Impact of acute and long-term exposure to oxybenzone on the *Caenorhabditis elegans* life history

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A thesis submitted in partial fulfilment for the award of the degree of Master's in Science by Research

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June 2022

I, Lynn de Miranda, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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List of abbreviations

3-BC- 3'-b-C-Methyluridine

4-MBC – 4-Methylbenzylidene

AVO – Avobenzone

BP-2 - Benzophenone-2

BP-3 – Benzonephenone-3

BP-4 Benzophenone-4

chl-*a* - Chlorophyll *a*

DMSO – Dimethylsulfoxide

EC_{50} – Effect concentration 50%

EDC - Endocrine disrupting compounds

EHMC – 2-Ethylhexyl 4-methoxycinnamate

EPA - American Environmental Protection Agency

HTS - Hypertonic stress

LC_{50} – Lethal concentration 50%

Log-K_{ow} - Octanol-water partition value

NGM - Nematode Growth media

Octinoxate- 2-Ethylhexyl 4-methoxycinnamate

OD-PABA - Octyl dimethyl p-aminobenzoate

OC – Octrocrylene

Oxybenzone - Benzonephenone-3

PCG -Protein-coding genes

PCP – Personal Care Products

ROS – Reactive Oxygen Species

RPM - Rotations per minute

SD - Standard deviation

SGP- Somatic gonad precursors

TiO_2 - Titanium dioxide

UV-filter – Ultra-violet filter

VTG – Vitellogenin

WWT – Waste-water treatment

ZNO – Zinc dioxide

Abstract

UV-filters are the active ingredients providing weatherproofing to industrial products and UV-light protection in personal care products such as sun-tan lotions. Sun-tan lotions products are commonly recommended to protect the skin from the sun's damaging rays however their increasing prevalence in drinking water and aquatic environments has raised concern regarding their effect on both human and environmental health. The common UV-filter Oxybenzone (BP-3) has been highlighted as a compound of particular concern due to its high prevalence, its known toxicity in aquatic species and its ability to accumulate in the active sludge of waste-water treatment plants. Here, we investigate for the first time the effect of BP-3 on the fecundity and growth of the terrestrial nematode *Caenorhabditis elegans* and develop a novel method of modelling the UV-filter pollution associated with the use of UV-filter contaminated active sludge as fertilizer. Our results show that four hour BP-3 exposure did not affect reproductive health at environmentally relevant concentrations (experimental concentrations of 50, 100, 250 and 500 µg/L) however, two of three replicates demonstrated a significant effect of BP-3 (experimental concentrations of 500, 1000, 1500, 2000 µg/L) on total *C. elegans* fecundity. One replicate also demonstrated that nematodes acutely exposed to 500 µg/L of BP-3 were significantly larger than those measured before the experiment, potentially suggesting an effect on the nematodes ability to control osmolarity. It was found that BP-3 did not influence the growth of *C. elegans,* which contradicts previous research. This highlights the need for further investigation into BP-3 toxicity. Finally, it was found that in one of the replicates, the nematodes exposed to *E. coli* contaminated with 1500 µg/L BP-3 were

significantly smaller than those exposed to *E. coli* contaminated with 1000 µg/L BP-3, suggesting the need for further investigation into the potential environmental risks associated with UV-filter pollution originating from active sludge. In conclusion the research conducted in this project provides a novel insight into BP-3 toxicity and the development of a universal toxicity model, which could provide reasoning for the implementation of restrictions on dangerous UV-filters both in localised areas but also internationally.

To Humphrey;

with love, admiration and gratitude.

Acknowledgments

I am indebted to many people who have supported me throughout my masters:

Firstly, I would like to thank past and present members of the research group at Canterbury Christ Church University, in particular, Hannah Shilling and Dave Belsom for their constant patience, kindness, support and advice.

I would also like to thank Lucy Vining, Alice Tirnoveanu and Sobha Tumbapo for providing me with a constant stream of invaluable support, comradery, friendship and entertainment despite warm days and long nights.

Personally, I would like to thank Rudy de Miranda, Petra Nevels and George Vano for their constant encouragement during this unprecedented year. I am particularly indebted to George for his constant support, discussions and endless supply of teas.

Finally, the largest debt of gratitude must fall to my supervisors, Kate and Simon Harvey. It has been a pleasure to work with them both as they have not only provided me with invaluable knowledge but also have guided me throughout this project.

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1 Introduction

1.1 Pollution: a global problem

The term pollution is defined as unwanted waste originating from human use, resulting in reduction in air, soil and / or water quality. Pollution equated to over nine million deaths worldwide in 2015, making it the leading environmental risk factor for disease and premature death (Landrigan *et al.*, 2018a). Although the effects of traditional types of pollution such as water pollution associated with extreme poverty are relatively well studied, more modern forms of pollution such as water pollution caused by chemicals used in pharmaceuticals and personal care products (PCP) is a much more novel field (Landrigan *et al.*, 2018a; Zhou *et al.*, 2019). It is believed that the number of world-wide deaths caused by these modern types of pollution, including the aquatic chemical pollution mentioned above, has increased by 66% since the year 2000 (Landrigan *et al.*, 2018a). This project will focus on one aspect of this chemical pollution deriving from the use of PCPs.

1.2 UV-filter worldwide uses and consumption

Ultra-violet light filter (UV-filter) is a collective name for the active ingredient providing sun protection in personal care products or the chemical additive used to add weather proofing to industrial products such as plastics, fabrics, and paints (Blüthgen, Zucchi and Fent, 2012; Molins-Delgado *et al.*, 2017). Although the current amount of UV-filters being used is unknown, the amount of UV-filters used worldwide in 2014 was between

16-25,000 tonnes (Díaz-Cruz and Barceló 2009; Osterwalder, Sohn and Herzog 2014; Wu et al. 2016). This amount is likely to be much larger now, as the yearly usage in the USA alone is now estimated to be between 9,000 and 32,000 tonnes (Narla and Lim, 2020). This growth in consumption is most likely due to an increase in the public's awareness of environmentally influenced skin conditions such as premature ageing, dark spots and skin cancers as well as more dermatologists recommending daily suntan use (Osterwalder, Sohn and Herzog, 2014; Leiter, Keim and Garbe, 2020). The fact that there is likely a very large amount of sunscreen being used and subsequently released into our water systems, has meant that many researchers have raised concerns as to what effects the chemicals within suntan lotion could have on both the environment and human health.

1.3 UV-filters pollution

There are two types of UV-filters: organic and inorganic UV-filters (Morlando *et al.*, 2016). The most commonly used inorganic UV-filters are titanium dioxide (TiO_2) and zinc oxide (ZnO) (Serpone, Dondi and Albini, 2007). These are large particles which reflect the light, providing weatherproofing to paints and plastics (Molins-Delgado *et al.*, 2017). Inorganic UV-filters are also used as cosmetic ingredient providing the UVA and UVB protection in personal care products (PCPs) such as moisturisers, sunscreen, perfume and makeup (Serpone, Dondi and Albini, 2007). Organic UV-filters differ to inorganic ones as these chemicals absorb UVA and UVB light with the wavelengths ranging between 280 nm to 400 nm, instead of reflecting them (Serpone, Dondi and Albini, 2007). Organic UV-filters are comprised of aromatic moieties comprised of

carbonyl-groups or methoxy-groups (Hopkins, Snowberger and Blaney, 2017). Oxybenzone (BP-3) (figure 1.1), octinoxate (ethylhexyl methoxycinnamate or EHMC), 4-methylbenzylidene camphor (4-MBC), octyl dimethyl p-aminobenzoate (OD-PABA), and octocrylene (OC) are some of the most prominent organic UV-filters used in PCPs (Tsui *et al.*, 2014). Due to the chosen field of study, this research paper will focus predominantly on organic UV-filters and so otherwise specifically mentioned, the term UV-filters refers to organic UV-filters.

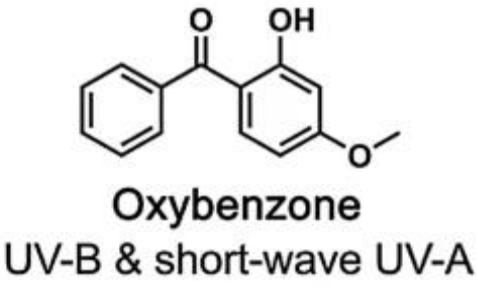

Figure 1.1: Chemical structure of Oxybenzone, a chemical UV-filter used to provide the protection against UVA and UVB rays needed for personal care products such as suntan lotions. Original image accessed from link.springer.com (Ginzburg et al., 2021).

Studies have found that UV-filter concentrations sampled from surface waters increased in line with population density of that area, suggesting human contribution to UV-filter pollution (Poiger *et al.*, 2004; Kaiser *et al.*, 2012; Tsui *et al.*, 2014). Further to this, they also found that the concentrations of organic UV-filters also increased in areas with outdated waste-water treatment (WWT) strategies (Tsui *et al.*, 2014). This supports the hypothesis that once applied to the skin, UV-filters largely remain on the skin due to their low skin permeability and are later washed off into the environment

or plumbing system (Gonzalez *et al.*, 2006). This entry method was further demonstrated by Labille *et al.* in 2020; here they sampled both surface and column water in France and found that recreational areas had higher levels of UV-filter pollution then offshore areas. They also found that UV-filter concentrations fluctuated in line with that of beachgoers (Labille *et al.*, 2020). These findings are in line with that of Ekpeghere et al (2016) who found that there was an 27% increase in organic UV-filter concentration being sampled in the summer months (Ekpeghere *et al.*, 2016).

As mentioned above, WWT facilities are thought to play a large role in UV-filter pollution (Tsui *et al.*, 2014; Ekpeghere *et al.*, 2016). Traditionally WWT plants rely on active sludge, which is comprised of flocs of micro-organisms and bacteria, to remove pollutants (Badia-Fabregat *et al.*, 2012). Many UV-filters have a high bioaccumulation factor represented by a high octanol-water partition value (Log-k_{ow})(Gago-Ferrero *et al.*, 2012). This high Log-K_{ow} value in combination with UV-filters lipophilic property, results in UV-filters tending to accumulate within this active sludge, making biodegradation ineffective (Badia-Fabregat *et al.*, 2012). This effect has been demonstrated with BP-3 for example, where only 28-51% is removed from the water (Vassalle *et al.*, 2020). UV-radiation treatment, a common physical WWT strategy, was also found to be ineffective in breaking down BP-1 and BP-3, with only minimal degradation being see before 24 hours, and none after this time (Gago-Ferrero *et al.*, 2012).

Taking the above into consideration, it is not surprising that UV-filters have been sampled in offshore waters across the globe (Cadena-Aizaga *et al.*, 2020). The sampling of UV-filters in very remote areas also suggest that these chemicals can travel through

the ocean to some extent (Domínguez-Morueco *et al.*, 2021). Samples taken from the rural Antarctic peninsula for example all contained organic UV-filters despite the peninsula being mostly uninhabited (Domínguez-Morueco *et al.*, 2021).

Once in the environment, the fate of UV-filters is uncertain. Traditionally, environmental forces heavily break down chemical pollutants (Ahmed *et al.*, 2017), however evidence suggests that some organic UV-filters are resistant to this (Rodil *et al.*, 2009; Badia-Fabregat *et al.*, 2012). When exposed to UV-light EHMC and OD-PADA for example, have half-lives between 20 and 57 hours while BP-3 was found to only degrade by 8% after 20 days (Rodil *et al.*, 2009; Liu *et al.*, 2011; Badia-Fabregat *et al.*, 2012). Inorganic UV-filters on the other hand are believed to be more efficiently removed using WWT strategies such as active sludge. TiO_2 for example is believed to be removed by upwards of 90% from influents passing through modern day WWT facilities (Mahlalela, Ngila and Dlamini, 2017). Despite this high removal rate TiO_2 has still been sampled in post-treatment effluents around the world at concentrations ranging from 0.5 and 6.5 $\mu g.L^{-1}$ (Neal *et al.*, 2011; Gondikas *et al.*, 2014). This has led some researchers to raise concerns regarding the potentially very high amount of TiO_2 in untreated waters as well as highlighting WWT overflow spills as major pollution hotspots (Wang *et al.*, 2020). In 2020 it was estimated that untreated waters could contain levels of TiO_2 as high as 150 $\mu g.L^{-1}$ and that due to TiO_2s ability to accumulate in sediments, terrestrial areas exposed to this contaminated water could reach pollution levels as high as 100 $mg.kg^{-1}$ (Gottschalk *et al.*, 2015; Hu *et al.*, 2020; de la Vega *et al.*, 2021). These levels of pollution could be a cause of concern for the fauna and flora found in these areas as TiO_2 is considered moderately toxic by the American

Environmental Protection Agency (EPA) and has an EC_{50} concentration (the concentration of a chemical at which a 50% reduction in tested endpoints is induced) of between 1 and 10 $mg.L^{-1}$ (Gottschalk *et al.*, 2015; Hu *et al.*, 2020; de la Vega *et al.*, 2021).

1.4 Effects of UV-filters on the environment

It is believed that as part of the degradation process, UV-filters release degradation products, of which some have be shown to cause damage to cells of a number of organisms (Zhang and Sun, 2004; Faria *et al.*, 2014; Haynes *et al.*, 2017; Zhao *et al.*, 2019). To make an assessment of the environmental dangers UV-filters and their derivatives could pose, research has focussed on the effects of these chemicals on ecologically key species.

Coral colonies not only provide essential food and habitat for many marine species but also provide structural protection to coastlines from storms (Harris *et al.*, 2018). It is estimated that over 40% of coastal coral colonies could be exposed to UV-filter pollution worldwide (Downs *et al.*, 2016). This UV-filter exposure could be detrimental to corals it has been found that, under the influence of UV-light, BP-3 for example can contribute to coral bleaching by causing oxidative stress to the thylakoid membrane in the zooxanthellae (Downs *et al.*, 2016). Zooxanthellae are the mutualistic dinoflagellates of the Dinophyceae order providing invertebrates such as corals, sea sponges and jellyfish with photosynthetic products (Ben-Zvi, Eyal and Loya, 2015). These dinoflagellates are brownish colour, and their absence leaves the white

exoskeleton of the corals exposed, this is what causes the white coral discolouring often associated with coral ''bleaching'' (Downs *et al.*, 2014; Corinaldesi *et al.*, 2018). The fact that the oxidative stress caused was influenced by UV-light exposure in combination with BP-3 supports the hypothesis that degradation products play a role in UV-filter toxicity (Rodil *et al.*, 2009; Lai, Chen and Lin, 2020).

