The potential for microplastics to cause harm in the marine environment

Submitted by

Stephanie Wright

To the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences, April 2015.

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

(Signature) …… (Stephanie Wright)


Current Biology
Volume 23
Number 23
December 2, 2013
www.cellpress.com
Microplastic Ecotoxicity

Abstract

Plastic debris is an emerging environmental issue, with >10 million tons of plastics debris per annum entering the sea. Exposure to marine conditions facilitates the exponential fragmentation of plastic to micro-sized particles (microplastics). Marine and coastal sediments are a sink for microplastic pollution. Consequently, the ingestion of microplastics by a range of benthic marine invertebrates, including polychaete worms, has been reported *in situ*. Microplastics are vectors for priority pollutants capable of eliciting adverse health effects. However, the particle and chemical toxicity which microplastics could incur to ecologically-important marine invertebrates is unknown.

This thesis aims to determine the potential for microplastics to cause harm in the marine environment, with a focus on benthic polychaete worms. Specifically, it assesses the potential particle toxicity which could arise from chemical-free microplastics; and the potential chemical toxicity which could arise from leached endogenous chemical additives or sorbed chemical pollutants. To address these, an integrative approach was employed, primarily using laboratory-based whole-sediment *in vivo* exposures, established cellular and whole-tissue toxicity assays, and analytical chemistry.

For the first time, this thesis reports that chemical-free microplastics cause particle toxicity in the lugworm *Arenicola marina*. Feeding activity was reduced during exposure to 5% microscopic unplasticised polyvinylchloride (UPVC) by sediment weight, whilst exposure to ≥1% UPVC by sediment weight significantly reduced energy reserves relative to control animals. Evidence for the transfer and toxicity of endogenous additives from PVC to lugworms is provided. Lugworms exposed to 1% PVC by sediment weight exhibited a 70% increase in additive concentration, coinciding with inhibited mucus production and enhanced lipid reserves and oxygen consumption, compared to control lugworms. Ragworms (*Hediste diversicolor*) exposed to leached toxicants from bioplastic cigarette debris were found to exhibit significantly longer burrowing times, >30% weight loss, and >2-fold increase in DNA damage compared to control ragworms. Bio-concentration factors for nicotine – the biomarker of exposure - were 500 fold higher from leachates in seawater than from microfibres in sediment.

Overall, this thesis provides evidence to suggest that the incorporation of microplastics into marine sediments can significantly impact the health of marine polychaete worms due to both particle and chemical effects. This emphasises the need to reconsider the classification of plastic as non-hazardous and questions whether we as humans are also at risk.

"The role of the infinitely small in nature is infinitely great."

-Louis Pasteur

Acknowledgements

First and foremost, I would like to thank Prof Tamara Galloway for her support and guidance throughout this project. Tamara's extensive knowledge and genuine passion for science, along with the supportive environment she provides is inspirational and greatly appreciated. She has been a mentor throughout this whole process and beyond, and I thank her for giving me the chance to have had this experience.

I'd also like to thank the DEFRA microplastics team – Prof Richard Thompson, Dr Adil Bakir, and Prof Steve Rowland – for their collaboration on this project, making it something truly unique and exciting. Their input has been invaluable and I'm proud of what we have all achieved. I am extremely grateful for the collaboration of Dr Kevin Thomas and Malcolm Reid at NIVA, whose analytical chemistry helped to complete this exciting story.

I'd like to thank Darren Rowe for his technical support throughout this project and for providing a dynamic environment to discuss scientific and experimental concepts. I'd also like to thank Steve and Jan for their back-breaking assistance whilst worm-digging at beautiful Mothecombe. I'd like to thank Dr Ceri Lewis for being supportive and enthusiastic about my science, for providing endless worm knowledge and for getting me on another boat.

I'd like to thank the Galloway laboratory group for being interactive and excited. Our lab meetings were always constructive, allowing me to progress, and filled me with interest and motivation. Thanks Tones and Suzie for saving me your cigarette butts for months and Tones for also being a great Cali travel pal. I am extremely grateful for the travel opportunities my PhD granted, and none would have been the same without my travel buddy, Matt Cole. We shared some fantastic experiences both around the globe and in the lab.

Thank you to my amazing friends who have enhanced this experience, scientifically and socially. I want to thank the Master for facilitating our avant-garde ways, being a feeder, and for all the eccentric memories we have shared since C4. Thank you Little One for being such a warm, honest, hilarious genius. Thank you Cockney for being so angry and uncultured; I sincerely respect and trust your scientific opinion. Thanks John, for being green and telling it like it is. Thanks Victoria, Josie, Jane, Emily, and Charlotte for being sassy. Thank you fellow

cave-dwellers; this little family made the final push something special. I also need to thank Mike, who had to put up with me for the best part; you were always a voice of reason, wisdom and serenity.

Thanks mum, dad, and Jon for all the support, love, and parties you continue to provide.

Thanks to everyone I have forgotten.

Contents

Title Page **1**

Abstract **5**

Acknowledgements **7**

Contents **9**

Abbreviations **15**

Species **17**

Research Papers and Author's Declaration **19**

General Introduction **21**

I.1 Marine Debris 23

I.2 Microplastics 24

I.3 *In situ* microplastic ingestion 25

I.4 The biological impacts of microplastics 28

I.4.1 Chemical Impacts 28

I.4.1.1 Additives 29

I.4.1.2 Environmental Persistent Organic Pollutants (POPs) 30

I.5 Bioplastics 37

I.6 The susceptibility of polychaete worms to microplastic exposure 38

I.7 Aim 39

I.7.1 Objectives 39

Chapter 1 ..... **41**

The physical impacts of microplastics on marine organisms: a review **43**

1.1 Abstract 44

1.2 Introduction 45

1.3 Factors affecting the bioavailability of microplastics 47

1.3.1 Size 47

1.3.2 Density 48
1.3.3 Abundance 48
1.3.4 Colour 49
1.3.5 Biological interactions 49
1.4 The susceptibility of marine organisms to microplastic ingestion 49
1.4.1 Detritivores and deposit feeders 50
1.4.2 Planktivores, filter feeders and suspension feeders 52
1.5 Factors influencing the physical impacts of microplastics 55
1.5.1 Accumulation 55
1.5.2 Translocation 56
1.5.3 Shape 58
1.5.4 Egestion 59
1.5.5 Population-level effects 59
1.5.6 Transfer to the food chain 60
1.6 Conclusions 63
1.7 Tables 65
1.8 Figure 68
Chapter 2 69
Microplastic ingestion decreases energy reserves in marine worms 71
2.1 Correspondence 72
2.1.1 Figure 1. 76
2.2 Supplemental Information: 78
2.2.1 Supplemental Results 78
2.2.2 Supplemental Experimental Procedures 84
2.2.2.1 Microplastics 84
2.2.2.2 Scanning Electron Microscopy 84

2.2.2.3 Animal Husbandry 84
2.2.2.4 Preparation of Sediment 85
2.2.2.5 Chronic exposure 85
2.2.2.6 Phagocytosis 86
2.2.2.7 Energy Reserves 87
2.2.2.8 Gut Residence Time 87
2.2.2.9 Food Concentration 88
2.2.2.10 Statistical Analysis 88
2.2.2.11 Sediment Reworking Calculations 91
I.7.1.1 The Potential Impacts of Size 91
Chapter 3 93
Microplastics transfer endogenous phthalates to marine worms 95
3.1 Abstract 96
3.2 Introduction 97
3.3 Materials and Methods 100
3.3.1 Microplastics 100
3.3.2 Animal Husbandry 101
3.3.3 Preparation of Sediment 101
3.3.4 Chronic exposure 102
3.3.5 Oxygen Consumption 103
3.3.6 Phagocytosis 103
3.3.7 Energy Reserves 104
3.3.8 Statistical Analyses 104
3.4 Results 106
3.4.1 Weight 106
3.4.2 Feeding rate 106

3.4.3 Energy Allocation 106
3.4.4 Oxygen Consumption 106
3.4.5 Mucus Production 107
3.4.6 Phagocytosis 107
3.4.7 Bioaccumulation 107
3.5 Discussion 107
3.6 Figures 114
3.7 Supplemental Information 116
I.7.2 The Potential Impacts of Size 117
Chapter 4 119
Cigarette litter impairs the ecological function of marine worms 121
4.1 Abstract 122
4.2 Introduction 123
4.3 Results 124
4.3.1 Nicotine Bioaccumulation 124
4.3.2 Nicotine Metabolism 125
4.3.3 Biological Endpoints 125
4.3.3.1 Relative Growth Rate 125
4.3.3.2 Burrowing Activity 125
4.3.3.3 DNA Damage 126
4.4 Discussion 126
4.5 Methods 130
4.5.1 Materials 130
4.5.1.1 Smoked Cigarette Filters 130
4.5.1.2 Chemicals and solutions 130
4.5.2 Exposures and Biological Endpoints 131

4.5.2.1 Animal Husbandry 131
4.5.2.2 Exposure to Smoked Cigarette Filter Toxicants in Sediment 131
4.5.2.3 Exposure to Smoked Cigarette Filter Microfibres in Sediment 132
4.5.2.4 Relative Growth Rate 132
4.5.2.5 Burrowing activity 133
4.5.2.6 Comet assay 133
4.5.2.7 Chemical Analysis 134
4.5.2.8 Chromatography and detection (MS/MS) parameters 134
4.5.2.9 Sample Preparation 134
4.5.3 Statistical Analyses 135
4.5.4 Bioaccumulation 136
4.6 Acknowledgments 136
4.7 Author Contributions 136
4.8 Additional Information 136
4.9 Tables 137
4.10 Figures 138
4.11 Supplementary Information 140
I.7.3 The Potential Impacts of Size 144
Chapter 5 149
General Discussion 151
5.1 Summary of Findings 151
5.1.1 Chapter 1 151
5.1.2 Chapter 2 152
5.1.3 Chapter 3 153
5.1.4 Chapter 4 154
5.2 Environmental and Human Health Implications 155

Bibliography **163**

Appendix **185**

Teaching 187

Outreach 187

Outreach Activities 187

Press Gang Articles 189

Presentations 193

Selected Posters 195

Accepted Manuscript 197

Abbreviations

ANOVA	analysis of variance
BPA	bisphenol A
DDT	dichlorodiphenyltrichloroethane
DEHP	Bis(2-ethylhexyl) phthalate
DOC	dissolved organic carbon
EDC	endocrine disrupting chemical
FTIR	Fourier transform infrared spectroscopy
PAH	polycyclic aromatic hydrocarbon
PBDE	polybrominated diphenyl ether
PCB	polychlorinated biphenyl
PE	polyethylene
POP	persistent organic pollutant
PP	polypropylene
PS	polystyrene
PVC	polyvinylchloride
WW	wet weight

Species

Amphipod	*Allorchestes compressa*
	Orchestia gammarellus
	Talitrus saltator
Antarctic fur seal	*Arctocephalus gazella*
Arrow worm	*Sagitta elegans*
Atlantic deep-sea scallop	*Placopecten magellanicus*
Blackworm	*Lumbriculus variegatus*
Calanoid copepod	*Acartia tonsa*
Common mussel	*Mytilus edulis*
Estuarine copepod	*Eurytemora affinis*
Epibenthic copepod	*Nitocra spinipes*
Fan worm	*Galeolaria caespitose*
Fin whale	*Balaenoptera physalus*
Florida sea cucumber	*Holothuria floridana*
Golden lanternfish	*Myctophum aurolaternatum*
Gooseneck barnacle	*Lepas anatifera*
	Lepas pacifica
Green sea urchin	*Lytechinus variegatus*
Grey sea cucumber	*Holothuria grisea*
Grubby	*Myoxocephalus aenaeus*
Harlequin fly	*Chironomus riparius*
Lugworm	*Arenicola marina*
Mediterranean mussel	*Mytilus galloprovincialis*
Norway lobster	*Nephrops norvegicus*

Orange-footed sea cucumber	*Cucumaria frondosa*
Ragworm	*Hediste diversicolor*
Rough lanternfish	*Electrona subaspera*
Rough woodlouse	*Porcellio scaber*
Salp	*Thetys vagina*
Saltwater clam	*Venerupis philippinarum*
Sea skater	*Halobates sericeus*
Striped sea cucumber	*Thyonella gemmate*
Subantarctic fur seal	*Arctocephalus tropicalis*
Water flea	*Daphnia magna*
Winter flounder	*Pseudopleuronectes americanus*

Research Papers and Author's Declaration

Research paper 1. Wright, S., Thompson, R.C. and Galloway, T.S. The physical impacts of microplastics on marine organisms: a review. *Environmental Pollution*. 178, 483-492. [Impact: 3.9; 91 citations; highest altmetric score, June-Dec 2013]

Research paper 2. Wright, S., Rowe. D., Thompson, R. C. and Galloway, T. S. Microplastic ingestion decreases energy reserves in marine worms. *Current Biology*. 23 (23). (Front cover) [Impact: 9.9; 24 citations]

Research paper 3. Wright, S. L., Rowe, D., Bakir, A., Rowland, S., Reid, M., Thomas, K., Thompson, R. C., and Galloway, T. S. 2015. Microplastics transfer endogenous phthalates to marine worms. *Manuscript in preparation*.

Research paper 4. Wright, S., Rowe, D., Reid, M., Thomas, K., and Galloway, T. S. 2015. Cigarette litter impairs ecological function in marine worms. *Manuscript submitted to Nature Scientific Reports*.

Declaration: I, Stephanie Wright, made the following contributions to the research papers presented in this thesis. For **research paper 1**, I conducted, prepared and wrote a critical literature review, with inputs from the listed authors on the manuscript. For **research papers 2, 3, and 4** I conceived, designed and performed the studies. Darren Rowe assisted in collecting worms in addition to the preparation and termination of experimental set-ups. Dr Adil Bakir and Prof Steve Rowland led the analytical chemistry in **research paper 3** with support from Malcolm Reid and Dr Kevin Thomas, analysing phthalate concentrations in sediment and tissue samples. Malcolm Reid and Dr Kevin Thomas led the analytical chemistry in **research paper 4**, analysing nicotine and cotinine concentrations in seawater, sediment, and tissue samples. For all papers, Prof Tamara Galloway supervised all aspects of the experimental work and manuscript preparation.

General Introduction

General Introduction

I.1 Marine Debris

The marine environment is crucial to the homeostasis of our planet. The oceans influence climate regulation; marine ecosystems play an important role in biogeochemical cycles; and a diverse range of species, some of which form an important food supply, inhabit these environments. Thus, the degradation of the marine environment will compromise many goods and ecosystem services, which may not only have a major impact on the planet, but also on humanity. Sediment is a vital component of the marine environment, forming the largest habitat on Earth based on areal coverage (Snelgrove, 1997). Its residents are fundamental to ecosystem function, impacting water column processes; trophic transfer; global biogeochemical cycles; and pollutant cycling (Snelgrove, 1997). However, the sediment has become a repository for pollution, accumulating heavy metals, Persistent Organic Pollutants (POPs), and marine debris.

Marine debris, defined as 'any persistent, manufactured or processed solid material discarded, disposed of, or abandoned in the marine and coastal environment', is a growing global conservation issue. Plastic accounts for approximately 80% of marine debris, pervading the sea surface, water column, seabed and coastlines, from the poles to the equator (see Figure 1).

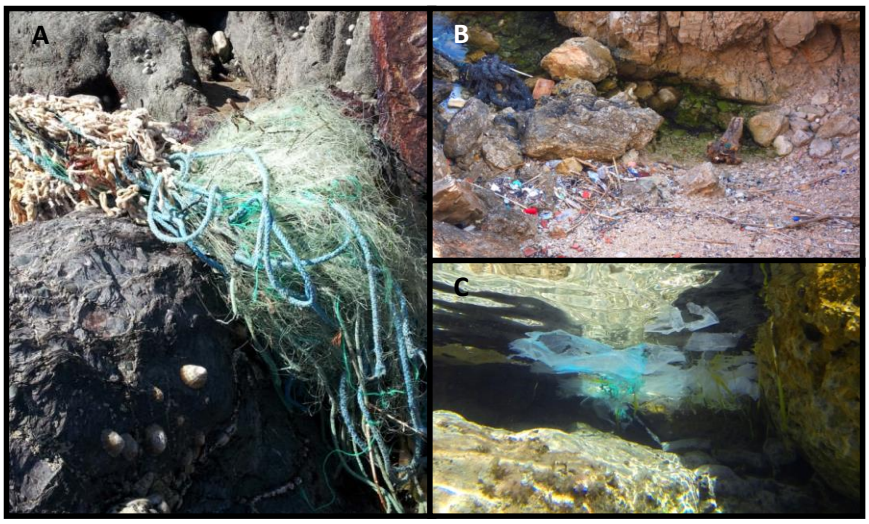


Figure 1. Plastic debris. Plastic is the most common type of marine debris and due to its low density, plastic debris can be transported over long distances by ocean currents, reaching shorelines around the globe. A) Discarded fishing gear entangled in

littoral rocks. B) Plastic debris which has washed up on the shore. C) Waterborne plastic debris. Wright (2013).

I.2 Microplastics

An emerging sedimentary pollutant of increasing global concern is small plastic debris (<5 mm), known as microplastics (see Figure 2). Predominantly formed through the continual degradation and fragmentation of larger plastic items including fishing gear, clothing and food packaging, microplastics have pervaded marine sediments due to: 1) the biofouling, and therefore increased density and sinking, of buoyant microplastics (Lobelle and Cunliffe, 2011); 2) the occurrence of high density plastic types sinking out e.g. polyvinylchloride (PVC); and 3) the deposition of buoyant microplastics onto shorelines. Concentrations reaching 3% (≤1 mm) by weight (Carson et al., 2011) and up to 10% by weight:volume (≤5 mm) (Baztan et al., 2014) have been reported for highly impacted beaches.

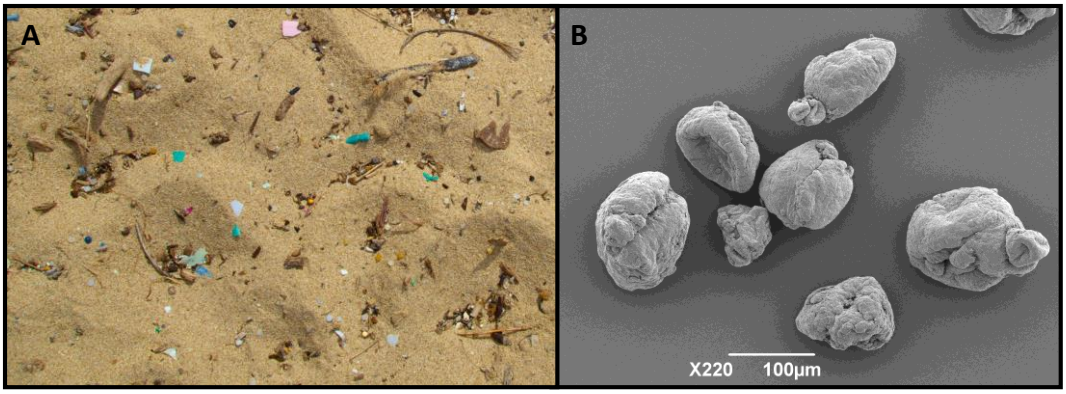


Figure 2. Microplastics A) Microplastics (fragments and pre-production pellets) on a beach in the Mediterranean. B) A micrograph of unplasticised polyvinylchloride using Scanning Electron Microscopy (SEM, x220, scale bar 100 µm). Wright (2013).

Microplastics (≤1mm) fall into the same size class as most sediment grains. Their introduction into marine sediments therefore presents a new substrate for benthic invertebrates. Subsequently, microplastics may be ingested by an array of marine invertebrates adopting detritus- and deposit-feeding strategies, with little discrimination. Laboratory studies have reported ingestion in several benthic species, including sea cucumbers (250 µm – 15,000 µm) (Graham and Thompson, 2009), sand hoppers (10 – 45 µm) (Ugolini et al., 2013), and polychaete worms (20 – 2000 µm)

(Besseling et al., 2012; Thompson et al., 2004). However, little is known of how these species process microplastics and whether interactions with this novel substrate result in biological harm.

I.3 *In situ* microplastic ingestion

A growing body of evidence suggests benthic marine invertebrates are feeding on microplastics, not only in the laboratory but in the environment (see Table 1). *In situ* microplastic ingestion has been reported for several invertebrate species adopting a range of different feeding modes. Mathalon and Hill (2014) found evidence of microplastic (microfibres) ingestion by deposit-feeding polychaete worms in Nova Scotia, Canada. This was indicated by the presence of microplastics in collected faecal casts, at an average concentration of 4-5.5 microplastics g^{-1} faecal casts. The concentration of microplastics in polychaete worm faecal casts and the inhabited sediment were statistically similar. This suggests that the consumption and excretion of microplastics by polychaete worms at these sites are in equilibrium; it is unlikely that selection for or bioaccumulation of microplastics is occurring and deposit-feeding polychaete worms will indiscriminately ingest microplastics at the concentration at which they occur *in situ* (Mathalon and Hill, 2014). However, this finding does not indicate the gut residence times of microplastics in polychaete worms.

Filter-feeding bivalves have also been found to ingest microplastics *in situ*. Both wild and farmed mussels collected from coastal sites in Nova Scotia, Canada, were contaminated by microplastics (microfibres). Wild mussels were contaminated with up to 126 microplastics per individual, whilst farmed mussels were contaminated with a significantly greater amount of microplastics; up to 178 microplastics per individual. Farmed mussels were cultured on deployed polypropylene (PP) lines, which may provide a source of microplastics as the line degrades (Mathalon and Hill, 2014). A similar study comparing wild mussels and mussels for human consumption (De Witte et al., 2014) found microplastic fibres (200-1500 µm) were the only shape of microplastic found to contaminate mussels, suggesting this shape has a longer gut residence time during periods of depuration. Concentrations of microplastic contamination ranged from 2.6 microplastics 10 g^{-1} in purchased mussels (for human

consumption), to 5.1 microplastics 10 g^{-1} in mussels collected from quaysides (De Witte et al., 2014). A case study concerning orange fibres (the only colour to not contaminate blank replicates) found a significantly higher concentration in mussels collected from quaysides, as opposed to mussels collected from groynes and purchased mussels. Orange fibres were linked to the presence of fishing-related orange polyethylene (PE) ropes in quayside sites (De Witte et al., 2014). Clearly, the consumption of contaminated mussels presents a pathway for human exposure to microplastics. Although the biological impacts of microplastics are relatively unknown, entry into the human food chain is a cause for concern.

The anatomical location of microplastics was not analysed in either study. Most bivalves capture and retain particles 3 – 4 µm in size/diameter with 100% efficiency (Gosling, 2003; Ward and Shumway, 2004). Given the size of microplastic fibres reported by De Witte et al. (2014) being 200 – 1500 µm, it is unlikely these microplastics are ingested. However, microplastic fibres could become entangled and lodge on the gill surface, increasing the time-window for trophic transfer to occur. A laboratory study investigating the role of microplastics (polystyrene (PS) and PE) in the transfer of pyrene found particles primarily localized in the digestive gland, with occurrence also in the haemolymph and gills (Avio et al., 2015). No qualitative difference between the two polymers and tissue localisation was observed. Furthermore, the several genes coding for proteins implicated in endosome maturation, endocytic trafficking and lysosomal degradation were up-regulated, suggesting that the uptake of microplastics occurs via endocytosis.

Lepedomorph barnacles are susceptible to microplastic ingestion due to their rafting lifestyle, occupying the sea surface, and their filter-feeding strategy, which targets neustonic zooplankton (Goldstein and Goodwin, 2013). At least one plastic particle was found in the gastrointestinal tracts of approximately 34% of opportunistically collected gooseneck barnacles (*Lepas anatifera* and *L. pacifica*) in the Pacific Ocean; a maximum of 30 plastic particles were ingested. The abundance of ingested plastic particles was correlated to capitulum length. Despite the frequency of microplastic ingestion, no blockages were observed. PE, polypropylene (PE), and PS were the most common types of plastic particles recovered from gooseneck barnacle gastrointestinal tracks and the median diameter of ingested particles was 1.4 mm.

Table 1. Microplastic ingestion by marine invertebrates

		Microplastic ingestion			
		Laboratory		Wild	
Organism	**Feeding mode**	**Evidence**	**Reference**	**Evidence**	**Reference**
Polychaete worms (larvae)	Deposit feeders (grazers)	✓	(Besseling et al., 2012; Setälä et al., 2014; Thompson et al., 2004)	✓	(Mathalon and Hill, 2014)
Bivalve molluscs	Suspension feeders	✓		✓	(Mathalon and Hill, 2014)
Barnacles	Suspension feeders	✓	(Thompson et al., 2004)	✓	(Goldstein and Goodwin, 2013)
Amphipods	Detritivores/scavengers	✓	(Chua et al., 2014)	✓	(Ugolini et al., 2013)
Isopods	Detritivores/grazers/predators	✓	(Hamer et al., 2014)		
Copepods	Filter feeders	✓	(Cole et al., 2013; Setälä et al., 2014)		
Decapods	Predators/scavengers	✓	(Farrell and Nelson, 2013; Watts et al., 2014)	✓	(Murray and Cowie, 2011)
Holothurians	Deposit feeders/scavengers	✓	(Graham and Thompson, 2009)		

Qualitative observations of the faeces of wild amphipods (*Talitrus saltator*) revealed the presence of suspicious, coloured fragments (Ugolini et al., 2013). Whilst FTIR revealed not all fragments were plastic, some were confirmed to be PE and PP. This highlights the potential for scavengers and detritivores to passively ingest microplastics (Ugolini et al., 2013).

The accumulation of microplastics in the digestive track has the potential to cause blockages, inducing satiation in the individual. This can ultimately decrease fitness, and lead to starvation. However, the biological impacts of microplastics are understudied.

I.4 The biological impacts of microplastics

Given the evidence for microplastic ingestion in marine invertebrates *in situ*, understanding potentially associated biological harm is paramount. Microplastics are a multi-stressor; they have the capacity to cause physical, chemical, and microbial harm. Firstly, the physicality of microplastic particles could cause harm through blockages, internal abrasions, poor nutrition, satiation, and inflammation. Secondly, microplastics could act as a vector for the introduction and transfer of toxic chemicals into aquatic food webs (Barnes et al., 2009; Thompson et al., 2009). They carry intrinsic chemical additives and concentrate water-borne Persistent Organic Pollutants (POPs), presenting a chemical hazard (Carpenter et al., 1972; Teuten et al., 2009). Thirdly, microplastics provide a novel hard substrate in the open ocean. Specific microbial assemblages have found to colonise the surface of microplastics, presenting a vector for the transfer of pathogenic and/or invasive species.

I.4.1 Chemical Impacts

There are two primary routes of marine invertebrate exposure to microplastic-associated additives and POPs. Upon entry to the marine environment or transport to new areas, microplastics could leach endogenous additives or adsorbed POPs to the surrounding environment. Following this, dermal sorption of the leachates could occur. Respiratory pathways also present a potential route of exposure (Watts et al., 2014). Microplastics which have become lodged on respiratory surfaces may leach associated chemicals. The most anticipated route of exposure is via ingestion (Teuten et al., 2009) with subsequent desorption of associated chemicals and transfer to tissues; however

the transfer of contaminants from ingested plastics to biota has received little attention.

I.4.1.1 Additives

During manufacture, chemical additives are incorporated into plastics, giving them flexible, flame-resistant, antioxidant, antimicrobial, and aesthetic properties, specific to plastic type and application. Such chemical additives have much lower molecular weights than plastics, and are incorporated in large quantities. Since they are not typically bound to the plastic polymer matrix, they are susceptible to leaching to the external medium. Some PVC medical devices may contain up to 80% by weight of the plasticiser bis(2-ethylhexyl) phthalate (DEHP) (Tickner, 1999), which is susceptible to leaching into blood or saline. The small molecular size (MW <1000) of these chemicals allows them to penetrate cells and therefore interact with biologically important molecules (Teuten et al., 2009). Many plastic additives are classified as endocrine disruptors, causing reproductive and developmental toxicity (see references within (Oehlmann et al., 2009)).

The toxicity of plastic additives has been assessed in a marine invertebrate. The leachates of 21 plastic products were screened for acute toxicity using the epibenthic copepod *Nitocra spinipes* (Bejgarn et al., 2015). Leachates were screened pre and post artificial weathering (irradiation), as experienced in the marine environment, to determine its effect on leachate toxicity. Thirty eight percent of the leachates elicited a toxic response, which was dependent on plastic type and the duration of weathering. Weathering increased the toxicity of leachates from 4 plastics, whilst it decreased the toxicity of leachates from 2 plastics. The toxicity of leachates from 2 plastics did not significantly change.

Given the ubiquity of marine microplastics and the capacity for a range of marine invertebrates to ingest them, it has been predicted that microplastics increase marine invertebrate exposure to chemical additives. However, it has been argued that low exposure is likely to occur given the slow diffusion rates of chemical additives (Berens, 1997). Alternatively, diffusion rates may increase for aged, brittle plastics, such as those found in the marine environment (Artham and Doble, 2009; Sajiki and Yonekubo, 2003). Additionally, the high levels of DOC and surfactants present in the

gastrointestinal fluids of marine invertebrates may facilitate further leaching of intrinsic chemical additives from ingested plastic; however, experimental and/or model-based evidence is lacking (Koelmans et al., 2014).

Recently, Browne et al. (2013) investigated the transfer of triclosan and PBDE-47 from the surface of microplastics to the tissues of the lugworm *A. marina*, and the associated impacts to ecophysiological function. Microplastics (PVC) constituting 5% by weight of the test sediment were pre-sorbed with chemical additives at concentrations of 5 – 30% (PBDE-47) and 0 – 5% (triclosan), as used commercially. Following 10 day sedimentary exposures, microplastics had transferred chemical additives to lugworm tissues via sorption to the gut. The body wall tissue concentrated up to 950% more chemical additives in comparison to the test medium. The gut wall tissue concentrated up to 3500% more additives in comparison to the test medium. Exposure to PBDE-contaminated microplastics reduced feeding activity by 30%, although not significantly. Exposure to triclosan-contaminated microplastics significantly reduced feeding activity by >65%. Moreover, >55% of lugworms died in the triclosan treatment. Thus, large concentrations of microplastics and their additives can harm ecophysiological function in marine worms (Browne et al., 2013).

However, additives are typically incorporated into the plastic polymer matrix during manufacture of macro-plastic items. Thus, chemicals additives are considered endogenous, migrating to the surface. Furthermore, the exposure was short-term. A sedentary species such as the lugworm may be exposed to localised pollution over prolonged periods of time. More research is therefore required to understand whether truly incorporated chemical additives are likely to leach and concentrate in tissues over longer-term exposures.

I.4.1.2 Environmental Persistent Organic Pollutants (POPs)

Seawater contains low-levels of POPs, however, the partitioning between seawater and plastic, combined with the increased surface area:volume ratio of microplastics, means they concentrate a range of POPs, including Polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDTs), and polycyclic aromatic hydrocarbons (PAHs) (Browne et al., 2007; Endo et al., 2005; Galgani et al., 2010). In a laboratory based study, the sorption rates of phenanthrene from seawater to PE, PP, and PVC greatly

exceeded sorption to natural sediments. Additionally, the desorption rates of phenanthrene from PE, PP, and PVC to seawater was much slower than desorption from natural sediment, highlighting the potential for microplastics to accumulate hazardous POPs. Accordingly, in the environment POPs can be up to six orders of magnitude greater on microplastics in comparison to the surrounding seawater (Mato et al., 2001). Some POPs are highly toxic, recognised for their endocrine disrupting, carcinogenic, mutagenic, and immunotoxic effects. The ingestion of microplastics could therefore facilitate the transport of POPs to marine organisms.

The digestive environment of deposit feeders, which has a range of potential solubilizing agents such as surfactants, enhances desorption of pollutants, including PAHs and PCBs, from sediment (Ahrens et al., 2001; Voparil and Mayer, 2000). Moreover, deposit feeding organisms desorb a greater concentration of POPs than suspension feeders or carnivores (Mayer et al., 1997). Recently, the potential for POPs to desorb from microplastics under simulated physiological conditions was studied (Bakir et al., 2014). Desorption rates were faster in gut surfactants than in seawater, and this increased under conditions simulating warm blooded organisms, up to 30 times greater than seawater. The plastic-POP combination presenting the greatest potential for POP transfer was PE and phenanthrene (Bakir et al., 2014). It is therefore apparent that the ingestion, egestion and re-ingestion of contaminated microplastics present a potential mechanism for the transport of POPs from the marine environment to organisms' tissues and, ultimately, marine food webs. What is more, as both marine macro- and microplastics continue to fragment, the potential for POPs transfer will increase, corresponding with both increased abundance and surface area. Sorption characteristics of POPs may also be liable to change as microplastics become weathered or biofouled.

Table 2. Reported POP concentrations on microplastics collected from the marine environment, including the type (shape/polymer type, where available) of plastic, type of POPs, POP concentrations, the location of collection, and references. Modified from Ivar do Sul and Costa (2014). NPCG = North Pacific Central Gyre, PET = polyethylene terephthalate, DDE = dichlorodiphenyldichloroethylene, HCH = hexachlorocyclohexane.

Type of plastic	Pollutants	Concentration	Location	Reference
Pellets (PP)	PCBs	4 – 117 ng g^{-1}	Japan	(Mato et al., 2001)
	DDE	0.16 – 3.1 ng g^{-1}		
	Nonylphenols	0.13 – 16 µg g^{-1}		
Pellets (PP, PE)	PCBs	<28 – 2300 ng g^{-1}	Japan	(Endo et al., 2005)
Pellets and fragments (PP)*	PCBs	27 – 980 ng g^{-1}	NPCG California	(Rios et al., 2007)
	PAHs	39 – 1200 ng g^{-1}		
	Aliphatic PAHs	1.1 – 8600 µg g^{-1}		
	DDTs	22 – 7100 ng g^{-1}		
Pellets (PP, PE, others)	PCBs	~300 – 600 ng g^{-1}	US	(Ogata et al., 2009)
		~50 – 400 ng g^{-1}	Japan, W Europe	

Type of plastic	Pollutants	Concentration	Location	Reference
		<50 ng g^{-1}	S Asia, Australia, S Africa	
	DDTs	~100 – 300 ng g^{-1}	US, Vietnam	
		<20 ng g^{-1}	Japan, W Europe, Australia, South Africa	
Pellets (PE, PP, PS, other)	PCBs	0.02 – 15.56 ng g^{-1}	Portugal	(Frias et al., 2010)
	PAHs	0.2 – 319.2 ng g^{-1}		
	DDTs	0.16 – 4 .05 ng g^{-1}		
Fragments*	PCBs	1 – 2566 ng g^{-1}	NPCG	(Rios* et al., 2010)
	PAHs	1 – 4395 ng g^{-1}		
	Aliphatic PAHs	1 – 6227 µg g^{-1}		
	Organochlorines	1 – 176 ng g^{-1}		
Pellets	PCBs	6000 – 290,000 pg g^{-1}	Greece	(Karapanagioti et al.,

Type of plastic	Pollutants	Concentration	Location	Reference
	PAHs	100 – 500 ng g^{-1}		2011)
	DDTs	0.56 – 25 ng g^{-1}		
	HCHs	0.09 – 1.41 ng g^{-1}		
Pellets (PE)	PCBs	41 – 113 (1980's) – 25 – 61 (2000's) ng g^{-1}	South Africa	(Ryan et al., 2012)
	HCHs	5 – 112 (1980's) – 2 – 5 (2000's) ng g^{-1}		
	DDTs	18 – 1281 (1980's) - 8 – 30 (2000's) ng g^{-1}		
Pellets and fragments	PAHs	30 – 1900 ng g^{-1}	San Diego, USA	(Van et al., 2012)
	PCBs	non-detect – 47 ng g^{-1}		
	Chlordanes	1.8 – 60 ng g^{-1}		
	DDTs	non-detect – 76 ng g^{-1}		
Pellets (PET, HDPE, PVC, LDPE, and PP)	PCBs	-	San Diego, USA	(Rochman et al., 2013b)
	PAHs			
Pellets	PAHs	386 – 1996 ng g^{-1}	Brazil	(Fisner et al., 2013)

Type of plastic	Pollutants	Concentration	Location	Reference
Pellets (PE, PP, other)	PCBs	10.5 – 307 ng g^{-1}	Portugal	(Mizukawa et al., 2013)
	PAHs	50 – 24,000 ng g^{-1}		
	HCHs	0 – 0.86 ng g^{-1}		
	DDTs	0 – 49 ng g^{-1}		
	Hopanes	8.3 – 71 µg g^{-1}		

The role of microplastics as a vector for chemical contaminants has been investigated with embryos and larvae of the sea urchin *Lytechinus variegatus* (Nobre et al., 2015). Larvae were exposed for 24 hours to the leachates of virgin and beach plastic pellets, either with or without a 30 minute agitation period. The greatest toxicity was found when embryos were exposed to the leachates of agitated virgin pellets. These leachates caused 67% of larvae to develop abnormally. Conversely, only 5% of larvae developed abnormally following exposure to the leachates of agitated beach pellets. Exposure to the leachates of static virgin pellets caused 35% of larvae to develop abnormally, while 58% of larvae showed abnormal development when exposed to the leachates of static beach pellets (Nobre et al., 2015). This highlights the capacity for microplastics to act as a vector for chemical contaminants.

Pyrene, a PAH commonly reported on the surface of environmental microplastics (Rios et al., 2007) has been shown to transfer from the surface of microplastics to the tissue of mussels. The mussel *Mytilus galloprovincialis* was exposed to both virgin and pyrene-contaminated (200 – 260 ng g^{-1} microplastic) PS and PE particles (<100 µm) for 7 days. There was a significant increase in pyrene in the gill tissue and bioaccumulation in the digestive gland tissue with concentrations 3 times greater than that on the surface of microplastics post-exposure to pyrene-contaminated microplastics. No difference between polymer types was observed. A Principal Component Analysis found a clear difference between the control and microplastic-exposed mussels, which was linked to immunological, lysosomal, cholinesterasic and antioxidant effects. However, this was not influenced by polymer type or contamination status. Only genotoxic responses (DNA strand breaks, nuclear aberrations, and micronuclei formation) were further separated by contamination status (Avio et al., 2015).

The amphipod *Allorchestes compressa* was exposed to PBDE congeners either in the presence of microplastics (11 – 700 µm), or via sorption to microplastics for 72 hours (Chua et al., 2014). Amphipods ingested microplastics with an average gut content of 18 particles, 0 hours post-exposure. Twelve hours post-exposure, 87% of amphipods had cleared their guts of microplastics, and after 36 hours amphipods contained <1 microplastic. PBDE concentrations were lower in amphipods exposed to PBDEs in the presence of microplastics than those exposed to PBDEs alone. Thus the adsorption of PBDEs onto the surface of microplastics is likely to reduce their bioavailability. The

greatest uptake of PBDEs via microplastics occurred in the microplastic treatment with highest concentration of PBDE contamination; however this was still lower than that accumulated from the same concentration of PBDEs in the presence of microplastics. This is likely due to a washing step of the contaminated microplastic pre-exposure (Chua et al., 2014).

I.5 Bioplastics

Given the ubiquity of marine plastic and microplastic debris, the durability of plastic has become a burden rather than an advantage. One solution is to replace synthetic plastic with biodegradable equivalents which undergo complete organic recycling, referred to as bioplastics. The onset of plastic degradation is distinguished by its disintegration into fragments. The final phase is its transformation into CO_2, as a result of aerobic biodegradation (Tosin et al., 2012). However, the rate of biodegradation in marine conditions is slow and there is little data concerning the mineralisation of plastics and bioplastics in the marine environment (Philp et al., 2013).

Bioplastic utilisation has been reported for the terrestrial isopod *Porcellio scaber* (Wood and Zimmer, 2014). Out of starch-, poly(3-hydroxybutyrate)-, and cellulose-based plastics, digestibility was greatest for the cellulose-based plastic, with isopods breaking it down into cellobiose. The greatest loss of biomass occurred in individuals being fed a cellulose-based plastic diet. Isopods increased the disintegration of starch- and cellulose-based plastics, suggesting this species is likely to consume these types of bioplastics in the environment (Wood and Zimmer, 2014). Currently, no study has considered the consumption and utilisation of bioplastics in marine invertebrates.

One of the most prolific types of bioplastic in the marine environment is cellulose acetate, due to its application in cigarette filters; the most commonly reported item in urban and beach cleans, globally (Novotny and Slaughter, 2014) (Figure 3A). The numerous chemicals both within tobacco and generated through tobacco combustion are included in the Environmental Protection Agency's Toxic Release Inventory Programme; these are chemicals which are considered a threat to human health

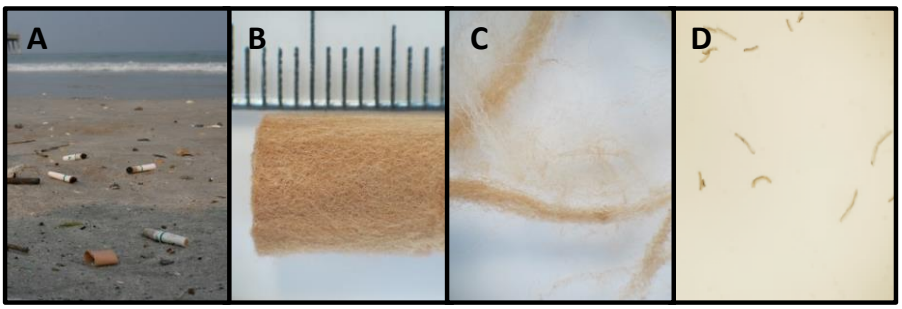


Figure 3. A) Cigarette filter debris on the beach (credit Danielle Richardet, marinedebrisblog.wordpress.com). B) An intact smoked cigarette filter free of excess tobacco and external paper C) The fibrous nature of cigarette filters (cellulose acetate) D) A micrograph of smoked cigarette filter microfibres. Wright (2013).

and/or the environment (Novotny and Slaughter, 2014). Few studies have assessed the potential toxicity of whole (Figure 3B) and fragmented smoked cigarette filters (Figure 3C-D) on wildlife. Particularly, inhabitants of aquatic ecosystems such as shorelines and waterways are anticipated to be most vulnerable due the prevalence of smoked cigarette filters in these habitats. Smoked cigarette filters therefore present a source of leaching POPs, in addition to a source of microplastics as the filter degrades and fragments into microfibres. Currently, no study has addressed the potential impacts of cigarette filter debris on a marine invertebrate.

I.6 The susceptibility of polychaete worms to microplastic exposure

Microplastics are most abundant on and within the seabed, from coastal sediments (Baztan et al., 2014; Carson et al., 2011) to the deep sea (Woodall et al., 2014), reaching concentrations 4 orders of magnitude greater than those reported in oceanic gyres. Consequently, benthic organisms are considered vulnerable to microplastic exposure. Polychaete worms inhabit almost all benthic marine and estuarine sediments (Fauchald, 1977). In terms of diversity and abundance, they commonly dominate the macrobenthos (Hutchings, 1998) and thus greatly contribute to the local productivity (Buchanan and Warwick, 1974; Warwick and Price, 1975).

Polychaetes exhibit a wide-range of feeding modes, including suspension, surface-deposit, and deposit feeding (Fauchald and Jumars, 1979), during which little selectivity is exhibited. Thus they are liable to ingesting microplastics of appropriate

size and have shown to do so both in the laboratory (Besseling et al., 2012; Thompson et al., 2004) and field (Mathalon and Hill, 2014; Van Cauwenberghe et al., 2015). Polychaetes form an ecologically-important component of marine ecosystems as they are predated on by a range of organisms, including molluscs, crustaceans (Hutchings, 1998), fish and birds (Goss-Custard et al., 1991). Moreover, their burrowing and feeding activity within the sediment influences the redistribution of organic matter (Lopez and Levinton, 1987), sediment metabolism (Ahrens et al., 2001), aeration (Kristensen, 1988), and animal respiration (Kristensen et al., 1992). Given their susceptibility to microplastic ingestion and their pivotal role in marine ecosystems, it is important to determine the potential impacts of microplastics and their associated pollutants on polychaetes.

I.7 Aim

The overall aim of this thesis is to examine the potential for microplastics to cause physical and chemical harm in the marine environment. Focussing on polychaete worms, an integrative approach employing biological endpoints at the physiological, cellular, and molecular level, in conjunction with analytical chemistry was adopted to address the following objectives:

I.7.1 Objectives

1) To review current literature, focussing on the potential for microplastics to negatively impact marine organisms with emphasis on the sensitivity of different feeding guilds, and identify key knowledge gaps. This is covered in Chapter 2: Wright, S. L., Thompson, R. C. and Galloway, T. S. 2013. The physical impacts of microplastics on marine organisms: a review. *Environmental Pollution*. 178, pp. 483-492.

2) To determine the extent to which the physical presence of chemically-inert microplastics can cause harm and in what quantities. This is examined in Chapter 3: Wright, S.L., Rowe, D., Thompson, R.C., and Galloway, T.S. 2013. Microplastic ingestion decreases energy reserves in marine worms. *Current Biology*. 23 (23).

3) To establish whether chemical additives commonly incorporated in plastic are bioavailable upon ingestion of microplastics, and whether bioaccumulation of chemical additives occurs at a level capable of causing toxicological harm. This is covered in Chapter 4: Wright, S. L., Bakir, A., Rowland, S., Reid, M., Thomas, K., Thompson, R., and Galloway, T. S. Manuscript in preparation.

