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CELLULAR AND PHYSIOLOGICAL IMPACTS OF NANOPLASTICS ON THE GREEN-LIPPED MUSSEL *PERNA VIRIDIS* UNDER OCEAN ACIDIFICATION

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Cellular and Physiological Impacts of Nanoplastics on the Green-Lipped Mussel *Perna viridis* under Ocean Acidification

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A thesis submitted in partial fulfilment of the requirements for the degree of Master of Philosophy

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Yuen Lai Shan

ABSTRACT

Plastics serve as an essential component of modern daily life worldwide, but a notable portion of these plastics eventually turn into waste and find their way into the ocean. Plastic waste breaks down into microplastics (1–5,000 μm) and nanoplastics (NPs; 1–1,000 nm) through processes like ultraviolet degradation. NPs, in comparison to larger-sized plastics, pose a greater biological threat as they can more easily enter cells, leading to immunological stress and oxidative damage that can hinder growth. Among marine organisms, bivalves are particularly susceptible to the impact of NPs due to their filter-feeding nature, which makes ingestion and bioaccumulation of NPs seemingly inevitable. Given the indispensable role of plastics in human society, it is expected that the environmental abundance of NPs will increase in the future. This escalation coincides with the occurrence of ocean acidification (OA), often referred to as the "evil twin" of climate change, which arises from anthropogenic carbon dioxide (CO_2) emissions. OA is associated with reduced seawater pH and decreased calcium carbonate saturation, posing a significant risk to marine life, especially calcifying organisms that rely on calcium carbonate for their growth. When OA intersects with NPs, it presents an unresolved understanding of the nature of their interaction and the subsequent biological and ecological consequences. In light of this, the aim of this thesis is to investigate the combined effects of OA and NPs, using the green-lipped mussel *Perna viridis* as the model species, a calcifying filter-feeder which is vulnerable to both OA and NPs. A factorial-design experiment of OA and NPs was performed for *P. viridis* over 30 days. Mussels were exposed to four OA scenarios at pH 8.1, 8.0, 7.8 and 7.7, with the partial pressure of CO_2 validated to be approximately 470 μatm, 590 μatm, 830 μatm and 1140 μatm, respectively. There were two treatments for each scenario, one without and one dosed with NPs. This experimental design led to eight treatments (4 levels of pH × 2 levels of NPs). The exposure level of NPs was environmentally realistic at 4×10^6 particles mL^{-1}, for 2 h daily. At the end of the experiment, a total of 12 biological endpoints were examined in *P. viridis* to evaluate the individual and combined effects of OA and NPs.

Four out of the 12 endpoints served as immunological biomarkers measured in haemocytes: Two of them, namely oxidative activity and apoptosis, exhibited significant increases in response to reduced pH, while the other two, lysosomal content and phagocytosis, decreased and increased significantly upon exposure to NPs, respectively. Another set of four endpoints focused on oxidative stress biomarkers assessed in soft tissue: Significantly higher activities of superoxide dismutase (SOD) and catalase (CAT), accompanied by decreased glutathione (GSH) content, were observed under the OA scenarios. Nevertheless, the levels of the four biomarkers related to oxidative stress, i.e. SOD, CAT, GSH and lipid peroxidation, all significantly increased in the presence of NPs. The remaining four endpoints centered around physiological changes at the individual level: The values of shell index, condition index and hepatosomatic index remained relatively consistent across all treatments. However, a significant increase was determined in the energy reserves of *P. viridis* under one of the OA scenarios. Among the 12 endpoints examined, there were no statistically significant interaction effects detected between pH and NPs in eight of them. In the remaining four endpoints (lysosomal content, phagocytosis, SOD and energy reserves), the obtained data trends across treatments did not support the presence of any synergistic or antagonistic interaction. Thus, it is likely that an additive interaction was predominantly at play in these cases. Furthermore, out of the 66 pairs of correlations analysed among the 12 endpoints in *P. viridis*, 11 pairs were statistically significant, and seven of them were specifically linked to condition index and energy reserves. These findings suggest the usefulness of the two higher-level physiological changes to integrate the cellular stress responses, particularly the activities of SOD and CAT, which, vice versa, can serve as early-warning biomarkers to reflect the overall health status under the impacts of OA and NPs.

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Chapter 1 - General Introduction

Our planet, Earth, can also be named as the “pale blue dot,” described by Sagan and Druyan (2011). Not only because of its small size compared to the solar system, but the incredible value of ocean toward planet. Ocean covered the majority of Earth’s surface, leading it to the role for maintaining the climatic condition for our planet, such as regulation of temperature and sustaining the water cycle that allows Earth to support the vast diversity of life, including human (Inniss et al., 2016). Furthermore, ocean and its diversity of lives can also support many other services toward people, ranging from food, transportation to recreation and even culture, but Barbier et al. (2014) proposed that the importance of ocean to human was highly underestimated in the past. However, due to the development in natural and social science, people have paid more attention on our impacts, including micro-/nano-plastic pollution and ocean acidification (OA), to ocean in recent decades because of the irreplaceability of ocean toward the planet and humankind (Inniss et al., 2016).

To better understand the design and aim for this study, a general introduction was provided in four sub-sections. First, two targeted marine pollution problems, which were ocean acidification and plastic pollution, were highlighted with their backdrops, definitions, sources, and entry pathways. Second, reasons for choosing mussels as targeted objects were illustrated. Lastly, an outline for introducing the aims of this study and the related parameters measured in aspects were presented.

1.1 Marine pollution problems: plastic contamination

Bakelite, the first synthetic plastic material, was invented in the middle of the 19 Century. Since then, based on their illimited innovative potential and scientific development, more plastics with divergent properties, such as electrically insulated, low-cost, durable, and lightweight, were created and the usage of them have become more prevalent, after World War 2 (Geyer et al., 2017). Until now, plastics have penetrated almost every sector of our society, including to produce packaging, transportation, consumer products, electrical and electronics, in building and construction, in textiles and industrial machinery (PlasticsEurope, 2019). With reference to PlasticsEurope (2019), fossil fuel based plastic production has risen from 359 to 368 million tons between 2018 to 2019 globally and most of the consumed plastic products in the world market can be classified into 6 major classes, which were polyethylene (29.8%), polypropylene (19.4%), polyvinylchloride (10%), polyurethane (7.9%), polyethylene terephthalate (7.9%) and polystyrene (6.2%).

Usage of plastics can indeed bring convenience to people's lives, accelerate urban development, and even contribute to social progress. However, everything is a double-edged sword, the subsequently pollution after plastic consumption is a problem that cannot be neglected. Unfortunately, at least 8 million tons of plastic waste end up in oceans every year, composed of 80% of all marine debris (Arthur et al., 2009). Thank to divergent properties of plastics, particularly for lightweight and durable, they can be observed in all regions globally, including polar regions and remote deep seas (Eriksen et al., 2014). As for micro- and nano-plastics, because of their small size, they can even be discovered in various biological systems, including human faecal sample, marine/plant life, and seafood (Arthur et al., 2009; Shahul Hamid et al., 2018).

1.1.1 Backdrop and definition of plastics

Plastic is a sub-category of polymers, a big class of materials. Polymers are large molecules with high molecular weight in average, contributed by the characteristically long chain-like molecular architecture. They are composed of identical repeating units to form homopolymers or various sub-units repeating in different feasible pattern to produce co-polymer. In general, plastics are considered as polymers that can be softened on heating and moulded into different shapes. Both pure plastic resin pellets (easily for transportation, compared to those manufactured plastic objects) and resins mixed with various additives to strength their performance of the material are included in the plastic family. And fillers, plasticizers, colorants, stabilizers, and processing aids are typical examples for additives used. In addition, plastic family can further be divided into 2 main sub-type, which are thermosetting plastic and thermoplastic (Kershaw and Rochman, 2015). Thermoplastics are defined as polymers that can be melted and recast nearly indefinitely and polypropylene (PP), polyvinylchloride (PVC) and polystyrene (PS) are commonly found plastics in marine debris. As for thermosetting plastics, they are cross-linked polymers that cannot be re-moulded upon heating and polyurethane foams and epoxy resins are typically found plastics in marine debris.

Microplastic is a sub-class of plastics, and its concept was first reported in the scientific literature in early 1970s (Carpenter and Smith Jr, 1972). Ryan and Moloney (1990) have first correlated the term ‘microplastic’ with marine debris to describe their results of research of South African beaches and Thompson et al. (2004) have also used ‘microplastic’ to illustrate the distribution of plastic fragment in seawater. The official size definition of microplastic has not been proposed at that time, but scientists have drawn the agreements that ‘microplastic’ meant material that could only be identified with the presence of microscope. In other words, microplastics are small pieces of plastics within the size range between millimetre to sub-millimetre (Carpenter and Smith Jr, 1972).Compared to the common definition mentioned in above, a more rigid and scientific definition of plastic debris based on their size range was suggested in Kershaw and Rochman (2015). Plastic can be classified into five sub-types, which are mega-plastic (>1,000,000 µm), macro-plastic (25,000 to 1,000,000 µm), meso-plastic

(1000 to 25,000 μm), micro-plastic (1 to 1000 μm) and nano-plastic (<1 μm). However, it has not yet been formally adopted by the international research community, due to the lack of agreement in nomenclature and the difficulties for measuring different size range in field work. Thus, microplastic has still be regarded as a generic name for 'small' pieces of plastic until now.

In 2008, the first international research workshop, focusing on marine microplastic pollution problem, was hosted by National Oceanic and Atmospheric Administration (NOAA). The upper size limit of microplastic was defined as 5000 μm in this forum, based on the assumption that particles smaller than or equal to 5000 μm are more readily ingested by biota and can subsequently cause unwanted threats toward them, compared to those larger plastic waste. Thus, in this study, microplastics are considered as particles in size range 1-5000 μm and nano-plastics are suggested as particles in size range <1 μm for the purpose of assessment on how micro-/nano-plastics can affect biological responses on mussels.

1.1.2 Origin and fate of plastics in marine

After understanding the backdrops of plastics and set definitions of micro- and nano-plastics in this study, the origin and fate of micro- and nano-plastic wastes in ocean were also explored. Micro- and Nano-plastics can be further distinguished into two subsets: primary and secondary (Sundt et al., 2014). This distinction is useful because it can be used for the indication of potential sources and identification of mitigation measures to lessen microplastics input to the environment. Primary micro- and nano-plastics are subsets of plastics that are directly released to the environment in form of small particulates. The presence of them in ocean can be attributed to two main reasons. First, small plastics are added voluntary to products or during industrial processes for various purposes, for example, industrial 'scrubbers' applied for blast cleaning of surface, micro-beads existed in cosmetic formulation, plastic powders used in moulding, spherical/cylindrical virgin resin pellets used as 'feedstock' for production of various plastic products and even nano-plastics required for various industrial processes. As for secondary micro- and nano-plastics, they are small particles that are resulted from the degradation of large plastic items once exposed to marine environment. They are originated from mismanaged waste (e.g., discarded plastic bags) or unintentional losses (e.g., fishing nets), through the photodegradation and other weathering processes. Also, the formation of those secondary plastics in ocean is influenced by a series of environmental factors and nature of the polymer.

Once microplastics entered the oceans, they can either sink or float, based on their densities. For micro- and nano-plastic lighter than seawater such as polypropylene, they can float and disperse broadly across the oceans and can subsequently accumulate in gyres with the presence of oceanic currents. With reference to the estimation of different scientists, 93 (Eriksen et al., 2014) to 268 (Sebille et al., 2015) kilotons of light-weighted microplastics are floating around the oceans currently. As for micro- and nano-plastic denser than seawater, such as acrylic, they may sink on the ocean floor, leading to the accumulation of significant amount of microplastics in the deep-sea sediments (Woodall et al., 2014) and can eventually enter the marine food chains (Seltenrich, 2015).

1.2 Marine pollution problems: Ocean acidification

Ocean acidification is the process of reduction of the oceanic pH and carbonate ion concentration over an extended period, primarily provoked by the uptake of atmosphere carbon dioxide ($CO_{2)}$ (Caldeira and Wickett, 2003; Liu and Shi, 2021). With the combined effort of many experts (Joos et al., 2001; Sabine et al., 2004), past, present and prediction of atmospheric CO2 level were summarized. In the 18th Century, before the industrial revolution, atmospheric CO_2 level was approximately 280 ppm. Nowadays, CO_2 concentration in atmosphere has approached to 380 ppm, due to various anthropogenic activities, including burning fossil fuels, disposal of domestic and industrial wastewater, improper land management (particular in agriculture), and industrialization. And at the end of 21st century, the atmospheric carbon dioxide level will even exceed 800 ppm.

Ocean plays an irreplaceable in carbon cycle by acting as the earth's largest carbon reservoir (Doney et al., 2009; Le Quéré et al., 2009), and hence around 30% to 50% of the atmospheric CO_2 has been absorbed by ocean (Sabine et al., 2004). Ellis et al. (2017) have further proved that the average surface seawater pH around the globe has approximately decreased from pH 8.21 to pH 8.10 over the past 200 years, representing a 30% increase in acidity (hydrogen ion concentration) in ocean. In accordance with current rate of fossil fuel emissions and carbon-sequestration, Caldeira and Wickett (2003) have even predicted that the average surface seawater pH will decrease by 0.4 and 0.7 in the end of 21 st century and around the year 2250 correspondingly. To alleviate the problem of rising atmospheric CO_2, Holloway (2005) has proposed well developed potential strategies and injection of CO_2 into underground porous reservoir rocks is one of his examples. However, these strategies may also provoke severe local pH reduction, further affecting offshore habitats, biota, and the related marine processes, due to occurrences of sub-seabed storage or pipes leakages. In short, atmospheric CO_2 molecules generated from anthropogenic activities are the key contributor toward ocean acidification. In other words, the atmospheric CO_2 concentration is directly proportional to the degree of ocean acidification. Based on the scientific prediction, ocean acidification will become more severe in future. Thus, ocean acidification is a

plight that cannot be ignored anymore, and its potential effects should also be considered for all marine creatures and even humans.

1.2.1 Linkage between atmospheric carbon dioxide and ocean acidification

As mentioned in above, atmospheric CO_2 molecules are the major culprit for deteriorating current marine environment. Raven et al. (2005) illustrated the mechanism behind the net dissolution of atmosphere CO_2 into ocean. In the atmosphere, CO_2 molecules exist in the chemically inert state, but they will become more active to take part in different chemical, physical, biological, and geological reactions once they have been dissolved into seawater. Once the atmosphere CO_2 molecules dissolved into marine, they will first react with water to form carbonic acid, H_2CO_3. However, not all the dissolved CO_2 molecules will react to produce H_2CO_3, leading to the presence of aqueous CO_2. With reference to Henry's Law, it could be suggested that the concentration of atmospheric CO_2 would be directly proportional to that in seawater of carbonic acid. And the formed H_2CO_3 can then decompose to bicarbonate (HCO_3^-) and hydrogen ions (H^+). H^+ generated can further react with carbonate ions (CO_3^{2-}) presented in ocean to produce more HCO_3^- ions. To sum up, the net dissolution of CO_2 can produce a net increase in concentrations of H^+ ions, H_2CO_3 & HCO_3^- ions and cause a net decrease in CO_3^{2-} ions and pH in ocean.

1.2.2 Potential consequences of ocean acidification toward all marine life

Ocean acidification is not only a term for describing the reduction of ocean pH, but also a 'seed' for a series of biological, physiological, and evolutionary catastrophes for all marine biota at different organizational levels (Caldeira and Wickett, 2003; Joos et al., 2001). It is because the net decrease in carbonate ions to counteract the reduction in oceanic pH could also reduce the fundamental building blocks of shells or skeletons among marine creatures. In order to predict the future of ocean acidification and marine biota, scientists have developed different animal models, including marine plankton, larvae and adults for silver seabream, and have also confirmed that the growth and development for those pelagic and intertidal organisms were disrupted after they have

been exposed to acidified seawater (Bibby et al., 2007; Ishimatsu et al., 2004; Pörtner et al., 2004; Riebesell et al., 2000).

Marine mussels, one members of calcifying organisms, have been receiving the particular attention because of two major reasons. First, they are the major victims suffering from the reduction of CO_3^{2-} ions in the marine, which is essential material for the formation of shells or skeletons of them. Second, their specific biological, ecological, and socio-economical characteristics were mentioned in Section 1.3. And alternations in growth, energy metabolism, antioxidant defence and haemolytic activities of mussels exposed to different pH values were investigated (Berge et al., 2006; Gazeau et al., 2007; Michaelidis et al., 2005; Petes et al., 2007).

In Michaelidis et al. (2005) study, marine mussels, *Mytilus galloprovincialis*, have been exposed to CO_2 induced seawater at pH 7.3 for 90 days (about 3 months) and some important findings in the aspects of mussel's acid-base balance and growth rate have been discovered. First, a permanent reduction in haemolymph pH was detected. To counter the degree of acidosis, the dissolution of shell $CaCO_3$ was performed by mussels to increase their haemolymph bicarbonate level for the compensation of intracellular pH in various tissues. Berge et al. (2006) have also supported the above finding by indicating that the shell growth rate of blue mussels could be inhibited by surface seawater pH equal to or below 7.4. Gazeau et al. (2007) have even suggested that the calcification rate of mussels may decrease by 25% at the end of 21 century. Second, the reduction in growth rate was confirmed by two following observations, which were the decreased rate of oxygen consumption indicating a lower metabolic rate and the increased nitrogen excretion implying a net degradation of protein (Michaelidis et al., 2005).

1.3 Targeted subject for study: *Perna virdis*

Throughout the upper sections, plastic pollution and ocean acidification were discussed and marine mussels have also been slightly mentioned. Thus, green-lipped mussels, *Perna viridis*, one of the major victims of marine pollutions would be indicated in detail in the aspects of biological, ecological, and socio-economical in this section.

1.3.1 An introductory to bivalves

Mollusca is one of the most diverse, important, and largest groups in the animal kingdoms, composed of over 5,000 species in its phylum and 60% of these species are discovered in the ocean (Gosling, 2008). It consists of 6 major classes: Gastropoda, Scaphopoda, Bivalvia, Cehalopoda, Polyplacophora and Monoplacophora. All its species are soft-bodied animals, which have also shared the basic body structure. First, they are covered by a hard protective shell in the outer layer. Second, mantle is presented to enclose all internal organs of animals. Third, a large muscular foot is existed for locomotion.

