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Ecotoxicological effects of polyethylene microplastics, with and without adsorbed contaminants, on the clam *Scrobicularia plana* (da Costa, 1778) using a multi-biomarker approach.

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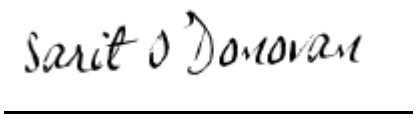

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ABSTRACT

The potentially detrimental impacts of plastic debris on the marine biota are numerous and diverse with deleterious physical effects recorded. Microplastics, defined as plastic particles ≤ 5mm, are of particular concern due to their ubiquitous distribution in the marine environment. Low density polyethylene accounts for ~17% of European plastic production, with use dominated by the packaging industry.

This study investigated the ecotoxicological effects of exposure of low density polyethylene microplastics, with and without added contaminants, in gill tissues and haemocytes of the peppery furrow shell clam, *Scrobicularia plana.* Environmentally relevant concentrations of contaminants, benzo[a]pyrene, oxybenzone, and perfluorooctane sulfonic acid, were adsorbed to microplastics to evaluate the potential role of plastic particles as a source of chemical contamination once ingested. *S. plana* were exposed to microplastics at a concentration of 1 mgL^{-1} for 14 days. To clarify any effects of exposure, a set of biomarkers were employed, including the quantification of antioxidant (superoxide dismutase, catalase, glutathione peroxidase) and biotransformation (glutathione-S-transferases) enzyme activities, oxidative damage (lipid peroxidation levels), genotoxicity (single and double strand DNA breaks) and neurotoxicity (acetylcholinesterase activity).

Neither a genotoxic nor an inhibitory neurotoxic effect was recorded. The dominant response in catalase and glutathione-S-transferase was attributed to virgin microplastics, suggesting the observed effects were due to physical ingestion and the potential mechanical injuries that result. Superoxide dismutase and lipid peroxidation, showed an increase in activity in contaminated microplastic treatments relative to virgin microplastic, suggesting a synergistic effect of physical ingestion and chemical exposure. A reduction in glutathione peroxidase activity was observed in contaminated microplastic treatments, compared to virgin microplastic, indicating an antagonistic effect may have occurred.

Further analysis is needed to confirm the bioavailability of microplastics and that the observed biomarker responses are a result of microplastic exposure, and chemical exposure in the case of superoxide dismutase and lipid peroxidation.

Keywords: microplastic, *Scrobicularia plana*, biomarker, benzo[a]pyrene, oxybenzone, perfluorooctane sulfonic acid.

RESUMO

Os potenciais impactos negativos de detritos de plástico nos organismos marinhos são diversos, e são também numerosos os impactos físicos que já foram observados. O impacto ambiental dos microplásticos (partículas de plástico com tamanho igual ou inferior a 5 mm) têm ganho especial atenção e preocupação tendo em conta a sua distribuição universal e a sua presença no meio marinho. As propriedades físico-químicas do plástico confere-lhe a capacidade de adsorver e concentrar contaminantes químicos hidrofóbicos presentes nas águas marinhas. A concentração de contaminantes em microplásticos é especialmente preocupante pois os organismos marinhos podem ingerir os microplásticos contaminados e estes contaminantes podem vir a ser dessorvidos e acumulados nos tecidos gordos dada a sua natureza lipofílica, o que representa um elevado e potencialmente prolongado risco ambiental. Uma vez ingeridos, os microplásticos podem-se acumular no sistema digestivo, ser excretados, ou sofrerem translocação entre tecidos.

O polietileno de baixa densidade é o segundo tipo de polímero mais produzido e representa cerca de 17% da produção Europeia de plástico. Este polímero é predominantemente utilizado na indústria de embalagem de alimentos, sendo também usado na produção de sacos reutilizáveis e revestimentos usados na agricultura. A maioria dos países da União Europeia recicla entre 15 a 30% dos plásticos, sendo que o restante é enviado para aterros sanitários, incinerado ou acaba descartado indiscriminadamente. São várias as vias de entrada de plástico no ambiente marinho e tanto a presença de macro- como de micro-plásticos está fortemente correlacionada com a densidade populacional do local.

O presente estudo investiga os efeitos ecotoxicológicos da exposição a microplásticos de polietileno de baixa densidade, com e sem contaminantes adsorvidos, nas brânquias da ameijoa *Scrobicularia plana*. Este estudo incide principalmente no estudo das brânquias, visto que em estudos anteriores verificou-se que este é o principal tecido por onde os microplásticos entram no organismo. A *S. plana* é considerada uma espécie fundamental na estrutura e funcionamento dos ecossistemas costeiros e estuarinos, e é também apropriada para ser utilizada como organismo bioindicador para avaliar a saúde ambiental dos ecossistemas estuarinos. Concentrações ambientalmente relevantes de poluentes orgânicos persistentes (benzo[a]pireno - BAP, oxibenzona - BP3 e ácido perfluorooctano sulfónico - PFOS) foram adsorvidos a microplásticos de forma a avaliar o potencial das partículas de plástico como fonte de contaminação química para os organismos uma vez ingeridas. Indivíduos de *S. plana* foram

expostos a 1 mg L^{-1} de microplásticos (11-13 µm) durante 14 dias. Durante este período não foi adicionado alimento de forma a minimizar a interacção dos microplásticos com o alimento, por exemplo fitoplâncton. Foi analisado um conjunto de biomarcadores de forma a clarificar o efeito da exposição, que incluíram a quantificação: da actividade enzimática de enzimas antioxidantes (superóxido dismutase, catalase, glutationa peroxidase) e de biotransformação (glutationa-S-transferase); do dano oxidativo (níveis de peroxidação lipídica); da genotoxicidade (quebra da cadeia de ADN simples ou dupla); e da neurotoxicidade (actividade da acetilcolinesterase). O índice de condição foi também analisado para avaliar o estado geral de saúde dos organismos ao longo do tempo e nos diferentes tratamentos.

Os parâmetros físico-químicos e a saúde geral dos indivíduos de *S. plana* permaneceram estáveis durante todo o período experimental e em todos os tratamentos. Existe alguma evidência que os microplásticos contaminados por benzo[a]pireno e oxibenzona induziram dano no ADN após 14 dias de exposição. No entanto, estes resultados devem ser interpretados com precaução pois não se verificou uniformidade na resposta entre os 3 parâmetros analisados relativos ao dano de ADN. Não se registou efeito genotóxico da presença de microplásticos virgem nem de microplásticos contaminados por ácido perfluorooctano sulfónico.

A actividade da glutationa peroxidase diminuiu nos tratamentos com microplásticos contaminados em comparação com os microplásticos virgem, indicando um provável efeito antagónico. A inibição da actividade da glutationa peroxidase pode ser uma resposta ao efeito tóxico da exposição a microplásticos contaminados.

Em relação à actividade das enzimas catalase e glutationa-S-transferase não se observaram efeitos sinérgicos entre a ingestão de microplásticos e exposição química. A resposta dominante foi atribuída aos microplásticos virgem, sugerindo que os efeitos observados possam estar relacionados com o efeito físico da ingestão das partículas e potencial dano físico nos tecidos.

Não se observaram efeitos neurotóxicos de inibição da actividade da acetilcolinesterase, cujos níveis se mantiveram estáveis ao longo do tempo e semelhantes entre os tratamentos controlo, microplásticos virgem e ácido perfluorooctano sulfónico. Após 14 dias de exposição observou-se um aumento significativo da actividade da acetilcolinesterase nos tratamentos com microplásticos contaminados por benzo[a]pireno e oxibenzona em relação ao controlo e microplásticos virgem.

Foi observado um aumento na actividade da superóxido dismutase e na peroxidação lipídica nos tratamentos com microplásticos contaminados em comparação com os tratamentos com os microplásticos virgem, sugerindo um efeito sinérgico entre danos fisicos nos tecidos e exposição química. O aumento da actividade da superóxido dismutase está relacionada com a sua função como primeira linha de defesa dos tecidos contra efeitos de stress oxidativo. O aumento da peroxidação lipídica pode resultar de uma ineficiente função dos mecanismos de redução dos efeitos de stress oxidativo aquando do processamento das espécies reactivas de oxigénio presentes em excesso no organismo.

A biodisponibilidade dos microplásticos e contaminantes deverá ser analisada por forma a verificar que os resultados obtidos com os biomarcadores no presente estudo são efectivamente uma resposta à exposição. A presença de microplásticos nos tecidos será quantificada utilizando técnicas como a espectroscopia de infravermelho com transformada de Fourier e reflectância difusa (DRIFT). A quantificação de cada contaminante (benzo[a]pireno, oxibenzona e ácido perfluorooctano sulfónico) nos tecidos será realizada por cromatografia líquida – espectrometria de massa (LC - MS), e permitirá verificar se estes podem ser dessorvidos após ingestão justificando por exemplo a aparente sinergia entre dano físico e químico, observada nos resultados da superóxido dismutase e da peroxidação lipídica.

É considerado necessário aprofundar os conhecimentos referentes aos efeitos ecotoxicológicos dos microplásticos no meio marinho, com particular foco em avaliar a possibilidade de ocorrer dessorção de contaminantes presentes nos microplásticos uma vez ingeridos e perceber o seu modo de acção no organismo. Estes conhecimentos serão essenciais para o estabelecimento de valores de referência necessários para a avaliação do impacto ambiental de detritos marinhos, de acordo com os objectivos delineados no âmbito da Directiva Quadro da Estratégia Marinha da União Europeia.

Palavras-chave: microplásticos, *Scrobicularia plana*, biomarcadores, benzo[a]pireno, oxibenzona, ácido perfluorooctano sulfónico.

INDEX

ABSTRACT……...i

RESUMO……..ii

INDEX……...v

FIGURE INDEX……...viii

TABLE INDEX……...ix

LIST OF ABBREVIATIONS……..x

1. INTRODUCTION 1

1.1 Plastic Production and Disposal 1

1.2 Sources of Plastics to the Oceans 3

1.2.1 Land Based Sources 3

1.2.2 Sea Based Sources 3

1.3 Macroplastic, Microplastic and Nanoplastic 4

1.3.1 Primary and Secondary Microplastics 5

1.4 Physico-Chemical Characteristics of Microplastics 5

1.5 Microplastic Distribution and Abundance in the Oceans 6

1.6 Biological Impacts of Microplastics in the Oceans 7

1.6.1 Providing a Vector for Invasive Species 7

1.6.2 Ingestion..... 8

1.6.3 Vector of Pollutants 13

1.7 Specific Marine Contaminants 14

1.7.1 Benzo[a]pyrene 14

1.7.2 Oxybenzone 15

1.7.3 Perfluorooctane Sulfonic Acid 16

1.8 Bioindicators 19

1.9 *Scrobicularia plana* 19

1.10 Biomarkers 22
1.10.1 Genotoxicity 23
1.10.2 Oxidative Stress 23
1.10.3 Oxidative Damage 25
1.10.4 Neurotoxicity 25
1.11 Objectives 26
2. MATERIALS AND METHODS 27
2.1 Experimental Design 27
2.2 Microplastics and Contaminants 29
2.3 Condition Index (CI) 29
2.4 DNA Damage 29
2.5 Enzyme Activity 31
2.5.1 Homogenisation of Tissues 31
2.5.2 Superoxide Dismutase (SOD) 31
2.5.3 Catalase (CAT) 32
2.5.4. Glutathione Peroxidase (GPx) 32
2.5.5 Glutathione-S-transferases (GST) 33
2.6 Acetylcholinesterase (AChE) Activity 34
2.7 Lipid peroxidation (LPO) 35
2.8 Total Protein Concentration 36
2.9 Statistical Analysis 36
3. RESULTS 37
3.1 Condition Index (CI) 37
3.2 Virgin LDPE MP 37
3.2.1 DNA Damage: Virgin LDPE MP 37
3.2.2 Enzyme Activity: Virgin LDPE MP 39
3.2.3 Acetylcholinesterase Activity: Virgin LDPE MP 41
3.2.4 Lipid peroxidation: Virgin LDPE MP 41

3.3 BAP Contaminated LDPE MP 42
3.3.1 DNA Damage: BAP Contaminated LDPE MP 42
3.3.2 Enzyme Activity: BAP Contaminated LDPE MP 44
3.3.3 AChE Activity: BAP Contaminated LDPE MP 46
3.3.4 Lipid peroxidation: BAP Contaminated LDPE MP 46
3.4 BP3 Contaminated LDPE MP 47
3.4.1 DNA Damage: BP3 Contaminated LDPE MP 47
3.4.2 Enzyme Activity: BP3 Contaminated LDPE MP 49
3.4.3 AChE Activity: BP3 Contaminated LDPE MP 51
3.4.4 Lipid peroxidation: BP3 Contaminated LDPE MP 51
3.5 PFOS Contaminated LDPE MP 52
3.5.1 DNA Damage: PFOS Contaminated LDPE MP 52
3.5.2 Enzyme Activity: PFOS Contaminated LDPE MP 54
3.5.3 AChE Activity: PFOS Contaminated LDPE MP 56
3.5.4 Lipid peroxidation: PFOS Contaminated LDPE MP 56
4. DISCUSSION 58
4.1 DNA Damage 58
4.2 Oxidative Stress 59
4.3 Neurotoxic Damage 61
4.4 Oxidative Damage 61
4.5 Experimental Challenges 62
4.6 Further Research 64
5. CONCLUSION 67
REFERENCES 69

FIGURE INDEX

1. INTRODUCTION

Figure 1.1 Exterior of a *Scrobicularia plana* shell with concentric growth rings visible…...21

Figure 1.2 *Scrobicularia plana* distribution……21

Figure 1.3 Schematic diagram of antioxidant ROS defence system……...25

2. MATERIALS AND METHODS

Figure 2.1 Aquarium set up and treatment…….....27

Figure 2.2 *S. plana* haemocyte cell, post comet assay electrophoresis……...31

3. RESULTS

Figure 3.1 DNA damage (mean ± SD) in the haemocytes of *S. plana*, expressed as Tail length (μm) (A), OTM (a.u.) (B) and Tail DNA (%) (C) for control (CT) and virgin LDPE (V) treatments……...38

Figure 3.2 SOD (A), CAT (B), GPx (C) and GST (D) activities (mean ± SD) in the gills of *S. plana* for control (CT) and virgin LDPE (V) treatments…….....40

Figure 3.3 AChE activity (mean ± SD) in gill tissues of *S. plana* for control (CT) and virgin LDPE MP (V) treatments…….41

Figure 3.4 LPO levels (mean ± SD MDA) in gill tissues of *S. plana* for control (CT) and virgin LDPE MP (V) treatments…….....42

Figure 3.5 DNA damage (mean ± SD) in the haemocytes of *S. plana* expressed as Tail length (μm) (A), OTM (a.u.) (B) and Tail DNA (%) (C) for control (CT), virgin LDPE (V) and benzo[a]pyrene contaminated LDPE (BAP) treatments…….....43

Figure 3.6 SOD (A), CAT (B), GPx (C) and GST (D) activities (mean ± SD) in the gills of *S. plana* for control (CT), virgin LDPE and benzo[a]pyrene contaminated LDPE MP (BAP) treatments…….45

Figure 3.7 AChE activity (mean ± SD) in gill tissues of *S. plana* for control (CT), virgin LDPE MP (V) and benzo[a]pyrene contaminated LDPE MP (BAP) treatments…….46

Figure 3.8 LPO levels (mean ± SD MDA) in gill tissues of *S. plana* for control (CT), virgin LDPE MP (V) and benzo[a]pyrene contaminated LDPE MP (BAP) treatments…….47

Figure 3.9 DNA damage (mean ± SD) in the haemocytes of *S. plana*, expressed as Tail length (μm) (A), OTM (a.u.) (B) and Tail DNA (%) (C) for control (CT), Virgin LDPE (V) and oxybenzone contaminated LDPE (BP3) treatments…….....48

Figure 3.10 SOD (A), CAT (B), GPx (C) and GST (D) activities (mean ± SD) in the gills of *S. plana* for control (CT), virgin LDPE MP (V) and oxybenzone contaminated LDPE MP (BP3) treatments……...50

Figure 3.11 AChE activity (mean ± SD) in gill tissues of *S. plana* for control (CT), virgin LDPE MP (V) and oxybenzone contaminated LDPE MP (BP3) treatments……...51

Figure 3.12 LPO levels (mean ± SD MDA) in gill tissues of *S. plana* for control (CT), virgin LDPE MP (V) and oxybenzone contaminated LDPE MP (BP3) treatments……...52

Figure 3.13 DNA damage (mean ± SD) in the haemocytes of *S. plana* expressed as Tail length (μm) (A), OTM (a.u.) (B) and Tail DNA (%) (C) for control (CT), virgin LDPE (V) and perfluorooctane sulfonate contaminated LDPE (PFOS) treatments…….....53

Figure 3.14 SOD (A), CAT (B), GPx (C) and GST (D) activities (mean ± SD) in the gills of *S. plana* for control (CT), virgin LDPE MP (V) and perfluorooctane sulfonate contaminated LDPE MP (PFOS) treatments…….....55

Figure 3.15 AChE activity (mean ± SD) in gill tissues of *S. plana* for control (CT), virgin LDPE MP (V) and perfluorooctane sulfonate contaminated LDPE MP (PFOS) treatments...56

Figure 3.16 LPO levels (mean ± SD MDA) in gill tissues of *S. plana* for control (CT), virgin LDPE MP (V) and perfluorooctane sulfonate contaminated LDPE MP (PFOS) treatments...57

4.DISCUSSION

Figure 4.1 Agglomeration of microplastic particles on a Pasteur pipette aeration tube due to electrostatic adherence…….63

TABLE INDEX

1. INTRODUCTION

Table 1.1 Laboratory microplastic exposure experiments on marine organisms……..8

Table 1.2 Environmental concentrations of BP3……16

Table 1.3 Environmental concentrations of PFOS…….....18

Table 1.4 Classification of *Scrobicularia plana* within the animal kingdom…….20

2. MATERIALS AND METHODS

Table 2.1 Concentration of adsorbed contaminants …….....28

Table 2.2 Percentage mortality of *S. plana* occurring during the two week experimental period…….....28

LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
ATC	Acetylcholine solution
a.u.	Arbitrary units
BAP	Benzo[a]pyrene
BHT	Butylated hydroxytoluene solution
BP3	Oxybenzone
CAT	Catalase
CDNB	1-chloro 2,4 dinitrobenzene
CI	Condition index
CT	Control
DAM	Daily assay mixture
DAPI	4,6-diamidino-2-phenylindole
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
DNTB	5,5' -dithio-bis (2- nitrobenzoic acid)
DRIFT	Diffuse reflectance infrared fourier transform spectroscopy
DTT	Dichlorodiphenyltrichloroethane
EDTA	Ethylenediaminetetraacetic acid
Etc.	*Et cetera*
GES	Good environmental status
GPx	Glutathione peroxidase
GR	Glutathione reductase
GS-DNB	Glutathione-S 2,4 dinitrobenzene
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione-S-transferase
HCL	Hydrochloric acid
HDPE	High density polyethylene
H_2O_2	Hydrogen peroxide
IARC	International Agency for Research on Cancer

KCL	Potassium chloride
LC – MS	Liquid chromatography - mass spectrometry
LDPE	Low density polyethylene
LMA	Low melting point agarose
LPO	Lipid peroxidation
MDA	Malondialdehyde
MP	Microplastic
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NMA	Normal melting point agarose
NOAA	National Oceanic and Atmospheric Administration
OTM	Olive tail moment
PAH	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PE	Polyethylene
PET	Polyethylene terephthalate
PFA	Perfluoroalkylated acid
PFOS	Perfluorooctane sulfonic acid
POP	Persistent organic pollutant
PP	Polypropylene
pp'DDE	2,2-bis-(p-chlorphenyl)-1,1-dichlorethylene
PS	Polystyrene
PVA	Polyvinyl acetate
PVC	Polyvinyl chloride
ROS	Reactive oxygen species
SD	Standard deviation
SOD	Superoxide dismutase
spp.	Species'

TBARS	Thiobarbituric-acid-reactive species assay
U	Units
UPVC	Unplasticised polyvinyl chloride
UV	Ultra violet
V	Virgin low density microplastic
4-HNE	4-hydroxyalkenals

1. INTRODUCTION

The human population has seen an unprecedented growth rate in the 20th century, increasing from 1.6 billion at the start of the century to 7.4 billion in 2016 (Curran *et al.*, 2002; World Population Data Sheet, 2016). Advances in technology coupled with the ever-increasing human population have led to a human dominated Earth system, often termed the Anthropocene, at the expense of both the environment and biodiversity. Human activities such as land use changes, the burning of fossil fuels and mass consumerism have had numerous detrimental impacts on the environment (Zalasiewicz *et al.,* 2016). Plastic debris has become one of the most abundant and persistent problems of anthropogenic origin effecting the Earth on a global scale. The cheap, light, durable and disposable nature of plastic, not only led to its commercial success, but also contributed to its persistence in the environment. It is theorised that all plastic ever produced, apart from that which has been incinerated, is still in circulation in the closed system of the Earth (Barnes *et al.,* 2009). As such, plastic has become a geological indicator of the Anthropocene (Zalasiewicz *et al.,* 2016).

1.1 Plastic Production and Disposal

Modern day plastics are synthetic materials derived from a wide range of organic polymers, mainly petroleum based (Leslie *et al.,* 2011). The first plastics were derived from biological sources such as natural resins and rubber. The invention of 'Bakelite', the first synthetic plastic in the early 1900's opened the door to the 'plastic age' (Crespy *et al.,* 2008). Since mass production of plastic began in the 1940's, plastic production has increased from approximately 5 million tonnes per year in 1950's to 322 million tonnes annually (in 2015). European production has slowed down in recent years, yet 1.5% growth is still predicted for 2017 (Plastics - the Facts, 2016). Globally the long-term growth of plastics is expected to be around 4% (Plastics - the Facts, 2010).

Advances in plastic production have resulted in more versatile, lightweight, durable and cheap plastics which have become incorporated in every part of our day to day lives. It is beyond doubt that plastics have greatly improved the quality of modern day life (Andrady and Neal, 2009). Yet plastic is now a ubiquitous, long lasting source of litter on the planet (Barnes *et al.,* 2009). As the history of mass production of plastic only spans the past ~ 60 years it is too early

to know exactly how long it will take plastic to degrade in environmental conditions (Barnes *et al.*, 2009). The relatively cold haline temperatures of the oceans slow down degradation further by preventing photo-oxidation (Cole *et al.*, 2011).

Consumer packaging accounts for almost one third of plastic production, replacing conventional materials such as paper and glass (Andrady, 2003). Due to the relative cheapness and versatility of producing plastic products, the past 50 years has seen a rapid increase in expendable, single use applications for plastics. Many plastic items, designed to be durable, are thrown away after one use. Plastic water bottles and shopping bags are such examples. Plastic debris is, and will continue to be in the foreseeable future, one of the biggest and most persistent contributors to anthropogenic debris worldwide.