Similar to the effects seen in corals zooxanthellae, photosynthetic pigment production in microalgae can also damaged by UV-filters (Paredes *et al.*, 2014; Mao *et al.*, 2017). In 2017, Moa *et al* found that environmentally relevant concentrations of BP-3 (*i.e.*, 0.1 and 1 $\mu g.L^{-1}$) was systematically absorbed and decreased chlorophyll *a* (chl-a) amounts in a number of algae species including *Chlamydomonas reinhardtii* (Mao *et al.*, 2017). When this concentration was raised above 10 $\mu g.L^{-1}$ chlorophyll *b* and carotenoid content were also decreased to the point that the photosynthetic pigment production was affected leading to subsequent growth rate reduction. (Mao *et al.*, 2017). Similar effects were seen in the algae species *Isochrysis galbana*, where BP-3 had an EC_{50} value (a value that represents the concentration needed to induce a 50% decrease in tested endpoints) of 13.87 $\mu g.L^{-1}$(Paredes *et al.*, 2014).

Besides effecting the pillars of marine food webs such as coral and algae, UV-filters have also been shown to have drastic effects on the reproductive health of several fish species (Zhang *et al.*, 2016; Alamer and Darbre, 2018; Tapper *et al.*, 2019). Preliminary studies found that the UV-filter Octocrylene (OC) caused an increase in vitellogenin (VTG) production in the fish model *Danio rerio,* similarly BP-3 caused the same in rainbow trout (Zhang *et al.*, 2016; Tapper *et al.*, 2019). VTG is found in high concentrations in oviparous female fish as it is a precursor for egg-yolk protein and is

expressed in response to oestrogen signalling within liver cells (Nagler *et al.*, 2010). Subsequently, male fish do not normally have VTG in their systems (Nagler *et al.*, 2010). One exception to this is when exposed male fish are exposed to external oestrogen, for this reason VTG is classically used as a biomarker for endocrine disrupting compounds (EDCs) (Nagler *et al.*, 2010; Yamamoto *et al.*, 2017). An unnatural amount of VTG in both male and female fish can be detrimental for fertility, negatively effecting spermatozoa count in males, altered social behaviours and gonad malformation in both sexes (Xu *et al.*, 2008). A study conducted by Kidd *et al.,* (2007) found that low level EDC exposure led to significant feminization in flathead minnows, this then led to their near extinction within the enclosed test lake. Besides effecting reproduction there is also evidence that organic UV-filters can cause birth defects in some fish (Li *et al.*, 2016). For example, 4MBC (at concentrations of 3.69 $mg.L^{-1}$ and higher) caused spinal malformation and motility issues for *Danio rerio* (Li *et al.*, 2016). Although these levels of 4MBC are more than 50 times higher than that currently being sampled in nature, 4MBC does have a high log-k_{ow} value and could potentially accumulate in the environment and in the fish themselves to unknown amounts (Balmer *et al.*, 2005; Li *et al.*, 2016).

Some scientists have also investigated what effect UV-filters could potentially have on marine mammals. In 2013 Gago-Ferrero *et al* first discovered that some Franciscana dolphins had been exposed to UV-filters and that their bodies did contain varying amounts of OCT contamination. Alonso *et al* (2015) later found that some *Pontoporia blainvillei* and *Sotalia guianens* also contain varying levels of UV-filters. This led further research to find that EHMC was sampled in placental tissue and breastmilk that UV-

filters were maternally transferred from mother to calf. Samples taken from foetus *P. blainvillei* contained significantly higher levels of EHMc then that found in their mothers (Alonso *et al.*, 2015; da Silva *et al.*, 2021).

1.5 UV-filters and the human body

It is believed that between 1-10% of applied UV-filters are absorbed into the body, this has led many researchers to investigate the potential effects this could have on the human body (Souza and Maia Campos, 2017; Cozzi, Perugini and Gourion-Arsiquaud, 2018). BP-3 for example is of particular concern as it has been detected very frequently within sampled urine (between 94% and 98% of samples taken in America and Denmark respectively) as well as being sampled in human breast milk (Calafat *et al.*, 2008; Molins-Delgado *et al.*, 2018).

Further to being sampled in urine and breast milk, BP-3 has also been sampled in human white brain matter, suggesting travel through the blood-brain barrier (Wang, Asimakopoulos and Kannan, 2015; Van Der Meer *et al.*, 2017). Studies have also found that BP-3 accumulated in such brain tissue, with post- mortem frozen human white-matter samples containing concentrations of up to 0.32 $ng.g^{-1}$ (Wang, Asimakopoulos and Kannan, 2015; Van Der Meer *et al.*, 2017). Although the exact effects of this type of exposure on humans is unknown, the effects on rats and mice are established. In rats BP-3 has been shown to cause oxidative stress and even apoptosis within the hippocampus and frontal cortex (Pomierny *et al.*, 2019) . In mice, BP-3 increased the concentration of extracellular glutamine in the brain. This change in glutamine

concentrations has been shown to also cause hippocampal athrophy in humans aswell as diseases such as medication-resistant temporal lobe epilepsy, and in Alzheimer's, Parkinson's and Huntington's disease (Cavus *et al.*, 2008).

UV-filters are also suspected to be EDCs and the endocrine disrupting effect can be detrimental to fish species. EDCs can also have very negative effects on human health (Alamer and Darbre, 2018; Krause *et al.*, 2018; Wnuk *et al.*, 2018; Huang *et al.*, 2020). A number of studies have used models to investigate the effects of these UV-filters on the endocrine balance (Schlumpf *et al.*, 2004; Alamer and Darbre, 2018). One such study found that the organic UV-filters 3,3,5-trimethylcyclohexyl or more commonly referred to as homosalate, EHMC, 4-MBC and BP-3 all caused an increase in invasiveness and cell migration in the MCF-7 breast cancer cell line (Alamer and Darbre, 2018). Worryingly, these effects were already seen at concentrations similar to that sampled in human breast milk (Schlumpf *et al.*, 2010). Studies also found that UV-filters 3-benzylidene camphor (3-BC) and 4-MBC both caused significant increase in uterine weight in immature rats (Schlumpf *et al.*, 2004; Alamer and Darbre, 2018). The increase in uterine weight has long been established as a bio-assay of osteogenic effect (Schlumpf *et al.*, 2004).

The fact that UV-filters have been sampled in breast milk suggests that UV-filters can cross cellular barriers and could reach foetuses and could pose a threat to foetal development and health. BP-3 for example has already been sampled in human placental tissue, amniotic fluid and even foetal blood however the effect of this exposure is still unknown (Vela-Soria *et al.*, 2011; Philippat *et al.*, 2012; Tang *et al.*, 2013; Krause *et al.*, 2018; Wnuk *et al.*, 2018; Song *et al.*, 2020).

BP-3 exposure during embryonic growth was found to cause abnormal migration of the human enteric neural crest cells, 293T and SH- SY5Y (Justus *et al.*, 2014; Huo *et al.*, 2016). Incomplete migration of these neural crest cells can lead to an absence of ganglia in the distal colon, often resulting issues with digestion such as severe constipation and in the life altering conditions, Hirschsprung's disease (Szylberg and Marszalek, 2014; Meinds *et al.*, 2019).

Prenatal BP-3 exposure has been shown to affect the major oestrogen receptors ESR1 and ESR2 as well as the G-coupled receptor GPER1 (Wnuk *et al.*, 2018). GPER1 is a crucial component in maintaining healthy neural function by moderating 17β-oestradiol concentration in cortical and hippocampal cells as well as regulating inter and intracellular communication (Liu, Lou and Fu, 2016; Lu *et al.*, 2016; Wnuk and Kajta, 2017). ESR1 and ESR2 receptors not only mediate a number of endocrine processes, but they also play a key role in the inhibition of CASPASE- mediated neural cell death by maintaining a steady BCL2-BAX ratio (Gingerich *et al.*, 2010; Foot, Henshall and Kumar, 2017; Wnuk and Kajta, 2017; Wnuk *et al.*, 2018; Zhou *et al.*, 2018). The altered function of both the oestrogen receptors or the G-coupled receptors can lead to abnormal neural apoptosis, severe anatomic abnormalities and potential mental disabilities (Roth and D'Sa, 2001).

BP-3 has also been found to inhibit global DNA methylation and decreased DNMT activity (Wnuk *et al.*, 2018). When an interruption in DNA methylation occurs, for example due to an EDC, it can result in a multigenerational epigenetic effect as the germ line can be impacted during the methylation process occurring in the premature gonad (Anway and Skinner, 2006; Wnuk *et al.*, 2018). This phenomenon was

demonstrated by Santamaria *et al* in 2019, here, rat models were used to demonstrate that BP-3 altered the germ line development resulting in a decreased follicle population and subsequently fewer mature oocytes (Santamaría *et al.*, 2019).

Another aspect of potential EDC effects caused by UV-filters currently being investigated is the effect these chemicals could have on puberty. One novel study assessed nearly 300 individuals using the Tanner staging method, this grading system is used as a standard in the medical field to assess puberty growth rates (Huang *et al.*, 2020; Koopman-Verhoeff *et al.*, 2020). A second assessment method was also conducted in this study by giving the participants questionnaires two years apart (in 2011 and 2013). Urine samples were also taken at the times of handing out the questionnaires and analysed for the presence of 12 commonly used UV-filers. Unsurprisingly, BP-3 was the most commonly found UV-filter and its prevalence was associated with an inhibition of testicular growth, pointing to an anti-androgenic effect on males (Huang *et al.*, 2020). Interestingly EHMC was found to also to delay male puberty development while OP-PABA was found to accelerate female puberty (Huang *et al.*, 2020). This study was the first to look at the real-life effects of UV-filter on human puberty growth, and although the results do give an interesting insight this study alone is not enough to pain a clear picture.

The field of UV-filter toxicology is a growing one with several studies finding adverse effects in some ecologically key species and some human health models, with many of these studies pointing at a potential EDC effect (Alamer and Darbre, 2018; Krause *et al.*, 2018; Wnuk *et al.*, 2018; Tapper *et al.*, 2019). Despite these studies BP-3 and other highlighted UV-filters are still widely used and have become more and more prevalent

in our environments (Tsui *et al.*, 2014). Some countries have started to introduce local restrictions against certain UV-filters. The small island of Palau banned the use of BP-3 in 2020 becoming the first country to do so (Narla and Lim, 2020). The popular island Aruba quickly followed suit and banned the import, sale and use of BP-3 (Gobierno Aruba, 2020). Other tourist destinations such as Hawaii, Bonaire and some areas of Mexico have also followed this example (Narla and Lim, 2020). Although these regulations might protect the localised areas that they are implemented in, UV-filters have been shown to travel great distances, these local legislations are likely not enough. Although the EU has not introduced a ban, it has stipulated a limit on the amount of BP-3 allowed in PCPs (from 10% to 6%) (EU, 2017), with other large governing bodies not yet following. Further research is needed to better understand the ecotoxic risks of UV-filter pollutions and to better understand the seriousness of BP-3 pollution compared other UV-filters so that an educated decision can be made if more aggressive legislation is needed or if a wider array of UV-filters need to be added to this ban. The establishment of a standardised UV-filter toxicity assay could allow better direct comparisons of UV-filters. In addition to this, one aspect of the eco-toxicity risk that has not yet been determined is the effects that BP-3 could have on the terrestrial ecosystem. In order to gain insight into the terrestrial eco-toxic potential, the terrestrial nematode *Caenorhabditis elegans* was used in this project as it has been long used as a model for toxicity research.

1.6 *C. elegans* model

Caenorhabditis elegans are multicellular eukaryotes belonging to the family *Rhabditidae*, order *Rhabditida*, class *Secenentea* and phylum *Nematoda* (Eisenmann, 2005). These nematodes are free-living, non-parasitic and are naturally found in humid shrubbery, compost and rotting fruit where they feed on a number of micro-organisms, including *Escherichia coli* (Schulenburg and Félix, 2017).

C. elegans have been used in research for decades with their use being linked to three Nobel prize winning projects, with the most recent award going to Martin Chalfie in 2008 for his research investigating green fluorescence in *C. elegans* (Chalfie, 2009). *C. elegans* lends themselves very well to being used for research for a number of reasons. Firstly, hundreds of nematodes can easily be kept on each plate and require minimal care, making them a cost-effective alternative to many other animal models that require specialist habitats, permits and care (Hunt, 2017). Secondly, compared to other animal models such as mice and rats, *C. elegans* have a short lifespan of two to three weeks and an even shorter reproductive period of a few days, this allows for a quick analysis of effects on both the tested individuals and also any subsequent progeny (Tralau *et al.*, 2012). Thirdly, *C. elegans* have a strong and transparent outer cuticle, this allows researchers to observe internal organs without the need to operate or dissections (Johnstone, 1994). Lastly, *C. elegans* have a very well documented and relatively simple body plan consisting of 959 somatic cells for hermaphrodites and 1031 cells for males, the apoptosis of cells during the growth process is also well documented with 131 of these cells perishing during specific stages (Driscoll, 1995).

1.6.1 *C. elegans* fertility

C. elegans has two sexes, modified females that act as hermaphrodites by producing a limited amount of sperm and males (Eisenmann, 2005). Once mature hermaphrodites are slightly longer and thicker than males and will have visible embryos in the gonad, while males can be distinguished by the presence of a small tail fan (Figure 1.2) (Eisenmann, 2005). The hermaphrodite nematodes have two X chromosomes while the male has a single X-chromosome (Ahringer *et al.*, 1992). The small number of males (<0.2% of the total population) that are produced by hermaphrodite self-fertilization are due to a rare disjunction of one of the X chromosomes (Frézal and Félix, 2015). If males are available the subsequent mating will result in male sperm preference over the hermaphrodite's sperm and the progeny's sex determination is based on a XX/XO ratio (Ahringer *et al.*, 1992).

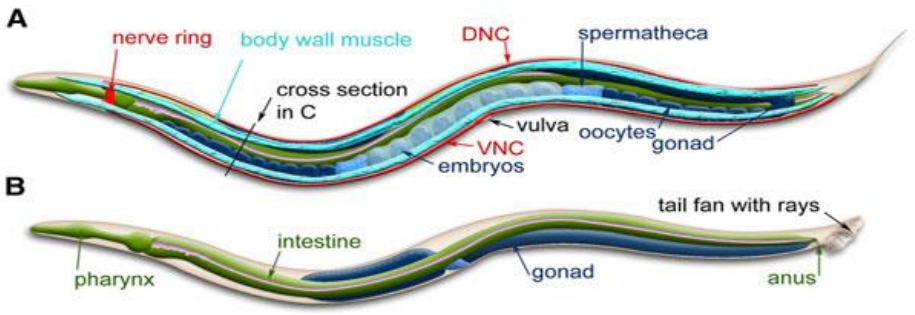

Figure 1.2: Lateral view of *C. elegans* anatomy of a hermaphrodite(A) and male (B) nematode. The ventral nerve cord (labelled as VNC), the dorsal nerve cord (labelled as DNC) and body wall muscle run along the nematode with the cuticle providing a barrier from the environment (represented here as a beige outline). The muscles and nerves have not been illustrated in B to better show the location of the intestinal track and

gonads in the male. Original image accessed from www.wormatlas.org (Albert Einstein College of Medicine, 2020).