Perna viridis, also known as Asian green mussel, belongs to the family Mytilidae, which is a typical type of Bivalvia, and has shared five typical physical characteristics as Mollusca. First, it is flattened in shape. Second, it is equipped with two mantle lobes responding for the secretion of two shell valves, hinging dorsally, and covering all body organs. Third, the anterior and posterior adductors are two muscles owned by *Perna viridis* for controlling the opening and closing of their shell valves. Fourth, it possesses two enormously enlarged gills for replacing mouth's role for catching food. Lastly, its wedge-shaped foot with reduced size is responsible for animal's movement.

1.3.2 Mussels as an ideal bioindicator for monitoring pollution in coastal regions

Thanks to the biological, ecological, and socio-economic specificity of mussels, they are considered as a virtually ideal sentinel for detectibg marine pollution (Commission, 1999). Biologically, mussels perform ideally in four criteria, which are sessility, size, number of samples and adaptivity.First, they are sessile and can provide location specific information. Second, each mussel individual is medium in size and can provide enough tissue for furthur analysis. Third, they often form large mussel beds in shallow waters, so they are easy to collect enough number of samples. Fourth, they are hardy creatures with strong adaptivity, which are suitable for ecotoxicological exposure tests in laboratory.

Mussels also conduct their monitoring role perfectly in the ecological aspect because of their feeding habit and niche in food web (Farrell and Nelson, 2013; Larsen et al., 2016; Wang and Fisher, 1999). Since mussels are filter-feeding on phytoplankton, they must pump and filter large volumes of sewater within their ciliated gills to uptake phytoplankton. Because of filtration action, microplastics and other artificial pollutants, can accumulate inside mussel's body efficiently, allowing the integrative measurement of the concentration and bioavailability of seawater pollutant in situ. Furthermore, mussels can act as food and habitat for other species in marine food chain too. As a primary consumer, mussel perform as a vehicle for transferring microplastics from abiota level to higher trophic level of the marine food chain by acting as the food source for those mussel eating invertebrates (crabs, sea stars, polychaeta & dog whelks) and vertebrates (sea birds, seals, walrus, and sea otters).

Apart from the role of bioindicator, blue mussel also has a high socio-economic value, since it is cultivated and consumed as food source for human for thousands of years. Thus, bioaccumulation of micro- and nano- plastics via seafood consumption may constitute a human health risk (Beyer et al., 2017).

1.4 Aims for this study

From above, it can be concluded that plastic pollution and ocean acidification were urgent problems nowadays, which have drawn the public attention. Many scientists have devoted their efforts on how micro-/nano-plastic pollution and ocean acidification affecting marine organisms separately by using mussels as the test model. However, combined effects on marine creatures provoked by nano-plastic pollution and ocean acidification is still a new area for study and not much investigation have been achieved in this topic. Since the immunolological alteration is a energy-demanding process (Lochmiller and Deerenberg, 2000), which have been correlated with the reproductive process (Brokordt et al., 2019), vital processes including escape response capacity and swimming ability (Brokordt et al., 2000a; Brokordt et al., 2000b) and even resistance toward environmental stress (Brokordt et al., 2015) in bivalves, it and the related stress & detoxification processes were first invesgiated deeply at cellular, tissue and individual levels in current study to figure out the combined interaction of nano-plastic pollution and ocean acidifcation and could serve as the stepping stone for the furture studies in molecular level.

Thus, in this study, a 30-day exposure experiment on marine mussels to polystyrene in nano-sized under the present-day scenario (pCO2 = 380 ppm) and three future OA scenarios, namely RCP4.5 (560 ppm), RCP6.0 (720 ppm) and RCP8.5 (1200 ppm) was designed. To investigate combined effects of nano-plastics and ocean acidification on the immune and physiological responses of mussels, four particuar organs, which are hepatopancreas, posterior adductor muscles, gills, and haemolymph, were chosen for further chemical analysis.

To investigate mussel's immunological alternation, four parameters were measured in haemolymph and they were lysosomal content, oxidative activity, phagocytotic ability and apoptosis percentage of cells. Cellular lysosomal content and phagocytotic ability were directly correlated with the immunological power of mussel. Oxidative activity and apoptotic ratio of cells can be used for inidcating the cell viability.

As for the investigation on the variations on mussel's celluar stress and anti-detoxification system, four biomarkers were studied by using mussel's soft tissues. Two well-studied antioxidant enzymes, which were catalase (CAT) and superoxide dismutase (SOD), were probed. The antioxidant protein, glutathione (GSH) and the level of lipid peroxidation (LPx) for representing the degree of cellular damage was measured.

For studying phosiological changes of mussels, four growth indexes were calculated, which were shell index (SI), condition index (CI), hepatosomatic index (HSI) and energy budget. As for SI, CI and HIS, they were caculated by mussel's biometric measurements, including shell length, total soft tissue weight and hepatopancreases weight for showing mussel health in individual level. As for the variation of energy budget, it was another parameter for representing mussel general health and was measured by using posterior adductor muscles.

Chapter 2 Literature Review

2.1 Haemolymph: key organ for investigating mussel's immunological alternation

Before investigating immunological responses provoked by combined effects of microplastic exposure and ocean acidification, the defence mechanism of marine invertebrate toward pathogens (virus, bacteria, and fungi) and even environmental stresses (mechanical stress, changes in salinity & pH, air exposure, algal toxins, elevated temperature, and presence of contaminants) needed to be introduced first. Unlike mammals, the adaptive immune system, ability for body to react based on the identification of specific antigen, is absent in all invertebrates. Innate immunity, also named natural immunity, is the only defence mechanism for mussels. It is a coherently cascade that relies on active immune cells (haemocytes) in collaboration with related humoral factors (lectins, complement & ProPo systems, and anti-microbial peptides) to provoke effective immune responses (Tanguy et al., 2013). Thus, it can be suggested that haemocytes are the crucial player for the general immune response by serving as the first line of defence to fight against xenobiotics and environmental stresses, based on their enrichments of components of innate immunity (Boraschi et al., 2017; Bouallegui, 2019). However, the external/physical defence barrier of mussels is out of the scope in this study.

2.1.1 Classification of haemocytes

To achieve a common haemocytotic classification system among invertebrates, different detecting approaches have been adopted, including cytochemical & fluorescent staining for the subtype identification and electron microscopy for ultrastructural analysis (Carballal et al., 1997; Cima, 2010; Tame et al., 2015). In brief, haemocytes can be divided into two populations, which are hyalinocytes and granulocytes. Hyalinocytes perform undifferentiated cell characteristics, which are large central nuclei and relatively small cytoplasm with some occasionally found pseudopodia, mitochondria, cisterna, organelles, vesicles of smooth and rough

endoplasmic reticulum, and free ribosomes. As for granulocytes, they are more differentiated than agranulocytes with the presence of membrane-bound, sub-cellular compartments named granules. They are ovoid in shape, commonly equipped with eccentric nucleus and thin pseudopodia, enriched cytoplasmic organelles, vesicles of smooth and rough endoplasmic reticulum, Golgi complex, mitochondria, and cisterna (Carballal et al., 1997). Despite the number of studies performed, an official classification for haemocytes has still not been confirmed, due to the absence of a clear hematopoietic site. However, morphological criteria of two mentioned haemocyte subpopulations were concluded and present in **Error! Reference source not found.**, which could be used as a general classification rule (Cima, 2010; Hine, 1999; Pipe et al., 1997).

Granulocytes are ranged between 10 to 15 μM with low nucleus to cytoplasm ratio and their nucleus can existed in various shapes, such as sphere, ellipse and ovoid, and even be considered as unnucleated or binucleated under the microscopic observation. Their key morphological feature is the presence of cytoplasmic granules with diverse size, tinctorial and shape. In addition, pseudopodia can be observed at the external hyaline region. As for hyalinocytes, they are around 6 μM with high nucleus to cytoplasm ratio, which can be further classified into two subtypes based on their nucleus. Common hyalinocytes are equipped with a large ovoid/irregular eccentric or reinform nucleus, surrounded by a little cytoplasm with organelles. Blast-like cells is a subtype of hyalinocytes with an ovoid/spherical nucleus in the centre surrounded by cytoplasm with no organelles. Compared to granulocytes, a few granules and even no granules can be observed in the cytoplasm of hyalinocytes

Apart from the morphological features of haemocytes, scientists have also attempted to establish functional correlations between mussel haemocytes subpopulation and their immune response capacities (Accorsi et al., 2013; De Vico and Carella, 2012a; Hine, 1999; Parisi et al., 2008; Pipe et al., 1997; Wootton and Pipe, 2003).

As for granulocytes, they can be further classified into two sub-groups, which are eosinophilic (bigger in cell size with more granules) and basophilic (smaller in cell size with more granules) granulocytes. Eosinophilic granulocytes have been considered as the key player for phagocytosis, due to the presence of cytoplasmic granules, that contain acid hydrolases needed for the formation of lysosome (Bayne et al., 1979; Carballal et al., 1997; GIAMBERINI et al., 1996; Lopez et al., 1997; Moore and Gelder, 1985; Moore and Eble, 1977) and they could engulf various pathogens and xenobiotics, including bacteria (Parisi et al., 2008), zymosan & heat killed yeast (Garcia-Garcia et al., 2008), algae (Galimany et al., 2008), parasites (Le Guernic et al., 2019), and even micro-plastics (Romdhani et al., 2022). In addition, eosinophilic granulocytes are the most active in releasing reactive oxygen species and show highest enzyme activities for phenol oxidase, peroxidase and arylsulphatase, which are needed for various kinds of microbicidal mechanisms (Pipe et al., 1997). As for basophilic granulocytes, catalase and superoxidase dismutase (SOD) are found in their granules and immunogold labelling can also be found between those mentioned enzymes and eosinophilic granulocytes confirming the assistant role of basophilic granulocytes during phagocytosis (Pipe et al., 1997). Hyalinocytes may not equip with (Carballal et al., 1997) or have weaker phagocytotic ability (Cajaraville and Pal, 1995), compared with granulocytes. They cannot produce reactive oxygen species but can generate nitric oxide (Garcia-Garcia et al., 2008). SOD molecules can be found on the plasma membrane or through the cytoplasm in hyalinocytes (Hine, 1999). Until now, specific immune functions for hyalinocytes are less clearly defined, but their participation in the clearance of bacteria can be confirmed (Parisi et al., 2008).

In short, eosinophilic granulocytes are the most active immune cells, due to their powers on proliferation, phagocytosis, releasement of reactive oxygen species and digestive enzymes. As for basophilic granulocytes and hyalinocytes, their participation in the immune response can been noted. However, the specific immune functions among each of them are less clear.

2.1.2 Synopsis of the molecular mechanism of mussel's immunity

In section 2.1.1, an overview of mussel's haemolymph was introduced in macroscopic point and the molecular mechanism of mussel's immunity was investigated and concluded into three main subdivisions., which are sensitive receptors, signalling pathways and selective effectors, 3S.

To eliminate pathogens or any other foreign materials precisely, mussel immune system must equip with the ability for identification of self (harmless body cells) or non-self (harmful foreign offenders) materials. Thus, pathogen recognition receptors (PRRs) are commonly existed in haemocytes and tissue of mussel, since they can recognize antigens presented on the pathogens or other foreign materials to achieve the job on identification of dangers and to further activate the downstream reactions with the help of intracellular mediators. After undergoing all the above steps, the selective effectors can be activated to perform a coordination of functional immune response, such as the releasement of anti-microbial peptides (AMPs) (Bouallegui, 2019). A variety of PRRs are present within mussels to help them to spot out different threats and can be classified into 3 main types based on their location in cells (Gerdol and Venier, 2015; Philipp et al., 2012). Extracellular (secreted) PRRs are targeting on pathogens or for foreign materials located outside the cells, and C1q contained domain proteins involved in the classical pathway of complement system (Gerdol et al., 2011; Liu et al., 2014), C-type lectins, fibrinogen-related proteins, and galectins (Gorbushin and Iakovleva, 2011; Jing et al., 2011; Romero et al., 2011a; Yang et al., 2011; Zhang et al., 2009) are some of their typical examples. Membrane-bound PRRs are also responsible for recognition of on extracellular pathogens ,and non-cell materials and toll-like receptors (Guo et al., 2018; Qi et al., 2018; Toubiana et al., 2013; Toubiana et al., 2014; Zhang et al., 2011) and peptidoglycan recognition receptors (Gerdol and Venier, 2015; Philipp et al., 2012) are two commonly found examples. Cytosolic (intracellular) PRRs are in charge of detecting intracellular pathogenic compounds ,and NOD-like receptors and RIG-like receptors are two classic examples of them (Gerdol and Venier, 2015).

After the successful PRRs recognition steps, varied downstream molecules are activated to transfer sensory signals into active and effective immune responses.

Cytokines are the dominant and diverse group of mediators for the coordination of immunity, which are responsible for intracellular and intercellular signalling (Hopkins, 2003; Ishihara and Hirano, 2002).Taking interleukin-8 as an example, it can enhance the phagocytotic activity in haemocytes (Ottaviani et al., 2000). As for Interleukin-1α (Haddad, 2002) ,transforming growth factor (TGF- β 1) (Franchini and Ottaviani, 2000), macrophage migration inhibitory factor (MIF) (Parisi et al., 2012) and allograft inhibitory factor-1 (AIF-1) (Li et al., 2013), they are cytokines taking part in inflammatory response. In addition, interleukin-16 is responsible for various receptors' cross-talking, immune cell's activation and clearance of extracellular bacteria (Canesi et al., 2003; Rosani et al., 2015).

Apart from cytokines, other mediators and intermediate elements are present and their roles in signalling pathway for innate immunity have been revealed with the information updated by the molecular database (Gerdol and Venier, 2015). Toll-like receptors (TLRs), one sub-class of membrane-bound PRRs, and its related signalling pathway are the most studied immune cascade for mussel. A TLR constituted by 3 main sections, which are an intracellular TIR domain, a transmembrane region and a variable extracellular region equipped with leucine-rich repeats (LRRs) with specific binding activity. In response to the ligand binding, TLR dimerization is performed to progressively hire intermediate elements (Myd88 adaptor, IRAK and IKK kinase) which can further provoke the activation of transcription factors, including NF-κB & interferon-regulatory factors, IRFs (Fitzgerald et al., 2001; Gerdol and Venier, 2015; Koch et al., 2015; Li et al., 2005; Martin and Wesche, 2002; Philipp et al., 2012; Toubiana et al., 2014). Different effector molecules (cytokines, chemokines, AMPs) can be expressed once such transcription factors have mounted on the DNA.

Four main signalling pathways can be controlled by TLRs, which are MyD88-dependent pathway, MyD88-independent pathway, mitogen-activated protein kinases (MAPK) pathway, JAK/STAT pathway (Gerdol and Venier, 2015; Guo et al., 2018; Kawai and Akira, 2010; Philipp et al., 2012). The presence of Toll pathways and their related components in mussel have been further confirmed by Toubiana et al. (2013). 23 TLRs are identified in *M.galloprovinciali,* which are classified into four different clusters, based on their LRRs. One cluster of mussels TLRs (MgTLR-I) was reported

to have up-regulation toward the bacteria exposure to enhance the expression of three Myd88 adaptors in different inducible and constitutive levels. Furthermore, MyD88-independent pathway is suggested to be absent in mussel, due to the absence of TRIF-like molecules in Toubiana's research. As for MyD88-dependent pathway consists of 4 key components, which are Mg-TOLLIP, Mg- ECSIT, Mg-TRAF-3, and Mg-TRAF-6. Mg-TOLLIP is a ubiquitin-binding protein responsible for interacting with varied TLR element and cooperating in the turnover of IL-1R-associated kinase. Mg- ECSIT is a signalling intermediate in Toll pathway. As for Mg-TRAF-3, and Mg-TRAF-6, they are TNF receptor-associated factors, and cell signalling proteins involved in inflammation. And, the mitogen-activated protein kinases (MAPK) pathway, it composed of MAP kinase kinases (MKKs) and theirs following activation elements. MKKs can activate TAK1, to further activate the c-JUN N-terminal kinases (JNK) pathway, which are necessary for the transcription of the targeted immune gene, such as NF-κB, AP1 and Relish. With reference to the name of the JAK/STAT pathway, it is composed of JAK-2 and STAT, which are two putative transcripts responsible for cooperating in the suppression of cytokine signalling and acting as the negative feedback-regulator of the cytokine production.

As mentioned in Section 2.1, haemocytes function as the indirect mediators in humoral immune response via the secretion of molecules in complement, ProPO system and even antimicrobial peptides to neutralize and eliminate foreign offenders. Complement system is a microbial destruction and pro-inflammatory system achieved by plasma and cell surface proteins within the haemolymph to perform pathogen neutralization and pro-inflammatory. As a evidence of the present of the complement system within *M. edulis* and *Ruditapes philippinarum,* the C3-like factor, a central element of 3 complement pathways, was discovered (Gerdol and Venier, 2015; Moreira et al., 2012; Philipp et al., 2012; Prado-Alvarez et al., 2009). As for the Pro-phenoloxidase (PPO) system, it is required for the process of melanisation to produce adhesive protein required by mussels allowing them to anchor on a wet surface. Furthermore, pro-phenoloxidase can be activated into phenoloxidase via proteolysis to perform as an immune effector in "self/non-self" recognition in haemocytes. (Cerenius et al., 2008; Cerenius and Soderhall, 2004; Hellio et al., 2007; Luna-Acosta et al., 2017). Diphenol oxidase and tyrosinase are two examples of the phenoloxidase observed in bivalves.

Microbicidal peptides, AMPs, is a group of heterogeneous gene-encoded peptides, equipped with antibiotic functions. Based on the structure and amino composition of AMPs, which are affected by the number of cysteine residues and disulfide bridges, they can be classified into various sub-groups. Most AMPs families discovered from *Mytilus edulis* belong to cysteine-rich subgroup and big defensins, lysozymes, mytimycins and mytimacins are some of the typical examples (Gerdol et al., 2015; Gerdol and Venier, 2015; Mitta et al., 1999; Mitta et al., 2000; Moreira et al., 2012; Venier et al., 2011).

