samples that originate from a biobank will be used in the study;
   1.4. No clinical studies will be performed in the target species, either in a laboratory or in the field;

2. In addition to the conditions mentioned in point 1, you are responsible for ensuring that your research project or study complies with all or part of the following, as applicable:

   2.1. Permission to perform research under Section 20 of the Animal Diseases Act, 1984 (Act no 35 of 1984) does not relieve the researcher of any responsibility which may be placed on him/her by any other Act of the Republic of South Africa, including the Veterinary and Para-Veterinary Professions Act, 1982 (Act No 19 of 1982), the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act, 1947 (Act No 36 of 1947), the Medicines and Related Substances Control Act, 1965 (Act No 101 of 1965), the Genetically Modified Organisms Act, 1997 (Act No 15 of 1997) and the National Environmental Management: Biodiversity Act, 2004 (Act No 10 of 2004);
   2.2. No part of the study may begin until valid ethical approval has been obtained in writing from the relevant South African authority;

2.3. Any incidence or suspected incidence of a controlled or notifiable disease in terms of the Animal Diseases Act 1984 (Act no 35 of 1984), must be reported immediately to the responsible state veterinarian;

2.4. Only 6500 zebrafish embryos and 700 zebrafish adults sourced from Petworld and Aquatics, 270 Victoria Road, Pietermaritzburg and/or Hillcrest Petshop, 9 Inanda Road, Hillcrest, Durban may be used for the study. All research project zebrafish must be euthanized at the end of the research project and their carcasses incinerated;

2.5. All potentially infectious material utilised or generated during or by the study is to be destroyed at completion of the study and only a registered waste disposal company may be used for the removal of waste generated during or by the study;

2.6. Records must be kept for five years for auditing purposes.

Written permission from the Director of Animal Health must be obtained prior to any deviation from the conditions. Application must be sent in writing to MarnaL@dalrrd.gov.za.

Failure to obtain written permission as above may be considered a contravention of the Animal Diseases Act, 1984 (Act no 35 of 1984).

Expiry date of this permit: 19 November 2024

Kind regards,

[redacted]

Dr Mpho Maja
DIRECTOR: ANIMAL HEALTH
Date:
2021 -11- 1 1

Figure B.1: Section 20 of the animal disease act, 1984 (ACT NO 35 OF 1984) stated by the Department of Agriculture, Land Reform and Rural Development in SA

APPENDIX C

Table C.1 Descriptive statistics for wastewater samples with high toxicity

Statistical analysis	SWFE (%)					JB (%)				
	8	**16**	**24**	**32**	**40**	**8**	**16**	**24**	**32**	**40**
P-value	0.1926	0.0003	0.0073	0.0003	<0.0001	0.0380	0.0005	0.0001	<0.0001	<0.0001
< 0.05 (Yes/No)	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
P-value summary	ns	***	**	***	****	*	***	***	****	****
COV	68.63 %	11.11 %	30.10 %	12.37 %	3.77 %	34.64 %	12.06 %	6.67	3.15 %	0.00 %

P-value less than 0.05 (*), 0.01 (**), 0.001 (***) or 0.0001 (****)
COV: Coefficient of variation.