Plastic production varies greatly with around 20 different main types manufactured globally. Of these, 5 polymers comprise roughly 75% of European production, these include: polyethylene (PE), polyethylene terephthalate (PET), polypropylene (PP), polyvinyl chloride (PVC), and polystyrene (PS) (Plastics - the Facts, 2010).

Low density polyethylene (LDPE) accounted for ~ 17% of European polymer production annually from 2013 - 2015, being the second highest polymer type produced. LDPE is used in the production of reusable bags and agricultural films, with its predominant use in the food packaging industry (Plastics - the Facts, 2016).

On average plastics contribute approximately 10% (mass) of municipal waste from various countries (Barnes *et al.*, 2009). The percentage of plastic recycled from country to country varies. Most countries in the EU recycle between 15-30% (Plastics - the Facts, 2010, 2016). The rest is either burned or sent to landfill sites where inevitably, due to its persistent and durable nature, the problem is not dealt with but merely stored and put off for future years. Inadequate management of landfill sites may lead to buoyant plastic rising and being blown onto land and into sea to constitute debris (Barnes *et al.*, 2009).

Natural marine debris have floated on the surface of the oceans for centuries, for example: drift wood, shells, pumice etc. It is only in the last 40-50 years with the evolution of the plastic age that anthropogenic sources of marine debris have started to accumulate. Plastic has been found to constitute the majority of marine debris (Coe and Rogers, 1997; Barnes and Milner, 2005). Many plastics are buoyant in nature and will float in the oceans and become deposited on strand lines. They can travel great distances, following oceanic circulation. Fouling by biofilms,

epibiota or water logging may increase their weight and cause flocculation, sinking to greater depth and settling in benthic sediments (Barnes *et al.*, 2009).

1.2 Sources of Plastics to the Oceans

1.2.1 Land Based Sources

There are numerous routes of plastic into the ocean including: indiscriminate disposal of litter, illegal dumping, blown in, blown from landfills (Leslie *et al.*, 2011), fibres from washing machines (Browne *et al.*, 2011), scrubbers and abrasives in cosmetics and commercial cleaning applications (Gregory, 1996), unintentional release during manufacture and transport (Mato *et al.*, 2001) as well as the unidirectional flow of rivers (Moore, 2008). Approximately 50% of the world population live within 50 miles of the coast. Abundance of both macro-and micro- plastics are highly correlated with population density (Barnes and Milner, 2005; Cole *et al.*, 2011).

Biodegradable plastics, seen as the 'green' alternative, may also be a source of microplastics (MPs). They are typically composed of pre-production plastic pellets and biodegradable additives such as starch or vegetable oil. If disposed of properly, such as in industrial composting plants, the vegetable component of bioplastic will readily decompose. Yet the synthetic polymer, raw material will not breakdown and may be considered a secondary source of MPs (Thompson *et al.*, 2004; Cole *et al.*, 2011; Leslie *et al.*, 2011).

1.2.2 Sea Based Sources

The fishing industry greatly contributes to the amount of plastic in the oceans. From the 1950's on, a switch from predominantly natural fibre ropes to synthetic plastic ropes was observed worldwide. Natural fibres such as hemp, manila and cotton, used to make fishing ropes and nets lose 50% of their strength when wet. Synthetic ropes do not (Henderson, 2001; Irish Underwater Council, 2010). In 1975 approximately 135,400 tonnes of plastic fishing equipment along with 23,600 tonnes of plastic packaging materials were dumped into the oceans by the world's fishing fleets (Derraik, 2002). Estimates of cargo lost at sea vary considerably. The World Shipping Council surveyed its members, which represent 90% of the global container shipping industry, and estimated on average 675 containers are lost at sea annually (World Shipping Council, 2011). Whereas, estimates of 2,000 – 10,000 containers lost annually have been speculated by popular media (Podsada, 2001). Littering and dumping

at sea by recreational boat users also contributes to this problem. International regulations are in place to reduce the amount of plastic litter in the oceans. Under Annex V of the MARPOL convention the disposal of plastic-derived garbage anywhere at sea is prohibited (Barnes *et al.*, 2009). This includes recreational as well as commercial vessels. This treaty came into effect in 1989 and is ratified by over 70 nations (Henderson, 2001). The LONDON convention, in force since 1975 also prohibits dumping of waste at sea and aims to protect the marine environment from pollution by human activities (Leslie *et al.*, 2011). The EU Marine Strategy Framework Directive (MSFD, 2008/56/EC) has established a framework for member States to achieve and maintain good environmental status (GES) of marine waters by 2020. One of the 11 qualitative descriptors upon which GES is based concerns marine litter, with the amount, distribution, composition and impacts of microplastics on the biota highlighted as a particular concern (Galgani *et al.*, 2013).

1.3 Macroplastic, Microplastic and Nanoplastic

Macroplastics (> 5 mm) and their effect on the marine environment have been studied for some years (Cole *et al.*, 2011). Due to their large size, being clearly visible they create 'eyesores' on the landscape and in the oceans as well as entangling and endangering marine life. In recent years MPs have become an area of concern. The term 'microplastic' was first used in 2004 by Thompson and colleagues (2004), in the paper 'Lost at sea: where is all the plastic?', to describe small fragments of plastic. In 2009 the first International Research Workshop on the Occurrence, Effects and Fate of Microplastic Marine Debris was held in America. The workshop defined MPs as plastic particles smaller than 5mm. The lower limit of MPs has not been set and is considered to be dependent on the equipment used during sampling and processing. A clear definition of the boundaries between MPs and nanoplastics has not been clarified (NOAA, 2009). It has been recommended that the conventional definition for non-polymer nanomaterials also be used for plastics. In this case, plastic < 100 nm, in at least one of its dimensions would be defined as nanoplastic. Knowledge on the occurrence and fate of nanoplastics in the marine environment is extremely limited due to the challenges posed in reliable detection (Bergmann, Gutow and Klages, 2015).

There are two main sources of microplastics to the environment; primary and secondary microplastics.

1.3.1 Primary and Secondary Microplastics

Primary MPs are microscopically sized plastic pellets used in production. Plastic scrubbers used in cosmetics, and preproduction pellets e.g. virgin material that is used in the production and moulding of plastic products, are two such examples of primary MPs. Secondary MPs result from the fragmentation of macroplastics by physical breakdown (weathering) and chemical UV-B degradation into consecutively smaller sizes (Hidalgo-Ruz *et al.,* 2012).

1.4 Physico-Chemical Characteristics of Microplastics

MPs behaviour and fate in the marine environment is dependent on their physico-chemical characteristics such as particle size, shape, surface charge and chemical composition. These characteristics influence their interaction with each other and with the surrounding environment. Environmental conditions which affect the behaviour and fate of MPs include ocean currents, horizontal and vertical mixing, temperature, pH, wind mixing, concentration and composition of natural organic matter, biofilm formation and UV exposure (Barnes *et al.,* 2009; Lusher, 2015).

Plastics are highly durable and long lasting due to their method of production, having various additives for such purposes. Plastic can persist in the environment for extensive time periods, with degradation being dependent on the chemical constituents of the plastic, combined with environmental factors such as wind, rain, temperature and UV exposure (Andrady and Neal, 2009). Thermal, photolytic (UV break down of polymers into smaller sizes), chemical and physical fragmentation (weathering) all aid in the breakdown of plastic. Macroplastics are broken down to progressively smaller pieces and eventually to MP sizes. Yet they remain unmineralised, never fully degrading (Thompson *et al.,* 2004). Biofilms may form on plastic debris comprising of microorganisms, viruses and bacteria. Their formation provides a barrier that inhibits degradation by UV-B and promotes settling within the water column (Barnes *et al.,* 2009). Estimates for the time period for plastic to degrade vary from 100's – 1,000's of years.

1.5 Microplastic Distribution and Abundance in the Oceans

In recent years MPs have become an area of concern due to their ubiquitous distribution in the marine environment. The occurrence of MPs have been recorded in all geographical regions of the oceans including ice cores from the Arctic (Obbard *et al.*, 2014) and visual identification in surface waters of Antarctica (Lusher, 2015). The presence of MPs have been reported in a wide range of marine habitats - beach sediments (Thompson *et al.*, 2004), subtidal and benthic sediments (Barnes *et al.*, 2009), deep-sea sediments (Fischer *et al.*, 2015), the water column, surface waters (Hidalgo-Ruz *et al.*, 2012), near densely populated areas (Barnes, 2005) and even in remote island atolls where no input or production of plastics occurs (McDermid and McMullen, 2004). MPs in freshwater habitats - estuaries (Browne, Galloway and Thompson, 2010), rivers (Sadri and Thompson, 2014), and lakes (Free *et al.*, 2014) have also been reported.

Properties of the plastic polymer itself (density, surface charge, aggregation potential) as well as abiotic (oxidation, weathering and vertical mixing) and biotic factors (biofouling) all contribute to the ubiquitous distribution of MPs within the oceans. MPs have been shown to accumulate in ocean gyres due to transportation by large scale ocean circulation and currents. A high concentration of plastics and MPs may converge and be captured in the centre of these regions (Lusher, 2015).

One recent study has estimated that there could be between 7,000 and 35,000 tonnes of plastic currently in the oceans (Cózar *et al.*, 2014), while another has estimated over 250,000 tonnes (Eriksen *et al.*, 2014). Yet the vast, three-dimensional nature of the world's oceans compared to the size of plastics being investigated complicates the quantification of both macro and micro plastics. This may be further confounded by ocean currents and by spatial and temporal variability caused by seasonal patterns (Cole *et al.*, 2011).

Varied methods of sampling, processing and reporting have led to problems in the accurate evaluation of MPs in the marine environment, with direct comparison between many published studies impossible. Although the size range of MP have been defined in recent years as 5mm and under, many studies report varying size ranges (Lusher, 2015). Experimental design may lead to plastics of a different size range being reported. For example, when investigating MP in sediments, 5 mm standardised sieves are generally not available in many laboratories as they are not used in sediment analysis, which is measured on a base two logarithmic scale, the phi scale (Φ). Sieve mesh sizes increase according to the phi scale (Blott and Pye, 2001). As such

MPs of 4 mm and under may be reported. The lower range of microplastic detection in water samples is constrained by the mesh size of nets used. Results of studies have also been reported in various dimensions. In water samples the number of MPs in a known water volume (particles m^{-3}), area measurements (particles km^{-2}), or concentration (mg L^{-1}) have been reported. While in sediment samples MP can be reported as the number of MPs in a known weight of dry or wet sediment (particles kg^{-1}), the number of MPs in a known area (particles km^{-2}) or the weight of MP in a known weight of dry or wet sediment (mg kg^{-1}) (Lusher, 2015).

The lowest reported estimates of MP from surface water samples (0.0000002 particles m^{-3}) come from the Bering Sea (Day, Shaw and Ignell, 1990) while the highest reported estimates (16,000 ($\pm 14 \times 10^3$) particles m^{-3}) come from Geoje Island, South Korea (Song *et al.*, 2014). Both are located in the Pacific Ocean. Some of the highest estimates of MPs from sediment samples include: 3,800 particles kg^{-1} in Spiekeroog, Germany (Liebezeit and Dubaish, 2012), > 40g kg^{-1} in the Canary Islands, Spain (Baztan *et al.*, 2014), 300,000 particles m^{-3} in Recife, Brazil (Ivar do Sul and Costa, 2007), 2,000-10,000 particles m^{-2} in Bermuda (Wilber, 1987) and > 5,000 particles m^{-1} also in Bermuda (Gregory, 1983).

Microplastics have been reported in 61% of Portuguese water samples with higher concentrations in Costa Vincentina (0.036 particles m^{-3}) and Lisbon (0.033 particles m^{-3}) compared to the Algarve (0.014 particles $^{-3}$) and Aveiro (0.002 particles $^{-3}$) (Frias, Otero and Sobral, 2014). Microplastic resin pellets (3-6 mm, 5% > 5 mm) represented 53% of total marine debris collected (1,289 items m^{-2}, 30 g m^{-2}) in another study on the Portuguese coastline, with 98% of marine debris being identified as plastic (Antunes *et al.*, 2013). In both cases, higher MP abundances were reported in proximity to urban, industrial and shipping areas.

1.6 Biological Impacts of Microplastics in the Oceans

1.6.1 Providing a Vector for Invasive Species

Marine organisms have had limited travel opportunities on natural, floating debris such as drift wood and pumice for much of the biotic history of earth. With the onset of shipping, dispersal ability increased. Organisms now had a greater opportunity to transverse vast areas of the globe attached to ships. The past 60 years have seen a huge increase in transport opportunities for marine organisms' due to the increased amount of plastic debris in the oceans.

Plastic debris may travel considerable distances in ocean currents. Drift plastic provides a surface for various marine organisms to grow. It can quickly become colonised by bryozoans, barnacles, polychaete worms, hydroids, molluscs, algae and bacteria (Barnes, 2002; Lobelle and Cunliffe, 2011). Plastics may serve as novel ecological habitats, being colonised by complex microbial communities, often referred to as the "Plastisphere". These communities are distinct from that of the surrounding surface waters (Zalasiewicz *et al.*, 2016). Plastic debris has increased the potential range of many marine organisms and provided an efficient vector for invasive species. Loss in biodiversity is highly correlated with the onset of colonisation by invasive species. This is a major concern of the 21st century. It is believed that the bryozoan, *Membranipora tuberculata*, invaded Australia from New Zealand encrusted on plastic pellets (Gregory, 1978). Much study has focused on the effect that surface type (wood, plastic, metal, etc.) has on the settlement of larvae (Barnes and Milner, 2005). Yet no clear consensus has emerged, highlighting the complexities of marine larvae settlement preferences.

1.6.2 Ingestion

MP ingestion has been reported in a variety of marine species including plankton, invertebrates, fish, sea birds, marine mammals and turtles. Although the majority of studies investigate interactions in controlled laboratory environments, field sampling studies have also demonstrated MP ingestion in wild populations (Lusher, 2015). See Table 1.1 for a review of laboratory MP exposure experiments on marine species.

Table 1.1 Microplastic exposure experiments on marine organisms

Organism	Ingested Material Size	Type and Exposure Concentration	Effect and Source
Phylum Annelida			
Class Polychaete			
Lugworm (*Arenicola marina*)	**20–2000 µm**	**1.5 g L^{-1} PP, PE, PVA**	**Ingested** **Thompson *et al.* (2004)**
Arenicola marina	130 µm	UPVC 0–5% weight	Ingested, feeding reduced, phagocytic activity increased, available energy reserves reduced, lipid reserves lowered. Wright *et al.* (2013)
Arenicola marina	230 µm	PVC 1,500 g of sediment mixture	Ingested, oxidative stress Browne *et al.* (2013)
Arenicola marina	400–1,300 µm	PS 0, 1, 10, 100 g L^{-1}	Ingested, feeding reduced, weight loss Besseling *et al.* (2013)
Fan worm (Galeolaria			Ingested

caespitosa)	3–10 μm	5 microspheres μL^{-1}, Ficoll inert polymer	Bolton and Havenhand (1998)
Galeolaria caespitose	3 and 10 μm	PS 635, 2,240, 3,000 beads mL^{-1}	size selectively ingested, egested Cole *et al.* (2013)
Mud worms (*Marenzelleria* spp.)	10 μm	PS 2,000 mL^{-1}	Ingested Setälä, Fleming-Lehtinen and Lehtiniemi (2014)
Phylum Arthropoda			
Subphylum Crustacea			
Class Maxillopoda			Ingested
Barnacle (*Semibalanus balanoides*)	20–2,000 μm	1 g L^{-1}	Thompson *et al.* (2004)
Subclass Copepoda	0.05 μm	PS 9.1×10^{11} mL^{-1}	Ingested, egested,
Tigriopus japonicas	0.5 μm	PS 9.1×10^{8} mL^{-1}	mortality, decreased fecundity
	6 μm	PS 5.25×10^{5} mL^{-1}	Lee *et al.* (2013)
Acartia (*Acanthacartia*) *Tonsa*	10–70 μm	3,000–4,000 beads mL^{-1}	Size selective ingestion, Wilson (1973)
Acartia spp.	10 μm	PS 2,000 mL^{-1}	Ingested Setälä, Fleming-Lehtinen and Lehtiniemi (2014)
Eurytemora affinis	10 μm	PS 1,000, 2,000, 10,000 mL^{-1}	Ingested, egested Setälä, Fleming-Lehtinen and Lehtiniemi (2014)
Limnocalanus macrurus	10 μm	PS 1,000, 2,000, 10,000 mL^{-1}	Ingested Setälä, Fleming-Lehtinen and Lehtiniemi (2014)
Temora longicornis	20 μm	PS 100 mL^{-1}	Ingested 10.7 ± 2.5 beads $individual^{-1}$ Cole *et al.* (2014)
Calanus helgolandicus	20 μm	PS 75 mL^{-1}	Ingested egested Cole *et al.* (2015)
Class Malacostraca		1 g $individual^{-1}$	Ingested
Orchestia gammarellus	20–2,000 μm	(n = 150)	Thompson *et al.* (2004)
Talitrus saltator	**10–45 μm**	**PE 10% weight food (0.06-0.09 g dry fish food)**	**Ingested, egested after 2 h Ugolini *et al.* (2013)**
Allorchestes compressa	11–700 μm	0.1 g	Ingested, egested within 36 hours Chua *et al.* (2014)
Neomysis integer	10 μm	PS 2,000 spheres mL^{-1}	Ingested Setälä, Fleming-Lehtinen and Lehtiniemi (2014)
Mysis relicta	10 μm	PS 2,000 spheres mL^{-1}	Ingested, egested Setälä, Fleming-Lehtinen and Lehtiniemi (2014)
Shore crab (*Carcinus maenas*)	8–10 μm	PS 4.0×104 L^{-1}ventilation 1.0×106 g^{-1}feeding	Ingested through gills and gut, retained and excreted, biological effects not measured Watts *et al.* (2014)
Norway lobster (Nephrops norvegicus)	5 mm	PP fibres 10 fibres cm^{-3} fish	Ingested Murray and Cowie (2011)
Nephrops norvegicus	500–600 μm	PE loaded with 10 μg of PCBs 150 mg microplastics in gelatin food	Ingested, egested fully PCB level in tissues increased but so did the positive control. No direct effect of microplastics observed Devriese *et al.* (2014)
Class Branchipoda			Ingested
Bosmina coregoni	10 μm	PS 2,000, 10,000 spheres mL^{-1}	Setälä, Fleming-Lehtinen and Lehtiniemi (2014)
Phylum Chlorophyta			
Scenedesmus spp.	20 nm	1.6–40 mg mL^{-1}	ROS increased, photosynthesis affected Bhattacharya *et al.* (2010)

Phylum Chordata			
Common goby (*Pomatoschistus microps*)	**1–5 µm**	**PE 18.4, 184 µg L^{-1}**	**Ingested, bioavailability modulated or pyrene biotransformed, energy decreased, AChE activity inhibited. Oliveira *et al.* (2013)**
Atlantic cod (*Gadus morhua*)	2, 5 mm	Concentration Not Available	Ingested, egested, 5 mm retained for prolonged periods, egestion improved by additional food consumption Santos and Jobling (1992)
Japanese medaka (*Oryzias latipes*)	**3 mm**	**LDPE 10% of diet, ground up**	**Liver toxicity, pathology, hepatic stress Rochman *et al.* (2013)**
Oryzias latipes		**PE pellets, two months chronic exposure**	**Gene expression altered, decreased choriogenin regulation (males), decreased vitellogenin and choriogenin (females) Rochman *et al.* (2014)**
Seabass larvae (*Dicentrarchus labrax*)	**10–45 µm**	**PE 0–105 g^{-1} incorporated with food**	**Ingested, no significant increase in growth, survival of larvae effected, gastric obstruction possible Mazurais *et al.* (2015)**
Phylum Ciliophora			
Strombidium sulcatum	0.41–10 µm	5–10% ambient bacteria concentration	Ingested Christaki *et al.* (1998)
Phylum Cryptophyta			
Rhodomonas salina	2 µm	PS 9 × 104 mL^{-1} Algae with Microspheres attached,	negative effect not observed Long *et al.* (2014)
Phylum Dinophyta			
Heterocapsa triquetra	2 µm	PS 9 × 104 mL^{-1} Algae with Microspheres attached,	negative effect not observed Long *et al.* (2014)
Phylum Echinodermata			
Class Holothuridea			
Giant Californian sea cucumber (*Apostichopus californicus*)	10, 20 µm	PS 2.4 $µL^{-1}$	Ingested, retained Hart (1991)
Stripped sea cucumber (*Thyonella gemmata*)	0.25–15 mm	PVC shavings (10 g), nylon line, resin pellets (60 g)	Selectively ingested Graham and Thompson (2009)
Grey sea cucumber (*Holothuria* (*Halodeima*) *grisea*)	0.25–15 mm	PVC shavings (10 g), nylon line, resin pellets (60 g)	Selectively ingested Graham and Thompson (2009)
Florida sea cucumber (*Holothuria floridana*)	0.25–15 mm	2 g nylon line added to 600 mL of silica sand	Selectively ingested Graham and Thompson (2009)
Orange footed sea cucumber (*Cucumaria frondosa*)	0.25–15 mm	2 g nylon line added to 600 mL of silica sand	Selectively ingested Graham and Thompson (2009)
Class Echinoidea			
Collector urchin (*Tripneustes gratilla*)	**32–35 µm**	**PE 1, 10, 100, 300 mL^{-1}**	**Ingested, egested Kaposi *et al.* (2014)**
Eccentric sand dollar (*Dendraster excentricus*)	10, 20 µm	PS 2.4 $µL^{-1}$	Ingested, retained Hart (1991)
Sea urchin (*Strongylocentrotus* sp.)	10, 20 µm	PS 2.4 $µL^{-1}$	Ingested, retained Hart (1991)
Class Ophiuroidea			
Crevice brittlestar (*Ophiopholis aculeata*)	10, 20 µm	PS 2.4 $µL^{-1}$	Ingested, retained Hart (1991)
Class Asteriodea			
Leather star (*Dermasterias*			Ingested, retained

imbricata)	10, 20 µm	PS 2.4 µL^{-1}	Hart (1991)
Phylum Haptophyta			
Isochrysis galbana	2 µm	PS 9 × 104 mL^{-1} Algae with Microspheres attached,	negative effect not observed Long *et al.* (2014)
Phylum Mollusca			
Class Bivalvia			Ingested, pseudofaeces
Blue mussel (*Mytilus edulis*)	30 nm	PS 0, 0.1, 0.2, and 0.3 g L^{-1}	produced, filtering reduced Wegner *et al.* (2012)
Mytilus edulis	**0−80 µm**	**HDPE 2.5 g L^{-1}**	**Ingested, retained in digestive tract transferred to lymph system, immune response** **von Moos, *Burkhardt-Holm and Köhler* (2012)** **Köhler (2010)**
Mytilus edulis	0.5 µm	PS 50 µL per 400 ml seawater	Ingested, trophic Transfer to *Carcinus maenas* Farrell and Nelson (2013)
Mytilus edulis	3, 9.6 µm	Fluorescently labelled PS, 0.51 g L^{-1}	Ingested, retained in digestive tract, transferred to lymph system Browne *et al.* (2008)
Mytilus edulis	10 µm	PS 2 × 104 mL^{-1}	Ingested, egested Ward and Tagart (1989)
	10 µm	PS 1,000 mL^{-1}	Ingested, egested Ward and Kach (2009)
Mytilus edulis	10, 30 µm	PS 3.10 × 105 mL^{-1} PS 8.65 × 104 mL^{-1}	Ingested Ingested Claessens *et al.* (2013)
Bay mussel (*Mytilus trossulus*)	10 µm	PS conc. Not Available	Ingested Milke and Ward (2003)
Atlantic Sea scallop (*Placopecten magellanicus*)	15, 10, 16, 18, 20 µm	PS 1.05 mL^{-1}	Ingested, retained, egested Brillant and MacDonald(2000, 2002)
Eastern oyster (*Crassostrea virginica*)	10 µm	PS 1,000 mL^{-1}	Ingested, egested Ward and Kach (2009)
Pacific oyster (*Crassostrea gigas*)	2, 6 µm	PS 1,800 mL^{-1} 200 mL^{-1}	Filtration and for the 2 µm size assimilation increased, for the 6 µm size gamete quality reduced (sperm mobility, oocyte number and size, fecundation yield), MP exposed parents display slower larval rearing. Sussarellu *et al.* (2014)
Peppery furrow shell clam (*Scrobicularia plana)*	20 µm	PS mg L^{-1}	Ribeiro *et al.* (2017) Uptake and accumulation in gills, oxidative stress, neurotoxic and genotoxic response to exposure.
Phylum Ochrophyta			
Chaetoceros neogracilis	2 µm	PS 9 × 104 mL^{-1}	negative effect not observed
Tintinnopsis lobiancoi	10 µm	Algae with Microspheres attached, PS 1,000, 2,000, 10,000 mL^{-1}	Long *et al* (2014) Ingested Setälä, Fleming-Lehtinen and Lehtiniemi (2014)

