Developmental Toxicity of Microplastic Leachates on Marine Larvae

Submitted by Flora Anna Rendell-Bhatti to The University of Exeter as a thesis of the degree of Master by Research in Biological Science, August 2019.

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

Signature: ……

Key Words: Ecotoxicology, microplastic, leachates, immunostaining, development, sea urchin, contaminants, marine pollution, additives, hydrophobic organic contaminants

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Abstract

Marine plastic pollution is now considered a diverse contaminant suite, differing in product origin, polymer composition, size, morphology, colour, additives and environmental co-contaminants. The environmental hazards associated with marine plastic pollution have been widely documented, however much of the existing research has yet to document developmental abnormalities observed when biota develop in plastic contaminated systems. The effect of microplastic leachate exposure on two marine echinoderms early developmental stages were investigated. *Psammechinus milaris* and *Paracentrotus lividus* embryonic and larval cultures were exposed to leachates derived from industrial or environmental exposed plastic pellets to investigate the effect of polymer additives and environmental contaminants. Toxicity was evaluated morphologically using images of live embryos and larvae, along with immunostaining of key developmental tissue groups to determine the extent of impact on a physiological level. This body of work suggests that leachates from pellets exposed to environmental contaminates (biobead and pre-production nurdle pellets) and highly plasticised industrial pellets (polyvinyl chloride) elicit severe, consistent and treatment-specific phenotypes in *P.* lividus embryonic and larval developmental stages, with impacts on morphogenic processes. Key differences in larvae morphology were documented between plastic types and environmental exposure. Industrial polyvinyl chloride pellets elicited the most pronounced abnormalities from the wild type at 24 hours post-fertilisation. However, leachates from un-plasticised industrial polyethylene pellets showed little differences from the wildtype with regards to developmental timing and abnormalities. Leachates from environmental sourced pellets elicited the most severe developmental delays and abnormalities at 48 hours post-fertilisation. Preliminary chemical analysis was also performed on industrial and beached pellet leachates, to investigate compound composition and to determine possible contributors to the developmental defects. To summarise, the findings suggest industrial and environmental microplastic leachate exposure elicits morphological malformations and specific abnormalities in neural, cilia and muscle tissues groups in both embryonic and larval stages of marine larvae of *P. lividus*. However, more research and investigation are needed to draw conclusive data.

Acknowledgements

I would like to thank my primary supervisor, Dr Eva Jimenez-Guri, for her continued support and guidance throughout the entirety of this research project. Huge thanks are also given to Professor Brendan Godley for invaluable academic support and advice. I would also like to thank Dr Tamara Galloway for her support. Great thanks go to Rob Arnold for his hard work and motivation in tackling marine plastic pollution, and for kindly contributing beached pellets from Tregantle used in studies. Thank you to Claire Wallerstein for your incredible knowledge, hard work and support. Thank you, Chris Mitchell, for your kind willingness to help and contribute to this body of work. Huge thanks go to Tim and John Hammond at Northern Polymers and Plastics. Great thanks also go to FIDRA and NGO for providing plastic pellets used in the preliminary studies. Thank you to ASSEMBLE Plus for funding the work carried out with *P.* lividus. To everyone at Stazione Zoologica Anton Dohrn, Napoli, you have been amazing. Dr Ina Arnone and Dr Salvatore D'Aniello thank you for hosting our visits, your time and the delicious parmigiana (thank you Rosa)! Thank you to Ines for hosting me and making sure we had a great time in Napoli. To the Sea Urchin Lab - Periklis, Ina, Danila, Maria, Ines and Yovanna your guidance has been invaluable.

The use of facilities and materials at the University of Exeter, Penryn and Streatham Campus during this project was greatly appreciated. Huge thanks go to Dr David Santillo, at the Greenpeace Laboratories, for all his guidance, patience and help. Many thanks to Matt Slater at The Cornwall Wildlife Trust for his advice on collecting Green Sea Urchins around Cornwall. To my family and Colin Mulvey for their assistance with many hours of field collections, in the wind and rain, and unwavering support and encouragement with everything I do - thank you. Finally, thanks go to all those who are working invaluably hard to protect and give a voice to ecosystems which we so deeply depend.

It is important to conduct scientific research and to advance our knowledge on anthropogenic threats. However, I believe we all have a duty to recognise and publish the carbon footprint of our work and the consequences of scientific research. To compose the final chapter of this thesis, air travel to and from Naples was necessary to gain access to *P. lividus* sea urchin larvae. Two return flights for myself and supervisor, Eva, were taken (643 kg of CO_2). As a result of samples being destroyed during transport from the first trip to Naples, I returned to SZN in May to re-run the experiments (292.8 of CO_2). The total calculated carbon footprint from air travel was 935.8 kg of carbon dioxide released into the atmosphere, which equates to two average adult male polar bears. These emissions were offset, however recognising and reducing air travel within the academic community needs to be at the forefront of our work.

Author's Declaration

The main body of the experimental work was carried out at Stazione Zoologica Anton Dohrn, Napoli, within the Laboratory of Cellular and Developmental Biology (Ina Arnone and Salvatore D'Aniello lab groups) and supported by ASSEMBLE plus grant. The instruments and knowledge available at the SZN were instrumental in the success of the study. I co-wrote the grant for the ASSEMBLE plus grant with Dr. Eva Jimenez-Guri. Eva Jimenez-Guri devised fundamental ideas, with additional contributions from myself. Chris Mitchell completed the method development and SPME analysis of the microplastic leachates. Under the supervision of Eva, I completed coordinated the project, completed the laboratory work, data analysis, thesis compilation and formatting. Eva aided in producing some of the figures within this report. Professor Brendan Godley provided guidance and comments throughout the project. Ina Arnone provided many of the morphological descriptions of the phenotypes observed.

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Definitions and Abbreviations

Antibody: proteins that are used by the immune system to identify and neutralise foreign objects

Blastocoel: fluid-filled cavity that forms in the embryo after the morula stage

Blastula: stage of embryonic development of animals near the end of cleavage but before gastrulation

Bioavailability: extent to which a compound enters tissues and reacts with biological molecules

Cleavage: first few cellular divisions of a zygote

Ecotoxicology: investigates the impact of toxic substances on the biota

Epitope: specific part of an antigen that is recognised by the immune system, where an antibody binds

Fluorophore: absorbs ultraviolet light and emits light of longer wavelength, visualised via fluorescent microscopy.

Gastrulation: process by which cells of the blastoderm are translocated to new positions in the embryo, producing the three primary germ layers

Germ Layer: primary layer of cells that forms during embryonic development

Ingression: individual cells leave an epithelial sheet and become freely migrating mesenchyme cells

Partitioning: transfer of chemical between any types of phases

Plasticisers: additives that enhance that plasticity or fluidity of a material

Plastisphere: prokaryotic and eukaryotic communities on microplastics

Pluteus: sea urchin larva

Syncytium: multinucleated cell that can result from multiple cell fusions of uninuclear cells

Sorb: to take up and hold by either absorption or adsorption.

Sorption Equilibrium: the final state, in which there is no net transfer of the chemical between the two phases

Vegetal plate: flattened plate of cells at the vegetal pole of the early gastrula (see definition figure of sea urchin)

Wildtype: the phenotype of the typical form of a species as it occurs in nature

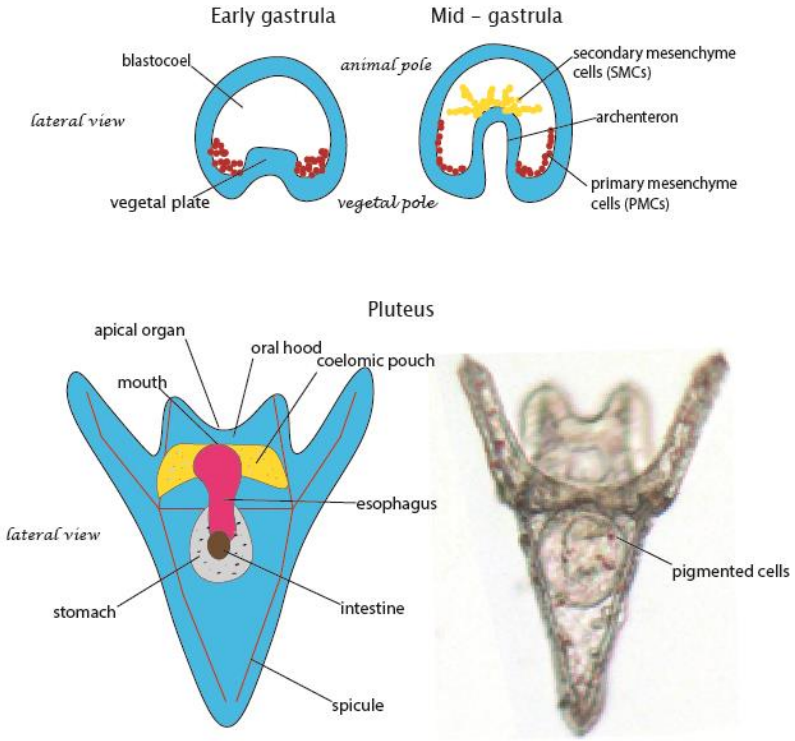
ASW: artificial sea water

PA: polyamide

PE: polyethylene

PET: polyethylene terephthalate

Sea Urchin Embryonic and Larval Stages



Definition fig.1: Sea urchin embryo (gastrula) and larva (pluteus) definition figure, annotations identify key structures referred to throughout the thesis. The final gastrula (late) gives rise to the pluteus larva of the sea urchin.

PS: polystyrene

PP: polypropylene

HOCs: hydrophobic organic contaminants

POPs: persistent organic pollutants

PBDE: polybrominated diphenyl esters

PBS: phosphate-buffered saline

PCB: polychlorinated biphenyl

PMCs: Primary mesenchyme cells

SMCs: Secondary mesenchyme cells

HPF: hours post fertilisation

DPF: days post fertilisation

FSW: filtered sea water

WWTP: wastewater treatment plant

WEEE: waste electrical and electronic equipment

Chapter One

Literature Review: Microplastics and Co-contaminants in the Marine Environment

1.1 Introduction: The Age of Plastic

Humanity is collectively mismanaging the ocean to the brink of collapse; fisheries stocks are under pressure, trophic webs are breaking down, plastics and pollution are choking ecosystems, the ocean is becoming a busy place and repercussions are already in effect (McRae, 2015). The pace of marine defaunation, or human-caused animal loss in the ocean is quickening. Yet, the ocean is fundamental to life on Earth, underpinning economies and business, as well as sustaining livelihoods and the well-being of billions of people worldwide (Gascon *et al*.,2015). Industries are intensifying to meet the demand of an ever-increasing human population, exploiting our marine resource and moving into deeper waters in search for fish and minerals. Human impact on the earth has been so profound that some believe a new planetary epoch needs to be declared (Zalasiewicz, 2012): The Anthropocene. Plastic pollution is one of the key candidates as evidence for the onset of the Anthropocene, along with increases in carbon dioxide and methane (Steffen, 2011). Anthropogenic pressures on our marine environment can be far reaching and devastating (Halpern *et al*., 2007), especially given the lack of knowledge we have of this vast, complex environment.

Marine debris, such as plastics and marine persistent pollutants, is seen as a key anthropogenic influence, which we are only just starting to truly understand (Gall, 2015). Plastic pollution is now recognised as a pervasive pollutant in both freshwater and marine aquatic systems; estimates suggest that plastic accounts for 73% of marine litter globally (Bergmann, 2017) However, of the estimated 8 million tons of plastic which moves from land to sea each year (Jambeck *et al*., 2015), only approximately 15% of marine debris is washed ashore (Marlin Baltic Marine Litter, 2014). This highlights that we are yet to identify some of the major sinks of oceanic plastic debris.

Large gaps remain in our knowledge on environmental plastic pollution (Syberg *et al*., 2015; Horton *et al*., 2017). To understand whether plastic pollution poses a threat to life on earth, we must understand the composition, quantity and physical form of the range of plastic pollution in the aquatic and marine environment (Rochman *et al*., 2016). We must twin this knowledge with understanding the individual-level biological effects of such a pollutant, and the subsequent far-reaching ecosystem level effects (Syberg *et al*., 2015). As a novel stimulus, plastic has the ability to threaten ecosystems and biological processes, and ultimately ecosystem services we rely on as a global community.

The aim of this review is to firstly to discuss the extent of microplastic pollution in the marine environment, followed by a discussion of our current understanding of the impact of microplastic pollution and associated co-contaminants on marine biota. Using the following literature review as a foundation, experiments were then devised and conducted to address evident knowledge gaps within the field of microplastic research. In combining the study of the effects of microplastic pollution with developmental biology, this work seeks to address knowledge gaps. I hope this body of work will aid in broadening our understanding of the impacts of microplastic pollution on key biological processes and the mechanisms behind the abnormalities microplastic pollution elicit.

1.2 Marine Microplastic Pollution

Marine microplastic pollution is a complex environmental pollutant, made up of many different constituent chemical mixtures and entering the marine environment from multiple different sources. Plastic is not readily degraded or processed by microorganisms (Yoshida *et al*., 2016). Instead plastics are broken down mechanically or by photo and thermal-oxidative degradation, into microplastics (<5mm; Arthur *et al*. 2009) and further into nanoplastics (<0.1 μm). It is important to note that the definition of microplastics and how they are defined as a pollutant is constantly changing within the field, based on the relatively young research area with frequent in-flow of scientific findings altering the way in which we see such a complex pollutant (Lusher *et al*. ,2017). Rochman *et al*. (2019), has recently suggested the necessity to rethink microplastics and consider them as a diverse contaminant suite. Rochman *et al*. (2019) and Hartmann *et al*. (2019) suggest the need for an establishment of universal criteria for identifying microplastics, with current ambiguous terminology resulting in confusion and miscommunication. Hartmann *et al*. propose the following categories: (i) nanoplastics: 1 to <1000 nm; (ii) microplastics: 1 to <1000 μm; (iii) mesoplastics: 1 to <10 mm; (iv) macroplastics: 1 cm and larger. However, for the purposes of this thesis the definition of microplastics as <5mm in diameter, proposed by experts at a NOAA workshop in 2008 will be used, as this was the most frequently used definition during the writing of this thesis.

Microplastics are classified as primary or secondary, depending on their origin. Primary microplastics are intentionally produced within the <5mm size range, the main identified sources are microbeads used in cosmetics, domestic and industrial-strength cleaners. The main polymer type for these purposes is PE and PP (Mrowiec, 2018). Primary microplastics enter marine environments mainly from terrestrial-based activities, these include wastewater treatment plants (WWTP) (Mrowiec, 2018), agriculture and green construction run-off. In contrast, secondary microplastics are formed during the degradation of macroplastics due to photolytic, mechanical and chemical degradation of larger plastic products or fragments. Key

sources of secondary microplastics contributing to marine plastic pollution, are loss from landfill sites and waste collection, due to inappropriate management, storm sewers, wind, currents, runoff (Auta *et al*., 2017; Mrowiec, 2018).

Microplastics have become ubiquitous in all marine environments, from the ocean surface, water column to deep sea and coastal sediments (Andrady, 2011; Cole *et al*., 2011; Van Cauwenberghe *et al*., 2013), and from the remote habitats in the Artic (Obbard *et al*., 2014; Lusher *et al*., 2015; Cozar *et al*., 2017; La Daana *et al*., 2018, Peekan *et al*., 2018) to the Antarctic Oceans (Isobe *et al*., 2017). Microplastic pollution has recently been evidenced at depths of 10,898 m in the Mariana trench (Chiba *et al*., 2018). The scale of global plastic pollution within our oceans is difficult to quantify, and therefore there are few data on the subject. The most robust global data set available uses a combination of sampling methods, over a 6-year period to produce an estimate for four class sizes of marine plastic pollution: marco (>200 mm), meso (3.76-200 mm), micro plastic (0.33-1.00 mm and 1.01-3.75 mm) (Erikson *et al*., 2013). Erikson *et al*. (2013) estimates suggest at least 5.25 trillion plastic particles dispersed throughout our oceans, with 92.4% of this being surface microplastics. Other estimates suggest between 15-51 trillion particles of floating plastics are present across the world's surface oceans (van Sebille *et al*., 2015).

Recent research by Tekman *et al*. (2018) highlight that microplastics can easily be vertically transported and prevail from the sea surface to the deep seafloor in western Svalbard. However, Tekman *et al*. witnessed a decrease in microplastics throughout the water column. Tekman *et al*. also identified 15 different polymer types, with polyamide accounting for the largest proportion at 28%. This research suggests that microplastic pollution is a complex pollutant and predicting the behaviour of such a heterogenous material in a diverse environment is difficult. In addition to the complex nature of microplastics, most literature base estimates on surface measurements using Neuston nets, often with a mesh size of 333 µm. Therefore, recent reviews highlight that we are underestimating the microplastic contamination in aquatic environments (Conkle *et al*., 2018). Smaller size classes than the standard 300 µm mesh size may beunrecorded, as Conkle *et al*. show that ~80% of 50 published aquatic surveys only account for plastics larger than 300 µm.

Microplastic particles in aquatic environments are highly heterogenous and differ in shape, size, colour, density and chemical composition. The most abundant types of polymers found in marine pollution are PE, PVC, PA and PET (Rehse *et al*., 2018; Murrell *et al*., 2018). Depending on the composite material and particle size, plastic pollution occupies different areas of the water column. Plastic with densities less than water, such as PE, tend to float on the water surface. Those plastics with densities heavier than water can sink to bottom sediments, these

include PVC, nylons and polyethylene terephthalate (PET). The persistent properties of microplastics accelerate dispersal in the marine environments through ocean currents and hydrodynamic processes (Claessens *et al*., 2011). The heterogeneity of microplastics makes not only quantification a challenge, but also hinders strategies to prevent microplastics entering our oceans (Syberg, 2015a).

In addition to direct physical impacts on biota, recent research suggests that global carbon dynamics of pelagic environments may be affected by the build-up of microplastics surfaces, acting as new niches for aquatic microorganisms. This emerging novel environment, the plastisphere, will inevitably increase with increasing microplastic pollution and could affect the dynamics of the carbon cycle by altering heterotrophic activities (Arias-Andres *et al*., 2018).

1.3 Biological Impacts of Marine Microplastics

Microplastic and synthetic particles are evidence to be ingested by organisms occupying six of the deepest marine ecosystems on Earth, suggesting that there are no marine ecosystems left that are not impacted by plastic pollution (Jamieson *et al*., 2019). Ubiquitous, persistent and heterogenous microplastics have been identified as one of the main anthropogenic threats to marine ecosystems, due to the effects they elicit through multiple mechanisms, throughout multiple life stages and levels of the food web (Messinetti,2017a; Hale, 2017). More than 600 species have been reported to have had some form of interaction with microplastics, with over 300 of these being found to have ingested plastic (Galloway *et al*., 2017). The heterogenous nature of plastics poses a significant problem for marine biota, the various shapes, sizes and polymer type of plastics all affect the way in which this contaminant interact with organisms. Microplastic and nanoplastics are of particular concern, due to their increased bioavailability to a larger range of organisms throughout the food chain given their small size (Cole *et al*., 2011). As a novel stimulus, ingestion of microplastic is most often accidental (Auta *et al*., 2017) and can therefore have significant impacts on marine life; from restricted feeding ability (Ogonowski *et al*., 2016), intestinal damage (Lei *et al*.,2018), reproductive impairment (Sussarellu *et al*., 2016), inflammation (Wright *et al*., 2013a; Wan *et al*., 2018), hepatic stress (Rochman *et al*., 2013), decreased growth (Setala *et al*., 2016; Ziajahromi *et al*., 2018) and even death (Butterworth *et al*., 2012; Cole *et al*., 2016).

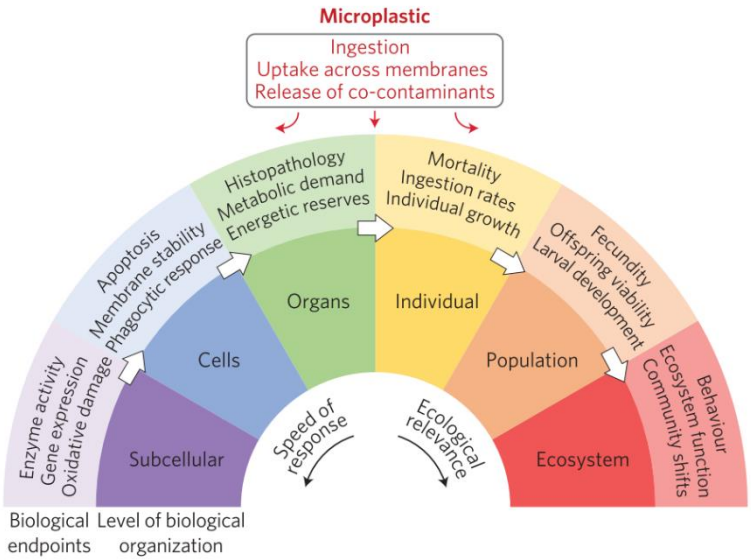


Fig.1.1 Microplastics act on multiple levels and elicit effects from subcellular to ecosystem effects. Figure from *Galloway et al., 2017.*

A complex pollutant, microplastics can have wide ranging implications on marine life, specifically when primary producers can be some of the worst affected. For example, Foley *et al*. (2018) meta-analysis suggests that zooplankton are among the most susceptible marine biota to microplastic exposure, which has wide implications for marine food webs. Foley *et al*. suggest that if recruitment of populations which play a role in structuring marine food webs, such as copepods, sea urchins and amphipods are negatively impacted, then this may have potential effects throughout local food webs.

Microplastics have the ability to act across all levels of biological organisation, even down to cellular and sub-cellular level (fig.1.3). It is important to document and understand these risks across all levels to inform management and conservation strategies of threatened marine species. Cellular level effects which microplastics elicit, include abnormal cell development and damage, cell death and an altered immune response (Galloway *et al*., 2017). Microplastics have been evidenced to act on a subcellular level, causing DNA damage and altered gene expression (Capolupo *et al*., 2018; Lei *et al*., 2018), enzyme activity and oxidative responses (Galloway *et al*., 2017; Jeong *et al*.,2017). For example, recent data from Capolupo *et al*. (2018) evidences the onset of transcriptional impairments elicited by microplastics at 50 and 500 microplastic mL^{-1} in mussel larvae, *Mytilus galloprovincialis*. Capolupo *et al*. report upregulation of genes involved in shell biogenesis and immunomodulation and the inhibition of those genes coding for lysosomal enzymes. However, the researchers did not observe morphological or feeding abnormalities within the mussel larvae. Research such as this highlights the sub-lethal effects which microplastics elicit, potentially increasing marine larvae vulnerability to other environmental stressors.

1.4 Microplastic Co-contaminants

In addition to the physical presence of a novel stimulus in the marine environment, microplastic pollution has been strongly linked to toxic contaminants. Microplastic pollution can contain two types of contaminants: (1) additives originating from the plastic itself, and (2) chemicals sorbed from the surrounding seawater. This section will provide an overview of these two types of contaminants associated with marine plastic pollution.

1.3.1 Toxic Plastic Additives

The plastics we use in our everyday lives are rarely pure substances. Instead, chemical additives, such as plasticisers, fillers, stabilisers, flame retardants, colourants, antioxidants, foaming agents and antimicrobials are intentionally added to the polymer matrix to give the plastic its desired physical, chemical or mechanical properties (Teuten *et al*., 2009; Groh *et al*., 2019). Many are added in substantial quantities, up to 60% by weight in some cases (Meeker *et al*., 2009; Navarro *et al*., 2010). Since additives are not covalently bound to the polymer matrix, they can leach out from polymer matrix under certain physiochemical conditions, such as a change in temperature, UV levels and/or pH (Lithner, Nordensvan and Dave, 2012; Law and Thompson, 2014).

Recent research by Groh *et al*. (2019) reveal the extent of hazardous chemicals associated with plastic packaging. Groh *et al*. complied a comprehensive database (CPPdb) and found 906 chemicals likely associated with plastic packaging, with 148 of these chemicals known to be hazardous, 68 of these were considered environmental hazards. Additives are potentially toxic and can have adverse effects in animal and human populations (BPA, phthalates). The central issue with additives relates to the uptake and accumulation by living organisms. Of particular concern are bisphenol A (BPA), phthalate plasticisers, anti-microbial agents and flame retardants.

The global plastics additives market is expected to stand at $57.8 billion dollars by 2020, growing at a rate of 3.4% between 2013-2020 (Allied Market Research, 2014). Despite their beneficial properties within the polymer matrix, certain chemicals may also act as endocrine disrupting compounds (EDCs), which could lead to adverse reproductive and developmental effects in marine life, and even humans (Meeker and Sathyanarayana, 2009). Chemical additives have been detected in human tissue (Talsness *et al*.,2009), causing concern for the potential impacts to human health. The diversity of microplastics and their constituents is complex and highly heterogenous in environmental samples.

One key example of a widely used additive is phthalates (PAEs) or phthalic acid esters, a group of man-made chemical compounds primarily used as plasticisers in the manufacture of everyday plastics, building materials, children's toys and cosmetics to improve their flexibility (Heudorf *et*

al., 2007). As an additive to the polymer matrix, PAEs are not covalently bound to the plastic, increasing likelihood of leaching and exposure of the hazardous chemical in the environment (Cacho *et al*., 2012; Frajzadeh *et al*. 2012). Di (2-ethylhxyl) phthalate (DEHP) is a type of phthalate most commonly used as a plasticiser to improve flexibility in polyvinyl chloride (PVC). DEHP is weakly bonded to the polymer material and also characterised as a highly lipophilic molecule, further enhancing the leaching of DEHP into the surrounding environment (Rose *et al*., 2012). PVC products are ubiquitous in our everyday lives, uses include a wide range of building materials, household furnishings, food packaging, cars, lubricants, waxes, insecticides and cleaning materials. In 2003, more than 800, 000 tons of PAEs were used in Western Europe (Heudorf *et al*., 2007). Some PVC plastics can contain close to 50% by weight of PAEs (Freire *et al*., 2006; Kwakami *et al*., 2011).

Once released into the environment, these compounds have the potential to be transported long distances (Mankidy *et al*., 2013). Environmental contamination of PAEs can therefore be detected in the aquatic environments, dust and in the air (Rudel *et al*., 2001, 2003). Recent research has highlighted toxicological concerns with regards to phthalates acting upon biological process. These include effects on reproduction (Ye *et al*., 2014), infertility (Tranfo *et al*., 2012; Mankidy *et al*., 2013), acting as endocrine disruptors (Gray *et al*., 2000), along with mutagenic and carcinogenic activity (Harrison, Holmes and Humfrey, 1997). In developmental studies by the National Toxicology Program Centre for the Evaluation of Risks to Human Reproduction, effects such as increases in prenatal mortality, reduced growth and birth weight, skeletal, visceral, and external malformations were discussed as possibly associated with PAE exposure (Heudorf, Mersch-Sundermann and Angerer, 2007). Because of their chemical properties, exposure to PAEs does not result in bioaccumulation. Nevertheless, health concerns are raised around the developmental toxicity of PAEs, not only on the basis of results of animal experiments but also under consideration of recently published human studies (Heudorf, Mersch-Sundermann and Angerer, 2007). Furthermore, when significant proportions of PAEs are added to the polymer matrix, the application of biocides is necessary, because PAEs are known to be prone to microbial attack (Groh *et al*., 2019). Most biocides are classified as environmental hazards, and although usually only added in small amounts, in the case of PVC, the use of biocides can be higher to prevent microbial degradation of PAEs (Groh *et al*., 2019).

1.3.2 Persistent Organic Pollutants

The marine environment is a complex mix of organic contaminants. Anthropogenic actions have increased levels of toxic contaminants, from polyaromatic hydrocarbons (PAHs) to polychlorinated biphenyls (PCBs) (Bakir,2012), in the ocean. Persistent organic pollutants (POPs), are organic compounds of natural or anthropogenic origin, with particular chemical and

physical properties which are able to persist in the environment for a long time. With the ability to migrate though air, water and soil, POPs accumulate in environmental media leading to levels which could harm to biota (Wania and MacKay, 1996; El-Shahawi, 2010). POPs are highly resistant to photolytic, chemical and biological degradation, which coupled with their long-range transport and deposition, results in POPs being of particular concern with regards to toxicity in food chains and polluting seemingly pristine environments (El-Shahawi, 2010; Jamieson *et al*.,2017).

POPs are divided into 3 categories; pesticides, chemicals and unintentionally produced by-products, such as polycyclic aromatic hydrocarbons (PAH). Certain POPs fall under the hydrophobic organic contaminate (HOC) class of chemicals due to a high hydrophobicity that results in a high binding affinity to organic or inorganic particles present in the water column (Jamieson *et al*., 2017). Specific POPs also have high lipophilicity, which results in these contaminants bioaccumulating in organisms, with cumulative increases at each trophic level (Jamieson *et al*., 2017).

POPs are of particular concern for human health and the environment, consequently being banned or phased out in many countries (Engler, 2012). An example of this is all 209 variations of PCBs are now known carcinogens (Lauby-Secretan *et al*., 2013). PCBs were commercially produced for a variety of applications, including dielectric fluids for capacitors and transformers, hydraulic fluids, lubricating and cutting oils, as well as additives in pesticides, paints, plastics and adhesives (Robertson and Hansen, 2015). This class of chemicals, which are known to be prolific in the marine environment (Wania and Mackay, 1996) are a key concern due to their known and unknown environmental effects. For example, PCBs, are known to cause cancer, suppress the immune system, disrupt hormonal signals, and impair reproduction (Robertson and Hansen, 2015). Despite PCBs being banned for more than thirty-five years, they have been leached into the ocean for decades and their stability has allowed them to continue to impact marine populations, even in the most remote and inaccessible habitats on earth. PCBs have been detected in *Hirondellea giga*, a tiny amphipod which resides in some of the deepest waters of Earth's ocean trenches (Jamieson *et al*., 2017), and linked to the decline in world populations of Orcas (Desforges *et al*., 2018).

Plastics are made of highly hydrophobic, lipid-rich, stable polymers, which increases the likelihood of environmental contaminants concentrating onto their surface (Liu *et al*.,2019). Once marine plastic pollution breaks down via biotic and abiotic factors, the overall surface area to volume ratio increases, which further increases the sorption of hydrophobic pollutants (Ivar do Sul and Costa, 2014) and metals (Ashton, Holmes and Turner, 2010; Cole *et al*., 2011) onto the surface. In addition, plastic pellets are most commonly composed of low-destiny polymers,

which causes them to float on the sea surface. This increases the affinity of pellets to sorb contaminants from the microlayer, where hydrophobic contaminants (Teuten *et al*., 2009; Ogata *et al*., 2009) and metals (Wurl and Obbard, 2004) are known to be enriched in relatively high concentrations.

Hydrophobic organic contaminates (HOCs) have been shown to display strong sorption behaviour, concentrating onto microplastic fragments from seawater up to six orders of magnitude greater than that of the surrounding seawater given the correct conditions (Mato *et al*., 2001; Endo *et al*., 2005; Rios *et al*., 2007; Hirai, 2011). Measured environmental concentrations of HOCs sorbed to plastics range from less than a part per billion (nanograms per gram) to parts per thousand (milligrams per gram) (Ziccardi, 2016). Variability of HOC types and concentrations between individual pellets can be high, even within a single environmental sample (Ziccardi, 2016). Most studies to date have focused on quantifying the best-known environmental contaminants, which include PCBs, DDTs, HCHs and PAHs (Rios *et al*., 2007; Ogata *et al*., 2009; Hong *et al*., 2017). Among others the main pollutants which have been shown to sorb onto marine plastic debris are polybrominated diphenyl ethers (PBDEs) and perfluorinated surfacants (PFCs) (Llorca *et al*., 2014). POPs sorb to plastic at a wide range of concentrations depending on the contaminate and location (Ogata *et al*., 2009; Hirai *et al*., 2011).

Different polymer types have variability in sorption of POPs, for example Endo *et al*. (2005) identified that PE pellets have an increased affinity for PCBs than other polymer types, which is consistent with long term field studies. Rochman et al. (2013) completed a 12-month field experiment to quantify the sorption rates and concentrations of PCBs and PAHs to five most common types of plastic. The authors found that concentrations of PCBs and PAHs sorbed to high and low-density PE and PP were consistently higher than concentrations sorbed to PET and PVC (Rochman et al., 2013). Rochman et al. (2013) data highlights the importance in identifying the polymer type of beached plastic being used in toxicity studies, with a greater risk of concentrated hazardous chemicals posed from those beached plastics composed of PE and PP. Frias *et al*. (2010) analysed black, white, coloured and aged pellets separately for PCBs, polycyclic aromatic hydrocarbons (PAHs) and DDTs. Black pellets exhibited higher concentrations of PCBs than aged pellets, possibly because they have higher adsorption rates (Frias *et al*., 2010).The "vector effect" is described as the ability for plastic pollution to act as a vector for the transport of POPs and other chemicals though ecosystems (Ziccardi *et al*., 2016). It is important to note that only hydrophobic compounds are shown to consistently sorb to particles (Seidensticker *et al*., 2018), and studies indicate a variable and complex interaction (Koelmans *et al*., 2013). Other substrates may provide a greater influence on the bioaccumulation of pollutants, however the

sorption of pollutants to plastics may aid in the transfer of pollutants over distances, when compared to those associated with denser sediment (Nizzetto *et al*., 2016a).

The dispersive nature of POPs and given that some are still used globally, presents difficultly with clean-up and prevention strategies of chemicals entering aquatic systems worldwide and adhering to microplastics. However, there is limited evidence to support the occurrence of ecologically significant adverse effects on aquatic marine life as a result of exposure to HOCs sorbed to microplastics (Ziccardi, 2016). Current research evidencing the effects of HOCs sorbed to microplastic are limited to seven experiments on four different taxa: Polychaetes (Besseling *et al*., 2012; Browne *et al*., 2013); Fish (Oliverira *et al*.,2013; Rochman *et al*., 2013,2014); Bivalves (Avio *et al*., 2015) and Sea Urchins (Nobre *et al*., 2015). Collectively these studies suggest the effects observed are likely to be caused by the plastics or plastic additives themselves as opposed to sorbed HOCs. Current data do not support a strong conclusion that HOCs sorbed to microplastic pose significant ecological risk, however a substantial data gap exists in our understanding of a mechanistic basis for both leaching potential of HOCs and resulting defects in marine organisms. To conclude, some predict that plastic debris and HOCs form a complex cocktail, which increases the overall bioavailability of HOCs to aquatic biota (Vethaak and Leslie, 2016). On the other hand, some suggest that microplastics play a minor role as a vector for contaminants when compared to natural exposure pathways, such as diet and sediments (Hartmann *et al*., 2017).