2.1.3 Functional defence mechanism of haemocytes

Apart from the humoral immunity, cellular mediated response is also a key component for the mussel's immunity and is deliberated into two main subsections, which are phagocytosis and haemocyte's infiltration & inflammatory response. Phagocytosis is one of the major functional defence mechanisms, which can be achieved by eosinophilic granulocytes. In mammals, phagocytosis and the corresponding respiratory burst have always been performed simultaneously by professional phagocytes to perform as complimentary roles for each other (Tanguy et al., 2013). Phagocytosis is the physical internalization of foreign molecules regarded as dangers for cells. And the related respiratory burst is a series of biochemical reactions generating microbicidal oxidative radicals, including peroxide, hydrogen peroxide and nitric oxide, for activating the NADPH-oxidase to digest phagocytosed compartments (Gosling, 2008). In short, the whole phagocytosis and respiratory burst process can be described in four steps. First, phagocyte identifies whether the foreign material is self or non-self. Second, endocytosis is performed, when the engulfed material is considered as non-self by phagocyte. Third, once the physical internalization is completed, oxidative radicals are released to the phagosome to perform the digestion. Lastly, residuals of the foreign material are expelled by cells via exocytosis, once the digestion process completed (Gosling, 2008).Many scientists have reported that the key defensive mechanism of mussel's haemocytes against a variety of pathogens, including marine pollutants, poisons, bacteria, and protists, was phagocytosis (Canesi et al., 2002; Carballal et al., 1997; Malagoli et al., 2008). Garcia-Garcia et al. (2008) have reported that the phagocytotic ability of mussel haemocytes and their related respiratory burst process

were complicated, but they have highlighted that phosphatidylinositol 3-kinase, protein kinase and extracellular signal regulated kinase have played important roles in intracellular signalling pathways for the regulation of phagocytosis.

Inflammatory is a highly conserved physiological process in innate immunity by inducing genomic and cellular damage to promote a sustained inflammatory microenvironment for body recovery across species, which can be provoked by infections, post-ischemic & toxic injuries and phagocytosis & haemolytic infiltration originated from pollutions (Barcia et al., 1999; Hughes et al., 1990; Isailovic et al., 2015; Novas et al., 2007; Ottaviani et al., 2000; Parisi et al., 2012; Roberts et al., 2008). Apart from phagocytosis, haemocytes have also played a significant role in mussel's inflammatory response by working as immune-effector cells, that can be differentiated and released directly in the connective tissue adjacent to the inflamed site (Ottaviani, 2006). With reference to morphological features of the inflammatory response in mussels, three inflammation subtypes can be concluded, which are infiltrative-type, nodular-type, and encapsulation-type (De Vico and Carella, 2012b; Galloway and Depledge, 2001; Kim and Powell, 2004, 2007; Villalba et al., 1997). As for the infiltrative-type inflammation, it is activated by living, pathogens, chemicals, and even physical causes, and can be further classified into two subtypes, which are focal invasion and diffuse invasion. In focal invasion, haemocytes will invade injured sites focally to form one or more immunocyte infiltration centres. In diffuse invasion, haemocytes will invade a large section of injured tissue broadly without forming any immunocyte infiltration centre. Nodular -type inflammation is another type of inflammatory response, which is provoked by numerous small particles, including bacteria and environmental pollutants. During the process, haemocytes will aggregate to form small to large clusters in both circulation and interstitial tissue, which can merge to the injured site for performing phagocytosis on degenerating haemocytes, foreign particles and other debris. Lastly, encapsulation-type is caused by the invasion of multicellular parasites, which are too large for phagocytosis and have entered the haemocoel. During this process, haemocytes will form a large capsule for enclosing the foreign body and cytotoxic products (e.g., free radical and degradative enzymes) are also released to destroy the invader.

2.1.4 Haemocytes death as immuno-strategies

Cell death is a fundamental reaction to immunological stress and several types of cellular damage and can be initially divided into 3 groups, which are apoptosis, autophagy, and necrosis. Both apoptosis and autophagy are well studied and examined pathways in mussel, particular for their roles in innate immunity (Estevez-Calvar et al., 2013; Philipp et al., 2012; Romero et al., 2011b).

In mammalian cell, apoptosis, the cellular suicidal process, has been activated via extrinsic (convergent on 'death receptors' expressed in plasma membrane) and intrinsic (dependent on mitochondria-controlling signalling) pathways (Fadeel and Orrenius, 2005) and both pathways can also be identified within mussels through the observation of nucleus fragmentation and translocation of phosphatidylserine to the outer membrane at cellular level. Extrinsic pathway is defined by the involvement of caspase, which is a family of intracellular cysteine-dependent and aspartate-specific proteases controlling the cellular apoptosis as well as differentiation and inflammation, while intrinsic pathway can be activated in presence or absence of caspase. Romero et al. (2011b) have suggested several initiator and executioner caspases in *M. galloprovincialis*. Two sequences, named caspase 2 and caspase 8, were classified into the initiator caspases group and four sequences, named caspase-3/7-1, -3/7-2, -3/7-3 and -3/7-4 were included into the executioner caspases group. For the mitochondrial apoptotic pathway in *M. galloprovincialis*, six mitochondrial apoptotic-related genes (p53, PDRP, Bcl2, Bax, Bl-1 and Dff-A) and their molecular mechanisms were investigated. (Estevez-Calvar et al., 2013).

Compared to apoptosis, autophagy performs a significant role in maintaining cellular homeostasis via the removal of damaged and dysfunctional organelles and can be characterized by the presence of autophagosomes, which are double membrane vesicles containing unwanted cell debris and lysosomes (Andon and Fadeel, 2013). Scientists have well depicted the autophagic mechanism in mussel by highlighting the key players in autophagic process, including mTOR, which is the major inhibitor, and the large group of auto-related genes (ATGs, Beclin 1, and mTOR transcripts), which are responsible for controlling autophagic induction and autophagosome formation (Gerdol and Venier, 2015; Murgarella et al., 2016; Philipp et al., 2012). Balbi et al. (2018) have first confirmed the protective function of autophagy toward a pathogenic infection in mussel's haemocytes. Their study illustrated that immune response could not be initiated by the invasion of *V.tapetis*, but a moderate decrease in lysosomal membrane stability, which implied lysosomal activation, was recorded in haemocytes via two specific markers, which were LC3-11 and p62.

Lastly, many studies have also confirmed that various environmental pollutants, including nanoparticles, pharmaceuticals, heavy metals, and chemicals, could interfere with apoptotic and autophagic pathways in haemocytes within mussels (Balbi et al., 2018; Bouallegui et al., 2017; Moore et al., 2007; Muttray et al., 2005).

2.1.5 Responses of mussel's haemocytes toward micro/nano-plastic contamination

Many scientists have devoted their efforts on investigating how can micro/nano-plastic affect the immunological response of mussel's haemocytes in different ways, ranging from concentration, size and type of plastic used, exposure time for the test subject and detection method used (Auguste et al., 2020a; Auguste et al., 2020b; Avio et al., 2015; Canesi et al., 2015; Capolupo et al., 2021; Cole et al., 2020; Katsumiti et al., 2021; Paul-Pont et al., 2016; Pittura et al., 2018; Sendra et al., 2020).

Various haemocyte parameters, including cell viability, phagocytotic ability, non-specific esterase activity, content of reactive oxygen species, lysosomal activity, ratio of granulocytes per hyalinocyte, and total circulating cells have been monitored under micro-/nano-plastic exposure. Most studies have drawn the agreements that cellular viability of mussel's haemocytes would reduce under high concentration of micro-/nano-plastic exposure (Canesi et al., 2015; Katsumiti et al., 2021; Paul-Pont et al., 2016; Sendra et al., 2020). The majority of studies have also suggested that lysosomal membrane stability was decreased (Auguste et al., 2020a; Avio et al., 2015; Canesi et al., 2015; Capolupo et al., 2021; Katsumiti et al., 2021) and lysosomal activity in serum was increased (Auguste et al., 2020b; Canesi et al., 2015; Capolupo et al., 2021) within haemocytes under plastic pollution in each study. However, no significant change in lysosomal membrane stability under plastic exposure was also noticed in some studies (Auguste et al., 2020a; Cole et al., 2020; Pittura et al., 2018).

Phagocytosis, the internalization of foreign molecules regarded as danger for cells, is the major functional defence mechanism in mussel's immunity achieved by eosinophilic granulocytes. The phagocytotic activity of haemocytes under plastic exposure has increased (Katsumiti et al., 2021; Pittura et al., 2018), decreased (Canesi et al., 2015; Capolupo et al., 2021; Pittura et al., 2018; Sendra et al., 2020) or remain unchanged (Avio et al., 2015; Paul-Pont et al., 2016) in different studies. Due to the variations in concentration, size and type of plastic used, exposure time for the test subject and detection method applied in each study, the conclusion of the change of phagocytotic ability under plastic exposure among haemocytes can still not be concluded. Like phagocytotic activity, the variation content of reactive oxygen species within haemocytes under plastic pollution has not yet been confirmed, and increase (Auguste et al., 2020b; Canesi et al., 2015; Paul-Pont et al., 2016), decrease (Sendra et al., 2020) and remained unchanged (Katsumiti et al., 2021) in ROS content have been indicated in different studies. Lastly, the ratio of granulocytes per hyalinocyte within haemocytes under plastic exposure has not yet been identified, and increase (Cole et al., 2020), decrease (Avio et al., 2015) and remained unchanged (Auguste et al., 2020b; Pittura et al., 2018) in it have been illustrated in various studies.

2.1.6 Responses of mussel's haemocytes toward ocean acidification

Apart from microplastic pollution, scientists have also performed their studies on investigating how can ocean acidification affect the immunological response of mussel's haemocytes with the usage of different immune parameters. Majority of studies have drawn the agreements that low pH would induce haemocyte parameter changes, including increase in haemocyte mortality and reactive oxygen species and decrease in phagocytic activity, esterase activity, intracellular lysosomal content and some mechanisms were suggested to explain the above observations (Bibby et al., 2008; Huang et al., 2016; Sui et al., 2016; Wu et al., 2016; Wu et al., 2018).

Reactive oxygen species have played a vital role in innate immunity in bivalves by generating oxygen metabolites (Pipe, 1992; Terahara and Takahashi, 2008). Once the intracellular ROS production exceeds antioxidant capability within mussel, cells might suffer from oxidative damage, including DNA rupture, change of calcium homeostasis, membrane lipid damage, amino acid oxidation, and enzyme inactivation, and even apoptosis (Cheng et al., 2004; Turrens, 2003), which can be used for the explanation of the increase in haemocyte mortality observed in those mentioned studies. Furthermore, Wu et al. (2018) even proposed the elevated level of ROS production might be a compensated strategy for the reduction of haemocytes for facing the low pH environment.

To counteract the decreased haemolymph pH value provoked by the ocean acidification, mussels would decrease their metabolic rate or dissolve their calcium carbonate shell for maintaining a constant internal pH for all tissues (Gazeau et al., 2007; Lindinger et al., 1984). However, the action of calcium carbonate shell dissolution could elevate the Ca2+ concentration in mussel's haemolymph at the same time. Since calcium ions are an important messenger at molecular level for transmitting signals for regulating haemocyte functions, including phagocytosis and cell spreading, the disruptions on them might lead to the decrease of phagocytotic level in each haemocytes within mussels and could be explained for the suppression of phagocytosis activity in response to the reduction of pH in all studies (Humphries and Yoshino, 2003; Massullo et al., 2006).

Non-specific esterase is a group of cellular enzymes, which are involved in the intracellular degradation of haemocytes (Mottin et al., 2010), and are affected by the oxidative stress (Paital and Chainy, 2010; Xian et al., 2014). The decrease of esterase activity observed in low pH environment could be attributed to the increase in haemocyte mortality and reactive oxygen species within mussels.

Lysosome is a key organelle, which has taken part in host defence and autophagy by releasing lysozymes during phagocytosis to kill bacteria or perform intracellular degradation (Andon and Fadeel, 2013). Thus, the intracellular lysosomal content could be regarded as an indicator of the health status and the defence system in mussels. The decrease of the intracellular lysosomal content observed in low pH environment could be contributed to the decrease in lysosomal membrane stability caused by overproduction of ROS and increase in lysosomal releasement toward serum for achieving immune responses.

Lastly, Bibby et al. (2008) suggested that no significant change in total cell count was observed and the circulating eosinophilic granulocytes only increased from 40 to 50%, when mussels were exposed to the low pH environment. The average number of circulating cells might be affected by physiological state and health of haemocytes (Cao et al., 2007). It could be suggested that the presence of stress could activate haemocytes, and the seawater acidity could damage their function and health. Thus, positive, and negative effects in haemocytes could cancel out each other and the total cell counts could remain stable.

2.1.7 Responses of mussel's haemocytes toward the combined interaction of micro/nano-plastic contamination and ocean acidification

The combined effect of microplastic contamination and ocean acidification toward the immunological response of mussel's haemocytes has been investigated by Huang et al. (2022)

In her study, 6 treatments were set up with two pH level (pH 7.7 and pH 8.1) and three concentrations of polystyrene microbead at size of 10 μm (0 μg/L, 2.5 μg/L and 25 μg/L) for studying three immune parameters, which were the concentration, viability, phagocytic activity, and esterase activity of haemocytes. Both haemolytic concentrations and viability were reduced, which might be attributed to the excessive ROS production (Canesi et al., 2015; Cheng et al., 2004) and this observation might further propose that microplastic contamination and ocean acidification might render marine creatures more vulnerable to infectious disease and even oxidative damage (Huang et al., 2022).

Aside from the concentration of viables haemocytes, the mussel's immuno-capability can also be measured by haemocytes phagocytotic power, which is an energy-demanding process and may be positive correlated with the energy budget of marine organisms (Chandak et al., 2010; Kuiper et al., 2008). Reduction in phagocytosis power recorded in Huang et al. (2022) might be attributed to the decrease of energy budget with marine organism under stress conditions (Gardon et al., 2020). As for the esterase activity of haemocytes, no significant impact was observed in Huang et al. (2022) study under the combined interaction of microplastic and low pH condition.

2.2 Hepatopancreas & gills: key organs for investigating cellular stress and detoxification system of mussels

2.2.1 Introduction and functions of gills

As a part of the Lamellibranchs, a modern bivalve's class, mussels have moved the water intake site to the posterior part of animals by replacing palps to gills as their feeding organ. In general, gills will follow the curvature of the margin of shell to achieve maximum surface area for the inhalation of incoming current of water (Gosling, 2015).

Lamellibranch gills, also named 'ctenidia,' are two large, curtain-like structures, which have been suspended from the ctenidial axis and connected to the mantle's dorsal margin. Furthermore, branchial nerves, afferent and efferent haemolymph vessels are located inside the ctenidial axis. Each gill is composed of numerous double V (W-shaped) filaments with the presence of collagen-richen internal skeletal rod for strengthening each filament. Each V is named as demibranch and each arm is called as lamella, formed by the inner descending and outer ascending lamella. The space between the descending and ascending lamella is the exhalant chamber, which has connected to the exhalant area of the mantle edge, and the space ventral to the gill's filament is the inhalant chamber, which has connected to the inhalant area of the mantle edge. Mussel is a more primitive lamellibranchs, because of its gills type, termed as the filibranch homorhabdic gills. The term 'fillibranch' implies that all neighboring gills filament in mussels are attached to one another simply via interlocking clumps of cilia. And the word 'homorhabic' suggests that the gills are made up of undifferentiated filaments (Gosling, 2015).

Cilia and the related neurological system on the gill filaments play a key role in controlling mussel's gills functions. A branchial nerve from a visceral ganglion is subdivided in each axis of gill for innervating individual groups of filaments and even controlling cilia presented on filaments. Lateral cilia are located along the sides of the filaments in filibranch gills, which are responsible for drawing water into mantle cavity and passing it through gill filaments, and then up to exhalant chamber and onward to

the exhalant opening. The large feather-like latero-frontal cilia lies between lateral and frontal cilia, which can flick particles from incoming current and convey them to the frontal cilia. The frontal cilia are distributed on the outer surface of the gill for facing incoming water current and can further convert aggregated particles downwards to the ciliated food grooves on the ventral side of each lamella (Gosling, 2015).

In mussels, gills play a vital role in respiration and feeding because of their large surface area and rich haemolymph supply. First, deoxygenated haemolymph travels from kidneys to gills via the afferent gill vein. The small, subdivided vein originated from the afferent vein is then received by filament, a hollow tube allowing the circulation of haemolymph. Thus, gas exchange can occur across the thin walls of filaments. Then, the oxygenated haemolymph collected from each filament is transferred to the kidney and then heart via the efferent gill vein. Because of the respiratory and feeding roles of gills, they fall victim to the process of bioaccumulation in various pollutants, including hydrocarbons, pesticides, and soluble heavy metals (Gosling, 2015). David et al. (2008) mentioned that the increase in turnover rate in gill epithelial cells was provoked by the mixtures of heavy metals and polycyclic aromatic hydrocarbons and can be considered as a compensation mechanism for cell injury and preventative measure taken by gill filaments for blockage of pollutants from entering the entire organism.

2.2.2 Introduction and functions of hepatopancreas

The hepatopancreas, also named as the digestive gland, is brown or black organ responding for intracellular digestion, which is consisted of blind-ending tubules that can connect to the stomach via ciliated ducts. With the presence of those ducts, a two-way flow can perform continuously, which means that substances can enter the gland for digestion and absorption and debris can leave to stomach at the same time. To achieve the mentioned purpose, digestive tubules are composed of two cell types, which are digestive cells and basophil secretory cells (Gosling, 2015).

Digestive cells are the most abundant cell type found in the digestive gland in columnar and vacuolated shape, which are responsible for intracellular digestion. Microvilli also exist on the surface of cells. Small particles are first taken up by microvilli via pinocytosis and stored in the form of phagosomes, large vesicles, within cells. Then, the formed phagosomes can fuse with lysosomes, vesicles containing hydrolytic enzymes. Once the digestion process is completed, the digested products are released into the haemolymph directly and waste is stored within residual bodies within cells. Lastly, digestive cells are eventually ruptured and those excretory spheres containing waste substances are swept toward stomach and even intestine via the ciliated secondary and primary ducts of the digestive gland. And the remaining digestive enzymes in the excretory sphere could also help in stomach for the extracellular digestion.(Gosling, 2015). Dimitriadis et al. (2004) have also confirmed that the digestive gland cells have a finite lifespan after which they disintegrate and are replaced. As for basophil secretory cells, they are in pyramidal shape and equipped with surface microvilli. Although the exact role of secretory cells are still unclear, rough endoplasmic reticulum and Golgi bodies are filled within the cytoplasm, which have implied their role in protein synthesis, particularly in enzyme secretion for extracellular digestion.