Phylum Rotifera			
Synchaeta spp.	10 μm	PS 2,000 mL^{-1}	Ingested Setälä, Fleming-Lehtinen and Lehtiniemi (2014)

As modified from Lusher (2015). Where: PS = Polystyrene, PE = Polyethylene, PVA = Polyvinyl Acetate, UPVC = Unplasticised Polyvinyl Chloride, PVC = Polyvinyl Chloride, PCB = Polychlorinated biphenyl, HDPE = High Density Polyethylene, and LDPE = Low Density Polyethylene. **Experiments with PE variants highlighted in bold.**

MP particle size plays an important role in their biological fate within the marine environment. Impacts on the marine biota may vary across the size spectrum of MPs. Large MPs (2-5 mm) could take more time to pass from the stomach of organisms having the potential to be retained in the digestive system. Toxicant adsorption, dependant on polymer type, may occur with increased exposure time to plastics. Feeding and digestion may occur with particles in the upper end of the size spectrum (1-2 mm) (Lusher, 2015). Small marine invertebrates have been shown to actively ingest and egest particles < 20 μm (Thompson *et al.,* 2004; Lee *et al.,* 2013). Smaller size MPs have larger effects on organisms at the cellular level. In the nanometre range MPs have been shown to translocate and pass into cell membranes (Browne *et al.,* 2008; Lusher, 2015).

Ingestion may occur in a variety of ways. Feeding mechanisms of some organisms may not allow for the discrimination between prey and anthropogenic debris (Moore *et al.,* 2001). In other cases, MPs may be mistaken for prey. Selective feeding on MPs over food may occur (Moore, 2008). MPs may enter the base of the food chain through ingestion or adsorption by phytoplankton and/or zooplankton (Lusher, 2015). Trophic transfer is an area of concern as are the potential human health impacts. Commercial marine species which are eaten whole, such as shrimps and bivalves, constitute a potential transference pathway of MPs from the marine environment to humans (Lusher, 2015).

Some organisms have been shown to reject MPs before digestion, with the production of pseudofaeces being one such example. Yet this requires extra energetic costs and prolonged production may lead to starvation (Lusher, 2015).

Ingested MP may accumulate in the digestive system, be egested or translocate between tissues. Once MPs are ingested, diminished food consumption may occur as MPs may occupy space in organism's stomachs, reducing storage volume and creating a false sensation of fullness. Reduced consumption diminishes the ability to gain fat deposits and leads to reduced fitness (Ryan *et al.,* 1988). In other cases, MPs may be egested, sometimes with no detrimental effects

observable in the organism. Such is the case in the majority of studies which report MPs in wild fish samples (Lusher, 2015). The ability of MP to translocate between tissues has been demonstrated in a variety of studies. In a laboratory study by Browne and colleagues (2008), MPs retained in the digestive tract of the mussel *Mytilus edulis* were transferred to the haemolymph after 3 days. von Moos and colleagues (2012) tracked HDPE MP particles in mussels from the gills to the digestive glands and ultimately to the lysosomal system, causing an inflammatory immune response. PS MPs were shown to translocate to the haemolymph of *S. plana* clams after 14 days exposure in a recent study by Ribeiro and colleagues (2017).

1.6.3 Vector of Pollutants

Many additives are added in the production of plastics, which give the plastics various desirable qualities and enhance their performance. Plasticisers are added to give plastic its flexible quality. Flame retardants to reduce the spread of fire. UV stabilisers to prevent photolytic degradation. Additives to enhance resistance to oxidation, modifiers to reduce breakage and surfactants may also be added during polymer production (Andrady and Neal, 2009). Many of the additives have known or suspected toxicity containing persistent organic pollutants (POPs), synthetic organic compounds of anthropogenic origin. POPs are chemically stable and do not easily degrade in the environment. Plastics are known to sorb hydrophobic chemical contaminants from the surrounding sea water and have been shown to concentrate them (Mato *et al.,* 2001; Rios, Moore and Jones, 2007; Barnes *et al.,* 2009). The high accumulation potential of plastic provides a transport medium for contaminants as well as being a potential source of contaminants themselves. Degradation of MPs to smaller plastic particle sizes adds more surface area to sorb contaminants. The combination of increased surface area due to weathering, long exposure times in the marine environment, and the hydrophobicity of organic xenobiotics may facilitate adsorption of these contaminants to MPs at concentrations significantly higher than those detected in seawater (Ogata *et al*., 2009; Antunes *et al*., 2013). Under laboratory conditions, various polymer particles have been shown to adsorb chemical pollutants from the surrounding environment, with PE, PVC, PP and PS displaying high sorption capacity for polycyclic aromatic hydrocarbons (PAHs), dichlorodiphenyltrichloroethane (DDT), hexachlorocyclohexanes and chlorinated benzenes (Bakir, Rowland and Thompson, 2012; Lee, Shim and Kwon, 2014). Various POPs including PAHs, polychlorinated biphenyls (PCBs), organo-halogenated pesticides, nonylphenol, and

dioxins have been detected in plastic pellets sampled from beaches worldwide (Ogata *et al*., 2009; Avio *et al.*, 2015). Since 2006 the International Pellet Watch has been monitoring the global distribution of POPs using beached plastic resin pellets stranded on beaches (Ogata *et al*., 2009).

Contamination in, and concentration of pollutants on MPs are of great importance as MPs may be ingested by marine organisms with contaminants having the potential to desorb and accumulate in fatty tissues due to their lipophilic nature, posing a long-term risk to the environment (Mato *et al.*, 2001; Rios, Moore and Jones, 2007). Although it has been demonstrated that MPs may act as a vector of contaminants to marine biota, it is still unclear as to the mode of action of these chemicals once ingested. This is an area that requires further research.

It has been established that a global marine debris problem exists on an International level, with microplastics being an area of concern (NOAA, 2009; Galgani *et al.*, 2010). Yet further research is needed to understand the ecological consequences of this emerging marine pollutant. The biological effects of ingestion, retention and egestion along with the potential for contaminant and trophic transfer are all areas which need to be addressed.

1.7 Specific Marine Contaminants

1.7.1 Benzo[a]pyrene

PAHs form a major class of marine pollutants**.** Listed as persistent toxic substances by United Nations Environment Program and by the European Union, they are formed through the incomplete pyrolysis of combustible organic material. Although this may occur naturally, e.g. through forest fires, the main sources are anthropogenically derived from fossil fuel combustion, waste incineration and oil spills**.** PAHs emitted, as soot or gas, to the atmosphere, enter the marine environment through rain and surface run off. Benzo[a]pyrene (BAP) is one such PAH which is ubiquitously distributed in coastal and marine environment (Antunes *et al.*, 2013; Liu *et al.*, 2015; Châtel *et al.*, 2017).

Known for its pro-carcinogenic properties, BAP ($C_{20}H_{12}$) is thought to be one of the most toxic PAHs and is classified by the International Agency for Research on Cancer as a group 1 human

carcinogen (IARC, 2011; Liu *et al.*, 2015; Châtel *et al.*, 2017). Recent studies have confirmed BAP as a reproductive and developmental toxicant, with endocrine disrupting properties (Corrales *et al*., 2014; Song *et al.*, 2016). The environmental effects of BAP contamination are of great concern. Due to its lipophilic nature, it resists degradation and has the potential to accumulate in organisms and biomagnify through the food web (Song *et al.*, 2016).

Environmental Concentration data for BaP in seawater is scarce, with values of 0.001 to 4.799 μg L^{-1} reported from seawater in China (Liu *et al.*, 2015) and values of 0.1 ng L^{-1} reported in Atlantic seawater (Toxnet.nlm.nih.gov., 2017). Sediment concentrations of 0.002 to 2.640 μg g^{-1} dry weight have been reported in China (Liu *et al.*, 2015). BaP has been used as a model to explore PAH effects in invertebrates and to investigate metabolic pathways of the known toxicant (Liu *et al.*, 2015).

1.7.2 Oxybenzone

Oxybenzone (2-hydroxy-4-methoxy-benzophenone), also known as benzophenone-3, is an organic UV filter present in sunscreen (average content 6-10%) and other personal care products such as cosmetics, skin creams, lipsticks, shower gels, hair sprays, hair dyes and shampoos. Organic UV filters are also found in plastics, such as plastic coverings that are used for the preservation of food, textiles, fabrics and household products (Balmer *et al.*, 2005; Fent *et al*., 2010; Bratkovics and Sapozhnikova, 2011). Concerns arise as to the toxicity of UV filters such as oxybenzone due to the daily use of products that contain them.

Contamination of the aquatic environment may occur through two main pathways. Direct inputs occur through recreational activities, being rinsed from the body while swimming or bathing. Indirect inputs occur through waste water treatment plants, being rinsed from the body while showering, and from clothes while washing. As such a seasonal variation in contamination may occur. It has been estimated that hundreds of tonnes of sunscreen products are released into the environment annually (Balmer *et al*., 2005).

Although recent studies have investigated the occurrence and environmental behaviour of BP3, data is scarce, with little know about its fate and transport in the marine environment. Table 1.2 displays environmental concentrations of BP3. A tentative preliminary environmental risk assessment of BP3 indicates that further ecotoxicological analysis is needed as an environmental risk cannot be ruled out (Fent *et al*., 2010).

Table 1.2 Environmental concentrations of BP3

Location	Concentration (ng $L^{-1)}$	Reference
Lake surface waters	35 – 125	Balmer *et al*., 2005
Switzerland	35 – 125	Poiger *et al*., 2008
Coastal seawater		
South Carolina, USA	10 – 2,013	Bratkovics and Sapozhnikova, 2011
Ionian Sea, Greece	8.2	Giokas *et al*., 2005
Remote Oceanic seawater		
Polynesia, Pacific Ocean	N.S	Goksoyr *et al*., 2009
Lake Fish, Switzerland	123	Balmer *et al*., 2005
Pool Shower Wastewater	8×10^6 - 10×10^6	Giokas *et al*., 2005
Wastewater Influent, Switzerland	up to 7,800	Balmer *et al.*, 2005
Wastewater Effluent, Switzerland	up to 700	Balmer *et al.*, 2005

Where N.S = Not Specified

Due to its lipophilic nature, BP3, like BaP has the potential to accumulate in aquatic organisms and to biomagnify to higher trophic levels. This is an under researched area of ecotoxicology (Balmer *et al.*, 2005; Brausch and Rand, 2011). BP3 has the potential to cause estrogenic effects and has been shown to induce vitellogenin production (used as a biomarker of exposure of fish to estrogen-active substances) in two species of fish (*Oncorhynchus. mykiss* and *Oryzias. Latipes*) as well as significantly decrease the hatchability of fertilized eggs of *O. latipes*. Acute toxicity (EC50 – median effective concentration) was recorded in *Daphnia magna* after 48 hours BP3 exposure, at a concentration of 1.9 mg L^{-1} (Fent *et al*., 2010).

1.7.3 Perfluorooctane Sulfonic Acid

Perfluoroalkylated acids (PFAs) are a class of perfluorinated alkylated substances which are produced synthetically in an electrochemical fluorination process. (Kannan *et al*., 2001, de Vos *et al.,* 2008). PFAs have been manufactured for over 50 years, being used in a variety of applications such as stain replants (in carpet, leather and textiles), fire-fighting foams and as paper protectors (Houde *et al.,* 2005; de Vos *et al.,* 2008). The industrial value of PFAs can be attributed to their physio-chemical properties such as thermal stability, high surface

activity, resistance to acidic and alkaline conditions, amphipathicity, density and weak intermolecular interactions. Yet these properties also contribute to the compounds' persistence in the environment and biota (Paul, Jones and Sweetman, 2009).

PFAs can degrade to the environmentally persistent perfluorooctane sulfonic acid (PFOS). Environmental contamination of PFOS may occur in two ways, either through direct release to the environment during manufacture and application, or indirectly, through degradation from precursor compounds. Under environmental conditions PFOS does not hydrolyse, photolyse, or biodegrade. The chemical properties of PFOS, including high water solubility and negligible vapor pressure, imply that it will reside in surface waters once released into the environment (Paul, Jones and Sweetman, 2009).

PFOS is a candidate POP under the Stockholm Convention and has been classed as an emerging chemical of concern, along with its precursors (Paul, Jones and Sweetman, 2009). Considered a widespread contaminant, PFOS has been recorded globally in seawater, human blood and in the biota. See table 1.3 for a summary of reported environmental concentrations of PFOS. Concentrations in wildlife and European river waters have been shown to correlate with human population densities (Giesy and Kannan, 2001; Houde *et al.,* 2005; Paul, Jones and Sweetman, 2009). Although limited information is available on the volume of PFOS, and its precursors, released into the environment, empirical oceanographic data estimates that approximately 235-1,770 tonnes of PFOS currently reside in oceanic surface waters (Paul, Jones and Sweetman, 2009).

PFOS has been shown to bioaccumulate and biomagnify to higher trophic levels. Concentrations of PFOS in fish eating predatory animals have been found in greater quantities than in that of their diets (Giesy and Kannan, 2001). Unlike other POPs, PFOS does not accumulate in fatty tissues, but binds to protein albumin, mainly present in blood, liver and eggs. As such the behaviour of PFOS within the body is similar to that of fatty acids, with hydrophobic interactions playing a role in bioaccumulation (de Vos *et al.,* 2008).

A lack of knowledge exists on the fate, transport, toxicity, and final sinks or loss mechanisms for PFOS and its precursors within the environment (Paul, Jones and Sweetman, 2009). Exposure to PFOS has been shown to induce neurobehavioral defects, mitochondrial dysfunction, and affect the metabolism of proteins and fats in marine Japanese medaka embryos (*Oryzias melastigma*) (Huang *et al.,* 2012). The toxic mode of action of PFOS and its effects on the health of organisms are still under investigation.

Growing awareness of the extent of environmental contamination, and its possible risks, led to the cessation of production of PFOS, and its precursor, in 2002 by the dominant manufacturer worldwide, 3M. Accumulated stocks were used until their total phase out by the European Union in 2011. Production continues in Southeast Asia. Volumes produced are unknown, yet very high levels of PFOS have been reported in the biota. Despite regulation PFOS emissions are expected to continue through the degradation of consumer products with landfill being a source of slow release to the environment (Paul, Jones and Sweetman, 2009).

Table 1.3 Environmental concentrations of PFOS

Species	**Location**	**Concentration**	**Reference**
AQUATIC MAMMALS			
Californian Sea Lion	West coast USA	4.6 – 49.4	Kannan *et al.*, 2001
Dolphin *spp*.	Florida, USA	36.3 – 1,520	Kannan *et al.*, 2001
Polar Bear	Alaska, USA	175 – 678	Kannan *et al.*, 2001
Pygmy Sperm Whale	Florida, USA	6.6 – 23	Kannan *et al.*, 2001
Seal *spp.*	West coast USA	< 5 – 113	Kannan *et al.*, 2001
Sea otter	West coast USA	< 5 – 35 113	Kannan *et al.*, 2001
BIRDS			
Albatross *spp.*	North Pacific	< 35	Giesy and Kannan, 2001
Albatross *spp.*	Korea	70 – 500	Giesy and Kannan, 2001
Brown Pelican	USA	290 – 620	Giesy and Kannan, 2001
Common Cormorant	Italy	33 – 470	Giesy and Kannan, 2001
Common Loon	USA	35 – 690	Giesy and Kannan, 2001
FISH			
Blue-fin Tuna	Mediterranean Sea	21 – 87	Giesy and Kannan, 2001
Brown Trout	USA	< 17 – 26	Giesy and Kannan, 2001
Chinook Salmon	USA	33 – 170	Giesy and Kannan, 2001
Lake Whitefish	USA	33 – 81	Giesy and Kannan, 2001
Yellow-fin Tuna	Northern PacificOcean	< 7	Giesy and Kannan, 2001
FROGS AND TURTLES			
Green Frogs	USA	< 35 – 290	Giesy and Kannan, 2001

YBM Turtle	USA	39 – 700	Giesy and Kannan, 2001
SEAWATER			
/	Atlantic Ocean	8.6 – 73	Yamashita *et al.*, 2005
/	Coastal China	23 – 9,680	Yamashita *et al.*, 2005
/	Coastal Hong Kong	70 – 2,600	Yamashita *et al.*, 2005
/	Coastal Korea	39 – 2,530	Yamashita *et al.*, 2005
/	Offshore Japan	40 – 75	Yamashita *et al.*, 2005
/	Pacific Ocean (surface water)	1.1 – 78	Yamashita *et al.*, 2005
/	Pacific Ocean (deep water, ~ 4000 m)	3.2 – 3.4	Yamashita *et al.*, 2005
/	South China Sea	8 – 113	Yamashita *et al.*, 2005
/	Tokyo Bay	338 – 57,700	Yamashita *et al.*, 2005

Where: YBM Turtle = Yellow-blotched map turtle. All tissue samples reported are from the liver and given in ng g^{-1} wet weight. All water samples are reported in pg L^{-1}.

1.8 Bioindicators

Bioindicators are species that are used to indicate adverse effects of contamination in the environment. These effects may be either measurable responses in the organism or changes in the number of species, or proportion of species within communities. Bioindicator species may be used as an estimation of ecosystem health if they occur in a wide range of environments, are tolerant and have a measured response to toxicants and no immigration or emigration from the studied environment occurs. The use of sessile organisms as bioindicators may be particularly relevant as they fulfil the last criteria mentioned while reflecting the *in situ* effects of contamination (Dixon *et al.*, 2002).

1.9 *Scrobicularia plana*

Invertebrates constitute a large and diverse group of organisms accounting for 90% of all animal species. They play an important role in ecosystem functioning as well as ecosystem monitoring, with sessile invertebrates being particularly applicable in the later (Dixon *et al.*,

2002). The class Bivalvia, phylum Mollusca, lie within this group and have an extensive distribution, from the Poles to the Tropics. Over 10,000 bivalve species have been described**.** Bivalves form an essential component of ecologically valuable intertidal mudflats. Only a handful of bivalve species account for the majority of bivalve biomass along the European coastline with one such species being the peppery furrow shell clam, *Scrobicularia plana* (da Costa, 1778) (Santos *et al.*, 2011). Table 1.4 describes the classification of *S. plana* within the animal kingdom.

Table 1.4 Classification of *Scrobicularia plana* within the Animal Kingdom

Kingdom	Animalia
Phylum	Mollusca
Class	Bivalvia
Subclass	Heterodonta
Infraclass	Euheterodonta
Superorder	Imparidentia
Order	Cardiida
Superfamily	Tellinoidea
Family	Semelidae
Genus	Scrobicularia
Species	*Scrobicularia plana*

(Marinespecies.org., 2017)

S. plana have thin, rounded, equivalve shells, being distinguishable from other bivalves' due to the shells flattened appearance. Concentric growth rings are visible on the shells exterior, which is a pale grey and yellow colour as can be seen in figure 1.1. Adults may grow up to 65 mm and have been known to live for up to 18 years (Green, 1957; Santos *et al.*, 2011).

Figure 1.1 Exterior of a *Scrobicularia plana* shell with concentric growth rings visible. Photo: Author.

The species displays an extensive geographical distribution in the Atlantic Ocean, from Norway in the north, to Senegal in the south, including the Baltic Sea and the Mediterranean, but excluding the Black Sea (Rodríguez-Rúa *et al.*, 2003). The distribution of *S. plana* is shown in figure 1.2. Environmental factors affecting distribution include: sediment type, salinity, temperature, hydrographic conditions and predation (Santos *et al.*, 2011).

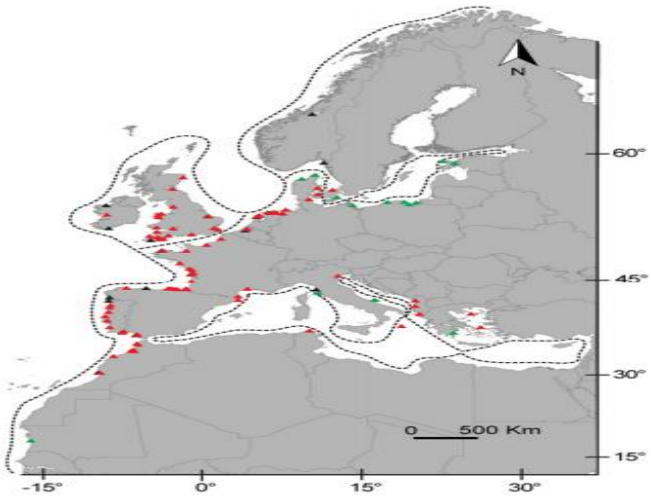

Figure 1.2 *Scrobicularia plana* distribution. Grey dotted line represents the assumed distribution based on references from Tebble (1976). Red symbols represent confirmed distribution based on records from literature of live organisms. Green symbols represent confirmed distribution based on records from literature of dead shells found in sediments. Black symbols represent confirmed distribution based on observations and/or comments (Santos *et al.*, 2011).

