DOCTORAL THESIS

Human Pharmaceuticals in the Marine Environment: Evidence of occurrence, direct impact and potential ecotoxicological effects on blue mussels, *Mytilus edulis*

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Dedicated to Octaviena R. A. W. Koagouw and Ingke M. W. Koagouw.
Two very different women, who to me share the same name: Mama

Didedikasikan untuk Octaviena R. A. W. Koagouw dan Ingke M. W. Koagouw.
Dua wanita yang sangat berbeda, yang saya sapa dengan sebutan yang sama: Mama

ABSTRACT

A growing body of literature suggests that pharmaceuticals used in human medicine pose an increasing contamination risk to non-target organisms and indeed to the overall health of marine ecosystems. However, data concerning the level and nature of that risk is lacking. This is particularly true for developing countries such as Indonesia, where the precarious service of wastewater treatment, especially domestic wastewater is threatening the coastal marine environment.

The present research investigates the biological responses and potential ecotoxicological effects of acetaminophen and metformin, two of the most commonly used human pharmaceuticals, on the marine bivalve *Mytilus edulis*. This is the first study to record the contamination of Indonesian marine waters (Jakarta Bay) with acetaminophen. Among the studied effects, this research focuses on biological responses recorded both in adults and at the early life stage, in mussels exposed to pharmaceuticals under laboratory conditions. Special emphasis was placed on histological and cytological effects, as well as the impact on molecular pathways, with several reproduction related genes such as *vitellogenin* and *estrogen receptor-2* being strongly modulated by the selected contaminants. The variation in mRNA expression of four other genes involved in apoptosis: *heat shock protein-70, caspase-8, B-cell lymphoma-2* and *Fas cell surface death receptor* was also investigated.

In summary, this study provides the first record to date of acetaminophen presence in Indonesian seawater, with one of the highest contamination levels published so far globally. Furthermore, it provides a comprehensive and integrative assessment of the short- and long-term effects of acetaminophen and metformin in marine mussels *Mytilus edulis*. Indeed, to date this is the first study recording the effects of acetaminophen in *Mytilus edulis*, and is also the first study recording the effects of both acetaminophen and metformin specifically on the early life stage of *Mytilus edulis*. Finally, it discusses the significance of these two most highly consumed pharmaceuticals in the context of marine pollution and the possible consequences for survival of coastal marine species.

Keywords: acetaminophen, metformin, blue mussels, transcriptomics, histopathology, marine pollution

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LIST OF ABBREVIATIONS

18S rRNA	*18S ribosomal RNA*
ADA	American Diabetes Association
AGC	automatic gain control
AM404	N-arachidonoylphenolamine
AMP	adenosine monophosphate
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
APHA	American Public Health Association
ATP	adenosine triphosphate
au	arbitrary unit
BCL2	*B-cell lymphoma-2* (gene)
Bak	Bcl-2-antagonist killer (protein)
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma-2 (protein)
Bcl-xL	B-cell lymphoma-extra-large (protein)
BH3	Bcl-2 homology 3 (protein)
BSM	binary solvent manager
CASP8	*caspase-8* (gene)
cDNA	complementary DNA
CECs	contaminants of emerging concern
COX	cyclooxygenase
Cq	cycle quantification
CYP	cytochrome P450
DDT	dichloro-diphenyl-trichloroethane
DISC	death-inducing signalling complex
DNA	deoxyribonucleic acid
DPX	distyrene plasticiser xylene

dsDNA	double-stranded DNA
EASD	European Association for the Study of Diabetes
EDCs	endocrine disrupting chemicals
EF1	*elongation factor-1 alpha*
ER2	*estrogen receptor-2* (gene)
ERs	estrogen (oestrogen) receptors
ESI	electrospray ionisation
EU	European Union
eV	electron volt
FA	formic acid
FADD	Fas associated via death domain
FAS	*Fas cell surface death receptor* (gene)
Fas	Fas cell surface death receptor (protein)
FasR	Fas receptor (protein)
GRL	government regulation limit
HEPES	N-2-hydroxyethylpiperazine-N-ethanesulfonic acid
HESI	heated electrospray ionisation
HSP70	*heat shock protein-70* (gene)
Hsp70	70-kDa heat shock protein (protein)
ISO/IEC	International Organization for Standardization / International Electrotechnical Commission
kV	kilovolts
LC	liquid chromatography
LC/MS	liquid chromatography - mass spectrometry
L/h	litre per hour
LMS	lysosomal membrane stability
µm	micrometre
µg/L	microgram per litre
MRM	multiple reaction monitoring
mRNA	messenger RNA
m/z	mass-to-charge ratio

NAPQI	n-acetyl-p-benzoquinoneimine
ng/L	nanogram per litre
NRRT	neutral red retention time
NSAIDs	nonsteroidal anti-inflammatory drugs
OTC	over the counter
PCBs	polychlorinated biphenyls
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
PhACs	pharmaceutically active compounds
PG	prostaglandin
qPCR	quantitative polymerase chain reaction
QTof	quadrupole time-of-flight
RNA	ribonucleic acid
ROS	reactive oxygen species
SD	standard deviation
SM-FTN	sample manager with flow-through needle
STPs	sewage treatment plants
TBT	tributyltin
TRPA1	transient receptor potential cation channel subfamily A member 1
TRPV1	transient receptor potential cation channel subfamily V member 1
WISE	Water Information System for Europe
WWTPs	wastewater treatment plants
V9	*vitelline envelope zona pellucida domain-9* (gene)
VTG	*vitellogenin* (gene)
UK	United Kingdom
UKPDS	United Kingdom Prospective Diabetes Study
UoM	unit of measurement
UPLC	ultra performance liquid chromatography
USA	United States of America
US-EPA	United States Environmental Protection Agency

UV	ultraviolet

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"If I have seen further, it is by standing on the shoulders of giants."
- Sir Isaac Newton

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At last but most importantly, to God, all the glory, honour and praises. As in Proverbs 3:13 (NIV) "*Blessed are those who find wisdom, those who gain understanding*".

DECLARATION

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Wulan Koagouw

13 July 2020

This thesis is a product of my own work and I have led the conception, data collection, analysis and writing of all chapters. The text conforms to a "papers style" format, in which chapters 2 – 5 take the form of discrete articles written in a style appropriate for publication in peer-reviewed journals. Chapter 1 provides a general overview of the field and research undertaken. Chapter 6 provides a summary of the outcomes of this research placed in the broader scientific context, alongside recommendations for policymakers based on these outcomes.

Chapter 2 is in preparation for submission to Journal of Marine Systems as "High concentrations of paracetamol in effluent dominated waters of Jakarta Bay, Indonesia" (authors: Wulan Koagouw, George Oliver, Zainal Arifin, Corina Ciocan).

A part of Chapter 3 has been published in Environmental Science and Pollution Research as "Effects of short-term exposure of paracetamol in the gonads of blue mussels *Mytilus edulis*" (authors: Wulan Koagouw and Corina Ciocan). The other part

is in preparation for submission to Ecotoxicology and Environmental Safety as “Long term exposure of marine mussels to paracetamol: disruption of reproduction-related processes” (authors: Wulan Koagouw, Nicolas Stewart, Corina Ciocan).

Chapters 4 and 5 are written in a style appropriate for submission to peer-reviewed journals. They will be adapted for publications in due course.

CHAPTER 1

Introduction

1.1 General introduction

The global human population is projected to rise to over 11 billion people by 2100 (Gerland et al., 2014). This continued growth over the coming decades is almost certain to be accompanied by a corresponding increase in the production, consumption and disposal of pharmaceutical products (Oosterhuis et al., 2013). This is likely to put coastal areas, especially those in close proximity to rapidly growing megacities in developing countries, at increasing risk of pharmaceutical contamination (Gaw et al., 2014). In order to take effective action to mitigate any such threats, it is important to first understand the level and nature of the risk posed to marine organisms from increased exposure to pharmaceuticals. This thesis aims to provide insight into this topic.

1.1.1 Fate and concerns over the contaminants present in seawater

The ocean serves as the ultimate receptacle of a massive amount of natural and anthropogenic waste continuously discharged from terrestrial and industrial sources (Norse and Crowder, 2005; Pereira et al., 2016). This waste typically contains a cocktail of pollutants, including industrial by-products and chemicals, pesticides, plastics, surfactants, hormones and pharmaceuticals, and many other substances that often exhibit toxic, carcinogenic, mutagenic, and/or endocrine-disrupting properties (Margot et al., 2015). Although precautionary measures (e.g. wastewater treatment systems) have been taken to reduce and/or tackle their environmental impact, in many instances, hazardous substances persist and are detected in the effluent, accumulating in the ocean (Pal et al., 2014; Margot et al., 2015). A plethora of adverse effects exerted by these contaminants on living organisms has been recorded, particularly regarding

reproduction, ontogenesis, and behaviour (Cole et al., 2011; Vasquez et al., 2014). As these contaminants are ubiquitous, there are growing concerns regarding their negative health impact both on human and wildlife populations (Ebele et al., 2017).

An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations. Meanwhile, a potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations (IPCS, 2002). The impact of endocrine disrupting chemicals or compounds (EDCs) on wildlife was recognised as an emerging issue in the early 1990s (Colborn and Clement, 1992). In Britain and Ireland, endocrine disruption has been recorded and scientifically proven in fish, invertebrates, birds and mammals. Affected animals are predominantly species living in a close relationship with aquatic environments (Tyler and Goodhead, 2010). A growing body of literature suggests that this range of substances interfere with hormonal systems in animals, such as androgen and oestrogen signalling pathways, and therefore can affect sex determination and sex ratio, gonadal and gamete development, viability and function of gonads, and development of early life stages (Smolarz et al., 2017). The most documented endocrine disruption effects include imposex in marine molluscs, where male characteristics develop in the female reproductive tract (Sternberg et al., 2010) and intersex in fish, where both male and female gametes are present in the same individual (Williams et al., 2009). Moreover, more dramatic effects have been documented, such as localised population extinction in marine molluscs (Tyler and Goodhead, 2010). Other results additionally highlight malformations and malfunctions of reproductive organs and gametes, histological and biochemical changes leading to gonad pathologies, variations in vitellogenin level, and steroid hormone balance (Ortiz-Zarragoitia and Cajaraville, 2006; Croll and Wang, 2007; Ketata et al., 2008; Ciocan et al., 2010; Zabrzańska et al., 2015).

1.1.2 Pharmaceuticals as emerging contaminants

Pharmaceuticals are a large group of diverse chemicals continuously discharged into the environment. The major proportion of these pharmaceuticals enter aquatic compartments, such as sewage outlets and rivers, and have recently been considered as emerging ubiquitous contaminants (Gaw et al., 2014; Fabbri and Franzellitti, 2016). The presence of pharmaceutically active compounds in water bodies has raised increasing concern in recent years (Scheurer et al., 2009; Gaw et al., 2014). According to a report released by IMS Institute for Healthcare Informatics (2015), global medicine use in 2020 will reach 4.5 trillion doses, an increase of 24 % from 2015. Furthermore, the report also predicts that in 2020 over 50 % of the world's population will consume more than 1 dose per person per day of medicines, up from one third of the world in 2005. The increased consumption is particularly evident in India, China, Brazil and Indonesia (IMS Institute, 2015).

Pharmaceuticals, including analgesics, antibiotics, lipid regulators, biguanides and oestrogens are continuously discharged into the environment mainly by production processes, inappropriate disposal, and municipal wastewater treatment plants (Scheurer et al., 2009; Gaw et al., 2014; Fabbri and Franzellitti, 2016). A review by Ebele et al. (2017) states that the main routes of pharmaceutical contamination are sewage treatment plants (STPs), wastewater treatment plants (WWTPs) and landfill leaching, while the major sources are industrial manufacturing, agriculture and veterinary use, and body excretion after medical use. Furthermore, Bound and Voulvoulis (2005) showed that household disposal of pharmaceuticals may be a prominent route for aquatic contamination in the UK that necessitates greater attention.

As a significant proportion of pharmaceuticals that pass through the body are not fully metabolised, these substances tend to be excreted into the environment and end up in aquatic compartments (Bound and Voulvoulis, 2005). Ebele et al. (2017) also highlight that pharmaceuticals are often persistent and frequently detected in surface waters at concentrations ranging from ng/L to mg/L even after the conventional removal processes in wastewater treatment. Inappropriate disposal of pharmaceuticals may add to the risk, particularly in the case of landfill leaching,

together with the use of sludge and effluent from STPs as fertiliser, as the run off of these substances can enter the surface water and groundwater (Gaw et al., 2014; Ebele et al., 2017).

1.1.3 Occurrence of pharmaceuticals in the environment

The presence of pharmaceuticals, including antibiotics, analgesics, antipyretics, steroids, illicit drugs and antiseptics has been recorded in sediment and sludge in Europe (Löffler and Ternes, 2003; Varga et al., 2010; Langford et al., 2011; Osorio et al., 2016), North and South America (Venkatesan et al., 2012; Klosterhaus et al., 2013; Yang et al., 2015), Africa (Matongo et al., 2015; Agunbiade and Moodley, 2016; Oluwatosin et al., 2016), Asia (Liu et al., 2011; Ramaswamy et al., 2011; Zhou et al., 2011), and Oceania (Stewart et al., 2014). Since the first contamination was reported in 1976 (Fent et al., 2006), a growing number of studies have confirmed the presence of pharmaceuticals – including, but not restricted to analgesics, antibiotics, antiretrovirals and antidiabetics – in surface water and wastewater all over the world (Kolpin et al., 2002; Ashton et al., 2004; Managaki et al., 2007; Ying and Kookana, 2007; Kasprzyk-Hordern et al., 2008; Fick et al., 2009; Loos et al., 2009; Watkinson et al., 2009; Gibson et al., 2010; Scheurer et al., 2012; Oosterhuis et al., 2013; Ghoshdastidar et al., 2014; Laak et al., 2014; Olatunde et al., 2014; Trautwein et al., 2014; Ma et al., 2016; Ngumba et al., 2016).

Recently, pharmaceuticals including antidepressants, analgesics and antibiotics have also been detected in biota (Brooks et al., 2005; Pojana et al., 2007; Fick et al., 2010; Schultz et al., 2010; Liu et al., 2011; He et al., 2012; Li et al., 2012; Gelsleichter and Szabo, 2013; Álvarez-Muñoz et al., 2015), as well as in the seawater around the Brazilian coastline (Pereira et al., 2016), the north Portuguese coast (Lolić et al., 2015), northern Taiwan (Fang et al., 2012), and China (He et al., 2012). All of these investigations indicate that a vast range of pharmaceutical compounds are often not removed completely and consistently by current water treatment methods, and thus might contaminate coastal waters and pose a risk to biota.

Although most contaminants enter water bodies and end up in the sea, ecotoxicological research focusing on the marine environment has been neglected due to the supposition that the dilution factor would negate their biological effects (Fabbri and Franzellitti, 2016). Interference of salt in seawater, as well as difficulty in employing the correct methods to quantify pharmaceuticals, are some technical factors which must be addressed in order to detect their presence in the ocean, as raised by some recent investigations (Verenitch et al., 2006; Xu et al., 2007; Chitescu et al., 2015). Consequently, the occurrence of pharmaceuticals in seawater and their effects on marine organisms are hardly addressed in the literature (Gaw et al., 2014).

In addition to this, and despite the fact that the presence of pharmaceuticals in aquatic compartments has been confirmed, the extent of their possible ecotoxicological combined effects on aquatic organisms is still less clear. As a result, there are gaps in the understanding of the potential risks faced by the marine organisms and their environment (Cleuvers, 2003; Boxall et al., 2012; Brodin et al., 2014; Fabbri and Franzellitti, 2016).

1.1.4 Effects of contaminants of emerging concern (CEC) on marine organisms

Pharmaceutically Active Compounds (PhACs) and Endocrine Disrupting Chemicals (EDCs) have been considered as Contaminants of Emerging Concern (CECs) (Kolpin et al., 2002) as a response to concerns that these substances may adversely affect aquatic organisms, and thus require additional consideration. Evaluation of the potential impacts of these compounds is essential in order for environmental agencies to implement protective measures for aquatic life (US-EPA, 2008).

Bioaccumulation is defined as the net accumulation of a contaminant in an organism from all sources including water, air, and solid phases in the environment (Newman, 2014). Meanwhile, Mackay et al. (2014) described persistence as the average time that a chemical is likely to reside in an environment before it degrades. Studies show that heavily prescribed medicines, such as carbamazepine, clofibric acid, diclofenac, ofloxacin, propranolol, and sulfamethoxazole are increasingly linked to

bioaccumulation, endocrine disruption and reduced survival rates in biota (Ferrari et al., 2004; Ebele et al., 2017). The concern is not only the extended persistence of pharmaceuticals in various aquatic compartments, but also their bioaccumulation in many organisms, as reported by Mimeault et al. (2005), Vernouillet et al. (2010), Wang and Gardinali (2013), Brodin et al. (2014), Du et al. (2015), and de Solla et al. (2016). Moreover, pharmaceuticals are able to interfere with biological systems at low doses, and thus cause unintentional detrimental effects in non-target organisms exposed to these substances (Arnold et al., 2014).

Most studies on adverse outcomes of pharmaceuticals are reported in freshwater organisms (Flammarion et al., 2000; Hoeger et al., 2005; Mimeault et al., 2005), whilst very limited data has been recorded in marine organisms. However, Franzellitti et al. (2013) reported that fluoxetine, a popular anti-depressant, was associated with many deleterious effects on major physiological systems in mussels *Mytilus galloprovincialis*, including reproduction, metabolism and locomotion even at concentrations below or approaching environmental levels. Koagouw and Ciocan (2018) also showed that metformin, a first line therapy drug for diabetes, could induce severe pathologies in gonads, destabilization of the lysosomal membrane in the haemocytes, and a potential increase in vitellogenin mRNA expression in the mussel *Mytilus edulis*, even at environmentally relevant concentrations.

Currently, pharmaceuticals are not included in routine monitoring programmes. Therefore, considering their suspected ecotoxicity, potential health effects, public perception and occurrence in the marine environment, pharmaceuticals may be listed as candidates for regulation in the near future (US-EPA, 2008).

1.1.5 Environmental regulations regarding the presence of pharmaceuticals in the marine environment

Generally, regulations governing marine environments are still few in number and only cover a few important parameters, mostly including heavy metal and industrial pollutants such as PCBs, DDT and TBT. Some of the most widely used legislative

instruments are the US-EPA National Recommended Water Quality Criteria – the Aquatic Life Criteria and EU 2008 Marine Strategy Framework Directive, in addition to other regulatory instruments that are specifically developed in each region and/or country (US-EPA, 2008; WISE Marine, 2008).

In spite of pharmaceutical contamination of water being recognised as an emerging global challenge, it is still not fully addressed by current clean water legislation (e.g. Water Framework Directive) or remediation strategies (Gaw et al., 2014). Although recent studies record the occurrence of pharmaceuticals and their potential adverse consequences on biota, the data are limited, and there is no routine monitoring of the fate and effects of pharmaceuticals in the marine environment (Donnachie et al., 2016).

This is also the case for Indonesia, the world's fourth largest country by population, composed solely of islands and heavily reliant on a stable marine environment for food, health and livelihood (US-CIA, 2017). According to the Indonesian Health Profile published by the Ministry of Health Republic of Indonesia (2014; 2015), the most commonly used pharmaceutical in Indonesia is acetaminophen, based on the national demand data. Oosterhuis et al. (2013) show that the consumption data of pharmaceuticals can be linked to the occurrence of the same pharmaceuticals in the environment. However, this assessment does not provide any possible ecotoxicological risk information.

1.1.6 Aim of this thesis

Building on the reasoning outlined above, this study investigates the ecotoxicological effects of acetaminophen and metformin on marine bivalves.

Chapter 2 provides a context for this research by presenting a snapshot of Indonesian seawater chemistry and the detection of acetaminophen. The chapter discusses the seawater quality of the most polluted coastal area in Indonesia, Jakarta Bay, which effectively acts as a repository for the megacity of Jakarta.

Chapters 3 – 5 then focus on the effects of two of the most widely used pharmaceutical products on marine biota, using the blue mussel *Mytilus edulis* as a bioindicator organism. Chapter 3 presents the effects of acetaminophen on adult mussels, whilst the effects of metformin are presented in Chapter 4. The consequences of exposure to both pharmaceuticals are studied extensively for both short- and long-term exposures. These effects are investigated at the cytological, histological, and molecular level with particular focus on the reproductive system. Chapter 5 then investigates the effects of both acetaminophen and metformin on early life stages of mussels. The discussions explore the potential impact of contaminants, present in seawater at environmentally relevant concentrations, on the development and survival of mussel gametes and larvae, focused at the molecular level.

Chapter 6 summarises the findings outlined in chapters 2 – 5, before providing a discussion which places them in the wider ecological context. It finally suggests recommendations for mitigating the effects of increased pharmaceutical discharge into marine environments.

1.2 Overview of methodology

1.2.1 Mussels and environmental pollution biomarkers

Blue mussels (*Mytilus edulis* Linnaeus, 1758) are known to be an ideal indicator organism for environmental monitoring (Cubero-Leon and Ciocan, 2014). Their characteristics as a sessile organism with high filtering rates, a wide geographical distribution and large and stable population sizes make them excellent model organisms. Mussels have been used widely for marine monitoring programs, such as International Mussel Watch (Tripp et al., 1992; Rittschof and McClellan-Green, 2005; Cubero-Leon and Ciocan, 2014). As they are also a commercially popular food source, information on their health status is also of public health interest (Rehnstam-Holm and Hernroth, 2005).

1.2.2 Lysosomal membrane stability

The lysosomal membrane stability (LMS) biomarker test has been proposed in monitoring programs as an indicator of marine pollution index (Martínez-Gómez et al., 2008). Decreased lysosomal membrane stability in living haemocytes is an indication of the harmful effects induced by exposure to environmental pollutants (Lowe and Pipe, 1994). The neutral red retention time (NRRT) assay is a test using cationic dyes (neutral red) which enter the cell and accumulate in lysosomes. Any damage to the lysosomal membrane, structural or functional, can be visualised microscopically when lysosomal contents leak into cytosol (Lowe and Pipe, 1994; Lowe et al., 1995). The lysosomal compartment is an excellent target with which to observe cytological-level responses in organisms like mussels, exposed to various stresses in the aquatic environment. NRRT is an ideal assay for detecting LMS damage in the haemocytes of bivalves (Martínez-Gómez et al., 2008). A lysosomal stability test was also employed by Minier et al. (2006) as a biomarker in a pollution-monitoring pilot study using zebra mussels (*Dreissena polymorpha*). The application of NRRT is extensively used in environmental monitoring of species in the genus *Mytilus* (Dailianis et al., 2003; Mamaca et al., 2005; Martínez-Gómez et al., 2008; Hu et al., 2015), and is employed in this study to assess the cytological effects of exposures at cytological level in *Mytilus edulis* under experimental exposures.

1.2.3 Histological observation

Histopathology analysis of mussels has been intensively used in marine monitoring in the last 30 years (Sunila, 1986; Akaishi et al., 2007; Cremonte et al., 2015; Yavaşoğlu et al., 2016) and in 1995 was included in the Mussel Watch Program, in order to evaluate the health status of mussels in potentially contaminated habitats (Kim et al., 2006). Histopathological analysis offers general information about the health status of an animal and shows direct effects of exposure to pollutants on the tissues (Sunila, 1987). In this study, histological observation is employed to determine the sex and the effects of exposures on the gonad tissue of *Mytilus edulis*.

1.2.4 Molecular analysis

The real-time quantitative PCR (qPCR) tackles the clear requirement for quantitative data analysis in a research, diagnostic, forensic and biotechnology setting and has become the benchmark technology for the quantification of mRNA (Bustin et al., 2006; Bustin, 2010). qPCR has become a decisive method for quantitation of gene expression levels in various samples (Taylor et al., 2010) and has been used widely to assess environmental effects in many species, including mussels (Yong Ki Choi et al., 2008; Ciocan et al., 2010; Anantharaman and Craft, 2012; Ragusa et al., 2012). Results showing the variation of gene expression offer important information, necessary to elucidate the complex mechanisms of activation or deactivation of target genes through physiological and pathological activities (Camacho et al., 2004). Comparatively small alterations in target gene expression patterns can be linked to substantial changes in the physiological processes of the organism, and in a broader sense, to the health state of the organism (Camacho et al., 2004). This study uses the qPCR method to investigate the mRNA expression level of target genes *vitellogenin* (*VTG*)*, vitelline envelope zona pellucida domain-9* (*V9*) and *estrogen receptor-2* (*ER2*), as genes involved in the reproductive system; and also *heat shock protein-70* (*HSP70*)*, caspase-8* (*CASP8*)*, B-cell lymphoma-2* (*BCL2*) and *Fas cell surface death receptor* (*FAS*), which are all involved in cell death or apoptosis.

Vitellogenin (*VTG*) mRNA expression is a sensitive indicator for early detection of exposure to endocrine disrupting chemicals (EDCs) in vertebrates, both in the laboratory and in wild populations (Ankley et al., 2001; Lattier et al., 2001; Muncke and Eggen, 2006). Ciocan et al. (2010) showed that *VTG* mRNA expression in *Mytilus edulis* gonads shows upregulation after oestrogen exposure at early stages of gonad development. Another recent study also reported that metformin induces transcription of mRNA for *VTG* in adult male fathead minnows *Pimephales promelas* (Niemuth et al., 2014).

The mRNA expression of *vitelline envelope zona pellucida domain-9* (*V9*) is also explored in this study. In vertebrates, the expression of *vitelline envelope zona pellucida* has been suggested as a biomarker for environmental oestrogens (Arukwe et

al., 1997; Ciocan et al., 2011). Arukwe et al. (1997) also indicate that changes in the vitelline envelope induced by xenoestrogen would likely have a high detrimental ecological effect, since it modulates crucial population parameters, because of the mechanical protection of eggshells and fertilisation.

Steroid receptors may likely be viewed as regulators of gametogenesis (Anantharaman and Craft, 2012). One of these steroid receptors, *estrogen receptor-2* (*ER2*) is investigated in this study. The data on oestrogen receptor variation of expression may suggest further impacts that could occur on gametogenesis and reproduction processes in bivalves (Ciocan et al., 2010). Several studies have investigated the expression of *ER2* in various invertebrates (Thornton et al., 2003; Kajiwara et al., 2006; Bannister et al., 2007; Matsumoto et al., 2007), including mussels (Puinean et al., 2006; Ciocan et al., 2010; Ciocan et al., 2011).

The 70 kilodalton heat shock proteins, expressed by the *HSP70* genes, are a family of conserved ubiquitous heat shock proteins. In nearly all living organisms, proteins with identical structure exist. The Hsp70s are an essential part of the cell's protein folding system and help protect cells from stress (Morano, 2007). The Hsp70 system works in conjunction with extended protein peptide segments as well as partially folded proteins to avoid aggregation, reshape folding pathways and control activity. Through strongly binding to partially synthesised peptide sequences (incomplete proteins), Hsp70 prohibits them from aggregating and becoming non-functional. Hsp70 also helps to transport proteins across the transmembrane by stabilising them in a partially folded state (Mashaghi et al., 2016).

Hsp70 proteins may act to protect cells from thermal or oxidative stress. Such stresses typically act to degrade proteins, causing partial unfolding and potential aggregation. By briefly binding to the hydrophobic residues exposed by stress, Hsp70 prevents the aggregation of these partially denatured proteins, and enables them to refold. Hsp70 appears to be able to assist in the processing of damaged or defective proteins. In addition to enhancing overall protein quality, Hsp70 inhibits apoptosis directly. Hsp70 may have an anti-apoptotic role in other steps but is not involved in Fas-ligand-

mediated apoptosis. As a result, Hsp70 not only saves important cell components (proteins) but also directly saves the cell as a whole (Beere et al., 2000).

The *CASP8* gene encodes a member of the family of cysteine-aspartic acid protease (caspase). Sequential caspases activation plays a critical role in the execution process of cell apoptosis. Activation of caspases includes proteolytic processing at conserved internal aspartic residues to create a heterodimeric enzyme comprising of large and small subunits. This protein is associated with programmed Fas-induced cell death and multiple apoptotic triggers. The N-terminal FADD-like death effector domain of this protein indicates that it can interact with Fas-interacting protein FADD (Salvesen and Walsh, 2014; NCBI, 2020).

Bcl-2 (B-cell lymphoma-2), encoded in humans by the *BCL2* gene, is the founding member of the Bcl-2 family of regulatory proteins that control cell death (apoptosis), by either inducing (pro-apoptotic) or inhibiting (anti-apoptotic) apoptosis. Bcl-2 is positioned in the outer membrane of mitochondria, where it plays a vital role in maintaining cell viability and inhibiting the action of pro-apoptotic proteins. Pro-apoptotic proteins in the Bcl-2 family, including Bax and Bak, typically function on the mitochondrial membrane to facilitate permeability and the release of cytochrome c and ROS, which are essential signals in the apoptosis cascade. These pro-apoptotic proteins are, in effect, activated by BH3-only proteins and are suppressed by the role of Bcl-2 and its relative Bcl-xL (Hardwick and Soane, 2013).

Fas or FasR is a protein that is encoded by the *FAS* gene in humans. The Fas receptor is a cell-surface death receptor that contributes to programmed cell death (apoptosis). It is one of the two pathways of apoptosis, the other being the mitochondrial pathway. Fas forms the DISC (death-inducing signalling complex) on ligand binding. Some studies have indicated that the extrinsic Fas pathway is adequate to trigger complete apoptosis in certain cell types by DISC assembly and corresponding caspase-8 activation (Wang et al., 2010).

CHAPTER 2

Trouble under the surface? - A snapshot of seawater chemistry in Jakarta Bay, Indonesia

Abstract

As a maritime country that relies heavily on marine resources, Indonesia's marine ecosystem is of high economic importance at both local and national levels, notwithstanding its global importance as a megabiodiversity hot spot. However, Jakarta, the capital and largest city of Indonesia, is well known as one of the most populous megacities in the world. Jakarta Bay is consequently the most polluted coastal area in Indonesia as an accumulated result of industrial and anthropogenic activities. Acting as a reservoir for the combined effluent of the so-called "sinking city", much data on marine pollution in Jakarta Bay has been generated alongside a decades-long debate on this topic. This chapter provides a snapshot of the seawater quality of Jakarta Bay and reveals the detection of acetaminophen in this area, giving an insight into this much-debated topic, as well as providing the broader context for this thesis. This is the first data on the presence of acetaminophen in Indonesian seawater to date. The high level of acetaminophen detected also contributes to growing concern towards the health of marine organisms in this area, highlighting the importance of ecotoxicological study on this topic.

Keywords: Jakarta Bay, seawater, acetaminophen, marine pollution, heavy metals, outflow

2.1 Introduction

Jakarta Bay has long been known for high levels of water contamination, as the coastal megacity of Jakarta is one of the largest metropolitan agglomerations in the world. As a wastewater reservoir for the 3rd most populated metropolitan area in the world, marine pollution is an ongoing and persistent problem. The unregulated rise in the urban population has resulted in increased demand on public services such as sewage treatment facilities, leading to significant contamination of surface waters (Dsikowitzky et al., 2018).

Indonesia has a tropical monsoon climate, with two major seasonal patterns: the rainy season and the dry season. During the rainy season in January and February, the Ciliwung River bursts its banks on a regular basis, resulting in the flooding of the lower stream in the Jakarta City area (Remondi et al., 2016). Model simulations built to analyse the impact of rapid and drastic changes in land use on the river's hydrological response have shown that unregulated urban development contributes to a significant increase in flood events during the rainy season and a decrease in baseline flow during the dry season (Rafiei Emam et al., 2016; Remondi et al., 2016).

More than 70,000 households live in riverside settlements along the lower Ciliwung River in the Jakarta City area (Padawangi et al., 2016). These residents live without sufficient infrastructure support, and are highly impacted by the frequent floods (Guest, 2019), the latest incident being recorded in early 2020 (Green and Reed, 2020). The combination of heavy rainfall, land use management in riverside settlements and repeated flood events may lead to water pollution by runoff from the ground (Rafiei Emam et al., 2016; Daksiya et al., 2017). As a result, the polluting mass fluxes discharged from Jakarta City to the tropical coastal environment may be exceptionally high compared to other cities.

Jakarta's problems with wastewater management are well-documented (Marshall, 2005). Dsikowitzky et al. (2016) detected a total of 71 organic contaminants present in Jakarta river water samples. Furthermore, Dsikowitzky et al. (2018) estimated that urban sewage components accounted for about 70 – 80 % of the flows of 32 quantified

water pollutants recorded in the Ciliwung River, which transported approximately 5 – 17 tonnes of these pollutants per year to the Java Sea. In addition, Costa et al. (2016) detected faecal coliform bacteria along the length of the Ciliwung River at concentrations of up to 3.2 MPN / 100 mL. These large pollutant fluxes from urban sources, an issue similar to many coastal megacity areas, may have a significant effect on fragile coastal ecosystems.

Indeed, all of the major rivers flowing through Jakarta discharge their pollutant loads into Jakarta Bay. As such, a wide range of different contaminants have been detected within the bay. Dsikowitzky et al. (2017) observed a total of 45 different organic contaminants in surface water samples at two sites located close to the outflow of the Cikarang and Bekasi Rivers, which carry wastewater from a number of industrial manufacturing facilities. High levels of nitrite (0.2 – 0.6 µM/L) and phosphate (1.5 – 4.2 µM/L) were detected by Baum et al. (2015) in Jakarta Bay, in contrast to sites further from the coast. Heavy metals have also been the subject of a number of studies. Lead, cadmium, copper and zinc were detected in Jakarta Bay both in solute form (at concentrations of up to 0.03 ppm) and in sediments (at concentrations of up to 480.5 mg/kg dry weight) by Arifin et al. (2003). More recently, Sindern et al. (2016) detected chromium, nickel, copper, zinc, arsenic and lead in coastal sediments at concentrations of up to 209.4 µg/g.

Tropical coasts receive most of the annual global inflow of freshwater, dissolved and particulate matter into the ocean. They include a wide range of highly complex habitats and a large proportion of the global human population, which in effect relies economically on their natural resources (Jennerjahn, 2012). The Southeast Asian area is regarded as a hot spot for marine biodiversity and is habitat to coral reefs that are highly vulnerable to water pollution and thus have the top priority for conservation actions (Roberts et al., 2002; Tittensor et al., 2010).

Currently, there is only limited information on the seawater chemistry profile in Indonesia and also little is known about the status of pharmaceuticals in Indonesian seawater, as well as their toxicological effects on marine organisms. Furthermore, at

the moment there is no monitoring program of any pharmaceutical in the environment, or regulations regarding the handling and disposal of unused pharmaceuticals.

2.2 Materials and methods

2.2.1 Sample collection

Seawater samples were collected in September 2017, from five sites in the Indonesian coastal areas: four sites in Jakarta Bay and one site in Eretan Bay on the north coast of Central Java. Figure 1 shows the sampling locations, while the coordinates and a brief general description of these sites are shown in Table 1. Photographs of the sampling sites are presented in Appendix 1.

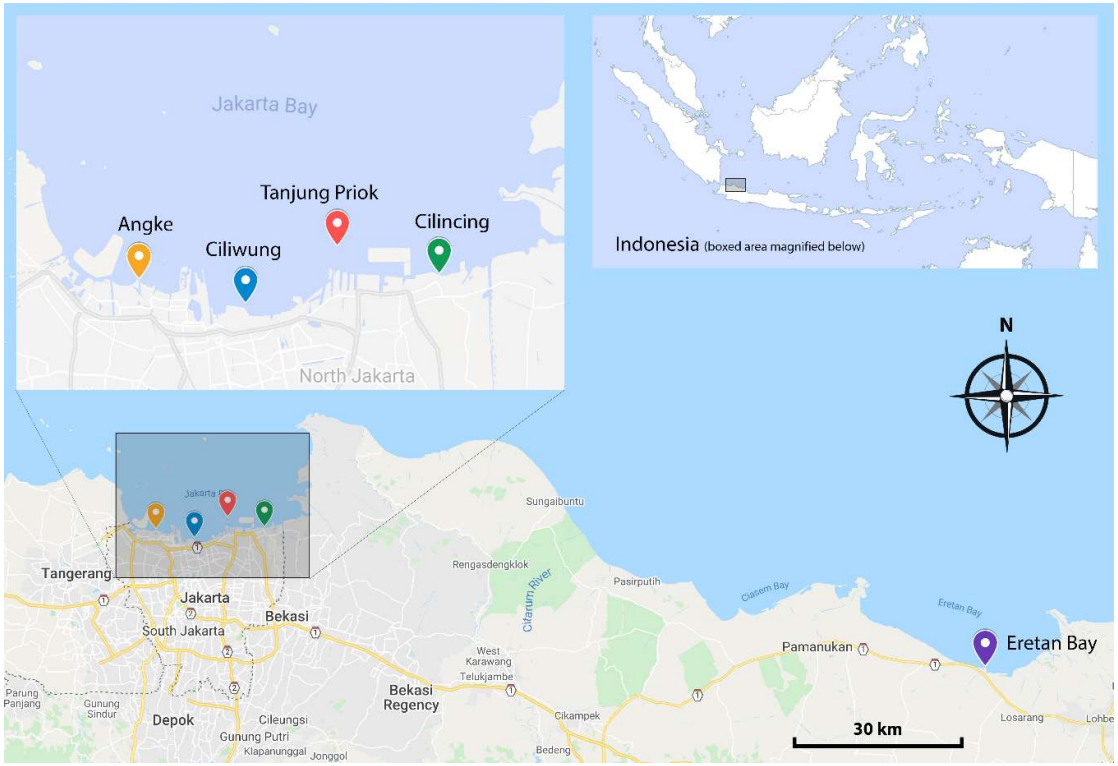


Figure 1 Map of sampling locations. Boxed inserts show a detailed map of the Jakarta Bay sampling locations (top left) and an overview displaying the location of the study area within Indonesia (top right). Generated using Google My Maps (Google, 2020).

Table 1 Coordinates and description of sampling locations.

Sites	Coordinates	General Description
Eretan Bay	6°19'10.1"S 108°05'20.1"E -6.319472, 108.088921	A rural area located on the northern coast of Central Java with poor sanitation in the local village.
Angke	6°06'01.7"S 106°46'00.7"E -6.100466, 106.766857	West Jakarta Bay, a highly populated area with poor sanitation, traditional market. Anthropogenic debris clearly observed along the coast and polluted river (Angke River).
Ciliwung	6°06'51.4"S 106°49'42.1"E -6.114275, 106.828360	Central Jakarta Bay, mouth of the main river crossing Jakarta (Ciliwung River), tourist destination, amusement park, local harbour.
Priok	6°04'53.4"S 106°52'52.3"E -6.081508, 106.881180	Central Jakarta Bay, the largest industrial harbour in Jakarta (Tanjung Priok), industrial area.
Cilincing	6°05'50.5"S 106°56'23.5"E -6.097354, 106.939851	A highly populated area in East Jakarta Bay. Anthropogenic debris clearly observed along the coast and river mouth. Poor sanitation, with a traditional market in the area.

The collection, storage and preparation of samples were conducted in accordance with good laboratory practice standards set by the Research Center for Oceanography, Indonesian Institute of Sciences (accredited by National Accreditation Committee for ISO/IEC 17025:2008). Prior to collection, all containers and equipment for the collections were cleaned and treated with methanol and dried to ensure sterility and non-contamination. Aluminium foil and other consumables (when used) were

discarded immediately after use. Each container was used for one sample only, plastic buckets were cleaned before and after each use, and the sample collection was performed in such a way as to minimise the risk of contamination, following guidelines provided by US-EPA (Decker and Simmons, 2013) and American Public Health Association (APHA) (Baird et al., 2017).

Samples were collected during the daytime in September 2017 and 2018 using clean plastic buckets immediately emptied into glass containers. After covering the containers with aluminium foil to keep them away from the light, all samples were kept in a cool box containing ice. Samples were then transported to the laboratory immediately after collection and stored in a -20 °C freezer until further analyses.

Further analyses were performed at laboratories which are accredited by National Accreditation Committee for ISO/IEC 17025:2008, meaning that the procedures used complied to international standards to deliver reliable results.

2.2.2 Acetaminophen analyses

2.2.2.1 Sample preparation and solid phase extraction

The method was adapted from Paiga et al. (2015), Alygizakis et al. (2016), and Pereira et al. (2016). Each 1 litre of all seawater samples was filtered through 1.2 µm Whatman grade GF/C microfiber glass filter paper (GE Healthcare, UK). The second filtration used 0.2 µm nylon membrane filter (Merck Millipore, UK) and filters were then washed with 1 mL methanol after each filtration. Each filter was single used only. Filtered seawater was then percolated through a Strata™-XL-C 100 µm polymeric strong cation 2 g / 20 mL giga tube cartridge (Phenomenex, USA), as per the manufacturer's recommendation. Prior to loading the sample, the cartridge was conditioned with 20 mL methanol followed by 20 mL water. A 20 mL solution of 5:95 methanol:water was used to wash the cartridge immediately after the sample, and it was then left for 10 – 15 minutes under full vacuum to dry the cartridge. The cartridge was then eluted with 10 mL methanol. All of these processes were performed with a vacuum pump system.

The extract was evaporated under a gentle nitrogen stream and reconstituted with 1:1 methanol:water.

2.2.2.2 LC/MS

The quantification of acetaminophen was performed at the Research Center for Chemistry, Indonesian Institute of Sciences (accredited by National Accreditation Committee for ISO/IEC 17025:2008). Liquid chromatographic separation was carried out using an Acquity UPLC® I-Class System (Waters®, USA), comprised of a binary solvent manager (BSM) and a sample manager with flow-through needle (SM-FTN), coupled with a Xevo G2-XS QTof quadrupole time-of-flight mass spectrometer (Waters®, USA) operated in electrospray ionization (ESI) mode. The separation used water-formic acid 0.1 % as solvent A and acetonitrile-formic acid 0.1 % as solvent B. The gradient elution was performed at a flow rate of 0.22 mL/min as shown in Table 2.

Table 2 Gradient elution condition of chromatographic separation.