4) To establish whether biodegradable plastics differ in their potential to cause harm, with focus on cigarette debris and the transfer of adhered persistent organic pollutants. This is addressed in Chapter 5: Wright, S. L., Rowe, D., Reid, M. J., Thomas, K. V., and Galloway, T. S. 2015. Cigarette litter impairs the ecological function of marine worms. *Nature Scientific Reports*. Manuscript in review.

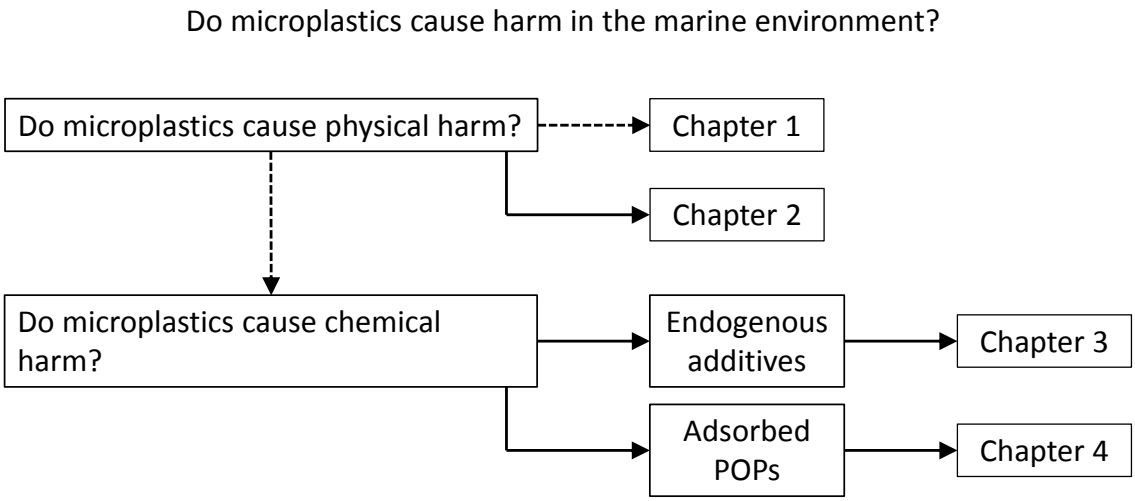


Figure 4. A flowchart outlining the basis of thesis chapters.

Overall, this project aimed to deliver scientific evidence contributing to the growing body of literature on anthropogenic debris and its potential to affect marine life in order to inform the scientific community, industry and policy-makers.

Chapter 1

The physical impacts of microplastics on marine organisms: a review

Environmental Pollution, 178, pp. 483-492

The physical impacts of microplastics on marine organisms: a review

Stephanie L. Wright[a*], Richard C. Thompson[b], Tamara S. Galloway[a]

[1]Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter, Devon EX4 4QD, UK.

[2]School of Marine Science and Engineering, University of Plymouth, Plymouth, Devon PL4 8AA, UK.

*E-mail: sw393@exeter.ac.uk

This manuscript has been modified to conform to the format of this thesis.

1.1 Abstract

Plastic debris at the micro-, and potentially also the nano-scale, are widespread in the environment. Microplastics have accumulated in oceans and sediments worldwide in recent years, with maximum concentrations reaching 100 000 particles m^{-3}. Due to their small size, microplastics may be ingested by low trophic fauna, with uncertain consequences for the health of the organism. This review focuses on marine invertebrates and their susceptibility to the physical impacts of microplastic uptake. Some of the main points discussed are (1) an evaluation of the factors contributing to the bioavailability of microplastics including size and density; (2) an assessment of the relative susceptibility of different feeding guilds; (3) an overview of the factors most likely to influence the physical impacts of microplastics such as accumulation and translocation; and (4) the trophic transfer of microplastics. These findings are important in guiding future marine litter research and management strategies.

Keywords: Microplastics; Plastic debris; Marine litter; Marine invertebrates; Food web.

1.2 Introduction

In contemporary society, plastic has achieved a pivotal status, with extensive commercial, industrial, medicinal and municipal applications. Demand is considerable; annual plastic production has increased dramatically from 1.5 million tonnes in the 1950s to approximately 280 million tonnes in 2011 (PlasticsEurope, 2012). Through accidental release and indiscriminate discards, plastic waste has accumulated in the environment at an uncontrollable rate, where it is subjected to wind and river-driven transport, ultimately reaching the coast. Due to its lightweight, durable nature, plastic has become a prevalent, widespread element of marine litter (Moore, 2008; Thompson et al., 2009); the most commonly produced and therefore encountered polymers being polypropylene (PP), polyethylene (PE) and polyvinylchloride (PVC) composing 24%, 21% and 19% of global plastic production in 2007, respectively (Andrady, 2011). Recently, inconspicuous microscopic plastic particles, referred to here as 'microplastics', have been identified as a ubiquitous component of marine debris. Defined as less than 5 mm in size by the National Oceanic and Atmospheric Administration (NOAA), microplastics can be of primary (purposefully manufactured to be of microscopic size) or secondary (derived from the fragmentation of macroplastic items) origin. They have been accumulating in oceans worldwide over the last four decades (Carpenter et al., 1972), from low background levels to localized 'hotspots' (see Table 1). Present on beaches, in surface waters, throughout the water column and within the benthos (Lattin et al., 2004; Moore et al., 2001; Thompson et al., 2004), microplastics have pervaded even the most remote marine environments (e.g. Ivar do Sul et al., 2009). Gyres are particular hotspots for microplastic accumulation.

Recently a maximum concentration and mass of 32.76 particles m^{-3} and 250 mg m^{-3} respectively have been recorded in the North Pacific Subtropical Gyre (Goldstein et al., 2012). Industrial coastal areas have also been identified as microplastic hotspots; concentrations of approximately 100 000 plastic particles m^{-3} of seawater have been reported in a Swedish harbour area adjacent to a PE production plant (Noren and Naustvoll, 2010). Sediment from densely populated coastal areas can be heavily contaminated with microplastics. Browne et al. (2011) found microplastics on eighteen shores across six continents, with a tendency towards fibrous shapes. Maximum concentrations of 124 fibres L^{-1} were reported and a significant relationship between

microplastic abundance and human population density was found (Browne et al., 2011). Thus as the human population continues to increase, the prevalence of microplastics will also most probably increase. Previous studies have found a predominance of fibrous microplastics (see Claessens et al., 2011; Thompson et al., 2004). Despite a variety of forms from irregular fragments to spherules, it seems likely that fibrous microplastics are most abundant in the marine environment.

A temporal increase in the abundance of marine microplastics has been indicated. Recently, combined data from peer-reviewed literature, publicly available data and new data sets revealed changes in the abundance and mass of microplastics in the North Pacific Subtropical Gyre. Abundance and mass increased by two orders of magnitude from a median of 0 – 0.116 particles m^{-3} and 0 – 0.086 mg m^{-3}, respectively from 1972 – 1987 to 1999 – 2010. This is believed to have been driven by a localised increase in microplastic abundance (Goldstein et al., 2012). Additionally, North Atlantic and North Sea surface samples collected by a Continuous Plankton Recorder (CPR, Sir Alister Hardy Foundation for Ocean Science), coincided with a growth in global plastic production (Thompson et al., 2004). Archived plastic samples from the west North Atlantic Ocean over the past 24 years have revealed a decrease in mean particle size from 10.66 mm in the 1990s to 5.05 mm in the 2000s. Sixty nine per cent of fragments were 2 – 6 mm (Morét- Ferguson et al., 2010), highlighting a prevalence of small plastic particles. Given the continual fragmentation of plastic items, particle concentrations are likely to increase with decreasing size.

The entanglement in and ingestion of macroplastic items is widely recognised in vertebrates. Over 250 marine species are believed to be impacted by plastic ingestion (Laist, 1997). The demise of higher organisms, typically vertebrates, is highly emotive and ultimately more conspicuous to observers. As a result, such instances are often subject to extensive scientific research and media coverage. Information regarding the biological impacts of microplastics on marine organisms, however, has received less attention and is only just emerging. A technical report considering the impacts of marine debris on biodiversity revealed that over 80% of reported incidents between organisms and marine debris was associated with plastic whilst 11% of all reported encounters are with microplastics (GEF, 2012). Since microplastics occupy the same size fraction as sediments and some planktonic organisms, they are potentially

bioavailable to a wide range of organisms. Microplastics can be ingested by low trophic suspension, filter and deposit feeders, detritivores and planktivores (Browne et al., 2008; Graham and Thompson, 2009; Murray and Cowie, 2011; Thompson et al., 2004). Therefore, they may accumulate within organisms, resulting in physical harm, such as by internal abrasions and blockages. In addition to the potential physical impacts of ingested microplastics, toxicity could also arise from leaching constituent contaminants such as monomers and plastic additives, capable of causing carcinogenesis and endocrine disruption (see Oehlmann et al., 2009; Talsness et al., 2009). Furthermore, microplastics are liable to concentrate hydrophobic persistent organic pollutants (POPs), which have a greater affinity for the hydrophobic surface of plastic compared to seawater. Due to their large surface area to volume ratio, microplastics can become heavily contaminated — up to six orders of magnitude greater than ambient seawater — with waterborne POPs (Hirai et al., 2011; Mato et al., 2001). This presents a possible route of exposure to marine organisms, whereby bioaccumulation and biomagnification could occur through the food chain. The transfer of POPs to marine organisms via microplastic vectors is not considered in detail in this review (for examples see Teuten et al., 2009); however the pathways and uptake of microplastic particles are clearly of relevance to chemical transfer, as well as physical harm.

Given the growing evidence outlined above, this review — focussing on marine invertebrates — aims to: (1) summarise the factors contributing to the bioavailability of microplastics; (2) outline the susceptibility of different feeding guilds to microplastic ingestion; (3) determine the factors likely to influence the physical impacts of microplastics; and (4) discuss microplastic transfer through the food chain.

1.3 Factors affecting the bioavailability of microplastics

1.3.1 Size

A key factor contributing to the bioavailability of microplastics is their small size, making them available to lower trophic organisms. Many of these organisms exert limited selectivity between particles and capture anything of appropriate size (Moore, 2008). Alternatively, higher trophic planktivores could passively ingest microplastics

during normal feeding behaviour or mistake particles for natural prey. Work by Fossi et al. (2012) investigated the impacts of microplastics on the Mediterranean fin whale *Balaenoptera physalus*, one of the largest filter feeders in the world. *B. physalus* can engulf approximately 70 000 L of water at one time, potentially risking microplastic ingestion both directly and indirectly from the water and plankton, respectively. Using phthalate contamination as a proxy for microplastic ingestion, the authors concluded that *B. physalus* could be consuming microplastics (Fossi et al., 2012).

1.3.2 Density

The density of the plastic particles will determine bioavailability in the water column; hence the type of plastic ingested may vary between organisms. Planktivores, filter feeders and suspension feeders inhabiting the upper water column are likely to encounter positively buoyant, low-density plastics, such as PE (specific gravity 0.91 – 0.94), on the sea surface (see Fig. 1). The buoyancy of plastic is influenced by biofouling, for example, PE food bags (20 × 28 cm) displayed a well-developed biofilm within one week, which continued to increase throughout a three week exposure period. By the third week, the PE food bags had started to sink below the sea surface, indicating neutral buoyancy (Lobelle and Cunliffe, 2011). The rate of biofouling depends on parameters such as surface energy and hardness of the polymer, as well as water conditions (Muthukumar et al., 2011). Defouling in the water column by foraging organisms is a potential pathway for microplastic particles to return to the sea-air interface (Andrady, 2011). This cyclic pattern may make microplastics available to organisms occupying different depths of the water column at different times (see Fig. 1). Alternatively, fouled microplastics could continue to sink, as would high-density plastics such as PVC (specific gravity 1.38). Such particles will become available to benthic suspension and deposit feeders and detritivores as they sink, eventually reaching the benthos (see Fig. 1).

1.3.3 Abundance

An increase in the abundance of microplastics in the marine environment will also affect its bioavailability, as the chance an organism will encounter a microplastic particle is enhanced. Therefore the progressive fragmentation of macroplastic items is likely to increase the amount of particles available for ingestion to a wider range of organisms (Browne et al., 2007, 2008; Thompson et al., 2009).

1.3.4 Colour

The colour of microplastics may potentially contribute to the likelihood of ingestion, due to prey item resemblance. Shaw and Day (1994) reported that plastic particles sampled from the North Pacific exhibited size variation related to colour; white plastic particles consistently decreased in abundance with decreasing size class. Some commercially important fish and their larvae are visual predators, preying on small zooplankton, and may feed on microplastics which most resemble their prey i.e. white, tan and yellow plastic (Shaw and Day, 1994). To further support the influence of colour on bioavailability, fish from the Niantic Bay area, New England had ingested only opaque, white polystyrene spherules. These were present in equal proportion with clear polystyrene spherules, indicating selectivity (Carpenter et al., 1972). Microplastic ingestion due to food resemblance may also be applicable to pelagic invertebrates, which are visual raptorial predators (Greene, 1985).

1.3.5 Biological interactions

Microplastic bioavailability could be enhanced by biological factors. The ingestion of polystyrene (PS) beads (100 nm) by suspension-feeding bivalve molluscs significantly increased when they were incorporated into manually-generated aggregates, formed by rolling natural seawater in the laboratory. The seasonal flocculation of natural particulates into sinking aggregates is an important pathway for energy transfer between pelagic and benthic habitats (Ward and Kach, 2009). Consequently, the potential for microplastics to become incorporated into marine aggregates may present a further mode of entry into the food chain. Once ingested, microplastics could sequentially be egested within fecal matter. Suspension feeders and detritivores may ingest such egested microplastics (see Fig. 1). Sediment-dwelling organisms, such as the lugworm *Arenicola marina*, are capable of bioturbation (cycling the upper layers of sediment). Microplastic particles which have settled on the benthos could be drawn into the sediment, where they would be available to infauna (see Fig. 1).

1.4 The susceptibility of marine organisms to microplastic ingestion

The potential for microplastics to cause harm in marine organisms is initially likely to be governed by the susceptibility of species to ingest and/or interact with them.

Selectivity is evident in particle ingestion of natural substances in a range of species and it is therefore likely that such selectivity will extend to microplastics. Various laboratory studies have reported the ingestion of microplastics by invertebrates from a range of feeding guilds.

1.4.1 Detritivores and deposit feeders

Since microplastics occur in sedimentary habitats, deposit- and detritus-feeding organisms are susceptible to exposure. Thompson et al. (2004) reported microplastic (20 – 2000 µm) ingestion in the omnivorous amphipod *Orchestia gammarellus* and the deposit-feeding polychaete *A. marina*. Amphipods may directly mistake microplastics as a natural food source and could therefore be regarded as primary consumers of microplastics (Murray and Cowie, 2011). The marine polychaete *A. marina* is capable of size-based selectivity, whereby smaller particles stick to the mucus-lined proboscis papillae and are retained, whilst larger particles are rejected (Zebe and Schiedek, 1996). Plastic particles within this size range are therefore likely to be retained and ingested (see Table 2). Morét-Ferguson et al. (2010) report a shift in the abundance of plastic debris to smaller size categories in the western North Atlantic Ocean. If this finding is extrapolated to other regions, then it is likely more particles are gradually becoming available to these organisms.

Microplastic ingestion has also been documented in the benthic holothurians (sea cucumbers) *Thyonella gemmate*, *Holothuria floridana*, *H. grisea* and *Cucumaria frondosa*. Generally scavengers, they feed on debris in the benthic zone of the ocean and adopt a non-selective feeding strategy whereby large volumes of sediment are ingested; the associated organic debris and microorganisms of which is retained. Graham and Thompson (2009) found individuals belonging to four species of two orders ingested significantly more plastic (0.25 – 15 mm) than expected — between 2- and 20-fold more PVC fragments and between 2- and 138-fold more nylon line fragments (up to 517 fibres per individual) — based on plastic to sand grain ratios from each sediment treatment. This suggests individuals were selectively ingesting plastic particles, which may be attributed to the feeding techniques adopted by each order. Species' exhibited either active foraging in the upper millimetres of the sediment (aspidochirotids), frequently encountering plastic particles, or less active foraging involving brushing tentacles over the surface of the sediment (dendrochirotids), thus,

only exposed and/or protruding particles were obtained. Both tentacle types could contact the plastic particles with limited shovelling and sand ingestion due to the large surface area of the plastic. Benthic holothurians displayed both random (the animals had to forage to encounter plastic particles) and selective (once encountered, plastic was separated from the sediment) feeding methods. This contradicts their indiscriminate feeding strategy; something which could potentially occur in all non-selective feeders when presented with microplastics. Size affected ingestion, as <0.5 mm PVC shavings were ingested 37 more times than the predicted quantity compared to <17 times more for other size categories. Moreover, ingestion was limited when individuals encountered PVC pellets (4.0 mm diameter), possibly due to a restriction imposed by mouth size or difficulty in grasping them with their tentacles. Whether there was an impact on the physiological condition of the organisms following plastic ingestion remains unknown.

The authors also analysed sediment samples from sites where the animals were collected, which were found to be contaminated with microplastics (105 – 214 plastic particles L^{-1}), predominantly in fibrous forms. This corresponds with recent studies, which have found a prevalence of microplastic fibres in coastal sediments (Browne et al., 2011; Claessens et al., 2011). Since Graham and Thompson (2009) found benthic holothurians mostly ingested plastic fibres, it is likely that microplastic ingestion is occurring in the natural environment (see Table 2).

The non-selective benthic scavenger and predatory crustacean *Nephrops norvegicus* has also been shown to ingest small plastic fragments. Gut content analysis found that 83% of animals collected from the Clyde Sea contained plastic, the majority of which took the form of tangled nylon-strand balls. This coincides with the dominance of plastic fibres contaminating sediments as previously mentioned. Additionally, laboratory-based feeding experiments using 'seeded' fish revealed 100% of individuals ingested and retained 5 mm nylon rope fragments (Murray and Cowie, 2011). These findings highlight the passive nature of plastic ingestion in *N. norvegicus*; whilst consuming sediment, or via the food it scavenges, suggesting a trophic link (Murray and Cowie, 2011; see Table 2).

1.4.2 Planktivores, filter feeders and suspension feeders

Due to the similarity between the specific gravity of plastic microspheres and algae, plastic microspheres have the potential to be prey analogues for planktivores and may be handled and ingested in a similar manner (Brillant and MacDonald, 2000). The common use of plastic microspheres in laboratory-based feeding experiments emphasises the likelihood for microplastic ingestion. Marine ciliates are capable of engulfing microplastics. Using plastic microspheres in laboratory experiments, Christaki et al. (1998) investigated ciliate ingestion as a function of particle size and surface characteristics. They found size played a key role, as clearance rates for plastic microspheres (0.75 mm) were indistinguishable from those for fluorescently labelled cells, indicating an absence of chemosensory-mediated selection. Thus, if ciliates encounter plastic particles of appropriate size in the marine environment, they present a potential pathway for plastic transfer within the food chain (see Table 2).

In a laboratory study investigating particle capture and suspension feeding methods, sea urchin, sea star, sand dollar, brittle star and sea cucumber larvae captured and ingested 10 – 20 µm PS divinylbenzene (dvb) microspheres. In echinoderm larvae, filter feeding is largely governed by the presence of a ciliated band which encircles the mouth. Particles are extracted from suspension by a short reversal in the direction of the cilia beat across the band. Cilia then transfer the accumulated particles to the mouth for ingestion (Hart, 1991). Particle capture and ingestion seems to be based on size selectivity, thus, if echinoderm larvae encounter microplastics of an appropriate size in the environment, they are likely to be captured and ingested (see Table 2). Whether the microspheres were subsequently egested or accumulated in the gut was not determined.

As well as echinoderm larvae, laboratory work on the larvae of the marine polychaete worm *Galeolaria caespitose* showed ingestion of 3 µm and 10 µm neutral-density polymer microspheres. The larvae ingested substantially more of the smaller 3 µm microspheres, emphasising the importance of size in microplastic ingestion (Bolton and Havenhand, 1998). Furthermore, this highlights the idea that the continuous fragmentation of plastic into smaller particles will increase its availability.

Wilson (1973) found the filter-feeding calanoid copepod *Acartia tonsa* ingested microplastics during food size selection experiments. Particle capture is achieved by creating currents which pass through a 'basket' formed from various appendages, or by sweeping net-like appendages through the water column (Wilson, 1973). *A. tonsa* selectively ingested plastic beads ranging from 13.9 to 59 µm. Selectivity was based on the size frequency distribution of available particles, choosing the largest abundant beads in conjunction with a passive filtering process. Wilson (1973) hypothesised that selectivity was attained through either discriminating between which particles were grasped or particles which were detected on feeding appendages yet disregarded. This reaffirms the capacity for zooplankton to ingest microplastics.

Marine zooplankton, particularly members of the herbivorous constituent, have proven to ingest microplastics in laboratory studies. The prevalence of low-density microplastics on the sea surface suggests euphotic zooplankton, including commercially important larvae, are susceptible to microplastic ingestion.

Benthic suspension feeders may additionally be susceptible to sinking microplastic particles; numerous bivalve mollusc species ingest microplastics (see Table 2). The suspension-feeding common mussel *Mytilus edulis* has been shown to capture and ingest microplastic particles ranging from 2 to 16 µm in size (Browne et al., 2008; Ward and Kach, 2009; Ward and Targett, 1989; Ward et al., 2003). In suspension-feeding bivalves, particle capture, retention and sorting occur prior to ingestion. In order to capture particles a current is created by the lateral cilia on the ctenidial filaments, which flows into the inhalant siphon. Particles encounter the frontal surfaces of filaments located on the ctenidium and become trapped in a fine mucus layer; cirral-trapping is fundamental to particle retention (Ward and Shumway, 2004). Most bivalves capture and retain 3-4 µm particles with 100% efficiency and are capable of withholding particles as small as 1 µm diameter with a reduced efficiency of approximately 50% (Gosling, 2003). Since microplastics >1.6 µm in size occur in coastal environments (Ng and Obbard, 2006), it is plausible that microplastics of optimum size for bivalve capture and retention exist and are consequently ingested.

As bivalves exert limited control on the types of particles captured, they can capture particles of low-nutritive value. However, bivalves have the capacity to sort particles

prior to ingestion, discriminating between similar-sized particles based on quality; unfavourable particles are subsequently rejected as pseudofaeces (Gosling, 2003; Ward and Shumway, 2004). Pre-ingestive sorting specifically concerning microplastics has so far not been described. Histological sampling and fluorescence microscopy have revealed the presence of 2 µm and 4 – 16 µm microspheres in the gut cavity and digestive tubules of *M. edulis* (Browne et al., 2008).

This suggests that *M. edulis* exerts selectivity based on size, shape, or density irrespective of particle quality as denoted by surface chemistry during pre-ingestive particle sorting. Due to their inherent feeding strategy, the apparent inability to sort and reject microplastics prior to ingestion may be applicable to all suspension-feeding bivalve molluscs.

The above studies used concentrations ranging from 1000 to 20 000 particles mL^{-1} (Bolton and Havenhand, 1998; Ward and Kach, 2009; Ward and Targett, 1989; Wilson, 1973). One of the highest microplastics concentration reported from the marine environment is 0.102 particles mL^{-1} in Swedish coastal waters adjacent to a PE production plant (Norén, 2008). Clearly, laboratory concentrations exceed reported environmental levels by several orders of magnitude, however the results do provide evidence that if encountered, microplastics may be captured and ingested by marine invertebrates.

Microplastics may not only enter the food chain via ingestion, as they have demonstrated a capacity to adsorb to organisms. At the base of the food web, the freshwater and freshwater/marine algal cells Chlorella and Scenedesmus, respectively, adsorbed charged nanoplastics (20 nm). A preference for positively charged particles was reported, probably due to the electrostatic attraction between the beads and cellulose constituent of the living cells. Nanoplastic sorption was further dependent on algal morphology and motility, with the flagellate Scenedesmus displaying a greater binding affinity to particles (Bhattacharya et al., 2010; see Table 2).

1.5 Factors influencing the physical impacts of microplastics

There is a wealth of literature regarding macroplastic ingestion in vertebrates (e.g. Denuncio et al., 2011; Laist, 1997; Lazar and Gracan, 2011; van Franeker et al., 2011; Yamashita et al., 2011), reporting global impacts including: internal and/or external abrasions and ulcers; and blockages of the digestive tract, which can result in satiation, starvation and physical deterioration. In turn this can lead to reduced reproductive fitness, drowning, diminished predator avoidance, impairment of feeding ability, the potential transfer of damaging toxicants from seawater and ultimately death (Gregory, 2009). Such detrimental effects are also likely to apply to smaller organisms including invertebrates, which ingest microplastics. For example, potentially fatal injuries such as blockages throughout the digestive system or abrasions from sharp objects. Other feasible impacts have been suggested by the Marine Strategy Framework Directive Task Group 10 (Galgani et al., 2010) and include: blockage of enzyme production; diminished feeding stimulus; nutrient dilution; reduced growth rates; lowered steroid hormone levels; delayed ovulation and reproductive failure; and absorption of toxins. There is potential for microplastics to clog and block the feeding appendages of marine invertebrates or even to become embedded in tissues (Derraik, 2002): plastic fragments and PP and/or monofilament line have been found in the tissues of two filter feeding salps — *Thetys vagina* — collected from neuston samples in the NPCG (Moore et al., 2001). Some of the factors likely to influence the physical and chemical impact of microplastics and their transfer through the food chain are discussed below.

1.5.1 Accumulation

The capacity for microplastics to accumulate within an organism is likely to affect the associated physical impact of microplastic ingestion. So far, there is limited literature regarding the accumulation of microplastics in marine invertebrates. A plankton tow in south New England coastal waters collected a 20 mm long chaetognath, *Sagitta elegans*, which had a 0.6 mm diameter spherule in its intestine (Carpenter et al., 1972). It was not confirmed whether this was plastic; however the spherule was described as being identical to PS spherules also collected in the tow. Nevertheless this highlights the ability for similar particles to accumulate in marine invertebrates.

In laboratory studies microplastics have been shown to accumulate in the digestive cavity and tubules of bivalve molluscs (Brillant and MacDonald, 2000; Browne et al.,

2008). Within 30 min of ingestion, 20 µm PS microspherules were observed in the primary ducts and tubules of the sea scallop *Placopecten magellanicus*' digestive gland where they persisted for up to 48 h. The microspherules were absent from the epithelial cells of the gut, implying they were not phagocytised. Despite being taken up by the digestive tubules, the microspherules were of a similar size to the epithelial cells and therefore may have been too large to permit phagocytosis (Brillant and MacDonald, 2000). In *M. edulis*, mid gut histological sections revealed 2 µm fluorescently-labelled and 4 – 16 µm non-labelled PS microspheres accumulating in the digestive cavity and tubules following a 12 h exposure (Browne et al., 2008).

Accumulation of microplastic particles in marine invertebrates could potentially cause blockages throughout the digestive system, suppressing feeding due to satiation. Alternatively, predation of microplastic-contaminated marine invertebrates may present a pathway for plastic transfer along the food chain.

Besides internal accumulation, the external adsorption of microplastics may also cause harm. Bhattacharya et al. (2010) found the binding of plastic beads (20 nm) to the algal species Chlorella and Scenedesmus inhibited photosynthesis, potentially due to the physical blockage of light and air. Moreover, it increased reactive oxygen species production, indicating a state of oxidative stress (Bhattacharya et al., 2010). Despite using extremely high concentrations — 1.4 – 40 mg mL^{-1} — relative to environmental levels, this study highlights the potential for microscopic plastic particles to adhere to algal cells, possibly impacting on photosynthesis. As algae play a key role in aquatic food webs, the productivity and resilience of ecosystems could be compromised if high concentrations occur due to the adverse effects of plastic particles.

1.5.2 Translocation

Andrady (2011) states that due to a lack of enzymatic pathways available to break down plastics in filter-feeding organisms, microplastics are unlikely to be digested or absorbed and can therefore be considered bio-inert. However, they may pass through cell membranes and become incorporated into body tissues following ingestion. Fluorescence and confocal microscopy revealed 3 µm and 9.6 µm fluorescent PS microspheres in the haemolymph and haemocytes of the suspension feeder *M. edulis*, three days after short (three hour) pulse exposures to 15 000 particles 350 mL^{-1}

(Browne et al., 2008). This implied the microspheres had translocated across the gut epithelial lining into the circularity system; however, the precise mechanism(s) for uptake across the epithelial lining remains unknown, as does the precise translocation time (Browne et al., 2008). In rats and humans, enterocytes are responsible for the transportation of particles across the epithelium by phagocytosis into the circularity fluid.

The smaller microspheres (3 µm) typically had the greatest abundance (>60 per cent) in both haemolymph and haemocytes. A similar pattern has been shown in rats whereby 14 nm latex particles were in contact with colonic enterocytes within 2 min of introduction compared to 30 min for 415 nm particles (Hussain et al., 2001). This implies the rapid translocation of smaller particles is applicable to both invertebrates and vertebrates. If phagocytosis is the primary mechanism for translocation of microplastics, it is conceivable that a greater abundance of small-sized particles are phagocytised due to the limited capacity of the phagosome within each cell (Browne et al., 2008). As plastic continues to fragment, the potential for it to accumulate within the circulatory fluid and phagocytic cells of an organism is likely to increase, as the smaller the microplastics, the greater the abundance available for translocation.

Despite the presence of microplastic particles in *M. edulis'* haemolymph and haemocytes, no toxicological effects were observed (Browne et al., 2008). Conversely, indications of granulocytoma formation (inflammation), an increase in haemocytes and a significant decrease in lysosome stability were observed in *M. edulis* after 48 h, following plastic particle (1 – 80 µm) uptake into the vacuoles of the digestive gland (GESAMP, 2010). Consequently, the energy allocated to immune function in such scenarios may compromise normal physiological processes. Over time, this could have a detrimental effect on the health of the organism, at both the individual and population level.

Once translocated from the gut to the circulatory system, microplastics can be retained for several weeks. PS microspheres persisted in *M. edulis* haemolymph and haemocytes for as long as 48 days (Browne et al., 2008). Such tenacity could be applicable across species and thus microplastics may be transported to various tissues

and organs via the haemolymph, potentially accumulating and causing harm. In turn, this could facilitate the transfer of microplastics to higher trophic organisms.

Presently, more research is required to determine the upper and lower size limits for translocation to occur in organisms. Additionally, the behaviour and fate of microparticles of different polymer types and shapes also needs to be established. In the natural environment, organisms may be exposed to microplastics throughout their lifetime as opposed to short experimental durations. Thus the continual ingestion and accumulation of such particles may incur chronic effects. Moreover, many different polymers occur in the environment, which may elicit a different response to a single polymer.

1.5.3 Shape

The potential adverse effects associated with the presence of microplastics are likely to vary with particle shape. Carbon nanotubules have exhibited lung damage; Warheit et al. (2004) found the lung tissue of rats exposed to single-wall carbon nanotubules displayed inflammation and cell injury. In mesoporous silica nanoparticles (MSNs), shape can influence the efficiency and ability of drug delivery irrespective of chemical composition, surface charge and diameter; rod-shaped MSNs showed increased cellular uptake and therefore a greater effect on apoptosis, migration and disruption of cytoskeleton organisation. Long rod-shaped nanoparticles severely reduced cell viability and apoptosis compared to sphere and short rod-shaped nanoparticles. An explanation for such shape-related toxicity is that the long rod-shaped MSNs are easily up-taken by cells due to the greater contact area and potential for interaction (Huang et al., 2010). Given that marine microplastics occur in a variety of shapes from fibres to irregular fragments to spheres and rods, there is potential for the physical adverse effects of polymers to alter depending on form. Along the Belgian coast, plastic fibres formed the majority (59%) of microplastic debris sampled (Claessens et al., 2011), with average concentrations of 81.0 ± 37.2, 65.6 ± 15.3 and 66.3 ± 28.6 fibres kg^{-1} for beach, harbour and sea sampling stations respectively. Concentrations of plastic fibres (<1 mm) ranging from 2 (Australia) to 31 (Portugal) fibres 250 mL^{-1} contaminated 18 shores across six continents, with concentration positively correlating with population density (Browne et al., 2011). Thus, benthic and sediment-dwelling organisms

inhabiting such areas are vulnerable to the shape-related toxicity of plastic fibres, if ingested.

1.5.4 Egestion

There is very little information available regarding the capacity for marine organisms to egest microplastics. Through fecal cast analysis, Thompson et al. (2004) found some microplastic particles were defecated by the lugworm *A. marina*. To assess the ingestion of microplastic fragments and pellets in benthic holothurians, faecal debris was collected from individuals held in the laboratory. The quantity of defecated microplastic particles was then enumerated (Graham and Thompson, 2009). Through egestion, it is possible that an organism will prevent any detrimental effects caused by the ingestion of plastic particles.

The estuarine copepod *Eurytemora affinis* demonstrated an ability to regurgitate latex beads (mean diameter 15 µm). Laboratory feeding trials were conducted with 3 – 90 beads mL^{-1} concentrations; beads were ingested at mean rates of up to 59 000 particles per copepod per hour. The capability for plastic microspherule ingestion was demonstrated, as was the potential for accumulation inside the gut cavity of *E. affinis*. However, following the initial microspherule ingestion, the particles were subsequently regurgitated between 1 and 3 h later. This was indicated by a decrease then sequential increase of latex microspherules in the feeding suspension coinciding with an absence of latex microspherules in the fecal pellets of *E. affinis*. Alternatively, bacterial-coated latex microspherules (15 µm) were retained and successively egested in fecal pellets (Powell and Berry, 1990), highlighting an ability to reject un-nutritious particles.

Egestion rates are likely to affect the capacity for potentially adhered contaminants to desorb in addition to the likelihood of transfer to the food chain. Predation could still occur within the timeframe. The diurnal vertical migration of zooplankton could further transport microplastics to predators occupying various depths of the water column.

1.5.5 Population-level effects

Aside from physical and chemical impacts, microplastics also have a potential role in providing a new hard-substrate habitat for rafting communities, which was previously

limited to items such as floating wood, pumice, and sea shells. In 2001, Moore et al. found monofilament line 10 cm below the sea surface to be colonised with diatoms and other microalgae. Recently, microplastics have been identified as an important oviposition resource for the pelagic insect *Halobates sericeus*, indicated by a positive correlation between *H. sericeus* eggs on microplastics and microplastic abundance. The pelagic invertebrate community represents a crucial link between primary producers and nektonic species. Thus, changes in the population structure of *H. sericeus* may lead to ecosystem wide consequences (Goldstein et al., 2012).

The increasing abundance of microplastics may be capable of modifying community-wide assemblages. Additionally, microplastics present a mechanism for long distance transport of rafting species, enhancing biogeographic connectivity. The most common rafting species are from the phyla Cnidaria, Crustacea and Ectoprocta (Thiel and Gutow, 2005). These species may be considered the most vulnerable to population-level microplastic-associated changes.

1.5.6 Transfer to the food chain

At present, there are few studies on the bioaccumulation of plastics and their associated POPs across marine trophic levels. Given that lower trophic organisms, specifically invertebrates, can ingest and accumulate microplastic particles, it is likely that microplastics will be introduced to the food web. Laboratory microplastic ingestion studies have mostly focused on invertebrates; however, *in situ* work has discovered microplastic ingestion in several vertebrate species.

Lusher et al. (2012) found microplastics in 36.5% of fish belonging to 10 species sampled from the English Channel, irrespective of habitat (pelagic vs. demersal). An average of 1.9 ± 0.1 particles were recovered from those which contained plastic, the main polymers being polyamide and polyester, which are materials commonly used in the fishing industry (Lusher et al., 2012). Whilst the biological consequences remain unclear, such findings are comparable to those from the North Pacific Central Gyre reported by Boerger et al. (2010); small plastic fragments were found in approximately one third of all fish caught. Individuals from the most common species caught (*Myctophum aurolanternatum*, Myctophidae) contained an average of six plastic pieces and the most frequently ingested size class across all species was 1 – 2.79 mm.

The majority of fish caught in this study belonged to the Myctophidae, a low trophic, mesopelagic family which adopts diurnal feeding behaviour, preying upon plankton near the surface at night. As the most commonly ingested plastic colours (white, clear and blue out of 12 reported colours) were similar to that of plankton species inhabiting the North Pacific Central Gyre, the Myctophidae may mistake small plastic fragments for their natural food source (Boerger et al., 2010). Alternatively, the myctophids could consume plankton which has previously ingested microplastics or ingest plastic passively. Since the most commonly occurring plastic colours in tow samples matched those ingested, it is likely the Myctophidae are not showing selectivity but ingesting particles in a more passive manner. The toxicological effects of plastic ingestion in myctophids remains to be determined, however if they are unable to egest small plastic fragments, the plastic may accumulate and compromise normal feeding activity. Additionally, the Myctophidae are preyed upon by tuna, squid, odontoceti whales, seabirds and fur seals (Boerger et al., 2010). Thus there are several routes of entry to various compartments of the marine food web.

Higher trophic level organisms have been found to ingest microplastics transported by prey items. Microplastic particles approximately 1 mm in diameter were recorded in the scat of fur seals and Hooker's sea lions (Goldsworthy et al., 1997; McMahon et al., 1999). The presence of plastic coincided with otoliths of the myctophid fish *Electrona subaspera*, suggesting a trophic link. Eriksson and Burton (2003) further investigated the transfer of plastic particles in Antarctic fur seals. Scats of *Arctocephalus tropicalis* and *A. gazella* were collected from Macquarie Island, Australia, during the periods 1990 – 1991 and 1996 – 1997. Out of 145 seal scats, 164 small plastic particles (generally ranging from 2 to 5 mm) were recovered. However, the sieves used to separate the plastic from the scat had a mesh size >0.5 mm, suggesting that any plastic particles <0.5 mm were not retained. Thus the quantities obtained are likely to be an underestimation. Interestingly, during 1990 – 1991, one plastic particle per scat was recorded, whilst during 1996 – 1997 up to four particles per scat (1%) were documented; possibly a result of the increasing abundance of plastic debris in the marine environment. PE (93%) was the primary polymer group identified from the samples, followed by PP (4%), which closely matched polymer type's identified in beach flotsam from the same location. Nearly all fragments were irregular in form and

approximately one third had one sharp edge, indicating there is potential for internal abrasion. Eriksson and Burton (2003) believed there was little possibility that the seal species' were directly ingesting plastic particles; a plastic-concentrating vector, such as fish, is a more likely explanation.

Several fish species caught in New England coastal waters contained plastic microspherules identical to those collected during plankton tows in the same area (0.1 – 2 mm); winter flounder (*Pseudopleuronectes americanus*) and grubby (*Myoxocephalus aenaeus*) larvae, approximately 5 mm in length, contained 0.5 mm diameter plastic microspherules (Carpenter et al., 1972). By extrapolating this data and that from Goldsworthy et al. (1997), Eriksson and Burton (2003) estimated that minimum concentration factors of plastic particles to seals ranges from 22 to 160 times. The narrow range of particle parameters (size and shape) observed, suggests that selectivity is being practiced. *E. subaspera*, a major component of the fur seal diet, is likely to consume copepods 1 – 9 mm in size near the surface waters. This size range has a 95 per cent overlap with the plastic particles found in scats, indicating the transfer of microplastics across trophic levels is plausible.

Microplastics have the potential to be ingested by baleen whale species through indirect consumption via planktonic prey. Mono-(2-ethylhexyl) phthalate (MEHP) contamination of the blubber of the Mediterranean fin whale *B. physalus* has recently been suggested as an indication that microplastic ingestion occurs, either from the water column or via a planktonic vector. Fifty six percent of neustonic and planktonic samples from the Mediterranean Sea contained microplastics e up to 9.67 particles m^{-3}. This coincided with high levels of phthalates in the water column, specifically di-2-ethylhexyl phthalate (DEHP) and MEHP, comparative to the levels found in the blubber samples. The use of phthalates and plastics additives such as antimicrobials, dyes or stabilisers as tracers for microplastic ingestion and bioaccumulation is certainly a promising avenue for future research (Fossi et al., 2012).

At present, there is limited information regarding the impacts of microplastics on food webs and no associated laboratory experiments have been conducted. Therefore, it remains undetermined whether plastic of any size can be transferred to higher trophic levels. There are well documented examples of trophic transfer for many POPs within

marine food webs, for example dioxins, PCBs and polybrominated diphenylethers, many of which have been reported to associate with oceanic plastics (Ogata et al., 2009) and some of which can biomagnify (Hu et al., 2005). Generally, the extent of trophic transfer is dependent on characteristics including the octanol/water partition coefficient (K_{ow}) and metabolic transformation rate of the compound under consideration (Wan et al., 2005). The effect of co-ingestion of microplastics on the trophodynamic behaviour of POPs and plastics additives remains an important topic for further study. Other important factors to consider for the transfer of microplastics and their associated POPs are organism-dependent gut retention times, as well as the fraction of consumed microplastics that are capable of moving across the gut epithelium and into other tissues or organs.

1.6 Conclusions

Low density microplastic debris is accumulating in ocean gyres and pelagic invertebrates inhabiting these regions may be susceptible to microplastic ingestion. In addition, the benthos is likely to be a sink for high density microplastics. Some organisms may have the capacity to egest microplastics, possibly leading to their incorporation into marine aggregates. Benthic suspension- and deposit-feeders are therefore likely to ingest sinking and sedimentary microplastics. Fibres are the most commonly encountered form of microplastics in the marine environment. Benthic holothurians were found to selectively ingest microplastics, showing a preference for fibrous shapes. Additionally, benthic scavengers are susceptible to fibrous microplastic exposure, as gut content analysis revealed nylon fibres in *N. norvegicus*. This implies their habitat is a sink for fibres. Since shape may play a role in the toxicity of ingested microplastics e long, rod-shaped nanoparticles are considered more toxic than spherules e these organisms can be considered sensitive to the potential physical toxicity of microplastics.

The presence of microplastics in myctophid fish and Hooker's sea lion and fur seal scats suggest microplastic transfer through pelagic food chains: microplastics-zooplankton-myctophid fish-Hooker's sea lions/fur seals. Such lower trophic organisms

therefore represent a vector for microplastic transfer and their associated contaminants.

Microplastics may not only affect species at the organism-level; they also have the capacity to modify population structure. Species which were once restricted by a lack of hard substrate, such as the marine insect *H. Sericeus*, are now able to proliferate. This may be applicable to a wide range of organisms with potential impacts on ecosystem dynamics.

The accumulation of microplastic debris has presented a new marine habitat where biological interactions are taking place. This habitat and its environmental impacts are still emerging areas of research. It is hoped that future work on this growing issue (see Table 3) will contribute to the development of better methods for controlling marine litter.

1.7 Tables

Table 1. The spatial distribution and abundance of microplastics, as summarised from a selection of reports.

Location	Maximum Observed concentration	Reference
Coastal waters, Sweden	102 000 particles m^{-3}	Noren and Naustvoll, 2010
Coastal Waters, California	3.141 particles m^{-3}	Doyle et al., 2011
Coastal waters, New England	2.58 m^3	Carpenter et al., 1972
Open ocean, North West Atlantic	67 000 pieces km^{-2}	Colton et al., 1974
Northwest Mediterranean Sea	0.892 particles m^{-2}	Collignon et al., 2012
Beach, Malta	>1000 particles m^{-2}	Turner and Holmes, 2011
Beach, UK	8 particles kg^{-1}	Thompson et al., 2004
Estuarine sediment, UK	31 particles kg^{-1}	Thompson et al., 2004
Subtidal sediment, UK	86 particles kg^{-1}	Thompson et al., 2004
Subtidal sediment, Florida	214 particles l^{-1}	Graham and Thompson, 2009
Subtidal sediment, Maine	105 particles l^{-1}	Graham and Thompson, 2009
Harbour sediment, Sweden	50 particles l^{-1}	Noren, 2008
Industrial harbour sediment, Sweden	3320 particles l^{-1}	Noren, 2008
Industrial coast sediment, Sweden	340 l^{-1}	Noren, 2008

Ship-breaking yard sediment, India	89 mg kg^{-1}	Reddy et al., 2006
Harbour sediment, Belgium	7.21 mg kg^{-1}	Claessens et al., 2011
Continental shelf sediment, Belgium	1.3 mg kg^{-1}	Claessens et al., 2011
Beach, Belgium	1.05 mg kg^{-1}	Claessens et al., 2011
Beach, Portugal	5.6 particles m^{-2}	Martins and Sobral, 2011
Beach, East Frisian Islands, Germany	621 particles $10g^{-1}$	Liebezeit and Dubaish, 2012

Table 2. Marine organisms susceptible to microplastic ingestion and their encounter pathways.

Species	**Encounter pathway**
Marine algae e.g. *Scenedesmus*	Adsorbs nanoplastics, especially when positively charged.
Grazing microzooplankton e.g. the marine ciliate *Strombidium sulcatum*	Size-based selectivity indicates potential to ingest microplastics of appropriate size.
Benthic deposit feeders e.g. the polychaete *Arenicola marina* and the holothurian *Holothuria floridana*	The sea bed is a sink for high-density microplastics; size-based, deposit- feeding strategies adopted by *A. marina* indicate potential to ingest microplastics of appropriate size; *H. Floridana* selectively ingests plastic particles, showing a preference for fibrous shapes.
Benthic scavengers e.g. the crustacean *Nephrops norvegicus*	Fibrous microplastics have been found to accumulate in marine sediments; gut content analysis has shown plastic microfibers are being ingested in the environment; ingestion is passive via food it scavenges or sediment.

