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ENVIRONMENTAL AND BIOLOGICAL CONSEQUENCES OF MICROPLASTIC WITHIN MARINE HABITATS

by

MARK ANTHONY BROWNE

A thesis submitted to the University of Plymouth
in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

School of Biological Sciences

October 2007

Frontispiece. Plastic debris on a beach in Looe, Cornwall, UK.

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October 2007

Environmental and biological consequences of microscopic plastic within marine habitats

Mark Anthony Browne

Abstract

Large pieces of plastic greater than a millimetre in diameter contaminate marine habitats worldwide and the associated environmental problems are well documented. In addition tiny fragments of plastic debris less than a millimetre in size have recently been reported. This thesis examines the distribution and environmental consequences of microscopic particles of plastic within marine habitats.

To quantify the relative influence of wind and depositional environment on the accumulation of plastic debris, a mensurative experiment was conducted in a macrotidal Estuary. The overall trend was that material accumulated in down-wind sites. However, the relative importance of wind as a transport agent depended on the size and density of the plastic. Natural sediments are transported according to their size; but the extent to which models of sediment dynamics could be applied to the transport of plastic debris remains untested. I examined relationships between the abundance of microplastic debris and sediment particle size, latitude and human population density using samples from sandy shores worldwide. Microplastic was found at every location, showing the global extent of this contamination and there was a significant positive correlation between human population density and microplastic abundance. Sewage sludge disposal grounds were examined as potential sources of microplastic. Replicate sediment grab samples showed that disposal grounds near Plymouth and Newcastle (UK) had greater abundance of microplastic debris compared to reference sites.

To investigate the biological consequences of ingesting clean microplastic particles the mussel, *Mytilus edulis* (L.) was used as a model organism. The fate of ingested plastic was tracked within the body tissues using a laboratory trial. Mussels were exposed to 3.0 and 9.6μm microplastic particles in seawater for 3 hours and then transferred to clean conditions. After 3 days ingested microplastic had accumulated in the circulatory fluid of *M. edulis*. Smaller particles 3.0 μm were present in the haemolymph in consistently higher numbers than larger particles, and both sizes were still present after 48 days. There were no measurable changes in organismal health from ingestion of this material. However, it has been frequently suggested that plastics debris may transfer chemical contaminants to marine life. To test this, the sorption-affinity of candidate environmental hydrophobic contaminants from aqueous solution onto microscopic particles of polyvinylchloride and similar sized particles of sand was compared. Chemical analysis confirmed that polyvinylchloride absorbed more contaminants than sand. A second experiment examined the bioavailability of sorbed contaminants and chemical additives that are incorporated into plastic during manufacture. Laboratory trials using *Arenicola marina* (L.) showed that the sorbed contaminants and additives bioconcentrated in gut tissues leading to deleterious biological effects.

In conclusion, microplastic debris is a ubiquitous form of contamination and when ingested, this material can translocate from the gut to the circulatory system and haemocytes, and can transfer chemicals into animal tissues, and reduce the health of animals near the base of the food chain. The implications of these findings are discussed in relation to potential measures to improve the management of plastics in society and to reduce the amount of plastic entering the environment.

List of contents

Abstract iii
List of figures viii
List of tables xi
Acknowledgements xii

Chapter 1. Microplastic an emerging contaminant of potential concern?

1.1 *Abstract* 2
1.2 What is plastic? 3
1.3 Brief history of plastic 5
1.4 Global plastic production, use and subsequent disposal 9
1.5 Degradation and persistence of plastic 10
1.6 What are the sources of microplastic in the environment? 14
1.7 Contamination of marine habitats by plastic debris 15
1.8 What is the extent of microplastic contamination in marine habitats? 16
1.9 Environmental consequences of microplastic in the environment 17
1.10 Does ingestion of microplastic have any toxicological consequences for animals? 19
1.11 *Aims of thesis* 21

Chapter 2. Influence of wind, depositional environment, and size and density of plastic debris on patterns of accumulation in the Tamar Estuary, UK

2.1 *Abstract* 24
2.2 *Introduction* 25
2.2.1 Hypotheses tested 27
2.3 *Materials and methods* 28
2.3.1 Field sampling 28
2.3.2 Extraction and identification of plastic debris 30
2.3.4 Sediment particle size analysis 31
2.3.5 Statistical analyses 31
2.4 *Results* 33
2.4.1 Characterisation of plastic debris within the Tamar Estuary (UK) 33
2.4.2 Influence of wind, depositional environment and plastic density on patterns of plastic debris accumulation 35
2.4.3 Influence of wind and depositional environment on the composition of plastic debris 38
2.4.4 Relationship between sediment grain composition and the abundance of microplastic debris 41
2.5 *Discussion* 41
2.5.1 Implications for marine habitats 43

Chapter 3. Global trends in microplastic abundance and the possible role of sewage disposal sites as sources of microplastic debris to marine habitats

3.1 *Abstract* 48
3.2 *Introduction* 49
3.2.1 Hypotheses tested 53
3.3 *Materials and methods* 53
3.3.1 Global sampling 53
3.3.2 Sewage disposal site sampling 55
3.3.3 Statistical analyses 56
3.4 *Results* 57
3.4.1 Global trends in the abundance of microplastic fragments in relation to mean sediment size, latitude and population density 57
3.4.2 Sewage disposal sites as potential sources of microplastic contamination in marine habitats 60
3.5 *Discussion* 61
3.5.1 Global trends in the abundance of microplastic fragments in relation to population density, mean sediment size and latitude 61
3.5.2 Sewage disposal sites as potential sources of microplastic contamination in marine habitats 63
3.5.2 Environmental implications of microplastic as a ubiquitous contaminant in marine habitats 64

Chapter 4. Biological consequences of microplastic in the mussel, *Mytilus edulis* (L.)

4.1 *Abstract* 67
4.2 *Introduction* 67
4.2.1 Potential toxicological consequences for marine animals ingesting microplastic debris 69
4.2.2 Ecotoxicological relevance of *Mytilus edulis* (L.) as a test organism for uptake of microplastic 70
4.2.3 Environmental relevance of polystyrene as a contaminant in marine habitats 71
4.2.4 Hypotheses tested 72
4.3 *Materials and methods* 73
4.3.1 Husbandry and experimental design 73
4.3.2 Biological assays 75
4.3.3 Statistical analyses 78
4.4 *Results* 80
4.4.1 Ingestion and translocation of polystyrene microspheres in *Mytilus edulis* (L.) 80

4.4.2 Biological effects of ingestion and translocation of polystyrene microspheres in *Mytilus edulis* (L.) 82
4.5. *Discussion* 84
4.5.1 Translocation of ingested polystyrene microspheres from the gut cavity to the circulatory system of *Mytilus edulis* (L.) 84
4.5.2 Influence of 'particle-size' on the translocation of ingested polystyrene microspheres from the gut cavity to the haemolymph and haemocytes of *Mytilus edulis* (L.) 86
4.5.3 Toxicological consequences of ingestion and translocation of polystyrene microspheres in *Mytilus edulis* (L.) 87
4.5.4 Implications for marine habitats 89

Chapter 5. Bioavailability and toxicity of chemicals associated with microplastic to the lugworm, *Arenicola marina* (L.)

5.1 *Abstract* 91
5.2 *Introduction* 92
5.2.1 Chemicals used as additives in plastic manufacture and their toxicity 93
5.2.2 Potential for hydrophobic contaminants to adsorb to plastic debris and their subsequent toxicity 96
5.2.3 Environmental relevance of microscopic polyvinylchloride as a contaminant in marine habitats 99
5.2.4 Ecotoxicological relevance of *Arenicola marina* (L.) as a test organism for the uptake of chemicals from plastic 99
5.2.5 Hypotheses tested 102
5.3 *Materials and methods* 103
5.3.1 Adsorption of persistent organic pollutants onto microplastic and sand particles 103
5.3.2 Incorporation of additives into microplastic 104
5.3.3 Exposure of *Arenicola marina* (L.) to toxified sediments 105
5.3.4 Biological assays 107
5.3.5 Chemical analyses 109
5.4 *Results* 111
5.4.1 Sorption of hydrophobic contaminants onto microplastic compared to sand particles 111
5.4.2 Uptake of chemicals from microplastic to the tissues of *Arenicola marina* (L.) 112
5.4.3 Bioavailability of adsorbed hydrophobic contaminants from sand and polyvinylchloride into the tissues of *Arenicola marina* (L.) 113
5.4.4 Biological consequences of ingesting microplastic with nonylphenol, phenanthrene, tetrabrominated diphenyl ether and triclosan for *Arenicola marina* (L.) 114

5.5 *Discussion* 120
5.5.1 Sorption of hydrophobic contaminants by polyvinylchloride and sand particles 120
5.5.2 Bioconcentration of hydrophobic contaminants from microscopic polyvinylchloride particles 121
5.5.3 Bioavailability of nonyphenol and phenanthrene from sand and polyvinylchloride particles 122
5.5.4 Biological consequences of ingesting polyvinylchloride particles with plastic with nonylphenol, phenanthrene, tetrabromodiphenylether and triclosan for *Arenicola marina* (L.) 124
5.5.5 Implications for food chains 126

Chapter 6. Environmental consequences of microscopic plastic in marine habitats

6.1 *Summary of findings* 129
6.2 Sustainable plastic use and waste prevention 130
6.3 Holistic life cycle assessments 134
6.4 Standardised monitoring of litter 135
6.5 Future research priorities 137
6.5.1. Spatial distribution of microplastic 137
6.5.2. Trophic distribution of microplastic 137
6.5.3 Degradation and persistence of plastic in natural habitats 138
6.5 *Where to from here?* 139

Appendices 140
List of references 143
Bound copies of publications 160

List of figures

Figure 1. Generalised production procedure for plastics (adapted from the Environment Agency 2001) 3

Figure 2. Tamar Estuary, U.K. (a) Sampling locations with up-wind sites; W1 Cargreen (50°26:34'N, 4°12:27'W), W2 Saltash 50°25:08'N, 4°12:40'W, W3 Cremyl (50°:21:37'N, 4°10:26'W) and down-wind sites; E1 Weir Quay (50°27:59N, 4°12:49'W), E2 Ernesettle (50°25:22'N, 4° 11:33'W) E3 Cremyll Street (50°:21:52'N, 4° 09:47'W). (b) Wind rose for Plymouth illustrating the speed and direction of winds and confirming the predominant southwestly winds (Adapted from the Met Office, http://www.metoffice.gov.uk/). (c) Percentage clay (0.00024 - 0.004 mm) in strandline sediments, values expressed as mean ± SE. Arrow indicates depositional environment stress based on clay content. 29

Figure 3. Identity composition of strandline plastic debris in Tamar Estuary, UK according to usage (a) and relative size (b). Data are presented as mean ± S.E. for all locations n = 15 (*N.B.* log scale for microplastic in part a). 34

Figure 4. Percentage composition of strandline (a) micro and (b) macroplastic debris in Tamar Estuary (UK), values expressed as percentages of total synthetic polymers identified using Fourier Transform Infrared Spectroscopy. 34

Figure 5. Abundance of microplastic debris (50 g sediment $^{-1}$) and macro- (0.25 m^{-2}) within the Tamar Estuary according to density. Values expressed as mean ± S.E. Significant different groupings from SNK tests are indicated by dissimilar letters ('a', 'b' and 'c'). 37

Figure 6. Abundance and composition of microplastic (< 1 mm) debris within the strandline of the Tamar estuary. (a) Nonparametric multidimensional scaling (nMDS) ordination plot of microplastic debris in relation to wind and depositional environment (Bray-Curtis similarity, no transformation), with two major superimposed clusters at 44 % Bray-Curtis similarity. 39

Figure 7. Abundance and composition of macroplastic (< 1 mm) debris within the Tamar Estuary. (a) Nonparametric multidimensional scaling (nMDS) ordination plot of macrodebris in relation to wind and depositional environment (Bray-Curtis similarity, no transformation), with two major superimposed clusters at 20 % Bray-Curtis similarity. 40

Figure 8. Locations of UK sewage-sludge disposal sites (1970 - 1998). Plymouth and Tyne disposal sites presented as filled black circles, whereas the other 11 sites are with open circles. 51

Figure 9. Composition (%) of microplastic debris collected from 18 intertidal sandy habitats around the world. 57

Figure 10. Examples of Fourier Transform Infrared spectra of microplastic debris and corresponding plastic reference material, vertical axis represents transmission in standard optical density units (Spectra courtesy of E. Teuten and A. Tonkin, University of Plymouth)........ 58

Figure 11. Relationship between (a) mean sediment particles size and number of microplastic debris in marine habitats (Spearman Rank 0.39, $P > 0.05$ ns), (b) latitude and number of microplastic items, Spearman Rank for Northern Hemisphere 0.28 and Southern Hemisphere 0.53, both $P > 0.05$ ns. (c) Linear regression analysis of log(population density) and log(number of microplastic items) in sediment from sandy habitats and the number of plastic items ($F_{1,16} = 8.36$, $P < 0.05$, $r^2 = 0.34$). Country codes in (c) indicated in table 5. 59

Figure 12. Abundance of microplastic in sediments from sewage disposal and reference sites at two locations in the UK (Tyne and Plymouth). Values expressed as mean ± SE and significant difference $P < 0.05$. 60

Figure 13. Fragments of polyethylene, polyvinylchloride, polypropylene, and polystyrene from the Tamar Estuary, UK. 62

Figure 14. Experimental design for second trial investigating the translocation of polystyrene microspheres and possible biological effects in *Mytilus edulis*. Time/treatment/replicate order was randomised using a replicated block design to remove bias associated with possible environmental gradients in a controlled temperature room. 75

Figure 15. Uptake of plastic particle by *Mytilus edulis* (L.) (a) Tissue section (4 µm thick) containing 2 µm and (b) 4 to 16 µm polystyrene microspheres in the gut cavity and digestive tubules. 3.0 (c) and 9.6 µm (d) polystyrene microspheres in the haemolymph and (e) haeomcytes. Significant differences in accumulation in the haemolymph according to (f) "particle-size" and (g) "time" at $P < 0.01$**. *N.B.* Values are expressed as mean ± SE and calculated from independent data from each time/treatment/replicate combination and are therefore are not cumulative. 81

Figure 16. Temporal changes at different levels of biological organisation within *Mytilus edulis*. Values are expressed as mean ± S.E. Differences between times marked with the same letter (a,b,c) were not significantly different; and statistical significance all at $P <$ 0.001 83

Figure 17. Scanning electron micrograph of polyvinylchloride and sand particles used for the study. 104

Figure 18. Adsorption of hydrophobic contaminant onto particles of polyvinylchloride compared to sand. Values expressed are dry mass mean ± S.E. *** and ** denotes statistical significance at $P < 0.001$ and $P < 0.01$ respectively. 111

Figure 19. Bioavailability of hydrophobic contaminants (nonylphenol, Cochran's C = 0.69 and phenanthrene, Cochran's C = 0.72) and additives (TBDE, Cochran's C = 0.45 and triclosan) from a mixture of sand and microscopic polyvinylchloride to the skin and gut tissues of the lugworm, *Arenicola marina*. For each chemical; treatments marked with the same letter (a,b,c) were not significantly different; '#' statistical comparisons with sediment not possible due to high mortality. Values are expressed are mean dry mass ± S.E for each chemical. 112

Figure 20. Contaminant uptake and tissue distribution within *Arenicola marina*. Values are expressed as mean ± S.E. ** and * denotes statistical significance at $P < 0.01$ and $P < 0.05$ respectively, whilst ns indicates not significant $P > 0.05$. 114

Figure 21. Percentage mortality across treatments. Statistical analysis not possible......... 115

Figure 22. Burrowing time of *Arenicola marina*, expressed by the number of seconds taken to achieve complete burial. Values are expressed as mean ± S.E and '*ns*' indicates not significant........ 116

Figure 23. Casting behaviour within *Arenicola marina*, expressed by the mean number of casts produced day $^{-1}$ individual $^{-1}$. Values are expressed as mean ± S.E. Casting behaviour was significantly depressed in triclosan treatment. Statistical significance at $P < 0.01$** and '*ns*' indicates not significant. 117

Figure 24. Coelomocyte phagocytosis from *Arenicola marina* expressed as number of zymosan particles (1 x 10^5) phagocytosed g^{-1} coelomocyte protein. Values expressed as mean ± S.E and * denotes statistical significance for presence of nonylphenol (Cochran's C =0.59), whilst '*ns*' indicates not significant $P > 0.05$. 118

Figure 25. Ferric reducing antioxidant potential (FRAP) of the coelomic fluid of *Arenicola marina*. Values expressed as mean ± S.E and statistical significance at $P < 0.05$*, $P < 0.001$*** and '*ns*' indicates not significant. 119

Figure 26. Sustainable (white arrows) and non-sustainable (black arrows) use of plastic in modern society, gray curved arrows represent reuse. Thickness of each arrow indicates proportion of total plastic........ 131

List of tables

Table 1. Scientific development of plastic materials (* No longer produced). 8

Table 2. Analysis of variance table showing the relative importance of wind, depositional environment, size and density on patterns on the abundance of plastic debris within the Tamar Estuary, UK. Post-hoc pooling carried out when $P > 0.25$ is indicated by a hyphon 36

Table 3. Differences in the composition of microdebris between (b) wind and (c) depositional environment from SIMPER analysis.. 39

Table 4. Difference in the composition of macrodebris between (b) wind and (c) depositional environment for SIMPER analysis.. 40

Table 5. Locations for samples taken as part of global programme to quantify microplastic debris in marine habitats. 55

Table 6. Analysis of variance table for uptake and tissue distribution of nonylphenol (Cochran's C= 0.43) and phenanthrene (Cochran's C = 0.59) within *Arenicola marina*....... 113

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- Basic course on design and analyses of ecological experiments using analyses of variance (2005). Taught by Tony Underwood and Gee Chapman (University of Sydney, Australia) at the University of Azores, Portugal.
- Advanced course on design and analyses of ecological experiments using analyses of variance (2005). Taught by Tony Underwood and Gee Chapman (University of Sydney, Australia) at the University of Algarve, Portugal.

Contribution to public education

- Appeared and helped prepare for BBC reports that appeared on Ten O'Clock News, Lunch time News, Radio 4 and Newsround on environmental issues relating to microscopic plastic debris within marine habitats
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Chapter 1. Microplastic an emerging contaminant of potential concern?

1.1 Abstract

Global plastic production is now estimated at 230 million tonnes per year (Plastic-Europe 2006) and 40 % of the plastics that are produced are disposable items that are discarded within a year. Hence, plastic debris is accumulating in landfill and in natural terrestrial and aquatic habitats worldwide. This debris appears to be progressively fragmenting into smaller pieces (Browne et al., 2007) and as the plastic breaks down, the potential for ingestion by animals increases. The biological consequences of macroplastic (≥5 mm) debris on wildlife have been well documented and include suffocation, entanglement and starvation. However, the potential impacts of microscopic debris, which is greater than 1 μm and less than 1 mm are poorly understood. Smaller fragments of plastic less than 1 μm (nanoplastic) are likely to be present in marine habitats; however the limit of detection for the smallest plastic fragment, using Fourier Transform Infra Red spectroscopy, is currently 20 μm

This thesis shall examine the sources, sinks and drivers of microplastic debris in marine habitats and using laboratory experiments to help establish the chemical and biological consequences for the marine animals that ingest this material. This chapter will start by defining plastic and associated terminology. Using relevant examples, the history of plastic in terms of its invention, development and its mass application in society will be explored. The mechanisms of degradation and associated time-scales will be described to illustrate the persistence of synthetic polymers. This synthesis will provide a foundation for evaluating the potential environmental concerns associated with the accumulation of microplastics in marine habitats.

1.2 *What is plastic?*

Plastics are synthetic materials representing over 500 different compounds based on polymers, with 13,000 grades and 25,000 trade names (Elias, 1997). Polymers are defined according to their intrinsic chain-like conformation, which determines their physical and chemical properties during and after manufacture. For instance, the molecular mass is determined by the average chain length of individual monomers and the sequence of these monomers within the plastic (Goodship, 2001).

The term plastic originates from the Greek words, *'plastikos'* and *'plastien'* which mean bendable, and to form or shape, respectively. A more coherent definition is that plastics are man-made polymers produced from synthetic resins that are moulded during manufacture and have the ability to pass through 'plastic states' during processing (Morton-Jones, 1989).

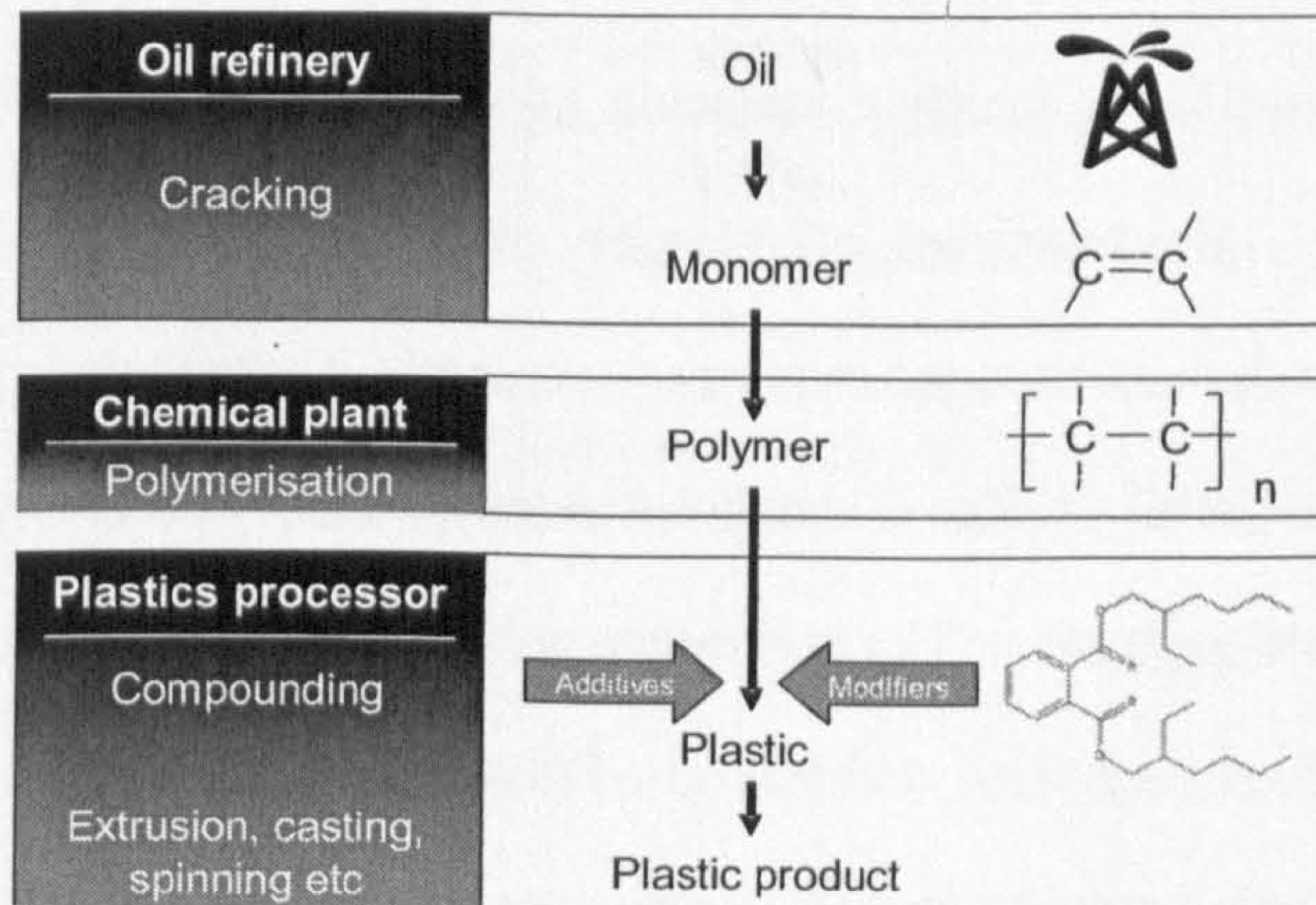


Figure 1. Generalised production procedure for plastics (adapted from the Environment Agency 2001).

There are two types of plastic; thermoplastic which softens when heated and hardens again on cooling (e.g. polyethylene, polystyrene, polyvinylchloride, polypropylene, polymethylmethacrylate, starch-based bioplastic) and thermosetting plastics which are produced by modifying natural polymers or by the polymerisation of petrochemicals (Figure 1) (Isaacs, 1981). These harden on heating to give a product that cannot then be softened by reheating (e.g. polyurethanes, polyesters, silicones and epoxy resins) and therefore cannot be processed by tertiary level recycling that uses high temperatures to reduce plastics into reusable chemicals, fuels or hydrocarbon fractions (McEwan et al., 2002).

Polymerisation involves the chemical combination of monomers to form polymers of repeating units (Figure 1). There are two methods for this, addition and condensation. In addition-polymerisation, the monomers combine together and no other compound is formed. Polyethylene is made from ethylene in this way. In condensation-polymerisation, water, alcohol, or other small molecules are released in the reaction, for example the production of polyamide (Nylon™), in which a molecule of water is released when the monomers join (Ulrich, 1982). However, not all plastics are made from petroleum and some called 'bioplastics' are derived from the monomers of plants. These include cellulose film, polyester and polyamide. Cellulose-film is starch-based and derived from wood cellulose and common uses include food packaging. The polyesters, polylactide acid and poly-3-hydroxybutyrate are used to make food packaging and bottles. Polyamide 11 is derived from vegetable oil and because of its high durability is used to make car fuel pipes, tubing, sport shoes and electrical cable insulation.

Plastic materials have replaced many conventional materials, such as glass, metal and wood, due to their light weight, durability, safety and aesthetics. For instance, containers

made of plastic are shatterproof and therefore safer than glass containers that can lead to injury to humans or to the product being spoilt if dropped. Products such as wine bottles made from plastic are 13 % lighter than glass bottles (Benjamin, 2007). This therefore reduces the energy required to transport them. In the construction industry, polyvinylchloride and polyethylene pipes have replaced the use of heavy metal pipes that are prone to corrosion (EVC, 2000). The use of plastic in computers has led to smaller circuit boards that reduce the size and increase the portability of computers.

1.3 Brief history of plastic

The first plastic, a phenol-formaldehyde resin (Bakelite) was invented in 1908 by Leo Baekeland and was used to make housings for radios and telephones, kitchenware, jewellery, pipe stems, and children's toys (Isaacs, 1981). The invention of these early polymers was unplanned and driven serendipitously by the accidental polymerisation of chemicals used to make other products. In 1912, Fritz Klatte combined acetylene with hydrochloric acid and produced vinyl chloride. This gas was left in glass containers which when exposed to sunlight polymerised and formed an off-white solid material, now known as polyvinylchloride (PVC). However, the solid material was often brittle and no use was initially found. In 1926, the American chemist Waldo Semon invented a method to produce flexible PVC by adding chemical additives that plasticised the PVC. This method was first used to make shower curtains and then shoe heels, golf balls, raincoats and wire insulation. PVC is still used today to make water pipes, electrical wiring, clothes, window frames and weatherboarding for building construction.

The 1930s saw the invention polychloroprene (Neoprene™), polystyrene, polyethylene, polyurethane and polyamide (Nylon™). The uses of these polymers are given in Table 1.

Investigation of these early polymers found many beneficial physico-chemical properties such as light weight, durability, and water and heat resistance. However, it was not until the 1940s that reproducible methods were developed to produce these plastics on an industrial scale. Polyethylene which is widely used today for food packaging was discovered by accident in 1898 by Hans von Pechmann. He was heating diazomethane and noticed a white waxy substance with his colleagues Eugen Bamberger and Friedrich Tschirner and termed it '*Polymethylene*'. In 1933, Eric Fawcett and Reginald Gibson, again accidentally produced the white waxy material when applying several hundred atmospheres of pressure to a mixture of ethylene and benzaldehyde. However, the methodology was not fully reproducible until 1935 when Michael Perrin discovered that Fawcett and Gibson's apparatus had leaked tiny quantities of oxygen into the reaction mixture and that these were then critical to enabling the formation of polyethylene. Using this knowledge, Perrin developed a high-pressure technique that was used to mass produce polyethylene from 1939 onwards (Brydson, 2001). Polytetrafluoroethylene (Teflon) was accidentally discovered by Roy Plunkett in 1938, after he attempted to produce a new chlorofluorocarbon refrigerant using perfluorethylene. In this case iron from the pressurised storage container acted as a catalyst causing the perfluorethylene to polymerise. Polytetrafluoroethylene has very low friction and it is used to make bearings, bushings, gears, sliding plates for machinery and coatings for cookware (e.g. frying pans) and armour-piercing bullets. Karl Ziegler patented a method for production of high density polyethylene (Campbell, 1994) and working with Giulio Natta, Ziegler also developed polypropylene in 1957. Polypropylene is now widely used for food packaging, ropes, textiles, and reusable food containers, car components and banknotes. During the next four decades petroleum was used as an inexpensive source of monomers, and developments in

polymerisation technologies and additives saw a rapid increase in plastic production and use, resulting in the diverse array of plastics used today (Elias, 1997).

Development of new types of plastics continues with degradable polymers for routine deployment in medicine. For instance polymethylmetacrylate is used in bone cement, and microscopic particles of polyglycolide and polylactide are being developed as drug delivery systems that can transfer chemicals from the gut to the circulatory system (Kjellstrand et al., 1994, Ikada and Tsuji, 2000).

To combat environmental concerns over consumer goods packaging (Chandra and Rustgi, 1998), the construction industry is using recycled high density polyethylene (HDPE) as building materials for houses and furniture (Weis et al., 1992). Plastic films made of polyethylene and polyvinylchloride are used in agriculture and horticulture as 'mulching' to cultivate plants, vegetables, fruits and crops. For example, maize grown under greenhouses produces 2.4 tonnes more yield per hectare than plants grown conventionally (Hussain and Hamid, 2003). Plastic covers are also used as 'mulching' to cover the soil around crops to reduce water loss from the soil and limit the use of chemical weed killers by reducing weeds. However, 'mulching' can be difficult to remove and current research is investigating the potential use of photo-degradable plastic films.

Table 1. Scientific development of plastic materials (* No longer produced).

Polymer	Discovery	Production	Main uses
Phenolic resins	1907	1910	Snooker balls, coatings, adhesives
Methyl rubbers	1912	1915	Car tyres*
Alkyd resins	1847	1926	Varnish
Amino resins	1915	1928	Treat water
Polymethyl methacrylate	1880	1928	Glass substitute, prosthetic limbs, drug delivery systems
Polybutadiene	1911	1929	Car tyres
Polyvinyl acetate	1912	1930	Adhesive, copolymer
Polystyrene	1839	1930	Vehicle licence plate frames, plastic cutlery, drinking cups, protective packaging
Polyvinylchloride	1838	1931	Water pipes, electrical wiring, clothes, window frames and weatherboarding
Polyethylene oxide		1931	Thickeners in cosmetic products
Polychloroprene	1925	1932	Wetsuits, laptop sleeves, electrical insulation, and car fan belts
Polyvinyl ethers	1928	1936	Adhesives, plasticisers
Polyisobutylene		1937	Lining of basket balls, additive to fuel
Polyvinylidene chloride	1838	1939	Packaging films*
Polyvinylpyrrolidone		1939	Blood plasma expander, binders, adhesive
Polycaprolactam	1938	1939	Tooth brush bristles, rope, nets, textiles
Polyurethane	1937	1940	Foam insulation
Polyacrylonitrile	1940	1941	Textiles and clothing
Silicone	1901	1942	Sealing, electrical components, breast implants
Epoxy resins	1938	1946	Adhesives
Polyterafluoroethylene	1939	1950	Coatings for bearings, gears, sliding plates for machinery, cookware (e.g. frying pans) and armour-piercing bullets
Polyethylene terephthalate	1941	1953	Food containers and packaging
Polycarbonate	1898	1953	Tubes, food containers, computers
Polyethylene	1898	1939	Bags, packaging
Polybutadiene-		1956	Car tyres
Polypropylene	1954	1957	Food containers, packaging, textiles
Aramid		1961	Flame resistant clothing, tyres, protective clothing
Styrene- block copolymers		1965	Adhesives
Polyhydroxybutyrate		1983	Biodegradable substitute polyolefins in plastic containers, films and bottles

1.4 Global plastic production, use and subsequent disposal

Worldwide plastic production has grown from 5 million to 230 million tonnes in the last 50 years (EA, 2001b, Plastic-Europe, 2006) and 8 % of oil production is used to manufacture polymers (Azapagic et al., 2003). In the US alone, plastic represents an industry worth over £50 billion (EPA, 1992). Plastics are primarily (40 %) used for packaging products of which most are thrown away within two years of manufacture. Plastic are generally disposed of through landfill, recycling and incineration. It is estimated that waste management programmes, such as domestic collection and recycling, collect up to 70 % of used plastic debris and these are disposed of in landfill (Azapagic et al., 2003). These materials form a fifth of municipal waste, in the EU this equates to half a billion tonnes per year (EA, 2001a). Few polymers are rapidly degradable and most persist for many years in the environment. It is, however, not known how long plastics take to degrade in the natural environment, as the procedures used to test the degradability of plastic do not use realistic environmental conditions that plastic articles will be exposed to in natural terrestrial or aquatic habitats; this is discussed in Section 1.5. The capacity of landfill sites to accommodate municipal waste is diminishing rapidly. Furthermore, reports suggest that additives used to improve polymer properties have the potential to leach out and contaminate the surrounding land (Azapagic et al., 2003).

1.5 Degradation and persistence of plastic

All polymers are susceptible to degradation to varying degrees, depending upon their production procedure, and the persistence of plastics is directly related to their ability to degrade. Plastics fragment in the environment as a consequence of photolytic, mechanical and biological degradation. The structure, morphology and molecular mass of plastics are intrinsic properties that determine the rate of degradation. High surface area to volume ratio has been shown to be important for polymer decomposition (Karlsson and Albertsson, 1998) as it increases the area open to external degradatory mechanisms.

During photo-degradation, sunlight oxidises the chemical structure, causing amide (polyamide), carbon-oxygen, (polycarbonate and polyester) carbon-carbon and carbon-chlorine (polyvinylchloride) bond cleavage in plastics. This reduces their molecular mass and as a result, plastics become brittle and disintegrate giving rise to tiny fragments. In the marine environment, plastics also fragment through the combined effects of wave action and abrasion from sediment particles (Searle, 2003).

In addition, some plastics are susceptible to biodegradation by bacteria, fungi (Gusse et al., 2006) and amphipods (Braybrook, 2006). Plastics remain relatively resistant to microbial attack whilst their molecular mass remains high. Physical degradation by invertebrate macrofauna can reduce the polymer's size and mass to undergo enzyme attack by naturally occurring bacteria in the wider marine environment (Chandra and Rustgi, 1998). The structure of polymers greatly affects their ability to be degraded by enzymes, in particular the hydrophilic-hydrophobic character of synthetic polymers and proficiency-of-fit within an enzyme's active site (Klemchuk, 1990). Flexible polymers are degraded more quickly than more rigid aromatic polymers (Tokiwa and Suzuki, 1977). Plastics are now engineered with

chemical bonds (e.g. hydrolysable, glycosidic and peptide) that can be broken down by naturally occurring bacteria and enzymes. Furthermore, copolymer blends of inert polymers (polyethylene and polyester) and biodegradable compounds such as starch have been intentionally designed so that the starch is consumed by microorganisms, leading to the disintegration of the plastic item (Klemchuk, 1990). Both of these measures help reduce the structural integrity of the plastic and thereby facilitate breakdown into smaller fragments (Roper and Koch, 1990). Biodegradable polymers have been proposed as an environmentally friendly alternative to conventional plastics that have low rates of degradation as they are more prone to breakdown by organisms. For example, plastics like polylactic acid, a plant-derived polyester are used for packaging and bottles, though Gross and Kalra (2003, , 2002) estimate it takes 40% more energy to produce polylactic acid than conventional petrochemical-based polyamide. In addition, the processes used to produce biodegradable polymers are considered by many to expend more carbon dioxide emissions and be more expensive than conventional bulk-produced, oil-based plastics, that are recyclable (Amass et al., 1998, Gerngross et al., 2003). Despite continually increasing oil prices, the annual production of petroleum-based plastics is much less that of non-oil derived biodegradable plastics (Gross and Kalra, 2002). A particular concern is that if biodegradable plastics do find their way into the waste management, the limited technology for their separation from petroleum-based plastics may lead to contamination and subsequent weakening of new recycled plastic products (Amass et al., 1998).

The Organisation for Economic Cooperation and Development (OECD) and American Standard Testing Methods (ASTM) have proposed a series of tests of biodegradability and these include the modified sturm test, closed bottle test, petri dish screen, environmental chamber method and soil burial test (Chandra and Rustgi, 1998). The 'Sturm test' involves

incubating the plastic material in a mineral nutrient solution (free of organic carbon) at ambient temperatures with sewage microorganisms for 28 days. The carbon dioxide produced is measured and used as an index of biodegradability. The 'Closed bottle test' is carried out by adding a known quantity of plastic to a salt solution which is incubated with sewage microorganisms and then transferred into closed bottles, which are incubated in the dark at 20 °C and the dissolved oxygen content measured. High levels of oxygen indicate high microorganism growth and a highly degradable plastic. The 'Petri dish screen' involves placing the plastic material inside a Petri dish with mineral salts agar and adding a known fungi or bacteria. The degradability of the plastic is estimated by measuring the number of microorganisms and determining changes in the mass and electrical conductivity of plastics. The 'Environmental chamber' assay works by hanging plastic strips in a humid chamber containing fungi. The percentage cover of fungi on the strips is used to assess the degradability of the plastic in question. In the 'Soil burial test' samples of plastic are buried in beakers containing sieved soil-based compost for up to a year at 20 to 30 % humidity. The biodegrability of plastic is assessed by quantifying the degree of microbial growth, the fragmentation and embrittlement of the plastic (Chandra and Rustgi, 1998).

According to the ASTM (2007), a degradable plastic "undergoes a significant change in its chemical structure under specific environmental conditions resulting in a loss of some properties", whilst a biodegradable plastic is one where the "degradation results from the action of naturally occurring microorganisms such as bacteria, fungi and algae". A compostable plastic undergoes degradation by biological processes during composting to yield carbon dioxide, water, inorganic compounds and biomass at a rate consistent with other compostable materials and leaves no visible, distinguishable or toxic residue (ASTM, 2007).

The ASTM also has a series of test to assess photo, hydrolytic, oxidative degradability of plastics using laboratory and out-door set-ups. Laboratory tests examine the effects of different components of solar radiation using different lamps (e.g. filtered xenon and carbon arcs, florescent ultraviolet and metal halide lamps), temperature and moisture. Whereas outdoor trials utilise racks that hold the plastic sample and, in conjunction with reflective panels, chambers and temperature-controlled apparatus, manipulate the influence of solar irradiance (sunlight), temperature and humidity on the degradation of plastic (Searle, 2003).

Unfortunately, the procedures used to test the biodegradability of plastic do not use realistic environmental conditions that simulate terrestrial or aquatic habitats, such as rivers, estuaries and the marine environment. Most tests for the biodegradability of plastic are not designed with due attention to the likely environment in which the plastic product may end up as litter. Therefore, the reliability of tests of biodegradability tests to provide realistic data for the environmental persistence plastic is low (Amass et al., 1998). The main problem is that aquatic and terrestrial habitats represent physically and biologically complex environments and are very different to the constant conditions of a laboratory or an outdoor fixed angle rack. Intrinsic variability at several biological, spatial and temporal scales will determine plastic degradation. For instance, the activity of micro- (bacteria) and macro-organisms (e.g. detritivorous worms and amphipods) that have been shown to display a broad range of polymer-degrading abilities will be influenced by physical factors (e.g. temperature, wind, wave action, substratum chemistry, and UV light), reducing plastic debris to biologically suitable sizes for biodegradation to take place. To develop environmentally realistic tests for the degradability of different types of plastic, research is needed to determine the relative influence of different physical, spatial and temporal factors on the rate of degradation of plastic in different natural habitats. Once completed, this research could form the basis by

which tests could be designed to estimate the degradability of plastic and therefore, make improved predictions for the likely persistence of plastic within natural habitats.

1.6 What are the sources of microplastic in the environment?

The two most likely sources of microplastic are from fragmentation of larger plastic items and the use of small particles of plastic as abrasive scrubbers in cleaning products (Zitko and Hanlon, 1991, Gregory, 1996, Derraik, 2002). Plastics fragment in the environment as a consequence of photolytic, mechanical and biological degradation and through the combined effects of wave action and abrasion from sediment particles. Regardless of the method of deterioration, the size and identity of plastic fragments that have been found in marine habitats indicates that microscopic particles can form from the breakdown of larger items. Microscopic fragments of plastic used in the manufacture of clothing (polyester, acrylic), packaging (polyethylene, polypropylene) and rope (polyamide) have been identified from beaches around the UK (Thompson et al., 2004). Another source of microplastic particles is from industrial and domestic cleansing products, including toilet, hand, body and facial cleansers (Derraik, 2002, Gregory, 1996) that contain tiny polyethylene and polystyrene particles less than 1 mm in diameter. In addition, larger particles of acrylic, melamine and polyester, ranging from 0.25 to 1.7 mm in diameter, are used to clean machinery and boat hulls in dockyards by a process known as 'media blasting'(Gregory, 1996, Abbott, 1992, Wolbach and McDonald, 1987, Anonymous, 1998). Microplastic particles are also used in range of medical applications including drug delivery systems (Matsusaki et al., 2001, Wen et al., 2003, Curley et al., 1996, Kockisch et al., 2003, Hussain et al., 2001). Small particles such as these are likely to be transported with waste water through sewage treatment works and subsequently to enter aquatic habitats. Up to 4 synthetic fibres per gram sediment have been

found in samples of sewage sludge taken from a sewage treatment works in New York, USA (Zubris and Richards, 2005, Habib et al., 1998). The likely source of these fibres to sewage is probably the laundering of clothes and the cleaning of fabrics including carpets. Hence, there is considerable potential for microscopic plastic debris to accumulate in both freshwater and marine environments. From the early 1970's to 1998 a quarter of UK sewage sludge was disposed of at designated marine sites around the UK (CEFAS, 1997, Emu, 1999). It is, however, not known whether these areas act as point sources of this material. Chapter 3 investigates whether sewage disposal sites (Plymouth and Tyne) have higher levels of microplastic compared to relevant control areas.