APPENDIX D

Table D.1: P-values compared at different pH of wastewater SWFE

SWFE (%)		P-value							
	pH	**3.02 vs. 4.02**	**3.02 vs. 5.02**	**3.02 vs. 6.02**	**3.02 vs. 7.02**	**3,02 vs. 8,02**	**3,02 vs. 9,02**	**3,02 vs. 10,02**	**3,02 vs. 11,02**
40		<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,0039	0,0002	>0,9999
32		<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,9970
24		<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	>0,9999
16		<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,5520
8		>0,9999	0,9970	<0,0001	<0,0001	<0,0001	0,2944	<0,0001	<0,0001
	pH	**4,02 vs. 5,02**	**4,02 vs. 6,02**	**4,02 vs. 7,02**	**4,02 vs. 8,02**	**4,02 vs. 9,02**	**4,02 vs. 10,02**	**4,02 vs. 11,02**	**-**
40		>0,9999	<0,0001	<0,0001	<0,0001	0,0039	0,0453	<0,0001	
32		0,9970	<0,0001	<0,0001	<0,0001	0,2944	0,8123	<0,0001	
24		>0,9999	<0,0001	<0,0001	<0,0001	0,9970	0,5520	<0,0001	
16		>0,9999	<0,0001	<0,0001	<0,0001	>0,9999	0,9598	<0,0001	
8		>0,9999	<0,0001	<0,0001	0,0010	0,8123	<0,0001	<0,0001	
	pH	**5,02 vs. 6,02**	**5,02 vs. 7,02**	**5,02 vs. 8,02**	**5,02 vs. 9,02**	**5,02 vs. 10,02**	**5,02 vs. 11,02**		
40		<0,0001	<0,0001	<0,0001	<0,0001	0,0010	<0,0001		
32		<0,0001	<0,0001	<0,0001	0,0002	0,0039	<0,0001		
24		<0,0001	<0,0001	<0,0001	0,2944	0,0140	<0,0001		
16		<0,0001	<0,0001	<0,0001	>0,9999	0,9970	<0,0001		
8		0,0039	0,0010	0,1263	>0,9999	<0,0001	<0,0001		
	pH	**6,02 vs. 7,02**	**6,02 vs. 8,02**	**6,02 vs. 9,02**	**6,02 vs. 10,02**	**6,02 vs. 11,02**			
40		>0,9999	>0,9999	<0,0001	<0,0001	<0,0001			
32		>0,9999	>0,9999	<0,0001	<0,0001	<0,0001			
24		>0,9999	>0,9999	<0,0001	<0,0001	<0,0001			
16		>0,9999	>0,9999	<0,0001	<0,0001	<0,0001			
8		>0,9999	>0,9999	0,2944	<0,0001	<0,0001			
	pH	**7,02 vs. 8,02**	**7,02 vs. 9,02**	**7,02 vs. 10,02**	**7,02 vs. 11,02**				
40		>0,9999	<0,0001	<0,0001	<0,0001				

32		>0,9999	<0,0001	<0,0001	<0,0001
24		>0,9999	<0,0001	<0,0001	<0,0001
16		>0,9999	<0,0001	<0,0001	<0,0001
8		>0,9999	0,1263	<0,0001	<0,0001
	pH	**8,02 vs. 9,02**	**8,02 vs. 10,02**	**8,02 vs. 11,02**	
40		<0,0001	<0,0001	<0,0001	
32		<0,0001	<0,0001	<0,0001	
24		<0,0001	<0,0001	<0,0001	
16		<0,0001	<0,0001	<0,0001	
8		0,9598	<0,0001	<0,0001	
	pH	**9,02 vs. 10,02**	**9,02 vs. 11,02**		
40		>0,9999	0,1263		
32		>0,9999	0,0453		
24		>0,9999	<0,0001		
16		0,9970	<0,0001		
8		<0,0001	<0,0001		
	pH	**10,02 vs. 11,02**			
40		0,0140			
32		0,0039			
24		<0,0001			
16		<0,0001			
8		>0,9999			

p-value less than: 0.05 (*), 0.01 (**), 0.001 (***) and 0.0001 (****).