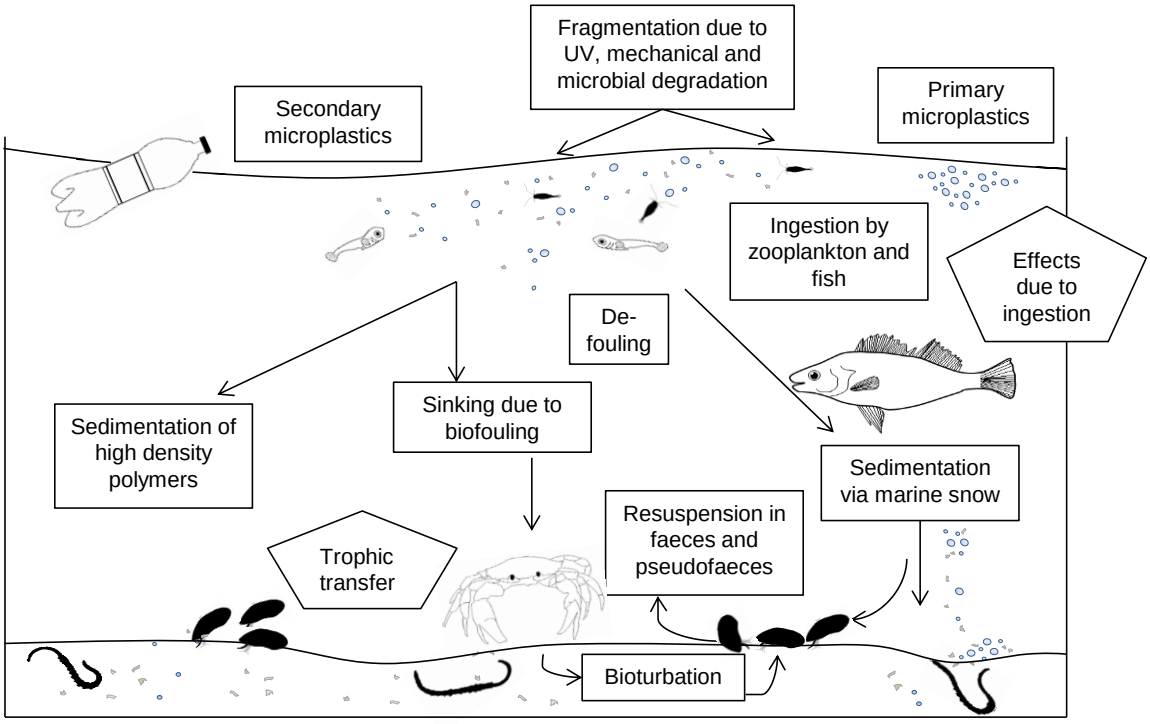
Mesozooplankton e.g. echinoderm larvae, calanoid copepods, chaetognaths	Low density microplastics present on the sea surface with greatest abundances in gyres and industrial harbours; size-based selectivity indicates potential to ingest microplastics of appropriate size.
Benthic suspension feeders e.g. the bivalve *Mytilus edulis*	Susceptible to sinking microplastics; have been found to ingest microplastics despite low qualitative value.

Table 3. Areas for future research.

The destination of ingested microplastics within marine invertebrates in addition to potential adverse effects remains unknown, emphasising a need for laboratory studies focussing on the physical impacts of microplastics
Given the occurrence of different shapes and plastic types in the marine environment, research into the impacts of these factors on marine organisms is should be conducted
The bioavailability of constituent contaminants is undetermined. This highlights a requirement for further laboratory studies to establish the effects of ageing on the concentration of microplastic additives, their bioavailability and the associated toxicological impacts.
The role of microplastics as a vector for environmental POPs is uncertain. Laboratory studies investigating the bioavailability and associated toxicological impacts of microplastic-associated POPs are required
There are presently no conclusive reports on the transfer of microplastics to higher trophic levels and whether they act as a vector for contaminants. Studies are needed to understand the capacity for microplastics and their associated contaminants to be transported along marine food webs via trophic interactions as well as an estimation of population and ecosystem level impacts.

1.8 Figure

Figure 1. Potential pathways for the transport of microplastics and its biological interactions.



Chapter 2

Microplastic ingestion decreases energy reserves in marine worms.

C*urrent Biology*. 23 (23), pR1031–R1033

Microplastic ingestion decreases energy reserves in marine worms

Stephanie L. Wright[1], Darren Rowe[1], Richard C. Thompson[2], and Tamara S. Galloway[1],*

[1]Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter, Devon EX4 4QD, UK.

[2]School of Marine Science and Engineering, University of Plymouth, Plymouth, Devon PL4 8AA, UK.

*E-mail: T.S.Galloway@exeter.ac.uk

This manuscript has been modified to conform to the format of this thesis.

2.1 Correspondence

The indiscriminate disposal of plastic to the environment is of concern. Microscopic plastic litter (<5 mm diameter; 'microplastic') is increasing in abundance in the marine environment, originating from the fragmentation of plastic items and from industry and personal-care products. On highly impacted beaches, microplastic concentrations (<1mm) can reach 3% by weight, presenting a global conservation issue (Carson et al., 2011). Microplastics are a novel substrate for the adherence of hydrophobic contaminants (Wright et al., 2013b), deposition of eggs (Goldstein et al., 2012), and colonization by unique bacterial assemblages (Zettler et al., 2013). Ingestion by indiscriminate deposit- feeders has been reported, yet physical impacts remain understudied (Wright et al., 2013b). Here, we show that deposit- feeding marine worms maintained in sediments spiked with microscopic unplasticised polyvinylchloride (UPVC) at concentrations overlapping those in the environment had significantly depleted energy reserves by up to 50% (Figure 1). Our results suggest that depleted energy reserves arise from a combination of reduced feeding activity, longer gut residence times of ingested material and inflammation.

Seabeds worldwide are composed of a range of organic and inorganic sediments that sustain a vast range of marine species. The polychaete worm *Arenicola marina* (lugworm) of the globally distributed family Arenicolidae is a keystone species inhabiting intertidal sediments in Northern Europe; it bioturbates and irrigates the sediment and is an important secondary producer, as a prey species for fish and wading birds. Using a laboratory mesocosm, we performed chronic (four weeks) and short-term (48 hours) experiments, exposing *A. marina* to natural sediments containing clean, chemically-inert UPVC ranging from 0 – 5% by weight. PVC is denser than seawater and sinks out of suspension to sediments; >25% of microplastics sampled from estuarine sediments inhabited by *A. marina* were PVC (Browne et al., 2010). Thus, we selected UPVC, mimicking the size and shape of sediment (130 µm mean diameter; Figure 1E). We assessed chronic effects on feeding activity, immunity and energy reserves and made short-term observations on gut residence times.

Worms chronically exposed to 5% UPVC by weight displayed significantly reduced feeding activity compared to control and 1% UPVC-exposed worms (Figure 1A), supporting recent findings whereby 7.4% polystyrene by weight inhibited feeding

activity in *A. marina* over 10 days (Besseling et al., 2012). Reduced feeding activity implies that either fewer particles are ingested overall or a lack of a protein coating on the clean UPVC weakens particle adhesion to the worm's feeding apparatus, reducing uptake efficiency. Suppressed feeding activity may decrease energy assimilation, compromising fitness. It could also decrease bioturbation and therefore oxygenation of the sediment, which is crucial for maintaining infaunal diversity.

Chronic UPVC exposure significantly increased the phagocytic activity of *A. marina's* immune cells, although this was not dose-dependent (Figure 1C). Enhanced phagocytic activity is indicative of an inflammatory response, which is a metabolically demanding process. Interestingly, the UPVC powder is classified as an irritant to human health following dermal contact.

The total available energy reserves in worms chronically exposed to 1% and 5% UPVC by weight were significantly reduced compared to pre- exposure and control animals. Worms exposed to 5% UPVC by weight had approximately 50% less total available energy reserves compared to controls (Figure 1D) and all UPVC-exposed animals had significantly lower lipid reserves than controls (Supplemental information). Jonker et al. (2009) found lipid reserves declined in a freshwater oligochaete worm (*Lumbriculus variegatus*) following chronic exposure to powdered activated carbon, presumably due to reduced feeding activity. In our experiments, depleted energy reserves, which closely followed the trend for lipid reserves, could compromise somatic maintenance and growth, maturity and reproduction.

We determined the time it took ingested material to traverse the gut and found intervals between egestion events were 1.5 times longer (mean 1086 seconds) in animals exposed to 5% UPVC by weight, with an average of 0.33 ± 1 (median ± range) hourly egestion events compared to control worms (1.33 ± 2.33 (median ± range); Figure 1B; Supplemental information). *A. marina's* digestion is characterised by material continuously entering and exiting the digestive tract, with negligible mixing during passage. Prolonged gut residence times imply that microplastics, which are of low nutritional value, are being retained and subjected to extensive digestion, at an energetic cost.

Polychaete worms exhibit positive correlations between organic content and feeding activity (Hymel and Plante, 2000). We therefore tested the hypothesis that UPVC reduced the organic content of the sediment to a level at which food concentration becomes a limiting factor. When *A. marina* was exposed to natural sediment of varying organic content — altered by adding clean silica sand — there was no significant reduction in feeding activity (Supplemental information). This suggests that the observed reduction in feeding activity of 5% UPVC- exposed worms is likely attributed to a characteristic of the UPVC and not the secondary effect of decreased food concentration.

At a density of 85 individuals per m^2, which is typical of a tidal flat habitat, *A. marina* is estimated to process 400 cm^3 of sediment annually (Cadée, 1976). Microplastic debris (<1 mm) comprising 3.17% by weight of the sediment has been reported, which when adjusted for density could represent up to 6.34% of the sediment volume at contamination hotspots (Carson et al., 2011). Using the Wadden Sea, where *A. marina* is a keystone species, as an example, if contamination accumulated to such levels *in situ*, *A. marina could* consume up to 33 m^3 of microplastics annually. We found overall feeding activity reduced by approximately 25% in worms exposed to 5% UPVC by weight for a month. Using the Wadden Sea example, this would result in 130 m^3 less sediment being reworked annually. Our current observations indicate that 1% microplastics by weight can reduce total energy reserves by approximately 30%, mainly linked to a reduction in lipid reserves. We propose a conceptual model (Figure 1F) whereby high concentrations of microplastics could induce suppressed feeding activity, prolonged gut residence times, inflammation and reduced energy reserves, impacting on growth, reproduction and ultimately survival. We have shown that microplastics can cause physical harm to an important marine species, emphasizing the need to reconsider how discarded PVC, polystyrene, polyurethane and polycarbonate (30% of global production) are classified in terms of hazard (Rochman et al., 2013a).

Supplemental Information

Supplemental Information including experimental procedures and two figures can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.10.068.

Acknowledgments

This work was funded by the Department for Environment, Food & Rural Affairs; 1-SW-P- N21-000-031-DN-A1-05102. We thank Peter Splatt for SEM imaging assistance, Professor Stuart Bearhop for invaluable comments on the manuscript and Dr. Adil Bakir for UPVC chemistry analyses.

2.1.1 Figure 1.

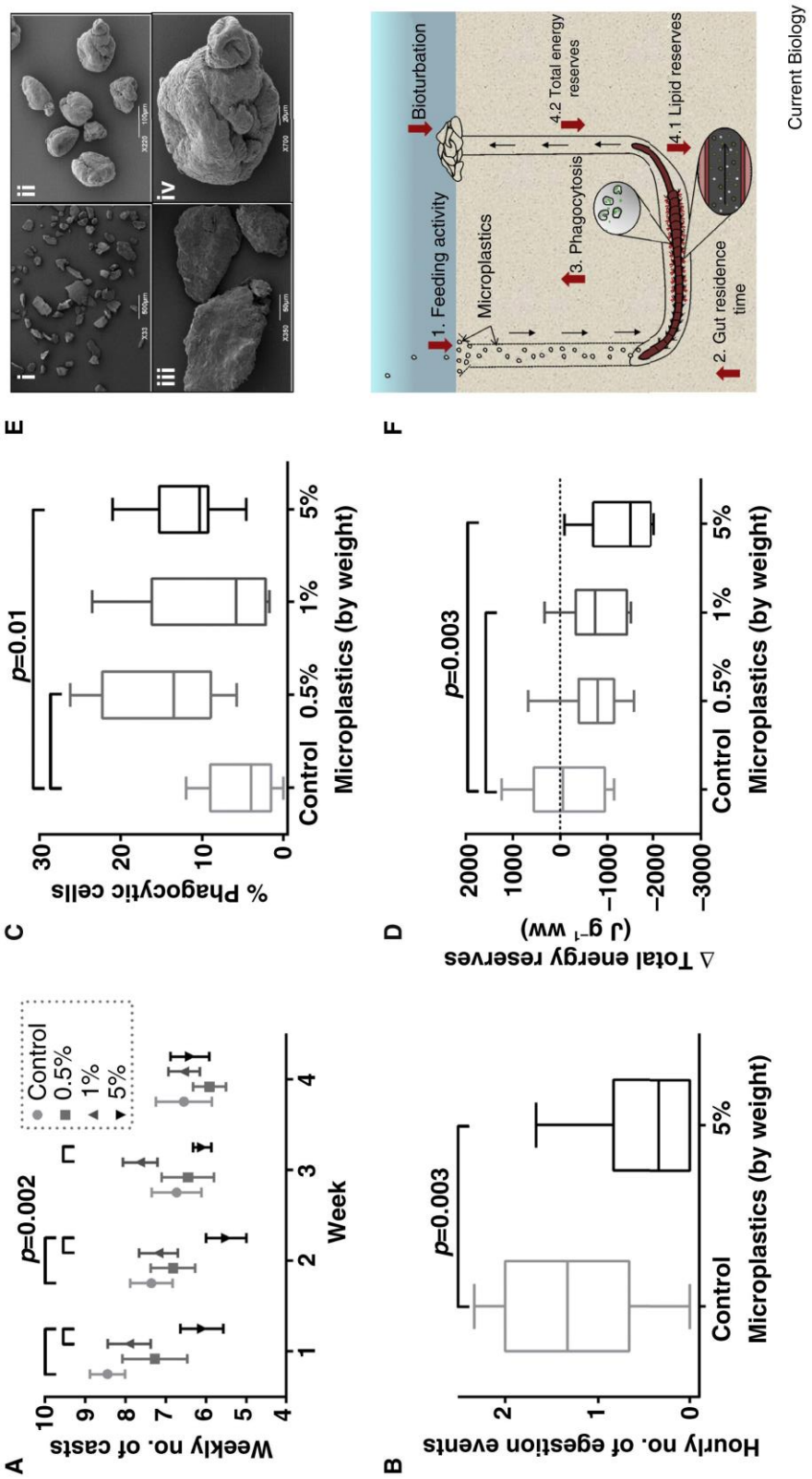

A
Weekly no. of casts
Week
Control
0.5%
1%
5%
p=0.002
B
Hourly no. of egestion events
Microplastics (by weight)
Control
5%
p=0.003
C
% Phagocytic cells
Microplastics (by weight)
Control
0.5%
1%
5%
p=0.01
D
Δ Total energy reserves (J g⁻¹ ww)
Microplastics (by weight)
Control
0.5%
1%
5%
p=0.003
E
i
ii
iii
iv
F
1. Feeding activity
Bioturbation
Microplastics
3. Phagocytosis
4.2 Total energy reserves
4.1 Lipid reserves
2. Gut residence time
Current Biology

Figure 1. The impacts of microscopic UPVC on *A. marina*. (A) The effects of UPVC exposure on weekly feeding activity (Generalised Estimating Equation (GEE); p = 0.002 for 'time*treatment'). Data are presented as weekly average (mean ± SE) per worm. (B) The average (median ± range) hourly number of egestion events following 48 h exposure (Mann Whitney U test, p = 0.003). (C) Effects of UPVC exposure on phagocytosis (one-way ANOVA, p = 0.01), which was enhanced for 0.5% and 5% exposed worms (Fisher's LSD test, p = 0.002 and p = 0.013 for 0.5% and 5%, respectively). (D) The effects of UPVC exposure on total available energy reserves in *A. marina*. Data presented as average (median ± range) compared with pre-exposure baseline (dotted line) (one way ANOVA, p=0.003). For 1% and 5% exposed worms, p = 0.036 and 0.001, respectively (Fisher's LSD test). (E) Scanning electron micrographs of i) natural sediment (x33, scale bar 500 µm); ii) UPVC (x220, scale bar 100 µm); iii) natural sediment (x350, scale bar 50 µm); iv) UPVC (x700, scale bar 20 µm). (F) A conceptual model of the effects of microscopic UPVC on *A. marina*: 1) suppressed feeding activity; 2) prolonged gut residence times; 3) inflammation; and 4) reduced lipid and total available energy reserves. Horizontal bars indicate a significant difference at the 0.05 confidence level. Data for the following can be found in Supplemental Information: statistical output; impacts on average feeding activity, cumulative number of casts, feeding status and egestion time; feeding activity in reduced food concentration; grain size distribution of natural sediment and UPVC; differences in weight (pre- and post-exposure); impacts on lipid, protein and sugar reserves.

2.2 Supplemental Information:

2.2.1 Supplemental Results

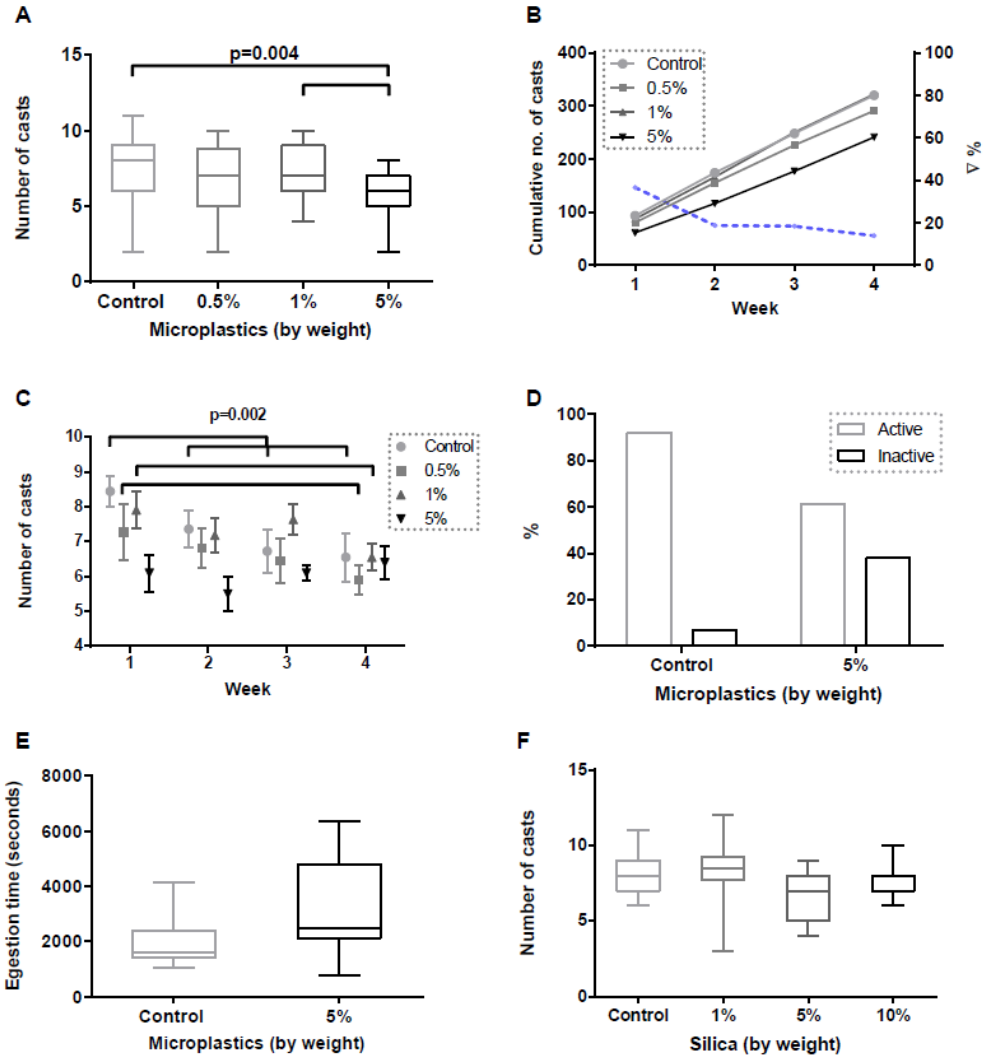


Figure S1. The impacts of microscopic UPVC on the feeding of *A. marina*. A) The effect of chronic UPVC exposure on feeding activity per worm per week. Data is presented as median ± range. (Generalised Estimating Equation (GEE); 'treatment', p=0.004). B) The effects of chronic UPVC exposure on the cumulative number of casts produced. The percentage difference between control and 5% exposed animals is indicated by the blue line on the right y axis. C) The effects of chronic exposure under laboratory conditions on the feeding activity of *A. marina*. Data is presented as mean ± SE (GEE, p=0.002). D) The feeding status of worms following 48 h exposure. Data is presented as a percentage of the sample population (n=13 per treatment). E) The average (median ±

range) egestion time following 48 h exposure. F) The effect of food concentration on the feeding activity of *A. marina* following a 7 day exposure.

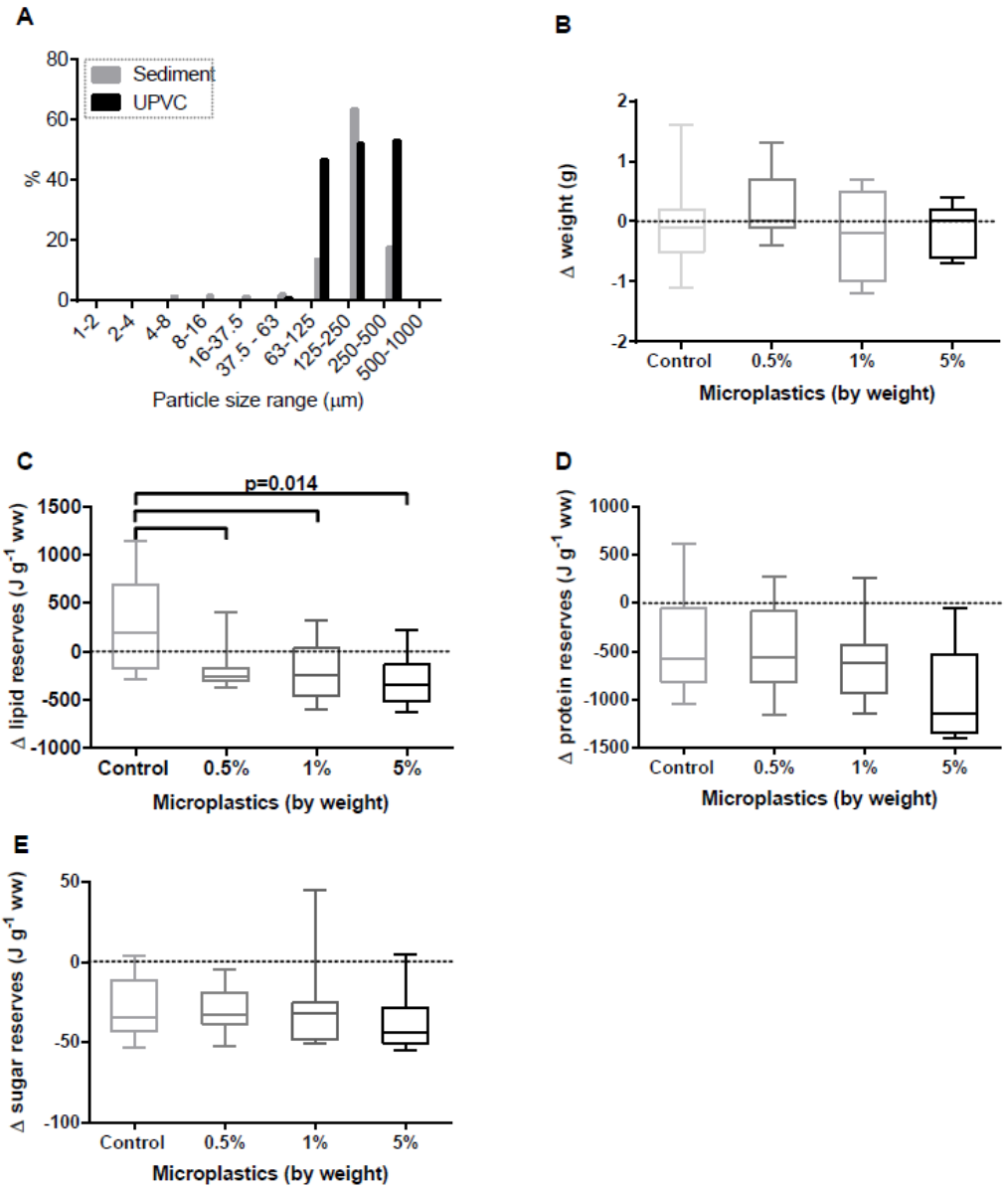


Figure S2. Microscopic UPVC and its impacts on *A. marina*. A) The grain size distribution of natural coastal sediment (Mothecombe, UK) and microscopic UPVC powder. B) The average (median ± range) difference between pre- and post-chronic-exposure weights for *A. marina*. C) The effects of chronic UPVC exposure on lipid reserves in *A. marina*. Data is presented as the average (median ± range) respective to a pre-exposure baseline (dotted line). All exposed worms had significantly reduced lipid reserves compared to controls (Kruskal Wallis, p=0.014, confirmed by Mann

Whitney U tests). D) The effects of chronic UPVC exposure on protein reserves in *A. marina*. Data is presented as the average (median ± range) respective to a pre-exposure baseline (dotted line). E) The effects of chronic UPVC exposure on sugar reserves in *A. marina*. Data is presented as the average (median ± range) respective to a pre-exposure baseline (dotted line).

Generalised Estimating Equation Output

Table S1. Empirical and estimated means and standard errors for weekly feeding activity.

Week	Treatment	Mean	Standard Error	Estimated Mean	Estimated Standard Error	Interval	
						Lower	Upper
1	Control	8.45	0.15	8.45	0.41	5.57	7.35
	0.5%	7.27	0.19	7.27	0.77	5.86	7.32
	1%	7.91	0.19	7.91	0.51	5.18	6.74
	5%	6.10	0.18	6.10	0.50	5.37	7.98
2	Control	7.36	0.18	7.36	0.50	5.68	6.55
	0.5%	6.82	0.15	6.82	0.53	6.87	8.49
	1%	7.18	0.17	7.18	0.46	5.34	7.80
	5%	5.50	0.18	5.50	0.47	5.66	7.99
3	Control	6.73	0.18	6.73	0.59	4.64	6.51
	0.5%	6.45	0.17	6.45	0.62	6.33	8.14
	1%	7.67	0.13	7.67	0.41	5.86	7.93
	5%	6.10	0.14	6.10	0.22	6.44	8.42
4	Control	6.55	0.17	6.55	0.66	5.20	7.16
	0.5%	5.91	0.17	5.91	0.40	6.98	8.96
	1%	6.55	0.16	6.55	0.37	5.91	8.96
	5%	6.40	0.16	6.40	0.45	7.68	9.31

Dependent Variable: Casting

Table S2. GEE parameter estimates for the effect of treatment on feeding activity. Parameters are respective to a baseline coded for by treatment and week. Only significant data are presented.

Parameter Estimates											
Baseline	**Parameter**	**B**	**SE**	**95% Wald Confidence Interval**		**Hypothesis Test**			**Exp(B)**	**95% Wald Confidence Interval for Exp(B)**	
				Lower	**Upper**	**Wald Chi-Square**	**df**	**Sig.**		**Lower**	**Upper**
Control W1	(Intercept)	2.00	0.07	1.86	2.13	856.22	1.00	0.000	7.36	6.44	8.42
	5%	-0.33	0.10	-0.51	-0.14	11.72	1.00	0.001	0.72	0.60	0.87
Control W2	(Intercept)	2.00	0.07	1.86	2.13	856.22	1.00	0.000	7.36	6.44	8.42
	5%	-0.29	0.11	-0.51	-0.08	7.04	1.00	0.008	0.75	0.60	0.93
1% W1	(Intercept)	2.07	0.06	1.94	2.19	1047.27	1.00	0.000	7.91	6.98	8.96
	5%	-0.26	0.10	-0.46	-0.06	6.26	1.00	0.012	0.77	0.63	0.95
1% W2	(Intercept)	1.97	0.06	1.85	2.10	946.27	1.00	0.000	7.18	6.33	8.14
	5%	-0.27	0.11	-0.48	-0.06	6.17	1.00	0.013	0.77	0.62	0.95
1% W3	(Intercept)	2.03	0.05	1.93	2.14	1419.33	1.00	0.000	7.64	6.87	8.49
	5%	-0.22	0.07	-0.35	-0.10	11.93	1.00	0.001	0.80	0.70	0.91

Dependent Variable: Casting

Table S3. GEE parameter estimates for the effect of duration under laboratory conditions on feeding activity. Parameters are respective to a baseline, coded for by treatment and week. Only significant data are presented.

Parameter Estimates											
Baseline	**Parameter**	**B**	**SE**	**95% Wald Confidence Interval**		**Hypothesis Test**			**Exp(B)**	**95% Wald Confidence Interval for Exp(B)**	
				Lower	**Upper**	**Wald Chi-Square**	**df**	**Sig.**		**Lower**	**Upper**
Control W1	(Intercept)	2.13	0.05	2.04	2.23	1901.51	1.00	0.00	8.45	7.68	9.31
	Control W2	-0.26	0.09	-0.44	-0.07	7.61	1.00	0.01	0.77	0.65	0.93
	Control W3	-0.23	0.10	-0.42	-0.04	5.73	1.00	0.02	0.80	0.66	0.96
	Control W4	-0.14	0.07	-0.28	0.00	3.91	1.00	0.05	0.87	0.76	1.00
0.5% W1	(Intercept)	1.98	0.11	1.78	2.19	349.05	1.00	0.00	7.27	5.91	8.96
	0.5% W4	-0.21	0.08	-0.37	-0.05	6.34	1.00	0.01	0.81	0.69	0.96
1% W1	(Intercept)	2.07	0.06	1.94	2.19	1047.27	1.00	0.00	7.91	6.98	8.96
	1% W4	-0.19	0.09	-0.36	-0.02	4.61	1.00	0.03	0.83	0.70	0.98

Dependent Variable: Casting

2.2.2 Supplemental Experimental Procedures

2.2.2.1 Microplastics

UPVC powder (mean particle size 230 µm, 1.4 g cm^3) was purchased from Goodfellow Cambridge Ltd. (UK). Further analysis using static light scattering (Mastersizer 2000, Malvern Instruments Ltd, UK) found the greatest proportion of particles to be 125 – 149 µm in size, with a mean size of 130 µm. According to the suppliers, the UPVC is a strong, inherently flame retardant thermoplastic with quite good chemical and ultra violet resistance and good barrier properties. It has applications in building products, bottles, film and Long Play records, whilst plasticised grades are widely used as cable insulation. Scanning electron micrographs show the grains to be heterogeneous and granular in form with crevices and irregular surfaces as common features (see scanning electron microscopy below and Fig. 1).

To ensure the UPVC was chemically-inert, it was screened for leaching of trace metals and organic compounds using elemental mapping with energy dispersive X-ray spectrometry and soxhlet extraction coupled with GC-FID (gas chromatography-flame ionization detection), respectively. This indicated the UPVC was non-toxic.

2.2.2.2 Scanning Electron Microscopy

The UPVC powder and marine sediments (sieved to 500 µm) were imaged using a JEOL JSM 6390 LV Scanning Electron Microscope. Briefly, dried samples were deposited onto a carbon tab, producing a monolayer. Sample surfaces were then sputter-coated with gold-palladium to a thickness of approximately 14 nm using a SC510 Sputter Coater (VG Microtech, UK). SEM micrographs were taken using 33x to 700x magnification. For both sediment and UPVC, several grains were deposited onto separate carbon tabs.

2.2.2.3 Animal Husbandry

Animals were collected from Mothecombe beach, England (50˚31’’23 N, -3˚94’’58 W), selecting for immature individuals. Animals were transferred to 16 litre holding tanks in a temperature-controlled room (15˚C) where they were maintained in natural sediment for a minimum of seven days. To void their gut content and mitigate any stress, animals were removed from their holding tanks and maintained in aerated artificial seawater (ASW) approximately 24 h prior to experiments.

2.2.2.4 Preparation of Sediment

Natural sediment (C:N 154.82) collected from Mothecombe, England was used throughout all experiments. The most common grain size was 125 – 250 µm, with a mean percent of 63.29 (±0.01 SE), which overlaps with the mean size class of UPVC particles. Sediment was sieved to 500 µm using ASW and stored at 5˚C until use (within 2 weeks of collection). Each concentration was prepared by weighing out a bulk amount of natural sediment and substituting a known weight for an equivalent amount of UPVC powder, achieving concentrations of 0.5 – 5% UPVC by weight. Natural sediment alone was used as a control (0%) for each experiment. All sediment mixtures and control sediment were homogenised for five minutes using an electric paint mixer.

Replicates were prepared by adding 1 kg of sediment to individual 1 L tall-form glass beakers in a temperature-controlled room (15 ˚C) 48 hours prior to the addition of animals. Beakers were randomly allocated a position to eliminate any possible temperature effects occurring throughout the room. Four hundred mL of ASW was added to each beaker (salinity 34.1 ± 0.2) 24 hours prior to the addition of animals and a glass pipette tip on the end of an airline provided gentle aeration.

2.2.2.5 Chronic exposure

Several animals were immediately snap-frozen as a baseline for energy reserve quantification. Remaining animals were weighed (mean 2.01 g ± 0.1 SE) and transferred to individual beakers containing microplastic concentrations ranging from 0 – 5% by weight (see *Sediment Preparation*), with 11 replicates per treatment. Animals were exposed for 28 days at 15 ˚C with an ambient photoperiod (14 h light: 10 h dark). Laboratory conditions were monitored throughout the 28 day exposure period (see table S4) and conformed to the International Council for the Exploration of the Sea (ICES) guidelines (Thain and Bifield, 2002).

Table S4. Laboratory conditions measured throughout the 28 day exposure period.

Parameter	Mean	±SE
Salinity	34.1	0.2

pH	8.1	0.1
Dissolve oxygen (%)	99.8	0.5
Temperature (˚C)	15	1
Ammonia (mg L^{-1})	0.8	0.2

Feeding rate was monitored daily by recording the number of faecal casts present in each beaker. Once casts were counted, the sediment surface was flattened using a clean, stainless steel spoon.

After 28 days, animals were removed from the exposure and weighed (mean 1.97 g ± 0.1 SE). A sample of coelomic fluid was extracted for use in the phagocytosis assay (see *Phagocytosis*). Briefly, coelomic fluid was withdrawn into a 1 mL syringe containing chilled anticoagulant using a 21G hypodermic needle. Coelomic fluid was stored on ice until use. Following coelomic fluid extraction, animals were snap-frozen in liquid nitrogen and stored at -80 ˚C for energy reserve analysis.

2.2.2.6 Phagocytosis

The phagocytosis of fluorescent zymosan by a coelomocyte monolayer was used to assess immune function. Briefly, coelomic fluid samples were incubated on a poly-L-lysine slide for 15 minutes, after which any excess fluid was gently rinsed away with phosphate-buffered saline (PBS). Coelomocyte monolayers were then incubated with Fluorescein isothiocyanate (FITC)-stained zymosan for one hour, before excess particles were gently rinsed away with PBS. Cells were fixed using Baker's Formol Calcium to stop the phagocytic process and Trypan Blue was used as a quenching agent to eliminate external fluorescence. Slides were mounted with DPX and covered with a coverslip which was sealed with clear nail varnish. The slides were later examined with a fluorescence microscope. From a minimum of 100 cells per slide, phagocytosis – indicated by fluorescence - was quantified and expressed as a percentage.

2.2.2.7 Energy Reserves

Energy reserves (carbohydrate, protein and lipids) were biochemically quantified following the methods of DeCoen and Janssen (1997). Snap-frozen animals were homogenised (diluted 20 times (w/v)) in a pestle and mortar with homogenisation buffer (0.1 M Tris-HCl buffer, pH 7.5, 0.4 M MgSO4, 15% (w/v) polyvinylpyrrolidone and 0.2% (v/v) Triton X-100). 200 µl aliquots of whole-body homogenate were then stored at -80 ˚C until use.

Total carbohydrates were measured by washing the sample in 15% and 5% trichloroacetic acid. 5% phenol and concentrated sulphuric acid were then added to the combined supernatant and absorbance was read at 492 nm (make/model), with glucose as a standard. Whole-body protein content was quantified with Bio-Rad reagent (Bio-Rad Laboratories, UK). Absorbance was measured at 592 nm, with bovine serum albumin (Sigma, UK) as a standard. Total lipid content was analysed following the method of Bligh and Dyer (1959). Lipids were extracted in chloroform/methanol then charred with sulphuric acid at 200 ˚C. Absorbance of charred samples was then measured at 340 nm, using tripalmitin (Sigma, UK) as a standard.

Energy equivalents were calculated based on Gnaiger's enthalpies of combustion: protein 24,000 J g^{-1}, carbohydrates 17,500 J g^{-1} and lipids 39,000 J g^{-1} (Gnaiger, 1983). The sum of these reserves provided the total available energy for each worm.

2.2.2.8 Gut Residence Time

Animals were weighed (mean 3.8 g ± 0.2 SE) and transferred to individual beakers containing either 0% or 5% UPVC by weight (see Sediment Preparation), with 13 replicates per treatment. Animals were left in exposure conditions at 15 ˚C with an ambient photoperiod (14 h light: 10 h dark) for 48 hours to ensure active feeding began. Laboratory conditions conformed to the International Council for the Exploration of the Sea (ICES) guidelines (Thain and Bifield, 2002).

After 48 hours, observations were made over three hours, recording the number and time of egestion events. Once a cast was produced it was recorded and immediately flattened. To standardize for 'fullness', the first egestion event from each individual was considered its 'time 0'. This ensured all individuals were in the same state of 'fullness' i.e. rectum had just been voided.

Following this, the time of every egestion event was recorded throughout the 3 hour period. An average egestion time for each individual was calculated, representing the average interval between rectum voidance. The frequency of egestion events was expressed as number per hour. Furthermore, the number of active and inactive feeders was quantified for each treatment.

2.2.2.9 Food Concentration

Substituting UPVC for clean silica, animals were weighed (mean 3.9 g ± 0.2 SE) and transferred to individual beakers containing either 0, 1, 5, or 10% silica by weight (see Sediment Preparation), with 11 replicates per treatment. Animals were exposed for 7 days at 15 ˚C with an ambient photoperiod (14 h light: 10 h dark). Laboratory conditions were monitored throughout the 7 day exposure period and conformed to ICES guidelines (Thain and Bifield, 2002).

Feeding rate was monitored daily by recording the number of faecal casts present in each beaker. Once casts were counted, the sediment surface was flattened using a clean, stainless steel spoon.

2.2.2.10 Statistical Analysis

Statistical analyses were carried out in SPSS Statistics 20 (IBM). For all continuous data, normality and equality of variances were tested using a Shapiro-Wilk test and Levene's Test of Homogeneity of Variance, respectively. Where necessary, data was transformed using a Log10 (x+1) transformation to conform to the assumptions of analysis of variance. For phagocytosis data, percentage was first converted into proportions, before being transformed to a continuous form using an arcsine square-root transformation. A one-way ANOVA was applied to determine the effect of UPVC on energy reserves and phagocytosis. If data did not conform to the assumptions of the test, a non-parametric Kruskal Wallis test was used. Where significant effects were found, Fisher's Least Significant Difference (LSD) test or a Mann Whitney U test was performed, indicating the specific differences, with significance accepted for $p<0.05$.

A Generalised Estimating Equation (GEE) was used to determine the effect of UPVC on feeding activity (number of casts excreted over time). This is because the average treatment effect of UPVC is of interest, regardless of change over time at the individual level (Hu et al., 1998). As the data were collected longitudinally, the responses within

individuals over time are expected to be correlated with each other. Therefore both autoregressive and unstructured working correlation structures were tested. Both structures yielded the same Pan's quasilikelihood under the independence model information criterion (QIC) statistic (QIC=90.268), suggesting they were an equally good fit. Since the unstructured model is considered less restrictive with regards to modelling the true correlation structure within individuals (Ballinger, 2004), it was employed in the final model. The model information, QIC and model effects output are summarised in table S5, S6 and S7, respectively.

Table S5. Model information for the Generalised Estimating Equation.

Dependent Variable	Number of casts
Probability Distribution	Poisson
Link Function	Log
Subject Effect	Individual
Within-Subject Effect	Week
Working Correlation Matrix Structure	Unstructured

Table S6. Pan's quasilikelihood under the independence model information criterion (QIC) statistics.

	Value
Quasi Likelihood under Independence Model Criterion (QIC)[b]	90.268
Corrected Quasi Likelihood under Independence Model Criterion (QICC)[b]	109.118

Dependent variable: Casting

Model: (Intercept), Week, Treatment, Week*Treatment

Information criteria are in small-is-better form.

Computed using the full log quasi-likelihood function.

Table S7. Model effects output.

Source	Type III		
	Wald Chi-Square	Df	Sig.
(Intercept)	5258.742	1	.000
Week	15.253	3	.002
Treatment	13.217	3	.004
Week*Treatment	26.607	9	.002

For egestion time, any individuals which did not cast during the 3 hour observation period were removed from the data set, as a value of 0 would imply rapid/continuous egestion times. A Mann Whitney U test was performed to determine the effect of 5% UPVC by weight on both egestion time and hourly egestion rate data, as both data sets were continuous but did not conform to normal distribution.

A generalised linear model, stating a Poisson probability distribution with a log link function was applied to food concentration data, as results were expressed as number of casts per week i.e. number per unit of time (see tables S8 and S9).

Table S8. Goodness of Fit for generalised linear model.

	Value	df	Value/df
Deviance	15.593	39	.400
Scaled Deviance	15.593	39	
Pearson Chi-Square	14.590	39	.374
Scaled Pearson Chi-Square	14.590	39	
[b]	-90.999		
Akaike's Information Criterion (AIC)	189.998		
Finite Sample Corrected AIC (AICC)	191.051		
Bayesian Information Criterion (BIC)	197.043		
Consistent AIC (CAIC)	197.043		

Dependent Variable: Casts

Model: (Intercept), Treatment

[a]Information criterion are in small-is-better form.

[b]The full log likelihood function is displayed and used in computing information criterion.

Table S9. Test of model effects for generalised linear model.

		Type III	
Source	Wald Chi-Square	df	Sig.
(Intercept)	1364.716	1	.000
Treatment	1.711	3	.634

Dependent Variable: Casts

Model: (Intercept), Treatment

2.2.2.11 Sediment Reworking Calculations

We calculated sediment reworking based on the total area of tidal flats in the Wadden Sea (Beukema, 1976).

I.7.1.1 The Potential Impacts of Size

The potential main and interactive effects of the size (weight) of worms on the reported dependent variables were analysed where the variable did not already account for the weight of the worm, such as with energy reserves.

Following 28 d exposure to UPVC, there was no significant main or interactive effect of of weight on the overall feeding activity of individuals (two way ANOVA, p=0.698 and p=0.091 for weight and weight*treatment, respectively) or on the proportion of phagocytically active cells of individuals (two way ANOVA, p=0.715 and p=0.340 for weight and weight*treatment, respectively). There was no significant main or interactive effect of weight on gut residence time following exposure to 5% PVC (two way ANOVA, p=0.910 and p=0.539 for weight and weight*treatment, respectively) or on the frequency of egestion events (two way ANOVA, p=0.527 and p=0.298 for weight and weight*treatment, respectively).

Following 7 d exposure to a reduced organic content (silica), there was no significant main effect of weight on feeding activity (generalised linear model, p=0.2437); however there was an interactive effect whereby weight significantly affected the feeding activity of individuals exposed to 1% silica by weight. Larger worms produced

more casts in this treatment group. This may be due to larger worms having a proportionately greater gut volume and therefore the capacity to egest more sediment. However, as this is only in the 1% UPVC by weight treatment group, it is probable that in this instance, this has occurred by chance.

Chapter 3

Microplastics transfer endogenous phthalates to marine worms

This manuscript is in preparation for publication

Microplastics transfer endogenous phthalates to marine worms

Wright, S. L.[1], Rowe, D.[1], Bakir, A.[2], Rowland, S.[2], Reid, M.[3], Thomas, K.[3], Thompson, R.[2], and Galloway, T. S[1*].

[1]Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter, Devon EX4 4QD, UK.

[2]School of Marine Science and Engineering, University of Plymouth, Plymouth, Devon PL4 8AA, UK.

[3]Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, N-0349 Oslo, Norway

[*]T.S.Galloway@exeter.ac.uk

This manuscript is in preparation for publication.