1.7 Contamination of marine habitats by plastic debris

The rise in plastic production is reflected in the contamination of macroscopic plastic debris in marine habitats which now stretches from the poles to the equator (Carpenter et al., 1972, Gregory, 1996, Ryan and Moloney, 1990, Derraik, 2002). There is, however, a significant gap in the knowledge about the size-spectra and polymer composition (e.g. polystyrene, polyamide, polyethylene, polypropylene, polyvinylchloride, polyester) of plastic debris on intertidal strandlines (Chapter 2). This litter accumulates along strandlines (Thornton and Jackson, 1998), in the open ocean (Shaw and Day, 1994) and on the seafloor (Stefatos et al., 1999, Galgani et al., 2000), and varies in shape and sizes from small fragments micrometres in length (Thompson et al., 2004) to larger items, including boat hulls and fishing nets that can be several metres in length. Plastic debris enters marine habitats from a variety of sources, including direct littering by beach visitors, riverine inputs (Galgani et al., 2000, Lecke-mitchell and Mullin, 1992, Stefatos et al., 1999), sewage discharge (Williams and Simmons, 1997), discarded fishing gear (Donohue et al., 2001) and illegal dumping (MCS,

2006). In the 1970's a survey of the north-west Atlantic found that there were over 160,000 macroplastic (≥5 mm) items per km^2 (Colton et al., 1974) and recent work in the North Pacific Central Gyre has found over 960,000 items per km^2 (Moore et al., 2001). In 2003, the Marine Conservation Society estimated that there were over 1,300 plastic items per km of beach in the UK (MCS, 2006). Whilst virgin plastic granules used as feedstock and to make plastic articles contaminate the Atlantic Ocean, Sargasso Sea, Pacific Ocean and coastal waters of South Africa and New Zealand at densities ranging from 20 to 334,000 items per km (Gregory and Andrady, 2003). This debris can be transported thousands of kilometres and can contaminate relatively isolated locations (Ebbesmeyer, 2003). However, there has been relatively little quantitative work to describe the main factors that influence the transport and subsequent spatial distribution of macroplastic debris (Moore et al., 2001, Thornton and Jackson, 1998) and no work on microplastic debris. Many studies have suggested the likely importance of physical factors, including wind (Williams and Tudor, 2001, Debrot et al., 1999a) and wave-action (Thornton and Jackson, 1998), in relation to plastic density (Lattin et al., 2004, Thiel et al., 2003) on the spatial abundance of plastic debris. Chapter 2 addresses this knowledge gap by examining the relative influence of size and density of plastic debris, wind and depositional environment on patterns of debris accumulation in the Tamar Estuary, a macrotidal estuary in south-west England.

1.8 What is the extent of microplastic contamination in marine habitats?

A study of archived plankton samples from the north-east Atlantic showed that the abundance of microscopic plastics in the water column has increased considerably over the last 40 years and this trend mirrors the global rise in plastic production (Thompson et al., 2004). Similar particles were also found on beaches throughout the UK and therefore

microplastic particles appear to be a widespread contaminant that has accumulated across a range of habitats (Thompson et al., 2004). Recently large accumulations of plastic (81 mg per kg sediment) have been reported at a ship-breaking yard in India and these included of microscopic fragments of polyurethane, polyamide, polystyrene and polyester (Reddy et al., 2006). Furthermore sediment and water samples from Singapore have been shown to contain microscopic fragments of polyethylene, polypropylene, polystyrene, polyamide, polyvinyl alcohol and acrylonitrile butadiene styrene (Ng and Obbard, 2007). In these studies, only fragments that differed in appearance from sediment grains or plankton were quantified. Therefore the amount of microplastic that was recorded is likely to be an underestimate of the microscopic plastic in the environment. For example, larger plastic granules (>3 mm) that are used as feedstock in factories to produce plastic can reach densities of 100,000 m^{-2} (Gregory, 1978). A major gap in our knowledge is the global extent of microplastic contamination in marine habitats and this is addressed in Chapter 3 using samples of strandline sediments collected from Europe, North and South America, Oceania, Asia and Africa.

1.9 Environmental consequences of microplastic in the environment

Large (>5 mm) macroplastic debris causes entanglement, suffocation and starvation of fish, turtles, birds and cetaceans (Derraik, 2002). Microplastic (<1 mm) is much smaller and occupies the same size range as plankton, and although it is not as unsightly as larger fragments of plastic debris, there is a greater potential for uptake by ingestion and respiration (e.g. gills) by a much wider range of animals. Uptake of microplastic by different feeding guilds will depend upon the size, shape and density of the particles, as these parameters determine the position of the debris in the water column and their potential availability. For a given size, low-density plastic will float and will be available for uptake by filter-feeders or

planktivores, whereas high density plastics such as PVC will tend to sink and accumulate in sediments where it is more likely to be ingested by deposit-feeders.

The uptake and retention of microplastic by animals in their natural habitats has received little attention, partly because quantifying tiny plastic fragments in the tissues of animals presents a range of methodological challenges. Laboratory trials have shown that amphipods (detritivores), barnacles (filter feeders) and lugworms (deposit feeders) ingest small fragments of polyvinylchloride (mean size 230 μm)(Thompson et al., 2004). In addition, filter-feeding polychaetes, echinoderms, bryozoans and bivalves have been shown to ingest 10 μm polystyrene microspheres during feeding assays (Ward and Shumway, 2004). Given that microplastic debris is accumulating in the environment (Ng and Obbard, 2007, Reddy et al., 2006, Thompson et al., 2004), these laboratory trials suggest that microplastic particles are probably also being ingested by organisms in their natural habitats.

If microplastic is ingested by animals it may be retained in the digestive tract, egested in the form of faeces, or absorbed via the epithelial lining of the gut by phagocytosis. Larger plastic debris is certainly retained in the digestive tracts of sea birds and mammals, whilst laboratory trials using lugworms *(A. marina)* kept in sediments containing microplastic have shown that these animals are capable of egesting this debris within their faecal casts (Thompson et al., 2004). An important aim of this thesis is to determine if ingested microplastic particles are taken up by the gut epithelial lining and whether further transport around the body is possible. This will be evaluated in Chapter 4 using laboratory trials with the mussel, *Mytilus edulis* as a model filter-feeding organism.

1.10 Does ingestion of microplastic have any toxicological consequences for animals?

A wide range of vertebrates and invertebrates with different feeding strategies have been shown to ingest and accumulate microscopic plastic debris in laboratory trials (Thompson et al., 2004, Ward et al., 1991, Ward et al., 2003, Ward and Shumway, 2004). However, little is known about the subsequent biological effects. The mechanisms of lung damage by micro- and nano-scopic (<1 μm) plastic and non-plastic particles, have been examined in medical studies looking at the effects of ultrafine material on workers in the plastic industry (see review by Hoet et al., 2004). These particles cause damage through the combined effect of their intrinsic toxicity and their large surface area. For example, inhalation of polyvinylchloride dust by humans can cause lung and liver damage through tissue fibrosis and cancer (Wagoner 1983). This research illustrated that the toxicity of plastic was influenced by its monomer composition and size. Polymers are composed of repeating sub-units called monomers and there is concern that toxic monomers (e.g. vinlychloride, styrene, bisphenol A, etc) may be released from plastics, especially as these compounds are linked with cancer and reproductive abnormities in humans, rodents and invertebrates (Vettori et al., 2005, Turner et al., 2005, Ryan et al., 1976, Gamer et al., 2004). Monomers are not the only chemicals that could be potentially transferred from plastics upon uptake by organisms. During manufacture, a range of chemical additives are incorporated into plastic. In addition, polypropylene pellets have been shown to adsorb and concentrate persistent organic contaminants from the marine environment at concentrations several orders of magnitude higher than those of the surrounding seawater (Mato et al., 2001, Rios et al., 2007). This concern may be particularly relevant for microplastics, since per unit mass, they have a much greater surface area to volume ratio than larger items and are therefore likely to have greater

potential to adsorb and transport contaminants. Chapter 5 introduces evidence from laboratory trials with the lugworm, *Arenicola marina*, to determine whether ingested plastics can facilitate the transport of contaminants into wildlife.

1.11 Aims of thesis

The overriding objective of the thesis is to examine the potential environmental and biological consequences of microplastic within marine habitats. Based on the literature reviewed, it is clear that an interdisciplinary approach involving environmental chemistry, toxicology and oceanography is required to better understand the sources, sinks and drivers of microplastic contamination within marine habitats and the likely biological consequences for animals capable of ingesting and accumulating microplastic debris. Throughout the thesis, the term 'hypothesis' is used describe a prediction about unknown events based on, or derived from a model to explain the original observations (Underwood, 1997). The aims of this thesis are:

- To quantify the relative influence of wind, depositional environment, size and density on patterns of plastic debris accumulation in the Tamar Estuary (UK). This is examined in Chapter 2.
- To test the relationship between the abundance of microplastic debris of strandline sediments from beaches around the world and mean sediment particle size, latitude and density population density, and to examine whether sewage disposal grounds can act as point sources of microplastic. This will be examined in Chapter 3 using data collected from a global sampling programme whereby sediment was made available from open coastal sandy beaches and the plastic extracted and quantified in terms of abundance and type.
- To investigate the biological consequences of ingesting microplastic debris using the mussel, *Mytilus edulis* (L.) as a model filter-feeding organism. Chapter 4 discusses an *in vivo* experiment to track the fate of ingested microplastic debris within the body

tissues (gut and haemolymph) and determine whether this material causes measurable changes in cellular function, oxidative status and/or feeding behaviour.

- To determine whether microplastics can transfer contaminants into the marine food chain. Chapter 5 uses *in vivo* experiments to examine the release of a selection of contaminants and plastic additives from polyvinylchloride particles to the tissues of the polychaete worm, *Arenicola marina* (L.). Toxicological consequences were assessed using a series of assays to measure changes in oxidative status, immune function, feeding and burrowing behaviour in *A. marina*.
- Chapter 6 will then (i) summarise the major findings of the thesis, (ii) introduce sustainable plastic use and waste prevention strategies, and (iii) examine the importance of holistic life style assessments and standardised litter monitoring strategies across aquatic and terrestrial habitats, directions and priorities to address gaps in the knowledge required to combat the environmental concerns related to microplastics within marine habitats.

Chapter 2. Influence of wind, depositional environment, and size and density of plastic debris on patterns of accumulation in the Tamar Estuary, UK

2.1 Abstract

Macro (>1 mm) and microscopic (< 1mm) fragments of plastic debris represent a substantial environmental problem to marine animals and boat traffic in coastal habitats. However, little is known about the relative importance of factors that influence the accumulation of this material in marine habitats. This chapter examines the influence of wind and depositional environment on the distribution of plastic debris within the macrotidal Tamar Estuary (UK). Fourier-Transform Infrared Spectroscopy (FT-IR) was used to identify the plastics found and group them according to their density. Both wind and depositional environment influenced patterns of accumulation plastic debris, and the relative strength of the wind dominated pattern depended on the size and density of the material. The overall trend was that material tended to accumulate on sites that were down-wind. For macroplastic, the clearest patterns of down-wind accumulation were for lower density material, whilst for smaller microplastic debris the clearest patterns were for high density material. In terms of abundance, microplastic debris accounted for 85 % of plastic found and this increasingly prevalent contaminant now constitutes a major component of beach debris. In contrast to predictions based on natural particulate material, correlative analyses showed no relationship between the abundance of small particles of plastic and depostional environment. Based on these findings, the chapter discusses the likely patterns of accumulation of plastic debris in wind-exposed locations and the environmental consequences for coastal habitats.

2.2 *Introduction*

Global plastic production has grown from 5 to 230 million tonnes between 1950 and 2006 (EA, 2001b, Plastic-Europe, 2006). The rapid increase in plastic consumption, coupled with high durability and low recycling rates (even some of the most developed and environmentally aware nations only recycle a fifth of this material), predisposes plastic to accumulate in the environment (Carpenter et al., 1972, Derraik, 2002, Ryan and Moloney, 1990). Plastic debris enters marine habitats from a variety of sources, including direct littering by beach visitors, riverine inputs (Stefatos et al., 1999, Lecke-mitchell and Mullin, 1992, Galgani et al., 2000), sewage discharge (Williams and Simmons, 1999), discarded fishing gear (Donohue et al., 2001) and illegal dumping (MCS, 2006). This debris can be transported thousands of kilometres and can contaminate relatively isolated locations (Ebbesmeyer, 2003). This debris may also transport adsorbed chemicals (Mato et al., 2001), sessile invertebrates, including invasive and non-species (Barnes, 2002) and potentially harmful micro-organisms (Maso et al., 2003) that have colonised the surface of the plastic.

There has however been relatively little quantitative work to describe the main factors that influence the transport and subsequent spatial distribution of plastic debris (Moore et al., 2001, Thornton and Jackson, 1998). Litter accumulates along strandlines (Thornton and Jackson, 1998), in the open ocean (Shaw and Day, 1994) and on the seafloor (Galgani et al., 2000, Stefatos et al., 1999), and many studies have suggested the likely importance of physical factors in transporting debris, including wind (Williams and Tudor, 2001, Debrot et al., 1999b) and wave-action (Thornton and Jackson, 1998), in relation to plastic density (Lattin et al., 2004, Thiel et al., 2003). Researchers in Hawaii have suggested that marine debris can accumulate under conditions of climatic forcing (which produces gradients of solar radiation

and stratospheric temperatures) and geostrophic winds (wind resulting from the balance between the Coriolis and pressure gradient forces) (Kubota, 1994), whilst research from South Australia suggests wind-driven water currents transports macroplastic debris within coastal areas (Edyvane et al., 2004).

In coastal areas, the role of wind (Nordstorm et al., 2006) and tidal processes transporting natural sediments according to their size, shape and density have been well described (see review by Le Roux 2005). However, the extent to which models of sediment dynamics could be applied to the transport of plastic debris remains untested. The transportation of particles, whether sand or plastic, is a function of their size, shape and density. These three properties largely determine the minimum velocity required to transport particles. Plastic debris occurs in many shapes and sizes from microscopic fragments micrometres in length (Thompson et al., 2004), to larger items, including packaging crates and rope that can be several metres in length. The density of plastic debris is not uniform and depends on the plastic type and the manufacturing process used to make the original article. For instance, the density of polyethylene ranges from 0.90 g cm^{-3} (very low density polyethylene or VLDPE) to 0.99 g cm^{-3} (very high density polyethylene or VHDPE), whilst polystyrene can vary from around 1.00 g cm^{-3} (high impact polystyrene) to less than 0.05 g cm^{-3} in expanded polystyrene. The size and density of plastic is likely to determine its vertical position within the water column and hence the transport potential of the plastic debris by wind and tide.

Estuaries provide a tractable model system to study the influence of wind and depositional environment on the spatial dynamics of plastic debris. In relatively linear estuaries there is typically a prevailing wind direction and obvious gradients of deposition of fine material in relation to tidal shear stress and waves. Gradients of tidal shear stress

structure estuarine sediments by suspending, transporting and depositing sediments according to their size. Fine sediment particles (e.g. clay) have lower settling velocities and are readily suspended and transported in areas of high energy and can accumulate in areas of low energy (Dalrymple et al., 1992). Therefore, particles of microplastic are likely to be differentially retained on shores with differing sediment characteristics due to variations in the depositional environment due to tidal shear stress, waves and because of interactions between the plastic particles and the sediment particles. Consequently, the proportion of clay particles within strandline sediments was used in this study as an index of the depositional environment.

2.2.1 Hypotheses tested

To assess the influence of wind and tide on the spatial dynamics of micro (<1 mm) and macroplastic (>1 mm) debris, the Tamar Estuary (UK) was used as a tractable model system to test the following hypotheses:

i. Mean abundance of plastic debris will be significantly higher on sites receiving onshore wind (down-wind sites), the pattern will be strongest for low density material and weakest for high density plastic

ii. Wind and depositional environment influence the abundance and composition of plastic debris on the strandline

iii. The abundance of microplastic debris will be positively correlated with the proportion of fine material such as clay, silt and sand within deposited sediments of the strandline.

2.3 *Materials and methods*

2.3.1 Field sampling

The Tamar Estuary is a partially mixed and moderately sheltered macrotidal estuary in the north-east Atlantic. It is approximately 32 km long and has a mean tidal range of 3.5 m, with clear gradients relating to wind and deposition of fine sedimentary material (Figure 2c), and sediment accumulation is particularly important in the lower reaches where dredging is required to maintain shipping channels (Tattersall et al., 2003). To examine spatial patterns of plastic debris in the Tamar Estuary, 3 locations representing a gradient of deposition of fine sedimentary material were chosen on the east and west banks in relation to the prevailing south-westerly wind (Figure 2). At each location, there was 2 replicate up-wind sites (on the bank from which the wind is blowing) located on the west bank of the estuary, and 2 replicated down-wind sites that receive the prevailing wind on the east bank. Sites on the east and west banks were selected in each of three locations with differing depostional environments, as indicated by percentage clay content within strandline sediments. At each location the 2 replicate sites were separated by 60 m and each site consisted of 50 m stretch of linear shoreline. Though wide enough part for microplastics that are very small, ideally for macroplastics the sites would have been further apart, however this was possible due to limited access to the shore across private land and fragmentation of the shore in urbanised areas by seawalls. At each site, areas of strandline with obvious accumulations of plastic debris were numbered and a list of random numbers was then used to place a series of 0.25 m^2 quadrat and collect replicate samples of the plastic debris for these areas (n = 5). To avoid differences in the abundance and composition of plastic debris being confounded by sampling at different tidal height causing differences in settlement rates of plastic material, the height at

which samples were taken across sites was standardised by collecting material deposited by the last high tide (5.30 m, 10.23 am, 26 July 2005). Visible macroplastic debris was collected and the underlying 3 cm of sediment (500 ml) was placed into foil containers for extraction of smaller debris and sediment particle size analysis.

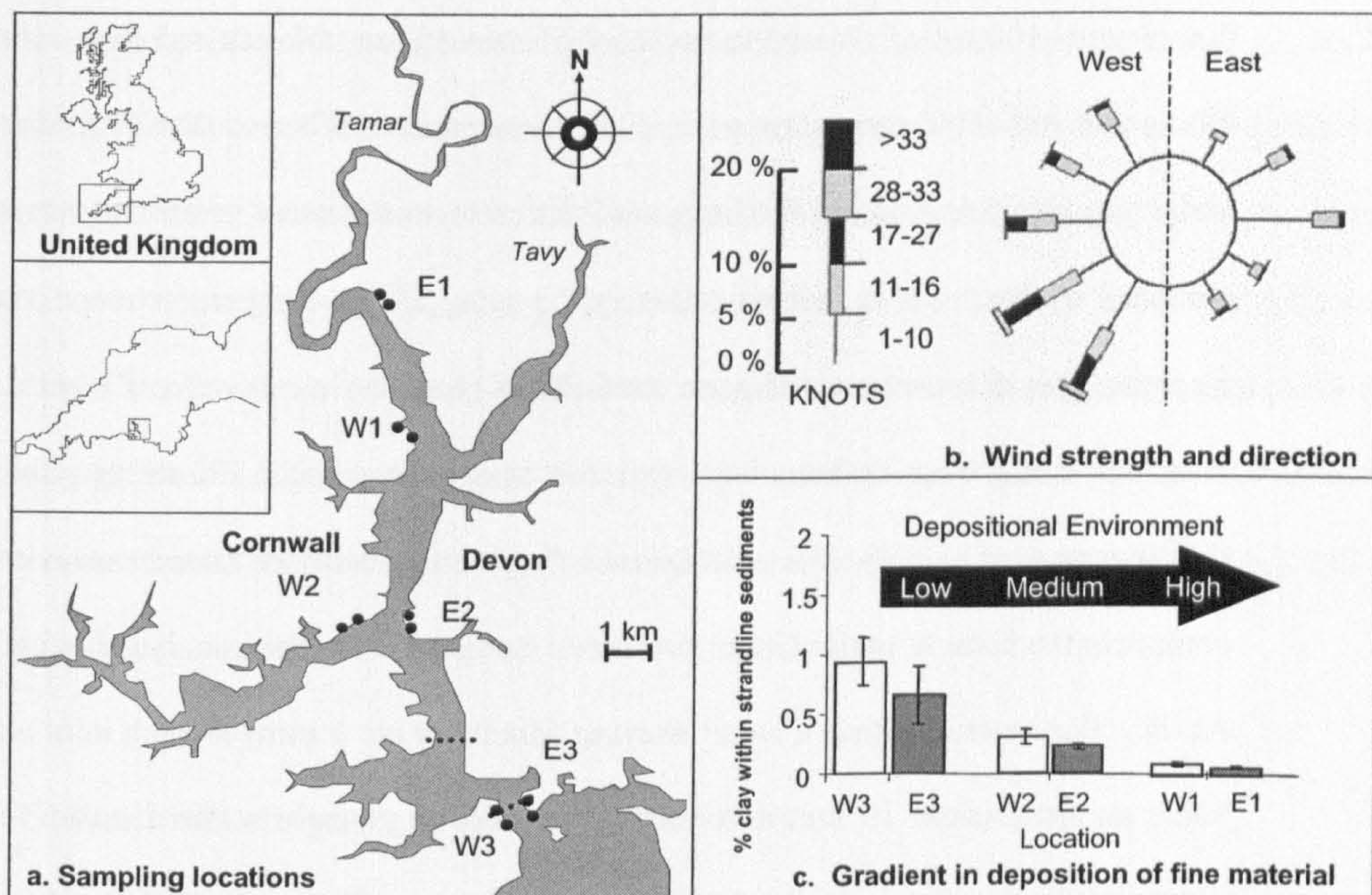


Figure 2 Tamar Estuary, U.K. (a) Sampling locations with duplicate up-wind sites; W1 Cargreen (50°26:34'N, 4°12:27'W), W2 Saltash 50°25:08'N, 4°12:40'W, W3 Cremyl (50°:21:37'N, 4°10:26'W) and duplicate down-wind sites; E1 Weir Quay (50°27:59N, 4°12:49'W), E2 Ernesettle (50°25:22'N, 4° 11:33'W) E3 Cremyll Street (50°:21:52'N, 4° 09:47'W). (b) Wind rose for Plymouth illustrating the speed and direction of winds and confirming the predominant southwestly winds (Adapted from the Met Office, http://www.metoffice.gov.uk/). (c) Percentage clay (0.00024 - 0.004 mm) in strandline sediments, values expressed as means ± SE. Arrow indicates gradient in deposition of fine material based on clay content from data collected within this study by M. A. Browne.

2.3.2 Extraction and identification of plastic debris

Microplastic debris was extracted from sediments using a modification of a published flotation separation method (Thompson et al., 2004) with an extra three repetitive rinses of saturated chloride solution. A 50 ml sub-sample of each replicate was placed in a separation funnel with 100 ml of filtered saturated 4.55 M sodium chloride solution. Buoyant smaller debris was extracted onto glass microfibre filter papers (Whatman® GF/A, diameter 47 mm) under vacuum. Any items that appeared different to sediment grains or natural debris were collected by hand under magnification (x45) using a dissecting microscope and watchmaker forceps. This debris was dried in an oven (40 °C) to remove moisture. Plastic debris was then identified using Fourier-Transform Infrared Spectroscopy (FT-IR) using published methods (Thompson et al., 2004). For microplastic fragments a diamond compression cell was used to compress the sample under finger pressure. Samples were then analysed using transmittance FT-IR. For macroplastics, a small shaving (diameter *ca.* 1 mm) of each item was compressed using an anvil under 10 metric tonnes of pressure to produce a thin sample. The identity of each sample was determined using a polymer database Brüker I26933 Synthetic fibres ATR-library. For items that were conclusively identified as plastic, the abundance, mass and cross sectional area was recorded. Fragments of polyester, acrylic, polypropylene, polyethylene, polypropylene-polyethylene blend, polystyrene, polyamide and polyurethane were identified and spectra of possible synthetic-natural polymer blends (e.g. polyester-cotton, "acrylic-wool"), modified cellulose, rayon and cellulose were not included as they could not be reliably differentiated from one another and/or determined as synthetic polymers.

2.3.3 Sediment particle size analysis

To make a preliminary assessment as to whether models of sediment dynamics could be applied to the transport of plastic debris, the relationship between the abundance of plastic and the clay content within the strandline was examined. Sediments were dried in an oven for 48 hours and processed through a series of Wentworth sieves (mean aperture sizes 32, 16, 8, 5.6, 4, 2.8 and 2 mm) and the mass of each fraction recorded. To accurately determine the contribution of smaller particles, natural organic substances that can aggregate particles were removed by heating (90 °C) in hydrogen peroxide for 24 hours. Each sample was then mixed with a small volume (1-2 ml) of distilled water to produce a homegenous paste and 3 sub-samples were analysed using laser diffraction (Malvern Laser Particle Sizer). These data were combined with the sieve data to determine the proportion of sediment (clay, silt and sand) within each sample.

2.3.4 Statistical analyses

To determine the relative influence of size, density, wind and depositional environment on patterns of plastic accumulation within the estuary, a three-factor ANOVA was used. The factor "wind" had two levels (up-wind and down-wind), "depositional environment" had three levels (high, medium and low), and "site" had two levels. The factors "wind" and "depositional environment" were each treated as fixed and orthogonal and the third factor "site" was treated as a random factor nested in "wind" and "depositional environment". Plastics identified by FT-IR were grouped according to density as being ultra-low (ULD) less than < 0.05 g cm^{-3} (e.g. expanded polystyrene), low (LD) 0.90 to 0.99 g cm^{-3} (e.g. polythene and polypropylene), medium (MD) 1.00 to 1.20 g cm^{-3} (e.g. polyamide, unexpanded

polystyrene) and high density (HD) > 1.3 g cm^{-3} (e.g. polyterephthalate, polybutlyphthalate and polyvinylchloride).

Prior to ANOVA, homogeneity of variances was examined using Cochran's tests. Variances of the HD macroplastic, ULD and total macro- and microplastic were heteroscedastic. This could not be resolved by transformation and so the analyses was conducted on untransformed data, as large balanced designs are considered robust to departures from variance assumptions (Underwood, 1997) and to reduce the likelihood of Type-I error, a more conservative significance level of $P < 0.01$ was used. For the analyses involving ULD and LD microplastic, and medium density macroplastic, the factor "site" was pooled ($P > 0.25$) to create a more powerful test against the residual. Analysis of significant effects was determined using post-hoc Student-Newman-Keuls (SNK) tests.

Differences in the composition of the plastic debris assemblage were examined using an established, non-parametric, multivariate analyses framework (Clarke, 1993, Anderson, 2001). Analysis of Similarities (ANOSIM) tested differences between the factors "wind" and "depositional environment". The relative contribution of different plastic types to the patterns of composition was assessed using Similarity Percentage Contribution (SIMPER).

Spearmans Rank correlation was used to examine the relationship between sediment grain composition (sand silt and clay) and the abundance of low, medium and high density microplastic debris, and a conservative P-value of 0.01 was chosen in order to adjust for multiple comparisons.

Statistical analyses was carried out using PRIMER (Plymouth Routines in Multivariate Ecological Research, Plymouth, UK) and GMAV (General Models of Analysis of Variance; EICC, University of Sydney, Australia).

2.4 *Results*

2.4.1 Characterisation of plastic debris within the Tamar Estuary (UK)

A total of 1998 items of debris were recorded with an average of 9 macro items per 0.25 m^2 and 13 micro items per 50 ml sediment. This is equivalent to 59 macro and 366 micro items m^{-2} in the top 3 cm of strandline sediment.

Of the larger items of debris that were recognisable, the main types of plastic were packaging (polystyrene foam 32 % and wrappers 18 %) sewage related (cotton wool bud sticks 4 % and sanitary towels 15 %), fishing related (fishing line 6 %) and shipping related (rope fragments 7 %) (Figure 3). This was reflected in composition of synthetic polymers present which was dominated by polypropylene (28 %), polytheylene (32 %) and polystyrene (23 %) (Figure 4).

However, the majority of the items were highly fragmented and their original use could not be determined (Figure 3). This was reflected in the size spectra of plastic debris, which was exponentially skewed towards smaller debris and in terms of numerical abundance, microplastic (<1 mm) accounted for 85 % of the total amount of debris found (Figure 3). In contrast to macroplastic debris, assemblages of smaller microplastic fragments were mainly composed of polyvinylchloride (27 %), polyester (24 %) and polyamide (19 %) debris.

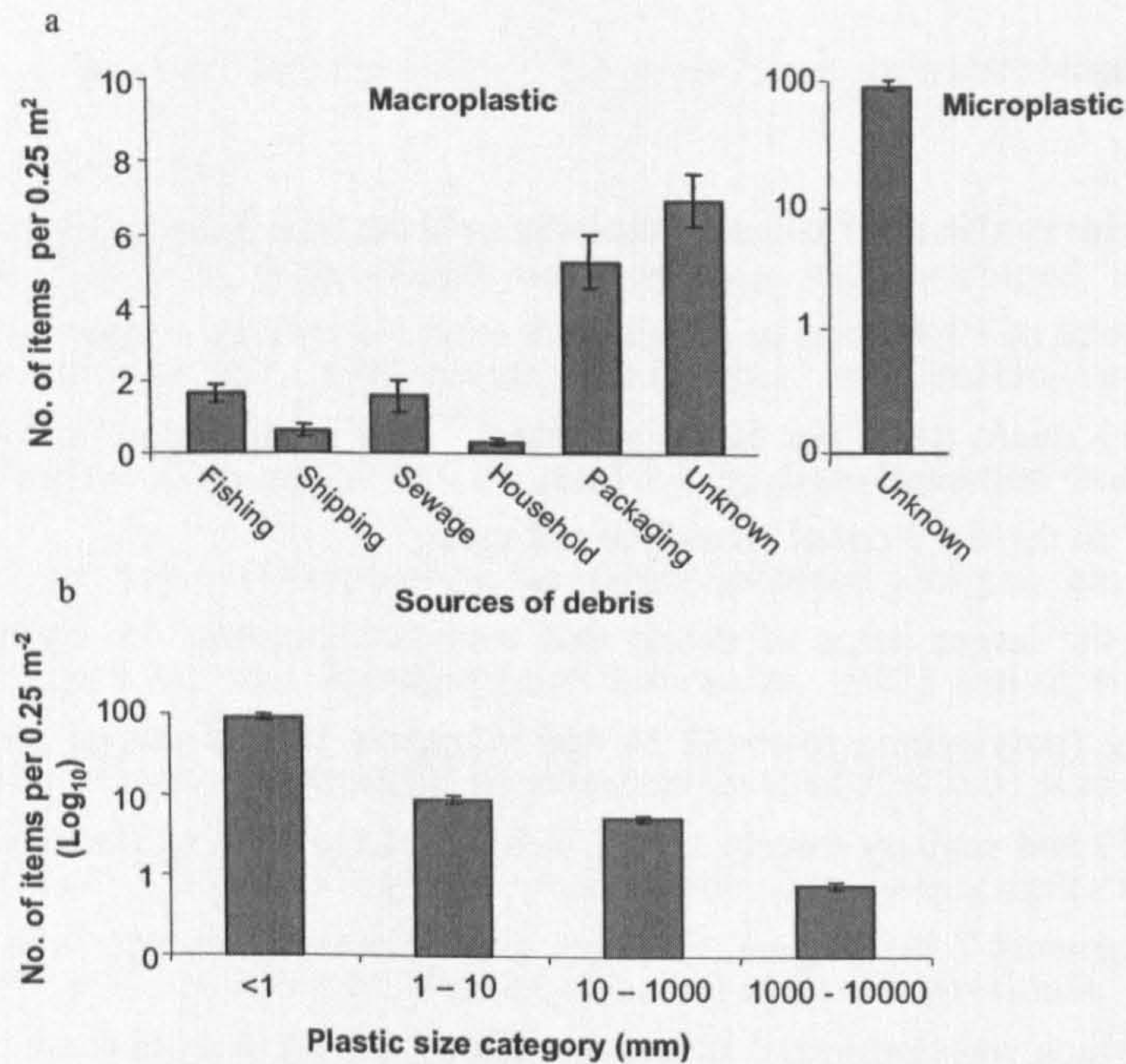


Figure 3. Identity composition of strandline plastic debris in Tamar Estuary, UK according to usage (a) and relative size (b). Data are presented as mean ± S.E. for all locations n = 15 (*N.B.* log scale for microplastic in part a).

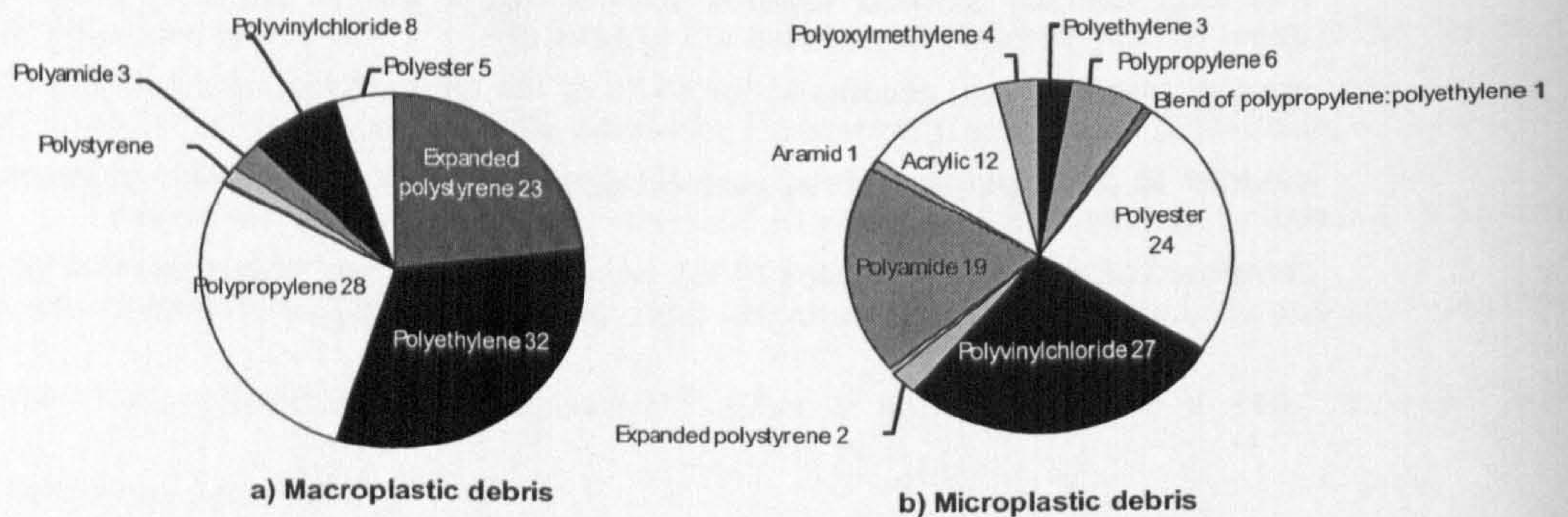


Figure 4. Percentage composition of strandline (a) macro and (b) microplastic debris in Tamar Estuary (UK), values expressed as percentages of total synthetic polymers identified using Fourier Transform Infrared Spectroscopy.

2.4.2 Influence of wind, depositional environment and plastic density on patterns of plastic debris accumulation

There were generally greater abundances of plastic material in down-wind sites; however the pattern varied with size, density, depositional environment and site. For microplastic debris, there were significantly greater accumulations of MD, HD and total microplastic debris in down-wind sites, however the relative strength of this pattern varied with sites and depositional (see ANOVA table 2 and graph in Figure 5). ULD microplastic was only present in down-wind (east) and high deposition sites, at 0.6 and 0.2 fragments 50 g sediment^{-1}. Whilst LD microdebris was distributed evenly throughout the estuary at abundances up to 0.6 fragments 50 g sediment^{-1}. The abundance of MD microdebris was over three-times higher on down-wind sites (east) at 0.6 - 1.0 fragments 50 g sediment^{-1} compared to down-wind sites (west) that ranged from 0 - 0.6 fragments 50 g sediment^{-1}. As polyester and polyvinylchloride dominated over 50 % of the composition of total microplastics, the abundance of HD and Total microplastic debris both showed significantly greater abundances of microplastic fragments in down-wind sites in low and high depositional environments. At these sites, there was up to 7 HD and 8 Total microplastic fragments 50 g sediment^{-1}, compared to other sites that varied from 0.2 to 2.6 fragments per 50 g sediment^{-1}.

As with microplastic debris, there were generally greater abundances of macroplastic material in down-wind sites, and again the strength of the pattern varied with density, depositional enviornmnet and site (see ANOVA table 2 and graph in Figure 5). The abundance of ULD debris was upto 140 times greater in down-wind and high deposition sites, with between 6.6 – 28 articles 0.25 m^{-2} compared to 0.2 – 1.2 articles 0.25 m^{-2} throughout the rest of the estuary. Articles of LD macroplastic debris was significantly more abundant in down-wind sites located in areas of low, medium and high sediment deposition (11.6 – 22

macrodebris 0.25 m^{-2}), compared to the other sites which ranged from 1.6 - 7 macrodebris 0.25 m^{-2}). The abundance of MD macroplastic remained constant throughout the estuary, irrespective of wind and deposiotnal enviornmnet. In contrast to patterns of distribution for microplastic, there was greater abundance of HD macrodebris at one of the up-wind sites in the area of high sediment deposition (7.2 macrodebris 0.25 m^{-2}) compared to the rest of the estuary (0 – 2.4 macrodebris per 0.25 m^{-2}). The composition of total macroplastic was dominated by LD (50 %) and ULD (23 %) articles, and similar patterns of accumulation of Total macroplastic were observed in down-wind sites in locations of low, medium and high sediment deposition (11.4 – 40.4 macrodebris per 0.25 m^{-2}), compared to the rest of the estuary (4 - 8.8 macrodebris per 0.25 m^{-2}).

Table 2. Analysis of variance showing the relative importance of wind, depositional environment, size and plastic density on the abundance of plastic debris within the Tamar Estuary, UK. Post-hoc pooling carried out when *P* > 0.25 is indicated by a hyphon.

			Microplastic			Macroplastic		
Plastic		df	MS	F	P	MS	F	P
Ultra-low density	Wind	1	0.27	3.27	>0.07	464.82	2.42	>0.1
	Depositional Environment	2	**0.27**	**3.27**	**<0.05**	456.62	2.38	>0.1
	Site (Wind X Depositional Environment)	6	0.07	-	-	**191.78**	**6.51**	**<0.001**
	Wind X Depositional Environment	2	**0.27**	**3.27**	**<0.05**	456.52	2.38	>0.1
	Residual	48	0.08			29.44		
Low density	Wind	1	0.82	3.37	>0.07	12.77	7.18	<0.05
	Depositional Environment	2	0.15	0.62	>0.5	1.22	0.69	>0.5
	Site (Wind X Depositional Environment)	6	0.18	-	-	**1.78**	**4.32**	**<0.01**
	Wind X Depositional Environment	2	0.52	2.13	>0.1	1.57	0.88	>0.4
	Residual	48	0.25			0.41		
Medium density	Wind	1	**4.27**	**10.57**	**<0.001**	0.60	0.61	>0.4
	Depositional Environment	2	0.45	1.11	>0.3	1.05	1.07	>0.3
	Site (Wind X Depositional Environment)	6	0.10	-	-	1.10	-	-
	Wind X Depositional Environment	2	0.02	0.04	>0.96	0.35	0.36	>0.7
	Residual	48	0.44			0.97		
High density	Wind	1	**120.42**	**8.93**	**<0.05**	9.60	0.83	>0.3
	Depositional Environment	2	11.82	0.88	>0.4	19.32	1.67	>0.2
	Site (Wind X Depositional Environment)	6	**13.48**	**3.85**	**<0.01**	**11.57**	**3.34**	**<0.01**
	Wind X Depositional Environment	2	19.02	1.41	>0.3	39.65	3.43	>0.1
	Residual	48	3.50			3.47		
Total	Wind	1	**13.80**	**18.48**	**<0.01**	**2381.40**	**7.25**	**<0.05**
	Depositional Environment	2	0.82	1.10	>0.3	813.65	2.48	>0.16
	Site (Wind X Depositional Environment)	6	**0.75**	**2.46**	**<0.05**	**328.50**	**3.43**	**<0.01**
	Wind X Depositional Environment	2	1.58	2.12	>0.2	460.05	1.40	>0.3
	Residual	48				95.64		

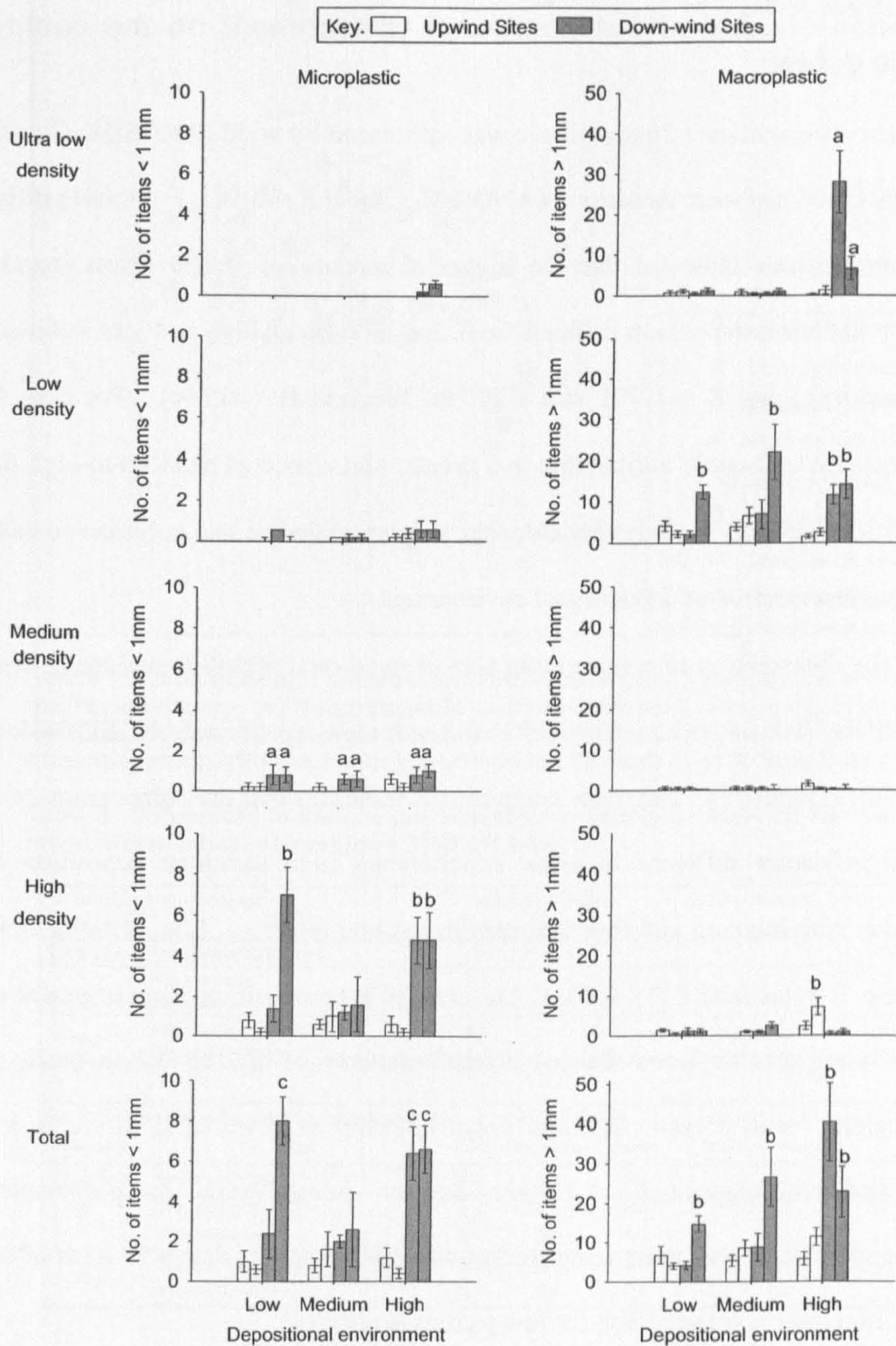


Figure 5. Abundance of micro- (50 g sediment $^{-1}$) and macroplastic debris (0.25 m^{-2}) within the Tamar Estuary according to density. Values expressed as means ± S.E. Significant differences indentified by S.N.K. tests are indicated by 'a' ($P < 0.001$), 'b' ($P < 0.01$) and 'c' ($P < 0.05$).

