MASTEROPPGAVE

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A study of the effects of copper-rich mine tailings and microplastics on *Mytilus edulis*

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ABSTRACT

The disposal of copper (Cu) mine tailings unto the seafloor after separation of target minerals from ore during mining results in the accumulation of heavy metals above background concentrations. Microplastics (MP) in the environment can adsorb metals, potentially leading to combined toxicological effects of MP and metals. The goal of the present study was, therefore, to investigate the effect of Cu and polyethylene MP on blue mussels (*Mytilus edulis*) using Cu-rich mine tailings and dissolved Cu ($CuCl_2$). Mussels were exposed for 3 days without feeding to eight different treatments. These were control (C), low (L) and high (H) concentrations of dissolved Cu, MP alone (M), old mine tailing sediments from Repparfjorden (T), a mixture of old mine tailings and MP (TM), newly processed drill core from the Nussir mine (FT), and a mixture of MP and high Cu (MH). Microplastics accumulation in the soft tissues and Cu accumulation in the digestive glands of mussels were measured with pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) and inductively coupled plasma - mass spectrometry (ICP-MS), respectively. Nitric oxide production and phagocytic activity of hemocytes were measured as biomarkers. The different treatments resulted in no significant effects on Cu accumulation, with highest levels found in mussels exposed to low levels of dissolved Cu (L). No significant effects were observed for the immune parameters, although exposure to L and H resulted in high and low production of NO, respectively. Furthermore, no significant combined effect of the Cu-mine tailings and microplastics were found. Factors such as lack of feeding and short exposure time might have influenced the results. Further studies on the combined and single effects of Cu-rich mine tailing and MP on mussels are recommended.

Keywords: Mytilus edulis, Microplastics, Copper, Phagocytosis, Nitric oxide, Submarine mine tailings

1. INTRODUCTION

1.1 Project and relevance

This project aimed to investigate the biological effects of copper (Cu)-rich mine tailings and microplastics (MP) on blue mussels (*Mytilus edulis*). The research involved laboratory exposure experiments and the use of immune responses such as nitric oxide production, and phagocytosis as biomarkers.

Repparfjorden is one of the fjords used for the disposal of mine waste in Norway. The fjord has been designated as a "national salmon fjord" by the Norwegian government. Due to this designation, many concerns were raised when a recent permit was granted for the disposal of Cu-rich tailings from the Nussir mine in the Repparfjorden. Among the opponents to this were various scientific institutions (e.g. High North Research Centre for Climate and the Environment; Framsenteret, 2018), civil organizations (e.g. Naturvernforbundet, 2019), local inhabitants, and the Sami Parliament representing indigenous Sami people (Hage, 2018).

Furthermore, the Environmental Impact Assessment (EIA) conducted by Nussir AS for the project indicated "medium negative consequences" on the marine environment in the near zone and minor consequences in the middle and far zones. Of particular interest in the EIA are the "medium negative consequence" of the submarine tailings disposal on species of soft-bottom fauna in the sublittoral zone, as well as the "slightly negative consequence" on species diversity among zooplankton, and its associated effects on plankton-eating fish. The EIA further anticipates a "medium / large negative" consequence for the spawning area and a "medium negative" consequence for the rearing area for fish in the Repparfjorden (Christensen et al., 2011).

Although the EIA indicated a very low impact on the environment with the establishment of an on-land landfill as compared to submarine disposal, a permit was granted for the establishment of submarine disposal of the mine tailings. The EIA identified the most important remediation/mitigation measures as the establishment of new benthic communities and habitats for benthic fish after cessation of operations, as well as limiting the spread of metals from the disposal site during operations. The EIA further proposed a program including leakage and aging tests, chemical monitoring, monitoring of the particle concentration in the fjord, metal concentrations in discharged sediments, and monitoring of metal levels in the marine food chain (Christensen et al., 2011).

Due to the ongoing agitations against the project, and to better understand and document the biological effects of xenobiotic exposure in the fjord, biomonitoring and scientific investigations of the fjord are highly relevant. This study is a contribution to the body of scientific knowledge on the ecological situation in the Repparfjorden.