Time (min)	Flow Rate (mL/min)	Composition A (%)	Composition B (%)
0.0	0.22	70.0	30.0
1.0	0.22	70.0	30.0
2.0	0.22	64.4	35.6
6.0	0.22	0.0	100.0
6.5	0.22	0.0	100.0
10.5	0.22	70.0	30.0

The chromatography analysis employed an Acquity UPLC HSS T3 column (Waters®, USA) of size 2.1 mm x 100 mm and particle size of 1.8 µm. The column temperature was set at 30.0 ˚C, while the sample temperature was 8.0 ˚C as operated by sample

manager. The sample manager (SM-FTN) used water:methanol:acetonitrile:isopropyl alcohol (1:1:1:1) as wash solvent, and methanol:water (1:9) as purge solvent.

Acetaminophen was analysed using a mass spectrometer Xevo G2-XS QTof operated in multiple reaction monitoring (MRM) mode and negative electron spray ionization (ESI). MRM transitions were set up as follows: acquisition time was 0 – 10.5 min, precursor mass was 150.0554 *m/z*, product mass was 107.0365 *m/z*, and collision energy was 10 eV. Other parameters were as follows: capillary voltage was 1.5 kV, cone voltage was 30 V, source temperature was 120 ˚C, desolvation temperature was 500 ˚C, cone gas flow was 50 L/h, and desolvation gas flow was 1,000 L/h.

2.2.3 Other physical and chemical parameters of seawater

Analyses were performed at Water Laboratory Nusantara, an accredited testing laboratory for water and wastewater analysis. All methods comply with ISO/IEC 17025:2008. Analyses were performed following APHA – Standard Methods for Examination of Water and Wastewater (Rice et al., 2012), US-EPA procedure, and standardised methods provided by instruments' protocols. Detail of the method of analysis for each parameter is shown in Table 3.

Table 3 Method reference of physical and chemical parameters.

No	Test Description	Method Reference
	Physicals Test	
1	Total Suspended Solids	APHA-2540-D(2012)
2	Salinity	APHA-2520-B(2012)
3	Conductivity	APHA-2510-B(2012)
	Chemical-Anion	
4	pH	APHA-4500H+.B(2012)

No	Test Description	Method Reference
	Nutrients	
5	Ammonia (N-NH3)	APHA-4500NH3-D(2012)
6	Nitrate (N-NO3)	APHA-4500-NO3-E(2012)
7	Total-Phosphate (P-PO4)	APHA-4500P-B&E(2012)
	Metals	
8	Chromium Hexavalent-Dissolved (Cr-VI)	APHA-3500Cr-B(2012)
9	Arsenic-Hydride Dissolved (As)	APHA-3120-B(2012) and WLN-ML-WI-09
10	Aluminium-Dissolved (Al)*	APHA-3120-B(2012)
11	Boron-Dissolved (B)*	APHA-3120-B(2012)
12	Barium-Dissolved (Ba)*	APHA-3120-B(2012)
13	Calcium-Dissolved (Ca)*	APHA-3120-B(2012)
14	Cadmium-Dissolved (Cd)	APHA-3120-B(2012)
15	Cobalt-Dissolved (Co)	APHA-3120-B(2012)
16	Chromium-Dissolved (Cr)*	APHA-3120-B(2012)
17	Copper-Dissolved (Cu)	APHA-3120-B(2012)
18	Iron-Dissolved (Fe)	APHA-3120-B(2012)
19	Potassium-Dissolved (K)*	APHA-3120-B(2012)
20	Lithium-Dissolved (Li)*	APHA-3120-B(2012)
21	Magnesium-Dissolved (Mg)*	APHA-3120-B(2012)
22	Manganese-Dissolved (Mn)	APHA-3120-B(2012)
23	Molybdenum-Dissolved (Mo)*	APHA-3120-B(2012)
24	Nickel-Dissolved (Ni)	APHA-3120-B(2012)
25	Sodium-Dissolved (Na)*	APHA-3120-B(2012)

No	Test Description	Method Reference
26	Lead-Dissolved (Pb)	APHA-3120-B(2012)
27	Selenium-Hydride Dissolved (Se)	APHA-3120-B(2012) and WLN-ML-WI-09
28	Silicon-Dissolved (Si)*	APHA-3120-B(2012)
29	Antimony-Hydride Dissolved (Sb)	APHA-3120-B(2012) and WLN-ML-WI-09
30	Silver-Dissolved (Ag)*	APHA-3120-B(2012)
31	Tin-Dissolved (Sn)*	APHA-3120-B(2012)
32	Titanium-Dissolved (Ti)*	APHA-3120-B(2012)
33	Vanadium-Dissolved (V)*	APHA-3120-B(2012)
34	Zinc-Dissolved (Zn)	APHA-3120-B(2012)
35	Mercury-Dissolved (Hg)	USEPA-245-7(2005)
	Organics	
36	Dissolved Oxygen	APHA-4500O-G(2012)
37	Total Poly Chlor Biphenyl	WLN-OL-WI-04
38	Total Pesticides as Organo Chlorine Pesticides	WLN-OL-WI-03

** Ten times dilution was performed due to high interferences*

2.3 Results

2.3.1 The detection of acetaminophen

Acetaminophen, or paracetamol, was detected in two sampling sites in Indonesia, both off the coast of Jakarta Bay. The highest acetaminophen concentration was in Angke with 610 ng/L, whilst its level in Ciliwung was 420 ng/L (Fig. 2). This pharmaceutical was not detected in other sites, or possibly present at low concentration, below the detection limit of the equipment (50 ng/L).

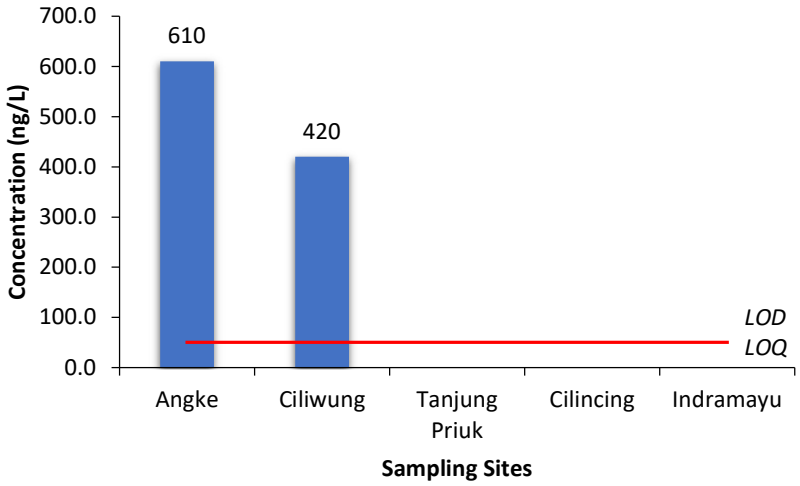


Figure 2 Concentration of acetaminophen detected at selected sampling sites, in Indonesian seawater (ng/L). Red bar shows limit of detection and quantification at 50 ng/L.

2.3.2 The levels of other physical and chemical parameters of seawater

Table 4 shows the recorded seawater quality parameters in all sites compared to the Indonesian government regulation limit (Decree of the State Minister of the Environment number 51 year 2004 regarding Standard Quality of Seawater). Results show that nutrient parameters exceed the upper limit, while some physical parameters of all sites are lower than the government regulation limit. The government regulation mainly set the limit for several heavy metals; however, the investigations presented here highlight some alarming concentrations of heavy metals detected in Indonesian seawater.

Table 4 Seawater quality parameters in selected sampling sites, compared with Indonesian government regulation limit (GRL).

No	Test Description	UoM[1]	Angke	Ciliwung	Tanjung Priok	Cilincing	Eretan Bay	GRL[2]
	Physicals Test							
1	Total Suspended Solids	mg/L	9	1	7	7	5	20-80
2	Salinity	0/00	6	16	32	32	25	33-34

No	Test Description	UoM[1]	Angke	Ciliwung	Tanjung Priok	Cilincing	Eretan Bay	GRL[2]
3	Conductivity	uS/cm	10500	25900	49100	48800	39800	n/a
	Chemical-Anion							
4	pH	n/a	7.32	7.19	7.68	7.55	7.54	7-8.5
	Nutrients							
5	Ammonia (N-NH3)	mg/L	8.59	2.25	0.2	0.35	0.32	0.3
6	Nitrate (N-NO3)	mg/L	0.012	0.012	0.040	< 0.005	0.023	0.008
7	Total-Phosphate (P-PO4)	mg/L	0.294	0.221	0.028	0.175	0.047	0.015
	Metals							
8	Chromium Hexavalent-Dissolved (Cr-VI)	mg/L	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	0.005
9	Arsenic-Hydride Dissolved (As)	mg/L	<0.0005	<0.0005	<0.0005	<0.0005	<0.0005	0.012
10	Aluminium-Dissolved (Al)*	mg/L	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	n/a
11	Boron-Dissolved (B)*	mg/L	1.30	2.88	4.49	4.13	3.67	n/a
12	Barium-Dissolved (Ba)*	mg/L	0.07	0.05	< 0.05	< 0.05	< 0.05	n/a
13	Calcium-Dissolved (Ca)*	mg/L	90.4	221	365	349	297	n/a
14	Cadmium-Dissolved (Cd)	mg/L	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.001
15	Cobalt-Dissolved (Co)	mg/L	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	n/a
16	Chromium-Dissolved (Cr)*	mg/L	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	n/a

No	Test Description	UoM[1]	Angke	Ciliwung	Tanjung Priok	Cilincing	Eretan Bay	GRL[2]
17	Copper-Dissolved (Cu)	mg/L	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	0.008
18	Iron-Dissolved (Fe)	mg/L	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	n/a
19	Potassium-Dissolved (K)*	mg/L	101	310	544	492	420	n/a
20	Lithium-Dissolved (Li)*	mg/L	< 0.1	0.1	0.2	0.2	0.2	n/a
21	Magnesium-Dissolved (Mg)*	mg/L	233	694	1170	1140	950	n/a
22	Manganese-Dissolved (Mn)	mg/L	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	n/a
23	Molybdenum-Dissolved (Mo)*	mg/L	< 0.01	< 0.01	0.01	0.01	< 0.01	n/a
24	Nickel-Dissolved (Ni)	mg/L	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.05
25	Sodium-Dissolved (Na)*	mg/L	1950	5720	8820	7490	7560	n/a
26	Lead-Dissolved (Pb)	mg/L	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.008
27	Selenium-Hydride Dissolved (Se)	mg/L	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005	n/a
28	Silicon-Dissolved (Si)*	mg/L	28	18	13	<5	15	n/a
29	Antimony-Hydride Dissolved (Sb)	mg/L	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005	n/a
30	Silver-Dissolved (Ag)*	mg/L	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	n/a

No	Test Description	UoM[1]	Angke	Ciliwung	Tanjung Priok	Cilincing	Eretan Bay	GRL[2]
31	Tin-Dissolved (Sn)*	mg/L	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	n/a
32	Titanium-Dissolved (Ti)*	mg/L	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	n/a
33	Vanadium-Dissolved (V)*	mg/L	< 0.05	< 0.05	0.06	< 0.05	< 0.05	n/a
34	Zinc-Dissolved (Zn)	mg/L	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	0.05
35	Mercury-Dissolved (Hg)	mg/L	<0.00005	<0.00005	<0.00005	<0.00005	<0.00005	0.001
	Organics							
36	Dissolved Oxygen	mg/L	8.30	7.14	8.29	8.40	8.28	>5
37	Total Poly Chlor Biphenyl	mg/L	<0.00001	<0.00001	<0.00001	<0.00001	<0.00001	0.01
38	Total Pesticides as Organo Chlorine Pesticides	mg/L	<0.00001	<0.00001	<0.00001	<0.00001	<0.00001	0.01

[1] *UoM: Unit of Measurement*

[2] *GRL: Government Regulation Limit, refer to Decree of the State Minister of the Environment number 51 year 2004 regarding Standard Quality of Seawater.*

2.4 Discussion

2.4.1 Acetaminophen pollution in Indonesia

Acetaminophen was detected at two sites in Jakarta Bay, which are Angke and Ciliwung. The concentrations of this pharmaceutical detected in both sites are considerably high, compared to other levels reported in scientific literature so far. These results exceed the maximum concentration of 34.6 ng/L as reported by Pereira et al. (2016), as 12 – 17 times higher than the acetaminophen detected in the seawater

around the Brazilian coastline. Lolic et al. (2015) also reported the presence of acetaminophen in the north Portuguese coast in a range of 51.2 – 584 ng/L with a mean of 95.2 ng/L and median of 62.8 ng/L. The results here in Jakarta Bay are 4 – 6 times the average concentration recorded in Portugal.

Acetaminophen at 610 ng/L detected at Angke is one of the highest concentrations recorded in seawater to date, after those recorded in the western Mediterranean Sea (Togola and Budzinski, 2008) and Aegean Sea (Nödler et al., 2014). From observations at the location, the Angke River estuary located in West Jakarta Bay is a highly populated area with poor sanitation. There is a traditional market and slum area in the location which dumps wastewater directly into the sea. Anthropogenic debris were clearly marked along the coast and the polluted river during the sample collection, as also reported by Purba et al. (2019) and van Emmerik et al. (2019). Given that the Angke River is also one of the rivers that flows through the city of Jakarta with heavy accumulation of urban and industrial waste before it discharges into Jakarta Bay, it can be suggested that this enormous accumulation of urban waste accounts for the high concentration detected here.

Acetaminophen was also detected at a concentration of 420 ng/L off the coast near the mouth of the Ciliwung River, which drains part of the Jakarta metropolitan area and most likely accounted for the domestic waste. The Ciliwung River flows through the centre of the city into the Jakarta Bay and is recorded as the river with one of the highest flow rates and nutrient levels in Jakarta (van der Wulp et al., 2016). As this is also one of the sites in Jakarta Bay acting as a recreational park as well as a modern residential and commercial area, this high concentration may be of concern in terms of environmental safety.

Dsikowitzky et al. (2018) presented data from pharmaceuticals and stimulants at several points along the Ciliwung River from its source 40 km south of Jakarta to its mouth in Jakarta Bay. The authors found that the pharmaceuticals tested (diclofenac, ibuprofen and mefenamic acid) were undetected or only detected in very low quantities (the highest being mefenamic acid at 10 ng/L) at sites upstream from

Jakarta. In contrast, all three pharmaceuticals were found to increase in prevalence along the course of the river after entering Jakarta, reaching maximum levels (50 – 1100 ng/L) at the river's mouth where it empties into Jakarta Bay. This data strongly suggests that almost the entirety of pharmaceutical contamination in the Ciliwung River occurs in Jakarta itself, and is likely therefore the result of anthropogenic activity and waste in the city.

Acetaminophen was not detected in other sites. However, in regard to this result, the limit of measurement has to be taken into account. This means that there is a possibility that acetaminophen could be present at other locations at low concentrations, below 50 ng/L, which is the detection limit of the equipment. In this particular analysis, the detection limit meets the condition as a limit of quantification. This is due to the nature of the measurement, which can only be carried out at levels at which the ions used for quantitation can already be determined with high precision, as described by Armbruster and Pry (2008) and Wenzl et al. (2016).

According to Dsikowitzky et al. (2018), only 2 % of households in Jakarta are linked to a centralised wastewater treatment system, 16 % are linked to individual wastewater treatment plants, 71 % of wastewater is contained in septic tanks and transferred to septic tank wastewater treatment facilities, and 11 % of urban residents, especially in slum areas, have to dispose of their wastewater directly into the rivers. These figures provide an overview of information on poor sanitation and wastewater management in Jakarta. Despite the limitations of this study in pointing to the source of the pollution, this fact can be related to Bound and Voulvoulis (2005) that show household disposal necessitates greater attention.

The two sites where acetaminophen was detected are on the mouth of two large rivers, acting as reservoirs for household and industrial waste, as well as residential and commercial areas. This is the first record to date of acetaminophen in Indonesian seawater, and it raises concerns regarding the safety of residents in slum areas, in terms of access to clean surface water, wastewater treatment and regulation, and also

the policy on monitoring pharmaceutical contamination and its disposal. Some photographs of these sites are presented in Appendix 1.

Another alarming inference of pharmaceutical pollution is the issue of food safety and of fisheries based in those polluted areas. Angke and Ciliwung are two popular sites known both as the main farms and the central markets for marine products that are heavily consumed by people living in Jakarta. Ebele et al. (2017) highlighted that one of the routes of contamination is the reaccumulation of contaminants through biota which enter the food chain.

From an environmental point of view, this is one of the highest concentrations of acetaminophen detected in seawater; therefore, it is important to investigate the potential ecotoxicological impacts. Previous studies recording acetaminophen in seawater with much lower concentrations than those uncovered by this study (Lolić et al., 2015; Pereira et al., 2016) have also recommended further investigations into the environmental risk posed to marine biota.

2.4.2 The level of contamination in Jakarta Bay

The results are presented in relation to the Indonesian government regulation limit, referred to by the Decree of the State Minister of the Environment, number 51, year 2004 regarding Standard Quality of Seawater. This government regulation only covers limited parameters in the seawater, e.g. total suspended solids, salinity, pH, ammonia, nitrate, total-phosphate, chromium hexavalent, arsenic, cadmium, copper, nickel, lead, zinc, mercury, dissolved oxygen, PCB, pesticide, and some microbiological parameters.

Angke and Ciliwung, two rivers flowing through the heavily populated metropolitan region of the Jakarta megacity, are vulnerable to high nutrient loads. This is due to the basis that the presence and the level of these parameters in the environments are directly related to anthropogenic activities. According to Dsikowitzky et al. (2018) the high occurrence of nutrients in the environment can be due to insufficient urban wastewater treatment. Van der Wulp et al. (2016) reported an average nitrogen load

of approximately 7 tonnes per day in October 2012, whilst this was 1.7 tonnes a day for total phosphorus. These amounts contributed for about 18 % and 12 % of the overall riverine flows to Jakarta Bay at that time, respectively.

Although the concentrations of contaminants in rivers receiving inputs from these large urban agglomerations can be considered to be moderate, the corresponding contaminant fluctuations are enormous and present a danger to marine environments. A sufficient wastewater treatment infrastructure will have to be established in place to minimise these large contamination fluctuations. However, this is a major problem in rapidly rising mega cities such as Jakarta, the capital city of the world's fourth largest country by population (US-CIA, 2017).

Only a comparatively small fraction of the households in Jakarta are linked to centralised modern wastewater treatment systems. The results presented here show that nutrient parameters (ammonia, nitrate, and total phosphate) mostly exceed the upper limit of the Indonesian government regulation, highlighting the importance of wastewater management in Jakarta. A very high result of ammonia in Angke might be attributed to poor sanitation at the sampling site where there are slum areas, a traditional market, and no access to wastewater treatment.

The Indonesian government regulations (e.g. Decree of the State Minister of the Environment, number 51, year 2004 regarding Standard Quality of Seawater) set a limit only for several heavy metal contaminants (chromium hexavalent, arsenic, cadmium, copper, nickel, lead, zinc, and mercury). Although the results from all sites included in this study did not exceed these limits, the present investigation highlights some interesting concentrations of other heavy metals detected in Indonesian seawater. The presence of these heavy metals in the seawater can be related to runoff carrying all contaminants from the land, as a prominent route of water contamination from terrestrial environments.

The adverse effects of pollution in Jakarta Bay include (but are not limited to): fish mortality, hypoxia, eutrophication and frequent toxic algal bloom, reduced total fish population and cases of contaminated fishery resources (Bengen et al., 2006;

Steinberg, 2007; Dwiyitno et al., 2016; Ladwig et al., 2016). Although it is difficult to construct a direct causal relationship in the aquatic environment given the impact of the interaction of pollutants, this study indicates that a cocktail of contaminants is present in the coastal waters of Jakarta, many of them at relatively high levels that might be related to the issues mentioned above.

2.4.3 Effects on mussel consumption

Consumption of mussels is highly variable globally, ranging for example in EU countries from 0 – 3.9 kg per capita annually (Monfort, 2014). In Indonesia meanwhile, it is well known anecdotally that the presence of contaminants in Jakarta Bay has made the consumption of mussels harvested from this area potentially hazardous to human health (Sufa, 2019). Indeed, Riani et al. (2018) found high concentrations of several heavy metals in the bodies of green mussels *Mytilus viridis* after being cultured in Jakarta Bay for seven months. With this in mind, it is worth considering the effects of mussel consumption on human physiology.

Acetaminophen toxicity develops in humans at 7.5 to 10 g/day or 140 mg/kg, and it can cause severe liver toxicity if taken in amounts above this threshold (Agrawal and Khazaeni, 2020). The level of contamination in mussels in therefore unlikely to reach doses toxic to humans, and may only have minimal possible effects on human physiology. In addition, this study investigates acetaminophen levels in seawater rather than mussel flesh itself. Nevertheless, the aforementioned unsuitability of mussels harvested from Jakarta Bay for human consumption may have an impact on food security in the area, especially considering the dependence of coastal communities on seafood consumption.

2.5 Summary

Acetaminophen was detected at two sites in Jakarta Bay: Angke and Ciliwung. Both sites show considerably high levels of acetaminophen compared to other levels reported in the scientific literature so far. As these two sites are situated at the mouths

of rivers that flow through the city of Jakarta with heavy accumulation of urban and industrial waste before it discharges into Jakarta Bay, it might indicate that this extensive accumulation accounts for the high concentrations detected. This the first record to date of acetaminophen in Indonesian seawater, and raises concerns regarding the safety of residents of slum areas in terms of access to clean surface water, wastewater treatment and regulation, and also the policy on monitoring pharmaceutical contamination and its disposal. Another alarming inference of pharmaceutical pollution is the issue of food safety and of fisheries based in those polluted areas.

High levels of nutrients detected in some sites can be attributed to a lack of municipal wastewater treatment, highlighting the importance of wastewater management in Jakarta. Whilst results from all sites did not exceed these limits set by the Indonesian government for seawater, the present investigations additionally reveal some interesting concentrations of other heavy metals detected in Indonesian seawater.

Overall, the results here suggest that it may be necessary to recommend the inclusion of more parameters in Indonesian government regulations for seawater, as well as routine monitoring programmes for the protection of marine ecosystems.

CHAPTER 3

Acetaminophen in marine mussels – Is time a healer or a killer?

Abstract

A growing body of literature suggests that pharmaceutical contamination poses an increasing risk to marine ecosystems. Acetaminophen or paracetamol is the most widely used medicine in the world and has recently been detected in seawater. This chapter presents the results of short- and long-term exposures of blue mussel adults to 40 ng/L, 250 ng/L and 100 µg/L of acetaminophen. Histopathology shows haemocytic infiltration and follicle dilatation to be the most observed conditions in the exposed mussels for short- and long-term exposures, respectively. After short exposure, *VTG* and *CASP8* mRNA expression show downregulation in all exposed mussels, irrespective of sex. The *V9, HSP70, BCL2* and *FAS* transcripts show a concentration-dependent response in gene expression and may therefore be considered good biomarker candidates. *ER2* mRNA expression displays a downregulated trend, with a clearer dose-response relationship in males than in all mussels combined. Following long-term exposure, the mRNA expression of all target genes shows less variation between exposed groups, indicating that even the lowest concentration of acetaminophen (40 ng/L) can illicit almost equal responses to the highest concentration (100 µg/L). The downregulation in this case does not show any concentration-related trend, as it is relatively similar between exposed groups. In conclusion, this study suggests that acetaminophen has the potential to alter the expression of several genes related to processes occurring in the reproductive system and may therefore impair reproduction in blue mussels.

Keywords: acetaminophen, blue mussels, gene expression, histology, seawater, pharmaceuticals, marine pollution

3.1 Introduction

3.1.1 Acetaminophen - the use, mode of action, and effects

Acetaminophen or paracetamol was first synthesised in 1878 from its precursor phenacetin (McCrae et al., 2018). After initial reports of a connection to methemoglobinemia were disqualified, it was commercialised in the 1950s as a better alternative to phenacetin (Jefferies et al., 2012; Antoni et al., 2014). In the early 1980s, acetaminophen overtook aspirin as the most commonly used over-the-counter (OTC) analgesic in the UK (Bertolini et al., 2006), and now as the most widely sold OTC medicine in the world (Warwick, 2008). Its availability without prescription makes this low-cost medicine the most frequently used analgesic in the world (Jozwiak-Bebenista and Nowak, 2014; Star and Choonara, 2015; Roberts et al., 2016), including in Indonesia (Ministry of Health Republic of Indonesia, 2014, 2015). Acetaminophen is currently being marketed as an analgesic and antipyretic, and should not be taken for more than 3 days without doctor's advice (McCrae et al., 2018).

The mechanism of action of acetaminophen is still not fully comprehended, but is likely to incorporate the inhibition of cyclo-oxygenase-2 (McCrae et al., 2018). Conventional nonsteroidal anti-inflammatory drugs (NSAIDs) block cyclooxygenase (COX) enzymes and prevent the conversion of arachidonic acid to prostaglandin (PG) G2. COX enzymes also have a distinct peroxidase function and metabolise prostaglandin G2 to prostaglandin H2, which is transformed by local tissues into many different PGs according to their individual needs (Jefferies et al., 2012; Graham et al., 2013; Aminoshariae and Khan, 2015). In contrast to closely related NSAIDs, acetaminophen interacts with the peroxidase activity of COX isoenzymes, primarily COX-2, especially when the cell environment is low in arachidonic acid and peroxide (Jefferies et al., 2012; Graham et al., 2013; Aminoshariae and Khan, 2015). The presence of COX-3 isoenzyme (exon splice version of COX-1 seen in insects and rodents) is another potential mechanism of action. Nevertheless, it has not been identified in humans, and further studies show that acetaminophen may not have clinically relevant impacts on

the variants of COX-1 exon splice detected so far in humans (Graham and Scott, 2005; Graham et al., 2013).

Mallet et al. (2010) suggested a possible mechanism of action involving direct activation of the capsaicin receptor identified as transient receptor potential cation channel subfamily V member 1 (TRPV1) by an active metabolite of acetaminophen N-arachidonoylphenolamine (AM404). Another hypothesis also mentioned the inhibition of the reuptake of anandamide (and subsequent activation of cannabinoid receptor CB1) by this metabolite, resulting from the conjugation of arachidonic acid and deacetylated acetaminophen (Högestätt et al., 2005). Other studies also propose the activation of transient receptor potential cation channel, subfamily A, member 1 (TRPA1) by acetaminophen metabolites as the mechanism of action (Andersson et al., 2011; Fresno et al., 2014).

Regardless of the mechanism, it is well known that acetaminophen is mainly metabolised to many conjugates (with glucuronide and sulphate) and excreted in the urine. Approximately 10 % of acetaminophen is metabolised by cytochrome P450 (CYP) enzymes to form n-acetyl-p-benzoquinoneimine (NAPQI), which is then conjugated with intracellular glutathione, and eventually excreted as cysteine and mercapturic acid conjugates (McCrae et al., 2018). In addition, the first human biomonitoring study by Modick et al. (2014) demonstrated pervasive presence of acetaminophen excreted in urine in a wide variety of concentrations in the general population.

While acetaminophen has less an analgesic effect in chronic use than commonly believed, the acute effects of acetaminophen ingestion in overdoses are well documented (Ferner et al., 2011). McCrae et al. (2018) summarised the adverse effects of long-term acetaminophen use with highlights on critical impacts related to the respiratory, cardiovascular, gastrointestinal, renal and central nervous systems, as well as the possible effects on the offspring of women consuming acetaminophen during their pregnancy.

Recent studies have increasingly correlated acetaminophen with endocrine-disrupting effects, although this medication is classified as a non-steroidal anti-inflammatory drug

(NSAID) (Kristensen et al., 2012; Mazaud-Guittot et al., 2013; Jégou, 2015; Kristensen et al., 2016). Snijder et al. (2012) indicated a link between exposure to acetaminophen during pregnancy and the elevated risk of cryptorchidism, a condition in humans where testes have failed to descend into the scrotum during the developmental stage of the foetus. Other studies also have recorded the anti-androgenic property of acetaminophen (Kristensen et al., 2012), and a decrease in testosterone production in foetal testes (van den Driesche et al., 2015).

3.1.2 Acetaminophen in the aquatic environment

In the US, the occurrence of acetaminophen has been detected in Mississippi river water in New Orleans at a concentration of 25-65 ng/L (Zhang et al., 2007), and showed the highest concentration in two WWTPs from Charleston, South Carolina over a period of one year (Hedgespeth et al., 2012). A recent study by Fairbairn et al. (2016) also recorded the presence of acetaminophen with a median concentration of 3.3 ng/L and detection frequency of 88 % in 68 grab water samples from the Zumbro River watershed, Minnesota over a period of one year.

Acetaminophen was reported in the UK by Kasprzyk-Hordern et al. (2008) and has been recorded in surface water of the Han River, Korea at concentration of 34.8 ng/L (Kyungho Choi et al., 2008). In the estuarine-receiving environment around Auckland, New Zealand, acetaminophen was detected at concentrations up to 7.7 ng/g (Stewart et al., 2014), while a national study of trace organic contaminants in Australian rivers revealed that the most frequently found contaminants were pharmaceuticals, one of the highest being acetaminophen at maximum concentrations of 7.15 µg/L (Scott et al., 2014). Olatunde et al. (2014) recorded the occurrence of acetaminophen at an average concentration of 2.57 µg/L in surface and groundwater samples from an irrigation canal and wells in a pharmaceutical industrial region of Sango Ota, Nigeria; while another study in South Africa reported an anomalous concentration of acetaminophen being much higher (16 µg/L) in surface water than in wastewater (Agunbiade and Moodley, 2014).

Evidence suggests that pharmaceuticals can exert some important toxic effects over non-target aquatic species, due to their chemical and biological properties (Franzellitti et al., 2013). The present study aims to assess the biological responses induced by acetaminophen, at environmentally relevant levels, in the blue mussel *Mytilus edulis*, namely focusing on reproductive parameters. After short-term (7 days) and long-term (24 days) laboratory exposures, seven potential target genes were selected to investigate toxicological effects in mussel gonads, and potential disturbances of the reproductive cycle. The study examines the modulation of some important reproduction-related genes: *vitellogenin* (*VTG*), *vitelline envelope zona pellucida domain-9* (*V9*) and *estrogen receptor-2* (*ER2*). The variation in mRNA expression of four other genes involved in apoptosis: *heat shock protein-70* (*HSP70*)*, caspase-8* (*CASP8*)*, B-cell lymphoma-2* (*BCL2*) and *Fas cell surface death receptor* (*FAS*) was also investigated. Histopathological alterations caused by acetaminophen, together with its potential to act as a non-traditional endocrine disruptor are discussed.

3.2 Materials and methods

3.2.1 Collection of mussels

Blue mussels *Mytilus edulis* were collected manually in April 2018 at low tide from Hove beach, East Sussex, UK (50.823797, -0.173423). A map and photograph of the sampling site are presented in Appendix 2. Mussels were taken from one population only, kept on ice after collection, and transferred immediately to the laboratory. Mussels were cleaned by removing the dirt and organic materials including algae and barnacles that attached to the surface of the shell. Cleaned mussels were then placed in a container with artificial seawater (Instant Ocean® Sea Salt, USA) for acclimatisation. During acclimatisation, the mussels were fed daily with a 500 µL dose of green algae *Tetraselmis* sp. culture suspension (ReefBoost, UK) per 5 litres of artificial seawater. The initial water temperature (15 °C) was then gradually increased over the next 6 days, until reaching room temperature 20 ± 2 °C.

3.2.2 Experimental exposure

Mussels used in the exposure experiments were selected based on their size range (30 mm to 50 mm) to ensure that they are in the similar stage of development. For all exposures, artificial seawater (Instant Ocean® Sea Salt, USA) was prepared and mussels were allocated with the volume proportion of approximately 1 litre per mussel per tank.

Mussels were exposed to acetaminophen (BioXtra, ≥ 99.0 %, Sigma-Aldrich) in three different concentrations. The four treatments consisted of a control group (artificial seawater only, without acetaminophen), and three exposed groups of nominal concentrations of 40 ng/L, 250 ng/L, and 100 µg/L. The selected nominal concentrations were chosen to reflect the levels found in the marine environment (Togola and Budzinski, 2008; Nödler et al., 2014; Bebianno et al., 2017). Ten mussels were used in each exposure group, with some extra mussels also reserved as back up. Artificial seawater was changed every 72 and 96 hours with the addition of acetaminophen for exposed groups only. These water changing periods were adopted to minimise the stress caused by disturbance from changing the water and to accommodate the accessible hours of the building where the experiment was conducted. The short-term exposure was terminated on the 7th day, whilst long-term exposure was carried out until day 24. Physical attributes of the seawater (temperature, salinity, conductivity, and resistance) were recorded daily.

At the end of the experiment 10 mussels from each group were collected, measured and dissected. For histology, approximately 1 cm square of each gonad was fixed in neutral buffered formaldehyde in clean tubes and stored at 4 °C. For the molecular analysis, the tissues were immediately placed in RNA*later*™ (Invitrogen, UK) and stored at –80 °C.

3.2.3 Water analysis

Water samples (1,000 mL) from each treatment group and control were collected 15 – 30 minutes after the addition of each contaminant (t_0) and immediately before

changing artificial seawater (t_1 and t_2). Water samples from short- and long-term exposures were collected and stored in the fridge until processed. In summary, there were eight samples from short-term exposures (four each from t_0 and t_1, representing each exposure group), and eight samples from long-term exposures (four each from t_0 and t_2, representing each exposure group). All samples underwent a solid phase extraction process, following two filtrations using 1.2 µm Whatman grade GF/C microfiber glass filter paper (GE Healthcare, UK) and 0.22 µm nylon membrane filter (GE Healthcare, UK). Methanol (LC/MS Grade, Optima™, Fisher Chemical, UK) 1 mL was used to wash the single-used filter at the end of each filtration. This procedure was adapted from Paiga et al. (2015), Alygizakis et al. (2016) and Pereira et al. (2016). Solid phase extraction was performed using a Strata™-XL-C 100 µm polymeric strong cation 2 g / 20 mL giga tube cartridge (Phenomenex, USA) following the method described in section 2.2.2.1. The extract was evaporated using a speed vacuum system and reconstituted with LC/MS grade water (LiChrosolv®, Merck KGaA, Germany).

Liquid chromatography-mass spectrometry (LC-MS) was performed using an Orbitrap QExactive (Thermo Scientific) coupled to an ultra-high performance LC system (Ultimate 3000, Thermo Scientific). Acetaminophen was separated using reversed phase chromatography using Kinetex XB-C18, 5µ, 100Å, 100 x 2.1 mm, with trap column (Phenomenex, UK) and a solvent gradient consisting of solvent A; water with 0.1 % formic acid (FA) (Hypergrade for LC-MS, LiChrosolv®, Merck KGaA, Germany) and solvent B; acetonitrile with 0.1 % FA (Hypergrade for LC-MS, LiChrosolv®, Merck KGaA, Germany) at a flow rate of 300 µL/min. The gradient started with 0 % B (0 - 1 minute), increased to 100 % B over 5 minutes, ramped to 100 % B in 1 minute, held at 100 % B for 1 minute, decreased back to 0 % B in 0.5 minute and equilibrated for 1.5 minutes. The column was kept at 50 °C in the column oven. Samples were stored at 4 °C in the autosampler and 10 µL was injected using a partial loop injection.

Mass spectrometry was performed in the positive mode using heated electrospray ionization (HESI). Spray voltage was set to 3,000 V, sheath gas set to 6 arbitrary unit (au), auxiliary gas set to 4 au, sweep gas set to 1 au and probe temperature set to 200 °C. The capillary temperature was 350 °C and S-Lens set to 50. Data was acquired using

parallel reaction monitoring with an inclusion list comprising one mass: 152.0700 *m/z* corresponding to the $[M+H]^+$ ion. Resolution was set to R = 140,000 at 200 *m/z*, with automatic gain control (AGC) target of 1×10^6, maximum injection time of 200 ms, an isolation window of 2.2 *m/z* and a collision energy of 20 eV. Total run time was 8 minutes and data were acquired from 2 to 5 minutes. The area under the peak was determined from the reconstructed ion chromatogram of the fragment at 110.0602 *m/z*.

3.2.4 Neutral red retention time (NRRT) assay

The assay was employed only on the haemocytes of mussels from the long-term exposure groups. The assay was performed following the methods described by Lowe and Pipe (1994) and Lowe et al. (1995). The chemical solutions used in this assay were prepared according to Mamaca et al. (2005). Neutral red (Sigma-Aldrich) 28.8 mg was dissolved in 1 mL of dimethyl sulfoxide (Sigma-Aldrich) to prepare the dye stock solution. The physiological saline solution was prepared as follows: 4.77 g HEPES (Sigma-Aldrich), 25.48 g NaCl (Fisher BioReagents), 13.06 g $MgSO_4$ (Sigma-Aldrich), 0.75 g KCl (Fisher Chemical), and 1.47 g $CaCl_2$ (Merck Millipore) were dissolved to a final volume of 1 L reverse osmosis water after adjusting the pH to 7.3. A working solution was freshly prepared by diluting 2 µL of neutral red stock solution to 1 mL of physiological saline solution. Haemolymph of mussels was withdrawn using a 2 mL syringe containing 0.2 mL of physiological saline solution and transferred to a clean tube. After a brief centrifugation, 30 µL of haemolymph saline mixture was transferred onto a poly-L-lysine coated microscope slide and left for 15 minutes at room temperature in a lightproof humid container to allow cell attachment. Neutral red working solution 30 µL was added to the area containing the haemolymph and a cover slip was applied. The slide was left for another 15 minutes for incubation. Observations were conducted using a light microscope with 40x/100x magnification. The first observation after incubation was recorded as t_0, followed by further observations at 30-minute intervals until a total time of 180 minutes. Slides were returned to the lightproof humid container after each observation. The test was terminated, and data

were recorded in the table at the point when the neutral red which had been taken up into lysosome was lost to the cytosol. This is determined by the neutral red dye of the small granular in haemocytes being more than 50 % evidently lost to the cytosol. The assay is based on the capability of lysosomes to accumulate pollutants; however, this concentration of toxic contaminants results in lysosomal damage and possible leakage of contaminants into the cytosol.

3.2.5 Histopathology

Histological analysis was employed to determine the sex of individuals and to examine the pathological conditions present in mussel gonads. Mussel tissues (n = 10 from each treatment group) were processed prior to histological examination following a technique adapted from Ciocan et al. (2010). Approximately 1 cm cube of each gonad were fixed in neutral buffered formaldehyde (Sigma-Aldrich) in clean tubes. Prior to wax infiltration, tissues were placed into labelled cassettes and processed through a series of dehydration and clearing using an automated tissue processor (Leica Biosystems, Germany) following the manual provided by Howard et al. (2004). After paraffin embedding, solid blocks were sectioned using a manual microtome (Thermo Scientific, UK). Each gonad tissue sample was sectioned 7 µm in three different parts from the edge to the centre, to ensure the sections captured not only the edge part of the tissue. About 6 – 10 intact sections were obtained from each part of the tissue, giving a total of 18 – 30 sections of each gonad. Sections were transferred to slides and placed in a 37 °C oven overnight to ensure the tissue fixation to slides. Histo-Clear™ (National Diagnostics™, UK) and descending concentrations of ethanol (95 %, 70 %, and 50 %) were used to remove paraffin from sections. After staining with haematoxylin (Sigma-Aldrich) and eosin (Sigma-Aldrich) for 5 and 2 minutes respectively, deparaffinized sections were then dehydrated and mounted in DPX (CellPath, UK). Histological examination of tissues from all slides was performed using a light microscope (Leitz Wetzlar, Germany) (40x/100x), and histopathological conditions were recorded along with micrographs corresponding to each condition using GXCam Hichrome-Lite (GT Vision, UK). The prevalence of each histopathological

condition was quantified by counting how many mussels exhibited the particular pathology, expressed as a percentage of all mussels in each group. The result only presented the occurrence, not the severity, of each histopathological condition in each treatment group. In many cases, several pathologies could be present in one individual, as captured by micrographs.

3.2.6 Gene expression analysis

3.2.6.1 RNA extraction and cDNA synthesis

Total RNA from the gonads (n = 10 for each experimental group) were individually extracted using SurePrep™ TrueTotal™ RNA Purification Kit (Fisher BioReagents™, UK), SurePrep™ RNA/Protein Purification Kit (Fisher BioReagents™, UK) and Monarch® Total RNA Miniprep Kit (New England Biolabs, UK) as per manufacturers' instructions. The extracted RNA concentration was measured with Qubit™ RNA HS Assay Kit (Invitrogen, UK) and Qubit® Fluorometer (Invitrogen, UK) according to the manufacturer's protocol. The reverse transcription was performed with Transcriptor High Fidelity cDNA Synthesis Kit (Roche, UK) using 1 μg of total RNA of each sample, following the protocol provided by the manufacturer. The complementary DNA (cDNA) quantification from each sample was measured using Qubit™ dsDNA HS Assay Kit and Qubit® Fluorometer (Invitrogen™, UK).

3.2.6.2 Quantitative real time PCR

Molecular analysis was employed in order to highlight changes in the expression pattern of selective mussel transcripts. This analysis was adapted from Ciocan et al. (2010) to examine the effects of exposure on mRNA expression of target genes *vitellogenin* (*VTG*)*, vitelline envelope zona pellucida domain-9* (*V9*)*, estrogen receptor-2* (*ER2*)*, heat shock protein-70* (*HSP70*)*, caspase-8* (*CASP8*)*, B-cell lymphoma-2* (*BCL2*) and *Fas cell surface death receptor* (*FAS*) in mussel gonads. The real time qPCR was performed using LightCycler® Nano Real-Time PCR System and FastStart Essential DNA Green Master (Roche, UK) following the manual guide from the manufacturer. All parameters were set as follows: 95 °C for 10 minutes, 45 cycles of 20 seconds at 95 °C,

20 seconds at 50 – 57 °C and 20 seconds at 72 °C. Melting curves were determined to analyse the specificity of the reaction and identify the presence of primer dimer by holding the reaction at 65 °C for 1 minute and 95 °C for 1 minute. A control lacking cDNA was included in each qPCR run in order to determine the specificity of target cDNA amplification, and all reactions were performed in duplicate. Two housekeeping genes, *18S ribosomal RNA* (*18S rRNA*) and *elongation factor-1 alpha* (*EF1*), were used as the internal control genes, as suggested by Cubero-Leon et al. (2012) (Table 5).