1.6.2 *C. elegans* development

During the early embryonic development *C. elegans* go through a two-part process (Sulston *et al.*, 1983). Firstly, the embryo goes through a proliferation process, here the embryo will go through mitotic cell divisions and will go from a single cell to more than 550 founder-cells (Sulston *et al.*, 1983). Within the first half of this process the embryo is still located inside of the hermaphrodite's uterus and the zygote and somatic founder cells are formed (Bucher and Seydoux, 1994). The egg is then laid and the approximately 28 cells will then reach gastrulation, it is also during this time that the first primordial germ line cells (PGC) are created (Bucher and Seydoux, 1994). Some of these cells then organise into the primary cell layers also known as the three germ layers endoderm, ectoderm and mesoderm (Eisenmann, 2005).

The second part of embryonic development is organogenesis, here for example, the germline layers develop into neurons, hypodermis and muscles (Eisenmann, 2005). The pharynx also develops, as well as the finalizing L1 cuticle shape (Anderson and Bird, 1972). The final nerve and muscle cells differentiate, and the nematode will start to move, soon after this the nematode will hatch from the egg (Ambros, 2000).

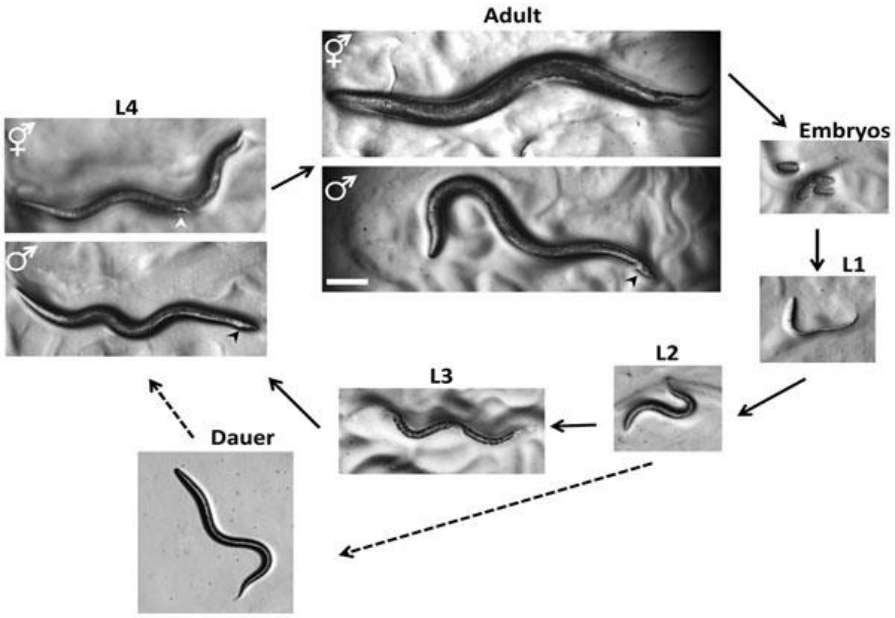

Figure 1.3: **Developmental lifecycle of *C. elegans*.** Sexes are difficult to distinguish until the L4 stage where the hermaphrodites develop a visible vulva (indicated by a white arrowhead), and the males develop a thickened tail (indicated by a black arrowhead). Nematodes also become significantly larger as they process through larval stages. All photographed nematodes were housed on Petri dishes with access to a bacterial lawn with the only exception being the dauer stage as this stage is often induced by starvation. Images acquired from Corsi, Wightman and Chalfie, (2015)

1.6.2.1 L1

In the presence of food, the nematodes enter a four-stage growth cycle, L1-L4 (Ambros, 2000)(Figure 1.3). If there is no food available, growth can be suspended at this phase for up to ten days(Johnson *et al.*, 1984). During L1 the number of somatic cells increases to around 1000. It is also in this stage that the protein-coding genes (PCGs) Z1 and Z3 as well as the somatic gonad precursors (SGPs) Z1 and Z4 start to

prolificate within the gonadal primordia (Kimble and Crittenden, 2005; Harris *et al.*, 2020).

1.6.2.2 L2

Not many cell divisions occur at this stage, however Z2 and Z3 daughter cells continue to divide (Kimble and Hirsh, 1979). Both somatic and germ line cells are intermingled, this remains the case until the nematode moults to reach L3, where they then start to organize into what will become the gonad (Kimble and Hirsh, 1979).

1.6.2.3 Dauer larvae

If the nematodes experience overcrowding, limited food and/or high temperatures during L2, they can turn into a dormant form often referred to as Dauer form (Golden and Riddle, 1984)(Figure 1.3). Here, the cuticle ridge changes slightly, their oral orifices become sealed off and the pharynx stops pumping (Cassada and Russell, 1975). During this period, larvae cannot feed, their movements are greatly reduced, their metabolism is greatly slowed down and aging is paused (Cassada and Russell, 1975). Once food becomes available again and conditions become favourable, it takes about an hour before the larvae will exit the dauer phase, within 2-3 hours they will start to feed and after about 10 hours they will molt into the L4 chase (Golden and Riddle, 1984). After this molt nematodes can continue to life a full lifecycle, however extended

arrest can then lead to several consequences including reduced reproductive success (Webster *et al.*, 2018).

1.6.2.4. L3

It is during this phase that the 143 cells required for the anterior and posterior gonadal sheaths and uterus are created by the somatic gonad precursors (Eisenmann, 2005). The posterior gonadal sheaths will continue to grow until mid L3 where the distal tip cells then reorientate into dorsal directions (Cassada and Russell, 1975). The vulva terminals and the muscles needed for egg-laying are also developed at this point and spermatozoa production starts. In males, this is the point where the gonad blast cells undergo cell division that will eventually turn into the seminal vesicle and the vas deferens (Cassada and Russell, 1975; Eisenmann, 2005).

1.6.2.5 L4

During the L4 phase the gonadogenis is completed and the gonadal sheaths continue to grow along the dorsal wall, completing towards the end of L4 (Eisenmann, 2005). Towards the end of L4 the hermaphrodite's spermatozoa production also halts and the remaining germline cells go through meiosis and start producing oocytes only (Félix *et al.*, 2000). Tissue morphogenesis leads to the finalization of the egg-laying apparatus which becomes visible at this point (Félix *et al.*, 2000)(Figure 1.3).For the males, it is at

this point where the seminal vesicle, ventricle midline and vas deferens become fully differentiated.

1.6.2.6 Adulthood

After about 3 days the hermaphrodite fully matures and will lay their first eggs. The hermaphrodite will continue to lay eggs for 3-4 days while the male stays fertile for about 6 days (Lewis and Fleming, 1995). A hermaphrodite fertilised by a male can lay upwards of 1400 eggs while a self-fertilised hermaphrodite only lays around 300, this is due to a limited amount of sperm (Meyer, 1988).

1.6.3 *C. elegans* suitability as a toxicity research model

According to the National Institute of Environmental health Sciences, the field of toxicology is sometimes referred to as the "Science of Safety" as it focusses on the effects of substances can have on the health and safety of humans, animals and the environment (Guston, 2012). Traditionally, models such as rats and mice are used to determine if a substance is toxic and if so at what concentrations and sometimes to better understand the methodology of this toxicity (Hunt, 2017). However, it is believed that only about 50% of the time these rodent models can predict specific toxic effect (Hartung *et al.*, 2013; Boyd *et al.*, 2016). This percentage can be increased when multiple models are used however, can sometimes be difficult as these models expensive and time consuming (Tralau *et al.*, 2012). It is for this reason that many

studies have focussed on predictive *in silico* modelling or *in vitro* cell assays however, these methods do provide valuable insight on a cellular level, these methods cannot determine the organismal level effect needed to assess the safety of a substance (Li *et al.*, 1999; Miranda *et al.*, 2009; Scott, Peters and Dragan, 2013). To still achieve this understanding of organismal level and to remain cost and time efficient, many studies have opted to use *C. elegans* as an alternative (Hunt, 2017). Beside the practicalities of using *C. elegans*, the fact that *C. elegans* has been fully sequenced and has shares many genes and signalling pathways with other animals including humans is also a benefit (C. elegans sequencing consortium, 1998). Many genetically modified strains are available with specific genetic knock-outs, allowing for precise molecular work (Honnen, 2017). Further to this, as the model is quickly becoming more popular, more specifically designed resources are also becoming available allowing for accurate analysis and wider comparisons within the scientific field (Kaletta and Hengartner, 2006).

1.6.3.1 Toxicity endpoints

Many studies have successfully used growth and fecundity and have found that these endpoints efficiently predicted LC_{50} (the concentration of a substance needed to cause a 50% lethality rate) values found in rat and mouse testing (Wang, Wick and Xing, 2009; Boyd et al., 2010; Campos, Fraceto and Fraceto, 2017; Hunt, 2017).

When *C. elegans become* stressed and later transform into their dauer state their cuticle ridge changes, their pharynx stops pumping and their oral opening seals off

(Cassada and Russell, 1975). During this phase the nematodes cannot eat, they greatly slow down their movements and their growth and aging halts (Cassada and Russell, 1975). After conditions return to normal and become favourable again, the nematodes can exit this phasein about an hour and after about 12 hours will shed into the L4 form. As this pause in growth is caused by a stressor, researchers can use body length after a set period of time as an endpoint to investigate negative effects a variable on nematode health.

Fecundity is also a commonly used endpoint for *C. elegans* toxicity tests as this can also be affected by external factors. For example, a decrease in VTG production in *C. elegans* has been shown to cause a decrease in brood size (Khanna, Johnson and Curran, 2014; Pradhan *et al.*, 2017)In order to better our understanding of BP-3 toxicity on the nematode model *C. elegans* several groups of nematodes were exposed to varying levels of BP-3 and the effect on body length and fecundity were measured as endpoints for developmental and reproductive inhibition.

1.7 Thesis aims

UV-filters are essential in protecting humans from the suns damaging rays however their wide usage has led to these chemicals being frequently being sampled in the environment as well as being sampled in the tissue if animals and humans (Tsui *et al.*, 2014, 2017). As can be concluded from the studies summarised in the paragraphs above, BP-3 shows potential to be both a risk to environmental health as well as human health (Asthana, Mishra and Pandey, 2016). When compared to other UV-filters BP-3 not only shows the strongest evidence of EDC activity but is also some of the most environmentally prevalent. Despite being the subject of several preliminary

studies, very little is known about the exact mechanism behind BP-3 toxicity or regarding the risk BP-3 poses to aquatic ecosystems and in regard to terrestrial ecosystems, the effects are nearly unknown.

In order to better understand the toxicity of BP-3 in both the aspects of human health and environmental health we used the model organism *C. elegans* to address the following research questions:

A) Does acute BP-3 exposure effect *C. elegans* fertility?

B) Does acute BP-3 exposure effect *C. elegans* growth?

C) Does long term BP-3 exposure differ from acute exposure in *C. elegans*?

D) Is ingestion a more severe form of BP-3 exposure for *C. elegans*?

2 Materials and methods

2.1 Microscopy

A Swiftcam SC500 digital camara mounted on a Zeiss STEMI-2000 microscope was used to acquire images of the test nematodes. The software ImageJ was used to then analyse the images (Schneider, Rasband and Eliceiri, 2012).

2.2 Reagents

2.2.1 C. elegans strain

Wild type Bristol (N2) strain of Caenorhabditis *elegans* were taken from frozen stocks at Canterbury Christ Church University (https://cgc.umn.edu/strain/N2(Brenner, 1974; Sterken et al., 2015).

2.2.2 Chemicals

Benzophenone-3 (BP-3,2-Hydroxy-4-methoxybenzophenone, Oxybenzone, CAS no: 131-57-7, purity ≥ 98%).

Dimethylsulfoxide (DMSO).

M9 buffer was used as a temporary liquid media for the nematodes. M9 buffer was made by combining 3 g of KH_2PO_4, 6 g Na_2HPO_4, 5 g $NaCl_2$, 1 mL 1M $MgSO_4$ and 1 L of distilled water. This solution was then autoclaved, sealed, labelled and stored to be used as needed.

KPO_4 buffer was made by combining 868 ml 1M KH_2PO_4 with 132 ml 1 M K_2HPO_4 and making this up to 1 L with distilled water.

2.3 General *C. elegans* maintenance and protocols

2.3.1 Nematode growth media preparation

Nematodes were maintained on Nematode Growth media (NGM) (Stiernagle, 2006). This media has been standardised within the field and is created by adding 34 g Agar, 5 g peptone, 6 g NaCl and 2 mL dissolved cholesterol to 2 L distilled water and stirred and autoclaved a stirrer and then autoclaved (Brenner, 1974). In order to avoid

contamination, the solution was moved to a laminar flow hood (Stiernagle, 2006). The solution was left to cool to 40 °C (Stiernagle, 2006). Once at this temperature the buffers 2 mL CaCl, 2 mL $MgSO_4$ and 50 ml of KPO_4 buffer was added and the solution was used to fill small (35 mm) sterile petri dishes (Stiernagle, 2006). The plates were then closed, stacked on top of each other and left to harden in the laminar flow (Stiernagle, 2006). Once solidified the plates were wrapped up in a plastic sleeve, sealed, labelled and then stored at 12 °C until needed (Stiernagle, 2006).

2.3.2 *E. coli* preparation

Escherichia coli is a bacterium that is easily accessible, can be grown and maintained in a sterile environment and is part of *C. elegans* natural diet. The *E. coli* strain OP50 has become the laboratory standard to effectively maintain *C. elegans* and has been shown to have less effects on their lifecycle and offspring when compared to other *E. coli* strains, and it is for this reason these reasons that OP50 was used in this project (Stuhr and Curran, 2020; de Sousa Figueiredo et al., 2021).

OP50 strain of *Escherichia coli* was ordered from the *Caenorhabdities* Genetics Centre (https://cgc.umn.edu/) and streaked on LB plates. Sterile technique was used to extract a single colony of *E. coli*, that colony was then used to inoculate sterile LB broth. The inoculated LB broth was then left to incubate at 37 °C for 24 hours. 50 µL of inoculated LB broth was then added to the centre of each NGM plate and left to dry overnight.