The cellular structure and functions of bivalves in the aspect of digestion have been investigated by some scientists and various digestive enzymes have been reported in bivalve's stomach, digestive glands, and even intestine and some lipid-digesting enzymes, including esterase and acid & alkaline phosphatases, were reported from three mentioned organs. Furthermore, bivalves have low proteolytic activity in general, but some endopeptidases, such as chymotrypsin and cathepsins, were reported from the digestive gland (Reid, 1968). Apart from the digestive role, hepatopancreas also contributes to the process of gametogenesis by acting as the energy source, once the adductor muscles, the original energy reserve, have been depleted (Le Pennec et al., 2001). Furthermore, hepatopancreas can also function as an energy reservoir during periods of physiological stress (Bayne, 1976).

2.2.3 Responses of mussel's hepatopancreas & gills toward micro/nano-plastic contamination

Researchers have focused on determining how micro/nano-plastic can influence the mussels' tissues in the aspect of oxidative-stress detoxification, range from the size and type of plastic used to the test subject's exposure time and the detection method (Avio et al., 2015; Magara et al., 2018; Magara et al., 2019; Paul-Pont et al., 2016; Revel et al., 2019; Wang et al., 2020).

Various biomarkers have been monitored during the investigation on mussels' tissues in the aspect of oxidative-stress detoxification under microplastic exposure and their mechanisms were suggested as follows. Oxidative stress is provoked by the imbalance between the pro-oxidants, including reactive oxygen species (ROS), and the antioxidant system. In mussels, antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidases (GPx), glutathione (GSH) and GSH-related enzymes, including glutathione S-transferase (GST) and glutathione reductase (GR), have played vital roles in protecting them from ROS-mediated oxidative damage.

SOD is responsible for the dismutation of superoxide radicals to hydrogen peroxide. During microplastic exposure, no significant change in SOD activity has been noted in gills (Magara et al., 2018; Magara et al., 2019) and the digestive gland (Magara et al., 2018; Wang et al., 2020) in mussel, which implied that superoxide anion production may not be the primary factor for causing the oxidative impairment. In contrast, increase has been observed in SOD activity in mussel's gills (Revel et al., 2019) and the digestive gland (Revel et al., 2019; Wang et al., 2020) under microplastic exposure, which supported the theory that SOD has taken part in mussel's antioxidant system by countering the excess superoxide anions into the less damaging hydrogen peroxide to prevent cellular damage. Compared to petroleum-based microplastics, Magara et al. (2019) has exposed one of the biodegradable plastics, PHB, to mussels for 4 days, and a decrease in SOD activity can be observed in mussel's digestive gland.

Increased SOD activity results in higher H_2O_2 production, which could further enhance the CAT activity, an enzyme responsible for catalysing the dismutation of H_2O_2 into

water and oxygen. Under microplastic exposure, an increase in CAT activity has been observed in gills (Magara et al., 2018; Magara et al., 2019; Revel et al., 2019) and the digestive gland (Magara et al., 2018; Revel et al., 2019; Wang et al., 2020) in mussel, which suggested that CAT may play a vital role in mussel's defence mechanism against MPs. In comparison, a decrease in SOD activity was noticed in mussel's digestive gland under microplastic exposure (Avio et al., 2015; Paul-Pont et al., 2016) and this phenomenon could be explained by the biphasic response of CAT in the neutralization of H_2O_2. Once the ROS accumulation in digestive gland exceeded the threshold value of CAT, a decrease could be detected in its activity. Like CAT, GPx is also an enzyme targeted on the catalysation of reducing H_2O_2 into water and oxygen. During microplastic exposure, no notable change in GPx activity has been noted in gills (Magara et al., 2019) and the digestive gland (Magara et al., 2018; Magara et al., 2019; Wang et al., 2020) in mussels.

Reduced GSH plays a significant role in the metabolism of endogenous substances and toxic compounds by acting as non-enzymatic scavenger of oxyradicals, such as hydrogen peroxide, superoxide anions and alkoxy radicals and maintenance of membrane protein thiols (Meister and Anderson, 1983). The glutathione transferases (GST) are one of the major phase 2 detoxification enzymes via catalysing the conjugation of electrophilic substrates to GSH for forming less-reactive material (Sheehan et al., 2001). Glutathione reductase (GR) is a flavoprotein, responding for the catalysation of reduction of glutathione disulfide to GSH with the participation of NADPH (Mander and Liu, 2010). Under microplastic exposure, the total GSH value has decreased in gills and increased the digestive gland (Magara et al., 2018) and these finding were supported by the increasing GR activity in the digestive tissue and the no significant change in GR activity in gills (Magara et al., 2019). However, Avio et al. (2015) have proposed that the total GSH value, GST and GR activities were not affected, even if mussels were exposed to microplastic contamination.

Lastly, the level of lipid peroxidation (LPx) is a biomarker of oxidative stress within cells. A slight increase (Avio et al., 2015) and a slight decrease (Paul-Pont et al., 2016) in LPx level in mussel's digestive glands were noted in different studies for microplastic pollution. Their studies implied that short-term exposure to microplastic could only induce a moderate pro-oxidant challenge on mussels, which could be coped up with the corresponding antioxidant enzymes and only a slightly modification in LPx level could be observed.

2.2.4 Responses of mussel's hepatopancreas & gills toward ocean acidification

Apart from microplastic pollution, scientists have also performed their studies on investigating how ocean acidification can affect the mussels' tissues on the aspect of oxidative-stress detoxification. Various biomarkers have been monitored during the investigation on mussels' tissues in the aspect of oxidative-stress detoxification under acidified environment and their mechanisms were suggested as follows. Oxidative stress faced by mussel's tissue was monitored by the LPx level and the related antioxidant response was measured by the activities of different enzymes, including SOD, CAT, GPx, GSH, GST, glutamic-pyruvic transaminase (GPT), acid phosphatase (ACP) and alkaline phosphatase (ALP).

Most studies agreed that the SOD activity within mussel's gills (Freitas et al., 2017; Hu et al., 2015; Huang et al., 2018; Matozzo et al., 2013; Sui et al., 2017) and the digestive gland (Hu et al., 2015; Matozzo et al., 2013) increased as pH decrease. A similar trend can be observed in the activity of CAT in mussel's gills (Hu et al., 2015; Huang et al., 2018; Matozzo et al., 2013; Sui et al., 2017) and digestive gland (Hu et al., 2015; Matozzo et al., 2013), and it has increased under low pH environment for converting the H_2O_2 produced from SOD into water and oxygen. GPx has shared a similar job with CAT as a reducing enzyme and its activity has also increased in mussel's gills (Hu et al., 2015; Huang et al., 2018) and digestive gland (Hu et al., 2015) under low pH environment. Hu et al. (2015) study also proposed that the GSH value has increased in both mussel's tissues under low pH condition. All these biomarkers supported the idea that more reactive oxidative species were produced under low pH environment and the related antioxidant responses were also activated for protecting mussel's tissues.

Apart from those typical antioxidant enzymes, some multifunctional enzymes and metabolic- related enzymes, including acid phosphate (ACP), alkaline phosphate (ALP) and glutamic pyruvic transaminase (GPT), have also been investigated as the biomarkers for mussels under stress-induced conditions. Both ACP and ALP can act as transphosphorylases, which have played the key role in the catalyzation of hydrolysis of different phosphate-containing compounds at acid and alkaline pH environments correspondingly, and are involved in various of metabolic processes, such as steroidogenesis, growth and cell differentiation and molecular permeability (Ram, 1985). Cajaraville et al. (2000) further suggested that ACP activity can be altered under the presence of xenobiotics, allowing it to act as a biomarker for the detection of lysosomes in cell fractions. Increased in ACP activities were observed in mussel's gills (Huang et al., 2018; Sui et al., 2017) and might suggest that the reduction of lysosomal membrane stability and the releasement of ACP under low pH environment (Lowe et al., 1995). However, Hu et al. (2015) showed that ACP activities in mussel's gills and digestive glands were reduced under low pH environment in their study.

As for ALPs, they are intrinsic plasma membrane enzymes participating in various biological process in marine animals including oxygen carrying system (Viarengo and Nott, 1993), osmoregulation (Lovett et al., 1994) and biomineralization (Zhang et al., 2012). Furthermore, ALP can be found on almost all animals cells and can function as a marker enzyme for xenobiotic-induced stress during ecotoxicological measurement (Blasco et al., 1993; Krajnovic-Ozretic and Ozretic, 1982; Reddy and Ramana Rao, 1990). As a multifunctional enzyme, ALP has also played a vital role in the immune response (Xing et al., 2002). However, no significant alternation can be observed in mussel's gills under low pH condition, and it has been further proposed that ALP might not be an ideal indicator for investigating the immunity of mussels (Sui et al., 2017). In contrast, increase in ALP activities were observed in mussel's gills (Hu et al., 2015; Matozzo et al., 2013) and digestive gland (Matozzo et al., 2013), which might imply that shell deposition of mussels could be altered under the low pH condition by affecting ALP activities.

GPT is well-known for its roles in mobilizing L-amino acids for gluconeogenesis, the pathway for glucose formation, and working as a bridge between carbohydrate and protein metabolisms under stress environments (Ramaswamy et al., 1999). Thus, its alternation can function as an indicator for tissue damage in gills and liver (Rao, 2006). Increased in GPT activities were noted in mussel's gills and digestive gland in low pH environment (Hu et al., 2015), which might imply an increase in turnover of amino acids (Li et al., 2008). In other words, protein might be used as the energy source with mussel's tissues under the stress condition.

Lastly, the level of LPx was also investigated for detecting oxidative stress within mussel's tissues. An increase (Freitas et al., 2017; Sui et al., 2017) and a decrease (Huang et al., 2018; Matozzo et al., 2013) in LPx level in mussel's gills were noted in different studies under low pH environment. Furthermore, a decrease in LPx activity was also noted in mussel's digestive gland (Matozzo et al., 2013). Since no agreement can be drawn within different, more studies can still perform on monitoring oxidative condition within different mussel's tissues under low pH condition.

2.2.5 Responses of mussel's hepatopancreas & gills toward the combined interaction of micro/nano-plastic contamination and ocean acidification

In addition, studies on the investigation of the combined interaction of microplastic contamination and ocean acidification toward the oxidative-stress detoxification system of mussels have also been performed by some researchers (Provenza et al., 2020; Wang et al., 2020). Five biomarkers have been monitored in their studies, which were activities of SOD, CAT and GPx, content of GSH and the level of LPx. Both Provenza et al. (2020) and Wang et al. (2020) agreed that no significant variation was noted in the SOD activity within mussel's tissue toward the combined interaction of microplastic and low pH condition. As for activities of CAT and GPx, no significant difference were noted (Wang et al., 2020).

In contrast to Wang et al. (2020) study, under microplastic exposure, the GPx activity has been affected significantly and a decrease was reported under the low pH condition with reference to natural marine pH condition (Provenza et al., 2020). This finding might be explained by the massive mucous protein production under low pH condition recorded in Provenza et al. (2020) study, since those produced proteins can inhibit the entry of microplastics and biological responses activated subsequently. The variation of GPx activity noted between Wang et al. (2020) and Provenza et al. (2020) studies might be explained by the different experimental condition applied, ranging from the concentration, size and type of plastic, exposure time and even the type of test subject.

As for the GSH content, a significant interaction between pH and microplastic was noted in Wang et al. (2020) study. A general increasing trend in the GSH content was detected in low pH environment under microplastic exposure and might further suggest that low pH condition may enhance the oxidative damage of mussel's tissue in the presence of micro-polystyrene. Lastly, under microplastic exposure, the LPx level has been reduced significantly under the low pH condition with reference to natural marine pH condition (Provenza et al., 2020), which could be attributed to the blockage of the entry of microplastic achieved by the formed mucus proteins under low pH condition.

2.2.6 Ideal organs for studying environmental stress and pollution

From section 2.2.1 to section 2.2.4, it could be concluded that mussel's gills and digestive gland tissues are the major responsers for micro-/nano-plastic pollution and ocean acidification, due to their physiological roles. However, it can be noticed that antioxidant enzymes have reacted differently in mussel's gills and digestive gland under microplastic exposure (Magara et al., 2018; Revel et al., 2019). Revel et al. (2019) have proposed that the natural differences in physiological roles of different organs and the size dispersity of plastic particles used for exposure were the possible reasons for explaining this phenomenon. Aggregational intensity of micro-/nano-plastics in water may correlated with the concentration of plastics positively, which means that the higher concentration of plastics will lead to the stronger the aggregation intensity among plastics and the larger size of the formed particles, and vice versa. Because of the respiration and feeding roles of mussel's gills, they may be the major victim for the bioaccumulation of plastic with larger size. For the microplastic that cannot be filtered by gills, they may accumulate and further provoke the upcoming antioxidant responses within this tissue. As for the digestive glands, they are responsible for the filter-feeding process of phytoplankton. Thus, microplastics in low concentration of suspensions are less aggregated, leading to the decrease in average size of plastic particles, which may allow them to enter and induce the oxidative stress inside mussel's digestive gland. Furthermore, Magara et al. (2019) have also suggested that antioxidant responses, particularly for the activities of CAT and SOD, occurred more likely in gills than in the digestive gland under microplastic contamination. As for studies focused on the antioxidant responses provoked by ocean acidification, a more consistent result can be noticed between mussel's gills and digestive gland, compared to those investigated on the microplastic pollution. To conclude, it can be suggested that mussel's gills and digestive gland are ideal organs for studying plastic contamination and ocean acidification. However, it could be suggested that the antioxidant responses of mussels should be investigated by organs rather than the whole homogenized soft tissue in future studies to better understanding the effects provoked by different concentration (size) of plastics toward mussels.

Chapter 3 Materials and method

3.1 Experimental design

The green-lipped mussel *Perna viridis* was collected from the area of Tung Lung Chau, a relatively less polluted site in Hong Kong waters (Fang et al., 2010), and acclimatised in the laboratory for a month prior to the exposure experiment. Fifty-six healthy individuals, with an average shell length of 70 mm, were used in this study. To simulate the four ocean acidification scenarios, we used four channels of a carbon dioxide (CO_2) automated control system, specifically the DAQ-M operated by CapCTRL Version 1.3.0 software (Loligo Systems, Denmark), to maintain the targeted seawater pH at 8.1 (control), 8.0, 7.8 and 7.7 in four reservoirs, respectively. The desired pH level in each reservoir (150 L) was maintained by injecting CO_2 gas into the seawater through a ceramic air stone, and the activation of gas injection was feedback-controlled by a solenoid valve according to the actual change in seawater pH monitored by a WTW pH3310 portable pH meter and a SenTix HWD pH probe calibrated with the NBS scale (Xylem Analytics, Germany). Each of the four pH-adjusted seawater reservoirs had its own circulation system connected to an aquarium filter filled with cotton and ceramic rings, and a water-table. Each water-table served two treatments, i.e. with and without NP. A standard of 50 nm polystyrene nanoparticles (17149-10, Polysciences, USA) was used as a test model of NP in this study. The exposure level of NP was set to be 4×10^6 particles mL^{-1}, for 2 h daily, and was environmentally realistic (Lenz et al., 2016). This experimental design led to eight treatments (4 levels of pH × 2 levels of NP).

There were seven beakers (1 L) per treatment, and each beaker contained one mussel as each replicable (n = 7). The mussels were maintained in circulated seawater, which was pumped from the reservoir into the seven beakers of each treatment and overflowed into the water-table before returning to the reservoir. The experimental seawater was set at 35‰ and was made with artificial sea salt (Instant Ocean, USA). The seawater temperature was maintained at 24 °C throughout the experiment, with a 9 h daily light period at 50 µmol m^{-2} s^{-1} (9:00 h–18:00 h). Mussels in all treatments were fed with mixed microalgae of genera *Tetraselmis*, *Thalassiosira* and *Schizochytrium* (LPB

Frozen Shellfish Diet, Reed Mariculture, USA) at 10^5 cells mL^{-1} for 2 h daily from 12:00 h to 14:00 h. During the feeding period, all 56 beakers of the eight treatments were isolated from the circulation, adjusted to 1 L of seawater and gently aerated. The required quantity of NP was dosed into 28 beakers of the four treatments with NP during the 2 h mixed microalgae feeding period to facilitate its uptake by mussels. After this daily routine of feeding and NP exposure, a portion of used seawater in all isolated beakers was kept for estimating the amount of nanoplastics taken by mussels and the remaining seawater was discarded, and the beakers were reconnected to the corresponding reservoirs to resume the water circulation. About one third of the seawater in all reservoirs was renewed weekly, and the exposure experiment lasted 30 days.

Seawater pH, temperature and salinity in the eight treatments were daily monitored throughout the experiment. To validate the acidification conditions, seawater samples (100 mL) were taken from selected beakers of the eight treatments on exposure days 0, 5, 10, 15, 20, 25 and 30. Total alkalinity was measured in these seawater samples following Fang et al. (2013a). Each seawater sample was filtered through 0.22 µm pores and titrated against 0.1 M hydrochloric acid using a 916 Ti-Touch potentiometric titrator with an 800 Dosino dosing system (Metrohm, Switzerland). The titrator was calibrated at the beginning of each run. Total alkalinity was determined from the titration results based on the Gran function as described by Dickson et al. (2007). The measured values of total alkalinity, pH, temperature and salinity were used to calculate other carbonate system parameters including the partial pressure of CO_2 (pCO_2), and the saturation states of aragonite (Ω_{Ar}) and calcite (Ω_{Ca}), in the eight treatments using the software CO_2 System v2.1(Pierrot et al., 2006). At the end of the experiment, all mussels were dissected to collect haemolymph, soft tissue and shells. These biological samples were used to determine 12 endpoints of sublethal biomarkers, physiological indices and energy reserves, aiming to assess the impacts of the eight treatments on *P. viridis*.