1.3.3 Metal Contaminants

The additives associated with plastics are wide-ranging and potentially hazardous. Research by Groh *et al*. (2019), revealed a prominent group of hazardous plastic additives consisting of substances that contained metals. These included cadmium, chromium, lead, mercury, cobalt, tin and zinc (Groh *et al*., 2019). Metal-containing substances are often used as colourants, antimicrobials and accelerators, for example, cadmium and zinc containing substances have been used as stabilisers in PVC (Groh *et al*., 2019). Despite regulations to prevent the use of hazardous metals, toxic metals such as cadmium, have been detected at levels exceeding the regulatory limits in some PVC samples (Groh *et al*., 2019). Groh *et al*. (2019) highlight that the presence of heavy metals in plastic packaging originates from areas of the world where regulation is non-existent, insufficient or there is a lack of enforcement. One example they point to, is the levels of cadmium, chromium, cobalt and lead contaminating food cooked in PE bags in Uganda (Groh *et al*., 2019).

Within the marine environment it was previously thought that marine plastic pollution was relatively inert with regards to sorption of aqueous metal ions. Metals enter aquatic environments through industrial waste, antifouling paints and fuel combustion (Auta *et al*.,

2017). However, recent research has highlighted the potential for microplastics to sorb heavy metals from aqueous environments (Ashton, Holmes and Turner, 2010; Holmes, Turner and Thompson, 2012; Rochman *et al*., 2013; Boucher, Morin and Bendell, 2016; Prunier *et al*., 2018; Li *et al*., 2019). Beached plastics, including PVC and PS, have been found to contain multiple heavy metals, including lead, mercury and cadmium (Filella and Turner, 2018; Munier and Bendell, 2018), whether these originate from the additives within the polymer matrix or from sorption at sea it is hard to conclude. Aged, beached pellets have been observed to accumulate trace metals to a significantly greater extent, than virgin PE pellets (Holmes, Turner and Thompson, 2012). Despite the knowledge that plastic fragments are able to sorb metals present in the environment, some authors have suggested that the metals detected could be ''legacy'' chemicals contained within the polymer matrix, originating before regulatory restrictions on metal additives (Filella and Turner, 2018; Groh *et al*., 2019).

Recent studies suggest that microplastic particles can accumulate metals at similar rates to both estuarine sediments and suspended sedimentary particles (Duarte *et al*., 2010; 2014). Metals have a high affinity to organic polymers, of which microplastics are composed (Ashton *et al*., 2010; Rios *et al*., 2007). Metal sorption kinetics can estimate adsorption of metals to plastic. Virgin and beached polethylene reached equilibrium within 25-100 h for adsorption of metals from seawater, with beached pellets having a greater adsorption capacity than virgin pellets (Holmes, Turner and Thompson, 2012). As with hydrophobic organic contaminates, the adsorption capacity of metals probably increases for beached pellets, due to increasing surface area and anionic active sites, as a result of increased weathering and fouling (Holmes *et al*., 2014; Endo *et al*., 2005; Ogata *et al*., 2009). PVC fragments and PS beads have been evidenced to adsorb zinc and copper, which had been leached from antifouling agents into seawater (Brennecke *et al*., 2016). Brennecke and collaborators showed a significant interaction between microplastic and heavy metals and the key role of microplastic as vectors for heavy metal ions in marine ecosystems. PVC fragments accumulated Cu and Zn to a greater extent without reaching equilibrium. In addition, black plastic, in particular, has been shown to contain a higher frequency of hazardous chemicals such as bromine, antimony and lead as a result of manufactures using recycled electrical equipment (Turner, 2018). Brominated compounds have been identified in non-electrical black consumer products, which suggested a high level of hazardous chemical could reside in the black biobeads so commonly found around our shores. This was confirmed by using x-ray fluorescence (XRF) spectrometry of beached black biobeads by Turner (2018), who identified Br (298 ppm), Cd (85.6 ppm), Cr (53.9 ppm), Pb (77.8 ppm) and Sb (784ppm) in 108 pellets collected from beaches in the southwest (Turner, 2018). This suggests

that black plastic pollution potentially poses a greater risk of chemical exposure to marine organisms, when compared to other types of plastic pollution.

Biofilms present on microplastic have recently been shown to facilitate metal accumulation onto microplastics in estuarine waters (Richard *et al*., 2019). This area of research is poorly understood, however the findings of Richard *et al*. indicate the complex nature of plastic within the marine environment, with the ability to potentially alter the fate of xenobiotics in aquatic ecosystems. The cumulative effect of both heavy metal and microplastic pollution, and their interaction could have significant implications for marine biota and ecosystems. Marine invertebrate larvae have been shown to be sensitive to metal compounds. The larvae of *Echinometra mathaei* (echinoidea) and *Isognomon californicum* (bivalvia) have been evidenced as very sensitive to cadmium toxicity (Ringwood, 1992). It has also been suggested that heavy metals adsorbed to plastic pellets are highly bioavailable (Holmes, Turner and Thompson, 2012). It is important to note that the leaching potential of metals from plastic and sorption to plastic is not strongly evidenced and the research is still in its infancy, however recent developments are highlighting this as a potential issue within the marine environment (Nakashima *et al*., 2016)

1.3.4 Co-contaminate Sorption and Leaching Capacity

The sorption of contaminants encompasses both the adsorption of the chemical to the surface of the particle, and the absorption of the chemical into the particle. However, information to differentiate between absorption and adsorption is often unavailable; therefore, the general term of sorption is used to refer to the relationship between the chemical and the microplastics (Ziccardi, 2016). The portioning of HOCs to plastics has been well documented in lab studies. Sorption and desorption of HOCs from microplastic is governed by the intrinsic properties of the interacting compartments. The factors that contribute to ability of microplastics to concentrate contaminants is the hydrophobicity and lipophilicity of organic compounds and the high surface-volume ratio of microplastics. (Liu *et al*., 2016). Variability in the chemical and physical properties of microplastic, which include surface area (diffusivity) and hydrophobicity (polarity), means sorption rates of contaminants to and from microplastic are expected to differ (Karapanagioti and Klontza, 2008; Teuten *et al*., 2007).

The migration potential of substances among interacting compartments is controlled by the differences in chemical activity and will only occur until the chemical activities in the interacting phases reach equilibrium (Ziccardi *et al*., 2016). Therefore, the environment in which pellets reside is key to the distribution of HOCs and additives among phases. Key factors which influence diffusion include: temperature, permeability of the polymer matrix; size; surrounding medium; size of gaps between polymer molecules compared to the migrant; and the solubility and volatility of the migrant (Brydson, 1999; Sheftel, 2000; Lithner *et al*., 2012). The sorption capacity

of microplastics is influenced by the type of polymer and its physical state (Rodrigues *et al*.,2018). Plastics are synthetic organic polymers, which contain crystalline (glassy) or amorphous (rubbery) regions, and these regions determine the size of the gaps in the polymer matrix (Brydson, 1999; Endo and Koelmans, 2016; Rodrigues *et al*.,2018). Rubbery polymers, such as LDPE, have larger gaps than crystalline polymers, which has a higher degree of molecular packing (Brydson, 1999; Lithner et a., 2012). PVC is a crystalline polymer and has a lower migration potential, than PE (Brydson, 1999). However, plasticised PVC has large gaps between polymer chains, which increases migration of molecules (Godwin and Jrauskopf, 2008). Larger gaps and an increase in concentration of additives in PVC means that large amounts of nonbound toxic substances may leach from the polymer matrix. This has been evidenced by Lithner *et al*. (2009;2012).

The weathering process has also been identified as an important factor for adsorption and leaching capacities (Karapanagioti and Klontza, 2008; Holmes *et al*., 2014; Rodrigues *et al*.,2018). Studies suggest that sorption of hydrophobic contaminants from seawater to pellets may take weeks to reach equilibrium (Ogata *et al*., 2009). Teuten *et al*. (2007) show preferential sorption of POPs onto plastic polymers compared to their sorption to natural sediments. Toxicity of plastic products can be related to the surface area. Therefore, this is of great importance for the outcome of leaching chemicals from plastic, since leaching mainly occurs from the surface (Lithner *et al*., 2012). The heterogeneity of plastic pollution in the marine environment, in reference to shape, thickness and densities makes it difficult to estimate the toxicity of leachates from plastic pollution. Marine plastic pollution is highly heterogeneous in terms of associated concentrations of HOCs, metals and additives which prevents general conclusions being drawn for environmental samples (Teuten *et al*., 2009; Endo *et al*., 2005; Rios *et al*., 2007; Ogata *et al*., 2009; Heskett *et al*., 2012; Hirai *et al*., 2011; Takada *et al*., 2006; Takada *et al*., 2006b; Karapanagioti *et al*., 2011).

1.5 Chemical Effects: Toxicity of Co-contaminants to Marine Biota

Marine microplastic pollution is frequently evidenced as causing adverse impacts to marine biota through physical interactions. However, detrimental impacts caused through toxicity of leachates from plastics into the environment is less well known. The transfer of contaminants to marine organisms can, in theory, occur through two main exposure pathways; exposure to the internal surface or organismal fluids from ingestion, or exposure from environmental media. Recent research suggests that in addition to contamination of marine organisms by plastic additives through ingestion, contamination via non-ingestion may also occur (Hermabessiere *et al*., 2017). Direct contact exposure of organisms with microplastics and the associated

contaminants leaching from the polymer matrix might be an important process which has so far been an uptake route which has been overlooked (Hartman *et al*., 2017).

Recent advances in understanding the role of microplastics as containment vector have led to the hypothesis that microplastics can potentially act as vectors of chemical pollutants of firstly additives added during polymer synthesis, and secondly of chemicals adsorbed directly from seawater, however large data gaps exist. Although microplastics are not the only route by which marine species are exposed to hazardous chemicals, there is some evidence to suggest that plastics may present an additional exposure pathway of chemical contaminants in the marine environment, in addition to the physical effects that microplastics elicit on biota (Hartmann *et al*., 2017). The topic carries controversy, with experimental studies and review papers, both supporting and challenging this hypothesis.

The toxicity of microplastic leachates could pose a threat to marine organisms, since a wide range of plastic additives have been identified as toxic compounds (Lithner *et al*. 2011; Bejgarn *et al*., 2015; Hermabessiere *et al*., 2017; Groh *et al*., 2019) and have been detected leaching from marine plastic (Teuten *et al*., 2009). Plastic additives are recently being identified in environmental samples. These include flame retardants (Zhang Liang and Haiqing Liao, 2011) and phthalate esters (PAEs) (Net *et al*., 2015; Baini *et al*., 2017; Zhang *et al*., 2019). Marine microorganisms have been found to metabolise contaminants associated with microplastic pollution (Chua *et al*., 2014; Wardrop *et al*., 2016). Contaminants and additives in plastics beached around coastlines have been investigated by XRF (Turner, 2016; Massos and Turner, 2017), gas chromatography-mass spectrometry (GC-MS) (Santillo *et al*., 2017) and liquid chromatography- mass spectrometry (LC-MS), allowing identification of key compound groups which might elicit such effects.

Phthalates or phthalate esters have been shown to induce effects on reproduction of marine organisms and have wider ecosystem effects (Andrady,2011; Kolena *et al*., 2014; Zhang *et al*., 2018a). Baini *et al*. (2017) detected seven phthalate esters, with frequencies as high as 78.9% in planktonic samples and four free-ranging cetaceans, in the north-western Mediterranean Sea. The authors highlight the potential toxicological threats for marine organisms by exposure to chemicals associated with plastic debris. PAEs have also been detected in areas of high biological importance and activity, such as seamounts. Zhang *et al*. (2019) analysed 14 phthalate esters from seawater samples taken from seamounts in the Tropical Western Pacific Ocean. The authors highlight the finding that DEHP, a common additive in plastic, posed a medium risk in seawater, suggesting marine plastic pollution has become an urgent environmental issue (Zhang *et al*.,2019). Another example of a polymer additive which has been evidenced to elicit defects is triclosan. Triclosan is a broad-spectrum antibacterial agent, which is added to polymers and is

commonly found in aquatic environments (Hwang *et al*., 2013). Triclosan is found in domestic sewage and wastewater treatment plants. Triclosan has been found to cause abnormal development and reduced hatching rates in sea urchin *Strongilocentrotus nudus* (Hwang *et al*., 2013). Hwang *et al*. also found that gametes exposed to triclosan reduces viability of sperm and fertilisation rate.

The body of literature experimentally researching the effects of microplastic leachates on marine biota suggests that co-contaminants elicit a toxic effect in the marine biota studied, with particularly consistent results from virgin polymers and associated additives. Li *et al*. (2015) quantified the impact of leachates from seven categories of everyday plastic items on the survival and settlement of barnacle *Amphibalanus amphitrite* larvae. The authors found that at the highest leachate concentration, (0.10 and 0.50m^2/L) for all plastics, survival rate was significantly lower, and leachates inhibit settlement on glass. Li *et al*. (2015) additionally found that PVC leachate was the most toxic to *Amphibalanus amphitrite* larvae.

Leachates from plasticised PVC have been identified to cause acute toxicity to Daphnia (Lithner *et al*., 2009, 2012). In the second study, Lithner *et al*. (2012) concluded that all plasticised PVC products caused acute toxicity (immobility after 24h and 48h), most likely due to phthalate leachates from the polymer matrix (Lither *et al*., 2009). Bejgarn *et al*. (2015) performed leaching experiments, using weathered and non-weathered plastics on the copepod *Nitocra sinipes*. Of the 21 plastics tested, 38 of them demonstrated toxicity, many of these included PVC based polymers, such as a garden hose and packaging. e Silva *et al*. (2016) evaluated the toxicity of leachates with beached and virgin polyethylene pellets present, on the development of brown mussel embryos, *Perna perna*. The exposure showed embryotoxicity and impaired larval development. These results highlight that microplastic pollution may be harmful even if ingestion is not the only or main pathway of interaction.

Nobre *et al*. (2015) hypothesised that virgin pellets would be more toxic than beached collected pellets, to developing larvae, due to leaching of toxic additives from the polymer matrix. Data supported this hypothesis with an increase in abnormal larvae development for industrial virgin polyethylene pellet treatments. This is consistent with Browne *et al*. (2013) study, that additives within the polymer matrix are potentially more toxic to early life stages than hydrophobic organic contaminates sorbed from the environment. Both Lithner *et al*. (2012,2009) and Bejgarn *et al*. (2015) carried out toxicity test using the plastic leachates alone, however e Silva *et al*. (2016) used both the plastic pellets and leachates. This was said to have been done, because Nobre *et al*. (2015) study concluded that sea urchin larvae exposed to leachates of beached pellets without the presence of pellets did not show any toxic response. When the larvae were exposed to the same leachate with the presence of pellets however, toxic effect was observed.

It is important to note that there are key differences in methodology and species used, however results from these experiments do suggest that toxicity of plastic leachates is of particular concern for early life stage development. It is also important to note that methodology of exposure can influence the conclusions of experiments, as shown in Nobre *et al*. (2015), with an elutriate treatment showing a higher toxicity than the pellet-water-interface.

Controlled laboratory experiments have explored the exposure of marine biota to dosed microplastics and the ability for such chemical pollutants to transfer to tissues and elicit a response following ingestion. Browne *et al*. (2013) exposed lugworms to sediment with 5% PVC, presorbed with high concentrations of nonylphenol (<700 mg/L) and phenanthrene (Phe) (<120 mg/L), in addition to triclosan and PBDE. The authors found that the chemicals desorbed from the plastic and transferred to the lugworm's epithelial tissue membrane. Avio *et al*. (2015) evidenced the ability for PAHs to adsorb to microplastics, and subsequently to transfer PAH to exposed Mediterranean mussels, *Mytilus galloprovincialis*. Following ingestion, exposure to both virgin and contaminated plastics induced transcriptional and cellular levels effects. Bussolaro *et al*. (2019) evidenced that microplastics can alter the genotoxicity of PAH contaminants in rainbow trout epithelial cells. Pittura *et al*. (2018) examined the effect of microplastics as a vehicle of environmental PAHs to *M. galloprovincialis.* Through histological evaluation, the group concluded that overall microplastics and absorbed contaminants induced slight cellular toxicity (Pittura *et al*., 2018). Batel *et al*. (2018) study highlights the ability of POPs to elicit effects even when no ingestion of microplastics has occurred. The group studied the accumulation pattern and transfer of toxic substances associated with microplastic exposure to zebra fish embryos and adult gill transfer. Exposure to loaded PAH benzo[a]pyrene (BaP) polymers indicate that POPs adsorbed to microplastics might be transferred by the simple attachment and re-solution of POPs into the water column from the plastic (Batel *et al*., 2018).

However, there is also research which does not support the hypothesis that microplastics can act as a vector for contaminants. Some experimental research suggests that microplastics are not ample transport vector of persistent organic pollutants (POPs) in comparison to the air or water, except for very high octanol-water partition coefficient chemicals (Rodrigues *et al*., 2018). The partitioning of POPs to seawater and natural organic matter is still predicted to be higher. The research also suggests that the relationship of microplastics with contaminants, especially those sorbed from the environment, is complex and requires further investigation to fill large knowledge gaps. A key example of a study exhibiting the complexity of microplastics is Kleinteich *et al*. (2018) who used a laboratory approach to assess the sensitivity of bacterial communities to exposure of microplastics and microplastics loaded with PAHs. Following a two-week exposure, microplastics altered bacterial community compositions from an unpolluted river,

however those communities sourced from downstream of a wastewater treatment plant remained unchanged. Those microplastics loaded with PAHs induced less pronounced response, when compared to the same total amount of PAHs alone. This study highlights that although microplastics can affect the bacterial composition in some areas, they also can reduce the bioavailability of chemicals to bacterial communities through sorption (Kleinteich *et al*., 2018). Scopetani *et al*. (2018) evidenced that ingestion of contaminated microplastics by a supralittoral amphipod, *Talitrus saltator*, moves organic pollutant to its tissues. However, uncontaminated microplastic ingestion removes organic pollutants. This research suggests that microplastics may act as both a carrier and scavenger of contaminants, further complicating the predicted effect of this pollutant.

Generic theoretical models, such as MICROWEB which simulate the transfer of microplastics and hydrophobic organic contaminates in food webs, conclude that microplastics can act as significant vectors of POPs to marine organisms (Diepens *et al*., 2018). However, the difference in mass between microplastics and other potential hydrophobic organic contaminates sources, such as the mass of water and sediments, has led to the relative role of microplastics as a vector for hazardous contaminants to organisms to be considered negligible compared to natural exposure pathways (Hartman *et al*.,2017). However, there may be specific scenarios in which microplastics act as an important pathway for contamination of toxic additives, HOCs and or metals. Current estimates of microplastic pollution across the globe are underestimated (Syberg *et al*., 2015; Conkle *et al*., 2018). In addition, microplastics are heterogeneously dispersed in the environment (Browne *et al*., 2011; Syberg *et al*., 2015) and estimates of marine microplastic pollution are only set to increase. A specific scenario in which microplastic pollution levels may be overlooked is within the plankton. Many polymers float on the surface, due to their low-polymer density, along with pollutants. This increases the worldwide transport of contaminated microplastic pollution alongside the dispersal of sensitive early life stages of planktonic larvae, which ride oceanic currents. Microplastics may also form a layer of debris and contaminants which could be difficult to identify and remove. Therefore, it is of key interest to identify potential developmental defects in planktonic life stages which may be elicited as a results of microplastic pollution. Bioavailability is the extent to which a compound enters tissues and reacts with biological molecules (Semple *et al*., 2004). This is particularly important for the interaction of microplastics and associated leachates from natural pathways and through external interaction. Many environmental pollutants show little or no bioavailability, such as metals sorbed on particulate matter (Lorenzo *et al*., 2002). The heterogeneity of marine pollutants associated with microplastics and lack of research surrounding the uptake of toxic

chemicals by organisms through the plasmatic membrane, makes it difficult to estimate the bioavailability of the cocktail of contaminants (Lorenzo *et al*., 2002).

1.6 General Conclusion – do microplastics and their co-contaminants present an ecological risk?

It is well evidenced that microplastics are ubiquitous across marine ecosystems and their potential to negatively interact with marine biota has been demonstrated across numerous phyla (Worm *et al*., 2017). However, research into the polymer additives and sorbed contaminants in marine microplastic is very limited (Hong *et al*., 2017). Non-target screening of marine plastic pollution has revealed more than 200 organic chemicals associated with microplastics (Gauquie *et al*., 2015; Rani *et al*., 2015). These organic chemicals include both plastic additives, such as plasticisers, lubricants, dyes, inks, flame retardants, antioxidants and UV stabilisers, and environmental pollutants such as HOCs. However, little is understood about the potential for such organic chemicals to pose a higher risk, than the exposure to such contaminants within the water column. To assess the potential impacts that microplastic and associated contaminants elicit, it is important to identify and understand the processes which govern sorption and desorption of substances from microplastic. Such particle-pollutant interactions govern the bioavailability of contaminants and the potential effects on individuals, populations and ecosystems. However, these processes are not yet well-understood, due to insufficient data to draw valid conclusions. However, the increase of marine microplastic pollution cannot be ignored since the volumes of such an emerging contaminate, with the potential for contaminant sorption ability are growing, and ecotoxicological effects are consistently being reported (Rodrigues *et al*., 2018).

To conclude, microplastic pollution is a highly complex emerging contaminant of concern, with some researchers proposing that microplastics should be considered as a suite or class of contaminants (Rochman *et al*., 2019). Increasingly evidence suggests that 1) co-contaminants are strongly associated with microplastic pollution, including both additives and HOCs; 2) co-contaminants can elicit defects in marine biota, however 3) large data gaps exist and more complete studies are needed to further understand the relationship between microplastics and their co-contaminants. We need further experimental research to understand the leaching potential of co-contaminants, together with their bioavailability to ultimately elicit biological and mechanistic effects, presenting an ecological risk. A broad chemical risk assessment of plastics and their associated contaminants is essential for assessing the impacts of plastic pollution on populations, ecosystems, and human health. Without vital scientific research assessing the relationship between microplastics and their co-contaminants and the resulting effects on marine biota, these risk assessments cannot be drawn.

1.7 Sea Urchins as a Model Organism for Microplastic Toxicity Studies

The coordinated response of cells through complex communications and interactions are essential events in the correct development of tissues and organs. These events receive an input from environmental stimuli and cells modify their behaviour throughout signalling pathways based on the integrated exchange of information. The sea urchin embryo has been an ideal study system for understanding the cellular basis of development, informing developmental studies since 1892 (Driesch, 1892). Sea urchins produce high quantities of transparent embryos, exhibit fast cell division and morphogenesis and are biochemically similar to vertebrates (Matranga *et al*., 2013). More recently, this flexible study system has allowed the investigation of the effects of toxicants on development and species of this taxa are now candidates for selection as test species in ecotoxicology (Lawrence, 2013). Echinoderms might share similar targets at both the organism and cell level, in terms of response to environmental contamination with vertebrates (Arslan and Parlak, 2008 and Sugni, 2007). Therefore, sea urchin embryos are now being used to research the effects of environmental contamination upon early development, reproduction and genetics (Gunduz *et al*., 2013).

1.8 Thesis Aims and Questions

The aim of this thesis is to investigate the contaminants liberated from virgin (industrial) and beached (environmentally exposed) microplastic and the developmental defects which they elicit in two sea urchin species, *Psammechinus miliaris* and *Paracentrotus lividus*. To complete this aim the following questions were addressed:

(1) Do industrial and environmentally exposed plastic pellets leach toxic contaminants?

(2) Are the embryonic and larval stages of sea urchin species (*P. miliaris* and *P. lividus*) negatively affected by such plastic leachates?

(3) Are there differences in developmental timing and morphology of key embryonic structures in *P. miliaris* and *P. lividus* cultures, as a result of exposure to different plastic treatments (industrial and environmentally exposed) and plastic leachate concentration?

Chapter Two

Developmental Toxicity of Microplastic Leachates to *Psammechinus miliaris*

2.1 Introduction

Understanding the mechanistic basis of changes in developmental processes which are elicited by microplastics and co-contaminants is of critical importance, to provide a thorough risk assessment of such a ubiquitous pollutant. However, the level of physiological impact that microplastic leachate exposure has on marine biota remains unclear. Evident from numerous assessments of marine plastic pollution is the extent of microplastic ingestion and physical interactions that marine biota has with this novel stimulus. However, the toxicological effects of microplastics are not well documented. As outlined in chapter one, microplastic pollution has been strongly linked to toxic contaminants, however research into the leaching potential of chemical additives and sorbed chemicals in marine microplastic is very limited. Recent research suggests that in addition to the well-studied hazards posed from plastic pollution to marine organisms such as ingestion, contamination via leaching of chemicals may also occur (See chapter one; Hermabessiere *et al*., 2017). Exposure of organisms to microplastics and the associated contaminants leaching from the polymer matrix might be an important process and has so far been overlooked as an uptake route (Hartman *et al*., 2017).

The present chapter uses the knowledge gained from the literate review (chapter 1) to compare the wildtype phenotype observed in *P. miliaris*, to the phenotype of embryos exposed to microplastic leachates from beached and raw industrial feedstock. The subsequent documentation of the wildtype phenotype for *P. miliaris* embryonic stages, provides an independent comparison between expected wildtype phenotype and treatment phenotypes within these toxicological experiments.

2.1.2 Microplastics and Marine Organisms

The negative impacts of marine microplastic pollution is well supported by scientific research. Physical impacts, such as ingestion (Cole and Galloway, 2015; Desforges *et al*., 2015; Nelms *et al*., 2017) can lead to growth inhibition (Au *et al*., 2015; Cole and Galloway, 2015; Watts *et al*., 2015; Sussarellu *et al*., 2016), reproductive problems (Besseling *et al*., 2014; Sussarellu *et al*., 2016), reduced feeding (Hart, 1991; Wright *et al*., 2013; Cole *et al*., 2015; Hall *et al*., 2015) and mortality (Au *et al*., 2015). Microplastic pollution has also been evidenced to cause oxidative damage (Donovan *et al*., 2018; Prokić *et al*., 2018) and alter gene expression (Sleight *et al*., 2017). Zooplankton exposed to microplastic fibres have been observed with antennal and carapace deformities resulting from external damage (Ziajahromi *et al*., 2017).

Microplastic ingestion has received substantially more attention than the hazards elicited from the leaching of chemicals from marine plastic pollution. Microplastics are associated with >78% of primary pollutants (Rochman *et al*., 2013a), which are introduced to the polymer matrix via two potential pathways. Firstly, the manufacturing process of plastic products incorporates additives such as plasticisers, metals, dyes, flame-retardants, UV stabilisers, antimicrobials and antioxidants (Hahladakis *et al*., 2018). Despite these additives ability to transform simple polymers into useable, versatile plastic products, their contamination of the marine and terrestrial environment is widely documented (Hahladkis *et al*., 2018). Secondly pollutants can be sorbed from the surrounding environmental media (Ogata *et al*., 2009).

In a wider context, microplastics do not just affect individual marine organisms and can have far-reaching consequences across multiple habitats and life stages. Al-Jaibachi *et al.* (2018), recently evidenced that fluorescent polystyrene (PS) microplastic beads can be transferred ontogenically between *Culex* mosquito larvae, pupa and adult life stages, under laboratory conditions. However, the authors highlight the dependence on particle size, with smaller 2 µm microplastic's transferring readily, whilst 15 µm microplastics transferred at a significantly reduced rate. This paper highlights that any organisms which feeds on terrestrial life phases of freshwater insects could be impacted by MPs found in aquatic ecosystems (Al-Jaibachi *et al*.,2018).

2.1.3 Microplastic and Developmental Pathways

A complex pollutant, microplastics can assault marine life on multiple levels. In addition to the physical impacts of microplastics, the less obvious ecotoxicity of microplastic pollution on biota is a relatively new field of research which we are only starting to uncover. Recent advances in scientific enquiry have highlighted clear evidence of the developmental effects of microplastics across a range of taxa, including sea urchins (Della Torre *et al*., 2014; Nobre *et al*., 2015; Martínez-Gómez *et al*., 2017; Messinetti *et al*., 2017) and mussels (e Silva *et al*., 2016; Wathsala *et al*., 2018). However, despite this, understanding into which developmental pathways are affected by microplastic pollution is still unknown. The developmental defects have not been thoroughly characterised, and the mechanistic basis of changes in developmental processes elicited by microplastics are not known.

The underlying machinery of development across all animal phyla, invertebrates and vertebrates, are largely conserved (Alberts *et al*., 2002). The DNA sequences of many individual regulatory modules and developmental genes are evolutionary conserved and homologous across distant animal phyla (Mallo and Alonso, 2013). These strong similarities in developmental pathways across the animal kingdom, results in findings from one species providing insight into the development of many other types of animals. Therefore, if microplastic pollution is affecting

organisms on a genetic level, this could have extensive consequences across the animal kingdom. Developmental pathways can be disrupted by mechanical (Farge, 2003), physical (Bonaventura *et al*., 2005) and chemical (Carballeira *et al*., 2012; Pinsino *et al*., 2013; Corinaldesi *et al*., 2017) environmental stressors, which can lead to transcriptional and epigenetic changes (Bonaventura *et al*., 2005; Gambardella *et al*., 2012; Pinsino *et al*., 2013; Corinaldesi *et al*., 2017). However, it is not clear which mechanisms are elicited by microplastic pollution. Plastic pollution is strongly associated with chemicals which have been shown to act at a transcriptional or physiological level, disrupting developmental processes. Recent research calls on future work to focus on whether microplastics may be affecting aquatic organisms through contaminate exposure at a molecular level (Foley *et al*., 2018).

Recent laboratory research follows a general pattern which suggest that plastic and associated leachates elicits a toxic effect in invertebrates. Virgin plastic pellet exposure is evidenced to cause toxic effects on the embryonic development of sea urchin (Nobre *et al*., 2015; Martinez-Gomez etal., 2017) and mussels (e Silva *et al*., 2016). Consumer products have also been studied with data from Bejgarn *et al*. (2015) suggesting that leachates from plastic products causes acute toxicity to the marine copepod *Nitocra spinipes*. Leachates from seven commercial plastics, including PS and HDPE significantly increase the mortality in acorn barnacle (*Amphibalanus amphitrite*) and inhibits settlement on glass with plastic leachates (Li *et al*., 2015). Recent research highlights that leachates from coloured plastic products exhibit different embryotoxic effects on *Paracentrous lividus* larvae. These differences were expected to stem from the different heavy metals present in the colouring agents of the PVC based children toys (Oliviero *et al*., 2019).

Contaminants such as hydrophobic organic contaminants (HOCs), metals and additives which are associated with microplastic pollution, can act as endocrine disruptors (Pannetier *et al*., 2018; Provencher *et al*., 2018) and developmental toxicants, causing mutagenicity and carcinogenicity (Rios, Moore and Jones, 2007).These contaminants often act at a genetic or physiological level disturbing developmental processes. Japanese medaka (*Oryzias laptipes*) fed virgin polyethylene (PE) fragments, express altered gene expression and signs of physiological stress in the liver. The effects observed were less severe than in fish fed plastic fragments that also contained HOCs (Rochman *et al*., 2014). The results from Rochman *et al*. (2014) suggest that ingestion of plastic may alter endocrine system function through altered gene expression, and the addition of HOCs produces a more severe phenotype. Wan *et al*. (2018) evaluated the effects of polystyrene microplastics on larval zebrafish over a 7-day period. The authors reported alterations in the microbiome and changes in glycolysis-related genes and lipid metabolism-related genes.

Therefore, it is evident that the presence of microplastics as foreign material and cocktail of chemicals introduced to organisms upon ingestion causes defects in the animals functioning. However, there is a lack of evidence that microplastic and associated pollutants can elicit developmental defects, without ingestion and instead through contact within the environmental media, through microplastic co-contaminant leaching. Research into the sorption and desorption of HOCs, metals and additives from microplastics into the surrounding environmental media, and subsequent interactions with marine larval tissue development is sparse.

2.1.4 Echinodermata and Microplastics

Sea urchin embryos are an ideal study species for understanding successful development and the effects of contaminants on early life stages. Therefore, sea urchin embryos are being selected as a suitable model system for ecotoxicological and environmental studies, aimed to the determination of the effects of chemical and persistent pollutants (Bonaventura *et al*., 2005). Sea urchins produce large quantities of transparent embryos, exhibit fast cell division and morphogenesis and are biochemically similar to vertebrates (Matranga *et al*., 2013). Sea urchin larvae are pelagic until they metamorphose after approximately 3 weeks. Suspension feeding commences during the pluteus stage, around 48h post-fertilisation, with larvae feeding in the plankton using external ciliary bands (Messinetti *et al*., 2017). This planktonic life stages of echinoderms may expose them to increased risk from toxic contaminants of microplastics, with the majority of lightweight microplastics residing in the surface layers. Marine benthic sediments are also considered a major sink for microplastic pollution (Coppock *et al*., 2017; Courtene-Jones *et al*., 2017; Tekman *et al*.,2018). Therefore, adults in close contact with the sediment, are likely to be exposed to microplastics and co-contaminants.

Evidence suggests that sea urchin plutei can efficiently ingest microplastics, with significant effects on development. Messinetti *et al*. (2017) observed polystyrene microplastic (diameter 10 µm), at four increasing concentrations (0.125, 1.25, 12.5, 25 µg/ml) have a significant effect on *Paracentrotous lividus* larvae (72 hpf) body length, width and post-oral arm length. The authors observed a large variability in measured responses at low microplastic concentrations, with increased variability in microplastic uptake. However, at higher concentrations a consistent microplastic uptake pattern was detected, resulting in plutei body and arm length being reduced and an increase in body width. They showed that the co-ingestion of beads and algae compromised the correct development of the larval body shape (Messinetti *et al*., 2017). The same study report that without co-ingestion with algae, no effect of polystyrene microplastic was observed on *P. lividus* survival. This study highlights that microplastic exposure can disrupt the physical development of invertebrate larvae.

Nobre *et al*. (2015) used the sea urchin, *Lytechinus variegatus*, as a model species to evaluate the toxicity of beached-collected and virgin plastic pellets (polyethylene, PE) on embryonic and larval stages. Using two ecotoxicological assays, pellet-water interface and elutriate. Nobre *et al.* studied both the toxicity of microplastic leachates and microplastic itself. Anomalous development was assessed via morphological abnormalities following 24 hours development via light microscope observations. In both assays, virgin PE was deemed toxic, with an increase in abnormal embryonic development by an average of 62.3% over both assays, when compared to controls. Toxicity of beached pellets was observed to be lower than virgin, in both assays. The authors concluded that microplastics can be a vector of pollutants, and leachates from pellets can be toxic through various exposure pathways.