Table 5 Primers sequences used for the molecular analysis.

Primer Name	Forward Primer	Reverse Primer
Housekeeping gene		
18S rRNA[1]	GTGCTCTTGACTGAGTGTCTCG	CGAGGTCCTATTCCATTATTCC
EF1[1]	CACCACGAGTCTCTCCCAGA	GCTGTCACCACAGACCATTCC
Target gene		
VTG[2]	GGACCTCCACCAGTGCTAATCC	ATCTCAGCGGTTCCGACTGC
V9[3]	TTCTGGACGAAATGCTAATGTGA	GGATTGAGCGTGACGAGACC
ER2[2]	GGAACACAAAGAAAAGAAAGGAAG	GCTGGATTAGGACTGCCACTTG
HSP70[4]	ATAACTACTGAGATATGGCAGGAA	TGGTCGTTGGCTATGATGT
CASP8[5]	CCCAACCAGTAGTAACACCAGAC	GTATGAACCATGCCCCTATATCA
BCL2[6]	GACAGTCCGTGGGATGTGAA	CTTAACGCCATTGCGCCTAT
FAS[6]	ACGCTGATGACATACATCGCA	CGGTACTGGCTGAGCTTGTT

[1]Cubero-Leon et al. (2012), [2]Ciocan et al. (2010), [3]Ciocan et al. (2011), [4]Pirrone et al. (2018), [5]Yavaşoğlu et al. (2016), [6]Zhu et al. (in preparation)

3.2.7 Data analysis

The raw data expressed as cycle quantification (*Cq*) from real-time qPCR analysis of each target gene mRNA expression was normalised against the average *Cq* of

housekeeping genes *18S rRNA* and *EF1*. The calculation of relative changes in expression of target genes determined from qPCR followed the comparative $2^{-\Delta\Delta Ct}$ method expressed as fold changes to the control group as described by Livak and Schmittgen (2001).

Statistical analysis was carried out using GraphPad Prism 8. For the NRRT assay and mRNA expression data, one-way analysis of variance (ANOVA) was performed, followed by a Tukey's post-hoc multiple comparison test to identify significant differences between the groups with $p < 0.05$. Two-way ANOVA and post-hoc Tukey's multiple comparison test ($p < 0.05$) were employed for the histopathological data.

3.3 Results

3.3.1 Water chemistry

The results of seawater analysis are presented in Figure 3, as percentage reduction of acetaminophen for each experimental group. A different pattern of reduction is displayed in each exposed group. After 72 hours, almost 100 % reduction of acetaminophen in water was observed in the group exposed to 100 µg/L, and an 83 % reduction in the group exposed to 40 ng/L, after 96 hours. The reduction after 72 hours shows more divergence between groups, while after 96 hours the reduction is more closely converged to an average of 72 %.

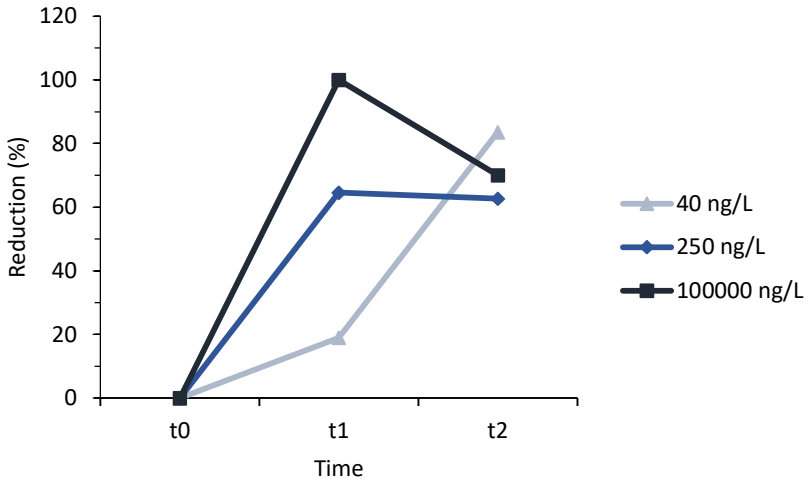


Figure 3 Percentage reduction of acetaminophen (n = 1) in exposed groups after 72 hours (t1) and 96 hours (t2).

3.3.2 Neutral red retention time (NRRT) assay

Lysosomal membrane integrity of haemocytes expressed as neutral red retention time is shown in Figure 4. All exposed groups have a reduced ability to retain the dye inside the lysosomes compared to the non-exposed group, however there is no significant difference between groups exposed to various concentrations of acetaminophen.

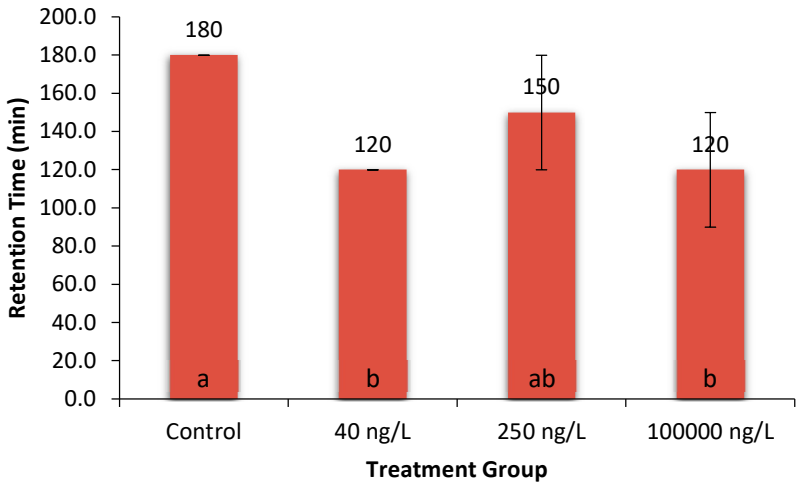
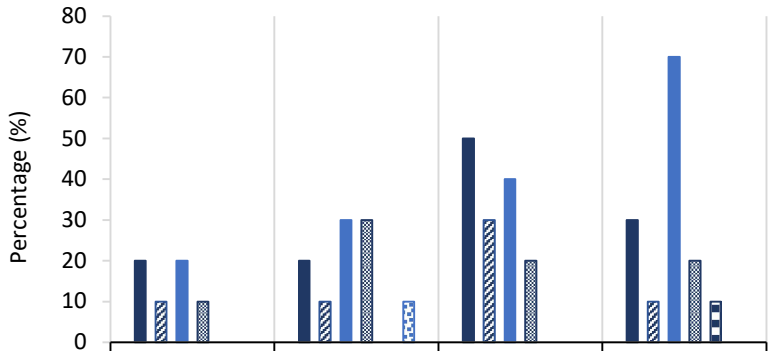


Figure 4 Neutral red retention time of haemocytes from mussels exposed to acetaminophen for 24 days (n = 3). Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

3.3.3 Histopathology

For the short-term exposure (Fig. 5), haemocytic infiltration, a condition typically occurring as a response to inflammation, was the most observed histopathological condition in mussel gonad tissue. This was followed by follicle dilatation (Fig. 6c-d), a pathological condition in which the follicle shows degenerating signs (e.g. dilation). These conditions were observed in all groups alongside gamete degeneration (Fig. 6e-f), and atresia (Fig. 7b) in female gonads. Gamete degeneration is a pathology where the gamete deteriorates, while atresia is a degenerative process with subsequent elimination and resorption of oocytes (García-Gasca et al., 2010).

Haemocytic infiltration (Fig. 7a) and atresia (Fig. 7b) were the most commonly occurring pathologies in the 40 ng/L group, with up to 30 % of mussels affected, while follicle dilatation was dominant in the 250 ng/L group, with up to 50 % occurrence. Haemocytic infiltration was the pathological condition with the highest occurrence across all groups, reaching up to 70 % incidence in the 100 µg/L group. Induced spawning (Fig. 7c) was also recorded in this group only, although with very low prevalence (10 %). Lipofuscin (Fig. 7d), an autofluorescent lipopigment strongly associated with lysosomal damage (Viarengo et al., 2007) was detected only in 10 % of mussels from the 40 ng/L group and was absent in other groups.



	Control	40 ng/L	250 ng/L	100000 ng/L
Follicle dilatation	20	20	50	30
Gamete degeneration	10	10	30	10
Haemocytic infiltration	20	30	40	70
Atresia	10	30	20	20
Induced spawning	0	0	0	10
Lipofuscin	0	10	0	0

Figure 5 The occurrence of histopathological conditions observed in the gonad tissue of mussels exposed to acetaminophen for 7 days (n = 10). The treatments were as follows: Control, 40 ng/L, 250 ng/L and 100 µg/L. No statistically significant differences were found between groups (two-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

The micrographs of histopathological conditions recorded in the observation are shown in Figure 6 and Figure 7. Figures 6a and 6b show healthy female and male gonads, respectively. Follicle dilatation in female (Fig. 6c) and male (Fig. 6d) mussels are characterised by dilation or breakdown of the follicles. When the oocyte (Fig. 6e) or spermatozoid (Fig. 6f) shows signs of defect or irregularity, it is recorded as gamete degeneration. Inflammatory reactions are also recorded here, whether as haemocytic infiltration (Fig. 7a) where haemocytes (which normally only occur in the connective tissue) permeate into and are present in follicles; or a more severe condition where haemocytes form a cluster known as haemocytic aggregate (Fig. 7f). Atresia, a condition marked by the breakdown of oocytes or a form of apoptosis before maturation is shown in Figure 7b. Figure 7c shows an induced spawning where a tubercle that only appears preceding spawning is present in the premature gonads. The appearance of accumulated lipid and protein that display fluorescent pigment is a characteristic of lipofuscin (Fig. 7d). Figure 7e shows some observations that possibly

indicate the presence of parasites in the mussels' gonads, although identification is required here.

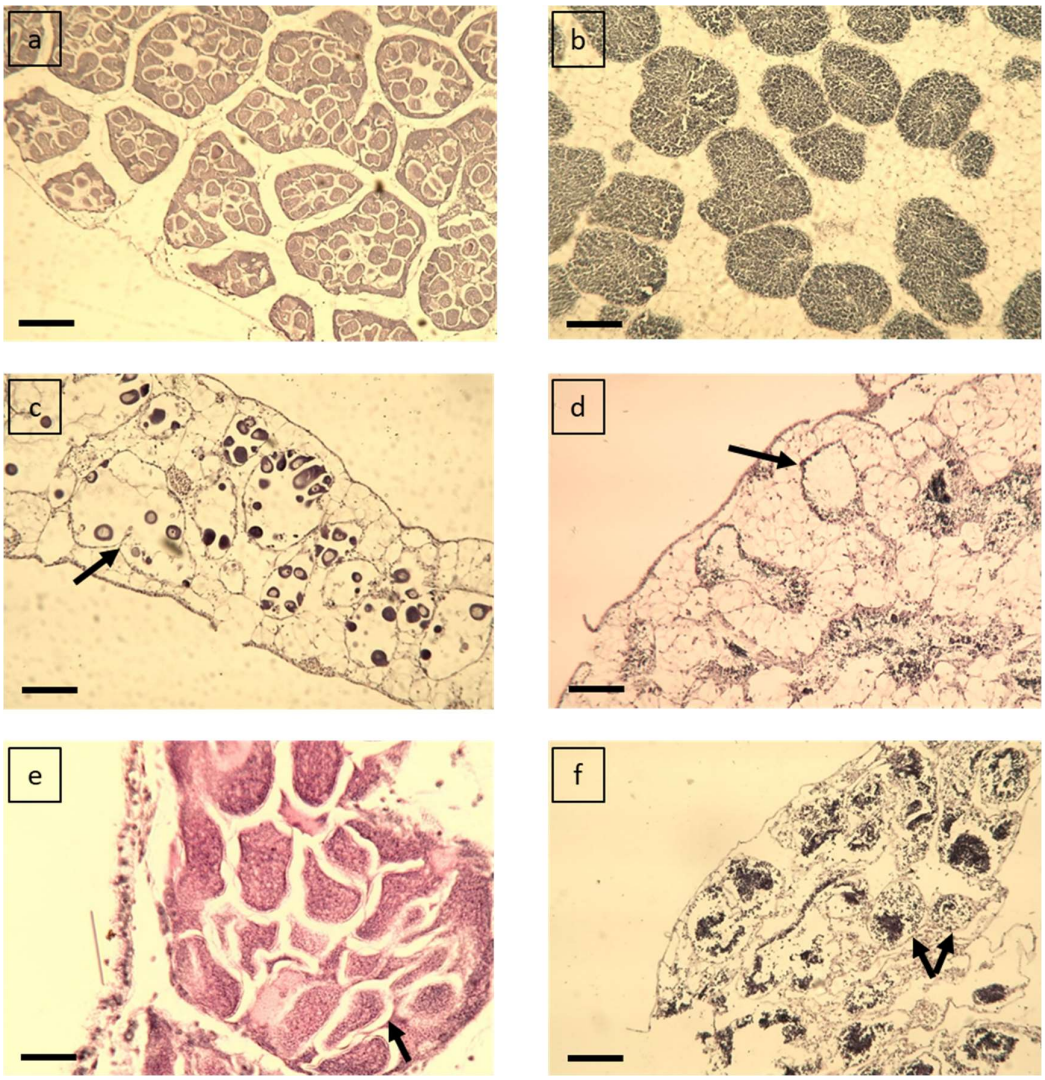


Figure 6 Histopathological conditions in mussel gonads after exposure to acetaminophen. Sections of 7 µm, stained with haematoxylin and eosin. (a) normal histology, female; (b) normal histology, male; (c) follicle dilatation, female; (d) follicle dilatation, male; (e) gamete degeneration, female; (f) gamete degeneration, male. Arrows point to each pathological condition. Scale bar = 100 µm (A-D, F-J), 40 µm (E).

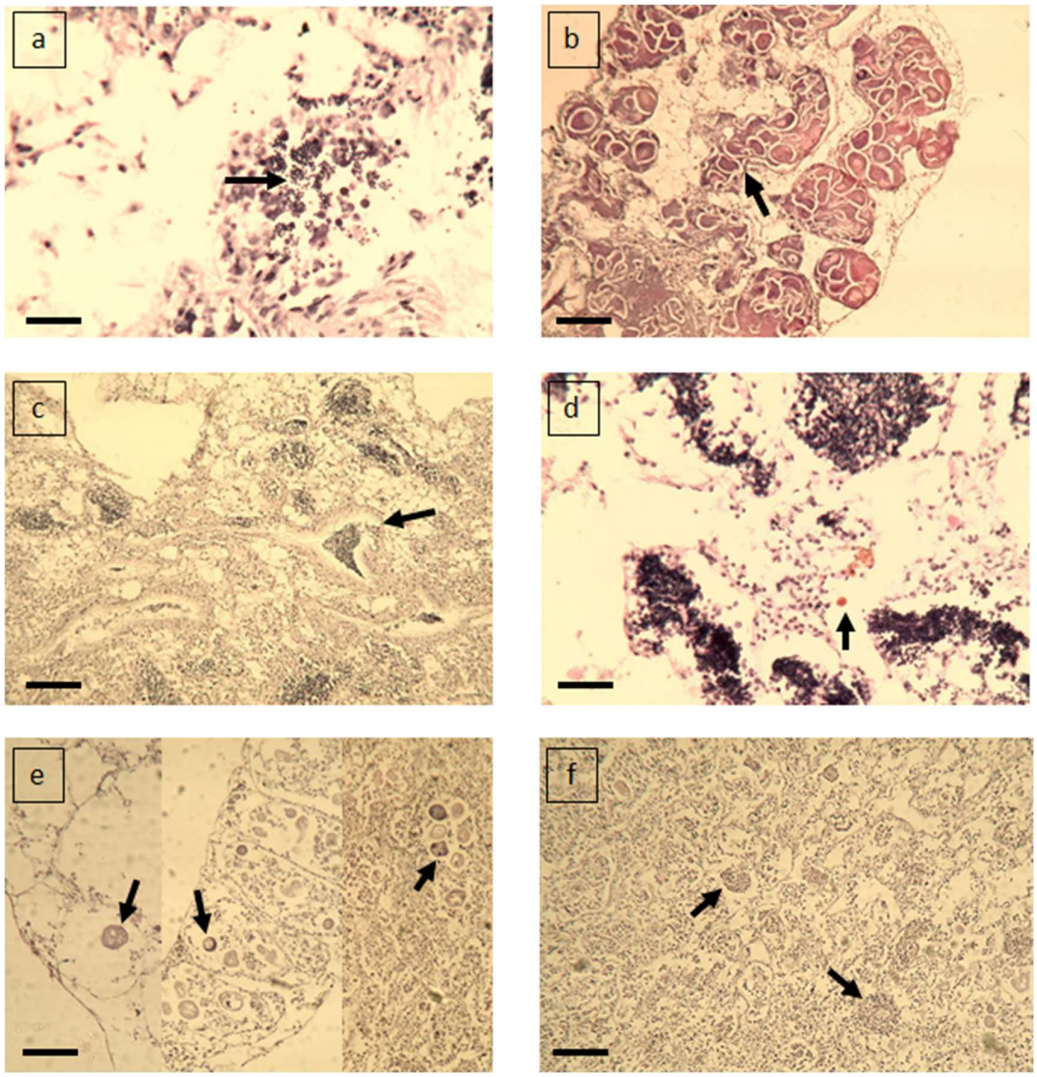
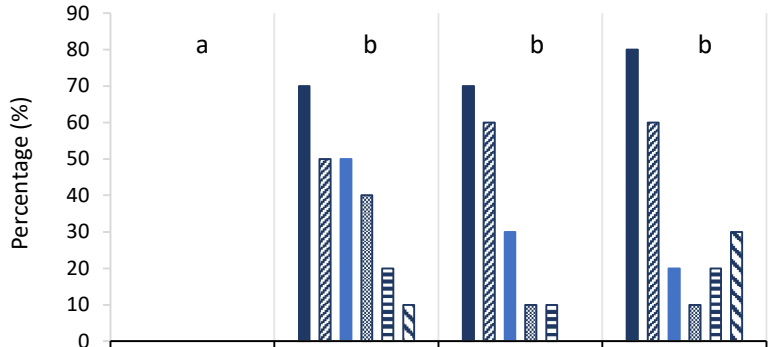


Figure 7 Histopathological conditions in mussel gonads after exposure to acetaminophen (continued). Sections of 7 µm, stained with haematoxylin and eosin. (a) haemocytic infiltration; (b) atresia; (c) induced spawning; (d) lipofuscin; (e) parasites; (f) haemocytic aggregate. Arrows point to each pathological condition. Scale bar = 100 µm.

Follicle dilatation was recorded as the most widespread pathology in the long exposure groups (Fig. 8). This pathological condition was observed in all exposed groups with very high prevalence (70 – 80 %). Gamete degeneration is another pathological condition that showed high occurrence, being present in 50 – 60 % of individuals in all exposed groups. Atresia, a condition occurring in female gonads, also showed high incidence, even in the group exposed to the lowest concentration of acetaminophen (40 ng/L) at 40 %. Inflammatory pathologies such as haemocytic infiltration and

haemocytic aggregate were also observed in as many as 50 % and 30 % of individuals, respectively. Another pathological condition of note is parasites, which were observed in all exposed groups although with low prevalence (10 – 20 %).



	Control	40 ng/L	250 ng/L	100000 ng/L
Follicle dilatation	0	70	70	80
Gamete degeneration	0	50	60	60
Haemocytic infiltration	0	50	30	20
Atresia	0	40	10	10
Parasites	0	20	10	20
Haemocytic aggregate	0	10	0	30

Figure 8 The occurrence of histopathological conditions observed in the gonad tissue of mussels exposed to acetaminophen for 24 days (n = 10). The treatments were as follows: Control, 40 ng/L, 250 ng/L and 100 µg/L. Different letters represent statistically significant differences between groups (two-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

3.3.4 mRNA expression

Mussels used in this study were confirmed as *Mytilus edulis* (a more detailed description is provided in Appendix 3). The mRNA expression of each target gene is shown in Figures 9 – 15, with data from all individuals in each group plotted alongside data for males only. The mRNA variation is expressed as fold changes to the control group, where the control group was standardised to 1; downregulation is represented by values below 1 and upregulation by values above 1.

In the short exposure, the mRNA expression of *VTG* (Fig. 9) shows significant downregulation in all treatment groups compared to the control group (approx. 50 fold), with no significant difference between treatment groups. A similar pattern was observed in all mussels and males only. The expression in the long exposure groups

also show similar downregulation in all exposed groups, although with a different pattern and less downregulated (4 – 11 fold). Again, there is little difference between data from all mussels and those from male mussels only.

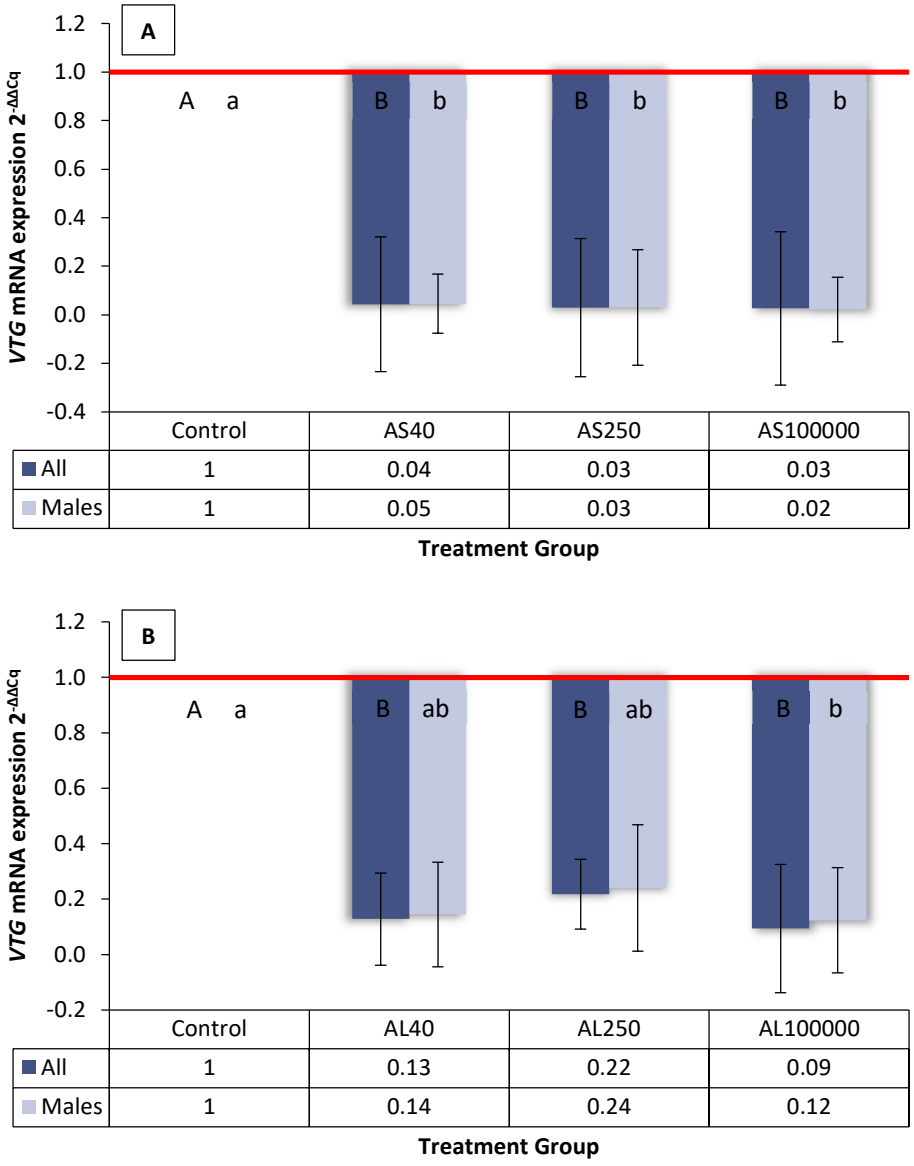
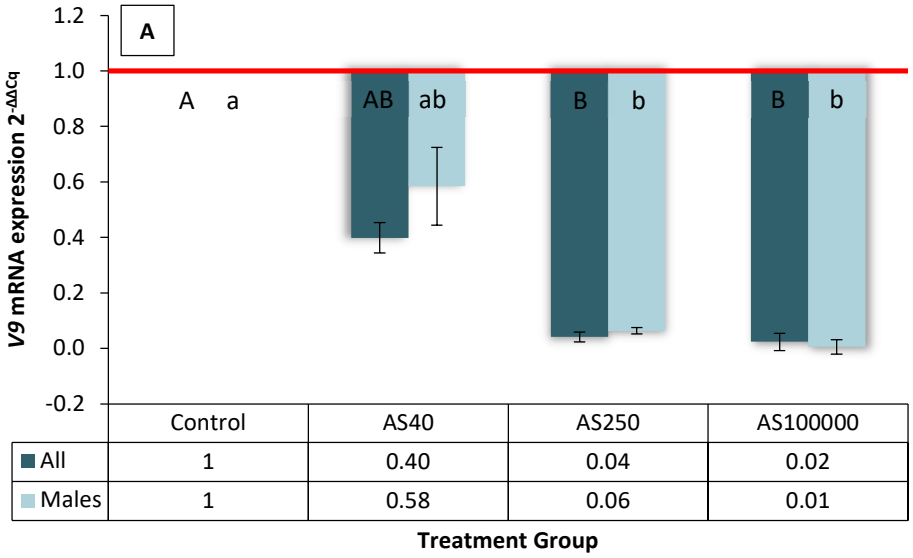


Figure 9 Summary of mRNA expression of *VTG* in fold changes compared to control group in mussel gonads (n = 10). Data from all individuals and male mussels only. Treatments are denoted by alphanumeric codes: AS, groups exposed to short term exposure (A); AL, groups exposed to long term exposure (B); each followed with nominal concentration of acetaminophen in ng/L. Different letters represent statistically significant differences between groups, with capitals used for all individuals and lower case letters for males only. Bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

After short exposure, *V9* mRNA expression (Fig. 10) shows downregulation with a concentration-dependent trend for both data from mixed sexes and from male mussels only. Only mussels in group 250 ng/L and 100 µg/L show a significantly different result to the control. The downregulation ranges from 2.5 to 50 fold changes in mixed sex mussels, and from nearly 2 to 100 fold changes in male mussels only. For long exposure, whilst the data again shows downregulation for all exposed groups, in this case the expression does not follow a concentration-dependent trend. The mRNA expression is almost equally downregulated in all exposed groups, displaying 4 – 16 fold changes compared to the control group, and mixed sex mussels in exposed groups show significantly different data to the control group statistically.

The expression of *ER2* (Fig. 11) in all treatment groups for short exposure was downregulated compared to unexposed mussels. While both sets of data show downregulation, a concentration-related trend is expressed only in male mussels. For mixed sex mussels data, all groups show significantly different results to the control group ranging from 4 to 12 fold changes. While in the male mussels alone, the significant difference relative to the unexposed group observed only in the group 250 ng/L and 100 µg/L. There is no concentration-dependent trend observed in exposed groups from long exposure, either in mixed sex mussels or male mussels only. The downregulation is 10 – 33 fold compared to control, almost equal between all exposed groups in this long exposure study.



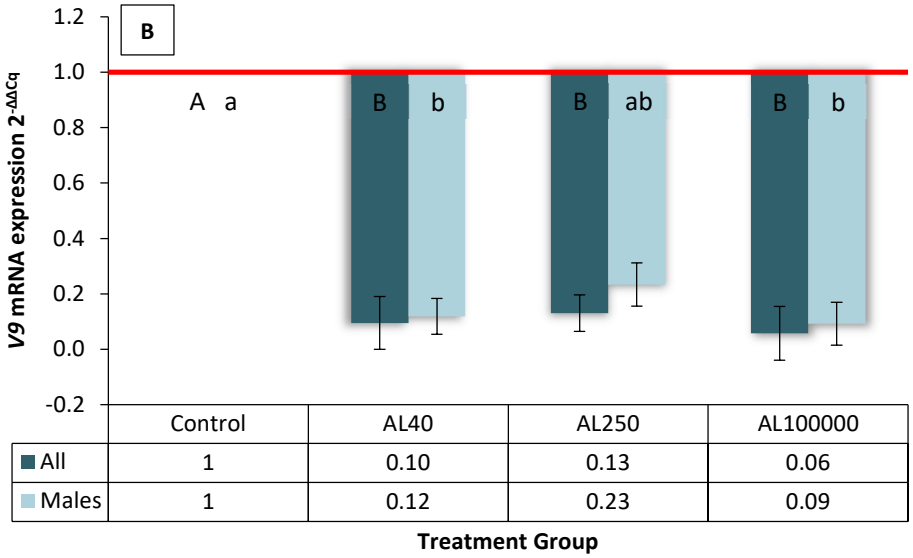
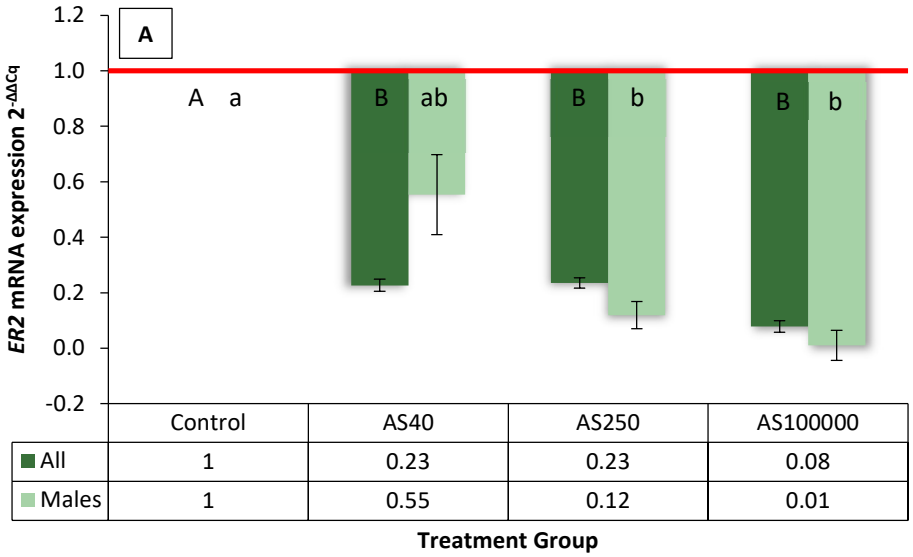


Figure 10 Summary of mRNA expression of *V9* in fold changes compared to control group in mussel gonads (n = 10). Data from all individuals and male mussels only. Treatments are denoted by alphanumeric codes: AS, groups exposed to short term exposure (A); AL, groups exposed to long term exposure (B); each followed with nominal concentration of acetaminophen in ng/L. Different letters represent statistically significant differences between groups, with capitals used for all individuals and lower case letters for males only. Bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).



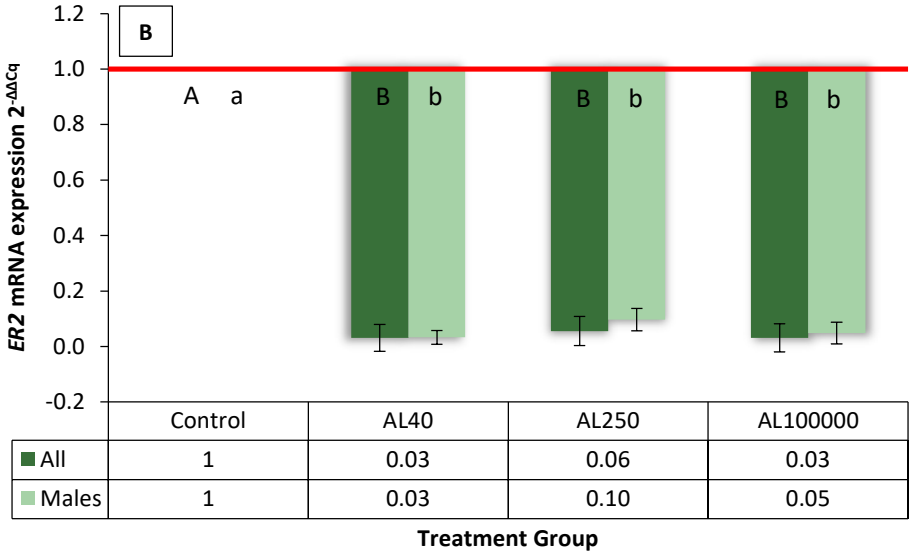


Figure 11 Summary of mRNA expression of *ER2* in fold changes compared to control group in mussel gonads (n = 10). Data from all individuals and male mussels only. Treatments are denoted by alphanumeric codes: AS, groups exposed to short term exposure (A); AL, groups exposed to long term exposure (B); each followed with nominal concentration of acetaminophen in ng/L. Different letters represent statistically significant differences between groups, with capitals used for all individuals and lower case letters for males only. Bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

The mRNA expression of *HSP70* was reduced in all treatment groups following a concentration-dependent trend both in data from all individuals and from male mussels only after short exposure to acetaminophen (Fig. 12). Although both data sets

show concentration-related changes, data from male mussels only show more variation between concentrations, with *HSP70* mRNA expression ranging from 2 to 50 fold changes (group 40 ng/L and 100 µg/L respectively) compared to data from all individuals displaying a range of 3 to 9 fold changes. Less variation is shown in long exposure data, as the downregulation does not show a different trend from low to higher concentration of acetaminophen, or between data of all mussels (14 – 25 fold) and male mussels only (10 – 14 fold). Both data sets, however, show statistically significant differences between exposed groups and the control group from this long exposure.

The expression of *CASP8* mRNA was highly downregulated in all groups exposed in the short term to acetaminophen, regardless of concentration (Fig. 13). The downregulation reveals that even the lowest concentration of acetaminophen (40 ng/L) reduced *CASP8* mRNA expression up to 33 fold changes in mixed sex mussels, with the highest concentration inducing 100 fold changes compared to the control group. All exposed groups show statistically significant different results compared to the control group, both in data from all mussels and male mussels only. The mRNA expression of *CASP8* after longer exposure to acetaminophen shows a similar pattern, with almost the same level of downregulation observed in all exposed groups (12 – 25 fold). Both data from all mussels and from male mussels only show similar downregulation, however downregulation is lower overall compared to the data from the short exposure.

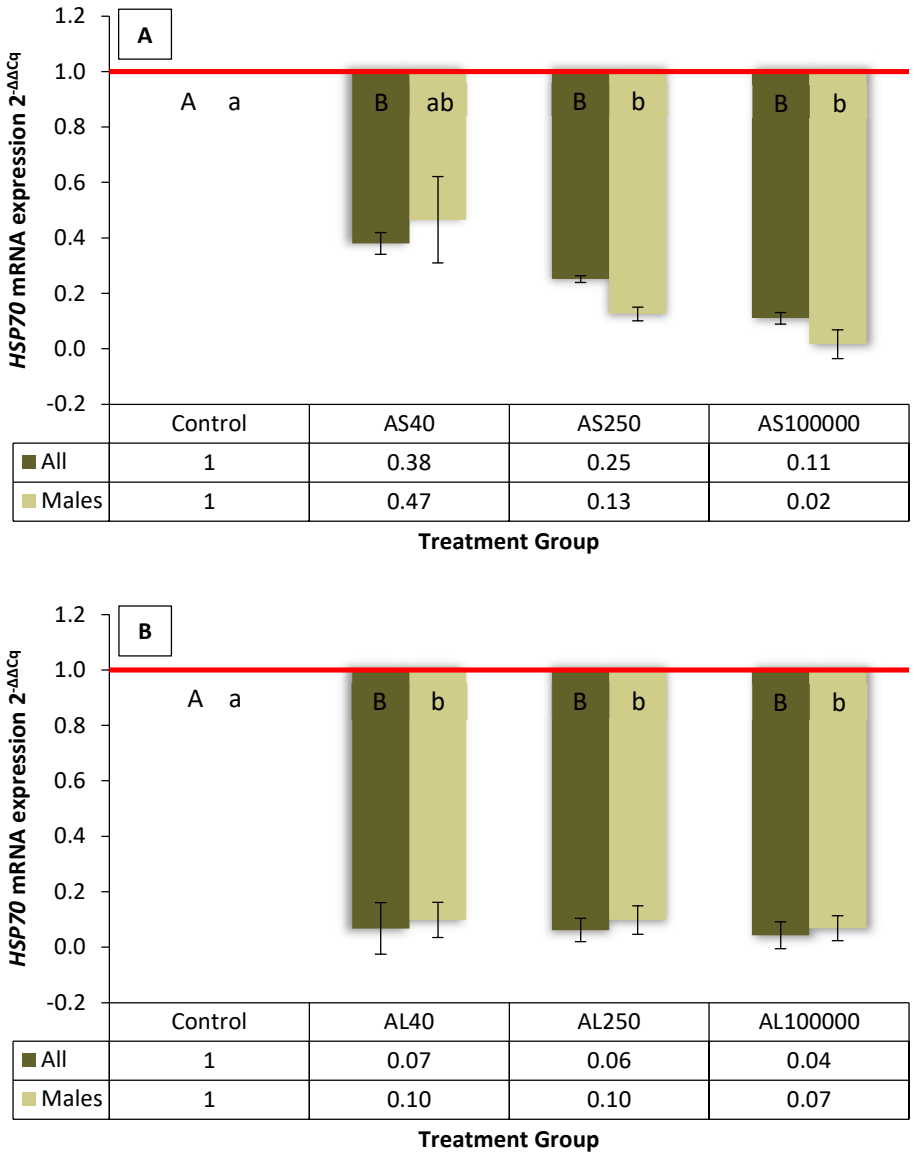


Figure 12 Summary of mRNA expression of *HSP70* in fold changes compared to control group in mussel gonads (n = 10). Data from all individuals and male mussels only. Treatments are denoted by alphanumeric codes: AS, groups exposed to short term exposure (A); AL, groups exposed to long term exposure (B); each followed with nominal concentration of acetaminophen in ng/L. Different letters represent statistically significant differences between groups, with capitals used for all individuals and lower case letters for males only. Bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

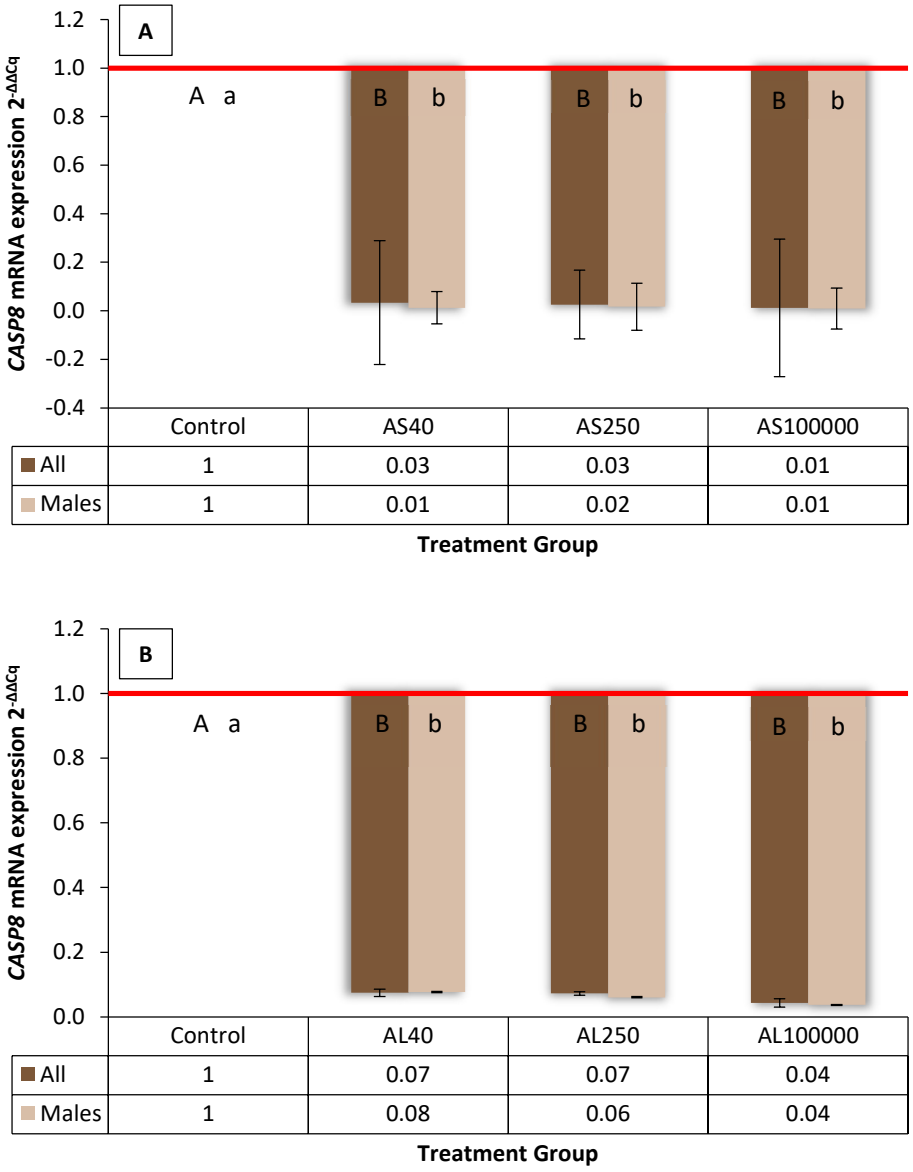


Figure 13 Summary of mRNA expression of *CASP8* in fold changes compared to control group in mussel gonads (n = 10). Data from all individuals and male mussels only. Treatments are denoted by alphanumeric codes: AS, groups exposed to short term exposure (A); AL, groups exposed to long term exposure (B); each followed with nominal concentration of acetaminophen in ng/L. Different letters represent statistically significant differences between groups, with capitals used for all individuals and lower case letters for males only. Bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

A concentration-related trend is also shown in the mRNA expression of *BCL2* after short exposure to acetaminophen (Fig. 14). The expression of *BCL2* in all exposed mussels is downregulated, in a direct relationship with the concentration of acetaminophen. The variation related to concentration of acetaminophen is more evident in the male mussels only, ranging from 1.5 to 33 fold changes compared to unexposed mussels, while all individuals data show 2 – 12.5 fold changes. However, after long exposure, no concentration related trend is observed in the exposed groups, with the *BCL2* mRNA expression downregulated in the range of 11 – 33 fold. The expression shows less variation between exposed groups, and there is very little difference between the data from mixed mussels and from male mussels only. Although it is very minor and not apparent, male mussels display slightly anomalous data whereby the lowest concentration 40 ng/L shows the most downregulation (25 fold changes), and higher *BCL2* mRNA expression or less suppression was observed in the group 250 ng/L (11 fold changes) compared to the control group.