2.4.3 Influence of wind and depositional environment on the composition of plastic debris

The composition of microplastic was influenced by wind (ANOSIM, Global R = 0.274, $P < 0.001$) and sediment deposition (ANOSIM, Global R = 0.143, $P < 0.001$) (Figure 6). Pair-wise comparisons indicated that the higher abundance of plastic debris found in areas of medium sediment deposition differed from that in areas of high and low sediment deposition (Medium vs. Low, R = 0.201 and High vs. Medium R = 0.144). For both factors these differences were mainly attributable to a greater abundance of medium-to-high density debris (e.g. polyamide, acrylic, polyvinylchloride, polyterephthalate and polyester) on all down-wind locations irrespective of depositional environment.

The composition of macroplastic also showed clear distributional trends related to wind (ANOSIM, Global R = 0.28, $P < 0.001$) and sediment deposition (ANOSIM, Global R = 0.18, $P < 0.001$) (Figure 7). Pair-wise comparisons indicated that the composition of macroplastic was significantly different in areas experiencing high sediment deposition compared to locations with medium and low sediment deposition (High vs. Low, R-value 0.328; High vs. Medium, R-value 0.435; $P < 0.001$). Differences in composition suggest greater wind and tide driven transport for lower density items/fragments of plastic debris (such as expanded polystyrene foam, polyethylene and polypropylene) to down-wind shores in locations with high sediment deposition. Higher density debris (such as polyvinylchloride and polyterephthalate) was more abundant on western (up-wind) shores in areas of high sediment deposition, however the reason for this pattern is not clear.

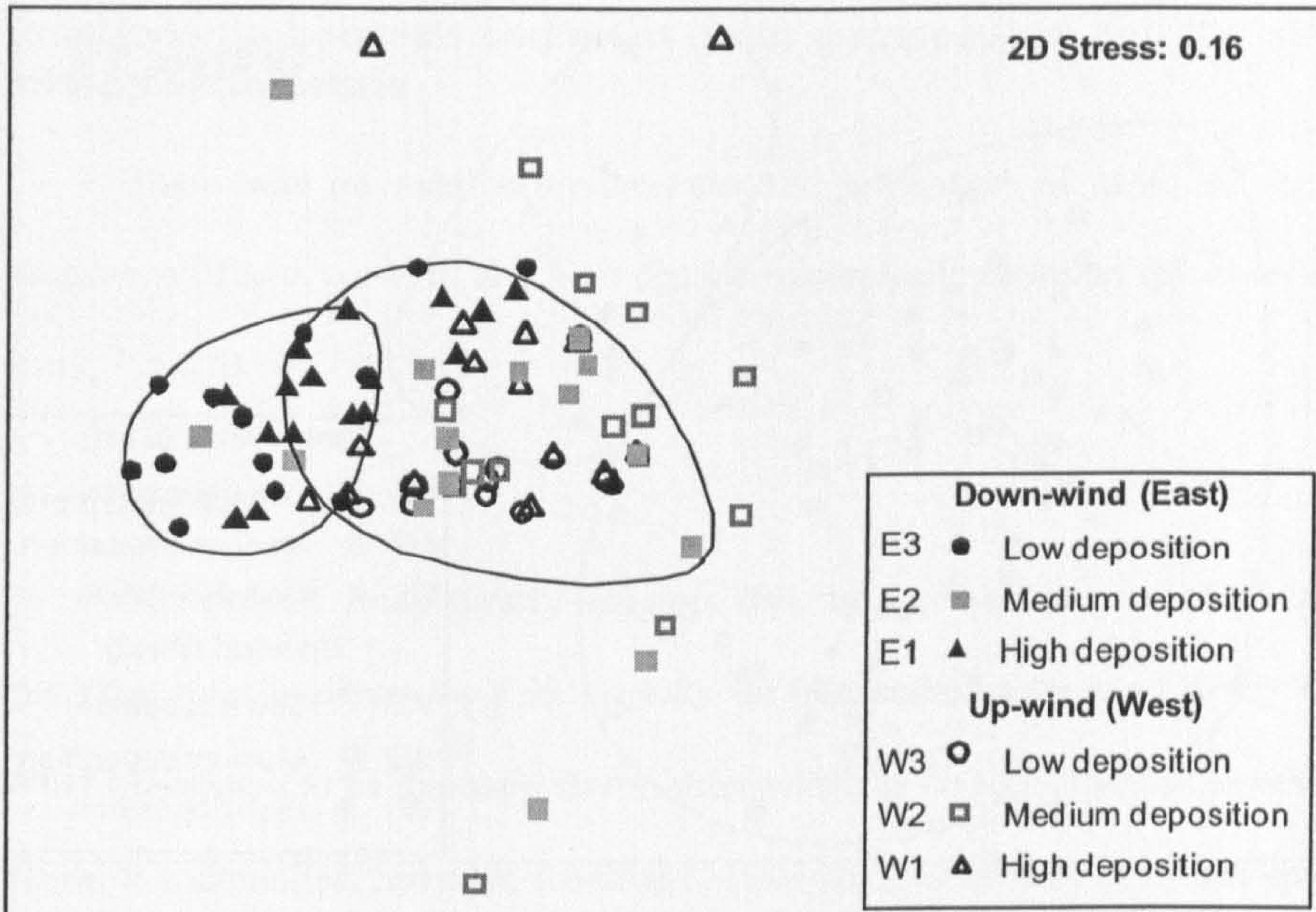


Figure 6 Abundance and composition of microplastic (< 1 mm) debris within the strandline of the Tamar estuary. (a) Nonparametric multidimensional scaling (nMDS) ordination plot of microplastic debris in relation to wind and depositional environment (Bray-Curtis similarity, no transformation), with two major superimposed clusters at 44 % Bray-Curtis similarity.

Table 3. Differences in the composition of microdebris between (a) wind and (b) depositional environment (shear stress) from SIMPER analysis.

a. Wind and macroplastic		Mean No. items		Contribution to compositional differences (%)
Density	Type	Up-wind	Down-wind	Up-wind vs. Down-wind
Ultra-Low	Expanded Polystyrene	0.5	5.3	19
Low	Polyethylene	2.4	6.2	28
	Polypropylene	1.9	5.0	27
High	Polyethylene terepht halate	0.6	0.4	5
	Polyvinylchloride	1.1	0.9	9

b. Depositional environment and macroplastic		Mean No. items			Contribution to compositional differences (%)	
Density	Type	Low	Medium	High	Low vs. Medium	Medium vs. High
Ultra-Low	Expanded Polystyrene	0.5	0.5	8.0	22	26
Low	Polyethylene	2.8	6.1	4.0	24	17
	Polypropylene	2.0	0.5	4.0	22	18
High	Polyethylene terephthalate	0.1	0.5	1.0	8	9
	Polyvinylchloride	0.7	4.5	2.0	12	16

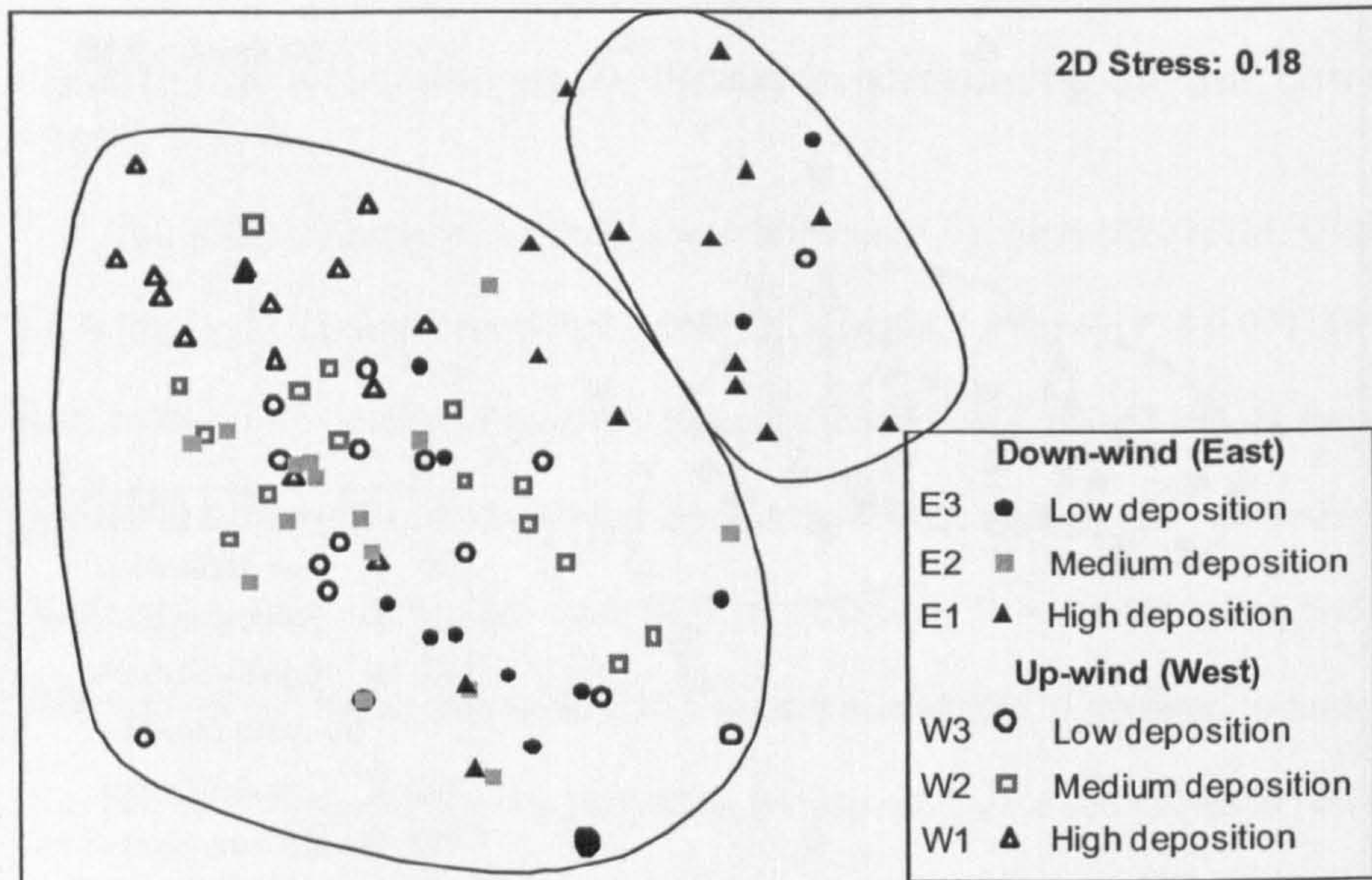


Figure 7 Abundance and composition of macroplastic (< 1 mm) debris within the Tamar estuary. (a) Nonparametric multidimensional scaling (nMDS) ordination plot of macrodebris in relation to wind and sediment deposition (Bray-Curtis similarity, no transformation), with two major superimposed clusters at 20 % Bray-Curtis similarity.

Table 4. Difference in the composition of macrodebris between (a) wind and (b) depositional environment for SIMPER analysis.

a. Wind and microplastic		Average No. items		Contribution to compositional differences (%)
Density	Type	Up-wind	Down-wind	Up-wind vs. Down-wind
Unknown	Acrylic wool	0.4	1.7	27
Ultra-Low	Expanded Polystyrene	0.0	0.1	2
Low	Polyethylene	0.0	0.1	3
	Polypropylene	0.2	0.2	6
Medium	Acrylic	0.2	0.4	10
	Polyamide	0.3	0.7	14
High	Polyester	0.1	0.2	5
	Polyethylene terephthalate	0.2	0.6	12
	Polyoxymethylene	0.0	0.2	2
	Polyvinylchloride	0.0	1.3	18

b.Depositional environment and microplastic		Average No. items			Contribution to compositional differences (%)	
Density	Type	Low	Med.	High	Low vs. Medium	Medium vs. High
Unknown	Acrylic wool	1.4	0.7	1.1	22	25
Ultra-Low	Expanded Polystyrene	0.0	0.0	0.2	0	2
Low	Polyethylene	0.0	0.1	0.1	3	2
	Polypropylene	0.1	0.2	0.2	7	8
Medium	Acrylic	0.4	0.4	0.1	16	12
	Polyamide	0.7	0.3	0.5	19	13
High	Polyester	0.1	0.1	0.3	4	7
	Polyethylene terephthalate	0.4	0.4	0.4	13	13
	Polyoxymethylene	0.0	0.0	0.3	0	3
	Polyvinylchloride	0.9	0.3	0.8	13	14

2.4.4 Relationship between sediment grain composition and the abundance of microplastic debris

There was no relationship between the proportion of sand, silt or clay and the abundance of low, medium and high density microplastic debris in the strandline (Spearman Rank, $P > 0.01$ ns).

2.5 *Discussion*

The present study clearly suggests that the size and density of plastic debris play important roles in determining its capacity for transportation by wind in the Tamar Estuary. Wind was shown to be the main factor influencing the composition and abundance of plastic debris on a strandline; however, the relative strength of the pattern depended upon the size and density of the debris. For macroplastic, the clearest patterns of down-wind accumulation were for lower density material (ULD and LD); whilst for smaller microplastic debris the clearest patterns were for higher density material (MD and HD).

Ultra low density macroplastic (expanded polystyrene) debris accumulated in greatest abundance at one of the down-wind high sediment deposition sites and similarly LD macroplastics (polyethylene and polypropylene) were more abundant on sites down-wind that experience medium to high sediment deposition. The results show that some densities of plastic are distributed evenly throughout the estuary and others are apparently affected by wind. One possible explanation for the accumulation of large numbers of ULD and LD macroplastic fragments on sites near the mouth of the Tamar Estuary could be that this material orginates from littering from adjacent urbanised areas in Plymouth. However, given the dominant role wind appears to play in the accumulation of plastic debris throughout the Tamar Estuary, another possible explanation is that lower density material is transported from

the wider ocean to the estuary by wind-induced currents. Indeed, similar patterns of transport have been reported in Hawaii and South Australia (Edyvane et al., 2004, Kubota, 1994), whilst the importance of wind and plastic density as factors regulating the transport of plastic debris has been suggested previously for North American oceanic and coastal habitats. A greater abundance of expanded polystyrene was found in wind-dominated upper margins of an estuarine shore (Thornton and Jackson, 1998). Lattin et al., (2004) hypothesised that oceanic plastic debris is likely to be vertically distributed within the water column in relation to density and that the influence of wind on mixing decreases with depth. Therefore greater abundances of lower density macroplastic on the strandline compared to higher density macroplastic may have resulted from ultra-low and low density macroplastic debris accumulating at the top of the water column and being rapidly transported by wind-induced currents, whereas HD macroplastic debris occur near the seabed where water velocity and transportation rates are slower. To help differentiate between these explanatory models, further research is needed using the same approach outlined within this chapter, but with multiple estuaries and carried out in conjunction deployment studies that track items of plastic from potential sources. In this present study, only one estuary was studied due to time constraints and therefore the data presented within this chapter may be confounded by inherent differences between the east and west shores of the Tamar Estuary, other than their orientation inrelation to prevailing winds. Therefore any further research should sample more than estuary.

2.5.1 Implications for marine habitats

These findings have implications for the plastic industry. Since the 1970s, the typical density of plastic items being produced has been reduced by manufacturers in some cases by up to 50 % to help minimise transportation costs (EA, 2001a, Plastics-Europe, 2004). Given the clear patterns of macroplastic in relation to wind presented within this chapter, it seems that lighter macroplastic debris is more likely to accumulate in sites that receive on-shore winds and this may partly explain the increasing accumulation of plastic debris in remote locations away from human populations (e.g. Edyvane et al., 2004). A better understanding of how wind and depositional gradients influence the accumulation of plastic debris is vital for the environmental agencies charged with protecting our shores from the hazards this debris poses to humans and wildlife. Many of the fastest growing cities in Europe, North America, Asia, Africa and Oceania are in sheltered estuaries and bays, and are subject to considerable contamination by plastic litter.

Manual beach cleaning is used to remove macroplastic debris from locations of high tourism to improve the aesthetic quality of beaches and protect wildlife and in 2001 it is estimated that this cost the UK government £2.5 million (MCS, 2006). The present work suggests that beach cleaning may be most effective if focused at locations exposed to the prevailing wind which accumulates the largest quantities of macroplastic debris. For microplastic, this debris accumulated in higher abundances on sites that receive on-shore winds, with the clearest patterns for MD and HD plastics such as polyamide, non-expanded polystyrene, polyvinylchloride and polyester. Although remediation schemes, like beach cleaning, remove large debris items before they fragment into tiny fragments, they are unable to remove microplastic. Microplastic fragments accounted for the majority (85 %) of the

plastic debris recorded here. Similarly, high levels of tiny plastic debris (< 2.8 mm) have been found in the Pacific Ocean and can constitute between 43 % to 96 % of the stranded plastic debris (McDermid and McMullen, 2004, Robards et al., 1997). Smaller particles of plastic within the nanometre (< 1μm) range are likely to be present; however, current methods only identify plastic debris using FT-IR down to 20 μm in size (Thompson et al., 2004).

The size-frequency distribution of plastic debris within the Tamar Estuary is exponentially skewed to smaller debris, with 85 % of the debris microscopic; it therefore seems likely that small debris is formed by the fragmentation of larger items. This is in agreement with Thompson et al., (2004) who suggested that fragments of polyester, acrylic, polyethylene, polypropylene and polyamide found on beaches around the UK probably formed from the fragmentation of clothing, packaging and rope. Although the exact underlying mechanisms for fragmentation are unclear, plastics are known to degrade by photolytic, biological and mechanical processes (Klemchuk, 1990, Andrady et al., 1998, Searle, 2003, Braybrook, 2006). Recent laboratory trials have also shown that amphipods from the strandline are able to shred plastic debris (Braybrook, 2006). Plastics may also fragment through the combined effects of wave action and abrasion by sediment particles, and this may explain the high abundance of microplastic in areas of high sediment deposition. Another source of microplastic debris is sewage discharges. Household laundering of clothes may act as a source of these microplastic fibres to treatment plants where this material accumulates in sewage sludge and effluents. Up to 4 microplastic fibres per gram sediment have been found in samples taken from a sewage treatment works in North America (Zubris and Richards, 2005, Habib et al., 1998). From the early 1970's to 1998 a quarter of UK sewage sludge was disposed of at designated marine sites (Emu, 1999). Chapter 3 investigates whether disposal

sites in the UK (Plymouth and Tyne) have higher levels of microplastic compared to control areas.

The lack of any clear relationship between the clay content and abundance of different densities of microplastic is somewhat surprising since these synthetic particles are similar in size and density to fine particles of sediments. As there was no relationship between the amounts of fine sedimentary material and plastic content, it is unlikely that interactions between the plastic and the sediment particles caused differences in the retaintion of plastic on on shores with differing sediment characteristics. However, fine-grained sediments do have a cohesive nature and regularly flocculate with organic material; therefore furtherwork is needed to understand the role flocculation may play in transporting particles of microplastic in marine habitats. Additionally, although the Tamar Estuary is comparable in terms of proportions of sand (6 – 46 %), silt (0.5 – 2 %) and clay (0.1 - 0.9 %) to numerous estuaries in Britain and France (Frost, 2003), open coasts are by definition more exposed with a higher proportion of sand (45 – 100 %), lower proportions of silt (0 - 0.2 %) and virtually no clay (Frost, 2003). Chapter 3 discusses a further study where sediment samples from open coastal beaches from around the world were used to test the relationship between the abundance of microplastic debris and the mean particle size of sediment within strandline sediments. Recently, Barnes (2005) has showed that the abundance of large (>1 mm) anthropogenic debris in the southern hemisphere, mostly plastic, is closely related to latitude with higher abundances at the equator compared to more southerly shores. Additionally, this research demonstrated that over 90 % of the variation in the abundance of these debris can be explained by the population density of the country where the samples was taken, with more debris in locations of high population density. It remains unknown whether there are latitudinal trends in the abundance of microplastic and whether population density can predict the number of microplastic fragments

in marine habitats. Chapter 3 tests these hypotheses using data from a global sampling programme, undertaken as part of a wider project looking at the global extent of microplastic contamination in marine habitats.

Over the last 40 years, the amount of microplastic contamination has increased in the Atlantic Ocean (Thompson et al., 2004) and the present study shows that this material is 5 times more abundant than macroplastic items, and that microplastic debris accumulates in wind-exposed locations than on sheltered shores. A range of marine organisms with differing feeding strategies including mussels, barnacles (filter-feeders), amphipods (detritivores) and lugworms (deposit-feeders) are capable of ingesting microplastic (Thompson et al., 2004); however the fate and biological consequences of this material remain unknown. As with previous studies in Greece and the Falklands Islands (Otley and Ingham, 2003, Stefatos et al., 1999) plastic food packaging was a major component of intertidal litter and legislation is now required to help reduce the amount of single use plastic packaging and to increase the amount of plastic that is reused and/or recycled. Looking to the future, unless this type of legislation is successful it seems likely that the amount of microplastic will increase in the environment. Research is therefore needed to assess the body burdens of microplastic in the tissue of natural animal populations and to investigate more fully the environmental consequences of this type of debris in marine habitats.

Chapter 3. Global trends in microplastic abundance and the possible role of sewage disposal sites as sources of microplastic debris to marine habitats

3.1 Abstract

The sources, sinks and drivers of microplastic debris within marine habitats are poorly understood. Previous research in the southern hemisphere has shown trends in the abundance of larger items of debris in relation to latitude and human population density. This chapter examines four key hypotheses that may help explain the distribution of microplastic debris: there is a relationship between the abundance of microplastic debris and (1) mean particle size of sediment, (2) latitude, and (3) human population density. Finally, (4) marine disposal sites for sewage sludge have significantly higher numbers of microplastic fragments compared to relevant control areas, and therefore act as potential sources of microplastic debris.

The first three hypotheses were investigated using data from a global sampling programme undertaken as part of a wider project looking at the global extent of microplastic contamination in marine habitats. Levels of microplastic contamination and mean particle size in sediment were determined in samples from sandy beach habitats in Australia, Japan, Oman, United Arab Emirates, Chile, Philippines, Portugal, Azores, USA, South Africa and Mozambique and from the Tamar Estuary (UK). Analyses using Spearman Rank Correlation found no relationship between the abundance of microplastic fragments and either sediment mean particle size or latitude. There however a significant positive relationship between human population density and the number of microplastic items.

To determine whether sewage sludge disposal grounds act as sources of microplastic, replicate sediment grab samples were taken from sewage sludge disposal grounds and reference sites in the English Channel and the North Sea (UK). Overall, there were significantly greater abundance of microplastics at the disposal sites than at references sites at each location.

Identification of fragments using Fourier Transform Infrared Spectroscopy (FT-IR) showed that polyester (including polyethylene terephthalate; PET) was a major component of microplastic in subtidal areas of the UK (88 %) and sandy shores globally (56 %). The environmental significance of microplastic debris as a persistent and globally ubiquitous form of contamination in marine habitats is discussed with regard to the potential for this material to be ingested by marine organisms.

3.2 Introduction

Understanding the sources, sinks and drivers of microplastic debris in marine habitats is crucial to assessing the environmental consequences of this new form of contamination. Over the last 50 years human population density has increased nearly three-fold from 19 to 48 individuals km^{-2} globally (UN, 2007), during this time the numbers of microscopic fragments of acrylic, polyethylene, polypropylene, polyamide and polyester have increased in surface waters of the north-east Atlantic (Thompson et al., 2004). This debris now contaminates sandy, estuarine and sub-tidal habitats in the United Kingdom (Thompson et al., 2004), Singapore (Ng and Obbard, 2007) and India (Reddy et al., 2006). Larger macroplastic items (> 1mm) are now routinely found contaminating marine habitats from the poles to the equator (Barnes, 2002, Barnes, 2005); however, our understanding of the spatial extent of smaller microplastic contamination is largely unknown. In the Southern Hemisphere, Barnes (2005) has shown that human population density can explain over 90 % of the variation in the abundance of macroplastic (>5 mm) debris, with the abundance of these items increasing with population density. Latitudinal patterns in the accumulation of plastic debris on shores were also evident, with over 2 plastic items m^{-1} $year^{-1}$ at equatorial shores compared to less than 0.01 plastic items m^{-1} $year^{-1}$ on southern shores. It is however not known if the number of

microplastic particles increase with human population density or whether there are any latitudinal patterns of distribution.

In order to examine whether models of sediment dynamics could be applied to the transport of plastic debris, Chapter 2 examined the extent to which models of sediment dynamics could be applied to the accumulation of microplastic debris in estuarine habitats. Data from these habitats indicated that there was no clear relationship between the clay content of sediment and abundance of microplastic debris. Sediment from open coastlines is mainly composed of sand, whereas macrotidal estuarine sediments contain higher proportions of silt and clay that give rise to comparatively lower overall mean particle size (Frost, 2003). Therefore, to determine whether there is a relationship between mean particle size of sediment and the number of microplastic debris present, examination of a broad range of sediment types from estuarine (silt and clay) to open coastal habitats (sandy) may be more informative.

Sources of microplastic are likely to include fragmentation of larger plastic items, the small particles of plastic present as abrasive scrubbers in cleansing products (Zitko and Hanlon, 1991, Gregory, 1996), plastic media blasting (Anonymous, 1998, Abbott, 1992, Wolbach and McDonald, 1987), drug-delivery systems (Wen et al., 2003, Kawashima et al., 1992, Curley et al., 1996, Matsusaki et al., 2001) the laundering of clothes (Habib et al., 1998) and cleaning of fabrics including carpets. Small particles such as these are likely to be transported with waste water, through sewage treatment works and subsequently enter aquatic habitats. In the UK alone, over 11 km^3 of water is discharged into inland waters, estuaries and the sea each year (DEFRA, 2002). Although this is treated to remove solids, (primary treatment), breakdown organic substances (secondary treatment) and sometimes phosphates and nitrates (tertiary treatment), there is no procedure to specifically remove microplastic debris (personal communication, Paul McNie, South West Water, UK). Consequently,

numerous microplastic fibres have been found in samples of sludge and effluent taken from a sewage treatment works in New York, USA (Habib et al., 1998) and up to 4 synthetic fibres per gram were detected in soil collected from terrestrial habitats to which sewage sludge had been applied (Zubris and Richards, 2005). From the early 1970's to 1998, a quarter of UK sewage sludge was disposed of at 13 designated marine sites around the UK (Figure 8), until this practice was phased out at the end of 1998 as a result of the Urban Waste Water Treatment Regulations 1994 (CEFAS, 1997).

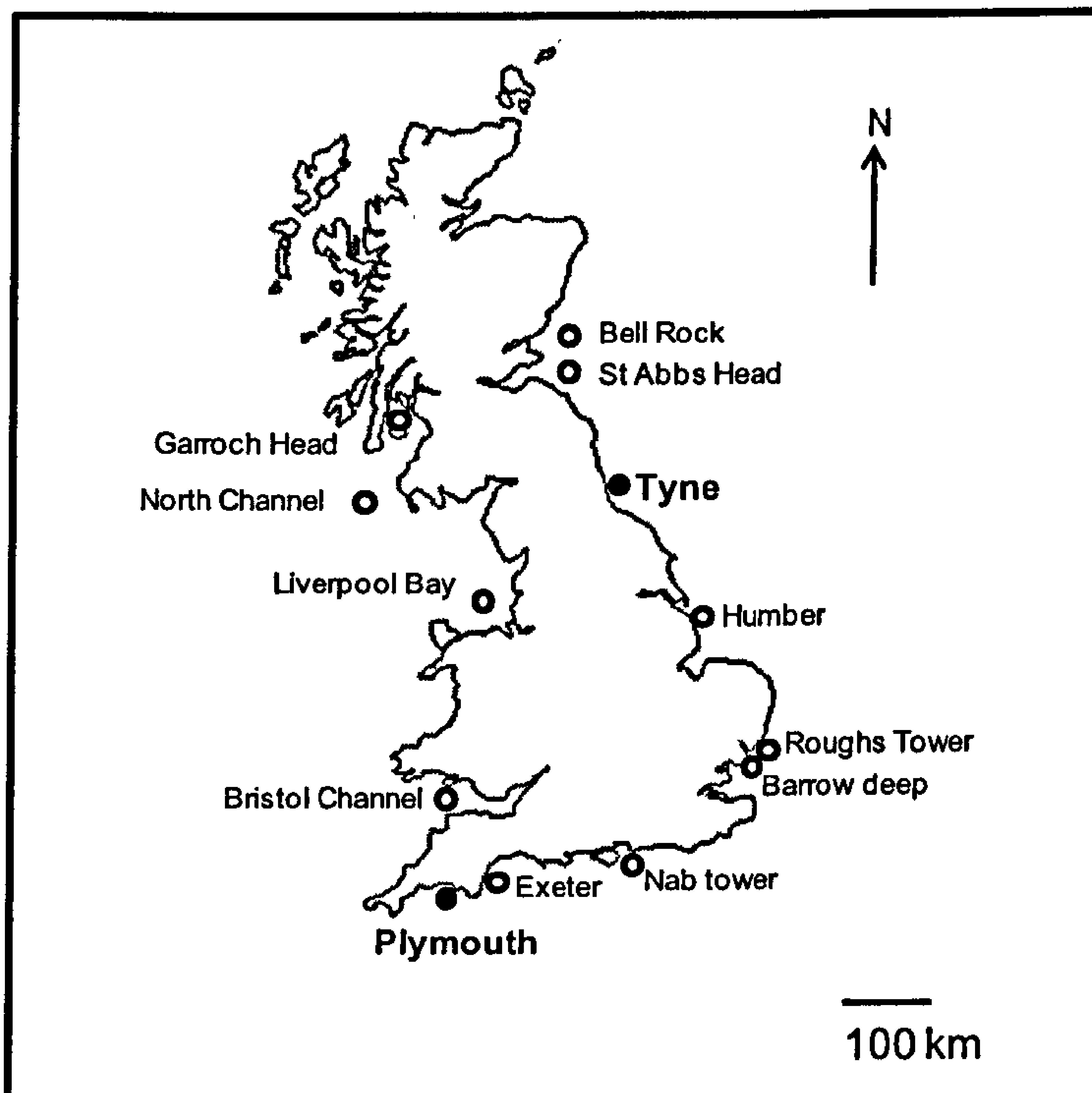


Figure 8. Locations of UK sewage-sludge disposal sites (1970 - 1998) (CEFAS, 1997). Plymouth and Tyne disposal sites presented as filled black circles, whereas the other 11 sites are with open circles.

Since substantial quantities of sewage sludge and effluent are discarded to the sea, there is considerable potential for microscopic plastic debris to accumulate in aquatic

environments, especially in countries with a high population density. Sewage sludge has been shown to contain high levels of toxic contaminants, such as organotin (Fent, 1996), nonylphenol (Ferguson et al., 2001) and triclosan (Aguera et al., 2003, EA, 2007), along with microplastic fibres of polyamide (Zubris and Richards, 2005). Furthermore, disposal areas have been shown to contain macroplastic debris, including shoes, elastic bands, textiles, packaging, sanitary towels and tampons (CEFAS, 1997). These items are liable to degradation into smaller fragments and it is therefore important to determine the potential for sewage disposal sites to act as sources of microplastic debris. The Tyne and Plymouth disposal sites are located 10 km offshore and were used for sewage sludge disposal from 1971 to 1998. The population of Newcastle (260,000) and Plymouth (240,000) are similar in size (National Statistics, 2001). Between 1979 and 1998 the Tyne sewage disposal ground received a total of 8,639,144 wet tonnes of sewage sludge compared to disposal ground at Plymouth which received 1,372,866 (personal communication from J. Rhodes, CEFAS 2007). Therefore sediments at the Tyne disposal ground may be expected to have over 6 times more synthetic fibres than sediments from the Plymouth disposal site.

3.2.1 Hypotheses tested

Samples collected from around the world;

i) There will be a relationship between mean sediment particle size and the number of microplastic fragments.

ii) There will be a relationship between latitude and the abundance of microplastic debris.

iii) Sediment collected from countries with high human population density will have greater abundance of microplastic fragments.

Samples from the sub-tidal areas around the UK;

iv) Sediment collected from sewage disposal sites (Plymouth and Tyne) will have significantly higher levels of microplastic compared to sediment collected from reference sites outside of the disposal area.

3.3 Materials and methods

3.3.1 Global sampling

To examine the relationship between the mean sediment size and the abundance of microplastic in strandline sediments worldwide, samples were collected from exposed sandy shores as part of programme looking at the global distribution of microplastic debris in marine habitats (Table 5).

During collection, cotton clothing was worn rather than synthetic items (such as fleeces) to avoid contamination by plastic fibres. Samples were collected by working down-wind to the particular part of the highest strandline deposited by the previous tide. Sediment was sampled to a depth of 1 cm deep by scooping sand at random positions in an arc to the

front of the person undertaking the sampling, until a 500 ml container was full. Once a container was full it was sealed and labelled (Appendix 1). This procedure was repeated at 25 m intervals along the strandline of the last tide until 5 independent replicate samples had been obtained. This avoided sampling replicates at different tidal heights and therefore minimised confounding due to potential differences in the abundance and composition of microplastic caused by sampling variations in settlement between repliactes. As the sampling was opportunistic, the sampling design was unable to remove this type of confounding between sites/locations, where tidal range and position of the strandline on the shore will vary. Each of the 5 replicate containers were wrapped in foil or paper, and sent to the University of Plymouth for extraction of microplastic and sediment analysis. Samples were collected by members of the Marine Biology and Ecology Research Centre or by individuals who were separately briefed and trained.

The extraction and identification of plastic debris was carried using methods decribed in Section 2.3.2, page 30 and analysis of sediment particle size was carried using methods decribed in section 2.3.3, page 31.

Table 5. Locations for samples taken as part of global programme to quantify microplastic debris in marine habitats.

Continent	Country	Code	Location	Latitude	Longitude
Asia	United Arab Emirates	AE1	Dubai	25°17N	55°18E
	Oman	OM1	Shinas	24°43N	56°28E
	Japan	JP1	Kyushu	32°24N	131°39E
	Philippines	PH1	Malapascua Island	01°18N	01°103E
South America	Chile	CL2	Vina Del Mar	32°56S	71°32W
		CL3	Punta Arenas	53°08S	70°53W
Europe	UK	UK1	Sennon Cove	50°04N	05°41W
	Finland	FI1	Korpo Island	21 °63N	60 °11W
	France	FR1	Roscoff	48°43N	03 °58W
	Portugal	PT1	Faro	36°59N	07°57W
	Portugal (Azores)	PT2	Ponta Delgado	37°44N	25°34W
North America	United States of America	US1	Virginia	36 56N	76 14W
		US2	Virginia	36°57N	76°14W
		US3	California	35°50N	118°23W
Oceania	Austrailia	AU1	Port Douglas	16°29S	145°28E
		AU3	Busselton Beach	33°39S	115°19E
Africa	South Africa	ZA1	Western Cape	33°06S	17°57E
	Mozambique	MZ1	Pemba	19°01S	36°01E

3.3.2 Sewage disposal site sampling

Five independent replicate grab samples were collected from each reference site (Plymouth 50°14N 04°10W and Tyne 55°06N 01°18W) and sewage sludge disposal sites (Plymouth 50°14N 04°18W; Tyne 55°03N 01°17W) using a van Veen grab in 2005/6 deployed from RV Bernicia (Newcastle University) and Aquatay (University of Plymouth). There is no data on how this material travels in sub-tial habitats, however if the controls were contaminated this would reduce the ability of the statistical analysis (Section 3.3.3) to detect

significant effects. The distance between the sewage sludge disposal sites and the reference sites was over 10 km and the main direction of currents were from the reference site to disposal sites. For each sample, the surface 5 - 10 cm of sediment was placed into 500 ml glass or foil containers. The extraction and identification of plastic debris was carried using methods described in Section 2.3.2, page 30.

3.3.3 Statistical analyses

Spearman Rank correlation was used to determine whether there was any relationship between mean sediment grain size and the number of microplastic fragments from open coast sandy (Table 5) and estuarine beaches (data from Chapter 2). A similar analysis was carried out for the number of microplastic fragments from sandy beach habitats globally (Table 5) and the latitude of the sites at which they were collected in both the northern and southern hemisphere. To test whether the population density of counties could be used as a predictor of the number of microplastic fragments found in sandy beach habitats globally, a linear regression analysis was performed on log transformed data.

To determine whether sewage disposal grounds contain higher levels of microplastic, material identified by FT-IR were grouped as total plastics per sample. The abundance of microplastic in sewage disposal and reference sites in the North Sea and English Channel were compared by 2 factor ANOVA, where the first factor "location" was fixed and randon with two levels (Tyne and Plymouth) and the second factor "site" was fixed and had two levels (disposal and reference). Prior to ANOVA, homogeneity of variances was examined using Cochran's tests. For this analyses the interaction term was pooled ($P > 0.25$) to create a more powerful test against the residual.

3.4 Results

3.4.1 Global trends in the abundance of microplastic fragments in relation to mean sediment size, latitude and population density

Microscopic fragments of plastic were found at all sites. On sandy shores the abundance ranged from 2 (Busselton Beach, Australia) to 31 synthetic fibres per 250 g sediment in Portugal and the UK, the Tamar Estuary contained over 55 fragments of microplastic debris per 250 g sediment. These fibres manly consisted of polyester (56 %), acrylic (23 %), polypropylene (7 %), polyethylene (6 %), polyamide (3 %), and in contrast to Chapter 2 (polyvinylchloride 27 %), polyvinylchloride contributed just 1%. There were no correlations between the abundance of microplastic items in sediments and mean sediment grain size (0.39, $P > 0.05$, n = 21) or latitude, in either the Northern (0.28, n = 18) and Southern hemispheres 0.53, n = 18). The number of microplastic debris items in the sediment of sandy beaches increased with population density, explaining 34 % of the variation in abundance of microplastic debris ($F_{1,16} = 8.36$, $P < 0.05$, $r^2 = 0.34$).

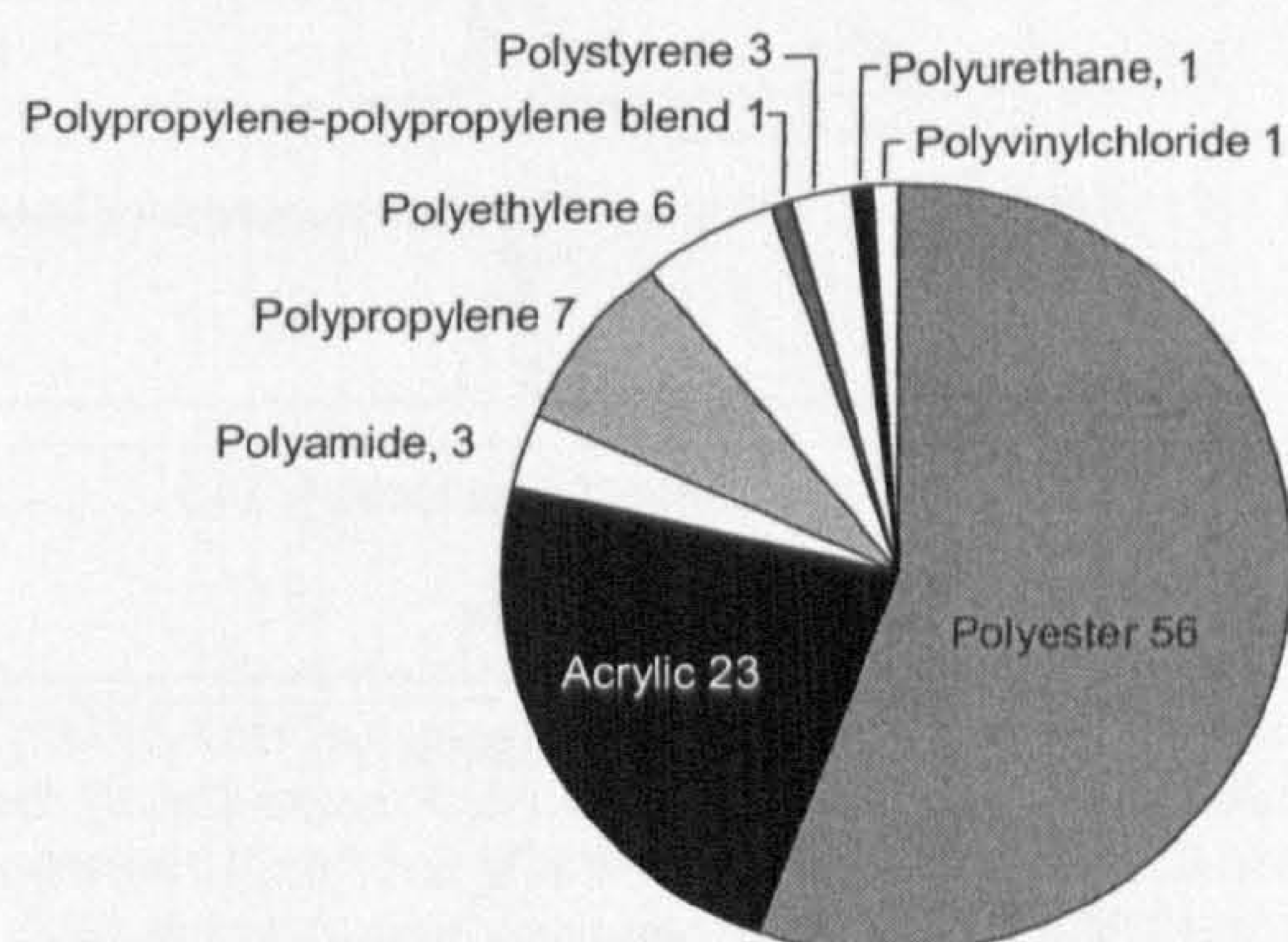


Figure 9. Composition (%) of microplastic debris collected from 18 sandy habitats around the world.