If a new batch of inoculated LB broth was used it would first be tested for quality and contamination. This was done by setting up five NGM plates, seeding them with the new inoculated LB broth and allowing the plates to dry for 24h. These plates were then inspected using a microscope. As OP50 is a self-limiting bacterial strain it was important for the lawn to not be "overgrown'', this meant that's the lawn was only deemed suitable if it had a smooth surface and self-contained boarder. The plates were also inspected for any visible fungal growth or foreign contamination. If the LB broth was deemed suitable then the need test plates were poured and left to dry for 24h and then used.

2.3.3. Nematode stock preparation

The N2 strain of *C. elegans* was used in this project as this is one of the most researched strains, is easily maintained in a lab environment and is used as the standard wild-type strain across the board) (Brenner, 1974; Weber *et al.*, 2010).

To gain the initial stock of nematodes, frozen nematodes were collected from the Canterbury Christ Church University -80 °C freezer and were thawed at room temperature. Once the liquid was fully defrosted the vial was emptied on a non-seeded NGM plate and left to dry in a laminar flow hood. Once all the liquid evaporated, the plates were analysed under a microscope and live L1 nematodes were transferred to fresh seeded plates. These nematodes were left to grow and reproduce in a 20 °C incubator. After four days L1 nematodes were moved onto fresh plates, these steps were then repeated twice more to reduce the chance that any effect seen could be caused by the thawing process and to ensure that the nematodes used in any tests were healthy and well acclimated to the lab conditions.

2.3.4 *C. elegans* transport

Nematodes used in this project were moved using two methods; firstly, large quantities of nematodes were moved by adding 2.5 mL of M9 buffer and gently swirling the liquid around. The plate was then tilted to allow the nematodes to settle at the bottom of the plate. The nematodes were then pipette up and released onto new un-seeded plates or into falcon tubes. A second method was used to move individual nematodes onto new plates. Here a platinum nematode pick was sterilised using a Bunsen burner and used to carefully pick up an individual nematode and then moved onto a new plate.

2.3.5 Population synchronization

To ensure the nematodes were all at the L1 life phase at the time of testing the nematodes were age synchronised using the common bleaching method (Stiernagle, 2006). This was done by rinsing well established plates of healthy nematodes with M9 buffer and moving the nematodes into 15 mL tubes. The tubes were then centrifuged for 1 minute at 1500 rotations per minute (rpm). The supernatant was removed and 9 mL of M9 was added again. This M9 wash was repeated, and the supernatant was reduced to 1 mL. 0.5 mL of 10% bleach solution was added, and the tube was vigorously shaken for 5 minutes or until all the nematode cuticles were dissolved. To stop the reaction and to avoid damage to the nematode eggs, 10 mL M9 was added as soon as the nematode cuticle were dissolved, and the eggs floated freely. The tube

was then centrifuged for two minutes at 2000 rpm to allow the nematode eggs to settle and adhere to the bottom of the tube. The supernatant was removed and 1 mL of M9 was added to the tube, this was then shaken and pipetted onto non seeded NGM plates. These plates were left to dry in a laminar flow and once dry were moved to an incubator set at 20 °C for 24 hours. After 24 hours each plate was then seeded by adding 50 µL OP50 *E. coli.*

2.4 Acute BP-3 exposure methodology

2.4.1 Nematode preparation

Using a M9 wash, age synchronised nematode colonies were removed from their original NGM plates and placed in 15 mL tubes. In order to remove any *E. coli* remnants from the nematodes, the falcon tubes were filled with 10 mL with M9 buffer. The tubes were then left for five minutes to allow the nematodes to settle to the bottom after which the supernatant was removed using a pipette. This M9 buffer rinse was repeated twice.

2.4.2 BP-3 stock preparation

In order to minimise the chance of measuring error an original stock solution was used to create the final test concentrations of 500, 1000, 1500 and 2000 µg/L. This base stock solution was created by adding 0.01 g BP-3 to 1 mL DMSO and shaken until fully dissolved.

This original stock solution was then further diluted with DMSO to create the four test stocks. 10, 20, 30 and 40 µL were made up to 1 mL with DMSO to create the concentrations 500, 1000, 1500 and 2000 µL stocks respectively. Finally, 5 µL of each stock solution was added to 995 µL M9 buffer containing the washed nematodes.
In order to ensure the effects of DMSO were negligible on the nematodes the concentration of DMSO used in the actual exposure test was kept under 0.5% of the total solution. Despite this a control concentration containing 5 µL DMSO was used to survey for any unexpected effects of DMSO on the nematodes. These concentrations were chosen following a pilot study (figure 3.1) where it was found that environmentally relevant concentrations (50, 100, 250, 500 µg/L) were too low to induce measurable changed in the nematodes. Due to this it was decided to use the higher concentrations of BP-3.

2.4.3 BP-3 dosage

As soon as the DMSO and BP-3 solution was added to the nematodes the falcon tubes were lightly closed and placed on a shaking platform and gently shaken at 150 rpm. This was done to ensure enough agitation of the surface of the solution allowing for sufficient aeration for the nematodes. After being continuously shaken for 4 hours the test tubes were removed from the shaking platform and 9 mL M9 buffer was added to stop the any potentially toxic effects of BP-3.
The nematodes were allowed to settle to the bottom of the test tube and the supernatant was removed leaving 200 mL of solution. In order to remove any traces of BP-3 or DMSO, another M9 wash was performed by adding and removing 10 mL of M9 buffer. The 200 mL of M9 buffer containing the nematodes was then pipetted onto a

seeded NGM plates labelled with the corresponding concentrations. The plates were then left in the sterile environment created by a laminar flow hood until all the liquid had evaporated.

2.4.4 Fecundity endpoint

The universally used method for fecundity was adapted for this project (Zhang, Mostad and Andersen, 2021). Once all the liquid was evaporated the plates were removed from the laminar flow hood. 15 nematodes were selected at random, removed from each concentration and added to their own individual plates. The plates were numbered randomly with numbers ranging between 1 and 75 and were placed in a 20 °C incubator. After 24h the plates were removed from the incubator. Using a platinum pick the adult nematodes were moved onto new individual plates with the same number as the previous plate. Both the new plates and the original plates were then stored at 20 °C. After 72 hours the original plates were observed under the microscope. Using a four square grid drawn on the back of the plates and a clicker, the number of juvenile nematodes were counted and noted. This step was repeated daily for four days. After the fourth day the adult nematodes were moved to new plates and left at 12 °C for a further four days and then any juveniles were counted.

2.4.5 Growth endpoint

Images were taken using a microscope mounted camara. Firstly, an image was made of a 1 mm scale, this was used to calibrate the program settings. Images were then made

of each nematode and saved according to their plate number. After all the images were taken, the program ImageJ was used to analyse the images (Schneider, Rasband and Eliceiri, 2012). The 1 mm scale was used to calibrate the program to the microscope scale. Fragmented lines were drawn over the nematodes and the program converted these lines to known distance.

2.5 Contaminated *E. coli* exposure methodology

2.5.1 *E. coli* pellet preparation

In order to ensure a chronic exposure to *C. elegans* and to model potential natural interactions between pollutant and species interactions within a food web, a novel methodology was formulated. Two 250 mL chronicle flasks containing standard LB broth were firstly inoculated with a colony of stock OP50 *E. coli* and left in a shaking incubator set at 200 rpm and 37 °C for 24 hours. An empty 50 mL test tube was then weighed and labelled. 40 mL of the this newly inoculated LB broth was then added to the test tube and centrifuged at 5000 rpms for five minutes. The supernatant was then removed in a laminar flow and discarded. This step was then repeated for the rest of the prepared inoculated LB broth. The tube containing the resulting *E. coli* pellet was then weighed and the pellet weight was calculated by deducting the original tube weight from the final weight. The appropriate amount of M9 buffer was then added to the pellet to produce a M9 solution with a 10% *E. coli* concentration.

2.5.2 BP-3 preparation

0.01 g of Oxybenzone was dissolved in 1mL DMSO, this mixture was then shaken vigorously. 10, 20, 30 and 40 µL of this original solution were diluted further with DMSO to create the same concentrations as used in the acute exposure tests (500, 1000, 1500 and 2000 µg/L). 5 µL of these solutions were added to 995 µL of the 10% *E. coli* mixture.

2.5.3 Plate seeding

NGM plates were prepared without the addition of Peptone to ensure uptake of BP-3 by the bacteria. Once plates had solidified 50 µL of *E. coli* and BP-3 solution was added to each plate. These plates were then left in the laminar flow to dry overnight.

2.5.4 *C. elegans* dosing

Established *C. elegans* were taken from the 20 ^{o}C incubator and were age synchronised (as described above). L1 nematodes were then collected by preforming a M9 wash, here 10 mL of M9 was used to wash three plates of established L1 nematodes. The nematodes were then allowed to settle, and 5 mL of the supernatant was removed. 205 µL of this nematode solution was then pipetted onto each *E. coli* and BP-3 plate. Care was taken to gently shake the test tube before the nematodes were taken so that the nematodes were evenly dispersed into the solution and roughly the same number

of nematodes would be placed on each plate. The plates were randomly numbered and were placed in a 20 ^{o}C incubator for three days.

2.5.5 Growth endpoint

After 3 days the plates were removed, and the now adult nematodes were measured. This was done in the same manner as mentioned above, using the same microscope mounted camara and the analysis program ImageJ (Schneider, Rasband and Eliceiri, 2012).

2.6 Data analysis

After the normality tests were conducted it was decided that the Kruskal-Wallis testing method was most suitable for the analysis of the fecundity and length data, as this test is more robust against non- normally distributed data (Ostertagová, Ostertag and Kováč, 2014). Datasets that showed a P-value under 0.05 were then further analysed using Dunn method for *post-hoc* testing. Additionally, due to the repeated dependant nature of the length data of the acute test, it was decided that the Two-way ANOVA method was most suitable (Holcomb and Cox, 2018). All the data was adjusted using the Bonferroni method to compensate for the multiple testing. All the statistical analysis and graphics mentioned in this project was conducted in RStudio (version R-4.2.0 (RStudio Team, 2021))

3 Results

3.1 Preliminary toxicity tests

As BP-3 has never been tested on nematodes, it was unknown how sensitive *C. elegans* would be. Because of this, it was decided to run a small preliminary experiment looking at the reproduction potential of nematodes exposed to a variety of environmentally relevant BP-3 concentrations (50, 100, 250 and 500 µg/L) for 4 hours. Reproduction potential was investigated by analysing the effect of BP-3 on both the early fecundity (first two days) and the total fecundity (fecundity over five days). Analysis of the acute test data indicated that the early and total nematode fecundity was not affected by the different concentration groups (50, 100, 250 and 500 µg/L)(Figure 3.1 and 3.2).

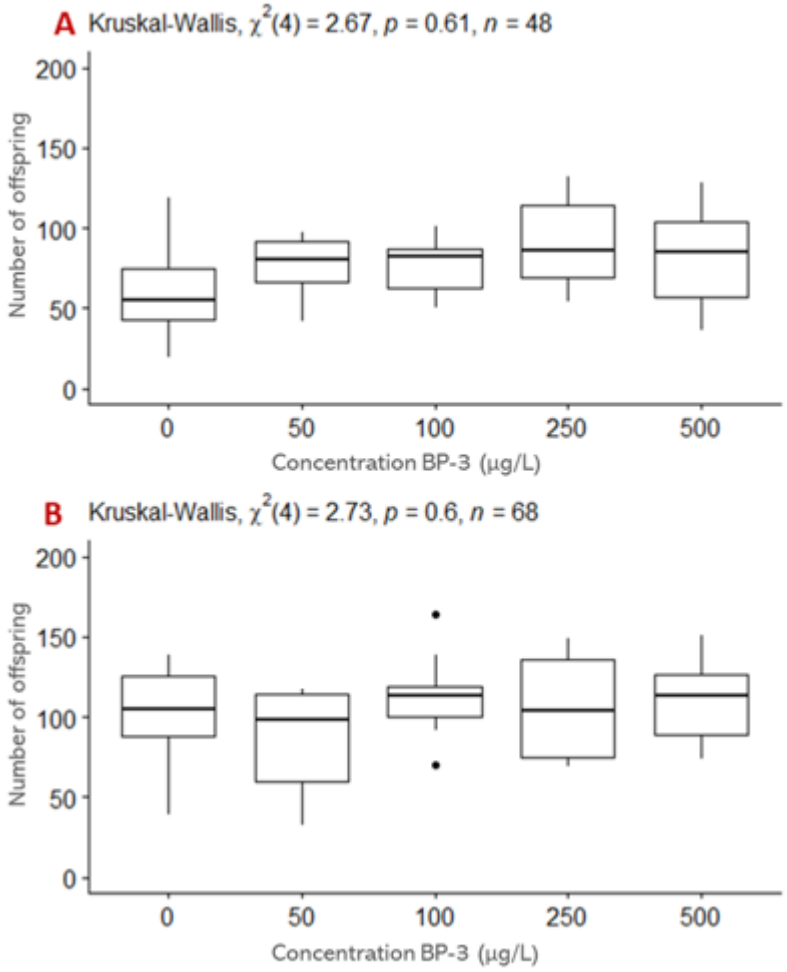

Figure 3.1: Comparison of early fecundity of *C. elegans* when acutely exposed to varying concentrations of BP-3. Nematodes were exposed to a range of concentrations of BP-3 (50, 100, 250, 500 µg/L) for four hours. There was also a control group that was not exposed to any amount of BP-3 (treatment group 0) however, they were exposed to the same amount of DMSO as the other groups. The number of offspring each adult nematode would produce within the first two days of maturity was presented by box plots showing the mean ± standard deviation (SD) with upper and lower quartiles as well as outliers represented as dots. The letters A and B

replicants the replicated conducted in this study. The letter n representing the number of nematodes used per replicate and p representing the significance for that replicate.