Martinez-Gomez *et al*. (2017) found that aged nano polystyrene (uncharged, 6nm) and HPDE fluff (additive-free, > 0-80 um) and their leachates elicit toxic effects on fecundation and embryonic development of *Paracentrotus lividus*. Using sea urchin embryotoxicity tests (SET), two toxicity parameters were calculated: percentage of pluteus larval abnormality and larval growth as a percentage of net response. Overall, the growth of larvae from exposed gametes was significantly lower than in controls after 48 hours incubation and in a dose-dependent manner. Toxic effects exceeding environmental assessment criterion (EAC) value, which is indicative of significant harm to the species (Davies *et al*., 2012), was only found for zygotes exposed to leachates from the lowest concentration. The authors proposed that, since the selected microplastic particles failed to aggregate in the exposure media, the toxicity observed is ascribed to the chemical leachate of the exposed microplastic. Furthermore, Della Torre *et al*. (2014) assessed the toxicity of polystyrene (PS) nanoplastics with two different surface charges (PS-COOH and PS-NH_2). *P. lividus* larvae from 48h exposure to PS-COOH exhibited severe developmental defects and up regulation of *Abcb1* gene. *Abcb1,* is related to P-glycoprotein efflux pump development in sea urchin species (Shipp and Hamdoun, 2012), which is a pump demonstrated to have a protective role during sea urchin embryo development (Hamdoun *et al*., 2004; Bošnjak *et al*., 2013). The authors report that PS-NH_2 exposure induced a significant up-regulation of *cas8* gene at 24hpf, which is an initiator of programmed cell death in vertebrates (Romano *et al*., 2011). The authors report that this suggests membrane damage as a result of toxicity caused by nano PS-NH_2 exposure (Della Torre *et al*.,2014). This research highlights that differences in surface charges and nanoplastic aggregation in seawater strongly affects embryotoxicity to invertebrate larvae. Additional research into amino-modified PS nanoplastic highlights disruption of sea urchin embryonic development through modulating protein and gene profiles (Pisino *et al*., 2017).

Complex gene networks, which act as an integral part of sea urchin developmental programs, also act as a defensive network. These integrated pathways allow sea urchins embryos to defend themselves against stressors. When an environmental stressor occurs, such as toxic metal exposure or ionizing radiation malformations in the development of key structures are prevalent (Matranga *et al*., 2013). This malformation is directly linked to the expression and regulation of expression of gene networks and proteins. Marine community structures depend on the ability for planktonic larvae to enter adult populations. However, it is well documented that echinoderm early life stages exhibit a high sensitivity to toxicants, including persistent organic pollutants, compared to other life stages (Pagano *et al*., 1982; Lithner *et al*., 2009, 20012; Anselmo *et al*.,2011; Beiras *et al*., 2012; Messinetti *et al*., 2018; Oliveiro *et al*., 2019). Therefore, research priorities must lie with the early life stages of developing marine larvae, to ensure recruitment into adult life stages and to prevent a population collapse and potentially far-reaching ecosystem impacts.

2.1.5 Sea Urchin Embryonic and Larval Development

'It is not birth, marriage, or death, but gastrulation which is truly the most important time in your life.' Lewis Wolpert

Early morphogenesis is particularly important in establishing differences in animal body plans and the correct development of tissue structures. However, one of the most dynamic periods of development is the time in which embryos undergo gastrulation. This important morphogenetic process involves multiple cell movements, which results in a reorganisation of the embryo from a simple hollow-spherical ball of cells, the blastula, into a multi-layer organism. The three primary germ layers, namely ectoderm, mesoderm and endoderm, are formed and organised during gastrulation.

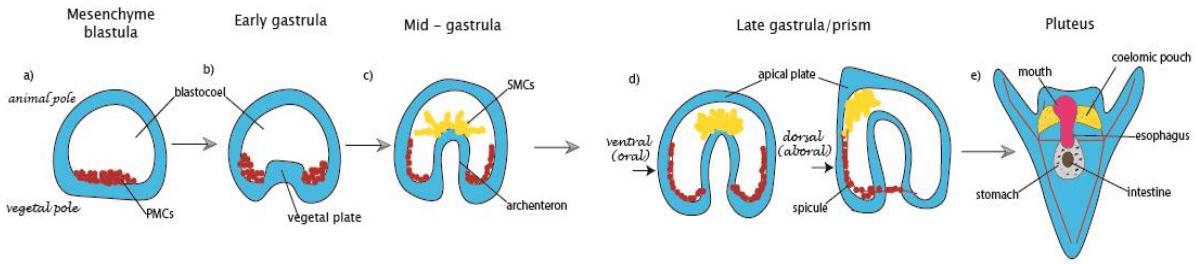


Fig.2.1. Gastrulation of the sea urchin embryo from mesenchyme blastula (a) to form the pluteus (e)**.** PMCs represented by red dots; SMCs represented by yellow dots. Figure adapted from Department of Integrative Biology, University of Wisconsin (*worms.zoology.wisc.edu).*

The blastula stage is characterised by the presence of a large fluid-filled blastocoel, surrounded by a single layer of cells (mesenchyme blastula; fig.2.2a). Morphogenetic movements following this stage completely rearranges the embryo. Three specific stages of gastrula form, namely early (fig.2.2b), middle (fig.2.2c) and late gastrula (fig.2.2d), during which there is the elongation

of the archenteron towards the animal pole. The primary mesenchyme cells (PMCs) detach from the vegetal plate and enter the blastocoel and begin to migrate. Following PMC ingression (see definitions), the vegetal plate undergoes primary invagination to form the archenteron, which will later become the gut (fig.2.2b). Following primary invagination and during gut elongation, a population of mesodermal cells, namely secondary mesenchyme cells (SMC) appear at the archenteron tip (fig.2.2.c). The SMCs are precursor cells for the coelomic pouch, a fluid-filled body cavity surrounding the digestive tract. Eventually the tip of the archenteron meets near the animal pole, through secondary invagination which involves the elongation of the archenteron. While the archenteron is elongating, the PMCs form patterned arrays, and ultimately secrete calcium-carbonate containing skeletal rods, termed spicules. The PMCs are therefore precursors for the skeletal system of the pluteus larvae.

The formation of the plutei larval stage occurs when changes in shape and structures such as the intestine and skeleton develop (fig.2.2e). Prismatic spicule development is integral for constructing the framework of the sea urchin pluteus. A normal well-developed pluteus exhibits four arms, a complex patterned skeleton and a tripartite intestine, encircled by muscles capable of peristaltic contraction. A good culture will have embryos that have developed synchronously with a morphology characteristic of their developmental stage.

2.1.6 Immunohistochemistry

Immunostaining allows for the identifiation and assessment of key tissue groups by staining specific proteins of intrest. An antibody is used to detect a specific protein epitope within the sample of interest, followed by a labelled secondary antibody, which allows the proteins localisation to be visulised. Following the fixing of a sample to preserve epitopes, cell

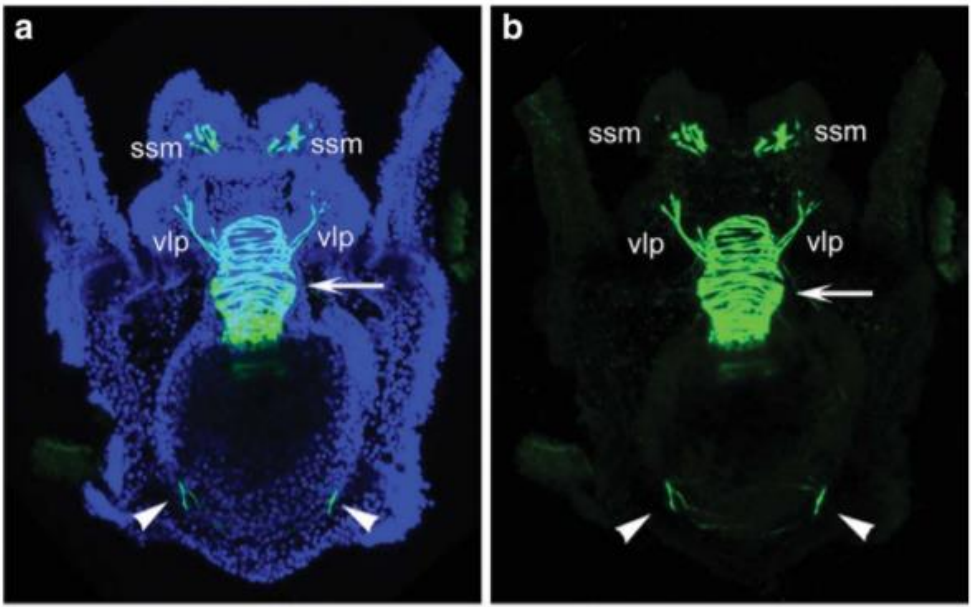


Fig. 2.2. The digestive systems of the 8-arm sea urchin pluteus larvae visualised via immunohistochemistry of a whole mount *Strongylocentrotus purpuratus* 3-week old larvae (a-b). The anti-MHC antibody (green) stains the circumesophageal muscle fibres (arrows). The distal oesophagus (des; arrow), the mouth (mo), the proximal oesophagus (pes), the left rudiment (lr; arrowhead) and the stomach (st) are indicated. Image from Annuziata *et al*., 2014.

morphology and tissue structure, the process of immunostaining can begin. The first step involves blocking, this process reduces non-specific background noise by preventing non-specific antibody binding, improving the signal of the antigen of interest. Following removal of the blocking, the sample is incubated with the primary antibody. The culture is washed, and the secondary antibody is added for incubation. For the antigen antibody immunoreaction to be visualised, the secondary antibody must be labelled. Immunofluorescence is an example of immunostaining which specifically uses fluorescent dyes, or fluorophores to target and visualise the location of the antibody. This process allows for the localisation of antigens in specific tissue groups during sea urchin development, visualising territories in a fluorescent microscope, which would not be seen without such techniques. Primary antibodies are raised against the antigen under study, whereas secondary antibodies are raised against the corresponding isotype of the primary antibody (Buchwalow and Bocker, 2010). Fluorescent immunohistochemistry of embryological features, such as neurogenesis, myogenesis and cilia, allows vital developmental processes to be visualised and assessed. Few studies within the field of ecotoxicology have employed immunostaining to characterise the extent of damage that environmental toxicants can cause (Kiyomoto et al., 2010; Morales et al., 2011; Gambardella et al., 2015). However, such a powerful technique can provide us with a deeper understanding of the mechanistic basis of the abnormalities resulting from environmental pollution.

The larval digestive system of the sea urchin pluetus form is tripartite, composed of a muscular oesophagus, a large sphical stomach and a short tubular intestine (Annuziata *et al*., 2014). The

muscle fibers associated with the digestive system in the pluetus stage, can be clearly visualised via microfilament labelling using phalloidin, a compound that binds to actin filaments known as F-actin (Cole *et al*., 2009; McDougall *et al*., 2006). Phalloidin labelling of F-actin allows the visualisation of the gut tissue and cellular junctions of a developing larva. Figure 3.1. shows the use of immunohistochemistry to visualise the digestive system of an 8-arm sea urchin pluteus, using myosin heavy chain (MHC) immunolocalization, which shows the same structure as phalloidin labelling. The circumesophageal muscles are a conspicuous feature in pluteus sea urchin larvae, which can be successfully visualised through immunostaining (fig.3.1, centre arrows). The development of these muscles marks the onset of the ability of the larvae to feed (Burke and Alvarez, 1988), therefore microfilament labelling using phalloidin, allows the crucial development of the digestive system to be assessed. Within my study phalloidin labelling was employed over MHC, due to ease of protocol and reduced costs associated with phalloidin labelling.

Acetylated tubulin is a molecule which is essential for larval development of a sea urchin larva. In vertebrates, the complete loss of cilia results in development stopping mid-gestation (Sreekumar and Norris, 2019). Acetylated tubulin is found in the microtubules that make up cilia axonemes, or the central core of a cilium. Cilia are an important organ in sea urchin embryos and larvae, required for motility (Hara *et al*., 2003), food capture (Emlet, 2002) and sensory abilities (Fujiwara *et al*., 1999; Perkins *et al*., 1986). The pluteus larvae of sea urchins feed on unicellular algae, though a ciliated band which runs along the body and out to each arm (Emlet, 2002). Using acetylated tubulin to visualise the cilia of embryos and larvae can aid in assessing the impact of toxicants on the development of a key sensory organ. Gambardella *et al*. (2015) used acetylated tubulin staining to investigate the toxicity of silica nanoparticles (SiO_2 NP) during sea urchin larvae development. The authors observed morphological anomalies in larvae at pluteus stage, with decreased immunostaining in the stomach and cilia at the surface, and ciliary damage in those larvae exposed to SiO_2 NP. This suggests treated embryos had a decreased lifespan as a result of a reduction in lifespan of acetylated microtubles, and therefore ability to feed. The nerve cell axons in invertebrates are also rich in tubulin, and therefore has been used as a tool for studying the nervous system in insects (Wolf *et al*., 1988; Thuerkauf, 1992), crustacean (Hazsch, Anger and Dawiris, 1997), molluscs (Voronezhskaya *et al*., 2002; Kempf and Page, 2005) and echinoderms (Garcia-Arra and Viruet, 1993). Therefore, anti-acetylated tubulin also allows the visualisation of developing neuron architecture within some marine invertebrate larvae.

Neuropeptides are used as signalling molecules in the nervous systems of a broad range of invertebrates and vertebrates (Li, Kim and Nelson, 1999), and play a role in the development of

the nervous systems (Kim and Li, 2004). FMRFamide is an important neuropeptide which is known to affect heart rate, blood pressure, gut motility, feeding behaviour and reproduction in invertebrates. There are more than a hundred FMRFamide-related peptides (Greenberg and Price, 1992), and they have been found in central larval ganglia and peripheral nerves in larval marine filter feeders (Hay-Schmidt, 1990). The use of antibodies against FMRFamide allows the visualisation of a subset of neurons and the development of the nervous system. Visualising these key developmental features via fluorescent immunohistochemistry permits the assessment of the underlying mechanistic basis of morphological anomalies, which environmental contaminants elicit in marine larvae development. Adopting techniques from molecular biology, such as immunohistochemistry aids in the assessment of the effects which microplastic pollution elicits on physiological processes.

2.1.7 Plastic Proxies: Environmental and Industrial Pellets

Nurdles, or virgin plastic pre-production pellets are a key source of primary microplastic pollution (Cole and Sherrington, 2016). Designed to be 3-5 mm in diameter, they are the industrial feedstock for the plastic industry (fig.2.3a; Mato *et al*., 2000). Approximately 27 million tonnes of nurdles are produced annually in the USA (Hammer *et al*., 2012). Transported all over the world, many of these pellets are lost accidently at sea during transportation. Despite difficulties in predicting the actual level to which are lost into the environment, recent estimates suggest that 5-53 billion nurdles may be lost in the UK alone (Cole and Sherrington, 2016; FIDRA, 2016). Environmental exposure, mainly photo-oxidation transforms a white, oval shaped

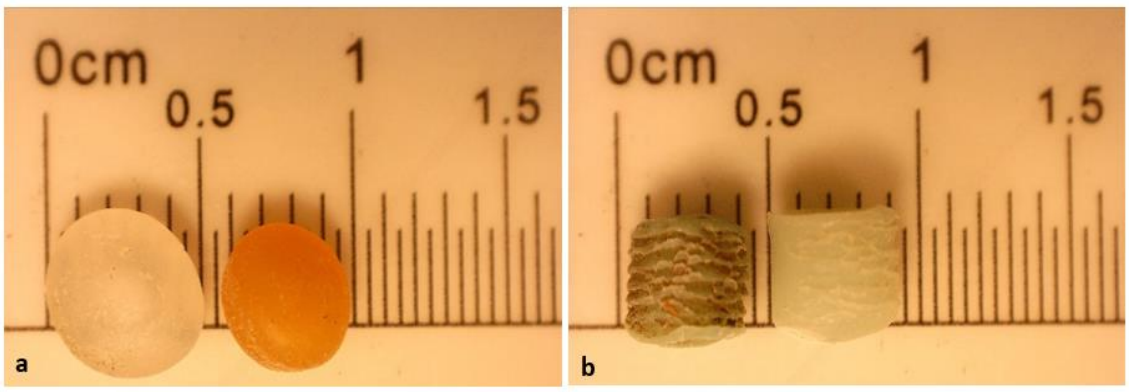


Fig.2.3 Beached microplastic pellets, sourced from Porthtowan beach, Cornwall, South West England. a) Nurdle pellets. Discoloured pellet on the right is due to increased environmental exposure, which indicates potentially higher hydrophobic organic contaminate load. b) Biobead pellets. Screw thread type profile increases surface area available for attachment of biofilm of bacteria.

polymer into a yellow-orange discoloured pellet (Endo *et al*., 2005). Weathering, ultraviolet light and oxidation causes pellets to yellow further, indicating the time spent in the marine environment and increased opportunity for persistent organic pollutants to be sorbed onto the surface (Ogata *et al*., 2009; Hong *et al*., 2017). Weathering and fouling by anthropogenic,

hydrogenous and biogenic accumulations increase the threat of plastic pellets, with higher PCB concentrations found on fouled pellets (Endo *et al*., 2005). Photodegradation and abrasion increases the surface area of pellets, further increasing the affinity of hydrophobic organic contaminates to sorb to plastics (Rehse *et al*.,2018; fig.2.3a).

Biobeads, or biological-aerated flooded filter (BAFF) are an emerging problematic primary microplastic pellet found on beaches of western Europe (Turner, Wallerstein and Arnold, 2019). Biobeads, 3- 4 mm in diameter, are designed to aid in filtration in waste water treatment plants (WWTP), acting as a buoyant, high surface area substrate for the attachment of a biofilm of bacteria used to digest compounds such as ammonia (fig.2.3b; Gray, 2004; Cornish Plastic Pollution Coalition, 2017; Turner, Wallerstein and Arnold, 2019). In the UK, the BAFF plant system is currently in use at 9 out of 600 plus South West Water WWTP. Nationally at least 55 plants, serving 2 million people employ this technology (Turner, Wallerstein and Arnold, 2019). This system is employed as an effective use of a smaller area, with wastewater passing through as a flooded condition and therefore requiring less area than an equivalent activated sludge process (Turner, Wallerstein and Arnold, 2019). The Cornish Plastic Pollution Coalition report that biobeads end up on our beaches through WWTP spills, transport and handling (Turner, Wallerstein and Arnold, 2019).

The concentration of pollution on beached pellets has allowed environmental scientists to passively sample areas for *in-situ* pollution concentrations. The International Pellet Watch (IPW) has been globally monitoring for hydrophobic organic contaminates, using plastic resin pellets since 2006 (Takada, 2006). Unpublished data from the IPW identified the levels of persistent organic pollutants in beached biobead (n=69) and nurdle (i.e. resin) (n=42) pellets from a sample from Tregantle beach. Concentrations of PCBs in pellets from Tregantle Beach (69 ng/g-pellet) was lightly to moderately polluted and consistent with those from UK southern coast which the project has previously analysed. In addition, concentrations of PCBs were similar between nurdle pellets (69 ng/g-pellet) and biobeads (42 ng/g-pellet). The similarity of PCB concentrations between pellets and biobeads are explained by the authors given that both are made of polyethylene (PE) and have similar sizes and, therefore, sorption capacity of PCBs to biobeads is similar to that of pellets. However, the data suggests that Tregantle biobeads contain higher levels of PAHs than nurdles, collected from the same area. The concentration of 16 major PAHs in biobeads was 3 to 4 times higher (929 ng/g-pellet) than that of nurdle pellets (263 ng/g-pellet). The authors highlight that these data suggest that biobeads may accumulate higher concentrations of PAHs as a result of sewage influents and effluents containing higher concentrations of PAHs than sea water (Hideshige Takada, pers. comm).

Turner, Wallerstein and Arnold (2019) are the first to publish on biobeads and their contamination with potentially toxic elements. This research suggests that biobeads are composed of a heterogenous mix of recycled plastic and end-of-life electrical and electronic plastic (WEEE) , with concentration of heavy metals in about 10% of biobeads sampled to be non-compliant with respect to current regulations on hazardous plastic waste (Turner, Wallerstein and Arnold, 2019). To date there has been no published peer-reviewed scientific research on the environmental impacts of biobeads, however they have strong potential to impact the marine environment in similar ways to nurdle beached pellets. Biobeads may also present an additional threat to marine life given their potential role in accumulating HOCs whilst in WWTP, and the increased content of recycled plastic within the biobeads, leading to a higher heavy metal content.

2.2 Chapter Three Aims and Hypotheses

The aims of this study were to determine and compare the toxicity of plastic pellets leachates from beached and virgin industrial plastic granules, which have the highest environmental relevance, or are composed of hazardous monomers and associated additives, to the development of key embryonic tissue structures of the green sea urchin (*Psammechinus miliaris*).

(1) What is the developmental timing and morphology of key embryonic and larval structures (blastula, gastrula, pluteus) for the green sea urchin (*Psammechinus miliaris*) under controlled laboratory conditions?

(2) Do environmental and industrial sourced plastic pellet leachates elicit defects in developmental timing and/or morphological abnormalities in *P. miliaris,* when compared to the wildtype development?

(3) Can such morphological abnormalities be identified in key embryonic tissue groups, specifically cilia, neural and muscle tissue using immunohistostaining of such structures?

2.3 Materials and Methods

2.3.1 Sample Collection and Maintenance

Adult *Psammechinus milaris* were collected during April 2018, at low tide by hand from subtidal and intertidal populations from Prisk Cove, Mawnan Smith, on the south-west coast of England (50.106832, -5.0866160). Brown macroalgae, *Laminaria Saccharina*, was collected from the same location as food source for adults. Animals were then transported back to the lab and placed in prepared glass aquaria with artificial sea water (ASW). Tanks were maintained at 9°C ± 1°C, with a photoperiod of 11:13 (light:dark), salinity of 32.5 ppt and pH of 7.5. Adults were maintained on a diet of *Laminaria Saccharina*, as suggested by Kelly (2000), until induced to

spawn. All tanks were environmentally enriched with pieces of rock and fauna from the sample collection site.

2.3.2 Spawning and Fertilisation

The eggs and sperm used in the present study were collected from a single adult female and two adult male *P.miliaris*. It is not possible to sex sea urchins by visual cues, therefore 6 sea urhcins were maintained in aquaria, with the anticipation that one of each sex was available. Spawning must be induced to distinguish between sexes via the colour of the gametes. Vigorous shaking of the sea urchins was undertaken to induce spawning, followed by an injection of 1ml 0.1 M KCL directly into the coelomic cavity through the membrane surronding the artistoles latern. Males were placed oral side down on a petri dish and sperm was allowed to collect in the dish. Sperm was then collected 'dry' using a pipette and samples were stored in an eppendorph on ice (<15 min) until use. Females were inverted over a 200ml beaker containing ASW and left to release eggs. 250µL standard sperm soloution (500µL sperm + 23.5mL ASW) was added to 300 mL egg culture. 10 mL zygotes were added to the beakers.

2.3.3 Embryonic Development

The embryo culture was incubated at 15°C±1°C for 150 hours post fertilisation (hpf) on a 11:13 (L:D) photoperiod. The developmental stages of *P. miliaris*, including blastula, gastrula and pluteus stages were documented, with a sub-sample of the culture photographed under a light microscope (Leica DFC 3000 G). Morphometric measurements were made on photographed live specimens from 13 different stages using Image J (table 2.1).

2.3.4 Microplastics Particles

The plastic pellets were selected based on (1) environmental relevance, beach pellets, or (2) plastic composed of hazardous monomers and high percentage of additives, PVC. A total of four pellet types were studied; two beached pellet types (pre-production nurdle resin pellets and biobeads) and two industrial pellet types (polyvinyl chloride and polyethylene) (fig.2.4).

Fig.2.4. Beached pellets were formed of two separate treatments; biobeads and nurdles (see text for characterisation). Industrial pellets were separated into raw industrial plasticised pellets, polyvinylchloride (PVC), and polyethylene pellets with no additives.

Environmental proxies included nurdles and biobeads collected from a local Cornish beach. Selective sampling was undertaken on multiple occasions during March-April 2018, at Porthtowan beach, on the North Coast of Cornwall. Beach-collected pellets and biobeads were sampled by sieving the surface sand. The pellets were collected using stainless steel tweezers and stored in sealed glass jars. (Note: fragment of plastic, fibres and foamed plastic were not considered in this present study). In the laboratory pellets were stored in aluminium foil in the dark at room temperature until further use. Nurdles and biobeads were separated based on visual identification. Biobeads were separated from nurdle pellets based on their 'screw thread' appearance. Pooling the colours removed the ability to ascertain abnormalities in phenotype to any particular contaminant; for example, black pellets have been associated with higher concentrations of PCBs and metals (Frias et al., 2010; Turner, 2018; Turner, Wallerstein and Arnold, 2019).

Industrial proxies included, high additive pellets and non-additive pellets, polyvinyl chloride (PVC) and low-density polyethylene (PE) respectively. PVC is renowned for its resistance to light, chemicals and corrosion, as a result being the 3rd largest selling commodity plastic worldwide (Babinsky, 2006). Based on previous research and the nature of the polymer, plasticized PVC pellets were chosen as a microplastic proxy with high levels of hazardous additives and therefore potentially leachates (Navarro *et al*., 2010; Stringer and Johnston, 2001). PVC is made from vinyl chloride and its composition requires high volumes of additives, accounting for 73% of the world

production of additives by volume (Murphy, 2001; Lithner *et al*., 2012). PVC comes in two forms, rigid and plasticised. Plasticised PVC, commonly used in the construction industry, can commonly be 35-40 % by weight plasticisers and can have amounts of up to 60 % by weight in some cases (Navarro *et al*., 2010). White PVC industrial plasticised pellets were kindly donated from Northern Polymers and Plastics (fig.2.4). The white PVC pellets were REACH and ROHS compliant, however the complete formulation was not known by Northern Polymers and Plastics as this information was the suppliers copyrighted information. However, what they could disclose was that the PVC had a K66 PVC resin base, a calcium zinc stabiliser and a diisononyl phthalate (DINP) plasticiser. Additionally, the pellets were said to have very small contents of other lubrication additives at very small percentages, zero $CaCO_3$ content and were lead free. The K-value is a measure of the molecular weight, the higher the K-value the higher the viscosity number and the lower rigidity of the product. K66 resin base equates to a medium molecular weight and the more widely used type of PVC due to its properties. Industrial low-density polyethylene granules were used to control for the presence of the polymer only. PE pellets were obtained from Sigma-Aldrich (cat number 428043; fig.2.4). Sigma's product manager was contacted to confirm the absence of additives in the pellets.

2.3.5 Fourier-Transformed Infrared Spectroscopy

The foundation of all plastic polymers is a series of repeating monomers, which forms the backbone of the polymer. This structure is the fundamental difference between polymer types, resulting in a fundamental differences in physical and chemical properties (Rochman *et al*., 2019). It is therefore important to know what polymer types are being used within the experimental design. In order to determine which polymer types predominate in beach pellet samples, approximately 50 randomly selected pellets within each of the beached sample categories were analysed using Fourier Transform Infrared spectroscopy (FT-IR) (Agilent Cary 630 FTIR spectrometer; PerkinElmer Spotlight 400 FT-IR Imaging System; PerkinElmer Spectrum software version 10.5.3.738). Polymer type was determined by comparing sample FT-IR spectra against known standard polymer spectra from PerkinElmer's Spectrum software, only those with scores 70% or greater, and considered to have reliable spectra matches (after visual inspection) were accepted. The FT-IR analyses was completed at the Greenpeace Science Laboratory at Exeter University, Streatham Campus.

2.3.6 Microplastic Leachate Exposure

Methods to obtain the microplastic leachate were adapted from Nobre *et al*. (2015); 20ml of pellets were placed in 30ml of artificial seawater (ASW) in 100ml beakers, remaining static to leach for 25 days. Following this the beakers were rocked for 17 hours on a horizontal platform shaker, with a continuous shaking orbit of 60 oscillations per minute in darkness at 18°C.

Leachates were then obtained by removing plastic pellets using a sterilised metal spoon. Test dilutions were prepared in ASW at 10 % of leachates and 10 mL of fertilised zygotes were added separate treatment beakers (fig.2.5).

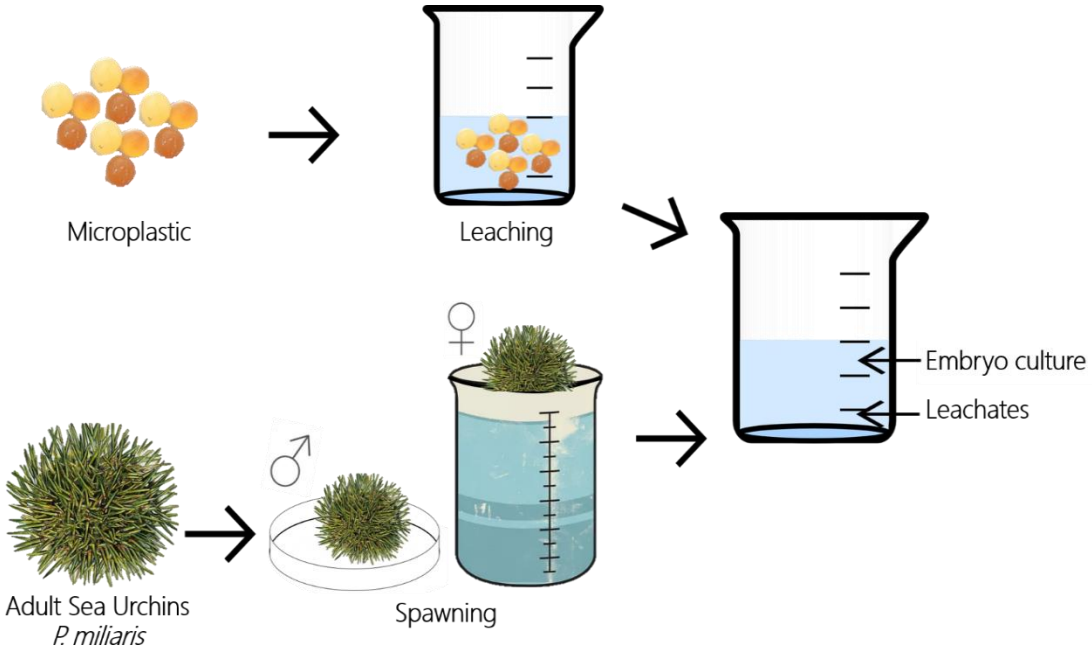


Fig.2.5 Experimental set up for toxicity assessment of microplastic leachates on *P. milaris* embryos.

2.3.7 Microplastic Toxicity Assessment: Morphological Assessment

Morphological abnormalities of live embryos were assessed by using a microscope (Leica DFC 3000 G) and classified on the basis of morphological features and timing of development compared with controls.

2.3.8 Microplastic Toxicity Assessment: Immunostaining Assessment

Fluorescent immunohistochemistry for key embryological features and processes namely neurogenesis (FMRFamide), myogenesis (phalloidin) and cilia (anti-acetylated tubulin) were carried out. Embryos from blastula, gastrula and pluteus stages (24,48 and 72hpf) were fixed in 4% paraformaldehyde (PFA) in ASW for 20 minutes at room temperature and washed in phosphate-buffered saline with 0.1% Triton (PSBTr). Following an initial wash with PBSTr, the cultures were split to allow differential staining. Embryos intended for analyses of muscle were washed twice in PBSTr. For analyses of neural and cilia tissue embryos were washed in methanol (MeOH) 50%, followed by two washes MeOH 100%. Methanol was avoided in Phalloidin intended samples, as it inhibits the staining. Embryo cultures intended for phalloidin and anti-acetylated staining were refrigerated at 4°C.Those intended for FMRFamide staining were kept at -20°C.

To visulse myogensisis the following protocol was completed on a sub-sample of embryos from 48 hpf. Fixed *P. miliaris* cultures from pluetus stage (48 hpf) were washed in phosphate-buffered saline with 0.1% Tween-20 (PBST) and incubated for 60 minuites, in darkness at room temperature (RT) with phalloidiin - 488 , diluted to a working concentration (1:100) in PBST. Following incubation, larvae were washed three times with PBST and mounted for imaging with a light microscope (Leica DFC 3000 G). Neurogenisis and cilia were visulaised using the following protocol on a sub-sample of embryos from 48 hpf. Whole-mount immunostaining was carried out according to the method of Romancino *et al*. (1998) and Zito *et al*. (2000) with some modifications. Fixed cultures from pluetus stage (48 hpf) were washed five times in PBST and blocked overnight at 4°C in 4% goat serum. The blocking was removed and incubated overnight at 4°C with primary antibody, depending on the desirded tissue stain: FMRFamide (aminergic neurons) raised in rabbit or acetylated tublin (cilia) raised in mouse diluted to 1:1000 in 4% goat serum in PBS. Followeing primary antibody incubation, larvae were washed 5 times in PBST and incubated for 2 hours at room temperature with the secondary antibody Alexa Fluro-488 goat anti-mouse or Alexa Fluro-488 goat anti-rabbit secondary antidodies. Larvae were washed in PBST five times and mounted for imaging with a light microscope (Leica DFC 3000 G).

2.4 Results

2.4.1 Wild Type Embryonic and Larval Development of P. miliaris

The morphological events occurring during the embryonic development of *P. miliaris* are depicted in table 2.1, while the developmental stages are shown in figure 2.6. The documentation of *P. miliaris* embryonic development provides evidence that this species follows the predicated pattern of embryonic development for echinoderms, as expected (Ettensohn, Wessel and Wray, 2004).

The fertilised embryos underwent successive radial cleavages to form a spherical hollow ball of cells at 6 hpf, representing a 32-cell stage embryo (fig.2.6a), prior to forming an epithelial monolayer, or a hatched blastula at 12 hpf (Table 1; fig.2.6b). After hatching from the fertilisation membrane, PMCs began to migrate into the blastocoel and vegetal plate began to form (fig.2.6c). From 24 hpf onwards, *P. miliaris* embryos began the process of gastrulation, typical of sea urchin species. At 26 hpf, embryos exhibited a thickening vegetal plate followed by primary invagination of the archenteron to form a mid-late gastrula at 31 hpf (Table 1; fig.2.6d). The archenteron tip developed to form the ventral surface of the prism stage of the embryo (fig.2.6e). Between approximately 36 and 48 hpf, secondary invagination underwent completion, and the gut and skeleton developed to form the two-arm pluteus at 54 hpf (fig.2.6f). The larvae continued to develop through the pluteus stage to form a digestive tract and 4 well-defined arms at 80 hpf (Table 2.1; fig.2.6g). At 150 hpf *P. miliaris* larvae exhibited four well-defined arms in late pluteus stage (fig.2.6h).