3.1 Abstract

The contamination of the environment by discarded plastic debris is a global health issue. Plastics can break down to microscopic fragments, enhancing the potential for entry of plastics and their endogenous additives into aquatic food webs. Many plastic additives are endocrine disrupting chemicals (EDCs) capable of causing reproductive, developmental, metabolic, and immunological disruption. Here, we show that marine worms (*Arenicola marina*, lugworm) maintained in sediments containing plasticised microscopic polyvinylchloride (PVC with 50% w/w bis(2-ethylhexyl) phthalate) accumulated phthalates in their tissues at concentrations capable of inducing adverse biological effects. Lugworms exposed to plasticised microplastics accumulated >70% more phthalates, coinciding with inhibited mucus production and enhanced lipid reserves and oxygen consumption (>30%), in comparison to lugworms exposed to unplasticised microplastics. These results suggest mechanisms of phthalate toxicity similar to mammalian systems and highlight a potential entry route for plastics and associated EDCs into aquatic food webs.

Keywords: Microplastics, plasticisers, DEHP, additives, worms

3.2 Introduction

Global plastic production has reached 300 million tonnes a^{-1}. Over a third of the plastic produced is incorporated into single-use packaging, which is typically discarded within a year (Thompson et al., 2009). This 'throw-away' culture, combining extreme consumption with rapid disposal of plastic via landfill, littering, and sewer systems, has led to an accumulation of plastic in the marine environment. Plastic debris presents widespread environmental, economic, and human health impacts; the rising world population, anticipated to reach 9 billion by 2050, is only likely to exacerbate these problems.

Once released into the marine environment, mechanical, photo and biological degradation of plastic occurs (Andrady, 2011), resulting in the exponential fragmentation of microscopic plastic particles (micro- or nanoplastics). Municipal waste streams also provide a source of microplastics, due to their occurrence as microbeads and/or -granules in personal-care products, or due to the release of textile microfibres in washing machine effluent (Browne et al., 2011). Collectively, these microplastics are emerging as a ubiquitous form of marine debris, polluting marine ecosystems from the poles (Obbard et al., 2014) to the equator (Ivar do Sul et al., 2013). In addition to the accumulation of buoyant microplastics at the sea surface (Song et al., 2014) and on coastlines, high, localised concentrations on and within the seabed have been reported, including even the deep ocean floor (Woodall et al., 20014). This is because over half of plastic polymers are denser than seawater and thus liable to sinking (http://www.dotmar.com.au/density.html). Microplastic density also increases with biofouling (Lobelle and Cunliffe, 2011). On impacted beaches in Hawaii and Lanzarote, plastic concentrations up to 3% (<1 mm) and 10% (<5 mm) by weight of the sediment, respectively, have been reported (Baztan et al., 2014; Carson et al., 2011).

Microplastics cause concern because their ubiquitous presence and small size make them vulnerable to ingestion by a wide range of aquatic and terrestrial organisms, with the potential for adverse health effects. Laboratory and field-based studies report microplastic ingestion by many organisms inhabiting the ocean floor, including invertebrates that ingest detritus or deposit feed with little discrimination e.g. sea cucumbers (0.25 mm to 15 mm) (Graham and Thompson, 2009), sand hoppers (≥25

µm) (Ugolini et al., 2013), and polychaete worms (20 – 2000 µm) (Besseling et al., 2012; Thompson et al., 2004). Once ingested, the physical presence of non-digestible plastic in the diet has the capacity to disrupt feeding activity (Besseling et al., 2012; Wright et al., 2013a), compromise energy storage (Wright et al., 2013a), translocate to tissues (Browne et al., 2008), and induce localised inflammatory responses (von Moos et al., 2012). In addition, microplastics have the potential to cause chemical toxicity. Persistent organic pollutants in seawater readily accumulate on the surface of microplastics (Hirai et al., 2011) and may be released to tissues upon ingestion (Browne et al., 2013; Rochman et al., 2013c). Additionally, chemical additives (e.g. plasticisers, flame retardants, and antimicrobials) are incorporated into plastics during manufacture, to inhibit photo-degradation; to improve strength, rigidity, or flexibility; and to prevent microbial growth. Additives are not covalently bound but dispersed throughout the plastic polymer matrix, and are therefore susceptible to leaching to the external medium. The small molecular size (MW <1000) of these chemicals allows them to penetrate cells and interact with biologically important molecules (Teuten et al., 2009).

Currently, no laboratory studies have explored microplastics as a pathway for endogenous chemical additives to transfer to animal tissue. Polyvinylchloride (PVC) has one of the greatest global production volumes. Its density is greater than seawater and the occurrence of microscopic PVC particles in marine sediments has been reported (Browne et al., 2010; Vianello et al.). PVC contains more additives than any other plastic type; the predominant additives incorporated being phthalates, which increase the flexibility of PVC by reducing the affinity between the molecules of the polymer chains (Teuten et al., 2009). Some PVC medical devices may contain up to 80% by weight of the plasticiser bis(2-ethylhexyl) phthalate (DEHP) (Tickner, 1999).

Phthalates are an important family of chemicals because they are linked to adverse health effects. Experimental studies have identified phthalates as endocrine disruptors, causing reproductive and developmental effects. Testosterone production was significantly inhibited in adult human testis explants and adrenocortical human cells cultured in DEHP or its metabolite mono-(2-ethylhexyl) phthalate (MEHP) (Desdoits-Lethimonier et al., 2012). Urinary concentrations of phthalate metabolites in men are inversely associated with serum testosterone concentrations (Meeker et al.,

2009) and significantly linked to poor semen quality and sperm immotility (Jurewicz et al., 2013), further highlighting the adverse reproductive effects of phthalate exposure. Additionally, phthalate exposure is negatively associated with thyroid hormone levels in men (Meeker et al., 2007), women (Huang et al., 2007) and children (Boas et al., 2010). Phthalates are established peroxisome proliferators, an effect consistent across a range of species. In organisms as diverse as rodents, clams, and crustaceans, exposure to DEHP alters glucose metabolic efficiency and energy use (Bernal et al., 2002; Knowles et al., 1987; Li et al., 2014). The binding to and activation of peroxisome proliferator activated receptors by MEHP leads to increased adipogenesis in rodents (Feige et al., 2007). Consequently, phthalates are also considered obesogenic. Phthalates can also interfere with inflammatory pathways. The up-regulation of nuclear transcription following the activation of peroxisome proliferator-activated receptors has the capacity to trigger a pro-inflammatory reaction. Subsequently, a causal link between the phthalate DEHP and systemic inflammatory response syndrome has been proposed (Latini et al., 2006). In DEHP-exposed ovalbumin-challenged mice, mucus production and inflammatory cell/interleukin counts were significantly reduced in conjunction with Th2 cytokines (Shin et al., 2014). Additionally, potassium intermediate/small conductance calcium-activated channel domains were identified as genetic targets of DEHP in rats (Kondo et al., 2012); the blockade of calcium-activated chloride channels has also been linked to inhibited mucus secretion (Kondo et al., 2012). The potential entry of phthalates into the food chain is thus of great concern for both the environment and human health.

The potential for migration of phthalates from the polymer matrix is influenced by many factors including the size and solubility of the phthalate, porosity of the polymer matrix (Teuten et al 2009) and nature of the surrounding medium; for example, leaching of high molecular weight lipophilic phthalates, such as DEHP, is likely to increase in the presence of lipids (Mazur et al., 1989). Studies to determine the exposure pathways of humans to phthalates highlight PVC flooring and the consumption of certain foods as risk factors (Larsson et al., 2014); however, the most acute exposures occur in hospital patients. Concentrations of DEHP in leachates from hospital equipment, such as infusion sets, can be up to 3.1×10^6 µg L^{-1} (Bagel et al., 2011) with the amounts of leached DEHP retained in patient's blood increasing in

proportion to their plasma lipid content (cholesterol and triglycerides) (Faouzi et al., 1999). The high levels of DOC and surfactants present in the gastrointestinal fluids of marine invertebrates may further facilitate leaching of residual chemical additives from ingested microplastic (Koelmans et al., 2014). Continuous fragmentation of microplastics by wind, tide and UV light will constantly expose new surfaces, facilitating the migration of phthalates and other additives from the core to the surface of the particle (Teuten et al., 2009).

Currently, no laboratory studies have explored microplastics as a pathway for endogenous chemical additives to transfer to animal tissues. Here we address this knowledge gap with the hypothesis that endogenous additives will be capable of leaching from microplastics to animal tissue at concentrations capable of inducing harm. We test this hypothesis focusing on marine worms (lugworms, *Arenicola marina*), which were maintained in sediments spiked with PVC microplastics containing endogenous phthalates. We measured bioconcentration factors and the effects on key life parameters, including growth, energy assimilation and immune function. We discuss the implications in relation to the marine food web and potential human health risks.

3.3 Materials and Methods

3.3.1 Microplastics

Samples of plastic with known concentrations of incorporated additives were unavailable for trade confidentiality reasons. Therefore, we manufactured our own plasticised PVC with a known concentration of additive (50% bis(2-ethylhexyl) phthalate (DEHP) by weight). This allowed for a controlled amount of additive to be incorporated, enabling the study of release of a single additive as opposed to the complex mixtures which may be present in commercially-produced plastics. Unplasticised polyvinylchloride (PVC) powder (mean particle size 230 µm, 1.4 g cm^3) was purchased from Goodfellow Cambridge Ltd. (UK). To incorporate the chemical additive, 50% by weight of DEHP (Sigma Aldrich UK) was added to the PVC powder and thoroughly homogenised. The material was then prepared using a CRC Clarke Schred press with constant monitoring of the internal temperature of the mould using an

electronic thermometer. This resulted in a flexible PVC disc. An aged treatment was prepared, representing a scenario whereby microplastics in the water column have lost a proportion of their chemical additives to the surrounding seawater before settling to the sediment. Previous work has found a rapid desorption of approximately 30% of additives over the first 24 hours in seawater, after which desorption stabilises. To mimic this loss, plasticised discs were placed in autoclaved, filtered (0.45 µm) seawater on a rotary shaker at 120 rpm for 48 hours. Unplasticised plastic was prepared with no added DEHP as a control. The plastic discs were cut into small pieces and then an electric blender was used to grind them. The particles were sieved to 200 – 250 µm.

3.3.2 Animal Husbandry

Lugworms were collected from Mothecombe beach, England (50˚31''23 N, -3˚94''58 W), selecting for immature individuals (mean 5.7 g ± 0.21). Lugworms were transferred to 16 L holding tanks in a temperature-controlled room (15 ˚C) where they were acclimated in natural sediment for a minimum of 7 days. To void their gut content and mitigate stress, lugworms were removed from their holding tanks and maintained in aerated artificial seawater (ASW) approximately 24 hours prior to experiments.

3.3.3 Preparation of Sediment

Natural sediment collected from Mothecombe beach, England was used throughout all experiments. Previous analysis of the sediment from the same site found the most common grain size to be 125 – 250 µm, with a mean percent of 63.29 (±0.01 SE), which overlaps with the mean size class of PVC particles. Sediment was sieved to 500 µm using ASW and stored at 5 ˚C until use. Sediment was prepared as reported previously (Wright et al., 2013). The final treatments used were 1% unplasticised PVC by weight (control) and 1% PVC + 50% DEHP (by weight of the PVC) by weight of the sediment. The concentration of DEHP was chosen based on up to 80% DEHP by weight of plastic occurring (Tickner, 1999). The concentration of microplastics used, whilst conservative in comparison to highly impacted areas (which have reached up to 10% by weight (Baztan et al., 2014)), is likely to reflect commonly encountered concentrations. All sediment treatments were homogenised for 5 minutes using an electric paint mixer.

Replicates were prepared by adding 1 kg of sediment to individual acid-washed 1 L tall-form glass beakers in a temperature-controlled room (15 ˚C) 48 hours prior to the

addition of animals. Beakers were randomly allocated a position to eliminate any possible temperature effects occurring throughout the room. Four hundred ml of ASW was added to each beaker (salinity 34) 24 hours prior to the addition of lugworms and a glass pipette tip on the end of an airline provided gentle aeration.

3.3.4 Chronic exposure

Lugworms were weighed (5.67g ± 0.21 SEM) and transferred to individual beakers containing the different microplastic treatments (see Sediment Preparation), with 10 replicates per treatment. Lugworms were exposed for 28 days at 15 ˚C with an ambient photoperiod (14 hours light: 10 hours dark). Laboratory conditions were monitored throughout the 28 day exposure period (see table S4) and conformed to the International Council for the Exploration of the Sea (ICES) guidelines (Thain and Bifield, 2002).

Table 1. Laboratory conditions measured throughout the 28 day exposure period.

Parameter	Mean	±SEM
Salinity	34.42	0.05
pH	8.07	0.02
Dissolve oxygen (%)	95.69	0.61
Temperature (˚C)	15	-
Ammonia (mg L^{-1})	0.82	0.10

Feeding rate was monitored daily by recording the number of faecal casts present in each beaker. Once casts were counted, the sediment surface was flattened using a clean, stainless steel spoon. Observations were made for mucus. These appeared as string-like structures protruding from the burrow opening, which were easily removed using clean forceps. After 28 days, lugworms were removed from the exposure and transferred to seawater to void their gut contents overnight.

3.3.5 Oxygen Consumption

Oxygen consumption rates were measured in all lugworms following 28 days exposure to plasticised microplastics. Lugworms were individually transferred to 200 mL of gently-aerated fresh ASW in glass 1 L beakers covered with Parafilm® in a temperature-controlled room (15 ˚C). Lugworms were left undisturbed for 2 hours. The aeration was then stopped and lugworms were left to consume oxygen for 1 hour. A 1 mL sample was taken at the start and end of this incubation period and the oxygen partial pressure was immediately measured via a Strathkelvin Instruments oxygen meter (model 781) and electrode. The oxygen electrode was calibrated before use with fully aerated ASW (100% oxygen saturation) and a saturated sodium sulphite solution (0% oxygen saturation). Following oxygen consumption measurements, lugworms were removed from the seawater. Blank readings were then taken at the start and end of an 18 hour period to account for any potential microbial oxygen consumption. To avoid compensatory responses associated with depleted dissolved oxygen levels, the chamber PO_2 values were always in excess of approximately 16 kPa (~ 120 mmHg), following (Toulmond, 1975).

Lugworms were weighed and a sample of coelomic fluid was extracted for use in the phagocytosis assay (see Phagocytosis). Coelomic fluid was withdrawn into a 1 mL syringe containing chilled anticoagulant at a 1:1 ratio using a 21G hypodermic needle. Coelomic fluid was stored on ice until use. Following coelomic fluid extraction, lugworms were snap-frozen, and stored at -80 ˚C until further use.

3.3.6 Phagocytosis

The phagocytosis of neutral red stained-zymosan by a coelomocyte monolayer was used to assess immune function. Briefly, coelomic fluid samples were incubated on a poly-L-lysine slide for 15 minutes, after which any excess fluid was gently rinsed away with phosphate-buffered saline (PBS). Coelomocyte monolayers were then incubated with neutral red-stained zymosan for one hour, before excess particles were gently rinsed away with PBS. Cells were fixed using Baker's Formol Calcium to stop the phagocytic process. Slides were mounted with DPX and covered with a coverslip which was sealed with clear nail varnish. Slides were carefully stored in the dark before examination under a microscope (40x). From a minimum of 100 cells per slide,

phagocytosis – indicated by internalised zymosan - was quantified and expressed as a percentage, and a phagocytic index was calculated:

3.3.7 Energy Reserves

Energy reserves (carbohydrate, protein and lipids) were biochemically quantified following the methods of DeCoen and Janssen (1997). Snap-frozen animals were homogenised in a pestle and mortar with homogenisation buffer (0.1 M Tris-HCl buffer, pH 7.5, 0.4 M MgSO4, 15% (w/v) polyvinylpyrrolidone and 0.2% (v/v) Triton X-100; diluted 20 times (w/v)). 200 µl aliquots of whole-body homogenate were then stored at -80 ˚C until use.

Total carbohydrates were measured by washing the sample in 15% and 5% trichloroacetic acid. Five per cent phenol and concentrated sulphuric acid were then added to the combined supernatant and absorbance was read at 492 nm (make/model), with glucose as a standard. Whole-body protein content was quantified with Bio-Rad reagent (Bio-Rad Laboratories, UK). Absorbance was measured at 592 nm, with bovine serum albumin (Sigma, UK) as a standard. Total lipid content was analysed following the method of Bligh and Dyer (1959). Lipids were extracted in chloroform/methanol then charred with sulphuric acid at 200 ˚C. Absorbance of charred samples was then measured at 340 nm, using tripalmitin (Sigma, UK) as a standard.

Energy equivalents were calculated based on Gnaiger's (1983) enthalpies of combustion: protein 24,000 J g^{-1}, carbohydrates 17,500 J g^{-1} and lipids 39,000 J g^{-1}. The sum of these reserves provided the total available energy for each lugworm.

3.3.8 Statistical Analyses

Statistical analyses were performed in R. Any change in the weight of lugworms during exposures was assessed using the method of (Crawley, 2005). First, relative growth rate (RGR) was calculated:

$$RGR = \ln \frac{fw}{iw}$$

where fw = final weight and iw= initial weight.

A bioconcentration factor (BCF) – the level of accumulation of a chemical in an organism from the surrounding medium - was calculated. This was quantified using the following calculation:

$$BCF = \frac{CB}{CM}$$

Where CB = biota concentration and CM = medium (leachates or sediment) concentration.

The effect of treatment on RGR, the percent of phagocytic cells (arcsine square root transformed), the phagocytic index of cells, biochemical energy reserves, oxygen consumption, and DEHP bioaccumulation were analysed using pairwise T-tests, comparing the unplasticised microplastics (plastic control) treatment group to aged plasticised microplastics.

To ensure correct specification of the tests used, the distribution of residuals was monitored using the Shapiro Wilks test for normality and Levene's test for homogeneity of variance. Where variances did not conform to homogeneity, a Welch (or Satterthwaite) approximation to the degrees of freedom was applied.

The frequency of individuals which produced mucus during exposure was analysed using Fisher's Exact test on 2x2 contingency tables. The effect of treatment on the amount of mucus a lugworm produced was analysed using a generalised linear model (glm, family = Poisson, link function = log). Over-dispersion was monitored by dividing the residual deviance by the degrees of freedom, and corrected for by including a quasi-poisson over-dispersion parameter in the model.

The effect of treatment on the casting activity of lugworms was analysed using a linear mixed effects analysis of the relationship between casting activity and treatment. Treatment was specified as a fixed effect. Intercepts for individual (lugworm) and week were random effects. Significance was determined using likelihood ratios tests between the full and null model.

3.4 Results

3.4.1 Weight

The Relative Growth Rate (RGR) of lugworms was not significantly affected following exposure to plasticised microplastics (T test, p=0.512). On average, lugworms from both treatment groups had a negative growth rate, indicating weight loss during the exposure period; a likely artefact of being maintained under laboratory conditions (see Figure S1 A).

3.4.2 Feeding rate

Throughout the exposure period, the casting activity of lugworms was not significantly affected by the presence of plasticised microplastics (glm, p=0.374). A trend towards a decrease in the casting activity of lugworms over time was observed in all treatments (see Figure S1 B). This is likely due to the depletion of organic matter whilst maintained under laboratory conditions for a prolonged period.

3.4.3 Energy Allocation

Following 28 day exposure to plasticised microplastics, no significant effects on the biochemical energy reserves of lugworms were observed. Lipid reserves were >30% greater in lugworms following exposure to plasticised microplastics than in lugworms following exposure to unplasticised microplastics (T-test, p=0.261; see Figure 1E). Total energy reserves were 12.5% greater in lugworms following exposure to aged plasticised microplastics than in lugworms exposed to unplasticised microplastics (T-test, p=0.395, see Figure S1 E). Protein and carbohydrate reserves were generally stable across all groups (see Figure S1 C and D).

3.4.4 Oxygen Consumption

Following exposure to plasticised microplastics, no significant effects on the oxygen consumption of lugworms was observed. However, with an average of 1.7 µmol g^{-1} h^{-1}, lugworms exposed to aged plasticised microplastics exhibited a >30% increase in oxygen consumption compared to lugworms exposed to unplasticised microplastics (1.3 µmol g^{-1} h^{-1}; T-test, p=0.821; see Figure 1F).

3.4.5 Mucus Production

Following exposure to plasticised microplastics, a significant effect on the amount of mucus produced by lugworms was observed (glm poisson model with a quasi-poisson over-dispersion parameter, p=0.04). Lugworms exposed to plasticised microplastics produced >70% less mucus on average than lugworms maintained with unplasticised microplastics (see Figure 1C).

Treatment did not significantly influence the outcome of whether a lugworm produced mucus or not throughout the exposure period. Thirty percent of lugworms maintained in the plasticised microplastic treatment produced mucus, whilst 80% of lugworms exposed to unplasticised microplastics produced mucus throughout the exposure (see Figure 1D).

3.4.6 Phagocytosis

Following exposure to plasticised microplastics, no effect on the percent of phagocytic cells of lugworms was observed (T-test, p=0.95; see Figure 1B). There was no significant difference in the phagocytic index of phagocytically active coelomocytes following exposure to plasticised microplastics (T-test, p=0.97; see Figure 1A).

3.4.7 Bioaccumulation

Lugworms exposed to plasticised microplastics accumulated >70% more DEHP than lugworms exposed to unplasticised microplastics, with 78.8 µg and 45.9 µg DEHP g^{-1} WW, respectively (T-test, p=0.81; see Figure 2). There was no difference when concentrations were normalised to lipid content, therefore we present concentrations based on whole-tissue wet weight.

3.5 Discussion

For the first time we have shown that endogenous chemical additives are released from microplastics to the tissues of an animal upon ingestion. There was a clear elevation in the bioaccumulation of DEHP, with levels being >70% greater on average, in lugworms exposed to plasticised microplastics than in lugworms exposed to unplasticised microplastics. This contradicts previous *in silico* and *in vivo* studies, which indicate that microplastics do not present a significant route of exposure to other chemical additives. The contribution of microplastic ingestion relative to total plastic

additive exposure was estimated for lugworms using a biodynamic modelling approach (Koelmans et al., 2014). Focussing on Nonylphenol (NP) and bisphenol A (BPA), concentrations in lugworms were predicted to be low. Ingestion of microplastics contaminated with 1000 µg kg^{-1} NP, based on a high environmental microplastic concentration (81 mg kg^{-1}), resulted in a steady-state concentration of 250 µg NP kg^{-1} lugworm lipids. At a concentration an order of magnitude less than NP, the ingestion of BPA-contaminated microplastics based on a high environmental microplastic concentration (81 mg kg^{-1}) would result in a steady-state concentration of 0.5 µg BPA kg^{-1} lugworm lipids, suggesting microplastic ingestion is not likely to constitute a relevant exposure pathway (Koelmans et al., 2014).

Lugworms are considered ecosystem engineers as their feeding activity, which overturns relatively large volumes of sediment, influences the abiotic conditions of the sediment, and therefore the infaunal assemblage and ecosystem functioning (Rossi et al., 2013). Previous studies have found microplastic exposure reduced lugworm feeding activity (Besseling et al., 2012; Wright et al., 2013a); however, this was observed at higher concentrations than that used in the present study. Consequently, such an adverse effect may only occur in response to high levels of microplastic contamination. In the current study, the feeding activity of lugworms was not significantly affected in the presence of plasticised microplastics. This suggests that microplastics comprising up to 1% by weight of the sediment plasticised with 50% DEHP by weight will not disrupt deposit-feeding processes. A recent study found that exposure to triclosan sorbed to the surface of PVC granules of a similar size to those used here reduced lugworm feeding activity by >65%, whilst exposure to PBDE in the same way reduced feeding activity by 30% (Browne et al., 2013). Unlike the present study, these additives were sorbed to the surface of the microplastics, which may enable rapid leaching at a higher concentration.

Phthalates and their metabolites are putative metabolic disruptors, compromising the energy balance between caloric intake and expenditure. This is believed to be primarily due to their role as peroxisome proliferators, binding to and activating Peroxisome Proliferator Activated Receptors (PPARs) *in vitro*. PPARs are lipid sensors, regulating gene expression to correspond to metabolic state. Peroxisome proliferators can have opposing effects *in vivo* depending on which isotype is activated, inducing

adipogenesis (PPARγ) or fatty acid oxidation (PPARα, PPARβ). Under these mechanisms, growth rate, oxygen consumption (as a proxy for metabolism), and energy reserves are all anticipated to be affected.

The Relative Growth Rate (RGR) of lugworms was not significantly affected following exposure to plasticised microplastics. On average, lugworms in both treatments exhibited a negative RGR, indicating weight loss between the start and end of exposure. This is likely due to the depletion of the organic content of the sediment whilst maintained in laboratory conditions. DEHP has shown to cause both weight gain (Manikkam et al., 2013; Schmidt et al., 2012) and weight loss in rodent models, depending on the extent and timing of exposure (Bernal et al., 2002; Gray et al., 2000; Mocchiutti and Bernal, 1997; Ward et al., 1998). In aquatic species, guppies exposed to 10 µg L^{-1} DEHP showed significantly reduced body lengths (by up to 40%) and weight (by up to 70%) from as early as 14 days, in comparison to control animals, highlighting the capacity for DEHP to inhibit development (Zanotelli et al., 2010).

Exposure to plasticised microplastics, resulting in 78.8 µg g^{-1} WW DEHP, increased the lipid reserves of lugworms by >30% compared to lugworms exposed to unplasticised microplastics (45.9 µg g^{-1} WW DEHP), which may be analogous to the onset of adipogenesis in vertebrates. DEHP has been identified as an obesogen, primarily due to its metabolite MEHP (Manikkam et al., 2013; Schmidt et al., 2012). Female mice exposed to up to 500 mg DEHP kg^{-1} body weight d^{-1} exhibited a significant increase in visceral adipose tissue in comparison to control mice. In addition, F1 offspring exposed to DEHP *in utero* and during lactation also had increased visceral adipose tissue (Manikkam et al., 2013).

The long-term lipid stores of invertebrates are typically located in the intestines or epidermis. While the lipid stores of invertebrates contribute to energy storage and homeostasis, as does the liver and adipose tissue of vertebrates, the role of invertebrate lipid stores in the pathophysiology of metabolic disorders has not been determined (Azeez et al., 2014). Consequently, the mode of action of DEHP and its metabolites in lugworms may differ to that in mammalian systems.

Oxygen consumption was measured as a proxy for lugworm metabolism in the current study. Whilst no significant effects were observed, lugworms exposed to plasticised

microplastics consumed over 30% more oxygen on average than lugworms exposed to unplasticised microplastics. This suggests DEHP could increase metabolism in lugworms, which has potential to impact energy budgets. Metabolic disruption following DEHP exposure has been measured in mammalian models. In rats, DEHP treatment reduced glycogenesis and the abundance of enzymes specific to glycogen metabolism, suggesting an overall reduction in glucose utilisation (Mushtaq et al., 1980). Moreover, male rats receiving a dietary dose of DEHP (2% by weight) over 21 days showed a reduction in energy efficiency and nitrogen utilisation, leading to a substantial decrease in body weight gain (Bernal et al., 2002).

Correlations between phthalate exposure and metabolic disorders have been observed. Short-term studies in rodents found a dose-dependent disruption to serum insulin, blood glucose, liver glycogen, thyroid-stimulating hormones, and cortisol (Gayathri et al., 2004). In humans, concentrations of phthalate metabolites were positively correlated with abdominal obesity and insulin resistance (Stahlhut et al., 2007). The metabolite with the highest concentration reported for the whole adult population was mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP, 771 ± 66.7 $\mu g\ g^{-1}$ creatinine). However, without definitive cause-and-effect relationships established through laboratory studies, this link is still hypothetical. At the molecular level, ERs and estrogens regulate many aspects of metabolism, including glucose transport, glycolysis, mitochondrial structure and activity, and fatty acid oxidation (Chen et al., 2009). Phthalates including DEHP may therefore act as a metabolic disruptor *in vivo*.

Throughout the exposure period, observations were made of mucus secretions, the so-called 'ventral shield' that is secreted by glandular epithelial cells and is periodically shed at the burrow opening (Stabili et al., 2009). Exposure to plasticised microplastics significantly reduced the number of lugworms secreting mucus and reduced the frequency of mucus shedding. Reduced mucus production has recently been observed in mice offspring following exposure to DEHP. Along with significant reductions in inflammatory cell counts and interleukins (ILs), decreased mucus production was observed in ovalbumin-challenged male and female offspring maternally-exposed to DEHP in comparison to ovalbumin-challenged control offspring (Shin et al., 2014). This was associated with a reduction in the abundance of Th2 cytokines. Th2 cells, when activated, produce pro-inflammatory mediators such as IL (cytokines). The increase in

pro-inflammatory mediators subsequently causes a localised flux of inflammatory cells, which produce pro-inflammatory mediators and chemo-attractants, aggravating the area and enhancing localised inflammation and therefore mucus secretion (Shin et al., 2014). DEHP or its metabolite MEHP could disrupt this pathway, and therefore suppress mucus secretion.

Cytokines are highly-conserved, biologically-important molecules present in a broad range of vertebrates and invertebrates (Ottaviani et al., 1996). IL-like molecules have been detected in several annelid species, including the ragworm *Hediste diversicolor* (Paemen et al., 1992); a close relative of the lugworm. Given the high conservancy of cytokines, it is likely lugworms possess cytokine-mediated IL pathways, which may be compromised by DEHP in a similar mechanism to that described for mammals.

There is also evidence to suggest mucus production is related to calcium-activated chloride channels. In the airway of asthmatics, human calcium-activated chloride channel (hCLCA1) is expressed in goblet cell hyperplasia. hCLCA1 expression and goblet cell metaplasia are induced by IL-13. Furthermore, the murine putative anion channel mCLCA3 localises in mucus granule membranes, suggesting it is related to goblet cell secretion. Niflumic acid has been shown to inhibit CLCA and IL-13-induced goblet cell metaplasia. In a guinea pig asthma model, niflumic acid inhibited goblet cell degranulation and morphological changes in mucus granules. The authors postulated this might be due to a blockade of granule CLCA. Moreover, as niflumic acid is capable of inhibiting cyclooxygenase, the inhibition of mucus secretion, may be mediated by a cyclooxygenase-dependent pathway (Kondo et al., 2012). Additionally, global gene expression analysis identified potassium intermediate/small conductance calcium-activated channel domains as DEHP targets in rats following *in utero* exposure to 1-300 mg kg^{-1}. A similar mechanism whereby DEHP interferes with ion channels and disrupts mucus secretion pathways may have occurred in lugworms following exposure to plasticised microplastics.

Mucus has several functional roles in marine invertebrates, including minimising sedimentation, preventing dehydration and facilitating locomotion. It also acts as a structural barrier against infection and is thus essential for survival (Davies and Viney, 1998). This mucosal defence mechanism protects against the continuous threat of

bacteria and debris accumulating on the body surface of marine invertebrates, which is particularly important for slow-moving and sessile species (Stabili et al., 2009). In polychaete worms, such as lugworms, mucus production – secreted from epidermal glandular (goblet) cells – constitutes a key factor determining their ability to survive in their environment, given their proximity to the rich assemblage of microorganisms in both the sediment and the surrounding seawater (Stabili et al., 2009). In addition to microbial protection, it also keeps the epidermis moist in order for it to function as a respiratory surface and can act as an adhesive in burrow formation (Healy, 1963). The accumulation of DEHP via a microplastic vector therefore threatens the microbial defence mechanism and overall survivability of lugworms. Mucus production requires a significant energetic cost in marine invertebrates (Wild et al., 2004). Given the elevated lipid and total energy reserves in lugworms exposed to plasticised microplastics compared to lugworms exposed to unplasticised microplastics, a trade-off may have occurred whereby suppressed mucus production allowed for increased lipid and total energy reserves.

Aside from the importance of mucus in microbial defence, it can alternatively provide a substrate for microbes to convert into biomass, thus forming the base of a food chain whereby microorganisms are consumed by higher organisms (Davies et al., 1992; Herndl and Peduzzi, 1989; Peduzzi and Herndl, 1991). A reduction in mucus production could therefore have knock-on effects on the surrounding microbial community with repercussions for food chains reliant on a microbial base.

Lugworms exposed to plasticised microplastics accumulated 78.8 µg g^{-1} DEHP, which was >70% greater than in lugworms exposed to unplasticised microplastics. Following 9 days of exposure (10 days post fertilisation) to 1 mg L^{-1} DEHP, Japanese rice fish (medaka) had accumulated 29 µg g^{-1} DEHP (BCF approx. 29) (Ye et al., 2014). This is high in comparison to the current study, considering the relatively low exposure concentration. The saltwater clam *Venerupis philippinarum* accumulated 1.7 µg g^{-1} following 24 hours exposure to 4 mg L^{-1} DEHP, which then decreased with time (up to 96 hours) (Li et al., 2014). The common mussel *Mytilus edulis* accumulated up to approximately 122.5 µg g^{-1} dry weight DEHP following a 28 day exposure to DEHP in seawater (42 µg L^{-1}) resulting in a BCF of approximately 3000 (Brown and Thompson, 1982). Crustaceans and molluscs appear to accumulate DEHP to a greater degree than

polychaete worms: polychaetes had a wet weight BCF of 422, compared to 1,469 and 1,164 for molluscs and crustaceans, respectively (Stales et al., 1997). A greater bioaccumulation observed in invertebrates in comparison to fish suggests invertebrates may have a lower metabolic capacity for DEHP. Given the high level of DEHP which accumulated in lugworm tissue, their capacity to metabolise DEHP to MEHP may be somewhat compromised compared to other species. Alternatively, this may be a result of long-term, continuous exposure of a sedentary animal to a localised pollutant.

Phthalate exposure has been found to cause various effects in invertebrate species. Exposure to 0.3 – 30 µg L^{-1} resulted in reproductive effects in the midge *Chironomus riparius* (Kwak and Lee, 2005). This was also the case for the model freshwater crustacean *Daphnia magna*, whereby 3 weeks exposure to 3 and 30 µg L^{-1} DEHP reduced the number of offspring produced by 40% and 83%, respectively, in comparison to individuals maintained in control conditions (Mayer and Sanders, 1973). The observation of this effect was not repeated in a similar study utilising concentrations ranging from 0 to 100 µg L^{-1} DEHP (Brown and E Thompson, 1982). The DEHP concentration to cause mortality in half the test population of *D. magna* was 1.82 g L^{-1} (Park and Choi, 2007), highlighting the varied toxicity this contaminant can have between populations. *M. edulis* exhibited no adverse effects in response to DEHP exposure at 5 and 50 g L^{-1} for 2 weeks, whilst concentrations of DEHP in excess of 100 g L^{-1} elicited cellular effects (Brown and Thompson, 1982).

The results presented here provide an important step towards understanding the potential for endogenous chemical additives to enter marine food chains via microplastics. Whilst phthalates are not considered persistent or likely to biomagnify, we have shown an elevation of phthalates in the tissues of lugworms following 1 month exposure to plasticised microplastics. Additionally, other chemical additives, which are known human toxicants, are capable of biomagnifying up the food chain. It is imperative to determine the contribution of microplastics to contaminant burdens in marine organisms and in humans due to seafood consumption, and the environmental health effects this could have.

3.6 Figures

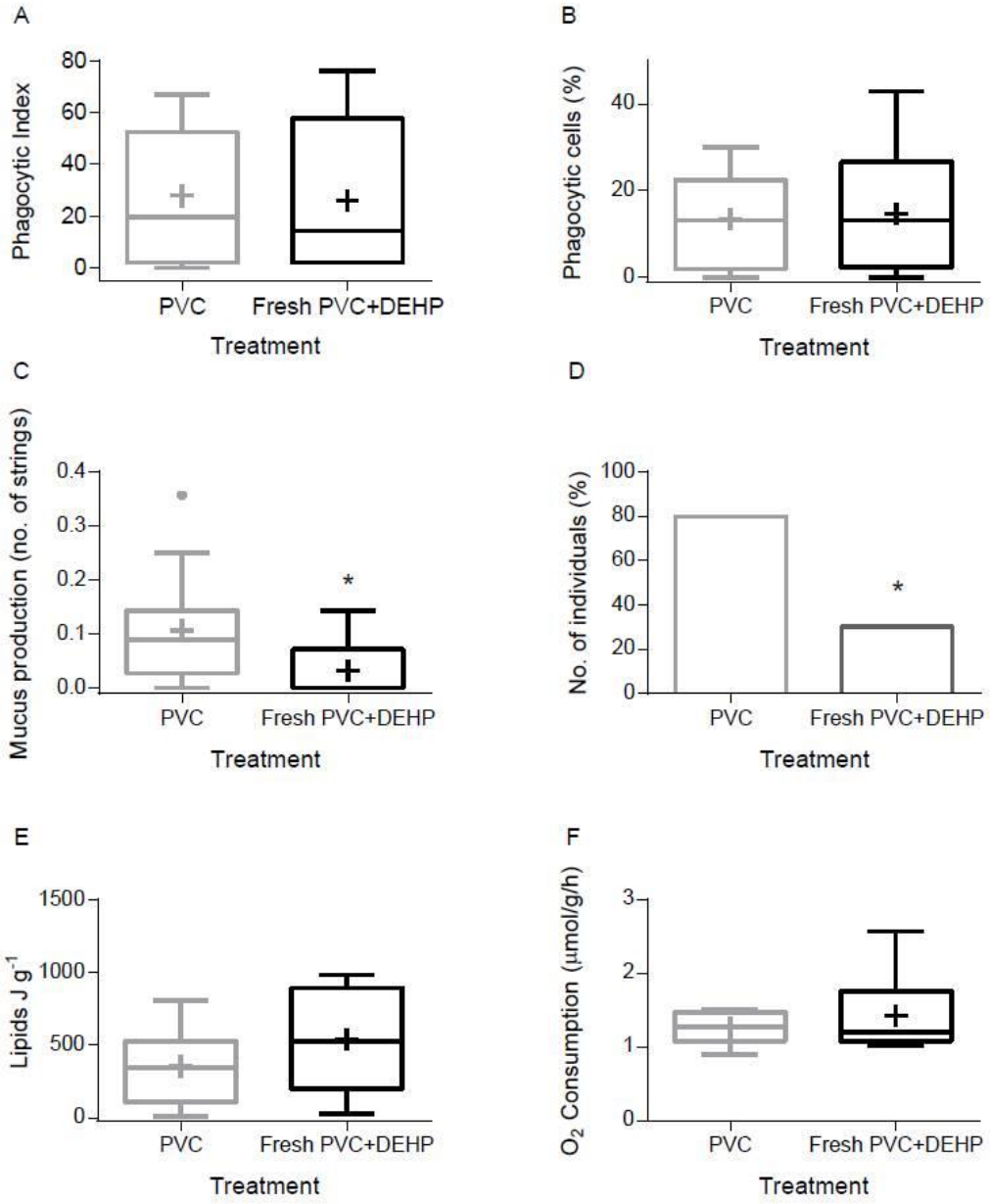


Figure 1. The biological effects of 4 week exposure to unplasticised (control) and plasticised (DEHP) microplastics (PVC) on A) the phagocytic index of lugworm coelomocytes; B) the percent of phagocytically-active lugworm coelomocytes; C) the

amount of mucus produced per lugworm; D) the proportion of lugworms which produced mucus; E) the lipid content of lugworm whole-tissue; and F) lugworm oxygen consumption. Tukey boxplots show the median ± range; the mean is indicated by a cross. Significance at a ≥95% confidence interval is indicated by *. Outliers are shown by a circle.

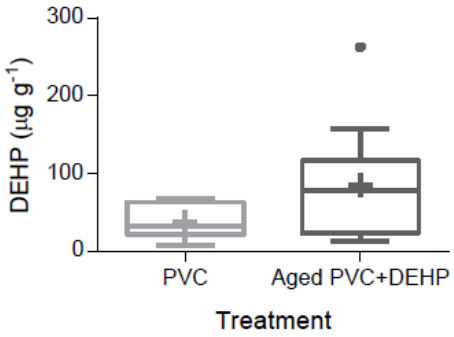


Figure 2. The concentration of DEHP in whole lugworm tissue (WW) after 4 weeks exposure to unplasticised (control) and plasticised (DEHP) microplastics (PVC). Tukey boxplots show the median ± range; the mean is indicated by a cross. Outliers are shown by a circle.

3.7 Supplemental Information

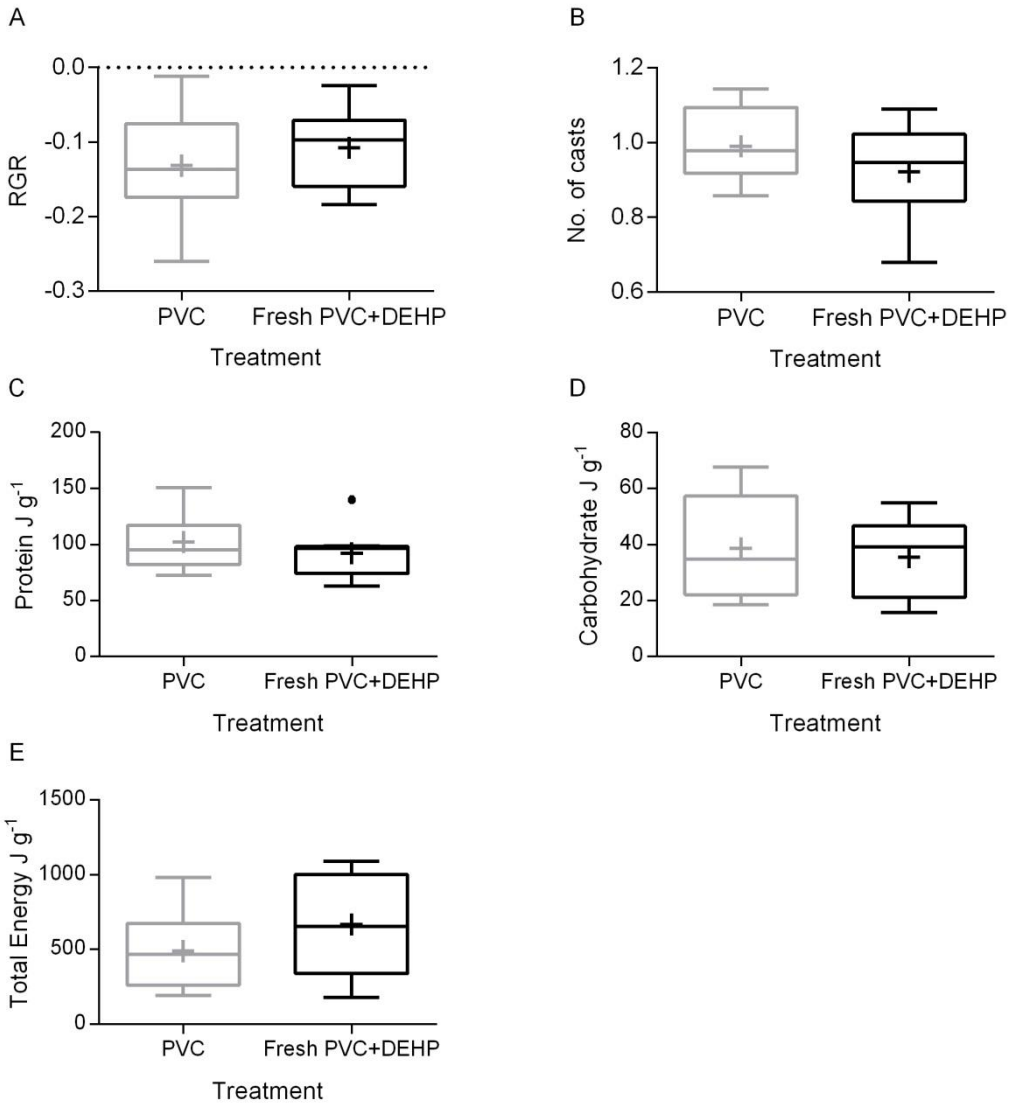


Figure S1. The biological effects of 4 week exposure to unplasticised (control) and plasticised (DEHP) microplastics (PVC) on A) the Relative Growth Rate (RGR) of lugworms; B) the feeding activity of lugworms shown as the average number of casts per lugworm per day; C) the protein reserves of whole lugworm tissue; D) the carbohydrate reserves of whole lugworm tissue; and E) the total energy reserves of whole lugworm tissue. Tukey boxplots show the median ± range; the mean is indicated by a cross. Significance at a ≥95% confidence interval is indicated by *. Outliers are shown by a circle.

I.7.2 The Potential Impacts of Size

The potential main and interactive effects of the size (weight) of worms on the reported dependent variables were analysed, where the variable did not already account for the weight of the worm, such as oxygen consumption and lipid content.

Following 28 d exposure to plasticised PVC, there was no significant main or interactive effect of weight on the proportion of phagocytically active cells of individuals (two way ANOVA, p= 0.0832 and p= 0.9525 for weight and weight*treatment, respectively) or on the phagocytic index of cells (two way ANOVA, p= 0.223 and p= 0.611 for weight and weight*treatment, respectively). There was no significant main or interactive effect of weight on mucus production (generalised linear model, p=0.09 and p=0.545 for weight and weight*treatment, respectively) and there was no significant main or interactive effect of weight on the number of casts produced by individuals based on likelihood ratio tests (linear mixed effects model, p=0.727).

Chapter 4

Cigarette litter impairs the ecological function of marine worms

Manuscript submitted to Scientific Reports

Cigarette litter impairs the ecological function of marine worms

Stephanie L. Wright[1], Darren Rowe[1], Malcolm J. Reid[2], Kevin V. Thomas[2], and Tamara S. Galloway[1]*

[1]Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter, Devon EX4 4QD, UK.

[2]Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, N-0349 Oslo, Norway

*T.S.Galloway@exeter.ac.uk +44 (0) 1392 723436

This manuscript has been modified to conform to the format of this thesis.