1.2 Problem

Mine tailings are by-products produced after the separation of target minerals from ore during mining. In countries that allow submarine tailings disposal, large volumes of tailings are produced from mines and deposited into marine environments for storage. This disposal method of mine tailings is practiced in Norway, especially in fjords. This method is known to have detrimental impacts on aquatic biota (Burd, 2002; Neira et al., 2015).

The characteristics and ubiquitous presence of MP (Wright et al., 2013; Anbumani & Kakkar, 2018) in the environment is a cause of concern for many environmental and scientific institutions. Its effect on marine fauna is an area of significant research and it has been postulated to have detrimental effects such as bioaccumulation, biomagnification, mortality, and loss of species diversity (Valko et al., 2005) when no mitigation and remediation measures are considered. Unpublished data from the Institute of Marine Research, Norway reported (the unintended discovery of) the presence of MP amongst fish eggs in about 25% of samples collected from Repparfjorden in a study on the quantification of fish eggs in the fjord (Dahl, 2018). Plastic debris of about 14 mm in size was discovered to be present amongst collected plankton samples indicating the presence of MP in fjords in northern Norway.

The combination of MP (from both long and short-range transport) and elevated metal concentrations from mine tailings potentially presents significant risks to marine flora and fauna. As the fjord is important for its fisheries potential for Norwegian and Sámi fishers, any long-term contamination may have adverse impacts not only on the aquatic biota but also have significant economic impacts on those that rely on it for their livelihoods should fish stock be depleted in the fjord.

1.3 Project Aim and objectives

Whereas there have been several studies on the effects of Cu and MP using mussels and fish as bioindicators, this present study is the first to consider the interplay between Cu from mine tailings and MP using mussels as bioindicators.

The main aim of this thesis was to study possible immunological effects of Cu-containing mine tailing sediments on blue mussels, and whether co-exposure with MP affect the biological responses.

To achieve this aim, the following objectives were set:

- measure the accumulation of Cu and MP in the mussels.
- measure the immune response of mussels exposed to Cu and MP.

The following hypotheses were tested:

- The immunological effect of Cu is not affected by MP in mussels co-treated with Cu-rich mine tailings and MP (H_{01}).
- Cu accumulation in the digestive glands of mussels exposed to high concentrations of dissolved Cu alone is not higher than the combined exposure of high concentrations of dissolved Cu and MP (H_{02}).

1.4 Mine waste disposal & microplastics

Mining is an essential industry involved with the extraction and processing of economically valuable minerals or geological materials from the earth. It has been important in the development of human society from antiquity to the present day; creating employment opportunities and providing metals needed for diverse use (Healy, 1979; Richards, 2009). The mining of minerals either in the artisanal or industrial fashion is done in most countries around the globe. The exploration of minerals in Norway is growing rapidly with new sites being commissioned for exploration (Kvassnes & Iversen, 2013). According to World Mining Data (2020), there was an 89% increase in the tonnage of materials mined globally (excluding bauxite) from 1984 to 2018 (see Fig. 1). This represents a remarkable growth in the industry and significant contributions to the economic growth and wellbeing of the countries (and corporations) involved. With this increase in global mining activity comes significant increases

in the volume of (contaminated) waste products which must be carefully managed to reduce its impact on the environment.