Table 2.1. Embryonic and larval developmental events of *P. miliaris* at 15°C ± 1°C. Asterisk (*) indicates where measurement of length is taken, as opposed to diameter (* Length (μm)).

Hours post fertilisation (hpf)	Developmental stages	Diameter/*Length (μm)
06.00	32-cell enclosed with fertilisation membrane	146.11
12.00	Mesenchyme blastula	153.61
24.00	Early gastrula exhibiting vegetal plate	164.24
26.00	Early gastrula with thickening vegetal plate	167.98
31.00	Mid-gastrula with primary invagination of archenteron	171.42
37.00	Early prism stage	167.64
48.00	Prism stage	*196.22
54.00	2-arm pluteus	*334.74
59.00	2-arm pluteus	*366.73
70.00	4-arm pluteus	*382.23
80.00	4-arm pluteus	*463.13
130.00	4-arm late pluteus	*461.10
150.00	4-arm late pluteus	*430.43

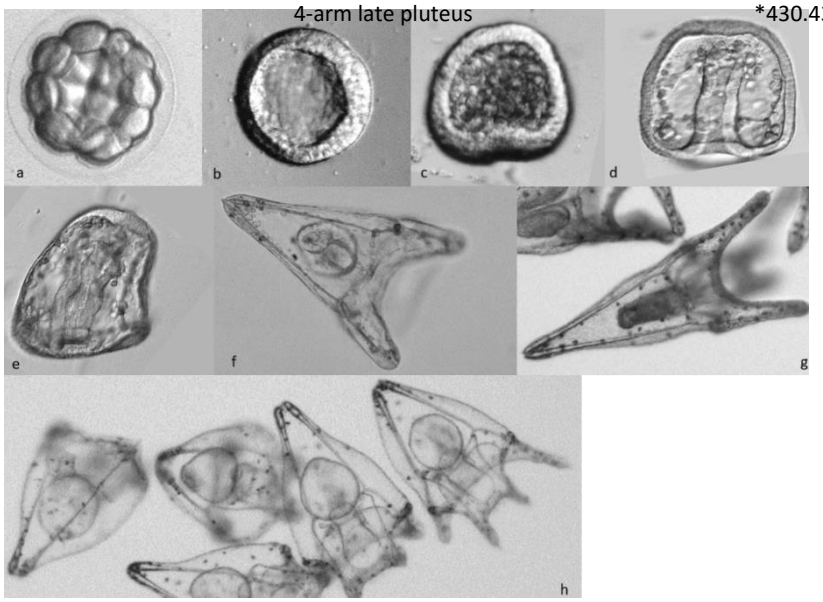


Fig.2.6. Development of the sea urchin embryo *Psammechinus miliaris* at 15°C ± 1°C. (a) 32-cell 6hpf; (b) mesenchyme blastula 12 hpf; (c) early gastrula 24hpf, blastopore visible; (d) mid-late gastrula 31 hpf; (e) prism 36 hpf (f) 2-arm pluteus 54 hpf; (g) pluteus 80 hpf; (h) 4 arm-pluteus 150 hpf.

2.4.2 Physical Characterisation of Microplastics

Colour coding revealed that the majority of beached nurdle pellets (fig.3.5; n=360) were white-clear (51.7%), with the next most abundant colours being aged-yellow (19.4%). The remaining pellets were a mixture of grey (15.3%), white (3.7%), black (3.4%), blue (2.2%), purple (1.9%) and green (0.3%). The aged pellets had a distinctive amber colouration. Within the beached biobead sample (fig.3.5; n=347) the most abundant pellet colour was blue (38.3%), followed by black (30.8%), with the remaining pellets clear (13.0%), grey (11.0%) and green (4.9%). All industrial pellets, PVC (n=491) and PE (n=383), were clear in colouration.

Table 2.2. Descriptive data on beached and industrial pellets used to produce leachate exposure to *P. miliaris* embryos.

	Polyethylene	**Polyvinyl Chloride**	**Beached nurdle**	**Beached biobead**
no. pellets/20ml	383	491	360	347
mean mass, mg	29	27	31	31
Dominant colour	white-clear	white-clear	white-clear	blue

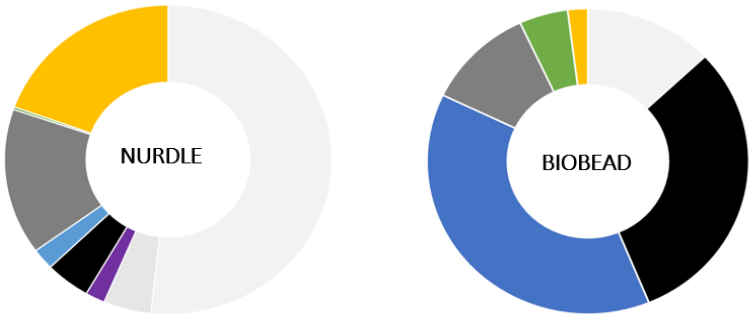


Fig.2.7. Colours of Porthtowan beached pellets split into nurdle (n=360) and biobead (n=347)

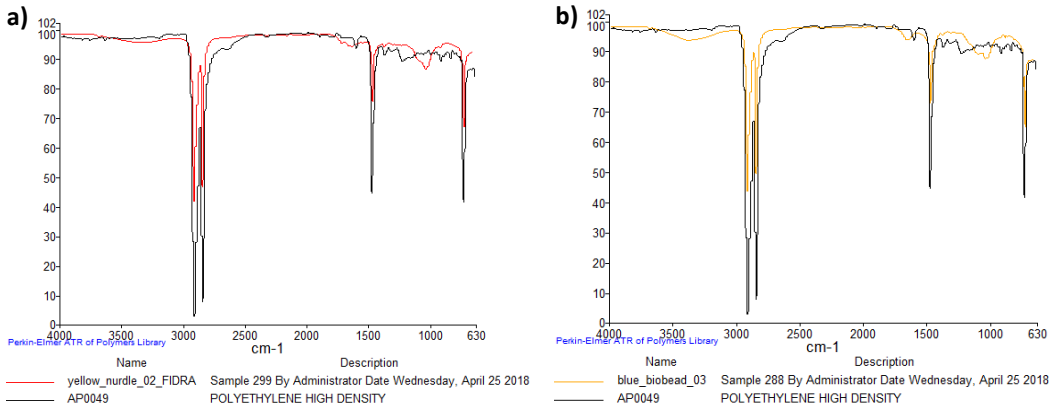


Fig.2.8. Example of FTIR spectra for (a) nurdle pellet and (b) biobead pellet, identified as high-density polyethylene (red and yellow lines) matching library search (black line).

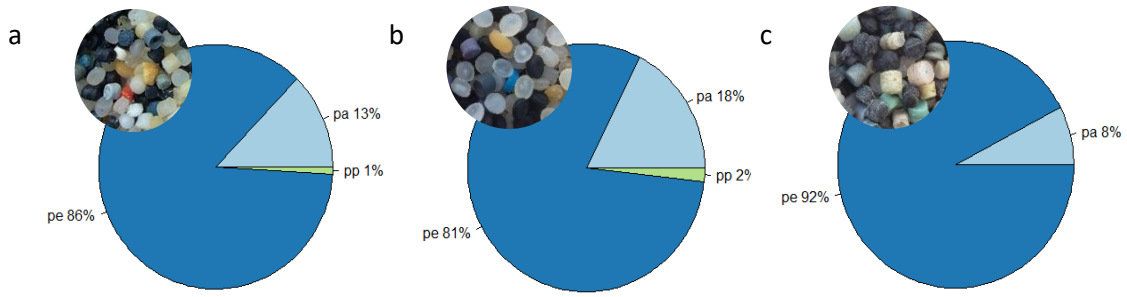


Fig.2.9. Polymer composition of Porthtowan beached pellets; polyethylene (pe), dark blue; polyamide (pa), light blue; polypropylene (pp), green. a) Beached pellets polymer composition (n=109). b) Nurdle polymer composition (n=57). c) Biobead polymer composition (n=52).

Measurements revealed that both beached biobeads and beached nurdles were, on average, 31 mg. Whereas industrial pellets, PE and PVC, were 29 and 27 mg, respectively. The microscopic images shown in figure 3.2 highlights the differences between beached bio-beads and nurdles, or pre-production pellets.

Results of FTIR analysis on a sub-sample of pellets revealed that the majority of beached pellets were polyethylene (86%; fig.2.9), followed by polyamide (13%) and polypropylene (1%) (fig.2.9a, n=109). The sub-sample of nurdles contained all three polymer types (fig.2.9b), however biobeads were composed of polyethylene and polyamide only (fig2.9c).

2.4.3 Microplastic Toxicity Assessment: Morphological Assessment

Unfortunately, it was not possible to reliably assess the impact of microplastic leachates on the morphology *of P. miliaris* as the fertilisation rate and culture was very poor, which lead to reduced numbers of embryos and larvae within treatments to give a reliable phenotype within each treatment. The key phenotypes observed in the embryos and larvae at five key time points, spanning blastula, gastrula and pluteus stages are depicted in figure.2.10.

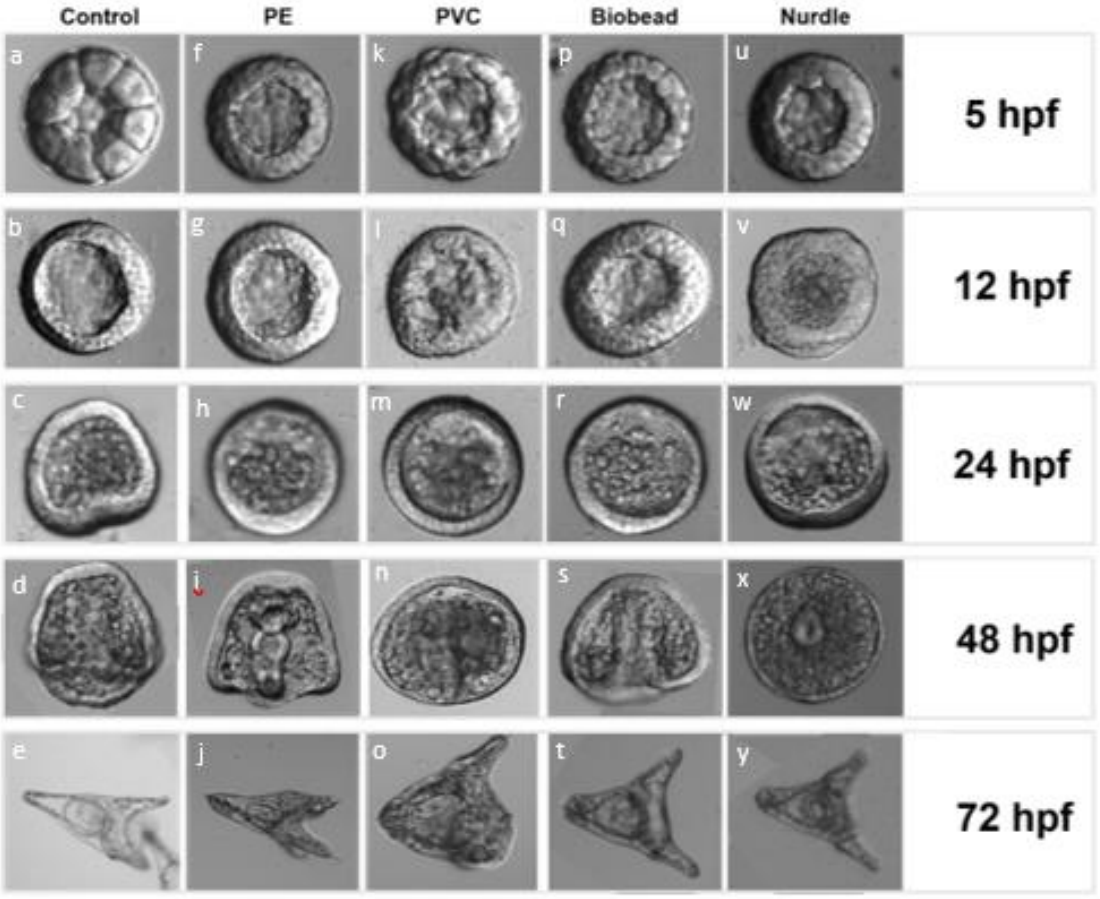


Fig.2.10 Phenotypic comparison of control and treatment *P. miliaris* embryos and larvae at 5,12, 24, 48- and 72-hours post fertilisation. Treatments plastic leached for 25days static and 17 hours shaking 72h at 10% microplastic leachate in ASW. Wildtype/control for comparison of key developmental time points.

Observing the above comparison, it could be suggested that developmental timing was delayed for all treatments, when compared to wildtype control (Fig.2.6a-e). Specifically, at 24 hpf the wildtype treatment was beginning to undergo gastrulation, with the blastopore starting to form. However, all other treatment lacked a blastopore and signs of archenteron elongation. However, the observations from this experiment are not very informative until 72 hpf, given the lack of embryos and therefore the view in which the embryo is placed prevents a full observation of developmental timing. At 48 hpf the wildtype larvae began to form an early pluteus (fig.2.10d), with PE also reaching this stage (fig.2.10i), with a well-developed gut. PVC and beached treatment groups however remained in the gastrula stage exhibiting a rounder phenotype, with the archenteron continuing to elongating across the blastocoel in the biobead treatment (fig.2.10n,s,x). The nurdle treatment embryo is positioned in the anal view (fig.2.10x), therefore it is difficult to assess how well-developed the archenteron is.

At 72 hpf, the wild type larvae exhibited four well-formed arms, a complete skeleton and gut (fig.2.10e). The PE treatment experienced some skeletal abnormalities, with spicule protruding from the arms (fig.2.10j). This could be due to a problem with the culture; however, the overall morphology was similar to that of the wildtype. The morphology of 72 hpf pluteus larvae treated

with PVC microplastic leachates exhibited an abnormal structure, with a shorter body length and rounder phenotype, when compared to the wildtype. The PVC treatment also gave rise to larvae with shorter arms, less developed and anomalous skeletal structure, with spicule extending from the arms (fig.2.10o). Those embryos exposed to beached microplastic leachates, biobeads and nurdles, exhibited a less severely altered phenotype when compared to PVC. They did however experience a developmental delay, with less defined structures compared to the wildtype control. Both biobead and nurdle treatment, show a shorter body length and splayed arms phenotype (fig.2.10t, y). However, all of these assessments are based on a very small sample size and therefore reliable and valid conclusions cannot be drawn, rather a trend in the images above can be speculated.

2.3.4 Microplastic Toxicity Assessment: Immunohistochemistry

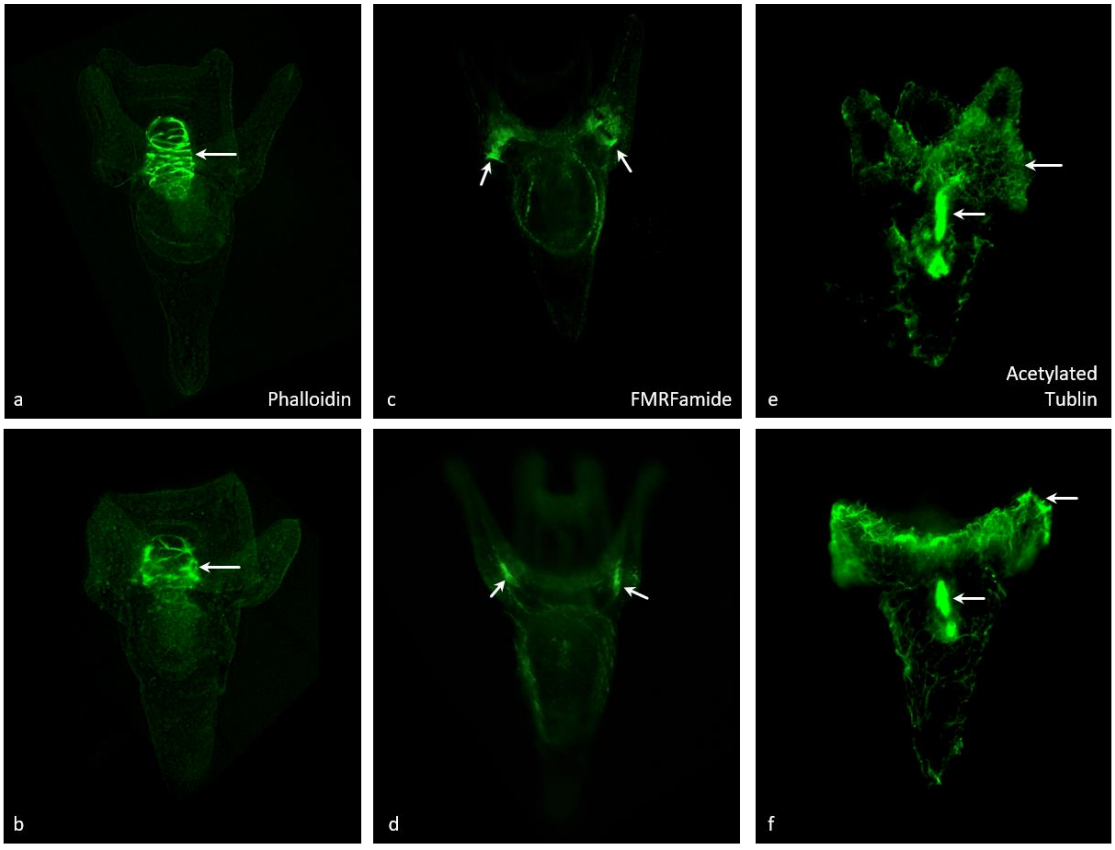


Fig. 2.11. Immunostaining of *P. miliaris* wildtype larvae at pluteus stage 48 hpf, oral and anal view. (a-b) phalloidin labelling of the circumesophageal muscle fibres (arrows in a-b). (c-d) FMRFamide antibody staining of neural tissue (arrows c-d). (e-f) acetylated tubulin antibody staining in the stomach (central) and on cilia surface (arrows e-f).

Given the poor fertilisation rate within the embryo culture, immunohistochemistry was not run on treatment embryos, instead the immunostaining protocols were learnt and practiced on control embryos. Figure 2.11. shows the immunostaining of circumesophageal muscles (phalloidin), immunoreactive neurons (FMRFamide), and cilia (anti-acetylated tubulin) in

wildtype *P. miliaris* larvae at 48 hpf. The circumesophageal muscle fibres can clearly be seen, as indicated by the arrow (fig.2.11 a-b). The neural staining with FMRFamide was very specific to the base of the arms in the larvae (fig.2.11 c-d, arrows). Acetylated tubulin antibody staining shows the cilia in the stomach, at the surface and along the ciliary band (fig.2.11, arrows e-f).

2.5 Discussion

Section *2.4.1* of this study documents the key stages in the early development of the green sea urchin, *P.* miliaris, from blastula to 4-arm pluteus. Development of *P. miliaris* embryo and larvae were similar to those reported in other echinoids with planktonic larvae (Strathmann, 1987; Ettensohn, Wessel and Wray, 2004). The study found that the husbandry of *P. miliaris* is relatively easy, when compared to larger species such as the common sea urchin, *Echinus esculentus.* However, lack of access to sufficient ripe animals prevented consecutive experiments to be run during the spawning season. Difficulty accessing *P. miliaris* lead to the collection of a more accessible species. Six specimens of *E. esculentus* were caught at Prisk Cove in September 2018 to be used for gamete collection and future experimental work. However, due to their size and lack of ideal facilities to house the larger urchins, most of the animals died or were returned to Prisk Cove. Setting up a novel experimental model for Penryn Campus, with little husbandry literature and the lack of ideal housing facilities to base our efforts on, lead to this piece of research to be a steep learning curve about the difficulties of working with live animals and basing the research around one spawning season, with access to few animals. Experimental issues and experience working with the study species prevented a full, clear record of the development of *P. miliaris* to be documented.

The increase in marine plastic pollution, resulting from a rapid increase in plastic production and subsequent incorrect disposal or accidental loss, presents microplastic pollution as an emerging contaminate of concern within the aquatic environment (Browne *et al*., 2007). The explosion of studies around the subject of microplastics and the effects in marine life is providing a deeper understanding of this complex problem (Anbumani and Kakkar, 2018). However, few studies have investigated the developmental effects of microplastic leachates, which are critical to understand the extent of damage microplastics elicit on marine life and ecosystems. A handful of studies have investigated the impact of microplastic or nanoplastics on sea urchins (Kaposi *et al*., 2014; Della Torre *et al*., 2014; Nobre *et al*., 2015; Beiras *et al*., 2018; Mesinetti *et al*., 2018; Oliviero *et al*. 2019), however to the best of our knowledge, there are no studies assessing the developmental impact at a tissue level. Here, I investigated the effects of four different types of microplastic leachates, with varying additive and organic contaminant load, to the development of *P. miliaris* at key stages of development. The results obtained in this chapter unfortunately do not address the developmental impact of microplastic leachates on a tissue level, however

chapter four completes this aim. Despite not gaining the results I would have hoped for, the experience taught me a huge amount about the interdisciplinary subject matter, working with sea urchin embryos and learning key molecular protocols, which I would have otherwise not have had a chance to study.

I was unable to quantitively assess the impacts of microplastic leachates on *P. miliars* larvae, however it was possible to identify phenotypes within each treatment. It is important to note that this assessment lacks reliability with less than 10 embryos for comparison within each treatment. Overall, the results indicate that there is a potential effect of microplastic leachates to the developmental timing and organisation of key tissue groups in *P. miliaris.* The extent to which this effect is reliable cannot be drawn from this data set, due to minimal sample sizes. Difficulties encountered culturing *P. miliaris* embryos, lead to two attempts to run this experiment, however both experiments lead to a very low fertilisation and survival rate.

The delay in development and anomalous features observed in the beached pellet treatments suggests that contaminants leached from the biobeads and nurdle pellets disrupted the developmental program of the embryo culture. This may have been as a result of the leached hydrophobic organic contaminates from the pellets. However, due to lack of information on the chemical composition of the leachates that the embryo cultures were exposed to, it is difficult to conclude. Building on this, further research in chapter four uses chemical analysis to characterise the additives and pollutants which leach from the pellet treatments. Interestingly, the industrial low-density polyethylene treatment, which was confirmed to have no additives from the manufacture process, produced a similar phenotype to that of the control. This strengthens the suggestion that the morphological abnormalities observed in the PVC and beached pellet treatments are a result of the additives and hydrophobic organic contaminates leaching from the polymer matrix.

The characterisation of the beached-collected pellets suggests that there is a higher variation in colours in beached nurdle pellets, with eight different colours observed. The biobead pellets consist of mainly blue and black polymers, which aligns with the recent research from Turner, Wallerstein and Arnold (2019), suggesting that biobeads are commonly composed of WEEE polymers, being mostly black in origin. Differences in the colour of plastics can reflect differences in polymer additives and/or dyes, and subsequently toxic effects from leaching of such additives. The FTIR analysis of the beached pellets identified the most common plastics from both pellet types as polyethylene, which reflects the most common polymer type found in previous studies of beached pellets (Frias *et al*., 2010; Turner, Wallerstein and Arnold, 2019).

The higher proportion of aged pellets within the beached nurdles indicates the length of time these pellets have been environmental contaminants, given that the yellowing is likely to have been caused by the photo-oxidative formation of by-products in originally white-clear pellets (Endo *et al*., 2005). Gorman *et al*. (2019) assessed the organic contamination of beached pellets along a 39-km stretch of Brazil's South Atlantic coastline and found PAH concentrations to exceed the threshold effect level for sediments defined by the United States Environmental Protection Agency. The study highlights the complexity of assessing toxicological effects from organic contaminant load on microplastics, given the heterogeneity of their data on PAH and PCB concentrations. Gorman *et al*. (2019) report that contamination gradients may not be linear, instead governed by location specific pellet inputs and local pollution sources. These data would align with the observations from the Tregantle pellets, given the increased PAH load on biobeads, in comparison to the beached nurdles, as a result of road-run off (Hideshige Takada, pers. comm). These data would therefore predict the biobead treatment to result in higher embryotoxicity than the nurdle beached pellets, given the higher PAH load, however this was not observed in this data chapter.

Immunostaining within wildtype larvae, matched those observed in previous studies on *Paracentrotus lividus* (Gambardella *et al*., 2015), and allowed for the practice of molecular techniques within the project. However, no specific conclusions can be drawn from the immunostaining of those larval tissues of *P. miliaris,* as only control larvae were stained. If more time was given, further research would seek to re-run the experiments and immunostaining to draw conclusive data. Furthermore, I would like to identify the transgenerational effects of microplastic co-contaminants on larvae. Clark *et al*. (2018) evidenced that adult conditioning to low pH critically pre-loads the embryonic transcriptional pool with antioxidants to prepare larvae for the new conditions. This study highlights the ability for microplastics to act as environmental pollutants and to contribute to the vulnerability of early life stages in marine invertebrate larvae. Following multiple attempts to culture and treat *P. milaris* embryos in Penryn, it became obvious that this was not going to give robust and reliable results needed to observe the effects of microplastic leachates on sea urchin larvae development, mainly due to the lack of adult animals.

Chapter Three

Developmental Toxicity of Microplastic Leachates to *Paracentrotus lividus*

3.1 Introduction

The challenges experienced working with *P. miliaris,* made it necessary to look for another sea urchin model. The main sea urchin models within developmental biology are *Strongylocentrotus purpuratus*, a Californian species, and *Paracentrotus lividus*, a Mediterranean species. We therefore sought a collaboration with a laboratory expert in sea urchin development at Stazione Zoologica Anton Dohrn (SZN), Napoli. Scientists at the SZN are experts in the study of the development of *P. lividus*. Therefore, this institute was chosen based on the knowledge and skills of the Arnone group, and access to key facilities and *P. lividus* to allow successful completion of the research proposed in chapter three. In addition to this, chapter three outlined that we do not have the expertise to consistently culture sea urchin embryos. The collaboration between myself, Eva Jimenez-Guri, and the Arnone lab was possible thanks to ASSEMBLE Plus funding. All experimental treatments with live animals and embryos were performed at SZN.

3.2 Chapter Three Aims and Hypotheses

Similarly, to chapter two, the aims of this study were to determine and compare the toxicity of plastic pellets leachates from beached and virgin plastic granules, to the development of key embryonic tissue structures. Chapter three focuses on the embryonic and larval stages of the purple sea urchin (*Paracentrotus lividus*). The following key questions were addressed with experimental work at the SZN laboratories in Naploi:

(1) What is the developmental timing and morphology of key embryonic and larval structures (blastula, gastrula, pluteus) of the purple sea urchin (*Paracentrotus lividus*) under controlled laboratory conditions?

(2) Do environmental and industrial sourced plastic pellet leachates elicit defects in developmental timing and/or morphological abnormalities in the embryonic and larval stages of *P. lividus,* when compared to the species wildtype development?

(3) Does length of leaching time and volume of plastic pellets result in differences in morphological abnormalities and developmental timing of embryonic and larval stages of *P. lividus*?

(4) Can such morphological abnormalities be identified in key embryonic tissue groups, specifically cilia, neural and muscle tissue using immunohistostaining of such structures?

3.3 Materials and Methods

To evaluate the toxicity of additives and sorbed pollutants leaching from the microplastics, embryos of *P. lividus* were exposed to the leachates of industrial and beach-collected pellets. The plastic pellets were selected based on (1) environmental relevance, beach pellets, or (2) plastic composed of hazardous monomers and high percentage of additives, PVC. A total of four pellet types were studied, alongside a control treatment consisting of filtered seawater. Four key developmental stages were tracked for embryo cultures, from blastula all the way through to larval development. However, for some of the analyses only 24 hpf and 48 hpf were used to compare developmental abnormalities and timing between treatments. Replication within embryo cultures was performed, with approximately 100 embryos or larvae from each treatment and time point assessed for developmental abnormalities. However, no replication within treatments (repeated measures) was performed. This was due to space, time and resource limitations. Therefore, the statistical framework of the analyses carried out are based on very simple proportional comparison (two-proportion z-test) between numbers of embryos or larvae with each morphological category, each performed for independent experiments.

3.3.1 Animal Husbandry and Embryo Culture

Adult *P. lividus* were collected by SZN drivers and housed in circulating seawater aquaria (18°C). Gamete release was induced by first vigorously shaking ripe animals, followed by an injection of potassium chloride for those who did not spawn. One male and one female were used in each experiment. A suspension of eggs was filtered through 100 µm mesh to remove the jelly coat and debris into a new beaker with a few ml of filtered sea water (FSW). FSW was used to rinse the mesh to remove any remaining eggs and the egg culture was kept on a cold plate at 13°C until used. Gametes were stored at the specified conditions for no longer than 120 minutes until fertilisation was induced. 5µl sperm was diluted in 12ml FSW and used immediately for fertilisation. Diluted eggs were fertilised in one batch, by 0.3 ml diluted sperm, and swirled to mix the gametes. Fertilisation success was checked by observing the elevation of the fertilisation envelope in > 90% of eggs, which represents a successful fertilisation. Embryos were then added to the treatment beakers at a density of 50 embryos.ml^{-1}, and transferred to an incubator at 18°C.

3.3.2 Microplastic Particles

Environmental proxies were composed of beached pellets from Tregantle beach, UK (50.339591, -3.240468), collected in March 2019 by Rob Arnold. Beached nurdles (pre-production pellets) and biobeads (WWTP) were separated based on visual identification, with expert advice from

Rame Peninsula Beach Care. Biobeads were separated from nurdle pellets based on their 'screw thread' appearance. Dubious particles were discarded. Two industrial proxies were used to assess the toxicity of polymer additives. White PVC prime virgin plasticised pellets were obtained from Northern Polymers and Plastics (see chapter three, *2.3.4*). Virgin low-density polyethylene granules (PE) were chosen as a non-additive proxy and obtained from Sigma-Aldrich (cat number 428043). Sigma's product manager was contacted to confirm the absence of additives in the PE pellets. PE pellets were used as analyses of the composition of beached pellets showed that the dominant polymer type is PE (see section *2.4.1*). Control treatments underwent the same leaching protocol, consisting of filtered sea water.

3.3.3 Fourier-Transformed Infrared spectroscopy

In order to determine which polymer type predominated the microplastic samples, a sub-sample of randomly selected biobead and nurdle pellets from Tregantle beach (50.339591, -3.240468) were analysed for polymer composition, using Fourier Transform Infrared Spectrometry (Perkin Elmer, FT-IR Spectrum, 30 scans). Individual samples were placed on the ATR crystal and FTIR absorption spectra were recorded as an average of 30 scans min^{-1} the mid-infrared range 4000 - 450 cm^{-1}. Polymer type was determined by comparing sample FT-IR spectra against known standard polymer spectra from PerkinElmer's Spectrum software (version 10.5.4).

3.3.4 Leachate Preparation

Microplastics were removed from direct interaction with the developing embryos to remove potential mechanical effects of the pellets. In addition, microplastics were removed from beakers due to the continuous leaching potential of the pellets throughout embryo development, potentially increasing toxicity for the larvae during later stages of development. Methods to obtain the microplastic leachate were adapted from Nobre *et al*. (2015) 0.1L of pellets were placed in 0.4L of filtered seawater (FSW, 0.22 µm filtered Mediterranean Sea water) in 1L glass bottles. Pellets were leached for either 24 or 72 hours on a Heidolph orbital platform shaker (Heidolph Unimax 2010), with a continuous shaking orbit of 54 min^{-1} (speed 2) in darkness at 18°C. Leachates were obtained by filtering the FSW with Whatman GF/C glass microfiber filters (1.2 µm pore size) in order to remove particles. Test dilutions were prepared in FSW at 2%, 4%, 5%, 10%, 15%, 20% of leachates. Between 400 – 600ml of test dilutions were transferred into glass beakers with aluminium foil caps and stored at 18°C for a maximum of 60 minutes, until *P. lividus* embryos were added.

3.3.5 Microplastic Leachate Chemical Analysis

In order to preliminary test to see if phthalates and polyaromatic hydrocarbons (PAHs) were present in plastic leachate water samples, Chris Mitchell ran the following extraction procedure.

Three separate water samples containing PVC, beached nurdle pellets and beached biobead pellets and one control sample were analysed using four different types of SPME fibre (65µm PDMS/DVB, 85µm Carboxen/PDMS, 50/30µm DVB/CAR/PDMS for phthalates, and 100µm PDMS for PAHs). The extraction and GC methods were based on the methods of Luks-Betlej et al (2001). Water samples were placed in 20ml GC vials, and sampled using a CTC PAL autosampler. The extraction was carried out at 30°C for 40 minutes, with the agitator set to 750rpm. Samples were injected into an Agilent 7890A GC coupled with a 5975B MSD. The inlet temperature was set at 270C, and the desorption time was 5 minutes with the inlet operating in splitless mode. The GC was fitted with a HP5-ms column (30m x 0.25mmx0.25µm). The initial oven temperature was 60°C for 5 minutes, increasing to 280°C at 15°C/min, with a 10-minute hold. The scan range of the MSD was set to m/z 45-450.

3.3.6 Microplastic Leachate Exposure

Embryo cultures remained static at 18°C under a 12:12 light dark cycle. Embryos were exposed to plastic leachates and the development of key stages were tracked to larval stage. Blastula, gastrula and pluteus stages were fixed with 4% paraformaldehyde for further analyses. Live image counts on approximately 100 unfixed embryos for each treatment under a light microscope were also obtained at these time points to characterise embryo morphology. Morphological abnormalities of embryos were assessed under the microscope (ZEISS Imager.Z2 and Lieca DMI 6000 B) and classified on the basis of morphological features and timing of development compared to controls.

Preliminary testing of the effect of microplastic leaching time and concentration, informed the use of 72-hour leaching protocol for all pellet types, along with microplastic leachate concentrations of; 5% and 10% PVC, 15% beached pellets (biobead and nurdle pellets) and 20% PE. These concentrations were chosen as these were the concentrations of microplastic leachates which gave consistent phenotypes in the embryo and larval cultures. An outline of the experiments undertaken, including different leaching times, microplastic leachate dilutions and embryonic and larval stages fixed is available in supplementary table.s.3.1. Most of the images and analyses in the following section focusses on experiment three, unless otherwise stated, as sufficient live images were taken at the key developmental stages assessed, under the chosen leachate concentrations and microplastic leaching time. Furthermore, consistent phenotypes were observed across experiments between corresponding leaching times and dilutions.