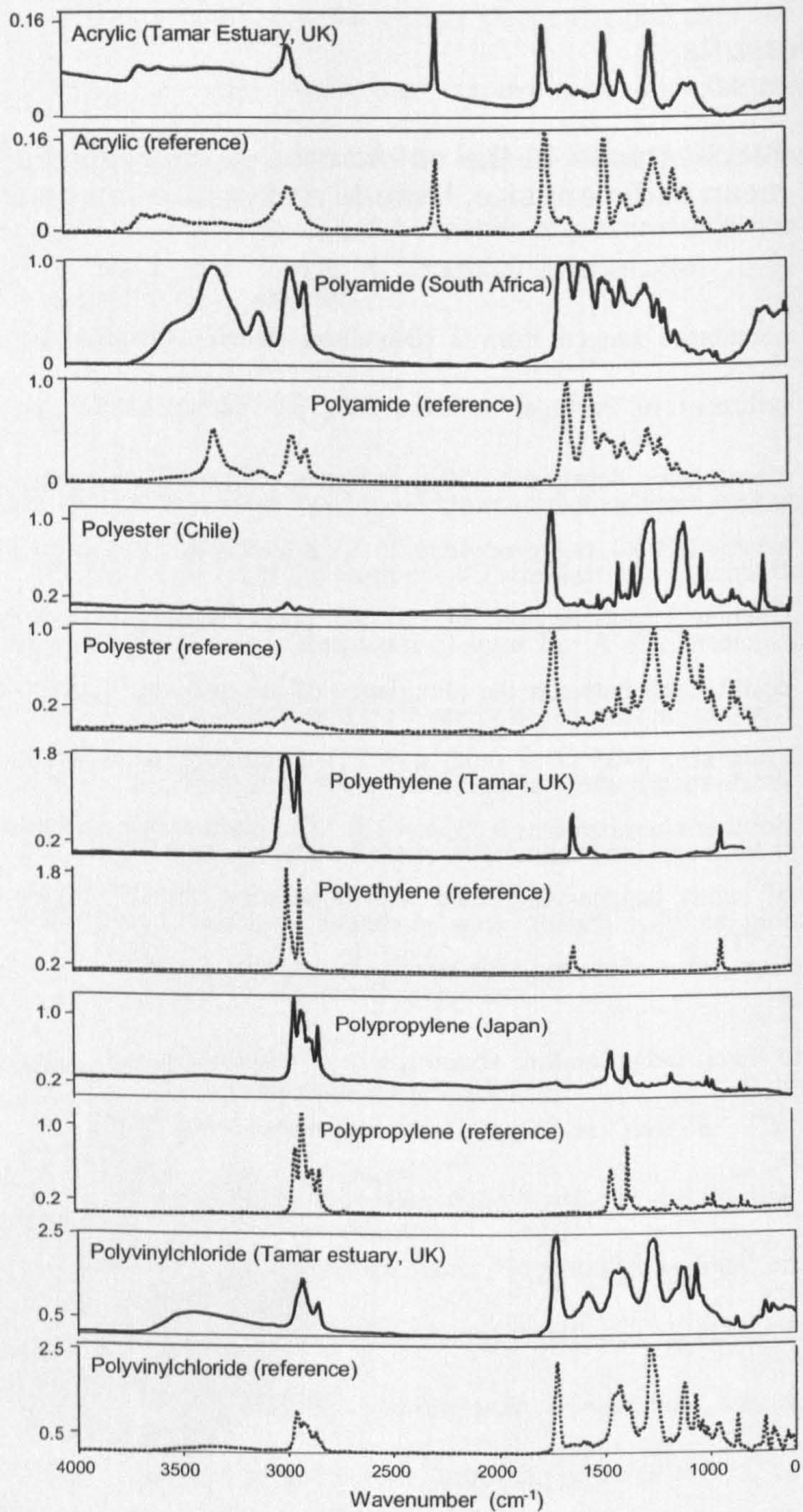


Figure 10. Examples of Fourier Transform Infrared spectra of microplastic debris and corresponding plastic reference material, vertical axis represents transmission in standard optical density units (Spectra courtesy of E. Teuten and A. Tonkin, University of Plymouth).

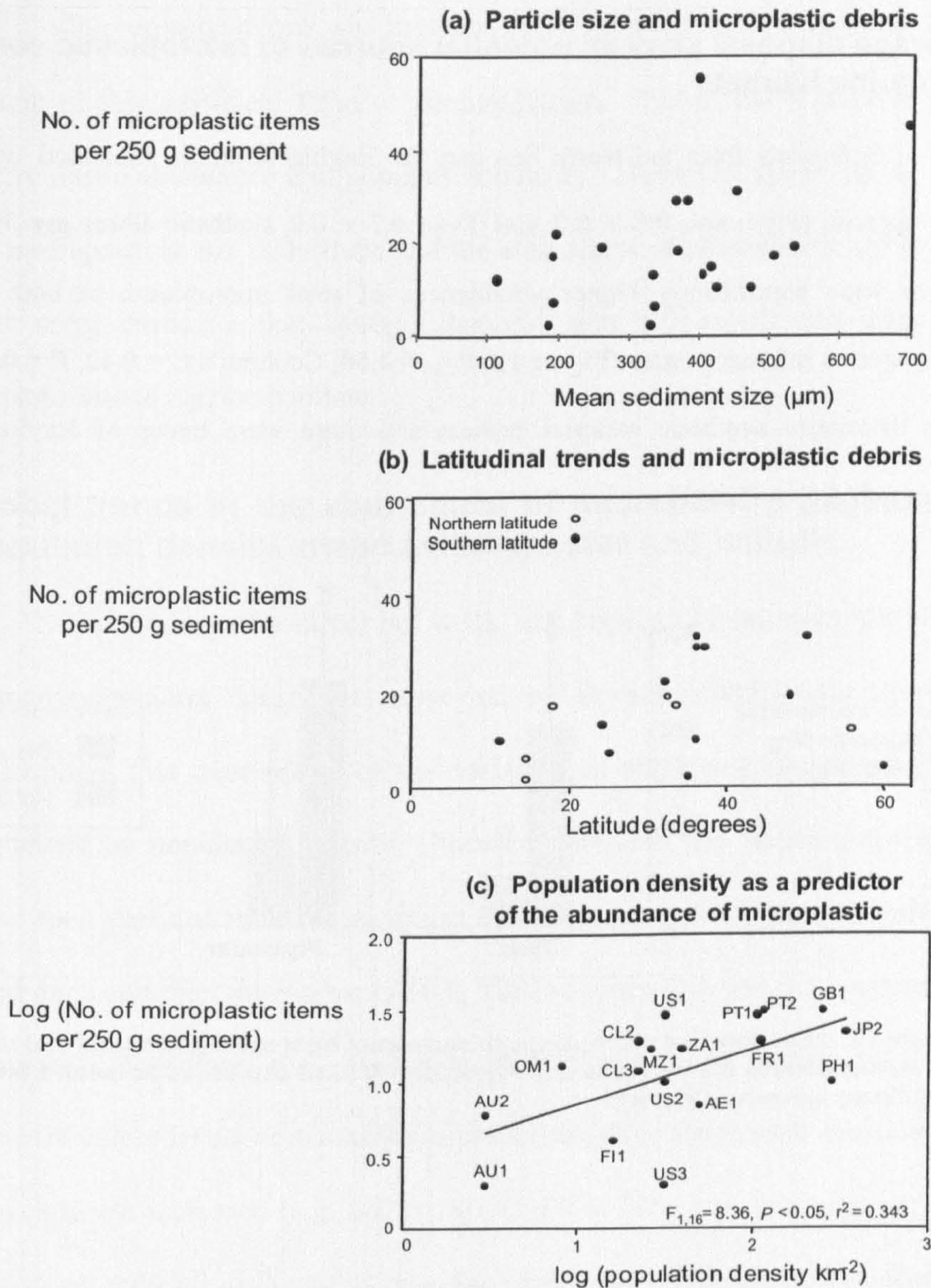


Figure 11. Relationship between (a) mean sediment particles size and number of microplastic debris in marine habitats (Spearman Rank 0.39, $P > 0.05$ ns), (b) latitude and number of microplastic items, Spearman Rank for Northern Hemisphere 0.28 and Southern Hemisphere 0.53, both $P > 0.05$ ns. (c) Linear regression analysis of log(population density) and log(number of microplastic items) in sediment from sandy habitats and the number of plastic items ($F_{1,16}$ = 8.36, $P < 0.05$, $r^2 = 0.34$). Country codes in (c) indicated in table 5.

3.4.2 Sewage disposal sites as potential sources of microplastic contamination in marine habitats

Sediments from the North Sea and the English Channel contained similar levels of microplastic (Plymouth 0.5 ± 0.2 and Tyne 0.7 ± 0.2 synthetic fibres per 50 g sediment). There were significantly higher abundances of total microplastic at both disposal sites compared to references sites (Figure 12; $F_{1,16} = 4.50$, Cochran's C = 0.42, $P < 0.05$). Polyester was the major synthetic material present and there were traces of acrylic found at the Plymouth disposal site.

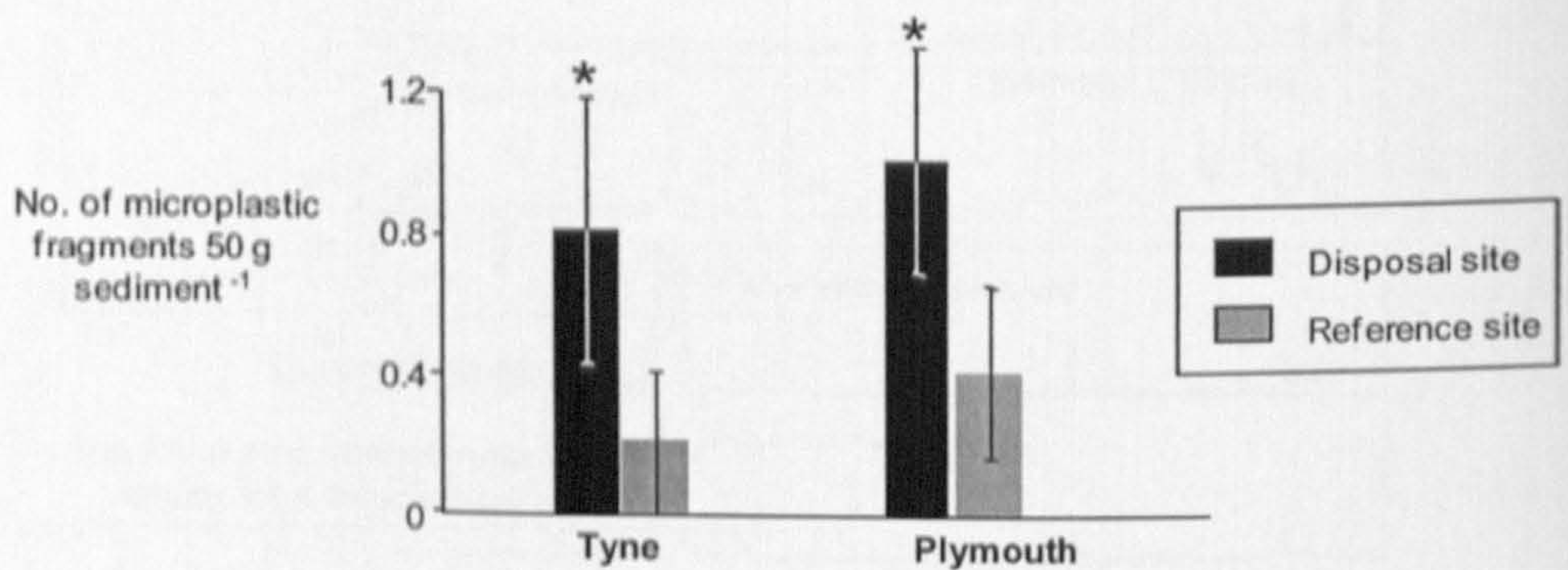


Figure 12. Abundance of microplastic in sediments from sewage disposal and reference sites at two locations in the UK (Tyne and Plymouth). Values expressed as mean ± S.E. and significant difference *$P < 0.05$.

3.5 Discussion

Microplastic debris was found at every sample location, showing the large-scale spatial extent of this persistent form of contamination. There was a positive relationship between microplastic abundance and population density. However, there was no relationship between its mean particle size or latitude and the abundance of microplastic debris. Furthermore, there was some evidence that sewage disposal sites (Plymouth and Tyne) act as sources of microplastic to marine habitats.

3.5.1 Global trends in the abundance of microplastic fragments in relation to population density, mean sediment size and latitude

The finding of a direct but weak link between global microplastic contamination and human population density is supported by Barnes (2005), who showed in the southern hemisphere that over 90 % of the variation in anthropogenic debris (mostly plastic), was explained by population density. Plastic production and population density have increased year upon year and most plastic items are single use packaging materials (Chapter 2) that are used once and then thrown away (EA, 2001a), since they are not accepted by many recycling schemes. Therefore, the abundance of microplastic in marine habitats will continue to increase unless levels of discharge of plastic debris to the marine environment are reduced and recycling are increased (e.g. UK 6 %) (DEFRA, 2004), or consumption of single-use plastic packaging reduced in favour of reusable plastic bags and containers. However, the results do need to be interepreted with caution, as the scale of measurment of human population density for the country as a whole is different to the local population density at which the samples were collected.

The lack of any clear relationship between the abundance of microplastic debris and mean particle size, together with the findings of Chapter 2 suggest that the suspension,

transportation and settlement of microplastic debris is not related to the size of natural sediments. Given the findings of Chapter 2, in which plastic density was found to affect patterns of its distribution, a possible explanation for is that differences in the density and between natural sediment particles and plastic debris may be more important than sediment size. Microplastic debris also varies in shape, from irregular fractual (e.g. rough or fragmented geometric) and spherical fragments to long thin fibres (Figure 13). A range of indices (e.g. the disk-rod index, rod-index, flatness index, oblate-prolate index and spherecity) have been used to quantify the shape of sediment particles (Le Roux, 2005) and these indicies could also be used to quantify the shape of plastic debris. Laboratory trials have shown that non-spherical fractual shaped aggregates of microscopic polystyrene between 100 – 1000 µm in diameter can settle up to 8.3 times fasters than equivalent spherical particles (Johnson et al., 1996). Since the majority of plastic fragments found in the present work were fibres, a research priority is to examine the relative influence of diameter, length and density on the settling velocity of microplastic fibres in seawater. These data could then be related to their transportation in marine habitats.

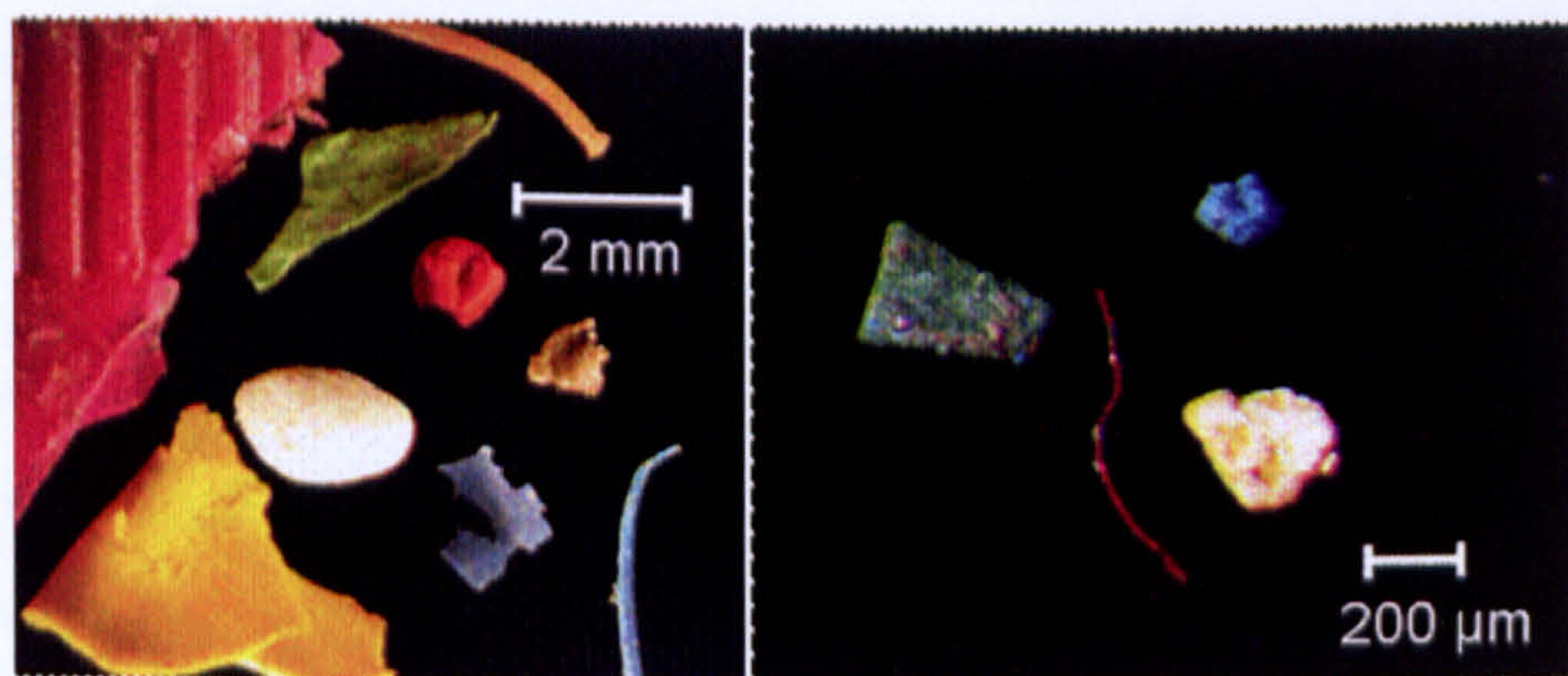


Figure 13. Fragments of polyethylene, polyvinylchloride, polypropylene, and polystyrene from the Tamar Estuary, UK.

3.5.2 Sewage disposal sites as potential sources of microplastic contamination in marine habitats

There were significantly greater abundances of microplastic fragments at both disposal sites compared to references sites. Research has shown that sewage effluent applied to terrestrial soils can contaminate the soil with synthetic polyamide fibres with the effects remaining for over 15 years (Zubris and Richards, 2005). However, there are limitations in the sampling design and a more extensive sampling programme is needed to conclusively asses whether abandoned sewage disposal sites can act as a source of microplastic debris in marine habitats. There is no data on the distance or rate of movement of microplastic in sub-tial habitats and it is impossible to determine without further work if the control sites were likely to have been contaminated. The distance between the sewage sludge disposal and reference sites was over 10 km, furthermore the main direction of currents were from the reference site to disposal sites. Therefore if the reference sites were contaminated this would have reduced the ability of the statistical analysis to detect significant effects. Any further work should employ a greater number of reference sites at different spatial distances from the disposal area in each location, and by taking deeper sediment samples using a core-sampler; it would also reduce the potential problems of contamination by drifting particles of microplastic from other sources.

Sewage sludge from the UK has not been discharged at sea since 1999 and is now disposed of on agricultural land (52 %) or to landfill (17 %), or is incinerated (21 %) (DEFRA, 2002). In other countries Sewage sludge that has been treated to remove pathogens, is applied to fruit, vegetable and animal feed crops, and to grassland for grazing (ADAS, 2001). Given that over a million tonnes of sewage sludge were applied to farmland in the UK (DEFRA, 2002) and that the sludge contains microplastic fibres (Zubris and Richards, 2005,

Habib et al., 1998), research is needed to asses whether microplastic material has any environmental or health implications.

Sewage effluent is continually discharged into freshwater and aquatic habitats, however the influence of seasonality and sewage treatment (primary, secondary, tertiary, etc) on the abundance and composition of synthetic fibres in sewage effluent and sludge is unknown. It has been suggested that the main source of fibres to sewage effluent is the laundering of clothes (Habib et al., 1998). Clothing provides protection against seasonal changes in temperature. A recent study in Australia has shown that office workers wear more clothes during the winter and than in the summer (Erlandson et al., 2005) and washing machine usage in residential homes is up to 7 times greater in the winter than to the summer (Takuma et al., 2006). Research is therefore needed to determine assess seasonal changes in clothing and washing results in significantly more plastic fibres in sewage effluent and sludge during the summer months compared to the winter.

In addition, analyses of these fragments using FT-IR as employed in other studies (Thompson et al., 2004, Reddy et al., 2006, Eriksson and Burton, 2003) rather than polarised light microscopy, would provide more detailed information on the composition of plastic debris entering the environment from sewage effluent.

3.5.2 Environmental implications of microplastic as a ubiquitous contaminant in marine habitats

Microscopic plastic debris was present in samples from all locations sampled. Most locations contained high levels of polyester. It is well established that microplastic debris is present in marine habitats of the North-East Atlantic Ocean (Thompson et al., 2004), UK (Chapter 2), India (Reddy et al., 2006) and Singapore (Ng and Obbard, 2007). Consequently, microplastic appears to be a ubiquitous form of contamination, across a range of habitats and

the number of microplastic fragments is influenced by population density. However, the potential environmental impacts of microscopic (<1 mm) plastic debris are still poorly understood. Laboratory experiments have shown that microscopic particles of polystyrene and polyvinylchloride have been ingested by a wide range of invertebrates, including the filter feeding mollusc, *Mytilus edulis* (Chapter 4). If microplastic is ingested by animals it may be retained in the digestive tract, egested in the form of faeces, or absorbed into the epithelial lining of the gut by phagocytosis. Laboratory trials using lugworms *(A. marina)* kept in sediments containing microplastic have shown that these animals are capable of egesting this debris within their faecal casts (Thompson et al., 2004). However, it is unknown if ingested microplastic particles are taken up by the gut epithelial lining of marine organisms and whether further transport around the body is possible. Therefore Chapter 4 investigates the internal transport and biological consequences of ingesting polystyrene microspheres using the mussel, *Mytilus edulis* (L.) as a model filter-feeding organism. Additionally, over the last 30 years there has been speculation that ingested may accumulate toxic chemicals from the environment and act as a transport mechanism for these chemicals and chemical additives incorporated during manufacture, into the tissues of marine animals. Up to now there has been no experimental evidence for this hypothesis, Chapter 5 address this gap in the knowledge using a series of laboratory trials using the lugworm, *Arenicola marina* (L.) as a model deposit-feeder.

Chapter 4. Biological consequences of microplastic in the mussel, *Mytilus edulis* (L.)

4.1 Abstract

Plastics debris is accumulating in the environment and is fragmenting into smaller pieces, as it does, the potential for ingestion by animals increases. The consequences of macroplastic debris for wildlife are well documented, however the impacts of microplastic (<1 mm) are poorly understood. The mussel, *Mytilus edulis* was used to investigate ingestion, translocation and accumulation of this debris. Initial experiments showed that upon ingestion, microplastic accumulated in the gut. Mussels were subsequently exposed to treatments containing seawater and microplastic (3.0 or 9.6 µm). After transfer to clean conditions, microplastic was tracked in the haemolymph. Particles translocated from the gut to the circulatory system within 3 days and persisted for over 48 days. Abundance of microplastic was greatest after 12 days and declined thereafter. Smaller particles were more abundant than larger particles and this indicates as plastic fragments, the potential for accumulation in the tissues of an organism increases. The short-term pulse exposure used here did not result in significant biological effects. However, plastics are exceedingly durable and so further work using a wider range of organisms, polymers and periods of exposure will be required to establish the biological consequences of this debris.

4.2 Introduction

Chapter 3 showed that microplastic appears to be a ubiquitous form of contamination, across a range of habitats and the number of microplastic fragments is influenced by population density. However, the potential environmental impacts of microscopic (<1 mm) plastic debris are still poorly understood. Large (>5 mm) items of plastic debris are ingested by species of fish (Derraik, 2002, Kartar et al., 1976), turtles (Mascarenhas et al., 2004, Tomas

et al., 2002), marine mammals (Baird and Hooker, 2000) and birds (Harrigan, 1992, Blight and Burger, 1997), however the biological fate and consequences of smaller, microscopic plastic debris (<1 mm) that are ingested by marine animals is poorly understood. This material has accumulated in the water column of the north-east Atlantic over the last 40 years (Thompson et al., 2004) and work in the Tamar Estuary (UK) has shown microplastic comprises 85% of stranded plastic debris (Chapter 2). As this debris occupies the same size range as sand grains and plankton, it is available to a wide range of organisms, including invertebrates at the base of the food chain. Many invertebrates feed by collecting, sorting and digesting particulate matter; however the uptake and retention of microplastic by animals in their natural habitats has received little attention, partly because quantifying the abundance of tiny plastic fragments in the tissues of animals presents a range of methodological problems. Laboratory trials have shown that amphipods (detritivores), barnacles (filter feeders) and lugworms (deposit feeders) ingest microplastic debris (Thompson et al., 2004). In addition, filter-feeding polychaetes, echinoderms, bryozoans and bivalves have been shown to ingest 10 µm polystyrene microspheres as part of research into particle selection (Ward and Shumway, 2004, Ward et al., 2003, Ward et al., 1991). However, the fate of this ingested material is poorly understood. Once microplastic is ingested it may be retained in the digestive tract, egested through defecation, or transferred through the epithelial lining of the gut into body tissues (translocation) (Browne et al., 2007, Hoet et al., 2004). Laboratory trials demonstrate that the lugworm, *A. marina* (L.) can egest polyvinylchloride (230 µm) particles within their faecal casts (Chapter 5). Medical studies report that tiny particles, made of titanium dioxide, plastic and carbon within the nano and micrometer range can translocate from the gut into the wider body tissues of rodents and humans (see Hoet et al., 2004 for a review). For example, particles of polyvinylchloride (Volkheimer, 1975) and polystyrene (see review by Hussain et

al., 2001) less than 150 μm can translocate from the gut cavity to the lymph and circulatory system. *In-vitro* experiments with segments of rat colonic tissue confirm that smaller particles undergo translocation more readily than larger particles (Szentkuti, 1997). However, it is not known whether microscopic plastic particles can translocate from the gut cavity to the circulatory system of marine animals or whether smaller particles translocate more readily than larger particles of plastic.

4.2.1 Potential toxicological consequences for marine animals ingesting microplastic debris

Polymers are composed of repeating sub-units called monomers. Polyvinylchloride (Marcilla et al., 2004), polystyrene (Garrigos et al., 2004) and polycarbonate (vom Saal et al., 2005, Howdeshell et al., 2003) have been shown to release toxic monomers which have been associated with cancer and reproductive abnormalities in humans, rodents and invertebrates. Exposure to fullerenes (carbon based nanoparticles) has been linked with cerebral oxidative stress within juvenile largemouth bass, *Micropterus salmoides* (Oberdorster, 2004). Work using mice injected intraperitoneally with microplastic has showed that phagocytic uptake of microscopic polylactic acid led to cellular damage, through changes in morphology and viability (Lam et al., 1993). Ingestion can lead to long-term accumulation of macroplastic by birds. This is hypothesised to reduce feeding behaviour by reducing the storage volume of the stomach (Ryan, 1988); however it is unknown whether ingestion of microplastic influences reduce feeding behavior of marine animals. The medical literature suggests that that micro- and nanometer sized (<1 μm) plastic (e.g. polyvinylchloride, polystyrene) and non-plastic particles (e.g. titanium dioxide, asbestos, fullerenes) exert damage in rodents through the combined effect of the chemical composition, shape (fibre, sphere, etc), surface area and overall size (Hoet et al., 2004). For example, inhalation of polyvinylchloride dust by humans

can cause, depending on monomer composition and size, lung and liver damage through tissue fibrosis and cancer (Wagoner, 1983). However, much of this work has come as a secondary outcome of medical trials and feeding studies and little is known about effects in natural habitats. Moreover, it remains unknown whether microscopic fragments of plastic can cause measurable changes in the 'health' of marine organisms near the base of the food chain.

4.2.2 Ecotoxicological relevance of *Mytilus edulis* (L.) as a test organism for uptake of microplastic

This chapter describes a study using *M. edulis* as a model filter-feeder to investigate the uptake, fate and biological consequences of ingesting microscopic particles of polystyrene. The mussel *Mytilus edulis L.* is a major component of benthic systems in the north Atlantic (Seed and Suchanek, 1992) and these animals are thought to act as ecosystem-engineers (Jones et al. 1994) via occupation of primary space, filtration and provision of secondary habitat (O'Connor and Crowe, 2007, Ragnarsson and Raffaelli, 1999, Seed, 2000). This species has a large geographic range and is an important component of the diet of intertidal predators (Menge et al., 1994, Cote and Jelnikar, 1999, Norman and Jones, 1992, Ebling et al., 1964, Freire, 1996, Mascaro and Seed, 2000, Du Preez, 1984) and humans (Nizzoli et al., 2005, CEFAS, 2005). The haemolymph can be easily sampled and its toxicology is well described (Brown et al., 2004, Hagger et al., 2002, Bloxham et al., 2004, Manduzio et al., 2004, Pipe et al., 1999, Pipe et al., 1995). Detailed work on the mussel, *M. edulis* has shown that polystyrene microspheres are drawn through the inhalant siphon and filtered using the gill (Ward and Shumway, 2004, Ward et al., 2003, Ward et al., 1991). Gill filaments composed of cilia capture particles and transport microplastic particles to the ventral groove. Once here mucus transports particles to labial palps, where cilia sort particles for ingestion or rejection (pseudofaeces) (Ward and Shumway, 2004, Ward et al., 2003, Ward et al., 1991). It remains

unknown whether microplastic can accumulate in the gut cavity of mussels and transfer to the circulatory system, however *in vitro* experiments using haemolymph from *M. galloprovincialis* have shown that haemocytes can phagocytose polystyrene micropsheres up to 800 nm (Cajaraville and Pal, 1995).

4.2.3 Environmental relevance of polystyrene as a contaminant in marine habitats

In 2003, over 170 items of polystyrene per km^2 were cleared from sandy beaches in the UK. Chapter 2 showed that, in terms of abundance, polystyrene accounted for 24 % of the macroplastic found in estuarine habitats. Polystyrene is primarily used (36 %) as food packaging and insulation in the construction industry (Plastic-Europe, 2006). In 2001, global production of polystyrene was more than 13.6 million tonnes and by 2010 it is expected to exceed 15 million tonnes (Plastics-Europe, 2004, Plastic-Europe, 2006). Spheres of polystyrene (0.1 - 2 mm) are known to accumulate in Atlantic fish species from coastal regions. Stomach content analysis has shown that over 20 % of snail fish (*Liparis liparis*) and commercially important flounder (*Platichthys flesus*) from the Bristol Channel contain polystyrene fragments (Kartar et al., 1976). Similar work in New England (USA) has shown that the stomach of contents of nearly 60 % of the fish species contain polystyrene fragments (Carpenter et al., 1972). However, the potential for microscopic fragments of this debris to translocate from the gut cavity to the circulatory system of any marine animal has not been investigated. Furthermore, the toxicity of polystyrene and its monomer, styrene to marine animals is poorly understood, with only one laboratory study examining the effects styrene on the viability and DNA damage to molluscan haemocytes (*M. edulis*) and to the blood cells of

the fish (*Symphodus melops*) (Mamaca et al., 2005). However, with only one exposure tank and one control tank, the experimental design suffered from pseudoreplication.

4.2.4 Hypotheses tested

To quantitatively test hypotheses relating to translocation and biological effects of particles of microplastic, animals were independently exposed to either a single pulse exposure of fluorescent labelled particles or to no plastic particles (control). These animals were then independently transferred to clean conditions to prevent further ingestion of microspheres. Exposed and control animals were then sampled over a logarithmic timescale to track labelled particles and measure biological effects. Histological and haematological techniques were used to assess translocation of particles, and toxicological assays were employed on different tissue samples to test specific hypotheses about biological effects. As each experimental unit is independent, analysis of variance was used to test hypotheses about translocation and quantitatively assess short-term (pulse) and long-term (press) toxicological effects, without confounding due to statistical non-independence of data.

To assess the fate and biological consequences of particles of microplastic ingested by *M. edulis*, the following hypotheses were tested:

i. After ingestion, polystyrene particles accumulate in the gut cavity of mussels,
ii. Ingested polystyrene microspheres translocate from the gut to the circulatory fluid (haemolymph), and when transferred to clean conditions, the abundance of microplastic in mussels will decline over time,
iii. After feeding mussels with the same number of microspheres, smaller (3.0 μm) polystyrene particles will occur in the haemolymph in greater quantities than larger (9.6 μm) particles,

iv. Ingestion and/or translocation of microplastic particles will reduce the viability and phagocytic function of haemocytes, reduce the oxidative status of haemolymph and reduce feeding activity of mussels.

4.3 Materials and methods

4.3.1 Husbandry and experimental design

Mytilus edulis (L.) (3.0 to 4.0 cm) were collected from Port Quinn (50°35'20.60" N 4°52'05.63" W), Cornwall, U.K. This site is free from point-sources of contamination and the mussels there have low contaminant burdens and high scope for growth (Widdows et al., 1995, Booth et al., 2007). Mussels were collected from the substrate by carefully cutting byssal threads to avoid damaging the abductor muscle. Epifauna were removed and mussels were transferred to temperature controlled conditions at the University of Plymouth. Individuals were numbered using plastic labels and cyanoacrylate glue, and randomly assigned to individual beakers according to the experimental design below, left for 48 hours to acclimatise in filtered seawater (15 °C) and fed using *Isochyrsis glabana* during this time (*www.reed-mariculture.com*). Two experiments were then conducted. The first experiment investigated whether mussels ingest polystyrene microspheres. A second then sought to investigate whether this material can translocate from the gut cavity to the circulatory system, whether there were differences in translocation according to particle size and whether translocation compromises the health of *M. edulis.*

In the first experiment to determine whether mussels were able to uptake microplastic from the water column to their gut cavity, mussels were exposed for 12 hours to separate treatments (each with three replicates) containing 2 x 10^8 particles of 2 μm fluorescently labeled polystyrene microspheres (excitation 365 nm / emission 447 nm) (Brookhaven

International, UK) and 0.5 g l^{-1} of non-labeled polystyrene (4-16 μm) microspheres (Sigma-Aldrich, UK) and a control treatment without plastic added. After exposure histological techniques were used to determine the presence of microplastic in the gut tissues of *M. edulis*.

In the second experiment, to establish whether ingested microplastic can translocate from the gut cavity to the circulatory system of mussels, clean glass beakers (400 ml) were filled with 350 ml of seawater, stirring bars added and the beakers were placed on magnetic stirrers. To avoid bias associated with possible environmental gradients in a controlled temperature room, mussels were randomly assigned to their time/treatment/replicate combination using a replicated block design. Two sizes of fluorescently dyed polystyrene particles (3.0 μm 490 nm excitation/ 515 emission nm and 9.6 μm 520 nm excitation/ 580 nm emission) were obtained from Molecular probes (USA) in a non-toxic, distilled water carrier. These were used to make plastic treatment groups each containing either 3.0 μm or 9.6 μm with 15,000 individual microscopic polystyrene spheres (as verified by coulter counter analysis). Mussels were exposed to these treatments for 3 hours, as preliminary work demonstrated that this was sufficient time for microspsheres to be ingested. After 3 hours exposure to microspheres, mussels were transferred to individual clean beakers using a 2 factor experimental design. Here, "time" since transfer to clean conditions had 5 levels 3, 6, 12, 24, 48 days) and "treatment" had three levels, control (no plastic), 3.0 μm polystyrene and 9.6μm polystyrene particles. There were 5 replicates of each time/treatment combination (Figure 14). Mussels were placed in separate 2 litre beakers and fed daily on *I. galbana*. Seawater was changed every-other day. Faeces was extracted by filtering the seawater under vacuum using a Whatman GFA filter.

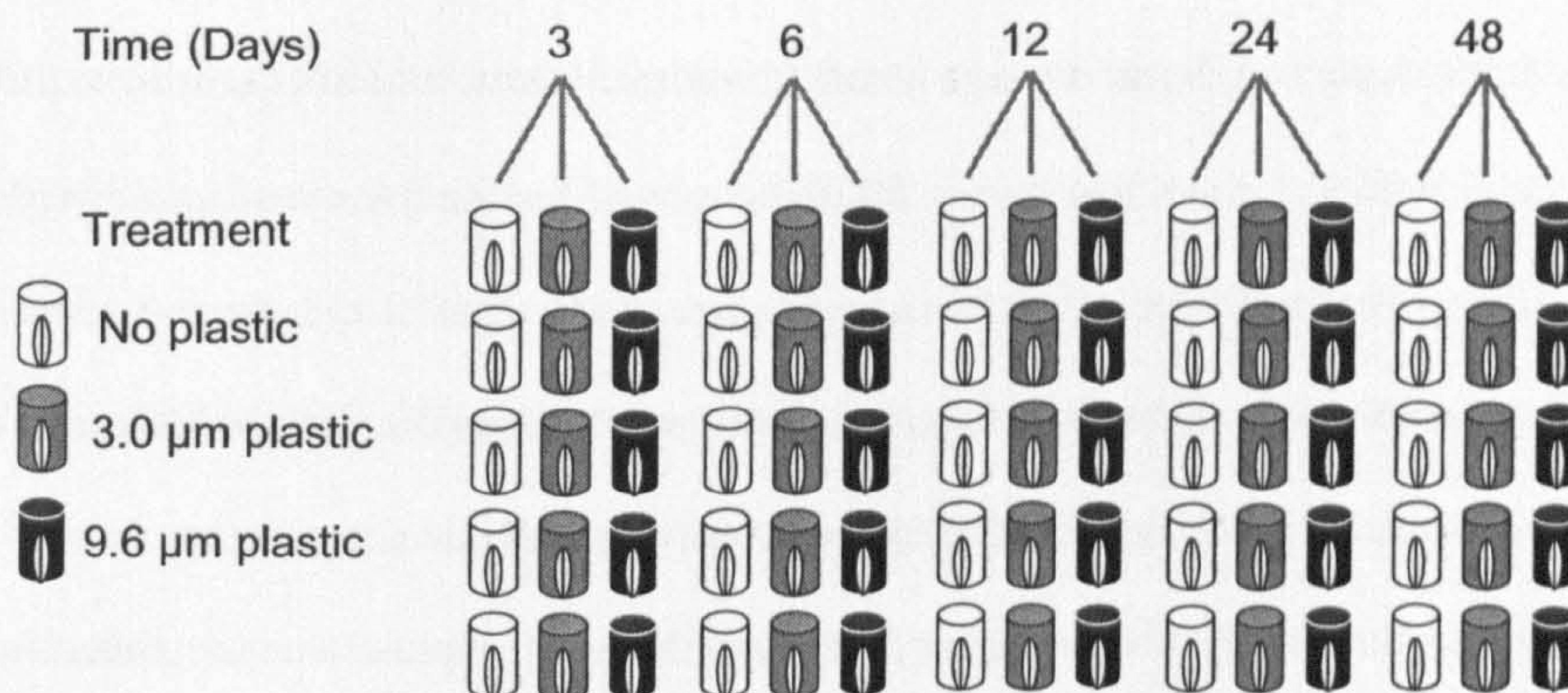


Figure 14. Experimental design for second trial investigating the translocation of polystyrene microspheres and possible biological effects in *Mytilus edulis*. Time/treatment/replicate order was randomised using a replicated block design to remove bias associated with possible environmental gradients in a controlled temperature room.

4.3.2 Biological assays

The cellular viability assay was used to examine whether the ingestion of microplastic leads to measurable reductions in the ability of mussel haemocytes to absorb neutral red dye (Bloxham et al., 2004, Cartwright et al., 2006). A 50 µl aliquot of haemolymph was pipetted into triplicate wells of microtitre plates and agitated using a plate shaker (1400 rpm for 60 seconds). The plate was then left for 50 minutes to allow haemocytes to adhere to the bottom of the wells. After this period, excess cells were discarded by rinsing the plate with phosphate buffer. Neutral red dye (0.4 %) was added, and cells were left in the dark for a further 3 hours to prevent photolysis. Wells were then washed with phosphate buffer solution once again before an acidified solution of 1 % acetic acid/20 % ethanol was added to resolubilise the dye. Absorbance was read at 550 nm using a spectrophotometer. The total protein in the haemolymph was determined following the method of Bradford (1976) and results were presented as optical density per gram protein.

To quantify whether the immune system of mussels had been compromised by ingesting microplastic, the phagocytosis assay was used to quantify ability of mussel

haemocytes to ingulf foreign yeast (zymosan) particles (Bloxham et al., 2004, Cartwright et al., 2006). To achieve this, a 50 µl sample of haemocytes was transferred in triplicate into a microtitre plate and agitated using a plate shaker (1400 rpm for 60 seconds). The plate was covered with a plate-sealer and incubated at 10 °C for 50 minutes. Aliquots of 50 µl of neutral-red-stained and heat-stabilised zymosan suspension (containing 1 $x10^5$ particles ml^{-1} in phosphate buffer) were added to each well, and the plate was incubated for 3 hours at 10°C. The cells were washed to remove residual haemocytes using 100 µl phosphate buffer (pH 7.4) and a series of zymosan standards were added. The dye was resolubilised via addition of 100 µl of acetic 1 % acetic acid/20 % ethanol. The microtitre plate was covered with a plate-sealer and incubated for 10 min at 20°C, and then read at 550 nm. The total protein in the haemolymph was determined (Bradford, 1976), and results were presented as the number of zymosan particles phagocytosed per gram haemocyte protein. Differences in cellular viability and phagocytosis were assessed using ANOVA.

To determine asses ingestion/translocation of microplastic can cause measurable reductions in the capacity of the haemolymph to deal with oxidative stress, the Ferric Reducing Antioxidant Potential (FRAP) assay was used (Hagger *et al.*, 2006). Haemolymph (30 µl) was pipetted in triplicate in microtitreplate wells. Aqueous solutions of known Fe^{II} concentrations in the range of 0 - 100 µmol l^{-1} were used for calibration. 200 µl of reagent (300 mM acetate buffer, TBTZ (2,4,6-tripyridyl-*s*-triazine) was placed into each well. The absorbance (595 nm) within each sample well was read immediately (time 0), incubated at 25 °C for 10 minutes and read again. Results were presented as the change in absorbance at 595 nm and differences were assessed using ANOVA.

Feeding behaviour was assessed using clearance rate (Widdows & Staff, 1997). An algal solution (*Isochrysis galbana*) was prepared by diluting an *I. galabana* concentrate with

100 ml of seawater to produce an initial concentration of algal cells in the feeding vessels at ca. 15,000 algal cells 0.5 ml^{-1}. Beakers (400 ml) were filled with 350 ml of seawater (15 °C), stirring bars (each 12 x 6 mm) added and the beakers were placed on the magnetic stirrers (RO 15 Power IKAMAGŽ). Individual mussels were carefully placed into beakers using forceps and positioned away from the moving bars. At any one time, the clearance rate of 15 individual mussels was independently measured, plus three 'no mussel controls'. The mussels were left *in situ* for at least 10 minutes to acclimatise and to ensure all the mussels had opened their valves. The algal solution (500 µl) was added to all the vessels and, using a glass syringe, 20 ml of seawater-algal mixture was immediately removed from all vessels (t_0) and placed into pre-labelled plastic vials for Coulter Counter analysis. Further water samples were taken at 30 minutes (t_2) after the initial feeding. The abundance of *I. galbana* within samples was quantified using the Z2 Coulter™ Particle Size and Count Analyser (100 µm aperture tube and 0.5 ml metered volume). The clearance rate of the individual mussels was then calculated using the equation below and differences were assessed using ANOVA. Clearance Rate ($l\ hr^{-1}$) = ($v \times 60 / t$) ($\ln t_0 - \ln t_2$), where: v = volume of water in feeding rate beaker (litres), t = duration of assay (in hours), t_0 = initial cell count and t_2 = final cell count.

To assess whether ingestion of microplastic can lead to translocation of microplastic from the gut cavity to the circulatory system of mussels, 500 µl of haemolymph was withdrawn from the posterior abductor mussel and placed onto pre-prepared poly-l-lysined glass microscope slides and spread. To prevent contamination, shell water was drained from each mussel prior to haemolymph extraction, and clean syringes and needles were used for each mussel, the needle was only ever placed in once. Prepared slides of haemolymph were left in a humidity chamber for 30 minutes for haemocytes to adhere to the slide. After incubation, the slides were inverted and allowed to dry at room temperature. The slides were

fixed in methanol for 15 minutes and allowed to dry at room temperature. A glass cover slip was then mounted on the slide using clean forceps and a few drops of aqueous polyvinyl alcohol resin mounting agent (Immuno-mount, Thermo Shandon, USA) added. The abundance of microplastic particles in haemolymph was counted under fluorescence microscopy (Leica DMR with N2.1 and G/R filter sets). Results were presented as the number of microspheres per µl haemolymph^{-1} and differences were assessed using ANOVA. To confirm whether polystyrene microspheres had accumulated within haemocytes or not, 500 µl of haemolymph was placed onto poly-l-lysined glass microscope slides and incubated with fluorescein isothiocyanate in a humidity chamber for 30 minutes. After incubation, the slides were inverted and washed with phosphate buffer, then left to dry at room temperature. A cover slip was then mounted and viewed under a Zeiss LSM 510 confocal microscope.

Mussels were dissected and the mid-gut extracted using a butterfly section. This was then presevered using Baker's formal calcium at 4 °C for a minimum of 24 hours. Following fixation, the tissues were dehydrated through an ascending alcohol series, cleared in xylene, impregnated in wax and blocked-up using the Tissue-Tec system. Sections of 10 µm were cut using Brights WM3050 microtone, mounted on APTS coated slides and stained using the Papanicolaou staining technique (Bloxham et al., 2004).