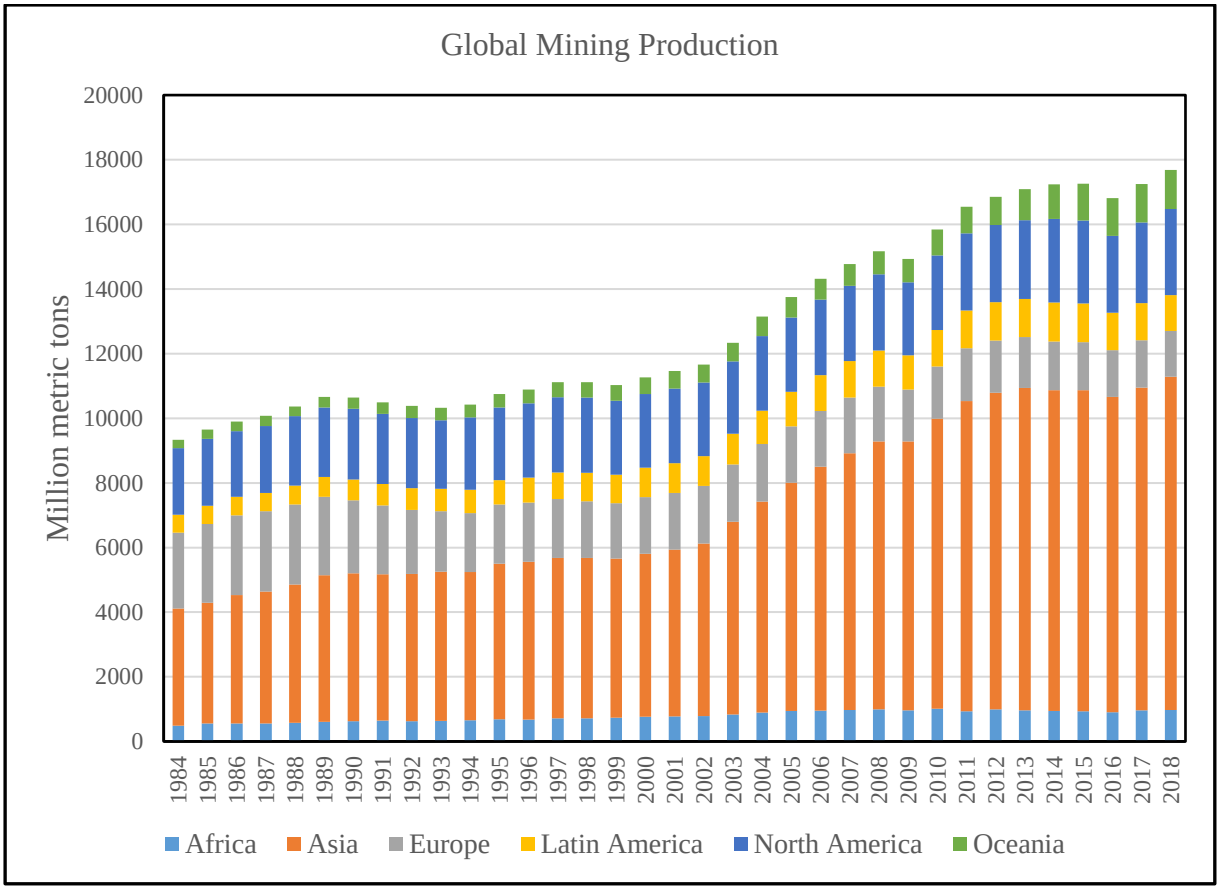


Figure 1. *Global mining production (excluding bauxite) from 1984 to 2018. Chart plotted from publicly available data at https://www.world-mining-data.info/?World_Mining_Data__Data_Section (World Mining Data, 2020).*

Issues on the volumes of plastic materials in the environment and the long term impacts it has on several earth systems have also been in sharp focus and the attention of much research in recent years (Barnes et al., 2009; Li et al., 2016; Hermabessiere et al., 2017). Data published by Geyer et al. (2017), show a marked increase in the global generation and disposal of plastics from 2000 to date, and this trend is predicted to accelerate over the next 30 years (see Fig. 2).

The global use, indiscriminate disposal, and large production of plastics have led to the widespread presence of MP in the marine environment, exacerbating already contaminated marine ecosystems.