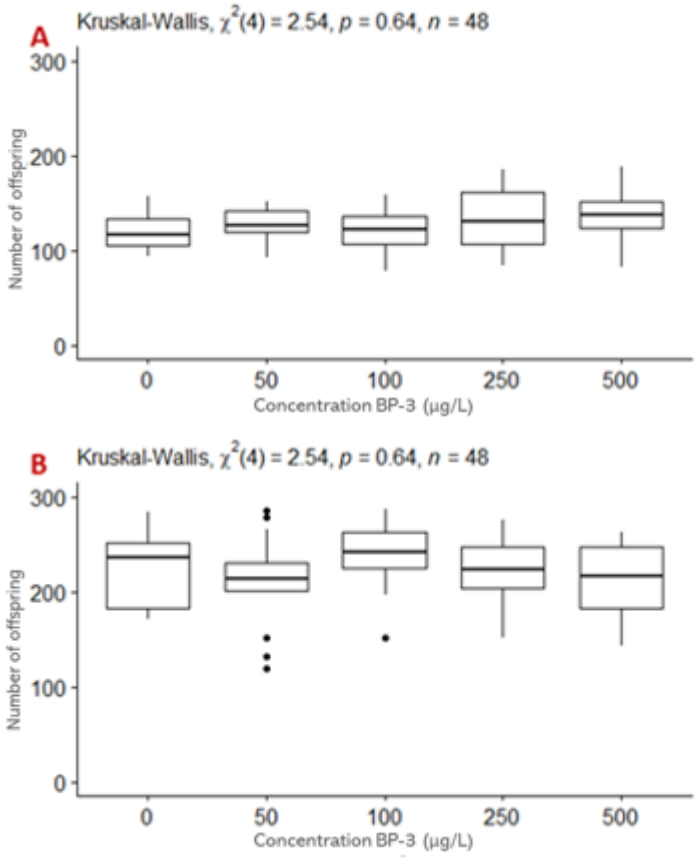

Figure 3.2: Comparison of total fecundity of *C. elegans* when acutely exposed to varying concentrations of BP-3. Nematodes were exposed to a range of concentrations of BP-3 (50, 100, 250, 500 µg/L) for four hours. There was also a control group that was not exposed to any amount of BP-3 (treatment group 0) however, they were exposed to the same amount of DMSO as the other groups. The total number of offspring produced by an adult nematode over the span of 5 days was presented by

box plots showing the mean ± standard deviation (SD) with upper and lower quartiles as well as outliers represented as dots. The letters A and B replicants the replicated conducted in this study. The letter n representing the number of nematodes used per replicate and p representing the significance for that replicate.

3.2 Acute exposure test

3.2.1 Effect of BP-3 treatment method

In order to better understand the initial effect of this experimental protocol on the nematodes, an analysis was conducted of the pre-test and post-test lengths of the different concentration groups (figure 3.3).

Analysis of the acute test data indicated that nematode lengths differed between treatment group in one of the three replicates (Replicate A, P=0.0081), but not in the other two replicates (Replicate B and C, P>0.05) (Figure 3.3). Post hoc testing indicated that in replicate A, the nematodes exposed to 500 µg/L of BP-3 for four hours were significantly larger than those measured before the treatment (PT)(Figure 3.3 A).

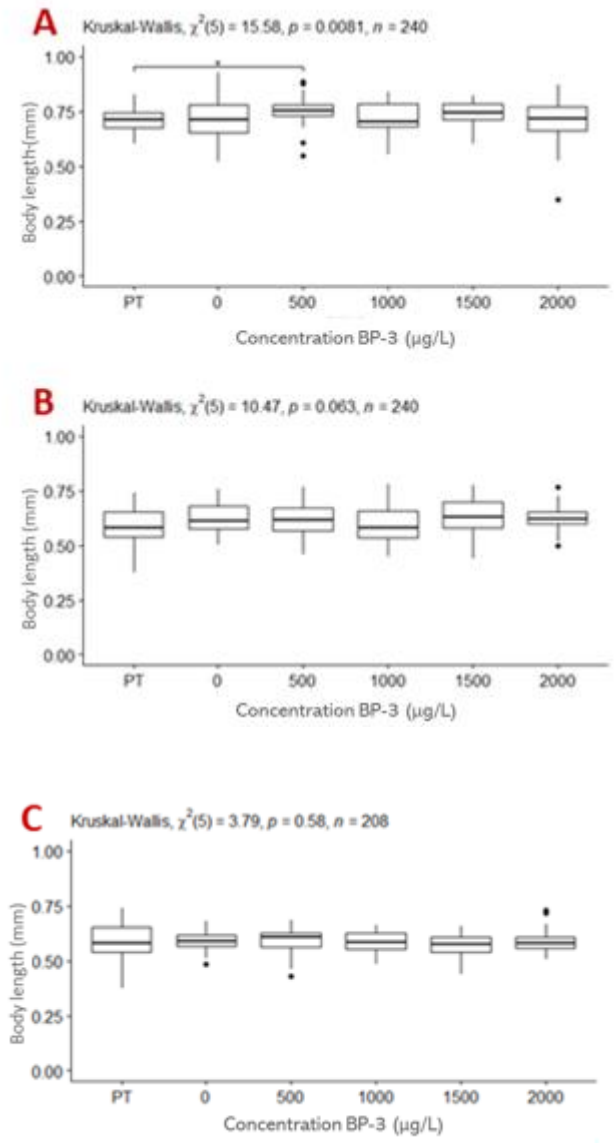

Figure 3.3: Comparison of length of *C. elegans* before and after 4-hour acute test. The growth of *C. elegans* was represented by their body-length before (PT) and after being exposed to BP-3 at a varying concentration (0, 500, 1000, 1500, 2000 µg/L) for four-hours. The length of each nematode in millimetres was presented by Box Plots showing the mean ± standard deviation (SD) with upper and lower quartiles as well as outliers represented as dots. Statistical significance was represented by linked boxes annotated with a star symbol. The letters A, B and C replicants the replicated

conducted in this study. The letter n representing the number of nematodes used per replicate and p representing the significance for that replicate.

3.2.2 Effect of acute BP-3 exposure on growth

In order to determine the effects of BP-3 on the growth of *C. elegans* over time, L1 nematodes were treated with different concentrations of BP-3 and their length measured on days 1-4 (Figure 3.4). Analysis of length indicated that there was a consistent effect of time on length ($F_{3,12}$= 28.600, P<0.001, $F_{3,12}$= 5.025, P<0.001, $F_{3,12}$= 48.55, P<0.001 for replicates 1-3, respectively). This analysis did not however identify any effect of BP-3 concentration on nematode length ($F_{3,12}$= 1.213, P>0.05, $F_{3,12}$= 2.460, P>0.05, $F_{3,12}$= 2.460, P>0.05 for replicates 1-3, respectively) or any interaction between BP-3 dosage and time ($F_{3,12}$= 1.006, P>0.05, $F_{3,12}$= 0.439, P>0.05, $F_{3,12}$= 1.201, P>0.05 for replicates 1-3, respectively). This indicates that for the dosage used here, BP-3 did not effect the growth of L1 nematodes.

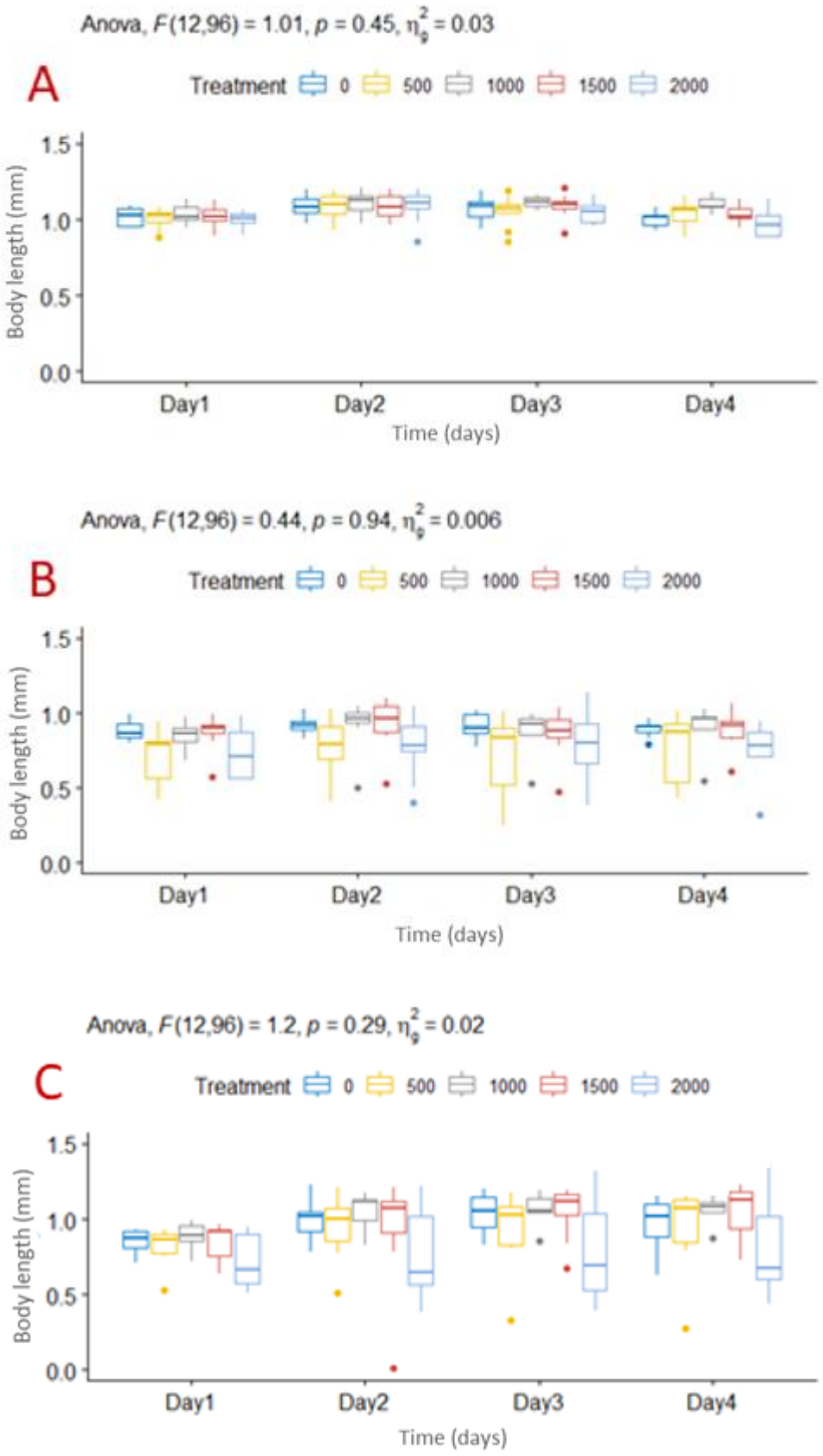
Anova, F(12,96) = 1.01, p = 0.45, η²p = 0.03
A
Treatment 0 500 1000 1500 2000
Body length (mm)
1.5
1.0
0.5
0.0
Day1
Day2
Day3
Day4
Time (days)
Anova, F(12,96) = 0.44, p = 0.94, η²p = 0.006
B
Treatment 0 500 1000 1500 2000
Body length (mm)
1.5
1.0
0.5
0.0
Day1
Day2
Day3
Day4
Time (days)
Anova, F(12,96) = 1.2, p = 0.29, η²p = 0.02
C
Treatment 0 500 1000 1500 2000
Body length (mm)
1.5
1.0
0.5
0.0
Day1
Day2
Day3
Day4
Time (days)

Figure 3.4: Daily growth of *C. elegans* after acute exposed to varying concentrations of BP-3. Over four days daily measurements were taken of nematodes that had been exposed to a range of concentrations of BP-3 (500, 1000, 1500, 2000 µg/L) for four hours. There was also a control group that was not exposed to any amount of BP-3 (treatment group 0) however, they were exposed to the same amount of DMSO as the other groups. The length (in millimetres) of each nematode was presented by coloured box plots showing the mean ± standard deviation (SD) with upper and lower quartiles as well as outliers represented as dots. The letters A, B and C replicants the replicated conducted in this study. The letter n representing the number of nematodes used per replicate and p representing the significance for that replicate.

3.2.3 Effect of acute BP-3 exposure on fecundity

In order to increase our understanding of the effect that BP-3 might have on fertility, an analysis was conducted of the fecundity potential of nematodes exposed to different concentrations of BP-3 (500, 1000, 1500, 2000 µg/L). Two aspects of nematode fecundity were investigated. Firstly, the effect of BP-3 on early fecundity was analysed. To do this the fecundity of each nematode was measured daily and the data from the first two days was summed up together (figure 3.5). The effect BP-3 might have on total fecundity history was also investigated by summing up all the fecundity data for each nematode into one total fecundity number (figure 3.5).

3.2.3.1 Early fecundity

Analysis of the acute test data indicated that early nematode fecundity did not differ between treatment group in the three replicates (Replicate A, B and C, P=>0.05) (Figure 3.5)

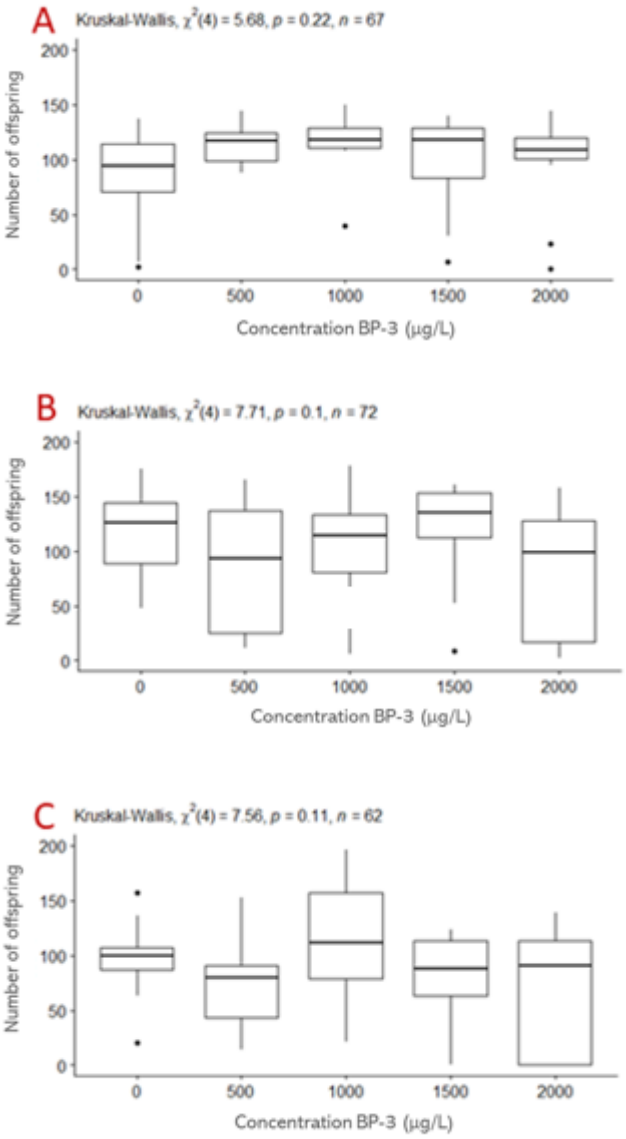

Figure 3.5: Comparison of early fecundity of *C. elegans* when acutely exposed to varying concentrations of BP-3. Daily measurements were taken of nematodes that had been Nematodes were exposed to a range of concentrations of BP-3 (500, 1000, 1500, 2000 µg/L) for four hours. There was also a control group that was not exposed to any amount of BP-3 (treatment group 0) however, they were exposed to the same amount of DMSO as the other groups. The number of offspring each adult nematode would produce within the first two days of maturity was presented by box plots showing the mean ± standard deviation (SD) with upper and lower quartiles as well as outliers represented as dots. The letters A, B and C replicants the replicated conducted in this study. The letter n representing the number of nematodes used per replicate and p representing the significance for that replicate.