Table D.2: P-values compared at different pH of wastewater JB

JB (%)	P-value							
	pH	**3,65 vs. 4,65**	**3,65 vs. 5,65**	**3,65 vs. 6,65**	**3,65 vs. 7,65**	**3,65 vs. 8,65**	**3,65 vs. 9,65**	**3,65 vs. 10,65**	**3,65 vs. 11,65**
40		>0,9999	>0,9999	<0,0001	<0,0001	<0,0001	<0,0001	>0,9999	>0,9999
32		0,9990	0,0885	<0,0001	<0,0001	<0,0001	<0,0001	>0,9999	>0,9999
24		0,0029	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001
16		>0,9999	0,0885	0,0004	<0,0001	0,0029	0,0182	<0,0001	<0,0001
8		0,9990	>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	<0,0001	<0,0001
	pH	**4,65 vs. 5,65**	**4,65 vs. 6,65**	**4,65 vs. 7,65**	**4,65 vs. 8,65**	**4,65 vs. 9,65**	**4,65 vs. 10,65**	**4,65 vs. 11,65**	**-**
40		>0,9999	<0,0001	<0,0001	<0,0001	<0,0001	>0,9999	>0,9999	-
32		0,9990	<0,0001	<0,0001	<0,0001	<0,0001	>0,9999	0,9990	-
24		>0,9999	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	-
16		0,6840	0,0182	<0,0001	0,0885	0,3067	<0,0001	<0,0001	-
8		0,6840	>0,9999	0,3067	0,9509	0,9990	<0,0001	<0,0001	-
	pH	**5,65 vs. 6,65**	**5,65 vs. 7,65**	**5,65 vs. 8,65**	**5,65 vs. 9,65**	**5,65 vs. 10,65**	**5,65 vs. 11,65**	**-**	**-**
40		<0,0001	<0,0001	<0,0001	<0,0001	>0,9999	>0,9999	-	-
32		<0,0001	<0,0001	<0,0001	<0,0001	0,9509	0,0885	-	-
24		<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	-	-
16		>0,9999	0,3067	>0,9999	>0,9999	<0,0001	<0,0001	-	-
8		>0,9999	>0,9999	>0,9999	>0,9999	<0,0001	<0,0001	-	-
	pH	**6,65 vs. 7,65**	**6,65 vs. 8,65**	**6,65 vs. 9,65**	**6,65 vs. 10,65**	**6,65 vs. 11,65**	**-**	**-**	**-**
40		>0,9999	0,6840	0,6840	<0,0001	<0,0001	-	-	-
32		0,6840	>0,9999	0,3067	<0,0001	<0,0001	-	-	-
24		0,9509	>0,9999	0,9509	<0,0001	<0,0001	-	-	-
16		0,9990	>0,9999	>0,9999	<0,0001	<0,0001	-	-	-
8		0,9990	>0,9999	>0,9999	<0,0001	<0,0001	-	-	-
	pH	**7,65 vs. 8,65**	**7,65 vs. 9,65**	**7,65 vs. 10,65**	**7,65 vs. 11,65**	**-**	**-**	**-**	**-**
40		0,0885	0,0885	<0,0001	<0,0001	-	-	-	-
32		0,0885	<0,0001	<0,0001	<0,0001	-	-	-	-
24		0,6840	0,0029	<0,0001	<0,0001	-	-	-	-
16		0,9509	0,6840	<0,0001	<0,0001	-	**-**	-	-
8		>0,9999	>0,9999	<0,0001	<0,0001	-	-	-	-
	pH	**8,65 vs. 9,65**	**8,65 vs. 10,65**	**8,65 vs. 11,65**	**-**	**-**	-	**-**	**-**
40		>0,9999	<0,0001	<0,0001	-	-	-	-	-
32		0,9509	<0,0001	<0,0001	-	-	-	-	-

24		0,9990	<0,0001	<0,0001	-	-	-	-	-
16		>0,9999	<0,0001	<0,0001	-	**-**	**-**	-	-
8		>0,9999	<0,0001	<0,0001	-	-	-	-	-
	pH	**9,65 vs. 10,65**	**9,65 vs. 11,65**	**-**	**-**	-	-	**-**	**-**
40		<0,0001	<0,0001	-	-	-	-	-	-
32		<0,0001	<0,0001	-	-	-	**-**	-	-
24		<0,0001	<0,0001	-	-	-	-	-	-
16		<0,0001	<0,0001	-	-	-	-	-	-
8		<0,0001	<0,0001	-	-	-	-	-	-
	pH	**10,65 vs. 11,65**	-	-	-	-	-	**-**	**-**
40		>0,9999	-	-	-	-	-	-	-
32		>0,9999	-	-	-	-	**-**	-	-
24		0,3067	**-**	**-**	**-**	**-**	-	-	-
16		0,0029	-	-	-	-	-	-	-
8		<0,0001	-	-	-	-	-	-	-

p-value less than: 0.05 (*), 0.01 (**), 0.001 (***) and 0.0001 (****).