3.3.7 Assessment of Toxicity: Morphological Assessment

Characterisation of embryos (24 hpf) were completed following methodology based on Gambardella *et al*. (2013) and Cordinaldesi *et al*. (2017). Live images of a sub-sample of embryos

(n=100) from each treatment were taken and the number of anomalous embryos, as well as their morphological characterisation were determined and compared with controls. At 24 hpf embryos were classified based on developmental timing and abnormalities into five defined categories (table.3.1); developed (D), delayed (DD), strongly delayed (SD), under-developed (UD) and anomalous (AD). Those embryos displaying normal development (D), showed well-structured, normal archenteron; delayed (DD) embryos showed a delayed archenteron or were positioned in an anal view; strongly delayed (SD) embryos displayed a blastopore and vegetal plate, but no archenteron; under-developed (UD) embryos remained as a mesenchyme blastula, exhibiting a strong delay in development and lacking key defining features, such as an archenteron, vegetal plate or blastopore. The anomalous class (AD) was used for embryos which exhibited abnormalities such as cell death. Wildtype embryos tend to rotate on a lateral plane which allows to clearly see the archenteron developing. However, embryos which were positioned in the anal view, so that the archenteron length could not clearly be seen, were classed as delayed embryos.

The embryos from each condition were mounted on slides and morphology was compared among the different microplastic leachates treatments and the controls. Categories were evaluated quantitatively, classifying embryos based on their category from level 0 (normal development) to level 4 (anomalous), to establish a quantitative measure of severity of the toxicity of microplastic leachate to embryonic development.

Pluteus larvae (48 hpf) were assessed in a similar way to embryos, with normal developed (D) larvae exhibiting complete skeletal rods and with well-formed four arms (table.3.1). Malformations such as fused arms, crossed or separated tips, and developmental delay were classified under malformed larvae (MF) or undeveloped larvae (UD). Malformed larvae included developed 4-arm larvae with abnormalities, while undeveloped larvae included all those which had not reached 4-arm pluteus stage after 48h (fertilised eggs, blastula, gastrula and prism). Similarly, to the 24 hpf category, the anomalous class was used for under-developed embryos or larvae which also exhibited abnormalities such as cell death. The toxicity was evaluated

Table 3.1. Classification of embryonic (24 hpf) and larval (48hpf) developmental stages

Embryonic Classification (24 hpf)		
Category	**Score**	**Feature**
Developed (D)	0	Well-structured, normal archenteron
Delayed (DD)	1	Delayed archenteron formation (lateral view) + radalised (anal view)
Strongly delayed (SD)	2	No archenteron, blastopore and vegetal plate visible
Under-developed (UD)	3	Mesenchyme blastula
Anomalous (AD)	4	Malformed and abnormal features
Larval Classification (48 hpf)		
Category	**Score**	**Feature**
Developed (D)	0	Normal, 4-arm, complete spicules
Malformed Larvae (MF)	1	4-arm, incorrect location of spicules
Under-developed (UD)	2	Reduced spicules, stunted arms visible
	3	No arms visible
Anomalous (AD)	4	Abnormal features

quantitatively, with methods adapted from Cordinaldesi *et al*. (2017). Classification spanned from level 0 (normal development) to level 4 (anomalous), to establish a quantitative measure of severity of the toxicity of microplastic leachate to larval development, as with embryos at 24 hpf. This allowed an index of microplastic impact (IMPI) to be calculated. At both 24 and 48 hpf, the frequency of anomalies for each degree of larval alteration were determined and IMPI score was calculated by multiplying the level number (e.g. 0,1,2…) by the percentage of embryos or larvae classified within that level, for example:

IMPI = [0 x % level 0 + 1 x % level 1 + 2 x % level 2 + 3 x % level 3 + 4 x % level 4] / 100

IMPI ranged from 0 (no impact) to 3 (high impact), including also the levels 1 (slight impact) and 2 (moderate impact).

3.3.8 Nickel Chloride Exposure

As a result of a high numbers of embryos expressing a specific radialised phenotype and positioned in an anal rotation in the beached pellet treatments, additional experiments were undertaken to compare this abnormal phenotype to that of embryos experiencing heavy metal exposure. For methods regarding animal husbandry and embryo culture refer to section *3.3.1*. Cultures of *P. lividus* embryos (24 hpf) and larvae (48 hpf) were exposed to either 0.05 mM or 0.1 mM Nickel salt ($NiCl_2$ hexahydrate), based on Hardin *et al*. (1992) lowest dose of $NiCl_2$. which gave a similar radialised phenotype in embryos exposed to beached pellet leachates.

3.3.9 Fixation of Sea Urchin Embryos and Larvae

Embryos and larvae were fixed in 4% PFA in ASW for 20 minutes at room temperature and washed in phosphate-buffered saline with 0.1% Tween (PSBT). Fluorescent immunohistochemistry and staining for key embryological features, namely neurogenesis (anti-serotonin), myogenesis (phalloidin), skeletal (1g8 and 1d5) and cilia (anti-acetylated tubulin) was carried out on the fixed embryos.

3.3.10 Phalloidin Staining

Fixed *P. lividus* cultures from pluetus stage (48 hpf) were incubated for 60 minuites, in darkness (RT) with Alexa fluor 488 Phalloidin (thermo fisher), diluted to a working concentration (1:100) in PBST. Following incubation, larvae were washed three times with PBST and mounted for imaging with a light (Leica DFC 3000 G) and confocal microscope (Leica CTR 7000).

3.3.11 Fluorescent immunohistochemistry of embryos and larvae

Anti-serotonin antibody was used to assess pleutus larvae for the presence of serotonergenic neurons. This antibody labels a specific subset of neurons in the epithelium of sea urchins (Wikramanayake and Klein,1997). The monoclonal antibody 1g8 (McClay *et al*., 1983) specific for ingressed primary meschyme cells (PMCs), was used to stain PMCs of treated and control embryos at 19hpf. Whole-mount immunostaining was carried out according to the method of Romancino *et al*. (1998) and Zito *et al*. (2000) with some modifications for anti-serotonin, acetylated tubulin and 1g8. Fixed cultures from 19 or 48 hpf were and blocked overnight at 4°C in 4% goat serum. The blocking was removed and incubated overnight at 4°C with primary antibody, depending on the desired tissue stain: anti-serotonin (1:1000, Sigma) raised in rabbit, acetylated tublin (1:1000, abcam) raised in mouse or 1g8 used undiluted raised in mouse, diluted in PBS containing 4% Goat Serum. Following primary antibody incubation, larvae were washed 5 times in PBST and incubated for 2 hours at room temperature with the secondary antibody goat anti-rabbit Dylight-488 (thermo fisher) or goat anti-mouse alexa-488 (abcam) secondary antidodies. Larvae were washed in PBST five times and mounted for imaging with a light microscope (Leica DFC 3000 G) and confocal microscope (Leica CTR 7000).

3.4 Results

3.4.1 Physical and chemical characterisation of microplastic

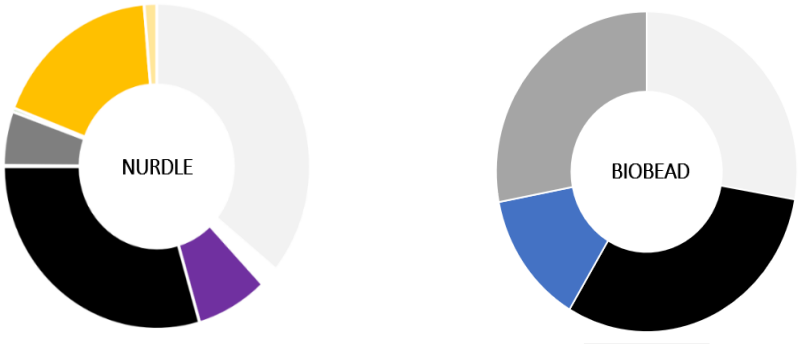


Fig.3.1. Colours of Tregantle beached pellets split into nurdle pellets (n=1132) and biobead pellets(n=1156), expressed as a percentage of the total.

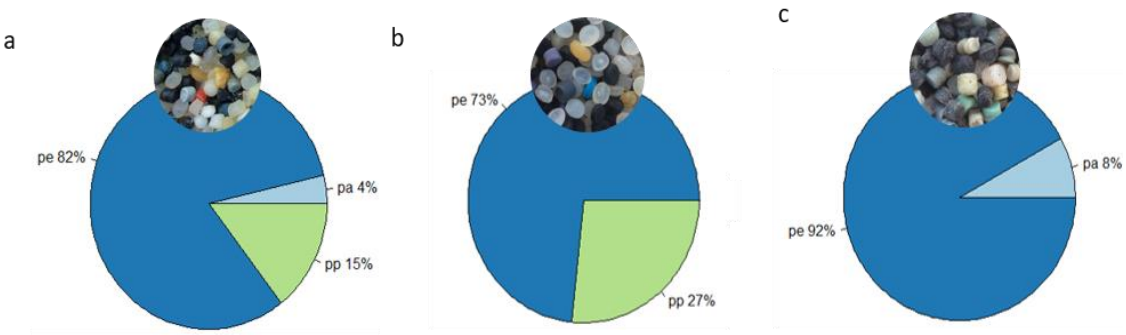


Fig.3.2. Polymer composition of Tregantle pellets (n=55), pe -polyethylene, pa – polyamide, pp – polypropylene. a) Nurdle and Biobead mix. b) Nurdle polymer composition (n=30). c) Biobead polymer composition (n=25).

Colour coding of Tregantle pellets leached for Experiment 3 revealed that the majority of beached nurdle pellets (fig.3.2; n=1132) were white-clear (35.9%), with the next most abundant colours being black (29.5%). The remaining pellets were a mixture of aged-yellow (17.8%), purple (7.6%), grey (5.4%), white (2.0%), yellow (1.3%), green (0.4 %) and blue (0.1 %). The aged pellets had a distinctive amber colouration. Within the beached biobead sample (fig.3.2; n=1156) the most abundant pellet colour was black (30.8%), followed by grey (28.0%), white (27.8%) and blue (13.4%).

Polymer characterisation using FT-IR spectra of a sub-sample of Tregantle beached pellets revealed that 73% of nurdle pellets polymer type as polyethylene and 27% were composed of polypropylene (n=30, fig.s.3.1b). Within the sub-sample of beached biobead, 92% of pellets were composed of polyethylene and 8% of pellets were composed of polyamide (n= 25, fig.s.3.1c).

3.4.2 Microplastic Leachate Chemical Analysis

Preliminary analysis of the presence of phthalates and PAHs in microplastic leachate samples, using solid phase micro-extraction (SPME) fibres revealed multiple compounds present in the beached and PVC pellets leachates, which were not present in the control. This analysis required a development of methods and from the four SPME fibres tested, the most effective at identifying the presence of phthalates was carboxen/PDMS and PDMS for the detection of PAHs. There was evidence of phthalates above control levels in all of the samples, particularly the PVC treatment. Additionally, pyrene and acenaphthylene was detected in PVC, with trace amounts of acenaphthylene identified in the beached pellets. Anthracene was detected in all the leachates of all pellet types.

3.4.3 Wildtype Development of P. lividus

To understand the process of development within this species and key stages of development which may be affected in the treatment groups, I had to familiarise in detail with the wildtype phenotype of *P. lividus.* Figure 3.3 shows the wildtype development of *P. lividus* from unfertilised egg (fig.3.3a) to 4-arm pluteus (fig.3.3m) under 18°C incubation in FSW. Control phenotypes in all experiments were consistent with the wildtype phenotype (fig.3.3), exhibiting normal

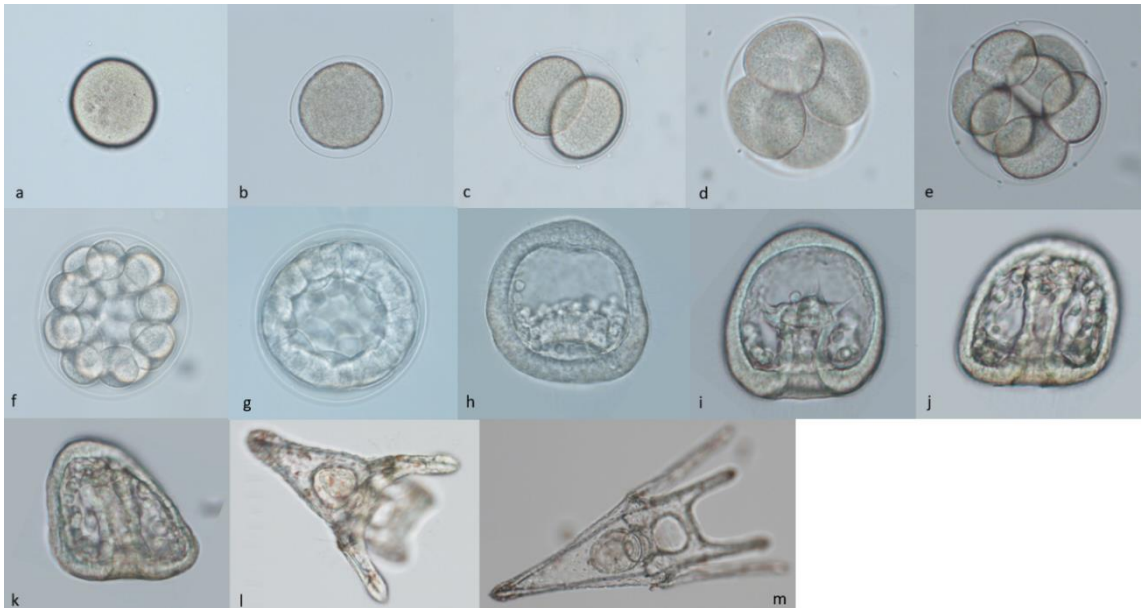


Fig. 3.3. Wildtype development series of my control *P. lividus*. (a) unfertilised egg (b) fertilised egg with fertilisation membrane (c) two-cell (d) 4-cell (e) 8-cell (f) 32-cell (g) early blastula (h) blastula with vegetal plate (i) gastrula (j) late gastrula (k) prism (l) two-arm pluteus (m) 4-arm pluteus.

development in 87% embryos (24 hpf) and 89% in larvae (48 hpf), with the remaining embryos and larvae falling in an anal view plane in which the extent of development was difficult to see, or slight abnormalities were observed, for example developmental delay or incorrect positioning of spicules. This falls within the normal expected developmental malformation.

3.4.4 Effect of Microplastic Leaching Time and Concentration

Using microscopic photographs of embryonic and larval stages, different types of malformations were distinguished and classified according to the degree of embryonic and larval alteration and developmental delay, to establish an index of severity of the microplastic leachate impact (IMPI), as described in the Materials and Methods section (table 3.1). Initial experiments were run to observe the effect of microplastic leaching time and leachate concentration on *P. lividus* phenotype. Significant differences in larval abnormalities between 24- and 72-hour leaching time of the microplastic were observed in larvae assessed at 62 hpf for 20% beached pellet and PVC leachates (fig.3.4,3.5, two proportion z-test, $p < 0.001$). Cultures exposed to microplastic leachates which were leached for 24 hours exhibited fewer abnormalities and a weaker delay in developmental times, with >50% of larvae in class 0, referring to normal development for both beached pellet treatments (fig.3.4,fig.3.5; biobead; n= 73; nurdle, n=35). Cultures exposed to 24 hour leached PVC exhibited phenotypes which reflected higher class categories, referring to a stronger developmental delay, compared to the beached pellet treatments (fig.3.4,3.5; PVC, n= 42).

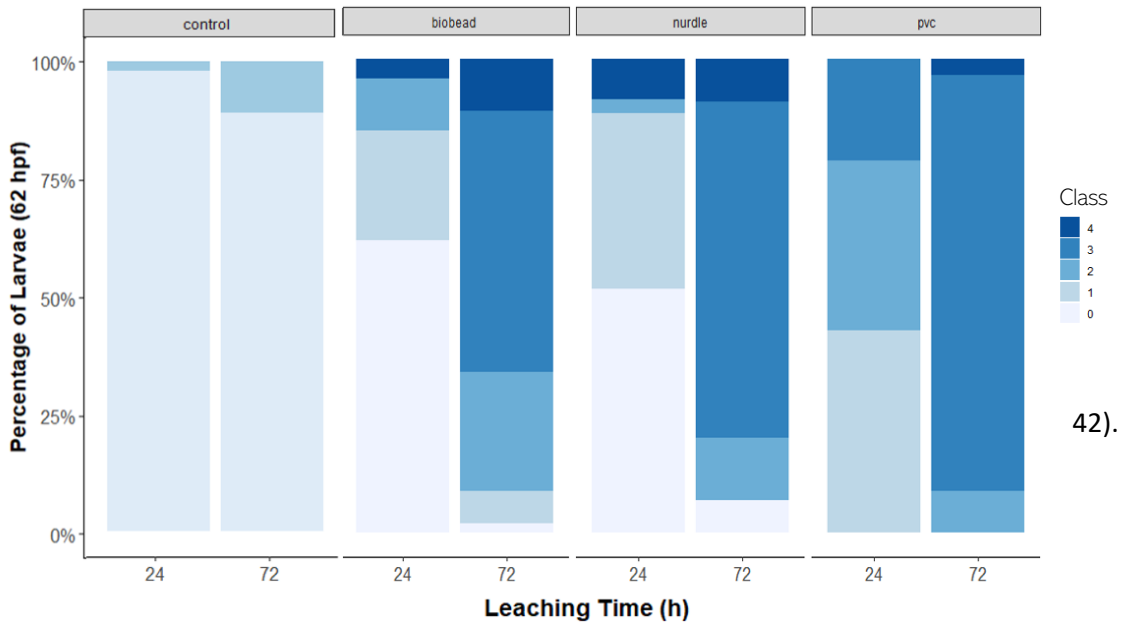


Fig.3.4 Effect of leaching time (24 and 72 hours) of beached and industrial microplastic leachates on the development of *P. lividus* larvae (62hpf). Percentage of *P. lividus* larvae within each IMPI class according to morphological delay and abnormalities at 20% plastic concentration leachate for all treatments. Larvae cultured in filtered sea water with no microplastic as a control.

However, all treatments produced severe phenotypes when exposed to the same initial concentration of microplastic leachate (20%), but with a longer leaching time of 72 hours. Both beached pellet treatments had increased numbers of larvae in classes 3 and 4, with 66% of embryos in classes 3 and 4 for the biobead treatment (n=100) and 80% falling in those classes for the nurdle pellet treatment (n=100). The 20% 72 hour leached PVC also elicited a strong phenotypic delay with >87.72% larvae categorised into class 3, referring to under-developed larvae with no arms visible (fig.3.4,3.5; PVC, n=57).There was no difference in the phenotype observed in the control treatments, between leaching times with >75% of larvae in class 0, for both leaching times (fig.3.5; 24h,n=100;72h,n=77).

Leaching concentration effects were assessed at 24 hours leaching (fig.3.6, fig.3.7). The exposure of beached and PVC pellets at increasing leachate concentrations (4%,10%,20%), produced an increase in anomalous larvae at 62 hpf. At 4% microplastic leachate, all treatments produced cultures in which the majority of larvae (>86.36%) exhibited a normal phenotype (Class 0: biobead, n=29; nurdle, n=32;PVC, n=38), as shown by the white bars in figure 3.6 representing class 0. At 10% microplastic leachates a slight increase in anomalous larvae was observed across all treatments, as shown in figure 3.6 with the increase in classes 1-4, however the majority of larvae (>79.41%) were classed as normal (class 0; biobead, n=39; nurdle, n=64; PVC, n=27; fig.3.6). At 20% microplastic leachates, the number of anomalous larvae increased for all treatments, with a more pronounced effect for cultures exposed to 20% PVC, with all larvae considered as abnormal (class 1-3: n=42; fig.3.6, fig.3.7).

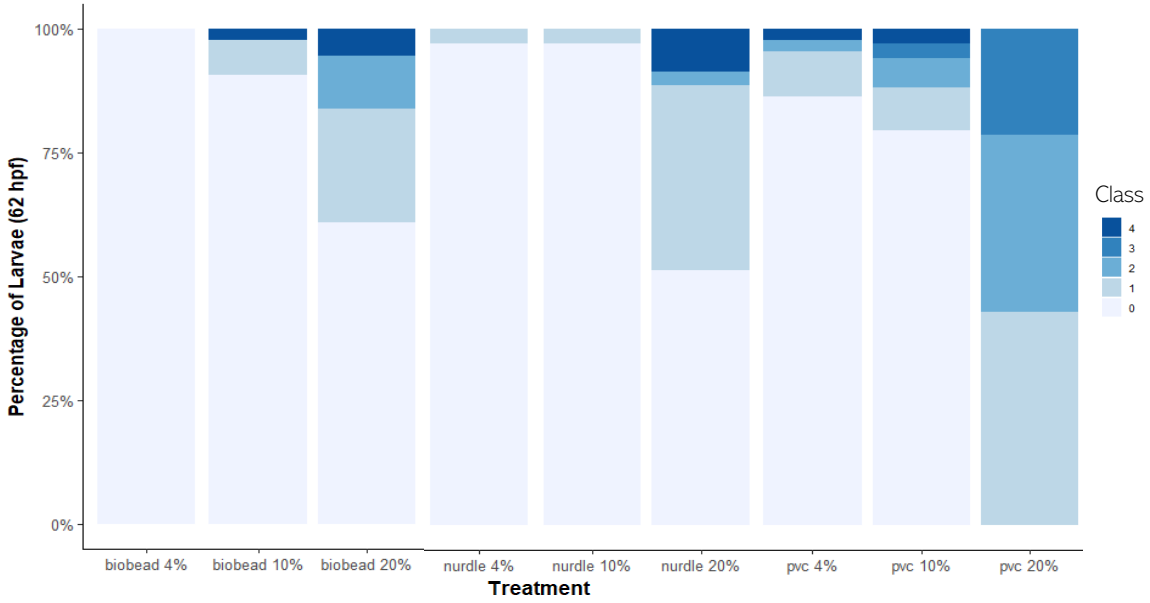


Fig.3.6. Comparison of IMPI classes between treatments and leachate dilutions (4,10,20%), assessed for larvae (62hpf) under 24-hour leaching protocol. (Biobead: 4%, n=29; 10%, n=43; 20%, n=73.Nurdle:4%, n=33;10%, n=66;20%, n=35. PVC: 4%, n=44;10%, n=34;20%, n=42)

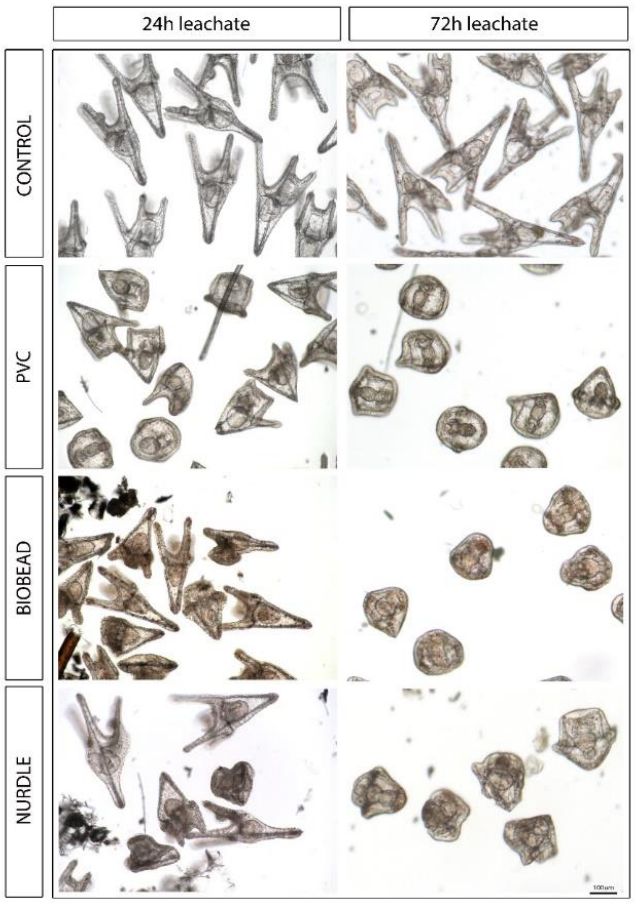


Fig. 3.5. Phenotypic comparison of the leaching time (24h vs. 72h) of microplastic on the morphology of *P. lividus* larvae, exposed to beached and PVC pellet leachates at 20% concentration.

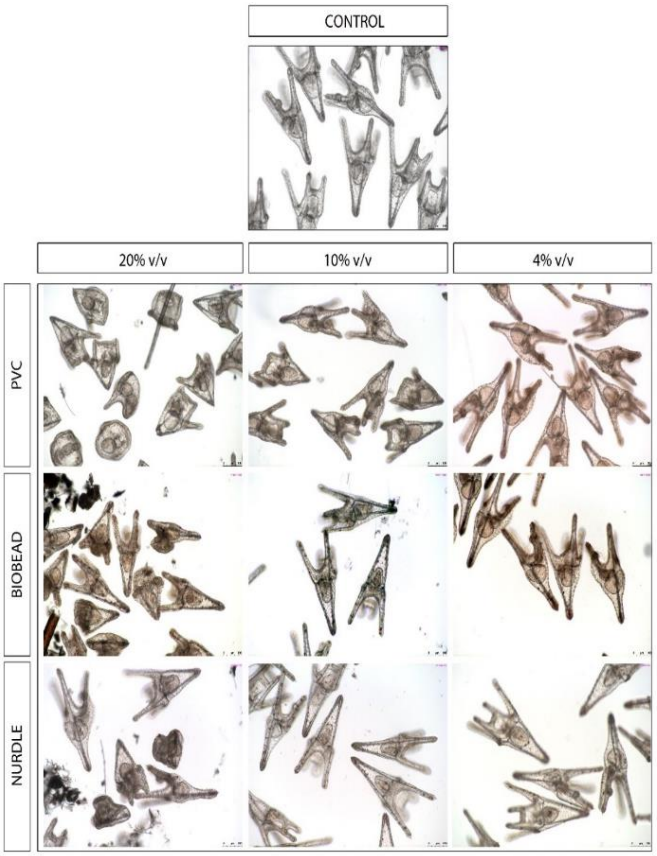


Fig.3.7. A comparison of microplastic leachate concentration (4,10,20%) on the morphology of *P. lividus* larvae at 62 hpf, exposed to beached and PVC pellet leachates. Note the microplastics were leached for a 24h protocol.

3.4.5 Assessment of Toxicity: Index of Microplastic Impact to P. lividus

Beached and PVC pellets elicited a strong increase in abnormal embryos and larvae, with >86% embryos classed in categories 1-4, across treatments and development stages (24,48 hpf; pvc 5%,10%, biobead 15%, nurdle 15%; table.s.3.2, fig.3.8a-b). On average most embryos (24hpf) across biobead (n=93) , nurdle (n=90) and PVC 5% (n=61) treatments were classed as delayed (category 1), with an index of microplastic impact (IMPI) of 1.3 for biobead (n=99), 1.2 for nurdle (n=100) and 1.5 for 5% PVC (n=100), referring to a moderate environmental impact for embryos at 24 hpf (table.s.3.2, fig.4.8a). Embryos exposed to 10% PVC were classed in a higher category, with most embryos in class 2 and 3 (n=25), referring to an IMPI of 2.5 and a high environmental impact (table.s.3.2, fig.4.8a).

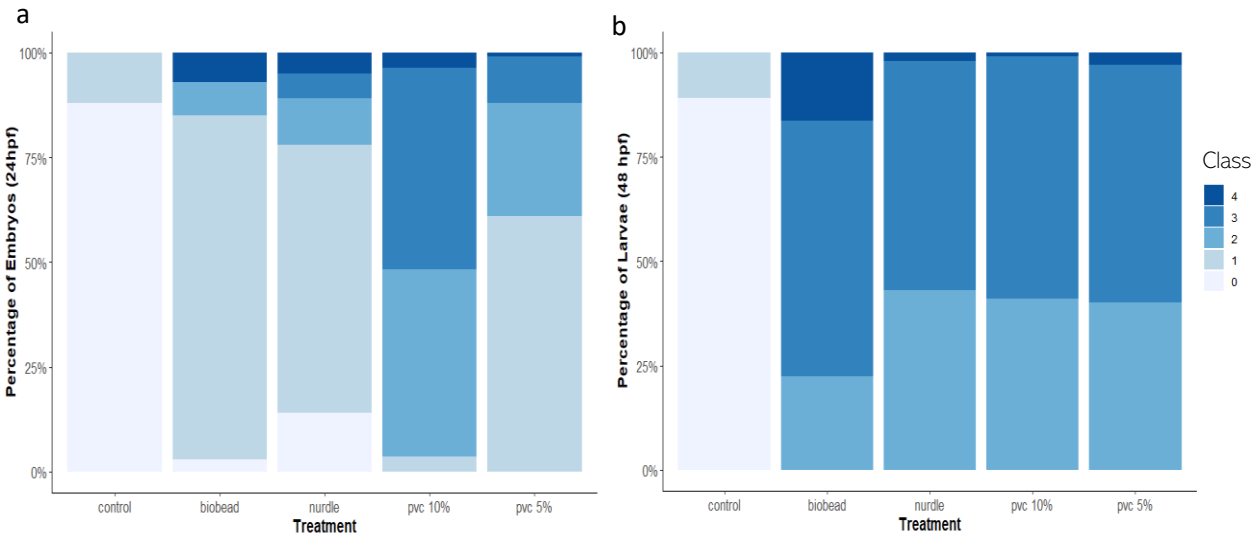


Fig.3.8. Effect of beached and industrial microplastic leachates on the development of embryos and larvae of *P. lividus*, with unexposed larva as a control. (a) Percentage of *P. lividus* embryos (24 hpf) classified according to IMPI class (b) Percentage of *P. lividus* larvae (48 hpf) classified according to IMPI class. Embryonic and larval data derived from the same treatment, with development tracked from embryonic (24 hpf) to larval stage (48 hpf) of the same embryo culture.

All larvae (48 hpf) exposed to beached and PVC treatments were classed in categories 2,3 and 4, referring to a strong phenotypic effect from all leachates (table.s.3.2, fig.4.8b). Larvae exposed to the beached microplastic leachates, had a higher IMPI score compared to the PVC treatments, with a score of 2.8 for cultures exposed to nurdle pellet leachates (n=92) and 2.9 for those exposed to biobead pellets (n=70) (table.s.3.2). Larvae exposed to both 5% PVC (n=100) and 10% PVC (n=100) leachates had a IMPI score of 2.6, relating to high environmental impact for both cultures (table.s.3.2, fig.4.8b).Furthermore, to gain a statistically comparative measure between the control and treatment cultures, embryos and larvae were categorised into abnormal, referring to classes 1-4, and normal phenotype, referring to class 0. The percentage of anomalous embryos, which include both delayed and abnormal phenotypes, in all treatments

(beached pellets, 15%; PVC, 5%, 10%); was statistically significantly higher than controls at both 24 hpf (two proportion z-test, $p < 0.001$) and 48 hpf (two proportion z-test, $p < 0.001$).

3.4.6 Assessment of Toxicity: Morphological Malformations

Figure 3.9 (a-o) shows a phenotypic comparison between control, beached and industrial microplastic pellet leachates upon *P. lividus*, across three developmental time points, blastula (19 hpf), gastrula (24 hpf) and pluteus (48 hpf). Severe phenotypic abnormalities were observed in cultures exposed to both beached plastic pellet leachates, namely nurdles (fig3.4 m-o) and biobeads (fig.3.9 j-l) (15% concentration), and industrial PVC pellet leachates (fig.3.9 g-j) (10%, 5% concentration). In contrast, cultures exposed to polyethylene (20% concentration) showed no delay in developmental timing, morphology or arm development at 19, 24 and 48 hpf (fig.3.9 d-f), emulating the wildtype phenotype across all developmental stages. The control and polyethylene cultures showed advanced ingression of the primary mesenchymal cells into the blastocoel at 19 hpf (fig.3.9a,d), however both beached pellet and PVC treatments show a delay in developmental timing at this stage, with fewer cells in the blastocoel (fig.3.9.g,j,m).

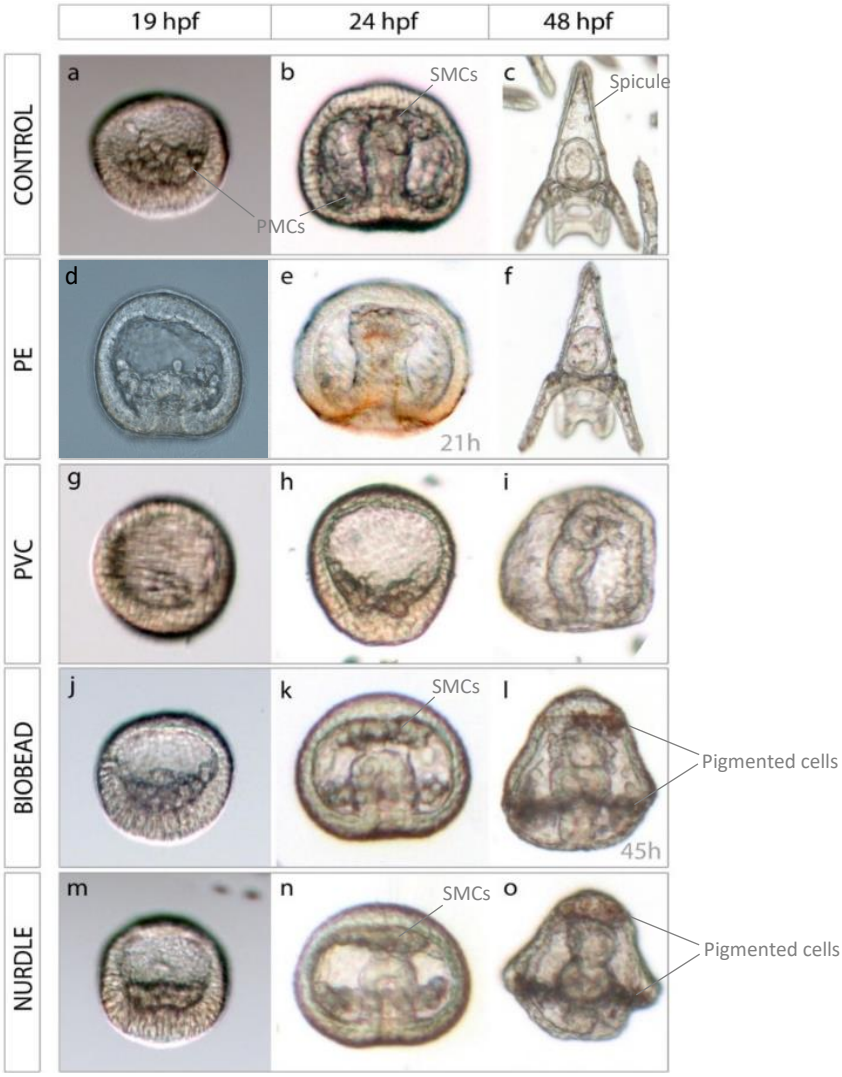


Fig.3.9 Phenotypic comparison of control and treatment *P. lividus* embryos and larvae at 19, 24 and 48h post fertilisation. Treatments plastic leached for 72h at leachate dilution of 20% (PE pellets), 15% (biobead and nurdle pellets) and 10% (PVC pellets). Key features highlighted, refer to definition fig.1.