3.2.3.2 Total fecundity

Analysis of the acute test data indicated that total nematode fecundity differed between treatment group in two of the three replicates (Replicate A, P=0.016 and replicate B, P=0.036), but not in the other one replicate (Replicate C, P>0.05) (Figure 3.6). After conducting a Post hoc analysis, it was concluded that we were unable to pinpoint which concentration group contributed to the overall significant difference as none of the individual groups themselves were significant (P>0.05).

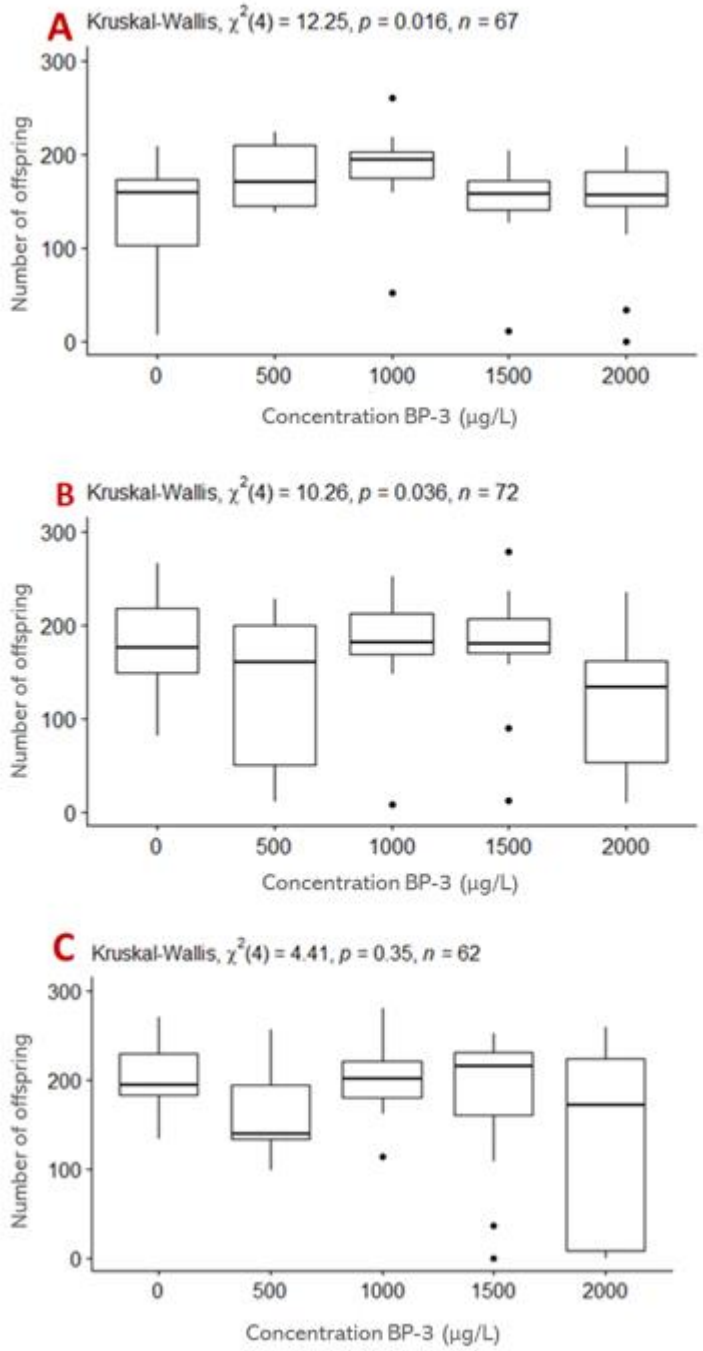

Figure 3.6: Comparison of total fecundity of *C. elegans* when acutely exposed to varying concentrations of BP-3. Nematodes were exposed to a range of concentrations of BP-3 (500, 1000, 1500, 2000 µg/L) for four hours. There was also a control group that was not exposed to any amount of BP-3 (treatment group 0) however, they were exposed to the same amount of DMSO as the other groups. The

total number of offspring produced by an adult nematode over the span of 5 days was presented by box plots showing the mean ± standard deviation (SD) with upper and lower quartiles as well as outliers represented as dots. The letters A, B and C replicants the replicated conducted in this study. The letter n representing the number of nematodes used per replicate and p representing the significance for that replicate.

3.3 Chronic exposure test

3.3.1 Effect of chronic BP-3 exposure on growth

An analysis of the body lengths of *C. elegans* fed *E. coli* that had been exposed to a variety of BP-3 concentrations (500, 1000, 1500, 2000 µg/L) was conducted (figure 3.7). This was done to better understand how BP-3 can affect nematodes over an extended amount of time.

Analysis of the test data indicated that nematode lengths differed between treatment group in one of the three replicates (Replicate B, P= 0.011), but not in the other two replicates (Replicate A and C, P>0.05) (Figure 3.7). Post hoc testing indicated that in replicate B, the nematodes fed *E. coli* exposed to 1500 µg/L of BP-3 for three days were significantly smaller than those fed *E. coli* exposed to 1000 µg/L for three days ($p<0.005$) (figure 3.7 B).

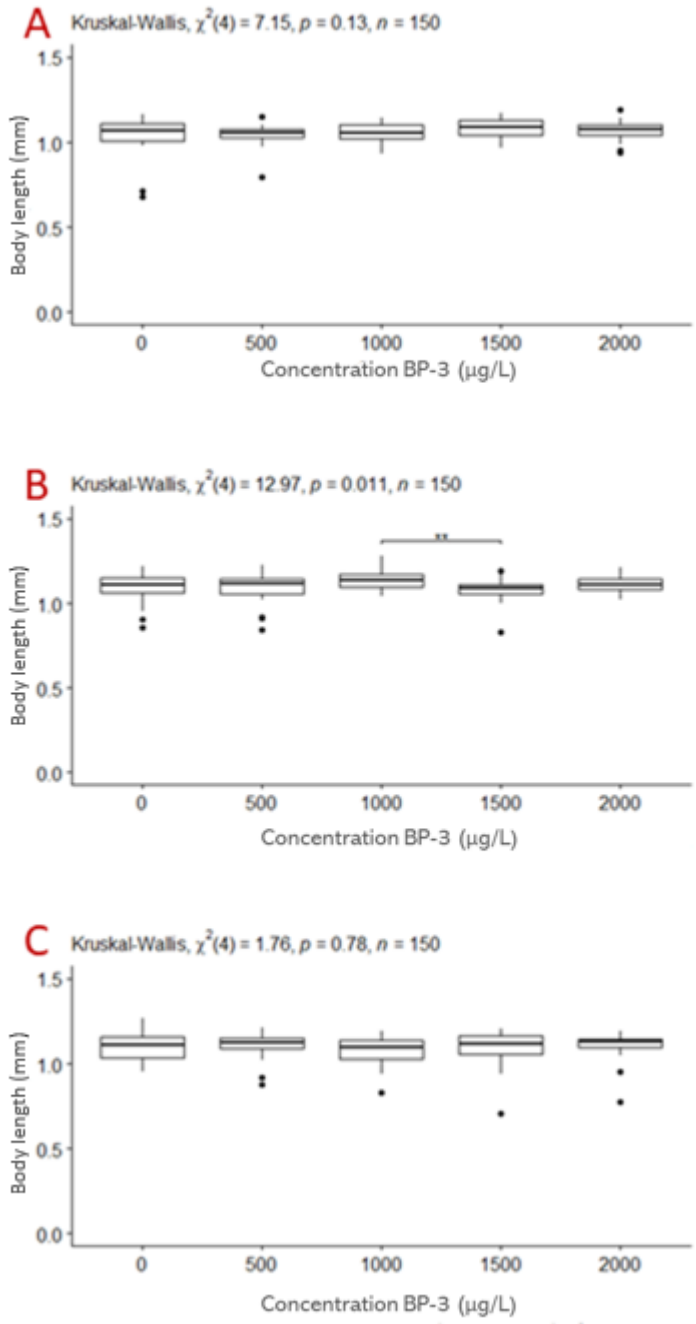

Figure 3.7: Comparison of length of *C. elegans* when exposed to varying concentrations of BP-3 for 3 days. The length measurements (in millimetres) of *C. elegans* were taken after three days of being fed *E. coli* contaminated with varying concentration (500, 1000, 1500, 2000 µg/L). There was also a control group that was fed *E. coli* that had not been exposed to BP-3 but was exposed to the same amount of

DMSO as the other groups (treatment group 0). The length of each nematode was presented by box plots showing the mean ± standard deviation (SD) with upper and lower quartiles as well as outliers represented as dots. Significance is represented by brackets connecting the boxplots and are noted with a star symbol. The letters A, B and C replicants the replicated conducted in this study. The letter n representing the number of nematodes used per replicate and p representing the significance for that replicate.

4 Discussion

Although the research into UV-filters is in its infancy, it is noticeable that BP-3 has been highlighted as a potential danger (Paredes *et al.*, 2014). BP-3 has been sampled very commonly both within the environment and even in living organisms, however what exactly happens there is relatively unknown. It is believed that some organic UV-filters act as EDCs and that EDCs can be detrimental however the limitation of this effect is unknown (Fent, Zenker and Rapp, 2010). Because one of the effects of BP-3 was the ability to cause alterations to the germline, the question arises if BP-3 can cause multigenerational effects (Anway and Skinner, 2006; Wnuk *et al.*, 2018). Multigenerational effects or epigenetic effects can be very difficult and costly to investigate using traditional models such as rats and mice, it is for this reason that we investigated the effects of BP-3 on the nematode *C. elegans* (Hunt, 2017). *C. elegans* is a small nonparasitic nematode which are naturally found in the soil or on decaying fruits and vegetables. *C. elegans* are a commonly used model within the field of toxicology as they are easily kept in a laboratory environment, are translucent, have a

very well documented life history and have a lifecycle of just a few weeks in which they can produce hundreds of offspring, potentially making it a suitable model for BP-3s multigeneration effect (Hunt, 2017). In order to further our understanding of BP-3s effects and the assess the suitability of *C. elegans* as a model for UV-filter toxicity, a number of experiments were conducted (Hunt, 2017).

As the aim of this project was to determine the effect of BP-3 on living nematodes it was immensely important to make sure that both the acute test method and the BP-3 concentrations did not outright kill the nematode but instead affected them to the point of inducing significant alterations to their life history. It was for this reason that a pilot study was conducted to determine the suitability of the experimental methodology and to determine the appropriate test concentrations.

Although this pilot was successful in the sense that there were little deaths during the exposure period during the subsequent days, it was discovered after statistical analysis of the fecundity data that the concentrations used did not invoke a response on fecundity (Figure 3.1 and 3.2). Due to this it was decided to increase the test concentrations to the higher concentrations of 500, 1000, 1500 and 2000 µg/L. Although this was no longer in line with the amounts of BP-3 currently being sampled within recreational areas it is still very much relevant as it has been shown that due to BP-3s low LOG_{KOW} value it can accumulate within tissues and sediments to unknown levels. This sediment is then often used as fertiliser in agriculture. It could thus be hypothesised that there are areas within the environment where organisms could be exposed to these very high levels of BP-3, similar to those used in this project.

Due to the fact that BP-3 has not been used in this manner before, we did not know how it would react to the test conditions. One of the unknowns is how BP-3 would affect the osmolarity of the M9 buffer or if BP-3 would alter *C. elegans* ability to acclimate to changing parameters. When *C. elegans* are exposed to fluctuations in extracellular osmolarity in their natural habitat or during test conditions, they experience hypertonic stress (HTS). When initially exposed to HTS the body cavity of *C. elegans* shirks rapidly due to water being pulled out of the cells (Lamitina *et al.*, 2004). This cell shrinkage activates the protein kinase 1 (WNK-1) pathway, this pathway is the initial cellular response that controls the influx of ions within the cell, restoring cell volume (Choe and Strange, 2007). The WNK-1 activation is initiated by cell shrinkage and results in the phosphorylation of the SPS1-related proline, alanine-rich kinase (SPAK) and oxidative stress responsive kinase 1 (OSR1). SPAK and OSR1 then activate the cotransporter NKCC1 which regulates the sodium-water influx to correct the osmolarity within the cells (Choe and Strange, 2007). It has been shown that several small molecules have been able to potently inhibit the function of this WNK1-SPAK - OSR1 pathway (AlAmri *et al.*, 2017). In order to investigate if such an inhibition could be caused by BP-3, an analysis was conducted looking at the body length of the nematodes directly before and after being exposed directly to BP-3.

It was found that two replicates showed no significant difference between the body lengths of the nematodes exposed to the varying BP-3 concentrations and the Pre-treatment group (Figure 3.3 B and C), while one replicate found that the nematodes exposed to 500 µg/L were significantly larger than those measured pre-treatment (Figure 3.3 A). Due to the inconsistencies within the replicated it is impossible to

confidently comment on how BP-3 effected the osmolality or the ability of *C. elegans* to accumulate to a change in osmolality through the WNK1-SPAK-OSR1 pathway within this study.

Although the exact reason for the inconsistencies is unknown, it is possible that the significant size difference could be caused by a failure to effectively divide the nematodes into the concentration groups with an even distribution of size as the PT group was an average body length of 40 randomly selected nematodes which were then divided into the test groups. Given the opportunity again it would be interesting to conduct this experiment again however I would measure the nematodes of each concentration group specifically before and after the experiment.

Acute BP-3 exposure has been shown to significantly inhibited the growth of the green algae *Desmodesmus subspicatus*, with similar results reported for *Chlorella vulgaris* (Du et al., 2017, *Sieratowicz et al., 2011),* the unicellular eukaryote *Tetrahymena thermophila* (Gao *et al.*, 2013) however, the effects of BP-3 have never been tested on the model *C. elegans* and so the effects it would have on the nematode are completely unknown. Due to the fact that many studies have reported inhibited growth in a host of different models when exposed to BP-3, it was hypothesised that BP-3 toxicity would induce a similar response in *C. elegans.* recently Huang *et al,.* (2022) conducted a similar study where *C. elegans* were exposed to 0, 1, 5, 10, 100, 500 µM of OMC. In this study, they found that the growth of *C. elegans* was inhibited when exposed to the minimum concentration of 100 µM OMC. However, after an analysis of our data, it was discovered within our 3 replicates there was no significant difference in body length between *C. elegans* exposed to BP-3 and not (Figure 3.4).