4.1 Abstract

Marine debris is a global environmental issue. Smoked cigarette filters are the predominant coastal litter item; 4.5 trillion are littered annually, presenting a source of bioplastic microfibres (cellulose acetate) and harmful toxicants to marine environments. Despite the human health risks associated with smoking, little is known of the hazards cigarette filters present to marine life. Here we studied the impacts of smoked cigarette filter toxicants and microfibres on the polychaete worm *Hediste diversicolor* (ragworm), a widespread inhabitant of coastal sediments. Ragworms exposed to smoked cigarette filter toxicants in seawater exhibited significantly longer burrowing times, >30% weight loss, and >2-fold increase in DNA damage compared to ragworms maintained in control conditions. In contrast, ragworms exposed to smoked cigarette filter microfibres in marine sediment showed no significant effects. Bio-concentration factors for nicotine were 500 fold higher from seawater than from sediment. Our results illustrate the vulnerability of organisms in the water column to smoking debris and its related toxicants, highlighting a need for remedial policy intervention to reduce the risks posed by smoked cigarette filter debris to aquatic life.

4.2 Introduction

Marine debris is a global conservation issue (Sutherland et al., 2010). Semi-synthetic bioplastic (rayon) and plastic materials are widely reported in the marine environment (Woodall et al., 2014). Environmental exposure causes these materials to degrade and fragment, resulting in micron-sized particles and fibres <1 mm (e.g. microplastics). Fibres are the most frequently reported type of particulate debris, not just in coastal ecosystems, but in deep ocean sediments, where recent estimates suggest over 2 billion rayon fibres km^{-2} contaminate the seabed (Woodall et al., 2014).

Smoked cigarette filters – the predominant item reported globally in coastal litter surveys – present a substantial source of rayon microfibres; each filter is comprised of >15,000 cellulose acetate (rayon) fibres, 20 µm in diameter (Hon, 1977; Novotny and Slaughter, 2014). Approximately 4.5 trillion smoked cigarette filters, equivalent to 750,000 tonnes, are littered annually. Despite the anti-littering laws operative in many countries, enforcement at the individual-level is impractical and has proved ineffective in preventing this debris from accumulating in the environment.

Smoked cigarette filters can cause harm in the marine environment in several ways. They present a vector for the transport and introduction of toxicants, including heavy metals, nicotine and known carcinogens (Moriwaki et al., 2009), to aquatic habitats. Exposure to such toxicants in seawater could occur following the dissolution of compounds from the bioplastic filter to the surrounding seawater (leaching). Dietary exposure could occur through the ingestion of smoked cigarette filter microfibers due to filter degradation. If ingested, there is potential for the transfer of adhered toxicants to tissues. These bioplastic microfibres and their associated toxicants may persist in the marine environment and continue leaching chemicals for up to 10 years (Novotny and Slaughter, 2014). Research into the impacts of smoked cigarette filters on marine life is therefore crucial for consolidating the evidence base for remedial policy (Novotny and Slaughter, 2014). Despite this, few studies have assessed their potential toxicity. This is particularly important in coastal sediments, where smoked cigarette filters dominate litter (Ivar do Sul and Costa, 2007; Mehlhart, 2012).

Sediment is a vital component of the marine environment, forming one the largest habitats on Earth. Its diverse residents are fundamental to marine ecosystem function,

impacting water column processes; trophic transfer; and global biogeochemical cycles (Snelgrove, 1997). Polychaete worms are widespread and abundant inhabitants of coastal sediments, where they rework and irrigate sediment and form a key prey species for birds and fish. They adopt a range of feeding strategies, including surface deposit feeding, and are thus vulnerable to smoked cigarette filter debris and toxicants via both oral and dermal exposure pathways.

For the first time we explore the impacts of smoked cigarette filter toxicants and microfibres on the polychaete worm *Hediste diversicolor* (ragworm). We address the hypothesis that 1) the toxicants desorbed from smoked cigarette filters are harmful to ragworms, and 2) that smoked cigarette filter microfibres present a physical hazard and/or vector for the associated toxicants. Our findings contribute to the growing evidence base for policy intervention on smoking-related debris.

4.3 Results

4.3.1 Nicotine Bioaccumulation

Nicotine and its metabolite cotinine were used as biomarkers of exposure to the toxicants associated with filters. The average cigarette contains 0.8 – 1.9 mg of nicotine. Based on an average adult human weight of 68 kg, this delivers a dose of 10 – 30 $\mu g\ kg^{-1}$, resulting in average peak plasma levels of 10 – 50 $ng\ ml^{-1}$ (Matta et al., 2007). Nicotine was detected in whole-ragworm tissue following all exposures. Ragworms received a higher dose of nicotine compared to the dose received by humans following cigarette smoking (see Table 1). Consequently, greater levels of nicotine accumulated in tissues compared to human plasma.

After 96 h, the greatest levels of nicotine were measured in ragworms exposed to the highest concentrations of both filter toxicants in seawater and microfibres in sediment (8 filters L^{-1}; Bio-Concentration Factors (BCFs) of 172.4 and 0.338, respectively). Ragworms accumulated several orders of magnitude less nicotine following both short- and long-term sediment exposures to filter microfibres than following exposure to filter toxicants in seawater (Fig. 1d and Supplementary Fig. 2a).

4.3.2 Nicotine Metabolism

The nicotine metabolite cotinine was detected in all ragworms following exposure to filter toxicants in seawater (Fig. 1c). Nicotine:cotinine ratios peaked (0.034) at 1 filter L^{-1}, before dropping with increasing concentration (Fig. 1c). This drop indicates impaired nicotine metabolism at concentrations in excess of 1 filter L^{-1}.

Following a 96 h sediment exposure to filter microfibres, cotinine was detected in ragworms exposed to 2 filters L^{-1} and above (Fig. 1d). The greatest nicotine:cotinine ratio was measured in ragworms exposed to 4 filters L^{-1} (0.018) (Fig. 1d). After 28 d in sediment, cotinine was detected in ragworms exposed to 4 filters L^{-1} and above (Supplementary Fig. 2a). These results indicate reduced bioavailability of nicotine in comparison to filter toxicants in seawater.

4.3.3 Biological Endpoints

4.3.3.1 Relative Growth Rate

Relative Growth Rate (RGR) was measured as a general health indicator. A significant effect on RGR was observed in ragworms following exposure to filter toxicants in seawater (one-way ANOVA, p=0.00005, Fig. 2a). The lowest concentration to cause a significant effect (LOEC) on RGR was 8 filters L^{-1} (-33% mean weight ± 2% s.e.m.). Following 96 h and 28 d sediment exposures to filter microfibres, no effect on the RGRs of ragworms was observed (Fig. 2b and Supplementary Fig. 2b, respectively).

4.3.3.2 Burrowing Activity

Given the neurotoxicity of nicotine (Matta et al., 2007), we selected burrowing time as a primary sublethal endpoint. Exposure to the two highest concentrations of filter toxicants in seawater (4 and 8 filters L^{-1}) inhibited the burrowing capacity of 100% of individuals during the assay observation period (Fig. 2c). The LOEC for the burrowing time of ragworms exposed to filter toxicants in seawater was 2 filters L^{-1} (Kruskal Wallis, p=0.0001).

Following a 96 h sediment exposure to filter microfibres, the LOEC for burrowing time was 8 filters L^{-1} (one-way ANOVA, p=0.04, Fig. 2d). Post hoc analysis showed that this result was significant at a confidence level of 0.1 (Tukey HSD Test, p=0.07). The

burrowing time of ragworms following 28 d sediment exposure to filter microfibres was not affected (Supplementary Fig. 2c).

4.3.3.3 DNA Damage

Exposure to filter toxicants in seawater significantly affected the median, 75th, and 90th percentile tail moment (TM, a measure of DNA fragmentation, see Methods) of ragworms (one-way ANOVA, p=0.016, p=0.003, and p=0.003, respectively). Ragworms exposed to 8 filters L^{-1} had significantly greater TMs than those exposed to 0.5, 2, and 4 filters L^{-1} (Fig. 2e, Supplementary Fig. 3a and d for 90th, median, and 75th percentiles, respectively). The 75th and 90th percentile tail intensities (TI, a measure of the relative fraction of DNA, see Methods) were also significantly greater in ragworms exposed to 8 filters L^{-1} than to ragworms exposed to 0.5 and 4 filters L^{-1} (Kruskal Wallis, p=0.04 and p=0.01, respectively; Supplementary Fig. 4a and d for 75th and 90th percentiles, respectively). Following 96 h and 28 d exposures to filter microfibres in sediment, there was no significant DNA damage (see Fig. 2f, Supplemetary Fig. 3 and 4 b and e; and Supplementary Fig. 2d and 3 and 4 c and f, for 96 h and 28 d, respectively).

An average increase in DNA damage of 2- to 3-fold from control to treatment is considered biologically relevant (Bright et al., 2011). Fold increases >2 from control to treatment were measured in median, 75th, and 90th TM percentiles (Fig 2e, and Supplementary Fig. 3a and d, for median, 75th, and 90th percentiles, respectively) and in 75th and 90th TI percentiles (Supplementary Fig. 4a and d, respectively) of ragworms exposed to filter toxicants in seawater. Thus, filter toxicants in seawater at a concentration of 8 filters L^{-1} caused biologically relevant DNA damage.

4.4 Discussion

This is the first study to assess the importance of smoked cigarette filter (filters) debris as both a source of hazardous bioplastic microfibres and a vector for associated toxicants in the marine environment. We found that exposure to filter toxicants in seawater at a concentration of ≥2 filters L^{-1} significantly inhibits burrowing behaviour in a marine worm, whilst greater concentrations lead to reduced growth rates and increased DNA damage. Of the few studies that have assessed the impacts of filter toxicants on aquatic species, water fleas and juvenile fish exhibited greater sensitivity

than ragworms did in the present study (Micevska et al., 2006; Slaughter et al., 2011). Further investigation is therefore required to determine the impacts of filters on other biotic components of marine ecosystems.

Ragworms accumulated considerably less nicotine – an established biomarker of exposure to the toxicants associated with smoking - following sediment exposure to filter microfibres than following exposure to filter toxicants in seawater. Notably, the nicotine dose delivered by just one filter L^{-1} via seawater is around 98 times that delivered to a human via smoking (Table 1). In seawater, exposure to filter toxicants occurs trans-dermally (Fig. 1a). Nicotine is unionized and bioavailable under alkaline conditions (Benowitz et al., 2009). The alkalinity of the seawater in this study (pH 8.06 mean ± 0.007 s.e.m.) indicates over 30% of nicotine was bioavailable, allowing for rapid systemic circulation (Benowitz et al., 2009).

Sediment exposure to filter microfibres and associated toxicants occurs predominantly via indiscriminate surface-deposit feeding. Post-ingestion, up to 70% of nicotine is metabolised before entering systemic circulation (Benowitz et al., 2009) (Fig. 1b). The pH of the sediment during low tide (7.5 mean ± 0.01 s.e.m., n=12) suggests over 30% of the nicotine is bioavailable in sediment exposures (Benowitz et al., 2009). However, the moderately acidic gut conditions of ragworms could counter this (Ahrens, 2001). These factors may explain the low concentration of nicotine detected in ragworms following sediment exposures. Additionally, ragworms are unlikely to encounter the entire sediment volume, thus contacting only a fraction of the contaminant. The worms' mucus-lined burrow may also act as a physical barrier, limiting encounter rates with nicotine (Fig. 1b). Following 96 h exposure to filter toxicants in seawater, nicotine metabolism was impaired. Nicotine metabolism is important in reducing toxicity: cotinine has a similar mechanism of action to nicotine, but binds to neuronal acetylcholine receptors with lower potency (Buccafusco et al., 2007).

If ingested, there is potential for micro- plastic and bioplastic debris to transfer adhered pollutants, which can accumulate on their surface up to several orders of magnitude greater than the surrounding water column (Hirai et al., 2011; Mato et al., 2001). Whilst sediment exposure to filter microfibres limited nicotine bioaccumulation, other types of particulate debris have been shown to transport chemical contaminants

to invertebrates. Microscopic polyvinylchloride (PVC) transferred adhered triclosan and nonylphenol to the gut tissue of sediment-dwelling lugworms, at levels which caused biological harm (Browne et al., 2013). Moreover, simulated gut conditions elicited greater desorption rates of chemical contaminants from microscopic polyethylene and PVC than seawater (Bakir et al., 2014). These studies employed higher concentrations of particles than the current study.

Using the biomarker nicotine, we have shown that filters can act as a vector for the transport and introduction of associated toxicants to seawater through leaching. Thus, whole-filter and filter microfibers in seawater are of concern. Epibenthic and pelagic species are considered vulnerable to the bioaccmumulation of these compounds, indicated by the substantial accumulation of nicotine from seawater exposures. However, the ingestion of filter microfibers within sediment by benthic species as a route of exposure to associated toxicants is considered a lower threat.

We show that exposure to filter toxicants in seawater has a significant negative effect on the RGR of ragworms. Similarly, the weight of earthworms was reduced by up to 40% following exposure to the neurotoxic insecticide imidacloprid, which is chemically similar to nicotine (Capowiez et al., 2005; Dittbrenner et al., 2010). The authors postulated this was due to decreased feeding, reduced assimilation efficiency, or the up-regulation of an energetically costly detoxification mechanism. Similar modes of toxicity could have also occurred in the present study. Weight loss indicates that important Darwinian fitness parameters such as growth and reproduction are being compromised, with repercussions at higher levels of biological organisation.

No effect on the RGRs of ragworms was observed following 96 h and 28 d sediment exposures to filter microfibres. The low nutritional value of the cellulose acetate microfibres may be anticipated to reduce RGR. Female rats showed a 14% reduction in growth following prolonged dietary exposure to high doses of cellulose acetate, linked to a nutritional reduction in the feed (Thomas et al., 1991). The polychaete worm *Arenicola marina* suffered significant reductions in energy reserves following exposure to ≥1% microscopic PVC by weight (Wright et al., 2013a). This was likely in-part due to a reduction in the nutritional quality of material consumed. The current study employed lower concentrations of microplastics, resulting in a higher proportion of

nutritious substrate. We consider the chemical toxicity of leached nicotine and associated toxicants from filters to seawater to be of greater concern than the ingestion of low-nutritive filter microfibers for impacting growth rate.

Exposure to filter toxicants in seawater at a concentration of ≥2 filters L^{-1} significantly affected burrowing activity in ragworms. The insecticide imidacloprid impaired burrowing behaviour in earthworms; burrows were smaller in area and shallower than control groups following a 6 day exposure (Capowiez and Bérard, 2006). Nicotine is neurotoxic, affecting the central and autonomic nervous system and neuromuscular junctions by agonistically binding to the nicotinic acetyl cholinergic receptors (nAChRs). This opens ion channels, causing an influx of sodium or calcium ions, increasing the release of neurotransmitters. Prolonged stimulation of nAChRs can lead to desensitization, impairing neurological function (Matta et al., 2007). Ataxia, muscle fasciculation (twitching), lethargy, and muscle paralysis were observed in exposed ragworms, suggesting the prolonged stimulation and consequent desensitization of nAChRs inhibited their burrowing capacity.

The burrowing behaviour of worms is central to their role as ecosystem engineers, reworking and aerating sediment to allow other organisms to thrive. Thus, nicotine exposure via filter debris presents a risk to ecosystem health through its detrimental neurotoxic effects on survival, growth and behaviour of worms. As sediment exposure to filter microfibres limited nicotine bioaccumulation, burrowing activity was minimally affected. Filter microfibres within sediment as a vector for nicotine are therefore anticipated to be less neurologically hazardous than filter toxicants in seawater.

Previous studies have highlighted a protective effect of nicotine on DNA damage at low concentrations through radical scavenging (Sobkowiak and Lesicki, 2009). At higher concentrations, DNA damage occurs due to oxidative stress (Crowley-Weber et al., 2003). Ragworms exposed to filter toxicants in seawater at concentrations up to 4 filters L^{-1} exhibited significantly lower levels of DNA damage than those exposed to 8 filters L^{-1}. This indicates that ragworms experienced the protective effect of low nicotine dosage. At lower nicotine doses, the neurotoxicity of nicotine may be of greater concern than potential molecular effects.

In conclusion, filter toxicants in seawater cause acute dose-dependent neurotoxicity in marine worms, which is linked to nicotine bioaccumulation. These results highlight the vulnerability of animals exposed to leached filter toxicants in the water column. In comparison, sediment exposure to filter microfibres – an anticipated route of exposure for ragworms in the marine environment - limits the bioaccumulation and toxicity of this harmful toxicant.

The protection, conservation and restoration of marine ecosystems increasingly rely on international legislation to curb anthropogenic impacts. Recently, statutory frameworks such as the European Union Marine Strategy Framework Directive (MSFD) have for the first time stipulated that the properties and quantities of marine litter, including microplastics, should not cause harm to the marine environment (Descriptor 10, MSFD, 2008/56/EC). Quantitative toxicological data is essential for supporting the implementation of such legislation; our results provide a first step towards setting guidance limits to curb smoking-related bioplastic debris. We encourage further research into the role of environmental and physiological pH, and different exposure pathways when considering the impacts of filter toxicants and bioplastic microfibers on biotic components of marine ecosystems.

4.5 Methods

4.5.1 Materials

4.5.1.1 Smoked Cigarette Filters

Smoked cigarette filters (‘filters’, nicotine content 0.7-0.9 mg) were collected and immediately kept in sealed falcon tubes in the dark. Before use, the outer paper and any excess tobacco was carefully removed. Filters were individually weighed to calculate an average filter weight.

4.5.1.2 Chemicals and solutions

Ethyl acetate (Chromasolv HPLC Grade, Sigma Aldrich), methanol (HPLC Super Gradient Reagent, VWR Chemicals), carbon dioxide (food grade, AGA), ammonium hydroxide (ACS Reagent, Sigma Aldrich), AOAC Method 2007.01 Extraction salts (DisQuE, Waters Corp, Milford USA), AOAC Method 2007.01 clean-up tubes (DisQuE, Waters Corp,

Milford USA), nicotine, nicotine-D4, cotinine and cotinine-D3 (all from Cerilliant, Round Rock Texas, USA), and 3'-hydroxycotinine (Toronto Research Chemicals, Ontario Canada).

4.5.2 Exposures and Biological Endpoints

4.5.2.1 Animal Husbandry

The ragworm *Hediste diversicolor* was hand collected from the Exe Estuary, Devon, UK (50˚66''76 N, -3˚44''40W) between February to April 2014. They were maintained in 4 cm of natural sediment with overlying artificial seawater (ASW, salinity of 22) in a temperature-controlled room (12˚C, 14 h light:10 h dark). Ragworms were acclimated for at least 1 week. Water changes were performed on alternate days. In all exposures, only healthy, complete ragworms were used.

4.5.2.2 Exposure to Smoked Cigarette Filter Toxicants in Sediment

To establish whether the toxicants associated with filters are harmful to ragworms, an initial aqueous exposure was performed following (Micevska et al., 2006) and (Slaughter et al., 2011). To produce increasing doses of leachates based on a filter L^{-1} concentration, a leachate stock was produced, also forming the highest concentration (8 filters L^{-1}). Smoked filters were placed in artificial seawater (salinity of 22) on an orbital shaker in a temperature-controlled room for 24 h. The leachates were then vacuum-filtered through Whatman cellulose filter paper (grade 1) to remove any particulates due to cigarette filter degradation. The remaining test concentrations were made by performing 0.5x dilution series with the filtered leachate and artificial seawater, achieving final concentrations of 8, 4, 2, 1 and 0.5 cigarette filters L^{-1}. Subsamples of each stock concentration were kept at -80˚C for chemical analysis.

300 mL aliquots of leachates were added to 400 mL glass beakers (acid-washed, 13% HNO_3) immediately before the addition of ragworms. Beakers were randomly allocated a position in a temperature-controlled room (12˚C). Each beaker contained a length of silicon tubing, providing refuge. Beakers were gently aerated and covered to minimise evaporation. Ragworms were individually weighed and transferred to a beaker. Observations were made daily. Following 48 h, a water change was performed using fresh leachate from which subsamples were again taken for chemical analysis. Water

parameters (salinity, pH, dissolved oxygen) were monitored throughout the exposure period. After 96 h, ragworms were removed from exposure.

4.5.2.3 Exposure to Smoked Cigarette Filter Microfibres in Sediment

To determine whether particulate debris from filters can transfer toxicants at levels capable of causing harm, the impacts of filter microfibers on ragworms were assessed. Filters free of outer paper and excess tobacco were ground under liquid nitrogen using a pestle and mortar until a fine powder formed. Subsamples were suspended in DI and observed under a microscope fitted with a camera for size analysis. Individual microfibres were randomly sized using image analysis software.

Microfibers (mean length 120.6 ± 5.1 µm s.e.m., median length 96.5 µm, Supplementary Fig. 1) were added to sediment in bulk by weights equivalent to the concentrations above (number of filters L^{-1}). The sediment was manually homogenised. Subsamples of each sediment stock concentration were kept at -80˚C for chemical analysis. 48 h prior to exposures, 225 mL of test sediment was added to 400 mL acid-washed, glass beakers (4 cm depth). Beakers were randomly allocated a position in a temperature-controlled room (12˚C), covered and left to acclimate overnight. 24 h prior to exposures, 150 mL of artificial seawater (salinity of 22) was poured into beakers over a clean, stainless steel spoon. Gentle aeration was provided and beakers were left covered.

Ragworms were individually weighed and transferred to a beaker. Observations were made daily and water parameters were monitored throughout the exposure period. Two exposures were conducted, lasting 96 h and 28 d. During the 96 h exposure, a water change was performed after 48 h using fresh ASW. After 96 h, ragworms were removed from the exposure. During the 28 d exposure, water changes were performed every 72 h. After 28 days, ragworms were removed from exposure. Following endpoint measurements, ragworms were individually maintained in seawater (salinity of 22) to void gut content in preparation for chemical analyses. After approximately 10 h, ragworms were snap-frozen and stored at -80º until use.

4.5.2.4 Relative Growth Rate

In addition to pre-exposure wet weights, post-exposure weights were also recorded. Following sediment exposure, any external sediment was carefully rinsed from

ragworms. Excess seawater was gently absorbed using a paper towel and ragworms were weighed to 0.01 g.

4.5.2.5 Burrowing activity

Individuals were transferred to 400 mL glass beakers containing 225 mL wet control sediment (corresponding to approximately 4 cm depth). Their burrowing time into clean sediment – from the moment their anterior end touched the sediment to being completely burrowed –was recorded within a 1 h observation period. The burrowing time of ragworms which did not burrow during this time was considered as 60 min.

4.5.2.6 Comet assay

DNA damage – measured as single-strand breaks in individual cells (Comet assay) – was quantified to assess potential carcinogenic and pro-oxidative effects, anticipated due to the constituent toxicants of smoked cigarettes. The Comet assay quantifies DNA damage as tail intensity (TI) and tail moment (TM) for individual cells. TI indicates the relative fraction of damaged DNA. TM is the product of TI and tail length (the migratory distance of broken DNA fragments from the nucleus of the cell), providing a descriptive assessment of DNA damage.

Ragworms were recovered from the burrowing assay and carefully rinsed. A sample of coelomic fluid was withdrawn with a 1 mL syringe containing chilled PBS at a 1:1 ratio, fitted with a 23 gauge needle. Samples were taken from the posterior region, taking care to avoid the gut, and stored on ice until use. 100 µL of sample was used per individual. Coelomic fluid was centrifuged at 1000 rpm for 3 min and the supernatant was discarded. The cell concentrate was then suspended in 1% low melting point agarose (37˚C) and two aliquots were dropped onto a slide pre-coated with 1% normal melting point agarose. Coverslips were placed on top of the sample and slides were left for 10 min at 4˚C. Once the gel was set, coverslips were carefully removed and the comet assay was conducted, following (Singh et al., 1988), modifying for alkaline conditions. Slides were placed in lysis solution for 1 hour, followed by 40 min denaturation in electrophoresis buffer (pH 13) and then electrophoresis for 30 min (25 V, 300 mA). The slides were then gently washed in neutralising buffer. 100 cells per slide (50 per gel) were scored within 48 h using sybr safe staining and a fluorescent

microscope (420-490 nm excitation filter and 520 nm emission filter) equipped with Kinetic COMET software.

4.5.2.7 Chemical Analysis

Nicotine and its metabolite cotinine were used as biomarkers of exposure to the toxicants associated with filters. Frozen ragworm tissue was thoroughly homogenised under liquid nitrogen using a pestle and mortar. For each exposure and concentration sub-aliquots of homogenised tissue from each individual were pooled.

4.5.2.8 Chromatography and detection (MS/MS) parameters

Analysis was carried out on an Acquity UPC2 system with a Quattro Premier XE Mass Spectrometer (MSMS) as detector (both from Waters Corp, Milford USA). See Supplementary Table 1 for details.

4.5.2.9 Sample Preparation

1. *Water Samples*

   500 µL samples of aqueous exposure media (water) were spiked with internal standard solution (25 µL of a solution containing 500 ng/mL nicotine-D4 and cotinine-D3) and then adjusted to pH 10 with ammonia. Liquid-liquid extraction was performed with 1 mL ethyl acetate. The upper (ethyl acetate) phase was removed and analysed.

2. *Sediment Samples*

   0.5 g sub-samples were weighed into 10 mL glass test-tubes and spiked with internal standard solution (100 µL of a solution containing 500 ng/mL nicotine-D4 and cotinine-D3) together with 3mL water (2 % ammonium hydroxide) and 4 mL acetonitrile. Samples were then extracted and cleaned according to AOAC Method 2007.01 for pesticide residues in foods by acetonitrile extraction and partitioning with magnesium sulphate (REFERENCE: available online at http://www.eoma.aoac.org/methods/info.asp?ID=48938).

3. *Ragworm Samples*

   50 mg ragworm samples were weighed into 2 mL tubes and spiked with internal standard solution (10 µL of a solution containing 500 ng/mL nicotine-D4 and cotinine-D3) together with 300 µL water (2 % ammonium hydroxide) and 400 µL acetonitrile. Samples were then extracted and cleaned according to AOAC Method

2007.01 for pesticide residues in foods by acetonitrile extraction and partitioning with magnesium sulphate (REFERENCE: available online at http://www.eoma.aoac.org/methods/info.asp?ID=48938).

4.5.3 Statistical Analyses

Statistical analyses were performed in R (R Core Team, 2013). To ensure correct specification of the models used (analysis of variance), the distribution of residuals was monitored using the Shapiro Wilks test for normality and Levene's test for homogeneity of variance. Where data did not conform to model assumptions, a $\log_{10}(x+1)$ transformation was performed. If this did not increase suitability, an equivalent non-parametric test was performed.

Any change in the weight of ragworms during exposures was assessed using the method of (Crawley, 2005). First, relative growth rate (RGR) was calculated:

$$RGR = \ln\left(\frac{fw}{iw}\right)$$

where fw = final weight and iw = initial weight. The effect of treatment on RGR was then analysed using a one-way ANOVA (n=6).

Any change in burrowing time due to exposure was determined using a one-way ANOVA or Kruskal Wallis test where appropriate (n=6). A change in tail intensity (TI) and tail moment (TM) was analysed using the methods of (Bright et al., 2011; Duez et al., 2003; Wiklund and Agurell, 2003), whereby the statistical analysis is performed by animal (as opposed to by gel or by cell) using a summary statistic calculated by:

Mean (across replicate gels) of the x percentile of the $\log_{10}(TI+1)$

where x is substituted for the median, 75th, or 90th percentile based on recommendations by (Bright et al., 2011; Duez et al., 2003). The effect of treatment on TI and TM was then analysed using a one-way ANOVA or Kruskal Wallis test (n=6).

Where a Kruskal Wallis was applied and a significant p-value obtained, post-hoc Wilcoxon rank sum tests were used on pairwise permutations, using a Bonferroni correction. Post-hoc analysis following a one-way ANOVA was conducted using a Tukey HSD test. The lowest concentration which elicited a significantly different response compared to the control was identified as the Lowest Observed Effect Concentration

(LOEC), whilst the highest concentration which did not cause a significantly different response compared to the control was identified as the No Observed Effect Concentration (NOEC).

4.5.4 Bioaccumulation

A bioconcentration factor (BCF) – the level of accumulation of a chemical in an organism from the surrounding medium - was calculated for both aqueous and sedimentary exposures. This was quantified using the following calculation:

$$BCF = \frac{CB}{CM}$$

Where CB = biota concentration and CM = medium (leachates or sediment) concentration.

4.6 Acknowledgments

Many thanks to Dr Yuktee Dogra and Steve Cooper for sieving sediment. Thanks to the volunteers who saved smoked cigarette filters. M.R.E. and K.V.T. acknowledge the financial support from NFR project 225203. S.L.W, D.R., and T.S.G acknowledge financial support from the Department for Environment, Food & Rural Affairs project 1-SW-P-N21-000-031-DN-A1-05102.

4.7 Author Contributions

S.L.W. and T.S.G. conceived the study. S.L.W. designed experiments with the input of T.S.G. S.L.W. carried out experiments. D. R. assisted in the collection of worms, and the preparation and termination of experiments. M.J.R. and K.V.T. conducted chemical analyses. S.L.W. performed statistical analyses. T.S.G. helped in the interpretation of data and preparation of the manuscript. S.L.W. wrote the manuscript, with contribution from all authors.

4.8 Additional Information

The authors declare no competing financial interests.

4.9 Tables

Table 1. The nicotine dose delivered to: a human smoker; a ragworm following 96 h exposure to smoked cigarette filter toxicants in seawater; and a ragworm following short- and long-term exposure to smoked cigarette filter microfibres in sediment, at equivalent concentrations (1 cigarette/filter L^{-1}).

Organism	Dose delivered (1 cigarette)	Dose accumulated (1 cigarette)	Nicotine exposure relative to humans
Human (smoking; 1 cigarette)	10-30 µg kg^{-1}	10-50 ng ml^{-1}	-
Ragworm (96 h exposure to smoked cigarette filter toxicants in seawater)	63 µg kg^{-1}	4912 ng g^{-1}	98x
Ragworm (96 h sediment exposure to smoked cigarette filter microfibres)	1400 µg kg^{-1}	374 ng g^{-1}	7.5x
Ragworm (28 d sediment exposure to smoked cigarette filter microfibres)	1000 µg kg^{-1}	129 ng g^{-1}	2.6x

4.10 Figures

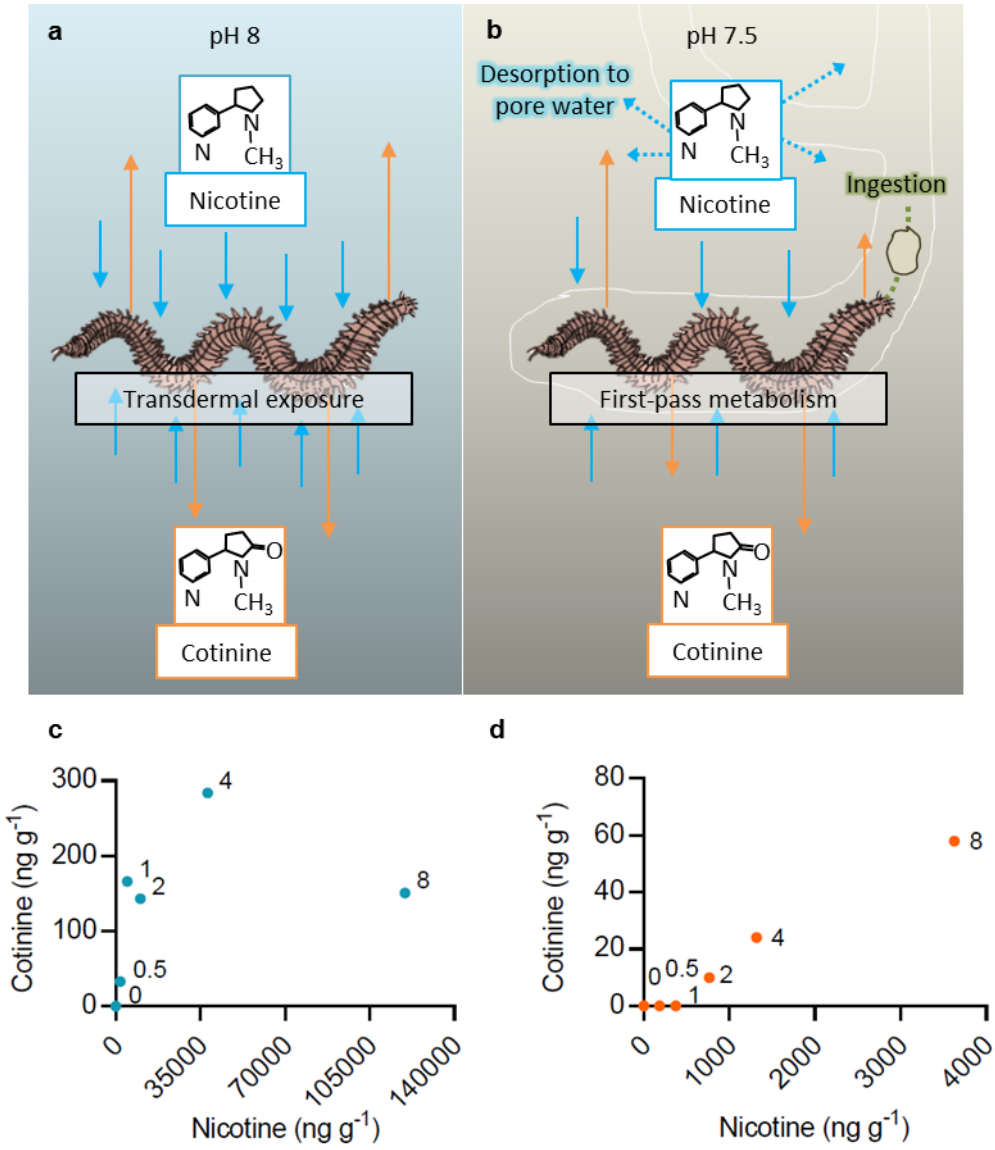


Figure 1. The bioaccumulation of nicotine in ragworms. The potential routes of nicotine transfer to ragworms from smoked cigarette filter a) toxicants in seawater, and b) microfibres in sediment. The bioconcentration and bioaccumulation of nicotine and cotinine for ragworms following 96 h exposure to smoked cigarette filter c) toxicants in seawater, and d) microfibres in sediment.

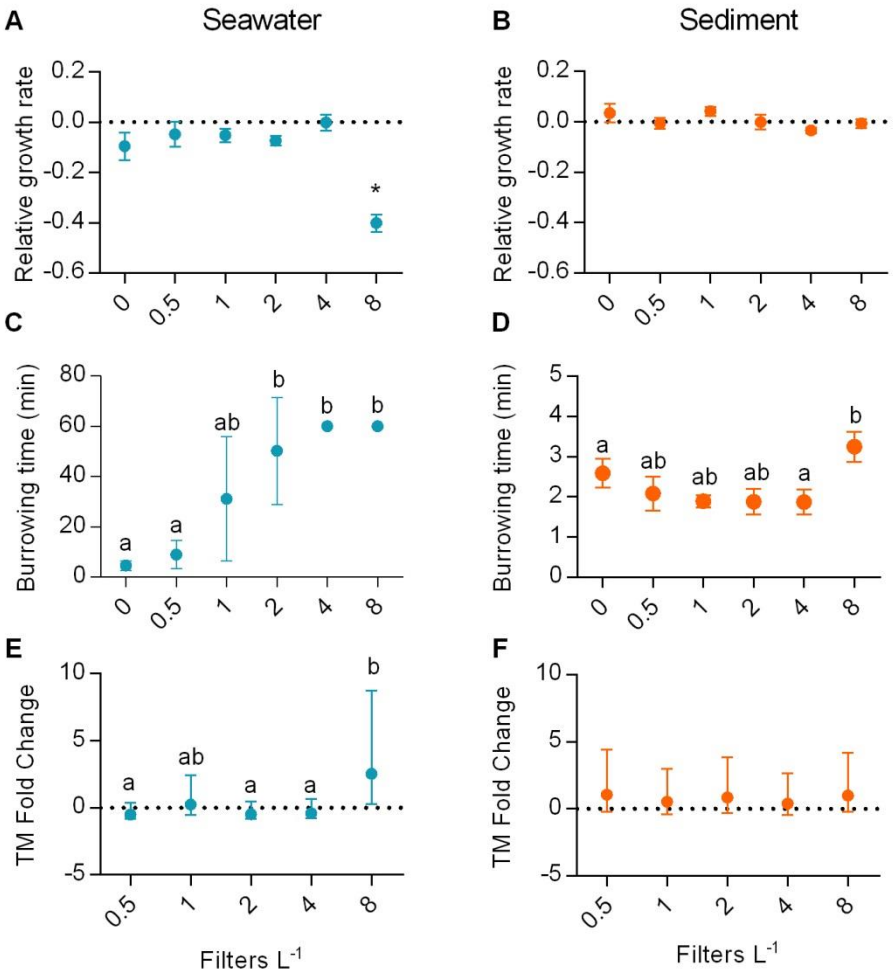
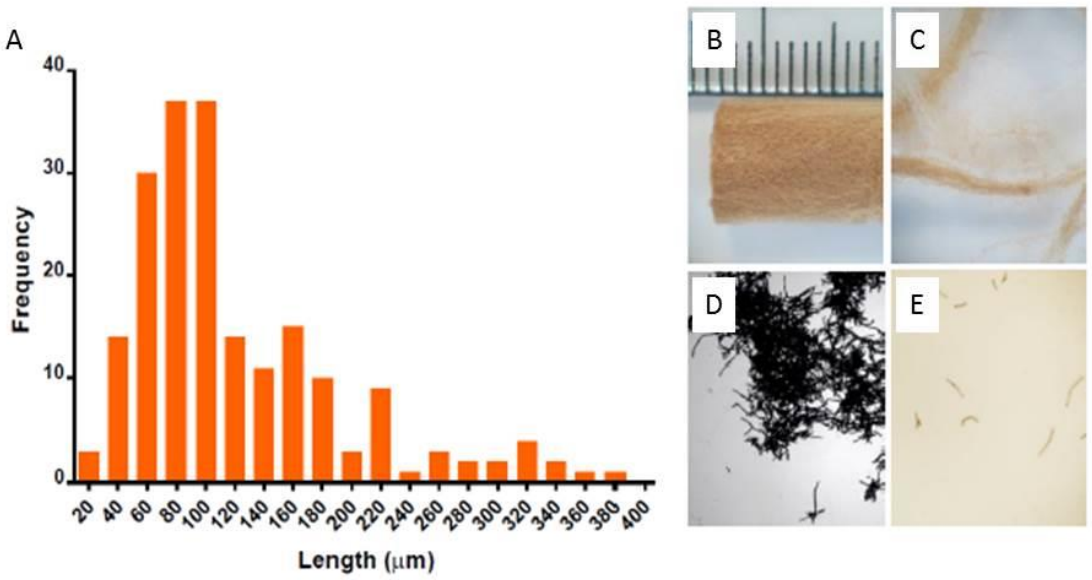


Figure 2. The biological impacts of smoked cigarette filter exposure on ragworms. The effect of 96 h exposure to smoked cigarette filter A) toxicants in seawater, and B) microfibres in sediment on the relative growth rate (RGR) of ragworms (mean ± s.e.m.). The effect of 96 h exposure to smoked cigarette filter C) toxicants in seawater, and D) microfibres in sediment on the burrowing time (minutes) of ragworms (mean ± s.e.m.). The effect of 96 h exposure to smoked cigarette filter E) toxicants in seawater, and F) microfibres in sediment on DNA damage in ragworms, measured as fold-change in the 90th percentile tail moment relative to control ragworms (indicated by the dotted line, mean ± s.e.m.). Significance between groups is indicated by different letters. * denotes significance compared to all other groups.

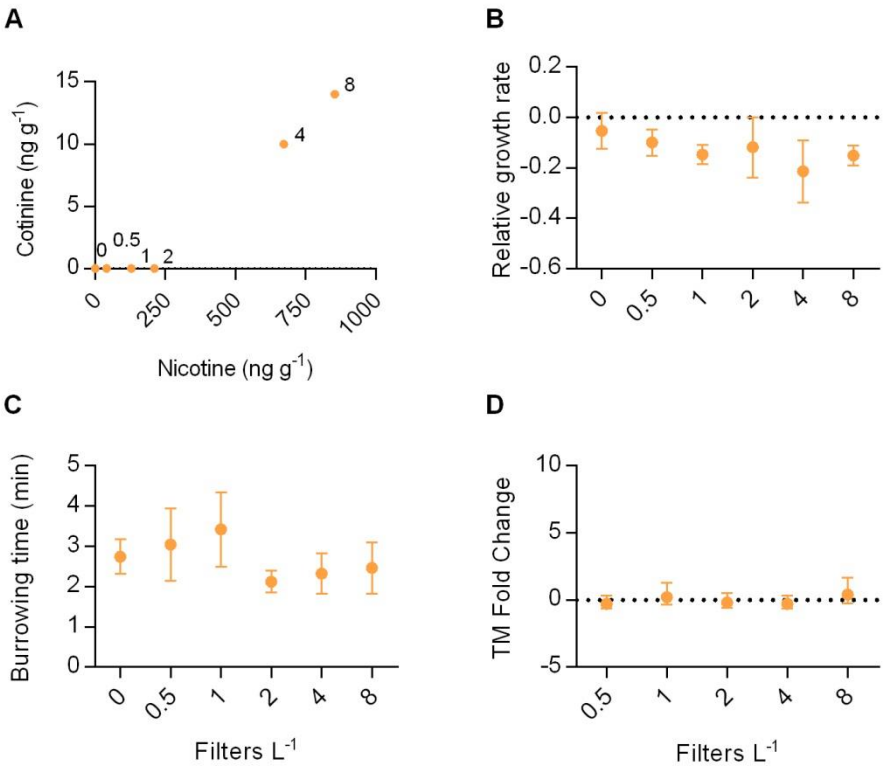
4.11 Supplementary Information

Supplementary Table 1. Chromatography and MS/MS detection parameters for the analysis of nicotine and metabolites

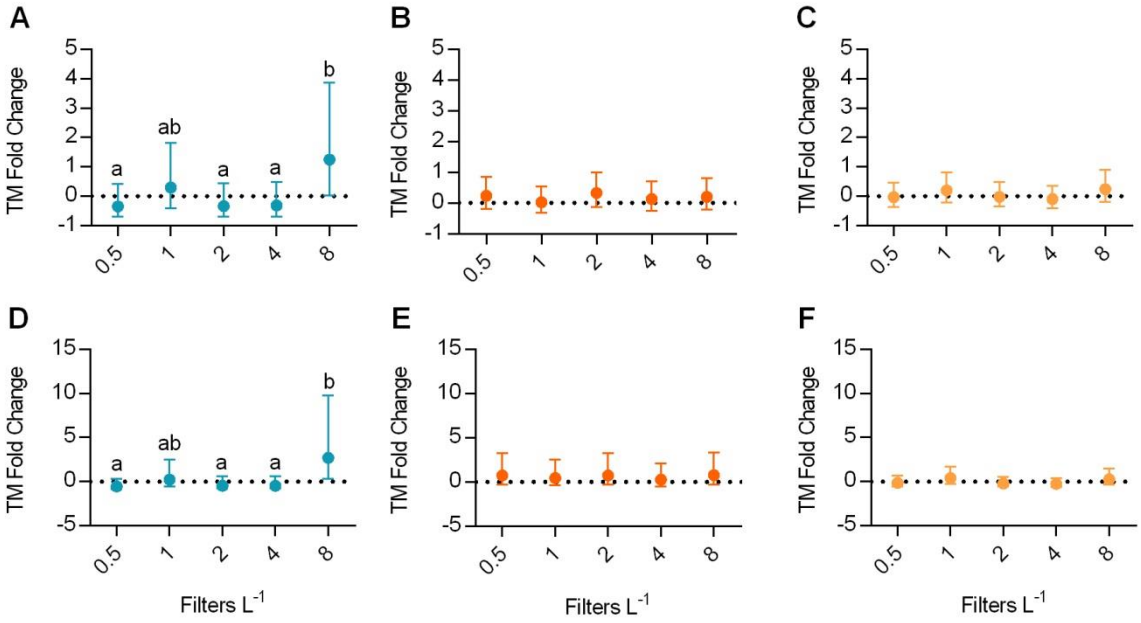
Chromatograhy Parameters		
Column	Waters Acquity BEH 2-EP column, 1.7 µm, 3x100 mm, at 50 °C	
Mobile phase	(A) Super-critical carbon dioxide	
	(B) methanol (0.5 % ammonia)	
Flow (Back-pressure)	1.0 mL/min (2000 PSI)	
Gradient	Initial 0.5 % phase (B) hold for 0.1 minutes then ramp up to reach 50 % (B) at t = 2.5 minutes. Hold to t = 3.0 minutes then step down to initial conditions at t = 3.01 minutes. Hold at initial conditions until completion. Total gradient program, t = 4.0 minutes.	
MS/MS Detection Parameters		
Compound	Precurser Ion > Fragment Ions (m/z)	Retention Time (mins)
Nicotine	163>106	1.93
	163>130	
Trans-3'-hydroxycotinine	193>80	2.16
	193>134	
Cotinine	177>80	2.06
	177>98	
Nicotine-D4	167>121	1.93
	167>134	
Cotinine-D3	180>80	2.06
	180>101	



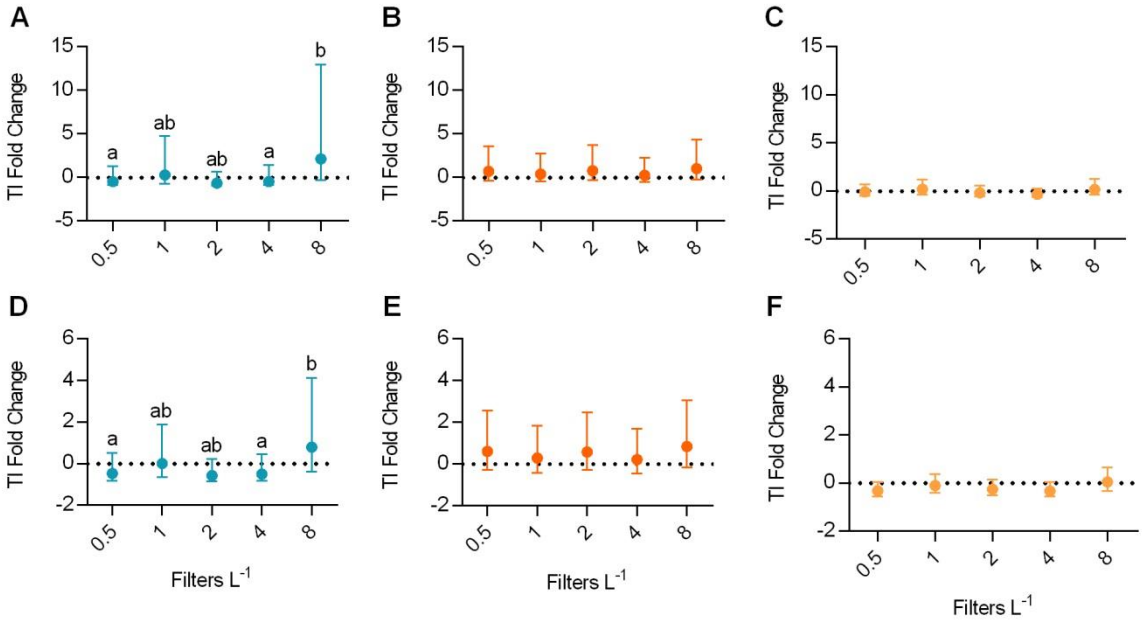
Supplementary Figure 1. a) The size-frequency distribution of stock smoked cigarette filter microfibers; b) An intact smoked cigarette filter free of excess tobacco and external paper; c) The fibrous nature of cigarette filters; d-e) micrographs of smoked cigarette filter microfibres following manufacture using liquid nitrogen.