3.2 Biological endpoints measured for indicating immunological alternations in haemolymph

Approximately 1.5 mL of fresh haemolymph was gently extracted from the posterior adductor muscle of each live mussel using a 2 mL sterile syringe with a 25 g needle (0.5 mm × 25 mm). After haemolymph collection, all mussels were frozen at –80 °C until further dissection for different organs (as described below). Extracted haemolymph was filtered through 41 µm pores to eliminate larger aggregates and detritus that could potentially affect the subsequent cytometric measurements. Filtered haemolymph was immediately kept in microtubes on ice to avoid aggregation of cells (da Silva et al., 2000). No anticoagulant agent was required, given that the cytometric assays were completed within 2 h (Wang et al., 2012).

Haemocytes in the samples were analysed using an Accuri C6 flow cytometer equipped with 488 nm and 640 nm lasers (BD Biosciences, USA). Milli-Q ultrapure water (Merck, Germany) prefiltered through 0.22 µm pores served as the sheath fluid. The flow rate and core size for all analyses were set at 66 µL min^{-1} and 22 µm, respectively. Data obtained from the analysis were presented as cell cytograms using the specific Accuri C6 software, which provided information on the relative size (FSC value) and granularity (SSC value) of the haemocytes under investigation, as well as fluorescence channels (FL1 and FL2) specific to the staining agents used. For each sample, 10,000 events were acquired to generate the fluorescence frequency distribution of the haemocyte population. Four biomarkers were selected to indicate the immunological responses in haemocytes of *P. viridis*. These biomarkers based on flow cytometry included lysosomal content, oxidative activity, phagocytosis and apoptosis.

3.2.1 Lysosomal content

The Invitrogen LysoTracker Yellow HCK-123 assay kit (L12491, Thermo Fisher Scientific, USA) was employed to assess the lysosomal content in haemocytes of *P. viridis*, according to the methods described by Gagnaire et al. (2008) and Wang et al. (2012). The LysoTracker Yellow HCK-123 is a cell-permeable yellow-fluorescent dye that specifically stains acidic cellular compartments, including lysosomes, with

emission at 535 nm. According to the manufacturer's instructions, 200 µL of filtered haemolymph was mixed with 0.5 µL of the LysoTracker reagent provided in the kit. The mixture was then incubated in darkness for 2 h at room temperature. The fluorescence developed in lysosomes was detected using the FL1 channel of the flow cytometer. This fluorescence signal served as a proxy for the relative lysosomal content and was expressed in arbitrary units (AU).

3.2.2 Oxidative activity

The oxidative activity in haemocytes of *P. viridis* was determined using a cell-permeant fluorogenic probe, 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich 35845, Merck), as described in Bass et al. (1983) and Delaporte et al. (2003). A stock solution of DCFH-DA was prepared in dimethyl sulfoxide (10 mM) and was further diluted in Milli-Q water to form the working solution (1 mM). In the assay, 200 µL of filtered haemolymph was added with 2 µL of the DCFH-DA working solution, and the mixture was incubated in darkness for 15 min at room temperature. Upon entering haemocytes, DCFH-DA was hydrolysed by intracellular esterase, forming an intermediate compound that was oxidised by reactive oxygen species to produce 2′,7′-dichlorofluorescein (DCF), which is fluorescent with emission at 512 nm. The fluorescence intensity of DCF was detected using the FL1 channel of the flow cytometer, and was used to quantify the haemocyte's oxidative activity, expressed in AU.

3.2.3 Phagocytosis

The extent of phagocytosis in haemocytes of *P. viridis* was evaluated following the methods of Xue et al. (2001) and Wang et al. (2012). To perform the assay, 200 µL of filtered haemolymph was dosed with 5 µL of 2 µm fluorescent polystyrene microbeads (emission at 515 nm; Invitrogen F8827, Thermo Fisher Scientific) at 10^5 particle μL^{-1}. The solution was then incubated in darkness at room temperature for a 1 h period, during which the proportion of haemocytes that had engulfed the microbeads indicated the relative strength of phagocytosis. The haemocytes that exhibited fluorescence from the microbeads were detected and identified using the FL1 channel of the flow

cytometer. The extent of phagocytosis was determined by calculating the percentage of these fluorescent cells in relation to the total haemocyte count.

3.2.4 Apoptosis

The extent of apoptosis in haemocytes of *P. viridis* was evaluated using the Dead Cell Apoptosis Kit with Annexin V for Flow Cytometry (V13241, Thermo Fisher Scientific), as described by Rieger et al. (2010). This assay kit included propidium iodide (PI), a red-fluorescent nuclear and chromosome counterstain for dead cells (emission at 520 nm), and fluorescein isothiocyanate (FITC) conjugated with Annexin V, a green-fluorescent probe for apoptotic cells (emission at > 575 nm). Following the manufacturer's instructions, 200 µL of filtered haemolymph was mixed with 1 µL of PI and 5 µL of FITC-Annexin V. The mixture was then incubated in darkness for 15 min at room temperature before being assessed using flow cytometry, in which the FL1 and FL2 channels were utilised to detect PI and FITC-Annexin V, respectively. The PI signal in haemocytes indicated cell mortality, while the extent of apoptosis was quantified as a percentage of haemocytes exhibiting the FITC-Annexin V signal relative to the total haemocyte count.

3.3 Biomarkers in soft tissue

Once the flow cytometry assays were completed, the frozen mussel samples were thawed and processed. The mussel shells were oven-dried at 40 °C to measure the shell length and dry weight. The whole soft tissue was gently blotted on tissue paper to remove excess water and weighed while still wet. The soft tissue of each mussel was further dissected to extract gills, hepatopancreas and posterior adductor muscle, and their respective wet weights were recorded. The gills and hepatopancreas were used to assess four cellular stress biomarkers, namely superoxide dismutase activity, catalase activity, glutathione content, and lipid peroxidation. The collected posterior adductor muscle was employed to evaluate the energy reserves (as described below).

3.3.1 Superoxide-dismutase (SOD) activity

SOD is an antioxidant enzyme (EC 1.15.1.1) that protects cells from oxidative damage caused by reactive oxygen species. SOD catalyses the transformation of superoxide into less reactive species such as hydrogen peroxide (H_2O_2; (Magara et al., 2018)). The enzymatic activity of SOD in the gills of *P. viridis* was assessed using a SOD assay kit (BC0175, Solarbio Science and Technology, China). The assay principle relies on the reaction between nitro-blue tetrazolium and superoxide, resulting in the formation of a product that absorbs at 560 nm. SOD inhibits this reaction by eliminating superoxide, and thus the SOD activity is proportional to the decrease in absorption at 560 nm. To perform the assay, approximately 100 mg of tissue was homogenised at 4 °C in 1 mL of the provided extraction solution using a Precellys Evolution Homogeniser (Bertin Technologies, France). The homogenate was then centrifuged at 8,000 × g and 4 °C for 10 min. The resulting supernatant was used in the assay and processed with the supplied reagents following the manufacturer's instructions. The specific activity of SOD was measured at 560 nm with a SpectraMax M3 microplate reader (Molecular Devices, USA), and the results were expressed as U g^{-1} of tissue wet weight.

3.3.2 Catalase (CAT) activity

CAT is another antioxidant enzyme (EC 1.11.1.6) that functions downstream of SOD to facilitate the breakdown of H_2O_2 to water and oxygen (Magara et al., 2019; Revel et al., 2019). The enzymatic activity of CAT in the gills of *P. viridis* was determined with a CAT assay kit (A007-1-1, Nanjing Jiancheng Bioengineering Institute, China). The working principle is based on the reaction between ammonium molybdate and H_2O_2, leading to the generation of a complex that can be measured at 405 nm. CAT hinders this reaction by removing H_2O_2, and thus the CAT activity is proportional to the reduction in absorbance at 405 nm. For the assay, approximately 100 mg of tissue was added to 0.9 mL of the provided buffer solution and homogenised at 4 °C using the Precellys Evolution Homogeniser. The homogenate was then centrifuged at 5,200 rpm and 4 °C for 10 min. The resulting supernatant was collected and treated with the supplied reagents according to the manufacturer's instructions. The specific activity of CAT was measured at 405 nm using the SpectraMax M3 reader, and the results were expressed as U g^{-1} of tissue wet weight.

3.3.3 Glutathione (GSH) content

GSH is a tripeptide antioxidant that modulates cellular homeostasis and neutralises reactive oxygen species by converting to its oxidised state, known as glutathione disulfide. A higher concentration of GSH is indicative of a stronger antioxidative capability (Meister and Anderson, 1983). Here, the GSH concentration in the hepatopancreas of *P. viridis* was determined using a GSH assay kit (BC1175, Solarbio Science and Technology). This kit operates based on the reaction between GSH and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), resulting in the formation of a product that can be measured at 412 nm. The GSH content is proportional to the absorbance at 412 nm. To perform the assay, approximately 100 mg of tissue was washed with phosphate-buffered saline and then homogenised at 4 °C in 1 mL of the provided extraction solution using the Precellys Evolution Homogeniser. The homogenate was subsequently centrifuged at 8,000 × g for 10 min at 4 °C. The resulting supernatant was collected and processed with the supplied reagents according to the manufacturer's instructions. The concentration of GSH was determined at 412 nm using the

SpectraMax M3 reader. The results of GSH content were expressed as μg g^{-1} of tissue wet weight.

3.3.4 Lipid peroxidation (LPX)

LPx is a process of oxidative degradation initiated by free radicals that extract electrons from lipids in cell membranes, resulting in cellular damage, and malondialdehyde (MDA) is one of the major end products (Sheehan et al., 2001). In this study, the concentration of MDA was measured in the hepatopancreas of *P. viridis* as a proxy for assessing the extent of LPx, using an MDA assay kit (BC0025, Solarbio Science and Technology). Approximately 100 mg of tissue was homogenised in 1 mL of the provided extraction solution at 4 °C using the Precellys Evolution Homogeniser. The homogenate was then centrifuged at 8,000 × g for 10 min at 4 °C. The resulting supernatant was collected and processed with the provided reagents following the manufacturer's instructions. The concentration of MDA was measured at 532 nm using the SpectraMax M3 reader. The results of LPx were expressed as nmol MDA g^{-1} of tissue wet weight.

3.4 Physiological indices and energy reserve

Three physiological indices of *P. viridis* were derived from the dissected mussels, namely shell index (g mm^{-1}), as shell dry weight divided by shell length, condition index (g mm^{-1}), as whole tissue wet wight divided by shell length, and hepatosomatic index (g g^{-1}), as a ratio of wet weight of hepatopancreas to that of the whole soft tissue. The shell index and condition index provided information about the energy available for growth of the shells and somatic tissue, respectively, standardised to the individual size. The hepatosomatic index indicated the relative size of hepatopancreas in the mussels, and a higher value can be interpreted as an increased investment of energy in the hepatopancreas and an increased antioxidant capacity to maintain cellular homeostasis (Cartier et al., 2004). In addition to these physiological indices, the level of energy reserves (kJ g^{-1}) was assessed in the posterior adductor muscle, an organ which serves as a crucial energy storage site for marine bivalves (Gosling, 2015). Each adductor muscle was divided into three portions with known wet weight, which were used to determine the contents of protein, lipid and ash, respectively. The data obtained from these measurements were used to calculate the glycogen content and the level of energy reserves.

3.4.1 Protein content

The first portion of the adductor muscle was used for protein analysis following Bradford (1976). To prepare the lysis buffer, 12.5 g of urea, 4.54 g of thiourea and 0.145 g of tris were dissolved in Milli-Q water to a final volume of 30 mL. Approximately 100 mg of tissue was homogenised in 1 mL of the lysis buffer at 4 °C using the Precellys Evolution Homogeniser. The homogenate was then incubated on ice for 10 min to solubilise the proteins, followed by centrifugation at 13,000 × g for 5 min at 4 °C. The resulting solution was kept on ice, and the supernatant was collected for protein analysis using the Quick Start Bradford Protein Assay Kit (5000201, Bio-Rad, USA), according to the manufacturer's instructions. Bovine serum albumin was used as the protein standard in this assay kit. The protein concentration in each sample was measured at 595 nm using the SpectraMax M3 reader. The measured protein content values were converted into mg g^{-1} of tissue wet weight.

3.4.2 Lipid content

The second portion of the adductor muscle was used for lipid analysis using the Sulphophosphovanillin assay (Frings et al., 1972). Approximately 50 g of tissue was homogenised in 500 µL of the provided buffer from a chloroform-free lipid extraction kit (ab211044, Abcam, UK), using the Precellys Evolution Homogeniser at 4 °C. The homogenate was centrifuged at 10,000 × g for 5 min at 4 °C. The resulting supernatant was carefully transferred to a test tube, sealed with parafilm, and agitated on an orbital shaker at room temperature for 20 min, leading to the formation of two layers. Only the upper layer was needed, and it was dried overnight at 37 °C. The dried sample was reconstituted in 200 µL of sulfuric acid and heated with a boiling marble at 100 °C for 10 min. The solution was then cooled in a water bath at room temperature for 5 min. Subsequently, 2.5 mL of a phosphoric acid-vanillin reagent, prepared by mixing 85% phosphoric acid and 0.6% vanillin in a 4:1 ratio, was added to the solution. Following a further incubation for 60 min, the lipid content in the solution was measured as cholesterol equivalents at 520 nm using the SpectraMax M3 reader. The measured lipid content values were converted into mg g^{-1} of tissue wet weight.

3.4.3 Water content and ash content

The last portion of the adductor muscle was used for measuring water content and ash content, following the method described by Fang et al. (2013b). Approximately 100 mg of wet tissue was initially weighed, oven-dried at 40 °C for 72 h and weighed again. The difference between the wet weight and dry weight yielded the water content. Subsequently, each dried sample was placed in a quartz crucible and burnt at 550 °C for 3 h using a Carbolite Gero ELF laboratory chamber furnace (Verder Scientific, Germany). All organic matter was removed in this burning process, after which the sample was cooled to room temperature and weighed to determine the amount of ash. The change in weight of each quartz crucible at 550 °C was corrected accordingly in the estimation of ash content.

3.4.4 Glycogen content and energy content

The protein content, lipid content, water content and ash content were expressed as percentages of tissue wet weight. The glycogen content in the adductor muscle was calculated using the equation proposed by Lemasson et al. (2019), i.e. % glycogen = 1 – (% protein + % lipid + % water + % ash). The protein content and lipid content, and the calculated glycogen content, were then converted into concentrations per unit weight (mg g^{-1}), and subsequently into energy equivalents using the conversion factors of 24 J mg^{-1} protein, 39.5 J mg^{-1} lipid and 17.5 J mg^{-1} glycogen (Yeung et al., 2016). The summation of these values yielded the energy reserves (kJ g^{-1}).

3.5 Statistical analysis

Two-way analysis of variance (ANOVA) was used to individually compare the 12 variables obtained from *P. viridis* at the conclusion of the 30-day experiment. These variables included lysosomal content (AU), oxidative activity (AU), phagocytosis (%), apoptosis (%), SOD activity (U g^{-1}), CAT activity (U g^{-1}), GSH content (µg g^{-1}), lipid peroxidation (nmol MDA g^{-1}), shell index (g mm^{-1}), condition index (g mm^{-1}), hepatosomatic index (g g^{-1}), and energy reserves (kJ g^{-1}). The analysis assessed the main effects of two independent factors, pH (with 4 levels) and NP (with 2 levels), as well as their interaction. Given that certain datasets violated the assumptions of normality and homogeneity of variance, we opted for a non-parametric approach and all datasets were aligned rank-transformed using ARTool (Wobbrock et al., 2011) before conducting the ANOVA procedures.

For each variable, if the interaction (pH × NP) did not yield any statistical significance in the two-way ANOVA, we examined the main effects of pH and NP individually. In the case of a significant main effect, Tukey's multiple comparison test was employed to pairwise-compare among all levels of the factor. However, if the interaction (pH × NP) was significant in the two-way ANOVA for a given variable, we proceeded to assess the simple effect of pH at each level of NP (without and with NP) using the Kruskal-Wallis test, if significant, followed by Dunn's multiple comparison test. The simple effect of NP at each level of pH (pH 8.1, 8.0, 7.8 and 7.7) was examined using Wilcoxon's rank-sum test. Moreover, Spearman's rank correlation test was used to elucidate the inter-relationships among the 12 variables. Statistical results were considered significant when $p < 0.05$. The data analysis described above was conducted using the SigmaPlot 14.0 statistical software package (Systat Software, USA).

Chapter 4 Result

The model animal *P. viridis* was subjected to four ocean acidification scenarios (pH 8.1, 8.0, 7.8 and 7.7), in the absence and presence of NP (4×10^6 particles mL^{-1}, 2 h daily), under laboratory conditions for 30 days. And the treatment of pH 8.1 without NP represents the control treatment of the present-day scenario. The summary of seawater carbonate chemistry in the eight mentioned treatments was listed in **Error! Reference source not found.**. The total alkalinity measured in the experimental seawater remained similar across the eight treatments during the entire exposure period, ranging from 2797 to 2867 µmol kg^{-1} on average. The mean levels of pCO_2 were estimated to be 467–481 µatm for pH 8.1, 585–594 µatm for pH 8.0, 834–834 µatm for pH 7.8, and 1132–1157 µatm for pH 7.7. As the pH decreased and pCO_2 increased, the values of Ω_{Ar} and Ω_{Ca} declined accordingly. Based on the values of pH, temperature, salinity and total alkalinity measured on selected days throughout the study period (mean ± standard deviation; n > 10), the partial pressure of carbon dioxide (pCO_2), saturation state of aragonite (Ω_{Ar}) and saturation state of calcite (Ω_{Ca}) were calculated using the software CO_2System v2.1 (Pierrot et al., 2006).

Table 1. Seawater carbonate chemistry in the eight treatments of four ocean acidification scenarios (pH 8.1, 8.0, 7.8 and 7.7) in the absence and presence of nanoplastics (NP).