At 24 hpf, embryo cultures within the control and polyethylene treatments express a phenotype corresponding to late gastrulation stage, with mesodermal cells populating at the tip of a well-formed archenteron elongating towards the animal pole (fig.3.9b, e). However, the PVC leachate treatment produced an embryo culture with a severe delay at 24 hpf (fig.3.9h), with the phenotype similar to that of a wildtype embryo at early gastrulation stage; the vegetal plate has begun to form, and the primary mesenchymal cells (PMCs) have detached and begin to migrate, however, there is lack of primary invagination to form the archenteron in this treatment. Both beached pellet treatments exhibit an archenteron elongating towards the animal pole, however both show a delay in developmental timing compared to the control, with a shorter archenteron (fig.3.9 k,n).

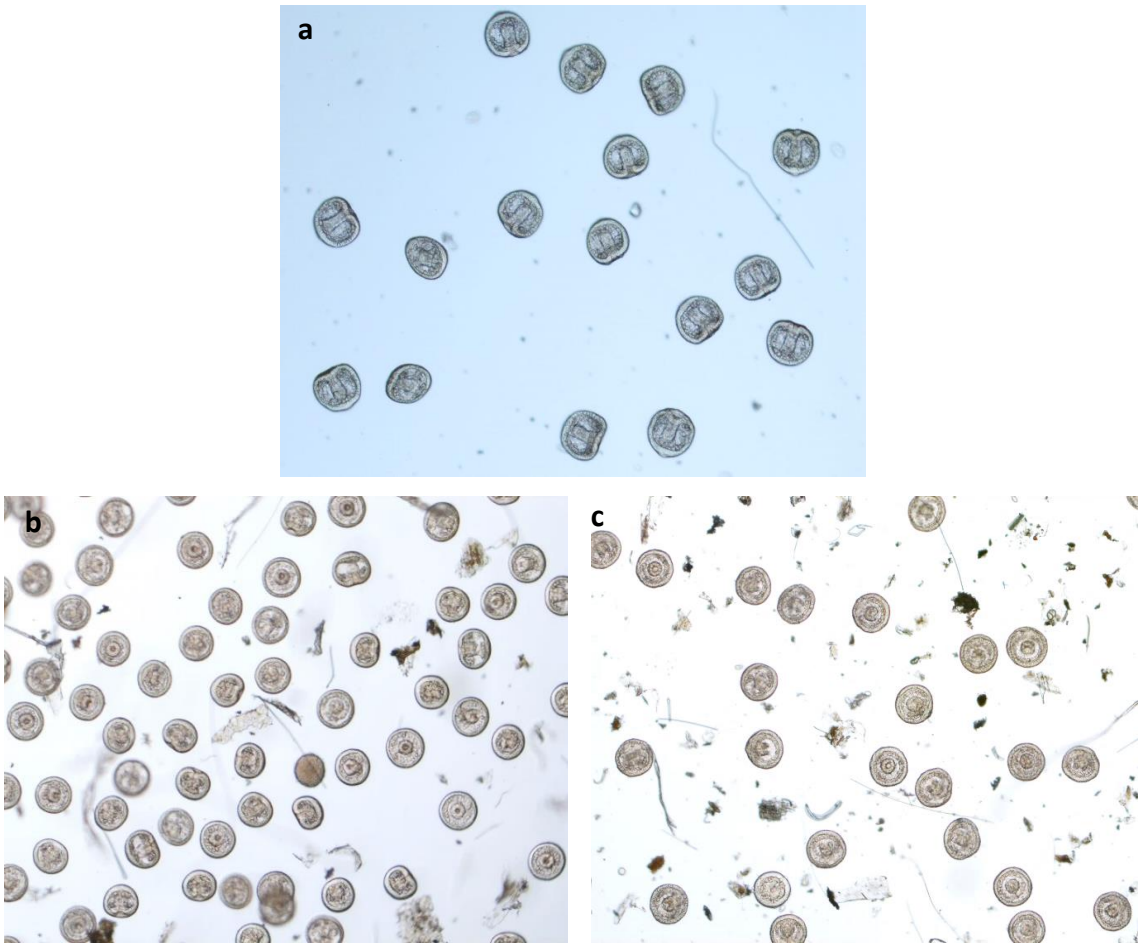


Fig.3.10. Radialised phenotype of *P. lividus* embryos at 24 hpf exposed to the beached pellet leachate treatments, with control wildtype control phenotype for reference. (a) control (b) nurdle (c) biobead.

In wildtype *P.* lividus cultures, the majority of embryos at 24 hpf are positioned in the lateral view (fig.3.10a, fig.3.11). However, 57% of embryos in the biobead treatment and 38% of the nurdle treatment at the same time were positioned in the anal view (fig.3.10;fig.3.11), which is statistically significantly higher than number of embryos in anal view in control cultures (two

proportion z-test; biobead, p = 1.357572^ {-14}; nurdle, p < 0.001). Both concentrations of PVC (5,10% concentration) did not show an anal view but were positioned laterally (fig.3.11).

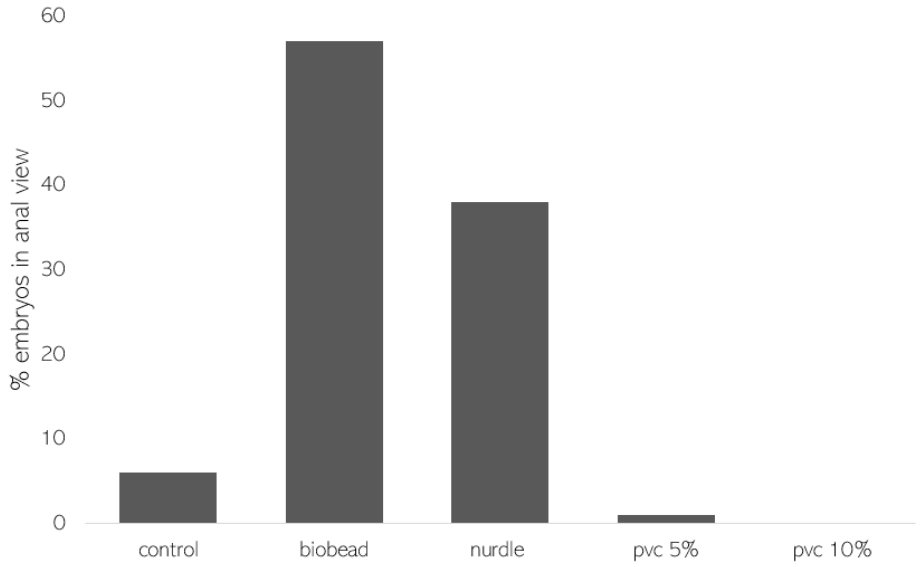


Fig.3.11. Percentage of *P. lividus* embryos in the anal view at 24 hpf by treatment (control. n=100; biobead, n=99; nurdle, n=100; PVC 5%, n=100; PVC 10%, n=27).

Figure 3.12 shows embryos exposed to beached pellets, during gastrulation (24hpf). The primary mesenchyme cells (PMCs) of beached pellet treatments did not form bilateral clusters, as they normally do in wildtype embryos, instead forming a complete ring of cells around the blastopore (fig.3.12b-c). Furthermore, the shape of the embryos is rounder than the prismatic shape of the control gastrulas, which form bilateral clusters of PMCs (fig.3.12a). When beached pellet treated embryos were in lateral view, the embryos exhibit a higher concentration of mesoderm cells (SMCs) above the archenteron at the animal pole, in beached pellet treatments, when compare with the control and wildtype phenotype (fig.3.9k, n).

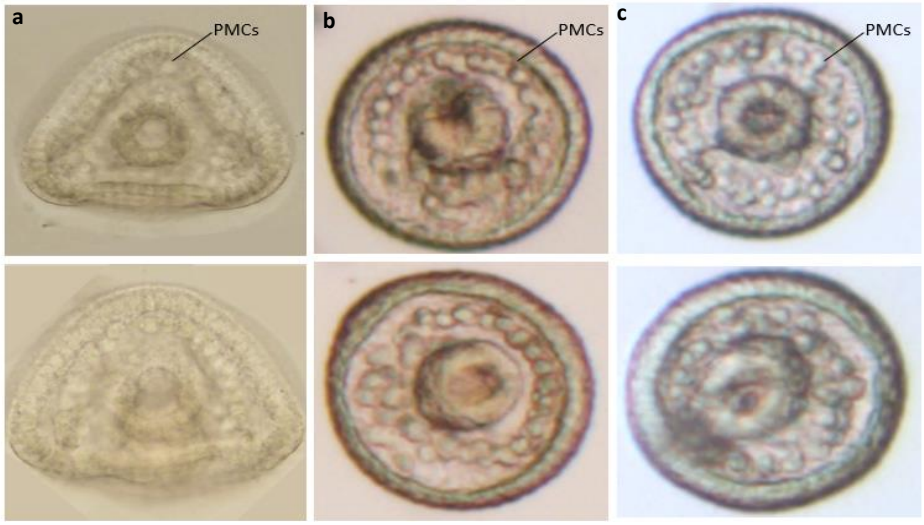


Fig.3.12. Radialised phenotype of *P. lividus* embryos exposed to beached pellet leachates in anal view. Aboral-oral polarity not shown here, with an absence of primary mesenchymal cells (PMCs) in a bilateral arrangement, suggesting radial symmetry exhibited in those embryos exposed to beached biobeads. (a) control (b) biobead (c) nurdle.

At 48 hpf, larvae exposed to the control and polyethylene treatments (fig.3.9.c,f) show an early 4-arm pluteus larvae with a complete skeleton, which mirrors the wildtype morphology (fig.3.3). However, PVC, nurdle and biobead pellet treatments show a strong delay in developmental timing and phenotypic abnormalities (fig.3.9, fig.3.13). The 10% PVC leachate treatment gave rise to larvae at 48 hpf which was similar to a wildtype embryo in prism stage or around 30-36 hpf, exhibiting a strong developmental delay, but with a consistent morphologically abnormal round shape (fig.3.13a-c). Despite forming a normal tri-part gut, the larvae consistently lacked complete arm and skeletal formation. Differential interphase contrast (DIC) images of larvae show the presence of the skeleton in the control larvae compared to larvae exposed to PVC leachates, which lack or have reduced spicules (fig.3.14). Failed elongation of the spicules or complete absence was common in the cultures exposed to PVC leachates at both 5% and 10% concentration. The pluteus phenotype of the beached pellet treatments, exhibited a similar morphology between both biobead and nurdle leachates, a bell-shaped larva opposing the wildtype phenotype (fig.3.9 j-o). The pluteus larvae exposed to biobead leachates developed short arms, a large apical organ, increased cilia length (fig.3.15), reduced spicules, an elongated shape and increased numbers of pigment cells (fig.3.9l). Leachates from the nurdle treatment gave rise to a slightly milder phenotype than that of the biobead treatment, however the larvae also exhibited a developmental delayed compared to the wildtype, an abnormal shape, reduced spicules, larger apical organ and an increase in pigment cells.

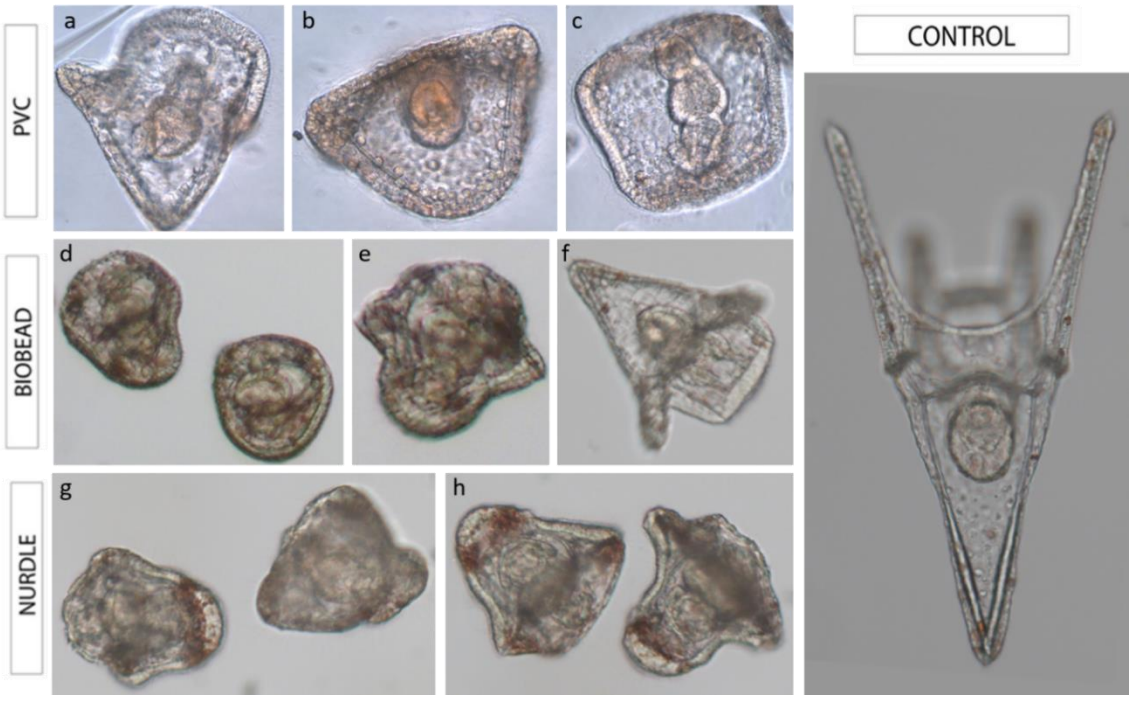


Fig. 3.13. Main abnormalities in treatments when with control for reference found at *P. lividus* pluteus stage (48hpf). (a-b) PVC 10% (d-f) beached biobead (g-h) beached nurdle. (a) anomalous pluteus larvae showing delayed development, short arms.

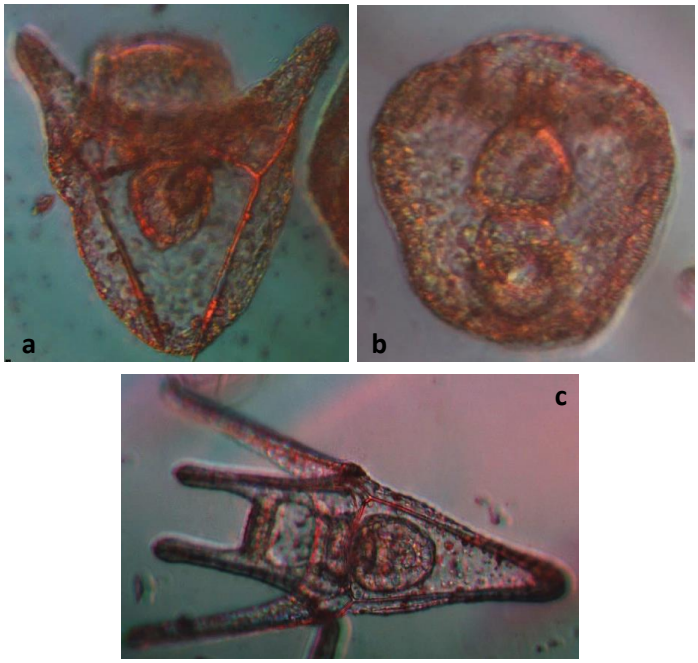


Fig.3.14. Differential interphase contrast (DIC) images of PVC 10% 72 hpf larvae showing (a) reduced spicules, (b) no spicules present and (c) fully developed spicules in control.

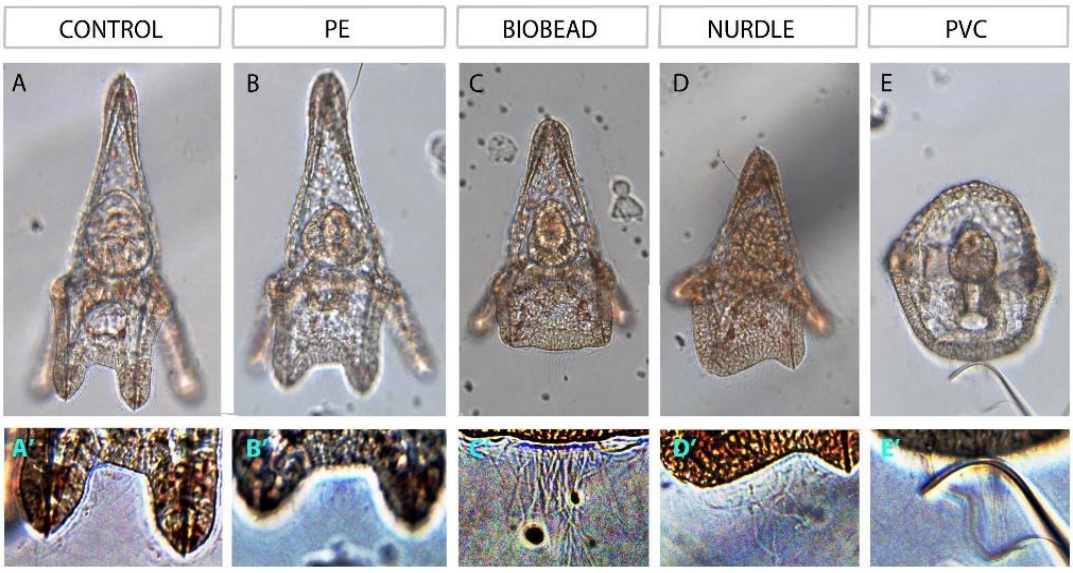


Fig. 3.15. Effect of beached and industrial microplastic leachates on cilia development of larvae of *P. lividus*. (A) control; (B) PE 20%) ;(C) beached biobead 15% ; (D) beached nurdle 15% (E) PVC 10%.

3.4.7 Nickle Chloride Exposure

Figure 3.16 shows the resulting embryonic (24 hpf) and larval (48 hpf) phenotypes from the $NiCl_2$ experiment, alongside the phenotype of both biobead and nurdle pellet leachates at 15% dilutions (fig.3.16 g-j). Embryos exposed to both $NiCl_2$ (fig.3.16.c, e) and beached pellet leachates (fig.3.12b, c; fig.3.16g, i) exhibit a characteristic round morphology in anal view, when compared to the prismatic shape of the control embryos (fig.3.12a). Additionally, the primary mesenchyme cells (PMCs) in all exposed embryos form a ring in the ectoderm, contrasting the expected development of bilateral clusters of PMCs, as observed in the wildtype embryo.

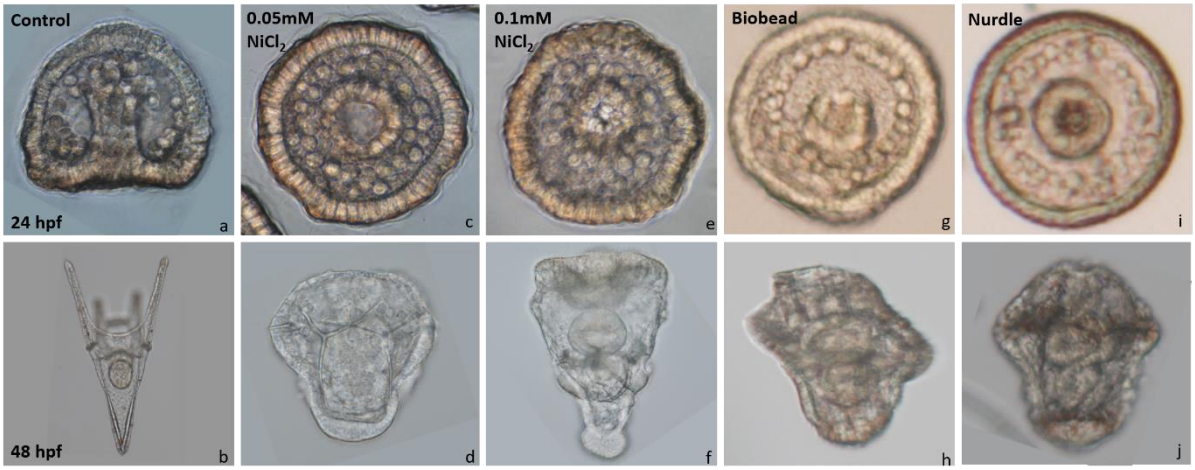


Fig. 3.16 Radialised phenotype of $NiCl_2$ and beached pellet leachate treated *P. lividus* cultures in anal view at 24hpf and 48hpf. (a) control embryo (lateral view) (b) control larva (c) 0.05mM $NiCl_2$ embryo (d) 0.05mM $NiCl_2$ larva (e) 0.1mM $NiCl_2$ embryo (f) 0.1mM $NiCl_2$ larva (g) 15% biobead embryo (h) 15% biobead larva (i) 15% nurdle embryo (j) 15% nurdle larva. Embryos from control cultures were predominantly positioned in the lateral view, therefore it was difficult to image control embryos in an anal view, refer to figure 3.12a for a control embryo intentionally moved into the anal view.

Larvae at 48 hpf exposed to 0.05 mM $NiCl_2$ (fig.3.16d) exhibit a bell-shaped morphology, with a strong developmental delay and abnormal features when compared to the control larvae (fig.3.16b). Those cultures exposed to the higher dose of 0.1 mM $NiCl_2$ exhibited a severely abnormal phenotype at 48 hpf (fig.3.16f), with a large proboscis at the animal pole of the larva. The radialised phenotype of the embryos of the beached pellet treatments was partially rescued, as the larvae exhibit a less severe phenotype than cultures exposed to 0.05 mM $NiCl_2$; exhibiting a specific bell-shape morphology, reduction in arm development and abnormal apical organ development.

3.4.8 Assessment of Toxicity: Immunohistochemistry

The sea urchin antibodies and micro labelling which were determined to be the most informative for larvae (48 hpf) were phalloidin, anti-serotonin, acetylated-tubulin and 1g8 for embryos (19hpf). This was decided baed on the morphological assessment of treated cultures and advice from the Arnone lab. To investigate the effects of beached and industrial microplastic pellet leachates on a specific tissue level, phalloidin, anti-serotonin, acetylated tubulin and 1g8 staining were used to monitor development of embryonic and larval tissue groups. The assessment of toxicity via immunohistochemistry of key embryonic tissue groups revealed some important differences between treatments (fig.3.17a-t). Phalloidin labelling of the muscle fibers associated with the digestive system, successfully allowed the visulisation of the circumesophageal muscles (fig.3.17). The development of these muscles marks the onset of the ability for the larvae to feed (Burke and Alvarez, 1988), therefore microfilament labelling using phalloidin, allows the crucial development of the digestive system to be assessed. The confocal laser scanning microscopy image of the control larvae exhibit well-developed circumesophageal muscle fibres (fig.3.17a). Larvae within the polyethylene, biobead and nurdle pellet treatments also exhibit well-formed digestive system (fig.3.17). However, the PVC treatment gave rise to larvae with underdeveloped circumesophageal muscles, with fewer muscular bands compared to the control (fig.3.17).

Localisation of serotonin expressing cells of pluteus larvae using anti-serotonin antibody for all treatments can be seen in figure 3.17. Wildtype sea urchin larvae have two vague groups of anti-serotonin immunoreactive cells, with a dense bundle of axons connecting the two within the oral hood, with axons extending laterally and into the larval arms (Nakajima, Burke and Noda, 1993). This can clearly be observed in the confocal microscopy image of the control larvae in figure 3.17b. Additionally larvae exposed to leachates from polyethylene (20%) also express serotonergic neurons, as described in wildtype larvae. However, those larvae exposed to beached and industrial microplastic leachates show abnormal expression of serotonergic cells (fig.3.17j, n,r). Larvae exposed to 10% PVC exhibit well-developed neural connections and anti-serotonin immunoreactive cells in the oral hood. However, there is a lack of axons projecting laterally into the posterior parts of the larvae and into the under-developed arms (see definition figure). Larvae exposed to the beached pellet treatments, biobead and nurdle pellet leachates, exhibited a reduction in serotonin expressing cells and a loss of interconnections between neural cells, in the oral hood, together with a complete lack of neural connections laterally or in the arms (fig.3.17n, r). Acetylated tubulin immunostaining is shown in figure 3.17, where control and polyethylene larvae are shown with cilia staining on the arm surface and in the stomach (fig.3.17c, g). Increased fluorescent cilia were observed on the surface of larvae exposed to the

beached and industrial PVC pellet leachates. Those larvae exposed to beached biobead leachates exhibited an increase in cilia number and length around the whole of the larvae structure (fig.3.17o, s).

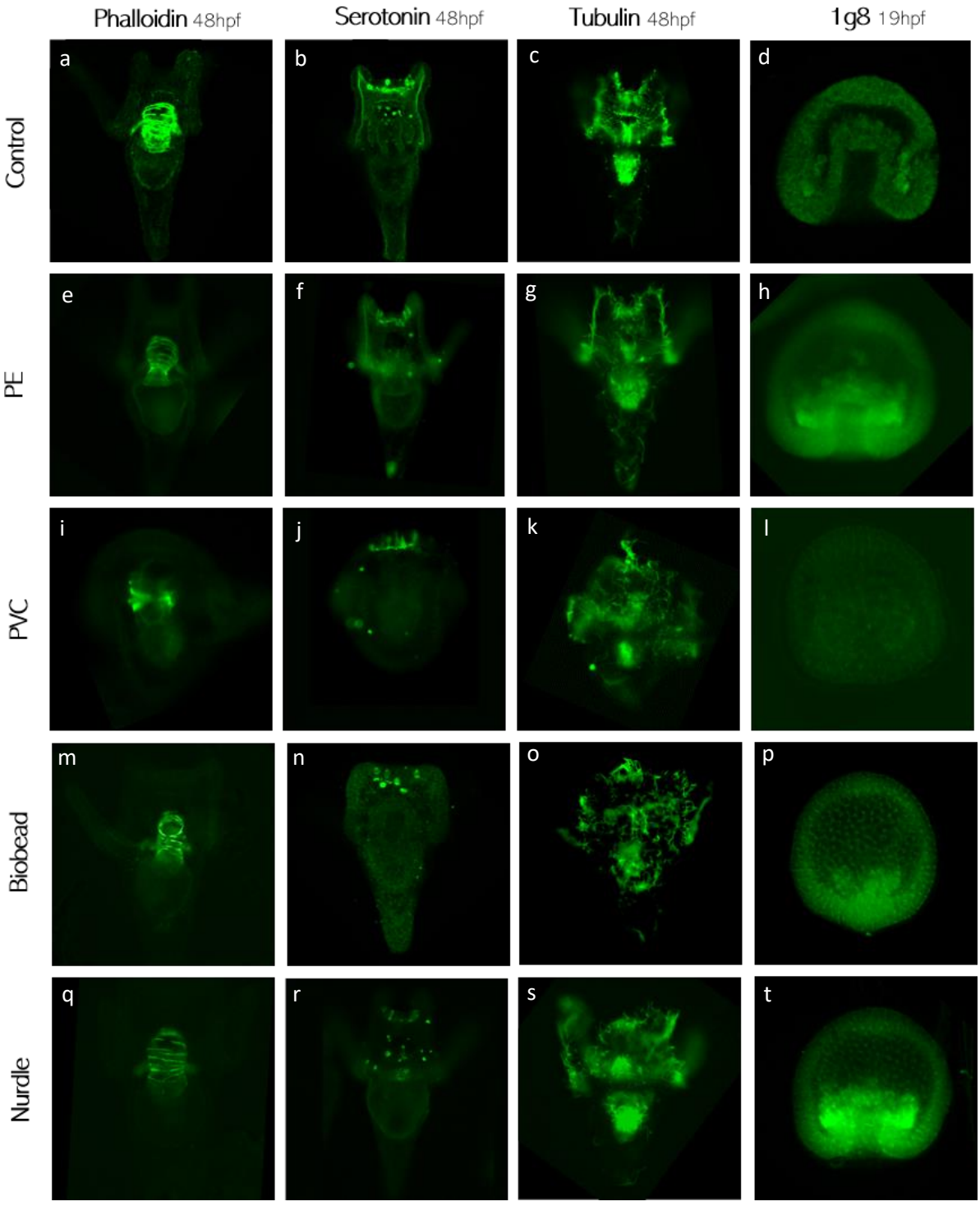


Fig.3.17. Microscopy images of phalloidin labelling, anti-serotonin, acetylated tubulin and 1g8 immunolocalization in *P. lividus* larvae (48 hpf; phalloidin, anti-serotonin, tubulin) and embryos (24 hpf; 1g8). (a) Confocal image of pluteus control with phalloidin labelling of the circumesophageal muscles (b) Confocal image of pluteus control with anti-serotonin staining of neural tissue (c) Pluteus control with acetylated tubulin staining in the stomach and on cilia surface (d) Control embryo with 1g8 staining of the PMCs (e-h) cultures exposed to 20% PE leachates (i-l) cultures exposed to 10% PVC leachates (m-p) cultures exposed to 15% beached biobead pellets (q-t) cultures exposed to 15% beached nurdle pellets.

The skeleton of sea urchin larvae is formed by the secretion of calcite by the PMCs. 1g8 immunostaining recognises msp130, which is a cell-surface glycoprotein, exhibiting a complex pattern of spatial regulation within the PMC syncytium during skeletogenesis (Guss and Ettensohn, 1997). Therefore, 1g8 immunostaining, which stains the PMCs, was carried out on embryos (19 hpf) to visualise PMC ingression and therefore identify abnormalities or delays in this process and subsequent skeletal problems (Ettensohn, Wray and Gary, 2004). The antibody used for this staining was expected to have little success in recognising the msp130 antigen as it was transported in non-ideal conditions to our lab. It is not possible to purchase this antibody or have a shipment made, therefore the antibody was kindly donated to us from the Arnone lab (originally from the McClay lab). Figure 3.17 shows some success in the immunostaining of the PMCs, with control, PE, biobead and nurdle embryos exhibiting some weak fluorescence in the area where PMCs are (fig.3.17d, h, p, t). The 1g8 staining provides some aid in the visualisation of gastrulation, with the archenteron clearly visible in figure 3.17d, h. However, no fluorescence was detected in the PVC treatment (fig.3.17l). The control embryo immunostaining suggests the PMCs are beginning to move through the blastocoel to form patterned arrays, as can be seen from the weak fluorescence of the cells near the base of the archenteron (fig.3.17d). The 1g8 staining in the PVC treatment was either unsuccessful or there was no recognition of the msp130 antigen of the PMCs (fig.3.17l). The beached biobead and nurdle treatments shows some staining of msp130 (fig.3.17). However, due to the state of the 1g8 antibody, little can be concluded or discussed about this immunostaining and therefore the position of the PMCs within each treatment.

3.5 Discussion

In this study we focus on the developmental effects of microplastic leachates from beached and industrial pellets, specifically beached nurdle pre-production pellets, beached biobead pellets, non-additive polyethylene and highly plasticised polyvinyl chloride pellets. We assessed the effects of leachates from these environmental and industrial proxies on key developmental stages and tissue groups of embryonic and larval cultures of *Paracentrotus lividus*. This body of work suggests that microplastic leachates from beached pellets (biobead and nurdle pellets) and highly plasticised industrial pellets (PVC) elicit severe, consistent and treatment-specific phenotypes in *P. lividus* embryonic and larval developmental stages. On the other hand, those cultures exposed to polyethylene leachates, with no co-contaminants, such as environmental contaminants or additives, exhibited a morphology emulating the wildtype phenotype of *P. lividus*. This suggests that the abnormalities observed are as a result of both the environmental adsorbed contaminants and/or pre-existing industrial additives within the polymer matrix. In general, a dose-dependent delay in the development schedule was found, along with phenotypic abnormalities in specific embryonic tissue groups, namely gut, cilia, neural and skeletal tissues. In general, the morphological assessment of toxicity revealed that those cultures exposed to beached and PVC pellet leachates, experienced delayed and abnormal gastrulation, abnormal shape, impaired spiculae, increased pigmented cells, larger apical organs, reduced arm growth, evidence of oral-aboral axis malformation. The immunostaining of key developmental tissue groups suggests that exposure to microplastic leachates affects sea urchin larvae at a tissue level, showing abnormal neural, skeletal and cilia development. However, this needs further investigation to confirm such an observation given the assessment being based on preliminary experiments.

Wildtype adult echinoderms are radially symmetrical animals; however, their contrasting larvae develop in a bilateral fashion, akin to almost all echinoderms (Ettensohn, Wray and Gray, 2004). A wild type sea urchin unfertilised egg is polarised along a single maternal axis; the animal-vegetal axis. Soon after fertilisation, a secondary axis develops, the oral-aboral axis (Duboc *et al*., 2004; Ettenshn and Sweet, 2000). The oral-aboral (OA) is used to define the axis running from the mouth region to the opposite side (Duboc *et al*., 2004). Correct establishment of the OA axis is key to the development of sea urchin embryos. Through the mis-expression of genes or signalling pathways, the formation of the oral-aboral axis can be disrupted. Several molecules have now been identified in the specification of the oral-aboral axis in sea urchins, specifically - goosecoid (gsc), BMP2/4, antivin, and nodal – all of which are exclusively expressed on the oral side of the embryos (Angerer *et al*., 2000, 2001; Duboc *et al*., 2004). Research by Duboc *et al*. (2004) provides evidence that misexpression of *nodal* by mRNA microinjection into the egg

provokes a strong radialisation of a developing embryo (fig.3.18c). This phenotype is known to be rescued by the microinjection of morpholino-oligonucleotide directed against nodal transcripts (MO-*nodal*) into the egg, followed by microinjection of nodal mRNA into a single blastomere at the 8-cell stage, rescues, to a large extent, the OA polarity in a large fraction of the embryos.

Figure 3.18a shows a wildtype embryo with two bilateral clusters of PMCs on the oral side (Duboc *et al*., 2004). Figure 3.18c, on the other hand presents an embryo developing in a radialised fashion with the central axis acting as the central point in which the body parts will radiate. This phenotype results in an embryo in which oral-aboral polarity is not established, resulting in a strongly radialised phenotype. The 48 hpf larvae forms a large proboscis at the animal pole (fig.3.18d). Results from Duboc *et al*. show a strongly radialised larvae, with a mouth opening (arrow in fig.3.18d) sometimes observed in embryos overexpressing *nodal.*

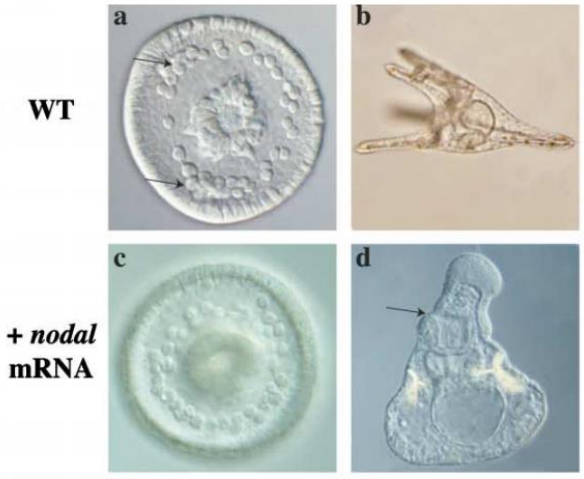


Fig.3.18 Radialised vs normal embryos from *Duboc et al., 2004* experiments outlined in text. Normal/WT (Aa) have two clusters of PCMs in the oral side (vs a continuous from the radialised embryo).