Supplementary Figure 2. The biological impacts of smoked cigarette filter exposure (28 d) on ragworms. A) The bioconcentration and bioaccumulation of nicotine and cotinine for ragworms following 28 d exposure to smoked cigarette filter microfibres in sediment. The effect of 28 d exposure to smoked cigarette filter microfibres in sediment on a); b) the relative growth rate (RGR) of ragworms (mean ± s.e.m.); c) the burrowing time (minutes) of ragworms (mean ± s.e.m.); and d) on DNA damage in ragworms, measured as fold-change in the 90th percentile tail moment relative to control ragworms (indicated by the dotted line, mean ± s.e.m.).



Supplementary Figure 3. The impacts of smoked cigarette filter exposure on DNA damage (tail moment). The effect of 96 h exposure to smoked cigarette filter a) toxicants in seawater, b) microbfibres in sediment, and c) 28 d exposure to microfibres in sediment, on DNA damage in ragworms, measured as fold-change in the median tail moment relative to control ragworms (indicated by the dotted line, mean ± s.e.m.). The effect of 96 h exposure to smoked cigarette filter d) toxicants in seawater, e) microbfibres in sediment, and f) 28 d exposure to microfibres in sediment, on DNA damage in ragworms, measured as fold-change in the 75th percentile tail moment relative to control ragworms (indicated by the dotted line, mean ± s.e.m.). Significance between groups, as identified by post-hoc analysis, is indicated by different letters.



Supplementary Figure 4. The impacts of smoked cigarette filter exposure on DNA damage (tail intensity). The effect of 96 h exposure to smoked cigarette filter a) toxicants in seawater, b) microfibres in sediment, and c) 28 d exposure to microfibres in sediment, on DNA damage in ragworms, measured as fold-change in the 75th percentile tail intensity relative to control ragworms (indicated by the dotted line, mean ± s.e.m.). The effect of 96 h exposure to smoked cigarette filter d) toxicants in seawater, e) microbfibres in sediment, and f) 28 d exposure to microfibres in sediment, on DNA damage in ragworms, measured as fold-change in the 90th percentile tail intensity relative to control ragworms (indicated by the dotted line, mean ± s.e.m.). Significance between groups, as identified by post-hoc analysis, is indicated by different letters.

I.7.3 The Potential Impacts of Size

The potential main and interactive effects of the size (weight) of worms on all of the reported dependent variables were analysed, except for RGR as this value is relative to original size. Where the data did not meet the assumptions of a parametric factorial analysis e.g. two way ANOVA, a Kruskal Wallis test was applied; however, this only allowed for the analysis of a main effect.

There was no significant main or interactive effect of 96 h exposure to smoked cigarette filter leachates in seawater (Kruskal Wallis, p=0.105) and of 96 h (two way ANOVA, p=0.179 and p=0.984 for weight and weight*treatment, respectively) and 28 d (two way ANOVA, p=0.141 and p=0.329 for weight and weight*treatment, respectively) exposure to smoked cigarette filter microfibers (fibres) in sediment on burrowing time.

Following 96 h exposure to smoked cigarette filter leachates in seawater, there was no significant main or interactive effect of weight on the median TM of cells (two way ANOVA, p=0.443 and p=0.124 for weight and weight*treatment, respectively). Weight had no significant main or interactive effect on the 75th percentile TM (two way ANOVA, p=0.873 and p=0.06 for weight and weight*treatment, respectively). However, the data violates the assumptions of ANOVA as the residuals of the data do not follow a normal distribution. A Kruskal Wallis test shows there is no main effect of weight on the 75th percentile TM of cells (p=0.197). There was no significant main and interactive effect of weight on the 90th percentile TM (two way ANOVA, p=0.394 and p=0.333 for weight and weight*treatment, respectively).

Following 96 h exposure to smoked cigarette filter leachates in seawater, there was no significant main or interactive effect of weight on the median TI of cells (two way ANOVA, p=0.414 and p=0.110 for weight and weight*treatment, respectively). Weight had no significant main or interactive effect on the 75th percentile TI (two way ANOVA, p=0.817 and p=0.096 for weight and weight*treatment, respectively); however the data violates the assumptions of ANOVA due to heterogenous variances. A Kruskal Wallis test found no significant main effect of weight on the 75th percentile TI of cells (p=0.361).There was no significant main or interactive effect of weight on the 90th percentile TI of cells (two way ANOVA, p=0.562 and p=0.411 for weight and weight*treatment, respectively). However, the data violated the assumptions of ANOVA due to heterogenous variances and non-normally distributed residuals. A Kruskal Wallis test found no main effect of weight on the 90th percentile TI of cells (p=0.207).

Following 96 h exposure to fibres in sediment, there was no significant main or interactive effect of weight on the median TM of cells (two way ANOVA, p=0.132 and

p=0.708, for weight and weight*treatment, respectively). There was no significant main or interactive effect of weight on the 75th percentile TM of cells (two way ANOVA, p=0.246 and p=0.695 for weight and weight*treatment, respectively) or on the 90th percentile TM of cells (two way ANOVA, p= 0.349 and p=0.842 for weight and weight*treatment, respectively); however, this data violates the assumptions of ANOVA. A Kruskal Wallis test also found no significant main effect of weight on the 90th percentile TM of cells (p=0.798).

Following 96 h exposure to fibres in sediment, there was no significant main or interactive effect of weight on the median TI of cells (two way ANOVA, p=0.192 and p=0.808 for weight and weight*treatment, respectively). There was no significant main and interactive effects of weight on the 75th percentile TI of cells (two way ANOVA, p=0.284 and p=0.812 for weight and weight*treatment, respectively) or on the 90th percentile TI of cells (two way ANOVA, p=0.435 and p=0.975 for weight and weight*treatment, respectively).

Following 28 d exposure to fibres in sediment, there was no significant main or interactive effect of weight on the median TM of cells (two way ANOVA, p=0.901 and p=0.346 for weight and weight*treatment, respectively), the 75th percentile TM of cells (two way ANOVA, p=0.632 and p=0.391 for weight and weight*treatment, respectively) and the 90th percentile TM of cells (two way ANOVA, p=0.833 and p=0.669 for weight and weight*treatment, respectively.

A 28 d exposure to fibres in sediment had no significant main effect on the median TI of cells (two way ANOVA, p=0.704 and p=0.751 for weight and weight*treatment, respectively); however, the data did not conform to the assumptions of ANOVA as the residuals did not follow a normal distribution. A Kruskal Wallis test found no significant main effect of weight on the median TI of cells (p=0.402). Weight had no significant main or interactive effect on the 75th percentile TI (two way ANOVA, p=0.839 and p=0.605 for weight and weight*treatment, respectively), however the residuals of the data did not follow a normal distribution. A Kruskal Wallis test found no significant main effect of weight on the 75th percentile TI of cells (p=0.300). Additionally, there was no significant main or interactive effect of weight on the 90th percentile TI (two way ANOVA, p=0.976 and p=0.707 for weight and weight*treatment, respectively);

however the residuals of the data did not follow a normal distribution. A Kruskal Wallis test found no significant main effect of weight on the 90^{th} percentile TI of cells ($p=0.276$).

Chapter 5

General Discussion

General Discussion

The contamination of the environment by discarded plastic debris is a global health issue. Plastics can break down to microscopic fragments (microplastics), enhancing the potential for entry of microplastics, their endogenous additives, and their adsorbed Persistent Organic Pollutants into the food web. The potential multi-stressor impacts of microplastics due to both particle and chemical toxicity, combined with their ubiquity in marine sediments, raises concern for the vulnerability of benthic marine invertebrates and ultimately the food chain to this novel pollutant. Little is known of how these species process microplastics and whether interactions with this novel substrate result in biological harm. Thus it is important to assess the potential harm microplastics present to this component of marine ecosystems. The primary objectives of this thesis, focussing on benthic polychaete worms, were to: 1) assess whether particle toxicity arises from the presence and ingestion of chemical-free microplastics; 2) determine whether microplastics can transfer endogenous chemical additives at concentrations capable of eliciting a response; and 3) address whether microplastics act as a vector for adhered Persistent Organic Pollutants (POPs), inducing chemical toxicity.

5.1 Summary of Findings

5.1.1 Chapter 1

A thorough review of the literature up to July 2011 (Wright et al., 2013b) found the following key knowledge gaps:

Areas for future research.
• The destination of ingested microplastics within marine invertebrates in addition to potential adverse effects remains unknown, emphasising a need for laboratory studies focussing on the physical impacts of microplastics (see Chapter 2)
• Given the occurrence of different shapes and plastic types in the marine environment, research into the impacts of these factors on marine organisms should be conducted

- The bioavailability of constituent contaminants is undetermined. This highlights a requirement for further laboratory studies to establish the effects of ageing on the concentration of microplastic additives, their bioavailability and the associated toxicological impacts (see Chapter 3)
- The role of microplastics as a vector for environmental POPs is uncertain. Laboratory studies investigating the bioavailability and associated toxicological impacts of microplastic-associated POPs are required (see Chapter 4)
- There are presently no conclusive reports on the transfer of microplastics to higher trophic levels and whether they act as a vector for contaminants. Studies are needed to understand the capacity for microplastics and their associated contaminants to be transported along marine food webs via trophic interactions as well as an estimation of population and ecosystem level impacts.

5.1.2 Chapter 2

To investigate the potential particle toxicity of microplastics, irrespective of their endogenous or adsorbed chemical pollutants, the benthic polychaete worm *Arenicola marina* (lugworms) was exposed to microplastics within sediment for short and long durations. Microplastics have been reported throughout the top 100 cm of sediment (Fisner et al., 2013), thus lugworms were exposed to a homogenous mix of microplastics throughout the total test sediment volume. Since microplastic ingestion has previously been reported in lugworms, it was assumed that exposure would primarily occur via ingestion during normal feeding behaviour.

These laboratory studies for the first time showed that clean, chemical-free microplastics are capable of causing biological harm in an ecologically-important marine invertebrate, at concentrations representing environmental and worst-case scenarios. High concentrations (5% by WW of the sediment) reduced the feeding activity of lugworms, indicating an adverse response. Further investigation found this was attributed to the plastic itself and not the secondary effect of decreased food concentration. Suppressed feeding activity may decrease energy assimilation, compromising fitness. It could also decrease bioturbation and therefore oxygenation of the sediment, which is crucial for maintaining infaunal diversity.

Lower concentrations (≥1% by WW of the sediment), overlapping those reported for impacted sites in the environment (Baztan et al., 2014; Carson et al., 2011), limited the lugworms ability to store energy. The space occupied by the low-nutritional microplastics in the gut may inhibit energy assimilation and therefore compromise somatic maintenance and growth, maturity and reproduction, with repercussions at the population level.

Long-term exposure also caused an inflammatory response, although this was not dose-responsive, indicating potential particle toxicity. This chapter of work has furthered our understanding of microplastic impacts by assessing the effects associated with the physical presence and ingestion of microplastics as a novel substrate at the physiological, cellular, and biochemical level.

5.1.3 Chapter 3

It has been predicted that microplastic ingestion increases marine invertebrate exposure to chemical additives, however experimental and/or model-based evidence is lacking. To establish whether chemical additives in plastic are bioavailable upon ingestion of microplastics, and whether the bioaccumulation of chemical additives occurs at a level capable of causing toxicological harm, lugworms were exposed (4 weeks) to plasticised (diethylhexyl phthalate (DEHP), 50% by weight of the plastic) microplastics (polyvinylchloride (PVC), 130 µm) within sediment (see Chapter 3). This included a microplastic control (unplasticised microplastics) and an aged microplastic treatment (plasticised microplastic), to accurately reflect the level of additive contamination of microplastics encountered in the marine environment.

Lugworms accumulated concentrations of phthalates >70% greater, on average, than in lugworms exposed to control microplastics. Lugworms exposed to plasticised microplastics showed inhibited mucus production; significantly fewer individuals produced mucus, and the overall amount of mucus produced was 70% lower than that produced by lugworms exposed to control microplastics. Mucus provides microbial protection and keeps the epidermis moist, enabling it to function as a respiratory surface. Inhibited mucus production therefore threatens the overall survivability of lugworms.

Oxygen consumption was 30% greater, on average, in lugworms exposed to plasticised microplastics than in lugworms exposed to control microplastics. This suggests DEHP could increase lugworm metabolism, impacting overall energy budgets. Lipid content also increased by >30% which may be analogous to the onset of adipogenesis, since DEHP is a recognised obesogen in vertebrates. The results in this chapter emphasise the potential for ingested microplastics to transfer endogenous additives to surrounding tissues in wildlife, and highlight the potential entry route for plastics and associated endocrine disrupting chemicals (EDCs) into the aquatic food web.

5.1.4 Chapter 4

Microplastics concentrate a high concentration of priority POPs, which can transfer to tissues upon ingestion. Currently, the most commonly reported litter item on beaches is smoked cigarette filters (filters), which are comprised of the bioplastic cellulose acetate. Despite their prevalence and evidence for the concentration of POPs and other toxicants on them, no study has assessed the role of filters as both a vector for POPs/toxicants and as a source of microfibres in the marine environment. Focussing on the benthic polychaete *Hediste diversicolor* (ragworm), laboratory studies were performed to determine the impacts of filter toxicants in seawater and microfibres in sediment.

When exposed to leached toxicants in seawater, ragworms exhibited adverse responses. Burrowing time into clean sediment – a key behavioural marker – was prolonged. The burrowing behaviour of worms, including ragworms, is central to their role as ecosystem engineers, reworking and aerating sediment to allow other organisms to thrive. Thus, exposure to leached toxicants via filter debris presents a risk to ecosystem health through its detrimental neurotoxic effects on worms.

The highest concentrations of filter leachates in seawater also elicited >30% weight loss compared to control ragworms, indicating that important Darwinian fitness parameters such as growth and reproduction are being compromised, with repercussions at higher levels of biological organisation. Ragworms exposed to the highest concentrations of filter leachates in seawater exhibited >2-fold increase in DNA damage compared to ragworms kept in control conditions, linked to oxidative stress.

Nicotine and its metabolite cotinine were employed as biomarkers of exposure. Nicotine bioconcentration factors (BCFs) were 500 fold higher from seawater than from sediment. We therefore linked the toxicity of filters to the bioavailability of nicotine, providing evidence to suggest that sedimentary exposure to filter microfibres reduced BCFs and toxicity of this harmful compound. The results of this chapter show that organisms exposed to leached toxicants from smoking debris via the water column are considered vulnerable to this form of marine debris, providing a first step towards setting guidance limits to curb smoking-related bioplastic debris.

5.2 Environmental and Human Health Implications

Microplastic concentrations reaching 3% (≤1 mm) by weight (Carson et al., 2011) and up to 10% by weight:volume (≤5 mm) (Baztan et al., 2014) have been reported for highly impacted beaches. The overlap of concentrations between those used in the current studies and those found in the environment indicate that benthic invertebrates occupying impacted sites may be suffering detrimental health impacts. Lugworms feed by ingesting large volumes of sediment, extracting the organic content (Zebe and Schiedek, 1996). Many other benthic marine invertebrates adopt similar deposit feeding modes, such as sea cucumbers, crustaceans, and bivalve molluscs. The limited selectivity these organisms exert means they are also likely to ingest microplastics if encountered *in situ*. The physical impacts of microplastic ingestion therefore threaten the benthic community.

In the current thesis, the model microplastic was PVC (plasticised or unplasticised). This was selected as PVC is denser than seawater and sinks out of suspension to sediments; >25% of microplastics sampled from sediments inhabited by lugworms were PVC (Browne et al., 2010). Plastic can leach constituent hazardous monomers. Polyurethanes, PVC, epoxy resins, and styrenic polymers have been identified as plastics of the greatest concern in terms of environmental and health effects, as their monomers are classified as carcinogenic, mutagenic, or both (Lithner et al., 2011). This could explain the biological impacts observed in the absence of endogenous additives and adsorbed POPs. Additionally, particle toxicity of the microplastics themselves could arise. Plastic particles elicit immunotoxicological responses (Avio et al., 2015; Nel

et al., 2006; von Moos et al., 2012), and can cause cell death (Nel et al., 2006). This has been studied extensively from a nanomedicine perspective, which may lend an understanding of the potential particle toxicity of microplastics once internalized.

A range of polymer types contaminate the marine environment. It is important to assess the toxicity of different polymer types ranked highly-hazardous due to their constituent monomers. All polymer types, even those which are positively buoyant, are anticipated to sink out and accumulate in the seabed, due to increased density from biofouling (Lobelle and Cunliffe, 2011). Additionally, a variety of shapes and sizes are also encountered. Such characteristics are likely to influence the level of harm induced. For example, the ingestion of smaller size classes may lead to engulfment by cells via endocytosis or pinocytosis, and subsequent translocation to the circulatory system (Browne et al., 2008). This may result in tissue localization (Farrell and Nelson, 2013).

Microplastics are a multistressor due to both the particle and chemical toxicity which may arise (Rochman, 2013). Plastic and microplastic debris has the potential to act as a vector for sorbed POPs and other contaminants to the surrounding seawater (see Chapter 4). Whilst the sorption of ambient POPs to clean microplastics may reduce their bioavailability (Chua et al., 2014), the leaching of POPs from contaminated microplastics can cause toxicity (Nobre et al., 2015). The primary route for POP exposure via microplastics is anticipated to be through desorption to tissues following ingestion; however, we show that leaching to seawater and subsequent dermal absorption is another plausible route for the transfer of POPs and other toxicants via plastic and microplastic vectors. This is in agreement with Nobre et al. (2015). Microplastics may therefore transport and leach POPs to otherwise uncontaminated areas. Bioconcentration factors (BCFs) for nicotine – the marker of exposure – were 500-fold higher from seawater than from sediment, highlighting the potential for microplastics to increase the body burden of POPs in marine organisms at a level capable of causing harm. The complex role of microplastics in enhancing or reducing the bioavailability of environmental POPs is an area requiring further research.

In addition to POPs, chemical additives have been reported on the surface of microplastics in both seawater and sediment samples, with indication that the

additives originated from the microplastics as opposed to sorption from the environment (Fries et al., 2013; Teuten et al., 2009). Whilst at least one laboratory study has explored the transfer of adsorbed chemical additives to tissues upon microplastic ingestion (Browne et al., 2013), Chapter 3 is the first study to consider the potential transfer of truly endogenous chemical additives over longer-term exposures.

Through collaboration with Plymouth University, it was possible to manufacture plastic, plasticised with a known amount of internalised additive (diethylhexyl phthalate (DEHP)). The subsequent aging of the manufactured plastic accurately reflects the plastic which occurs in the environment; studies by Plymouth University (unpublished) found approximately 30% of plasticiser is lost during the initial 24 h in seawater, following which desorption remained relatively stable. This was believed to be due to the rapid loss of surface-associated plasticiser, with approximately 70% remaining internalised. Thus a slow migration from core to surface is anticipated; however, the digestive environment of many marine invertebrates may facilitate the leaching of chemical contaminants (Voparil and Mayer, 2000; Weston and Mayer, 1998). This permitted an extremely novel study, which found lugworms accumulated phthalates in their tissues at concentrations (78.8 DEHP µg g^{-1} WW), inducing adverse biological responses. This confirms the potential for ingested microplastics to transfer endogenous additives at concentrations capable of causing harm.

Consequently, microplastics represent a source of hazardous POPs and additives to marine food webs. A key area requiring further study is whether biomagnification of chemicals introduced via ingested microplastics occurs in marine organisms, given the risk this presents to seafood and food security. Further studies into the trophic transfer and bioaccumulation of microplastics and their associated chemical contaminants will determine the threat microplastics pose to human dietary exposure.

Many of the species which have been observed to ingest microplastics – both *in situ* and in laboratory studies – are lower trophic species with important ecosystem roles. Polychaete worms, for example, are recognized ecosystem engineers which conduct key ecological processes via their burrowing and deposit-feeding activity, influencing the physical, chemical and biological properties of sediment. In turn, this creates an environment which supports a network of infaunal inhabitants. Other species known

to ingest microplastics are also recognized as ecosystem engineers, such as bivalve molluscs. The potential physical and chemical risks associated with microplastic ingestion therefore have the capacity to disrupt the physiology and behavior of ecosystem engineers. At heavily contaminated sites, such as beaches located in ocean currents, there may be repercussions for the infaunal community, resulting in decreased biodiversity and overall ecosystem health.

Moreover, the species which are vulnerable to microplastic ingestion also form important trophic links in marine food webs. Thus, there is potential for microplastics to impact the health of important prey species, which could disrupt the food web and lead to a decline in higher trophic species. Additionally, prey species represent a transfer pathway for microplastics and their associated chemical contaminants; particle and chemical toxicity could establish similar effects up the food chain. There is some *in situ* evidence to suggest microplastics are being transferred along food chains e.g microplastics have been found in fur seal and hooker sea lion scat, attributed to a fish vector. However, there is currently a lack of controlled laboratory studies to show this, thus the level of microplastic bioaccumulation is unknown.

Since microplastics can transfer endogenous additives and adhered POPs, their bioaccumulation up the food chain may result in increased body burdens of these chemicals. Most plastic-associated additives and POPs are ubiquitous in aquatic habitats, but the high concentrations of additives and POPs associated with microplastics may create a gradient, allowing for transfer and thereby presenting a significant exposure pathway. Many of the pollutants associated with microplastics are known endocrine disruptors. Thus the exposure to and bioaccumulation of such pollutants could affect reproduction, altering the timing of reproductive events or skewing the sex ratio of populations. However, the impacts of microplastics and their associated pollutants on higher trophic levels currently remain unknown.

Table 1. Areas for future research.

Areas for Future Research
The mechanisms and extent of microplastic translocation, and therefore bioaccumulation, in relation to size/shape/polymer type.

The bioaccumulation of microplastics will enhance the time-window for the leaching and desorption of additives and POPs to occur. The capacity for microplastics to lodge internally or translocate will greatly enhance this. The translocation of microplastics across cell membranes and epithelial layers, and the subsequent redistribution of microplastics in the body is an area deserving of further research.
The particle toxicity of micro- and nanoplastics. Micro and nanoplastics have the capacity to induce immunological responses. They may be internalised in cells and tissues via endocytosis or pinocytosis, which can be influenced by size, shape, and surface chemistry. The localization of a foreign particle can subsequently cause inflammation. Whilst the immunotoxicology of nanoplastics has been studied extensively from a therapeutic perspective, little is known as to the particle toxicity which may arise from the micro- and nanoplastics which occur in and are exposed to the marine environment i.e. plastic which was never intended for animal and human exposure.
The role of microplastics in enhancing or reducing the bioavailability of environmental POPs. Microplastics are believed to be a vector for POPs which have sorbed from the ambient environment. This may happen due to the ingestion of contaminated microplastics and subsequent release of sorbed POPs or due to the desorption of POPs to the surrounding environment and subsequent exposure via the environmental medium. Further study is therefore required to understand the relative importance of each exposure route.
The transfer and bioaccumulation of different polymer/additive combinations, especially in marine organisms for human consumption. Specific polymer-additive combinations are considered hazardous to human health. Additionally, different polymer-additive combinations have different rates of leaching. It is therefore necessary to determine the level of transfer and bioaccumulation of those additives predicted to have rapid rates of leaching and be hazardous to human health to organisms destined for human consumption. This will contribute towards determining the microplastic contribution to human body burdens of plastic-associated contaminants.
The potential for microplastics to increase POP/additive body burdens in marine organisms, at what magnitude, and at what biological cost. Controlled laboratory and environmental studies exploring the transfer of additives and POPs from microplastics to marine organisms in comparison to environmental and dietary pathways

will provide an understanding of the relative importance of microplastics as a vector for contaminants.

The occurrence of microplastics in seafood for human consumption (Mathalon and Hill, 2014; Van Cauwenberghe and Janssen, 2014) warrants further studies concerning the potential human health effects. Plastic is already of concern with regards to human health, primarily due to the additives it harbours. However, it is difficult to discern between contaminant exposure and bioaccumulation which stems from food or from environmental sources. An essential factor is whether plastic additives transfer to marine organisms, as indicated in this thesis. It could be assumed that additive transfer from ingested microplastics to marine organisms for human consumption is occurring, resulting in possible exposure through seafood; however this requires further investigation.

The global issue of microplastics is only likely to worsen. This is due to the continual degradation of the legacy of plastic litter already contaminating the marine environment. Additionally, high plastic production volumes, rapid use and disposal, and a current lack of legislation to prevent plastic loss from land suggests there is no active remediation being exercised. The widespread distribution of microplastics means that their impact on food webs and ecosystems will not be limited to their sources; they are a global problem, effecting all marine habitats.

It is difficult to estimate the relative importance of microplastics as an environmental stressor in comparison to others such as global warming, ocean acidification, and the increased occurrence of hypoxic zones. One problem is that the current methods employed to identify and quantify microplastics are limited by size and are therefore underestimating microplastic abundance. Additionally, there are few studies which quantify the level of harm microplastics present to marine organisms, and what that harm might be. The most pressing issue facing the marine environment cannot be linked to a single stressor, but the suite of perturbations it faces, the combination of which may elicit a greater effect than any alone.

This thesis presents evidence for the potential harm microplastics can cause in the marine environment. It shows that particle toxicity can arise following microplastic

ingestion, that microplastics have the capacity to increase body burdens of endogenous additives, causing harm; and that microplastics can carry and leach adsorbed chemical contaminants, rendering them bioavailable and toxic. It is now imperative to determine the contribution of microplastics to contaminant burdens, not only in marine organisms, but in humans due to seafood consumption, in relation to health biomarkers (see Figure 6.1). Future research could also consider microplastics in a mulit-stressor design to understand their impact in combination with other global marine issues. Ultimately, new laws to ensure responsible handling of plastics during recycling and disposal should be considered, whilst smarter material choices and conscientious consumer attitudes should be encouraged.

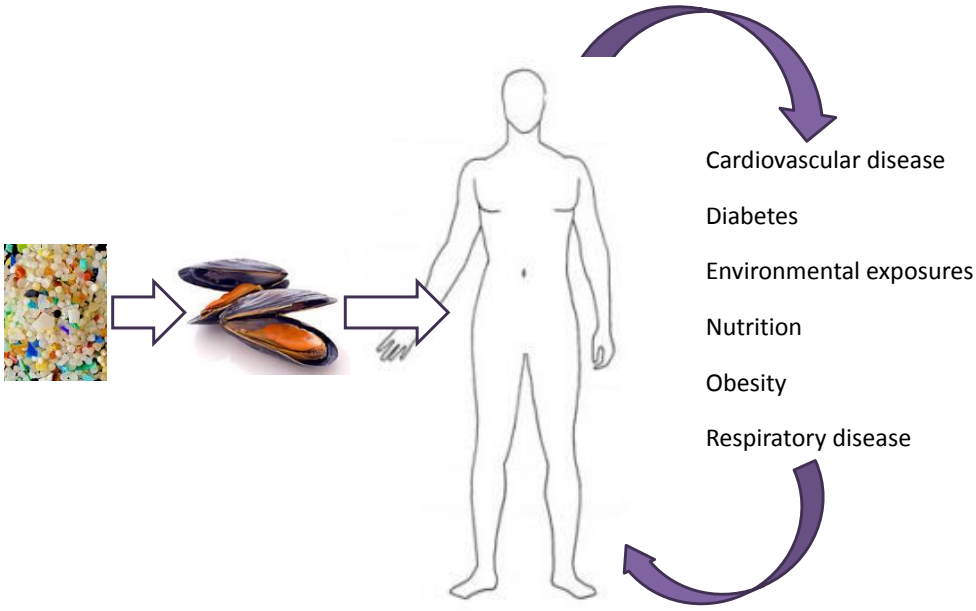


Figure 6.1 The potential for microplastics to contribute to human contaminant exposure through seafood consumption and related health effects.

Bibliography

Ahrens, J. M., Hertz, J., Lamoureux, M. E., Lopez, R. G., Mcelroy, E. A., and Brownawell, J. B. 2001. The role of digestive surfactants in determining bioavailability of sediment-bound hydrophobic organic contaminants to 2 deposit-feeding polychaetes. Marine Ecology Progress Series. 212, 145 – 157.

Ahrens, M.J. and Lopez, G. R. 2001. *In vivo* characterization of the gut chemistry of small deposit-feeding polychaetes, in: Aller, J.Y., Woodin, S. A., and Aller, R. C. (Ed.), Organism-sediment interactions. University of South Carolina Press, Columbia, S.C.

Andrady, A.L., 2011. Microplastics in the marine environment. Marine Pollution Bulletin. 62 (8), 1596 – 1605.

Artham, T. and Doble, M. 2009. Fouling and Degradation of Polycarbonate in Seawater: Field and Lab Studies. Journal of Polymers and the Environment. 17, 170-180.

Avio, C.G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., d'Errico, G., Pauletto, M., Bargelloni, L., and Regoli, F. 2015. Pollutants bioavailability and toxicological risk from microplastics to marine mussels. Environmental Pollution. 198, 211-222.

Azeez, O.I., Meintjes, R., and Chamunorwa, J.P. 2014. Fat body, fat pad and adipose tissues in invertebrates and vertebrates: the nexus. Lipids in Health and Disease. 13, 71-71.

Bagel, S., Dessaigne, B., Bourdeaux, D., Boyer, A., Bouteloup, C., Bazin, J.E., Chopineau, J., and Sautou, V. 2011. Influence of lipid type on bis (2-ethylhexyl)phthalate (DEHP) leaching from infusion line sets in parenteral nutrition. Journal of Parenteral and Enteral Nutrition. 35, 770-775.

Bakir, A., Rowland, S.J., and Thompson, R.C. 2014. Enhanced desorption of persistent organic pollutants from microplastics under simulated physiological conditions. Environmental Pollution. 185, 16-23.

Ballinger, G.A., 2004. Using generalized estimating equations for longitudinal data analysis. Organizational Research Methods. 7, 127-150.

Barnes, D.K.A., Galgani, F., Thompson, R.C., and Barlaz, M. 2009. Accumulation and fragmentation of plastic debris in global environments. Philosophical Transactions of the Royal Society B: Biological Sciences. 364, 1985-1998.

Baztan, J., Carrasco, A., Chouinard, O., Cleaud, M., Gabaldon, J.E., Huck, T., Jaffrès, L., Jorgensen, B., Miguelez, A., Paillard, C., and Vanderlinden, J.P. 2014. Protected areas in the Atlantic facing the hazards of micro-plastic pollution: First diagnosis of three islands in the Canary Current. Marine Pollution Bulletin. 80, 302-311.

Bejgarn, S., MacLeod, M., Bogdal, C., and Breitholtz, M. 2015. Toxicity of leachate from weathering plastics: An exploratory screening study with *Nitocra spinipes*. Chemosphere. 132, 114-119.

Benowitz, N.L., Hukkanen, J., and Jacob, P. 2009. Nicotine chemistry, metabolism, kinetics and biomarkers. Handbook of Experimental Pharmacology. 192, 29-60.

Berens, A.R. 1997. Predicting the migration of endocrine disrupters from rigid plastics. Polymer Engineering & Science. 37, 391-395.

Bernal, C.A., Martinelli, M.I., and Mocchiutti, N.O. 2002. Effect of the dietary exposure of rat to di(2-ethyl hexyl) phthalate on their metabolic efficiency. Food Additives and Contaminants. 19, 1091-1096.

Besseling, E., Wegner, A., Foekema, E.M., van den Heuvel-Greve, M.J., and Koelmans, A.A. 2012. Effects of microplastic on fitness and PCB bioaccumulation by the lugworm *Arenicola marina* (L.). Environmental Science & Technology. 47, 593-600.

Beukema, J.J., 1976. Biomass and species richness of the macro-benthic animals living on the tidal flats of the Dutch Wadden Sea. Netherlands Journal of Sea Research. 10, 236-261.

Bligh, E.G. and Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology. 37, 911-917.

Boas, M., Frederiksen, H., Feldt-Rasmussen, U., Skakkebaek, N.E., Hegedus, L., Hilsted, L., Juul, A., and Main, K.M. 2010. Childhood exposure to phthalates: associations with

thyroid function, insulin-like growth factor I, and growth. Environmental Health Perspectives. 118, 1458-1464.

Bright, J., Aylott, M., Bate, S., Geys, H., Jarvis, P., Saul, J., and Vonk, R. 2011. Recommendations on the statistical analysis of the Comet assay. Pharmaceutical Statistics. 10, 485-493.

Brown, D. and Thompson, S. 1982. Phthalates and the aquatic environment: Part I The effect of di-2-ethylhexyl phthalate (DEHP) and di-isodecyl phthalate (DIDP) on the reproduction of *Daphnia magna* and observations on their bioconcentration. Chemosphere. 11, 417-426.

Brown, D. and Thompson, R.S. 1982. Phthalates and the aquatic environment: Part II The bioconcentration and depuration of di-2-ethylhexyl phthalate (DEHP) and di-isodecyl phthalate (DIDP) in mussels (*Mytilus edulis*). Chemosphere. 11, 427-435.

Browne, M.A., Crump, P., Niven, S.J., Teuten, E., Tonkin, A., Galloway, T., and Thompson, R. 2011. Accumulation of microplastic on shorelines woldwide: sources and sinks. Environmental Science & Technology. 45, 9175-9179.

Browne, M.A., Dissanayake, A., Galloway, T.S., Lowe, D.M., and Thompson, R.C. 2008. Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). Environmental Science & Technology. 42, 5026-5031.

Browne, M.A., Galloway, T., and Thompson, R. 2007. Microplastic—an emerging contaminant of potential concern? Integrated Environmental Assessment and Management. 3, 559-561.

Browne, M.A., Galloway, T.S., and Thompson, R.C. 2010. Spatial patterns of plastic debris along estuarine shorelines. Environmental Science & Technology. 44, 3404-3409.

Browne, M.A, Niven, S.J., Galloway, T.S., Rowland, S.J., Thompson, R.C. 2013. Microplastic moves pollutants and additives to worms, reducing functions linked to health and biodiversity. Current Biology. 23, 2388-2392.

Buccafusco, J.J., Shuster, L.C., Terry, A.V., Jr. 2007. Disconnection between activation and desensitization of autonomic nicotinic receptors by nicotine and cotinine. Neuroscience Letters. 413, 68-71.

Buchanan, J.B. and Warwick, R.M. 1974. An estimate of benthic macrofaunal production in the offshore mud of the Northumberland coast. Journal of the Marine Biological Association UK. 54, 197-222.

Cadée, G.C. 1976. Sediment reworking by *Arenicola marina* on tidal flats in the Dutch Wadden Sea. Netherlands Journal of Sea Research. 10, 440-460.

Capowiez, Y. and Bérard, A. 2006. Assessment of the effects of imidacloprid on the behavior of two earthworm species (*Aporrectodea nocturna* and *Allolobophora icterica*) using 2D terraria. Ecotoxicology and Environmental Safety. 64, 198-206.

Capowiez, Y., Rault, M., Costagliola, G., Mazzia, C. 2005. Lethal and sublethal effects of imidacloprid on two earthworm species (*Aporrectodea nocturna* and *Allolobophora icterica*). Biology and Fertility of Soils. 41, 135-143.

Carpenter, E.J., Anderson, S.J., Harvey, G.R., Miklas, H.P., and Peck, B.B. 1972. Polystyrene spherules in coastal waters. Science. 178, 749-750.

Carson, H.S., Colbert, S.L., Kaylor, M.J., and McDermid, K.J. 2011. Small plastic debris changes water movement and heat transfer through beach sediments. Marine Pollution Bulletin. 62, 1708-1713.

Chen, J.Q., Brown, T.R., and Russo, J. 2009. Regulation of energy metabolism pathways by estrogens and estrogenic chemicals and potential implications in obesity associated with increased exposure to endocrine disruptors. Biochimica et Biophysica Acta. 1793, 1128-1143.

Chua, E.M., Shimeta, J., Nugegoda, D., Morrison, P.D., and Clarke, B.O. 2014. Assimilation of polybrominated diphenyl ethers from microplastics by the marine amphipod, *Allorchestes compressa*. Environmental Science & Technology. 48, 8127-8134.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J., and Galloway, T.S. 2013. Microplastic ingestion by zooplankton. Environmental Science & Technology. 47, 6646-6655.

Crawley, M.J., 2005. Statistics: An Introduction using R. Wiley.

Crowley-Weber, C.L., Dvorakova, K., Crowley, C., Bernstein, H., Bernstein, C., Garewal, H., and Payne, C.M. 2003. Nicotine increases oxidative stress, activates NF-kappaB and GRP78, induces apoptosis and sensitizes cells to genotoxic/xenobiotic stresses by a multiple stress inducer, deoxycholate: relevance to colon carcinogenesis. Chemico-biological Interactions. 145, 53-66.

Davies, J.M. and Viney, C. 1998. Water–mucin phases: conditions for mucus liquid crystallinity. Thermochimica Acta. 315, 39-49.

Davies, M.S., Hawkins, S.J., and Jones, H.D. 1992. Pedal mucus and its influence on the microbial food supply of two intertidal gastropods, *Patella vulgata* (L.) and *Littorina littorea* (L.). Journal of Experimental Marine Biology and Ecology. 161, 57-77.

De Coen, W.M. and Janssen, C.R. 1997. The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular Energy Allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. Journal of Aquatic Ecosystem Stress and Recovery. 6, 43-55.

De Witte, B., Devriese, L., Bekaert, K., Hoffman, S., Vandermeersch, G., Cooreman, K., and Robbens, J. 2014. Quality assessment of the blue mussel (*Mytilus edulis*): Comparison between commercial and wild types. Marine Pollution Bulletin. 85, 146-155.

Desdoits-Lethimonier, C., Albert, O., Le Bizec, B., Perdu, E., Zalko, D., Courant, F., Lesné, L., Guillé, F., Dejucq-Rainsford, N., and Jégou, B. 2012. Human testis steroidogenesis is inhibited by phthalates. Human Reproduction. 27 (5), 1451-9.

Dittbrenner, N., Triebskorn, R., Moser, I., and Capowiez, Y. 2010. Physiological and behavioural effects of imidacloprid on two ecologically relevant earthworm species (*Lumbricus terrestris* and *Aporrectodea caliginosa*). Ecotoxicology. 19, 1567-1573.

Duez, P., Dehon, G., Kumps, A., and Dubois, J. 2003. Statistics of the Comet assay: a key to discriminate between genotoxic effects. Mutagenesis. 18, 159-166.

Endo, S., Takizawa, R., Okuda, K., Takada, H., Chiba, K., Kanehiro, H., Ogi, H., Yamashita, R., and Date, T. 2005. Concentration of polychlorinated biphenyls (PCBs) in beached resin pellets: Variability among individual particles and regional differences. Marine Pollution Bulletin. 50, 1103-1114.

Faouzi, M.A., Dine, T., Gressier, B., Kambia, K., Luyckx, M., Pagniez, D., Brunet, C., Cazin, M., Belabed, A., and Cazin, J.C. 1999. Exposure of hemodialysis patients to di-2-ethylhexyl phthalate. International Journal of Pharmaceutics. 180, 113-121.

Farrell, P. and Nelson, K. 2013. Trophic level transfer of microplastic: *Mytilus edulis* (L.) to *Carcinus maenas* (L.). Environmental Pollution. 177, 1-3.

Fauchald, K. 1977. The Polychaete Worms., definitions and keys to the Orders, Families and Genera. Natural History Museum LA County Museum Science Series. 28, 1-190.

Fauchald, K. and Jumars, P.A. 1979. Diet of worms: a study of polychaete feeding guilds. Oceanography and Marine Biology. 17, 193-284.

Feige, J.N., Gelman, L., Rossi, D., Zoete, V., Métivier, R., Tudor, C., Anghel, S.I., Grosdidier, A., Lathion, C., Engelborghs, Y., Michielin, O., Wahli, W., and Desvergne, B. 2007. The endocrine disruptor monoethyl-hexyl-phthalate is a selective peroxisome proliferator-activated receptor γ modulator that promotes adipogenesis. Journal of Biological Chemistry. 282, 19152-19166.

Fisner, M., Taniguchi, S., Moreira, F., Bícego, M.C., and Turra, A. 2013. Polycyclic Aromatic Hydrocarbons (PAHs) in plastic pellets: Variability in the concentration and composition at different sediment depths in a sandy beach. Marine Pollution Bulletin. 70, 219-226.

Frias, J.P.G.L., Sobral, P., and Ferreira, A.M. 2010. Organic pollutants in microplastics from two beaches of the Portuguese coast. Marine Pollution Bulletin. 60, 1988-1992.

Fries, E., Dekiff, J.H., Willmeyer, J., Nuelle, M.-T., Ebert, M., and Remy, D. 2013. Identification of polymer types and additives in marine microplastic particles using

pyrolysis-GC/MS and scanning electron microscopy. Environmental Science: Processes & Impacts. 15, 1949-1956.

Galgani, F., Fleet, D., van Franeker, J., Katsanevakis, S., Maes, T., Mouat, J., Oosterbaan, L., Poitou, I., Hanke, G., Thompson, R., Amato, E., Birkun, A., and Janssen, C. 2010. Task Group 10 Report: Marine Litter, in: Zampoukas, N. (Ed.), Marine Strategy Framework Directive. JRC, Ifremer & ICES.

Gayathri, N.S., Dhanya, C.R., Indu, A.R., and Kurup, P.A. 2004. Changes in some hormones by low doses of di (2-ethyl hexyl) phthalate (DEHP), a commonly used plasticizer in PVC blood storage bags & medical tubing. The Indian Journal of Medical Research. 119, 139-144.

Gnaiger, E. 1983. Calculation of energetic and biochemical equivalents of respiratory oxygen consumption, in: Gnaiger, E., Forstner, H. (Eds.), Polarographic Oxygen Sensors. Springer, Berlin Heidelberg, 337-345.

Goldstein, M.C. and Goodwin, D.S. 2013. Gooseneck barnacles (Lepas spp.) ingest microplastic debris in the North Pacific Subtropical Gyre. PeerJ. 1, e184.

Goldstein, M.C., Rosenberg, M., and Cheng, L. 2012. Increased oceanic microplastic debris enhances oviposition in an endemic pelagic insect. Biology Letters. 8, 817-820.

Gosling, E., 2003. Bivalve molluscs: biology, ecology and culture. Blackwell Publishing.

Goss-Custard, J.D., Warwick, R.M., Kirby, R., McGrorty, S., Clarke, R.T., Pearson, B., Rispin, W.E., Dit Durell, S.E.A.Le V., Rose, R.J. 1991. Towards predicting wading bird densities from predicted prey densities in a post-barrage Severn Estuary. Journal of Applied Ecology. 28, 1004-1026.

Graham, E.R. and Thompson, J.T. 2009. Deposit- and suspension-feeding sea cucumbers (Echinodermata) ingest plastic fragments. Journal of Experimental Marine Biology and Ecology, 368, 22-29.