Treatment	pH (NBS scale)	Temperature (°C)	Salinity (‰)	Alkalinity (µmol kg^{-1})	pCO_2 (µatm)	Ω_{Ar} (no unit)	Ω_{Ca} (no unit)
pH 8.1, no NP	8.05 ± 0.03	24.0 ± 0.24	35.0 ± 0.00	2867 ± 75.3	480.7 ± 13.1	4.23 ± 0.11	6.44 ± 0.17
pH 8.1, with NP	8.06 ± 0.07	24.0 ± 0.25	35.0 ± 0.00	2864 ± 95.4	466.9 ± 16.1	4.30 ± 0.15	6.55 ± 0.23
pH 8.0, no NP	7.97 ± 0.03	23.9 ± 0.11	34.9 ± 0.40	2797 ± 87.7	585.3 ± 18.9	3.55 ± 0.11	5.40 ± 0.17
pH 8.0, with NP	7.97 ± 0.03	24.0 ± 0.12	34.9 ± 0.40	2838 ± 82.9	594.0 ± 17.9	3.62 ± 0.11	5.50 ± 0.17
pH 7.8, no NP	7.84 ± 0.03	24.0 ± 0.10	34.9 ± 0.42	2807 ± 85.3	833.5 ± 25.9	2.79 ± 0.09	4.24 ± 0.13
pH 7.8, with NP	7.84 ± 0.03	23.9 ± 0.10	34.9 ± 0.42	2808 ± 101	834.0 ± 30.8	2.78 ± 0.10	4.23 ± 0.16
pH 7.7, no NP	7.72 ± 0.02	23.9 ± 0.14	35.0 ± 0.00	2851 ± 122	1157 ± 50.4	2.18 ± 0.10	3.33 ± 0.14
pH 7.7, with NP	7.73 ± 0.02	23.9 ± 0.15	35.0 ± 0.00	2861 ± 79.1	1132 ± 31.9	2.23 ± 0.06	3.41 ± 0.10

No mortality of mussels was observed throughout the exposure period. At the conclusion of the experiment, 12 sublethal biological variables, including four immunological biomarkers in haemocytes, four oxidative stress biomarkers in soft tissue, and four physiological changes at the individual level, were assessed in *P. viridis* and compared across the eight treatments. The treatment of pH 8.1 without NP served as the control treatment. The values of mean and standard deviation for these 12 variables, based on seven replicates, are presented in **Error! Reference source not found.**. AU, SOD, CAT, GSH, LPx and MDA are abbreviations for arbitrary units, superoxide dismutase, catalase, glutathione, lipid peroxidation and malondialdehyde, respectively. In addition, median and other descriptive statistics for the 12 variables are reported in **Figures 1–3**.

Table 2. Summary of data, in terms of mean and standard deviation, for 12 variables determined in the green-lipped mussel *Perna viridis* upon exposure for 30 days to four ocean acidification scenarios at pH 8.1, 8.0, 7.8 and 7.7, in the **(a)** absence and **(b)** presence of nanoplastics (NP).

(a) Twelve variables in *P. viridis*	Four treatments without NP (mean ± standard deviation; n = 7)			
	pH 8.1	pH 8.0	pH 7.8	pH 7.7
Lysosomal content (AU)	2.381 ± 0.432	2.043 ± 0.479	1.644 ± 0.175	2.017 ± 0.444
Oxidative activity (AU)	1.593 ± 0.546	2.624 ± 0.457	2.704 ± 1.346	1.896 ± 0.654
Phagocytosis (%)	2.787 ± 1.178	4.079 ± 1.337	5.481 ± 2.454	4.974 ± 2.606
Apoptosis (%)	33.94 ± 11.85	38.47 ± 8.694	53.40 ± 11.52	59.19 ± 15.19
SOD activity (U g^{-1})	24.59 ± 16.10	73.68 ± 23.51	36.08 ± 15.83	59.10 ± 6.613
CAT activity (U g^{-1})	5.216 ± 3.737	7.287 ± 5.773	9.498 ± 8.042	13.14 ± 4.121
GSH content (µg g^{-1})	23.27 ± 5.722	20.87 ± 5.174	19.32 ± 10.69	21.48 ± 5.378
LPx extent (nmol MDA g^{-1})	115.1 ± 26.26	100.4 ± 49.24	156.6 ± 96.37	136.6 ± 50.24
Shell index (g mm^{-1})	0.143 ± 0.029	0.144 ± 0.023	0.148 ± 0.034	0.144 ± 0.020
Condition index (g mm^{-1})	0.056 ± 0.012	0.041 ± 0.011	0.043 ± 0.008	0.037 ± 0.008
Hepatosomatic index (g g^{-1})	0.123 ± 0.028	0.169 ± 0.017	0.141 ± 0.028	0.154 ± 0.025
Energy reserves (kJ g^{-1})	3.232 ± 0.400	4.770 ± 0.773	3.505 ± 0.666	3.996 ± 0.440
(b) Twelve variables in *P. viridis*	Four treatments with NP (mean ± standard deviation; n = 7)			
	pH 8.1	pH 8.0	pH 7.8	pH 7.7
Lysosomal content (AU)	1.570 ± 0.397	1.785 ± 0.350	1.932 ± 0.665	1.545 ± 0.403
Oxidative activity (AU)	2.990 ± 3.077	3.970 ± 2.614	2.448 ± 1.238	2.191 ± 1.365
Phagocytosis (%)	6.836 ± 3.394	8.939 ± 5.210	5.104 ± 2.478	4.726 ± 1.392
Apoptosis (%)	39.23 ± 9.254	40.44 ± 15.90	40.10 ± 6.279	50.73 ± 8.304
SOD activity (U g^{-1})	58.95 ± 8.460	81.08 ± 17.65	39.22 ± 9.747	53.15 ± 11.71
CAT activity (U g^{-1})	10.79 ± 6.900	12.47 ± 6.673	7.206 ± 9.674	26.94 ± 13.65
GSH content (µg g^{-1})	30.09 ± 4.814	24.96 ± 9.304	20.05 ± 8.368	25.75 ± 4.603
LPx extent (nmol MDA g^{-1})	135.7 ± 47.41	159.6 ± 77.36	154.7 ± 64.65	229.2 ± 83.95
Shell index (g mm^{-1})	0.155 ± 0.024	0.147 ± 0.026	0.141 ± 0.027	0.134 ± 0.020
Condition index (g mm^{-1})	0.048 ± 0.018	0.041 ± 0.011	0.052 ± 0.014	0.040 ± 0.010
Hepatosomatic index (g g^{-1})	0.147 ± 0.051	0.146 ± 0.028	0.126 ± 0.019	0.156 ± 0.029
Energy reserves (kJ g^{-1})	4.481 ± 0.380	4.205 ± 0.445	3.327 ± 0.353	4.306 ± 0.261

In **Figures 1–3,** additional descriptive statistics for the 12 mentione variables are depicted in box and whisker plots. The box and whisker plot for each treatment indicates the median (middle line in the box), mean (cross marker), 75th percentile (top of the box) and 25th percentile (bottom of the box), as well as the largest data point that is ≤ 1.5 times the interquartile range (IQR; top of the upper whisker), and the smallest data point that is ≥ 1.5 times the IQR (bottom of the lower whisker). Any data point that falls outside the range between the two whiskers is considered an outlier (dot). Statistical results regarding the treatment effects on these biomarkers are reported in **Tables 2–6.**

Four immunological biomarkers, including lysosomal content and oxidative activity expressed in arbitrary units (AU), as well as phagocytosis and apoptosis expressed in percentage (%), were determined in the haemocytes of *Perna viridis* exposed to the eight treatments of pH and nanoplastics and results were indicated in **Error! Reference source not found.**.

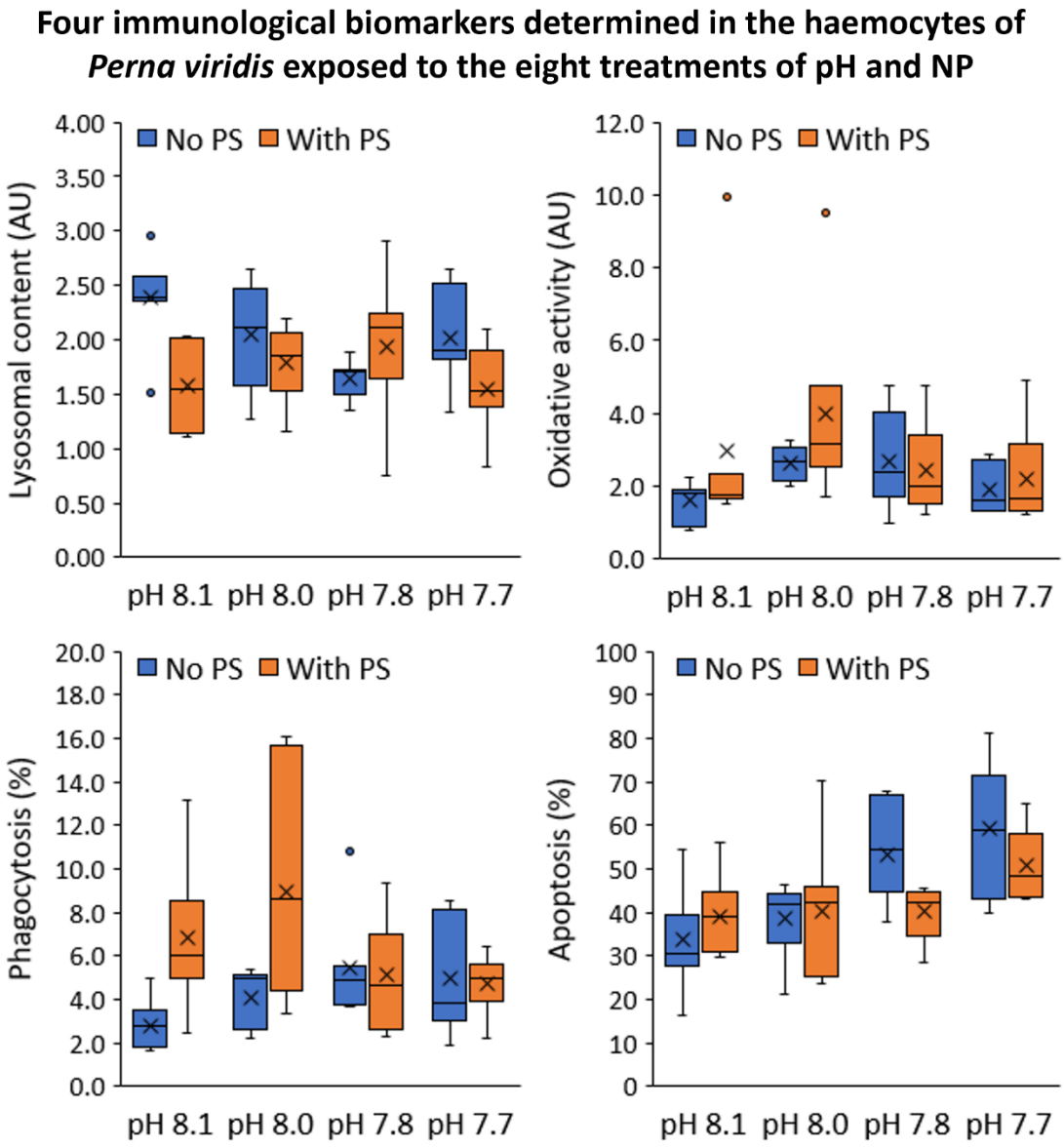

Figure 1. Box and whisker plots for four immunological biomarkers determined in the haemocytes of *Perna viridis* exposed to the eight treatments of pH and nanoplastics (NP).

Four oxidative stress biomarkers, including the activities of superoxide dismutase (SOD) and catalase (CAT), and the content of glutathione (GSH), as well as the extent of lipid peroxidation (LPx) indicated by the concentration of malondialdehyde (MDA) were assessed in the soft tissue of *Perna viridis* exposed to the eight treatments of pH and nanoplastics and results were illustrated in **Figure 2**.

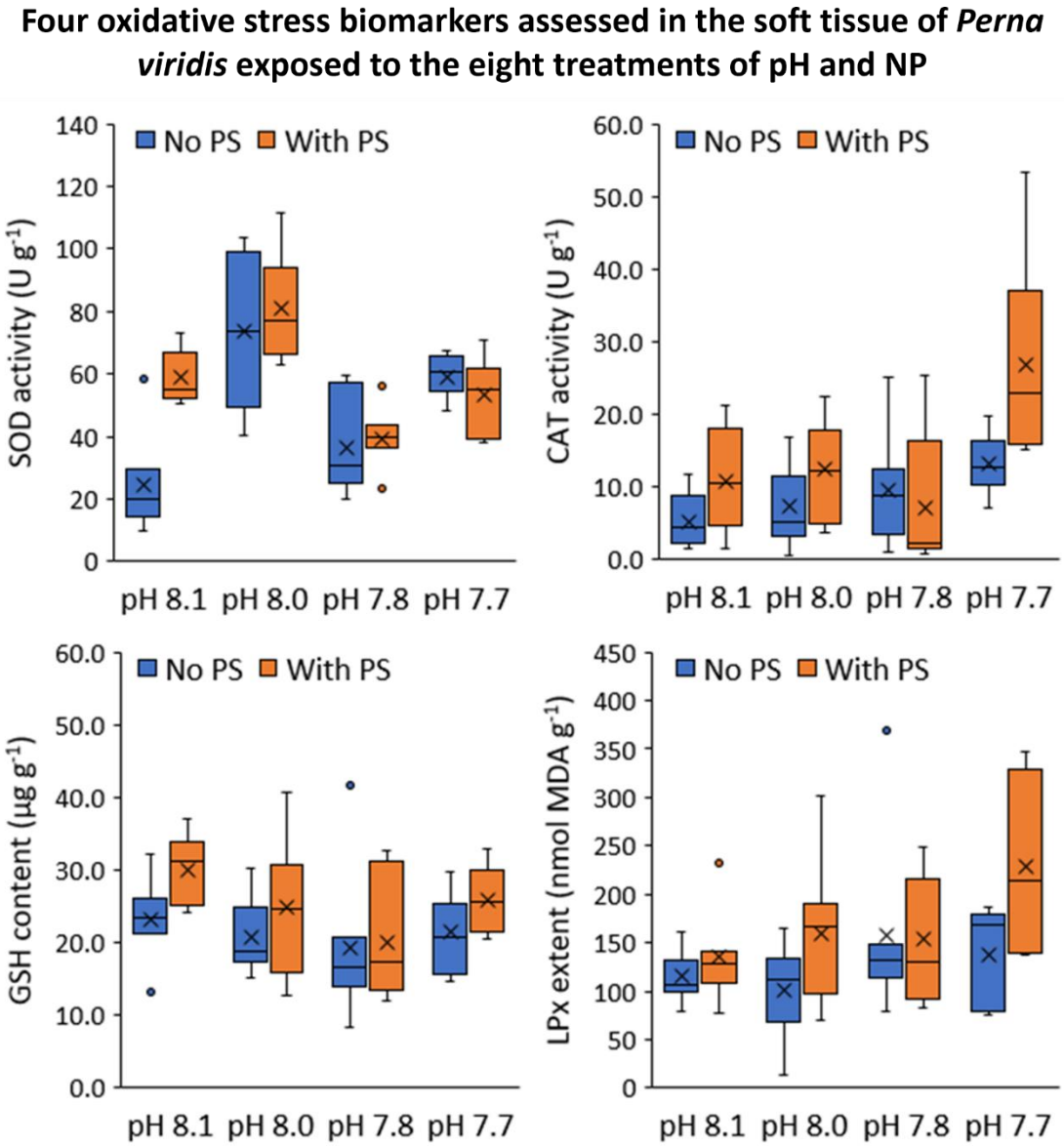

Figure 2. Box and whisker plots for four oxidative stress biomarkers assessed in the soft tissue of *Perna viridis* exposed to the eight treatments of pH and nanoplastics (NP).

Four physiological changes at the individual level of *Perna viridis* exposed to the eight treatments of pH and nanoplastics were measured and results were shown in **Error! Reference source not found.**. These physiological responses include shell index, condition index, hepatosomatic index and energy reserves.

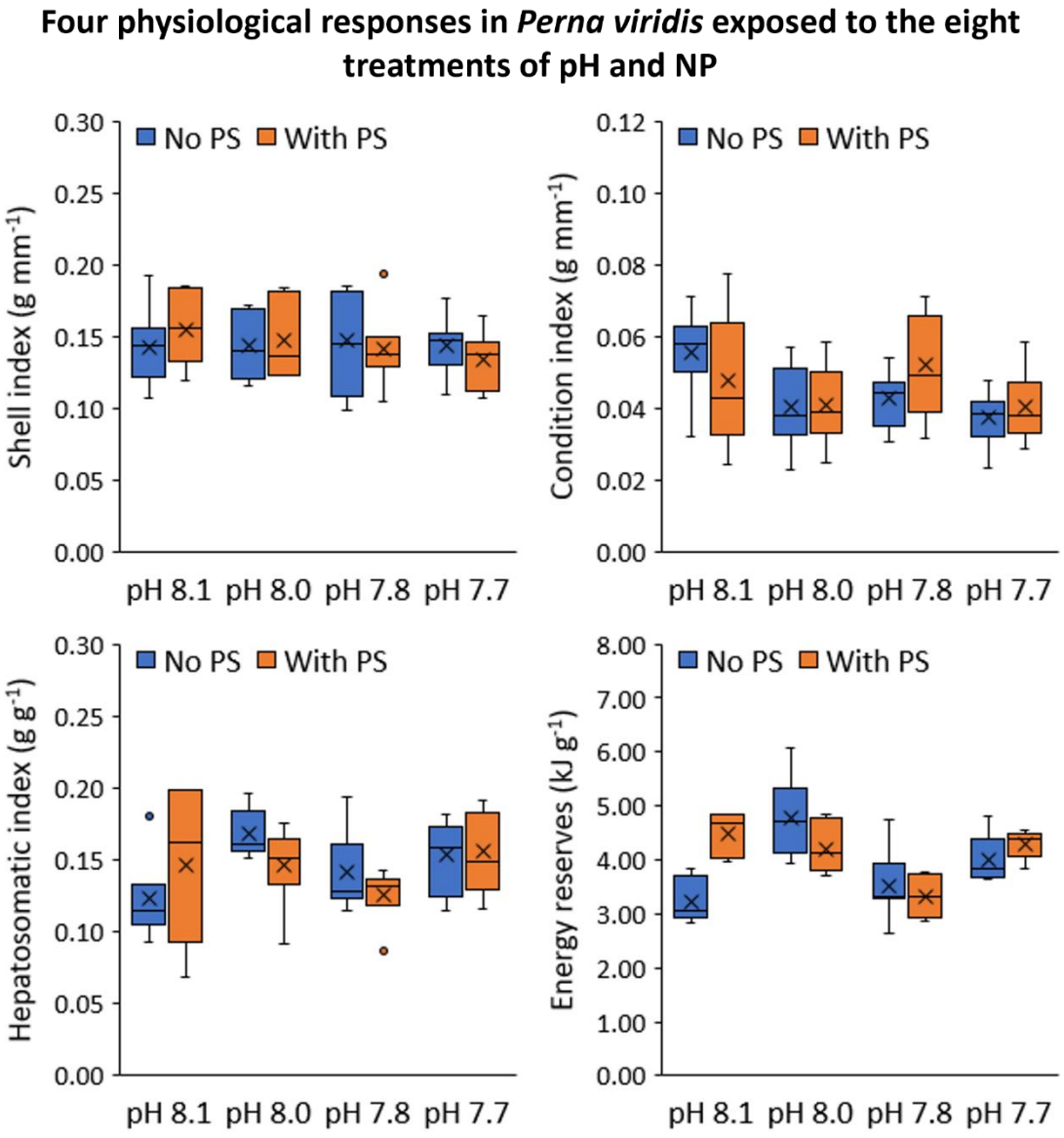

Figure 3. Box and whisker plots for four physiological changes observed at the individual level of *Perna viridis* exposed to the eight treatments of pH and nanoplastics (NP).