S. plana are sedentary infaunal, species. They inhabit intertidal, muddy sediments, rich in organic matter, occupying burrows (5-20 cm deep) in estuarine and coastal locations. As maintenance of burrows for inhalant siphons is not possible in coarser motile substrate, the species is normally absent from locations with this type of substrate. This may somewhat explain their patchy distribution along the European coastline (Santos *et al.*, 2011).

S. plana are deposit filter feeders, also capable of obtaining food by siphoning suspended matter from the surrounding water. Particles from 4-40 µm have been shown to be filtered with up to 100% efficiency. Although much of the filtered material is ingested, *S. plana* also produce pseudofaeces (Hughes, 1969).

S. plana is a gonochoristic species which reaches sexual maturity between 2-3 years with a corresponding shell length of ~ 20 mm (Hughes, 1971). Gametogenesis is initiated at the beginning of Spring (Santos *et al.*, 2011). Spawning occurs during summer months. A prolonged spawning period is observed at the southern limits of its distribution (Spain, Portugal and Northern Africa). Environmental factors including increased food availability and water temperatures are thought to promote spawning. Direct correlation between spawning percentages and water temperature has been reported (Rodríguez-Rúa *et al.*, 2003). Spawning is followed by a period of sexual inactivity from October to January during which the sex of the majority of organisms cannot be determined (Rodríguez-Rúa *et al.*, 2003; Mouneyrac *et al.*, 2008).

The estuarine clam, *S. plana* is an environmentally relevant species to use as a bioindicator for evaluating the health status of estuarine ecosystems (Mouneyrac *et al*., 2008). The clam's physiology and ecology have been well studied. The euryhaline species' sessile nature and estuarine habitat allows contact with multiple contaminants. As a burrowing deposit filter feeder, it can assimilate particles, and associated contaminants, from both the sediments and the water column. Being positioned at the base of the food web, the clam is an importance food source for crabs, fish, birds and increasingly for human consumption (Rodríguez -Rúa *et al.*, 2003; Langston *et al.*, 2005; Langston, Burt and Chesman, 2007). *S plana* are key species in the structure and functioning of both coastal and estuarine ecosystems (Châtel *et al.*, 2017).

1.10 Biomarkers

Biomarkers are defined as 'a change in biological response, ranging from molecular through cellular and physiological responses to behavioural changes, which can be related to exposure to or toxic effects of environmental chemicals' (Peakall and Walker, 1994). Thus, a biomarker measures a disturbance to the normal function of an organism responding to a toxicant. Knowledge of the normal physiology of an organism along with understanding how this is

disturbed by contaminants is required. Biomarkers can be used as sensitive, early warning indicators and monitors of the potentially toxic effects of environmental contamination. Properties of good biomarkers include; (a) that it is fast, cheap and easy to measure, (b) the measurement is specific to the toxicant, and (c) a concentration- or dose- response relationship is shown. There are two types of biomarkers: biomarkers of exposure which indicate the organism has been exposed to a toxicant; and biomarkers of effect which measure the disturbance resulting from exposure (van der Oost, Porte-Visa and van den Brink, 2005; Nikinmaa, 2014).

1.10.1 Genotoxicity

The integrity of cellular DNA is often compromised as one of the first effects of an organism exposed to genotoxic contaminants. Alterations including modified bases, DNA-DNA crosslinks, DNA-protein crosslinks and single and double strand breaks may result. DNA strand breaks may form as a result of oxidative stress due to interactions with reactive oxygen species (ROS). Other mechanisms inducing strand breaks include excision repair enzymes and apoptotic or necrotic processes (Oliveira *et al.,* 2009). The use of haemocytes as a robust cell for assessing genotoxic screening through the Comet assay has been verified by Petridis and colleagues (2009).

1.10.2 Oxidative Stress

Molecular oxygen consumed by organisms is tetravalently reduced to water while being coupled to the oxidation of food and the production of energy. Partial reduction leads to the formation of ROS, with an estimated 1-3% of O_2 consumed in animal cells being converted to ROS. Radical ROS species include: the superoxide anion radical ($O_2^{\bullet -}$), the hydroxyl radical ($OH^{\bullet}$), the peroxyl radical ($RO_2^{\bullet}$), the alkoxyl radical ($RO^{\bullet}$) and the hydroperoxyl radical ($HO^{\bullet}$). Non-radical ROS species include: hydrogen peroxide (H_2O_2), hypochlorous acid (HOCL) single oxygen (O) and peroxynitrite ($ONOO^-$, which can alternatively be termed a reactive nitrogen species). Such oxygen species are continually produced, mainly as unwanted by products of endogenous sources and processes including certain enzymes, haem proteins, auto-oxidation and electron transport occurring in the mitochondria, endoplasmic reticulum and the nuclear membrane **(**Livingston, 2001**;** van der Oost, Porte-Visa and van den Brink, 2005).

Defence systems which inhibit the formation of ROS include the production of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Non-enzymatic antioxidant defence namely involving glutathione, vitamin E, ascorbate, β-carotene, and urate also plays a role (Valavanidis *et al.,* 2006).

Antioxidant enzymes have been used as biomarkers in previous studies to investigate the effect of environmental contaminants in aquatic organisms (Valavanidis *et al.,* 2006; Silva *et al.,* 2012; Bebianno *et al.,* 2015). These enzymes are produced in the cytoplasm, in membrane bound organelles - peroxisomes, which function in ROS homeostasis. Peroxisome proliferation has been found to occur in various invertebrates as a result of contaminant exposure (Viarengo *et al,* 2007).

Superoxide dismutases (SODs) are a family of enzymes that catalyse the partitioning of the superoxide anion radical ($O_2^{\bullet -}$) into hydrogen peroxide and water. They typically contain metal ions eg: CuZnSODs, MnSODs, FeSODs, and NiSODs (Nikinmaa, 2014). The antioxidant enzyme catalase (CAT), prevents cellular damage from ROS by reducing H_2O_2 to H_2O (Oliveira *et al.,* 2009; Solé, Kopecka-Pilarczyk and Blasco, 2009). Glutathione peroxidases (GPxs) are a family of antioxidant enzymes which catalyse H_2O_2 into H_2O with GSH oxidation (reduced glutathione) (Oliveira *et al.,* 2009; Silva *et al.,* 2012). H_2O_2 is the main cellular precursor of the most toxic ROS, the hydroxyl radical ($OH^{\bullet}$). As antioxidant enzymes show poor efficiency towards removal of the hydroxyl radical, H_2O_2 removal by both CAT and GPx, plays an important role in counteracting toxicity (Oliveira *et al.,* 2009). The phase two enzymes, glutathione-S-transferases (GSTs) are mainly involved in detoxification but also have an antioxidant role with some isoforms inhibiting lipid peroxidation (Solé, Kopecka-Pilarczyk and Blasco, 2009; Nikinmaa, 2014).

Biomarkers of oxidative stress rely on the fact that a change in the activity of the ROS defence system, i.e. antioxidant enzymes, can occur as a result of environmental contamination. Figure 1.3 displays this relationship. Cells experience oxidative stress when the production of ROS exceeds antioxidant defences. Membrane lipid peroxidation may result and can be taken as a sign of oxidative damage (Viarengo *et al.,* 2007).

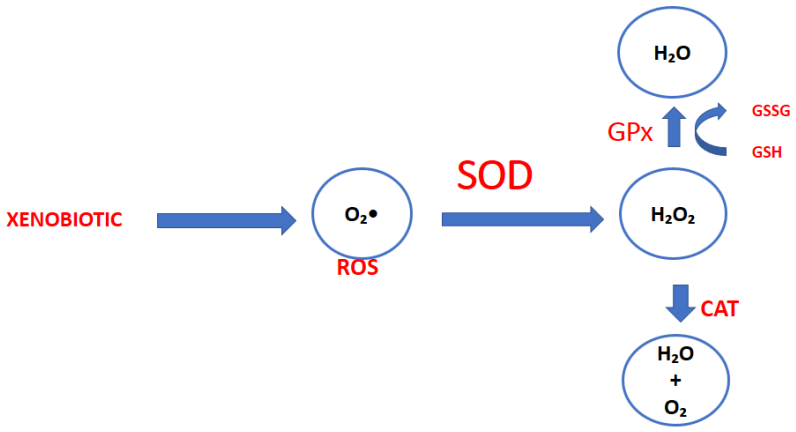

Figure 1.3 Schematic diagram of antioxidant ROS defence system.

1.10.3 Oxidative Damage

Prolonged oxidative stress may cause oxidative damage to DNA, proteins, carbohydrates and lipid membranes. Lipid peroxidation (LPO) occurs when free radicals react in membranes and form lipid hydroperoxides. The hydroperoxides decompose unsaturated fatty acid double bonds and dismantle membrane lipids. The end products of LPO include aldehydes such as malondialdehyde which may be assayed using thiobarbituric acid (or thiobarbituric-acid-reactive species assay - TBARS) (Oliveira *et al.*, 2009).

1.10.4 Neurotoxicity

Exposure to chemicals may affect the nervous system, peripheral nerves or neuromuscular junctions function within an organism. Such chemicals are said to be neurotoxic (Nikinmaa, 2014). The enzyme acetylcholinesterase (AChE) is involved in neuro and neuromuscular transmission and has been shown to respond to low levels of contaminants in the environment, being inhibited by organophosphorus and carbamate pesticides, some metals, surfactants and petrogenic compounds in invertebrates (van der Oost, Porte-Visa and van den Brink, 2005; Solé, Kopecka-Pilarczyk and Blasco, 2009). AChE forms part of a large group of serine hydrolases and functions to remove acetyl esters such as acetylcholine from synaptic clefts. This ubiquitous enzyme plays a role in detoxifying natural compounds (Viarengo *et al.*, 2007).

1.11 Objectives

This work aims to assess the ecotoxicological effects of exposure of low density polyethylene microplastics, with and without added contaminants, in gill tissues and haemocytes of the peppery furrow shell clam, *Scrobicularia plana.* Environmentally relevant concentrations of contaminants, benzo[a]pyrene (BAP), oxybenzone (BP3) and perfluorooctane sulfonic acid (PFOS), were adsorbed to microplastics in order to evaluate the potential role of plastic particles as a source of chemical contamination once ingested.

To clarify any effects of exposure, a set of biomarkers were employed, including the quantification of antioxidant (superoxide dismutase - SOD, catalase - CAT, glutathione peroxidase - GPx) and biotransformation (Glutathione-S-transferases - GST) enzyme activities, which play a role in detoxification under conditions of oxidative stress; lipid peroxidation (LPO) levels, indicative of oxidative damage; single and double strand DNA breaks to evaluate genotoxicity; and activity of the enzyme acetylcholinesterase (AChE), involved in neuro and neuromuscular transmission. The condition index was evaluated to assess the overall health status of the organisms.

2. MATERIALS AND METHODS

2.1 Experimental Design

Clams (*S. plana*) were collected at low tide from Cabanas, Tavira, Ria Formosa, Southern Portugal (37.130092, -7.610814) over 2 days and transported to the laboratory alive surrounded by ambient seawater in thermally insulated boxes. Collection occurred in early February, during the period of sexual inactivity. A total of 850 clams (4 cm ± 0.5 cm) were collected. Clams were acclimatised for 5 - 7 days, in Ria Formosa seawater at a constant (air) temperature of 18 ºC and photoperiod of 12 hours light to 12 hours dark. After the acclimation period, clams were transferred to 25 L aquaria containing 4 L of sediments and 16 L of seawater. Sediment was previously collected from the top 30 cm at the same site and passed through a 4 mm sieve to remove any macro-organisms and debris. Sediments were subsequently dried at 65 ºC for 48 hours in an effort to further reduce organic matter and volatile compounds. Sediment was rehydrated, to the same original sediment moisture content (%) as when collected, calculated by the difference in the wet weight of a known volume of sediment and after reaching constant dry weight at 65 ºC.

The exposure experiment consisted of 10 aquaria, with 5 treatments, 2 replicates per treatment as depicted in figure 2.1. All aquaria, excluding the two controls, were exposed to LDPE microplastics (11-13 µm) at a concentration of 1 mg L^{-1}. Table 2.1 displays the chemical concentrations adsorbed to LDPE MPs.

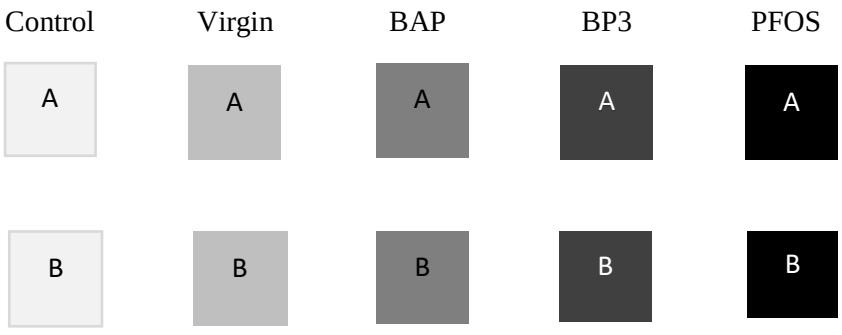

Figure 2.1 Aquarium set up and treatment. No addition of plastics occured in the control aquaria. Virgin LDPE MP was added in the 'Virgin' aquaria. LDPE MP with Benzo[a]pyrene adsorbed to the plastic was added to the 'BAP' aquaria. LDPE MP with oxybenzone adsorbed to the plastic was added to the 'BP3' aquaria. LDPE MP with perfluorooctane sulfonate adsorbed to the plastic was added in 'PFOS' aquaria. A and B represent replicate aquaria.

Table 2.1 Concentration of adsorbed contaminants

Code Name	Contaminant Adsorbed	Concentration of Adsorbed Contaminant
Virgin	/	/
BAP	Benzo[a]pyrene	16.87 (± 0.22) µg g^{-1}
BP3	Oxybenzone	82 ng g^{-1}
PFOS	Perfluorooctane sulfonate	70.22 (± 12.41) µg g^{-1}

Water was changed every 72 hours, with the routine application of microplastics. Abiotic parameters (temperature, oxygen saturation, salinity and pH) were checked using a multiparametric probe (ODEON V3.3.0). Physical and chemical parameters remained stable for the duration of the experiment with average water temperatures of 19.31 ± 0.21 ºC, average O_2 concentrations of 95.78 ± 1.77%, average salinity of 34 ± 1 ppt, and average pH of 8.05 ± 0.04. Additional food was not supplied during the acclimatisation or exposure period in order to minimise the interaction of microplastics with other organisms, namely phytoplankton. The use of plastic material was avoided, where possible, throughout the duration of the experiment. Glass Pasteur pipettes were attached to the bottom of plastic aeration tubes in order to minimise plastic contamination.

Mortality was observed in all aquaria, with the highest being in the control and virgin treatments and the lowest in BP3 treatments. Actual mortality rate may differ from the observed rate, with results being confounded by the burrowing nature of the species. See table 2.2 for percentage mortality occurring during the two week experimental period.

Table 2.2 Percentage mortality of *S. plana* occurring during the two week experimental period

TREATMENT:	**Control**	**Virgin**	**BAP**	**BP3**	**PFOS**
% MORTALITY:	7	7	3	1	5

Where: Virgin = LDPE virgin MP, BAP = benzo[a]pyrene contaminated LDPE MP, BP3 = oxybenzone contaminated LDPE MP, PFOS = perfluorooctane sulfonate contaminated LDPE MP.

Individuals were randomly sampled from each aquarium before the addition of plastics, at the beginning of the experiment (day 0), and after 3, 7 and 14 days of exposure. Tissues - gills, digestive gland and remaining tissue (foot, mantle and adductor muscles) - were dissected immediately, placed in micro-centrifuge tubes, flash-frozen in liquid nitrogen and stored at -80 ºC for analysis at a later stage. Haemolymph was extracted from the posterior adductor muscle of *S. plana* at each sampling time, using a 1.5 mm sterile hypodermic syringe with an attached needle.

2.2 Microplastics and Contaminants

LDPE Microplastics (MPP-635G) were obtained from MicroPowders Inc. (USA). Chemicals were obtained from Sigma Aldrich. Sorption of contaminants (BAP, BP3 and PFOS) to microplastic particles was conducted by the Man-Technology-Environment Research Centre, Department of Natural Science, Örebro University, Sweden.

2.3 Condition Index (CI)

The gravimetric condition index (CI) was assessed in 96 individuals to determine the physiological status of both control and exposed clams during the experiment. Individuals were sampled from each aquarium (3 individuals per aquarium, 6 per treatment) at day 3, 7 and 14 of exposure. 6 individuals were randomly sampled pre-exposure (day 0). The CI was estimated by calculating the percentage (%) of the ratio between dry weight of the soft tissues (g) and the dry weight (g) of the shell (Walne, 1976). Tissues were dried at 80 °C until a constant dry weight was achieved.

2.4 DNA Damage

The alkaline comet assay was used to determine DNA damage, through single and double DNA strand breaks, in 36 individuals. 6 individuals were randomly sampled pre-exposure at day 0.

30 individuals were sampled at day 14 (3 per aquarium, 6 per treatment). Protocol was followed as described by Almedia and colleagues (2013), originally adapted from Singh *et al.* (1988).

150 µl of haemolymph was extracted from the posterior adductor muscle of *S. plana* samples with a 1.5 ml sterile hypodermic syringe, placed in micro-centrifuge tubes and centrifuged at 3,000 rpm for 3 minutes at 4 ºC. Supernatant was removed and the DNA pellet re-suspended in 300 µl of phosphate buffered saline (PBS) solution. The suspended DNA and PBS solution was mixed with low melting point agarose (LMA 0.65%, dissolved in Kenny's salt solution). DNA cell suspensions were cast on microscope slides previously coated with normal melting point agarose (NMA 0.65%, dissolved in Tris-acetate EDTA). One slide, with two replicate agarose gels embedded with cells, was prepared per sample. Slides were immersed in Lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Sarcosil, 10% Dimethylsulfoxide, 1% Triton X 100, pH 10) at 4 ºC in the dark for 1 hour, enabling cell lysis. Microscope slides were rinsed with ultrapure water (Milli-Q), placed in an electrophoresis chamber, submerged in buffer (300 mM NaOH, 1 mM EDTA, ultrapure water (Milli-Q), pH > 13, at 4 ºC) for 15 minutes prior to running the current, to allow DNA to unwind. Electrophoresis was run under the following conditions: 25 V, 300 mA, for 5 minutes. Slides were removed, immersed in neutralisation solution (0.4 mM Tris, pH 7.5), rinsed with ultrapure water (Milli-Q), and allowed to dry, in the dark at room temperature. Once dry, slides were stained with 4,6-diamidino-2-phenylindole (DAPI). Each well was examined using an optical fluorescence microscope (Axiovert S100), under x 400 magnification. 25 photographs of individual cell nuclei were taken from each well (50 for each slide/sample), using a camera (Sony) attached to the microscope. Photographs were analysed using the Komet 5.5 image analysis system (Kinetic Imaging Ltd). DNA damage was quantified by measuring the displacement between the genetic material of the cell nucleus ('comet head') and the migrating comet 'tail', as displayed in figure 2.2. The following parameters were analysed in an effort to make results comparable to previously published studies: percentage DNA in comet tail, comet tail length (measured in µm) and olive tail moment (measured in arbitrary units, a.u.), calculated using the following formula:

*(Tail mean – Head mean) *(Tail % DNA/100).*

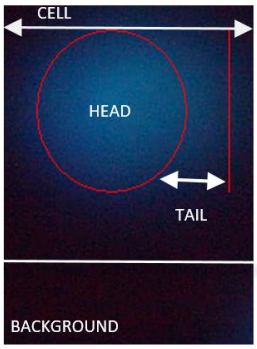

Figure 2.2 *S. plana* haemocyte cell, post comet assay electrophoresis. Parameters used to calculate DNA damage, including cell nucleus (head), migrated DNA (tail) and background reference, are indicated. 'Head' and 'Tail' defined by Komet 5.5 image analysis system. Photo: Author.

2.5 Enzyme Activity

2.5.1 Homogenisation of Tissues

Enzyme activities were assessed in the gill tissues of 384 individuals, with 96 samples investigated per enzyme (SOD, CAT, GPx and GST). Gills were defrosted, weighed and homogenised, on ice, in 5 ml of Tris sucrose buffer (Sucrose 0.5 M, Tris 20 mM, KCL 0.5 M, DTT 1 M, EDTA 1 mM, at pH 7.6). The homogenate was centrifuged at 500 g, at 4 ºC for 15 minutes. The cytosolic fraction was separated and centrifuged a second time at 12,000 g, at 4 ºC for 45 minutes. Supernatant was divided into 5 aliquots, stored in Eppendorf tubes and frozen at -80 ºC for the determination of SOD, CAT, GPx and GST activities as well as total protein concentrations.