After short exposure to acetaminophen (Fig. 15), *FAS* mRNA shows a lower expression in all exposed groups, and all data sets, with male mussels in the 100 µg/L group presenting a 100 fold downregulated expression. The downregulation shows a concentration-related trend both in all mussels and in male mussels only. The opposite trend is observed after long exposure of acetaminophen. Although only very little variation is apparent between exposed groups, the lowest concentration group 40 ng/L shows the most downregulation with 100 fold change relative to the control group, whilst in the higher concentration groups the mRNA expression of *FAS* is higher, or in other words, is less suppressed. There is almost no variation between the data from all mussels and male mussels only.

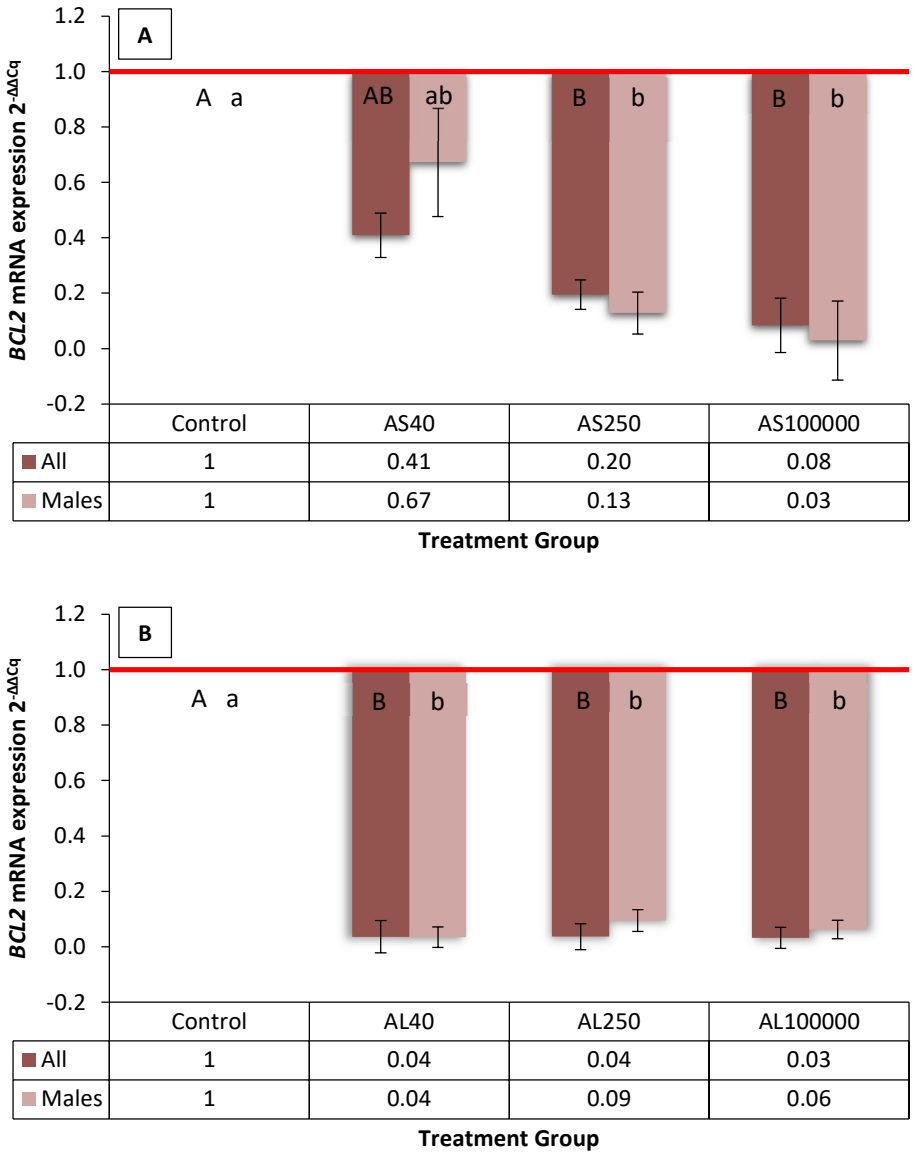


Figure 14 Summary of mRNA expression of *BCL2* in fold changes compared to control group in mussel gonads (n = 10). Data from all individuals and male mussels only. Treatments are denoted by alphanumeric codes: AS, groups exposed to short term exposure (A); AL, groups exposed to long term exposure (B); each followed with nominal concentration of acetaminophen in ng/L. Different letters represent statistically significant differences between groups, with capitals used for all individuals and lower case letters for males only. Bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

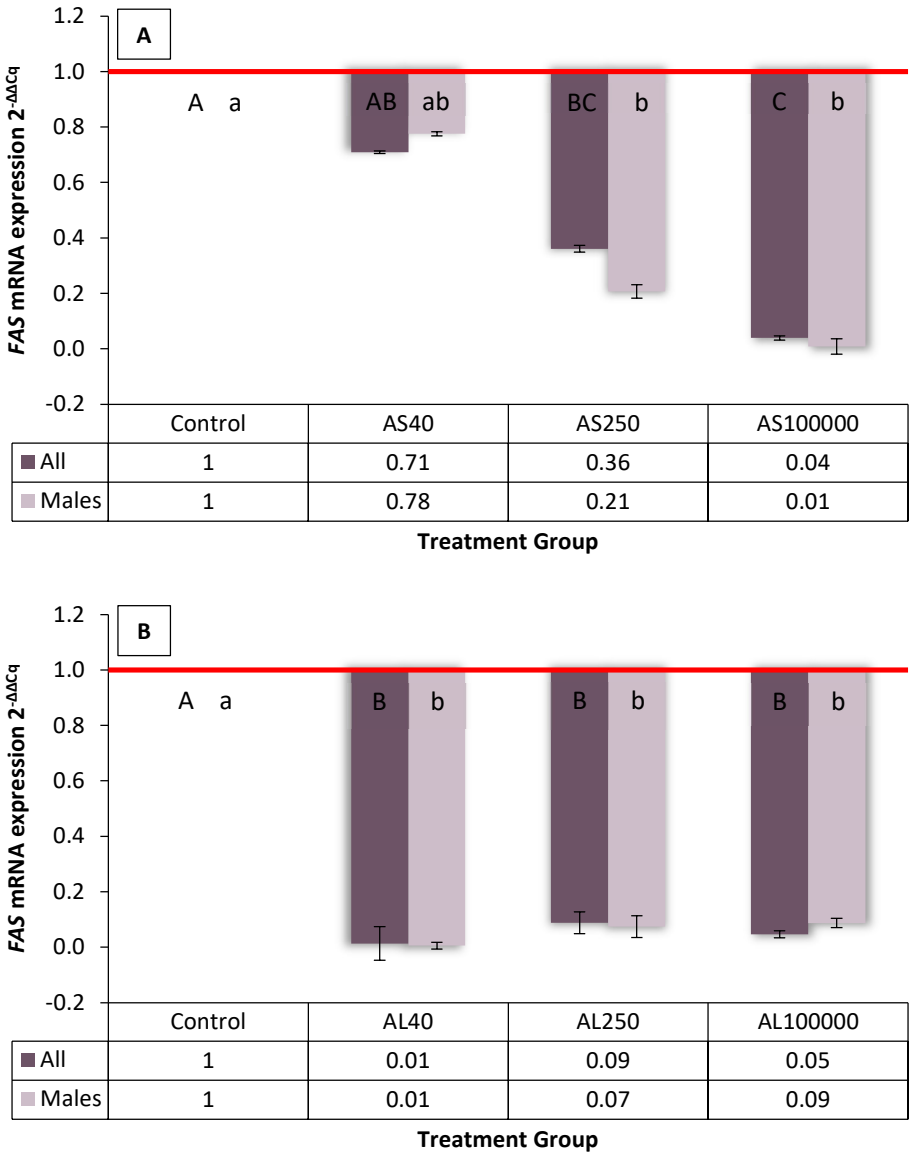


Figure 15 Summary of mRNA expression of *FAS* in fold changes compared to control group in mussel gonads (n = 10). Data from all individuals and male mussels only. Treatments are denoted by alphanumeric codes: AS, groups exposed to short term exposure (A); AL, groups exposed to long term exposure (B); each followed with nominal concentration of acetaminophen in ng/L. Different letters represent statistically significant differences between groups, with capitals used for all individuals and lower case letters for males only. Bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

3.4 Discussion

3.4.1 Water chemistry

The analysis of artificial seawater from each treatment group (Fig. 3) revealed a decrease in the level of acetaminophen at t_1 and t_2 of approximately 60 to 70 %. After 72 hours (t_1), a concentration-related trend was observed, in which higher concentration groups show higher reduction rates. However, at 96 hours after changing water (t_2), each exposed group showed a different pattern of reduction with the likelihood that the reduction is close to an equilibrium, where absorption occurs at a more constant rate at this point in time. This variation might be related either to different pharmacokinetics of each concentration in mussels that may lead to different absorption rates or simply to the sensitivity of analyses, or a combination of both factors. Another possible explanation for the more constant rate of absorption after 96 hours is the absorption capacity of mussels might reach the maximum at this point of time, and combine with the excretion might create a balance in the solution, hence more close to an equilibrium. Nevertheless, the kinetics of this substance in the solution remain to be elucidated.

The reduction rate was calculated at 18 – 20 % per 24 hours, which may correlate with the level of acetaminophen that can be taken up by mussels per day. Active uptake of acetaminophen in bivalves has been recently confirmed by Burket et al. (2019), who also demonstrated accumulation of pharmaceuticals and the trend towards a more constant concentration within 7 days, although the kinetics is still unclear. However, the data recorded here show the presence and the reduction pattern only and further analyses are required to validate the quantification of the contaminant in mussel flesh, to confirm the uptake of acetaminophen.

3.4.2 Neutral red retention time (NRRT) assay

Lysosomal membrane stability is a very sensitive indicator of cellular damage as lysosomes constitute the main cellular sites for sequestration and detoxification of metal and organic contaminants (Dailianis et al., 2003). The principle of NRRT assay is as follows: neutral red dye enters the cell and is taken up and retained by lysosomes

in healthy cells. The response to the uptake of any contaminant may lead to lysosomal membrane damage, and result in leakage of lysosomal contents into the cytosol. In stressed mussels, this process is measured using NRRT, and depicts a physiological process following membrane breakage. The assay measures the ability of cytological processes to adjust to stress conditions (Lowe and Pipe, 1994). In other words, mussels that have low immunity will respond by showing a lower retention time of the toxicant, in this case neutral red, as a result of the alteration to lysosomal membrane integrity of the organism.

For acetaminophen exposure experiments, exposed groups show less retention time as compared to non-exposed groups, but there is no significant difference between groups exposed to various concentrations of pharmaceutical acetaminophen (Fig. 4). This suggests that variations in the concentration of acetaminophen did not confer a substantial difference in its toxicity level in mussels. A low concentration of acetaminophen (as low as 40 ng/L in this study) had a similar effect on lysosomal membrane integrity of mussels' haemocytes when compared with higher concentrations. A slight difference is only seen between the haemocytes of non-exposed and exposed mussels, as non-exposed mussels show healthier haemocytes based on their longer retention time.

Moore et al. (2006) state the threshold values of NRRT assay in marine bivalves assumed as healthy at or more than 120 minutes; classified as moderately stressed or in a low to mild level of stress if NRRT is less than 120 minutes but equal to or more than 50 minutes; and considered severely stressed and tending towards pathology if the NRRT value is lower than 50 minutes. According to these thresholds, all mussels in the acetaminophen exposure groups can be assumed to be healthy.

3.4.3 Histopathology

Some pathological conditions were observed in the control group after short-term exposure to acetaminophen (Fig. 5), which might be due to the presence of certain stressors in the sampling area at the time of collection. However, in comparison, other

groups show higher incidences of histological damage, indicating a correlation between higher concentrations of contaminant as a stressor and the higher prevalence of pathologies in gonad tissue. In the group exposed to 40 ng/L acetaminophen for a short time, up to 30 % of mussels displayed haemocytic infiltration; a similar percentage of atresia was recorded in female gonads from the same group (Fig. 5). The data suggest that even the lowest concentration of acetaminophen has the potential to induce atresia in female mussels, alongside inflammatory reactions. The most frequently occurring pathology in the group exposed to 250 ng/L was follicle dilatation, followed by haemocytic infiltration; the results therefore suggest that acetaminophen may have caused degeneration in gonad follicles in 50 % of the samples. Haemocytic infiltration is notably the highest pathology in the group with 100 µg/L acetaminophen, where up to 70 % of the sample group were affected (Fig. 5).

Haemocytic infiltration is a histopathological condition frequently described in animals following stress-inducing exposure, such as in eastern oysters *Crassostrea virginica* exposed to graphene oxide (Khan et al., 2019), in the apple snail *Pomacea canaliculata* exposed to the insecticide cypermethrin (Arrighetti et al., 2018), and on early life stages of marbled crayfish *Procambarus virginalis* after exposure to metolachlor oxanilic acid (Velisek et al., 2018). Haemocytic infiltration is also recorded in brown mussel *Perna perna* samples from a natural location as a result of toxins produced by marine benthic dinoflagellate *Prorocentrum lima* in the same area (Neves et al., 2019). The result here indicates that short-term exposure to acetaminophen can induce inflammation as a response to stress-related events and adversely affect mussels' health. It also suggests that acetaminophen presents a comparable or even higher risk than the other substances mentioned above, as it contributes to a high prevalence of inflammatory reaction in gonads of up to 70 % of the samples.

Haemocytic infiltration shows a very interesting pattern after long-term exposure (Fig. 8), whereby the occurrence displays the opposite trend to the concentration. While the 7-day exposure to acetaminophen resulted in a dose-related trend, with a higher incidence of haemocytic infiltration in higher concentration groups, the longer exposure results suggest a change in the prevalence of this condition. While the

adaptability of mussels to tolerate the contaminant might be a factor, this result is more likely linked to the fact that mussels from the highest concentration of acetaminophen are more susceptible to suffer haemocytic aggregate condition (30 %). This implies that the inflammatory response to a longer contact to acetaminophen might simply have shifted to the more severe condition.

In mussels that were exposed for a greater length of time to acetaminophen (24 days), follicle dilatation was observed as the most commonly occurring pathological condition in their gonads (Fig. 8). This condition is recorded in all exposed groups at a prevalence of 70 – 80 %, which raises concerns regarding mussels' reproductive health. Moreover, even the lowest concentration of acetaminophen investigated here (40 ng/L) can induce this most observed pathology with similar rates of occurrence to the highest concentration.

More than 90 % of follicles in mammalian ovaries undergo a degenerative process as part of their developmental cycle (Kerr et al., 2013), with some indications that this degenerative stage is induced by apoptosis of granulosa cells, which are influenced by a precarious balance of pro-survival factor withdrawal and pro-apoptotic factors (Manabe et al., 2004; Hatzirodos et al., 2014; Zhang et al., 2018). A study by García-Gasca et al. (2010) has also suggested that this condition might be a useful predictor of environmental stress for coastal ecosystems, as it is directly related to the reproductive system and the success of reproduction. The high prevalence of follicle dilatation in the results presented here imply considerable potential for acetaminophen to cause disturbance in the reproductive systems of mussels and possibly to interfere with population sustainability.

An interesting result worth noting here is that all the pathological conditions observed in the highest concentration group (100 µg/L) are also recorded in the lowest concentration group, only displaying different levels of prevalence (Fig. 8). Overall, the results here present a concerning picture, suggesting acetaminophen concentrations as low as 40 ng/L can induce almost the same adverse effects caused by a concentration 2,500 times higher, given only longer exposure.

The overall results also highlighted the role of the duration of acetaminophen exposure. Shorter exposure time to acetaminophen is likely to alter the immune state of mussels and thus increase the incidence of inflammatory responses; whilst after a longer contact to acetaminophen, mussels are more susceptible to suffer pathological conditions related to the apoptosis and cell death mechanisms. Further investigations are encouraged to confirm this hypothesis.

3.4.4 mRNA expression of selected transcripts

The mRNA expression of *VTG* after short exposure to acetaminophen is significantly downregulated both in data from all individuals and from male mussels only (Fig. 9). It is worth noting that this downregulation of expression occurred in all treatment groups without any difference regarding the concentration, showing that even the lowest acetaminophen concentration (40 ng/L) can have an effect on *VTG* expression. This gene is also expressed in a similar pattern in mussels' gonads after long-term exposure to acetaminophen. The expression is equally downregulated in all exposed groups without following a monotonic trend, again emphasising that the lowest concentration of acetaminophen is potentially as potent as a concentration 2,500 times higher (100 µg/L).

According to Gagné and Blaise (2005), vitellogenin is the egg yolk precursor and is regulated by the oestrogen receptor. Normally, oocytes produce estradiol-17β to initiate vitellogenesis, inducing the production of vitellogenin for developing embryos (Gagné and Blaise, 2005). Thus, vitellogenin expression in male vertebrates is used as a biomarker for early detection of the biological effects of oestrogens. Vitellogenin (*VTG*) mRNA expression is a sensitive indicator for early detection of exposure to endocrine disrupting chemicals (EDCs) in vertebrates, both in laboratory and in wild populations (Ankley et al., 2001; Lattier et al., 2001; Muncke and Eggen, 2006). Another more recent study reported the induction of *VTG* mRNA transcription in adult male fathead minnows *Pimephales promelas* following exposure to metformin (Niemuth et al., 2014). In invertebrates, *VTG* mRNA expression in the gonads of *Mytilus edulis* shows upregulation after exposure to EDCs during the early stages of gonad

development (Ciocan et al., 2010), although its mechanism of action, synthesis and role are still unclear and require further investigation (Porte et al., 2006; Matozzo et al., 2008).

In vertebrates, the expression of vitelline envelope zona pellucida domain has been suggested as a biomarker for environmental oestrogens (Arukwe et al., 1997; Ciocan et al., 2011). Arukwe et al. (1997) also indicate that changes in the vitelline envelope induced by xenoestrogen would likely have further detrimental effects at higher levels, because of the direct involvement in the protection of eggshell and fertilization. Thomas-Jones et al. (2003) highlighted the high sensitivity of vitelline envelope genes to oestrogen in rainbow trout exposed to 17β-estradiol (E2) and 17α-ethinylestradiol (EE2).

In this study, the result of *V9* mRNA expression (Fig. 10) shows downregulation in all treatment groups compared to the control group, both in datasets of mixed sex and of male mussels only after short-term exposure to acetaminophen. The downregulation of this gene shows a monotonic response, with higher concentrations of contaminant resulting in more marked suppression of *V9* mRNA expression. A different pattern of downregulation is shown in the mussels exposed to acetaminophen for a longer period. The data display similar levels of downregulation in all exposed groups and do not follow a concentration-related trend, both from all individuals and male mussels only. This emphasises the consequences of longer exposure which can magnify the effects of this pharmaceutical at a very low concentration to show similar effects to that of the highest concentration. This also indicates that a longer exposure of acetaminophen may induce a notably much lower mRNA expression of *V9* and subsequently to the changes of vitelline envelope potentially, which might cause further adverse effects due to direct interference in reproduction.

Another pharmaceutical, metformin, was shown by Koagouw and Ciocan (2018) to have the potential to alter the expression of *V9* in gonads of *Mytilus edulis*, raising concerns regarding their impact on the reproductive success of the organisms. The important role of vitelline envelope or zona pellucida in fertilisation, such as facilitating

sperm binding and protection against polyspermy (Tian et al., 1997), supports these concerns, as the alteration of *V9* in this study might lead to reproductive impairment in mussels.

The data on oestrogen receptor variation of expression suggests potential further impacts that may occur on gametogenesis and reproduction processes in bivalves (Ciocan et al., 2010). Several studies have investigated expression of *ER2* in various invertebrates (Thornton et al., 2003; Kajiwara et al., 2006; Bannister et al., 2007; Matsumoto et al., 2007), including mussels (Puinean et al., 2006; Ciocan et al., 2011).

Similarly to *VTG* and *V9*, the expression of *ER2* (Fig. 11) in the short-term exposure experiment also shows downregulation in all treatment groups compared to unexposed mussels. Interestingly, a monotonic response is present only in male mussels. Meanwhile, after a longer exposure to acetaminophen, the *ER2* mRNA expression is downregulated in all treatment groups, and no difference in trend is observed between the data from all individuals and from male mussels only. This suggests that longer exposure to acetaminophen completely alters the trend of downregulation of this gene. Blalock et al. (2018) highlighted two pathways involved in the mechanism of endocrine disruption in bivalves: the steroidogenesis pathway and the non-genomic oestrogen signalling pathway activated by oestrogen binding to ERs, in the cytosol and plasma. Moreover, the report also indicates that this interaction of EDCs and ER in the plasma cause subsequent molecular events which have a greater effect on male mussels, resulting in feminisation. The mRNA expression of *ER2* in this study after short-term acetaminophen exposure is in agreement with Blalock et al. (2018), in that a stronger response was identified in male samples. This suggests that *ER2* expression in mussel males is more responsive to acetaminophen than in females, and therefore should be further investigated as a potential biomarker candidate.

In the short exposure groups, the mRNA expression of *HSP70* was downregulated in all treatment groups following a concentration-dependent trend both in data from all individuals and male mussels only (Fig. 12). After long-term exposure however, the expression is downregulated similarly in all exposed groups without any concentration-

related trend, and significantly different compared to the unexposed group, both in mixed sex and male only datasets. *HSP70* is involved in response to physicochemical sublethal stressors and acts to protect cells from thermal or oxidative stress (Beere et al., 2000). Pirrone et al. (2018) reported that *HSP70* is overexpressed in digestive glands and gills of *Mytilus galloprovincialis* exposed to oily wastewater discharge, providing evidence of their protection response to this stressor. Thermal and oxidative stress usually act by damaging protein structure, whereas *HSP70* maintains protein integrity and directly inhibits apoptosis. This suggests that the suppression of *HSP70* in mussels exposed to acetaminophen can induce a higher risk of apoptosis and cell death. This gene was expressed equally regardless of sex and showed a concentration-based trend in the short exposure, which suggests that *HSP70* might also be useful as a marker in a dose-dependent experiment. Animals express *HSP*s as a response to stress stimulation (Eissa et al., 2017). High mRNA expression of *HSP70* was recorded in fish as a response to heat stress (Bertotto et al., 2011), overcrowding (Gornati et al., 2005) and transport stress (Poltronieri et al., 2007). *HSP*s act as molecular chaperones to regulate protein homeostasis, to prevent aggregation, and to assist in the repair of malformed proteins in a stressful event (Ranford et al., 2000). These results, therefore, support Rungrassamee et al. (2010), Zhou et al. (2010), Sinha et al. (2012) and Eissa et al. (2017) in suggesting that since the gene expression of stress proteins is regulated in response to stress, then transcriptional expressions of these genes can be harnessed as sensitive biomarkers in biomonitoring in aquatic environments.

Unlike *HSP70*, the mRNA expression of *CASP8* (Fig. 13) was significantly downregulated in all groups exposed to acetaminophen, in both sexes, and in both short- and long-term exposures. The downregulation of *CASP8* can result in changes in the regulation of degeneration of cellular components, usually associated with the inhibition of apoptosis (Salvesen and Walsh, 2014; Allavena et al., 2018). Alteration of *CASP8* mRNA expression was also observed in mussels *Mytilus galloprovincialis* from a coastal area of Turkey with heavy metal pollution, where this transcript is highly expressed in mussels' hepatopancreas, suggesting a metal-induced apoptosis process (Yavaşoğlu et al., 2016).

The downregulation of *CASP8* can also be linked with the alteration of programmed cell death, leading to cancer-related pathologies in tissues (Shi, 2002; Hassan et al., 2014). The mRNA expression of *CASP8* is apparently sensitive enough to be downregulated by very low concentrations of acetaminophen present in seawater, from either short- or long-term exposure. This provides an indication that acetaminophen could potentially be implicated in carcinogenic mechanisms, or any pathological condition initiated by a downregulation of *CASP8* in blue mussels.

BCL2 and *FAS* play an important role in apoptosis through the modulation of their expression (Kawahara et al., 1998). The modulation of the transcripts of apoptosis regulatory genes maintains the healthy death balance of cells either by induction or inhibition of apoptosis (Hassan et al., 2014). Alterations to this mechanism can lead either to a defective cell's survival or contribute to carcinogenesis. The variation of expression of those transcripts has been reported by Yang et al. (2002) following an exposure to cyclosporine in mice. The authors suggested that changes in the cell death mechanisms as a result of *BCL2* and *FAS* modulation by various stressors can lead to subsequent cell death in renal tubular cells in the model animal. In invertebrates, Estévez-Calvar et al. (2013) reported a significant increase of *BCL2* in *Mytilus galloprovincialis* after exposure to pifithrin-α hydrobromide (PFT-α) and UV irradiation. The authors suggest a connection between this result and reduced apoptotic levels, although they also highlight that the increase of *BCL2* expression in mussel haemocytes might be a response to the occurrence of pro-apoptotic Bax.

In this study, the expression and the modulation of *BCL2* (Fig. 14) and *FAS* (Fig. 15) were driven by the concentration of acetaminophen when mussels were exposed over a short period of 7 days. The concentration dependent results of the mRNA expression of *BCL2* and *FAS* are very interesting, considering that they have antagonistic (with *BCL2* anti-apoptotic and *FAS* pro-apoptotic) actions (Aref et al., 2004). The expression of these two genes, however, are downregulated in a similar manner in all treatment groups after a longer period of exposure. These results are in agreement with previous research, suggesting that both *BCL2* and *FAS* are equally impacted by stressors as part of their interaction, and that their modulation can induce either apoptosis or cancer-

related conditions in metazoan cells. The identification of the expression of these apoptotic regulatory genes is important to understand their interactions in programmed cell death mechanisms in less documented species, such as mussels.

Furthermore, the variation of mRNA expression presented here, overall, provides substantial information regarding the vital role of exposure duration on the effects of acetaminophen in blue mussels. The expression of all transcripts signifies that longer exposure periods, when compared to shorter exposure, may result in much greater effects that could be equivalent to the effects of much higher concentrations.

The results of this study show that male mussels display a more evident response to acetaminophen exposure than females, especially following a short-term period of exposure. This inference is also supported by several other studies in bivalves (Matozzo and Marin, 2005; Ortiz-Zarragoitia and Cajaraville, 2006; Liu et al., 2017), thus supporting the recommendation of male mussels as organisms of choice in biomonitoring programs (Blalock et al., 2018). Therefore, this study strongly suggests that future research on biomarkers in mussels should avoid interpretation of the results without considering the sex of animals.

3.5 Summary

This acetaminophen exposure study in adult mussels has indicated the potential of acetaminophen to induce adverse effects in blue mussel gonads, with haemocytic infiltration and follicle dilatation recorded as the most widespread pathologies in short- and long-term exposures, respectively. Overall water analysis has shown that there is a reduction in the level of acetaminophen in treatment groups, and the reduction is consistent over the first 72 hours of the experiment.

The mRNA expression of several transcripts, such as *VTG, V9, ER2, HSP70, CASP8, BCL2*, and *FAS* in mussel gonads showed downregulation after 7 days of exposure to acetaminophen, with several transcripts potentially qualifying as biomarkers of acetaminophen exposure (*V9*, *HSP70*, *BCL2* and *FAS*). In contrast, *ER2* mRNA variation

in expression showed a marked difference between sexes, suggesting that acetaminophen has a dose related impact on males rather than females.

The patterns observed in mRNA expression after long exposure to acetaminophen demonstrate the importance of exposure period on the effects of this contaminant in adult blue mussels. The expression of all transcripts revealed that longer exposure periods may result in much greater effects, sometimes at levels similar to the effects of much higher concentrations.

CHAPTER 4

The effects of short- and long-term exposure to metformin on adult marine mussels

Abstract

The capability of selected pharmaceuticals to act as non-traditional endocrine disrupting chemicals in the environment has drawn growing concerns. The widely prescribed antidiabetic drug metformin has the potential to accumulate and persist in the water bodies, with little-known consequences for aquatic biota. This chapter reports the biological responses of a sentinel organism, the blue mussel *Mytilus edulis*, exposed to a range of concentrations of metformin commonly found in the aquatic environment, and also exceeding those levels, representing a worst-case scenario. In addition to this, data on exposure of mussels to a single concentration of metformin coupled with heat stress is also presented. The results indicate that metformin can alter the immune response in blue mussels, initiate pathological conditions in gonads, including apoptosis-related and inflammatory responses, and induce variation of mRNA expression of *VTG*, *V9*, *ER2*, *HSP70*, *CASP8*, *BCL2* and *FAS*. These alterations can be linked with the level of metformin in the environment; thus these findings suggest the potential of metformin to be considered an emerging contaminant of concern, and further investigations are required to clarify the mechanism of action.

Keywords: metformin, mRNA expression, blue mussel, histopathology, non-traditional EDCs

4.1 Introduction

4.1.1 Metformin - the use, mode of action, and effects

Metformin is originally a natural remedy extracted from the French lilac which appears in herbal manuals dated from the 17th century and was first synthesised in 1922 (Thomas and Gregg, 2017). Due to its mild side effect profile, affordability, ease of administration and positive effects on body weight, metformin has been widely used whether as monotherapy or in combination with other medications in the treatment of diabetes mellitus (Ferrannini, 2014). It has been used in Canada and Europe for 40-50 years, while in the US market it was not introduced until 1995 (Samson and Garber, 2015). Following the improvement in morbidity and mortality rates in type 2 diabetes demonstrated in the United Kingdom Prospective Diabetes Study (UKPDS), metformin has been recommended as the first line oral treatment for type 2 diabetes by the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) since 2009 (Bailey, 2017). Consequently, it is now the most commonly prescribed anti-diabetes medicine throughout the world (Samson and Garber, 2015; Thomas and Gregg, 2017). Jones et al. (2002) reported that metformin is one of the top three prescribed compounds in the UK and that usage here was more than 100 tons per year in 2000.

Metformin is also used as treatment for polycystic ovary syndrome (PCOS) in human patients, where it works by improving insulin sensitivity and thus decreasing glucose levels, as well as reducing LH-stimulated testosterone (Kurzthaler et al., 2014). In this way, metformin debilitates insulin-induced androgenesis in the ovaries of PCOS patients. Metformin has also been successfully prescribed as a treatment for hyperinsular obesity and weight gain induced by antipsychotic therapy (Thomas and Gregg, 2017).

The mechanisms underlying the effects of metformin are complex, and despite long-term clinical experience and active investigation, the exact mechanisms of action remain unclear and not fully understood. Metformin acts to enhance glucose uptake by promoting catabolism and impacting the cellular energy balance (Rena et al., 2017). In therapeutic doses, metformin appears to decrease blood glucose levels by several

mechanisms. It enhances suppression of gluconeogenesis by insulin and reduces glucagon-stimulated gluconeogenesis (Bailey and Turner, 1996). Additionally, it can positively affect insulin receptor phosphorylation and tyrosine kinase activity. It can also increase translocation of the glucose transporter-1 and -4 isoforms as well as prevent the development of insulin resistance in hepatocytes and adipocytes (Wang and Hoyte, 2018). Metformin can inhibit complex I of the electron transport chain and decrease cellular ATP level, resulting in activation of regulatory AMP kinase (AMPK), thus elevating glucose uptake and breakdown as well as fatty acid oxidation. These routes increase insulin sensitivity, reducing basal hepatic glucose output and fasting glucose concentrations, as well as impacting pathways regulated by insulin signalling, such as steroidogenic pathways (Viollet et al., 2012; Rena et al., 2017). Metformin therapy has been shown to influence the expression and activity of steroidogenic enzymes such as cytochrome p450 (CYP) llA and CYP17 (Viollet et al., 2012).

However, in a therapeutic setting, metformin toxicity has been recorded to potentiate hyperlactatemia, metabolic acidosis, and cellular hypoglycaemia (Wang and Hoyte, 2018). In addition to this, despite many positive effects of metformin, including lessening hyperandrogenism and generating ovulation by acting as an insulin-sensitiser, Dupont and Scaramuzzi (2016) show evidence pointing to a direct effect of metformin on ovarian steroidogenesis.

4.1.2 Metformin in the environment

In the human body, metformin is circulated essentially unbound and is eventually eliminated unchanged (Ferrannini, 2014). Graham et al. (2011) reported that a notable percentage (20 – 30 %) of a metformin dose is detected in the faeces as a consequence of the lack of absorption. These details indicate that this substance likely enters aquatic compartments in high quantities, as reported by Scheurer et al. (2009), Trautwein and Kümmerer (2011), Scheurer et al. (2012) and Trautwein et al. (2014). Indeed, recent studies (Kot-wasik et al., 2016; de Jesus Gaffney et al., 2017; Elizalde-Velázquez and Gómez-Oliván, 2020) showed that concentrations of metformin detected in the environment range from 0.5 ng/L to 325 µg/L. Moreover, Laak et al. (2014) indicated

that metformin and its transformed product guanylurea account for 53 – 80 % of pharmaceuticals identified in the Meuse River on the Belgian-Dutch border, highlighting its significance purely in terms of mass. An investigation by Ghoshdastidar et al. (2014) revealed that metformin was present in the highest concentrations among pharmaceuticals found in municipal wastewater treatment plant effluents across southwest Nova Scotia, Canada.

Metformin has been reported as the most frequently detected compound in surface water and sediment from Lake Michigan by Blair et al. (2013). Recent studies conducted on US streams (Bradley et al., 2017), waste waters in Poland (Kot-wasik et al., 2016), subarctic locations in Faroe Islands, Iceland, and Greenland (Huber et al., 2016) and freshwater environments in China (Asghar et al., 2018) and Vietnam (Chau et al., 2015) showed that metformin is one of the 10 most frequently detected designed bioactive contaminants.

Despite the elevated concentrations of metformin recorded in water bodies, relatively few studies have investigated the ecotoxicological effect of this pharmaceutical on marine organisms. The aim of this chapter is to evaluate the biological responses induced by metformin in the blue mussels *Mytilus edulis* at a range of concentrations commonly found in the aquatic environment and also, at a higher level, representing a worst-case scenario. Seven potential target genes were selected to examine toxicological effects in mussel gonads, and possible disruptions in the reproductive system following short-term (7 days) and long-term (21 days) laboratory exposures.

The study investigates the variation of several essential genes linked to reproduction: *vitellogenin* (*VTG*), *vitelline envelope zona pellucida domain-9* (*V9*), and *estrogen receptor-2* (*ER2*). This chapter also discusses the variability in mRNA expression of four other apoptosis-involved genes: *heat shock protein-70* (*HSP70*)*, caspase-8* (*CASP8*)*, B-cell lymphoma-2* (*BCL2*) and *Fas cell surface death receptor* (*FAS*). Histopathological changes caused by metformin, along with its potential to act as a non-traditional endocrine disruptor were also investigated. In addition to this, data on exposure of mussels to a single concentration of metformin coupled with heat stress is presented.

4.2 Materials and methods

4.2.1 Collection of mussels and acclimatisation

Mussels were collected in January 2016 and June 2018. Blue mussels *Mytilus edulis* were collected manually at low tide from Hove beach, East Sussex, UK. A map and photograph of the sampling site are presented in Appendix 2. Mussels were taken from one population only, kept on ice after collection, and transferred immediately to the laboratory. Mussels collected in January 2016 were then cleaned by removing the material attached to the shells' surfaces and placed in a container with a cooling system for acclimatisation at 10 ± 1 °C prior to exposures for 6 days. The temperature of acclimatisation (10 ± 1 °C) was equal to the seawater temperature when mussels were collected. Mussels from the collection in June 2018 were directly placed in a container, after cleaning, for acclimatisation at room temperature (23 ± 2 °C) for 6 days. Artificial seawater (Instant Ocean® Sea Salt, USA) was used for this purpose and the solution was prepared as per manufacturer's instructions.

4.2.2 Experimental exposure

Mussels used in exposure experiments were selected on the basis of their size range (30 mm to 50 mm) to ensure that they were at approximately the same developmental stage. For all exposures, artificial seawater (Instant Ocean® Sea Salt, USA) was prepared and mussels were allocated with the volume proportion of approximately 1 litre per mussel per tank. Two exposure experiments were carried out: one in 2016 and another in 2018.

4.2.2.1 Exposure to single concentration of metformin and high temperature

Mussels collected in January 2016 were separated into four treatment groups: control group at 10 °C, metformin at 10 °C, control group at 20 °C, and metformin at 20 °C. The nominal concentration of metformin (European Pharmacopoeia Reference Standard, Sigma-Aldrich) in artificial seawater was set at 40 µg/L, an environmentally relevant concentration as described in previous studies by Niemuth et al. (2014) and Niemuth and Klaper (2015). A cooling system was used to maintain the low temperature groups

at 10 °C, and other groups (20 °C) were placed in water baths at room temperature. Temperature was recorded every day during exposure to ensure that it was maintained as intended. Each exposure group contained at least seventeen mussels to be used in further analyses, with extra individuals as backup maintained with exactly the same exposure treatments. The exposure was conducted over seven days.

4.2.2.2 Short- and long-term exposure to selected levels of metformin

Mussels collected on June 2018 were split into four groups, with ten mussels per group. Short-term exposure was conducted over 7 days, and long-term exposure was ended after 21 days. Metformin exposures were performed as follows: a control group (artificial seawater only, without metformin) and three exposed groups with nominal water concentrations of 100 ng/L, 80 µg/L, and 150 µg/L. Water was changed and re-spiked with metformin (European Pharmacopoeia Reference Standard, Sigma-Aldrich) every 72 and 96 hours. Temperature, salinity and conductivity of the seawater were recorded daily.

4.2.3 Neutral red retention time (NRRT) assay

The assay was employed only on the haemocytes of mussels from the long-term exposure groups. The assay was performed following the methods described in section 3.2.4.

4.2.4 Histopathology

Following the termination of exposures, all samples were dissected for histological and molecular analyses. The procedure for histopathology analysis used to highlight the potential pathological conditions observed in gonad tissue was adapted from Ciocan et al. (2010). About 1 cm cube of each gonad (n = 10 from each treatment group) was kept in fixative neutral buffered formaldehyde (Sigma-Aldrich) in clean tubes. Tissues were then placed into labelled cassettes and processed through a series of dehydration and clearing following wax infiltration using an automated tissue processor (Leica Biosystems, Germany) following the manual provided by Howard et al. (2004). Tissues

were embedded in paraffin and then solidified prior to sectioning using a manual microtome (Thermo Scientific, UK). Each gonad tissue sample was sectioned 7 µm in three different parts from the edge to the centre, to ensure the sections captured not only the edge part of the tissue. About 6 – 10 intact sections were obtained from each part of the tissue, giving a total of 18 – 30 sections of each gonad. Sections were transferred to slides and kept in a 37 ° C oven overnight to ensure that the tissues were affixed to the slides. A series of concentrations of dish washing liquid (Fairy, Procter & Gamble) coupled with hot water were used to remove paraffin from sections, following the procedure described by Buesa and Peshkov (2009). As demonstrated by the authors and by Pandey et al. (2014), this method has proven effective in eliminating the risk posed by the use of the chemicals without compromising the quality of the tissue. After deparaffinization, tissues were stained with haematoxylin (Sigma-Aldrich) and eosin (Sigma-Aldrich) for 5 and 2 minutes respectively. Stained sections were then dehydrated and mounted in DPX (CellPath, UK) prior to histological examination of tissues from all slides using a light microscope (Leitz Wetzlar, Germany) (40x/100x). Histopathological conditions were recorded and micrographs corresponding to each condition were captured using GXCam Hichrome-Lite (GT Vision, UK). The prevalence of each histopathological condition was quantified by counting how many mussels exhibited the particular pathology, expressed as a percentage of all mussels in each group. The result only presented the occurrence, not the severity, of each histopathological condition in each treatment group. In many cases, several pathologies could be present in one individual, as captured by micrographs.

4.2.5 Gene expression analysis

Molecular analysis was employed in order to highlight changes in the expression pattern of selective mussel transcripts *vitellogenin* (*VTG*)*, vitelline envelope zona pellucida domain-9* (*V9*)*, estrogen receptor-2* (*ER2*)*, heat shock protein-70* (*HSP70*)*, caspase-8* (*CASP8*)*, B-cell lymphoma-2* (*BCL2*) and *Fas cell surface death receptor* (*FAS*) in mussel gonads. The procedures are identical to the methods described in section

3.2.6, except for several reagents used in these particular experiments. The species confirmation of *Mytilus edulis* is described in Appendix 3.

Prior to molecular analyses, fragments of mussels' gonads (n = 10 for each group) were dissected and stored in RNA*later*™ (Invitrogen, UK). Total RNA was extracted using High Pure RNA Tissue Kit (Roche, UK), SurePrep™ TrueTotal™ RNA Purification Kit (Fisher BioReagents™, UK), SurePrep™ RNA/Protein Purification Kit (Fisher BioReagents™, UK) and Monarch® Total RNA Miniprep Kit (New England Biolabs, UK) as per manufacturer's instructions. The quantification of extracted RNA concentration was performed with Qubit™ RNA HS Assay Kit (Invitrogen, UK) using Qubit® Fluorometer following the instructions from the manufacturer. For the reverse transcription of extracted total RNA, Transcriptor High Fidelity cDNA Synthesis Kit (Roche, UK) and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, UK) were used following the protocol provided by the manufacturers. The first strand cDNA was quantified using Qubit™ dsDNA HS Assay Kit and Qubit® Fluorometer (Invitrogen™, UK).