Table D.3: P-values compared at different pH of tank water

Tank water (%)	P-value								
	pH	**3,00 vs. 4,00**	**3,00 vs. 5,00**	**3,00 vs. 6,00**	**3,00 vs. 7,00**	**3,00 vs. 8,00**	**3,00 vs. 9,00**	**3,00 vs. 10,00**	**3,00 vs. 11,00**
40		>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	<0,0001	<0,0001	<0,0001
32		>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	0,0014	<0,0001	<0,0001
24		>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	<0,0001	<0,0001	<0,0001
16		>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	<0,0001	<0,0001
8		>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	<0,0001	<0,0001
	pH	**4,00 vs. 5,00**	**4,00 vs. 6,00**	**4,00 vs. 7,00**	**4,00 vs. 8,00**	**4,00 vs. 9,00**	**4,00 vs. 10,00**	**4,00 vs. 11,00**	**-**
40		>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	<0,0001	<0,0001	-
32		>0,9999	>0,9999	>0,9999	>0,9999	0,0126	<0,0001	<0,0001	-
24		>0,9999	>0,9999	>0,9999	>0,9999	<0,0001	<0,0001	<0,0001	-
16		>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	<0,0001	<0,0001	-
8		>0,9999	>0,9999	>0,9999	>0,9999	>0,9999	<0,0001	<0,0001	-
	pH	**5,00 vs. 6,00**	**5,00 vs. 7,00**	**5,00 vs. 8,00**	**5,00 vs. 9,00**	**5,00 vs. 10,00**	**5,00 vs. 11,00**	**-**	**-**
40		>0,9999	>0,9999	>0,9999	<0,0001	<0,0001	<0,0001	-	-
32		>0,9999	>0,9999	0,7720	<0,0001	<0,0001	<0,0001	-	-
24		>0,9999	>0,9999	>0,9999	<0,0001	<0,0001	<0,0001	-	-
16		>0,9999	>0,9999	>0,9999	>0,9999	<0,0001	<0,0001	-	-
8		>0,9999	>0,9999	>0,9999	0,9856	<0,0001	<0,0001	-	-
	pH	**6,00 vs. 7,00**	**6,00 vs. 8,00**	**6,00 vs. 9,00**	**6,00 vs. 10,00**	**6,00 vs. 11,00**	**-**	**-**	**-**
40		>0,9999	>0,9999	<0,0001	<0,0001	<0,0001	-	-	-
32		>0,9999	0,7720	<0,0001	<0,0001	<0,0001	-	-	-
24		>0,9999	>0,9999	<0,0001	<0,0001	<0,0001	-	-	-
16		>0,9999	>0,9999	>0,9999	<0,0001	<0,0001	-	-	-
8		>0,9999	>0,9999	>0,9999	<0,0001	<0,0001	-	-	-
	pH	**7,00 vs. 8,00**	**7,00 vs. 9,00**	**7,00 vs. 10,00**	**7,00 vs. 11,00**	**-**	**-**	**-**	**-**
40		>0,9999	<0,0001	<0,0001	<0,0001	-	-	-	-
32		0,7720	<0,0001	<0,0001	<0,0001	-	-	-	-
24		>0,9999	<0,0001	<0,0001	<0,0001	-	-	-	-
16		>0,9999	>0,9999	<0,0001	<0,0001	-	**-**	-	-
8		>0,9999	>0,9999	<0,0001	<0,0001	-	-	-	-
	pH	**8,00 vs. 9,00**	**8,00 vs. 10,00**	**8,00 vs. 11,00**	**-**	**-**	-	**-**	**-**
40		<0,0001	<0,0001	<0,0001	-	-	-	-	-
32		0,3371	<0,0001	<0,0001	-	-	-	-	-
24		<0,0001	<0,0001	<0,0001	-	-	-	-	-
16		>0,9999	<0,0001	<0,0001	-	**-**	**-**	-	-
8		>0,9999	0,0014	<0,0001	-	-	-	-	-
	pH	**9,00 vs. 10,00**	**9,00 vs. 11,00**	**-**	**-**	-	-	**-**	**-**
40		<0,0001	<0,0001	-	-	-	-	-	-