4.3.3 Statistical analyses

To determine the influence of size on the translocation of microplastic from the gut to the haemolymph of mussels, formal comparisons were made using two-factor ANOVA where "time" had 5 levels (3, 6, 12, 24, 48 days) and "treatment" had 2 levels (3.0 and 9.6 µm polystyrene particles). Potential toxicological consequences resulting from ingestion/translocation of polystyrene microspheres were investigated using a similar

experimental design whereby "treatment" had 3 levels (no plastic, 3.0 and 9.6 μm polystyrene plastic). These factors were treated as fixed and orthogonal, and there were 5 replicates of each time/treatment combination. Prior to ANOVA, homogeneity of variances was examined using Cochran's tests. The variances of the microspheres in the haemolymph, the oxidative status of haemolymph (FRAP assay), viability and phagocytic activity of haemocytes were heteroscedastic. This could not be resolved by transformation and so the analysis was conducted on untransformed data, as large balanced designs are considered robust to departures from variance assumptions (Underwood, 1997). Additionally, a conservative *P*-value of 0.01 was used to test significance. Comparisons among levels of significant terms were done using post-hoc Student-Newman-Keuls (SNK) tests. Computations were done using GMAV (General Models of Analysis of Variance; EICC, University of Sydney, Australia).

4.4 Results

4.4.1 Ingestion and translocation of polystyrene microspheres in *Mytilus edulis* (L.)

For the first experiment, histological analyses showed that within 12 hours, mussels exposed to both particle size treatments had accumulated polystyrene microspheres in their gut cavity and digestive tubules (*Hypothesis 1*, Figure 15a-b). After 3 days, ingested microplastic accumulated in the circulatory fluid of mussels and both sizes of polystyrene particles were found in the haemolymph and haemocytes (Figure 15c-e). Microspheres were not found in the haemolymph of mussels from the control treatment. Statistical analysis showed effects of 'time' ($F_{4,40}$=13.33, $P < 0.01$**) and 'particle-size' ($F_{1,40}$=9.87, $P < 0.01$**). The abundance of both sizes of microplastic was greatest after day 12 and declined thereafter (*Hypothesis II*, Figure 15f), smaller 3.0 µm particles occurred in consistently greater abundances than larger particles throughout the experimental duration (*Hypothesis III*, Figure 15g) and these effects were both significant. Furthermore, both sizes of microplastic were still present in the haemolymph after 48 days. Microplastic was present within faecal pellets from the first time of examination after 3 days in clean conditions and for the remainder of the experiment.

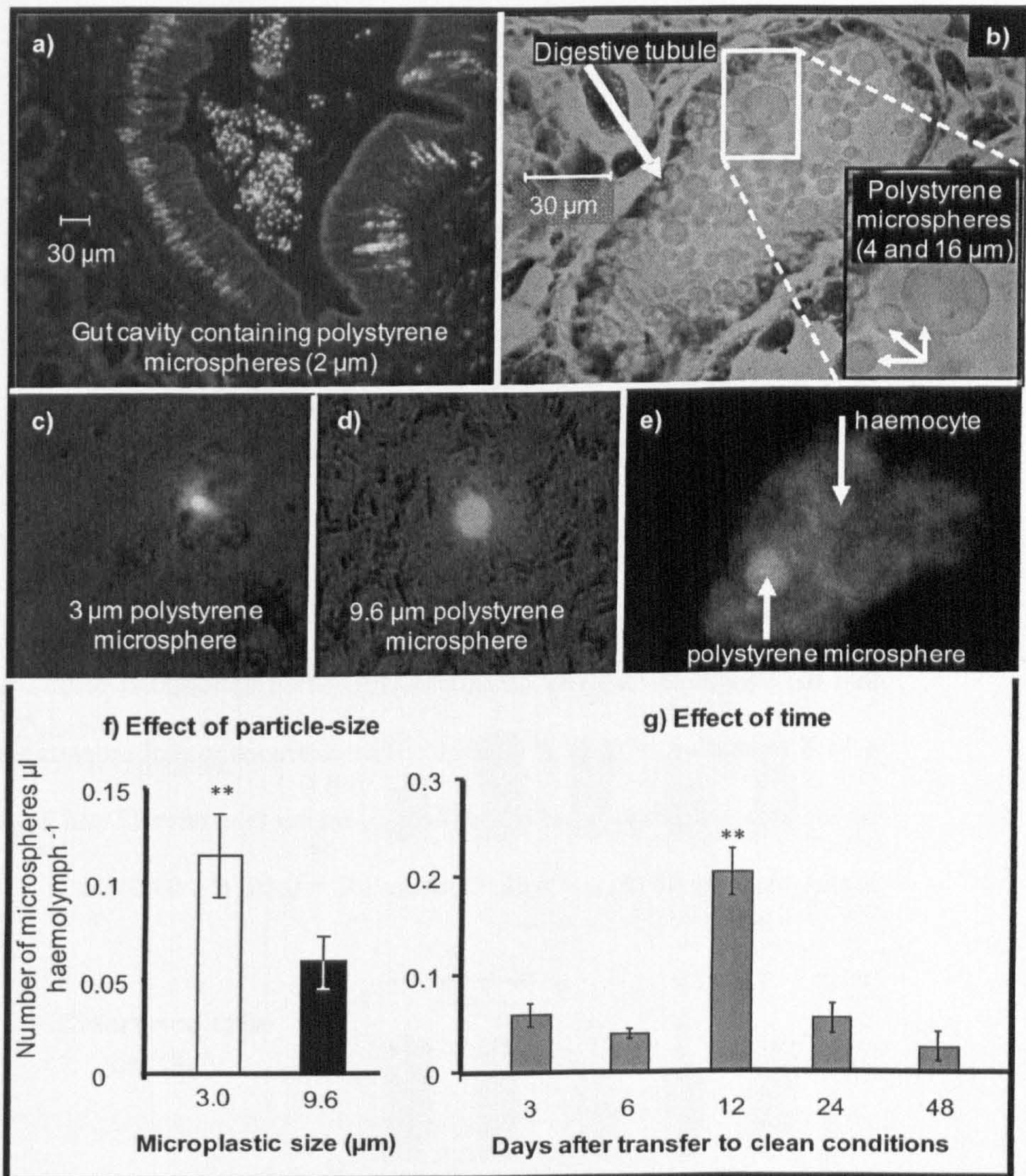


Figure 15. Uptake of plastic particle by *Mytilus edulis* (L.) (a) Tissue section (4 µm thick) containing 2 µm and (b) 4 to 16 µm polystyrene microspheres in the gut cavity and digestive tubules. 3.0 (c) and 9.6 µm (d) polystyrene microspheres in the haemolymph and (e) haemocytes. Significant differences in accumulation in the haemolymph according to (f) "particle-size" and (g) "time" at $P<0.01$. *N.B.* Values are expressed as mean ± SE and calculated from independent data from each time/treatment/replicate combination and are therefore are not cumulative.**

4.4.2 Biological effects of ingestion and translocation of polystyrene microspheres in *Mytilus edulis* (L.)

The ingestion and translocation of polystyrene microspheres by mussels did not cause any measurable changes in the oxidative status of haemolymph, the viability and phagocytic activity of haemocytes or filter-feeding activity (Figure 16). There was a main effect of 'time' in all biological assays. The oxidative status of haemolymph was over 35 % higher from mussels sampled on days 3, 12 and 48 compared to days 6 and 24 ($F_{4,60} = 15.03$, Cochran's C $= 0.34$, $P < 0.001$). The viability of mussel haemocytes was up to 64 % greater on day 6 and 12 compared to all other times ($F_{4,60} = 8.16$, Cochran's C $= 0.30$, $P < 0.001$). Comparisons of mussels sampled between days 3 and 6, and between day 24 and days 6, 12 and 48 showed that the phagocytic activity of haemocytes varied by over 50 % throughout the trial ($F_{4,60} = 6.33$, Cochran's C $= 0.25$, $P < 0.001$). The clearance rate of mussels also varied over time by up to 70 %, with days 3 and 6 significantly higher than days 12 and 24, and day 6 significantly higher than day 48 ($F_{4,60} = 6.62$, Cochran's C $= 0.16$, $P < 0.001$).

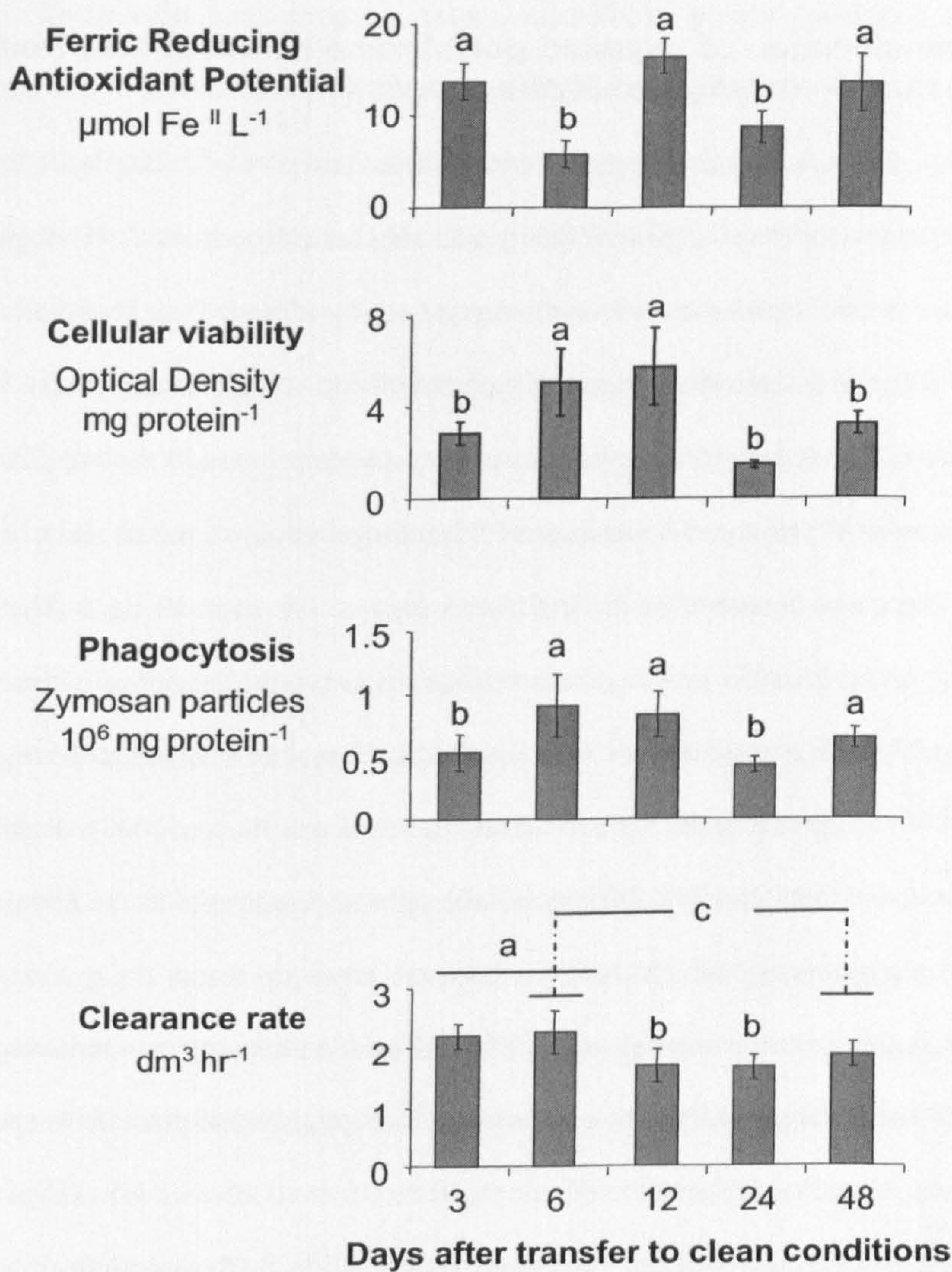


Figure 16. Temporal changes at different levels of biological organisation within *Mytilus edulis*. Values are expressed as mean ± S.E. Differences between times marked with the same letter (a,b,c) were not significantly different; and statistical significance all at $P < 0.001$.

4.5 Discussion

4.5.1 Translocation of ingested polystyrene microspheres from the gut cavity to the haemolymph of *Mytilus edulis* (L.)

Mytilus edulis ingested and transported particles of microplastic to the gut, where they accumulated in the digestive cavity and tubules (*Hypothesis I*). Though particles of plastic have been shown to accumulate in the gut cavity of birds (van Franeker, 1985), fish (Derraik, 2002) and polychaete worms (Carpenter et al., 1972), translocation from the gut to the circulatory system of an invertebrate has not been previously shown. This chapter shows that particles of polystyrene translocated from the gut cavity to the circulatory system in as little as 3 days and persisted in the circulatory system for over 48 days (*Hypothesis II*). This is important because previous research investigating the ingestion of macroplastic debris (>1 mm) by marine animals has only showed that it may be retained in the digestive tract (Derraik, 2002) or egested in the form of faeces (Eriksson and Burton, 2003). Further work is needed to determine how quickly particles of microplastic translocate to the circulatory system and the mechanism(s) by which these particles are taken up across the gut/extracellular barrier and accumulate in the haemolymph. Without detailed histological sectioning and examination of all tissues/organs using fluorescence microscopy, it is not possible to speculate as to why the peak abundance of small and large particles occurred after 12 days (*Hypothesis II*). In humans and rodents, the kinetics of particle translocation in the intestine depend on diffusion and accessibility of the plastic particles through the mucus, initial contact with the enterocyte, cell trafficking and post-translocation events (Hoet et al., 2004). It will therefore be important to characterise different cell types within the gut of mussels and investigate their ability to phagocytose and traffic particles of plastic through the epithelial lining. In particular, it is

important to investigate the possible presence of specialised enterocytes such as '*Microfold*' cells. These cells have irregular shaped microfolds, poorly developed microvilli, lectin binding, and in humas and rodents are known to transport plastic particles from the gut lumen, through the epithelium, towards follicles via phgaocyosis, from which particles can then migrate to circulatory system (Buda et al., 2005). The abundance of microspheres in the haemolymph of mussels showed the same pattern of accumulation for both sizes, it therefore seems likely that both sizes translocate by the same mechanism(s) (Figure 15g). Confocal microscopy showed that both sizes of particles of plastic were present inside haemoctytes (Figure 15e) and *in vitro* trials using the closely related species, *Mytilus galloprovincialis* showed that granulocytic haemocytes are responsible for the phagocytosis of polystyrene particles up to 800 nm in size (Cajaraville and Pal, 1995). Therefore for post-translocation events at least, phagocytosis could play a key role. The persistence of particles of microplastic in the haemolymph of *M. edulis* for over 48 days has implications for predators, including birds, crabs, starfish, predatory whelks (Petraitis, 1987, Reimer, 1999) and humans (CEFAS, 2005). For larger pieces of plastic, found in seal faeces, it has been suggested that the source of the material was from fish in their diet which have previously accumulated plastic; however it remains unknown whether plastic of any size can be transferred along food chains (Eriksson et al., 2002). An alternative source of microplastic is through the abrasion of ingested plastic in the gut cavity as a result of the digestive process. For instance, some seabirds (de Villiers and de Bruyn, 2004) and fish (Mauchline and Gordon, 1984) contain particles of sediment in their gut, which combined with muscular contractions, may abrade plastic and give rise to particles of microplastic in their gut.

4.5.2 Influence of 'particle-size' on the translocation of ingested polystyrene microspheres from the gut cavity to the haemolymph and haemocytes of *Mytilus edulis* (L.)

Particle size influenced the capacity of polystyrene to translocate from the gut cavity to the haemolymph, with over 60 % more smaller (3.0 μm) microspheres in the circulatory fluid than larger (9.6 μm) microspheres and (*Hypothesis III*). These results are in agreement with *in vitro* experiments using segments of colonic tissue from rats, which also showed rapid translocation of smaller (14 nm) compared to larger (415 and 1000 nm) polystyrene particles (Szentkuti, 1997). If phagocytosis is involved, a possible model to explain the greater number of small microplastic particles is that the smaller particles are more easily phagocytosed, possibly because the phagosomes within each cell, which are finite in space, can accommodate more smaller particles. Further research is required to test these predictions and to determine the upper and lower size boundaries for ingestion and translocation of plastic debris in other organisms. The greater accumulation of smaller particles suggests that as an item of plastic degrades, the potential for it to accumulate and translocate within the tissues of the organism increases. Other physical properties of plastic particles may also affect ingestion and translocation, medical studies using humans and rodents indicate that both the shape and charge of the particles is likely to play an important role in translocation (Donaldson et al., 2004). The composition of microplastic debris in the marine habitats varies in shape (e.g. fibres, spheres, irregular fragments), polymer type (e.g. polystyrene, polyethylene, polyester), and as an article of plastic debris degrades it will decrease in size, monomer content and its surface properties will also change. For example, medical studies with human white blood cells have shown that the styrene content of polystyrene microspheres strongly influences their ability to be phagocytised (Urakami et al., 1994). Further research is therefore needed to

investigate the factors influencing ingestion and translocation of microplastic in a wider range of organisms.

4.5.4 Toxicological consequences of ingestion and translocation of polystyrene microspheres in *Mytilus edulis* (L.)

The ingestion and translocation of polystyrene microspheres by mussels did not cause any measurable changes in the oxidative status of haemolymph, the viability and phagocytic activity of haemocytes or filter feeding activity (*Hypothesis IV*, Figure 16). It is, however, premature to suggest that ingestion and or translocation of plastic does not cause toxicological effects. Our laboratory trial exposed mussels for 3 hours to 15,000 particles of one type of plastic (polystyrene) and possible biological effects were monitored for only 48 days. In their natural environment mussels will be exposed over their life-time to various types of microplastic, including polyester, polyethylene, polypropylene, polyvinvylchloride and acrylic. Medical studies using mice injected intraperitoneally with microplastic indicate that phagocytic uptake of microparticles of polyester (polylactic acid) can lead to cellular damage, through changes in the morphology and viability of phagocytes (Lam et al., 1993). Consequently, research is needed to examine the toxicological consequences of longer term exposure to various plastic types routinely found in marine habitats. Particular attention is needed to assess whether microplastic can damage vital organs such as the heart and hepatopancreas. Given the open circulatory system of molluscs, haemocytes circulate throughout the animal and the connective tissue of major organs, and so microplastic could be transported to these important organs. Laboratory trials with hamsters injected with 60 nm particles of polystyrene have shown in the blood stream that these particles can induce thrombosis (Nemmar et al., 2003). The presence therefore of particles of microplastic in the circulatory system may restrict blood flow causing damage to the vascular tissues and changes

in cardiac activity. In addition, fragments of plastic found in marine habitats in Japan, Mexico and North America have shown high concentrations of polychlorinated biphenyls (PBCs), dichlorodiphenyl trichloroethane (DDT), nonylphenol polyaromatic and aliphatic hydrocarbons (Mato et al., 2001, Carpenter et al., 1972, Rios et al., 2007). Recent laboratory evidence has shown that the that sorption of phenanthrene onto microscopic particles of polyethylene, polypropylene and polyvinvylchloride was up to an order of magnitude higher than that to particles of natural sediment (Teuten et al., 2007). Although this chapter does not provide evidence of uptake of these chemicals, it does show that ingested particles of microplastic can persist in the haemolymph of mussels for over 48 days and therefore could provide a route for the transport of chemicals to various tissues.

The effect of 'time' observed which in all of the biological assays (Figure 16) used this chapter is consistent with previous field studies for mussels (Bloxham et al., 2004) and limpets (Cartwright et al., 2006) which have shown temporal variability on a scale of weeks to months in the viability and phagocytic activity of haemocytes and oxidative status of haemolymph. These small-scale temporal changes in the health of organisms kept under controlled laboratory conditions, support the views of Underwood (1995) who questions the ability of laboratory conditions to minimise temporal variability in the biological responses of aquatic invertebrates in toxicity trials. Hence the possibility of using field exposure experiments to gain more ecologically robust data needs to be more widely explored in ecotoxicology (Cartwright et al., 2006, Lindegarth and Underwood, 2002).

4.5.5 Implications for marine habitats

The data presented in this chapter and that of other medical studies with rodents and humans, indicates that fragments of microplastic may translocate from the gut cavity to the blood stream in a wide range of organisms. To establish the extent to which this occurs in natural populations, and to identify any adverse effects, it will be necessary to further refine techniques to quantify small plastic fragments. For example, current methods to identify plastic debris using Fourier Transform Infra Red spectroscopy only permit particles of plastic down to approximately 20 μm in size to be conclusively identified (Thompson et al., 2004), but it is entirely feasible that plastic particles are now present in the environment at the much smaller nanometre scale.

Chapter 5. Bioavailability and toxicity of chemicals associated with microplastic to the lugworm, *Arenicola marina* (L.)

5.1 Abstract

It has been suggested that plastic could act as a transport medium for toxic chemicals within the marine environment. There are two mechanisms by which chemicals might be transferred from plastic, (1) through adsorption of contaminants that are already present in the environment from other sources and (2), by release of chemicals that are incorporated in plastic during manufacture. In particular, microplastics are a possible route of these chemicals into marine food-chains due to their increased abundance over the last 40 years and their substantial surface area for sorption and desorption. Therefore, laboratory trials were conducted to assess the likelihood of microplastic particles absorbing and releasing toxic substances to marine organisms.

The sorption-affinity of candidate environmental contaminants (nonylphenol, phenanthrene and tributyltin) from aqueous solution onto microscopic particles of polyvinylchloride and similar sized particles of sand was compared. Chemical analysis confirmed that polyvinylchloride sorbed significantly more hydrophobic contaminants than sand particles. Laboratory trials using *A. marina* were then used to assess the bioavailability and toxicity of chemicals released from plastics. To establish the biological consequences of compounds that are incorporated into plastic during manufacture, the bioavailability of the flame retardant, tetrabrominated diphenyl ether (TBDE) and the antimicrobial, triclosan from microplastic were examined.

Tissue analyses confirmed the bioaccumulation of phenanthrene, nonylphenol, triclosan and TBDE into the skin and gut of *A. marina* from microscopic polyvinylchloride. The results of the toxicity trials indicated that chemicals released from the microplastic particles could

lower immune function (66 %), increase mortality (61 %) and that the plastic itself can reduce the antioxidant status (67 %) of the coelomic fluid of *A. marina.*

On the basis of previous work and the evidence from these laboratory trials, the wider environmental implications of the toxicological consequences of plastics in the environment are discussed. In particular the potential for microscopic plastic particles to act as a transport mechanism of toxic hydrophobic compounds from the environment and chemical additives used in the manufacture of plastic to transfer into the body tissues of other marine organisms, and the consequences for food chains.

5.2 Introduction

There is increasing concern that plastic debris may pose a toxicological challenge to wildlife. However, there is a major gap in our understanding as to whether chemicals can be transferred from plastics into body tissues if animals ingest plastics. There are two mechanisms by which toxic chemicals can be associated with plastic debris: (1) chemicals incorporated as additives during the manufacture of plastics and (2) adsorption of contaminants from the environment onto plastic debris (Mato et al., 2001).

Work in Japan has shown that that polypropylene pellets can adsorb and concentrate polychlorinated biphenyls (PBCs), dichlorodiphenyl trichloroethane (DDT) and nonylphenol from the marine environment at concentrations up to 10^6 higher than those of the ambient seawater (Mato et al., 2001). Furthermore, pellets and fragments of polypropylene and polyethylene floating in the North Pacific Sea and stranded on beaches of North America and Mexico have been shown to contain detectable levels of PCB, DDT, aliphatic and polyaromatic hydrocarbons (Rios et al., 2007). Over the last 30 years there has been continued speculation that plastic may function as a transport medium for toxic chemicals to

biota (Thompson et al., 2004, Rios et al., 2007, Mato et al., 2001), including seabirds (Day, 1980, Bourne and Imber, 1982, Pettit et al., 1981, van Franeker, 1985). Early work has shown that the mass of plastic ingested by Great Shearwaters (*Puffinus gravis)* was positively correlated with the concentration of PCBs in their fat tissue (Ryan et al., 1988). There has however been hitherto no experimental evidence for plastic to function as a transport medium for toxic chemicals to biota.

In addition to absorbed contaminants, a range of potentially toxic chemicals are used in manufacture of plastics. These include catalysts (organotin), antioxidants (nonylphenol), flame retardants (polybrominated diphenyl ethers) and antimicrobials such as triclosan. There has been no previous work evaluating the release of these chemicals in the natural environment or *in-vivo.* However, laboratory trials have shown that plastic can release additives to human serum (Wams, 1987, Brevik, 1976, Rettenmeier and Mettang, 1997, Mettang et al., 1997), food (Inoue et al., 2001), liquid beverages (Song et al., 2003, Jetten and de Kruijf, 2002) and other plastics that are in contact (Marcilla et al., 2004).

5.2.1 Chemicals used as additives in plastic manufacture and their toxicity

During the manufacture of plastics, nonylphenol is used to as an antioxidant to prevent the polymer reacting with oxygen during thermal processing and when exposed to ultra-violet light (Loyo-Rosales et al., 2004). Brominated flame retardants are added to plastic materials to prevent ignition or retard the spread of flames so that plastic products can comply with fire safety regulations. Globally, flame retardants are the second largest additive used by the plastics industry (Tullo, 2003) and in 1999 global consumption was estimated at 204,000 tonnes, with polybrominated diphenyl ethers (PBDEs) accounting for a third of this (Darnerud, 2003). PBDE additives can make up between 5 to 30 % of plastic products (Darnerud et al.,

2001) and, like many additives, are not chemically bound to the plastic. Consequently, they are susceptible to transfer from the plastic to the environment. It has been estimated that between 1997 and 2004 up to 1.2 million tonnes of PBDEs were released into the environment from plastic electronic waste (Martin et al., 2004). Work on leaching of PBDEs from plastic in landfill sites suggests that dissolved humic and organic matter may enhance the transfer of PBDEs from plastic items (Osako et al., 2004). The persistence of these chemicals is enhanced by their high chemical stability and high liposolubility, which bears strong resemblance to persistent organic contaminants like PCBs and DDT (Darnerud et al., 2001). PBDEs can biomagnify within food chains, with tissue concentrations up to 1.6 ng g^{-1} in the commercially important salmon, *Salmo salar* (Burreau et al., 2006). Marine animals have all been shown to accumulate plastic of varying sizes (Browne et al., 2007, Derraik, 2002) and ingestion of plastic may serve as a potential source of PBDE to marine animals.

Chemical additives are also incorporated into plastics to enhance performance. For example, phthalates allow the polymer chains within plastics to slide against one another. However, these compounds can also act as an additional source of carbon and promote the growth of micro-organisms which limit the useful lifespan of plastic, through staining, discolouring, odour, loss of aesthetics, and loss of mechanical and electrical insulating properties. To combat this, antimicrobial pesticides are incorporated into plastic. Antimicrobials are also used to reduce the risk of infections associated with medical goods, for example dental trays, surgical drapes and wound dressings (*www.microban.com*). Out of the 5000 or more products that are registered as antimicrobials with the EPA, one of the most common is triclosan, 2,4,4'-trichloro-2'-hydroxydiphenyl ether. This has been added to non-plastic items including antibacterial soaps, deodorant, skin cream and toothpaste for over 30 years (Sabaliunas et al., 2003). In 1998, human intake through toothpaste alone was estimated

to be around 2 tonnes in Sweden (Tan et al., 2002). However, triclosan is increasingly being used as a plastic additive in medical items, kitchen-ware (e.g. chopping boards), carpets (Iconomopoulou et al., 2005), sports clothing and footwear (www.microban.com). Products containing triclosan are also available with microscopic plastic particles as 'scrubbers' (toilet cleaner, shower gel, toothpaste and facial wash) and recent research has developed polystyrene-divinyl benzene beads that offer controlled release of triclosan (Iconomopoulou et al., 2005). Therefore, it is entirely possible that microscopic plastic with triclosan could enter the marine environment through the degradation of larger products or through release of cosmetic and cleansing products. The principle mechanism of toxicity of triclosan to microbes is through inhibition of the enzyme, enoyl-reductase, which is responsible for microbial fatty acid synthesis (McMurry et al., 1998). However, our knowledge of the toxicity of triclosan to non-target taxa is limited to the crustacean, *Daphnia magna* (Orvos et al., 2002), the fish *Oryzias latipes* (Ishibashi et al., 2004) and amphibians, *Bufo americanus* (Smith and Burgett, 2005), *Rana pipiens* (Fraker and Smith, 2004) and *Xenopus laevis* (Fraker and Smith, 2005). Only one study has investigated the toxicological challenge to a marine organism. Here, Canesi et al, (2007b) injected 50 μl of triclosan at three concentrations (0.29, 2.9 and 29 ng g^{-1}) into the posterior adductor muscle of the mussel, *Mytilus galloprovinciallis* and after 24 hours found changes in the levels of digestive enzymes in particular, decreases in catalase and increases in glycolytic enzyme activity. Given that triclosan can accumulate in natural marine sediments at concentrations up to 0.13 μg g^{-1} (Aguera et al., 2003), it is vital to the evaluate the toxicological consequences for marine animals ingesting plastic debris that contain triclosan and to determine how the transport of triclosan after ingestion varies between plastic and natural sediment.

5.2.2 Potential for hydrophobic contaminants to adsorb to plastic debris and their subsequent toxicity

Organotin, nonylphenol and phenanthrene are toxic hydrophobic contaminants that have the potential to sorb onto plastic debris. Organotins are used as biocides in anti-foulant paints for boats (Voulvoulis et al., 2002) and this has resulted in concentrations up to 1.24 μg g m^{-2} in natural marine sediments and 1.4 $\mu g\ l^{-1}$ in the water column (Page et al., 1996). However, the potential for plastic to adsorb the organotin, tributlytin from the water column and transport it into the body tissues of animals upon ingestion remains unexamined. Organotins suppress the immune system in a concentration-dependent manner in fish cells (O'Halloran et al., 1998), rats (Funahashi et al., 1980) and marine invertebrates (Matozzo et al., 2004, Cima et al., 2002, Cima and Ballarin, 2000, Cima et al., 1999, Cima and Ballarin, 1999, Cima et al., 1998d, Cima et al., 1998c, Cima et al., 1998b, Cima et al., 1998a, Cima et al., 1995). For marine molluscs, bioaccumulation of TBT from the water column, at aqueous concentrations less than 0.5 $\mu g\ l^{-1}$ has been shown to damage the DNA of haemocytes by causing strand breaks, micronuclei formation (Hagger et al., 2005) and growth inhibition in *Mytlius edulis* (Salazar and Salazar, 1991) and development of imposex in *Nucella lapillus* (Gibbs et al., 1997). Exposure of TBT to infaunal gammaridean amphipods has been shown to inhibit burrowing behaviour and cause mortality (Meador et al., 1997). Sedimentary concentrations of TBT less than 1 ng g^{-1} range have been shown to inhibit the growth of juvenile polychaete worms, *Armandia brevis* (Meador and Rice, 2001).

Nonyphenol is used in a wide range of applications. Here parent compounds of nonylphenol known as Alkylphenol polyethoxylates (APEs) are employed to process raw materials such as textiles, paper, coal, metal and leather. These processes draw impurities from these materials into aqueous solution which is then disposed of via waste water effluents.

In Canada, the annual consumption of APEs is estimated at 23,800 tonnes (Berryman et al., 2004), whilst in the UK it is around 15,000 – 19,000 tonnes (Blackburn et al., 1999). Consequently, the chemical breakdown of APEs to nonylphenol predisposes water treatment plants to concentrate nonylphenol and in doing so these plants can act as sources of this contamination in aquatic ecosystems. Coastal sediments appear to act as transitory sinks for nonylphenol. In the UK concentrations of nonylphenol in marine sediments have been recorded ranging from 1,000 to 15,000 $\mu g\ kg^{-1}$ (Blackburn et al., 1999), whilst in North America and Japan similar values (up to 13,700 $\mu g\ kg^{-1}$) have been recorded (Ferguson et al., 2001, Isobe et al., 2001). In Japan, plastic pellets < 5 mm in diameter collected from the shore have been shown to contain concentrations of nonylphenol up to 16 $\mu g\ g^{-1}$ (Mato et al., 2001). Hence, if plastics release nonylphenol to biota, then they could provide a transport mechanism facilitating the transfer of nonylphenol to the food chain. Laboratory trials have shown that exposure to nonylphenol can cause developmental abnormalities in the barnacle, *Elminius modestus* (Billinghurst et al., 2001), oxidative stress and reduced burrowing behaviour in the mollusc, *Tapes philippinarum* (Matozzo et al., 2004), and endocrine disruption in wild populations of freshwater fish (Beresford et al., 2004, Jobling and Tyler, 2003, Tyler et al., 1998).

Polycyclic aromatic hydrocarbons (PAHs) are major components of crude oil and are derivatives of fossil fuel combustion. They are not used as additives in plastic production; however, the widespread application of PAHs has resulted in broad-scale contamination of marine habitats with sources ranging from, roadside run-off from combusted fuel from automobiles, to industrial discharges leading atmospheric deposition, and accidental (Boehm et al., 1998) and deliberate oil spills (Readman et al., 1996, Readman et al., 1992). The low solubility of PAHs facilitates their persistence in sediments (Woodhead et al., 1999) and the

sea-surface micro layer (Wurl and Obbard, 2004). Therefore, plastic debris present within the sediments or sea-surface microlayer could accumulate and concentrate PAHs in a similar way to that described for other hydrophobic contaminant. Recent work has shown detectable levels of PAHs, including phenanthrene on polypropylene and polytheylene debris collected from the Pacific ocean and intertidal habitats in the US and Mexico (Rios et al., 2007). Another route by which PAHs could absorb onto plastic particles is from plastic media blasting. This process is used in dockyards around the world to remove PAHs and other compounds from metallic surfaces using acrylic, melamine, polyester and urea plastic particles (0.25 - 1.7 mm) (Wolbach and McDonald, 1987, Gregory, 1996, Abbott, 1992, Anonymous, 1998). However, the likelihood of plastic media blasting acting as a source of plastic particles with sorbed contaminants entering the marine environment has yet to be investigated.

PAHs are known to bioaccumulate in marine organisms (Law et al., 1999) and laboratory exposure of the polychaetes, *Cirriformia grandis*, *Clymenella torquata* and the mollusc *Macoma balthica* to sediments containing PAH-rich rubber demonstrated that PAHs associated with rubber are biologically available to infauna (Rust et al., 2004). Ingestion of sediment particles with phenanthrene has been reported to reduce egestion rates, burrowing behaviour and reproduction and increases mortality in the oligochaete, *Limnodrilus hoffmeisteri* (Lotufo and Fleeger, 1996). Exposure of PAHs to marine bivalve molluscs has been shown to reduce phagocytosis and impair cell functioning in the circulating haemocytes (Wootton et al., 2003, Grundy et al., 1996b, Grundy et al., 1996a). Given that these toxic compounds are present in the environment together with plastic particles, it is vital to determine if these compounds can adsorb onto plastic and if so whether the PAHs are able transfer into animal tissues once plastic is ingested

5.2.3 Environmental relevance of microscopic polyvinylchloride as a contaminant in marine habitats

In 2004, 7 million tonnes of polyvinylchloride was produced in Europe to make pipes for delivery of drinking water and as computer housings, footwear, flooring and phone cards (EVC, 2000, Plastics-Europe, 2004). Chapter 2 showed that, in terms of abundance, smaller plastic debris, less than 1mm, can represent over 85% of the debris found in soft-sediment estuarine habitats and over a quarter of the material described was PVC fragments, 20 μm to 1 mm in size. Laboratory trials have shown that the lugworm, *Arenicola marina* can ingest microscopic polyvinylchloride particles up to 230 μm in size (Thompson et al., 2004). However, the bioavailability of chemicals from ingested plastic, including polyvinylchloride remains unknown. Given that larger (1 to 150 mm) pellets and fragments of polypropylene, polyethylene and acrylonitrile butadiene styrene plastic have been shown to concentrate persistent organic pollutants in marine habitats in Japan, USA and Mexico (Mato et al., 2001, Rios et al., 2007), it is important to establish whether hydrophobic contaminants and chemical additives from the microscopic polyvinylchloride can transfer into the tissues of marine organisms capable of ingesting them. This is examined in this chapter using the polychaete worm, *Arenicola marina* as a test organism.

5.2.4 Ecotoxicological relevance of *Arenicola marina* (L.) as a test organism for the uptake of chemicals from plastic

Benthic invertebrates are important components of marine food webs that support higher vertebrates, including humans. The lugworm, *A. marina* is widely distributed across European and North American marine habitats (Thain and Bifield, 2001). Ecologically this organism is an important member of marine ecosystems and can account for 20 % of the total macrobenthos in tidal soft sediment habitats (Beukema, 1976), and can influence the

abundance of other macrobenthic species by ingesting and reworking large quantities of sediment (Philippart, 1994, Riisgard and Banta, 1998, Flach and Beukema, 1994). Average faecal production of *A. marina* has been reported at 29.6 ml sediment day^{-1} individual^{-1} with mean time between defecations ranging from 36 - 40 minutes (Wells, 1950). It has been estimated that a density of 30 lugworms m^{-2} can rework and ingest a 15 cm layer of sediment each year (Riisgard and Banta, 1998). This bioturbation modifies the physical and chemical nature of sediments (Kure and Forbes, 1997), influencing the bioavailability of particulate-bound contaminants by removal, uptake, enhanced microbial degradation, graded bedding within sediments (Kure and Forbes, 1997), or by resuspension to surface waters. The lugworm, *A. marina* is known to accumulate a diverse range of contaminants, including PAHs (Timmermann and Andersen, 2003, Kaag et al., 1998), metals (Chen and Mayer, 1999, Lawrence et al., 1999, Rasmussen et al., 1998), PCBs (Kaag et al., 1998) and pesticides (Thain et al., 1997) and is known to ingest plastic particles (Thompson et al., 2004). Therefore *A. marina* is highly suitable as a candidate for investigating the bioavailability of contaminants associated with ingested material (Chen and Mayer, 1999, Christensen et al., 2002, Voparil et al., 2003).

The lugworm *A. marina* is readily utilised in toxicity tests at national (Environment Agency) and international levels (OSPAR) and it is relatively easy to maintain in the laboratory. Survival and feeding behaviour (number and mass of casts) are conventional measures of toxicity and these have been well described (Bat and Raffaelli, 1998, Thain and Bifield, 2001, Thain et al., 1997) for a broad range of toxicants. In addition to conventional toxicity measures, a range of biochemical and cellular levels assays have also been shown to be valuable in studying sub-lethal responses to contaminants in other invertebrates (Bloxham et al., 2004, Galloway et al., 2004). Phenanthrene, nonylphenol and tributyltin are known

immunosuppressants of marine organisms and the phagocytic activity of coelomocytes has provided an amenable method to determine effects on the nematode immune system (Galloway and Goven, 2005). Exposure of zinc, lead and cadmium to the terrestrial annelid, *Dendrobaena venetato* has been shown to reduce coelomocyte numbers and increase the abundance of bacteria leading to mortality (Wieczorek-Olchawa et al., 2003). Therefore, ingesting plastic debris containing biologically available chemicals may compromise the immune system and increase the risk of infections from marine pathogens. There has been evidence that exposure to plastic metabolites (Turner et al., 2005, Gamer et al., 2004) and ultrafine particles (e.g. polyvinylchloride, titanium dioxide and carbon) could induce oxidative stress in rats, mice (Oberdorster et al., 2005) and fish (*Micropterus salmoides*) (Oberdorster, 2004). However problems in experimental design (e.g. lack of procedural controls, 'pseudo-replication') have limited the conclusions that can be reached from these studies. In *A. marina*, antioxidant status is an important physiological defence mechanism that deals with seasonal variations in oxidative stress (Keller et al., 2004, Buchner et al., 1996) and any reduction in antioxidant status may compromise the survivorship potential of *A. marina*. However, laboratory experiments using the mussel, *M. edulis*, presented in Chapter 4 failed to show changes in the oxidative status as a result of ingesting polystyrene microspheres (3.0 and 9.6 μm). Therefore, it is important to test this hypothesis in relation to *A. marina* which lives and ingests the sediments where microscopic PVC debris is known to accumulate.

5.2.5 Hypotheses tested

To assess the possibility and biological consequences of microplastic particles adsorbing toxic contaminants from the environment and releasing these and/or plastic additives to marine food chains, laboratory trials were conducted to test the following hypotheses:

i. When placed in separate aqueous solutions of persistent organic contaminants (phenanthrene, nonylphenol and tributyltin), particles of microscopic PVC will adsorb significantly higher concentrations of contaminants than similar-sized sand particles.

ii. Ingestion of sediments containing microscopic PVC with sorbed persistent organic contaminants (nonylphenol and phenanthrene) and chemical additives (TBDE and triclosan) will lead to bioconcentration of these chemicals in the tissues of *A. marina.*

iii. Ingestion of sediments containing sand+sorbed-hydrophobic-contaminants (nonyphenol and phenanthrene), will lead to significantly lower tissue burdens than similar treatments containing clean sand together with PVC + sorbed contaminant.

iv. Uptake of contaminants and chemical additives will lead to increased mortality and also significantly reduce the health of *A. marina*, in terms of casting rate, burrowing behaviour and immune function.

v. Ingestion of sediments containing PVC without contaminants or additive chemicals will significantly reduce the capacity of *A. marina* to deal with oxidative stress compared to animals from sediments with no PVC.

5.3 Materials and methods

Initially, a sorption trial examined the sorption-affinity of candidate hydrophobic contaminants (phenanthrene, nonylphenol and tributyltin) from aqueous solution onto microplastic and sand particles using chemical analyses. Then, a first laboratory trial examined the bioavailability and toxicological consequences for *A. marina* of ingesting microplastic and sand particles contaminated with sorbed persistent organic pollutants. A second trial examined the bioavailability and toxicological consequences of ingesting microplastic particles containing plastic additives (triclosan and TBDE).

5.3.1 Adsorption of persistent organic pollutants onto microplastic and sand particles

To examine the capacity of polyvinylchloride to adsorb hydrophobic contaminants from the environment, laboratory experiments exposed microscopic PVC particles (mean particle size 230 μm, Goodfellow Cambridge Ltd, UK) and similar sized (certified clean) sand particles (Figure 19) (mean grain size 260 μm, Fisher Scientific, UK) to aqueous solutions of phenanthrene, nonylphenol and tributyltin. For each treatment, 375 g of sand or polyvinylchloride was spiked with either 7.524 mg phenanthrene, 0.3415 mg tributyltin or 7.429 mg nonylphenol, using absolute ethanol (Fisher Scientific, UK) as the solvent-carrier to minimise between-sample variability. The ethanol was allowed to evaporate in a fume cupboard at room temperature until constant mass was obtained. When complete, the toxified polyvinylchloride or sand was washed three times in Milli-Q-purified water in order to remove contaminants not bound to the particulates. Chemical analyses tracked the adsorption and concentration of these contaminants onto the two different types of particles. The adsorption and concentration of phenanthrene, nonylphenol and tributyltin onto the separate treatments of

sand and polyvinylchloride from aqueous media was compared using a one-factor ANOVA, where "sediment" had two levels (sand and PVC).