The real time qPCR was performed using LightCycler® Nano Real-Time PCR System with FastStart Essential DNA Green Master (Roche, UK) and PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, UK) as described in section 3.2.6.2. The internal control genes were *18S ribosomal RNA* (*18S rRNA*) and *elongation factor-1 alpha* (*EF1*), and sequences of primers of each gene used in the quantitative real-time PCR are shown in Table 5.

4.2.6 Data analysis

For the mRNA expression analysis, the raw data expressed as cycle quantification (Cq) from quantitative real-time qPCR of each target gene was normalised using the average Cq of housekeeping genes *18S rRNA* and *EF1* as a normalisation factor, corresponding to each sample. The relative changes in target gene expression determined from quantitative real time PCR were calculated following the comparative $2^{-\Delta\Delta Ct}$ method expressed as fold changes (Livak and Schmittgen, 2001). Statistical

analysis was conducted using GraphPad Prism 8. Tukey's post-hoc multiple comparison tests were carried out after one-way analyses of variance (ANOVA), to identify the significant difference between the groups with $p < 0.05$, for the NRRT assay and mRNA expression data. For the histopathology data, two-way ANOVA was performed followed by a post hoc Tukey's multiple comparison test with $p < 0.05$.

4.3 Results

Mussels used in this study were confirmed as *Mytilus edulis* (a more detailed description of species confirmation is presented in Appendix 3).

4.3.1 Neutral red retention time (NRRT) assay

Metformin exposure (Fig. 16) shows an evident concentration-dependent trend, with higher concentrations of contaminant drastically affecting the retention time of haemocytes. Metformin shows a high toxicity effect at a concentration of 150 µg/L, as all lysosomal material burst into the cytosol in less than 30 min incubation time.

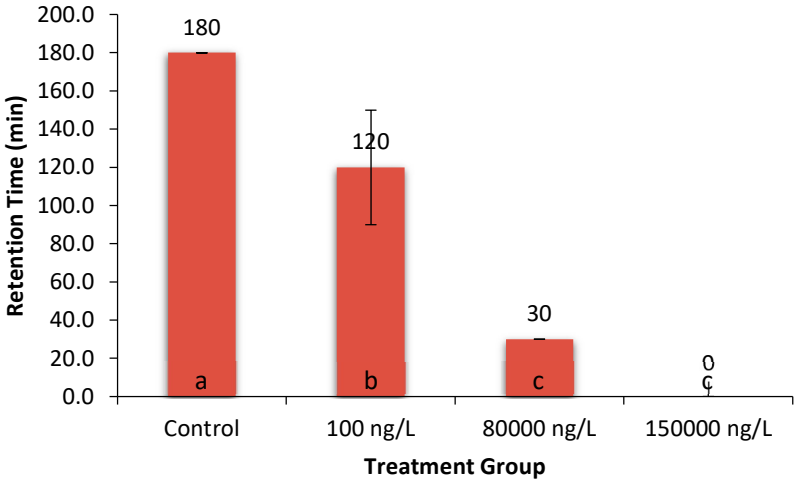
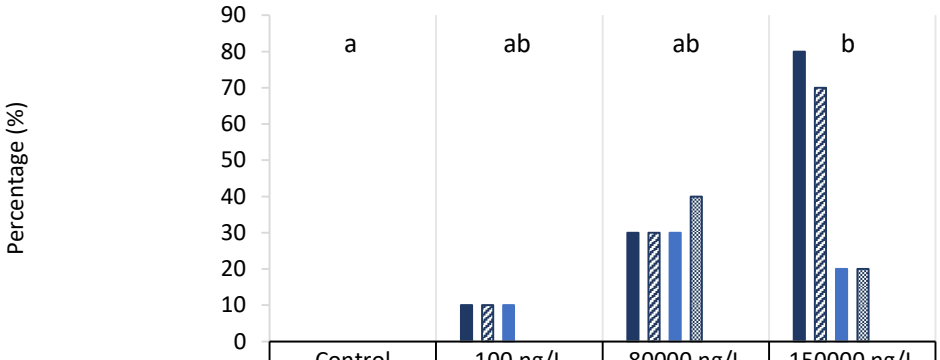


Figure 16 Neutral red retention time of haemocytes from mussels exposed to metformin for 21 days (n = 3). Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, *p < 0.05*).

4.3.2 Histopathology

Short exposure to metformin induced several pathologies in mussels' gonads (Fig. 17). Follicle dilatation was the most observed pathology overall across treatment groups, followed by gamete degeneration. The occurrence of both conditions also showed a trend related to concentration of metformin, where higher concentrations induced higher pathological prevalence. Follicle dilatation was also the condition with the highest incidence, up to 80 % in the group exposed to metformin at 150 µg/L. Mussels in this highest concentration group displayed a very high prevalence of pathologies in gonads, with gamete degeneration also observed in 70 % of mussels. Atresia was observed only in the two highest concentration groups, with 40 % of mussels in the group exposed to 80 µg/L exhibiting this condition. Haemocytic infiltration was the only inflammatory reaction observed here, and was present in all exposed groups, including a prevalence of 30 % in the group 80 µg/L.



	Control	100 ng/L	80000 ng/L	150000 ng/L
Follicle dilatation	0	10	30	80
Gamete degeneration	0	10	30	70
Hemocytic infiltration	0	10	30	20
Atresia	0	0	40	20
Parasites	0	0	0	0
Hemocytic aggregate	0	0	0	0

Figure 17 The occurrence of histopathological conditions observed in the gonad tissue of mussels exposed to metformin for 7 days (n = 10). The treatments were as follows: Control, 100 ng/L, 80 µg/L and 150 µg/L. Different letters represent statistically significant differences between groups (two-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

All histopathological conditions observed here are described briefly in section 3.3.3, and the micrographs corresponding to each condition are shown in Figure 18 and Figure 19.

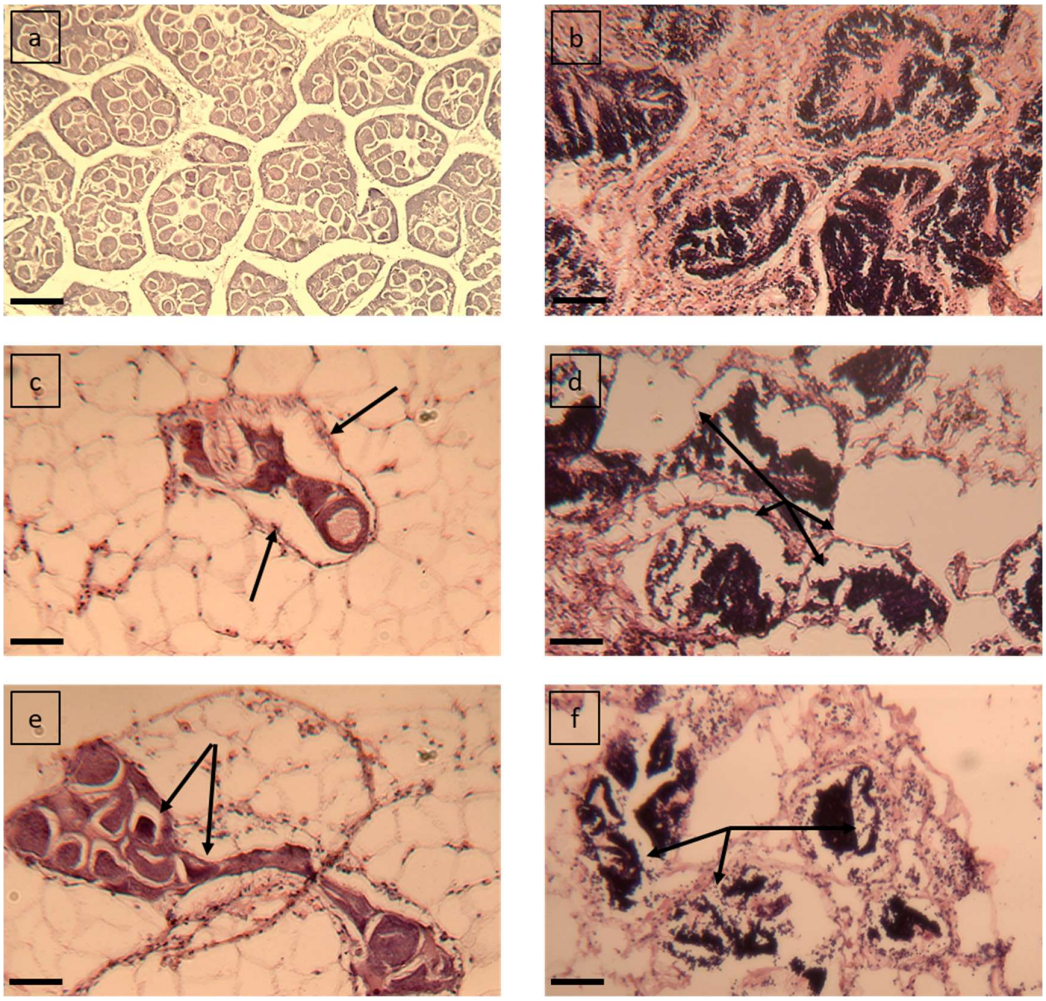


Figure 18 Histopathological conditions in mussel gonads after exposure to metformin. Sections of 7 µm, stained with haematoxylin and eosin. (a) normal histology, female; (b) normal histology, male; (c) follicle dilatation, female; (d) follicle dilatation, male; (e) gamete degeneration, female; (f) gamete degeneration, male. Arrows point to each pathological condition. Scale bar = 100 µm.

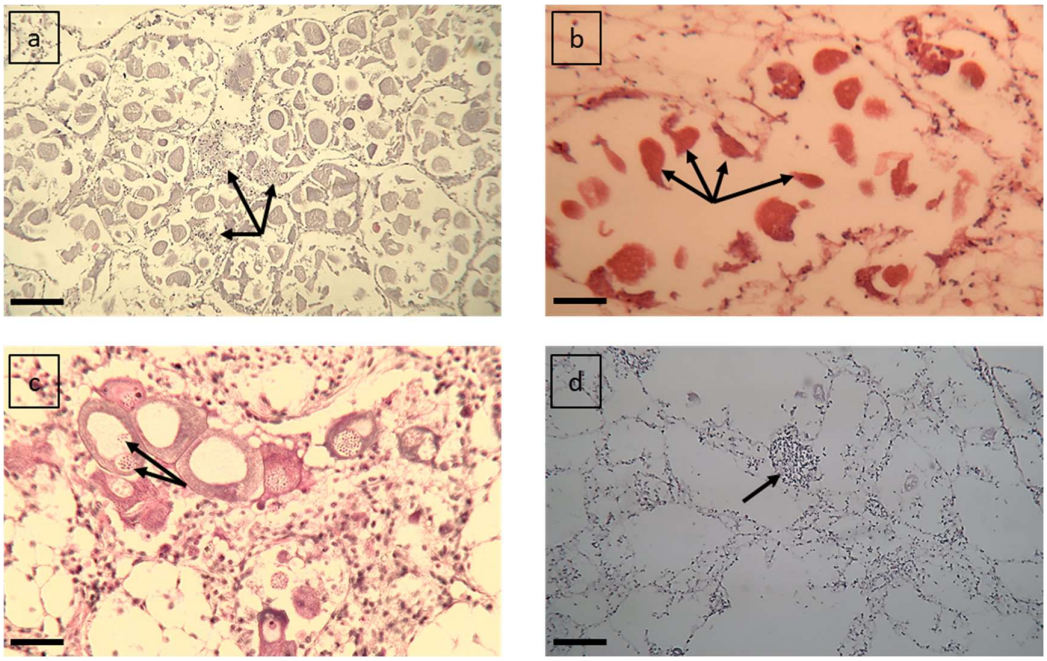
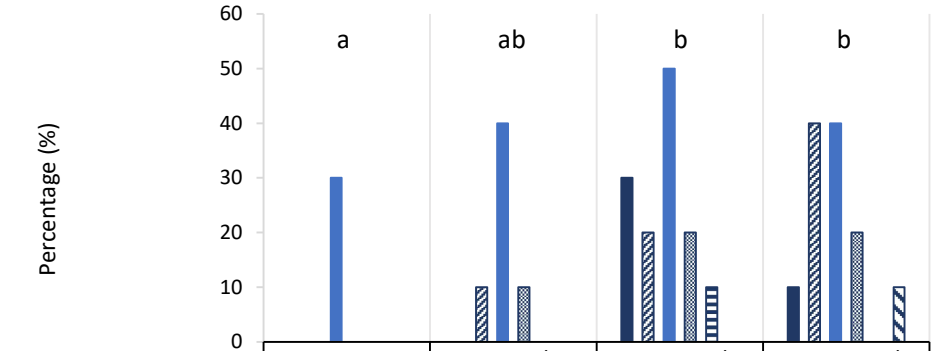


Figure 19 Histopathological conditions in mussel gonads after exposure to metformin (continued). Sections of 7 µm, stained with haematoxylin and eosin. (a) haemocytic infiltration; (b) atresia; (c) parasites; (d) haemocytic aggregate. Arrows point to each pathological condition. Scale bar = 100 µm.

A longer exposure to metformin evoked different prevalences of pathologies in mussels' gonads, as shown in Figure 20. In this case, haemocytic infiltration appeared in all groups and was the pathology with the highest incidence in every group. The occurrence was almost even across groups (30 – 50 %) and the highest incidence was observed in the group 80 µg/L. Gamete degeneration and atresia were present in all exposed groups with incidences up to 40 % and 20 %, respectively. Parasitic infiltration and haemocytic aggregate, both of which were not present in the short exposure, were observed here after 21 days of metformin exposure, although only in 10 % of the samples. The most observed pathology in the short exposure, follicle dilatation, occurred only in the two higher concentration groups here, affecting up to 30 % of mussels in the group 80 µg/L.



	Control	100 ng/L	80000 ng/L	150000 ng/L
Follicle dilatation	0	0	30	10
Gamete degeneration	0	10	20	40
Hemocytic infiltration	30	40	50	40
Atresia	0	10	20	20
Parasites	0	0	10	0
Hemocytic aggregate	0	0	0	10

Figure 20 The occurrence of histopathological conditions observed in the gonad tissue of mussels exposed to metformin for 21 days (n = 10). The treatments were as follows: Control, 100 ng/L, 80 µg/L and 150 µg/L. Different letters represent statistically significant differences between groups (two-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

4.3.3 Gene expression analysis

4.3.3.1 Exposure to single concentration of metformin and high temperature

The results regarding the variation in expression of apoptosis-related genes in mussel gonads are presented for: *HSP70* (Fig. 21), *CASP8* (Fig. 22), *BCL2* (Fig. 23) and *FAS* (Fig. 24). Variations in mRNA expression are calculated as fold changes compared to control group C10 (seawater only at 10 °C).

HSP70 mRNA expression was upregulated in all treatment groups (Fig. 21). Only high temperature exposure groups presented statistically significant ($p < 0.05$) different results from the control group. Figure 22 indicates that whilst *CASP8* mRNA expression is suppressed by exposure to metformin only, high temperature is seen to significantly upregulate the expression in both groups (C20 and M20) with $p < 0.05$.

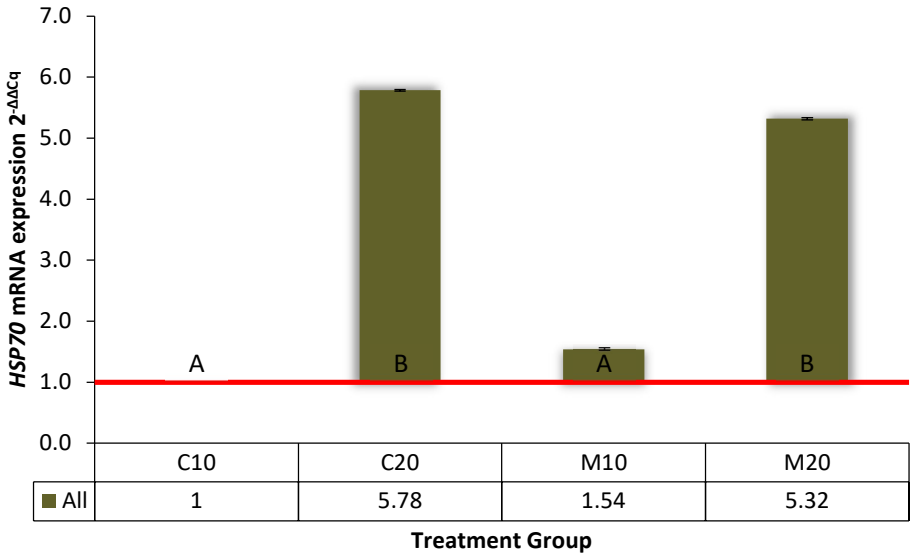


Figure 21 Summary of mRNA expression of *HSP70* in fold changes compared to control group in mussel gonads (n = 12). C10, control group without metformin, seawater at 10 °C; M10, group exposed to metformin at 10 °C; C20, control group without metformin, seawater at 20 °C; M20, group exposed to metformin at 20 °C. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

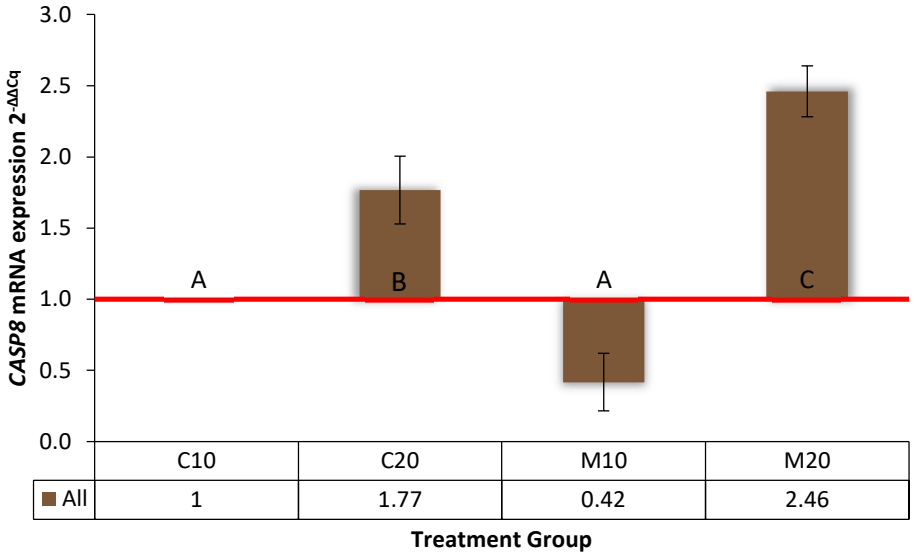


Figure 22 Summary of mRNA expression of *CASP8* in fold changes compared to control group in mussel gonads (n = 12). C10, control group without metformin, seawater at 10 °C; M10, group exposed to metformin at 10 °C; C20, control group without metformin, seawater at 20 °C; M20, group exposed to metformin at 20 °C. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

BCL2 mRNA expression is recorded as being upregulated in high temperature groups (C20 and M20) and slightly suppressed in the group exposed to metformin only (Fig. 23). Each exposed group showed a significant different result to other exposures, while only mussels exposed to metformin and higher temperature (M20) displayed a statistical difference to the control group ($p < 0.05$). Figure 24 highlights the expression of *FAS* mRNA, which is only upregulated in group M20 (metformin at 20 °C) and downregulated in C20 (control group without metformin, seawater at 20 °C) and M10 (metformin at 10 °C). All exposed groups showed significantly different results when compared to the control group with $p < 0.05$.

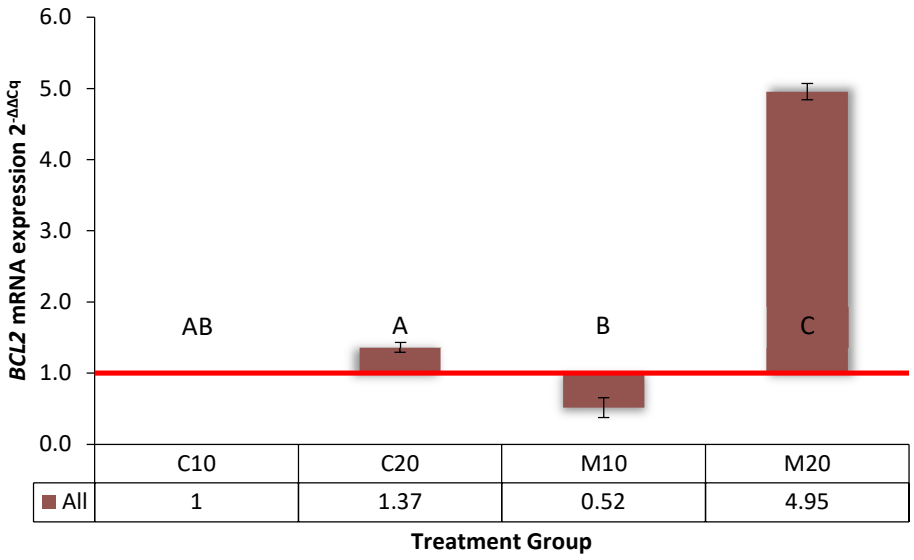


Figure 23 Summary of mRNA expression of *BCL2* in fold changes compared to control group in mussel gonads (n = 12). C10, control group without metformin, seawater at 10 °C; M10, group exposed to metformin at 10 °C; C20, control group without metformin, seawater at 20 °C; M20, group exposed to metformin at 20 °C. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

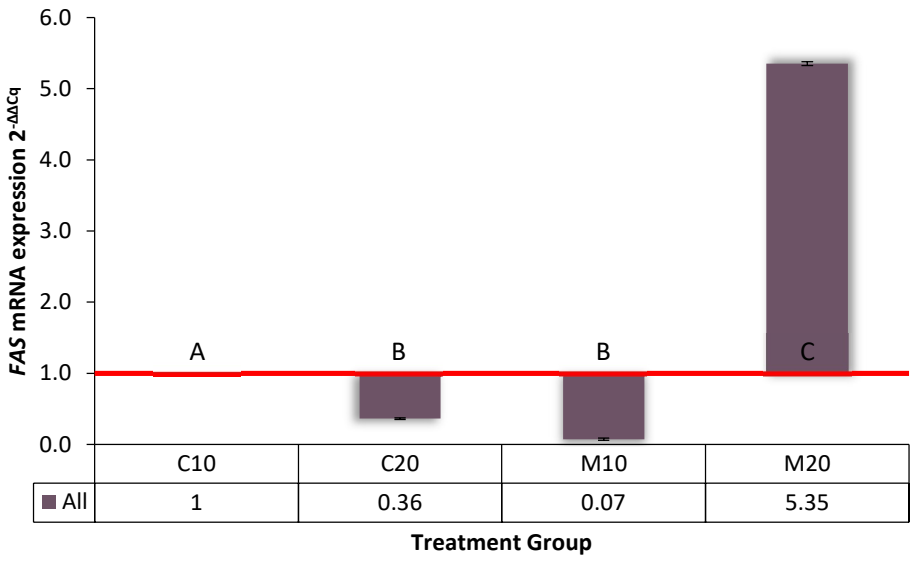


Figure 24 Summary of mRNA expression of *FAS* in fold changes compared to control group in mussel gonads (n = 12). C10, control group without metformin, seawater at 10 °C; M10, group exposed to metformin at 10 °C; C20, control group without metformin, seawater at 20 °C; M20, group exposed to metformin at 20 °C. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

4.3.3.2 Exposure to selected levels of metformin

The mRNA expression of *VTG* showed atypical trends after short- and long-term exposure to metformin (Fig. 25). While short term exposure increased the expression of this gene, a longer exposure to metformin downregulated the expression. In the short-term exposure, more prominent results are shown by male mussels where *VTG* is expressed as 50 – 700 fold changes compared to unexposed mussels. In the long-term exposure, only male mussels in the highest concentration group (150 µg/L) displayed a more marked result than mixed mussels, as the expression was downregulated 3 times compared to the control group. Mixed sex mussels tended to show similar results between exposed groups. However, no significant difference was observed between exposed groups statistically, either in mixed mussels or male mussels only.

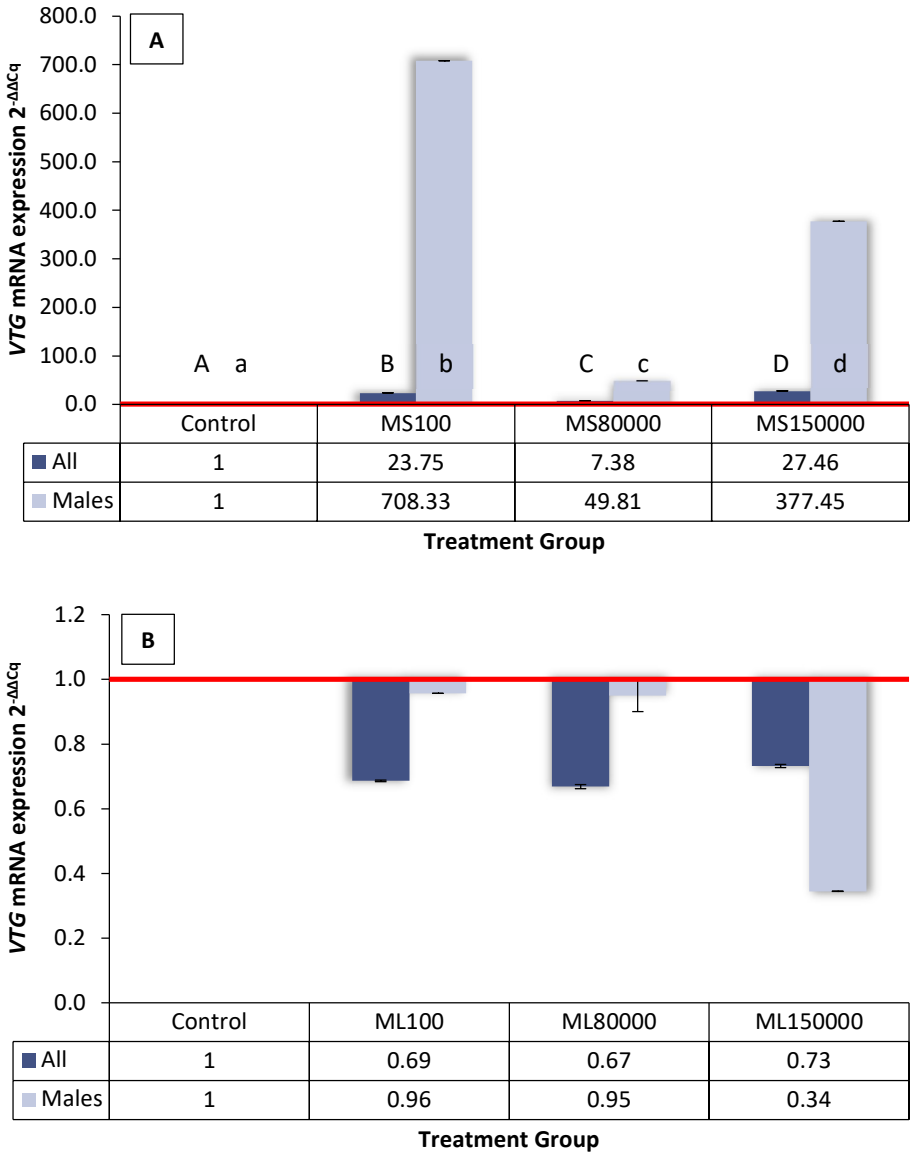


Figure 25 Summary of mRNA expression of *VTG* in fold changes compared to control group in mussel gonads (n = 10). Data from all individuals and male mussels only. Treatments are denoted by alphanumeric codes: MS, groups exposed to short term exposure (A); ML, groups exposed to long term exposure (B); each followed with nominal concentration of metformin in ng/L. Different letters represent statistically significant differences between groups, with capitals used for all individuals and lower case letters for males only. Bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

V9 also exhibited a different pattern of mRNA expression depending on the duration of metformin exposure (Fig. 26). Whilst short exposure downregulated the expression of this gene in all exposed groups, up to 3 fold compared to the control group, the

modulation of *V9* mRNA expression was more varied after long exposure. The data from male mussels in short exposure groups showed a more concentration-related trend, compared to data from all individuals in which no related trend was observed, although the differences here were not statistically significant. More fluctuating data were obtained after a long exposure to metformin, although the variations in each treatment group were not significantly different to the non-treatment group.

The exposure period to metformin appeared to alter the trend of *ER2* mRNA expression (Fig. 27), in which a longer exposure induced more fluctuation in the expression level compared to the short exposure. In the long exposure, only mussels exposed to 80 µg/L of metformin showed statistically different results compared to unexposed mussels, both for mixed sex and male mussels only. All mussels exposed to metformin for 7 days showed downregulated *ER2* mRNA expression, both in the data from all individuals and from male mussels only. Mixed sex mussels displayed a more dose-dependent trend, even though not statistically significant between exposed groups, only 2 – 3 fold downregulated compared to the control in the same dataset.

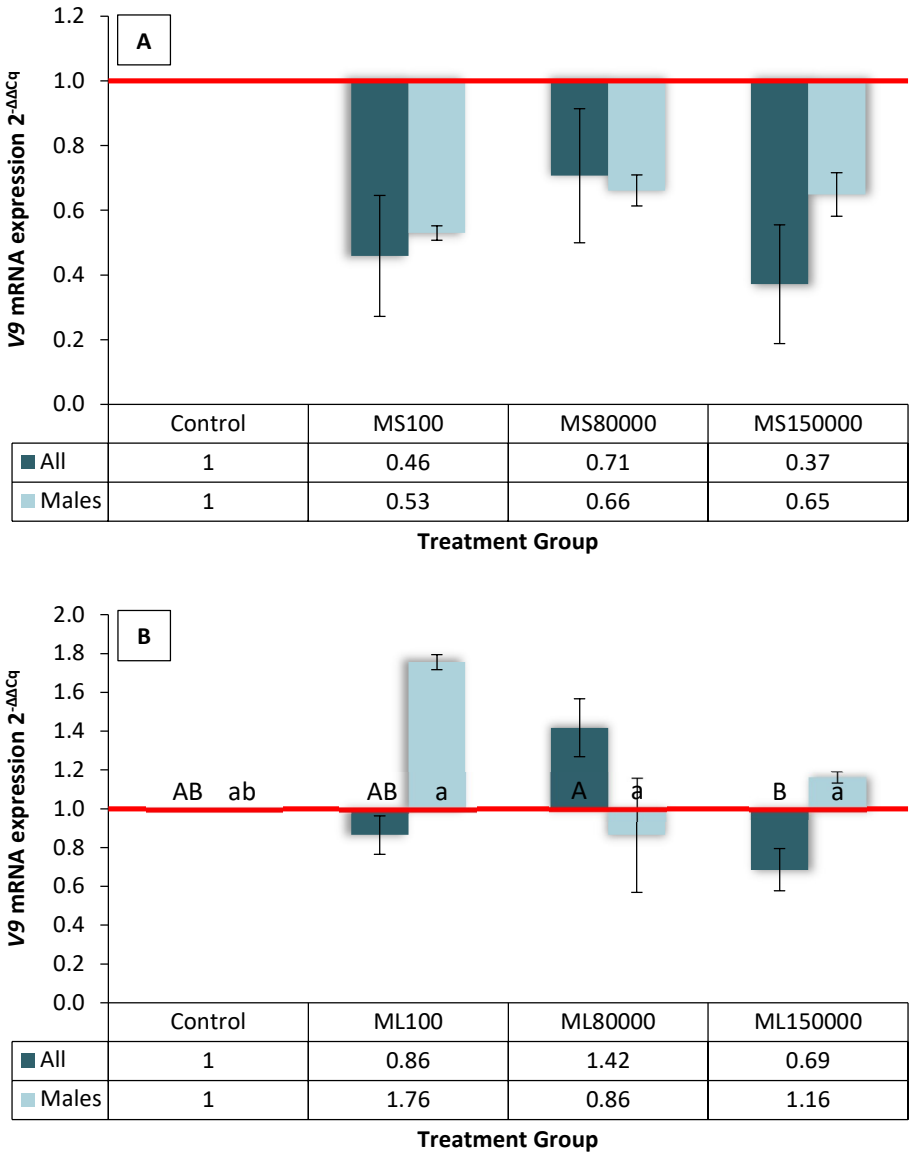


Figure 26 Summary of mRNA expression of *V9* in fold changes compared to control group in mussel gonads (n = 10). Data from all individuals and male mussels only. Treatments are denoted by alphanumeric codes: MS, groups exposed to short term exposure (A); ML, groups exposed to long term exposure (B); each followed with nominal concentration of metformin in ng/L. Different letters represent statistically significant differences between groups, with capitals used for all individuals and lower case letters for males only. Bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

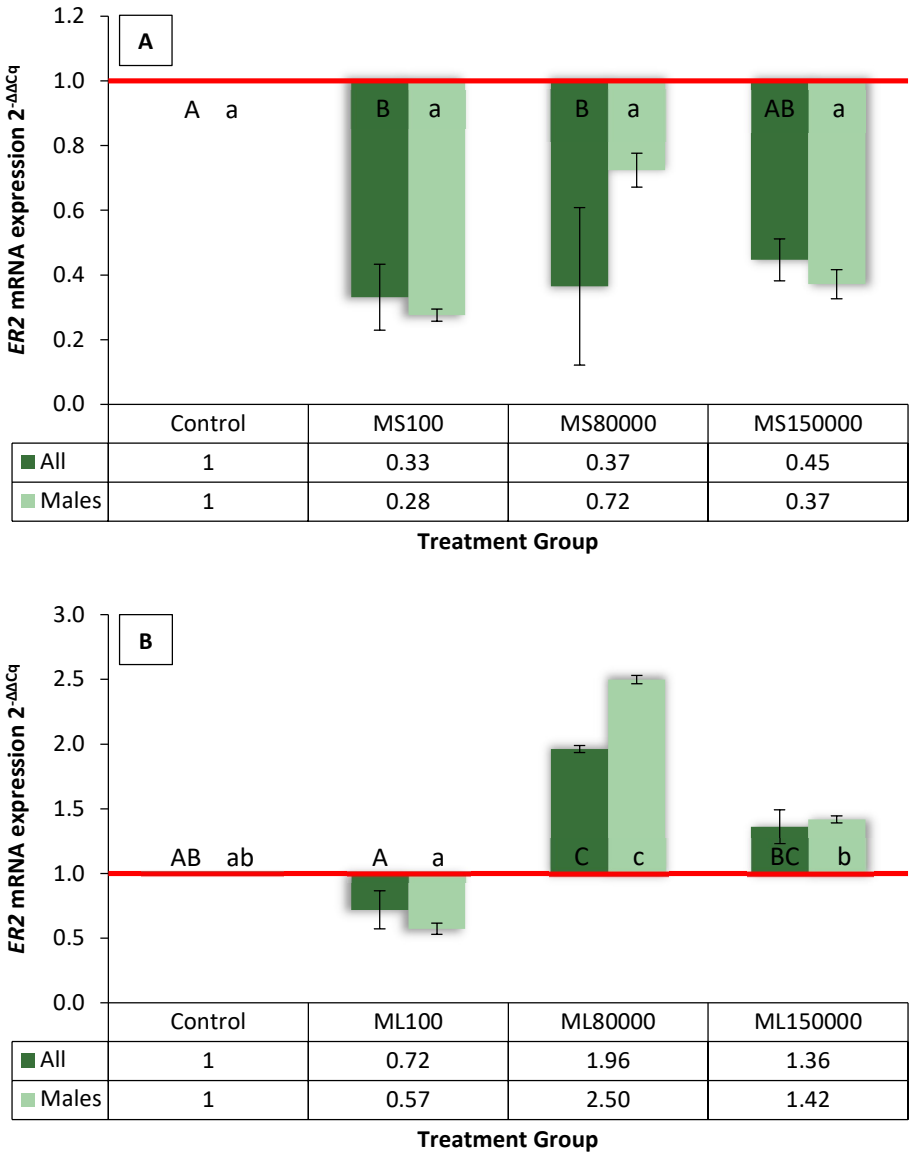


Figure 27 Summary of mRNA expression of *ER2* in fold changes compared to control group in mussel gonads (n = 10). Data from all individuals and male mussels only. Treatments are denoted by alphanumeric codes: MS, groups exposed to short term exposure (A); ML, groups exposed to long term exposure (B); each followed with nominal concentration of metformin in ng/L. Different letters represent statistically significant differences between groups, with capitals used for all individuals and lower case letters for males only. Bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

HSP70 mRNA expression was downregulated in all mussels exposed to metformin over 7 days (Fig. 28). A monotonic response was observed in the mixed mussels data, ranging from 1.5 to 4 fold downregulation compared to non-exposed mussels, while

there was no significant difference between groups observed in the male mussels data. Similarly to the short-term exposure, results from mixed mussels in the long-term exposure also showed a concentration related trend. The mRNA expression of *HSP70* in the two higher concentration groups (80 µg/L and 150 µg/L) ranged from 2 to 4 fold changes compared to the non-treatment group, and were statistically different to the control group ($p < 0.05$), both in male mussels and all individuals.

The short-term exposure data of *CASP8* mRNA expression exhibited a quite unique result, as shown in Figure 29. In this case, both datasets exhibited opposite responses to a short exposure of metformin, as male mussels displayed upregulation, whilst combined mussels showed downregulation. However, this variation was insignificant, as the expression showed only very small deviations from the control group and were not statistically different. Meanwhile, mixed mussels from all treatment groups showed significant upregulation compared to unexposed mussels after a long-term exposure to metformin. The expression level of *CASP8* in these exposed groups was almost invariable regardless of the different concentrations of metformin, upregulated around 2 fold changes compared to the unexposed group.