With reference to the results of Spearman's correlation analysis among the 12 variables in *Perna viridis* reported in **Figures 1–3**, the Draftsman's plot was provided in **Error! Reference source not found.**. Statistically significant correlation was detected between certain variables, but the strength of these relationships appeared to be weak or mild ($p < 0.05$; $\rho \leq 0.59$ in absolute values, Spearman's rank correlation).These variables include lysosomal content (LYS), oxidative activity (OXI), phagocytosis (PHG), apoptosis (APO), superoxide dismutase activity (SOD), catalase activity (CAT), glutathione content (GSH), lipid peroxidation (LPX), shell index (SI), condition index (CI), hepatosomatic index (HSI) and energy content (ENG). For each pair of variables, the table displays the rank correlation coefficient (ρ), p value and the number of replicates (n) in the first, second and third rows, respectively. Statistically significant results ($p < 0.05$) are highlighted in bold.

Table 3. Draftsman's plot showing results of Spearman's correlation among the 12 variables in *Perna viridis* reported in **Figures 1–3**.

	OXI	PHG	APO	SOD	CAT	GSH	LPX	SI	CI	HSI	ENG
LYS	0.098	–0.114	0.042	–0.076	–0.155	–0.217	**–0.306**	–0.133	0.141	–0.126	–0.123
	0.470	0.401	0.756	0.576	0.253	0.107	**0.022**	0.326	0.298	0.355	0.366
	56	56	56	56	56	56	**56**	56	56	56	56
OXI	*ρ* =	0.166	0.001	0.183	–0.104	–0.040	0.087	0.049	0.149	–0.060	0.241
	p =	0.220	0.997	0.176	0.442	0.768	0.524	0.719	0.270	0.659	0.074
	n =	56	56	56	56	56	56	56	56	56	56
PHG		*ρ* =	0.221	0.163	0.092	0.003	0.230	0.214	0.110	–0.031	0.222
		p =	0.101	0.228	0.500	0.981	0.089	0.113	0.419	0.820	0.100
		n =	56	56	56	56	56	56	56	56	56
APO			*ρ* =	–0.033	**0.337**	–0.260	0.114	–0.137	**–0.275**	0.040	0.116
			p =	0.806	**0.011**	0.053	0.401	0.315	**0.040**	0.769	0.391
			n =	56	**56**	56	56	56	**56**	56	56
SOD				*ρ* =	0.215	0.225	–0.008	–0.009	**–0.271**	0.196	**0.590**
				p =	0.112	0.096	0.952	0.947	**0.044**	0.148	**< 0.001**
				n =	56	56	56	56	**56**	56	**56**
CAT					*ρ* =	0.142	0.213	–0.034	**–0.266**	0.154	**0.314**
					p =	0.296	0.114	0.804	**0.047**	0.256	**0.019**
					n =	56	56	56	**56**	56	**56**
GSH						*ρ* =	**0.345**	–0.043	–0.012	–0.105	**0.304**
						p =	**0.009**	0.752	0.929	0.439	**0.023**
						n =	**56**	56	56	56	**56**
LPX							*ρ* =	0.104	0.257	–0.132	0.201
							p =	0.444	0.056	0.330	0.137
							n =	56	56	56	56
SI								*ρ* =	**0.487**	–0.224	0.050
								p =	**< 0.001**	0.096	0.714
								n =	**56**	56	56
CI									*ρ* =	**–0.593**	–0.090
									p =	**< 0.001**	0.510
									n =	**56**	56
HSI										*ρ* =	0.215
										p =	0.112
										n =	56

Results of the aligned rank transformation (ART) two-way analysis of variance (ANOVA) for the 12 variables in *Perna viridis* described in **Table 2** were shown in **Error! Reference source not found.** to study the interaction between pH and nanoplastics. Eight out of 12 variables showed no significant interaction effect between pH and NP, which were oxidative activity, apoptosis, CAT activity, GSH content, LPx extent, shell index, condition index and hepatosomatic index, and their main effects of pH and NP were further assessed in **Error! Reference source not found.**. As for variables showing a significant effect (pH × NP), including lysosomal content, apoptosis, SOD activity and energy reserves, further analysis was reported in **Table 6** to examine the simple effect of pH at each level of NP (without and with NP) and the simple effect of NP at each level of pH (pH 8.1, 8.0, 7.8 and 7.7). Other abbreviations used in **Tables 4-6** include source of variation (SV), degree of freedom (df), sum of squares (SS), mean square (MS), superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and lipid peroxidation (LPx). Statistically significant results ($p < 0.05$) are highlighted in bold.

Table 4. Results of the interaction analysis between pH and nanoplastics using ART two-way ANOVA for the 12 variables in *Perna viridis* described in **Error! Reference source not found.**.

	ART two-way ANOVA, for interaction of pH × NP						Main effect (**Error! Reference source not found.**) and simple effect (Table 6)
Biological variables	SV	df	SS	MS	*F* statistic	*p* value	
Lysosomal content	pH × NP	3	3364	1121	4.800	**0.005**	Tables 5–6
Oxidative activity	pH × NP	3	348.4	116.1	0.434	0.730	Table 5
Phagocytosis	pH × NP	3	2466	822.1	3.309	**0.028**	Tables 5–6
Apoptosis	pH × NP	3	1400	466.5	1.695	0.181	Table 5
SOD activity	pH × NP	3	3983	1328	6.000	**0.001**	Tables 5–6
CAT activity	pH × NP	3	1998	666.0	2.585	0.064	Table 5
GSH content	pH × NP	3	277.9	92.64	0.314	0.815	Table 5
LPx extent	pH × NP	3	784.9	261.6	0.913	0.442	Table 5
Shell index	pH × NP	3	477.4	159.1	0.540	0.657	Table 5
Condition index	pH × NP	3	1428	475.9	1.745	0.170	Table 5
Hepatosomatic index	pH × NP	3	1628	542.8	2.013	0.125	Table 5

Energy reserves	pH × NP	3	5140	1713	8.795	< **0.001**	Tables 5–6

In **Error! Reference source not found.**, post hoc Tukey's multiple comparison test was conducted on eight variables shown significant changes in *Perna viridis* in the main effect of pH or NP to compare pairwise among all levels of the corresponding factor. For the pairwise comparison among the four levels of pH, significant differences are indicated by unrepeated lowercase letters between two levels. Statistically significant results ($p < 0.05$) are highlighted in bold.

Regarding the main effect of pH, significant changes were observed in six variables. Increases in oxidative activity, apoptosis, SOD activity, CAT activity and energy reserves were identified, while the GSH content exhibited a significant decrease, under the acidification scenarios (pH 7.7–8.0) compared to the present-day scenario of pH 8.1 ($p < 0.05$, ART two-way ANOVA and post hoc Tukey's test; Error! Reference source not found.**a).** In terms of the main effect of NP, significant decrease in the lysosomal content, but significant increases in phagocytosis, SOD activity, CAT activity, GSH content and the extent of lipid peroxidation, were detected in *P. viridis* upon exposure to NP ($p < 0.05$, ART two-way ANOVA and post hoc Tukey's test; Error! Reference source not found.**b).**

Table 5. Results of ART two-way ANOVA regarding the main effects of **(a)** pH and **(b)** NP on the 12 variables in *Perna viridis*.

	ART two-way ANOVA, for factor pH						Main effect (Tukey's pairwise comparison)			
(a) Biological variables	SV	df	SS	MS	*F* statistic	*p* value	pH 8.1	pH 8.0	pH 7.8	pH 7.7
Lysosomal content	pH	3	741.9	247.3	0.859	0.469	Not significant			
Oxidative activity	pH	3	2435	811.8	3.535	**0.022**	**a**	**b**	**ab**	**ab**
Phagocytosis	pH	3	762.1	254.0	0.906	0.445	Not significant			
Apoptosis	pH	3	4754	1585	7.748	**<0.001**	**a**	**ab**	**bc**	**c**
SOD activity	pH	3	8357	2788	21.427	**<0.001**	**a**	**c**	**a**	**b**
CAT activity	pH	3	4503	1501	7.229	**<0.001**	**a**	**a**	**a**	**b**
GSH content	pH	3	2367	789.1	3.096	**0.035**	**b**	**ab**	**a**	**ab**
LPx extent	pH	3	1771	590.3	2.223	0.098	Not significant			
Shell index	pH	3	207.9	69.29	0.232	0.874	Not significant			
Condition index	pH	3	1926	642.1	2.467	0.073	Not significant			
Hepatosomatic index	pH	3	2305	768.4	3.030	**0.038**	Not significant			
Energy reserves	pH	3	6446	2149	12.78	**<0.001**	**ab**	**c**	**a**	**bc**

	ART two-way ANOVA, for factor NP						Main effect (Tukey's pairwise comparison)	
(b) Biological variables	SV	df	SS	MS	*F* statistic	*p* value	No NP	With NP
Lysosomal content	NP	1	1945	1945	7.419	**0.009**	**Higher**	Lower
Oxidative activity	NP	1	97.79	97.79	0.352	0.556	Not significant	
Phagocytosis	NP	1	2188	2188	8.733	**0.005**	Lower	**Higher**
Apoptosis	NP	1	591.5	591.5	2.036	0.160	Not significant	
SOD activity	NP	1	1921	1921	7.381	**0.009**	Lower	**Higher**
CAT activity	NP	1	1629	1629	6.160	**0.017**	Lower	**Higher**
GSH content	NP	1	1523	1523	5.670	**0.021**	Lower	**Higher**
LPx extent	NP	1	1694	1694	6.450	**0.014**	Lower	**Higher**
Shell index	NP	1	37.79	37.79	0.125	0.726	Not significant	
Condition index	NP	1	8.643	8.643	0.029	0.866	Not significant	
Hepatosomatic index	NP	1	31.50	31.50	0.105	0.748	Not significant	
Energy reserves	NP	1	928.3	928.3	3.254	0.078	Not significant	

In **Table 6**, the further analysis for the simple effects of pH and NP on four variables variables in *Perna viridis* showing a significant interaction effect (pH × NP) in ART two-way ANOVA was reported. The simple effect of pH at each level of NP (without and with NP) was examined using Kruskal-Wallis test and, if significant, post hoc Dunn's multiple comparison test. Significant differences are indicated by unrepeated lowercase letters among the pH levels. Likewise, Wilcoxon's rank-sum test was employed to assess the simple effect of NP at each level of pH (pH 8.1, 8.0, 7.8 and 7.7). Statistically significant results ($p < 0.05$) are highlighted in bold.

The four variables that showed a significant interaction effect (pH × NP) in **Error! Reference source not found.**, namely lysosomal content, phagocytosis, SOD activity and energy reserves, were further analysed for the simple effects of pH and NP in **Table 6**. The responses of these variables to the four tested levels of pH varied in the absence and presence of NP, with the patterns of significance outlined in **Table 6**a ($p < 0.05$, Kruskal-Wallis test and post hoc Dunn's test). On the other hand, significant effects of NP on the four variables were only observed at pH 8.1 ($p < 0.05$, Wilcoxon's rank-sum test), while no such effects of NP were detected at the three reduced pH levels (**Table 6**b).

Table 6. Assessment of the simple effects of **(a)** pH and **(b)** NP on lysosomal content, phagocytosis, SOD activity and energy reserves, the four variables in *Perna viridis* showing a significant interaction effect (pH × NP) in ART two-way ANOVA.

	Kruskal-Wallis test, for factor pH			Simple effect (Dunn's pairwise comparison)			
(a) Biological variables	SV	*H* statistic	*p* value	pH 8.1	pH 8.0	pH 7.8	pH 7.7
Lysosomal content	pH, no NP	8.348	**0.039**	**b**	**ab**	**a**	**ab**
	pH, with NP	4.322	0.229	Not significant			
Phagocytosis	pH, no NP	8.237	**0.041**	**a**	**ab**	**b**	**ab**
	pH, with NP	3.988	0.263	Not significant			
SOD activity	pH, no NP	16.34	< **0.001**	**a**	**b**	**ab**	**b**
	pH, with NP	16.95	< **0.001**	**ab**	**b**	**a**	**ab**
Energy reserves	pH, no NP	15.31	**0.002**	**a**	**b**	**a**	**ab**
	pH, with NP	15.42	**0.001**	**b**	**ab**	**a**	**b**
	Wilcoxon's rank-sum test, for factor NP			Simple effect			
(b) Biological variables	SV	*U* statistic	*p* value	No NP		With NP	
Lysosomal content	NP, at pH 8.1	4.000	**0.007**	**Higher**		Lower	
	NP, at pH 7.9	14.50	0.209	Not significant			
	NP, at pH 7.8	14.50	0.209	Not significant			
	NP, at pH 7.7	12.50	0.128	Not significant			
Phagocytosis	NP, at pH 8.1	5.000	**0.011**	Lower		**Higher**	
	NP, at pH 7.9	10.00	0.073	Not significant			
	NP, at pH 7.8	22.50	0.805	Not significant			
	NP, at pH 7.7	23.00	0.902	Not significant			
SOD activity	NP, at pH 8.1	4.000	**0.007**	Lower		**Higher**	
	NP, at pH 7.9	22.00	0.805	Not significant			
	NP, at pH 7.8	18.00	0.456	Not significant			
	NP, at pH 7.7	18.00	0.456	Not significant			
Energy reserves	NP, at pH 8.1	0.000	< **0.001**	Lower		**Higher**	
	NP, at pH 7.9	13.00	0.165	Not significant			
	NP, at pH 7.8	23.50	0.902	Not significant			
	NP, at pH 7.7	11.00	0.097	Not significant			

Chapter 5 Discussion and Conclusion

As highlighted in the introduction section, the combined effect of plastic pollution and ocean acidification on the ecophysiology of marine lifes has not been extensively investigated experimentally. In this study, mild effects of one month exposure of NP and pH were noted in three following aspects, which were alternations on immunological responses in mussel's haemolymph, variations on cellular stress and anti-detoxification system in mussel's gills and hepatopancreases, and the overall health condition of mussels at individual level.

5.1 Responses on the mussels's immunological system

Haemocytes, particularly for eosinophilic granulocyes, are the key component for the cellur-mediated responses in mussel's immunity, based on their functions on performing phagocytosis and the complemetary respiratory burst process (Pipe et al., 1997). Thus, four parameters, including intracellular lysosomal content, phagocytotic ability, oxidative activity, and apoptotic ratio were measured by using flow cytometry for investigating the combined effect on pH and NP on mussel's haemolymph samples.

Lysosome is a key organelle responsible for host defense and autophage via the releasement of lysozymes during phagocytosis for bacteria elimination and intracellular degradation (Andon and Fadeel, 2013). Hence, the intracellular lysosomal content is used as an indicator of the health status and the defence system in mussels commonly. In the present study, the intracellular lysosomal content in mussel was statistically affected by NP, pH and their interaction. A reduced trend was detected in the intracellular lysosomal content in the NP group, compared to the control group in the experimental condition at pH 8.1. This finding was supported by various studies, which showed the decrease in lysosomal membrane stability (Auguste et al., 2020a; Avio et al., 2015; Canesi et al., 2015; Capolupo et al., 2021; Katsumiti et al., 2021) and increase in lysosomal content in serum (Auguste et al., 2020b; Canesi et al., 2015; Capolupo et al., 2021) in mussel's haemocytes under plastic exposure. In addition, a general decreasing trend was observed in the intracellular lysosomal content was observed in

low pH environment with the absent of NP and this reduction observed in low pH conditions in mussel's haemocytes was also supported by several studies (Bibby et al., 2008; Huang et al., 2016; Sui et al., 2016; Wu et al., 2016; Wu et al., 2018). The decrease of intracellular lysosomal content observed in above could be attributed to the overproduction of reactive oxidative species (ROS) under low pH and exposure to NP (stress conditions), which might cause the damage on lysosomal membrane and further cause the leakage of enzymes inside lysosomes toward serum (Canesi et al., 2015; Wu et al., 2016). The degranulation process occurred in haemocytes for releasing lysosomal content toward serum for achieving immune purposes might also be the reason for explaining the decrease in intracellular lysosomal content detected (Pipe, 1992). Although both NP exposure and pH reduction could decrease the intracellular lysosomal content, their interaction has not performed the synergistic effect toward the lysosomal content with mussel's haemocytes in this study. Wang et al. (2020) also suggested that haemolytic viability was reduced significantly under the combined interaction of microplastic exposure and pH reduction, since the haemocytes viability is partially related to the intracellular lysosomal content (Pipe, 1992). Thus, reduced lysosomal content in additive form measured in current study might be enhanced by the reduction in haemolytic viability reported in Wang et al. (2020) study. Compared to the low pH, polystyrene exposure might impact the haemocyte's lysosomal content with higher intensity during their interaction, and this finding might be explained by the nodular inflammation and degranulation processes performed by eosinophilic granulocyes provoked nano-sized polystyrene plastics exposure.