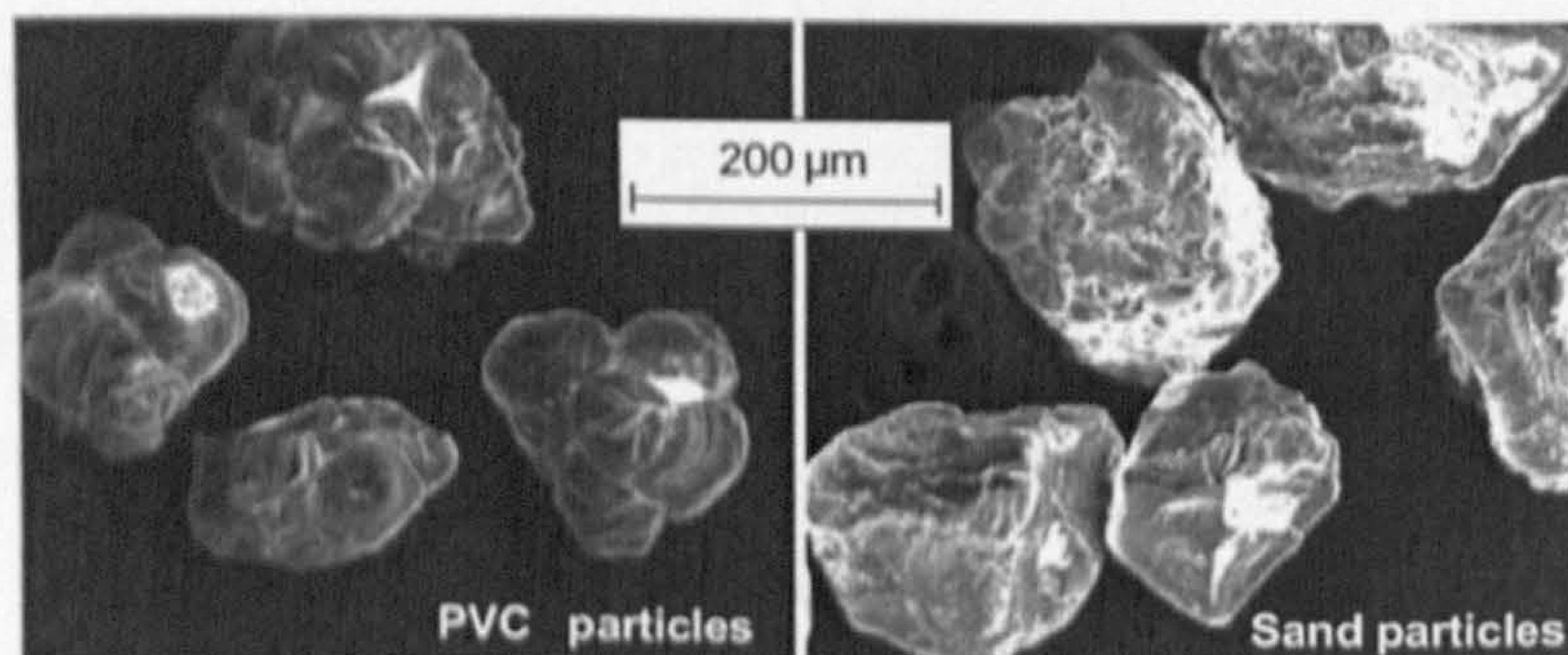


Figure 17. Scanning electron micrograph of polyvinylchloride and sand particles used for the study.

5.3.2 Incorporation of additives into microplastic

Additives (triclosan and TBDE) used in plastic manufacture were adsorbed onto microscopic polyvinylchloride using a laboratory method that closely mirrors how many additives are applied during plastic production (Martin et al., 2004). Microscopic polyvinylchloride particles were toxified using working stock solutions of triclosan and TBDE. Working solutions were prepared by adding a measured volume of each stock solution (411.7 mg triclosan and 60.5 mg TBDE) into absolute ethanol (Fisher Scientific, UK) to act as a solvent-carrier, 375 g of polyvinylchloride was added to the additive solution to form a slurry. To ensure near total evaporation, the ethanol was allowed to evaporate in a fume cupboard at room temperature until constant mass was obtained. When complete, the polyvinylchloride was washed three times in Milli-Q-purified water to remove residual

additives not bound to the particulate PVC. Chemical analyses confirmed the substantial sorption of additives to plastic with 98 and 100 % retention for TBDE and triclosan, respectively. The levels of additives added to plastic were realistic to levels of TBDE (5 to 30 %) (Martin et al., 2004) and triclosan (up to 5 %) used as an antimicrobial (Braid and Wale, 2002) by the plastics industry.

5.3.3 Exposure of *Arenicola marina* (L.) to toxified sediments

Two exposure trials examined the bioavailability and toxicity of chemicals from microscopic polyvinylchloride particles. The first trial investigated the potential for microscopic plastic to act as transport-mechanism for phenanthrene and nonylphenol to transfer from the environment to *A. marina* and the second examined the transfer of the plastic additives TBDE and triclosan from microscopic polyvinylchloride to the tissues of *A. marina.*

In each trial the ethanol/contaminant solutions were shaken with 375 g polyvinylchloride and the ethanol evaporated to constant mass as descried previously. Previous work had shown that residues of ethanol did not have any toxic effect upon *A. marina*. This produced contaminant of nonylphenol and phenanthrene at concentrations similar to those on plastic debris or natural sediments (Mato et al., 2001, Rios et al., 2007). Contaminated PVC particles were mixed with commercially available and certified clean sand (mean size 260 μm). The abundance of plastic debris varies among habitats and densities, ranging from 366 (Chapter 2) to 100,000 items m^{-2} (Gregory, 1978). A ratio of 19:1 (sand:contaminated PVC, w/w) was chosen for these experiments.

For the first trial involving phenanthrene and nonylphenol, there were the following treatments: sand only (sand-control), sand + PVC only (PVC-control), sand + contaminant, sand + PVC + contaminant, and there were five replicates of each treatment. The second trial,

involving the additives TBDE and triclosan did not contain a sand-control treatment as this trial examined the potential for additives used in the manufacture to transfer from plastics and was not relevant to sediment. This trial contained treatments consisting of sand + PVC (PVC-control), sand + PVC + contaminant and there were six replicates of each treatment.

For both experiments each replicate was prepared in acid washed 2 L Pyrex© beakers by adding 1500 g of the appropriate sediment mixture. To maintain the animals throughout the exposure, 750 µl of *Isochrysis galbana* (Class: *Prymnesiophyceae*) food supplement (Reed Mariculture, USA) was mixed into the sand and 500 ml of clean filtered seawater to form a homogenous slurry. A further 1000 ml of seawater was added over a clean stainless steel spoon to avoid disturbing the sediment mixture. For each trial, treatment order was randomised using a replicated block design to remove bias associated with possible environmental gradients in a controlled temperature room. Tanks were then covered with pre-cleaned (acetone/dichloromethane) ceramic tiles and aerated, via a glass pipette inserted through a hole in the centre of each tile. Treatments were kept in a temperature controlled room at 15 °C under 12 hour light/dark cycle for 10 days. For each replicate, three *A. marina* were randomly chosen, their mass was recorded and they were carefully added to each beaker. For the first exposure trial involving phenanthrene and nonylphenol, the mass of individual worms was 2.9 ± 0.4 g which were obtained from Seabait Ltd, Ashington, Northumberland, UK. For the second exposure trial involving TBDE and triclosan, the size cohort of *A. marina* was 4.6 ± 0.8 g and were obtained from the Fowey estuary, Cornwall, UK. Background body burdens of chemicals in skin and guts samples were analysed prior to commencement. Salinity was maintained, via addition of Mili-Q-purified water, to a pre-marked level. Dissolved oxygen, pH and salinity were monitored daily using a portable meter. Casts were counted and collected each day, freeze-dried and mass determined to quantify the sediment through-put.

After 10 days the worms were transferred from the glass exposure beakers to clean glass beakers containing clean seawater, so the animals could exude residual sediment from their guts. On day 11, the animals were carefully removed. Coelomic fluid was sampled using a syringe the needle (21G) of which was inserted into the worm and the coelomic fluid drawn out, this was then transferred into a siliconised centrifuge tube to prevent adherence of coelomocytes. Where possible a series of biological assays quantified mortality, total cast number and mass, burrowing rate, phagocytosis and FRAP using established techniques as described below. Tissue and gut samples were then taken using a clean scalpel and the tissues stored in clean glass vials which were frozen at - 80 °C for chemical analyses of contaminant load.

5.3.4 Biological assays

The number of casts produced and any mortality was recorded daily. At the end of exposure trial one, which investigated the bioavailability and toxicity of nonylphenol and phenanthrene, individuals were placed in triplicate onto clean sediment and the time taken for worms to completely bury was recorded. Due to time and logistical constraints this was not possible for the second trial involving triclosan and TBDE.

The phagocytosis assay was performed using a modification of previous methods (Pipe et al., 1999, Coles et al., 1995). This assay measures the ability of coelomocytes to phagocytise zymosan particles to give an indication of immune function. Haemolymph containing coelomocytes (10 μl) were transferred in triplicate into a microtitreplate and agitated using a plate shaker (1400 rpm for 60 seconds). The plate was covered with a plate-sealer and incubated at 10 °C for 50 minutes. Aliquots of 50 μl of neutral-red-stained and heat stabilised zymosan suspension (containing 1×10^5 particles ml^{-1} in phosphate buffer) were

added to each well and the plate incubated for 2.5 hours at 10 °C. The cells were washed to remove residual haemocytes using 100 µl phosphate buffer (pH 7.4) and a series of zymosan standards were added. The dye was resolublised via addition of 100 µl of acetic acid in 50% ethanol. The microtitreplate was covered with a plate-sealer and incubated for 10 minutes at 20 °C, and then read at 550 nm. 190 µl was removed from each well and series of protein standards (0, 0.2, 0.6, 1.0, 1.4, 2 g l^{-1}) added. BSA protein reagent (200 µl) was added to each well and left for 20 - 30 minutes. Protein assays were used to determine the number of zymosan particles phagoctyosed per g^{-1} coelomocyte protein (Bloxham et al., 2004).

The ferric reducing antioxidant potential (FRAP) assay was chosen within this study as it offers an inexpensive tool for measuring antioxidant status and has been used in marine corals (Griffin and Bhagooli, 2004), molluscs (Hagger et al., 2005) and crustaceans (Bloxham et al., 2004). The FRAP assay was carried out as described by Benzie and Strain (1996). In this study, the assay determines the antioxidant capacity of coelomic fluid to cope with oxidative stress, e.g. reactive oxygen species that can reduce survivorship potential. Coelomic fluid (10 µl) was pipetted in triplicate in microtitreplate wells. Aqueous solutions of known Fe^{II} concentrations in the range of 0 - 600 µmol l^{-1}, were used for calibration. 200 µl of reagent (300 mM acetate buffer, TBTZ (2,4,6-tripyridyl-s-triazine) was placed into each well. The plate was incubated at 25 °C for 10 min and read at 593 nm.

The phagocytosis and FRAP assay were performed for treatments involving nonylphenol, phenanthrene and TBDE, however due to high mortality in the triclosan treatments it was not possible to conduct these assays. For the exposure involving phenanthrene and nonylphenol, formal comparisons of toxicity were made using two-factor ANOVA, where "toxicant" had two levels (present and absent) and "sediment" had two levels (sand and PVC). These were treated as fixed orthogonal factors. Formal comparisons of

toxicity for TBDE and Triclosan were made using one-factor ANOVA. For both triclosan and TBDE here "toxicant" had two levels (present or absent). All biological and chemical data were transformed to achieve homogeneity of variance, however in the comparison of FRAP data, the variances were heteroscedastic. This could not be resolved by transformation and so the analysis was conducted on untransformed data, as large balanced designs (in this case n=15) are considered robust to departures from variance assumptions (Underwood, 1997). Additionally, a more conservative *P*-value of 0.01 was used to test significance. Statistical analysis was carried out using GMAV (General Models of Analysis of Variance; EICC, University of Sydney, Australia). *Post-hoc* analysis of significant interactions was carried out using SNK tests.

5.3.5 Chemical analyses

The amount of tributyltin taken up into sediments, gut and skin samples was determined by digesting the sample in 10 ml of concentrated nitric acid (Aristar grade, Fisher Chemicals) under gentle heat. For tributyltin, sediments and tissues were processed using a solvent extraction procedure (Langston et al., 1990; Langston and Burt 1991). Certified reference material was extracted using the same procedure and used to verify concentrations of TBT (Bryan et al., 1986). The samples were then allowed to cool and analysed by Dr A. Fisher (University of Plymouth) using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

The amount of nonylphenol, phenanthrene, triclosan and TBDE in sediments, gut and skin samples was quantified by Dr S. J. Niven (University of Plymouth) using Gas Chromatography-Mass Spectrometry (GC-MS). Analysis of triclosan and nonylphenol followed Blackburn (1999). Extracts were evaporated to dryness under nitrogen. Analysis of

TDBE and phenanthrene followed Kelly et al. (2000). Final samples were re-dissolved into 1 ml dichloromethane prior to gas GC-MS analysis. Extraction efficiency was over 80% and was determined using standards and spiked sediments (triclosan 95 %, TBDE 82 %, nonylphenol 90 % and phenanthrene 91 %).

The adsorption of nonylphenol, phenanthrene and tributyltin onto sand and PVC was compared using a 1 factor ANOVA, where "sediment" had 2 levels (sand and PVC). The bioavailability of the phenanthrene and nonylphenol from microplastic was examined using a using two-factor ANOVA, where "tissue" had two levels (skin and gut) and "toxicant" had two levels (present and absent). The bioconcentration of nonylphenol, phenanthrene and TBDE from sediments containing chemicals sorbed to microplastic into skin and gut tissues was assessed using one-factor ANOVA, where "treatment" had 3 levels (sand containing plastic with sorbed contaminant, gut and skin). All factors were treated as fixed and (where necessary) orthogonal factors. There was high mortality of *A. marina* in treatments containing triclosan, therefore there were no statistical comparisons.

5.4 Results

Background levels of nonylphenol, phenanthrene, TBDE and triclosan in the tissues of *A. marina* were below the limit of detection in PVC and control animals. However, levels of tributyltin in gut tissue were 0.95 ± 0.67 μg g^{-1} and 0.09 ± 0.01 μg g^{-1} in skin tissues. Consequently, the bioavailability and toxicity of tributyltin from sand and PVC particles was not investigated.

5.4.1 Sorption of hydrophobic contaminants onto microplastic compared to sand particles

Uncontaminated microscopic particles of polyvinylchloride adsorbed significantly more phenanthrene ($F_{1,8}$ = 120.53, Cochran's C = 0.87, $P < 0.001$***), nonylphenol ($F_{1,8}$ = 149.17, Cochran's C = 0.77, $P < 0.01$**) and tributyltin ($F_{1,8}$ = 42.65, Cochran's C = 0.80, $P < 0.01$**) compared to similar sized sand particles (Figure 18). Per unit gram mass, polyvinylchloride adsorbed up to 57.5 times more phenanthrene, 1.3 times more nonylphenol and 2.4 times more tributyltin than similar sized sand particles.

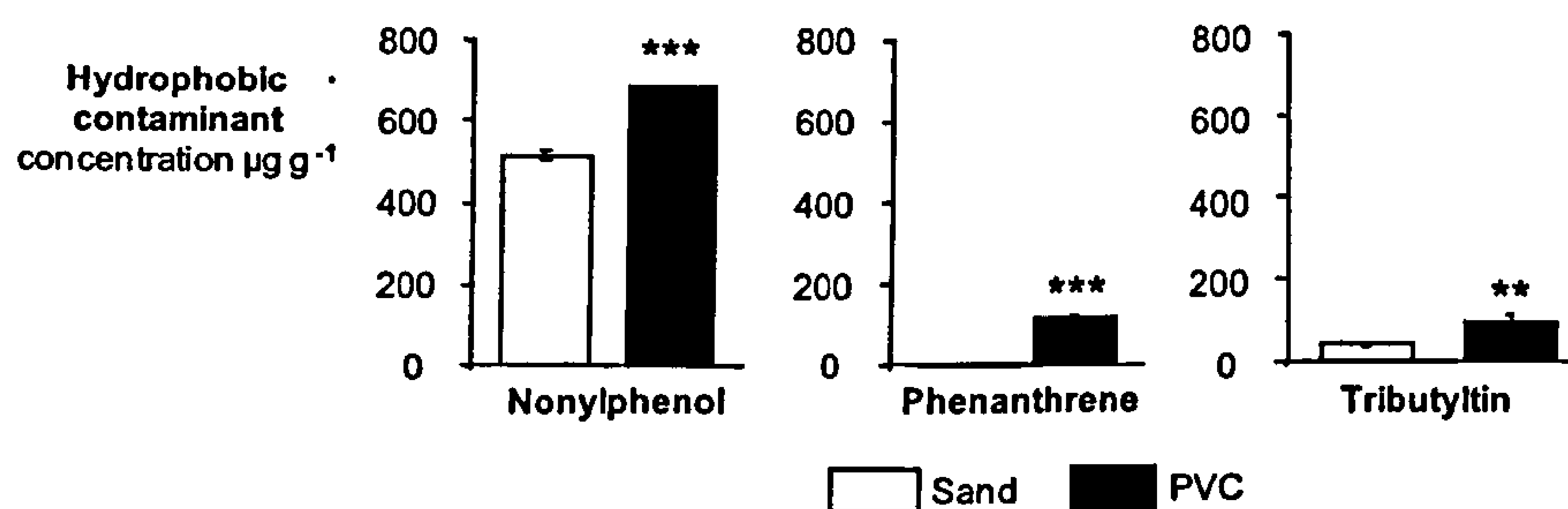


Figure 18. Adsorption of hydrophobic contaminant onto particles of polyvinylchloride compared to sand. Values expressed are dry mass mean ± S.E. * and ** denotes statistical significance at P < 0.001 and P < 0.01 respectively.**

5.4.2 Uptake of chemicals from microplastic to the tissues of *Arenicola marina* (L.)

Nonylphenol ($F_{2,12} = 14.69$, $P < 0.001$), phenanthrene ($F_{2,12} = 7.19$, $P < 0.01$** after log(x+1) transformation), TBDE ($F_{2,15} = 116.84$, $P < 0.001$ after log(x+1) transformation) and triclosan were released from the PVC and accumulated in the tissues of *A. marina* (Figure 19). Tissue concentrations were consistently greater than in the sand+PVC mixture that the worms had been exposed to. There was a consistent pattern of bioconcentration across all chemicals with concentrations of each chemical in the gut tissue compared to skin tissue. Polyvinylchloride particles transported TBDE and triclosan additives into the body tissues of *A. marina* (Figure 19). However, *A. marina* in the triclosan treatments suffered 61 % mortality and statistical analysis was not possible due to low number of individuals surviving at the end of the trial.

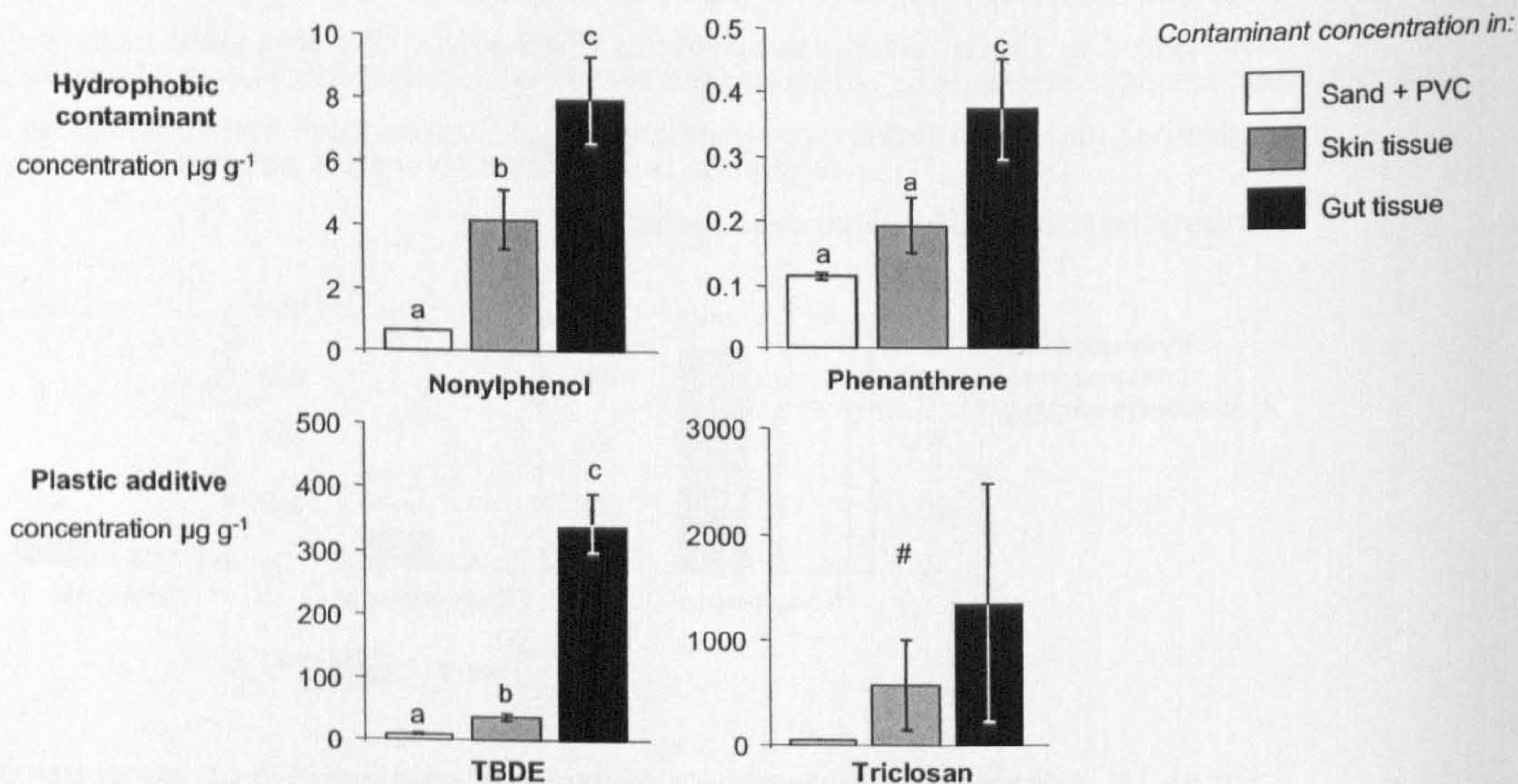


Figure 19. Bioavailability of hydrophobic contaminants (nonylphenol, Cochran's C = 0.69 and phenanthrene, Cochran's C = 0.72) and additives (TBDE, Cochran's C = 0.45 and triclosan) from a mixture of sand and microscopic PVC to the skin and gut tissues of the lugworm, *Arenicola marina*. For each chemical; treatments marked with the same letter (a,b,c) were not significantly different; # statistical comparisons with sediment not possible due to high mortality. Values are expressed are mean dry mass ± S.E for each chemical.

5.4.3 Bioavailability of adsorbed hydrophobic contaminants from sand and polyvinylchloride into the tissues of *Arenicola marina* (L.)

Polyvinylchloride and sand particles transported phenanthrene and nonylphenol into the body tissues of *A. marina*. The bioavailability of nonylphenol and phenanthrene was dependent upon sediment type and tissue type (Table 6, Figure 20). Over the short duration of this experiment, sand particles transported significantly (over 2.5 times) more phenanthrene (0.69 µg; $P < 0.01$**) and nonylphenol (17.1 µg; $P < 0.01$**) into the tissues of *A. marina* compared to polyvinylchloride particles (0.28 µg phenanthrene and 6.1 µg nonylphenol). For both particles there was over 1.8 times the amount of phenanthrene ($P < 0.05$*) and nonylphenol ($P < 0.05$*) taken up through gut tissue (0.37 µg phenanthrene and 14.8 µg nonylphenol) compared to skin (0.19 µg phenanthrene and 8.4 µg nonylphenol).

Table 6. Analysis of variance for uptake and tissue distribution of nonylphenol (Cochran's C= 0.43) and phenanthrene (Cochran's C = 0.59) within *Arenicola marina*.

		Nonylphenol			Phenanthrene		
	df	MS	F	*P*	MS	F	*P*
Sediment type	1	**608.64**	**14.68**	**<0.01**	**0.30**	**10.55**	**<0.01**
Tissue type	1	**201.80**	**4.87**	**<0.05**	**0.17**	**6.15**	**<0.05**
Sediment type X Tissue type	1	32.84	0.79	NS	0.00	0.02	NS
Residual	*16*	14.46			0.03		

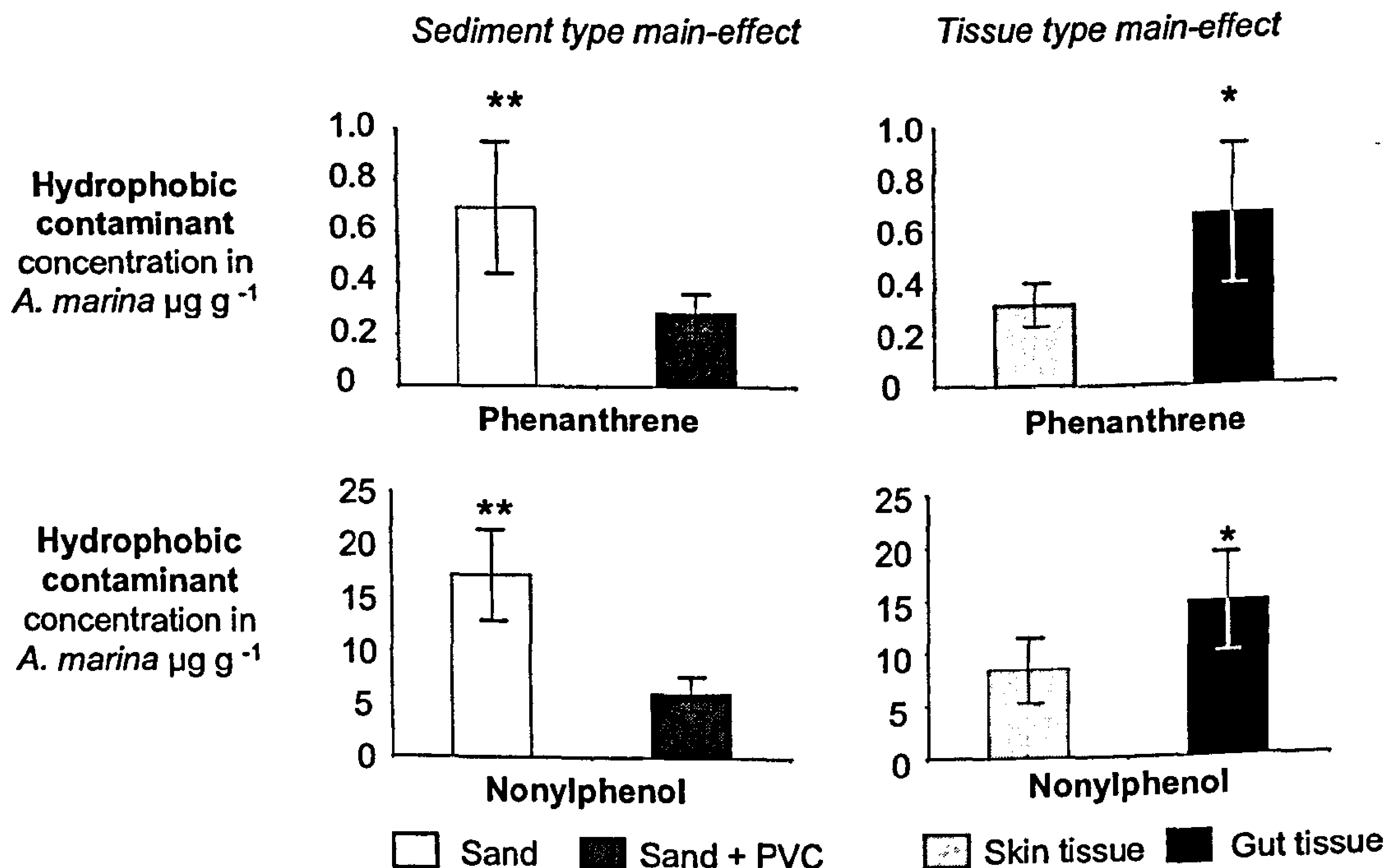


Figure 20. Contaminant uptake and tissue distribution within *Arenicola marina*. Values are expressed as mean ± S.E. ** and * denotes statistical significance at $P < 0.01$ and $P < 0.05$ respectively, whilst ns indicates not significant $P > 0.05$.

5.4.4 Biological consequences of ingesting microplastic with nonylphenol, phenanthrene, tetrabrominated diphenyl ether and triclosan for *Arenicola marina* (L.)

Nonylphenol, phenanthrene, triclosan and TBDE were all transferred from plastic to the tissues of *A. marina*. Of these, nonylphenol, phenanthrene and triclosan all had toxicological effects. In addition there were effects of PVC itself on the capacity of coelomic fluid to deal with oxidative stress. There was considerable mortality in exposures to the triclosan and this rendered sub-lethal measures (phagocytosis and FRAP) impossible.

5.4.4.1 Mortality

In treatments containing PVC with triclosan, there was considerable mortality (61%, Figure 21); however it was not possible to test this formally. Exposure to TBDE did not induce mortality above ambient levels in control treatments. There was no mortality in the trials with nonylphenol and phenanthrene.

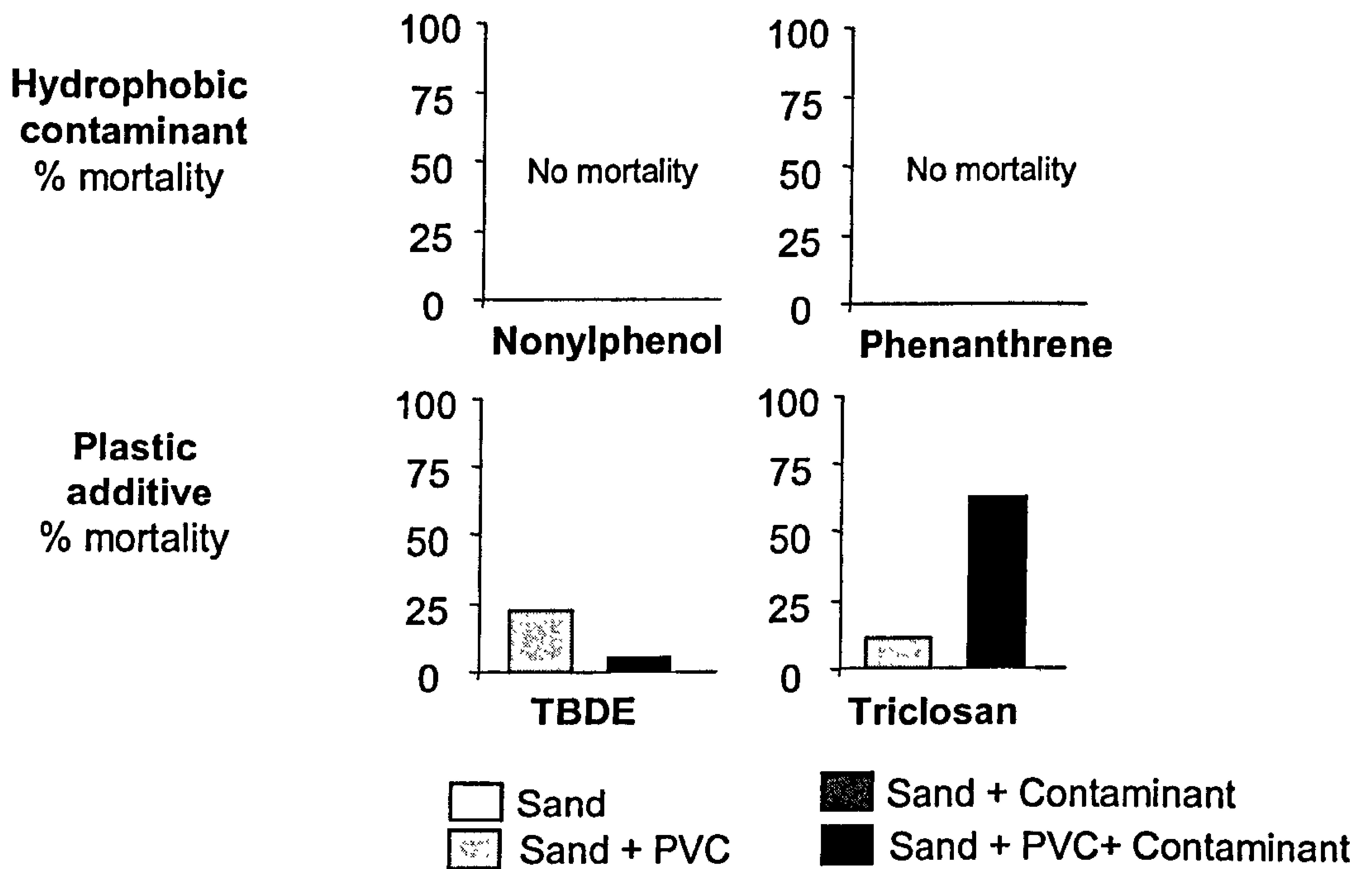


Figure 21. Percentage mortality across treatments. Statistical analysis not possible.

5.4.4.2 Burrowing behaviour

The presence of phenanthrene and nonylphenol did not significantly alter burrowing time compared to relevant controls (Figure 22).

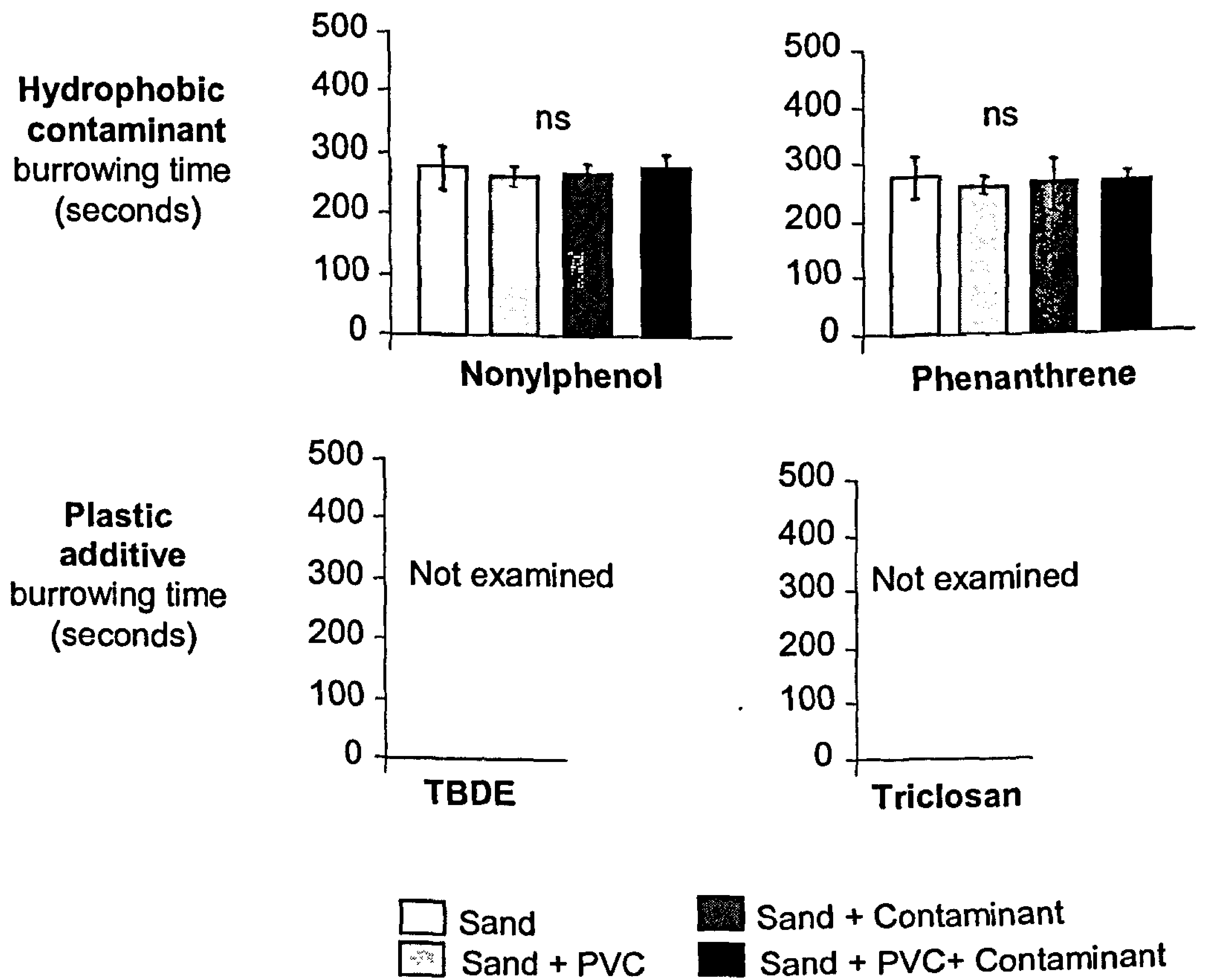


Figure 22. Burrowing time of *Arenicola marina*, expressed by the number of seconds taken to achieve complete burial. Values are expressed as mean ± S.E and '*ns*' indicates not significant.

5.4.4.3 Cast production

Triclosan significantly reduced mean cast production by 66 % from 2.1 casts day^{-1} inidviudal^{-1} in control animals, to 0.7 casts day^{-1} individual^{-1} in exposed animals ($F_{1,11} = 19.94$, Cochran's C = 0.86, $P < 0.01$**). Exposure to nonylphenol, TBDE and phenanthrene did not cause significant reductions in cast production (Figure 23).

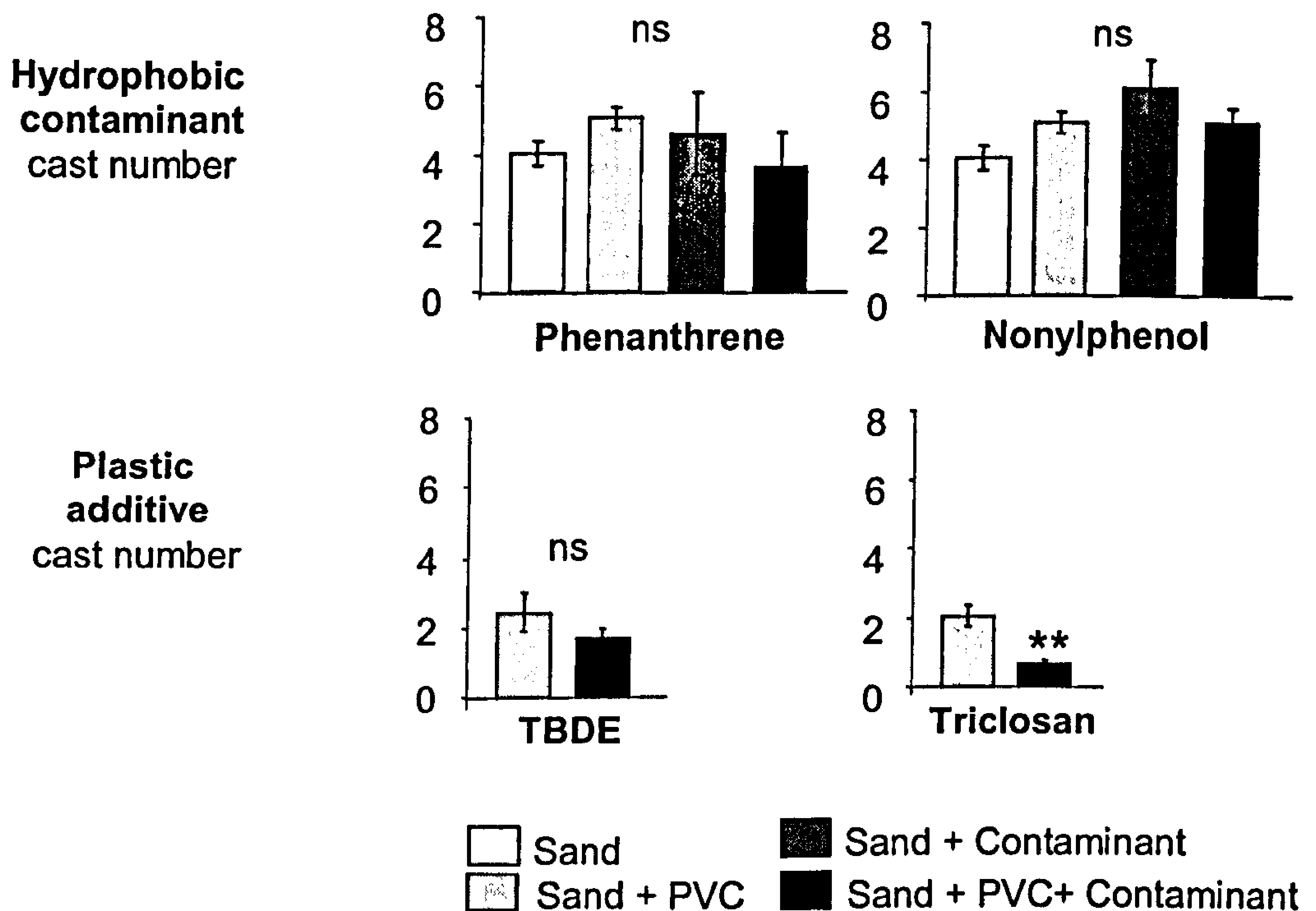


Figure 23. Casting behaviour within *Arenicola marina*, expressed by the mean number of casts produced day $^{-1}$ individual $^{-1}$. Values are expressed as mean ± S.E. Casting behaviour was significantly depressed in triclosan treatment. Statistical significance at $P < 0.01$ and '*ns*' indicates not significant.**

5.4.4.4 Phagocytic activity of coelomocytes

Treatments containing nonylphenol significantly reduced the phagocytic activity of coelomocytes from 16 x 10^5 to 6 x 10^5 zymosan particles phagocytosed g^{-1} coelomocyte protein in exposed animals (Figure 24; $F_{1,19}$ = 6.70, P <0.05*). However, exposure to sediment particles containing TBDE and phenanthrene had no effect upon phagocytic activity of coelomocytes. The phagocytic activity of coelomocytes from worms exposed to triclosan was not measured as samples were accidentally contaminated.