2.5.2 Superoxide Dismutase (SOD)

SOD activity was determined using the colorimetric method described by McCord and Fridovich, (1969). Percentage inhibition in the reduction of cytochrome *c* by the superoxide anion generated by the xanthine/hypoxanthine system was used to measure of the amount of SOD present. Samples were defrosted on ice and vortexed. 2,650 µL of phosphate buffer (50 mM, with EDTA 0.1 mM, at pH 7.8), 100 µL Hypoxanthine (1.5 mM), 100 µL of cytochrome *c* oxidase (0.15 mM), 50 µL of sample and 100 µL of Xanthine Oxidase (56 mU/ml) were added to a quartz cuvette, respectively. Absorbance was read at 550 nm for 1 minute using a spectrophotometer. Samples were run in triplicate. Activity is expressed in Units (U) mg^{-1} protein, where 1 U of activity corresponds to the amount of sample required to cause 50% inhibition. The following formula was used to determine SOD activity:

$$\% I = | 1 - (average\ \Delta OD_{Sample}) / (average\ \Delta OD_{Xanthine\ Oxidase}) |$$

Where:

I = Inhibition

ΔOD = Variation in Absorbance

2.5.3 Catalase (CAT)

CAT activity was determined by measuring the consumption of hydrogen peroxide (H_2O_2) at 240 nm, as described by Greenwald (1987). Samples were defrosted on ice and vortexed. 1,900 µL of phosphate buffer (pH 7.5), 1,000 µL H_2O_2 and 100 µL of sample were added to a quartz cuvette respectively. Absorbance was read spectrophotometrically at 240 nm for 1 minute, with two replicates per sample. The following formula was used to determine CAT activity (µmol min^{-1} mg^{-1} of total protein concentration):

$$CAT\ activity = \frac{\left(\frac{\Delta OD}{40}\right) * \left(\frac{Vol\ total}{Vol\ sample}\right)}{prot\ (mg/ml)}$$

Where:

V total = volume of cuvette, 3 mL

Vol sample = 100 mL

40 M^{-1} cm^{-1} = extinction coefficient of H_2O_2

ΔOD = Variation in absorbance

2.5.4. Glutathione Peroxidase (GPx)

GPx activity was measured indirectly following the colorimetric method described by Lawrence and Burk (1978). Samples were defrosted on ice and 40 µL added, in triplicate, to a 96 well microplate. 20 µL of DAM solution (Daily Assay Mixture: 3 mM GSH, 0.25 mM NADPH, 0.67 U/mL GR) was added to each well and incubated at 28 ºC for two minutes. 50 µL of Cumene Hydroperoxide probe (1 mM) was added to initiate the following reactions: GPx converts reduced glutathione (GSH) to oxidized glutathione (GSSG) while reducing the Cumene Hydroperoxide probe. As NADPH is consumed by glutathione reductase (GR) the generated GSSG is reduced to GSH. The decrease in NADPH, measured at 340 nm, is directly proportional to GPx activity. The absorbance was measured at 30 second intervals, over 5

minutes, at 28 ºC. The following formula was used to determine GPx activity (nmol min^{-1} mg^{-1} of total protein concentration):

$$GPx\,activity = \left(\frac{(\Delta Abssample - \Delta Absblank) \times Vtotal \times 1000}{6.22 \times 0.8085 \times Vsample \times [prot\,ein(mg/mL)]} \right) \times 1000$$

Where:

ΔAbsblank = Variation in absorbance of the blank

ΔAbssample = Variation in absorbance of the sample

Vtotal = Total volume per well: 0.290 mL (x 1000, correct to µL)

6.22 $mM^{-1}cm^{-1}$ = Molar extinction coefficient (ε) of the NADPH

0.8085 cm = Light path

Vsample = Sample volume (0.04 mL)

2.5.5 Glutathione-S-transferases (GST)

GST activity was determined on the cytosolic fraction following methods described by Habig and Jakoby (1981), adapted to the microplate by McFarland *et. al.* (1999). GST catalyses the conjugation of 1-chloro 2,4 dinitrobenzene (CDNB) with reduced glutathione (GSH) resulting in the formation of Glutathione-S 2,4-dinitrobenzene (GS-DNB). Dinitrophenyl thioether is produced as a result, which can be detected by spectrophotometry at 340 nm. Samples were run in triplicate, and absorbance was read every 30 seconds over a three minute period, at ambient temperature, using Tecan (Infinite 200 Pro) microplate reader. The following formula was used to calculate GST activity (in nmol CDNB min^{-1} mg $protein^{-1}$):

$$GST\,activity = \left(\frac{(Abss - Absb) \times Voltotal \times DF}{(9.6 \times 0.6135 \times Vols \times [prot\,mg/mL]) \times 1000)} \right)$$

Where:

Abss = Absorbance of the sample (OD/min)/t

Absb = Absorbance of the blank (OD/min)

Voltotal = Total volume per well (0.225 ml)

DF = Dilution Factor

9.6 = CDNB Extinction coefficient (mM^{-1} cm^{-1})

0.6135 (cm) = Light path

Vols = Sample volume (0.025 ml)

2.6 Acetylcholinesterase (AChE) Activity

AChE activity was assessed in the gills of 96 individuals following protocol modified from Ellman's colorimetric method (Ellman *et al.,* 1961). Principle behind the reaction: Thiocoline is produced as AChE hydrolyses to acetylcholine. Thiocoline reacts non- enzymatically with DNTB releasing 5-mercapto-2-nitrobenzoato compound which is yellow in colour. The increase in this yellow compound is measured at 405 nm, with an extinction co-efficient of $\varepsilon = 13.6\ mM^{-1}\ cm^{-1}$ in order to estimate the amount of thiocoline produced which is proportional to the activity of AChE (Colovic *et al.*, 2013).

Gills were defrosted, weighed and homogenised, on ice, in 5 ml of Tris HCL buffer (100 mM, pH 8.0) and 50 µL of Triton – X 100 (0.1%). The homogenate was centrifuged at 12,000 g, at 4º C for 30 minutes. The supernatant, containing the cytosolic fraction, was subdivided into two aliquots, stored in Eppendorf tubes: Aliquot 1 for the determination of AChE activity and Aliquot 2, which was frozen at -80 ºC, for the determination of total protein concentration.

50 µl of sample was added, in triplicate, to a 96 well microplate. 200 µl of 5,5' -dithio-bis (2-nitrobenzoic acid) (DNTB, 0.75 mM) solution was added to each well and incubated for 5 minutes at ambient temperature. 50 µl of acetylcholine solution (ATC, 3 mM) was added to each well to trigger the reaction, described below. The microplate was incubated for 10 minutes at ambient temperature.

Absorbance was read using Tecan (Infinite 200 Pro) microplate reader at 405 nm, in 30 second intervals for 5 minutes, also at ambient temperature.

AChE activity, measured in (nmol ACTC min^{-1} mg $proteins^{-1}$), was quantified using the following formula:

$$AChE\ Activity = (\Delta A405 \times Volt) / (\varepsilon \times Lightpath \times Vols \times [proteins]) \times 1{,}000$$

Where:

$\Delta A405$ = The variation in absorbance at 405 nm per minute.

Volt = 0.300 ml, the total volume of mixture per well.

ε = DNTB Extinction Co-efficient = 13.6 mM cm^{-1}

Lightpath = The length of light passing through the microplate wells = 0.8385 cm

Vols = Volume of sample = 0.05 ml

[Proteins] = Concentration of proteins in the supernatant (S12) fraction quantified using the Bradford assay.

2.7 Lipid peroxidation (LPO)

LPO levels were quantified in the gills of 96 individuals following the colorimetric method described by Erdelmeier *et al.* (1998). Gills were defrosted, weighed and homogenised on ice, in 5 ml of Tris HCL buffer (0.02 M, pH 8.6) and 50 µL of butylated hydroxytoluene solution (BHT). The homogenate was centrifuged at 30,000 g, at 4 ºC for 45 minutes. The supernatant, containing the cytosolic fraction, was separated from the pellet and subdivided into two aliquots, stored in Eppendorf tubes: Aliquot 1 for the determination of lipid peroxidation activity and Aliquot 2, which was frozen at -80 ºC, for the determination of Total Protein concentration.

200 µL of supernatant was mixed with both, 650 µL of 1-methyl-2-phenylindone diluted in methanol, and 150 µL of methanesulfonic acid (15.4 M), and incubated at 45 ºC for 60 minutes. Following incubation, the mixture was centrifuged at 15,000 g, at 4 ºC for 10 minutes. 150 µL of resulting supernatant was added, in quadruplicate to a 96 well microplate. Absorbance was read using Tecan (Infinite 200 Pro) microplate reader at 386 nm, over 30 seconds at ambient temperature.

The following reaction was used to determine lipid peroxidation through the quantification of Malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) concentrations upon decomposition by polyunsaturated fatty acid peroxides:

Two moles of N-methyl-2-phenylindole (chromogenic reagent) + one mole of MDA incubated at 45 ºC for 60 minutes = a stable chromophore with maximum absorbance at 586 nm.

Malonaldehyde bis (dimethyl acetal) was used as a standard.

MDA was quantified, using the following formula:

$$MDA\ (nmol.mg^{-1}\ protein) = \frac{\frac{Abs-b}{a}(\mu mol/l) \times volume\ Tris\ (ml)}{Weight\ tissue\ (g)} \Big/ Total\ protein\ (mg/g)$$

Where:

Abs = Absorbance of the sample

a & *b* are obtained from the standard curve equation

2.8 Total Protein Concentration

Total protein concentrations were determined in the cytosolic fraction of gill tissues, post homogenisation, using the Bradford Assay (Bradford, 1976). Concentrations were used to normalise enzyme activities and LPO levels. The principle of the assay is based on the absorbance shift of Coomassie Brilliant Blue G-250 dye. Binding of the dye to proteins causes a conversion, from it's doubly protonated red cationic form (maximum absorbance = 470 nm) to a stable un-protonated blue form (maximum absorbance = 595 nm) to occur. A series of standard protein dilutions were prepared, from 0.005 - 1.0 mg ml^{-1}, using bovine serum albumin (BSA). Ultrapure water (Milli-Q), was used as a blank (0 mg ml^{-1} protein). Samples were defrosted on ice, diluted 1/5 with ultrapure water (Milli-Q) and vortexed gently. 5 µL of sample, blank, or standard was added, in quintuplet, to a 96 well microplate reader. 200 µL of diluted Bradford solution (1:5) was added to each well. Microplates were incubated for 20 minutes. Absorbance was measured at 595 nm. The increase in absorbance is proportional to the amount of bound dye, therefore also to the amount of protein present in the sample. The following formulae were used to determine total protein concentrations, which are expressed as mg g^{-1} of wet tissue weight:

*Concentration (mg ml^{-1}) = ((Average Abs * b)/(a))*

And

*Protein (mg g^{-1}) = Concentration * (vol Tris(ml)/ W tissue (g))*

Where:

Concentration: takes into account the dilution factor used

a & b are obtained from the standard curve equation

vol Tris = Buffer volume used to dilute the sample before homogenisation = 5 ml

W tissue = Wet weight of gill tissue sample before homogenisation

2.9 Statistical Analysis

Statistical analyses were performed using R 3.3.1 (R Core Team, 2016). Data are expressed as mean ± standard deviation (SD). Results were compared using two-way ANOVA, with polymer type and time as variables. Significant ANOVA results were analysed using Tukey's HSD test. Any difference with a p value ≤ 0.05 was considered significant.

3. RESULTS

3.1 Condition Index (CI)

The CI of the organisms' pre-exposure, (day 0), was 7.7 ± 2.7%. No significant differences were found between control (7.6 ± 0.4%) and virgin MP treatments (7.7 ± 0.3%) ($p > 0.05$), nor between virgin MP and contaminated MP treatments (BAP 7.4 ± 0.5%, BP3 7.1 ± 0.8%, PFOS 7.4 ± 0.9%, $p > 0.05$). No significant changes were observed between sampling times of the same treatment ($p > 0.05$). Results, expressed as treatment means ± SD, indicate that *S. plana* clams remained in good health for the duration of the experiment.

3.2 Virgin LDPE MP

3.2.1 DNA Damage: Virgin LDPE MP

Figure 3.1 presents the results of the Comet assay for unexposed and virgin MP exposed clams. No significant differences were found between virgin LDPE and control treatments, nor between times of the same treatment, when quantifying DNA damage through the resulting comet tail length (Figure 3.1A) or the percentage of DNA (Figure 3.1C) in the migrating tail ($p > 0.05$). A significant difference occurred between day 0 and day 14 of exposure to virgin LDPE when quantifying DNA damage through OTM (Figure 3.1B), with cells exposed to virgin LDPE displaying a higher OTM pre-exposure than after 14 days of exposure ($p < 0.05$). Average OTM was higher pre-exposure, than 14 days of exposure, in both control and virgin treatments (Figure 3.1B).

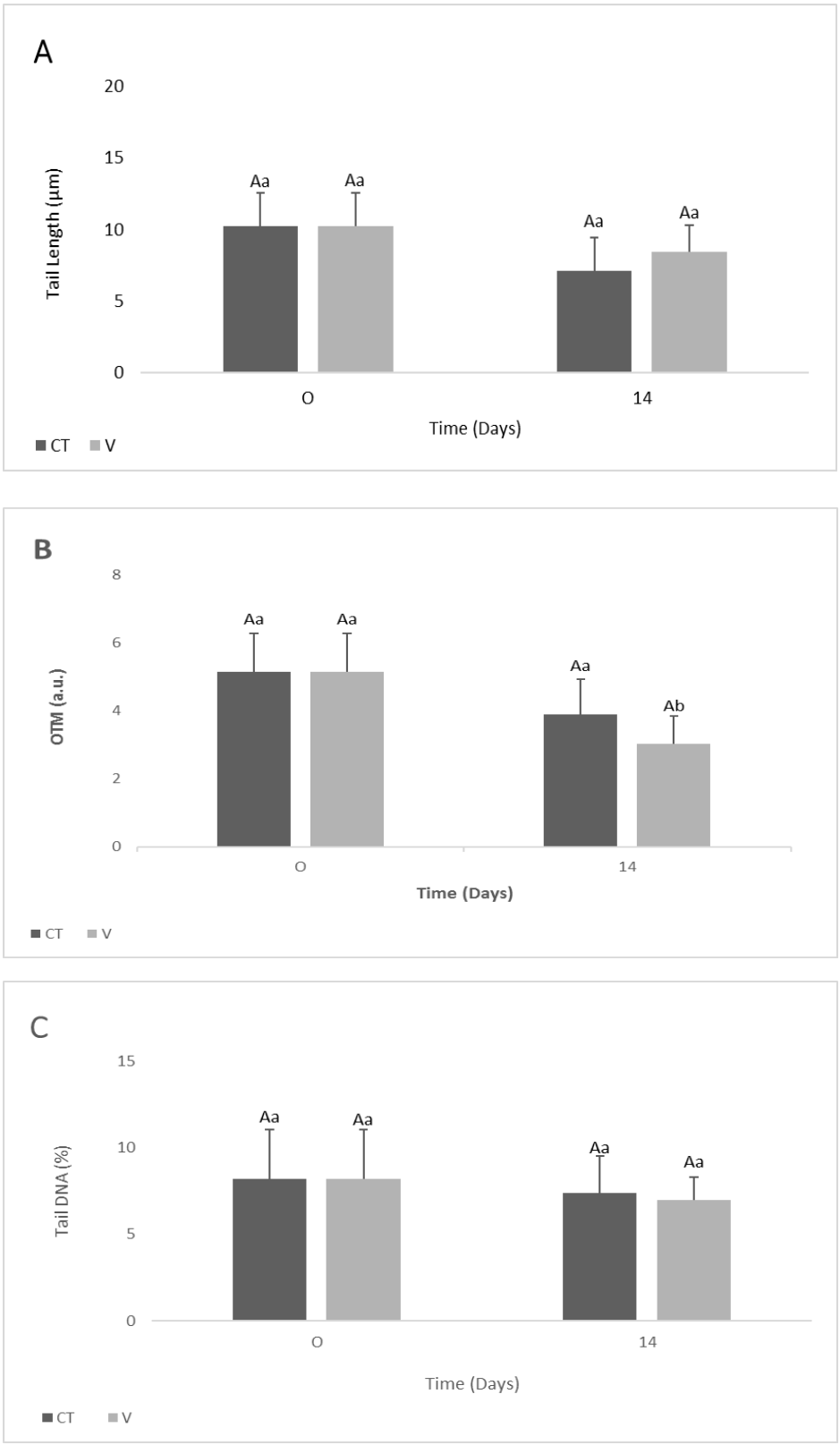

Figure 3.1 DNA damage (mean ± SD) in the haemocytes of *S. plana*, expressed as Tail length (µm) (A), OTM (a.u.) (B) and Tail DNA (%) (C) for control (CT) and virgin LDPE (V) treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.2.2 Enzyme Activity: Virgin LDPE MP

Antioxidant (SOD, CAT, GPx) and biotransformation (GST) enzyme activities for control and virgin treatments are displayed in figure 3.2. Responses vary per activity of enzyme investigated and per time.

No significant differences were found between virgin LDPE and control treatments when quantifying SOD activity in the gills of *S. plana* ($p > 0.05$) (Figure 3.2A). SOD activity did not change among the control or virgin LDPE treatments during the experiment ($p > 0.05$).

CAT activity in the gills of control organisms remained stable during the experiment ($p > 0.05$) (Figure 3.2B). Exposure to virgin LDPE caused a significant increase in CAT activity after 3 days ($p < 0.05$), with a significant difference between control and virgin treatments occurring at this time ($p < 0.05$). CAT activity subsequently decreased at day 7 and 14, with a significant difference occurring between tissues exposed to virgin LDPE after 3 days of exposure and both these times ($p < 0.05$).

Variation in GPx activity was investigated after 14 days of exposure (Figure 3.2C). A significant decrease in activity is observed in control treatments after 14 days ($p < 0.05$). GPx activity is significantly increased in virgin MP treatments after 14 days of exposure, relative to both pre-exposure and the control at day 14 ($p < 0.05$).

Control treatments increase in GST activity 3 days into the experiment and remain stable after that (Figure 3.2D). A significant difference in controls is observed between day 0 and all other sampling times ($p < 0.05$). GST activity increases steadily in gill tissues of organisms exposed to virgin LDPE. A significant increase in GST activity is first observed after 7 days of exposure ($p < 0.05$), with a significant difference in activity occurring between day 14 and all other previous sampling times ($p < 0.05$). A significant difference in GST activity only occurs between the control and virgin LDPE treatments after 14 days of exposure ($p < 0.05$).

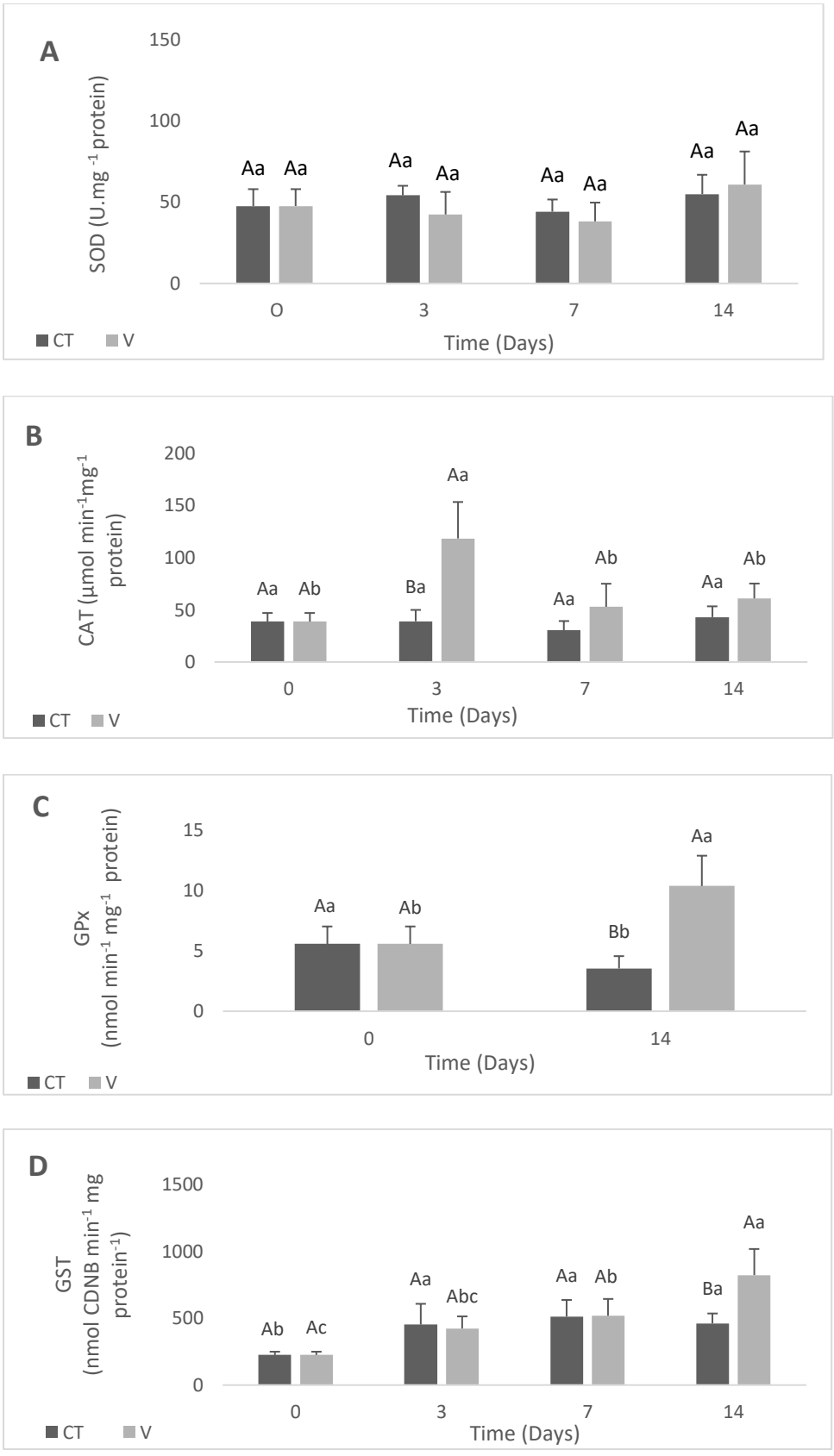

Figure 3.2 SOD (A), CAT (B), GPx (C) and GST (D) activities (mean ± SD) in the gills of *S. plana* for control (CT) and virgin LDPE MP (V) treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.2.3 Acetylcholinesterase Activity: Virgin LDPE MP

AChE activity remained stable in the control treatment until a significant decrease was observed between day 7 and 14 ($p < 0.05$) (Figure 3.3). No significant difference in AChE activity occurred between control and virgin LDPE MP treatments during the experiment ($p > 0.05$). See figure 3.3.

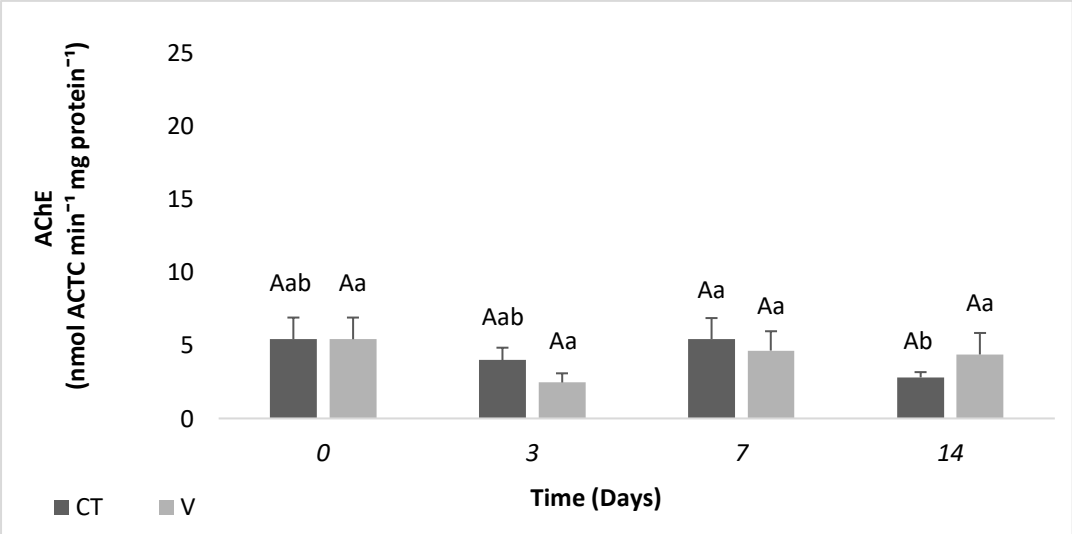

Figure 3.3 AChE activity (mean ± SD) in gill tissues of *S. plana* for control (CT) and virgin LDPE MP (V) treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.2.4 Lipid peroxidation: Virgin LDPE MP

LPO levels remained stable for both control and virgin exposed treatments, until day 14 when a significant increase occurred, with both increasing simultaneously ($p < 0.05$) (Figure 3.4). Levels significantly differed at this time for both treatments in respect to all previous times ($p < 0.05$). No significant difference in LPO levels occurred between control and virgin LDPE MP treatments over the two-week experimental period ($p > 0.05$). See figure 3.4.