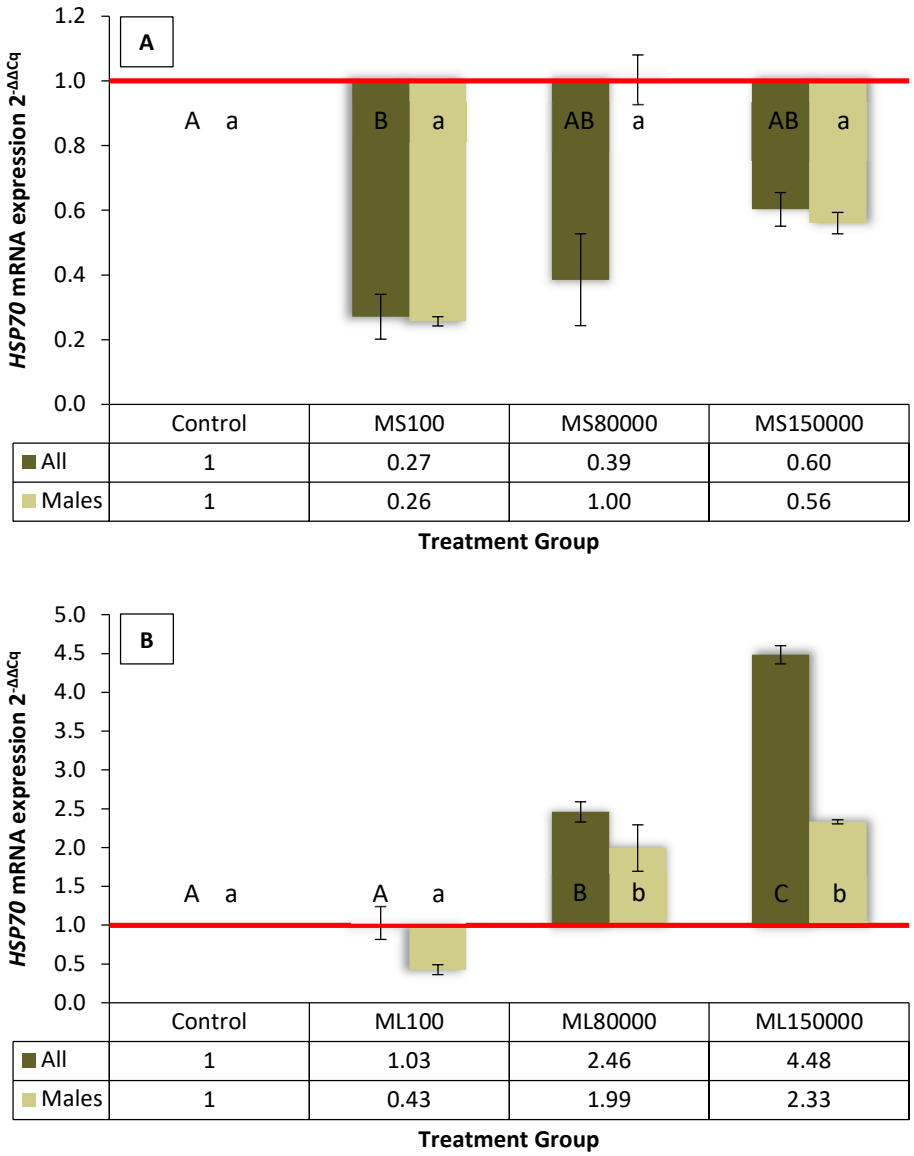


Figure 28 Summary of mRNA expression of *HSP70* in fold changes compared to control group in mussel gonads (n = 10). Data from all individuals and male mussels only. Treatments are denoted by alphanumeric codes: MS, groups exposed to short term exposure (A); ML, groups exposed to long term exposure (B); each followed with nominal concentration of metformin in ng/L. Different letters represent statistically significant differences between groups, with capitals used for all individuals and lower case letters for males only. Bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

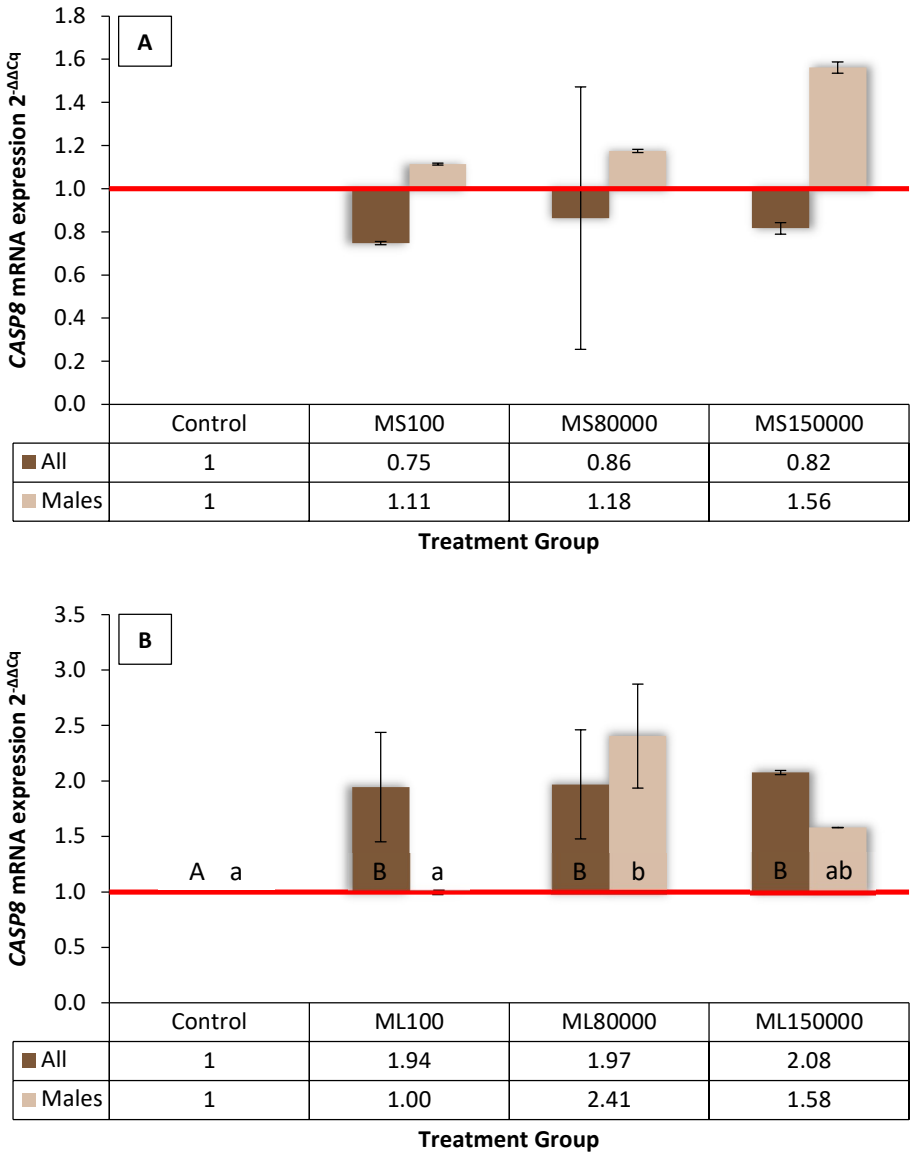
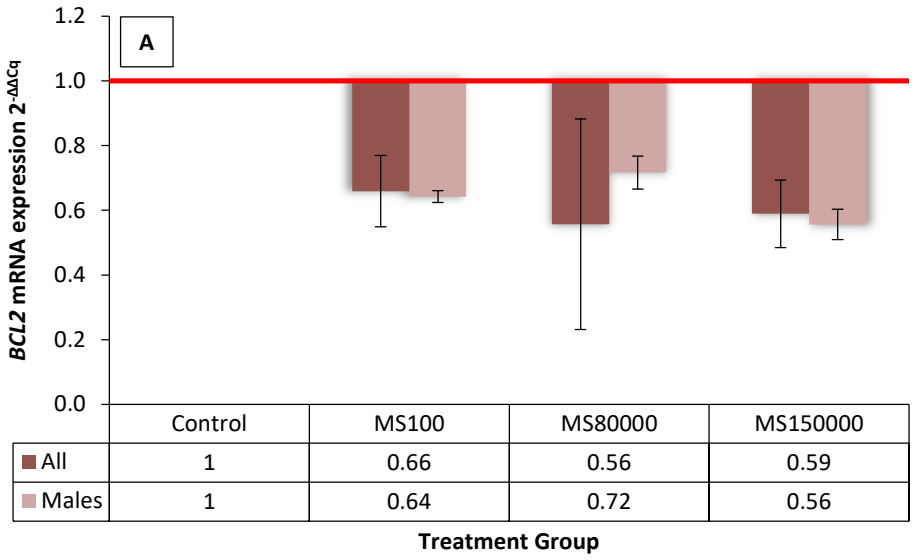


Figure 29 Summary of mRNA expression of *CASP8* in fold changes compared to control group in mussel gonads (n = 10). Data from all individuals and male mussels only. Treatments are denoted by alphanumeric codes: MS, groups exposed to short term exposure (A); ML, groups exposed to long term exposure (B); each followed with nominal concentration of metformin in ng/L. Different letters represent statistically significant differences between groups, with capitals used for all individuals and lower case letters for males only. Bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

Figure 30 presents the mRNA expression of *BCL2* after short- and long-term exposure to metformin. Although showing a lower mRNA expression in all exposed groups and in both datasets (all individuals and male mussels only), the downregulation of *BCL2* in

this short-term exposure was insubstantial and not significantly different from the control group. The long-term exposure, however, changed the variation of expression of this gene. Both mixed and male mussel datasets displayed concentration-dependent results, with the mRNA expression in the highest concentrations of metformin (150 µg/L) being significantly different by 2 – 3 fold changes compared to the control group ($p < 0.05$).

Male mussels showed remarkable responses in the *FAS* mRNA expression level following 7 days exposure to metformin (Fig. 31). The expression of this gene was upregulated in male mussels in all exposed groups, up to 358 fold changes compared to non-exposed mussels. However, after being exposed to metformin for a longer period, male mussels showed contrasting responses, as the expression was downregulated in all exposed groups, up to 100 fold in the group 150 µg/L. The data from all individuals showed varying responses to the duration of exposure to metformin, with upregulation to 18 fold in the short exposure, and more divergent variation of the expression in the long exposure.



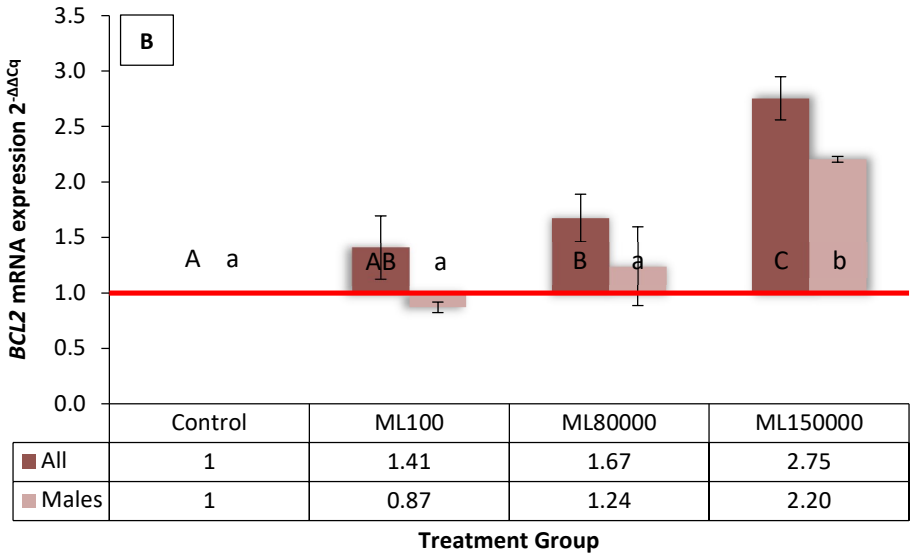


Figure 30 Summary of mRNA expression of *BCL2* in fold changes compared to control group in mussel gonads (n = 10). Data from all individuals and male mussels only. Treatments are denoted by alphanumeric codes: MS, groups exposed to short term exposure (A); ML, groups exposed to long term exposure (B); each followed with nominal concentration of metformin in ng/L. Different letters represent statistically significant differences between groups, with capitals used for all individuals and lower case letters for males only. Bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

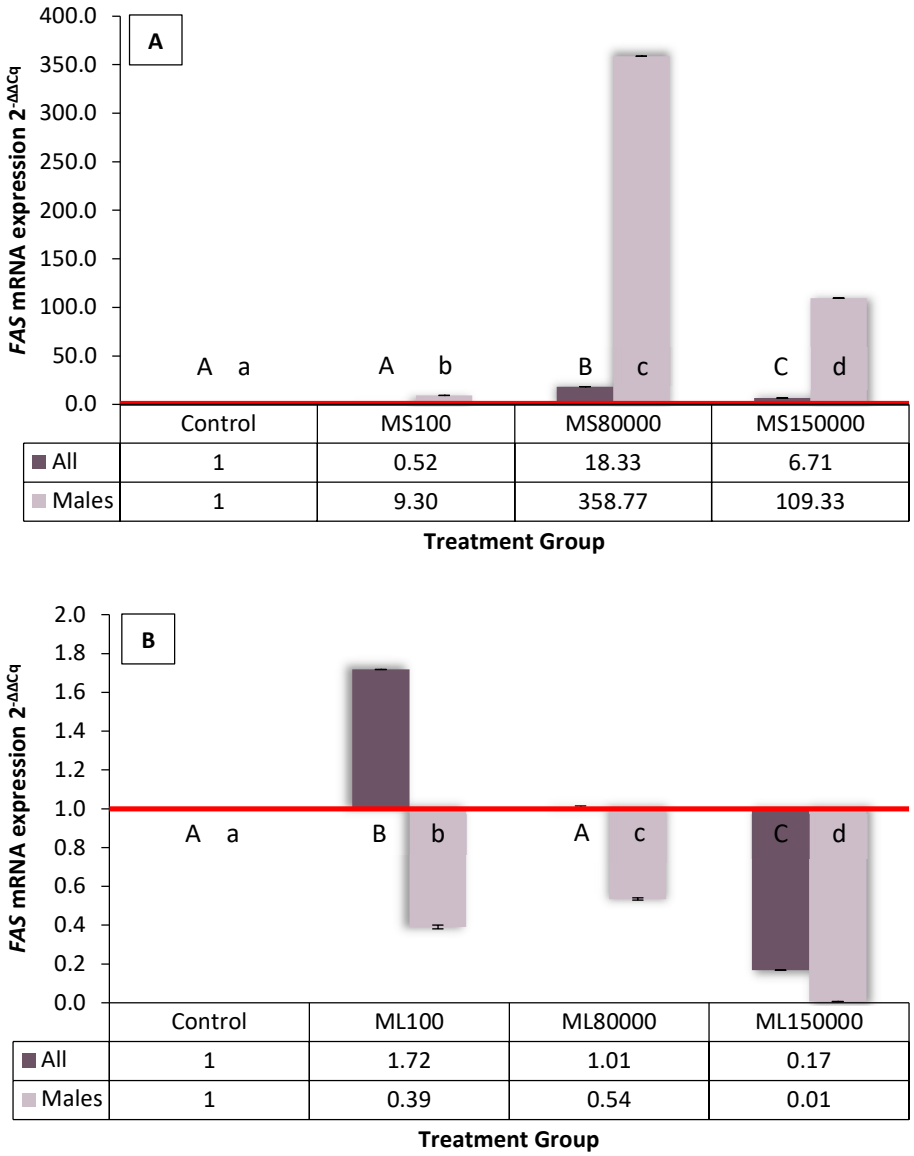


Figure 31 Summary of mRNA expression of *FAS* in fold changes compared to control group in mussel gonads (n = 10). Data from all individuals and male mussels only. Treatments are denoted by alphanumeric codes: MS, groups exposed to short term exposure (A); ML, groups exposed to long term exposure (B); each followed with nominal concentration of metformin in ng/L. Different letters represent statistically significant differences between groups, with capitals used for all individuals and lower case letters for males only. Bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

4.4 Discussion

4.4.1 Neutral red retention time (NRRT) assay

When compared with the results from acetaminophen exposure in Chapter 3 (Fig. 4), metformin exposure showed a more evident concentration-dependent trend, with higher concentrations of contaminant inducing a lower retention time (Fig. 16). Whilst non-exposed mussels showed very good lysosomal membrane integrity in their haemocytes, mussels exposed to 100 ng/L metformin had a shorter retention time (120 minutes). While mussels in these two groups still can be categorised as healthy according to the threshold defined by Moore et al. (2006), the other two exposed groups displayed more dramatic results. Mussels exposed to 80 µg/L metformin showed very short neutral red retention time with an average of 30 minutes and were classified as severely stressed according to the threshold. Metformin had a high toxicity effect on mussels exposed at a concentration of 150 µg/L, as the disintegrated lysosomal membrane released all lysosomal material to cytosol in less than 30 min incubation time (Fig. 16).

The result presented here is in agreement with the previous study (Koagouw and Ciocan, 2018) where mussels exposed to metformin displayed destabilization of the lysosomal membrane. Mussels exposed to 40 µg/L showed that the neutral red retention time was decreased to 87 minutes on average (compared to 138 minutes in the control), so that mussels were considered in a low to mild level of stress, according to the threshold developed by Moore et al. (2006). Increasing the concentration two times higher from 40 µg/L to 80 µg/L was able to illicit a distinct stress-inducing factor in mussels exposed to these concentrations, as they were classified as ranging from moderately stressed to severely stressed and tending towards pathology, in accordance with the aforementioned threshold. These results are an indication of a concentration-dependent trend, with higher concentrations of metformin inducing a lower retention time, and therefore a more severe destabilisation of the lysosomal membrane.

As NRRT has been adopted as an indicator for pollution or contaminant monitoring, this result shows that metformin can induce the alteration of immune response in

mussels, in a more dose-dependent manner than acetaminophen (Chapter 3). Decreasing immunity may lead to populations becoming susceptible to other diseases or infections, and in general can lead to vulnerability of the population itself.

4.4.2 Histopathology

The result from the histopathology analysis indicates that metformin inflicts several pathologies in the mussels' gonads even after only 7 days of exposure (Fig. 17). The pathological conditions observed here are mostly related directly to the reproductive system, such as follicle dilatation and gamete degeneration. Follicle dilatation is reportedly related to reduced fecundity in zebrafish *Danio rerio* (Hong et al., 2019), while Williams and Bentley (2002) demonstrated that the health status of gametes has a direct effect on fertilisation success in marine invertebrates. The authors suggest that any alteration to the oocytes or sperm can result in a decreased fertilisation ability or abnormalities in embryo development. The occurrence of these pathologies recorded in this study represents a direct impairment of the reproductive system, as successful reproduction is very likely dependent on the quality of gametes. Any alteration to the development stage of gametes, or gametogenesis in the follicles, is very likely to interfere with the quality of gametes. This is in agreement with García-Gasca et al. (2010), who proposed follicle dilatation as a useful predictor of environmental stress for coastal ecosystems, as it is directly related to the reproductive system and the success of reproduction.

Recent studies have linked metformin with some reproduction-related alterations in tissues (Niemuth and Klaper, 2015; Koagouw and Ciocan, 2018). The high prevalence of follicle dilatation and gamete degeneration in the results presented here thus imply considerable potential for metformin to cause disturbance in the reproductive systems of mussels and possibly to interfere with population sustainability.

It is worth noting that these two reproduction-related pathologies were observed in all exposed groups and showed a concentration-related trend (Fig. 17). This indicates that the level of metformin in the environment plays a vital role in the level of toxicity

of this substance to marine mussels. The very high prevalence of reproduction-related pathologies in the two groups in which metformin concentrations are in µg/L suggests a very concerning picture, should this substance reach those high levels in the environment, with follicle dilatation and gamete degeneration afflicting 80 % and 70 % of mussels, respectively.

There is also concern around the high percentage (30 – 40 %) of mussels exposed to high levels of metformin showing atretic condition in their gonads (Fig. 17). According to Krysko et al. (2008), atresia can negatively affect fertility and may eventually lead to irreversible premature ovarian failure. Atresia was also recorded in the gonads of mussels exposed to 40 µg/L metformin in a previous study with a prevalence of 8 – 17 % (Koagouw and Ciocan, 2018). The result presented here also suggests a correlation between the concentration of metformin and the prevalence of atresia, as higher prevalences are observed in line with the increase in metformin concentration.

Another important assumption is that metformin in concentrations as low as 100 ng/L could induce similar pathologies to those induced when the level is much higher (150 µg/L), albeit at lower incidence (Fig. 17). This highlights the present danger faced by the marine organisms inhabiting areas where this level of metformin is actually recorded. As mentioned earlier, metformin has been quantified in the environment in a range from 0.5 ng/L to 325 µg/L (Kot-wasik et al., 2016; de Jesus Gaffney et al., 2017; Elizalde-Velázquez and Gómez-Oliván, 2020), and has been detected in surface water at concentrations as high as 33.6 µg/L (Elliott et al., 2017). The results here therefore indicate a very concerning picture whereby these pathologies are actually developing in real life situations.

Whilst haemocytic infiltration was recorded in all groups in the short-term exposure, this condition was notably more prevalent after longer exposure (Fig. 20). The result here indicates that longer exposure to metformin can induce inflammation as a response to stress-related events and adversely affect mussels' health. Haemocytic infiltration has been frequently recorded in animals as a result of stress-inducing experiments as documented by Arrighetti et al. (2018), Velisek et al. (2018) and Khan

et al. (2019). The results here thus call the attention to the role of exposure duration in determining the level of risk to marine organisms, as longer exposure contributes to lower immunity in mussels, represented by high prevalence of haemocytic infiltration in the gonads of up to 50 % of samples.

Although observed only in 10 % of the samples, parasitic infiltration and haemocytic aggregate were detected here after 21 days of metformin exposure (Fig. 20). Neither pathology was present in the short exposure, thus the longer exposure to metformin seems to induce the incidence of these inflammatory reactions. It is possible however that parasites may have been present in the mussels at the beginning of the experiment, and the conditions for these mussels may have deteriorated as a consequence of the contaminant effect by reducing immunity, and thus this parasitic infection became more evident. Gamete degeneration, atresia, and follicle dilatation, all pathologies directly related to reproductive mechanisms, were still present after long exposure, albeit at a moderate level of occurrence. These apoptotic-related conditions seem to represent the first or primary reactions to metformin exposure, as they were observed with higher prevalence after the short-term exposure. However, after longer exposure, these pathologies occurred only mildly, while inflammatory-related conditions were more dominant.

These overall results, whereby metformin seems to induce apoptosis-related responses at first, while later prompting a shift towards more inflammatory reactions, also highlight the significance of the duration of metformin exposure. It is possible that different mechanisms are involved in the biological responses to metformin toxicity in non-target organisms in the environmental context, although further investigations are needed to confirm this. Metformin seems to alter the mechanisms or events related to programmed cell death reactions at first, and then later target the immune system of the organism as the time of exposure increases.

4.4.3 Gene expression analysis

4.4.3.1 Synergistic effects of metformin and temperature

The results discussed here are the mRNA expression of *HSP70* (Fig. 21), *CASP8* (Fig. 22), *BCL2* (Fig. 23) and *FAS* (Fig. 24) in gonads of control and exposed mussels from the 2016 short term exposure to metformin (40 µg/L) and high temperature (20 °C). The lysosomal stability, histopathological changes and the variation of expression of *VTG*, *V9* and *ER2* after a short-term exposure to metformin coupled with a heat stressor have been reported in Koagouw and Ciocan (2018). *HSP70*, *CASP8*, *BCL2* and *FAS* are genes potentially involved in the regulation of apoptosis, and therefore their expression in gonad tissue could affect reproduction. The mRNA expression of these genes is discussed in relation to their role in apoptotic changes in cells and tissues, such as atresia and follicle degeneration, as reported in the previous study (Koagouw and Ciocan, 2018).

Heat shock protein 70 (Hsp70) is involved in the response to physicochemical sublethal stressors that act to protect cells from thermal or oxidative stress (Beere et al., 2000). In this result, heat exposure distinctly upregulated the mRNA expression of *HSP70*. Both groups exposed to high temperature (C20 and M20) showed upregulation to more than a 5 fold change compared to the control group (Fig. 21). This demonstrates that the heat stressor clearly causes a response through the expression of this gene, as expected. Metformin exposure potentially also induces the mRNA expression of *HSP70* slightly, however the upregulation was not statistically significant. Thermal and oxidative stress usually act by damaging protein structure, whilst Hsp70 maintains the protein integrity and directly inhibits apoptosis to protect cells from these stressors (Beere et al., 2000). This suggests that in this study, Hsp70 has potentially been highly expressed in high temperature groups in order to protect cells and the tissue by inhibiting apoptosis. This result supports a study by Minier et al. (2000) that reported a significant correlation between Hsp70 and seasonal variation, with the maximum protein accumulation being recorded during the summer period, suggesting a direct role of temperature in the expression of this protein. Further investigation is needed to elucidate the mechanism by which this protein displays any direct effect on cells and

tissues of mussel gonads, or indeed whether it plays an indirect role in causing the observed effects, in combination with other regulations.

Temperature likely shows an impact on apoptosis by increasing the mRNA expression of *CASP8*, an initiator caspase in the apoptotic process, as shown in Figure 22. The *CASP8* mRNA expression only appeared upregulated in high temperature groups (C20 and M20). Upregulation of *CASP8* mRNA expression in high temperature groups was by 2 fold changes on average compared to the control group C10. Caspase-8 may likely be associated with the regulation of hydrolysis of cellular components in the apoptosis process (Shi, 2002), perhaps suggesting that temperature can affect apoptotic conditions in mussels, such as atresia. The result also showed that exposure to metformin alone seems to suppress mRNA expression of *CASP8*: mussels in group M10 (exposed to metformin at 10 °C) showed downregulation of *CASP8* mRNA expression compared to the control group C10 (Fig. 22). Interestingly, while inverse effects were expressed by a single exposure of temperature (upregulation of mRNA expression) and metformin (downregulation of mRNA expression), there is an indication of synergism in their combined exposure. Mussels in group M20 which were exposed to metformin coupled with heat exposure of 20 °C showed a notable increment compared to the control group, signifying the synergistic interaction of these exposures.

This result indicates that apoptotic initiation by *CASP8* is more likely upregulated by temperature than by metformin, and that metformin might only actively upregulate this initiator caspase protein at higher temperatures. However, as the apoptosis process involves another caspase initiator (caspase-2) and effector to generate the effects, any apoptotic change in the cell might be caused by the interaction of these proteins (Salvesen and Walsh, 2014). Another possibility is that the regulation of apoptosis is different depending on the cells in question.

Another synergistic interaction is shown in the mRNA expression result for *BCL2* (Fig. 23). While exposure to temperature only induced a slight upregulation, and exposure to metformin only induced a suppression, higher temperature was shown to reverse and intensify the effects of metformin by upregulating the mRNA expression of *BCL2*

up to nearly 5 fold changes compared to the control group (Fig. 23). Bcl-2 acts to promote cell survival by inhibiting the actions of pro-apoptotic proteins (Hassan et al., 2014). The result here suggests that metformin induces apoptosis by the suppression of *BCL2* mRNA expression, allowing proapoptotic proteins to regulate cell death. This stimulation of apoptosis in gonad cells suggests a possible means by which atresia may be induced by metformin. The upregulation of *BCL2* mRNA expression in high temperature groups indicates that high temperature might improve the inhibition action of apoptosis in this case. However, as apoptosis is a complex mechanism orchestrated by many regulatory genes and pathways, the role of *BCL2* here whether as inhibitor or inducer of cell death is highly dependent on its modulation with many other interacting genes, such as *FAS*.

Kawahara et al. (1998) demonstrated the modulation of Bcl-2 and Fas as having a substantial role in apoptosis. The Fas antigen (Fas) is a cell surface receptor that may be involved in the initiation and progression of follicle cell apoptosis during atresia. Fas initiates apoptosis in sensitive cells after binding Fas ligand (FasL) (Wang et al., 2010). The result of *FAS* mRNA expression analysis (Fig. 24) shows a noteworthy synergistic interaction of exposures. Exposure to metformin only, as well as to temperature only, apparently downregulated the *FAS* mRNA expression. In contrast, when these exposures were combined, the mRNA expression was upregulated to more than 5 fold changes compared to the non-exposed group (Fig. 24). This shows that while metformin alone, as well as temperature alone, might inhibit the apoptosis initiation and progression of follicle cells by affecting the cell surface receptor, the presence of metformin in higher temperatures might actually cause the initiation and notable apoptosis progression in follicles.

From the aforementioned results and subsequent discussion, it can be derived that apoptotic changes such as atresia and follicle degeneration recorded in the previous study (Koagouw and Ciocan, 2018) are likely regulated by many different interactions between these regulatory genes. These apoptotic-related pathologies, when caused by temperature, may be induced by *CASP8* acting as an initiator. Conversely, it seems that in this study metformin may cause atresia and follicle degeneration by suppressing

the anti-apoptotic gene *BCL2*, thus promoting the apoptotic process. In conditions where metformin and high temperature are combined, apoptotic changes such as atresia and follicle degeneration are likely to be *FAS*-mediated.

4.4.3.2 Short- and long-term exposures to selected concentrations of metformin

The duration of exposure to metformin seems to have evident impacts on the expression of *VTG*. Contrasting results were observed following the two exposures, where metformin increased the expression of *VTG* after 7 days, and then reduced the mRNA expression after a longer exposure (Fig. 25). *Vitellogenin* (*VTG*) mRNA expression has been recognised as a sensitive indicator for early detection of exposure to endocrine disrupting chemicals (EDCs) in vertebrates, both in laboratory and in wild populations (Ankley et al., 2001; Lattier et al., 2001; Muncke and Eggen, 2006). In invertebrates, Ciocan et al. (2010) recorded an upregulation of *VTG* mRNA expression in the gonads of *Mytilus edulis* after exposure to EDCs during the early stages of gonad development. However, it is also worth pointing out that *VTG* expression in invertebrates is a debatable topic as discussed by Porte et al. (2006) and Matozzo et al. (2008). The contrasting results observed here seem to highlight the complex mechanisms involved in this gene's expression. The divergent expression may however shed some light on the issue, as it is possible that the different contact time with the contaminant is one of the factors that generates these contrasting results. Nevertheless, it is relevant to recall Porte et al. (2006) and Matozzo et al. (2008), who pointed out that the mechanism of action, synthesis and role of *VTG* in invertebrates are still unclear and require further investigation.

Another noteworthy interpretation of the results is that male mussels showed more prominent expression, both by upregulation in the short term exposure and downregulation in the long term exposure, compared to mixed sex mussels that tended to express *VTG* in a similar manner (Fig. 25). The induction of *VTG* mRNA transcription in adult male organisms was also demonstrated by Niemuth et al. (2014) in fathead minnows *Pimephales promelas* exposed to metformin. This result highlights

the conclusion that male mussels might be more sensitive than females regarding the modulation of VTG expression.

The *V9* mRNA expression result (Fig. 26) in which male mussels showed a more concentration-related trend in the short-term exposure is also perhaps worth noting, as it again provides an indication that male mussels are more reactive than females to the level of metformin in the environment. However, the variations here were not significantly different.

The mRNA expression of *V9* also exhibited different responses to the duration of exposure (Fig. 26). Mussels exposed to a longer contact time to metformin showed a more varied pattern, although the variations are not significantly different. The *V9* expression in mussels exposed to metformin for a shorter period, however, was downregulated in all treatment groups. This result is in agreement with the previous result showing the downregulation of *V9* mRNA expression in adult mussels exposed to 40 µg/L metformin (Koagouw and Ciocan, 2018). Changes in the vitelline envelope induced by xenoestrogen would likely have further detrimental effects at higher levels, because of the direct involvement in the protection of eggshell and fertilisation as indicated by Arukwe et al. (1997). The suppression of *V9* mRNA expression in this study thus might indicate a severe reproductive impairment in mussels. A study by Tian et al. (1997) demonstrated the important role of vitelline envelope or zona pellucida in fertilisation, such as in facilitating sperm binding and protection against polyspermy, further confirming this concern.

A long-term exposure to metformin induced more fluctuation in the expression level of *ER2* compared to the short exposure (Fig. 27), suggesting that the exposure period is very likely to further alter the trend of mRNA expression of this gene. However, despite the variation of expression in the long exposure, only mussels in the 80 µg/L group were statistically different to the control group (Fig. 27). The fluctuation in the *ER2* mRNA expression observed here was also observed in the previous study (Koagouw and Ciocan, 2018), where mussels were exposed to 40 µg/L metformin and a heat stressor. The fluctuation recorded here might be related to the natural variation

in the *ER2* expression during different stages of gametogenesis as reported by Ciocan et al. (2010). The downregulation of *ER2* expression in all treatment groups in the short-term exposure was also observed in mussels exposed to acetaminophen, as described in Chapter 3. The result in mixed sex mussels here also showed a monotonic expression, even though the trend was inverse.

Blalock et al. (2018) highlighted two pathways involved in the mechanism of endocrine disruption in bivalves: the steroidogenesis pathway and the non-genomic oestrogen signalling pathway activated by oestrogen binding to ERs, in the cytosol and plasma. Moreover, data on expression variability in oestrogen receptors indicate possible further impacts that may occur in gametogenesis and reproduction processes in bivalve species (Ciocan et al., 2010). The results presented here thus raise a concern over the potential of metformin to induce endocrine disruption by altering the level of expression in the oestrogen receptor. However, as discussed earlier, the natural variation in the expression level of *ER2* during gametogenesis also has to be taken into account, and further investigation into this natural variation of *ER2* is therefore needed.

HSP70 is involved in the reaction to physicochemical sublethal stressors that act to protect cells from heat or oxidative stress as indicated by Beere et al. (2000). The overexpression of *HSP70* was recorded in the gastrointestinal glands and gills of *M. galloprovincialis* exposed to oily wastewater discharge, presenting evidence of their protective response to this pollutant (Pirrone et al., 2018). Thermal and oxidative stress typically operate by weakening protein structure, while Hsp70 preserves protein integrity and directly inhibits apoptosis. This indicates that suppression of *HSP70* in mussels exposed to metformin (Fig. 28) in the short-term exposure may lead to a higher risk of apoptosis and cell death.

The downregulation of *HSP70* here also showed concentration-related effects after short-term exposure to metformin (Fig. 28), both in mixed mussels and male mussels only. Whilst the concentration-dependent trend suggests that this transcript may be potentially used as a marker for mussels exposed to metformin, this also implies a risk

of apoptotic and cell death related incidents in mussels that might be associated with the level of metformin in the environment. The variation of the transcript in this study indicates that metformin may induce apoptotic-related pathologies in the gonads, and *HSP70* is expressed more in the higher concentration groups in order to provide more protection by directly inhibiting apoptosis. The role of *HSP70* here may be in providing the mechanism to inhibit cell death progression in the mussels' gonads, as indicated by the histopathology result, where the pathological condition was shifted from apoptotic-related to inflammatory reactions between 7 and 21 days' exposure. The significantly different result in the groups 80 µg/L and 150 µg/L after long term exposure (Fig. 28) supports this inference, as the upregulation of *HSP70* mRNA expression here can be associated with the protective role of Hsp70.

The alteration of *CASP8* expression can result in regulatory changes in the degeneration of cellular components, either by induction or inhibition of apoptosis as indicated by Salvesen and Walsh (2014) and Allavena et al. (2018). In this study, short exposure to metformin is likely to induce different responses in mussel males and females. The mRNA expression of *CASP8* (Fig. 29) was upregulated in male mussels, suggesting that male mussels are more susceptible to conditions relating to apoptotic-related incidents. In contrast, mussels of both sexes combined showed downregulation of *CASP8*, indicating a higher prevalence of inhibition of apoptosis or interfered cell death, such as cancer-related pathologies in the gonads of female mussels. The different modulation of the *CASP8* expression levels in male and female mussels indicates the sex-dependent response induced by the exposure to metformin.

In the long exposure experiment, the *CASP8* mRNA expression was upregulated in a similar manner regardless of the different concentrations of metformin (Fig. 29). The upregulation of *CASP8* can be linked with the caspase activation and induction of the cell death programme, conducing apoptosis-related pathologies in tissues as suggested by Shi (2002) and Hassan et al. (2014). This indicates that metformin could potentially be implicated in degenerative diseases, or any pathological condition initiated by enhanced apoptosis in adult blue mussels after longer contact time. The invariable expression level of *CASP8* regardless of different concentrations of

metformin demonstrates the sensitivity of this transcript and also signifies the importance of the length of exposure, rather than concentration of the contaminant. Indeed, the upregulation induced by metformin at concentrations as low as 100 ng/L could be at a similar level to that induced by a concentration 1500 times higher (150 µg/L), given only two weeks longer exposure time (Fig. 29).

The *BCL2* mRNA expression was downregulated in all treatment groups after short-term exposure, although the suppression was not significant compared to the control group (Fig. 30). The longer exposure to metformin changed the expression pattern by showing a dose-related trend of upregulation, in male and mixed sex mussels. The modulation of apoptosis-regulatory gene transcripts maintains a healthy cell death balance either by activation or inhibition of apoptosis (Hassan et al., 2014). The metformin concentration in this result can directly impact the upregulation pattern after long exposure, which suggests that inhibition of apoptosis in the gonads might be altered by the level of metformin in the environment. However, it should be taken into account that *BCL2* is only one of many regulatory genes, and that the mechanism of apoptosis is regulated through the expressions of these genes as part of a highly complex network. A study by Estévez-Calvar et al. (2013) recorded a substantial increase of *BCL2* in *M. galloprovincialis* following exposure to pifithrin-α hydrobromide (PFT-α) and UV irradiation. The authors imply a correlation between this result and reduced apoptotic rates, although they also indicate that the increase of *BCL2* expression in mussel haemocytes could be a reaction to the presence of pro-apoptotic Bax.

BCL2 and *FAS* play a significant role in apoptosis by modulating their expression (Kawahara et al., 1998). Alterations to this process may either impact the survival of a defective cell or contribute to carcinogenesis. The variability in expression of these transcripts was documented by Yang et al. (2002) following exposure to cyclosporine in mice. The authors found that alterations in cell death mechanisms as a result of *BCL2* and *FAS* modulation by various factors may lead to consequential cell death in the model animal renal tubular cells.

In this study, *FAS* mRNA expression displayed more remarkable responses in male mussels (Fig. 31). *FAS* was overexpressed in all groups exposed to metformin over a short period of time, while it was underexpressed in treatment groups after a long-term exposure. These contrasting responses again emphasise the importance of the contact time between the animal and the contaminant and also the sex of mussels. The varying responses in the mixed sex mussels indicate that male mussels potentially show more clear and dependable results in *FAS* expression levels after metformin exposure. The dissimilar variation of expression in male mussels after the short- and long-term exposures can be directly linked to *FAS*-mediated induction or inhibition of apoptosis, notwithstanding the fact that apoptosis is a very complex mechanism involving various pathways and regulatory genes. However, the results here also show a clear connection between the mRNA expression of *FAS* (particularly in male mussels) and *BCL2*, as pro-apoptotic and anti-apoptotic, respectively. This result is in agreement with Aref et al. (2004), indicating that these transcripts work through antagonistic actions. Their expressions here suggest that both *BCL2* and *FAS* are impacted by metformin, and that their modulation as part of their interaction can induce either cancer-related or progressed apoptotic conditions in the cells of mussel gonads.

4.5 Summary

Metformin can induce the alteration of immune response in mussels with an indication of a concentration-dependent trend; higher concentrations of metformin promote a lower retention time in neutral red analysis.

The overall histopathology results highlight the significance of the duration of metformin exposure. Metformin seems to induce apoptosis-related responses during short term exposure (follicle dilatation, gamete degeneration, atresia). After a longer contact with the contaminant, a shift towards a range of inflammatory reactions (haemocytic infiltration, haemocytic aggregate, parasitic infiltration) is apparent. This provides an important insight into the various mechanisms affected by metformin exposure in non-target organisms, although further investigations are needed to confirm this. Metformin seems to alter the mechanisms or events related to

programmed cell death reactions at first, and then targets the immune system of the organism over longer periods of exposure.

Metformin also induces variations in the mRNA expression of all transcripts investigated here. Some transcripts (*VTG*, *V9*, *CASP8*, *FAS*) display fluctuating, and in some cases contrasting results, in a sex-dependent manner, indicating that metformin induces different responses in males and females. Several transcripts follow concentration-dependent trends, mostly in the short-term exposure, as shown by the expression of *V9* in male mussels, *ER2* in mixed mussels and *HSP70* in both mixed and male mussels. *BCL2* expression also shows a dose-dependent result after long-term exposure, in males and mixed sex mussels. The monotonic responses exhibited by some transcripts indicate their potential as biomarkers of metformin exposure. The remarkable variation in expression also observed in short- versus long-term exposures reflects the importance of contact time between the metformin and the organism, in the natural environment, as shown by all transcripts. In addition, higher sensitivity to metformin is more apparent in males, particularly in the *VTG* and *FAS* transcripts after short-term exposure.

Overall, this study shows that the antidiabetic pharmaceutical metformin can alter the immune response in blue mussels, initiate pathological conditions in gonads including apoptosis-related and inflammatory responses, and induce variation of mRNA expression in selected transcripts. Furthermore, these alterations can be linked with the level of metformin present in the environment. Thus, these findings suggest the potential of metformin to act as a non-traditional EDC, and further investigations are required to clarify its mechanism of action.

CHAPTER 5

The effects of exposure to pharmaceuticals on the early life stage of mussels: a molecular perspective

Abstract

Recent studies have detected the presence of acetaminophen and metformin in seawater, posing questions regarding the potential consequences for marine biota. However, the impacts of these pharmaceuticals may have consequences for marine organisms at any life stage. This chapter discusses the effects of both acetaminophen and metformin on early life stages of mussels. Specifically, it focuses on the likely survival of zygotes following exposure to pharmaceuticals during the fertilisation process, depicting a scenario that may occur in the natural environment. Female and male gametes obtained from mature blue mussels *Mytilus edulis* during the spawning period were exposed to acetaminophen and metformin. The exposures were terminated after fertilisation was confirmed. The mRNA expression of *vitellogenin* (*VTG*)*, vitelline envelope zona pellucida domain-9* (*V9*)*, estrogen receptor-2* (*ER2*)*, heat shock protein-70* (*HSP70*)*, caspase-8* (*CASP8*)*, B-cell lymphoma-2* (*BCL2*) and *Fas cell surface death receptor* (*FAS*) in mussel zygotes was assessed. The variation in expression of these target genes in adult and in early life stages of mussels is discussed herein.

Keywords: early life stage, reproduction, acetaminophen, metformin, mRNA expression, blue mussel

5.1 Introduction

5.1.1 Ontogeny of blue mussels *Mytilus edulis*

Blue mussels (*Mytilus edulis* Linnaeus, 1758) are probably the most widely studied marine organisms. As a filter feeder, this species feeds on microalgae, detritus and organic material and also ingests other substances, potentially pollutants, in the process (Cubero-Leon and Ciocan, 2014). The ontogeny of the blue mussels starts with external fertilisation, as summarised by Tyler-Walters (2008) in the following description. When the egg is fertilised, in the external environment, it develops into a ciliated larva. The trochophore larva is then transformed into a veliger that lasts for 1 to 1.5 months. At this stage, the larva features ciliated fan-like protrusions before developing into a juvenile and identifying the primary settlement site. The primary settlement site is often established in substrate pores, or between bryozoans or other filamentous structures, and is usually located away from mature mussels, likely to minimise competition. After a few weeks, the juvenile has increased in size and detaches in order to migrate again to find a new suitable substrate. A young adult mussel may attach the byssus thread to the sea floor, or if such an open substrate is not secure, can bind it to another mussel, forming a mussel bed (Carl et al., 2012; Gosling, 2015). Usually dioecious, although there have been reports of hermaphrodites, blue mussels mature after 1 year, but can postpone the maturity if growing conditions are not optimal (Duinker et al., 2008; Tyler-Walters, 2008).

The growth rates of *Mytilus* spp. are highly variable, however most variations are likely to be determined by the environment as noted by Tyler-Walters (2008). Temperature, salinity, availability of food, tidal exposure, intra-specific competition for space and food, and parasitism are factors that have been posited to influence the growth rate of *Mytilus* spp. In addition, the author also stressed that a variety of different factors such as environmental conditions or the presence of pollutants can have a major influence.

Bivalves encounter an annual reproductive cycle, which includes a period of gametogenesis, followed by a single, prolonged or even multiple spawning phase, followed in turn by a period of gonad reconstitution (Gosling, 2015). In

spermatogenesis, primary spermatogonia initiate multiple mitotic divisions in order to create secondary spermatogonia, which complete meiosis to become spermatocytes. These spermatocytes then develop into spermatids that are eventually differentiated to flagellated spermatozoa of 25 – 60 µm in length (Franco et al., 2008).

Oogenesis works in a similar manner to spermatogenesis, whereby primary oogonia perform repetitive mitosis to produce secondary oogonia that are ready for meiosis (Gosling, 2015). The process is then halted at the prophase stage of meiosis-I, as the subsequent meiotic stages are completed during fertilisation. The oocytes enter the process of vitellogenesis, which includes the deposition of lipid globules and small amounts of glycogen (Chung, 2007). The formation of these deposits is followed by an increase in oocyte size to 70 µm (Gosling, 2015). Oocyte lysis (atresia) is generally expected at the end of the vitellogenesis, although previtellogenic oocytes may be influenced by the existence of hydrolytic enzymes produced from lysed oocytes. Lysed oocytes are believed to be metabolic materials and resources which produce energy at a time when the energy reserves in the adductor muscle are at their lowest levels (Beninger and Le Pennec, 2006).

5.1.2 Reproduction in mussels and affecting factors

Mytilus edulis spawns from April to September, relying on the temperature of the water, tides and other environmental conditions (Tyler-Walters, 2008). For most populations, resting gonads start developing from October to November, with gametogenesis taking place in the winter, such that the gonads mature early in the spring (Duinker et al., 2008). The phytoplankton blooms are a great advantage for the larvae spawned in the spring (Tyler-Walters, 2008). A rapid gametogenesis generally following the partial spawning in the spring, resulting in the maturation of gonads in the early summer, leads to exhaustive secondary spawning at the end of August or September (Mladineo et al., 2007; Enríquez-Díaz et al., 2009). The event of the secondary spawning is uncertain, determined by optimal environmental conditions and availability of food (Gosling, 2015).

Geographical location determines the strategy adopted for gametogenesis, spawning and fertilisation (Hassan et al., 2017). In general, a female mussel can release between 5 and 8 million eggs, while larger individuals are able to produce up to 40 million eggs (Tyler-Walters, 2008). In addition, larval development can be completed in no more than 20 days in optimal conditions, but could take up to 1 month at temperatures of 10 °C between spring and early summer. The metamorphosis of pediveligers can be postponed for up to 40 days at 10 °C, or in some exceptional cases, for up to 6 months (Tyler-Walters, 2008).