32		<0,0001	<0,0001	-	-	-	**-**	-	-
24		<0,0001	<0,0001	-	-	-	-	-	-
16		<0,0001	<0,0001	-	-	-	-	-	-
8		0,0014	<0,0001	-	-	-	-	-	-
	pH	**10,00 vs. 11,00**	-	-	-	-	-	**-**	**-**
40		>0,9999	-	-	-	-	-	-	-
32		>0,9999	-	-	-	-	**-**	-	-
24		<0,0001	**-**	**-**	**-**	**-**	-	-	-
16		<0,0001	-	-	-	-	-	-	-
8		<0,0001	-	-	-	-	-	-	-

p-value less than: 0.05 (*), 0.01 (**), 0.001 (***) and 0.0001 (****)

Table D.4: P-values compared at different alkalinity of SWFE, JB and tank water

Wastewater (%)		**P-value**
1. JB	**Total alkalinity (mg/L)**	**0.00 vs. 120.00**
40		<0.0001
32		<0.0001
24		<0.0001
16		<0.0001
8		>0.9999
2. SWFE	**Total alkalinity (mg/L)**	**0.00 vs. 120.00**
40		<0.0001
32		<0.0001
24		<0.0001
16		<0.0001
8		0.4132
3. Tank water	**Total alkalinity (mg/L)**	**0.00 vs. 120.00**
40		0.9850
32		>0.9999
24		>0.9999
16		0.9850

APPENDIX E

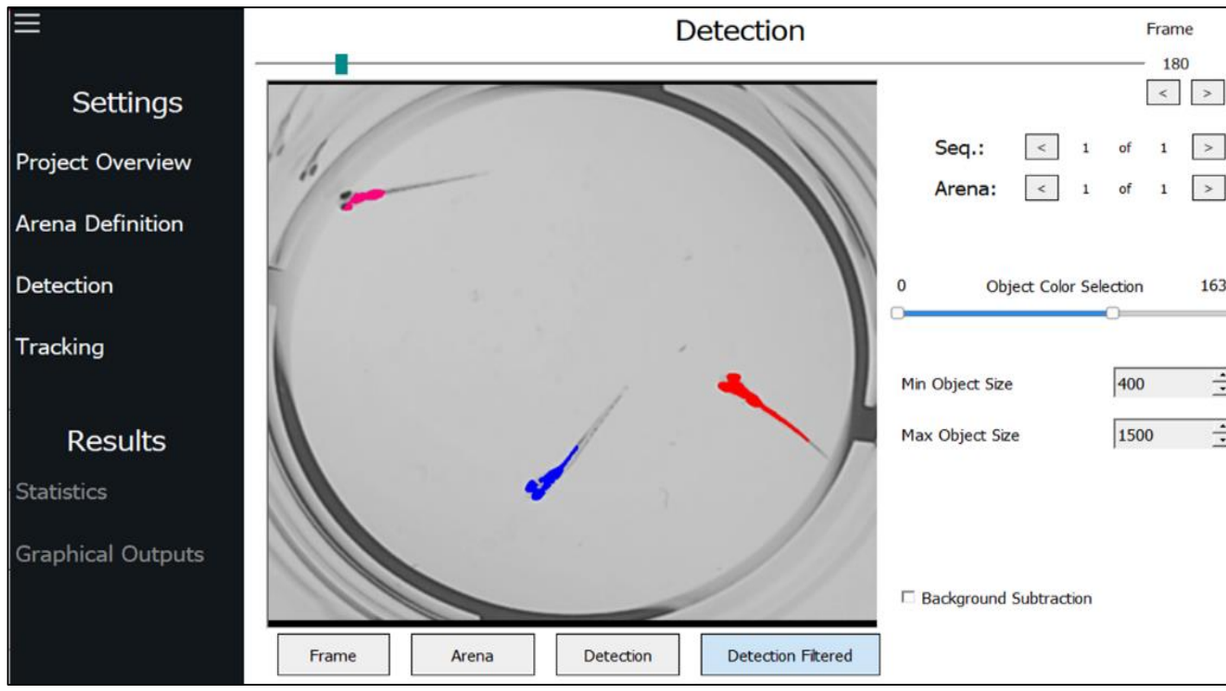

Figure D.1: Toxtrac monitoring swimming behaviour of zebrafish larvae (144 hpf) in wastewater. Three free-swimming larvae were tracked per concentration of each effluent sample. Detection of individual fish is shown by the different colours unique for each fish.