Phagocytosis is the physical internalization of foreign molecules regarded as dangers for cells (Gosling, 2008), which is directly related to the mussel's immunological power. Thus, phagocytotic power can be used as the index for investigating mussel's immune response. In this study, phagocytotic ability in mussel was statistically affected by NP, pH and their interaction. At pH 8.1 condition, a significant increase in the phagocytotic ability was observed in the NP exposed group, compared to the control group, and this observation was supported by some studies (Katsumiti et al., 2021; Pittura et al., 2018). However, the variation in phagocytotic ability in haemocytes under plastic exposure has not been aligned with each other and the result of decrease (Canesi et al., 2015; Capolupo et al., 2021; Pittura et al., 2018; Sendra et al., 2020) and remain unchanged

(Avio et al., 2015; Paul-Pont et al., 2016) were measured in other studies and this difference may result from the different experimental condition applied in each study. The increasement in phagocytotic power measured in present study may be contributed by the usage of polystyrene spheres in nano-sized that could provoke the immunostimulatory effect, which is commonly reported in mussel's haemocytes exposed to nanoscale particles (Katsumiti and Cajaraville, 2019; Katsumiti et al., 2014; Katsumiti et al., 2021). In addition, an increase trend in the phagocytotic ability was observed in low pH condition in the NP – absent group. However, this increasing trend was contradicted to other studies focusing on the effect of pH on mussel's haemolymph (Bibby et al., 2008; Huang et al., 2016; Sui et al., 2016; Wu et al., 2016; Wu et al., 2018). The increasing trend in phagocytotic ability under low pH condition measured may be explained by the biphasic effect of pH, since the lowest pH applied in present study was pH 7.7 and the lowest pH used in other studies were pH 7.3 and even pH 6.5. Although both NP exposure and pH reduction could increase the phagocytotic activity, their interaction has not performed the synergistic effect within mussel's haemocytes in this study. Compared to the low pH, polystyrene exposure might impact the haemocyte's phagocytotic ability with higher intensity during their interaction. Furthermore, a statistical significantly changes were noted in haemocyte phagocytic ability upon the combined interaction of pH and NP in Huang et al. (2022) and current studies. However, the reduced trend noted in Huang et al. (2022) study could not been observed in current study, which might be attributed to experimental conditions, including concentration and size of plastic used and the species of the test subject, applied in each studies.

Complementary to phagocytosis, the respiratory burst is performed simultaneously to achieve the immunological target (Tanguy et al., 2013), which is a series of biochemical reactions generating microbicidal oxidative radicals, including peroxide, hydrogen peroxide and nitric oxide, for activating the NADPH-oxidase to digest phagocytosed compartments (Gosling, 2008). However, overproduction of those oxidative radicals may lead to unwanted cellular damage. Hence, oxidative activity can be considered as the marker for studying haemocyte's viability and immune intensity. pH was the only factor affecting the oxidative activity within haemocytes statistically in this investigation, which illustrated that an increase trend in ROS content in low pH condition, compared to the pH 8.1 group in NP-absent environment. The finding on the

increasement of ROS content in haemocytes under low pH environment was proved by several studies (Bibby et al., 2008; Huang et al., 2016; Sui et al., 2016; Wu et al., 2016; Wu et al., 2018). As for the combined interaction in NP and low pH toward mussel's haemocytes, no significant variation in current study. This finding was contradicted to the synergistic effect in the increasement in ROS content observed in Athulya et al. (2023) study toward tissues of Artemia salina and might be explained by the application of different experimental conditions, ranging from concentration, size and type of plastic used, organs of species measured, exposure time for the test subject and detection method used.

Apoptosis is the cellular suicidal process, which is also an immunological strategy for facing environmental stress and pollutants in mussel's immunity. Like the oxidative activity, the apoptotic ratio with mussel's haemolymph samples was only affected by the variation of pH. An increasing trend in apoptotic ratio was observed in low pH condition, compared to the pH 8.1 group in NP-absent environment. This finding was similar to previous reports Bibby et al. (2008); (Huang et al., 2016; Sui et al., 2016; Wu et al., 2016; Wu et al., 2018). Furthermore, the increase in apoptotic ratio might be explained by the increase in ROS content in haemocytes in low pH condition in this study, which implied that overproduction of ROS occurred in low pH environments could further cause cellular damage and drive haemocytes death.

In short, polystyrene exposure, low pH condition and their interaction could activate haemocyte's immune system in mussels. As for parameter of intracellular lysosomal content and phagocytotic activity, polystyrene exposure may take the major lead for causing those mentioned variations. And pH was the only factor for changing the oxidative activity and apoptotic ratio in mussel's haemocytes.

5.2 Responses on the mussel's cellular stress and anti-detoxification system

Due to the physiological roles of gills and the digestive gland of mussels, they fall victim to plastic pollution and ocean acidification (Gosling, 2015). To better understand how plastic pollution and ocean acidification the mussel's cellular stress and anti-detoxification system can, four biomarkers, which are SOD, CAT, GSH and LPx have been used for investigation in gills and the digestive gland.

SOD is vital for a variety of life, ranging from cells, to plants, and even animals, because of its role in the biological detoxification system. Being a superoxide anion scavenging enzyme, SOD is responsible for the dismutation of superoxide radicals to hydrogen peroxide and oxygen molecules (Magara et al., 2018). Thus, the SOD activity can be used for studying mussel's detoxification system. In this study, SOD activity was affected by NP, pH and their interaction.

At pH 8.1, a significant increase in the SOD activity was detected in the polystyrene-exposed group, compared to the control group. The increasing trend of SOD activity in mussel's gills under plastic exposure has been confirmed by Revel et al. (2019) study, suggesting the theory that SOD may be a key player in mussel's antioxidant response by countering the excessive superoxide radicals into less active hydrogen peroxide molecules for cellular protection. However, other studies have mentioned that no significant change in SOD activity has been noted in gills (Magara et al., 2018; Magara et al., 2019) under plastic exposure, and have further proposed that superoxide radical production may not be the primary cause of oxidative impairment. Since experimental conditions applied in each study were different, the variations of size, concentration and exposure time of plastics applied may be the reasons for causing different trends in SOD activity under plastic exposure (Revel et al., 2019). Furthermore, a statistical increase in SOD activity was observed in the pH 8.0 and pH 7.7 group with reference to the pH 8.1 group in the NP-absent condition. The increasing trend of SOD activity in gills under low pH conditions was also supported by several studies (Freitas et al., 2017; Hu et al., 2015; Huang et al., 2018; Matozzo et al., 2013; Sui et al., 2017) and further suggested that low pH condition could enhance ROS productions to future

activate SOD for antioxidant responses. Although both polystyrene exposure and pH reduction could increase the SOD activity, their interaction has not performed the synergistic effect in this study. Both low pH and polystyrene exposure could share their impacts on the mussel's gills during their interaction. Surprisingly, a significant decrease in SOD activity was noted in pH 7.8 group, compared to pH 7.9 group in polystyrene-present condition and this observation was not sustain in pH 7.7 group with reference to pH 7.8 group under polystyrene exposure. Thus, it could be proposed that the decreasing trend observed might contribute to individual differences of experimental objects rather than the combined effect of plastic pollution and low pH condition. Although both NP exposure and pH reduction could increase the intracellular lysosomal content, their interaction has not performed the synergistic effect, but present in additive form toward the lysosomal content with mussel's tissue in this study. The statistical difference noted in SOD activity in current study toward the combined interaction of pH and NP was different from Provenza et al. (2020), Wang et al. (2020) and Athulya et al. (2023) studies, and could be explained by variations in experimental conditions, including concentration, size and type of plastic used, species and exposure time for the test subject and detection method used, applied in each studies.

CAT is another antioxidant enzyme that is required by all aerobic organisms, due to its role in protection of cells from oxidative damage by the dismutation of H_2O_2 molecules into harmless water and oxygen molecules (Magara et al., 2019; Revel et al., 2019). Hence, the CAT activity is measured commonly as the index for investigating mussel's detoxification system. In the present study, the CAT activity was influenced by NP and pH, but not with their interaction. Consistent to Wang et al. (2020) study, no significant alternation was noted in response to the combined interaction of MP and pH. However, a synergistic impact of MP and low pH condition was noted in Artemia salina (Athulya et al., 2023), and this variation might be explained by the experimental conditions applied in each study. A significant increase in CAT activity in mussel's gills was present in NP-present group with reference to the NP-absent group and this finding has been supported by various studies (Magara et al., 2018; Magara et al., 2019; Revel et al., 2019). Furthermore, the increasing trend in CAT activity was detected in pH 7.7 group, compared to pH 8.1, pH 8.0 and pH 7.8 groups. The increasing trend in CAT activity in gills under low pH condition were also noted in other studies (Hu et al., 2015;

Huang et al., 2018; Matozzo et al., 2013; Sui et al., 2017). Since increased SOD activity can yield a larger H_2O_2 production, the increased trend in SOD activity measured could be a possible reason for the enhanced CAT activity detected in this study.

Apart from two antioxidant enzymes mentioned in above, reduced GSH also plays a vital role in the metabolism of endogenous substances and toxic compounds by acting as the non-enzymatic scavenger of oxyradicals (Meister and Anderson, 1983). Thus, the reduced GSH content can also be used as the indicator for measuring the alternation in antioxidant system. In the current study, the reduced GSH content in the digestive gland was altered by NP and pH, but not with their interaction. A significant increase in reduced GSH content in the digestive gland was detected in the NP-present group with reference to the NP-absent group and this increasing trend was supported by Magara et al. (2018) study. However, no significant change in reduced GSH content has been noted in mussel's tissue under plastic exposure in Avio et al. (2015) study. Until now, the effect of the reduced GSH content under plastic exposure have not been confirmed and this variation might be provoked by the difference in the experimental set-up. A significant decrease in reduced GSH content in the digestive gland was noted in pH group 7.8 with reference to pH group 8.1 in NP-absent group. And, this result has been contradicted to Hu et al. (2015) study, which proposed that the reduced GSH content has increased in mussel's tissue under low pH condition. This difference could be explained by the two following reasons. First, it is the biphasic effect of pH toward the reduced GSH content in the mussel's digestive gland, since the lowest pH applied in present study was pH 7.7 and the lowest pH used in Hu et al. (2015) study were pH 7.3. Second, the decreasing trend observed might contribute to individual differences of experimental objects rather than the effect of low pH condition. In Wang et al. (2020) study, a statistically increase in the GSH content was detected in low pH environment under microplastic exposure. The statistical difference noted in the GSH content in current and Wang et al. (2020) studies upon the combined interaction of nano-/microplastic exposure and pH could be attributed to variations in experimental conditions applied.

LPx is a chain reaction of oxidative degradation, provoked by free radicals taking electrons from lipids in cell membranes, leading to cellular damage (Sheehan et al., 2001). Thus, the level of LPx can be used to represent the cellular oxidative stress. In this study, the level of LPx was only affected by NP, but not with low pH and their combination. A significant increase in the level of LPx in mussel's digestive gland was present in polystyrene-present group, compared to the polystyrene-absent group. The increasement in the level of LPx in mussel's digestive system after NP exposure has been supported by Avio et al. (2015) study, which could proposed that the exposure of mussels toward plastic spheres might lead to the overproduction of ROS and create oxidative stress toward mussel's digestive gland. Furthermore, the increase in the LPx level was supported by the increase in the GSH content in mussel's digestive gland in this study, which implied that the increase in GSH content cannot cope with the overproduction of ROS, leading to the increase in LPx level. Similar to current study, no significant impact was observed on the combined interaction of NP and pH in oyster tissue (Sui et al., 2022).In Provenza et al. (2020) study, the LPx level has been reduced significantly under the low pH condition with reference to natural marine pH condition. The statistical difference noted in the LPx level in present study and Provenza et al. (2020) studies studies upon the combined interaction of nano-/microplastic exposure and pH could be attributed to variations in experimental conditions applied.

In short, the SOD activity in gills was the only parameter influenced by NP, pH and their interaction. As for activities of CAT and GSH in mussel's gland, they were altered by the factors of NP and pH. Furthermore, the level of LPx in mussel's tissue was only affected by NP.

5.3 Responses on the mussels's individual health condition

As compared to the variations observed in the immunological response and the cellular stress & anti-detoxification system, milder concequences have been shown in the overall health condition of mussel's at individual level. First of all, mortality was not occurred during the adaptation and experimental periods, indicating that experimental conditions applied in present were not lethal for the targeted subjects. Furthermore, all three growth parameters measured, including shell index, condition index and hepatosomatic index have not shown significantly different among the control group and all sample groups.

Surprisingly, statistically changes were observed in the level of energy reserve in this study. To adapt to environmental and physiological stresses, including food shortage, gametogenesis, pH reduction and tempertaure elevation, metabolic reserve stored within mussel's tissues, such as the digestive gland and posterior adductor muscle, are mobilized for maintaining homestaticsis in bivalves (Ansell, 1974; Matoo et al., 2021; Shang et al., 2023; Zandee et al., 1980). Thus, the level of energy reserve can be used for representing mussel's health in individual level. In this study, the level of energy reserve was statistically influnced by NP, pH and their interaction. A significant increase in the level of energy reserve was detected in the NP exposed group with reference to the control group at pH 8.1. This finding was contradicted with previous studies performed, showing that the level of energy reserve in mussels remained unchanged after plastic exposure (Shang et al., 2023; Weber et al., 2020). Furthermore, an increased trend was noted in the level of energy reserve under low pH conditions with reference to the control group (pH 8.1) in NP-absent environment. This result has consistent with Freitas et al. (2017), which suggested that a protective behaviour might be taken by mussel via valve closure for achieving a lower metabolic activity and energetic expenditure to store more glycogen, protein and lipid (Gosling, 2008). The energy stored though low metabolic activity might even exceed the energy consumed by the immune and antioxidant responses, leading to the increase in the energy reserve. It can be also observed that pH reduction might impact the energy reserve in mussel with higher intensity during the combined effect of NP and pH. In addition, a significant synergistic reduction on protein content was observed in Athulya et al. (2023) study by

using brine shrimp tissue, which enhanced the suggestion that cellular and metabolic functions might be altered by the combined interaction of NP and pH. In short, the level of energy reserve was the only parameter significant affected by NP, pH and their interaction in this study. As for the shell index, condition index and hepatosomatic index, no stastistics difference were observed between the control group and all sample groups, indicating that the overall health condition of mussel's at individual level affected by NP and pH exposure was mild.

Lastly, it can be observed that out of the 66 pairs of correlations analysed among the 12 endpoints in *P. viridis*, seven of them, particularly for the SOD and CAT activities and apoptotic cell ratio, were specifically linked to condition index and energy reserves, which implied that the two higher-level physiological changes could serve as early-warning biomarkers to reflect the overall health status of marine organisms under the impacts of OA and NPs. To further confirm the specific correlation between early-warming biomarkers (SOD and CAT activities) and two mentioned higher-level physiological changes, investigations focused on molecular level could be for measuring alternations in expression of SOD and CAT related recognition receptors in future studies.

5.4 Improvements and Suggestions

To have better and deeper understanding on the combined effects of NP and pH toward marine lives, following improvements and suggested can be considered in future. First, four pH levels (pH 8.1, pH 7.9, pH 7.8 and pH 7.7) were originally designed, but the real applied pH levels were pH 8.1, pH 8.0, pH 7.8 and pH 7.7 in the current study. This variation of pH might be attributed to the fluctuation of carbon dioxide automated control system and might need to be concerned for the future study.

Second, investigations targeting on the variation of gene expression might be applied to have deeper understanding on the combined effects of ocean acidification and nano-plastic pollution toward mussels in molecular level in future. In this study, alternations in mussel's immunological and detoxification systems in cellular, tissue and individual levels of mussels have been studied under the co-exposure in nanoplastic and low pH condition and show that SOD activity in mussel tissues has been affected by the combined effect of NP and pH statistically in additive effect. Alternations in expression of SOD related genes, such as AP1 and NF- κB, might be studied with real-time PCR (Miao and St Clair, 2009). In short, mussel tissues obtained could first be used for RNA extraction, and the cDNA could be produced from the purified RNA and designed primer via reverse transcription. And the synthesized cDNA could be used as the template for real-time PCR for quantitative measurement.

Apart from the real-time PCR measurement, a field experiment might also be performed in future. With reference to EPD (2022), the surface water pH in Hong Kong water regions have been ranged between pH 6.5 to pH 8.5 in 2022. In addition, the annual average surface water in Tung Lung Chau area has decreased from pH 8.1 in 2018 to pH 7.7 in 2022, which implied that Hong Kong water regions have been suffered from ocean acidification (EPD, 2024). Tsang et al. (2020) reported that the average microplastic concentration in Hong Kong coastal waters and sediments were ranged from 51 to 27909 items/100m^3 and 49 to 279 items/kg in respectively, which proposed that microplastic contamination has also existed in Hong Kong water regions. Since both ocean acidification and micro-/nanoplastic pollution are present in Hong Kong

water regions, a field experiment might be designed in Tung Lung Chau area for studying the long-term combined effects of NP and pH toward mussels.

5.5 Conclusion

In this study, the individual and combined effects of OA and NPs in P. viridis were investigated in cellular, tissue and individual level by 12 biological endpoints measured. In cellular level, four immuno-related biological endpoints measured in haemocytes have shown significant alternations. Two of them, oxidative activity, and apoptosis, have exhibited significant increase in response to reduced pH. Upon the exposure of NP, the lysosomal content and phagocytic ability were statistically decreased and increased in respectively. In tissue level, four stress-related biomarkers measured in tissue level have exhibited significant changes. Under ocean acidification conditions, activities of superoxide dismutase (SOD) and catalase (CAT) have increased significantly, and the content of glutathione (GSH) has decreased statistically. As for the exposure of NP, all four stress-related biomarkers, including activities of SOD and CAT, content of GSH and the level of lipid peroxidation, have increased statistically. In individual level, four physiological changes have also been measured. Three of them, including values of shell index, condition index and hepatosomatic index, have remained relatively consistent across all treatments. A significant increase was only measured in the energy reserves of P. viridis upon one of the ocean acidification scenarios. Furthermore, four out of 12 endpoints (lysosomal content, phagocytosis, SOD and energy reserves) have been statistically impacted by pH and NP in form as additive interactions in all cases. Lastly, seven out of 66 pairs of correlations analysed among the 12 endpoints in P. viridis, were specifically linked to condition index and energy reserves and these findings might suggest that the cellular stress responses, particularly the activities of SOD and CAT, may be integrated with the two higher-level physiological and might serve as early-warning biomarkers to reflect the overall health status under the impacts of OA and NPs

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