The regulation of reproduction encompasses complex relationships between external factors such as temperature, food, salinity and light, and intrinsic determinants like genotypes and neuroendocrine cycles (Barber and Blake, 2006; Enríquez-Díaz et al., 2009). The function of these factors in determining the induction and duration of gametogenesis, a phase that stretches over many months, most likely varies from their role in scheduling and synchronisation of spawning, a much shorter process (Mladineo et al., 2007; Gosling, 2015).

Water temperature is the most important factor involved in the spawning initiation of mussels in wild, laboratory and hatchery populations (Duinker et al., 2008; Enríquez-Díaz et al., 2009). Ciliary activity and muscle contraction of the gonoducts are also known to induce the release of gametes into the water (Bayne, 2017). Physical stimulation by shaking or scratching the shell, pulling or removing the byssus thread may also trigger spawning in *Mytilus edulis* as documented by Gosling (2015). Moreover, the author also indicates that these physical stimuli represent forms of environmental signals acquired during storms that could also trigger the activation of spawning. Other natural factors that may play a role include variations in salinity, lunar phase, and tidal fluctuations (Barber and Blake, 2006; Bernard et al., 2016). When spawning is initiated, gametes are released, and their presence in the water provides a strong chemical signal to other mature individuals and thus increases the probability of fertilisation (Barber and Blake, 2006).

5.1.3 Effects of various contaminants in the early life stage of bivalves

Several recent publications have shown that embryos and larvae of marine organisms are more susceptible to toxic compounds than adults, and therefore early life stages of bivalves are commonly used in bioassays for quality assessment of seawater and sediment (Gosling, 2015). Geffard et al. (2002), Wang et al. (2009) and Fathallah et al. (2011) suggested that heavy metals essentially restrain growth in larval bioassays. A study by Meyer and Manahan (2010) reported over 180 candidate genes for differential larval growth in the oyster *Crassostrea gigas,* which might prove valuable as ontogenic biomarkers. Half of these genes were identified to be involved in protein and energy metabolism and feeding regulation. Fabbri et al. (2014) also reported high-performance screening of emerging pollutants such as endocrine-disrupting chemicals (EDCs), brominated and perfluorinated compounds (BFRs and PFCs) and pharmaceuticals adapted from the larval toxicity study.

Biological responses have been recorded in early life stages of mussels after exposure to anti-inflammatory medicines, with developmental parameters mainly affected (Almeida et al., 2020). Diclofenac and ibuprofen have been reported by Fabbri et al. (2014) to affect larval development and shell formation of *Mytilus galloprovincialis*, with diclofenac showing an inverted U-shaped dose response, while ibuprofen displayed a dose-dependent influence. Balbi et al. (2018) also recorded adverse impacts of diclofenac on shell formation and on the expression of the genes involved in the process, in the embryo of *Mytilus galloprovincialis*. The impaired development of the embryo-larval stage of mussels *Perna perna* as a result of exposure to a wide range of diclofenac has also been reported by Fontes et al. (2018). Ibuprofen was documented by Pusceddu et al. (2018) – the authors highlighted the slow embryo-larval development in mussels *Perna perna* after 48 hours of exposure. Finally, Munari et al. (2016) recorded that diclofenac altered larval development in clams *Ruditapes philippinarum*.

Metformin and its metabolite, guanylurea, have been demonstrated by Ussery et al. (2018) and (2019) respectively, to reduce the length and wet weight of the larvae of Japanese medaka fish *Oryzias latipes* and also to induce significant changes in some

metabolites related to cellular energetics and proliferation. Other alterations such as scoliosis and abnormal pigmentation in zebrafish embryos (Godoy et al., 2018) and an increase in hepatic glycogen as well as intestinal bacteria in exposed brown trout *Salmo trutta fario* embryos (Jacob et al., 2018) have also been reported.

Despite the sensitivity of the early life stages of bivalves being harnessed for environmental monitoring assessments as outlined earlier, so far there is no record regarding the effects of one of the most widely used pharmaceuticals, acetaminophen, on the early life stages of marine bivalves. In addition, the effects of metformin have to date only been recorded in fish.

5.1.4. Identification of selected transcripts in early life stages

A recent study by Moreira et al. (2018) presented the first oligo-microarray of *Mytilus galloprovincialis* which includes sequences across oocytes, larval and juvenile stages. The authors found thousands of differentially expressed genes, with significantly regulated biological processes and clear evidence of maternal RNA transfer to offspring. In other molluscs, Kitano et al. (2017) documented that several *VTG*s were detected in the embryo of the squid *Uroteuthis edulis*, and were possibly going through a breakdown process at different stages of embryogenesis. Maternally supplied *VTG* in bivalves was also reported by Li et al. (2014), who detected *VTG* mRNA expression in unfertilised eggs, fertilised eggs, embryos and early-stage larvae of the scallop *Chlamys farreri*. Ali et al. (2018) reported alterations in the expression of androgen receptor and oestrogen receptor in larvae of fathead minnows *Pimephales promelas* following early life exposure to water and sediment found within agricultural runoff. In addition to this, Heyland and Moroz (2005) suggested that while marine invertebrate larvae may synthesise hormones endogenously at very low levels, the exogenous route is a primary source, thus indicating the presence of the hormone receptors in early life stages.

Romero et al. (2011) characterised caspase genes in mussels *Mytilus galloprovincialis* and indicated that apoptosis in molluscs is involved in the larval developmental process

and seems to constitute an important immune response that can be initiated by different triggers. Moreover, the authors also highlighted that the apoptotic process in molluscs has a similar complexity to that of vertebrates, suggesting the involvement of many others regulatory genes in this highly complex network.

Research on the larval development and metamorphosis mechanisms of bivalves are scarce, however Blalock et al. (2018) constructed a comprehensive transcriptome database by de novo sequencing of a mixed RNA pool from *Mytilus edulis* larvae and zygotes. The role of *FAS* and *HSP70* transcripts in cellular response to stress and sperm-egg recognition was highlighted after exposure to EDCs.

These findings thus suggest the importance of the new genomic tools in ontogenic studies, in particular in blue mussels *Mytilus edulis*.

This present chapter discusses the effects of acetaminophen and metformin on the very early life stages of mussels. After external fertilisation, it is likely that zygotes (and then larvae) follow natural motions dictated by regular daily waves and currents, tidal changes, storms or other environmental factors. In this case, there is a high chance for these early life stages to encounter various contaminants, such as acetaminophen and metformin. This study attempts to elucidate the likely consequences of pharmaceutical exposure during fertilisation, depicting a scenario that may occur in the marine environment. The mRNA expressions of *vitellogenin* (*VTG*)*, vitelline envelope zona pellucida domain-9* (*V9*)*, estrogen receptor-2* (*ER2*)*, heat shock protein-70* (*HSP70*)*, caspase-8* (*CASP8*)*, B-cell lymphoma-2* (*BCL2*) and *Fas cell surface death receptor* (*FAS*) in mussel zygotes were investigated. The variation of these target genes is presented here to allow a comparison between adult and early life stages of mussels.

This chapter provides the first data on the effects of acetaminophen and metformin on the early life stage of blue mussels *Mytilus edulis*, from a molecular perspective.

5.2 Materials and methods

5.2.1 Gamete acquisition through spawning induction and dissection

The parental mussels for this experiment were collected manually from Hove beach, East Sussex, UK (50.823797, -0.173423) at low tide in March 2019. Mussels were transferred immediately to the laboratory, kept on ice during the transport, and stored in the freezer (-20 ± 2 °C) until spawning induction. Each mussel was cleaned promptly prior to the stimulation, by removing the byssus and materials attached to the shell. The clean mussel was then placed in a beaker with approximately 50 – 100 mL filtered artificial seawater, commencing the spawning induction. The artificial seawater (Instant Ocean® Sea Salt, USA) used for these techniques was prepared as per manufacturer's instructions and filtered through 1.2 µm Whatman grade GF/C microfiber glass filter paper (GE Healthcare, UK). The induction of spawning was performed using the heat shock technique (Sreedevi et al., 2014; Capolupo et al., 2018) by placing the mussels in a -20 °C freezer for few minutes and then straight to room temperature 16 ± 2 °C. Other physical stimuli, such as stirring and cutting of the byssus were additionally applied, in combination with the temperature shock. Once the spawning was completed (successful rate ± 50 %), the concentrated gametes at the bottom of the beaker were transferred, using a pipette, into a clean tube.

In cases where spawning had not occurred even after induction, gametes were obtained manually by performing a direct dissection on the mussel's gonads. A sterile scalpel was used to create deep incisions in the gonads, allowing the gametes to be released. Incised gonads were collected into a clean tube with a small amount of artificial seawater. All equipment used in the procedure was cleaned and washed after each spawning induction or dissection of each mussel.

An exact amount of 1 mL of gametes' suspension was pipetted to a Sedgewick rafter counting cell (Camlab, UK) and examined using light microscopy, with 40× magnification. This observation was performed to determine the sex, the concentration and the maturity of gametes, for the fertilisation process.

5.2.2 Fertilisation

Only adequately mature gametes were used for the fertilisation, as indicated by microscopic analysis. The gamete suspension of each male and female mussel went through filtration using sieves with fine mesh, size 50 µm and 100 µm respectively (Capolupo et al., 2018), to minimise the presence of debris in the following step. The filtered gamete suspensions were then combined at a ratio of 1:10 egg to sperm (Franzellitti et al., 2017), calculated from the concentration of gametes determined in the previous step. Each combined suspension was derived from only one male and one female mussel to ensure the progeny resulted from a single pairing only. The combined suspension was then kept in an incubator shaker at low speed at a temperature of 16 ± 2 °C to allow optimum conditions for fertilisation, as adapted from Franzellitti et al. (2017). After 30 minutes, each combined suspension was checked to confirm fertilisation (successful rate ≥ 75 %). The fertilisation was confirmed to have occurred when fertilised eggs with a polar body or a clear layer in the outer circle of eggs was observed (Capolupo et al., 2018).

5.2.3 Exposure to acetaminophen and metformin

Once fertilisation was confirmed, the combined suspension was distributed evenly to clean tubes representing experimental treatments. A control group, containing artificial water only, without acetaminophen or metformin, was arranged alongside the exposed groups. Fertilised eggs were exposed to acetaminophen, at three different concentrations of 40 ng/L, 250 ng/L, and 100 µg/L. The metformin exposure consisted of three exposed groups with nominal water concentrations of 100 ng/L, 80 µg/L, and 150 µg/L. These experiments used the exact nominal concentrations previously applied to adult mussels (see Chapters 3 and 4). Each contaminant, acetaminophen (BioXtra, ≥ 99.0 %, Sigma-Aldrich) or metformin (European Pharmacopoeia Reference Standard, Sigma-Aldrich), was added to the treatment corresponding to each concentration, in a final volume of 25 mL. Only artificial seawater (Instant Ocean® Sea Salt, USA), prepared in accordance with the instructions from the manufacturer, was used in this experiment for dilution purposes, to make up the volume of 25 mL. The fertilised eggs

were then incubated in a shaking incubator (New Brunswick, Germany) at low speed, at a temperature of 16 ± 2 °C for 30 minutes following the addition of the contaminant to each tube. After 30 minutes incubation, the exposures were terminated, as indicated by Capolupo et al. (2018), and samples were centrifugated at 1,000 × *g* for 5 minutes. The supernatant of each sample was discarded, and each sample was reconstituted and gently mixed with 1 mL RNA*later*™ (Invitrogen, UK).

5.2.4 RNA extraction and reverse transcription

The procedures followed here were identical to the methods described in section 3.2.6.1, except for several reagents used in these particular experiments. Prior to the analyses, the samples containing fertilised eggs stored in the RNA*later*™ solution were centrifuged at 1,000 × *g* for 5 minutes. The supernatant was discarded, and the pellet went through the RNA extraction process. Total RNA was extracted from each sample (n = 12 for each experimental group) separately using SurePrep™ TrueTotal™ RNA Purification Kit (Fisher BioReagents™, UK) and SurePrep™ RNA/Protein Purification Kit (Fisher BioReagents™, UK) following the procedures provided by the manufacturers. The RNA concentration extracted was quantified with Qubit™ RNA HS Assay Kit (Invitrogen, UK) and Qubit® Fluorometer (Invitrogen, UK) as per manufacturers' instructions. For the complementary DNA (cDNA) synthesis, total RNA of each sample underwent reverse transcription following manufacturer's protocol of High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems™, UK) and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, UK). Qubit™ dsDNA HS Assay Kit and Qubit® Fluorometer (Invitrogen™, UK) were used to quantify the first strand of cDNA according to the instructions from the manufacturer.

5.2.5 Quantitative real-time PCR

The quantitative real-time PCR was performed using LightCycler® Nano Real-Time PCR System with PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, UK) as described in section 3.2.6.2. The primer sequence of each gene used in the real time qPCR are shown in Table 5, and the internal control genes were *18S ribosomal RNA*

(*18S rRNA*) and *elongation factor-1 alpha* (*EF1*) as suggested by Cubero-Leon et al. (2012).

5.2.6 Data analysis

The relative changes in target genes' expression resulting from qPCR were calculated following the comparative $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001), expressed as fold changes to the control group. Prior to the calculation, the raw data expressed as cycle quantification (*Cq*) from real-time qPCR analysis of each target gene mRNA expression was standardised against the average *Cq* of housekeeping genes *18S rRNA* and *EF1* as normalisation factor.

Statistical analysis was performed using GraphPad Prism 8. One-way analyses of variance (ANOVA) were performed, followed by Tukey's post-hoc multiple comparison tests to determine significant differences between the groups with $p < 0.05$.

5.3 Results

5.3.1 Exposure to acetaminophen

The mRNA expression of each target gene in zygotes, after exposure to acetaminophen, is displayed in Figures 32 – 38. Figure 32 shows that the expression of *VTG* in all exposure groups was significantly upregulated compared to the control group ($p < 0.05$). Whist *VTG* was similarly expressed in the groups exposed to 40 ng/L and 250 ng/L of acetaminophen to almost 3 fold changes to the unexposed group, the highest concentration group (100 µg/L) displayed the highest upregulation to about 4.5 fold changes.

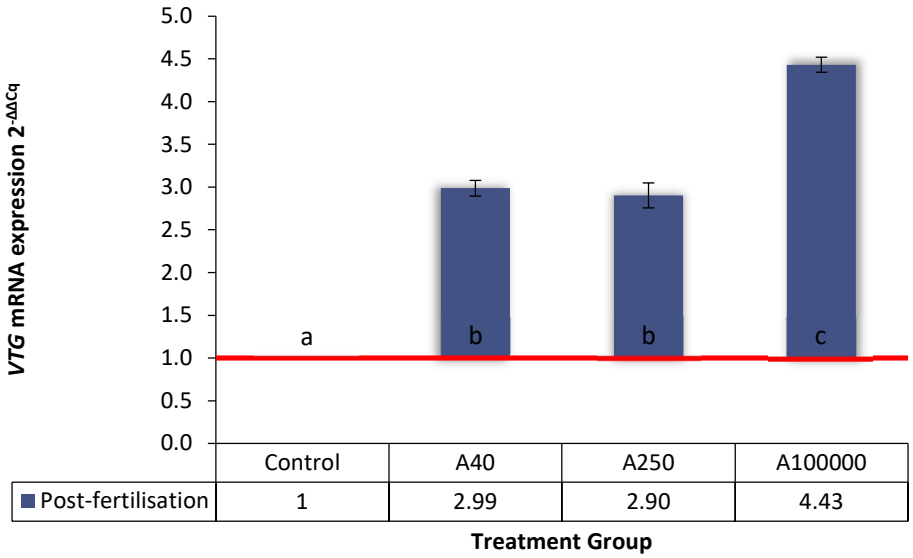


Figure 32 Summary of mRNA expression of *VTG* in fold changes compared to control group in early life mussels (n = 12). A40, group exposed to acetaminophen at 40 ng/L; A250, group exposed to acetaminophen at 250 ng/L; A100000, group exposed to acetaminophen at 100000 ng/L. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

The mRNA expression of *V9* was downregulated in all exposed groups as shown in Figure 33, although the downregulation was not significant compared to the control group. Still, the wide error bars (representing SD) also hint at a wide distribution of data, suggesting a highly varied expression of *V9* in the groups exposed to acetaminophen.

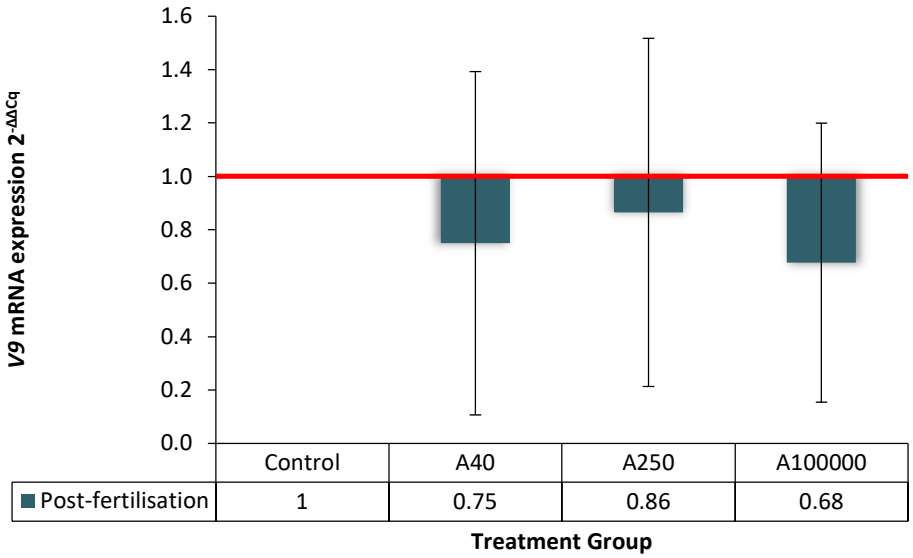


Figure 33 Summary of mRNA expression of *V9* in fold changes compared to control group in early life mussels (n = 12). A40, group exposed to acetaminophen at 40 ng/L; A250, group exposed to acetaminophen at 250 ng/L; A100000, group exposed to acetaminophen at 100000 ng/L. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

A more concentration-related trend was observed in the mRNA expression of *ER2* (Fig. 34). Here, the level of *ER2* transcripts in the groups exposed to acetaminophen was suppressed compared to the control group. The *ER2* mRNA expression in the groups exposed to 40 ng/L and 250 ng/L displayed similar levels of downregulation, almost 3 folds down, which were slightly lower than the highest concentration group (100 µg/L). In addition, only these two low concentration groups (40 ng/L and 250 ng/L) displayed significantly different expression of *ER2* compared to the control group ($p < 0.05$).

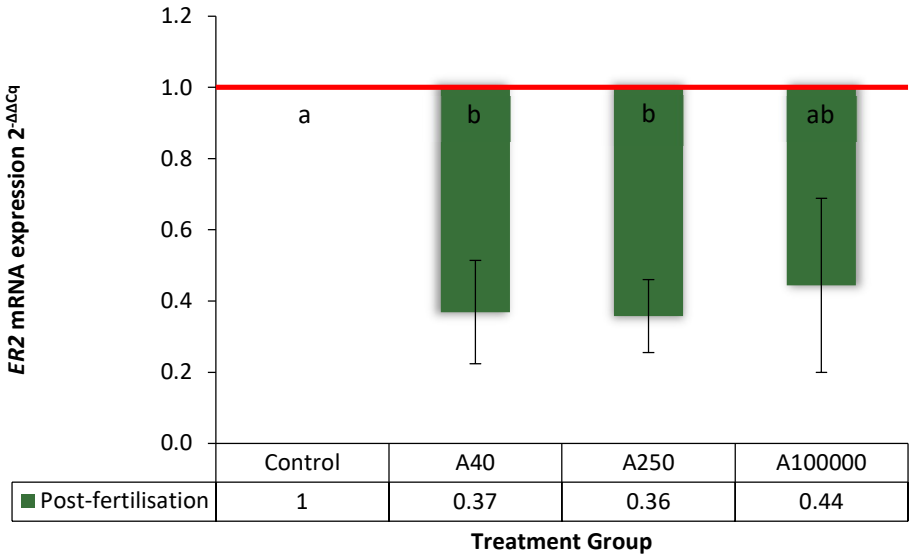


Figure 34 Summary of mRNA expression of *ER2* in fold changes compared to control group in early life mussels (n = 12). A40, group exposed to acetaminophen at 40 ng/L; A250, group exposed to acetaminophen at 250 ng/L; A100000, group exposed to acetaminophen at 100000 ng/L. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

A brief exposure of acetaminophen also induced downregulation of the expression of *HSP70* (Fig. 35), although not statistically significant compared to non-exposed zygotes. In addition, there was no significant variation in the expression of this transcript between the different acetaminophen treatments. Nevertheless, a wide variation of expression (shown by the error bars) is also observed here, suggesting that *HSP70* levels fluctuate between individuals.

Downregulation in all groups exposed to acetaminophen was also observed in the mRNA expression of *CASP8* as displayed by Figure 36. The downregulation, however, did not follow a monotonic response and was not significantly different to the non-treatment group. Again, the result shows a highly variable expression of *CASP8* in the exposed early life mussels, as shown by a wide range of SD.

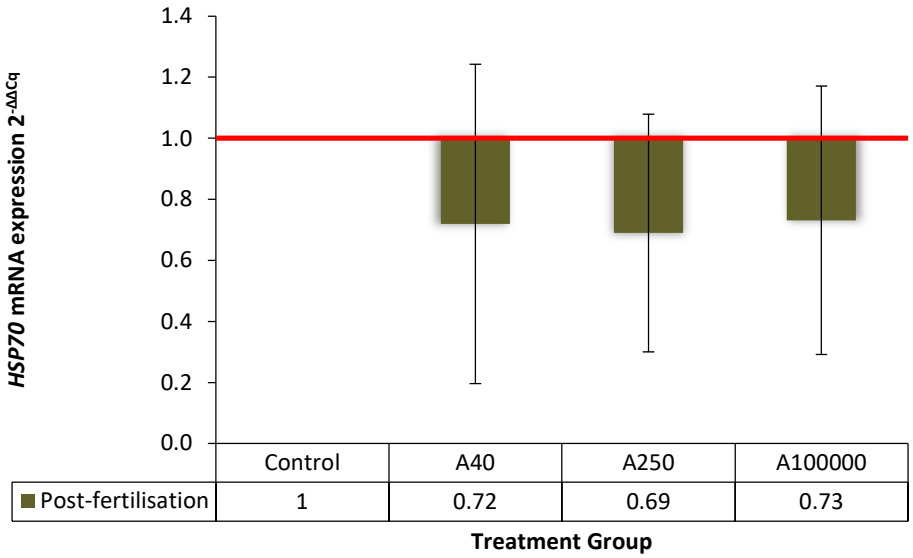


Figure 35 Summary of mRNA expression of *HSP70* in fold changes compared to control group in early life mussels (n = 12). A40, group exposed to acetaminophen at 40 ng/L; A250, group exposed to acetaminophen at 250 ng/L; A100000, group exposed to acetaminophen at 100000 ng/L. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

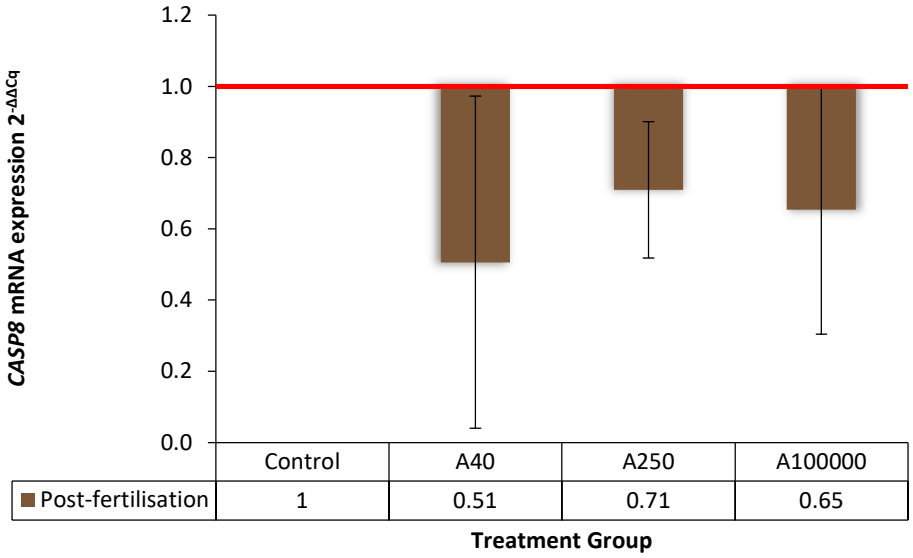


Figure 36 Summary of mRNA expression of *CASP8* in fold changes compared to control group in early life mussels (n = 12). A40, group exposed to acetaminophen at 40 ng/L; A250, group exposed to acetaminophen at 250 ng/L; A100000, group exposed to acetaminophen at 100000 ng/L. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

Figure 37 and 38 show the mRNA expression of *BCL2* and *FAS*, respectively. Acetaminophen exposure induced lower expression compared to the non-exposed group in both target genes. Early life mussels exposed to 40 ng/L and 250 ng/L acetaminophen showed similar levels of *BCL2* mRNA expression, lower than those exposed to the highest concentration of acetaminophen, although the difference here is not significant. The downregulation of this transcript in exposed mussels was also not significantly different to the control mussels. The mRNA expression of *FAS* showed a more concentration-related response, with the lowest concentration of acetaminophen (40 ng/L) inducing more suppression of this target gene. Only early life mussels in the group 40 ng/L showed a result statistically different to the control group ($p < 0.05$), with *FAS* transcription underexpressed 3 fold.

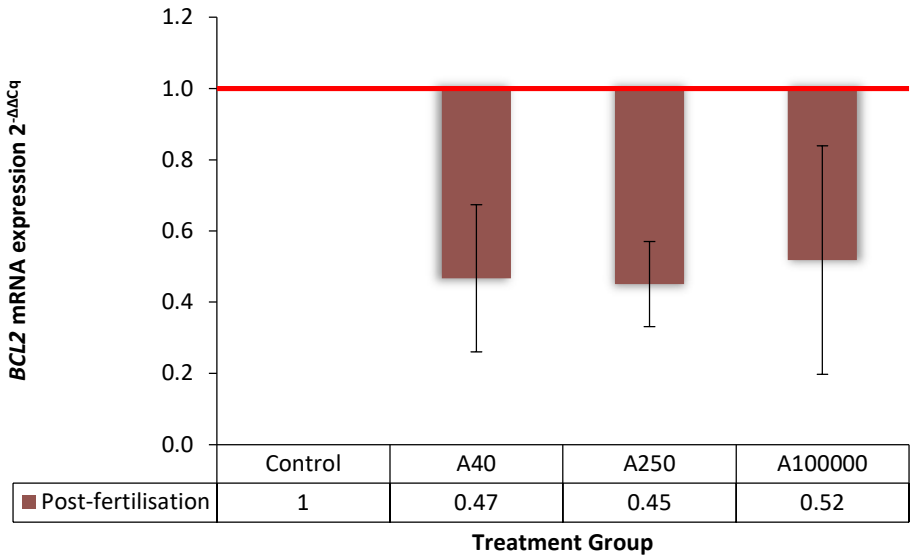


Figure 37 Summary of mRNA expression of *BCL2* in fold changes compared to control group in early life mussels (n = 12). A40, group exposed to acetaminophen at 40 ng/L; A250, group exposed to acetaminophen at 250 ng/L; A100000, group exposed to acetaminophen at 100000 ng/L. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

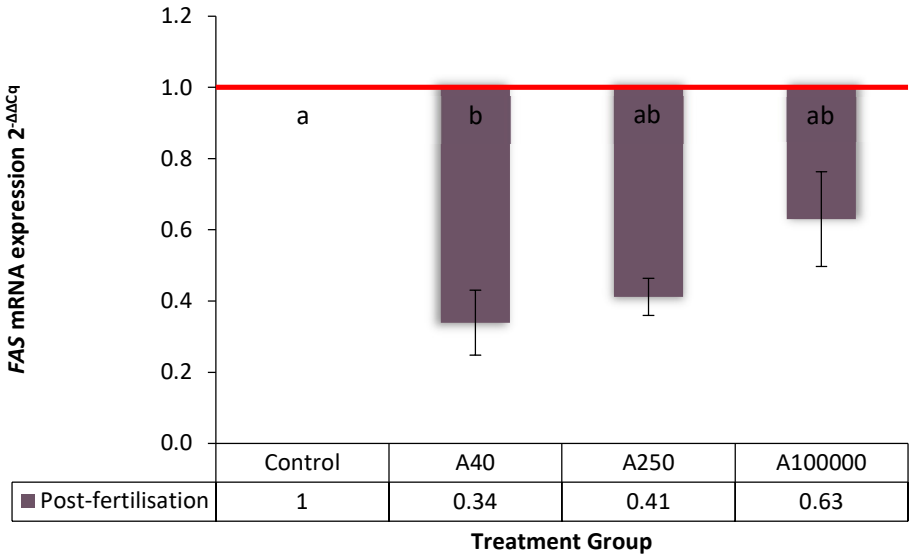


Figure 38 Summary of mRNA expression of *FAS* in fold changes compared to control group in early life mussels (n = 12). A40, group exposed to acetaminophen at 40 ng/L; A250, group exposed to acetaminophen at 250 ng/L; A100000, group exposed to acetaminophen at 100000 ng/L. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

5.3.2 Exposure to metformin

The variation of expression of target genes in early life stage mussels after exposure to metformin is displayed in Figures 39 – 45. The *VTG* mRNA expression was overexpressed in early life stages, exposed to various concentrations of metformin (Fig. 39). All metformin-treated groups showed significant upregulation to almost 4 fold changes compared to the non-treated group ($p < 0.05$). The result does not show a dose-dependent response, with the transcript expressed highest in mussels exposed to 80 µg/L metformin.

On the other hand, metformin did not induce significant alterations in the expression of *V9* (Fig. 40). Only a very slight downregulation was observed in all groups exposed to metformin, and this was not significantly different to the control group. The result, however, shows a wide distribution of data, represented by wide error bars.

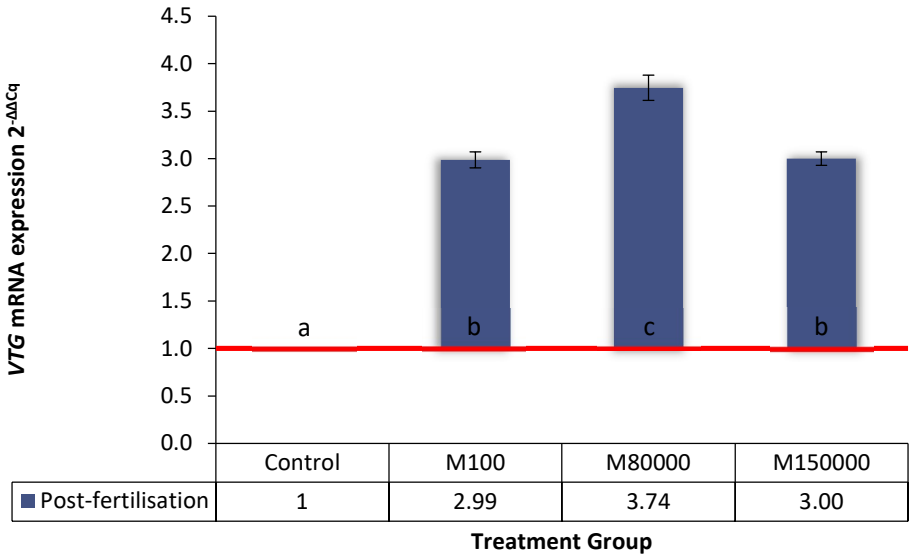


Figure 39 Summary of mRNA expression of *VTG* in fold changes compared to control group in early life mussels (n = 12). M100, group exposed to metformin at 100 ng/L; M80000, group exposed to metformin at 80000 ng/L; M150000, group exposed to metformin at 150000 ng/L. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

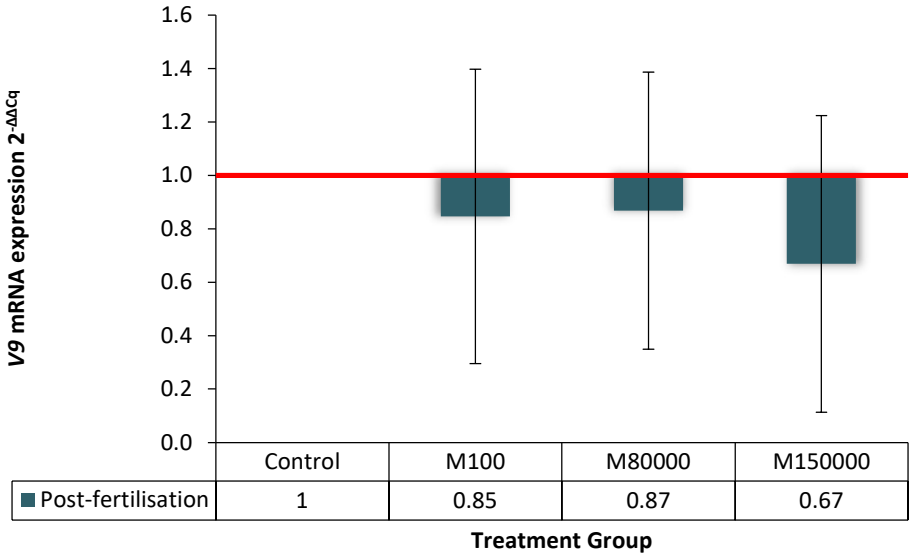


Figure 40 Summary of mRNA expression of *V9* in fold changes compared to control group in early life mussels (n = 12). M100, group exposed to metformin at 100 ng/L; M80000, group exposed to metformin at 80000 ng/L; M150000, group exposed to metformin at 150000 ng/L. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

A very brief exposure to metformin was shown to downregulate the mRNA expression of *ER2* in early life mussels (Fig. 41). Only mussels in the groups 100 ng/L and 150 µg/L displayed significantly different results to the unexposed mussels, with the expression being 2 to 3 fold downregulated, respectively.

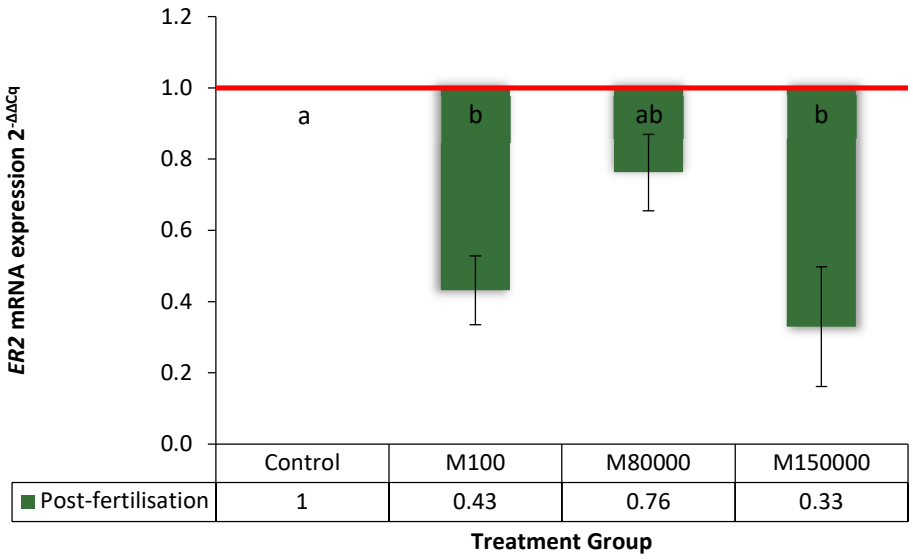


Figure 41 Summary of mRNA expression of *ER2* in fold changes compared to control group in early life mussels (n = 12). M100, group exposed to metformin at 100 ng/L; M80000, group exposed to metformin at 80000 ng/L; M150000, group exposed to metformin at 150000 ng/L. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

The mRNA expressions of *HSP70* (Fig. 42) and *CASP8* (Fig. 43) were also downregulated in early life mussels after exposure to metformin over a very short time. Both transcripts showed downregulation in all treatment groups, even though this was not statistically different to the control group. However, each transcript displayed a different pattern of expression. *HSP70* was expressed at a similar level in the groups exposed to 100 ng/L and 80 µg/L, whilst the highest concentration group of metformin (150 µg/L) displayed the lowest level of mRNA expression. The expression pattern of *CASP8*, on the other hand, showed a dose-related trend, with the lowest concentration of metformin (100 ng/L) expressing the lowest level of this transcript.

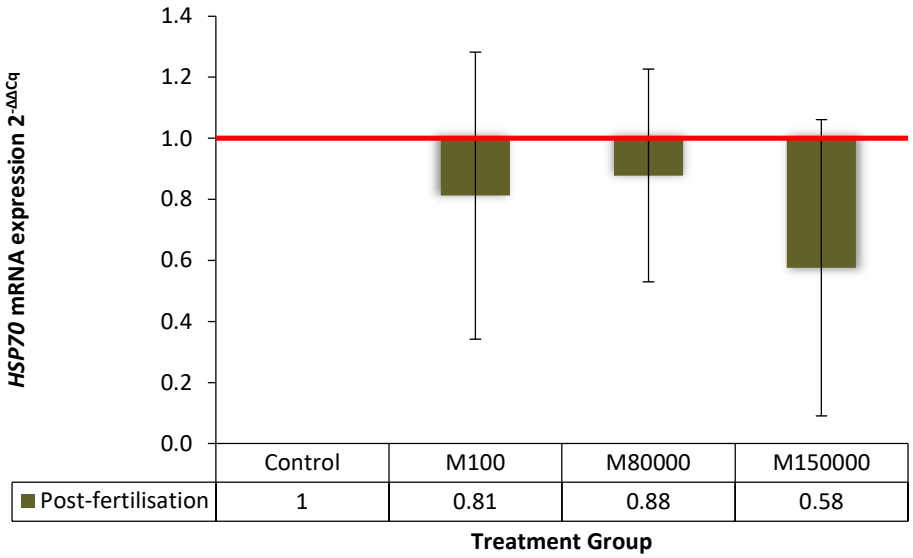


Figure 42 Summary of mRNA expression of *HSP70* in fold changes compared to control group in early life mussels (n = 12). M100, group exposed to metformin at 100 ng/L; M80000, group exposed to metformin at 80000 ng/L; M150000, group exposed to metformin at 150000 ng/L. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

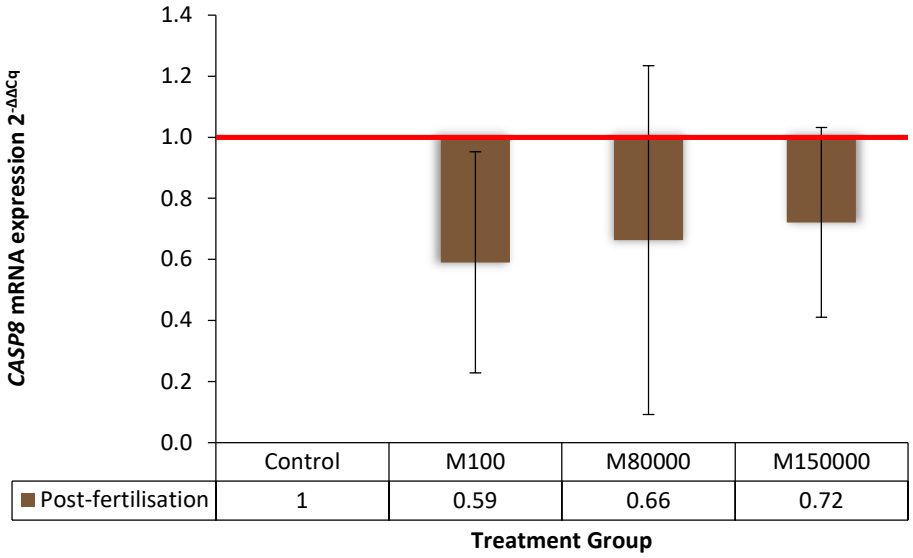


Figure 43 Summary of mRNA expression of *CASP8* in fold changes compared to control group in early life mussels (n = 12). M100, group exposed to metformin at 100 ng/L; M80000, group exposed to metformin at 80000 ng/L; M150000, group exposed to metformin at 150000 ng/L. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

The downregulation of *BCL2* and *FAS* mRNA expression was also observed in the early life mussels after exposure to a range of concentrations of metformin, as shown respectively in Figures 44 and 45. In Figure 44, only the 150 µg/L of metformin, the highest concentration used in this exposure, induced a significantly different result, 3 folds downregulated compared to the non-exposed group. The results in other concentration groups were not significantly different, and the variation in the mRNA expression of *BCL2* did not follow a dose-related trend. *FAS* mRNA expression (Fig. 45), however, displayed a concentration-dependent result, with the lowest concentration of metformin (100 ng/L) inducing the most suppression to the expression of this transcript. The mRNA expression of *FAS* in the exposed mussels was roughly 2 – 3 folds downregulated, with only mussels in the group 100 ng/L showing a statistically significant result ($p < 0.05$).