Gray, L.E., Ostby, J., Furr, J., Price, M., Veeramachaneni, D.N.R., and Parks, L. 2000. Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicological Sciences. 58, 350-365.

Hamer, J., Gutow, L., Kohler, A., and Saborowski, R. 2014. Fate of microplastics in the marine isopod *Idotea emarginata*. Environmental Science & Technology. 48, 13451-13458.

Healy, E.A. 1963. Mucous secretions of abarenicolid lugworms. Annals of the New York Academy of Sciences. 106, 444-450.

Herndl, G.J. and Peduzzi, P. 1989. Potential microbial utilization rates of sublittoral gastropod mucus trails. Limnology and Oceanography. 34, 780-784.

Hirai, H., Takada, H., Ogata, Y., Yamashita, R., Mizukawa, K., Saha, M., Kwan, C., Moore, C., Gray, H., Laursen, D., Zettler, E.R., Farrington, J.W., Reddy, C.M., Peacock, E.E., and Ward, M.W. 2011. Organic micropollutants in marine plastics debris from the open ocean and remote and urban beaches. Marine Pollution Bulletin. 62, 1683-1692.

Hon, N.-S. 1977. Photodegradation of cellulose acetate fibers. Journal of Polymer Science: Polymer Chemistry Edition. 15, 725-744.

http://www.dotmar.com.au/density.html.

Hu, F.B., Goldberg, J., Hedeker, D., Flay, B.R., and Pentz, M.A. 1998. Comparison of population-averaged and subject-specific approaches for analyzing repeated binary outcomes. American Journal of Epidemiology. 147, 694-703.

Huang, P.C., Kuo, P.L., Guo, Y.L., Liao, P.C., and Lee, C.C. 2007. Associations between urinary phthalate monoesters and thyroid hormones in pregnant women. Human Reproduction. 22, 2715-2722.

Hutchings, P. 1998. Biodiversity and functioning of polychaetes in benthic sediments. Biodiversity and Conservation. 7, 1133-1145.

Hymel, S.N. and Plante, C.J. 2000. Feeding and bacteriolytic responses of the deposit-feeder *Abarenicola pacifica* (Polychaeta: Arenicolidae) to changes in temperature and sediment food concentration. Marine Biology. 136, 1019-1027.

Ivar do Sul, J.A. and Costa, M.F. 2007. Marine debris review for Latin America and the Wider Caribbean Region: From the 1970s until now, and where do we go from here? Marine Pollution Bulletin. 54, 1087-1104.

Ivar do Sul, J.A. and Costa, M.F. 2014. The present and future of microplastic pollution in the marine environment. Environmental Pollution. 185, 352-364.

Ivar do Sul, J.A., Costa, M.F., Barletta, M., and Cysneiros, F.J. 2013. Pelagic microplastics around an archipelago of the Equatorial Atlantic. Marine Pollution Bulletin. 75, 305-309.

Jonker, M.T.O., Suijkerbuijk, M.P.W., Schmitt, H., and Sinnige, T.L. 2009. Ecotoxicological effects of activated carbon addition to sediments. Environmental Science & Technology. 43, 5959-5966.

Jurewicz, J., Radwan, M., Sobala, W., Ligocka, D., Radwan, P., Bochenek, M., Hawuła, W., Jakubowski, L., and Hanke, W. 2013. Human urinary phthalate metabolites level and main semen parameters, sperm chromatin structure, sperm aneuploidy and reproductive hormones. Reproductive Toxicology. 42, 232-241.

Karapanagioti, H.K., Endo, S., Ogata, Y., and Takada, H. 2011. Diffuse pollution by persistent organic pollutants as measured in plastic pellets sampled from various beaches in Greece. Marine Pollution Bulletin. 62, 312-317.

Knowles, C.O., McKee, M.J., and Palawski, D.U. 1987. Chronic effects of DI-2-ethylhexyl phthalate on biochemical composition, survival and reproduction of *Daphnia magna*. Environmental Toxicology and Chemistry. 6, 201-208.

Koelmans, A.A., Besseling, E., and Foekema, E.M. 2014. Leaching of plastic additives to marine organisms. Environmental Pollution. 187, 49-54.

Kondo, M., Nakata, J., Arai, N., Izumo, T., Tagaya, E., Takeyama, K., Tamaoki, J., and Nagai, A. 2012. Niflumic acid inhibits goblet cell degranulation in a guinea pig asthma model. Allergology International : official journal of the Japanese Society of Allergology. 61, 133-142.

Kristensen, E. 1988. Benthic fauna and biogeochemical processses in marine sediments: microbial activities and fluxes, in: Blackburn, T.H., Sorensen, J. (Eds.), Nitrogen in coastal marine environments. London, Wiley & Sons, 276-299.

Kristensen, E., Andersen, F.Ø., and Blackburn, T.H. 1992. Effects of benthic macrofauna and temperature on degradation of macroalgal detritus: The fate of organic carbon. Limnology and Oceanography. 37, 1404-1419.

Kwak, I.S. and Lee, W. 2005. Endpoint for DEHP exposure assessment in *Chironomus riparius*. Bulletin of Environmental Contamination and Toxicology. 74, 1179-1185.

Larsson, K., Ljung Björklund, K., Palm, B., Wennberg, M., Kaj, L., Lindh, C.H., Jönsson, B.A.G., and Berglund, M. 2014. Exposure determinants of phthalates, parabens, bisphenol A and triclosan in Swedish mothers and their children. Environment International. 73, 323-333.

Latini, G., Del Vecchio, A., Massaro, M., Verrotti, A., and De Felice, C. 2006. *In utero* exposure to phthalates and fetal development. Current Medicinal Chemistry. 13, 2527-2534.

Li, C., Chen, X., Zhang, P., Lu, Y., and Zhang, R. 2014. Di-(2-ethylhexyl) phthalate mediates glycolysis and the TCA cycle in clam *Venerupis philippinarum*. ISJ. 11, 174-181.

Lithner, D., Larsson, A., and Dave, G. 2011. Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. The Science of the Total Environment. 409, 3309-3324.

Lobelle, D. and Cunliffe, M. 2011. Early microbial biofilm formation on marine plastic debris. Marine Pollution Bulletin. 62, 197-200.

Lopez, G.R. and Levinton, J.S. 1987. Ecology of deposit-feeding animals in marine sediments. The Quarterly Review of Biology. 62, 235-260.

Manikkam, M., Tracey, R., Guerrero-Bosagna, C., and Skinner, M.K. 2013. Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic

transgenerational inheritance of obesity, reproductive disease and sperm epimutations. PLoS ONE. 8, e55387.

Mathalon, A. and Hill, P. 2014. Microplastic fibers in the intertidal ecosystem surrounding Halifax Harbor, Nova Scotia. Marine Pollution Bulletin. 81, 69-79.

Mato, Y., Isobe, T., Takada, H., Kanehiro, H., Ohtake, C., and Kaminuma, T. 2001. Plastic resin pellets as a transport medium for toxic chemicals in the marine environment. Environmental Science & Technology. 35, 318-324.

Matta, S.G., Balfour, D.J., Benowitz, N.L., Boyd, R.T., Buccafusco, J.J., Caggiula, A.R., Craig, C.R., Collins, A.C., Damaj, M.I., Donny, E.C., Gardiner, P.S., Grady, S.R., Heberlein, U., Leonard, S.S., Levin, E.D., Lukas, R.J., Markou, A., Marks, M.J., McCallum, S.E., Parameswaran, N., Perkins, K.A., Picciotto, M.R., Quik, M., Rose, J.E., Rothenfluh, A., Schafer, W.R., Stolerman, I.P., Tyndale, R.F., Wehner, J.M., and Zirger, J.M. 2007. Guidelines on nicotine dose selection for *in vivo* research. Psychopharmacology. 190, 269-319.

Mayer, F.L. and Sanders, H.O. 1973. Toxicology of phthalic acid esters in aquatic organisms. Environmental Health Perspectives. 3, 153-157.

Mayer, L.M., Linda, L.S., Robert, F.L.S., Peter, A.J., Robert, H.F., Zhen, C., and Stephen, S. 1997. Digestive environments of benthic macroinvertebrate guts: Enzymes, surfactants and dissolved organic matter. Journal of Marine Research. 55, 785-812.

Mazur, H.I., Stennett, D.J., and Egging, P.K. 1989. Extraction of diethylhexylphthalate from total nutrient solution-containing polyvinyl chloride bags. Journal of Parenteral and Enteral Nutrition. 13, 59-62.

Meeker, J.D., Calafat, A.M., and Hauser, R. 2007. Di(2-ethylhexyl) phthalate metabolites may alter thyroid hormone levels in men. Environmental Health Perspectives. 115, 1029-1034.

Meeker, J.D., Calafat, A.M., and Hauser, R. 2009. Urinary metabolites of di(2-ethylhexyl) phthalate are associated with decreased steroid hormone levels in adult men. Journal of Andrology. 30, 287-297.

Mehlhart, G. and Blepp, M. 2012. Study on land-sourced litter (LSL) in the marine environment. Institute for Applied Ecology, Germany.

Micevska, T., Warne, M.S.J., Pablo, F., and Patra, R. 2006. Variation in, and causes of, toxicity of cigarette butts to a cladoceran and microtox. Archives of Environmental Contamination and Toxicology. 50, 205-212.

Mizukawa, K., Takada, H., Ito, M., Geok, Y.B., Hosoda, J., Yamashita, R., Saha, M., Suzuki, S., Miguez, C., Frias, J., Antunes, J.C., Sobral, P., Santos, I., Micaelo, C., and Ferreira, A.M. 2013. Monitoring of a wide range of organic micropollutants on the Portuguese coast using plastic resin pellets. Marine Pollution Bulletin. 70, 296-302.

Mocchiutti, N.O. and Bernal, C.A. 1997. Effects of chronic di(2-ethylhexyl) phthalate intake on the secretion and removal rate of triglyceride-rich lipoproteins in rats. Food and Chemical Toxicology. 35, 1017-1021.

Moriwaki, H., Kitajima, S., and Katahira, K. 2009. Waste on the roadside, 'poi-sute' waste: Its distribution and elution potential of pollutants into environment. Waste Management. 29, 1192-1197.

Murray, F. and Cowie, P.R. 2011. Plastic contamination in the decapod crustacean *Nephrops norvegicus* (Linnaeus, 1758). Marine Pollution Bulletin. 62, 1207-1217.

Mushtaq, M., Srivastava, S.P., and Seth, P.K. 1980. Effect of di-2-ethylhexyl phthalate (DEHP) on glycogen metabolism in rat liver. Toxicology. 16, 153-161.

Nel, A., Xia, T., Mädler, L., and Li, N. 2006. Toxic potential of materials at the nanolevel. Science. 311, 622-627.

Nobre, C.R., Santana, M.F.M., Maluf, A., Cortez, F.S., Cesar, A., Pereira, C.D.S., and Turra, A. 2015. Assessment of microplastic toxicity to embryonic development of the sea urchin *Lytechinus variegatus* (Echinodermata: Echinoidea). Marine Pollution Bulletin. 92, 99-104.

Novotny, T. and Slaughter, E. 2014. Tobacco Product Waste: An environmental approach to reduce tobacco consumption. Current Environmental Health Reports. 1, 208-216.

Obbard, R.W., Sadri, S., Wong, Y.Q., Khitun, A.A., Baker, I., and Thompson, R.C. 2014. Global warming releases microplastic legacy frozen in Arctic Sea ice. Earth's Future. 2, 315–320.

Oehlmann, J.r., Schulte-Oehlmann, U., Kloas, W., Jagnytsch, O., Lutz, I., Kusk, K.O., Wollenberger, L., Santos, E.M., Paull, G.C., Van Look, K.J.W., and Tyler, C.R. 2009. A critical analysis of the biological impacts of plasticizers on wildlife. Philosophical Transactions of the Royal Society B: Biological Sciences. 364, 2047-2062.

Ogata, Y., Takada, H., Mizukawa, K., Hirai, H., Iwasa, S., Endo, S., Mato, Y., Saha, M., Okuda, K., Nakashima, A., Murakami, M., Zurcher, N., Booyatumanondo, R., Zakaria, M.P., Dung, L.Q., Gordon, M., Miguez, C., Suzuki, S., Moore, C., Karapanagioti, H.K., Weerts, S., McClurg, T., Burres, E., Smith, W., Velkenburg, M.V., Lang, J.S., Lang, R.C., Laursen, D., Danner, B., Stewardson, N., and Thompson, R.C. 2009. International Pellet Watch: Global monitoring of persistent organic pollutants (POPs) in coastal waters. 1. Initial phase data on PCBs, DDTs, and HCHs. Marine Pollution Bulletin. 58, 1437-1446.

Ottaviani, E., Franchini, A., Kletsas, D., and Franceschi, C. 1996. Presence and role of cytokines and growth factors in invertebrates. Italian Journal of Zoology. 63, 317-323.

Paemen, L.R., Porchet-Hennere, E., Masson, M., Leung, M.K., Hughes, T.K., Jr., and Stefano, G.B. 1992. Glial localization of interleukin-1 alpha in invertebrate ganglia. Cellular and Molecular Neurobiology. 12, 463-472.

Park, S.Y. and Choi, J. 2007. Cytotoxicity, genotoxicity and ecotoxicity assay using human cell and environmental species for the screening of the risk from pollutant exposure. Environment International. 33, 817-822.

Peduzzi, P. and Herndl, G.J. 1991. Mucus trails in the rocky intertidal: A highly active microenvironment. Marine Ecology Progress Series. 75, 267-274.

Philp, J.C., Bartsev, A., Ritchie, R.J., Baucher, M.A., and Guy, K. 2013. Bioplastics science from a policy vantage point. New Biotechnology. 30, 635-646.

R Core Team, 2013. R: a language and environment for statistical computing R Foundation for Statistical Computing, Vienna, Austria.

Rios, L.M., Moore, C., and Jones, P.R. 2007. Persistent organic pollutants carried by synthetic polymers in the ocean environment. Marine Pollution Bulletin. 54, 1230-1237.

Rios, L.M., Jones, P.R., Moore, C., and Narayan, U.V. 2010. Quantitation of persistent organic pollutants adsorbed on plastic debris from the Northern Pacific Gyre's "eastern garbage patch". Journal of Environmental Monitoring. 12, 2226-2236.

Rochman, C.M., 2013. Plastics and priority pollutants: a multiple stressor in aquatic habitats. Environmental Science & Technology. 47, 2439-2440.

Rochman, C.M., Browne, M.A., Halpern, B.S., Hentschel, B.T., Hoh, E., Karapanagioti, H.K., Rios-Mendoza, L.M., Takada, H., Teh, S., and Thompson, R.C., 2013a. Policy: Classify plastic waste as hazardous. Nature. 494, 169-171.

Rochman, C.M., Hoh, E., Hentschel, B.T., and Kaye, S., 2013b. Long-term field measurement of sorption of organic contaminants to five types of plastic pellets: implications for plastic marine debris. Environmental Science & Technology. 47, 1646-1654.

Rochman, C.M., Hoh, E., Kurobe, T., and Teh, S.J. 2013c. Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. Scientific Reports. 3.

Rossi, F., Gribsholt, B., Gazeau, F., Di Santo, V., and Middelburg, J.J. 2013. Complex effects of ecosystem engineer loss on benthic ecosystem response to detrital macroalgae. PLoS One. 8, e66650.

Ryan, P.G., Bouwman, H., Moloney, C.L., Yuyama, M., and Takada, H. 2012. Long-term decreases in persistent organic pollutants in South African coastal waters detected from beached polyethylene pellets. Marine Pollution Bulletin. 64, 2756-2760.

Sajiki, J. and Yonekubo, J. 2003. Leaching of bisphenol A (BPA) to seawater from polycarbonate plastic and its degradation by reactive oxygen species. Chemosphere. 51, 55-62.

Schmidt, J.S., Schaedlich, K., Fiandanese, N., Pocar, P., and Fischer, B. 2012. Effects of di(2-ethylhexyl) phthalate (DEHP) on female fertility and adipogenesis in C3H/N mice. Environmental Health Perspectives. 120, 1123-1129.

Setälä, O., Fleming-Lehtinen, V., and Lehtiniemi, M. 2014. Ingestion and transfer of microplastics in the planktonic food web. Environmental Pollution. 185, 77-83.

Shin, I.S., Lee, M.Y., Cho, E.S., Choi, E.Y., Son, H.Y., and Lee, K.Y. 2014. Effects of maternal exposure to di(2-ethylhexyl)phthalate (DEHP) during pregnancy on susceptibility to neonatal asthma. Toxicology and Applied Pharmacology. 274, 402-407.

Singh, N.P., McCoy, M.T., Tice, R.R., and Schneider, E.L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research. 175, 184-191.

Slaughter, E., Gersberg, R.M., Watanabe, K., Rudolph, J., Stransky, C., and Novotny, T.E. 2011. Toxicity of cigarette butts, and their chemical components, to marine and freshwater fish. Tobacco Control. 20, i25-i29.

Snelgrove, P.V.R. 1997. The importance of marine sediment biodiversity in ecosystem processes. Ambio. 26, 578-583.

Sobkowiak, R. and Lesicki, A. 2009. Genotoxicity of nicotine in cell culture of *Caenorhabditis elegans* evaluated by the comet assay. Drug and Chemical Toxicology. 32, 252-257.

Stabili, L., Schirosi, R., Licciano, M., and Giangrande, A. 2009. The mucus of *Sabella spallanzanii* (Annelida, Polychaeta): Its involvement in chemical defence and fertilization success. Journal of Experimental Marine Biology and Ecology. 374, 144-149.

Stahlhut, R.W., van Wijngaarden, E., Dye, T.D., Cook, S., and Swan, S.H. 2007. Concentrations of urinary phthalate metabolites are associated with increased waist circumference and insulin resistance in adult U.S. males. Environmental Health Perspectives. 115, 876-882.

Stales, C.A., Peterson, D.R., Parkerton, T.F., and Adams, W.J. 1997. The environmental fate of phthalate esters: A literature review. Chemosphere. 35, 667-749.

Sutherland, W.J., Clout, M., Côté, I.M., Daszak, P., Depledge, M.H., Fellman, L., Fleishman, E., Garthwaite, R., Gibbons, D.W., De Lurio, J., Impey, A.J., Lickorish, F., Lindenmayer, D., Madgwick, J., Margerison, C., Maynard, T., Peck, L.S., Pretty, J., Prior, S., Redford, K.H., Scharlemann, J.P.W., Spalding, M., and Watkinson, A.R. 2010. A horizon scan of global conservation issues for 2010. Trends in Ecology & Evolution. 25, 1-7.

Teuten, E.L., Saquing, J.M., Knappe, D.R.U., Barlaz, M.A., Jonsson, S., Bjorn, A., Rowland, S.J., Thompson, R.C., Galloway, T.S., Yamashita, R., Ochi, D., Watanuki, Y., Moore, C., Viet, P.H., Tana, T.S., Prudente, M., Boonyatumanond, R., Zakaria, M.P., Akkhavong, K., Ogata, Y., Hirai, H., Iwasa, S., Mizukawa, K., Hagino, Y., Imamura, A., Saha, M., and Takada, H. 2009. Transport and release of chemicals from plastics to the environment and to wildlife. Philosophical Transactions of the Royal Society B: Biological Sciences. 364, 2027-2045.

Thain, J. and Bifield, S. 2002. Biological effects of contaminants: sediment bioassay using polychaete *Arenicola marina*. ICES Techniques in Marine Environmental Science. 29, 17.

Thomas, W.C., McGrath, L.F., Baarson, K.A., Auletta, C.S., Daly, I.W., and McConnell, R.F. 1991. Subchronic oral toxicity of cellulose acetate in rats. Food and Chemical Toxicology. 29, 453-458.

Thompson, R.C., Moore, C.J., vom Saal, F.S., and Swan, S.H. 2009. Plastics, the environment and human health: current consensus and future trends. Philosophical Transactions of the Royal Society B: Biological Sciences. 364, 2153-2166.

Thompson, R.C., Olsen, Y., Mitchell, R.P., Davis, A., Rowland, S.J., John, A.W.G., McGonigle, D., and Russell, A.E. 2004. Lost at Sea: Where Is All the Plastic? Science. 304, 838.

Tickner, J. 1999. The use of Di-2-Ethylhexyl Phthalate in PVC medical devices: exposure, toxicity, and alternatives. Lowell Centre for Sustainable Production.

Tosin, M., Weber, M., Siotto, M., Lott, C., and Degli-Innocenti, F. 2012. Laboratory test methods to determine the degradation of plastics in marine environmental conditions. Frontiers in Microbiology. 3.

Toulmond, A. 1975. Blood oxygen transport and metabolism of the confined lugworm *Arenicola marina* (L.). The Journal of Experimental Biology. 63, 647-660.

Ugolini, A., Ungherese, G., Ciofini, M., Lapucci, A., and Camaiti, M., 2013. Microplastic debris in sandhoppers. Estuarine, Coastal and Shelf Science. 129, 19-22.

Van, A., Rochman, C.M., Flores, E.M., Hill, K.L., Vargas, E., Vargas, S.A., and Hoh, E. 2012. Persistent organic pollutants in plastic marine debris found on beaches in San Diego, California. Chemosphere. 86, 258-263.

Van Cauwenberghe, L., Claessens, M., Vandegehuchte, and M.B., Janssen, C.R. 2015. Microplastics are taken up by mussels (*Mytilus edulis*) and lugworms (*Arenicola marina*) living in natural habitats. Environmental Pollution. 199, 10-17.

Van Cauwenberghe, L. and Janssen, C.R. 2014. Microplastics in bivalves cultured for human consumption. Environmental Pollution. 193, 65-70.

Vianello, A., Boldrin, A., Guerriero, P., Moschino, V., Rella, R., Sturaro, A., and Da Ros, L. Microplastic particles in sediments of Lagoon of Venice, Italy: First observations on occurrence, spatial patterns and identification. Estuarine, Coastal and Shelf Science. 130, 54-61.

von Moos, N., Burkhardt-Holm, P., and Köhler, A. 2012. Uptake and effects of microplastics on cells and tissue of the blue mussel *Mytilus edulis* L. after an experimental exposure. Environmental Science & Technology. 46, 11327-11335.

Voparil, I.M. and Mayer, L.M. 2000. Dissolution of sedimentary polycyclic aromatic hydrocarbons into the lugworm's (*Arenicola marina*) digestive fluids. Environmental Science & Technology. 34, 1221-1228.

Ward, J.E. and Shumway, S.E. 2004. Separating the grain from the chaff: particle selection in suspension- and deposit-feeding bivalves. Journal of Experimental Marine Biology and Ecology. 300, 83-130.

Ward, J.M., Peters, J.M., Perella, C.M., and Gonzalez, F.J. 1998. Receptor and nonreceptor-mediated organ-specific toxicity of di(2-ethylhexyl)phthalate (DEHP) in peroxisome proliferator-activated receptor α-null mice. Toxicologic Pathology. 26, 240-246.

Warwick, R.M. and Price, R. 1975. Macrofaunal production in an estuarine mudflat. Journal of the Marine Biological Association UK. 55, 1-18.

Watts, A.J.R., Lewis, C., Goodhead, R.M., Beckett, S.J., Moger, J., Tyler, C.R., and Galloway, T.S. 2014. Uptake and retention of microplastics by the shore crab *Carcinus maenas*. Environmental Science & Technology. 48, 8823-8830.

Weston, D.P. and Mayer, L.M., 1998. Comparison of *in vitro* digestive fluid extraction and traditional *in vivo* approaches as measures of polycyclic aromatic hydrocarbon bioavailability from sediments. Environmental Toxicology and Chemistry. 70, 830-840.

Wiklund, S.J. and Agurell, E. 2003. Aspects of design and statistical analysis in the Comet assay. Mutagenesis. 18, 167-175.

Wild, C., Huettel, M., Klueter, A., Kremb, S.G., Rasheed, M.Y.M., and Jorgensen, B.B. 2004. Coral mucus functions as an energy carrier and particle trap in the reef ecosystem. Nature. 428, 66-70.

Wood, C.T. and Zimmer, M. 2014. Can terrestrial isopods (Isopoda: Oniscidea) make use of biodegradable plastics? Applied Soil Ecology. 77, 72-79.

Woodall, L.C., Sanchez-Vidal, A., Canals, M., Paterson, G.L.J., Coppock, R., Sleight, V., Calafat, A., Rogers, A.D., Narayanaswamy, B.E., and Thompson, R.C. 2014. The deep sea is a major sink for microplastic debris. Royal Society Open Science. 1, 140317.

Wright, S.L., Rowe, D., Thompson, R.C., and Galloway, T.S. 2013a. Microplastic ingestion decreases energy reserves in marine worms. Current biology : 23, R1031-R1033.

Wright, S.L., Thompson, R.C., and Galloway, T.S. 2013b. The physical impacts of microplastics on marine organisms: A review. Environmental Pollution. 178, 483-492.

Ye, T., Kang, M., Huang, Q., Fang, C., Chen, Y., Shen, H., and Dong, S. 2014. Exposure to DEHP and MEHP from hatching to adulthood causes reproductive dysfunction and endocrine disruption in marine medaka (*Oryzias melastigma*). Aquatic Toxicology. 146, 115-126.

Zanotelli, V.R.T., Neuhauss, S.C.F., and Ehrengruber, M.U. 2010. Long-term exposure to bis(2-ethylhexyl)phthalate (DEHP) inhibits growth of guppy fish (*Poecilia reticulata*). Journal of Applied Toxicology. 30, 29-33.

Zebe, E. and Schiedek, D. 1996. The lugworm *Arenicola marina*: A model of physiological adaptation to life in intertidal sediments. Helgolander Meeresunters. 50, 37-68.

Zettler, E.R., Mincer, T.J., and Amaral-Zettler, L.A. 2013. Life in the “Plastisphere”: microbial communities on plastic marine debris. Environmental Science & Technology. 47, 7137-7146.

Appendix

Teaching

- **2011-2013, Mentored student projects**

  Assisted in the supervision of BSc and MSc projects linked to my research; helped with experimental design, demonstrated laboratory techniques, and provided support for statistical analyses and writing;
- **2011-2014, Demonstrating**

  Provided undergraduate teaching support in laboratory practical classes; ensured methods were carried out correctly and safely; assisted students when required;
- **Journal Club**: Presented my research to undergraduates and helped them to read and evaluate scientific literature;
- Successfully completed the **Learning and Teaching in Higher Education Stage 1 and 2.**

Outreach

Outreach Activities

- Live radio interviews on the Naked Scientists show and BBC Radio Cornwall;
- Rozalia Project Green Drinks event, Portland, ME;
- Biosciences Press Gang: I am a member of the Biosciences Press Gang, a forum for media training whereby members produce press releases for internal research;
- 2012/2014 Big Bang Science Festival: Designed and implemented scientific experiments for secondary school students during science week; promoted an interest in science; communicated current research (personal and departmental), to both students and the general public.
- AS/A Level/Y9 microplastics workshops: helped to coordinate and run workshops with school students.
- Primary school science workshop: assisted in conducting a marine invertebrate workshop with primary school children.
- Gyre to Gaia: during a field trip with Pangaea Explore I presented my work to the boat crew, and discussed my field of research on a daily basis whilst conducting field work in an extreme environment. I also feature in an educational video resource, which was made during the trip.

Press Gang Articles

As part of the Biosciences Press Gang, I have authored and co-authored several press articles.

Feminised Fish Populations in Polluted English Rivers Remain Self Sustaining

Research by scientists at the University of Exeter in collaboration with Brunel University has revealed that some populations of wild roach, *Rutilus rutilus* L (a common fish in European rivers) are stable, despite their exposure to feminising chemicals in oestrogenic effluents.

The study, published in this week's BMC Biology compared the number of breeding roach in polluted sites to those in clean sites within multiple UK river systems, including the River Thames in southeast England. These rivers serve major cities and are consequently sites of extensive effluent pollution, largely originating from sewage treatment works. Oestrogenic compounds in these effluents are known to mimic the effect of natural oestrogen and feminise male fish in many species, including roach, both in the laboratory and in the wild. A common symptom of feminisation includes the appearance of eggs within testicular tissue, which led scientists to believe this would have an effect on their reproductive output.

Due to the widespread occurrence of this phenomenon the Government is so concerned about the impact of oestrogenic chemicals on UK rivers that £40 M has been invested in a programme to evaluate the effectiveness of the removal and treatment of these chemicals.

Scientists have been worried about the effect of oestrogenic chemicals as previous laboratory based studies revealed drastic population declines as all the fish became female. However, in the current study on wild roach populations, the number of breeding roach at each sample site was not found to be adversely affected by the oestrogenic chemicals present in the rivers. Interestingly, using DNA analysis, this study found that several populations in the most polluted rivers were self sustaining and did not rely on immigration from adjacent healthy populations as may have been the case.

Dr. Patrick Hamilton, the lead researcher in this study, believes that this is because “even in some of the most polluted rivers in the country, less than 10% of males contain moderately feminised gonads”. Individuals with gonads any less severely feminised are considered as reproductively competitive and unaffected males. He added “gonad feminisation increases with age, thus preventing domination by the oldest and largest males allowing a greater number of small fish a chance to reproduce”. Research at the University of Exeter is now continuing to investigate any adaptations these populations may have developed to become more resistant to oestrogenic chemicals after having being exposed for many generations.

Science under sail in the hunt for microplastics

A team of scientists from the University of Exeter recently embarked on a joint research programme with the Rozalia Project - a US-based NGO – to quantify microplastics in the Gulf of Maine.

Microplastics are microscopic plastic fragments, fibres, beads and granules that litter the marine environment worldwide. The team are optimising detection methods as well as determining whether key invertebrate species co-occur and interact with microplastics in this highly productive ecosystem.

The field study forms part of 3 UK and EU government-funded projects (NERC, Defra and CleanSea) led by Professor Tamara Galloway at the University of Exeter. These laboratory-based projects address the topical issue of microplastics and whether they cause harm in the marine environment, focussing on ecologically-important invertebrates such as zooplankton, crabs, mussels and worms. Results from the field will determine whether these animals are naturally ingesting microplastics.

The collaboration was set up with the Rozalia Project – an NGO dedicated to removing marine litter through action, outreach, technology and research - to address some key knowledge gaps. Currently, there is little data documenting the occurrence of particles <1/3 mm due to sampling and processing limitations. However, it is this size fraction which is most likely ingested by marine invertebrates, as the team's laboratory studies have shown. Through novel sample-processing protocols, the scientists hope to detect and quantify <1/3 mm microplastics from surface water and sediments around the Gulf, and determine whether animals with key roles in marine food webs, such as zooplankton and worms, are ingesting microplastics. The team sampled and preserved these animals onboard research vessel the *American Promise* – a 60ft yacht - and brought them back to the UK to process using optimised methods.

Professor Tamara Galloway from the College of Life and Environmental Sciences at the Streatham campus said: "*This research cruise has been a great opportunity for us to study how zooplankton and microplastics interact in the ocean and to see for ourselves how much plastic debris there really is in remote ocean locations far from the nearest*

shoreline. We will be using the samples we collected to study how much plastic has been ingested and the impact this may be having on the organisms themselves"

Rachael Miller, co-founder and director of the Rozalia Project and captain of the *American Promise* research vessel said: *"Rozalia Project was very excited to work with the University of Exeter. Their team was well prepared, dynamic and did an excellent job involving our interns while conducting leading edge marine debris research. It was a pleasure to have them onboard American Promise. We are looking forward to learning from their results and continuing to work with this great team for and toward a clean and healthy ocean."*

Stephanie Wright, a PhD student researching the impacts of marine microplastics, from the College of Life and Environmental Sciences at the Streatham campus said: *"Being able to witness the mounting issue of my research on microplastic debris in the marine environment has been an invaluable experience. It's highlighted how widespread this pollutant has become and emphasised the importance of determining whether microplastics are entering marine food chains as well as the consequences this could have."*

Prof. Tamara Galloway's team are now busy developing extraction methodologies in the laboratory to identify <1/3 mm microplastic particles in their seawater, sediment and biota samples. They hope to have the first set of results this Christmas as an initial step towards addressing some of the key questions in this emerging field.

Presentations

- A plastic diet: microplastics at the bottom of marine food chains. Unpackage Me debrief, University of Exeter (Falmouth), October 2014, **invited speaker**.
- The impacts of microplastics in the marine environment. The University of the Azores, 2nd October 2014, **invited speaker**
- The biological impacts of microplastics in the sand. Ocean Plastic – Consequences and Solutions, National Maritime Museum Falmouth, July 21st, 2014
- The physical impacts of microplastics on marine organisms. Micro International Workshop: fate and impacts of microplastics in marine ecosystems, Plouzane, France, January 13th-15th, 2013
- Exploring the effects of microplastics on ecologically-important benthic invertebrates. Oral presentation, SETAC Europe 23rd Annual Meeting, Glasgow, May 12th-16th, 2013
- Exploring the physical effects of microscopic plastic particles in a sediment-dwelling marine polychaete. Oral presentation, SETAC North America 33rd Annual Meeting, Long Beach, CA, Nov 11th-15th, 2012

Selected Posters

Cigarettes in the sand: butt what about worms?

Stephanie Wright and Prof Tamara Galloway (CLES)

Aim: To investigate the toxicity of smoked cigarette filters to an important marine worm.

1. Introduction

Animals living on and in the seabed have important roles in marine ecosystems. However, the seabed has become a sink for pollution, accumulating many harmful metals, chemicals, and litter.

Cigarette filters are the most common item of beach litter. Made from a bioplastic, they may take several years to break down. Consequently, cigarette filters accumulate in the marine environment and potentially fragment into microscopic fibres, a form of microplastic.

An estimated 4.5 trillion cigarette filters are released into the environment annually

Cigarette filters capture tar and nicotine along with other toxic chemicals. Initial reports have shown that these chemicals have a lethal effect on aquatic life. We are addressing whether cigarette filter micro-fibres and their associated chemicals have a negative impact on an important marine worm, the common rag worm.

2. Methods

In the aquarium, we exposed worms for 4 days to either 1) seawater containing chemicals from smoked cigarette filters or 2) sediment containing microscopic fibres from smoked cigarette filters.

3. Results

We found that:

- In seawater, worms lost weight and suffered DNA damage when exposed to the highest concentration of chemicals (8 filters L^{-1}; fig.1 A and C);
- Their burrowing activity was very sensitive, with significantly longer burrowing times in worms exposed to as few as 1 filter L^{-1} (fig.1 B);
- This suggests a toxic effect on their nervous system.
- When exposed to micro-fibres in the sediment, there were no observable toxic effects (fig.1 D-F).

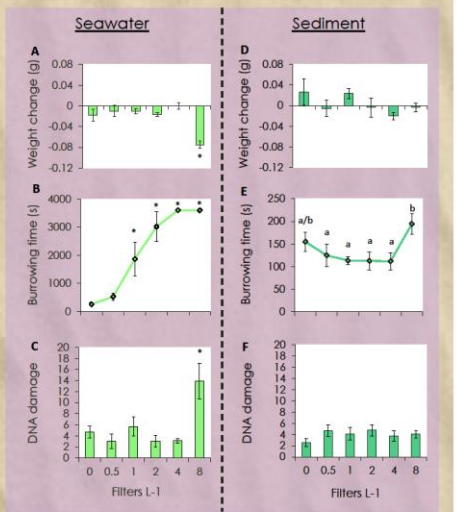


Figure 1. The toxicity of smoked cigarette filters on the common rag worm. A-C shows the change in weight, burrowing time and DNA damage, respectively, of worms exposed to cigarette filter chemicals in seawater. D-F shows the change in weight, burrowing time and DNA damage, respectively, of worms exposed to cigarette filter micro-fibres.
* shows a significant difference to the control (0 fibres L^{-1}, confidence level = 0.05). Letters indicate significant differences between groups (confidence level = 0.05).

- **Future work will consider what happens over prolonged periods of time: does this cause toxicity in the seabed?**

4. Discussion

In the short term, rag worms were sensitive to the chemicals of smoked cigarette filters in seawater, but not to micro-fibres in the sediment. This may be due to:

- Differences between the routes of exposure:
  - Across the skin in seawater vs. ingestion in sediment;
  - Following ingestion, certain chemicals may be broken down into less harmful forms.
- Differences in the behaviour of chemicals:
  - Nicotine is water soluble, and was therefore the likely cause of toxicity in seawater. In sediment it may not be released so easily;
  - In the sediment, many chemicals can stick to sediment even after ingestion, reducing their harm.

For further information, please see **Wright et al. (2013)** The physical impacts of microplastics on marine organisms: A review. *Environmental Pollution*. & **Wright et al. (2013)** Microplastic ingestion decreases energy reserves in marine worms. *Current Biology*.

sw393@exeter.ac.uk @MarineSteph

University of Exeter Postgraduate Research Showcase, April 28th-30th, 2014

Biological impacts of microplastics on a benthic polychaete

Stephanie Wright, Adil Bakir, Steven J. Rowland, Richard C. Thompson, Tamara S. Galloway

Aim

To determine the effects of unplasticised polyvinylchloride (uPVC) on the health of an ecologically important marine invertebrate.

Introduction

Plastic debris at the micro-scale is a widespread element of marine litter. Microplastics have accumulated in oceans and sediments worldwide from low densities to localized 'hotspots'.

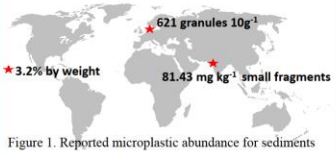
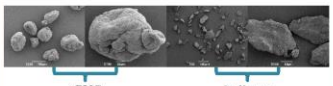


Figure 1. Reported microplastic abundance for sediments

The sediment is a sink for microplastics. Benthic invertebrates have already shown the capacity to ingest microplastics, with uncertain health consequences.

Methods



Natural sediment mixed with:
uPVC: mean size 130 µm, analysed by static light scattering; purchased from Goodfellow, UK.

Control: clean sediment

1kg sediment, 400 ml overlying artificial seawater, gentle aeration, 15˚C.

1 organism per beaker, 11 replicates

Arenicola marina, the lugworm, is:
- Widely distributed and intertidal
- Lives in a burrow
- Feeds by ingesting sediment, extracting the organic content
- Forms an important component of marine food webs
- Plays a vital role in mixing sediment.

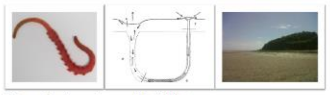
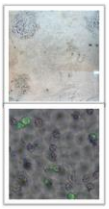


Figure 2. *A. marina* and its habitat.

28 day exposure to uPVC (% by weight):
Control 0.5% 1% 5%

Observations for feeding and mucus production

Measure endpoints:
- Feeding rate
- Immune response
- Energy reserves



Results

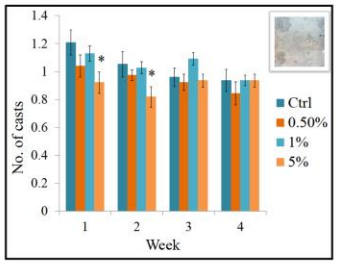


Figure 3. The effect of uPVC on the feeding rate of *A. marina*. The figure shows the mean daily number of casts per individual with SE.

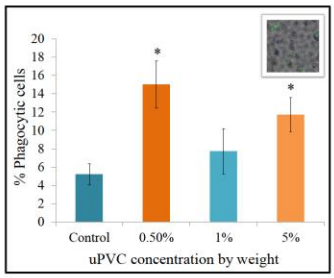


Figure 4. The effect of uPVC on the immune response of *A. marina*. The figure shows the mean percentage of phagocytic cells per individual with SE.

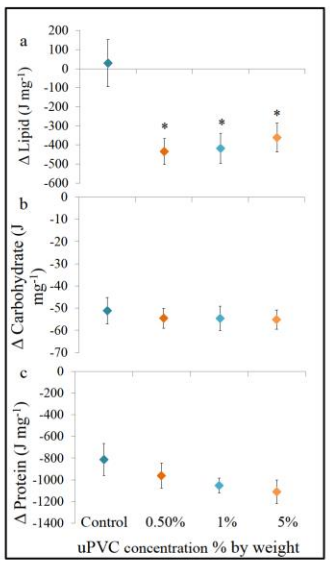


Figure 5. The effect of uPVC on the energy reserves of *A. marina*. The figure shows the mean energy reserve per individual with SE, for a) lipids; b) carbohydrate; and c) protein.

- 5% uPVC by weight significantly reduced feeding rate in the first 2 weeks (Repeated measures ANOVA, $F(3,40)=2.933$, $p=0.045$)
- uPVC significantly increased phagocytic activity (One-way ANOVA, $F(3,32)=4.113$, $p=0.014$), suggesting an inflammatory response
- Exposed animals had significantly reduced lipid stores (One-way ANOVA, $F(4,46)=5.263$, $p=0.001$).

Conclusion

- **Microscopic uPVC significantly effected the health of *A. marina* at the physiological and cellular level**
- **Reduced lipid stores may have repercussions at higher levels of biological organisation as less energy is available for growth and reproduction**
- **These results contribute to our understanding of the environmental risk microplastics present.**

sw393@exeter.ac.uk; College of Life & Environmental Sciences, University of Exeter, Exeter, UK

Thank you to the Department for Environment, Food & Rural Affairs, UK for funding this work; 1-SW-P-N21-000-031-DN-A1-05102.

Best Poster, PlyMSEF, Plymouth, Dec 18th, 2012

Accepted Manuscript

Bioaccumulation and biological effects of cigarette litter in marine worms

Stephanie L. Wright[1], Darren Rowe[1], Malcolm J. Reid[2], Kevin V. Thomas[2], and Tamara S. Galloway[1]*

[1]Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter, Devon EX4 4QD, UK.

[2]Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, N-0349 Oslo, Norway

*T.S.Galloway@exeter.ac.uk +44 (0) 1392 723436

Marine debris is a global environmental issue. Smoked cigarette filters are the predominant coastal litter item; 4.5 trillion are littered annually, presenting a source of bioplastic microfibres (cellulose acetate) and harmful toxicants to marine environments. Despite the human health risks associated with smoking, little is known of the hazards cigarette filters present to marine life. Here we studied the impacts of smoked cigarette filter toxicants and microfibres on the polychaete worm *Hediste diversicolor* (ragworm), a widespread inhabitant of coastal sediments. Ragworms exposed to smoked cigarette filter toxicants in seawater at concentrations 60 fold lower than those reported for urban run-off exhibited significantly longer burrowing times, >30% weight loss, and >2-fold increase in DNA damage compared to ragworms maintained in control conditions. In contrast, ragworms exposed to smoked cigarette filter microfibres in marine sediment showed no significant effects. Bioconcentration factors for nicotine were 500 fold higher from seawater than from sediment. Our results illustrate the vulnerability of organisms in the water column to smoking debris and associated toxicants, highlighting the risks posed by smoked cigarette filter debris to aquatic life.

Introduction

Marine debris is a global conservation issue[1]. Semi-synthetic bioplastic (rayon) and plastic materials are widely reported in the marine environment[2]. Environmental exposure causes these materials to degrade and fragment, resulting in micron-sized particles and fibres <1 mm (e.g. microplastics)[3]. Fibres are the most frequently reported type of particulate debris, not just in coastal ecosystems, but in deep ocean sediments where recent estimates suggest over 2 billion rayon fibres km^2 contaminate the seabed[2].

Smoked cigarette filters – the predominant item reported globally in coastal litter surveys – present a substantial source of rayon microfibres; each filter is comprised of >15,000 cellulose acetate (rayon) fibres, 20 µm in diameter[4,5]. Approximately 4.5 trillion smoked cigarette filters, equivalent to >750,000 tonnes, are littered to the environment annually[4]. Despite the anti-littering laws operative in many countries,

enforcement at the individual-level is impractical and has proved ineffective in preventing this debris from accumulating in the environment[4].

Smoked cigarette filters can cause harm in the marine environment in several ways. They present a vector for the transport and introduction of toxicants , including heavy metals, nicotine and known carcinogens[6], to aquatic habitats. Exposure to such toxicants in seawater could occur following the dissolution of compounds from the bioplastic filter to the surrounding seawater (leaching). Dietary exposure could occur through the ingestion of smoked cigarette filter microfibers due to filter degradation. If ingested, there is potential for the transfer of adhered toxicants to tissues. These bioplastic microfibres and their associated toxicants may persist in the marine environment and continue leaching chemicals for up to 10 years[4]. Despite this, few studies have assessed their potential toxicity. This is particularly important in coastal sediments, where smoked cigarette filters dominate litter[7,8].

Sediment is a vital component of the marine environment, forming one the largest habitats on Earth. Its diverse residents are fundamental to marine ecosystem function, impacting water column processes; trophic transfer; and global biogeochemical cycles[9]. Polychaete worms are widespread and abundant inhabitants of coastal sediments, where they rework and irrigate sediment and form a key prey species for birds and fish[10,11]. They adopt a range of feeding strategies, including surface deposit feeding[11,12], and are thus vulnerable to smoked cigarette filter debris and toxicants via both oral and dermal exposure pathways.

For the first time we explore the impacts of smoked cigarette filter toxicants and microfibres on the polychaete worm *Hediste diversicolor* (ragworm). We address the hypotheses that 1) the exposure to toxicants desorbed from smoked cigarette filters affect the behaviour and physiology of ragworms, and that 2) smoked cigarette filter microfibres present a physical hazard and/or vector for these associated toxicants. We measure this by quantifying the relative growth rate, burrowing time and level of DNA damage in ragworms exposed to smoked cigarette filter toxicants in seawater or microfibres in sediment, in relation to the bioaccumulation of the biomarker nicotine and it derivative cotinine.