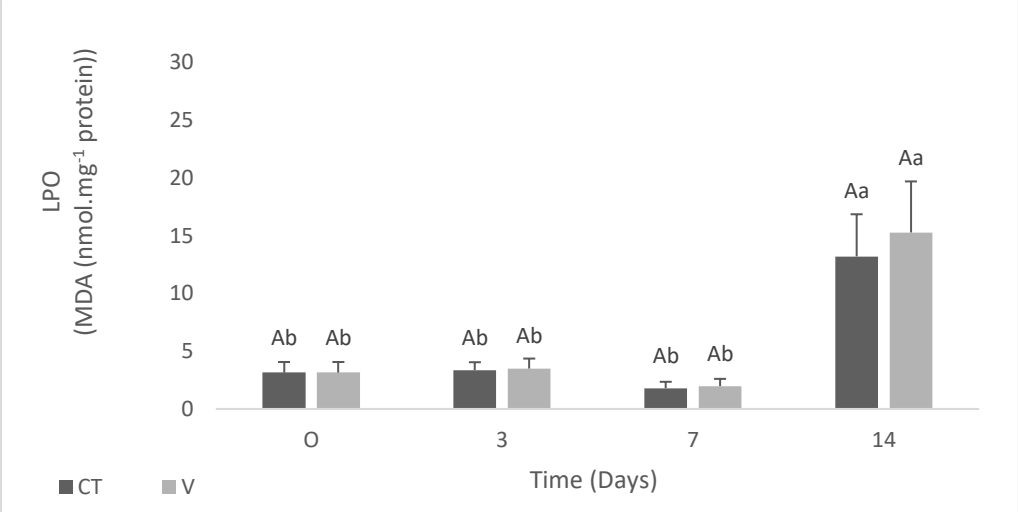

Figure 3.4 LPO levels (mean ± SD MDA) in gill tissues of *S. plana* for control (CT) and virgin LDPE MP (V) treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.3 BAP Contaminated LDPE MP

3.3.1 DNA Damage: BAP Contaminated LDPE MP

No significant differences were found between control and BAP contaminated LDPE treatments, nor between virgin LDPE and BAP contaminated LDPE treatments when quantifying DNA damage through the resulting comet tail length or the percentage of DNA in the migrating tail ($p > 0.05$) (Figure 3.5A, C). No significant differences were found between times for BAP contaminated LDPE treatments when investigating these two respective DNA damage parameters ($p > 0.05$). When quantifying DNA damage through OTM, a significant difference occurred between virgin and BAP contaminated LDPE treatments after 14 days of exposure, with average OTM being higher in the haemocyte cells of organisms exposed to LDPE contaminated with BAP ($p < 0.05$) (Figure 3.5B).

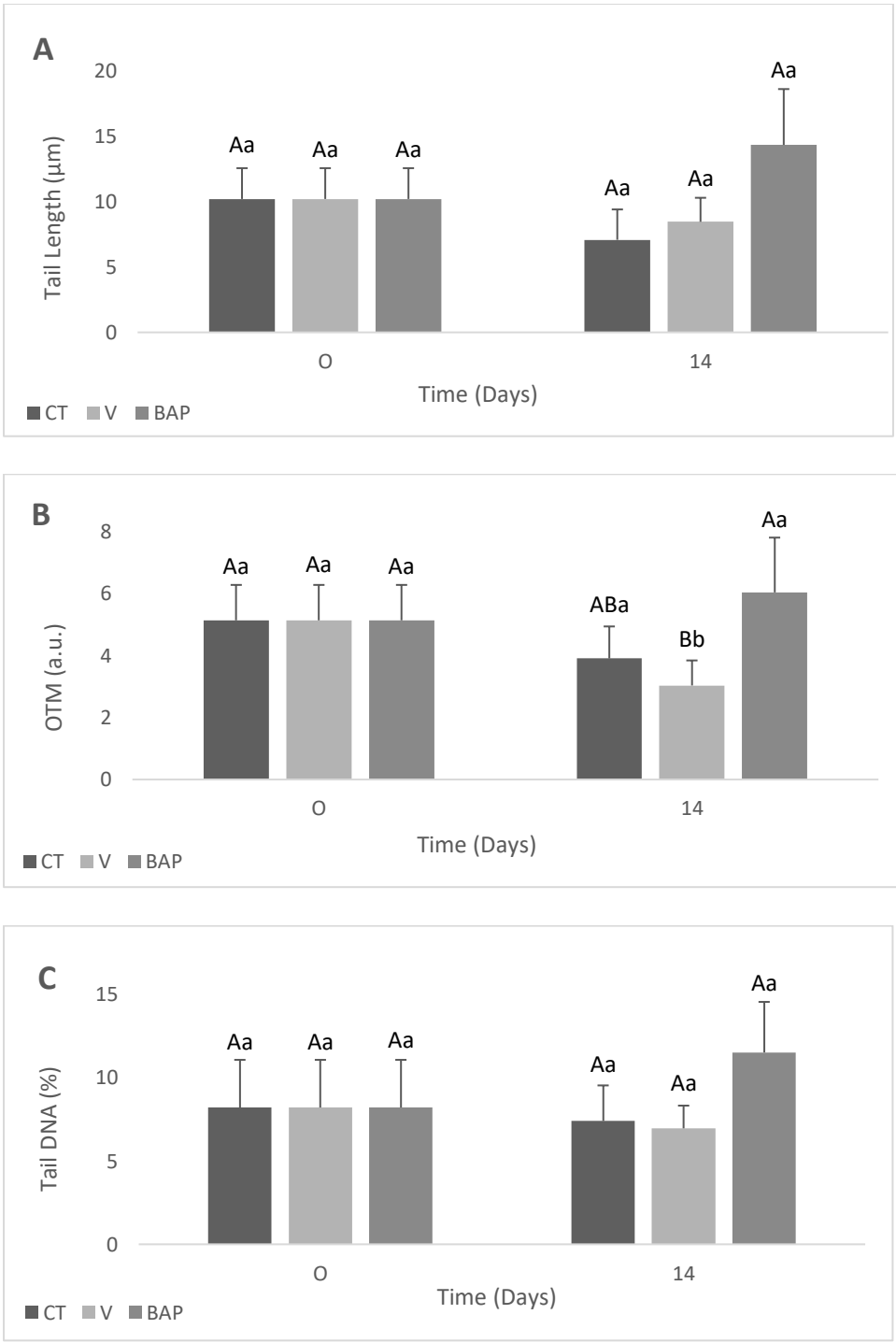

Figure 3.5 DNA damage (mean ± SD) in the haemocytes of *S. plana*, expressed as Tail length (µm) (A), OTM (a.u.) (B) and Tail DNA (%) (C) for control (CT), virgin LDPE MP (V) and benzo[a]pyrene contaminated LDPE MP (BAP) treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.3.2 Enzyme Activity: BAP Contaminated LDPE MP

Antioxidant (SOD, CAT, GPx) and biotransformation (GST) enzyme activities for control, virgin and BAP treatments are displayed in figure 3.6. Responses vary per activity of enzyme investigated and per time.

An increase in SOD activity in the gills of *S. plana* was induced following 7 days of exposure to BAP contaminated LDPE MP, with a significant difference occurring between tissues exposed to BAP contaminated MP and both control and virgin MP treatments ($p < 0.05$) (Figure 3.6A). This increase in activity was first observed between virgin LDPE and BAP contaminated LDPE following 3 days of exposure ($p < 0.05$).

CAT activity in the gills of organisms exposed to BAP contaminated LDPE MP remained stable during the experiment ($p > 0.05$) (Figure 3.6B). A significant difference in activity occurs between virgin and BAP treatments after 3 days of exposure, following an increase of CAT activity in tissues of *S. plana* exposed to virgin LDPE ($p < 0.05$).

Variation in GPx activity was only investigated after 14 days of exposure (Figure 3.6C). GPx activity in virgin and BAP treatments significantly differ after 14 days of exposure, with virgin MP inducing an increase in activity relative to BAP contaminated MP ($p < 0.05$). Exposure to BAP contaminated MP did not induce a significant response in GPx activity after 14 days ($p > 0.05$).

Exposure to BAP contaminated LDPE MP induces a steady increase in GST activity, with a significant increase in activity first observed after 7 days ($p < 0.05$) (Figure 3.6D). Activity does not significantly increase between 7 and 14 days exposure ($p < 0.05$). After 14 days GST activities are significantly higher than the control values at the same time ($p < 0.05$). No significant differences between GST activity in the gills of exposed organisms occurs between virgin and BAP contaminated treatments ($p > 0.05$).

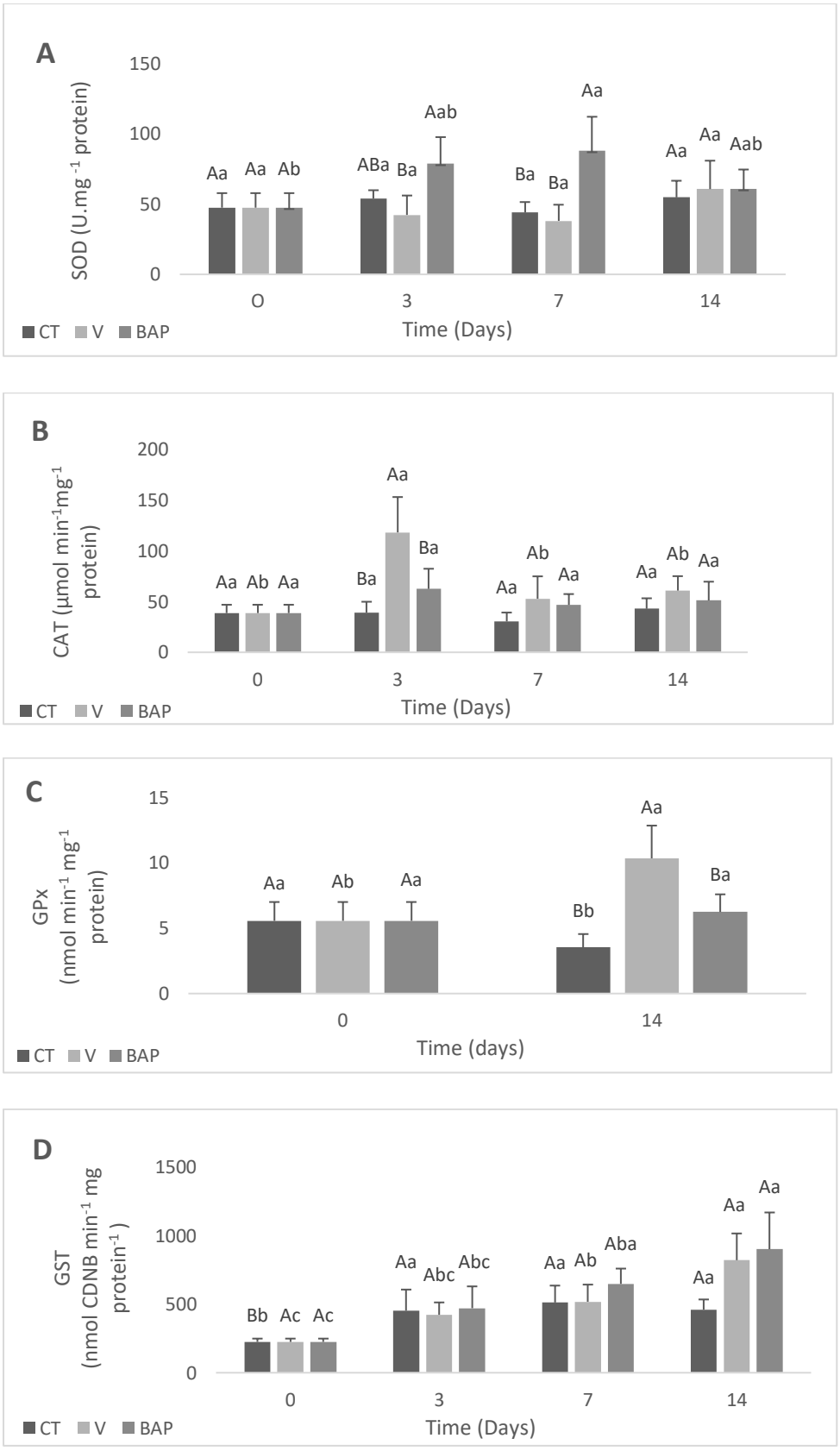

Figure 3.6 SOD (A), CAT (B), GPx (C) and GST (D) activities (mean ± SD) in the gills of *S. plana* for control (CT), virgin LDPE MP (V) and benzo[a]pyrene contaminated LDPE MP (BAP) treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.3.3 AChE Activity: BAP Contaminated LDPE MP

AChE activity remains stable in BAP contaminated LDPE MP treatments until day 14 of exposure, when values significantly increase ($p < 0.05$, relative to all previous sampling times), see figure 3.7. A significant difference is observed between both BAP and virgin, and BAP and control treatments at this time ($p < 0.05$). No significant difference in activity occurred previous to this between BAP contaminated LDPE MP and virgin LDPE MP treatments ($p > 0.05$) (Figure 3.7).

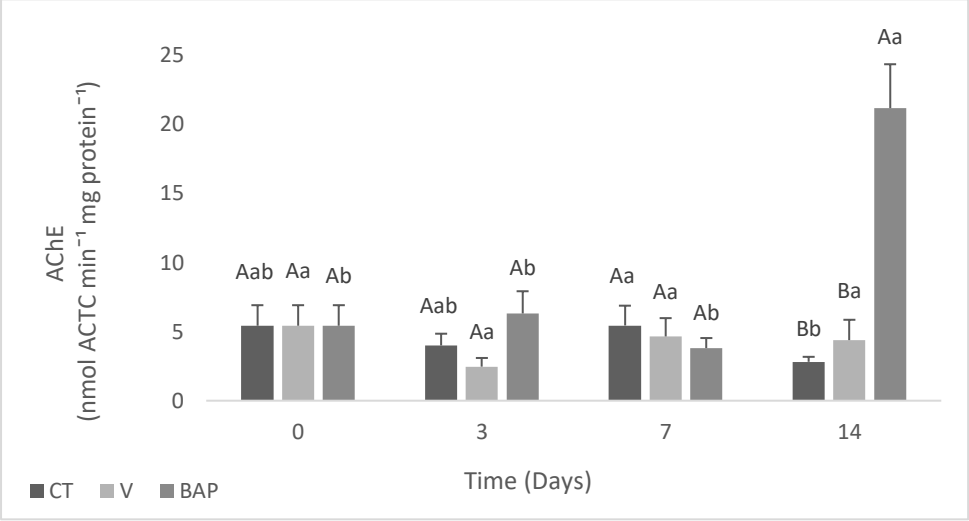

Figure 3.7 AChE activity (mean ± SD) in gill tissues of *S. plana* for control (CT), virgin LDPE MP (V) and benzo[a]pyrene contaminated LDPE MP (BAP) treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.3.4 Lipid peroxidation: BAP Contaminated LDPE MP

Exposure to BAP contaminated MP induced a significant increase in LPO levels after 14 days ($p < 0.05$) (Figure 3.8). Levels at this time significantly differ to all previous times of BAP exposure ($p < 0.05$). A significant difference in LPO levels after 14 days occurred between the control and BAP contaminated MP treatments ($p < 0.05$). No significant difference in LPO occurred between virgin MP and BAP contaminated MP treatments over the two-week experimental period ($p > 0.05$). See figure 3.8.

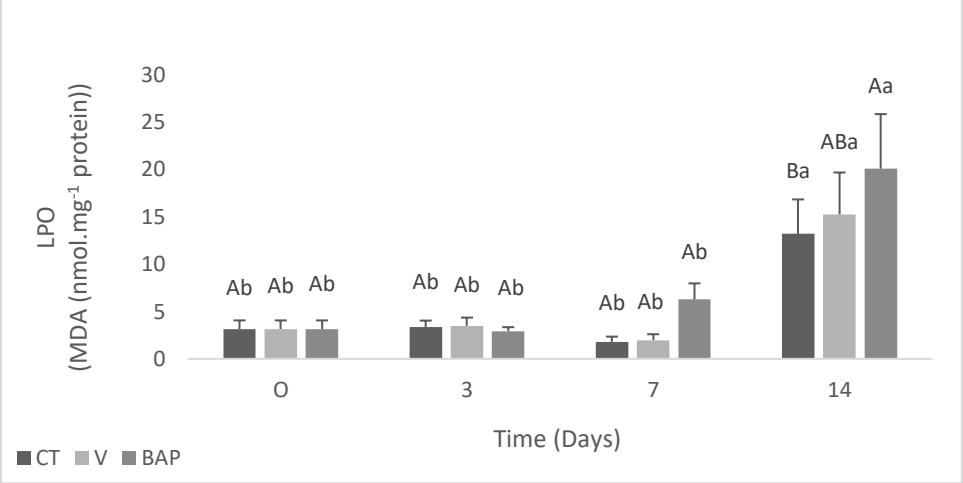

Figure 3.8 LPO levels (mean ± SD MDA) in gill tissues of *S. plana* for control (CT), virgin LDPE MP (V) and benzo[a]pyrene contaminated LDPE MP (BAP) treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.4 BP3 Contaminated LDPE MP

3.4.1 DNA Damage: BP3 Contaminated LDPE MP

No significant differences were found between control and BP3 contaminated LDPE treatments, nor between virgin LDPE and BP3 contaminated LDPE treatments when quantifying DNA damage through the resulting OTM or the percentage of DNA in the migrating tail ($p > 0.05$) (Figure 3.9B, C). No significant differences were found between pre- and 14 days post- exposure to BP3 contaminated LDPE, when investigating these two respective DNA damage parameters ($p > 0.05$). When quantifying DNA damage through the resulting comet tail length a significant difference occurred between the control cells and BP3 contaminated LDPE cells, both at day 14, with tail length increasing in cells exposed to LDPE MP contaminated with BP3 ($p < 0.05$) (Figure 3.9A).

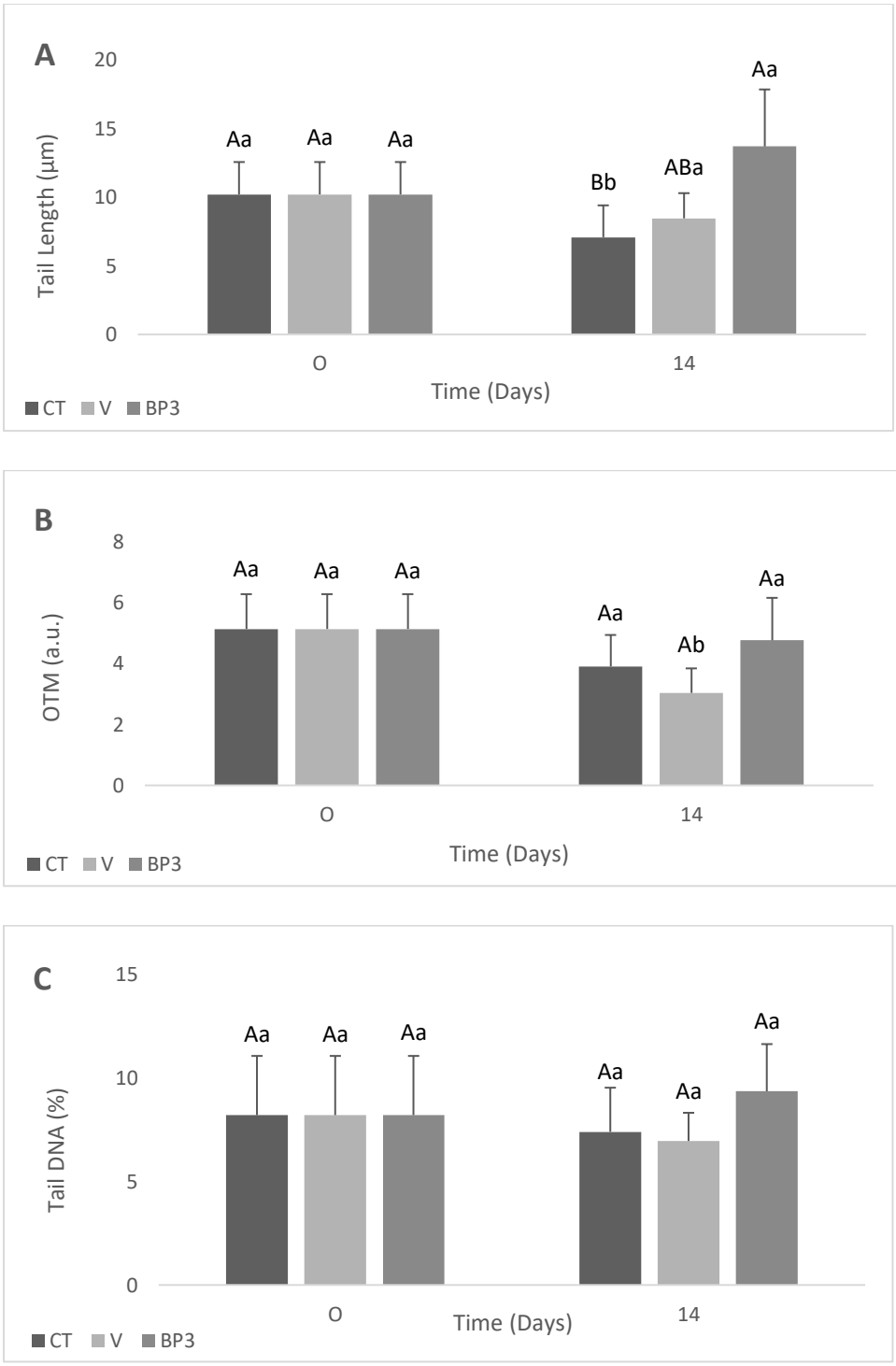

Figure 3.9 DNA damage (mean ± SD) in the haemocytes of *S. plana*, expressed as Tail length (µm) (A), OTM (a.u.) (B) and Tail DNA (%) (C) for control (CT), virgin LDPE MP (V) and oxybenzone contaminated LDPE MP (BP3) treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.4.2 Enzyme Activity: BP3 Contaminated LDPE MP

Antioxidant (SOD, CAT, GPx) and biotransformation (GST) enzyme activities for control, virgin and BP3 treatments are displayed in figure 3.10. Responses vary per activity of enzyme investigated and per time.

An increase in SOD activity in the gills of *S. plana* was induced following 7 days exposure to BP3 contaminated LDPE MP ($p < 0.05$), with a significant difference occurring between tissues exposed to BP3 contaminated LDPE and both control and virgin LDPE treatments at this time ($p < 0.05$) (Figure 3.10A). SOD activity subsequently decreased by day 14 of exposure, with a significant difference occurring between day 7 and 14 ($p < 0.05$).