The predominate phenotype at 24 hpf within the beached biobead leachate treatment was embryos positioned in the anal view exhibiting a phenotype very similar to that of the radialised embryo exhibited in Duboc *et al*. (2004) experiments (fig.18c). Furthermore, this phenotype is also very similar to that of embryos undergoing development under heavy metal exposure (Hardin *et al*., 1992). Heavy metals have been shown to have inhibitory effects on the cleavage of sea urchin embryos and on pluteus formation during development (Kobayashi and Okamura, 2004). In nickel-treated embryos of *Lytechinus variegatus*, Hardin *et al*. (1992) report radialised embryos and further skeletal problems. The authors found that the PMCs formed a ring in the lateral ectoderm and the skeletal pattern formed by the cells was completely radialised (Hardin *et al*.,1992). They also report nickel-treated embryos to under-express transcripts of the dorsal (aboral) gene LvS1, and to overexpress the ventral (oral) ectodermal gene product, EctoV. This

study suggests that nickel chloride ($NiCl_2$) exposure alters commitment of ectodermal cells along the dorsoventral axis. Kobayashi and Okamura (2004) also report radialised pluteus and skeletal malformation in *Anthocidaris crassispina*, sea urchin embryos exposed to zinc and surface waters containing metals from Japan.

The embryonic and larval phenotypes observed in the $NiCl_2$ experiment are very similar to those observed in Hardin *et al*. (1992) and the cultures exposed to beached pellet leachates. In particular interest is the rounder embryonic shape and abnormal position of the primary mesenchyme cells. Comparing the larval phenotypes of the $NiCl_2$ experiments to that of the beached pellet leachates, suggests that the radialised phenotype of the embryos of the beached pellet treatments was partially rescued, given that the larvae exhibit a less severe phenotype than cultures exposed to 0.05 mM $NiCl_2$; exhibiting a specific bell-shape morphology, reduction in arm development and abnormal apical organ development. The similarity in phenotype, although less severe, observed between the beached pellet treatments and the $NiCl_2$ experiment, could therefore indicate a high metal content within the beached pellet leachates. This would align to the current understanding of this type of microplastic, particularly in the case of biobeads. Turner (2018) highlights the increased levels of metals, such as bromine, cadmium, chromium, lead and antimony in beached black biobeads. This increase in hazardous chemicals is suggested to be as a result the use of a high content of recycled electronic-related material in the manufacture of biobead pellets. Additionally Turner, Wallerstein and Arnold (2019) provide further evidence that about 10% of sampled beached biobeads have concentrations of heavy metals which are non-compliant with respect to regulations on hazardous plastic waste, again the authors report as a result of biobeads being composed of a heterogeneous mix of recycled plastic and end-of-life electrical and electronic plastic (WEEE). However, further analysis to characterise the metal content of the water in these treatments would be needed to clarify this suggestion. With the most abundant colour of biobead pellets (>30%) being black (fig.3.2), leaching of potentially toxic metals from the biobeads could explain the dominant radialised phenotype and subsequent larval abnormalities observed within this treatment. However, further analyses on the pellets would need to be undertaken to determine the content of WEEE and leached heavy metals.

The increase in secondary mesenchyme cells (SMCs) above the archenteron at the animal pole in embryos exposed to leachates from the beached treatments in contrast to the wildtype and control phenotype. The SMCs are released from the tip of the archenteron, subsequently forming a heterogenous set of cells, which differentiate into a variety of mesodermal tissues in the pluteus larvae, including pigment cells, muscle and coeloms (Gibson and Burke, 1985). An increase in pigmented cells in the beached treatments is subsequently observed in the larvae,

which is not exhibited in the control or industrial pellet treatments (PE and PVC). This suggests that the increase in SMCs during gastrulation in beached pellet treatments, could be precursor pigmented cells, with increased pigment cells resulting in the larvae (fig.3.9l, o). Pigment cells are cells filled with granules containing red pigment and are evidenced to play a role in 'patrol and protect', as immune cells, within the larval ectoderm and gut from microbial colonisation and invasion (Smith *et al*., 2006). Despite pigmented cells being present in wildtype larvae, the increased prevalence of pigmented cells in the beached treatments, suggests an additional stressor within these treatments. Evidence has recently shown that the surface of microplastics acts as an ideal habitat to harbour microbial communities (Oberbeckmann *et al*., 2014; Keswani *et al*.,2016; Lu *et al*., 2019). The plastisphere is used to define the prokaryotic and eukaryotic communities which hitchhike on microplastic (Zettler, Mincer and Araral-Zettler, 2013; Amaral-Zettler *et al*., 2015; Arias-Andres *et al*., 2018). Biobead pellets themselves are actively used in WWTPs to form a biofilm of bacteria and aid in treating contaminated water (Turner, Wallerstein and Arnold, 2019). The plastisphere has also been suggested to be a reservoir for pathogenic microbes, faecal indicator organisms and harmful algal bloom species (Keswani *et al*., 2016). Therefore, the increase in cells with immune function in the pluteus larvae exposed to beached pellets could potentially be as a result of increased microbial invasion from the biofilm on the beached pellets.

The beached pellet treatments present an interesting phenotype in pluteus larvae, with a reduction in the number of, and interconnections between serotonergic cells, but a severe increase in cilia development and apical organ size. The apical ganglion or organ is a tufted cilia structure common in invertebrate larva, which aids in the sensory perception of the presence of a suitable habitat in which to metamorphose (Nakajima, Burke and Noda, 1993; Kempf and Page, 2005). Duboc *et al*. (2004) highlight that larvae undergoing misexpression of *nodal* by mRNA microinjection, which exhibited a bell-shaped phenotype, also presented an abnormally thick layer of cuboidal cells covered with long cilia which resembled the apical tuft or ciliary band of wildtype embryos. This phenotype is very similar to that observed in the larvae exposed to beached pellet leachates. The authors suggest that the ciliated epithelium, may represent a default fate on the absence of *nodal* signalling. The increased size of the apical organ in the larvae of the beached pellet treatments, could suggest that these cultures would exhibit an increase in expression of neural cells. However, contrary to this suggestion, the immunolocalization of serotonergic neurons revealed a smaller number of serotonergic cells and interconnections present in the oral hood of pluteus larva exposed to beached pellet treatments. Research highlights that the sensory serotonergic neurons of the apical organ are involved in modulating muscular and ciliary activity (Kempf,Page and Pires, 1997; Marois and

Carew, 1997). This abnormality in neural tissue development, could lead to a reduction in ability for larvae to sense cues for settlement and metamorphosis (Privitera et al., 2011). The abnormality in cilia length and number, along with the increased size of the apical organ could be acting as compensation for the reduction in neural tissue in the oral hood. The contaminates within the microplastic leachates could be interrupting neural signalling pathways and therefore the ability to detect environmental cues. The increase in cilia concentration and length, therefore, may aid in sensory perception, compensating for the lack of alternative sensory abilities. On the other hand, the increase in cilia could be as a result of misexpression of *nodal*, as seen in Dobuc et al. (2004) findings. Given that there is normal development of neural and cilia tissue in larvae exposed to polyethylene leachates, this finding suggests that environmental contaminants within the leachates from beached microplastics, as opposed to the polymer itself, may elicit a response in the signalling pathways of cilia and neural development.

The skeleton of sea urchin larvae forms a structure, which is under very tight developmental control (Cheers and Ettensohn, 2005). If this complex process is disrupted, this can lead to an abnormal skeleton and therefore abnormal shape of the larvae and ability to develop further, producing a developmental block (Cheers and Ettensohn, 2005). All larvae exposed to the leachates from PVC, biobeads and nurdles appear to experience a problem with skeletogenesis, a key morphogenetic event in the development of the embryo (Decker and Lennarz, 1988). Wolpert and Gustafson (1961) first reported that the growth of the arms depends on the pushing action of the growing skeleton over the ectoderm. However, since then, research has shown that skeletogenesis begins as early as the 16-cell stage embryo (Decker and Lennarz, 1988). The skeleton forms as a biomineral structure composed of calcite, which is secreted by the primary mesenchyme cells (PMCs) in the early sea urchin gastrula (Wilt and Ettensohn, 2007). PMCs arrange around the blastopore, on the vegetal side of the larvae, first in a circle, but then forming a prismatic symmetry within the blastopore (Decker and Lennarz, 1988). If these clusters do not form in a prismatic fashion, spiculogenisis will not be initiated (Decker and Lennarz, 1988). The lack of skeletal formation is most evident in those embryos exposed to PVC leachates. Figure 3.4g,h shows a severe developmental delay in the embryos exposed to PVC, with both 19 and 24 hpf not showing the expected clusters of PMCs, as seen in the wildtype embryo (fig.3.9a).The morphology of the PVC treatment could therefore be as a result of an abnormality in the early patterning of the skeleton from the ectoderm, namely the PMCs or precursor cells of the skeleton. The immunostaining of PMCs via 1g8 antibody was completed in the hope of visualising the number and position of the skeletogenic precursor cells, when compared to the control. However, the state of the antibody gives little reliability and validity to the conclusions drawn from this immunostaining.

At the end of gastrulation, wildtype sea urchin embryos have a gut which is formed as a straight tube. However, following the completion of the larval skeleton, which gives rise to a prismatic shape , the gut undergoes a series of morphological changes which leads to the formation of three clearly sperate compartments and the circumesophageal muscle fibre, which can be clearly seen using phalloidin labelling on wildtype embryos (fig.3.16a). Cultures exposed to PVC leachates produced a pluteus at 48 hpf with a normal tri-part gut. However, the larvae showed reduced circumesophageal muscle fibres (fig.3.16i). The larvae also exhibited a morphologically abnormal round shape, which suggests the spicule were not well-formed or missing. Differential interphase contrast (DIC) images of the spicule revealed that many of the PVC treated cultures indeed had reduced spicules (fig.s.3.8). The reduced development of the circumesophageal muscle fibres and spicules suggests either a strong developmental delay or that spiculogenisis has been impaired and may underpin the embryos consistently abnormally round shape. It is difficult to deduce whether the reduction in circumesophageal muscle fibre and spicules, is a result of the delayed stage or abnormalities in the formation of the gut and skeleton. Additionally, larvae exposed to PVC leachates also expressed multiple serotonergic cells and neural connections within the oral hood, but a reduction in neural connections laterally, which is also most likely as a result of a delay in developmental timing, with these neural connections not yet being formed. To inform this further, wildtype embryos which match the developmental stage that the cultures exposed to PVC leachates represent, would need to be fixed for further analyses. This would inform us whether the level of muscle fibres, spicule and neural connections exhibited in the PVC treated cultures is expected at this corresponding wild type developmental stage.

Chapter Four

General Discussion

There is an urgent need for plastic pollution to be assessed, in a broad sense, as a diverse suite of contaminants, with potentially multiple impacts to ecosystems worldwide (Rochman *et al*., 2019). As outlined throughout this thesis, a handful of studies have investigated the impact of microplastic leachates on larval developmental (Nobre *et al*., 2015; e Silva *et al*., 2016; Olivero *et al*., 2019). However, to our knowledge, there are no studies assessing the effects of microplastic leachates on specific developmental tissue groups of marine larvae. This work investigates the developmental toxicity of two types of beached microplastic pellets (biobead and nurdle) and two industrial pellets, one with high additive load (polyvinyl chloride) and another with no additives in the polymer matrix (polyethylene).

The overall arching questions and aims of the thesis were partly addressed, with preliminary results suggesting that industrial and environmentally exposed plastic pellets may leach toxic contaminates. This question was addressed via the leachate chemical analyses; however, questions remain around concentrations of a huge range of contaminates and a more vigorous attempt is needed here to quantify leachate composition and subsequent toxicity. The wild type development of *P. miliaris* and *P. lividus* was demonstrated, which allowed the assessment of how microplastic leachates may elicit developmental abnormalities from wildtype embryonic and larval development. Morphological assessment of both sea urchin species exposed to plastic leachates revealed stark differences in developmental timing and morphology of key embryonic and larval structures. However, this assessment is limited and requires further investigation with a more rigorous experimental design, repeated independently across developmental stages and within treatments. An index of microplastic impact assessment (IMPI) was used to assess the extent of toxicity of polymer types, which gave an indication of potential impacts from each polymer and environmental history. However, this approach again needs to be more rigorous and be repeated to give a more reliable set of data, to draw conclusive results. Lastly, morphological abnormalities were identified in key embryonic tissue groups using immunohistochemistry. Cilia and neural tissue groups elicited the highest variation from wildtype development and could offer a means of visually assessing the impact of plastic leachates within early stages development to key embryonic structures. However, the underlying mechanism for such variation is speculated and further assessment is needed to link the chemical composition of microplastic leachates and subsequent variation in key embryonic structures.

Drawing conclusive results from this data set is difficult given the low reliability, however preliminary suggestive assessments based on the developmental responses are outlined. Morphological assessments of embryos and larvae exposed to leachates from polyvinyl chloride, and beached pellets show severe developmental delays and morphological abnormalities, with a high environmental impact score for all cultures assessed at larval stage. Embryos exposed to polyethylene with no additives or environmental contaminants revealed no embryotoxicity and developed in tandem with the control embryos. The immunolocalization of embryonic structures allowed the assessment of specific tissue level toxicity elicited by microplastic leachates and revealed that vital embryonic structures, such as cilia and neural pathways are affected by highly plasticised and beached microplastic leachates. The findings suggest that the leaching of additives from the polymer matrix and environmental contaminants could negatively affect the developmental biology of marine invertebrate embryonic life stages. However, further assessment is needed to make reliable and valid conclusions, given the experimental design of current experiments lack rigorous replication.

The physical characterisation of beached pellets revealed the pellets on Cornish beaches occur in a diverse colour range, particularly for pre-production nurdle pellets. As highlighted in chapter one, colourants and dyes are not bound to the polymer matrix and can leach into the environmental media under the correct conditions (Lithner, Nordensvan and Dave, 2012; Law and Thompson, 2014). The most abundant colour within the biobeads pellets was black. Turner (2018) highlights the increase in metal contamination as a result of end-of-life waste electronic and electrical equipment (WEEE) used in the manufacture of black plastic. Biobeads have been found to be manufactured from WEEE and are subsequently associated with relatively high concentrations of potentially toxic heavy metals, with about 10% of sampled beached biobeads with concentrations of heavy metals which are non-compliant with respect to regulations on hazardous plastic waste (Turner, Wallerstein and Arnold, 2019). The FTIR analysis additionally revealed that the majority of beached pellets are composed of polyethylene, which accumulates higher concentrations of persistent organic pollutants, compared to other polymer types (Endo et al., 2005; Rochman et al., 2013). The data provided by Takada (2019) on the chemical analyses of beached pellets from Tregantle beach also suggest an increase in PAHs in biobeads, when compared to beached pre-production nurdle pellets. Hideshige Takada, from the Laboratory of Organic Geochemistry (Tokyo University of Agriculture and Technology), analysed the composition of the PAHs and suggest that the large amounts of PAHs could be derived from auto-mobile related sources, which are introduced to sewage treatment plants via street runoff (Hideshige Takada, pers. comm). Once the biobeads are released into the marine or terrestrial environment via accidental spillage or storm-surges, these pre-loaded biobeads might present

an additional risk with higher PAH loads. Therefore, beached biobead pellets could present a higher risk to developing larvae over the beached pre-production nurdle pellets, with increased metal and HOC load. This is observed in the data, with biobead leachates producing a more severe phenotype and higher IMPI than nurdle pellets.

The phenotypic abnormalities observed in the beached pellet treatments could be as a result of polymer additives or environmental pollutants, but without the complete chemical analysis of the microplastic leachates it is impossible to attribute the phenotypes observed to any particular contaminates. The development of methods to analyse the compounds present in microplastic leachates using SPMEs is still ongoing and the results presented in chapter four are preliminary, qualitive data. The data suggests that there are environmental and industrial compounds present in all of the microplastic leachates, with increased phthalate presence in the PVC leachates. With the addition of standards, the analysis of leachates from the experiments presented here, are currently kindly being completed by Chris Mitchell. This will give us a quantative understanding of the compounds present in the microplastic leachates and the candidate compounds which may be eliciting the phenotypes documented here.

The differences in phenotypes between 24 hour and 72-hour leaching time was significant and the longer leaching time lead to stronger phenotypes in *P. lividus* cultures. Therefore, it can be inferred that leaching time affects the concentration of leachates released from the microplastic, which was found across PVC, biobead and nurdle treatments. This finding calls for studies investigating the toxicity of microplastic leachates to account for this and ensure leaching procedures are sufficient to reflect environmental leaching and the resulting phenotype. However, this is still an area of research which lacks data and large variabilities between plastic pollution leachates are expected. Lee *et al*. (2018) used experimental data to test the desorption model of hydrophobic organic contaminates desorbing from plastic polyethylene and polypropylene sheets and found the experimental data to agree with the model simulation. This indicates the desorption of environmental contaminants from plastic particles can be predicted by partition coefficient between plastic and water. Further studies need to estimate this for the additives associated with the polymer. There is a critical need to identify the additives and environmental contaminants leaching from microplastic and whether these leachates present an environmental risk to marine populations.

Assessing all the information gained from this body of work, these data highlight the necessity for microplastic toxicity research to shift focus from assessing microplastic pollution as a single pollutant, towards a broader approach which assesses microplastics as a diverse range of particulates and chemical compounds, as suggested by Rochman *et al*. (2019). The data also calls for research to address the mechanistic basis of changes in developmental process which are

disrupted under microplastic leachate contamination. Such data will improve our understanding of the impacts of microplastic pollution in the marine environment, and potentially far-reaching effects across a broad range of taxa. The disruption of cellular processes which are under very tight developmental control, such as spiculogenisis, may lead to serious ramifications in individuals and populations. The phenotypes produced from the industrial and beached pellets are very specific to the treatment, with strong consistencies observed between experiments. The differences in severity of the phenotype observed within the beached pellets between experiments, aligns strongly with our understanding about this contaminant as each sample of beached pellets may contain a different complex mixture of hydrophobic contaminants and environmental history (Mato *et al*., 2001).

Recruitment of planktonic larvae into adult populations is an essential process (Booth and Brosnam, 1995; Menge and Sutherland,1987). Environmental perturbations and stressors can have a substantial effect on plankton larvae entering adult populations, through impacts on larval availability, settlement and subsequent juvenile and adult survival (Cameron and Schroeter, 1980). Benthic organism, with planktonic larval stages, including sea urchin larva have been shown to receive chemical and physical cues to prompt settlement by the sensory neural net of the larvae (ascidians: Coniglio et al., 1998; barnacles: Faimali et al., 2003; sea urchins: Aluigi et al., 2010). The results from chapter 4 highlight a reduction in the development of neural tissue in the oral hood of *P. lividus* Larvae (48 hpf), as a result of leachates from beached microplastic. If this abnormality is not recovered by the time the larvae undergo metamorphosis, which is approximately 18 days post fertilisation (Gosselin and Jangoux, 1998), the larvae may forgo detection of such a stimuli, with settlement and subsequently metamorphosis prohibited. To understand the real relevance of a reduction in neural tissue development at this stage, requires later endpoints, which will provide us with a more realistic understanding of how marine microplastic contamination is affecting the developmental of critical tissue groups of marine larvae.

The data obtained in this study contribute to the limited knowledge of the impacts of microplastic leachates on the development of marine larvae, and these new insights could be used to assess the risk of marine plastic pollution. It is crucial to obtain information on the effect of emerging contaminants on developmental biology, as developmental genes are, in many cases, conserved across all animal phyla. This vital information allows us to comprehensively evaluate the toxic influence of such substances on marine ecosystems. Robust and in-depth laboratory observations of populations, communities and ecosystems will aid in our predictions of possible future effects of microplastic pollutants and to evaluate observed effects in natural systems. Longer term research with more replication, multiple generations and later endpoints,

will provide us with a more realistic understanding of how this contaminate is affecting marine larvae on a population scale.

Appendix

Ethics Statement

Husbandry of *P. miliaris* and *P. lividus* was performed in accordance with the best practices developed for the echinoderm species in order to optimize animal health. No specific permissions were required for the locations/activities because *P. miliaris* and *P. lividus* are invertebrate species, not classified as endangered or protected. All facilities and procedures were compliant with the guidelines of European Union (Directive 609/86).

Supplementary Materials

Table.s.3.1 Summary of experiments performed on *P. lividus* embryos outlining leaching time, microplastic leachate dilution and stages of embryos and larvae fixed for further analyses.

Exp#1	Exp#2	Exp#3	Exp#4	Exp#5
Date: 13/03/19 – 16/03/19	**Date: 15/03/19-18/03/19**	**Date:18/03/19-21/03/19**	**Date: 25/03/19-27/03/19**	**Date: 20/05/19-24/05/19**
Microplastic leaching	**Microplastic leaching**	**Microplastic leaching**	**Microplastic leaching**	**Microplastic leaching**
72 hours shaking	24 hours shaking	72 hours shaking	72 hours shaking	72 hours shaking
Dilutions	**Dilutions**	**Dilutions**	**Dilutions**	**Dilutions**
PVC 20%, 10%	PVC 20%, 10%, 4%	PVC 10%, 5%	PVC 10%, 5%	PVC 10%, 5%
Nurdle 20%, 10%	Nurdle 20%, 10%, 4%	Nurdle 15%	Nurdle 15%	Nurdle 15%
Biobead 20%	Biobead 20%, 10%, 4%	Biobead 15%	Biobead 15%	Biobead 15%
Control	Control	Control	PE 20%	PE 20%
			Control	Control
4 stages fixed	**4 stages fixed**	**3 stages fixed**	**4 stages fixed**	**4 stages fixed**
Blastula 17.5 hpf	Blastula 17.5 hpf	Gastrula 24 hpf	Blastula 18 hpf	Blastula 18 hpf
Gastrula 24 hpf	Gastrula 24 hpf	Pluteus 48 hpf	Blastula 21 hpf	Blastula 21 hpf
Pluteus 48 hpf	Pluteus 48 hpf	Late Pluteus 64 hpf	Gastrula 24 hpf	Gastrula 24 hpf
Late Pluteus 67 hpf	Late Pluteus 67 hpf		Pluteus 48 hpf	Pluteus 48 hpf

Table.s.3.2 Index of microplastic impact (IMPI) and corresponding environmental impact for each microplastic leachate treatment.

Control	Class	Number of Larvae	IMPI	Environmental Impact
24 hpf (n=100)	0	88.00	0.1	-
	1	12.00		
48 hpf (n=100)	0	89.00	0.1	-
	1	11.00		
PVC 5%				
24 hpf (n=100)	0	0.00	1.5	Moderate
	1	61.00		
	2	27.00		
	3	11.00		
	4	1.00		
48 hpf (n=100)	2	40.00	2.6	High
	3	57.00		
	4	3.00		
PVC 10%				
24 hpf (n=27)	0	0.00	2.5	High
	1	3.70		
	2	44.44		
	3	48.10		
	4	3.70		
48 hpf (n=100)	2	41.00	2.6	High
	3	58.00		
	4	1.00		
Biobead 15%				
24 hpf (n=99)	0	3.00	1.3	Moderate
	1	82.00		
	2	8.00		
	3	0.00		
	4	7.00		
48 hpf (n=70)	2	22.39	2.9	High
	3	61.19		
	4	16.41		
Nurdle 15%				
24 hpf (n=100)	0	14.00	1.2	Moderate
	1	64.00		
	2	11.00		
	3	6.00		
	4	5.00		
48 hpf (n=92)	2	43.00	2.8	High
	3	55.00		
	4	2.00		

References

Al-Jaibachi, R., Cuthbert, R. N., & Callaghan, A. (2018). Up and away: ontogenic transference as a pathway for aerial dispersal of microplastics. Biology letters, 14(9), 20180479.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). Universal Mechanisms of Animal Development. In Molecular Biology of the Cell. 4th edition. Garland Science.

Allied Market Research (2014) Plastic Additives Market by Functions (Property Modifiers, Property Stabilizers, Property Extenders, Processing Aids), Product Types (Plasticizers, Flame Retardants, Impact Modifiers, Antioxidants, Antimicrobials, UV Stabilizers) and Applications (Plasticizers, Flame Retardants, Impact Modifiers, Antioxidants, Antimicrobials, UV Stabilizers) - Global Opportunity Analysis and Industry Forecast, 2012 – 2020

Aluigi, M. G., Falugi, C., Mugno, M. G., Privitera, D., & Chiantore, M. (2010). Dose-dependent effects of chlorpyriphos, an organophosphate pesticide, on metamorphosis of the sea urchin, Paracentrotus lividus. Ecotoxicology, 19(3), 520-529.

Amaral-Zettler, L. A., Zettler, E. R., Slikas, B., Boyd, G. D., Melvin, D. W., Morrall, C. E., ... & Mincer, T. J. (2015). The biogeography of the Plastisphere: implications for policy. Frontiers in Ecology and the Environment, 13(10), 541-546.

Anbumani, S. & Kakkar, P. (2018). Ecotoxicological effects of microplastics on biota: a review. Environ. Sci. Pollut. Res., 25, 14373–14396

Andrady, A.L. (2011). Microplastics in the marine environment. Mar. Pollut. Bull., 62, 1596–1605

Angerer, L. M., Oleksyn, D. W., Logan, C. Y., McClay, D. R., Dale, L., & Angerer, R. C. (2000). A BMP pathway regulates cell fate allocation along the sea urchin animal-vegetal embryonic axis. Development, 127(5), 1105-1114.

Angerer, L. M., Oleksyn, D. W., Levine, A. M., Li, X., Klein, W. H., & Angerer, R. C. (2001). Sea urchin goosecoid function links fate specification along the animal-vegetal and oral-aboral embryonic axes. Development, 128(22), 4393-4404.

Annunziata, R., Perillo, M., Andrikou, C., Cole, A.G., Martinez, P. & Arnone, M.I. (2014). Pattern and Process During Sea Urchin Gut Morphogenesis : The Regulatory Landscape, 268, 251–268

Anselmo, H. M., Koerting, L., Devito, S., van den Berg, J. H., Dubbeldam, M., Kwadijk, C., & Murk, A. J. (2011). Early life developmental effects of marine persistent organic pollutants on the sea urchin Psammechinus miliaris. Ecotoxicology and environmental safety, 74(8), 2182-2192.

Arthur, C.; Baker, J.; Bamford, H. Proceedings of the international research workshop on the occurrence, effects and fate of microplastic marine debris,Sept9−11, 2008; National Oceanic and Atmospheric Administration: 2009.

Ashton, K., Holmes, L., & Turner, A. (2010). Association of metals with plastic production pellets in the marine environment. Marine pollution bulletin, 60(11), 2050-2055.

Au, S. Y., Bruce, T. F., Bridges, W. C., & Klaine, S. J. (2015). Responses of Hyalella azteca to acute and chronic microplastic exposures. Environmental Toxicology and Chemistry, 34(11), 2564-2572.

Auta, H.S., Emenike, C.U. & Fauziah, S.H. (2017). Distribution and importance of microplastics in the marine environmentA review of the sources, fate, effects, and potential solutions. Environ. Int., 102, 165–176

Avio, C.G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., D'Errico, G., *et al*. (2015). Pollutants bioavailability and toxicological risk from microplastics to marine mussels. Environ. Pollut., 198, 211–222

Babinsky, R. (2006). PVC additives: a global review. Plastics, Additives and Compounding, 8(1), 38-40.

Bakir, A., Rowland, S.J. & Thompson, R.C. (2012). Competitive sorption of persistent organic pollutants onto microplastics in the marine environment. Mar. Pollut. Bull., 64, 2782–2789

Batel, A., Borchert, F., Reinwald, H., Erdinger, L. & Braunbeck, T. (2018). Microplastic accumulation patterns and transfer of benzo[a]pyrene to adult zebrafish (Danio rerio) gills and zebrafish embryos. Environ. Pollut., 235, 918–930

Beaumont, N.J., Aanesen, M., Austen, M.C., Börger, T., Clark, J.R., Cole, M., *et al*. (2019). Global ecological, social and economic impacts of marine plastic. Mar. Pollut. Bull., 142, 189–195

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

Beiras, R., Durán, I. & Bellas, J. (2012). Biological effects of contaminants : Paracentrotus lividus sea urchin embryo test with marine sediment elutriates. ICES Tech. Mar. Environ. Sci., 51, 13pp

Bergmann, M., Tekman, M.B. & Gutow, L. (2017). Marine litter: Sea change for plastic pollution. Nature, 544, 297

Besseling, E., Wegner, A., Foekema, E. M., Van Den Heuvel-Greve, M. J., & Koelmans, A. A. (2012). Effects of microplastic on fitness and PCB bioaccumulation by the lugworm Arenicola marina (L.). Environmental science & technology, 47(1), 593-600.

Bonaventura, R., Poma, V., Costa, C., Matranga, V., Nazionale, C., Molecolare, I., *et al*. (2005). UVB radiation prevents skeleton growth and stimulates the expression of stress markers in sea urchin embryos, 328, 150–157

Booth, D. J., & Brosnan, D. M. (1995). The role of recruitment dynamics in rocky shore and coral reef fish communities. In Advances in Ecological Research (Vol. 26, pp. 309-385). Academic Press.

Borrelle, S. B., Rochman, C. M., Liboiron, M., Bond, A. L., Lusher, A., Bradshaw, H., & Provencher, J. F. (2017). Opinion: Why we need an international agreement on marine plastic pollution. Proceedings of the National Academy of Sciences, 114(38), 9994-9997.

Bošnjak, I., Zaja, R., Klobučar, R. S., Šver, L., Franekić, J., & Smital, T. (2013). Identification of ABC transporter genes in gonad tissue of two Mediterranean sea urchin species: black, Arbacia lixula L., and rocky, Paracentrotus lividus L. Bulletin of environmental contamination and toxicology, 91(4), 415-419.

Boucher, C., Morin, M., & Bendell, L. I. (2016). The influence of cosmetic microbeads on the sorptive behavior of cadmium and lead within intertidal sediments: A laboratory study. Regional Studies in Marine Science, 3, 1-7Browne MA, Galloway T, Thompson R (2007) Microplastic- an emerging contaminant of potential concern? Integr Environ Assess Mgmt 3:559–566

Browne, M.A., Niven, S.J., Galloway, T.S., Rowland, S.J. & Thompson, R.C. (2013). Microplastic moves pollutants and additives to worms, reducing functions linked to health and biodiversity. Curr. Biol., 23, 2388–2392

Buchwalow, I. B., & Böcker, W. (2010). Immunohistochemistry. Basics and Methods, 1, 1-149.

Burke, R. D., & Alvarez, C. M. (1988). Development of the esophageal muscles in embryos of the sea urchin Strongylocentrotus purpuratus. Cell and tissue research, 252(2), 411-417.

Cacho, J.I., Campillo, N. & Vi, P. (2012). Determination of alkylphenols and phthalate esters in vegetables and migration studies from their packages by means of stir bar sorptive extraction coupled to gas chromatography – mass spectrometry, 1241, 21–27

Cameron, R. A., & Schroeter, S. C. (1980). Sea urchin recruitment: effect of substrate selection on juvenile distribution. Mar. Ecol. Prog. Ser, 2(3), 243-247.

Carballeira, C., Ramos-Gómez, J., Martín-Díaz, L. & DelValls, T.A. (2012). Identification of specific malformations of sea urchin larvae for toxicity assessment: Application to marine pisciculture effluents. Mar. Environ. Res., 77, 12–22

Cheers, M. S., & Ettensohn, C. A. (2005). P16 is an essential regulator of skeletogenesis in the sea urchin embryo. Developmental biology, 283(2), 384-396.

Chiba, S., Saito, H., Fletcher, R., Yogi, T., Kayo, M., Miyagi, S., Ogido, M., & Fujikura, K. (2018). Human footprint in the abyss: 30 year records of deep- sea plastic debris. Marine Policy.

Claessens, M., Meester, S. De, Landuyt, L. Van, Clerck, K. De & Janssen, C.R. (2011). Occurrence and distribution of microplastics in marine sediments along the Belgian coast. Mar. Pollut. Bull., 62, 2199–2204

Cole, A.G. & Arnone, M.I. (2009). Fluorescent in situ hybridization reveals multiple expression domains for SpBrn1/2/4 and identifies a unique ectodermal cell type that co-expresses the ParaHox gene SpLox. Gene Expr. Patterns, 9, 324–328

Cole, M., Lindeque, P., Halsband, C. & Galloway, T.S. (2011). Microplastics as contaminants in the marine environment: A review. Mar. Pollut. Bull., 62, 2588–2597

Cole, M., Lindeque, P., Fileman, E., Halsband, C. & Galloway, T.S. (2015). The impact of polystyrene microplastics on feeding, function and fecundity in the marine copepod Calanus helgolandicus. Environ. Sci. Technol., 49, 1130–1137

Coniglio, L., Morale, A., Angelini, C., & Falugi, C. (1998). Cholinergic activation of settlement in Ciona intestinalis metamorphosing larvae. Journal of Experimental Zoology, 280(4), 314-320.

Coppock, R. L., Cole, M., Lindeque, P. K., Queirós, A. M., & Galloway, T. S. (2017). A small-scale, portable method for extracting microplastics from marine sediments. Environmental Pollution, 230, 829–837.

Corinaldesi, C., Damiani, E., Marcellini, F., Falugi, C., Tiano, L., Brugè, F., & Danovaro, R. (2017). Sunscreen products impair the early developmental stages of the sea urchin Paracentrotus lividus. Scientific reports, 7(1), 7815.

Courtene-Jones, W., Quinn, B., Gary, S. F., Mogg, A. O., & Narayanaswamy, B. E. (2017). Microplastic pollution identified in deep-sea water and ingested by benthic invertebrates in the Rockall Trough, North Atlantic Ocean. Environmental pollution, 231, 271-280.

Cózar, A., Martí, E., Duarte, C.M., García-de-lomas, J., Sebille, E. Van, Ballatore, T.J., *et al*. (2017). The Arctic Ocean as a dead end for floating plastics in the North Atlantic branch of the Thermohaline Circulation, 1–9

Decker, G.L. & Lennarz, W.J. (1988). Skeletogenesis in the sea urchin embryo. Development, 103(2), 231-247.