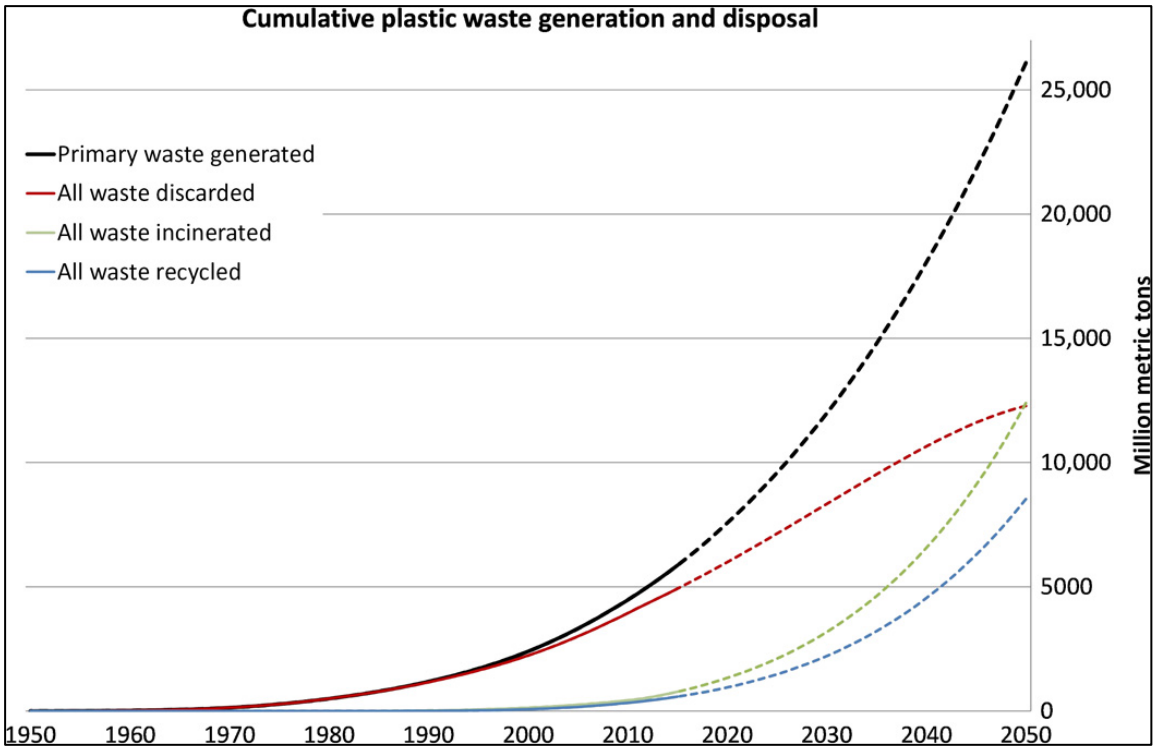


Figure 2*. Cumulative plastic waste generation and disposal showing historical data and projections to 2050. Image reproduced from Geyer et al. (2017).*

1.4.1 Mine tailings & microplastics

The waste produced after the separation of target minerals is called mine tailings. Mine tailings are often a slurry with high-water content and fine sediments (Lindsay et al., 2015; Starke, 2016). The tailings may contain trace metals, residual reagents added to separate and concentrate the target metals, and reagents added to increase the flocculation of the tailings upon discharge. A visual representation of submarine mine tailings disposal and MP presence in the marine environment is presented in (Fig. 3).