No significant differences were observed in CAT activity in the gills of *S. plana* exposed to BP3 contaminated LDPE MP during the experiment ($p > 0.05$) (Figure 3.10B). A significant difference in activity occurs between virgin and BP3 treatments after 3 days of exposure, following an increase of CAT activity in tissues of organisms exposed to virgin LDPE MP ($p < 0.05$).

Variation in GPx activity was only investigated after 14 days of exposure (Figure 3.10C). Activity in virgin and BP3 treatments significantly differ after 14 days, with virgin MP inducing an increase in activity relative to BP3 contaminated MP ($p < 0.05$). Exposure to BP3 contaminated MP did not induce a significant response in GPx activity after 14 days ($p > 0.05$).

Exposure to LDPE MP contaminated with BP3 induces a significant increase in GST activity after 7 days ($p < 0$ .05) (Figure 3.10D). GST activity stabilise between 7 and 14 days of exposure, with significant differences observed between 3 and 7, and 3 and 14 days ($p < 0.05$). No significant difference occurs in GST activity in the gills of exposed organisms between virgin and BP3 contaminated treatments ($p > 0.05$).

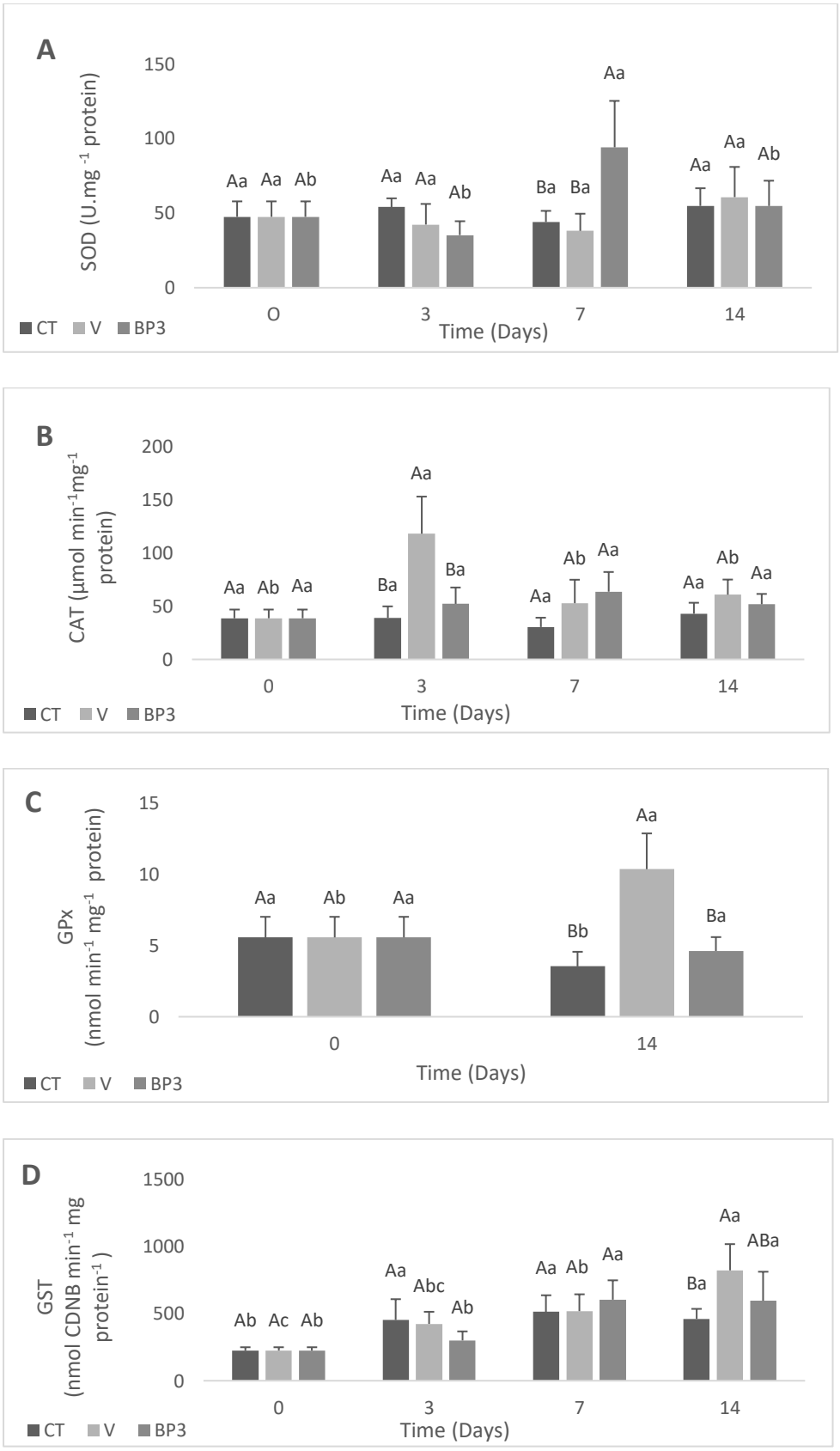

Figure 3.10 SOD (A), CAT (B), GPx (C) and GST (D) activities (mean ± SD) in the gills of *S. plana* for control (CT), virgin LDPE MP (V) and oxybenzone contaminated LDPE MP (BP3) treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.4.3 AChE Activity: BP3 Contaminated LDPE MP

No significant difference in AChE activity occurs in the gills of organisms exposed to LDPE MP contaminated with BP3, until day 14, when a significant increase is observed with respect to all previous sampling times ($p < 0.05$) (Figure 3.11). A significant difference occurs at this time between both BP3 and control, and BP3 and virgin treatments ($p < 0.05$). No significant difference in activity occurred previous to this between BP3 contaminated LDPE MP and virgin LDPE MP treatments ($p > 0.05$). See figure 3.11.

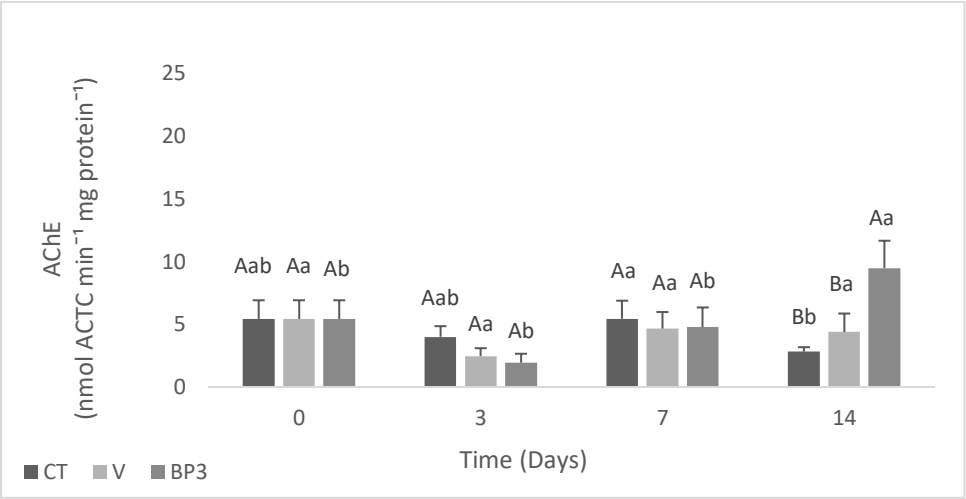

Figure 3.11 AChE activity (mean ± SD) in gill tissues of *S. plana* for control (CT), virgin LDPE MP (V) and oxybenzone contaminated LDPE MP (BP3) treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.4.4 Lipid peroxidation: BP3 Contaminated LDPE MP

An increase in LPO levels occurred in gill tissues following 7 days of exposure to MP contaminated with BP3 (Figure 3.12). A significant difference in levels is observed at this time with respect to all other BP3 MP treatment times ($p < 0.05$). A significant difference between LPO levels in BP3 and virgin, and BP3 and control treatments exists at day 7 ($p < 0.05$). LPO levels subsequently decreased after 14 days exposure, with both control and virgin MP treatment activities being significantly higher at this time ($p < 0.05$). See figure 3.12.

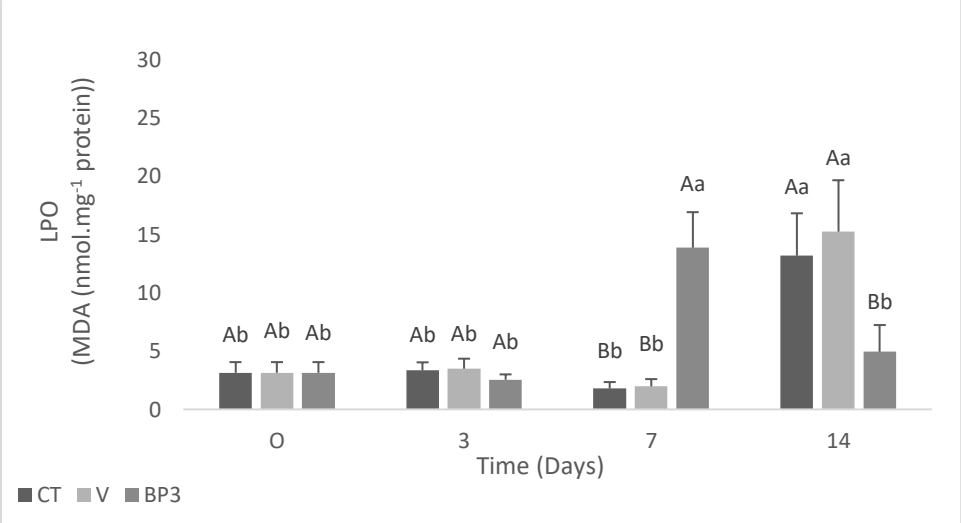

Figure 3.12 LPO levels (mean ± SD MDA) in gill tissues of *S. plana* for control (CT), virgin LDPE MP (V) and oxybenzone contaminated LDPE MP (BP3) treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.5 PFOS Contaminated LDPE MP

3.5.1 DNA Damage: PFOS Contaminated LDPE MP

No significant differences were found between control and PFOS contaminated LDPE treatments, nor between virgin LDPE and PFOS contaminated LDPE treatments when quantifying DNA damage through the resulting comet tail length, the percentage of DNA in the migrating tail, nor OTM ($p > 0.05$) (Figure 3.13A, B, C). No significant differences were found between pre-exposure, and 14 days of exposure to PFOS contaminated LDPE, when investigating either respective DNA damage parameter ($p > 0.05$). See figure 3.13.

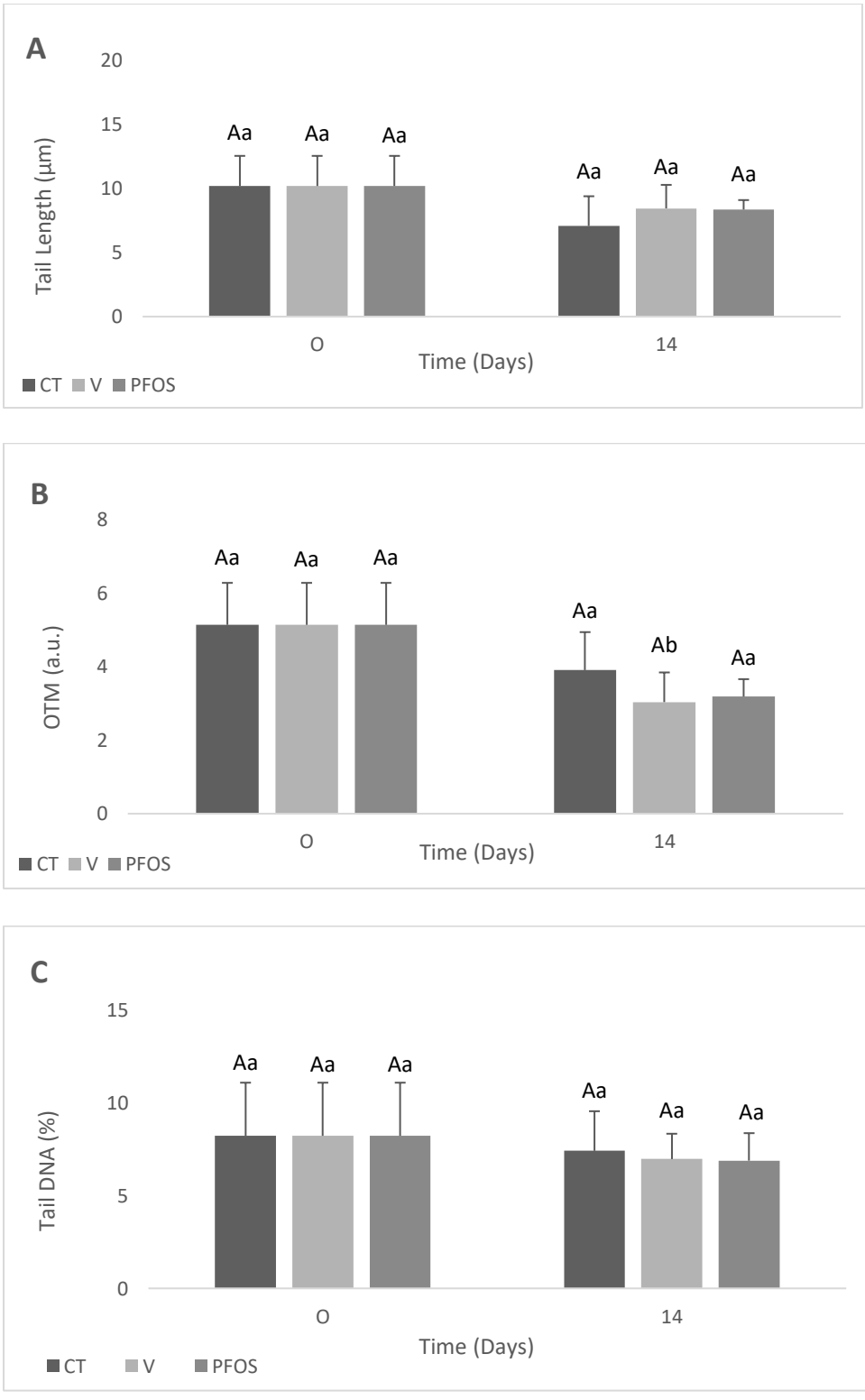

Figure 3.13 DNA damage (mean ± SD) in the haemocytes of *S. plana* expressed as Tail length (µm) (A), OTM (a.u.) (B) and Tail DNA (%) (C) for control (CT), virgin LDPE MP (V) and perfluorooctane sulfonate contaminated LDPE MP (PFOS) treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.5.2 Enzyme Activity: PFOS Contaminated LDPE MP

Antioxidant (SOD, CAT, GPx) and biotransformation (GST) enzyme activities for control, virgin and PFOS treatments are displayed in figure 3.14. Responses vary per activity of enzyme investigated and per time.

An increase in SOD activity in the gill tissues was induced following 7 days exposure to PFOS contaminated LDPE ($p < 0.05$), with a significant difference occurring between tissues exposed to PFOS contaminated LDPE and both control and virgin LDPE treatments at this time ($p < 0.05$) (Figure 3.14A). SOD activity subsequently decreased by day 14 of exposure, with a significant difference occurring between day 7 and days 0 and 14 ($p < 0.05$).

CAT activity in the gills of organisms exposed to LDPE MP contaminated with PFOS remained stable during the experiment ($p > 0.05$) (Figure 3.14B). A significant difference in activity occurs between virgin and PFOS treatments after 3 days of exposure, following an increase of activity in tissues exposed to virgin LDPE ($p < 0.05$). After 7 days exposure, a significant difference is observed in CAT activity between the control and LDPE contaminated with PFOS ($p < 0.05$). This is due to the combined slight increase of CAT activity in the tissues of the PFOS treatment and the slight decrease of CAT activity in control tissues.

Variation in GPx activity was investigated only after 14 days of exposure (Figure 3.14C). GPx activity in virgin and PFOS treatments significantly differ after 14 days, with virgin MP inducing an increase in activity relative to PFOS contaminated MP ($p < 0.05$). Exposure to PFOS contaminated MP did not induce a significant response in GPx activity after 14 days ($p > 0.05$).

Exposure to LDPE MP contaminated with PFOS induces a significant increase in GST activity after 7 days ($p < 0 .05$) (Figure 3.14D). Activity stabilises between 7 and 14 days of exposure, with activities on day 7 and 14 being significantly higher than pre-exposure levels ($p < 0.05$). A significant difference in GST activities in the gills of exposed organisms only occurs between virgin and BAP contaminated treatments after 14 days ($p <0.05$).

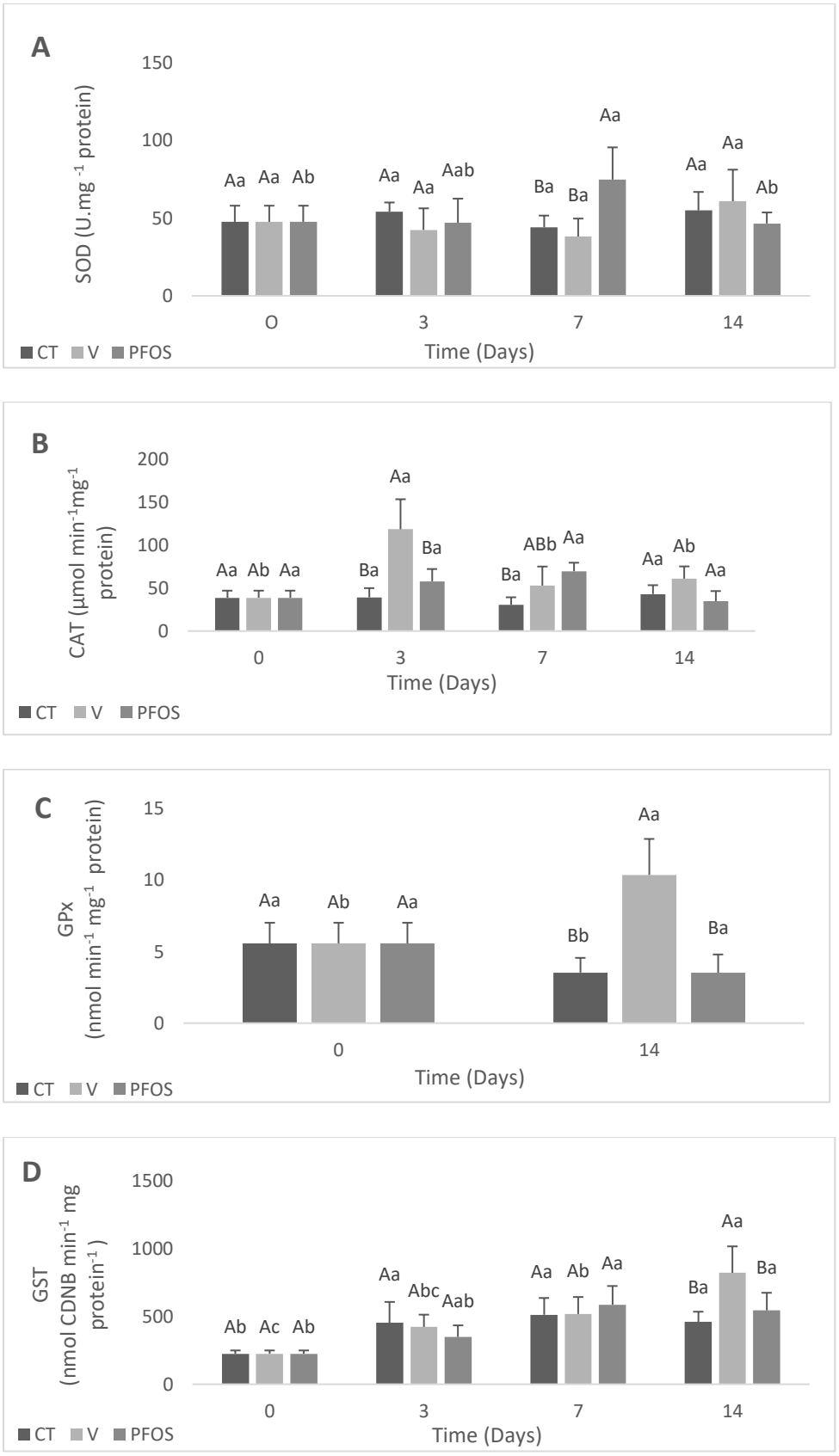

Figure 3.14 SOD (A), CAT (B), GPx (C) and GST (D) activities (mean ± SD) in the gills of *S. plana* for control (CT), virgin LDPE MP (V) and perfluorooctane sulfonate contaminated LDPE MP (PFOS) treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.5.3 AChE Activity: PFOS Contaminated LDPE MP

No significant difference in AChE activity in the gills of *S. plana* exposed to PFOS Contaminated LDPE MP occurred during the experimental period ($p > 0.05$) (Figure 3.15). No significant differences were observed in AChE activity between virgin and PFOS Contaminated LDPE MP treatments ($p > 0.05$). See figure 3.15.

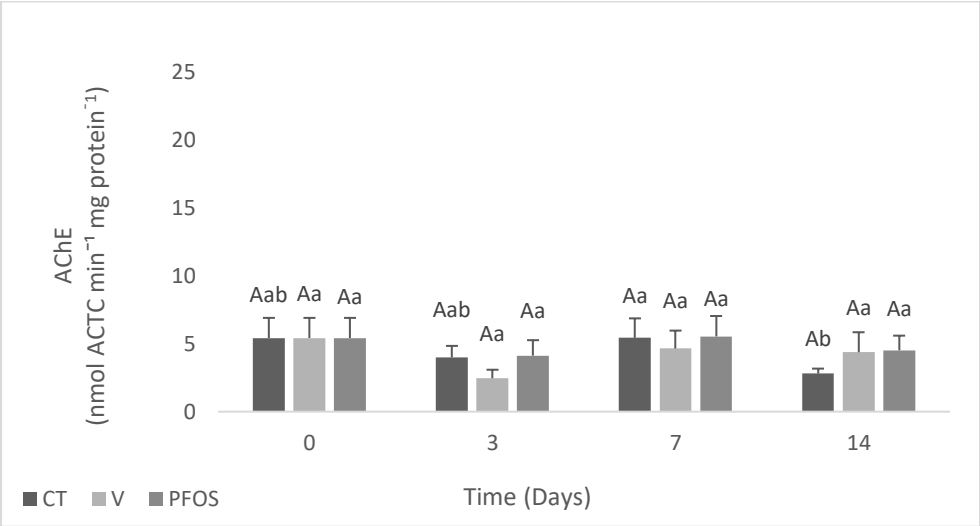

Figure 3.15 AChE Activity (mean ± SD) in gill tissues of *S. plana* for control (CT), virgin LDPE MP (V) and perfluorooctane sulfonate contaminated LDPE MP (PFOS) treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

3.5.4 Lipid peroxidation: PFOS Contaminated LDPE MP

A significant increase in LPO levels occur in the gills of *S. plana* after 7 days of exposure to PFOS contaminated MP ($p < 0.05$, with respect to both day 0 and day 3) (Figure 3.16). LPO levels at this time are significantly different to both control and virgin MP treatments ($p < 0.05$). LPO stabilises between day 7 and day 14 of exposure to PFOS contaminated MP. A significant difference in LPO levels occur between both pre-exposure and day 14, as well as at day 3 and day 14, in PFOS contaminated MP treatments ($p < 0.05$). See figure 3.16.