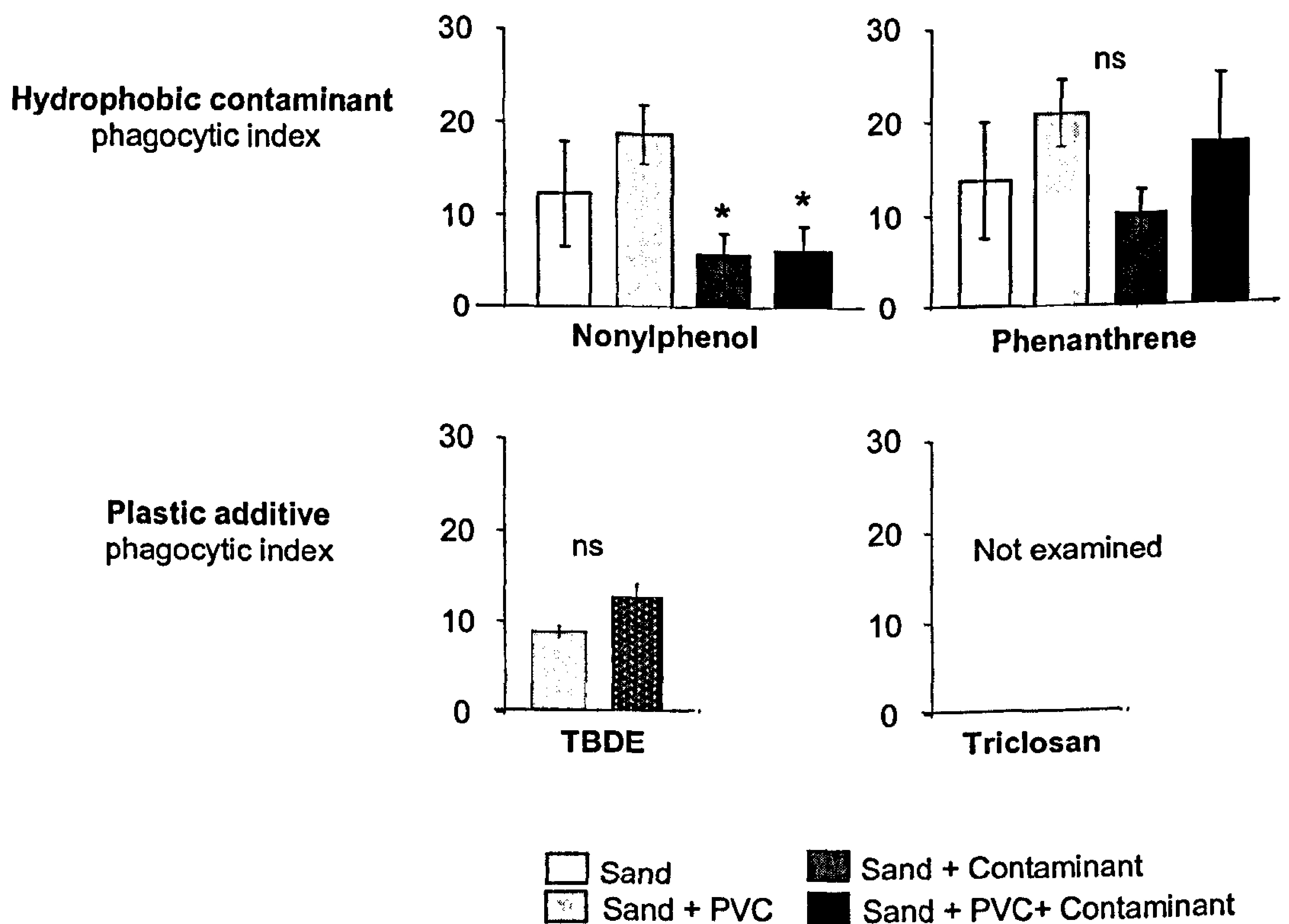


Figure 24. Coelomocyte phagocytosis from *Arenicola marina* expressed as number of zymosan particles (1 x 10^5) phagocytosed g^{-1} coelomocyte protein. Values expressed as mean ± S.E and * denotes statistical significance for presence of nonylphenol (Cochran's C =0.59) whilst '*ns*' indicates not significant P > 0.05.

5.4.4.5 Antioxidant capacity of coelomic fluid

Ingestion of sediment containing PVC significantly reduced the oxidative status of the coelomic fluid of *A. marina* by over 60 % (Phenanthrene data, $F_{1,19} = 7.64$, Cochran's C = 0.59, $P < 0.05$*) and, comparing just treatments containing 'sand' and 'sand+PVC' across the whole hydrophobics trial ($F_{1,28} = 30.25$, Cochran's C = 0.91, $P < 0.001$***) (Figure 25), exposure to nonylphenol, phenanthrene and TBDE did not significantly reduce antioxidant status within *A. marina*.

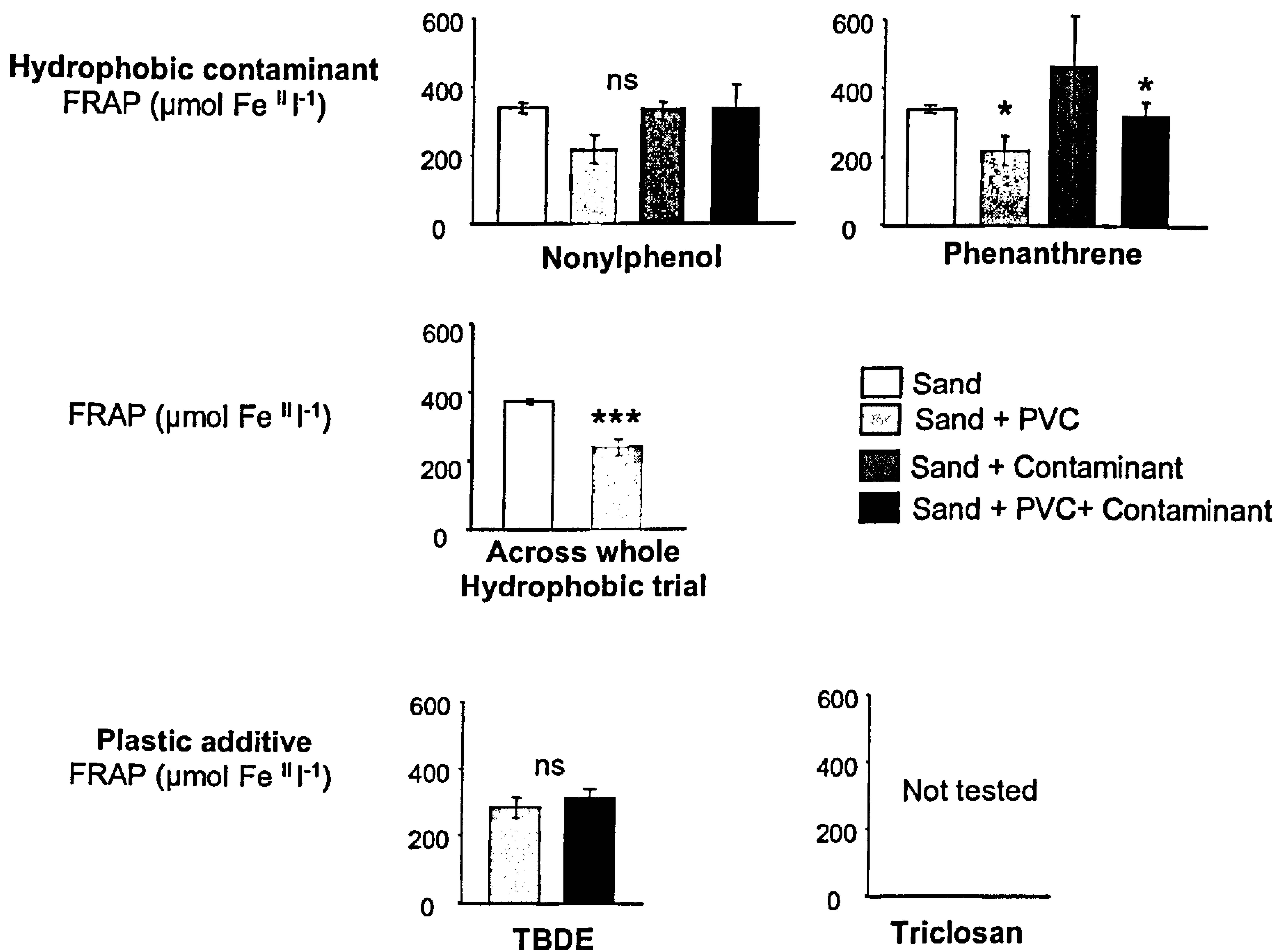


Figure 25. Ferric reducing antioxidant potential (FRAP) of the coelomic fluid of *Arenicola marina*. Values expressed as mean ± S.E and statistical significance at $P < 0.05$*, $P < 0.001$* and '*ns*' indicates not significant.**

5.5 Discussion

5.5.1 Sorption of hydrophobic contaminants by polyvinylchloride and sand particles

Microplastic particles adsorbed and concentrated significantly more hydrophobic contaminants than sand particles. Sorption of contaminants to sand and plastic particles was greatest for tributyltin and nonylphenol. This is in agreement with Teuten et al., (2007) who showed that sorption of phenanthrene onto microscopic particles of polyethylene, polypropylene and polyvinylchloride was up to an order of magnitude higher than that to particles of natural sediment. This has implications for geochemical analyses of hydrophobic contaminants in the sediments of marine habitats, toxicity testing and the remediation of contaminated habitats.

The spatial pattern of hydrophobic contaminants is often investigated to determine the sinks of these chemicals in the environment. Given the strong affinity of tributlytin, phenanthrene and nonyphenol to PVC it is likely that small amounts of plastic will increase the sorption of hydrophobic contaminants into marine sediments. Chapter 2 showed that the abundance of microplastic in estuarine sediments is affected by wind, with down-wind shores accumulating 3.6 times more microplastic than shores up-wind. It therefore seems likely that sediments from down-wind shores in the Tamar Estuary will contain significantly higher concentrations of hydrophobic contaminants than equivalent sediments from shores up-wind. Consequently, levels of microplastic in samples should be quantified as these are likely to influence the sorbative capacity of sediments for hydrophobic contaminants.

To investigate the bioavailability and toxicity of chemicals from the water column or sediments to aquatic organisms, aquaria made of plastic or made up of glass held together with

plastic-based glues (e.g. polysiloxanes) are often used in exposure trials (Bach et al., 2005, Inouea et al., 2006, Thain and Bifield, 2001), due to their light-weight, durability and safety. However, given the data presented in this chapter, it seems highly likely that aquaria made of plastic will adsorb high concentrations of contaminants and reduce the concentrations exposed to the animals, leading to an underestimation of the bioavailability and toxicity of many contaminants. Consequently, all-glass aquaria should be used where possible.

The high adsorption affinity of contaminants to plastic has a potentially positive outcome and indicates the possibility for plastic filters to be used as a remediation tool to remove contaminants from the environment. The data presented in this chapter show that PVC has a high affinity for tributyltin, which is one of the most acutely toxic contaminants used primarily as an anti-foulant on the hulls of large boats globally (Goldberg, 1995). Field experiments have also shown that deployed macroscopic polypropylene pellets can concentrate other pesticides (e.g. PCBs and DDE) from the environment (Mato et al., 2001). Therefore filters made of specific plastics could be used to remove chemical contaminants from sediments and water-column and hence remediate marine habitats at threat from high boating activity or oil spills.

5.5.2 Bioconcentration of hydrophobic contaminants from microscopic polyvinylchloride particles

Despite the considerable sorption capacity of plastics and the fact that only 1 % of the available sediment was ingested, this chapter shows that ingestion of plastic particles with sorbed hydrophobic contaminants and chemical additives, led to consistent patterns of bioconcentration of each chemical in the body tissues. Concentrations of nonylphenol, phenanthrene and TBDE were significantly greater in the gut compared to the skin tissue, and although it was not possible to analyse statistically, the same trend was evident for triclosan.

Over the short duration of trial (10 days) the gut tissue bioconcentrated each chemical by up to two orders of magnitude compared to the sediments to which the organisms had been exposed. Recent, *in-vitro* experiments suggest that gut surfactants could be responsible for this increased rate of phenanthrene desorption from polyvinylchloride (Thompson et al., 2008) and that the digestive system and gut surfactants are therefore likely to play a key role in the bioavailability of toxic chemicals from microscopic plastic particles.

5.5.3 Bioavailability of nonyphenol and phenanthrene from sand and polyvinylchloride particles

Microscopic particles of polyvinylchloride transferred significantly less phenanthrene and nonylphenol, to the tissues of *A. marina* than they did to sand particles. This effect is interesting since polyvinylchloride particles had significantly larger contaminant burden compared to sand. Microscopic plastic and sand particles released phenanthrene and nonylphenol into the body tissues of *A. marina*, with the main route of uptake for all contaminants being through gut tissues. Given that casting rates across treatments (e.g. phenanthrene, nonyphenol and controls) were constant, it seems that bioavailability of contaminants from sand and polyvinylchloride was not likely to be influenced by differences in gut retention between treatments. Therefore it is likely that the bioavailability of ingested hydrophobic contaminants may depend on an interaction between the type of material with which the contaminant is associated and the digestive environment of *A. marina*. Work on the bioavailability of PAHs to lugworms has shown that the ability of hydrophobic contaminants from ingested particles to cross from particles of sediment to the tissues of *A. marina* depends on the solubility within gut fluids and not aqueous solubility (Voparil and Mayer, 2000). However, physical and chemical differences, e.g. hydrophobicity (Jetten and de Kruijf, 2002) between sand and plastic particles, may be an important factor influencing sorption and

desorption of contaminants to and from plastic. Therefore, with the data from this chapter, it seems likely that the sorption-affinity of phenanthrene and nonylphenol for microscopic particles of polyvinylchloride limits the release of these compounds to digestive ligands, whilst the same contaminants were more readily bioavailable from sand particles. Similarly, Teuten et al., (in press), has showed that extent of transport by polyvinylchloride depends on the proportions of sand and polyvinylchloride. A little plastic increases transport potential whilst a lot of plastic decreases transport due to completive sorption. Therefore using the equilibrium partitioning method, the authors indicated that adding as little 1 μg of contaminated polyethylene to a gram of sediment would give a significant increase in phenanthrene accumulation by *A. marina.*

Furthermore it is important to realise that the increased availability from sediments compared to polyvinylchloride shown within this chapter is only relevant to infaunal organisms and there are many organisms including birds (Ryan, 1987a, Ryan, 1987b), fish (Kartar et al., 1976), seals (Eriksson and Burton, 2003), barnacles (Thompson et al., 2004) and mussels (Chapter 4) that have been shown to ingest plastic but do not ingest and/or live in the sediment. Chapter 4 shows compelling evidence that the residence time of polystyrene particles in mussels is over 48 days, therefore chemicals are more likely to transport from plastic particles into the tissues.

5.5.4 Biological consequences of ingesting polyvinylchloride particles with plastic with nonylphenol, phenanthrene, tetrabromodiphenylether and triclosan for *Arenicola marina* (L.)

Given that particles of microplastic debris are transported by wind (Chapter 2), it is very likely that when plastic particles are moved to a clean habitat, a small amount of contaminated plastic may release sorbed compounds. Therefore determining whether compounds from plastic pass to marine organisms is of great importance to predicting the biological consequences of plastic within marine habitats. This study demonstrates that ingestion of microplastic by marine organisms is likely to have pronounced toxicological consequences at different levels of biological organisation. Biological effects within *A. marina* manifested as changes in survival, casting and burrowing behaviour, immune function and antioxidant status.

Although it was not possible to analyse body burdens of triclosan statistically there was high mortality and therefore it seems likely when present at concentrations used in plastic manufacture, triclosan was highly bioavailable. Exposure of *A. marina* to sediments containing triclosan sorbed to PVC significantly reduced casting behaviour by 66% and caused 61% mortality over the ten-day exposure. Research on the toxicity of triclosan is limited to freshwater organisms (Smith and Burgett, 2005, Orvos et al., 2002, Ishibashi et al., 2004) and only one study has examined a marine animal (Canesi et al., 2007b). The present work represents the first study showing that exposure of triclosan to a marine organism causes serious toxicological effects. The use of antimicrobials in plastic remains questionable and the APIC (Association for Professionals in Infection Control and Epidemiology) found "no scientific data supporting the use of antimicrobial agents in household products as a means to prevent infection" (Slater, 1999), including the use of triclosan in plastics (Levy and McMurry, 1999). Despite this, new applications for triclosan in plastic products, including

polyvinylchloride-based medical implants (Zhang et al., 2006) and polystyrene-divinyl benzene beads that offer controlled release of triclosan are still being developed (Iconomopoulou et al., 2005). These add to the number of potential sources of microplastic with antimicrobial compounds entering the environment. With the levels of toxicity and the ease of transfer of triclosan to organism it may be more appropriate to apply the precautionary principle. More work is required to determine whether ingestion of microscopic plastic particles with triclosan reduces the survivorship potential of other marine organisms that have been shown to ingest microplastic.

Exposure to nonylphenol significantly reduced the phagocytic function of the coelomocytes of *A. marina*. These coelomocytes are used by *A.marina* and other polychaetes to identify and eliminate potential pathogens that can cause disease (Braunbecka and Dales, 1984, Dales and Cummings, 1987). Therefore the present study suggests that ingestion of microplastic with sorbed nonylphenol may increase the risk of infection by marine pathogens. The immunological results of this study are in agreement with the generalised mechanism of toxicity for nonyphenol (Gushiken et al., 2002, Canesi et al., 2007a).

The ingestion of sediment containing polyvinylchloride by *A. marina* significantly reduced the capacity of the coelomic fluid to deal with oxidative stress by over 60 %, compared to worms that ingested clean sand. Therefore, ingestion of microplastic may reduce the physiological competency of antioxidants (e.g. Vitamin E and antioxidant enzymes, including superoxide dismutase, glutathione reductase and catalase) within the coelomic fluid of *A. marina* to deal with high concentrations of hydrogen peroxide in the seawater (up to 4 $\mu mol\ l^{-1}$) (Abele-Oeschger et al., 1997) and coelomic fluid (up to 250 $\mu mol\ l^{-1}$) during daytime summer emersion periods (Buchner et al., 1996).

Biological effects in *A. marina* manifested as reductions in survival, cast production, immune function and antioxidant status. Given that exclusion of *A. marina* can lead to changes in the faunal assemblage of mudflats through increases in the abundance of the polychaete, *Nereis diversicolor* and reductions in numbers of the polychaete, *Scoloplos cf. armiger* (Volkenborn and Reise, 2006), it follows that ingestion of microplastic by *A. marina* that reduced the survival of lugworms may have potential consequences for the ecological structure and functioning of marine habitats where *A. marina* is a dominant organism.

5.5.5 Implications for food chains

Microscopic plastic debris and hydrophobic chemicals are increasingly common contaminants in marine habitats. This debris is likely to contain chemical additives and sorbed hydrophobic contaminants. This chapter presents the first data showing that toxic organic chemicals can be transported by plastic particles into living benthic marine organisms at the base of the food chain and that these chemicals can then give rise to adverse effects on the organisms. The lugworm, *A. marina* was used as a model deposit feeder and other species are likely to accumulate toxins from plastics in the same way. It is well established from laboratory trials that other phyla incorporating filter and detritivorous feeding guilds can ingest and accumulate microplastic. Chapter 4 showed that ingestion of smaller microscopic polystyrene by mussels translocates from the gut cavity to the circulatory system, where it can persist for over 48 days. However, the bioavailability and toxicity of chemicals from microplastic over these longer time-scales remains unknown. Given that lugworms are important components of the diet of wading birds (e.g. *Numenius arquata, Limosa lapponica, Haematopus ostralegus*) (Goss-Custard et al., 1991), fish (*Pleuronectes platessa*) (Beyst et al., 1999) and *A. marina* is collected commercially for angling bait, it follows that the data

presented in this chapter have potential food chain implications for predators that eat *A. marina*, in particular commercially important fish species such as *Scophthalmus rhombus, Solea vulgaris, Platichthys flexus, Pleuronectes platessa* and *Psetta maxima* that are consumed by humans.

Chapter 6. Environmental consequences of microscopic plastic in marine habitats

6.1 Summary of findings

This thesis goes some way to addressing significant gaps in our knowledge of the sinks and drivers of microplastic particles in the marine environment and also the possible toxicological effects on animals that ingest this material. The abundance of microplastic in intertidal sediments was 5 times greater than larger macroplastic material. Wind and depositional environment were found to be important drivers of spatial patterns of microplastic in the Tamar Estaury. Plastic debris accumulated on shores down-wind. For micro debris the pattern was strongest for high density material such as polyester and polyvinylchloride, for larger macro debris that pattern was strongest for lower density material such as polyethylene, polypropylene and expanded polystyrene, especially in areas of high sediment deposition. Globally, fragments of microplastic were found at every site and there was a weak positive correlation with population density.

Laboratory trials showed the potential hazards for marine animals ingesting microplastic. Ingested polystyrene microspheres accumulated in the gut of *M. edulis* and translocated into the circulatory system where they persisted for over 48 days, with no observed biological effects. For nearly 30 years it has been hypothesised that plastic may be a transport mechanism for chemicals into for toxic chemicals to marine animals (Mato et al., 2001, Rios et al., 2007, Thompson et al., 2004), including seabirds (Day, 1980; Pettit et al., 1981; Bourne & Imber, 1982; van Franeker, 1985). Data from Chapter 5 showed that microscopic fragments of polyvinylchloride can sorb and concentrate tributyltin, nonylphenol and phenanthrene from aqueous solutions. Subsequent ingestion of polyvinylchloride with sorbed hydrophobic contaminants and chemical additives led to bioconcentration of these chemicals in the tissues of *A. marina*, and this manifested as measurable increases in mortality and, reductions in

casting and burrowing behaviour, suppression of the immune system. Whilst ingestion of microscopic polyvinylchloride also led to reductions in the antioxidant status of the coelomic fluid of *A. marina.*

Therefore microplastic debris which is prevalent in marine habitats throughout the world may be a significant hazard to marine organisms. The major source of this material is likely to be the fragmentation of larger items of macroplastic that litter the environment. Therefore waste management legislation that encourages sustainable plastic use is required to reduce to the source of this material to marine habitats.

6.2 Sustainable plastic use and waste prevention

It is estimated that 8 % of world oil production is used in the manufacture of plastic and more 230 million tonnes are produced each year (Plastic-Europe, 2006). Forty percent of this material is used for single-use packaging that is thrown away within a year (EA, 2001b) and even in some of the most developed and environmentally aware nations, municipal waste contains up to 22 % plastic (OECD, 2007) and only a fifth of this material is recycled (Figure 26). Therefore a significant proportion of the world's non-renewable oil resource is consumed by plastic packaging that can only be used once (Thompson, 2007). Consequently, sustainable strategies are required to keep plastic waste in a continual loop of recycling and reuse so that the amount of plastic manufactured is equal to the amount of plastic reused and recycled (Figure 25). This would reduce the amounts of this material accumulating in landfill and marine habitats worldwide. The use of packaging resulted from increases in convenience food packed, which in turn has been brought about by the increased inexpensive and lightweight nature of plastic, the need to reduce transportation costs and the typical consumer having less time available for home preparation of food (Belcher, 2006, Eilert, 2005) .

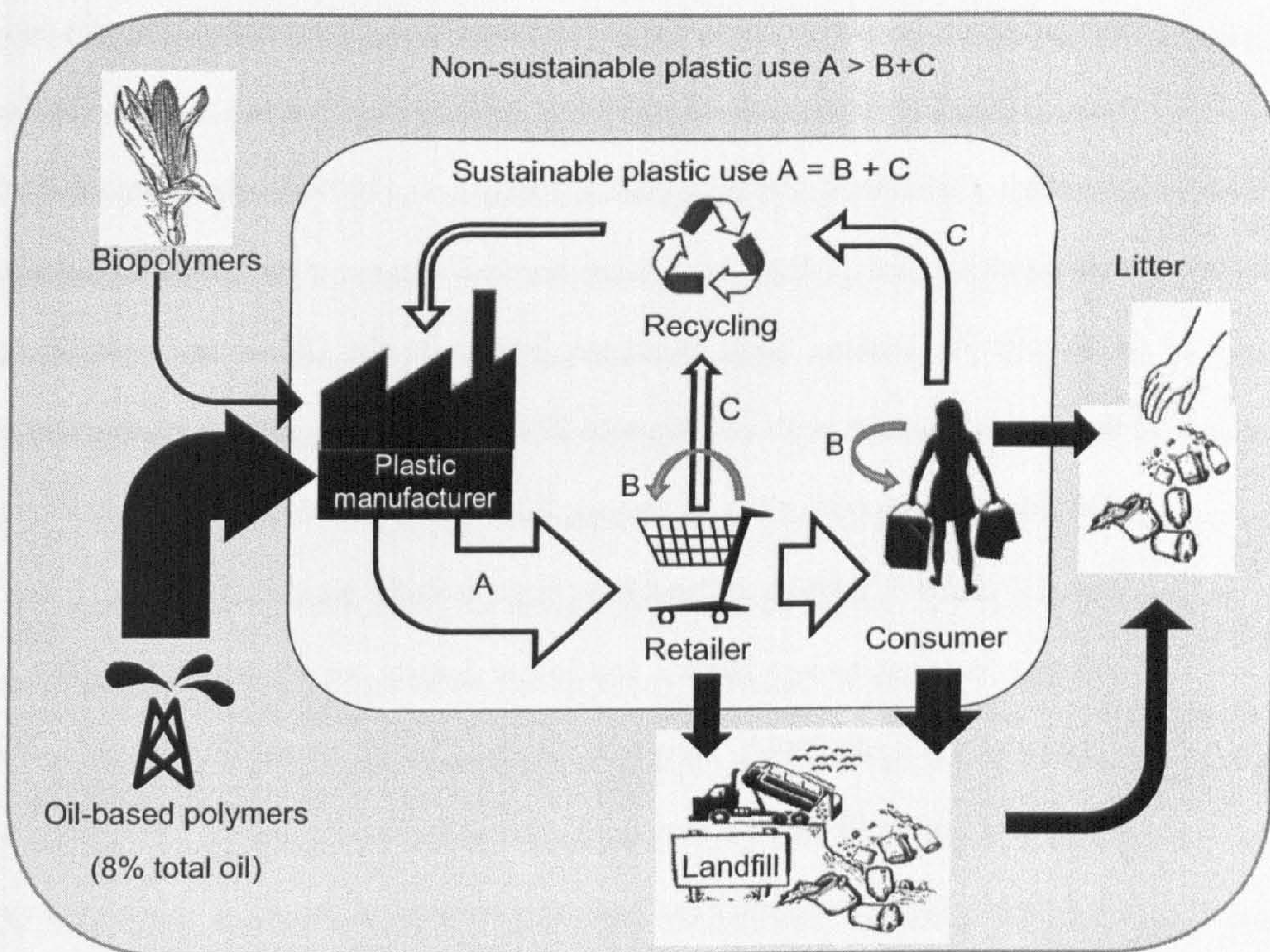


Figure 26. Sustainable (white arrows) and non-sustainable (black arrows) use of plastic in modern society, gray curved arrows represent reuse. Thickness of each arrow indicates proportion of total plastic.

There is over 500 different compounds based on polymers, with 13,000 grades and 25,000 trade names (Elias, 1997). Therefore governments around the world are now taking steps to reduce the amount of plastic packaging used by retailers and consumers by restricting the types of packaging that pose a significant environmental issue (Scottish Parliament, 2005a) and persuading manufacturers to lower the amount of plastic used in an item by making it lighter (WRAP, 2007b). For example the European Union has set recycling and recovery targets for packaging waste to reduce the environmental impact of packaging

(Packaging Waste Directive 2004). Individual governments have also taken action to reduce the use of plastic carrier bags. In Ireland, Denmark, Switzerland, Taiwan and South Africa a levy on plastic bags is charged to the consumer and in Ireland this has cut their use by 90 % (Scottish Parliament, 2005a, Scottish Parliament, 2005b). In Mumbai (India) and Dhaka (Bangladesh) plastic bags have been banned because they block the sewerage system and cause floods (Badam, 2005, Barkham, 2007). In the UK, over 13 billion bags are used by shoppers each year, with an estimated 220 given to each per person annually (DEFRA, 2001). The cost to local authorities to remove litter and refuse that contains plastic is £540 million a year (ENCAMS, 2006) and there have been calls for a carrier bag ban. In the UK the current strategy is to encourage thinner and lower density recycled bags (DEFRA, 2001) and food packaging (WRAP, 2006). However, Chapter 2 showed that thinner and lower density plastic articles are more likely to accumulate in wind exposed locations. Therefore, without accurate estimates of plastic degradation in natural habitats (Chapter 1), a ban or levy on carrier bags may be a better option and may create a greater demand for reusable plastic bags. For other types of packaging, some supermarkets have encouraged the reusable options by charging less for reusable bags and refillable packaging compared to single-use packaged items (WRAP, 2007b).

Current rates of recycling range from 6 to 25% (OECD, 2007) and these values need to be increased if plastics are to be used in a more sustainable manner. However, there are a number of barriers that need to be addressed. To increase recycling, the diversity of colour and polymer types used in plastic packaging needs to reduced and single-use packaging that cannot be recycled needs to be substantially reduced or eliminated (Thompson, 2007). It is widely recognised in the motor and food industry that a barrier to recycling is the difficulty in differentiating between plastics.

Recycling could be further facilitated by changing the present method of identification codes for plastic articles to colour (Thompson, 2007) or bar-coded plastics, which could then be more easily and precisely sorted. In Germany barcodes have been introduced on car parts that allow the identity to be established of the material used in a given part (Duval and MacLean, 2007). With barcodes present on all plastic packaging sold, simple bar code readers, which are routinely employed by customers at supermarkets, could be used to facilitate the separation of plastics. Furthermore the loyalty cards systems used by retailers in the UK (e.g. Nectare™) could also be used to incentivise consumers with additional points for each item of plastic returned. As each item has a bar code, this would also enable more accurate statistics to be gathered on the recycling habits of the population.

Clear or white plastics are easier to recycle and more valuable for recycling, than dark or unusual colours (WRAP, 2007b). In the UK, only 6 % of polyethylene terephthalate used for soft drink bottles is recycled and until recently the recycled material could not be incorporated back into food packaging due to health concerns about contaminants migrating into the food or drink within. However, there have been recent advances in tertiary recycling where recycled and virgin polyethylene terephthalate is mixed and washed in caustic soda at 90 °C, and then extruded as a film through a vacuum. This has reduced the threat of chemical migration from recycled polyethylene terephthalate packaging into food (FDA, 2006) and has led to a limited number of packaged products (Coca-Cola, Mark & Spencer and Boots) on sale in the UK. At the moment they only contain between 25 - 50 % recycled polyethylene terephthalate, with the rest from virgin polyethylene terephthalate and there has been no reduction in sales (WRAP, 2006).

6.3 *Holistic life cycle assessments*

One role of scientists in dealing with the problems of plastic is to educate the consumers, retailers and the plastic industry about the true costs and benefits to the environment of plastic. Life cycle and carbon foot print assessments of the environmental effects of plastic have in the past largely only taken into account green-house gas emissions from the burning of oil and natural gas, and have ignored issues such as acid rain, loss of biodiversity, human health effects, eutrophication and ecotoxicity due to a lack of data (Ross and Evans, 2003) . Plastic bottles have replaced glass bottles, mainly to reduce the fuel costs and green house gas emissions associated with transportation. However, glass still has several advantages over polyethylene terephthalate bottles and these are not taken into account during many life cycle assessments. Glass bottles have been reused and recycled into new bottles for decades (e.g. milk, wine and soft drinks), whereas polyethylene terephthalate can only be recycled into new bottles by combining them with virgin material and using additional tertiary treatment. Therefore, most recycled polyethylene terephthalate is used to not make new bottles and packaging but to make soil drainage pipes, fleece jackets, outdoor furniture, insulation for sleeping bags, carpets and even car parts. Glass bottles are less permeable to oxygen that can degrade the packaging and its contents (Cladman et al., 1998), and plastic bottles are known to transfer potentially toxic chemicals used during manufacture in the product (Song et al., 2003). Recent work by Grosch™ has shown that glass beer bottles can be reduced in mass by 13 % (WRAP, 2007a). Therefore if plastic bottles are used in preference to glass they should be made from materials that pose minimal risks to human health and that can be reused and easily recycled using current recycling programmes. However, currently there have been no life cycle assessments that incorporate the costs of

cleaning up litter, the impacts upon human and environmental health of chemical transfer of toxicants from plastic, and acid rain and green house gas emissions from the burning of oil and natural gas. Therefore, funding is urgently needed to address these knowledge gaps.

6.4 Standardised monitoring of litter

To determine whether environmental policies such as the European Packaging Waste Directive (EU, 2004) are effective at reducing plastic litter, further monitoring across terrestrial and aquatic habitats is required. In marine habitats there has been extensive monitoring and beach cleaning of plastic debris (Song and Andrady, 1991, Galgani et al., 2000, Hess et al., 1999, Thiel et al., 2003, Claereboudt, 2004, Aliani et al., 2003, Mascarenhas et al., 2004, McDermid and McMullen, 2004). In the UK routine monitoring to quantify the amount of plastic litter in cities and rural areas is carried out by ENCAMs (ENCAMS, 2006). Intertidal habitats are monitored through large-scale, yearly surveys of the removal of debris which have been undertaken for thirteen years (MCS, 2006). However, quantitative comparisons between studies are very difficult as they done in different locations on different dates with different sampling methodologies. Therefore standardised monitoring strategies are required with agreed environmental quality standards (Thompson, 2007). Using the same amount of effort and incorporating advances in statistical frameworks (Underwood, 1997), the design of the litter monitoring programmes could be improved in order to provide greater spatial and temporal information and to provide a method to test the effectiveness of different waste management programmes in reducing the quantities of plastic litter in the environment. Recently the Convention for the Protection of the Marine Environment of the North-East Atlantic has suggested that a monitoring programme is needed to establish spatial and temporal trends of microplastic debris in the North East Atlantic (OSPAR, 2006). A pilot

project by OSPAR is being used to assess the use of debris found in the stomachs of dead seabirds (Fulmars) found washed ashore in North Europe to set an Ecological Quality Objective for the amount of plastic debris in the sea. A target of less than 2 % of dead birds should have less than 0.1 g of plastics in their stomach has been proposed (van Franeker and Meijboom, 2007). Fulmars feed over large geographical areas of sea (kilometres) and are known to ingest plastic. Data collected over the last 25 years has shown that 98 % of fulmars have plastic in their stomachs and that the abundance of packaging material has increased (van Franeker, 1985, van Franeker and Meijboom, 2007). Although the abundance of plastic in their stomach will be a useful integrative measure of the abundance of plastic debris over large areas of the sea, the data will be not be representative of current levels of plastic debris (*the ghost of plastic contamination past*) and researchers have suggested that birds may ingest certain colours and shapes of plastic debris more than others (Shaw and Day, 1994, Moser and Lee, 1989). Therefore, Thompson (2007) suggests that research is needed to develop approaches that are sensitive to changes in the amount of 'new' plastic material that is entering the oceans each year. Governmental agencies, scientists, packaging manufacturers and retailers need to work together to explore ways in which packaging could be labelled to give important information to scientists who find the item as litter. One option is to use and develop the bar code to determine the date of production or sale, the location of sale and the type of plastic. Many of this information is already collected by retailers and loyalty card schemes for market research and would also remove the need for time consuming FT-IR to determine the types of plastic in litter surveys across habitats.

6.5 *Future research priorities*

6.5.1. Spatial distribution of microplastic

Research presented in this thesis has shown that microplastic accumulates across the globe in open water, sandy, estuarine, sub-tidal and deep sea habitats, and that abundance of microplastic can be higher in wind-exposed locations. However, the abundance of microplastic varied considerably over smaller (metres) and larger (kilometres +) spatial scales. Therefore further work is needed to better understand how the abundance of microplastic varies at different spatial scales using components of variation analyses (Underwood, 1997). Plastic debris have been identified by FT-IR as acrylic, polyamide, polyethylene, poly(ethylene: propylene), polyester, polyethylene terephthalate, polybutylene terephthalate, polyoxylmethylene, polypropylene, polystyrene, polyurethane, and polyvinylchloride. However, since only fragments that differed in appearance from sediment grains or plankton were quantified, the amount of microplastic recorded in this thesis is only likely to represent a small proportion of the microscopic plastic in the environment. Further research is required to optimise identification methods using FT-IR so that the abundance and composition of all microplastic can be assessed quickly and efficiently.

6.5.2. Trophic distribution of microplastic

A major gap in our knowledge is the extent of uptake and retention of microplastic by animals in their natural habitats, partly because quantifying tiny plastic fragments in the tissues of animals presents a range of methodological problems. For larger pieces of plastic found in seal faeces it has been suggested that the source of the material is from fish in their diet which have accumulated plastic; however, it remains unknown whether plastic of any size can biomagnify within marine food chains (Eriksson and Burton, 2003). Research reviewed

and carried out as part of this thesis has shown that that amphipods (detritivores), polychaete worms, echinoderms, bryozoans, barnacles, molluscs (filter feeders; Chapter 4) and polychaete worms (Chapter 5:deposit feeders) ingest fragments of microplastic (2 to 230 μm) (Thompson et al., 2004, Ward and Shumway, 2004). Laboratory trials reported in this thesis have shown that mussels (*Mytilus edulis*) can ingest and accumulate polystyrene beads (2 and 10 μm) in their gut cavity, where they can translocate into the circulatory system and persist for over 48 days (Chapter 4). With microplastic debris is accumulating in the environment on a global scale, these laboratory trials suggest that microplastic particles are probably also being ingested by animals in their natural habitats. Chapter 2 suggests that the main source of microplastic is likely to be the fragmentation of larger items, however the current methodology only allows particles down to 20 μm to be identified using FT-IR. Fragments less than 1 μm in size (nanoplastic) is likely to be present in marine habitats and their organisms, therefore it is vital to develop chemical methods to analyse body burdens of nano and microscopic plastic material in marine animals, perhaps using blood samples. This would allow rapid allow non-destructive sampling of larger animals including marine mammals and also to find other more appropriate test organisms for trials to investigate the toxicological consequences of accumulating tiny fragments of plastic.

6.5.3 Degradation and persistence of plastic debris in natural habitats

The persistence of plastic litter in marine habitats is poorly understood and Chapter 1 has discussed the lack reliability of biodegradability or compostability tests to provide realistic data for the environmental persistence plastic is low (Amass et al., 1998). The main issue is that aquatic and terrestrial habitats represent physically and biologically complex environments and are very different to the constant conditions of a laboratory or outdoor trials.

Therefore it is important to develop environmentally realistic tests for the degradability of plastic packaging. Field deployment experiments of different types of plastic packaging commonly found as litter are required and these need to be carried out across a range of marine habitats including open-water, inter- and sub-tidal habitats. Once completed, the data could be used to make ecologically reliable estimates about the likely persistence of plastic litter in marine habitats.

6.5 Where to from here?

Global plastic production has grown from 5 to over 230 million tonnes in just over 50 years (EA, 2001b, Plastic-Europe, 2006). The rapid increase in plastic consumption coupled with high durability and unsuitable plastic use, has led to caused widespread accumulation of plastic material in landfill and the environment (Carpenter et al., 1972, Derraik, 2002, Ryan and Moloney, 1990). Therefore scientists, government agencies, retailers, packaging manufacturers and the plastic industry need to work together to develop effective methods at managing plastic waste and reducing litter in the environment. However, even if effective measures are introduced, there are still many potential long term problems with large accumulations of plastic debris in the environment, therefore more work is required to understand the continued consequences that plastic debris pose in the environment today.

Appendices

Browne, M. A., Thompson, R. C, & S. Niven

Date: 12.06.04

Microscopic plastics sampling standard operating procedure (PLASSOP)

1. Objective

To confirm the key sources and sinks of microscopic plastic in marine habitats, we need to first provide a reliable and coherent method of sampling plastics. This standard operating procedure (PLASSOP) aims to achieve this by reducing the risk of procedural contamination to a minimum.

2. Personnel, Training and Responsibilities

This method is restricted to use by or under the supervision of professionals experienced with this protocol.

3. Materials

- Aluminium foil sampling vessel and foil covered cardboard lid
- or glass jars with metal tops (5 per site)
- Paper or aluminum foil to wrap the samples in and labels
- Cardboard box
- Small metal scoop (tablespoon) or use foil-covered lid

4. Procedure

- Cotton clothing is to be worn and synthetic clothing items (such as fleeces) are to be avoided due to contamination risk. Kneel down on the shore, down-wind of the part of strandline you intend to sample.

- Working in an area within the strand line of the last tide, collect separate amounts of sediment within the radius which you can reach without moving position.

- Within this radius collect material at random, but spread throughout the area of the strand that you can easily reach i.e. one spoon then, relocate spoon and again and so on until the first container is full.

- Each sample should be roughly 1 cm deep into the sediment. It does not matter that each separate spoonful gets mixed together. The point of this method is to integrate over small-scale variability in the distribution of microplastics.

- Once the first sample container is full seal with lid and label*. Move along the strand at least 25 m and repeat the operation until you have 5 independent replicates samples (each ≈ 500 ml) per site.

- Wrap each of the 5 replicate containers using foil, newspaper, paper bag or paper (no plastic), and place filled containers into the Parcel Pak provided.

- If an opportunity presents to do more than one site please do so (2 or 3 would be plenty as far apart as possible).

5. Labelling procedure*

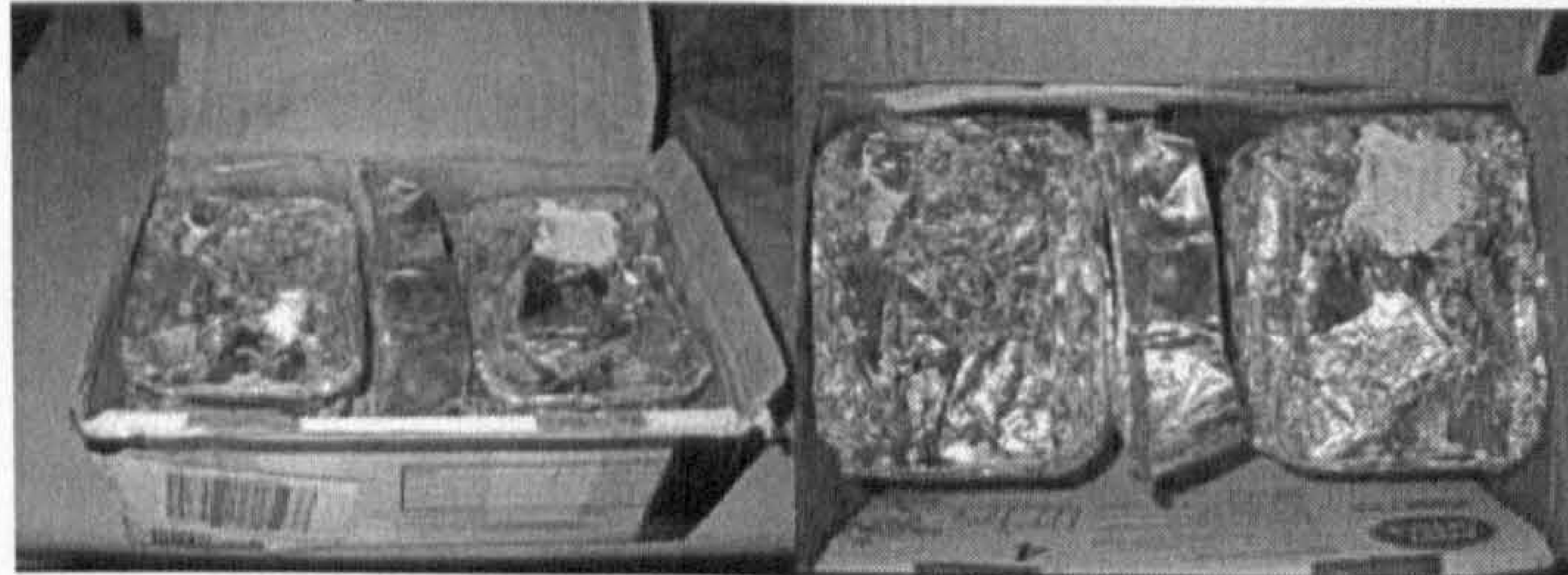
This utilises a 12-digit identifier code, e.g. PH1A150403MB

- PH = ISO code for the country where it was sampled, i.e. Philippines
- 1A = 1st sub-site, replicate A
- 150403 = sampling date i.e. March 15th 2003
- MB = collector initials i.e. Mark Browne

Identifier:												
Position: Long....................Lat..................												
Action required/Comments:												

6. Posting and Packing

Place the 5 replicate containers inside Postal Pak as shown in diagram below:



Please send Parcel Pak(s) complete with replicate containers to:

Mark A. Browne
Davy 616, MBERC, University of Plymouth, Drake Circus, Plymouth, PL4 8AA, United Kingdom

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Peter M. Chapman
Golder Associates Ltd.
195 Pemberton Avenue
North Vancouver, BC V7P 2R4, Canada
pmchapman@golder.com

In a Nutshell...