Results

Nicotine Bioaccumulation

Nicotine and its metabolite cotinine were used as biomarkers of exposure to the toxicants associated with smoked cigarette filters (from here on referred to as filters). Nicotine was detected in whole-ragworm tissue following all exposures (see Table 1). After 96 h, the greatest levels of nicotine were measured in ragworms exposed to the highest concentrations of both filter toxicants in seawater (119,654 ng g^{-1} tissue, Bioconcentration Factor (BCF) of 172.4) and microfibres in sediment (3,629 ng g^{-1} tissue, Bioaccumulation Factor (BAF) of 0.338) (see Table 1 and Fig. 1c and d). Ragworms accumulated several orders of magnitude less nicotine following both short- and long-term (854 ng g^{-1} tissue, BAF of 0.123) sediment exposures to filter microfibres than following exposure to filter toxicants in seawater (Fig. 1d and Supplementary Fig. 2a). The average cigarette contains 0.8-1.9 mg of nicotine. For comparison, this delivers a dose of 10-30 μg kg^{-1} based on an average adult weight of 68 kg, resulting in average peak plasma levels of 10-50 ng ml^{-1} [13] (see Table 2).

Nicotine Metabolism

The nicotine metabolite cotinine was detected in all ragworms following exposure to filter toxicants in seawater (Fig. 1c). Nicotine:cotinine ratios of worm tissues dramatically increased with filter concentration; worms exposed to 8 filters L^{-1} had the greatest ratio (792, see Table 1). Following a 96 h sediment exposure to filter microfibres, cotinine was detected in ragworms exposed to 2 filters L^{-1} and above (Fig. 1d). The greatest nicotine:cotinine ratio was measured in ragworms exposed to 2 filters L^{-1} (76.6, Table 1). After 28 d in sediment, cotinine was detected in ragworms exposed to 4 filters L^{-1} and above (see Table 2 and Supplementary Fig. 2a). The nicotine:cotinine ratio was 67.2 and 61, for 4 and 8 filters L^{-1}, respectively. These are similar to the ratios observed in worms exposed to microfibres in sediment over 96 h. These ratios indicate a reduced bioavailability of nicotine via microfibres in the sediment in comparison to filter toxicants in seawater.

Biological Endpoints

Relative Growth Rate

Relative Growth Rate (RGR) was measured as a general health indicator. A significant effect on RGR was observed in ragworms following exposure to filter toxicants in seawater (one-way ANOVA, p=0.00005, Fig. 2a). The lowest concentration to cause a significant effect (LOEC) on RGR was 8 filters L^{-1} (-33% mean weight ± 2% s.e.m.). Following 96 h and 28 d sediment exposures to filter microfibres, no effect on the RGRs of ragworms was observed (Fig. 2b and Supplementary Fig. 2b, respectively).

Burrowing Activity

Given the neurotoxicity of nicotine[13], we selected burrowing time as a primary sublethal endpoint. Exposure to the two highest concentrations of filter toxicants in seawater (4 and 8 filters L^{-1}) inhibited the burrowing capacity of 100% of individuals during the assay observation period (Fig. 2c). The LOEC for the burrowing time of ragworms exposed to filter toxicants in seawater was 2 filters L^{-1} (Kruskal Wallis, p=0.0001).

Following a 96 h sediment exposure to filter microfibres, the LOEC for burrowing time was 8 filters L^{-1} (one-way ANOVA, p=0.04, Fig. 2d). Post hoc analysis showed that this result was significant at a confidence level of 0.1 (Tukey HSD Test, p=0.07). The burrowing time of ragworms following 28 d sediment exposure to filter microfibres was not affected (Supplementary Fig. 2c).

DNA Damage

Exposure to filter toxicants in seawater significantly affected the median, 75^{th}, and 90^{th} percentile tail moment (TM, a measure of DNA fragmentation, see Methods) of ragworms (one-way ANOVA, p=0.016, p=0.003, and p=0.003, respectively). Ragworms exposed to 8 filters L^{-1} had significantly greater TMs than those exposed to 0.5, 2, and 4 filters L^{-1} (Fig. 2e, Supplementary Fig. 3a and d for 90^{th}, median, and 75^{th} percentiles, respectively). The 75^{th} and 90^{th} percentile tail intensities (TI, a measure of the relative fraction of DNA, see Methods) were also significantly greater in ragworms exposed to 8 filters L^{-1} than to ragworms exposed to 0.5 and 4 filters L^{-1} (Kruskal Wallis, p=0.04 and p=0.01, respectively; Supplementary Fig. 4a and d for 75^{th} and 90^{th} percentiles,

respectively). Following 96 h and 28 d exposures to filter microfibres in sediment, there was no significant DNA damage (see Fig. 2f, Supplemetary Fig. 3 and 4 b and e; and Supplementary Fig. 2d and 3 and 4 c and f, for 96 h and 28 d, respectively).

Discussion

This is the first study to assess the impacts of smoked cigarette filter (from here on referred to as filters) debris on a marine invertebrate. We found that exposure to leached filter toxicants in seawater at a concentration of ≥2 filters L^{-1} (172 µg L^{-1} nicotine) significantly inhibits burrowing behaviour in a marine worm, whilst greater concentrations lead to reduced growth rates and increased DNA damage. Of the few studies that have assessed the impacts of filter toxicants on aquatic species, water fleas and juvenile fish exhibited greater sensitivity than ragworms did in the present study[14,15]. Further investigation is therefore required to determine the impacts of filters on other biotic components of coastal and marine ecosystems.

Ragworms accumulated considerably less nicotine – an established biomarker of exposure to the toxicants associated with smoking - following sediment exposure to filter microfibres than following exposure to filter toxicants in seawater. Notably, the nicotine dose delivered by just one filter L^{-1} via seawater is around 98 times that delivered to a human via smoking (Table 1). Since ragworms were not fed during exposure to filter toxicants in seawater, uptake is anticipated to primarily occur via the epidermis (Fig. 1a). Nicotine is unionized and bioavailable under alkaline conditions[16]. The alkalinity of the seawater in this study (pH 8.06 mean ± 0.007 s.e.m.) indicates over 70% of nicotine was bioavailable, allowing for rapid systemic circulation[16].

Sediment exposure to filter microfibres and associated toxicants occurs predominantly via indiscriminate surface-deposit feeding. Post-ingestion, up to 70% of nicotine is metabolised before entering systemic circulation[13] (Fig. 1b). The pH of the sediment measured during low tide (7.5 mean ± 0.01 s.e.m., n=12) suggests that over 90% of the nicotine is bioavailable in sediment exposures[16]. However, the moderately acidic gut conditions of ragworms could counter this[17]. These factors may explain the low concentration of nicotine detected in ragworms following sediment exposures.

Additionally, ragworms are unlikely to encounter the entire sediment volume, thus contacting only a fraction of the contaminant. The worms' mucus-lined burrow may also act as a physical barrier, limiting encounter rates with nicotine (Fig. 1b). Following 96 h exposure to filter toxicants in seawater, the nicotine concentration of ragworm tissue dramatically increased with increasing filter concentration, suggesting metabolism becomes impaired. Nicotine metabolism is important in reducing toxicity: cotinine has a similar mechanism of action to nicotine, but binds to neuronal acetylcholine receptors with lower potency[18].

If ingested, there is potential for microplastic and bioplastic debris to transfer adhered pollutants, which can accumulate on their surface up to several orders of magnitude greater than the surrounding water column[19,20]. Whilst sediment exposure to filter microfibres limited nicotine bioaccumulation, other types of particulate debris have been shown to transport chemical contaminants to invertebrates: microscopic polyvinylchloride (PVC) transferred adhered triclosan and nonylphenol to the gut tissue of sediment-dwelling lugworms, at levels which caused biological harm[21]. Moreover, simulated gut conditions elicited greater desorption rates of chemical contaminants from microscopic polyethylene and PVC than seawater[22]. These studies employed higher concentrations of particles than the current study.

Using the biomarker nicotine, we have shown that filters can act as a vector for the transport and introduction of associated toxicants to seawater through leaching. This may pose an ecological risk to species which could encounter and bioaccumulate these toxicants from the surrounding seawater. However, the ingestion of filter microfibers within sediment by benthic species as a route of exposure to associated toxicants is considered a lower threat.

We show that exposure to filter toxicants in seawater has a significant negative effect on the RGR of ragworms. Similarly, the weight of earthworms was reduced by up to 40% following exposure to the neurotoxic insecticide imidacloprid, which is chemically similar to nicotine[23,24]. The authors postulated this was due to decreased feeding, reduced assimilation efficiency, or the up-regulation of an energetically costly detoxification mechanism. Similar modes of toxicity could have also occurred in the present study.

No effect on the RGRs of ragworms was observed following 96 h and 28 d sediment exposures to filter microfibres. The low nutritional value of the cellulose acetate microfibres may be anticipated to reduce RGR. Female rats showed a 14% reduction in growth following prolonged dietary exposure to high doses of cellulose acetate, linked to a nutritional reduction in the feed[25]. The polychaete worm *Arenicola marina* suffered significant reductions in energy reserves following exposure to ≥1% microscopic PVC by weight[26]. This was likely in-part due to a reduction in the nutritional quality of material consumed. The current study employed lower concentrations of microplastics, resulting in a higher proportion of nutritious substrate. We consider the chemical toxicity of leached nicotine and associated toxicants from filters to seawater to be of greater concern than the ingestion of low-nutritive filter microfibers for impacting growth rate.

Exposure to filter toxicants in seawater at a concentration of ≥2 filters L^{-1} significantly affected burrowing activity in ragworms. The insecticide imidacloprid impaired burrowing behaviour in earthworms; burrows were smaller in area and shallower than control groups following a 6 day exposure[27]. Nicotine is neurotoxic, affecting the central and autonomic nervous system and neuromuscular junctions by agonistically binding to the nicotinic acetyl cholinergic receptors (nAChRs)[13,28]. This opens ion channels, causing an influx of sodium or calcium ions, increasing the release of neurotransmitters. Prolonged stimulation of nAChRs can lead to desensitization, impairing neurological function[13]. This may be explain the inhibited burrowing capacity of ragworms in the current study.

The burrowing behaviour of worms is central to their role as ecosystem engineers, reworking and aerating sediment to allow other organisms to thrive[11]. Nicotine exposure via filter debris presents a potential risk to ecosystem health through its detrimental effects on the burrowing behaviour of worms; this is deserving of further assessment to determine the extent of the risk to the benthic community. As sediment exposure to filter microfibres limited nicotine bioaccumulation, burrowing activity was minimally affected. Filter microfibres within sediment as a vector for nicotine are therefore anticipated to be less neurologically hazardous than filter toxicants in seawater.

An average increase in DNA damage of 2- to 3-fold from control to treatment is considered biologically relevant[29]. A fold increase >2 from control to treatment was observed in the median, 75th, and 90th TM percentiles (Fig 2e, and Supplementary Fig. 3a and d, for median, 75th, and 90th percentiles, respectively) and in 75th and 90th TI percentiles (Supplementary Fig. 4a and d, respectively) of ragworms exposed to filter toxicants in seawater. Thus, filter toxicants in seawater at a concentration of 8 filters L^{-1} caused biologically relevant DNA damage, likely due to oxidative stress[30]. However, previous studies have highlighted a protective effect of nicotine on DNA damage at low concentrations through radical scavenging[31]. Ragworms exposed to filter toxicants in seawater at concentrations up to 4 filters L^{-1} exhibited significantly lower levels of DNA damage than those exposed to 8 filters L^{-1}. This indicates that ragworms experienced the protective effect of low nicotine dosage. At lower nicotine doses, the neurotoxicity of nicotine may be of greater concern than potential molecular effects.

In conclusion, filter toxicants in seawater caused adverse dose-dependent effects on behaviour and high concentrations of filter toxicants effected growth in ragworms, which were linked to nicotine bioconcentration. The concentration of nicotine in the aquatic environment is variable; up 32 µg L^{-1} in effluent and 11,400 µg L^{-1} in urban run-off have been reported [32,33]. It was recently estimated that one smoked cigarette filter could contaminate 1000 L of water at a concentration exceeding the predicted no effect concentration (24 µg L^{-1})[33]. Reported urban run-off concentrations are over 60 times greater than the effective concentration of nicotine in the current study (≥172 µg L^{-1}/2 filters L^{-1}). Therefore aquatic species in proximity to urbanised areas are at risk of nicotine exposure via run-off contaminated with smoked cigarette filters and their leachates. In comparison, sediment exposure to filter microfibres – an anticipated route of exposure for ragworms in the marine environment - limits the bioaccumulation and toxicity of nicotine. Up to 3.5 cigarette filters m^{-2} has been reported on beaches[34]. Particulate smoked cigarette filter debris is therefore predicted to be of lower risk than leachates. However, it is unknown how the aging of filters and their microfibres would affect nicotine bioaccumulation and toxicity. The quantification of filters in coastal environments as well as the role of aging on filter toxicity are areas deserving of further research.

The protection, conservation and restoration of marine ecosystems increasingly rely on international legislation to curb anthropogenic impacts. Recently, statutory frameworks such as the European Union Marine Strategy Framework Directive (MSFD) have for the first time stipulated that the properties and quantities of marine litter, including microplastics, should not cause harm to the marine environment (Descriptor 10, MSFD, 2008/56/EC). Quantitative toxicological data is essential for supporting the implementation of such legislation; our results provide a first step towards setting guidance limits to curb smoking-related bioplastic debris. We encourage further research into the role of environmental and physiological pH, and different exposure pathways when considering the impacts of filter toxicants and bioplastic microfibers on biotic components of marine ecosystems. Research into the impacts of smoked cigarette filters on marine life is crucial for consolidating the evidence base for remedial policy[4].

Methods

Materials

Smoked Cigarette Filters

Smoked cigarette filters ('filters', nicotine content 0.7-0.9 mg) were collected and immediately kept in sealed falcon tubes in the dark. Before use, the outer paper and any excess tobacco was carefully removed. Filters were individually weighed to calculate an average filter weight.

Chemicals and solutions

Ethyl acetate (Chromasolv HPLC Grade, Sigma Aldrich), methanol (HPLC Super Gradient Reagent, VWR Chemicals), carbon dioxide (food grade, AGA), ammonium hydroxide (ACS Reagent, Sigma Aldrich), AOAC Method 2007.01 Extraction salts (DisQuE, Waters Corp, Milford USA), AOAC Method 2007.01 clean-up tubes (DisQuE, Waters Corp, Milford USA), nicotine, nicotine-D4, cotinine and cotinine-D3 (all from Cerilliant, Round Rock Texas, USA), and 3'-hydroxycotinine (Toronto Research Chemicals, Ontario Canada).

Exposures and Biological Endpoints

Animal Husbandry

The ragworm *Hediste diversicolor* was hand collected from the Exe Estuary, Devon, UK (50˚66''76 N, -3˚44''40W) between February to April 2014. Stock worms were maintained collectively in 4 cm of natural sediment with overlying artificial seawater (ASW, salinity of 22) in a temperature-controlled room (12˚C, 12 h light:12 h dark). Ragworms were acclimated for at least 1 week. Water changes were performed on alternate days. In all exposures, only healthy, complete ragworms were used.

Exposure to Smoked Cigarette Filter Toxicants in Sediment

To establish whether the toxicants associated with filters are harmful to ragworms, an initial aqueous exposure was performed following [14] and [15]. To produce increasing doses of leachates based on a filter L^{-1} concentration, a leachate stock was produced, also forming the highest concentration (8 filters L^{-1}). Smoked filters were placed in artificial seawater (salinity of 22) on an orbital shaker in a temperature-controlled room for 24 h. The leachates were then vacuum-filtered through Whatman cellulose filter paper (grade 1) to remove any particulates due to cigarette filter degradation. The remaining test concentrations were made by performing 0.5x dilution series with the filtered leachate and artificial seawater, achieving final concentrations of 8, 4, 2, 1 and 0.5 cigarette filters L^{-1}. Subsamples of each stock concentration were kept at -80˚C for chemical analysis.

Three hundred mL aliquots of leachates were added to 400 mL glass beakers (acid-washed, 13% HNO_3) immediately before the addition of ragworms. Beakers were randomly allocated a position in a temperature-controlled room (12˚C). Each beaker contained a length of silicon tubing, providing refuge. Beakers were gently aerated and covered to minimise evaporation. Ragworms were weighed and individually transferred to a beaker (n=6 per treatment group). Observations were made daily. Following 48 h, a water change was performed using fresh leachate from which subsamples were again taken for chemical analysis. Water parameters (salinity, pH, dissolved oxygen) were monitored throughout the exposure period. After 96 h, ragworms were removed from exposure.

Exposure to Smoked Cigarette Filter Microfibres in Sediment

To determine whether particulate debris from filters can transfer toxicants at levels capable of causing harm, the impacts of filter microfibers on ragworms were assessed. Filters free of outer paper and excess tobacco were ground under liquid nitrogen using a pestle and mortar until a fine powder formed. Subsamples were suspended in deionised water and observed under a microscope fitted with a camera for size analysis. Individual microfibres were randomly sized using image analysis software.

Microfibers (mean length 120.6 ± 5.1 µm s.e.m., median length 96.5 µm, Supplementary Fig. 1) were added to sediment in bulk by weights equivalent to the concentrations above (number of filters L^{-1}). The sediment was manually homogenised. Subsamples of each sediment stock concentration were kept at -80˚C for chemical analysis. Forty eight hours prior to exposures, 225 mL of test sediment was added to 400 mL acid-washed, glass beakers (4 cm depth). Beakers were randomly allocated a position in a temperature-controlled room (12˚C), covered and left to acclimate overnight. Twenty four hours prior to exposures, 150 mL of artificial seawater (salinity of 22) was poured into beakers over a clean, stainless steel spoon. Gentle aeration was provided and beakers were left covered.

Ragworms were weighed and individually transferred to a beaker (n=6 per treatment group). Observations were made daily and water parameters were monitored throughout the exposure period. Two exposures were conducted, lasting 96 h and 28 d. During the 96 h exposure, a water change was performed after 48 h using fresh ASW. After 96 h, ragworms were removed from the exposure. During the 28 d exposure, water changes were performed every 72 h. Ragworms were not fed during this time as it was assumed they were surface-deposit-feeding on the test sediment. After 28 days, ragworms were removed from exposure. Following endpoint measurements, ragworms were individually maintained in seawater (salinity of 22) to void gut content in preparation for chemical analyses. After approximately 10 h, ragworms were snap-frozen and stored at -80º until use.

Relative Growth Rate

In addition to pre-exposure wet weights, post-exposure weights were also recorded. Following sediment exposure, any external sediment was carefully rinsed from ragworms. Excess seawater was gently absorbed using a paper towel and ragworms were weighed to 0.01 g.

Burrowing activity

Individuals were transferred to 400 mL glass beakers containing 225 mL wet control sediment (corresponding to approximately 4 cm depth). Their burrowing time into clean sediment – from the moment their anterior end touched the sediment to being completely burrowed –was recorded within a 1 h observation period. The burrowing time of ragworms which did not burrow during this time was considered as 60 min.

Comet assay

DNA damage – measured as single-strand breaks in individual cells (Comet assay) – was quantified to assess potential carcinogenic and pro-oxidative effects, anticipated due to the constituent toxicants of smoked cigarettes. The Comet assay quantifies DNA damage as tail intensity (TI) and tail moment (TM) for individual cells. TI indicates the relative fraction of damaged DNA. TM is the product of TI and tail length (the migratory distance of broken DNA fragments from the nucleus of the cell), providing a descriptive assessment of DNA damage[35].

Ragworms were recovered from the burrowing assay and carefully rinsed. A sample of coelomic fluid was withdrawn with a 1 mL syringe containing chilled PBS at a 1:1 ratio, fitted with a 23 gauge needle. Samples were taken from the posterior region, taking care to avoid the gut, and stored on ice until use. One hundred µL of sample was used per individual. Coelomic fluid was centrifuged at 1000 rpm for 3 min and the supernatant was discarded. The cell concentrate was then suspended in 1% low melting point agarose (37˚C) and two aliquots were dropped onto a slide pre-coated with 1% normal melting point agarose. Coverslips were placed on top of the sample and slides were left for 10 min at 4˚C. Once the gel was set, coverslips were carefully removed and the comet assay was conducted, following [36], modifying for alkaline conditions. Slides were placed in lysis solution for 1 hour, followed by 40 min

denaturation in electrophoresis buffer (pH 13) and then electrophoresis for 30 min (25 V, 300 mA). The slides were then gently washed in neutralising buffer. 100 cells per slide (50 per gel) were scored within 48 h using sybr safe staining and a fluorescent microscope (420-490 nm excitation filter and 520 nm emission filter) equipped with Kinetic COMET software.

Chemical Analysis

Nicotine and its metabolite cotinine were used as biomarkers of exposure to the toxicants associated with filters. Frozen ragworm tissue was thoroughly homogenised under liquid nitrogen using a pestle and mortar. For each exposure and concentration sub-aliquots of homogenised tissue from each individual were pooled.

Chromatography and detection (MS/MS) parameters

Analysis was carried out on an Acquity UPC2 system with a Quattro Premier XE Mass Spectrometer (MSMS) as detector (both from Waters Corp, Milford USA). See Supplementary Table 1 for details.

Sample Preparation

4. *Water Samples*
   Five hundred µL samples of aqueous exposure media (water) were spiked with internal standard solution (25 µL of a solution containing 500 ng/mL nicotine-D4 and cotinine-D3) and then adjusted to pH 10 with ammonia. Liquid-liquid extraction was performed with 1 mL ethyl acetate. The upper (ethyl acetate) phase was removed and analysed.
5. *Sediment Samples*
   Sub-samples (0.5 g) were weighed into 10 mL glass test-tubes and spiked with internal standard solution (100 µL of a solution containing 500 ng/mL nicotine-D4 and cotinine-D3) together with 3mL water (2 % ammonium hydroxide) and 4 mL acetonitrile. Samples were then extracted and cleaned according to AOAC Method 2007.01 for pesticide residues in foods by acetonitrile extraction and partitioning with magnesium sulphate (REFERENCE: available online at http://www.eoma.aoac.org/methods/info.asp?ID=48938).

6. *Ragworm Samples*

   Fifty milligram ragworm samples were weighed into 2 mL tubes and spiked with internal standard solution (10 µL of a solution containing 500 ng/mL nicotine-D4 and cotinine-D3) together with 300 µL water (2 % ammonium hydroxide) and 400 µL acetonitrile. Samples were then extracted and cleaned according to AOAC Method 2007.01 for pesticide residues in foods by acetonitrile extraction and partitioning with magnesium sulphate (REFERENCE: available online at http://www.eoma.aoac.org/methods/info.asp?ID=48938).

Statistical Analyses

Statistical analyses were performed in R[37]. To ensure correct specification of the models used (analysis of variance), the distribution of residuals was monitored using the Shapiro Wilks test for normality and Levene's test for homogeneity of variance. Where data did not conform to model assumptions, a $\log_{10}(x+1)$ transformation was performed. If this did not increase suitability, an equivalent non-parametric test was performed.

Any change in the weight of ragworms during exposures was assessed using the method of [38]. First, relative growth rate (RGR) was calculated as shown in equation (1).

$$RGR = \ln\left(\frac{fw}{iw}\right)$$

Equation (1) where fw = final weight and iw = initial weight. The effect of treatment on RGR was then analysed using a one-way ANOVA (n=6).

Any change in burrowing time due to exposure was determined using a one-way ANOVA or Kruskal Wallis test where appropriate (n=6). A change in tail intensity (TI) and tail moment (TM) was analysed using the methods of [29,39,40], whereby the statistical analysis is performed by animal (as opposed to by gel or by cell) using a summary statistic as calculated in equation (2).

$$\text{Mean (across replicate gels) of the x percentile of the } \log_{10}(TI+1)$$

Equation (2) where x is substituted for the median, 75th, or 90th percentile based on recommendations by [29,40]. The effect of treatment on TI and TM was then analysed using a one-way ANOVA or Kruskal Wallis test (n=6).

Where a Kruskal Wallis was applied and a significant p-value obtained, post-hoc Wilcoxon rank sum tests were used on pairwise permutations. Post-hoc analysis following a one-way ANOVA was conducted using a Tukey HSD test. The lowest concentration which elicited a significantly different response compared to the control was identified as the Lowest Observed Effect Concentration (LOEC), whilst the highest concentration which did not cause a significantly different response compared to the control was identified as the No Observed Effect Concentration (NOEC).

Bioaccumulation

A bioconcentration factor and bioaccumulation factor (BCF and BAF) – the level of accumulation of a chemical in an organism from seawater and sediment, respectively - was calculated using equation (3).

$$BCF = \frac{CB}{CM}$$

Equation (3) where CB = biota concentration and CM = medium (leachates or sediment) concentration.

References

1 Sutherland, W. J. *et al.* A horizon scan of global conservation issues for 2010. *Trends in Ecology & Evolution* **25**, 1-7, doi:http://dx.doi.org/10.1016/j.tree.2009.10.003 (2010).

2 Woodall, L. C. *et al.* The deep sea is a major sink for microplastic debris. *Royal Society Open Science* **1** (2014).

3 Andrady, A. L. Microplastics in the marine environment. *Marine Pollution Bulletin* **62**, 1596-1605, doi:http://dx.doi.org/10.1016/j.marpolbul.2011.05.030 (2011).

4 Novotny, T. & Slaughter, E. Tobacco Product Waste: An Environmental Approach to Reduce Tobacco Consumption. *Curr Envir Health Rpt* **1**, 208-216, doi:10.1007/s40572-014-0016-x (2014).

5 Hon, N.-S. Photodegradation of cellulose acetate fibers. *Journal of Polymer Science: Polymer Chemistry Edition* **15**, 725-744, doi:10.1002/pol.1977.170150319 (1977).

6 Moriwaki, H., Kitajima, S. & Katahira, K. Waste on the roadside, 'poi-sute' waste: Its distribution and elution potential of pollutants into environment. *Waste Management* **29**, 1192-1197, doi:http://dx.doi.org/10.1016/j.wasman.2008.08.017 (2009).

7 Mehlhart, G., and Blepp, M. Study on land-sourced litter (LSL) in the marine environment. 8 (Institute for Applied Ecology, Germany, 2012).

8 Ivar do Sul, J. A. & Costa, M. F. Marine debris review for Latin America and the Wider Caribbean Region: From the 1970s until now, and where do we go from here? *Marine Pollution Bulletin* **54**, 1087-1104, doi:http://dx.doi.org/10.1016/j.marpolbul.2007.05.004 (2007).

9 Snelgrove, P. V. R. The Importance of Marine Sediment Biodiversity in Ecosystem Processes. *Ambio* **26**, 578-583, doi:10.2307/4314672 (1997).

10 Dhainaut, A. & Scaps, P. Immune defense and biological responses induced by toxics in Annelida. *Canadian Journal of Zoology* **79**, 233-253, doi:10.1139/z00-196 (2001).

11 Hutchings, P. Biodiversity and functioning of polychaetes in benthic sediments. *Biodiversity and Conservation* **7**, 1133-1145, doi:10.1023/A:1008871430178 (1998).

12 Scaps, P. A review of the biology, ecology and potential use of the common ragworm Hediste diversicolor (O.F. Müller) (Annelida: Polychaeta). *Hydrobiologia* **470**, 203-218, doi:10.1023/a:1015681605656 (2002).

13 Matta, S. G. *et al.* Guidelines on nicotine dose selection for in vivo research. *Psychopharmacology* **190**, 269-319, doi:10.1007/s00213-006-0441-0 (2007).

14 Micevska, T., Warne, M. S. J., Pablo, F. & Patra, R. Variation in, and Causes of, Toxicity of Cigarette Butts to a Cladoceran and Microtox. *Archives of Environmental Contamination and Toxicology* **50**, 205-212, doi:10.1007/s00244-004-0132-y (2006).

15 Slaughter, E. *et al.* Toxicity of cigarette butts, and their chemical components, to marine and freshwater fish. *Tobacco Control* **20**, i25-i29, doi:10.1136/tc.2010.040170 (2011).

16 Benowitz, N. L., Hukkanen, J. & Jacob, P., 3rd. Nicotine chemistry, metabolism, kinetics and biomarkers. *Handbook of experimental pharmacology*, 29-60, doi:10.1007/978-3-540-69248-5_2 (2009).

17 Ahrens, M. J., and Lopez, G. R. in *Organism-sediment interactions The Belle W. Baruch library in marine science* (ed J. Y. Aller, Woodin, S. A., and Aller, R. C.) (University of South Carolina Press, 2001).

18 Buccafusco, J. J., Shuster, L. C. & Terry, A. V., Jr. Disconnection between activation and desensitization of autonomic nicotinic receptors by nicotine and cotinine. *Neuroscience letters* **413**, 68-71, doi:10.1016/j.neulet.2006.11.028 (2007).

19 Mato, Y. *et al.* Plastic Resin Pellets as a Transport Medium for Toxic Chemicals in the Marine Environment. *Environmental Science & Technology* **35**, 318-324, doi:10.1021/es0010498 (2001).

20 Hirai, H. *et al.* Organic micropollutants in marine plastics debris from the open ocean and remote and urban beaches. *Marine Pollution Bulletin* **62**, 1683-1692 (2011).

21 Browne, Mark A., Niven, Stewart J., Galloway, Tamara S., Rowland, Steve J. & Thompson, Richard C. Microplastic Moves Pollutants and Additives to Worms, Reducing Functions Linked to Health and Biodiversity. *Current Biology* **23**, 2388-2392, doi:http://dx.doi.org/10.1016/j.cub.2013.10.012 (2013).

22 Bakir, A., Rowland, S. J. & Thompson, R. C. Enhanced desorption of persistent organic pollutants from microplastics under simulated physiological conditions. *Environmental Pollution* **185**, 16-23, doi:http://dx.doi.org/10.1016/j.envpol.2013.10.007 (2014).

23 Capowiez, Y., Rault, M., Costagliola, G. & Mazzia, C. Lethal and sublethal effects of imidacloprid on two earthworm species (Aporrectodea nocturna and Allolobophora icterica). *Biol Fertil Soils* **41**, 135-143, doi:10.1007/s00374-004-0829-0 (2005).

24 Dittbrenner, N., Triebskorn, R., Moser, I. & Capowiez, Y. Physiological and behavioural effects of imidacloprid on two ecologically relevant earthworm species (Lumbricus terrestris and Aporrectodea caliginosa). *Ecotoxicology* **19**, 1567-1573, doi:10.1007/s10646-010-0542-8 (2010).

25 Thomas, W. C. *et al.* Subchronic oral toxicity of cellulose acetate in rats. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* **29**, 453-458 (1991).

26 Wright, S. L., Rowe, D., Thompson, R. C. & Galloway, T. S. Microplastic ingestion decreases energy reserves in marine worms. *Current biology : CB* **23**, R1031-R1033 (2013).

27 Capowiez, Y. & Bérard, A. Assessment of the effects of imidacloprid on the behavior of two earthworm species (Aporrectodea nocturna and Allolobophora icterica) using 2D terraria. *Ecotoxicology and Environmental Safety* **64**, 198-206, doi:http://dx.doi.org/10.1016/j.ecoenv.2005.02.013 (2006).

28 Yildiz, D. Nicotine, its metabolism and an overview of its biological effects. *Toxicon : official journal of the International Society on Toxinology* **43**, 619-632, doi:10.1016/j.toxicon.2004.01.017 (2004).

29 Bright, J. *et al.* Recommendations on the statistical analysis of the Comet assay. *Pharmaceutical statistics* **10**, 485-493, doi:10.1002/pst.530 (2011).

30 Crowley-Weber, C. L. *et al.* Nicotine increases oxidative stress, activates NF-kappaB and GRP78, induces apoptosis and sensitizes cells to genotoxic/xenobiotic stresses by a multiple stress inducer, deoxycholate: relevance to colon carcinogenesis. *Chemico-biological interactions* **145**, 53-66 (2003).

31 Sobkowiak, R. & Lesicki, A. Genotoxicity of nicotine in cell culture of Caenorhabditis elegans evaluated by the comet assay. *Drug and chemical toxicology* **32**, 252-257, doi:10.1080/01480540902882184 (2009).

32 Huerta-Fontela, M., Galceran, M. T., Martin-Alonso, J. & Ventura, F. Occurrence of psychoactive stimulatory drugs in wastewaters in north-eastern Spain. *The Science of the total environment* **397**, 31-40, doi:10.1016/j.scitotenv.2008.02.057 (2008).

33 Roder Green, A. L., Putschew, A. & Nehls, T. Littered cigarette butts as a source of nicotine in urban waters. *Journal of Hydrology* **519, Part D**, 3466-3474, doi:http://dx.doi.org/10.1016/j.jhydrol.2014.05.046 (2014).

34 Ariza, E., Jimenez, J. A. & Sarda, R. Seasonal evolution of beach waste and litter during the bathing season on the Catalan coast. *Waste management (New York, N.Y.)* **28**, 2604-2613, doi:10.1016/j.wasman.2007.11.012 (2008).

35 Fairbairn, D. W., Olive, P. L. & O'Neill, K. L. The comet assay: a comprehensive review. *Mutation Research/Reviews in Genetic Toxicology* **339**, 37-59, doi:http://dx.doi.org/10.1016/0165-1110(94)00013-3 (1995).

36 Singh, N. P., McCoy, M. T., Tice, R. R. & Schneider, E. L. A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental cell research* **175**, 184-191 (1988).

37 R: a language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria, 2013).

38 Crawley, M. J. *Statistics: An Introduction using R*. (Wiley, 2005).

39 Wiklund, S. J. & Agurell, E. Aspects of design and statistical analysis in the Comet assay. *Mutagenesis* **18**, 167-175, doi:10.1093/mutage/18.2.167 (2003).

40 Duez, P., Dehon, G., Kumps, A. & Dubois, J. Statistics of the Comet assay: a key to discriminate between genotoxic effects. *Mutagenesis* **18**, 159-166, doi:10.1093/mutage/18.2.159 (2003).

Acknowledgments

Many thanks to Dr. Yuktee Dogra and Steve Cooper for sieving sediment. Thanks to the volunteers who saved smoked cigarette filters. M.R.E. and K.V.T. acknowledge the financial support from NFR project 225203. S.L.W, D.R., and T.S.G acknowledge financial support from the Department for Environment, Food & Rural Affairs project 1-SW-P-N21-000-031-DN-A1-05102.

Author Contributions

S.L.W. and T.S.G. conceived the study. S.L.W. designed experiments with the input of T.S.G. S.L.W. carried out experiments. D. R. assisted in the collection of worms, and the preparation and termination of experiments. M.J.R. and K.V.T. conducted chemical analyses. S.L.W. performed statistical analyses. T.S.G. helped in the interpretation of data and preparation of the manuscript. S.L.W. wrote the manuscript, with contribution from all authors.

Additional Information

The authors declare no competing financial interests.

Tables

Filters L^{-1}	Nicotine (ng ml^{-1}/g^{-1})			Nicotine (ng g^{-1} tissue)			BCF/BAF			N:C ratio		
	Leachates	Sediment		Leachates	Sediment		Leachates	Sediment		Leachates	Sediment	
	96 h	96 h	28 d	96 h	96 h	28 d	96 h	96 h	28 d	96 h	96 h	28 d
0	5.5	0	0	0	0	0	0	0	0	0	0	0
0.5	23.5	787	350	1901	186	41	80.89	0.24	0.12	57.6	0	0
1	62.5	1399	971	4912	374	129	78.59	0.27	0.13	29.6	0	0
2	172	3124	1759	10193	766	211	59.26	0.25	0.12	71.3	76.6	0
4	235.5	5287	3743	38072	1318	672	161.66	0.25	0.18	134.1	54.9	67.2
8	694	11159	6964	119654	3629	854	172.41	0.33	0.12	792.4	62.6	61

Table 1. Nicotine concentrations measured in the stock exposure medium and whole ragworm tissue following 96 h exposure to smoked cigarette filter toxicants in seawater, and 96 h and 28 d exposure to smoked cigarette filter microfibres in sediment.

Organism	Dose delivered (1	Dose accumulated (1	Nicotine exposure

	cigarette)	cigarette)	relative to humans
Human (smoking; 1 cigarette)	10-30 µg kg^{-1}	10-50 ng ml^{-1}	-
Ragworm (96 h exposure to smoked cigarette filter toxicants in seawater)	63 µg kg^{-1}	4912 ng g^{-1}	98x
Ragworm (96 h sediment exposure to smoked cigarette filter microfibres)	1400 µg kg^{-1}	374 ng g^{-1}	7.5x
Ragworm (28 d sediment exposure to smoked cigarette filter microfibres)	1000 µg kg^{-1}	129 ng g^{-1}	2.6x

Table 2. The nicotine dose delivered to: a human smoker; a ragworm following 96 h exposure to smoked cigarette filter toxicants in seawater; and a ragworm following short- and long-term exposure to smoked cigarette filter microfibres in sediment, at equivalent concentrations (1 cigarette/filter L^{-1}).

Figures

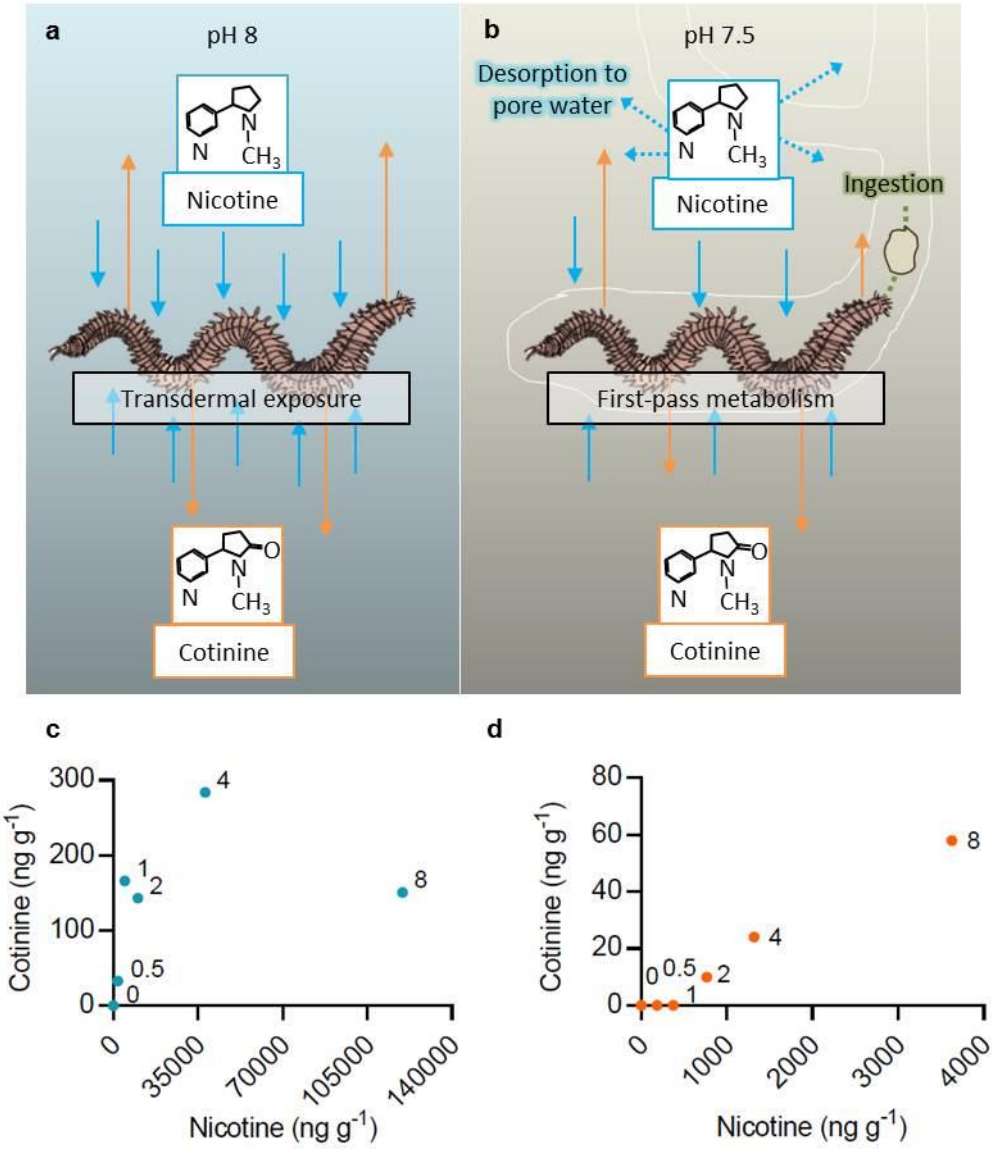


Figure 1. The bioaccumulation of nicotine in ragworms. The potential routes of nicotine transfer to ragworms from smoked cigarette filter a) toxicants in seawater, and b) microfibres in sediment (ragworm drawn by S. Wright). The bioconcentration and bioaccumulation of nicotine and cotinine for ragworms following 96 h exposure to smoked cigarette filter c) toxicants in seawater, and d) microfibres in sediment.

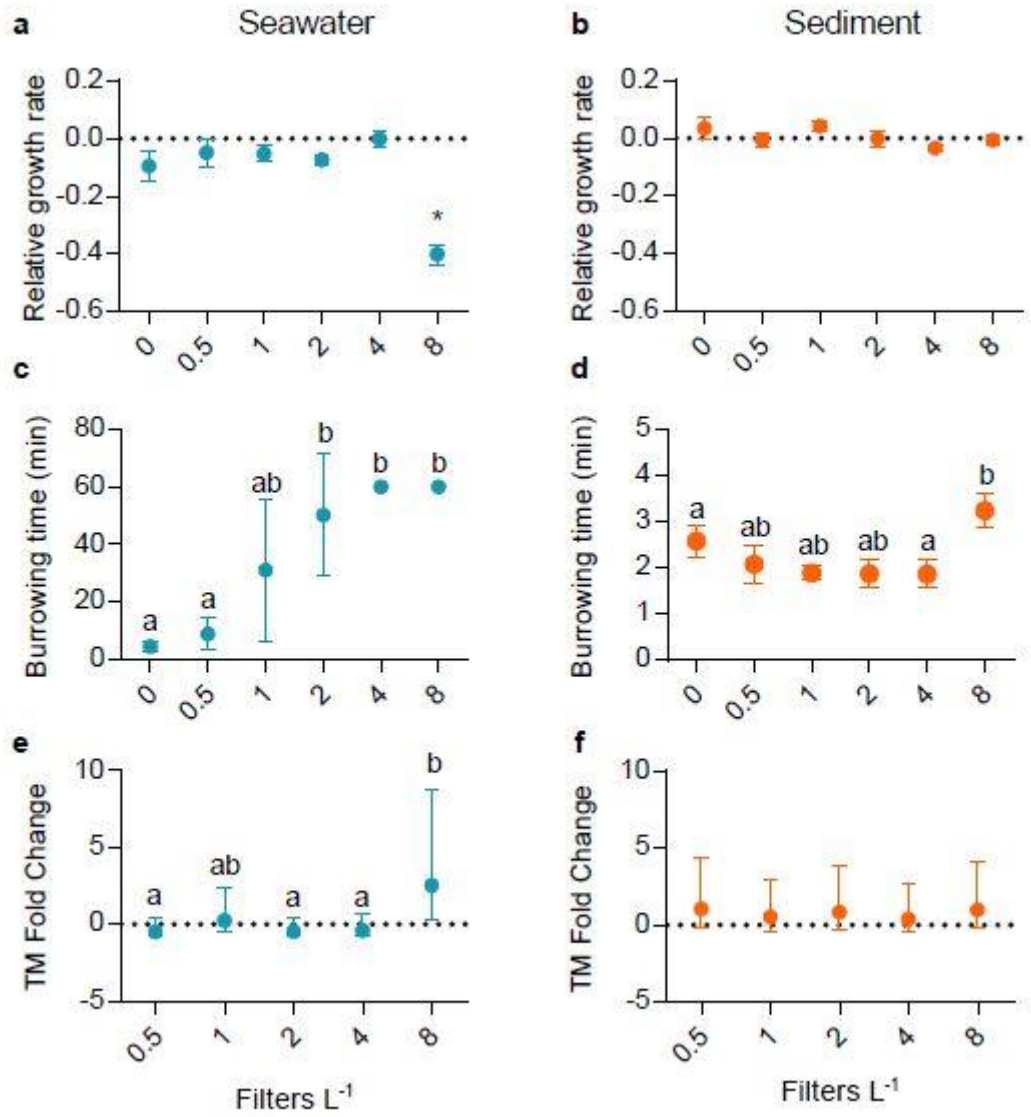


Figure 2. The biological impacts of smoked cigarette filter exposure on ragworms. The effect of 96 h exposure to smoked cigarette filter a) toxicants in seawater, and b) microfibres in sediment on the relative growth rate (RGR) of ragworms (mean ± s.e.m.). The effect of 96 h exposure to smoked cigarette filter d) toxicants in seawater, and e) microfibres in sediment on the burrowing time (minutes) of ragworms (mean ± s.e.m.). The effect of 96 h exposure to smoked cigarette filter g) toxicants in seawater, and h) microfibres in sediment on DNA damage in ragworms, measured as fold-change in the 90th percentile tail moment relative to control ragworms (indicated by the dotted line, mean ± s.e.m.). Significance between groups, as identified by post-hoc analysis, is indicated by different letters. * denotes significance compared to all other groups.