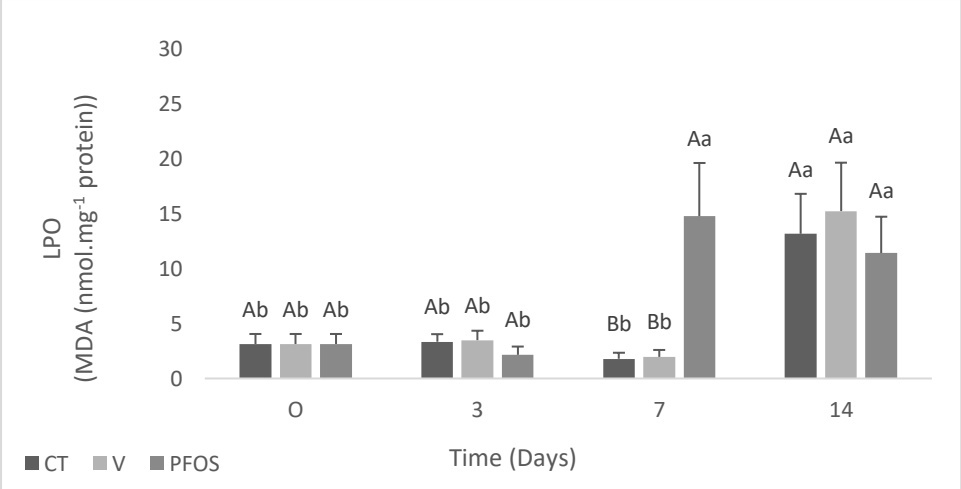

Figure 3.16 LPO levels (mean ± SD MDA) in gill tissues of *S. plana* for control (CT), virgin LDPE MP (V) and perfluorooctane sulfonate contaminated LDPE MP (PFOS) treatments. Different capital letters indicate a significant difference between treatments within the same time. Different lowercase letters indicate a significant difference for the same treatment between times ($p < 0.05$).

4. DISCUSSION

A battery of biochemical, cellular and physiological biomarkers were analysed in order to characterise the ecotoxicological potential of both virgin and contaminated LDPE microplastics. Environmentally relevant concentrations of contaminants were adsorbed to microplastics to address both the potential for microplastics to act as a vector for chemical exposure, and the effect of each respective contaminant. Efforts were focused on the gills of *S. plana* as previous studies, have indicated the gills to be the first site of MP particle uptake (Browne *et al*., 2008; von Moos, Burkhardt-Holm and Köhler, 2012; Ribeiro *et al*., 2017).

4.1 DNA Damage

No significant enhancement in DNA strand breaks were detected in the haemocytes of *S. plana* clams exposed to virgin MP or to PFOS contaminated MP (Figures 3.1, 3.13). There is some evidence to suggest that exposure to both BAP contaminated MP and BP3 contaminated MP induces DNA damage after 14 days (Figures 3.5B, 3.9A, 3.9B), yet the strength of these results are low.

Congruence between different parameters of the comet assay analysis - tail DNA, tail length and OTM - did not occur (Figures 3.1, 3.5, 3.9, 3.13, A – C). Results indicate OTM be a slightly more sensitive index of induced DNA damage compared to tail length and tail DNA (Figures 3.1B, 3.5B, 3.9B, 3.13B). This is due to OTM measurements including both the relative amount of DNA in the tail as well as the migration of genetic material.

The comet assay has been proven to be a robust, sensitive and cost-effective tool for assessing genotoxicity in haemocyte cells of *S. plana* (Petridis *et al*., 2009). Studies have indicated the genotoxic potential of both virgin polystyrene MP in *S. plana*, and virgin PE MP in *M. galloprovincialis*, through a significant increase in DNA strand breaks in haemocyte cells (Avio *et al*., 2015; Ribeiro *et al*., 2017). While Ribeiro and colleagues used similar MP concentrations to those described here, a significantly higher concentration of MP, 1.5 g L^{-1}, were used by Avio and colleagues. Although this study does not concur with those described above, results must be viewed with caution due to questions that arise from the experimental design used. See section 4.5 for further discussion.

4.2 Oxidative Stress

Results indicate a time dependant oxidative stress response in the gills of *S. plana* that varies depending on treatment used and biomarker investigated. Enhanced SOD activity was observed in the gills of organisms exposed to each of the contaminated MP by day 7 of the experiment (Figures 3.6A, 3.10A, 3.14A). An increase in SOD activity is indicative of the first line of defence in protecting tissues against oxidative stress. SOD catalyses the partitioning of the superoxide anion radical ($O_2^{\bullet -}$) into hydrogen peroxide and water thus reducing the potential for oxidative damage to occur. By day 14 a significant decrease in activity was observed in both BP3 (Figure 3.10A) and PFOS contaminated MP (Figure 3.14A), while SOD activity in BAP contaminated MP remained stable (Figure 3.6A). SOD activity remained stable for the duration of the experiment in gill tissues exposed to virgin MP, and did not significantly differ to that of the control (Figure 3.2A). Results in contaminated MP are comparable to those reported for virgin PS exposure in the gills of *S. plana* by Ribeiro and colleagues (2017). Exposure to virgin PE MP does not induce the same time dependant increase in SOD activity as exposure to virgin PS MP at the same concentration, 1 mg L^{-1} (Ribeiro *et al.,* 2017). A significant increase in SOD activity is observed during various sampling times for each respective contaminated MP treatment when compared with virgin MP treatment. A synergistic effect is implied, due to both physical ingestion and increased toxicity of MP with adsorbed contaminants.

A significant induction in CAT activity occurred after 3 days exposure to virgin MP (Figure 3.2B). The antioxidant enzyme CAT, prevents cellular damage from ROS by reducing both endo- and exo- genous sources of H_2O_2 to H_2O (Oliveira *et al.,* 2009; Solé, Kopecka-Pilarczyk and Blasco, 2009). CAT activity in virgin MP treatments is subsequently reduced, and comparable to pre-exposure levels by day 7 and 14 (Figure 3.2B). Exposure to polystyrene MP also induced an increase in CAT activity in the gills of *S. plana* after 3 days, yet in contrast levels did not reduce in subsequent days but remained elevated (Ribeiro *et al*., 2017). This suggests that PS MP may have an increased toxicity relative to PE MP in the peppery furrow shell clam. CAT induced activity was not observed in any of the contaminated MP treatments with CAT remaining stable relative to pre-exposure levels (Figures 3.6B, 3.10B, 3.14B). Previous studies have observed an inhibition of CAT activity in response to MP exposure, both in the digestive gland of *S. plana* exposed to PS MP, and in the digestive gland of *M. galloprovincialis* exposed to both virgin PE and PS, and pyrene contaminated PE and PS (Avio

et al., 2015; Ribeiro *et al.,* 2017). It may be argued that CAT is not the antioxidant defence mechanism used by *S. plana* in response to PE MP exposure or, that two weeks exposure to both virgin and contaminated LDPE MP, under the experimental conditions observed, is not sufficient to induce a significant response.

GPx activity does not respond to any of the contaminated MP treatments (Figures 3.6C, 3.10C, 3.14C). A defence mechanism reaction to oxidative stress is suggested by the significant increase activity in virgin MP treatments by day 14 (Figure 3.2C). If the observed increase in GPx activity in virgin treatments were to result from the physical ingestion of MP it would be expected that each respective contaminated MP treatment show a similar response, yet this does not occur (Figures 3.2C, 3.6C, 3.10C, 3.14C). Previous studies have shown that an inhibition in GPx may occur in response to elevated toxicity levels. Such inhibition was demonstrated in mussels (*M. galloprovincialis*) exposed to virgin and pyrene contaminated PE and PS MP (Avio *et al*., 2015). A similar trend was observed in whole tissues of *S.plana* exposed to mercury, with higher mercury levels inhibiting GPx activity (Ahmad *et al.,* 2011). This hypothesis, that an inhibition of the activity occurs due to the inability to process excess ROS, may explain the antagonistic effects observed in the contaminated MP treatments with respect to the virgin treatments. The experiment design used also needs to be considered when analysing such results, see section 4.5 for further discussion.

An increase in GST activity is observed in all MP treatments over time (Figures 3.2D, 3.6D, 3.10D, 3.14D). A similar increase in GST activity in the gills of *S. plana* has been described by Ribeiro and colleagues (2017) in response to PS MP exposure. GST activity has also been reported to increase in the gills of *M. galloprovincialis* following exposure to the persistent organic pollutant pp'DDE (2,2-bis-(p-chlorphenyl)-1,1-dichlorethylene) a metabolite of DDT (Khessiba *et al*., 2001). The phase two enzyme, glutathione-S-transferases (GSTs), play an important role in cellular protection against various xenobiotics and toxic endogenous substances by converting reactive lipophilic molecules into nonreactive water-soluble molecules which can be excreted by the organism (Hoarau *et al*., 2002). An increase in GST activity also occurs in the control between pre-exposure and day 3. GST activity in the control treatments remain similar to day 3 for the remainder of the experiment (Figure 3.2D). Although experimental conditions are kept as stable as possible, the influence of multiple stressors may affect GST activity in the control treatments. Removal of organisms on sampling days was complicated by the burrowing nature of the species and such perturbations in a controlled

environment may increase stress levels, see section 4.5 for further discussion. A significant increase in GST activity in contaminated MP treatments compared to virgin MP is not observed (Figures 3.2D, 3.6D, 3.10D, 3.14D). Synergistic effects of ingestion and chemical exposure did not occur, indicating that the overall increase in activity with time results from the physical ingestion of MP rather than the chemical toxicity of the respective contaminants.

4.3 Neurotoxic Damage

A significant reduction in AChE activity in control treatments occurs following 14 days of exposure (Figure 3.3). This result is hard to explain, leading to doubts about the reliability of the data at this time, see section 4.5 for further discussion. Inhibition of activity did not occur in any of the MP exposed treatments (Figures 3.7, 3.11, 3.15), with both BAP and BP3 contaminated MP treatments significantly increasing after 14 days of exposure. In contrast, a reduction in AChE activity in gill tissues of *S. plana* was observed following two weeks exposure to polystyrene microplastics at similar concentrations (Ribeiro *et al.*, 2017). Previous studies have indicated that a reduction in AChE activity in the gills of mussels (*Mytilus galloprovincialis*) occurred upon 7 days exposure to polystyrene and polyethylene microplastics, with and without pyrene contamination (Avio *et al*., 2015). Comparisons between experiments are confounded by numerous factors: different species used, different polymer concentrations used (1.5 g L^{-1} by Avio and colleagues (2015) compared to 1 mg L^{-1} in this experiment, a difference of 1500x), different polymer type (when comparing polyethylene, densities and sizes differ), different contaminants used and different exposure times. Exposure to polyethylene microplastics, with and without added pyrene have also shown to significantly reduce AChE activity in juveniles of the common goby, *Pomatoschistus microps* (Olivera *et al.*, 2013). No significant changes occur in AChE activity in either virgin or PFOS contaminated MP treatment (Figures 3.3, 3.15), indicating that a neurotoxic potential of PE MP does not exist under the experimental conditions observed for these treatments.

4.4 Oxidative Damage

LPO levels remain stable for the duration of the experiment in virgin MP treatments until day 14 when a significant increase is observed (Figure 3.4). Yet the same changes occur in the

control treatments indicating that the induced activity is not a result of MP exposure alone. LPO levels in membrane lipids of gill tissues in contaminated MP treatments show an increase after 7 days (Figure 3.8, 3.12, 3.16). As LPO levels in control treatment remain stable up to and including this time, it can be suggested with greater confidence that the observed increase in LPO is due to the exposure of contaminated MP. A synergistic effect of exposure is observed, with each respective contaminant having a greater effect on lipid peroxidation than the virgin microplastic alone. An increase in lipid peroxidation may result from inefficient oxidative stress reduction mechanisms in the processing of excess ROS. By day 14 of exposure each contaminated MP treatment shows a different response, with LPO levels significantly increasing in BAP treatments (Figure 3.8), staying the same in PFOS treatments (Figure 3.16) and significantly decreasing in BP3 treatments (Figure 3.12). Significant variation in the control at this time confounds the analysis of observed results. In contrast to the results of this experiment, levels of oxidative damage were reduced in the gills of *S. plana* exposed to PS MP at similar concentrations (Ribeiro *et al*., 2017). No increase in LPO levels was reported in tissues of the common goby, *P. microps*, following exposure to both PE MP and pyrene contaminated PE MP, at a concentration of 18.4 and 184 µg L^{-1} (Olivera *et al.*, 2013).

4.5 Experimental Challenges

Questions arise due to the experimental design, with the bioavailability of MP being the main concern. Both the limitations and assumptions of this experiment need to be addressed.

The use of LDPE, with a density of 0.96, which is lower than seawater density (1.025), raises doubts as to the bioavailability of the MP. During the exposure, it was assumed that once the plastic was applied to the aquaria, the aeration supply and constant mixing promoted the dispersal of the plastic within the water column and some setting would occur. Although the sedimentation rate of the microplastics used was investigated, (through the change of turbidity with time, (Sousa and Teixeira, 2013)) results remained inconclusive. Feeding mechanisms of *S. plana,* were assumed to increase the exposure of microplastics in the water column to the organism. Water and suspended particles are ingested through the inhalant siphons, which were observed up to 5cm above the sediment surface.

Water was changed every 72 hours, with the routine application of microplastics following. It was assumed that a concentration of 1 mg L^{-1} of microplastics was maintained throughout the experiment. Only 14 L of water, out of a total of 16 L was changed per aquarium, per time, in an effort to reduce the resuspension of sediment. As such 14 mg of plastics were reapplied each time following the changing of water. Distribution anomalies may have affected the actual concentration of particles during the course of the experiment. Microplastics were observed to cling to aquarium walls, just above the water line, and to the glass Pasteur pipette aeration tubes, as shown in figure 4.1.

Figure 4.1 Agglomeration of microplastic particles on a Pasteur pipette aeration tube due to electrostatic adherence. Photo: Author

Particle behaviour of microplastics (agglomeration and electrostatic adherence) may have affected the bioavailability of the particles. Although microplastics of 11-13 μm were applied, if dispersal within the water column did not occur, the size of the plastics may have been larger. Particles from 4-40 μm have been shown to be filtered by *S. plana* with up to 100% efficiency (Hughes, 1969). If agglomeration caused the particles to be larger than 40 μm, the polymers may not have been bioavailable. Agglomeration of microplastics have been observed and demonstrated in previous studies (von Moos, Burkhardt-Holm and Köhler, 2012; Ribeiro *et al*., 2017). Various methods for applying MPs were investigated. MP particles were sonified in ultrapure water in an effort to promote dispersion and reduce aggregation. Yet this method proved ineffective as re-aggregation of particles occurred during the transfer time between sonification equipment and the aquaria.

Although efforts were taken to reduce the resuspension of sediments when changing water in aquaria, significant resuspension occurred on sampling days, due to complication in removing organisms from their burrows. Resuspension could possibly increase the bioavailability of contaminants originating from the sediments, such as semi volatile persistent organic pollutants

which were not removed during sediment dehydration. Such perturbations in a controlled environment could also significantly increase *S. plana* stress levels. This hypothesis may explain the variations observed in some of the control treatments - GPx, AChE and LPO displayed variations between day 7 and day 14, but remained stable up until this, and an increase in GST activity occurred between pre-exposure and day 3, but subsequently remained stable. Yet, it must be noted that control treatments remained stable when analysing SOD, CAT and the comet assay.

Uncertainties in the sedimentation rate of LDPE MP are a cause of concern in this study. Yet such particles are a major contaminant in the marine environment and are bioavailable to benthic organisms. Deposition of low density plastics may be biologically mediated, through the formation of biofilms, through excretion in faecal pellets or caught in marine snow (Lobelle and Cunliffe, 2011; Zalasiewicz *et al.,* 2016). Deposition of low density plastics may also occur because of density changes resulting from mineral adsorption while in the water column (Corcoran *et al.,* 2015). A more appropriate experimental design could be used, such as one that mimics the tidal nature of an estuarine habitat, thus increasing the bioavailability of LDPE MP.

4.6 Further Research

Further reserch is needed in order to determine whether the observed effects of MP exposure in this study were induced by the physical ingestion or chemical toxicity of the MP particles.

Whole soft tissue samples were taken, both pre exposure and at day 14, and frozen at -20 ºC in order to investigate the potential for chemical accumulation to occur in *S. plana* as a result of desorption from ingested MP. Due to time constraints comprehensive chemical analysis was beyond the remit of this study.

Both SOD and LPO showed an increase in contaminated MP treatments relative to virgin MP treatments, sugesting a synergistic effect occured due to microplastic ingestion and chemical exposure. An initial increase in SOD activity was induced in all contaminated MP treatments relative to virgin MP treatment. LPO levels were increased in both BP3 and PFOS contaminated MP treatments relative to virgin MP treatments. Analysis of tissue samples at a later stage, using liquid chromatography - mass spectrometry (LC - MS), is needed to address

the potential for contaminants to desorb from MP once ingested and to confirm that the observed biomarker responses are a result of chemical contamination in tissues of *S. plana.*

Yet, increased activity was not observed in the majority of biomarkers used to investigated the effects of contaminated MP, with the dominant response atributed to virgin MP. This would suggest that the effects observed are due to the physical ingestion of the particles and the potential mechanical injuries that result.

Ingested MP may accumulate in the digestive system and gills, be egested or translocate between tissues. Impacts may vary according to the size range ingested with smaller size MPs have larger effects on organisms at the cellular level (Lusher, 2015). The ability of MP to translocate between tissues has been demonstrated in a variety of studies (Browne *et al*., 2008; von Moos, Burkhardt-Holm and Köhler, 2012; Ribeiro *et al*., 2017). Due to time constraints, an investigation into the potential for plastic to accumulate in the tissues of *S. plana* exposed to MP was beyond the scope of this study. Tissue samples were taken, both pre exposure and at day 14, dissected into gills, digestive gland and remaining tissues, and frozen at -20 ºC for this purpose. Further analysis at a later stage, using Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFT) to identify any polymers that may be present in tissues, is needed to confirm both the bioavailability of the MP and that the observed biomarker repsonses are a result of MP exposure.

Before guideline values for the environmental risk assessment of plastic debris can be produced further research is needed into the adsorption and desorption kinetics of common marine contaminants to various plastic polymers, and their effects on the marine biota once ingested. It is important that concentrations of MP particles used in future laboratory experiments reflect environmental values.

The extent to which microplastics act as a vector for contaminants once ingested by organisms within the marine environment is still unclear, as in field conditions organisms may accumulate the same chemical contaminants from other sources (Oliveria *et al.*, 2013). Environmental contaminants will rarely exist solely, but as a mixture with varying concentrations. Experiments involving the *in situ* adsorption of contaminants from various marine, coastal and estuarine environments to microplastic polymers of varying type, shape and density, could be used to evaluate both environmental concentrations of contaminants and also sorption kinetics to respective polymers. Such microplastic particles could subsequently be used in laboratory

exposure eperiments to investigate the effects of ingestion and chemical exposure while reflecting ecologicaly relevant scenarios.

Biomarkers, such as the ones employed in this experiment have been sucessfully used to assess the effects of MP exposure, with and without added contaminants, in previous studies (Oliveria *et al.*, 2013; Avio *et al*., 2015; Ribeiro *et al*., 2017). The varied results, both in this, and previous studies, highlight the need to use a multitude of biomarkers in order to accurately assess the effects of exposure, as results may vary according to species used and experimental design.

5. CONCLUSION

Detrimental impacts of plastic debris on the marine biota are numerous and diverse with deleterious physical effects recorded. Microplastics have become an area of concern due to their ubiquitous distribution in the marine environment. Under laboratory condition, various polymer particles have been shown to adsorb chemical pollutants from the surrounding environment, yet the potential for microplastics to act as a vector of contaminants once ingested by marine organisms is still under investigation. Contamination in, and concentration of pollutants on MPs are of great importance due to the potential for bioaccumulation of contaminants to occur, as a result of desorption from MP. Resulting bioaccumulation and biomagnification to higher trophic levels pose a long-term risk to the environment.

The following summarises the main conclusions from this study.

Under the experimental conditions observed:

- The organisms remained healthy for the duration of the experiment.
- There is some evidence to suggest that exposure to both benzo[a]pyrene and oxybenzone contaminated microplastic induces DNA damage after 14 days yet, the strength of these results are low. A genotoxic effect of exposure was not recorded in either virgin or perfluorooctane sulfonic acid contaminated microplastic treatments.
- A synergistic effect, from microplastic ingestion and chemical exposure, was not observed in contaminated microplastic treatments with respect to the enzymes, CAT or GST. The dominant response was atributed to virgin microplastics, suggesting the observed effects are due to the physical ingestion of particles and the potential mechanical injuries that result.
- Two biomarkers, SOD and LPO, did show an increase in activity in contaminated microplastic treatments relative to virgin microplastic, suggesting a synergistic effect of physical ingestion and chemical exposure.
- A reduction in GPx activity was observed in contaminated microplastic treatments with respect to virgin microplastics, indicating an antagonistic effect may have occurred. Such an inhibition in glutathione peroxidase may be in response to elevated toxicity levels of contaminated microplastic exposure.

- An inhibitory neurotoxic effect of exposure was not observed, with AChE activity remaining stable in the control, virgin and perfluorooctane sulfonic acid contaminated treatments, both over time and between treatments. A significant increase in activity was induced after 14 days exposure in benzo[a]pyrene and oxybenzone contaminated microplastic treatments with respect to control and virgin microplastic treatments.
- The physico-chemical conditions for the duration of the experiment remained stable, yet, significant variations in some control treatments lead to questions about the reliability of the data and whether the observed effects were a result of MP exposure alone.
- Questions arise due to the experimental design, with the bioavailability of LDPE MP, and the concentration of MP particles being the main concern. Further analysis, using Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFT) to identify any polymers that may be present in tissue samples, is needed to confirm both the bioavailability of the MP and that the observed biomarker repsonces are as a result of MP exposure and ingestion.
- Further analysis of tissue samples, using liquid chromatography - mass spectrometry (LC - MS), is needed to address the potential for contaminants to desorb from MP once ingested and to confirm that the observed biomarker responses are as a result of chemical contamination in tissues of *S. plana.*

It is clear that further research is need, both in this study and within the scientific comminity, into the ecotoxicological effects of MP in the marine environment with the potential for contaminants to desorb once ingested and their subsequent mode of action within organisms being of particular concern.

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