Emerging Contaminants

Microplastic—An Emerging Contaminant of Potential Concern?, by Mark A. Browne, Tamara Galloway, and Richard Thompson.

Organisms with a range of feeding strategies can ingest and accumulate microplastics.

Critical Body Residues

A Call for Scientific Rigor in the Development of Critical Body Residues: A Case Study, by Judi Durda, Jennifer Sampson, and Leslie Williams.

Berkvar et al.'s (2005) contaminant body residue for DDT is examined and found lacking.

Toxicogenomics

Toxicogenomic Assessment of the Population Level Impacts of Contaminants, by Edward Perkins, Kurt Fust, and Jeffrey Steevens.

Toxicogenomics can assess not only stressor mechanisms but also population susceptibility to stressors.

Ecological Risk Assessment

Assessment of Cumulative Ecological Effects of Agricultural Stressors on Aquatic Communities: An Elaboration of the Sediment Quality Triad, by Eric Luiker, Joseph Culp, and Glenn Benoy.

Individual effects of stressors are compared to overall cumulative effects.

MICROPLASTIC—AN EMERGING CONTAMINANT OF POTENTIAL CONCERN?

Mark A. Browne, Tamara Galloway, and Richard Thompson
University of Plymouth, Plymouth, UK
mabrowne@plymouth.ac.uk

Introduction

Global plastic production is now estimated at 225 million tons per year (Plastic-Europe 2006). Plastic debris is accumulating in terrestrial and aquatic habitats worldwide. This debris is progressively fragmenting into smaller pieces. As the plastic breaks down, the potential for ingestion by animals increases. The biological consequences of macroplastic (≥5 mm) debris on wildlife have been well documented and include suffocation, entanglement, and starvation. However, the potential impacts of microscopic (<1 mm) plastic debris remain poorly understood.

What are the sources of microplastic to the environment?

The 2 most likely sources of microplastic are from fragmentation of larger plastic items and the use of small particles of plastic as abrasive scrubbers in cleaning products. Plastics fragment in the environment as a consequence of photolytic, mechanical, and biological degradation. During photodegradation, sunlight oxidizes the chemical structure, causing bond cleavage that reduces the molecular mass of polymers, and as a result plastics become brittle and disintegrate, giving rise to tiny fragments. Within the marine environment, plastics also fragment through the combined effects of wave action and abrasion from sediment particles. In addition, some plastics are susceptible to biodegradation by bacteria and fungi (Gregory and Andrady 2003). Regardless of the method of deterioration, the size and identity of plastic fragments found in marine habitats clearly indicate that microscopic particles can form from the breakdown of larger items. Recent work in the Tamar Estuary (UK) has shown that the size frequency of plastic debris on the strandline is highly skewed toward smaller debris and that, in terms of abundance, microscopic fragments account for over 80% of the stranded plastic (Figure 1c; MA Browne, T Galloway, and R Thompson, unpublished data). Furthermore, microscopic fragments of materials used for clothing (polyester, acrylic), packaging (polyethylene, polypropylene), and rope (polyamide) have also been identified from beaches around the United Kingdom (Thompson et al. 2004).

Another source of microplastic particles is from industrial and domestic products, including toilet, hand, body, and facial cleansers (Derraik 2002; Thompson et al. 2004), that contain tiny polyethylene and polystyrene particles less than 1 mm in

diameter. In addition, larger particles of acrylic, melamine, and polyester, ranging from 0.25 to 1.7 mm in diameter, are used to clean machinery and boat hulls in dockyards by a process known as "media blasting." Microplastic particles are also used in a range of medical applications, including drug delivery systems. Small particles from all these sources are likely to be transported with wastewater and through sewage treatment works and subsequently enter aquatic habitats. Hence, there is considerable potential for microscopic plastic debris to accumulate in freshwater and marine environments.

What is the extent of microplastic contamination in habitats?

A study of archived plankton samples from the northeast Atlantic showed that the abundance of microscopic plastics in the water column has increased considerably over the last 40 y, and this trend mirrors the global rise in plastic production (Figure 1b). Similar particles were also found on beaches throughout the United Kingdom, and therefore microplastic particles appear to be a widespread contaminant that has accumulated across a range of habitats (Thompson et al. 2004). Recent work on plastic debris found within the Tamar Estuary (UK) has identified acrylic, polyamide, polyethylene, poly(ethylene: propylene), polyester, polyethylene terephthalate, polybutylene terephtalate, polyoxylmethylene, polypropylene, polystyrene, polyurethane, and polyvinylchloride (M.A. Browne, T. Galloway, and R. Thompson, unpublished data). Since only fragments that differed in appearance from sediment grains or plankton were quantified, the amount of microplastic recorded in this study is likely to represent only a small proportion of the microscopic plastic in the environment. Further research is required to optimize identification methods using Fourier transform infrared spectroscopy so that the abundance and composition of all microplastic can be assessed quickly and efficiently.

Do animals ingest microplastic?

Large (>5 mm) plastic debris is frequently ingested by a range of species, including fish, turtles, birds, and cetaceans (Derraik 2002). Microplastic is much smaller, occupying the same size range as plankton. Hence, there is a greater potential for ingestion by a wide range of animals. Uptake of microplastic by different feeding guilds will depend on the size, shape, and density of the particles, as these parameters determine the position of the debris in the water column and potential availability. For a given size, low-density plastic will float and will be available for uptake by filter feeders or planktivores, whereas high-density plastics, such as polyvinyl chloride (PVC), will tend to sink and accumulate in sediments where they are more likely to be ingested by deposit feeders.

The uptake and retention of microplastic by animals in their natural habitats has received little attention, partly because quantifying tiny plastic fragments in the tissues of animals presents a range of methodological problems. Laboratory trials have shown that amphipods (detritivores), barnacles (filter feeders), and lugworms (deposit feeders) ingest small PVC plastic fragments (mean size 230 µm; Thompson et al. 2004). In addition, filter-feeding polychaetes, echinoderms, bryozoans, and bivalves have been shown to ingest 10-µm polystyrene microspheres during feeding assays (Ward and Shumway 2004). Recently, mussels (*Mytilus edulis*) have been show to ingest and accumulate polystyrene beads as small as 2 µm in their gut cavity (Figure 1d). Given that microplastic is accumulating in the environment, these laboratory trials suggest that microplastic particles are probably also being ingested by organisms in their natural habitats.

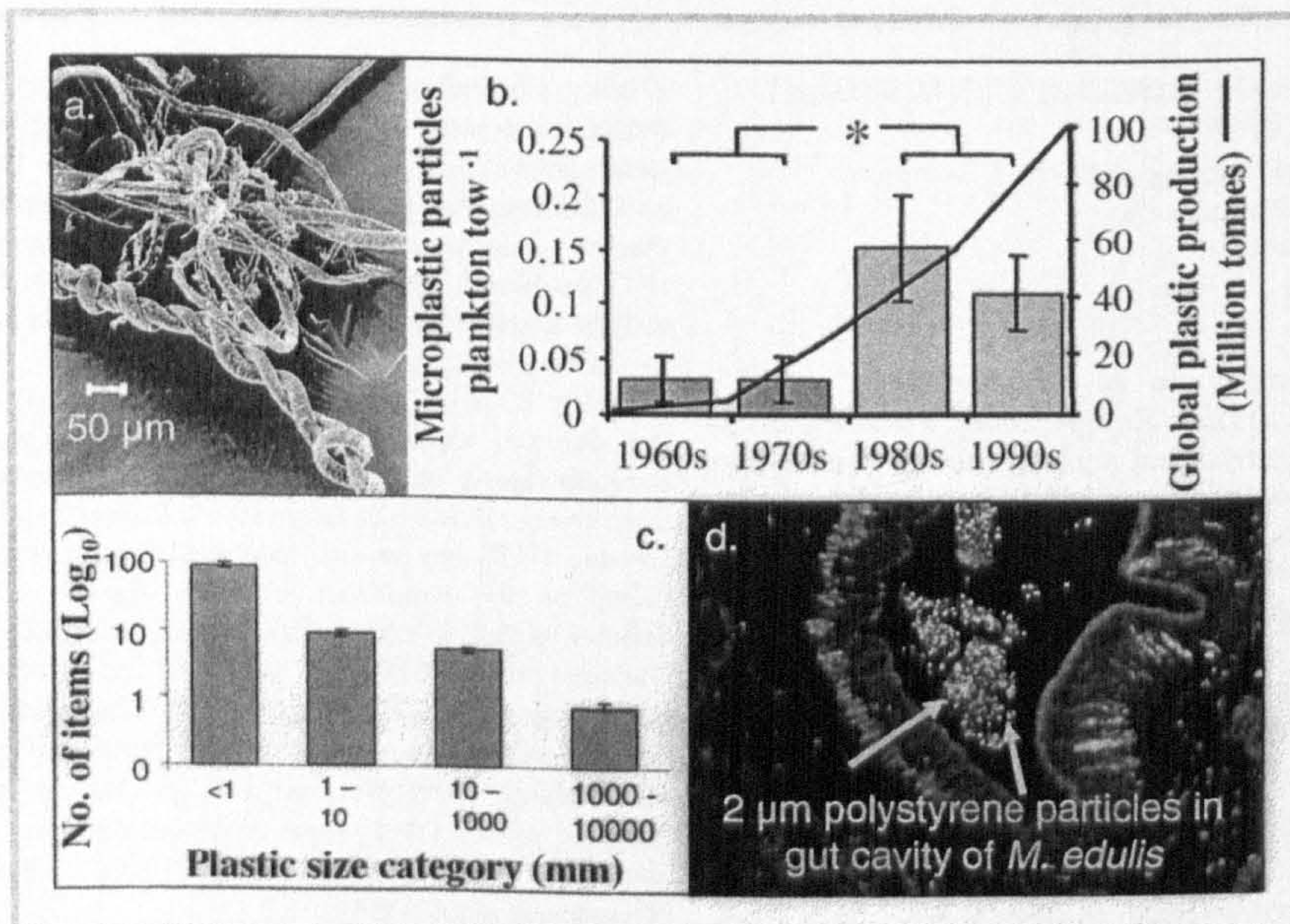

Figure 1. (a) Electron micrograph of microplastic fiber from the shoreline. (b) Accumulation of microplastic in the water column in the northeast Atlantic, with the global plastic production figures for the same period superimposed for comparison (adapted from Thompson et al. 2004). (c) Size composition of plastic debris in Tamar Estuary, United Kingdom (note log scale; unpublished data) (d) Tissue section of the gut of *Mytilus edulis* containing 2 µm fluorescent polystyrene particles (365-nm excitation, 477-nm emission) ingested during a laboratory trial (M.A. Browne, T. Galloway, and R. Thompson, unpublished data).

If ingested can microplastic transfer from the gut to the other body tissues?

Once microplastic is ingested by animals, it may be retained in the digestive tract, egested in the form of feces, or absorbed into the epithelial lining of the gut by phagocytosis. Retention of larger plastic debris certainly occurs in the digestive tracts of seabirds and mammals. Laboratory trials using lugworms (*Arenicola marina*) kept in sediments containing microplastic have shown that these animals are capable of egesting this debris within their fecal casts. If microplastic particles are taken up by the gut epithelial lining, then further transport around the body is possible. Qualitative research in rodents has shown that solid polystyrene microspheres can readily transfer (translocation) from the gut to the lymphoid system (Hussain et al. 2001). The lymphoid system supplies the circulatory system, and hence these particles will then have the potential to be transferred to other tissues around the body. Given that the rodent digestive system is similar to many other organisms, translocation of ingested microplastic from the gut around the body of aquatic animals is likely. Indeed, recent laboratory trials involving mussels (*M. edulis*) have shown that ingested polystyrene microspheres can translocate from the gut cavity to the hemolymph within 3 d (M.A. Browne, T. Galloway, and R. Thompson, unpublished data).

Does ingestion of microplastic have any toxicological consequences for animals?

A wide range of vertebrates and invertebrates have been shown to ingest and accumulate plastic debris; however, little is known about the biological effects. Micro- and nanoscopic (<1 μm) plastic and nonplastic particles exert damage through the combined effect of their intrinsic toxicity and their large surface area. For example inhalation of PVC dust by humans can cause, depending on monomer composition and size, lung and liver damage through tissue fibrosis and cancer (Wagoner 1983).

Polymers are composed of repeating subunits called monomers. Polyvinylchloride (Marcilla et al. 2004), polystyrene (Garrigos et al. 2004), and polycarbonate (vom Saal and Hughes 2005) have been shown to release toxic monomers that are linked with cancer and reproductive abnormities in humans, rodents, and invertebrates. Monomers are not the only chemicals that could be potentially transferred from plastics upon uptake by organisms. During manufacture, a range of chemical additives are incorporated into plastic, including catalysts (organotin), antioxidants (nonylphenol), flame retardants (polybrominated diphenyl ethers), and antimicrobials (triclosan). In addition to chemicals used in manufacture, plastic has been shown to adsorb and concentrate hydrophobic contaminants, including polychlorinated biphenyls, dichlorodiphenyl trichloroethane, and nonylphenol, from the marine environment at concentrations several orders of magnitude higher than those of the surrounding seawater (Mato et al. 2001). If plastics are ingested, they could act as a mechanism facilitating the transport of chemicals to wildlife. This may be particularly relevant for microplastics since they will have a much greater ratio of surface area to volume than larger items and hence are likely to have greater potential to transport contaminants.

Conclusion

Given the rapid rise in plastic production, the disposable nature of many plastic items and the durability of plastic, contamination of the environment by microplastic is likely to increase. Laboratory trials have shown that organisms with a range of feeding strategies are capable of ingesting and accumulating microscopic particles. More work is now required to determine the potential toxicological consequences of this new form of contamination.

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A CALL FOR SCIENTIFIC RIGOR IN THE DEVELOPMENT OF CRITICAL BODY RESIDUES: A CASE STUDY

Judi L. Durda,† Jennifer R. Sampson,‡ and Leslie G. Williams‡
†*Integral Consulting, Annapolis, MD, USA*
‡*Integral Consulting, Mercer Island, WA, USA*
jdurda@integral-corp.com

Tissue residue–response relationships are increasingly used in ecological risk assessments (ERAs) and natural resource damage assessments (NRDAs) to predict the toxicological consequences of accumulated chemical body burdens. Given that chemical body burden provides an integrated measure of exposure for certain chemicals, the use of these data as the dose metric in the overall risk equation is both practical and scientifically supportable in some situations. The utility of these data to reliably assess toxicological response is, however, tightly tied to the availability of data that reliably characterize the exposure–response relationship and can be used to establish a critical body residue (CBR) associated with a specified level of effect. We believe that scientific rigor and a sound toxicological understanding are necessary to develop reliable dose–response metrics based on body burden. CBRs developed without that foundation are not reliable and should not be used.

Meador (2006) reviewed several key technical issues to consider when using toxicological data to derive CBRs for aquatic species. While we don't agree with Meador's (2006) suggestion to use CBRs to derive sediment quality guidelines,

Ingested Microscopic Plastic Translocates to the Circulatory System of the Mussel, *Mytilus edulis* (L)

MARK A. BROWNE,*,† AWANTHA DISSANAYAKE,† TAMARA S. GALLOWAY,‡ DAVID M. LOWE,§ AND RICHARD C. THOMPSON†

School of Biological Sciences, University of Plymouth, Drake Circus, Plymouth, PL4 8AA, U.K., University of Exeter, Prince of Wales Road, Exeter, EX4 4PS, U.K., and Plymouth Marine Laboratory, Prospect Place, Plymouth, PL1 3DH, U.K.

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Plastics debris is accumulating in the environment and is fragmenting into smaller pieces; as it does, the potential for ingestion by animals increases. The consequences of macroplastic debris for wildlife are well documented, however the impacts of microplastic (<1 mm) are poorly understood. The mussel, *Mytilus edulis*, was used to investigate ingestion, translocation, and accumulation of this debris. Initial experiments showed that upon ingestion, microplastic accumulated in the gut. Mussels were subsequently exposed to treatments containing seawater and microplastic (3.0 or 9.6 μm). After transfer to clean conditions, microplastic was tracked in the hemolymph. Particles translocated from the gut to the circulatory system within 3 days and persisted for over 48 days. Abundance of microplastic was greatest after 12 days and declined thereafter. Smaller particles were more abundant than larger particles and our data indicate as plastic fragments into smaller particles, the potential for accumulation in the tissues of an organism increases. The short-term pulse exposure used here did not result in significant biological effects. However, plastics are exceedingly durable and so further work using a wider range of organisms, polymers, and periods of exposure will be required to establish the biological consequences of this debris.

Introduction

Global plastic production has increased from 5 million tonnes in the 1950s to over 230 million tonnes in 2005 (*1*). This production volume coupled with their high durability has led to widespread accumulation of discarded plastic in landfills and as litter in terrestrial and aquatic habitats worldwide (*2*). This debris progressively fragments into smaller pieces and as it does so the potential for it to be ingested by animals increases. Fragments of plastic less than 1 mm (microplastic debris) have accumulated in the northeast Atlantic over the last 40 years (*3*). In terms of numerical abundance, recent work in the Tamar Estuary (UK) has shown microplastic can comprise as much as 85% of stranded plastic debris (*4*). Large (>5 mm) items of plastic debris are ingested by over 180 species, including fish, turtles, marine mammals, and birds (*2*). The effects are well documented and include suffocation and starvation (*2*). However, ingestion of microplastic has received little attention, partly because quantifying tiny plastic fragments in the animal tissues presents a number of methodological challenges. As this debris occupies the same size range as sand grains and planktonic organisms, it is available to a wide range of invertebrates near the base of the food chain. Laboratory trials have shown that amphipods (detritivores), barnacles (filter-feeders), and lugworms (deposit-feeders) can ingest particles of microplastic (*3*). Many invertebrates feed by collecting, sorting, and digesting particulate matter and laboratory experiments focused on particle selection have also shown that filter-feeding polychaetes, echinoderms, bryozoans, and bivalves will ingest 10 μm polystyrene microspheres (*5*). Once microplastic is ingested it may be retained in the digestive tract, be egested through defecation, or transferred through the epithelial lining of the gut into body tissues (translocation). Laboratory trials demonstrate that the lugworm, *Arenicola marina* (L.), egests (230 μm) particles of polyvinylchloride within their fecal casts (*3*). However, medical studies on both rodents and humans have also shown that particles of polyvinylchloride (*6*) and polystyrene (*7*) less than 150 μm can translocate from the gut cavity to their lymph and circulatory systems. In vitro experiments with segments of colonic tissue from rats indicate that smaller particles undergo translocation more readily than larger particles (*8*). It is however not known whether ingested particles of microplastic can translocate from the gut cavity to the circulatory system of invertebrates, or whether smaller particles translocate more readily than larger particles of plastic.

In the present study, the bivalve mollusc *M. edulis* was used as a model organism to investigate the uptake, fate, and biological consequences of ingesting microscopic particles of polystyrene. Mussels are an important component of benthic assemblages worldwide (*9*) and are thought to act as ecosystem-engineers via occupation of primary space, filtration, and provision of secondary habitat (*10*). *Mytilus edulis* was selected as a model organism as it has a large geographic range and is an important component of the diet of various intertidal predators including humans. The hemolymph can be easily sampled and its toxicological responses to many contaminants are well described (*11–14*). Studies examining feeding in *M. edulis* have shown that microspheres of polystyrene are drawn through the inhalant siphon and filtered via the gill. On the gill, filamentous cilia capture plastic particles and rapidly transport them to the ventral groove and on to the labial palps, where cilia sort particles for ingestion or rejection as pseudofeces (*15*).

Global production of polystyrene in 2001 was more than 13.6 million tonnes and by 2010 it is expected to exceed 15 million tonnes (*1*). Polystyrene is primarily used (36%) as food packaging and insulation in the construction industry (*1*). Recent work has shown that, in terms of numerical abundance, polystyrene accounted for 24% of the macroplastic found in a estuarine habitat (*16*). Spherical particles of polystyrene (0.1–2 mm) are known to accumulate in fish, and stomach content analysis has shown that over 20% of snail-fish (*Liparis liparis*) and flounder (*Platichthys flesus*) from the Bristol Channel (UK), contained fragments of

* Corresponding author phone: +44 (0)1752 232941; fax: +44 (0)1752 232970; e-mail: mabrowne@plymouth.ac.uk.
† University of Plymouth.
‡ University of Exeter.
§ Plymouth Marine Laboratory.

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polystyrene (17). Similar work on the New England coast (United States) has shown that the stomach contents of nearly 60% of fish species including *Myoxocephalus aenus*, *Pseudopleuronectes americanus*, *Roccus americanus*, *Menidia menidia*, and the polychaete worm, *Sagitta elegans*, contained fragments of polystyrene (18). Ingestion of large fragments of plastic can lead to accumulation in the gut and is likely to compromise feeding [e.g., ref (19)]; however, it is unknown whether ingestion of microplastic will influence feeding behavior of marine animals or whether this material can translocate from the gut cavity to their circulatory system. Furthermore, the toxicity of polystyrene and its monomer, styrene, to marine animals is poorly understood, with only one laboratory study examining the effects of styrene on viability and DNA damage in molluscan and fish hemocytes (20). However, with only one exposure tank and one control tank, the experimental design suffered from pseudoreplication.

In the present study we quantitatively test hypotheses relating to translocation and biological effects of microplastic particles. Mussels were independently exposed to a single pulse of either fluorescently labeled particles in seawater or to seawater without particles of plastic (control). Animals were individually transferred to clean conditions to prevent further ingestion of microspheres. Exposed and control animals were then sampled over a logarithmic time scale to track the labeled particles and quantify any biological effects. Histological and hematological techniques were used to assess translocation of particles, and toxicological assays were employed to examine specific biological effects on *M. edulis*. The following hypotheses were examined: (I) After ingestion, polystyrene particles accumulate in the gut cavity of mussels. (II) Ingested polystyrene microspheres translocate from the gut to the circulatory fluid (hemolymph), and when transferred to clean conditions, the abundance of microplastic in mussels will decline over time. (III) After feeding mussels with the same number of microspheres, smaller (3.0 μm) polystyrene particles will occur in the hemolymph in greater quantities than larger (9.6 μm) particles. (IV) Ingestion and/or translocation of microplastic particles will reduce the viability and phagocytic function of hemocytes, reduce the oxidative status of hemolymph, and reduce feeding activity of mussels.

Materials and Methods

M. edulis (3–4 cm) were collected from Port Quinn (50°35′20.60″ N, 4°52′05.63″ W), Cornwall (UK), a site free from point sources of chemical contamination (21, 22). Mussels were collected from the rocky platforms by carefully cutting byssal threads to avoid damaging the underlying soft tissues. Epifauna were removed and mussels were transferred to temperature-controlled conditions (15 °C). Individuals were numbered using plastic labels, randomly assigned to separate beakers, and left for 48 h to acclimatize in filtered seawater containing *Isochyrsis galbana* food supplement. Two experiments were then conducted. The first investigated whether mussels ingest and accumulate polystyrene microspheres in their gut cavity (*Hypothesis I*). A second experiment then investigated whether this material could translocate from the gut cavity to the circulatory system, determined if the abundance of microplastic in mussels would decline over time when transferred to clean conditions (*Hypothesis II*). This experiment also examined whether there were differences in translocation according to particle size (*Hypothesis III*), and assessed if ingestion/translocation compromised organism health (*Hypothesis IV*).

In the first experiment to determine whether mussels were able to uptake microplastic from the water column into their gut cavity, animals were exposed for 12 h to separate treatments (each with three replicates) containing 0.51 gL^{-1} of either 2 μm fluorescently labeled microspheres of polystyrene (excitation 365 nm/emission 447 nm; Brookhaven International, UK), nonlabeled (4–16 μm) microspheres of polystyrene (Sigma-Aldrich, UK), or a control treatment without plastic. After exposure, histological techniques were used to determine the presence of microplastic in the gut.

A second experiment was conducted to establish whether ingested microplastic could translocate from the gut cavity to the circulatory system. Clean glass beakers (400 mL) were filled with 350 mL of filtered seawater, stirring bars were added, and the beakers were placed on magnetic stirrers. Mussels were randomly assigned to their time/treatment/replicate combination using a replicated block-design. Two sizes of fluorescent polystyrene particles, 3.0 μm (490 nm excitation/515 nm emission) and 9.6 μm (520 nm excitation/580 nm emission) were obtained from Molecular probes (USA) in a distilled water carrier. These particles are colored as part of manufacture through adsorption. As part of physiological experiments, these particles have been injected into the circularity and pulmonary systems of rodents and dogs where they can remain for months without loss of resolution or detectability (23, 24). In our exposure study we used 15,000 individual polystyrene microspheres for each plastic treatment (verified by Coulter Counter analysis) of either small or large size particles per mussel, in each time/treatment/replicate combination. The size of plastic chosen was representative of the smaller size range of particles known to be ingested by marine invertebrates (3, 5, 25) and the smallest diameter of plastic debris found in marine habitats (3). Mussels were exposed to these treatments for 3 h, as preliminary work demonstrated that this was sufficient for microspheres to be ingested. Animals were then transferred to separate clean beakers and fed daily on *I. galbana*. Seawater was changed every other day. To check for the presence of polystyrene microspheres in fecal matter, seawater was vacuum filtered using Whatman GFA filters, which were subsequently examined under fluorescence microscopy.

Biological Assays. A cell viability assay was used to examine whether the ingestion of microplastic led to reductions in the ability of mussel hemocytes to absorb neutral red dye (26), similarly a phagocytosis assay was used to determine whether the immune system of mussels had been compromised by ingesting microplastic. This assay quantifies the ability of mussel hemocytes to engulf yeast (zymosan) particles (26). To assess whether ingestion and translocation of microplastic reduced the capacity of the hemolymph to deal with oxidative stress, the ferric reducing antioxidant potential (FRAP) assay was used (27). Changes in feeding behavior were assessed by examining the rate of clearance of food particles from an algal stock solution (28). For detailed protocols please see Supporting Information S1.

Tracking the Uptake of Polystyrene Microspheres. To determine whether polystyrene microspheres had accumulated in the gut, mussels were dissected and the mid gut was extracted using a butterfly section and preserved in Baker's formal calcium (24 h, 4 °C). Following fixation, the tissues were dehydrated through an ascending alcohol series, cleared in xylene, impregnated in wax, and blocked-up using the Tissue-Tec system. Sections were cut using Brights WM3050-microtome, mounted on APTS-coated slides, and stained using the Papanicolaou technique to differentiate the tissues of the gut cavity and smaller digestive tubules (11).

To assess whether ingested microplastic was translocated from the gut cavity to the circulatory system of mussels, 500 μL of hemolymph was placed onto preprepared poly L-lysined glass slides which adds a positive charge that helps living cells adhere to surface of the slides. To prevent contamination, shell water was drained from each mussel prior to hemolymph extraction from the posterior abductor muscle, and a new

syringe/needle was used for each animal to avoid the possibility of cross contamination. Slides were left in a humidity chamber for 30 min while hemocytes adhered to the slides, and then inverted and allowed to dry at room temperature. Slides were then fixed in methanol (15 min) and allowed to dry at room temperature (*27*). A glass coverslip was then mounted on each slide using clean forceps and a few drops of aqueous polyvinyl-alcohol resin mounting agent (Immuno-mount, Thermo-Shandon, USA) were added. The abundance of microplastic particles was counted under fluorescence microscopy (Leica DMR). Microspheres were distinguishable due to their sizes (3.0 or 9.6 μm) and fluorescence at the appropriate spectral wavelength, and there was no observed leaching of the fluorescent chemical label.

To confirm whether polystyrene microspheres had accumulated within hemocytes, 500 μL of hemolymph was placed onto poly L-lysined glass-slides and incubated in a humidity chamber (30 min) with fluorescein-isothiocyanate. The slides were inverted and washed with phosphate buffer, then dried at room temperature. A coverslip was then mounted and the hameocytes on slides were viewed under a Zeiss LSM confocal microscope. The resulting 3-dimensional image of the hemocytes was used to assess whether polystyrene microspheres had accumulated within hemocytes.

Statistical Analyses. To determine the influence of particle size on the accumulation of microplastic in the hemolymph, formal comparisons were made using 2-factor ANOVA where "time" had 5 levels (3, 6, 12, 24, 48 days) and "treatment" had 2 levels (3.0 and 9.6 μm polystyrene particles), the analysis excluded the control animals as they did not contain microspheres. Times were specifically selected to represent a semilogarithmic series. Both factors were therefore treated as fixed and orthogonal, with 5 replicates of each time/treatment combination. As each experimental unit was independent, ANOVA was used to test hypotheses about translocation and quantitatively assess pulse and press toxicological effects. Prior to ANOVA, homogeneity of variance was examined using Cochran's tests. The variances of the microspheres in the hemolymph, oxidative status, viability, and phagocytic activity of hemocytes were heteroscedastic, and as this could not be resolved by transformation the analysis was conducted on untransformed data. Large balanced ANOVA designs are considered robust to departures from variance assumptions (*29*), and to reduce the likelihood of type-I error, a more conservative significance level of $P < 0.01$ was used. Significant effects were examined using posthoc Student−Newman−Keuls (SNK) tests. Statistical analysis was carried out using winGMAV 5 for windows (EICC, University of Sydney).

Results

For the first experiment, histological analyses showed that within 12 h, mussels exposed to both particle size treatments had accumulated polystyrene microspheres in their gut cavity and digestive tubules (*Hypothesis I*, Figure 1a and b).

After 3 days, ingested microplastic accumulated in the circulatory fluid of mussels and both sizes of polystyrene particles were found in the hemolymph and hemocytes (Figure 1c−e). Microspheres were not found in the hemolymph of mussels from the control treatment. Statistical analysis showed significant effects of "time" ($F_{4,40} = 13.33$, $P < 0.01^{**}$) and "particle size" ($F_{1,40} = 9.87$, $P < 0.01^{**}$). The abundance of both sizes of microplastic was greatest after day 12 and declined thereafter (*Hypothesis II*, Figure 1f), smaller 3.0 μm particles occurred in consistently greater abundances than larger particles throughout the experimental duration (*Hypothesis III*, Figure 1g), and these effects were both significant. Furthermore, both sizes of microplastic were still present in the hemolymph after 48 days. Microplastic was present within fecal pellets from the first time of examination after 3 days in clean conditions and for the remainder of the experiment.

The ingestion and translocation of polystyrene microspheres by mussels did not cause any significant reduction in the oxidative status of hemolymph, the viability and phagocytic activity of hemocytes, or filter-feeding activity (*Hypothesis IV*, see Figure S1 in Supporting Information). However, there was a significant effect of "time" in all biological assays. The oxidative status of hemolymph was over 35% higher from mussels sampled on days 3, 12, and 48 compared to those sampled on days 6 and 24 ($F_{4,60} = 5.03$, $P < 0.001$). The viability of mussel hemocytes was up to 64% greater on day 6 and 12 compared to all other times ($F_{4,60} = 8.16$, $P < 0.001$). Comparisons of mussels sampled between days 3 and 6, and between days 24 and 6, days 12 and 48 showed that the phagocytic activity of hemocytes varied by over 50% throughout the trial ($F_{4,60} = 6.33$, $P < 0.001$). Clearance rate varied over time by up to 70%, with days 3 and 6 significantly higher than days 12 and 24, while day 6 was significantly higher than day 48 ($F_{4,60} = 6.62$, $P < 0.001$).

Discussion

Mytilus edulis ingested and transported particles of microplastic to the gut, where they accumulated in the digestive cavity and tubules (*Hypothesis I*). Though particles of plastic have been shown to accumulate in the gut cavity of birds (*30*), fish (*2*), and polychaete worms (*18*), translocation from the gut to the circulatory system of an invertebrate has not been previously shown. In our study particles of polystyrene translocated from the gut cavity to the circulatory system in as little as 3 days and persisted in the circulatory system for over 48 days (*Hypothesis II*). This is important because previous research investigating the ingestion of macroplastic debris (>1 mm) by marine animals has only showed that it may be retained in the digestive tract (*2*) or egested in the form of feces (*31*). Further work is needed to determine how quickly particles of microplastic translocate to the circulatory system and the mechanism(s) by which these particles are taken up across the gut/extracellular barrier and accumulate in the hemolymph. Without detailed histological sectioning and examination of all tissues/organs using fluorescence microscopy, it is not possible to speculate as to why the peak abundance of small and large particles occurred after 12 days (*Hypothesis II*). In humans and rodents, the kinetics of particle translocation in the intestine depends on the diffusion and accessibility of the plastic particles through the mucus, initial contact with the enterocyte, cell trafficking, and post-translocation events (*32*). It will therefore be important to characterize different cell types within the gut of mussels and investigate their ability to phagocytose and traffic particles of plastic through the epithelial lining. In particular, it is important to investigate the possible presence of specialized enterocytes such as "*microfold*" cells. These cells have irregular-shaped microfolds, poorly developed microvilli, lectin binding, and in humans and rodents are known to transport plastic particles from the gut lumen, through the epithelium, toward follicles via phagocytosis, from which particles can then migrate to the circulatory system (*33*). In our experiments, the abundance of microspheres in the hemolymph showed the same pattern of accumulation for both sizes, it therefore seems likely that both sizes translocate by the same mechanism(s). Confocal microscopy showed that both sizes of particles of plastic were present inside hemoctytes (Figure 1e) and *in vitro* trials using the closely related species *Mytilus galloprovincialis* showed that granulocytic hemocytes are responsible for the phagocytosis of polystyrene particles up to 800 nm (*34*). Therefore for post-translocation events at least, phagocytosis could play a key

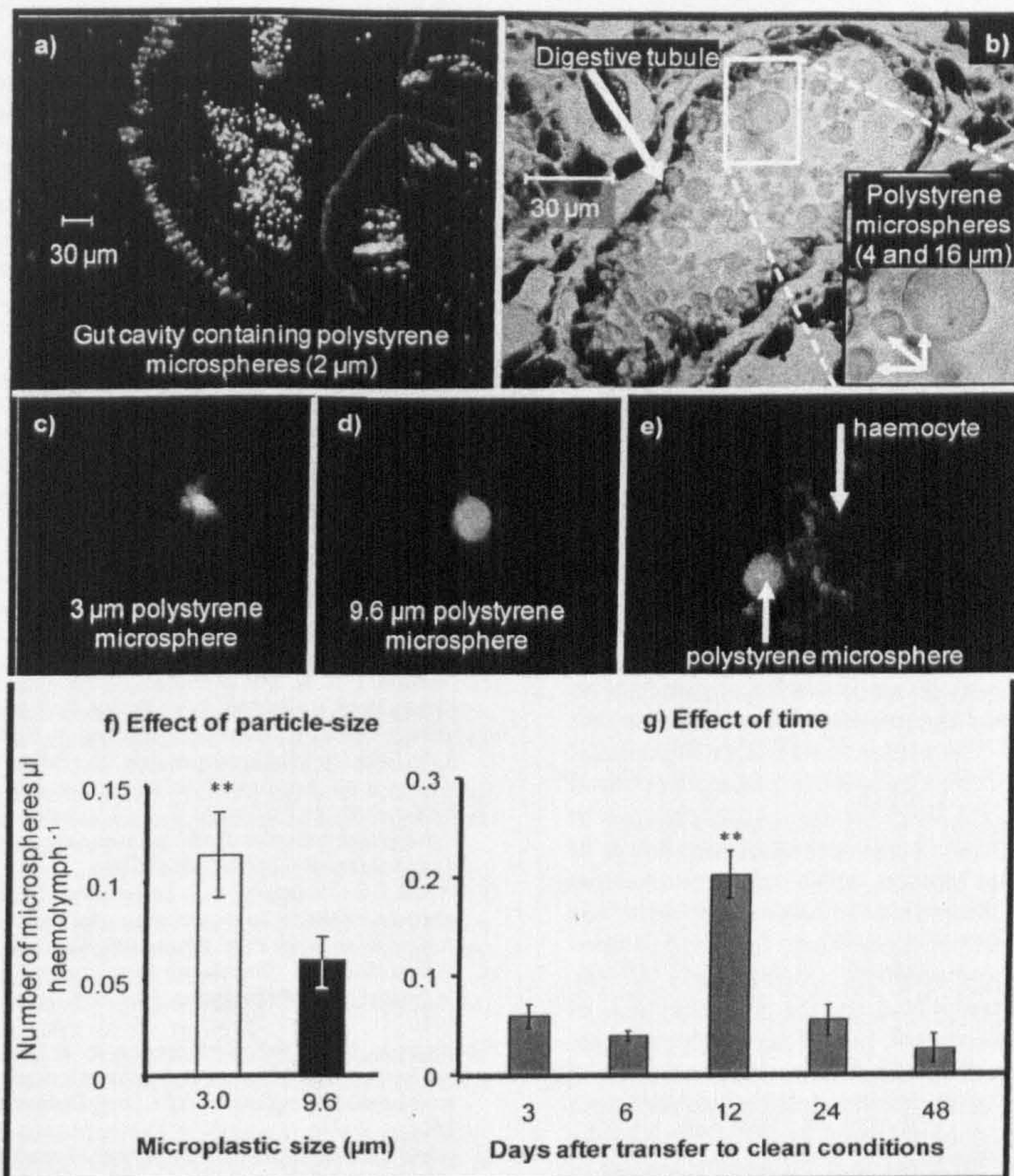

FIGURE 1. Uptake of plastic particle by *Mytilus edulis* (L.). (a) Tissue section (4 μm thick) containing 2 μm and (b) 4−16 μm polystyrene microspheres in the gut cavity and digestive tubules. 3.0 (c) and 9.6 μm (d) polystyrene microspheres in the hemeolymph and (e) hemocytes. Significant differences in accumulation in the hemeolymph according to (f) "particle-size" and (g) "time" at $P <$ 0.01. *N.B.* Values are expressed as mean ± SE and calculated from independent data from each time/treatment/replicate combination and are therefore are not cumulative.**

role. The persistence of particles of microplastic in the hemolymph of *M. edulis* for over 48 days has implications for predators, including birds, crabs, starfish, predatory whelks (*35, 36*), and humans (*37*). For larger pieces of plastic, found in seal feces, it has been suggested that the source of the material was from fish in their diet which have previously accumulated plastic; however, it remains unknown whether plastic of any size can be transferred along food chains (*38*). An alternative source of microplastic is through the abrasion of ingested plastic in the gut cavity as a result of the digestive process. For instance, some seabirds (*39*) and fish (*40*) contain particles of sediment in their gut, which combined with muscular contractions, may abrade plastic and give rise to particles of microplastic in their gut.

Particle size influenced the capacity of polystyrene to translocate from the gut cavity to the hemolymph, with over 60% more smaller (3.0 μm) microspheres in the circulatory fluid than larger (9.6 μm) microspheres (*Hypothesis III*). These results are in agreement with *in vitro* experiments using segments of colonic tissue from rats, which also showed rapid translocation of smaller (14 nm) compared to larger (415 and 1000 nm) polystyrene particles (*41*). If phagocytosis is involved, a possible model to explain the greater number of small microplastic particles is that the smaller particles are more easily phagocytosed, possibly because the phagosomes within each cell, which are finite in space, can accommodate more smaller particles. Further research is required to test these predictions and to determine the upper and lower size boundaries for ingestion and translocation of plastic debris in other organisms. The greater accumulation of smaller particles suggests that as an item of plastic degrades, the potential for it to accumulate and translocate within the tissues of the organism increases. Other physical properties of plastic particles may also affect ingestion and translocation; medical studies using humans and rodents indicate that both the shape and charge of the particles are likely to play an important role in translocation (*42*). The composition of microplastic debris in the marine habitats varies in shape (e.g., fibers, spheres, irregular fragments) and polymer type (e.g., polystyrene, polyethylene, polyester), and as an article of plastic debris degrades it will decrease in size, and monomer content and its surface properties will also change. For example, medical studies with human white blood cells have shown that the styrene content of polystyrene microspheres strongly influences their ability to be phagocytised (*43*). Further research is therefore needed to investigate the factors influencing ingestion and translocation of microplastic in a wider range of organisms.

The ingestion and translocation of polystyrene microspheres by mussels did not cause any measurable changes in the oxidative status of hemolymph, the viability and phagocytic activity of hemocytes, or filter feeding activity

(*Hypothesis IV*). It is, however, premature to suggest that ingestion and or translocation of plastic does not cause toxicological effects. Our laboratory trial exposed mussels for 3 h to 15,000 particles of one type of plastic (polystyrene) and possible biological effects were monitored for only 48 days. In their natural environment mussels will be exposed over their lifetime to various types of microplastic, including polyester, polyethylene, polypropylene, polyvinylchloride, and acrylic. Medical studies using mice injected intraperitoneally with microplastic indicate that phagocytic uptake of microparticles of polyester (polylactic acid) can lead to cellular damage, through changes in the morphology and viability of phagocytes (*44*). Consequently, research is needed to examine the toxicological consequences of longer term exposure to various plastic types routinely found in marine habitats. Particular attention is needed to assess whether microplastic can damage vital organs such as the heart and hepatopancreas. Given the open circulatory system of molluscs, hemocytes circulate throughout the animal and the connective tissue of major organs, so microplastic could be transported to these important organs. Laboratory trials with hamsters injected with 60 nm particles of polystyrene have shown in the blood stream that these particles can induce thrombosis (*45*). The presence therefore of particles of microplastic in the circulatory system may restrict blood flow causing damage to the vascular tissues and changes in cardiac activity. In addition, fragments of plastic found in marine habitats in Japan, Mexico, and North America have shown high concentrations of polychlorinated biphenyls (PBCs), dichlorodiphenyl trichloroethane (DDT), and nonylphenol polyaromatic and aliphatic hydrocarbons (*46–48*). Recent laboratory evidence has shown that sorption of phenanthrene onto microscopic particles of polyethylene, polypropylene, and polyvinylchloride was up to an order of magnitude higher than that to particles of natural sediment (*49*). Although our study does not provide evidence of uptake of these chemicals, we do show that ingested particles of microplastic can persist in the hemolymph of mussels for over 48 days and therefore could provide a route for the transport of chemicals to various tissues.

The effect of "time" observed in all of the biological assays used in our study is consistent with previous field studies for mussels (*11*) and limpets (*26*). These studies have shown temporal variability on a scale of weeks to months in the viability and phagocytic activity of hemocytes and oxidative status of hemolymph. These small-scale temporal changes in the health of organisms kept under controlled laboratory conditions support the views of Underwood (*50*) who questions the ability of laboratory conditions to minimize temporal variability in the biological responses of aquatic invertebrates in toxicity trials. Hence the possibility of using field exposure experiments to gain more ecologically robust data needs to be more widely explored in ecotoxicology (*26, 51*).

The data presented here, and that of other medical studies with rodents and humans, indicate that fragments of microplastic may translocate from the gut cavity to the blood stream in a wide range of organisms. To establish the extent to which this occurs in natural populations, and to identify any adverse effects, it will be necessary to further refine techniques to quantify small plastic fragments. For example, current methods to identify plastic debris using Fourier transform infrared spectroscopy only permit particles of plastic down to approximately 20 μm in size to be conclusively identified (*3*), but it is entirely feasible that plastic particles are now present in the environment at the much smaller nanometer scale.

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Supporting Information Available

Protocols for biological assays and temporal changes at different levels of biological organization within mussels during second exposure trial. This material is available free of charge via the Internet at http://pubs.acs.org.

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