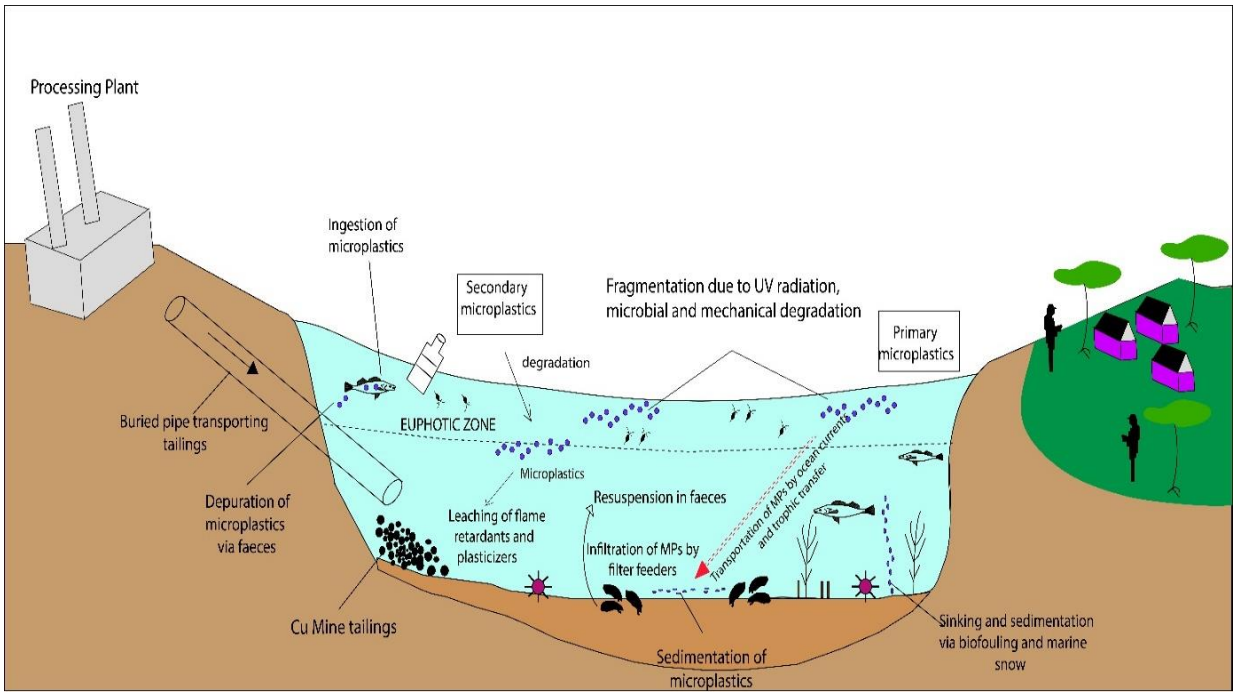


Figure 3. *A model illustrating the disposal of Cu mine tailings from a mine processing plant and the transport and sedimentation of microplastics in the marine environment.*

1.4.2 Submarine tailings disposal

Submarine mine tailing disposal (STD) is one of the three different types of mine tailing disposal in the sea including coastal tailing disposal and deep-sea tailing placement, which results in the accumulation of heavy metals above background concentrations in the marine environment (Ramirez-Llodra et al., 2015). It refers to the disposal of tailings unto the seafloor through underground pipelines at relatively shallow (between 50 and 150 m) but submerged depths (Skei & SMC, 2014). The general principle for disposal is to avoid the tailings having an impact on the upper water layer, where primary production occurs (Dold, 2014; Ramirez-Llodra et al., 2015). Norway is one of the few countries including Chile, Indonesia, and the Philippines that currently practices STD regardless of its environmental issues and the uncertainties in predicting its long-term effects (Cornwall, 2013; Dold, 2014; Ramirez-Llodra et al., 2015). Currently, Norway has several active and inactive mine tailings disposal sites (see Fig. 4) (Kvassnes & Iversen, 2013; Ramirez-Llodra et al., 2015). The main objective of mine tailing storage is to minimize environmental and social impacts with negligible public health and safety risks by ensuring that tailings are physically stable and chemically inert (Dold, 2014). The disposal of mine tailings into the marine environment has both positive and negative consequences.

It is advantageous because mine tailings are geotechnically stable on the ocean floor if deposited in a depression or canyon and require minimal land usage in situations of limited land space as observed in Norway. Furthermore, little long-term maintenance is required after disposal, and they are less vulnerable to oxidation resulting in reduced release of toxic metals to the environment (Franks et al., 2011; Cornwall, 2013; Dold, 2014).

Conversely, submarine mine tailings disposal impacts the marine environment negatively by destroying habitats, changing the topography of the seafloor, smothering biota especially benthic sessile fauna and infauna such as bivalves and polychaetes, risk of bioaccumulation of heavy metals affecting species abundance and diversity with potential human health risk in fish and shellfish consumption (Armstrong et al., 2012; Dold, 2014; Ramirez-Llodra et al., 2015)