In this project the effect of BP-3 on reproductive health was also investigated by measuring both early fecundity (over the first 2 days) and total fecundity (Figure 3.5 and 3.6, respectively). This two-part fecundity analysis was chosen as it has been reported in another study that when exposed to a stressor the total amount of hatched offspring of *C. elegans* was not affected, however after further investigation it was found that there was indeed a significant delay in the fecundity in the first few days, this demonstrated a delay in the start of fecundity and that this was later recuperated (Gruber, Soon and Halliwell, 2007). In our study it was discovered that although there was no effect of BP-3 exposure on the early fecundity (Figure 3.5) there was a significant effect of BP-3 on the total fecundity in one of the three replicates (Figure 3.6). Unfortunately, we were unable to determine which groups were affected as the post hoc P-values did not highlight any specific group as being significantly affected. In contrast, Huang *et al,.* (2022) also looked at how OMC affected the fecundity of *C. elegans*, here they found OMC exposure clearly reduced the total fecundity of the nematodes. The clear results that Huang *et al,.(*2022) achieved stand in contrast with our results, this could be due to a number of reasons;

Firstly, when comparing the two studies it can be noted that there are a number of slight differences in methodology. One such slight difference in methodology worth mentioning is that there was a difference between the amounts of DMSO used to dissolve the UV-filters. Hueng *et al ,.*(2022) used a total concentration of 0.1% DMSO while we used a total concentration of 0.5% DMSO. Many studies have found that the use of DMSO can in some circumstances extend the life of *C. elegans*, however if the

concentrations are kept under 0.5% the effects on life span and progeny are not significant (Boyd et al., 2010; Wang et al., 2010).

Another one of these differences in methodology for example is the fact that Huang *et al,.* (2022) dissolved the OMC and added this to NGM plates, while in our study it was decided to dissolve the BP-3 and add the pollutant directly into the M9 media that the nematodes were temporarily housed in. This was done to quicken the time between BP-3 preparation and *C. elegans* exposure as it is unknown if the DMSO might quicken the decomposition of BP-3. Although this difference in methodology might have contributed to the difference between the two studies it is worth mentioning that the use of liquid media (including M9 buffer) for acute toxicity studies with *C. elegans* is relatively common as it allows for more precise quantification of the chemical being tested (Boyd *et al.*, 2003; Meyer and Williams, 2014).

One such example is a study conducted by Chen *et al*., in 2019, where nematodes were exposed to a toxin using a liquid medium for a short amount of time. In our project specifically nematodes were housed in M9 buffer dosed with varying concentrations of BP-3 for four hours. In contrast to the study conducted by *Chen et al,.(2019)* the nematodes used in this project did not have access to bacterial food, this decision was made as live *E. coli* could potentially metabolise the BP-3 and thus alter the toxicity, and feeding sterilised *E. coli* was deemed not preferable, partly due to a lack of resources available, making UV-sterilising *E. coli* impossible as well as the possibility of decreasing the quality of food due to heat denaturing or UV-radiation (Eisenmann, 2005; Chen *et al.*, 2019).

One consequence of not using a bacterial food source during the experiment is that the nematodes were essentially exposed to a four-hour starvation period. Starvation for more than 12 hours has been shown to cause developmental arrest in *C. elegans* while acute exposure of several hours caused a hyper state in the nematodes characterised by an increased amount of pharynx pumping (Ahmadi and Roy, 2016). It is believed that the AMP-activated protein kinase (AMPK) acts as a trigger for the AMPA-type glutamate receptor (GLR-1) and the metabotropic glutamate receptor (MGL-1) which initiated this behavioural change, and that this aroused state was developed as an evolutionary response with the aim of increasing the possibility of locating food(Ahmadi and Roy, 2016; Wu *et al.*, 2018). In order to minimise the starvation period that the nematodes experienced, they were removed as quickly as possible after the four-hour experiment, washed with M9 buffer and placed on NGM plates seeded with *E. coli*. Another aspect to consider is how much light the nematodes were exposed to during the experiment. Some studies have found that the toxicity of UV-filters is significantly enhanced when pollutant exposure is accompanied by UV-radiation (Downs *et al.*, 2014). Considering that the test concentration was only briefly exposed to UV-light during measuring and dosing and nematodes used in this project were left in the dark during the four-hour test period, it could be assumed that the amount of UV-radiation that directly affected the samples was minimal. In the environment it would be highly likely that animals exposed to BP-3 would also be exposed to UV-light radiation from the sun. Downs *et al,.*(2016) found that BP-3 caused ossification in coral planula, causing skeletal malformation which in turn resulted in a coral bleaching. They also found that this effect was over three times stronger when exposure was accompanied by eight hour of UV-radiation, the reaction

was over five times higher when exposed to 24 hours of UV-radiation (Downs *et al.*, 2016).

Some UV-filters degrade relatively fast, with EHMC and OD-PADA with having half-lives of 20 and 57 hours respectively (Rodil *et al.*, 2009; Badia-Fabregat *et al.*, 2012), while BP-3 degrades slower with about 8% degradation seen in 20 days (Liu *et al.*, 2011). Regardless of this slower degradation it is still possible that degradation under different conditions could cause a significant difference in toxicity between co-exposure with UV-radiation or without. It is believed that the degradation of BP-3 in darkness is due to a process called ozonation (Wang *et al.*, 2022). Here, O_3 oxidises the unsaturated compounds via selective addition reaction (Wang *et al.*, 2021). When under the effects of UV-radiation it was found that degradation of BP-3 was largely caused by non- selective OH radical (OH·) attack (Gong *et al.*, 2015; Semones *et al.*, 2017). The different types of degradation could result in different concentrations of varying degradation products. Although research in area is very much in its infancy, there have been some studies looking into the toxicity of these derivatives, not only to assess them as potential secondary pollutants but also to assess their potential use as UV-filter alternatives in PCPs. The most recent of these BP-3 derivatives to be investigated is BP-4. BP-4 was found to be slightly less toxic than BP-3 with an EC50 value near 10.000 µg.L-1 (slightly toxic) in the algae *Isochrysis galbana*, the crustacean *Scorpaenopsella armata*, the sea-urchin *Paracentrotus lividus* and the bivalve *Mytilus galloprovincialis* (Paredes *et al.*, 2014). In contrast, the BP-3 derivative, BP-2 (which has previously been suggested as a “environmentally friendly” option (Du *et al.*, 2017)) was found to have an increased ability to bind to oestrogen receptors causing BP-2 to be an

even more potent EDC as BP-3 (Thia, Chou and Chen, 2020). These studies would suggest that toxicity of BP-3 solution could be altered depending on the type of derivatives released as well as the concentration released.

As neither our study nor the comparable study conducted by Huang *et al,.*(2022) investigated exactly which derivatives were created during the experimental period we cannot confirm if derivatives played a part in the toxicity of either OMC or BP-3 used in these studies. This issue is only exacerbated when considering that there was a significant difference between exposure times; being that the nematodes Huang *et al,.* (2022) analysed had been exposed to OMC for 72 hours while our nematodes were only exposed to BP-3 for four hours.

As BP-3 gains more notoriety as a potential pollutant it is become more and more important to find suitable alternatives while still providing sufficient UV-absorption capabilities to protect the wearer from sun damage. One approach has been to produce and test a UV-filter called Avobenzone (AVO), a synthetic UV-filter. The U.S Food and Drug Administration (FDA) found that AVO had a significantly lower skin absorption capability than BP-3, with AVO having an overall maximum plasma concentration of 7.1 ng/mL compared to BP-3 with a concentration of 258.1 ng/mL (Matta *et al.*, 2020; Zhong *et al.*, 2020). A lower overall plasma concentration is favourable as this means that less of the UV-filter is penetrating the dermis and thus reducing the amount of direct exposure the wearer faces. That said even AVOs substantially lower overall plasma concentration was significantly higher than the 0.5 ng/mL threshold the FDA has set, leading them to call for further investigation (Matta *et al.*, 2020; Zhong *et al.*, 2020). It is also worth mentioning that the overall plasma

concentrations used in the FDA study were derived from test subjects applying each UV-filter once on 75% of their body, while it has been reported that beachgoers apply UV-filters an average of 2.6 times in a day (Ahn *et al.*, 2019; Labille *et al.*, 2020). Taking discrepancy into account, it could be assumed that the amount of AVO that people would directly be exposed to would be much higher.

Another alternative approach has been to focus on using natural compounds derived from plants and algae as a sustainable UV-filter. Several potential extracts have been identified as having promising UV-light blocking capabilities as well as being capable of acting as an antioxidant protecting the skin from ROS. Almeida *el al.,* (2015) found that extracts (at a dosage of 1 µg/mL) taken from the leaves of the *Castanae sativa* plant was able to reduce UV-mediated DNA damage in the human keratinocyte cell line by 66.4% (Almeida *et al.*, 2015). Although this seems promising, there are currently no sun protection products approved that contain natural alternatives as the active ingredient. This is most likely due to several factors. One factor is the fact that adding natural components to formulations can drastically change the finished product. This was observed in the case where the seed oil of the green *Coffea arabica* plant was added to formulations as it was shown to have UV-protective capabilities, the additive caused the solution to become highly viscous, making application difficult likely resulting in insufficient skin coverage, as well as likely being unfavourable with consumers (Wagemaker *et al.*, 2015). Another reason for the lack of natural active ingredients being used in sun protection products is likely due to the fact that toxicity can be difficult to predict. One example of this was with the extract of the red seaweed *Eucheuma cottonii*, it was found to have a beneficial antioxidant effect on human

keratinocyte cell lines however, it was later on discovered that this extract was cytotoxic at higher concentrations (Lim *et al.*, 2015).

Although there has definitely been a push for the discovery of a suitable and sustainable alternative to the currently used UV-filers including BP-3, it seems that the discovery of such alternatives has been hindered by the fact that many of these studies are not directly comparable. The research conducted in this study, albeit not strong enough on its own to stand as a reliable example it does highlight the importance of a stable test environment, consideration of degradation products and a wide range of endpoint selection that could be used to optimise a *C. elegans* toxicity assay that could then be used to accurately compare UV-filters.

Due to the large volumes of UV-filters believed to pass through the WWT facilities and the fact that BP-3 has a high log-Kow value which allows BP-3 to accumulate within this sludge it could be possible that this sludge could be contaminated with high levels of BP-3 (Tsui et al., 2014; Ekpeghere et al., 2016).Traditionally, active sludge is disposed of by incineration, ocean dumping and even by dumping into landfills, while more recently active sludge has also been used as animal feed, construction materials and fertilizer (Ni et al., 2017). As a number of these disposal methods can result in secondary pollution of both soil and aquatic environments and the amounts of UV-filter contained in this active sludge is expected to be high but in ultimately unknown, it is becoming more and more important to understand how chronic exposure of these UV-filters effects the organisms that inhabit these areas (Ni et al., 2017).

If BP-3 contaminated active sludge would be used for example to fertilize the soil it could be hypothesised that the bacteria in this active sludge would also be

contaminated with BP-3, the bacterivore organisms would then consume these contaminated bacteria.

To model this, we decided to create a novel experiment where we exposed *E. coli* to varying concentrations of BP-3 and then allowed *C. elegans* to feed on this contaminated *E. coli* for several days, the nematodes were then analysed for body growth inhibition. It is hypothesised that this form of exposure is more potent than through the liquid exposure demonstrated in the previous experiments, this is due to the fact that this form of exposure ensures that the BP-3 enters the body through the pharynx pump, while the liquid exposure does in theory not insure this. As nematodes breath through gas diffusion and the nematodes used in the other studies in this project did not have access to food, the pharynx might not be pumping regularly (Bretscher *et al.*, 2011; Ortiz *et al.*, 2018).

The novel methodology used in this project consisted of dosing a 10% live *E. coli* solution with the same concentrations of BP-3 as was used in the previous studies conducted in this project (500, 1000, 1500 and 2000 µg/L). This contaminated *E. coli* were then used to seed the NGM plates, once dried 20 nematodes were added to each plate and stored in a 20 ^{o}C incubator after 36 hours nematodes were measured. After the results were analysed, it was discovered that in one replicate, the nematodes that had been fed *E. coli* contaminated with 1500 µg/L BP-3 were significantly smaller than the nematodes fed *E. coli* contaminated with 1000 µg/L BP-3 (Figure 3.7 B).Although it is interesting that there is a significant difference within these groups, as these results

were not seen in the other replicates (Figure 3.7 A and C), we are unable to confidently comment on how the contaminated *E. coli* affected the nematodes.

It was expected that the fact that the nematodes were ingesting the BP-3 through contaminated *E. coli* would significantly affect the body growth however our results did not definitely show this. The fact that live *E. coli* was used could have contributed to this unexpected result. The decision was made to use live *E. coli* as to avoid a phenomenon known as ''leaving'', here *C. elegans* will ignore food if it is deemed poor quality and will go searching for higher quality food (Shtonda and Avery, 2006). One possible explanation for the lack of toxicity could be that the live *E. coli* contributed significantly to the degradation of the BP-3 during the 3-day exposure time and created a less harmful BP-3 derivative. Several studies have investigated the use of novel micro-organism species to improve the efficiency of WWT facilities in removing UV-filters. Many of these studies have made use of the method known as "reverse discovery'', where individual strains of micro-organism are identified within a sample of bio-solid that is demonstrating bio-degradation capabilities(Suleiman *et al.*, 2019). Using this method Badia-Fabregat *el al,.* (2012) discovered that the fungus species *Tinae versicolor* was able efficiently break down 100% of the UV-filter 4-MBC within 24 hours while the bacteria species *Mycobacterium agri* broke down 19% of OC in 10 days (Badia-Fabregat *et al.*, 2012; Suleiman *et al.*, 2019). Currently work is also being done to identify the specific micro-organism responsible for the degradation of EHMC found in sediment samples collected from China (Zhang *et al.*, 2021). One bacterium that is of particular interest is the BP14P strain of *Sphingomonas wittichii* as it has been discovered that this bacterium actively used BP-3 as a carbon source which resulted in

a 95% decrease in BP-3 concentration after four days, and with the addition of R2B media, was also able to slowly degrade both 2-ethylhexyl salicylate (ES) and homosalate, but at low rates (28% and 18% within 14 days respectively)(Fagervold *et al.*, 2021) Most recently, a novel S12- 17 strain of the bacterial genus *Rhodococcus* was discovered, this strain was able to completely degrade BP-3 within ten days (Baek *et al.*, 2022). It is believed that S12- 17 uses a mixture of demethylation, hydroxylation, dioxygenation, reduction, and hydrolysis reactions to create several derivatives including BP-1, 2, 3, 4 and benzoic acid (Baek *et al.*, 2022). And as we also not test for the presence of any derivative it is impossible to say if *E. coli* was able to metabolise the BP-3 and if so, which derivatives were created. Further to this and as mentioned in the previous chapter, the research into the toxicities of these derivative is still in its infancy and with the effects of these on *C. elegans* being completely unknown.