Della Torre, C., Bergami, E., Salvati, A., Faleri, C., Cirino, P., Dawson, K. A., & Corsi, I. (2014). Accumulation and embryotoxicity of polystyrene nanoparticles at early stage of development of sea urchin embryos Paracentrotus lividus. Environmental science & technology, 48(20), 12302-12311.

Desforges, J. P., Hall, A., McConnell, B., Rosing-Asvid, A., Barber, J. L., Brownlow, A., ... & Levin, M. (2018). Predicting global killer whale population collapse from PCB pollution. Science, 361(6409), 1373-1376.

Donovan, S.O., Mestre, N.C., Abel, S., Fonseca, T.G., Carteny, C.C., Cormier, B., *et al*. (2018). Ecotoxicological Effects of Chemical Contaminants Adsorbed to Microplastics in the Clam Scrobicularia plana, 5, 1–15

Duboc, V., Röttinger, E., Besnardeau, L., & Lepage, T. (2004). Nodal and BMP2/4 signaling organizes the oral-aboral axis of the sea urchin embryo. Developmental cell, 6(3), 397-410.

e Silva, P. P. G., Nobre, C. R., Resaffe, P., Pereira, C. D. S., & Gusmão, F. (2016). Leachate from microplastics impairs larval development in brown mussels. Water research, 106, 364-370.

El-Shahawi, M.S., Hamza, A., Bashammakh, A.S. & Al-Saggaf, W.T. (2010). An overview on the accumulation, distribution, transformations, toxicity and analytical methods for the monitoring of persistent organic pollutants. Talanta, 80, 1587–1597

Emlet, R. B. (2002). Sea urchin larval ecology: food rations, development, and size at metamorphosis. The sea urchin: from basic biology to aquaculture. Swets & Zeitlinger, Lisse, 105-111.

Endo, S., Takizawa, R., Okuda, K., Takada, H., Chiba, K., Kanehiro, H., *et al*. (2005). Concentration of polychlorinated biphenyls (PCBs) in beached resin pellets: Variability among individual particles and regional differences. Mar. Pollut. Bull., 50, 1103–1114

Engler, R. E. (2012). The complex interaction between marine debris and toxic chemicals in the ocean. Environmental science & technology, 46(22), 12302-12315.

Eriksen, M., Lebreton, L.C.M., Carson, H.S., Thiel, M., Moore, C.J., Borerro, J.C., *et al*. (2014). Plastic Pollution in the World's Oceans: More than 5 Trillion Plastic Pieces Weighing over 250,000 Tons Afloat at Sea. PLoS One, 9, 1–15

Ettensohn, C. E., Wessel, G. M., & Wray, G. (2004). Development of sea urchins, ascidians, and other invertebrate deuterostomes: experimental approaches (Vol. 74). Elsevier.

Faimali, M., Falugi, C., Gallus, L., Piazza, V., & Tagliafierro, G. (2003). Involvement of acetyl choline in settlement of Balanus amphitrite. Biofouling, 19(S1), 213-220.

Farajzadeh, M. A., & Mogaddam, M. R. A. (2012). Air-assisted liquid–liquid microextraction method as a novel microextraction technique; Application in extraction and preconcentration of phthalate esters in aqueous sample followed by gas chromatography–flame ionization detection. Analytica chimica acta, 728, 31-38.

Farge, Emmanuel. "Mechanical induction of Twist in the Drosophila foregut/stomodeal primordium." Current biology13.16 (2003): 1365-1377.

Foley, C.J., Feiner, Z.S., Malinich, T.D. & Höök, T.O. (2018). A meta-analysis of the effects of exposure to microplastics on fish and aquatic invertebrates. Sci. Total Environ., 631–632, 550–559

Frias, J. P. G. L., Sobral, P., & Ferreira, A. M. (2010). Organic pollutants in microplastics from two beaches of the Portuguese coast. Marine Pollution Bulletin, 60(11), 1988-1992.

Fujiwara, M., Ishihara, T., & Katsura, I. (1999). A novel WD40 protein, CHE-2, acts cell-autonomously in the formation of C. elegans sensory cilia. Development, 126(21), 4839-4848.

Gall, S. C., & Thompson, R. C. (2015). The impact of debris on marine life. Marine Pollution Bulletin, 92 (1-2), 170-179.

Gallo, F., Fossi, C., Weber, R., Santillo, D., Sousa, J., Ingram, I., ... & Romano, D. (2018). Marine litter plastics and microplastics and their toxic chemicals components: the need for urgent preventive measures. Environmental Sciences Europe, 30, 1-14.

Galloway, T. S., Cole, M., & Lewis, C. (2017). Interactions of microplastic debris throughout the marine ecosystem. Nature Ecology & Evolution, 1(5), 0116.

Gambardella, C., Aluigi, M. G., Ferrando, S., Gallus, L., Ramoino, P., Gatti, A. M., ... & Falugi, C. (2013). Developmental abnormalities and changes in cholinesterase activity in sea urchin embryos and larvae from sperm exposed to engineered nanoparticles. Aquatic Toxicology, 130, 77-85.

Gambardella, C., Morgana, S., Ferrando, S., Bramini, M., Piazza, V., Costa, E., ... & Faimali, M. (2017). Effects of polystyrene microbeads in marine planktonic crustaceans. Ecotoxicology and environmental safety, 145, 250-257.

García-Arrarás, J., & Viruet, E. (1993). Enteric nerve fibers of holothurians are recognized by an antibody to acetylated α-tubulin. Neuroscience letters, 157(2), 153-156.

Gascon, C., Brooks, T.M., Contreras-Macbeath, T., Heard, N., Konstant, W., Lamoreux, J., *et al*. (2015). The importance and benefits of species. Curr. Biol., 25, R431–R438

Gibson, A. W., & Burke, R. D. (1985). The origin of pigment cells in embryos of the sea urchin Strongylocentrotus purpuratus. Developmental biology, 107(2), 414-419.

Gosselin, P., & Jangoux, M. (1998). From competent larva to exotrophic juvenile: a morphofunctional study of the perimetamorphic period of Paracentrotus lividus (Echinodermata, Echinoida). Zoomorphology, 118(1), 31-43.

Gregory, M. R. (1978). Accumulation and distribution of virgin plastic granules on New Zealand beaches. New Zealand Journal of Marine and Freshwater Research, 12(4), 399-414.

Groh, K.J., Backhaus, T., Carney-Almroth, B., Geueke, B., Inostroza, P.A., Lennquist, A., *et al*. (2019). Overview of known plastic packaging-associated chemicals and their hazards. Sci. Total Environ., 651, 3253–3268

Guss, K. A., & Ettensohn, C. A. (1997). Skeletal morphogenesis in the sea urchin embryo: regulation of primary mesenchyme gene expression and skeletal rod growth by ectoderm-derived cues. Development, 124(10), 1899-1908.

Hahladakis, J.N., Velis, C.A., Weber, R., Iacovidou, E. & Purnell, P. (2018). An overview of chemical additives present in plastics : Migration , release , fate and environmental impact during their use , disposal and recycling. J. Hazard. Mater., 344, 179–199

Hale, R.C. (2017). Analytical challenges associated with the determination of microplastics in the environment. Anal. Methods, 9, 1326–1327

Hall, N. M., Berry, K. L. E., Rintoul, L., & Hoogenboom, M. O. (2015). Microplastic ingestion by scleractinian corals. Marine Biology, 162(3), 725-732.

Halpern, B. S., Selkoe, K. A., Micheli, F., & Kappel, C. V. (2007). Evaluating and ranking the vulnerability of global marine ecosystems to anthropogenic threats. Conservation Biology , 21 (5), 1301-1315.

Hamdoun, A. M., Cherr, G. N., Roepke, T. A., & Epel, D. (2004). Activation of multidrug efflux transporter activity at fertilization in sea urchin embryos (Strongylocentrotus purpuratus). Developmental biology, 276(2), 452-462.

Hara, Y., Kuraishi, R., Uemura, I., & Katow, H. (2003). Asymmetric formation and possible function of the primary pore canal in plutei of Temnopleurus hardwicki. Development, growth & differentiation, 45(4), 295-308.

Hardin, J., Coffman, J.A., Black, S.D. & Mcclay, D.R. (1992). Commitment along the dorsoventral axis of the sea urchin embryo is altered in response to NiCl 2, 685, 671–685

Harrison, P.T.C., Holmes, P. & Humfrey, C.D.N. (1997). Reproductive health in humans and wildlife: Are adverse trends associated with environmental chemical exposure? Sci. Total Environ., 205, 97–106

Hartmann, N.B., Rist, S., Bodin, J., Jensen, L.H.S., Schmidt, S.N., Mayer, P., *et al*. (2017). Microplastics as vectors for environmental contaminants: Exploring sorption, desorption, and transfer to biota. Integr. Environ. Assess. Manag., 13, 488–493

Hartmann, N.B., Hu, T., Thompson, R.C., Hassello, M., Verschoor, A., Daugaard, A.E., *et al*. (2019). Are We Speaking the Same Language? Recommendations for a De fi nition and Categorization Framework for Plastic Debris

Hay-schmidt, A. (1990). c and processes in the nervous system of the actinotroch larva of Phoronis muelleri (Phoronida), 105–118

He, H. (2012). Effects of environmental policy on consumption: lessons from the Chinese plastic bag regulation. Environment and Development Economics, 17(4), 407-431.

Heudorf, U., Mersch-Sundermann, V. & Angerer, J. (2007). Phthalates: Toxicology and exposure. Int. J. Hyg. Environ. Health, 210, 623–634

Hirai, H., Takada, H., Ogata, Y., Yamashita, R., Mizukawa, K., Saha, M., *et al*. (2011). Organic micropollutants in marine plastics debris from the open ocean and remote and urban beaches. Mar. Pollut. Bull., 62, 1683–1692

Horton, A.A., Walton, A., Spurgeon, D.J., Lahive, E. & Svendsen, C. (2017). Microplastics in freshwater and terrestrial environments: Evaluating the current understanding to identify the knowledge gaps and future research priorities. Sci. Total Environ., 586, 127–141

Hong, S.H., Shim, W.J. & Hong, L. (2017). Methods of analysing chemicals associated with microplastics: A review. Anal. Methods, 9, 1361–1368

Hughes, A. D., Catarino, A. I., Kelly, M. S., Barnes, D. K., & Black, K. D. (2005). Gonad fatty acids and trophic interactions of the echinoid Psammechinus miliaris. Marine Ecology Progress Series, 305, 101-111.

Isobe, A., Uchiyama-Matsumoto, K., Uchida, K. & Tokai, T. (2017). Microplastics in the Southern Ocean. Mar. Pollut. Bull., 114, 623–626

Ivar Do Sul, J.A. & Costa, M.F. (2014). The present and future of microplastic pollution in the marine environment. Environ. Pollut., 185, 352–364

Jambeck *et al*., 2015. (2015). The ocean. Clim. Chang. 2014 Impacts, Adapt. Vulnerability Part B Reg. Asp. Work. Gr. II Contrib. to Fifth Assess. Rep. Intergov. Panel Clim. Chang., 1655–1734

Jamieson, A. J., Malkocs, T., Piertney, S. B., Fujii, T., & Zhang, Z. (2017). Bioaccumulation of persistent organic pollutants in the deepest ocean fauna. Nature ecology & evolution, 1(3), 0051.

Kawakami, T., Isama, K., & Matsuoka, A. (2011). Analysis of phthalic acid diesters, monoester, and other plasticizers in polyvinyl chloride household products in Japan. Journal of Environmental Science and Health, Part A, 46(8), 855-864.

Kempf, S. C., Page, L. R., & Pires, A. (1997). Development of serotonin-like immunoreactivity in the embryos and larvae of nudibranch mollusks with emphasis on the structure and possible function of the apical sensory organ. Journal of Comparative Neurology, 386(3), 507-528.

Kempf, S. C., & Page, L. R. (2005). Anti-tubulin labeling reveals ampullary neuron ciliary bundles in opisthobranch larvae and a new putative neural structure associated with the apical ganglion. The Biological Bulletin, 208(3), 169-182.

Kershaw, P. J., & Rochman, C. M. (2015). Sources, fate and effects of microplastics in the marine environment: part 2 of a global assessment. Reports and studies-IMO/FAO/Unesco-IOC/WMO/IAEA/UN/UNEP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP) eng no. 93.

Keswani, A., Oliver, D. M., Gutierrez, T., & Quilliam, R. S. (2016). Microbial hitchhikers on marine plastic debris: human exposure risks at bathing waters and beach environments. Marine environmental research, 118, 10-19.

Kim, K. & Li, C. (2004). Expression and Regulation of an FMRFamide-Related Neuropeptide Gene Family in Caenorhabditis elegans, 550, 540–550

Kiyomoto, M., Morinaga, S., & Ooi, N. (2010). Distinct embryotoxic effects of lithium appeared in a new assessment model of the sea urchin: the whole embryo assay and the blastomere culture assay. Ecotoxicology, 19(3), 563-570.

Kobayashi, N. & Okamura, H. (2004). Effects of heavy metals on sea urchin embryo development . 1 . Tracing the cause by the effects, 55, 1403–1412

La Daana, K. K., Gårdfeldt, K., Lyashevska, O., Hassellöv, M., Thompson, R. C., & O'Connor, I. (2018). Microplastics in sub-surface waters of the Arctic Central Basin. Marine pollution bulletin, 130, 8-18.

Law, K. L., Morét-Ferguson, S., Maximenko, N. A., Proskurowski, G., Peacock, E. E., Hafner, J., & Reddy, C. M. (2010). Plastic accumulation in the North Atlantic subtropical gyre. Science, 329(5996), 1185-1188.

Law, K. L., & Thompson, R. C. (2014). Microplastics in the seas. Science, 345(6193), 144-145.

Lauby-Secretan, B., Loomis, D., Grosse, Y., El Ghissassi, F., Bouvard, V., Benbrahim-Tallaa, L., *et al*. (2013). Carcinogenicity of polychlorinated biphenyls and polybrominated biphenyls. Lancet Oncol.

Lebreton, L., & Andrady, A. (2019). Future scenarios of global plastic waste generation and disposal. Palgrave Communications, 5(1), 6.

Laist, D. W. (1997). Impacts of marine debris: entanglement of marine life in marine debris including a comprehensive list of species with entanglement and ingestion records. In Marine Debris (pp. 99-139). Springer, New York, NY.

Li, C., Kim, K. & Nelson, L.S. (1999). FMRFamide-related neuropeptide gene family in Caenorhabditis elegans, 26–34

Li, H. X., Getzinger, G. J., Ferguson, P. L., Orihuela, B., Zhu, M., & Rittschof, D. (2015). Effects of toxic leachate from commercial plastics on larval survival and settlement of the barnacle *Amphibalanus amphitrite*. Environmental science & technology, 50(2), 924-931.

Lithner, D., Nordensvan, I. & Dave, G. (2012). Comparative acute toxicity of leachates from plastic products made of polypropylene, polyethylene, PVC, acrylonitrile-butadiene-styrene, and epoxy to Daphnia magna. Environ. Sci. Pollut. Res., 19, 1763–1772

Liu, X., Xu, J., Zhao, Y., Shi, H. & Huang, C. (2019). Chemosphere Hydrophobic sorption behaviors of 17 b -Estradiol on environmental microplastics. Chemosphere, 226, 726–735

Llorca, M., Farré, M., Karapanagioti, H.K. & Barceló, D. (2014). Levels and fate of perfluoroalkyl substances in beached plastic pellets and sediments collected from Greece. Mar. Pollut. Bull., 87, 286–291

Lu, L., Luo, T., Zhao, Y., Cai, C., Fu, Z. & Jin, Y. (2019). Science of the Total Environment Interaction between microplastics and microorganism as well as gut microbiota : A consideration on environmental animal and human health. Sci. Total Environ., 667, 94–100

Luks-Betlej, K., Popp, P., Janoszka, B., & Paschke, H. (2001). Solid-phase microextraction of phthalates from water. Journal of Chromatography A, 938(1-2), 93-101.

Lusher, A.L., Tirelli, V., Connor, I.O. & Officer, R. (2015). Microplastics in Arctic polar waters : the first reported values of particles in surface and sub-surface samples. Nat. Publ. Gr., 1–9

Lusher, A.L., Welden, N.A., Sobral, P. & Cole, M. (2017). Sampling, isolating and identifying microplastics ingested by fish and invertebrates. Anal. Methods, 9, 1346–1360

Mallo, M. & Alonso, C.R. (2013). The regulation of Hox gene expression during animal development, 3963, 3951–3963

Marois, R., & Carew, T. J. (1997). Fine structure of the apical ganglion and its serotonergic cells in the larva of Aplysia californica. The Biological Bulletin, 192(3), 388-398.

Matranga, V., Pinsino, A., Bonaventura, R., Costa, C. & Karakostis, K. (2013). Cellular and molecular bases of biomineralization in sea urchin embryos

Martínez-Gómez, C., León, V. M., Calles, S., Gomáriz-Olcina, M., & Vethaak, A. D. (2017). The adverse effects of virgin microplastics on the fertilization and larval development of sea urchins. Marine environmental research, 130, 69-76.

Mato, Y. & Isobe, T. (2001). Plastic Resin Pellets as a Transport Medium for Toxic Chemicals in the Marine Environment, 35, 318–324

Mankidy, R., Wiseman, S., Ma, H., & Giesy, J. P. (2013). Biological impact of phthalates. Toxicology Letters, 217(1), 50-58.

Marlin Baltic Marine Litter, 2014. Final Report of Baltic Marine Litter Project Marlin – Litter Monitoring and Raising Awareness 2011–2013.

McDougall, C., Chen, W.C., Shimeld, S.M. & Ferrier, D.E.K. (2006). The development of the larval nervous system, musculature and ciliary bands of Pomatoceros lamarckii (Annelida): Heterochrony in polychaetes. Front. Zool., 3, 1–14

McRae L, Freeman R, Shirkhorshidi M, & Tresize E. (2015) 'The state of our blue planet' in: Living Blue Planet Report. Species, habitats and human well-being. (eds. Tanzer J, Phua C, Lawrence A, Gonzales A, Roxburgh T, & Gamblin P). WWF, Gland, Switzerland.

Meeker, J.D., Sathyanarayana, S. & Swan, S.H. (2009). Phthalates and other additives in plastics: human exposure and associated health outcomes. Philos. Trans. R. Soc. B Biol. Sci., 364, 2097–2113

Menge, B. A., & Sutherland, J. P. (1987). Community regulation: variation in disturbance, competition, and predation in relation to environmental stress and recruitment. The American Naturalist, 130(5), 730-757.

Messinetti, S., Mercurio, S., Parolini, M., Sugni, M. & Pennati, R. (2017). Effects of polystyrene microplastics on early stages of two marine invertebrates with different feeding strategies. Environ. Pollut., 1–8

Mirza, M. M. Q. (2003). Climate change and extreme weather events: can developing countries adapt?. Climate policy, 3(3), 233-248.

Morales, M., Planelló, R., Martínez-Paz, P., Herrero, O., Cortés, E., Martínez-Guitarte, J. L., & Morcillo, G. (2011). Characterization of Hsp70 gene in Chironomus riparius: expression in response to endocrine disrupting pollutants as a marker of ecotoxicological stress. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 153(1), 150-158.

Murrell, K.A., Ghetu, C.C. & Dorman, F.L. (2018). The combination of spectroscopy, microscopy, and profilometry methods for the physical and chemical characterization of environmentally relevant microplastics. Anal. Methods

Mrowiec, B. (2018). Plastics in the circular economy (CE), Environmental Protection and Natural Resources; The Journal of Institute of Environmental Protection-National Research Institute., 29(4), 16-19.

Nakajima, Y., Burke, R. D., & Noda, Y. (1993). The Structure and Development of the Apical Ganglion in the Sea Urchin Pluteus Larvae of Strongylocentrotus droebachiensis and Mespilia globulus: (apical ganglion/anti-serotonin/sea urchin larvae/animalization/immuno histochemistry). Development, growth & differentiation, 35(5), 531-538.

Navarro R, Pérez Perrino M, Gómez Tardajos M, Reinecke H (2010) Phthalate plasticizers covalently bound to PVC: plasticization with suppressed migration. Macromol 43:2377–2381.

Nazareth, M., Marques, M. R., Leite, M. C., & Castro, Í. B. (2019). Commercial plastics claiming biodegradable status: Is this also accurate for marine environments?. Journal of hazardous materials, 366, 714-722.

Nelms, S.E., Galloway, T.S., Godley, B.J., Jarvis, D.S. & Lindeque, P.K. (2018). Investigating microplastic trophic transfer in marine top predators *. Environ. Pollut., 1–9

Nobre, C.R., Santana, M.F.M., Maluf, A., Cortez, F.S., Cesar, A., Pereira, C.D.S., *et al*. (2015). Assessment of microplastic toxicity to embryonic development of the sea urchin Lytechinus variegatus (Echinodermata: Echinoidea). Mar. Pollut. Bull., 92, 99–104

Nizzetto, L., Bussi, G., Futter, M.N., Butterfield, D. & Whitehead, P.G. (2016). A theoretical assessment of microplastic transport in river catchments and their retention by soils and river sediments. Environ. Sci. Process. Impacts, 18, 1050–1059

Oberbeckmann, S., Loeder, M. G., Gerdts, G., & Osborn, A. M. (2014). Spatial and seasonal variation in diversity and structure of microbial biofilms on marine plastics in Northern European waters. FEMS microbiology ecology, 90(2), 478-492.

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

Oberbeckmann, S., Loeder, M. G., Gerdts, G., & Osborn, A. M. (2014). Spatial and seasonal variation in diversity and structure of microbial biofilms on marine plastics in Northern European waters. FEMS microbiology ecology, 90(2), 478-492.

Ogata, Y., Takada, H., Mizukawa, K., Hirai, H., Iwasa, S., Endo, S., *et al*. (2009). International Pellet Watch : Global monitoring of persistent organic pollutants (POPs) in coastal waters . 1 . Initial phase data on PCBs , DDTs , and HCHs. Mar. Pollut. Bull., 58, 1437–1446

Oliveira, M., Ribeiro, A., Hylland, K., & Guilhermino, L. (2013). Single and combined effects of microplastics and pyrene on juveniles (0+ group) of the common goby Pomatoschistus microps (Teleostei, Gobiidae). Ecological indicators, 34, 641-647.

Oliviero, M., Tato, T., Schiavo, S., Fernández, V., Manzo, S. & Beiras, R. (2019). Leachates of micronized plastic toys provoke embryotoxic effects upon sea urchin Paracentrotus lividus. Environ. Pollut., 247, 706–715

Pahl, S., Wyles, K. J., & Thompson, R. C. (2017). Channelling passion for the ocean towards plastic pollution. Nature Human Behaviour, 1(10), 697.

Pagano, G., Esposito, A. & Giovan, G. (1982). Fertilization and Larval Development in Sea Urchins Following Exposure of Gametes and Embryos to Cadmium, 55, 47–55

Pannetier, P., Cachot, J., Clérandeau, C., Faure, F., Van, K., Alencastro, L.F. De, *et al*. (2018). Toxicity assessment of pollutants sorbed on environmental sample microplastics collected on beaches: Part I-adverse effects on fish cell line. Environ. Pollut.

Peeken, I., Primpke, S., Beyer, B., Gütermann, J., Katlein, C., Krumpen, T., *et al*. (n.d.). Arctic sea ice is an important temporal sink and means of transport for microplastic. Nat. Commun.

Perkins, L. A., Hedgecock, E. M., Thomson, J. N., & Culotti, J. G. (1986). Mutant sensory cilia in the nematode Caenorhabditis elegans. Developmental biology, 117(2), 456-487.

Plastics Europe. (2015). Plastics-the Facts 2015: An analysis of European plastics production, demand and waste data for 2014. Brussels, Belgium.

Pittura, L., Avio, C.G., Giuliani, M.E., d'Errico, G., Keiter, S.H., Cormier, B., *et al*. (2018). Microplastics as Vehicles of Environmental PAHs to Marine Organisms: Combined Chemical and Physical Hazards to the Mediterranean Mussels, Mytilus galloprovincialis. Front. Mar. Sci., 5

Provencher, J.F., Avery-gomm, S., Liboiron, M., Braune, B.M., Macaulay, J.B., Mallory, M.L., *et al*. (2018). Are ingested plastics a vector of PCB contamination in northern fulmars from coastal Newfoundland and Labrador ? Environ. Res., 167, 184–190

Prokić, M.D., Radovanović, T.B., Gavrić, J.P. & Faggio, C. (2018). Ecotoxicological effects of microplastics: examination of biomarkers, current state and future perspectives. TrAC Trends Anal. Chem.

Prunier, J., Maurice, L., Perez, E., Gigault, J., Pierson Wickmann, A.-C., Davranche, M., *et al*. (2019). Trace metals in polyethylene debris from the North Atlantic subtropical gyre. Environ. Pollut., 245, 371–379

Rehse berg, M. J., & Price, D. A. (1992). Relationships among the FMRFamide-like peptides. In Progress in brain research(Vol. 92, pp. 25-37). Elsevier.

Rehse, S., Kloas, W., & Zarfl, C. (2018). Microplastics reduce short-term effects of environmental contaminants. Part I: Effects of bisphenol a on freshwater zooplankton are lower in presence of polyamide particles. International journal of environmental research and public health, 15(2), 280.

Richard, H., Carpenter, E.J., Komada, T., Peter, T. & Rochman, C.M. (2019). Biofilm Facilitates Metal Accumulation onto Microplastics in Estuarine Waters PT. Sci. Total Environ.

Rios, L.M., Moore, C. & Jones, P.R. (2007). Persistent organic pollutants carried by synthetic polymers in the ocean environment. Mar. Pollut. Bull., 54, 1230–1237

Robertson, L. W., & Hansen, L. G. (Eds.). (2015). PCBs: recent advances in environmental toxicology and health effects. University Press of Kentucky.

Rochman, C. M., Hoh, E., Hentschel, B. T., & Kaye, S. (2013). Long-term field measurement of sorption of organic contaminants to five types of plastic pellets: implications for plastic marine debris. Environmental Science & Technology, 47(3), 1646-1654.

Rochman, C.M. (2013). Plastics and priority pollutants: A multiple stressor in aquatic habitats. Environ. Sci. Technol., 47, 2439–2440

Rochman, C.M., Kurobe, T., Flores, I. & Teh, S.J. (2014). Early warning signs of endocrine disruption in adult fish from the ingestion of polyethylene with and without sorbed chemical pollutants from the marine environment. Sci. Total Environ., 493, 656–661

Rochman, C. M., Browne, M. A., Underwood, A. J., Van Franeker, J. A., Thompson, R. C., & Amaral-Zettler, L. A. (2016). The ecological impacts of marine debris: unraveling the demonstrated evidence from what is perceived. Ecology, 97(2), 302-312.

Rochman, C.M., Brookson, C., Bikker, J., Djuric, N., Earn, A., Bucci, K., *et al*. (2019). FOCUS, 38, 703–711

Romano, G., Costantini, M., Buttino, I., Ianora, A., & Palumbo, A. (2011). Nitric oxide mediates the stress response induced by diatom aldehydes in the sea urchin Paracentrotus lividus. PLoS One, 6(10), e25980.

Rose, R. J., Priston, M. J., Rigby-Jones, A. E., & Sneyd, J. R. (2012). The effect of temperature on di (2-ethylhexyl) phthalate leaching from PVC infusion sets exposed to lipid emulsions. Anaesthesia, 67(5), 514-520.

Seidensticker, S., Grathwohl, P., Lamprecht, J. & Zarfl, C. (2018). A combined experimental and modeling study to evaluate pH-dependent sorption of polar and non-polar compounds to polyethylene and polystyrene microplastics. Environ. Sci. Eur., 30, 1–12

Seltenrich N (2015) Newlink in the food-chain? Marine plastic pollution and seafood safety. Environ Health Perspect 123:34–41

Shahul Hamid, F., Bhatti, M.S., Anuar, N., Anuar, N., Mohan, P. & Periathamby, A. (2018). Worldwide distribution and abundance of microplastic: How dire is the situation? Waste Manag. Res., 0734242X1878573

Shipp, L. E., & Hamdoun, A. (2012). ATP-binding cassette (ABC) transporter expression and localization in sea urchin development. Developmental Dynamics, 241(6), 1111-1124.

Sleight, V. A., Bakir, A., Thompson, R. C., & Henry, T. B. (2017). Assessment of microplastic-sorbed contaminant bioavailability through analysis of biomarker gene expression in larval zebrafish. Marine pollution bulletin, 116(1-2), 291-297.

Smith, L. C., Rast, J. P., Brockton, V., Terwilliger, D. P., Nair, S. V., Buckley, K. M., & Majeske, A. J. (2006). The sea urchin immune system. Invertebrate Survival Journal, 3(1), 25-39.

Steffen, W. L., Rockström, J., & Costanza, R. (2011). How defining planetary boundaries can transform our approach to growth. Solutions: For A Sustainable & Desirable Future.

Strathmann, M. F. (1987). Reproduction and development of marine invertebrates of the northern Pacific coast: data and methods for the study of eggs, embryos, and larvae. University of Washington Press.

Sreekumar, V., & Norris, D. P. (2019). Cilia and development. Current Opinion in Genetics & Development, 56, 15-21.

Sussarellu, R., Suquet, M., Thomas, Y., Lambert, C., Fabioux, C., Pernet, M. E. J., ... & Corporeau, C. (2016). Oyster reproduction is affected by exposure to polystyrene microplastics. Proceedings of the National Academy of Sciences, 113(9), 2430-2435.

Syberg, K., Khan, F.R., Selck, H., Palmqvist, A., Banta, G.T., Daley, J., *et al*. (2015). Microplastics: Addressing ecological risk through lessons learned. Environ. Toxicol. Chem., 34, 945–953

Talsness, C. E., Andrade, A. J., Kuriyama, S. N., Taylor, J. A., & Vom Saal, F. S. (2009). Components of plastic: experimental studies in animals and relevance for human health. Philosophical Transactions of the Royal Society B: Biological Sciences, 364(1526), 2079-2096.

Tekman, M. B. , Gerdts, G. , Lorenz, C. , Primpke, S. , Hasemann, C. , Wekerle, C. and Bergmann, M. (2018): From the sea surface to the deep seafloor: Microplastics prevail at all ocean depths of the HAUSGARTEN observatory (Arctic) , Sixth International Marine Debris Conference

Teuten, E.L., Saquing, J.M., Knappe, D.R.U., Barlaz, M.A., Jonsson, S., Bjorn, A., *et al*. (2009). Transport and release of chemicals from plastics to the environment and to wildlife. Philos. Trans. R. Soc. B Biol. Sci., 364, 2027–2045

Thet, M. M., Noguchi, M., & Yazaki, I. (2004). Larval and juvenile development of the echinometrid sea urchin Colobocentrotus mertensii: emergence of the peculiar form of spines. Zoological Science, 21(3), 265-275.

Thompson, R. C., Swan, S. H., Moore, C. J., & vom Saal, F. S. (2009). Our plastic age. Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 364(1526), 1973.

Theuerkauf, W.E., Smiley, S., Wong, M.L., Alberts, B.M., 1992. Reorganization of the cytoskeleton during Drosophila oogenesis: implications for axis specification and intercellular transport. Development 115, 923–936.

Van Cauwenberghe, L. & Janssen, C.R. (2014). Microplastics in bivalves cultured for human consumption. Environ. Pollut., 193, 65–70

Van Sebille, E., Wilcox, C., Lebreton, L., Maximenko, N., Hardesty, B.D., Van Franeker, J.A., Eriksen, M., Siegel, D., Galgani, F., & Law, K.L. (2015). A global inventory of small floating plastic debris. Environmental Research Letters, 10(12), 124006.

Vethaak, A.D. & Leslie, H.A. (2016). Plastic Debris is a Human Health Issue. Environ. Sci. Technol.

Vellutini, B. C., & Migotto, A. E. (2010). Embryonic, larval, and juvenile development of the sea biscuit Clypeaster subdepressus (Echinodermata: Clypeasteroida). PLoS One, 5(3), e9654.

Voronezhskaya, E. E., Tyurin, S. A., & Nezlin, L. P. (2002). Neuronal development in larval chiton Ischnochiton hakodadensis (Mollusca: Polyplacophora). Journal of Comparative Neurology, 444(1), 25-38.

Wan, Z., Wang, C., Zhou, J., Shen, M., Wang, X., Fu, Z., *et al*. (2018). Effects of polystyrene microplastics on the composition of the microbiome and metabolism in larval zebrafish. Chemosphere

Wania, F., & Mackay, D. (1996). Peer reviewed: tracking the distribution of persistent organic pollutants. Environmental science & technology, 30(9), 390A-396A.

Wathsala, R. H. G. R., Franzellitti, S., Scaglione, M., & Fabbri, E. (2018). Styrene impairs normal embryo development in the Mediterranean mussel (Mytilus galloprovincialis). Aquatic toxicology, 201, 58-65.

Wolpert, L., & Gustafson, T. (1961). Studies on the cellular basis of morphogenesis of the sea urchin embryo: Development of the skeletal pattern. Experimental cell research, 25(2), 311-325.

Wikramanayake, A. H., & Klein, W. H. (1997). Multiple signaling events specify ectoderm and pattern the oral-aboral axis in the sea urchin embryo. Development, 124(1), 13-20.

Wilt, F. H., & Ettensohn, C. A. (2007). The morphogenesis and biomineralization of the sea urchin larval skeleton. Handbook of biomineralization: biological aspects and structure formation, 182-210.

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

Yoshida, S., Hiraga, K., Takanaha, T., Taniguchi, I., Yamaji, H., Maeda, Y., *et al*. (2016). A bacterium that degrades and assimilates poly(ethyleneterephthalate). Science (80-.)., 351, 1196–1199

Zalasiewicz, J. (2012). The epoch of humans. Nature Geoscience, 6(1), 8.

Zettler, E. R., Mincer, T. J., & Amaral-Zettler, L. A. (2013). Life in the "plastisphere": microbial communities on plastic marine debris. Environmental science & technology, 47(13), 7137-7146.

Ziajahromi, S., Kumar, A., Neale, P. A., & Leusch, F. D. (2017). Impact of microplastic beads and fibers on waterflea (Ceriodaphnia dubia) survival, growth, and reproduction: implications of single and mixture exposures. Environmental science & technology, 51(22), 13397-13406.

Ziccardi, L.M., Edgington, A., Hentz, K., Kulacki, K.J. & Kane Driscoll, S. (2016). Microplastics as vectors for bioaccumulation of hydrophobic organic chemicals in the marine environment: A state-of-the-science review. Environ. Toxicol. Chem., 35, 1667–1676