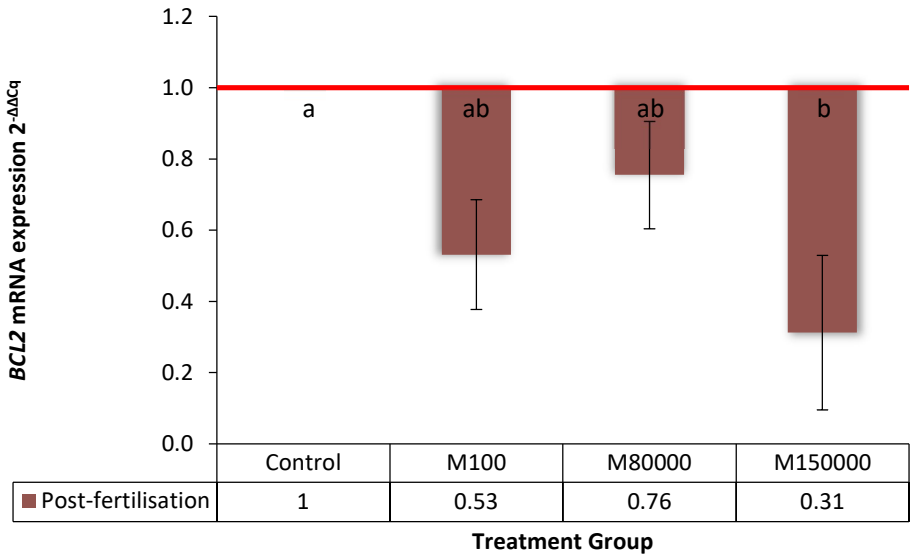


Figure 44 Summary of mRNA expression of *BCL2* in fold changes compared to control group in early life mussels (n = 12). M100, group exposed to metformin at 100 ng/L; M80000, group exposed to metformin at 80000 ng/L; M150000, group exposed to metformin at 150000 ng/L. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

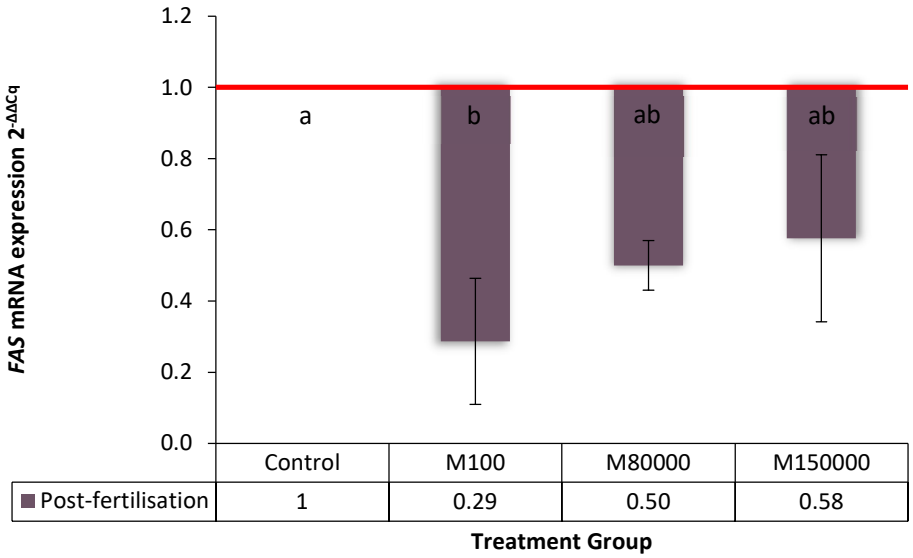


Figure 45 Summary of mRNA expression of *FAS* in fold changes compared to control group in early life mussels (n = 12). M100, group exposed to metformin at 100 ng/L; M80000, group exposed to metformin at 80000 ng/L; M150000, group exposed to metformin at 150000 ng/L. Different letters represent statistically significant differences between groups, bars represent SD (one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$).

5.4 Discussion

5.4.1 Acetaminophen exposure to post-fertilisation early life mussels

The results presented in this study demonstrate that even a very short exposure to acetaminophen in the early life stage of mussels, immediately after fertilisation, can induce an overexpression of *VTG* up to about 4.5 fold changes (Fig. 32). The significant upregulation of this transcript in all exposed groups relative to the control group highlights the capability of acetaminophen to cause a significant alteration even at low concentrations.

Vitellogenin provides the precursor for yolk proteins in developing oocytes and is a sensitive biomarker of oestrogen activity, as its synthesis is controlled via oestrogenic pathways (Meng et al., 2010; Nagler et al., 2010). The upregulation here indicates that acetaminophen may promote vitellogenesis in this post-fertilisation stage of mussels, the function that in vertebrates is regulated by the oestrogen receptor. In male vertebrates, vitellogenin expression is used as a biomarker for early detection of the biological effects of oestrogens (Jastrow et al., 2017). The results thus indicate that the

upregulation of *VTG* here might be associated with the role of oestrogen receptor to initiate vitellogenesis, suggesting the potential for acetaminophen to have an effect similar to EDC-like substances. However, further research is required to confirm whether the vitellogenesis induction here is achieved via oestrogen receptor or a non-genomic pathway.

The result also suggests that acetaminophen may induce different responses in adult and early life stages of mussels. The mRNA expression of *VTG* was significantly downregulated in adult mussels after short and long exposures to acetaminophen, in sharp contrast to the upregulation observed in early life mussels. As mentioned above, *VTG* mRNA expression is a sensitive indicator for early detection of exposure to endocrine disrupting chemicals (EDCs) in vertebrates, both in laboratory and in wild populations (Leusch et al., 2005; Shanthanagouda et al., 2013; Gilannejad et al., 2016). Nevertheless, its presence in invertebrates including its role and mechanism of action is subject to further investigation on this much-debated topic (Porte et al., 2006; Matozzo et al., 2008). For example, in the tobacco hornworm *Manduca sexta*, vitellogenin and its mRNA are first detectable in the prepupal stage; and production of both can be enhanced by methoprene, a juvenile hormone analogue, as demonstrated by Schafellner et al. (2008).

The contrasting responses here in very young and adult mussels, after exposure to acetaminophen, might provide new insights into the role of *VTG* in mussels and the possible target mechanism of this contaminant. The different response of *VTG* in adult and early life stages of bivalves was documented previously by Li et al. (2014). The authors reported the expression pattern of *VTG* in the zhikong scallop *Chlamys farreri*, which was detected in the very early life stages, before being supressed in the developed larval and juvenile stages, and then upregulated in the young adult.

The different life stage of mussels not only dictates the *VTG* responses to acetaminophen exposure (such as upregulation in early life versus downregulation in adult mussels), but also the sensitivity of mussels to the concentration of acetaminophen. Whilst the *VTG* expression was equally downregulated in all exposed

adult mussels without any differences relating to acetaminophen concentration, the expression in the early life mussels showed the highest upregulation in the group exposed to 100 µg/L, the highest concentration of acetaminophen used in the experiment. These results provide an indication of the changes that could be inflicted by acetaminophen at concentrations found in the environment, to both adult and early life mussels. Analysing these results in the context of the natural pattern of *VTG* expression documented by Li et al. (2014), it can be confirmed that acetaminophen exposure drastically modulates this transcript, which then exhibits high expression in zygotes and supressed levels in adults.

Wu et al. (2018) indicated the significance of vitelline envelope in early life stages and fertilisation, as it is involved directly in the protection of eggshells. Notwithstanding the important role of this transcript, the downregulation of *V9* mRNA expression observed here is not significant (Fig. 33), suggesting that acetaminophen is unlikely to induce significant impacts through this lower expression of this transcript. The data is widely distributed however, implying that *V9* is expressed at various levels in early life mussels exposed to acetaminophen. There is a possibility that this gene is only induced in the later stage of early life mussels, as indicated by Wu et al. (2018) who reported that the expression of zona pellucida genes in the Nile tilapia *Oreochromis niloticus* only started from 30 days after hatching. This suggests that these genes may be supressed or only expressed at a very low level at the very early life stage.

The expression of *ER2* has been studied in various invertebrates (Kajiwara et al., 2006; Keay and Thornton, 2009; Ni et al., 2013; Jones et al., 2017), including mussels (Puinean et al., 2006; Balbi et al., 2016). Here, acetaminophen seems to supress the expression of *ER2*, resulting in the downregulation of this transcript in all exposed early life mussels (Fig. 34). This transcript is also downregulated in adult mussels exposed to acetaminophen in both the short and long term, as recorded in Chapter 3. There is a suggestion of a dose-related response in the *ER2* mRNA expression in the early life mussels, although the expression level is similar in the two groups exposed to lower concentrations of acetaminophen (40 ng/L and 250 ng/L). This result suggests that acetaminophen concentrations detected in seawater could affect the downregulation

level of *ER2* in early life mussels, which could lead to subsequent changes in any mechanism associated with oestrogen receptor. This is supported by Nagasawa et al. (2015), who characterised oestrogen receptors in *Mytilus edulis* and *Mytilus galloprovincialis*, and revealed that these genes may have the capacity to autoregulate their expression and are also highly responsive to oestrogenic substances in the environment.

The data on variation of expression of oestrogen receptor suggests potential further impacts that may occur on gametogenesis and reproduction processes in bivalves (Ciocan et al., 2010). As the changes in *ER2* expression here are recorded in the early life stage of mussels, and oestrogen receptors are part of many regulatory mechanisms in bivalves, the results here thus pose a concern over the potential of acetaminophen to disrupt these regulatory mechanisms involving oestrogen receptors, which might lead to other adverse effects in the later developmental stages.

The *ER2* downregulation observed here also indicates that the initiation of vitellogenesis, which generally involves oestrogen receptor in vertebrates, might not occur through the *ER2* signalling pathway in the case of early life mussels, as the acetaminophen exposure does not induce the expression of this transcript. Instead vitellogenesis in early life mussels is more likely associated with the upregulation of *VTG* only. This supports previous research showing that *VTG* is maternally supplied in bivalves, as indicated by Li et al. (2014), who reported that the *VTG* mRNA was expressed in unfertilised eggs, fertilised eggs, embryos and early-stage larvae of the scallop *Chlamys farreri*.

Acetaminophen apparently does not cause substantial changes in the expression of *HSP70* and *CASP8* in early life stage mussels. Both target genes play important roles in the regulation of apoptosis. The expression of *HSP70* as a response to various stress-related exposures has been documented in mussels (Minguez et al., 2013; Giannetto et al., 2017; Gendron et al., 2019), indicating the protective function of this gene in mussel defence mechanisms. In addition, the modulation of caspases' expression by different stimuli in different apoptotic pathways in bivalves has been documented by

Romero et al. (2011). Although the mRNA expression of *HSP70* (Fig. 35) and *CASP8* (Fig. 36) are downregulated here in early life mussels after exposure to acetaminophen, the changes are not significant, and neither transcript displays a direct correlation with the concentration of acetaminophen in this very brief exposure. A recent study by Mlouka et al. (2020) also documented a similar pattern of *HSP70* expression, which was not significant in *Mytilus edulis* larvae (but highly expressed in *Mytilus galloprovincialis* larvae), even though the embryotoxicity test revealed a higher sensitivity of *Mytilus edulis* D-larvae after exposure to increased temperature. In a study by Perry and Lynn (2009), embryos and early larval stages of two invasive bivalves, the Mediterranean mussel (*Mytilus galloprovincialis*) and zebra mussel (*Dreissena polymorpha*) displayed variations in physiological and stress-induced apoptotic values after exposure to a commercial biocide. However, in the majority of exposures, the authors found out that there was a threshold at which apoptosis could no longer be used as a protective measure against damaged cells, and the apoptotic values (TUNEL assay) were back to normal.

Sokolova (2009) highlighted the importance of apoptosis in playing a key role in the homeostasis and function of the immune system in molluscs, including in the defence against parasites and pathogens. The mRNA expression of both *BCL2* (Fig. 37) and *FAS* (Fig. 38), two genes involved in apoptosis, were downregulated after a very brief acetaminophen exposure in early life mussels. Acetaminophen induced a significant downregulation in the expression level of *FAS* only, as the *BCL2* expression was not significantly different to the control group. The results presented here indicate that whilst the presence of acetaminophen slightly decreases the expression of *BCL2* in post-fertilised mussels, the level of this mRNA downregulation is not influenced by the acetaminophen concentration in the environment.

On the contrary, the concentration of acetaminophen is very likely to influence the expression of *FAS* according to the result here, although only early mussels exposed to 40 ng/L showed a significant downregulation. Although these genes have been recorded to work together via antagonistic actions (Botros et al., 2010) as is also supported by the results here, this result also highlights the possibility that in early life

mussels, acetaminophen might have the highest impact on *FAS* mRNA expression among all apoptosis-related target genes investigated in this study. The identification of the expression of these apoptotic regulatory genes is important to understand their interactions in programmed cell death mechanisms in less documented species such as mussels, particularly in their early life stages.

5.4.2 Metformin exposure to post-fertilisation early life mussels

The *VTG* mRNA expression was upregulated in early life mussels exposed to various concentrations of metformin (Fig. 39). Vitellogenesis is long known to be associated with the oestrogen receptor, as its initiation involves estradiol-17β released by oocytes (Gagné and Blaise, 2005). The significant upregulation observed here thus hints at the potential of metformin to act as a non-traditional EDC in the early life stage of mussels. This is not the first time the upregulation of *VTG* has been recorded. Niemuth et al. (2014) reported the induction of *VTG* mRNA transcription in adult male fathead minnows *Pimephales promelas* following exposure to metformin. In invertebrates, Ciocan et al. (2010) recorded an upregulation of *VTG* mRNA expression in the gonads of *Mytilus edulis* after exposure to EDCs during the early stages of gonad development. The previous study also recorded an upregulation of *VTG* in adult mussels *Mytilus edulis* exposed to metformin (Koagouw and Ciocan, 2018).

It is important to note that the expression of *VTG* shows different responses depending on the duration of exposure to metformin as recorded in Chapter 4. In adult mussels, metformin increased the expression of *VTG* over a short time, while after a longer exposure the contrary effect was observed: mRNA expression was reduced. The results here, combined with those recorded in adult mussels, highlight that metformin exposure can generate different patterns of *VTG* expression as a response both to the duration of exposure and to the life stage of exposed mussels. Different responses in adult animals and early life stages to a stressor have been documented in several studies (Baussant et al., 2011; Domingues et al., 2013; Baurand et al., 2015), as mentioned before, demonstrating the sensitivity and importance of the life stage of animals when exposure occurs in determining their response.

The significant upregulation of *VTG* recorded in the result here also implies that early life mussels are likely more susceptible to the alterations induced by pharmaceuticals, as a similar response was recorded in the acetaminophen exposure. The level of *VTG* expression, however, is not related to the concentration of metformin, with the transcript expressed highest in mussels exposed to 80 µg/L metformin. Nevertheless, it is possible that this could be due to hormetic effects, which are very common in toxicology, as also reported by Fabbri et al. (2014).

The result of *V9* mRNA expression (Fig. 40) shows that metformin did not induce significant alterations in the expression of this transcript. Despite the importance of vitelline envelope in the development of zygotes as documented by Arukwe et al. (1997), a very slight downregulation observed here in the exposed group was not significant, and is thus unlikely to have an impact. However, it is still worth noting that the result shows a wide distribution of data, implying that *V9* response varied significantly between individuals. The expression of vitelline envelope zona pellucida genes was reported only 30 days after hatching in Nile tilapia *Oreochromis niloticus* (Wu et al., 2018) and has been proposed as biomarker in young adults and juveniles of Pacific abalone *Haliotis discus discus* (Yu et al., 2018), suggesting that the expression of this gene may be more evident in the later stages of early life mussels. In addition, the downregulation of *V9* expression is also documented in adult *Mytilus edulis* exposed to metformin as reported by Koagouw and Ciocan (2018).

A rapid exposure of metformin to early life mussels induced downregulation of *ER2* mRNA expression at all concentrations of metformin (Fig. 41). Downregulation of *ER2* is also recorded in all adult mussels exposed to metformin for a short period, as discussed in Chapter 4. The concentration of metformin here seems to be unrelated to the level of *ER2* expression in early life mussels. The pattern of *ER2* expression displayed an inverted U-shape, which in Fabbri et al. (2014) is also discussed as a hormetic effect of the contaminant, where the response does not follow the concentration. However, fluctuation in *ER2* mRNA expression is also recorded in adult blue mussels as reported in a previous study (Koagouw and Ciocan, 2018), and also in the long-term exposure result from Chapter 4. Whilst the fluctuation recorded in adult

mussels is more likely related to the natural variation in *ER2* expression during different stages of gametogenesis as reported by Ciocan et al. (2010), the pattern of expression here is more likely a result of hormetic effects of metformin in the early life stage of mussels. The fluctuation in the expression of oestrogen receptor was also recorded in fertilised eggs of *Mytilus galloprovincialis* exposed to bisphenol-A (BPA), which showed upregulation after 24 hours and then downregulation after 48 hours (Balbi et al., 2016).

Apart from the pattern of expression, the results presented here contribute to concern over the potential of metformin to induce endocrine disruption by altering the level of expression in the oestrogen receptor, particularly in the early life of mussels. The downregulation of *ER2* here also complements the *VTG* expression result in this study, in that it provides an important indication regarding the mechanism of vitellogenesis. The combined results thus suggest that in the early life stage of mussels exposed to metformin, upregulation of *VTG* might be related to the initiation of vitellogenesis, but this mechanism may not be associated with the oestrogen receptor and may be achieved via another pathway.

The expression of *HSP70* has been recorded in marine mussels (Cellura et al., 2006; Aleng et al., 2015; Dallas et al., 2016) and harnessed as a biomarker (Ratkaj et al., 2015; Rossi et al., 2016). In bivalves, *HSP* family genes play an important role in the stress protection mechanism induced by various factors, including thermotolerance, host defence and chemical exposure (Buckley et al., 2001; Paulton et al., 2012). Despite the downregulation in the mRNA expression of *HSP70* (Fig. 42) and *CASP8* (Fig. 43) in early life mussels after exposure to metformin, the variations were not significant compared to unexposed mussels. Hence, this result indicates that metformin may not generate critical alterations in the expression of *HSP70* and *CASP8*. Whilst Zhang et al. (2014) suggested that *CASP8* may play an important role in responding to temperature stress, Romero et al. (2011) indicated that the apoptotic process could be regulated in bivalve molluscs by modulating caspase genes and also provided evidence of caspase-specific responses to pathogens and pollutants. Although not significant, the expression pattern of *CASP8* in this study hints at a dose-related trend, which may link the alteration to this transcript with the level of metformin in the environment,

considering both target genes play vital roles in apoptosis mechanisms. Zhang et al. (2015) have also indicated that caspase-dependent apoptotic pathways may significantly contribute to tetracycline-induced apoptosis in the early life stages of zebrafish.

The modulation of apoptosis-regulatory gene transcripts maintains a healthy cell death balance either by activation or inhibition of apoptosis, which is an important mechanism to maintain molluscan immune systems (Sokolova, 2009). Romero et al. (2011) and Estévez-Calvar et al. (2013) documented that *BCL2* and *FAS*, together with other apoptosis genes, play a significant role in apoptosis by modulating their expression. In the early life mussels after exposure to a range of concentrations of metformin, the mRNA expression of *BCL2* (Fig. 44) and *FAS* (Fig. 45) were downregulated. These results, together with the expression of both transcripts in adult mussels (as described in Chapter 4), again emphasise the connection between *BCL2* and *FAS* that work together through their modulation in regulating cell death. However, there are two differences observed in the variation of these transcripts. Firstly, *FAS* mRNA expression followed a concentration-dependent trend, whilst *BCL2* did not. Secondly, only mussels in the highest concentration group (100 µg/L) showed significantly different *BCL2* mRNA expression to the control group, while *FAS* mRNA expression was only significantly different in the group exposed to the lowest concentration of metformin (40 ng/L). This provides an indication of the way in which the level of metformin in the environment could possibly affect apoptosis-related events in the early life stage of mussels. Taken together, the expression of *BCL2* and *FAS* here suggest a strongly related modulation that is potentially linked to the concentration of metformin in this study, as these transcripts work through antagonistic actions. Several studies have recorded the expression of *BCL2* family genes in *Mytilus galloprovincialis* (Châtel et al., 2011) and in *Crassostrea gigas* (Medhioub et al., 2013), modulating apoptosis through their interactions with other apoptosis-related genes. This suggests that each of these apoptosis-related transcripts is highly dependent on other genes in apoptosis networks.

5.4.3 Differential responses to contaminants in adult and early life stages

Many studies have shown that a contaminant could induce different responses in adults compared to the early life stage. Domingues et al. (2010) highlighted the importance of early life stage assays in the assessment of chemical effects, after showing that biomarkers used in their study were much more responsive in larvae than in adults of zebrafish *Danio rerio* exposed to hexavalent chromium. Prášková et al. (2011) reported a low acute toxicity of ketoprofen in juvenile stages of zebrafish, however highlighted that the substance seems to be toxic for embryonic stages. Similarly, Prášková et al. (2012) documented the acute toxicity of acetylsalicylic acid in zebrafish and revealed higher sensitivity in the embryonic stages of zebrafish compared to its juveniles. Following early life exposure to water and sediment from agricultural runoff, Ali et al. (2018) reported alterations in the expression of androgen receptor and oestrogen receptor in larvae of fathead minnows *Pimephales promelas*. Furthermore, the authors also reported feminisation of adult males after the same exposure.

In invertebrates, Baussant et al. (2011) conducted a study on adult and early life stages of *Mytilus edulis* exposed to dispersed oil and revealed that severe effects were more evident in the larval stage. A study by Pineda et al. (2012) analysed stress sensitivity across four developmental stages of two ascidians, *Styela plicata* and *Microcosmus squamiger*, and revealed that fertilisation and larval development were the most sensitive stages. Baurand et al. (2015) documented the different expression of metallothionein isoforms in adults and embryos of the terrestrial snail *Cantareus aspersus* exposed to cadmium, exhibiting a comparatively more important role in embryos compared to adult individuals.

Although mostly reported in fish, these findings emphasise that adult individuals and early life stages could exhibit different responses to exactly the same stressor, with more dramatic effects observed in the early life stages. Again, this highlights the susceptibility of early life stages to various environmental contaminants. With this in mind, the results presented here might depict a particularly concerning picture, indicating that alteration in the expression of some transcripts recorded here might

generate more detrimental consequences in the later stages of mussels' development. This also suggests that alterations caused by acetaminophen and metformin here might produce more adverse effects related to the mussels' ontogeny.

5.5 Summary

Seven target genes were investigated in this study, in order to observe their modulation after a very brief exposure to acetaminophen and metformin immediately after fertilisation in blue mussels. Acetaminophen exposure generated substantial changes in the mRNA expression of *VTG*, *ER2* and *FAS*, whilst the expression level of *VTG*, *ER2*, *BCL2* and *FAS* were significantly affected by metformin. This provides information on the likely effects of those specific contaminants on the morphology and physiology of the larvae and juveniles.

Different contaminants also induce different patterns of gene expression in adult and early life mussels. Most target genes were expressed in a similar manner after exposure to acetaminophen, except for *VTG* which was upregulated in the early life stage of mussels, but downregulated in adult mussels. While metformin induced more fluctuating results between short- and long-term exposure in adult mussels, the expression of most target genes in this study is more similar to that of short-term exposure in adult mussels, with the only exception being *FAS* expression. Overall, the different patterns of expression in adult and early life mussels here emphasises the importance of life stage at the time when exposure occurs in inflicting different responses generated by these target genes.

Interestingly, some transcripts display unusual patterns of expression, not necessarily showing strict or random responses to the concentration of contaminant, or following a dose-related trend, but more likely showing hormetic effects instead. The expression of *V9* and *CASP8* follow this pattern after being induced by acetaminophen, although the variation was not significant. In response to metformin exposure, several target genes (*VTG*, *ER2* and *BCL2*) display significantly different results to the control group while displaying hormetic effects. This effect, in which the response to the

contaminant does not follow its concentration but displays a U-shaped or inverted U-shaped result, is quite common in toxicology studies. Nevertheless, these results also suggest that in the early life stage of mussels, metformin is able to induce certain molecular responses which are not strictly related to the concentrations detected in the environment, unlike acetaminophen.

CHAPTER 6

General conclusions

6.1 Summary of findings

Chapter 2 laid out the foundation of this thesis by providing a snapshot of the seawater quality of Jakarta Bay, the most polluted coastal area in Indonesia. Specifically, it revealed the detection of acetaminophen at two sites in this area: Angke and Ciliwung. This finding represents the first data on the presence of acetaminophen in Indonesian seawater. The relatively high concentrations recorded at both sites are likely to have resulted from extensive accumulation of urban and industrial waste into watercourses that discharge directly into Jakarta Bay. In addition to the detection of acetaminophen, high levels of nutrients as well as unusual concentrations of some heavy metals were recorded, suggestive of a lack of municipal wastewater treatment within Jakarta itself. The results of this chapter have important implications regarding human health, both in terms of access to safe water for those living in close proximity to affected watercourses, and in terms of food safety of fisheries located in potentially contaminated areas of Jakarta Bay. More directly relevant to the aims of this thesis, the levels of acetaminophen detected also contribute to growing concern surrounding the health of marine organisms in this area, which has been explored in more detail in Chapters 3 and 5. The results presented in this chapter suggest the need for the inclusion of stricter regulation of wastewater treatment, particularly regarding the disposal of pharmaceutical products. In addition, they highlight the need for the Indonesian government to consider the inclusion of pharmaceutical contamination in regulations addressing seawater quality.

Chapter 3 built on the ecotoxicological aspect of the findings from chapter 2 by focusing on the effects of acetaminophen on marine organisms, specifically the blue mussel *Mytilus edulis*. This chapter presented the results of both short- and long-term

exposures of blue mussel adults to three different concentrations of acetaminophen (40 ng/L, 250 ng/L and 100 µg/L), focusing on both histopathological and molecular-level effects. Water analysis showed that mussels readily and consistently took up acetaminophen from seawater. All exposed groups displayed a number of different pathologies, the most widespread of which were haemocytic infiltration in short-term exposed groups, and follicle dilatation in groups exposed over a longer period. The molecular analysis, which focused on the mRNA expression of selected genes (*VTG, V9, ER2, HSP70, CASP8, BCL2*, and *FAS)* in *Mytilus edulis* gonads, revealed differing patterns of downregulation in response to their exposure to acetaminophen. After short exposure, the mRNA expression of all genes showed downregulation, with some transcripts (*V9*, *HSP70*, *BCL2* and *FAS*) showing concentration-dependent trends and thus potentially qualifying as biomarkers for acetaminophen exposure. In the case of *ER2*, the variation in expression was suggestive of a dose-dependent trend in males only. Finally, *VTG* and *CASP8* mRNA expression showed similar levels of downregulation in all exposure groups, regardless of sex. After long-term exposure to acetaminophen, the overall pattern across gene transcripts was one of more uniform downregulation between exposed groups, without any apparent concentration-dependent trends. This indicates that the lowest concentrations of acetaminophen can induce similar responses to the highest concentrations, given only a longer exposure time. This pattern clearly demonstrates the importance of exposure time on the effects of acetaminophen in mussels, suggesting that longer exposure may produce effects of equivalent magnitude to those of a shorter exposure at much higher concentrations. Overall, the observed patterns of downregulation in several reproduction-related genes indicates the potential for acetaminophen to impair reproduction in *Mytilus edulis*.

Chapter 4 focused on the effects of another widely used pharmaceutical, metformin, on *Mytilus edulis*. In this case mussels were exposed to a range of concentrations of contaminant (100 ng/L, 80 µg/L, and 150 µg/L), as well as a single concentration (40 µg/L) coupled with heat stress. Histopathological and molecular-level effects were again recorded. The results suggest that metformin is able to induce the alteration of

immune response in mussels, indicating a concentration-dependent trend. Additionally, they reveal a range of pathologies initiated by exposure to metformin, the nature of which seem to be related to exposure time. After short exposure, the majority of responses were apoptosis-related (follicle dilatation, gamete degeneration, atresia), while longer exposure prompted a shift towards the occurrence of more inflammatory reactions (haemocytic infiltration, haemocytic aggregate, parasitic infiltration). These results highlight the significance of the duration of environmental exposure to metformin on the specific mechanisms targeted in non-target organisms, such as *Mytilus edulis*. As with acetaminophen exposure, exposure to metformin also induced variations in the mRNA expression of all genes investigated (*VTG*, *V9*, *ER2*, *HSP70*, *CASP8*, *BCL2* and *FAS*). Metformin was shown to induce contrasting patterns according to the mussels' sex in a number of transcripts (*VTG*, *V9*, *CASP8*, *FAS*). Several transcripts followed concentration-dependent trends, mostly in groups exposed over a short period, indicating the potential for the variation of these transcripts in the gonads of *Mytilus edulis* to be affected by the level of metformin in the environment. As with acetaminophen, the variation in expression observed after different periods of exposure signifies the importance of exposure duration to metformin's impact in the environment, as shown by all transcripts. Overall, these findings suggest the potential of metformin to act as a non-traditional EDC.

Chapter 5 followed on from chapters 3 and 4 by investigating the effects of both acetaminophen and metformin on *Mytilus edulis*, although this time focusing specifically on the early life stage (zygotes), following a brief exposure after fertilisation. As in the previous 2 chapters, the mRNA expression of *VTG, V9, ER2, HSP70, CASP8, BCL2* and *FAS* was measured, allowing a comparison between the variation of these target genes in adult and in early life stages of mussels. Exposure to acetaminophen generated substantial changes in the mRNA expression of *VTG*, *ER2* and *FAS*, while metformin exposure significantly altered the expression level of *VTG*, *ER2*, *BCL2* and *FAS*. Regarding the differences between adult and early life stages, all but one of the target genes were expressed similarly after exposure to acetaminophen. The exception was *VTG*, which was upregulated in the early life stage of mussels but

downregulated in adult mussels. The comparison was less straightforward for metformin, which induced fluctuating results between short- and long-term exposure in adult mussels, as demonstrated in chapter 4. Nevertheless, the expression of most target genes in this study was similar to that of short-term exposure in adult mussels, with the exception being *FAS* expression. Overall, the different patterns of expression in adult and early life mussels highlight the importance of life stage at the time of exposure in eliciting different responses in these genes. Finally, a number of transcripts displayed trends suggesting hormetic effects. This was particularly evident in mussels exposed to metformin, in which *VTG*, *ER2* and *BCL2* displayed hormetic effects which were significantly different from control groups. These results suggest that in the early life stage of mussels, unlike acetaminophen, metformin is more likely to induce biological responses which are not necessarily related to the level of contaminant detected in seawater.

6.2 Implications of the study

Over recent years, there has been growing concern regarding chronic pharmaceutical pollution of the aquatic environment. As rising human populations coincide with a higher demand for pharmaceuticals, their continuous emission is very likely to persist. The situation is likely to deteriorate further in the current pandemic, as pharmaceutical companies have seen an enormous demand for pain relief medicine, particularly acetaminophen. Some of these will be ingested and then excreted, but it is predicted that a lot of supply medicines will be disposed of, and will most likely end up in the natural environment in vast quantities. This study is therefore environmentally relevant, and the results here demonstrate that the presence of acetaminophen and metformin in concentrations recorded in the environment have the potential to cause several major changes related to the reproductive system of mussels. These findings also indicate that at least for acetaminophen, the effects are exacerbated after prolonged exposure.

A global pharmaceutical monitoring system would be an ideal initiative that could be used to address this issue. This study may provide some valuable information on the

ecotoxicological data that may be useful for establishing a baseline as part of an ecotoxicological assessment of pharmaceuticals, or possibly, material for any regulation constructed for this purpose. Given that regulation is a policy implementation tool, the need to use these pharmaceuticals as criteria for assessing the health status of marine ecosystems may be urgent. The findings of this study indicate the ecotoxicological impact of human pharmaceuticals on marine life, suggesting a strong need for environmental protection action. For a developing country like Indonesia, further necessary outcomes will include a robust environmental surveillance system linked to regulations as repressive and preventive measures.

Currently, there is only limited information on the seawater chemistry profile in Indonesia, in particular the status of acetaminophen in Indonesian seawater, as well as its effects on marine organisms. This study identifies that the concentration of acetaminophen found in Jakarta Bay is one of the highest levels recorded to date in seawater globally. This finding signifies its importance with regards to regulations in Indonesia relating to seawater or marine coastal areas. Moreover, as also presented in this study (Chapter 2), some heavy metals were present in Indonesian seawater with a quantity that might require attention. Nevertheless, the level of need for further investigation in this area is also unclear since these parameters are not included in the existing regulations. The Decree of the State Minister of the Environment Republic of Indonesia number 51 year 2004 regarding Standard Quality of Seawater, the current government regulation limit, is out of date and unfortunately only covers very few parameters in seawater. As addressed in this study, regulation regarding seawater covers numerous areas that are closely related to public interest, including marine-based food products, recreational water and impoverished coastal communities. Updated regulation covering these criteria is therefore urgently required.

Finally, it is known that fish and marine invertebrates have been used to measure the impacts of pharmaceuticals in seawater. The development of new biomarkers and relevant test to assess the impact of contaminants in sentinel organisms through the evaluation of sublethal effects is currently ongoing. Signalling pathways involved in apoptosis are mostly studied in vertebrates, and comparatively very little attention is

given to invertebrates, especially molluscs (Kiss, 2010). Moreover, this review highlights the lack of studies of *CASP8* and *FAS* in molluscs despite their important roles in apoptosis. Although apoptosis is a highly complex mechanism involving many different extrinsic and intrinsic pathways, this study has provided new information regarding this mechanism in marine bivalves. Because apoptosis might be considered a biomarker (Kiss, 2010; Estévez-Calvar et al., 2013), the data in this study therefore serves as a first step not only in understanding the ecotoxicological effects of acetaminophen and metformin on marine organisms, but also in the development of potential new tests. An understanding of the mechanisms of apoptotic-related modulation in marine bivalves may provide a framework with which to assess the genotoxic risk for marine organisms exposed to environmental stressors.

6.3 Further investigation

Further investigations exploring and confirming some related aspects, especially the mechanisms of action of both acetaminophen and metformin in mussels, are encouraged. The detailed points are mentioned as part of the discussion in each chapter, with main points as follows.

Regarding the presence and the reduction pattern of acetaminophen, further analyses are required to validate the quantification of the contaminant in mussel flesh, to confirm the uptake of acetaminophen. Further investigations are also recommended to confirm the findings in this study on the role of duration of acetaminophen exposure, as shorter exposure time to acetaminophen is likely to alter the immune state of mussels and thus increase the incidence of inflammatory responses; whilst after a longer contact to acetaminophen, mussels are more susceptible to suffer pathological conditions related to the apoptosis and cell death mechanism. The data in this study also suggest that *ER2* expression in mussel males is more responsive to acetaminophen than in females, and therefore should be further investigated as a potential biomarker candidate. As *HSP70* was expressed equally regardless of sex and showed a concentration-based trend in the short exposure, *HSP70* might also be useful

as a marker in a dose-dependent experiment. Studies investigating the impact of pharmaceuticals on *HSP70* expression are recommended.

The findings in this study document alterations that can be linked with the level of metformin in the environment, thus suggesting the potential of metformin to be considered an emerging contaminant of concern, and further investigations are required to clarify its mechanism of action. Metformin seems to alter the mechanisms or events related to programmed cell death reactions at first, and then later targets the immune system of the organism as the time of exposure increases. Further investigations are needed in order to confirm this sequential pattern of affected mechanisms in non-target organisms, in the environmental context. As suggested in Chapter 4, *HSP70* has potentially been highly expressed in high temperature groups in order to protect cells and the tissue by inhibiting apoptosis. Further investigation is needed to elucidate the mechanism by which this protein displays any direct effect on cells and tissues of mussel gonads, or indeed whether it plays an indirect role in causing the observed effects, in combination with other regulating factors. In relation to *ER2* expression, as discussed earlier, the natural variation in its expression level during gametogenesis has to be taken into account and further investigation is therefore encouraged to clarify and confirm findings in this study.

The *VTG* upregulation induced by acetaminophen may or may not be associated with the oestrogen receptor regulation in promoting vitellogenesis, therefore further research is required to confirm whether the vitellogenesis induction is achieved via oestrogen receptor or via a non-genomic pathway.

6.4 Recommendations

Although it is understood that the present situation is far from ideal, a number of recommendations could be made, mainly to the Indonesian government, following the explanations given in the preceding section.

A new revised and updated legislation, forming an Indonesian government regulation regarding standard seawater quality criteria, is essential for immediate

implementation. This revised and updated regulation is recommended to cover more parameters, including pharmaceuticals. This regulation should be strong enough to enforce the implementation of environmental monitoring programmes and a sufficient wastewater system for the public and industries.

Moreover, since household disposal is documented as one of the prominent routes of pharmaceutical contamination (Bound and Voulvoulis, 2005), there is an urgent need for regulation on the handling and disposal of unused pharmaceuticals to be widely introduced to the public. This regulation would need to be disseminated to the Indonesian public in order to make it easier to implement it as part of preventive measures.

There is currently no monitoring programme for any pharmaceuticals in Indonesia. This lack necessitates the need for a comprehensive monitoring programme to be part of environmental policy in Indonesia. Again, the addition of pharmaceuticals to the parameters of water quality criteria is recommended.

An adequate wastewater system is imperative in order to reduce further contamination. As the fourth most populous country in the world (US-CIA, 2017), a robust wastewater treatment system is required to address the waste problem for both the public and industry.

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APPENDICES

Appendix 1. Photographic depictions of sampling locations in Jakarta Bay

a. Angke

b. Ciliwung

Appendices 1-a, b, c, d show photographs of the sampling locations in Jakarta Bay. The complete description of sites is presented in Chapter 2 Table 1.

These photographs accompany this information in describing the sampling sites.

c. Tanjung Priok

d. Cilincing

e. Traditional fishing boats supplying seafood products to Angke market

f. Local fishermen trading in a traditional market in Angke

Appendices 1-e, f, g show one of the sampling sites, Angke, in more detail.

These photographs accompany the information describing the area as presented in Chapter 2, depicting: one of the main seafood markets (1e), a traditional market (1f), and a residential area (1g).

g. Local residences in Angke market district

h. Sample collection activities

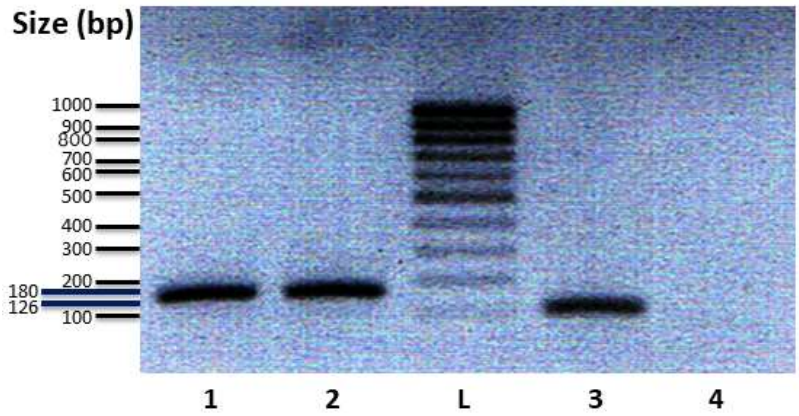
Appendix 1-h depicts the process of collecting seawater samples.

Appendix 2. Map and photograph of mussel sampling location in the UK

Appendix 2 shows a map of the mussel collection site in Hove, UK (top). The exact location of the site is represented by the blue marker, and illustrated in the photograph (bottom).

Appendix 3. Species confirmation of *Mytilus edulis*

The species confirmation of mussel samples was described in Koagouw and Ciocan (2018). The mussel species *Mytilus edulis* is confirmed by amplification of the *Glu* gene. According to Inoue et al. (1995), the sequence lengths of *Mytilus edulis* and *Mytilus galloprovincialis* are 180 bp and 126 bp, respectively. The PCR reactions were performed using Crimson Taq DNA Polymerase (New England BioLabs) as instructed by manufacturer guidelines, with the following parameters: initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of 30 seconds at 95 °C, 1 minute at 55 °C, and 1 minute at 68 °C, and a final extension at 68 °C for 5 minutes. A known sample of *Mytilus galloprovincialis* and a "no template" sample (without DNA, replaced with water) were also amplified in the same batch reaction. Amplified PCR products and exACTGene DNA ladder were electrophoresed in 0.8 % (w/v) agarose/TBE gel containing ethidium bromide. Photographs were captured to record the electrophoresed gel visualisation under UV light.



The figure above shows the result of *Glu* gene amplification visualised on 0.8 % agarose gel electrophoresis. Samples (lanes 1 and 2) exhibiting a single band size 180 bp identified the species as *Mytilus edulis*. A sample of *Mytilus galloprovincialis* was also amplified in the same PCR reaction and showed a single band size of 126 bp, as expected. A control with no DNA template was also included in the same PCR reaction and showed no band, confirming the non-contamination of the reactions.

Appendix 4. Ethics approval

University of Brighton

Animal Welfare and Ethics Review Board

School of Pharmacy and Biomolecular Sciences
Huxley Building
Moulsecoomb
Brighton
BN24GJ

22/06/2020

Ref: 2020-7150-Koagouw THE IMPACT OF PHARMACEUTICALS ON MARINE BIVALVES

Dear Wulan

The Animal Welfare and Ethics Review Board are happy to offer a favourable ethical opinion for this study.

Favourable ethical opinion is given on the basis of the information provided in your application, including a project end date of 02/01/2021. Please note that the Animal Welfare and Ethics Review Board must be informed of any changes to the research process after a favourable ethical opinion has been given. If you need to make changes to your proposal, including requesting an extension, please complete and submit a change request form in order that the Animal Welfare and Ethics Review Board can determine whether the changes will necessitate any further ethical review.

We wish you all the best with your research and hope that your research study is successful.

Best wishes

Dr Charley Chatterjee

Chair, Animal Welfare and Ethics Review Board

Appendix 5. Publications and disseminations from this thesis

Koagouw, W. & Ciocan, C. 2019. Effects of short-term exposure of paracetamol in the gonads of blue mussels *Mytilus edulis*. *Environmental Science and Pollution Research International*. https://doi.org/10.1007/s11356-019-06861-w

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Koagouw, W., 2019. Evaluation of Reproduction-Related Genes as Possible Targets of Paracetamol in the Blue Mussel, *Mytilus edulis*. In: SETAC (Society of Environmental Toxicology and Chemistry), *SETAC Latin America 13th Biennial Meeting*. Cartagena, Colombia, 15 – 18 September 2019. Cartagena: SETAC Latin America.

Koagouw, W., 2019. Alterations in the gonads of blue mussels *Mytilus edulis* exposed to paracetamol. In: SETAC (Society of Environmental Toxicology and Chemistry), *SETAC North America 40th Annual Meeting*. Toronto, Canada, 3 – 7 November 2019. Pensacola: SETAC North America.