Unfortunately, due to the inconsistencies within the replicates we are unable to assess how *E. coli* contaminated with BP-3 effects the nematodes. That said, this experiment has highlighted the need for further investigations into the relationship between micro-organisms and UV-filters and what derivatives are created and highlights the importance of subsequent toxicity tests for these derivatives. Due to the chemical characteristics of UV-filters many WWT strategies are ineffective, leading to WWT plants being one of the main contributors to UV-filter pollution (Tsui *et al.*, 2014; Ekpeghere *et al.*, 2016; Salthammer, 2020). This issue is further exacerbated when considering the other forms of pollution also associated with WWT plants including overflows, use as fertilizer and disposal in landfills (Ni *et al.*, 2017; Ji *et al.*, 2020) One promising solution to this issue would be to improve the effectiveness of the strategies

used by these WWT plants (Vasilachi *et al.*, 2021). The implementation of new strategies such as biofilms, membrane filters and constructed wetlands in combination with bio-sludge that has been specially inoculated with micro-organisms proven to degrade UV-filters could make a significant difference in the amounts of UV-filters entering the environment and thus reducing the environmental risk (Chen *et al.*, 2016; Sehar and Naz, 2016; Khan *et al.*, 2020; Vasilachi *et al.*, 2021).

The number of studies highlighting UV-filters as emerging pollutants is concerning however the number of studies focussing on the effects of different UV-filters on different models makes it difficult to compare UV-filters to each other. The ability to compare the toxicity of UV-filters to each other could be immensely beneficial when considering potential alternative UV-filters to be used in PCPs. One way to resolve this issue is to create a standardised assay that could be used to directly compare UV-filters. Considering their natural habitat, homology with humans and suitability as models for EDC effects, *C. elegans* could be suggested as a suitable model organism for this assay. Hueng *et al.,* (2022) demonstrated that *C. elegans* could definitely be a suitable model for this UV-filter toxicity assay, however our study also highlights some current constraints with this model. Besides the previously mentioned issue of potential natural degradation of BP-3 and the potential metabolization of BP-3 by bacteria, there were also several other things worth mentioning.

Firstly, although the nematodes were housed in a stable 12 and 20 °C incubators there were brief periods that the nematodes were exposed to higher temperatures. These instances would for example be during the four hour exposure test, when the nematodes were moved and briefly when images were taken using the microscope.

During the summer months the laboratory averaged a temperature between 21-25 °C. Although these temperatures are within the range deemed physiological for the nematodes (15-25 °C), the temperatures observed were on the higher end of this scale and would surpass this optimum to by a several degrees on occasions. It is possible that this and/or the change of temperature from incubator temperature to laboratory temperature could have caused some level of stress response in all the nematodes, thus effecting the results found. One way to limit the effect of heat could be to use a *C. elegans* strain that is more heat- stress resistant. In 1988, Friedman and Johnson successfully discovered the *age-1* strain of C. elegans, which could live 65% longer at 25^{o}C then the wild type of *C. elegans* used in this project (Friedman and Johnson, 1988). Another way to potentially reduce the amount of stress that *C. elegans* experience from the heat could be by altering their diet. In 2018 a study found that nematodes fed with *Bacillus subtilis* had less of a stress response when compared to nematodes fed with *E. coli* (Gómez-Orte *et al.*, 2018). Were this experiment to be repeated an alternative food source could potentially be utilised to optimise the acute exposure experiment in warmer environments.

This project of course was set in a very controlled setting and exposed the nematodes only to BP-3 and takes away all other aspects of the natural life C. elegans might face in the wild. One example of this is how the nematodes were only exposed to BP-3 while in within the environment they would likely be exposed to an array of different UV-filters at once. An area of further study that has not yet been explored is what effect a number of UV-filters could have on an organism. In the field of air pollution for example, the “multi-pollutant” approach has been deemed the preferable option over

the study of a single pollutant (Billionnet, Sherrill and Annesi-Maesano, 2012). It would also be interesting to investigate how BP-3 effects *C. elegans* immunity response as some studies have shown that exposure to a harmful chemical could go relatively unnoticed until an infection acquires, where the subtle toxic effect of the chemical becomes far more noticeable (Zala and Penn, 2004). That said, although the approach used in this study might not be an accurate representation of the environmental conditions faced by organisms in the wild, it does give us an insight as to the part effect of this pollutant.

Finally, this project could have benefitted from and would definitely be considered were this experiment be repeated, is the use of more extensive selection of endpoints. A number of our experiments both in the acute test (Figure 3.3 A, Figure 3.6 A and B) as well as the chronic exposure test (figure 3.7 B), had replicates that showed significance, however due to inconsistencies we were not able to draw solid conclusions from there. It could be that using more endpoints could have painted a clearer picture. One of these endpoints for example could have been to analyse the nematodes for in increased amount of reactive oxygen species (ROS). Naturally, ROS are produced by the mitochondria during the cellular metabolism process and is an important part of the energy cycling of cells (Zhang, Liu and Liang, 2018). However, excessive ROS concentrations, due to exposure to a toxin or external ROS exposure, can induce cell apoptosis and even death (Henrotin, Kurz and Aigner, 2005; Ghavami *et al.*, 2014) It is believed that when BP-3 starts to degrade in the environment, ROS are released (Wang et al., 2022). In *C. elegans* an excessive amount of ROS can be visualised using the CM-H_2DCFDA label and examined using either excitation

wavelength at 488 nm or emission filter at 520 mm under a laser scanning confocal microscope (Zhao *et al.*, 2020). It is also possible to use staining to demonstrate any apoptosis accruing in the germline that could be caused by the UV-filter (Huang, Kung and Wei, 2022). This could be done by using Acridine Orange staining, this will then allow the stained apoptotic cells in the germ line to be visible within the gonads of the nematode (Huang, Kung and Wei, 2022). Another endpoint that could be used it the concentration of VTG. Some studies have suggested that VTG concentrations are increased when the animal is exposed to BP-3 due to its potential EDC effect (Zhang *et al.*, 2016; Tapper *et al.*, 2019). The increase of VTG levels can be measured in *C. elegans* by measuring the levels of the mRNA of the vitellogenin precursor gene vit-2 and vit-6 (Li, How and Liao, 2018; Huang, Kung and Wei, 2022). As many studies have suggested reproductive inhibition caused by BP-3 and our results hint to this aswell it could be that the addition of these other endpoints could give us a clearer insight into the full effects of BP-3 on *C. elegans.*

5 Conclusion

Global pollution contributed to over 9 million deaths a year worldwide. One of the main contributors of this pollution is the pollution originating from pharmaceuticals and personal care products. UV-filters are the chemicals in suntan lotion (a common PCP) that provide the protection from the suns UVA and UVB rays. The global suntan lotion market is steadily increasing yearly with an estimated usage in the USA alone being between estimated to be between 9 and 32,000 tonnes. UV-filters enter the environment in one of two ways, one way is by direct exposure through people using

suntan lotion and directly depositing this into the environment through activities such as swimming and water sports. This direct exposure is made possible by the fact that between 90 to >99% of UV-filter sit on top of the skin and will eventually be washed off. Another place where these UV-filters can be washed off is in the shower, which leads us to the second exposure route; the plumbing system. Studies have found that UV-filter concentrations sampled in surface waters would increase in line with city population and would also be much higher in areas where waste-water treatment facilities were of low quality. Wastewater treatment plants are inefficient when it comes to removing UV-filters due to the fact that many UV-filters have high bioaccumulation potential and tend to accumulate in the bio-sludge used by these plants. UV-filters can be split into two groups; inorganic metals (inorganic UV-filters) that reflect UV-rays and organic chemicals that absorbed these rays (organic UV-rays). Organic UV-filters especially have a high accumulation factor and are believed to be very slowly broken down within these systems. The UV-filter BP-3 is highlighted to be of especial concern as only between 28-51% of it is ever actually removed from the pumping system. This stands in strong contrast the 90% removal rate of the inorganic UV-filter TiO_2. Another difference between the two types of UV-filters is their ability to permeate the skin. It is believed that due to the particle size, the amount of inorganic UV-filters that can penetrate the skin barriers is negligible, while as much as 9% of organic UV-filters could enter through the skin.

Once in the body, organic UV-filters can penetrate cellular barriers and accumulate in tissue. BP-3 has been highlighted as particularly concerning as it has been sampled in 94% and 98% of the urine samples taken in America and Denmark respectively. BP-3

has also been sampled in human white-matter at concentrations reaching 0.32 $ng.g^{-1}$. In rats BP-3 exposure caused an increased oxidative stress response and apoptosis in the hippocampus and frontal cortex, aswell as causing an increase in intercellular glutamine concentrations in the brains of mice which can also lead to hippocampal damage. BP-3 has also been sampled in placental tissue, amniotic fluid and foetal blood, meaning that humans are exposed to BP-3 from a young age. BP-3 exposure during early embryotic development has shown to cause abnormal neural crest development.

Besides this direct effect it is also believed that BP-3 acts as an EDC and alters the bodies hormone regulation. This was demonstrated when BP-3 was found to inhibit global DNA methylation and decreased DNMT activity, something associated with endocrine disruption and has been shown to cause multigenerational epigenetic consequences as the germ line can become altered. Another aspect of endocrine disruption is the effect of this on puberty rates. BP-3 was shown to be the most prominently sampled UV-filter among patients as well as being associated with an inhibition of testicular growth. BP-3s ability to alter hormone levels was also demonstrated when it was found to affect the major oestrogen receptors ESR1 and ESR3 as well as GPER1 G-coupled receptor as well as increasing the invasiveness and migration of the MCF-7 breast cancer cells.

Besides the negative effects of UV-filters on humans, they have also been shown to have detrimental effects on some key ecological species. Fish for example have been found to be particularly vulnerable to the endocrine altering effects that BP-3 has demonstrated, as this leads to an increase of VTG concentrations. This increased VTG

concentration has been shown to cause detrimental feminization in male fish and has even been associated with the near extinction of whole fish colonies. Micro-algae have also been shown to be affected by BP-3, with exposure leading to varying levels of chlorophyll production inhibition and decreases in overall plant growth. Further to this, scientists have highlighted that corals might be risk aswell, with over 40% of coastal colonies expected to be effected. BP-3 exposure has been linked to coral bleaching by inducing oxidative stress in the mutualistic zooxanthellae that provide corals with essential photosynthetic products.

Considering all the studies that have highlighted BP-3 as a potential ecological threat it is not surprising that some countries have introduced legislations limiting the sale and use of BP-3. That said, these legislations have mostly been on a relatively small scale and have been limited to small select areas. Further to this, many other UV-filters remain completely unregulated in terms of sales and usages. These limitations in legislation are likely because there are some key questions left unanswered. Firstly, there is very limited knowledge on how UV-filter effect terrestrial organisms. This question becomes of even more importance when taking into consideration that one major area of potential UV-filter pollution is likely to come from contaminated active sludge being used as fertiliser or being discarded in landfills. Active sludge is a bio-solid comprising of bacteria and other micro-organisms that traditionally breakdown pollutants in wastewater however due to their chemical properties, UV-filters tend to accumulate in this active sludge.

Another area of research that is in very much in its infancy is the comparative toxicity of BP-3 compared to other UV-filters. There are a number of studies that test toxicities

of different models however, there is no common toxicity assay used to directly compare UV-filters to each other. The establishment of such an assay could potentially allow governments to make informed decisions as to which UV-filter should be restricted and which should be encouraged as the UV light protection is still necessary.

The goal of this project was to gain novel understanding in how UV-filters could affect terrestrial micro-organisms as well as assess *C. elegans* as a potential model for the UV-filter toxicity assay. This goal was broken down into two parts. We investigated the direct acute effects of BP-3 on *C. elegans*. We attempted to model the type of BP-3 exposure terrestrial organisms might face due to the introduction of BP-3 contaminated active sludge to their habitat, by exposing *C. elegans* to contaminated *E. coli*.

We discovered that acute BP-3 exposure at environmentally relevant concentrations did not inhibit the early or total fecundity of the nematodes while acute exposure at higher concentrations of BP-3 did induce a significant effect on the total fecundity between groups in two of the three replicates. Unfortunately, due to the nature of the experimental design, we were not able to determine which of the concentration groups were significantly affected.

It was also discovered that in one of the three replicates, nematodes exposed to 500 µg/L for four hours were significantly larger than those measured before the experiment. One possible explanation for this could be that the nematodes natural ability to acclimate to osmolarity changes might have been diminished.

It was also found that acute BP-3 exposure did not have an effect on the growth of *C. elegans.* These results stand in contrast to that found in similar studies, where the UV-filter OMC induced both consistent growth inhibition as well as fecundity inhibition. One possible reasoning for this difference in results could be that BP-3 is less toxic than OMC, however this would stand in contrast to what many studies suggest. Another possibility is that BP-3s toxicity was reduced because the experiments were all conducted in mostly dark conditions, limiting the potential production of dangerous derivatives. Our chronic exposure study showed that in one of the three replicates nematodes exposed to *E. coli* contaminated with 1500 µg/L BP-3 for three days were significantly smaller than those exposed to *E.coli* contaminated with 1000 µg/L BP-3 for three days. It was hypothesised that this form of chronic exposure would cause a much stronger effect on the nematodes than acute exposure due to longer exposure times as well as the potential direct ingestion of BP-3, however two replicates did not show any significant effect between groups. One possible explanation for this could be that the *E. coli* partly metabolised the BP-3 into less toxic derivatives.

As there is a high level of inconsistency between replicates it is impossible for us to confidently comment on the effects of acute BP-3 exposure on *C. elegans* fecundity and growth as well as the effect of exposing *C. elegans* to contaminated *E. coli* on nematode fecundity. That said, this study does highlight the fact that there are still many uncertainties when it comes to this emerging pollutant and that there is a need for further investigation into BP-3 toxicity as well as the need to consider the creation of derivatives when conducting such studies.

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