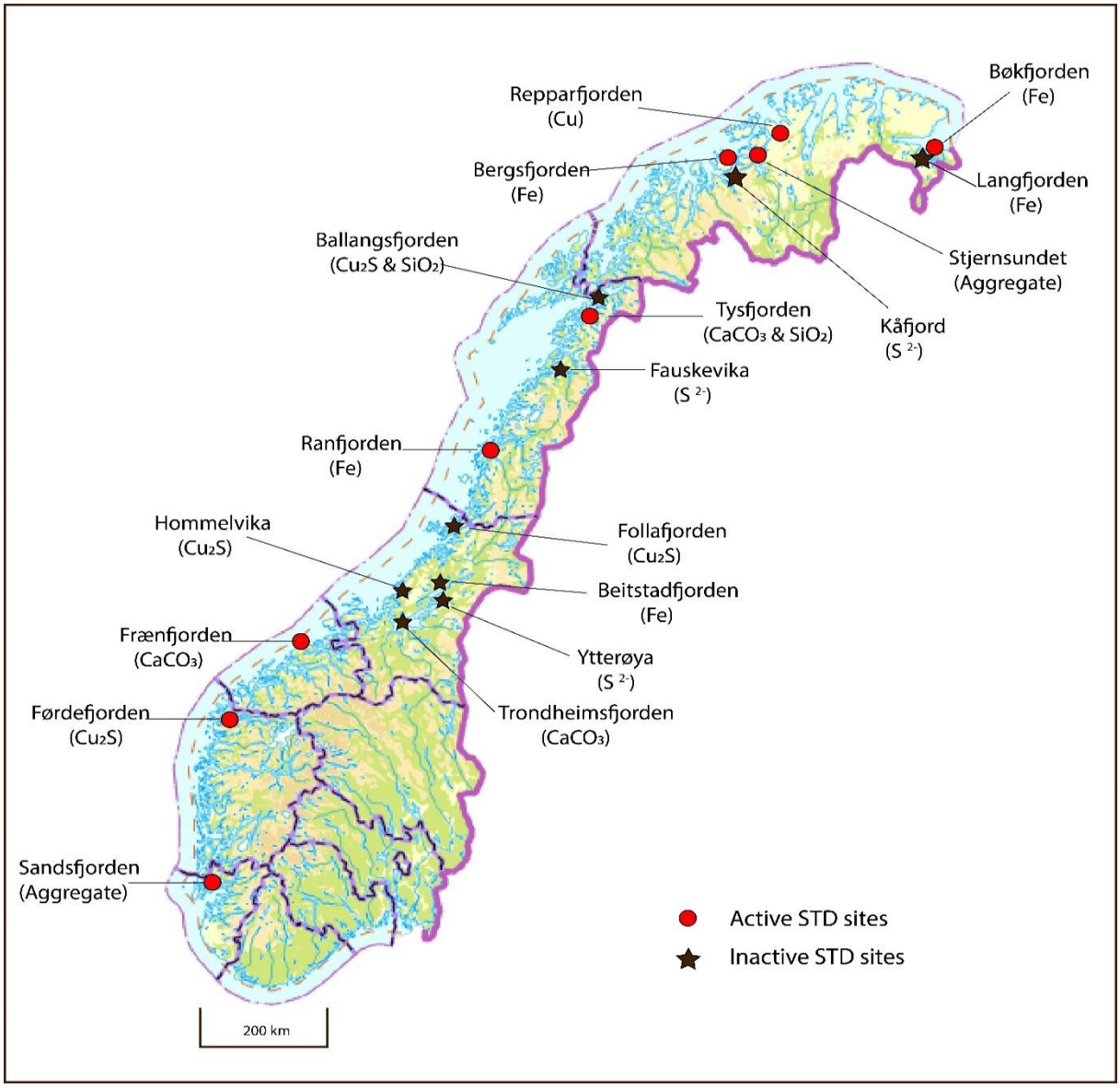


Figure 4. *Map of Norway showing locations of active and some inactive STD sites across the country based on Kvassnes & Iversen (2013) and Ramirez-Llodra et al. (2015).*

1.5 Study area

Repparfjorden is a fjord located in the Hammerfest municipality of Finnmark county, Northern Norway. There has been Cu mining at different periods in the municipality, however, large-scale operations started in the 1970s with approximately one million tonnes of Cu-enriched tailings being subsequently discharged at the inner fjord of Repparfjorden (Kvassnes & Iversen, 2013; Mun et al., 2020). The fjord is influenced by a river called Repparfjordelva which forms the fjord head and flows out to the open ocean through Kvalsundet and Sammelsundet. The length, width, and area of Repparfjorden are approximately 13 km, 4 km, and 37 km² respectively. The fjord is characterized by an inner and outer basin separated by a sill (Marthinussen, 1961). A map of the study area is shown in Fig. 5.

A permit has been granted to a local mining company, Nussir ASA by the Norwegian Environment Agency for the continual disposal of mine tailings into a new restricted area in the fjord. The permit allows for the annual discharge of up to two million tons of mine tailings over a 30-year production period to have started from the year 2019 (Pedersen et al., 2017). The project has, however, been delayed and is expected to begin in 2021.

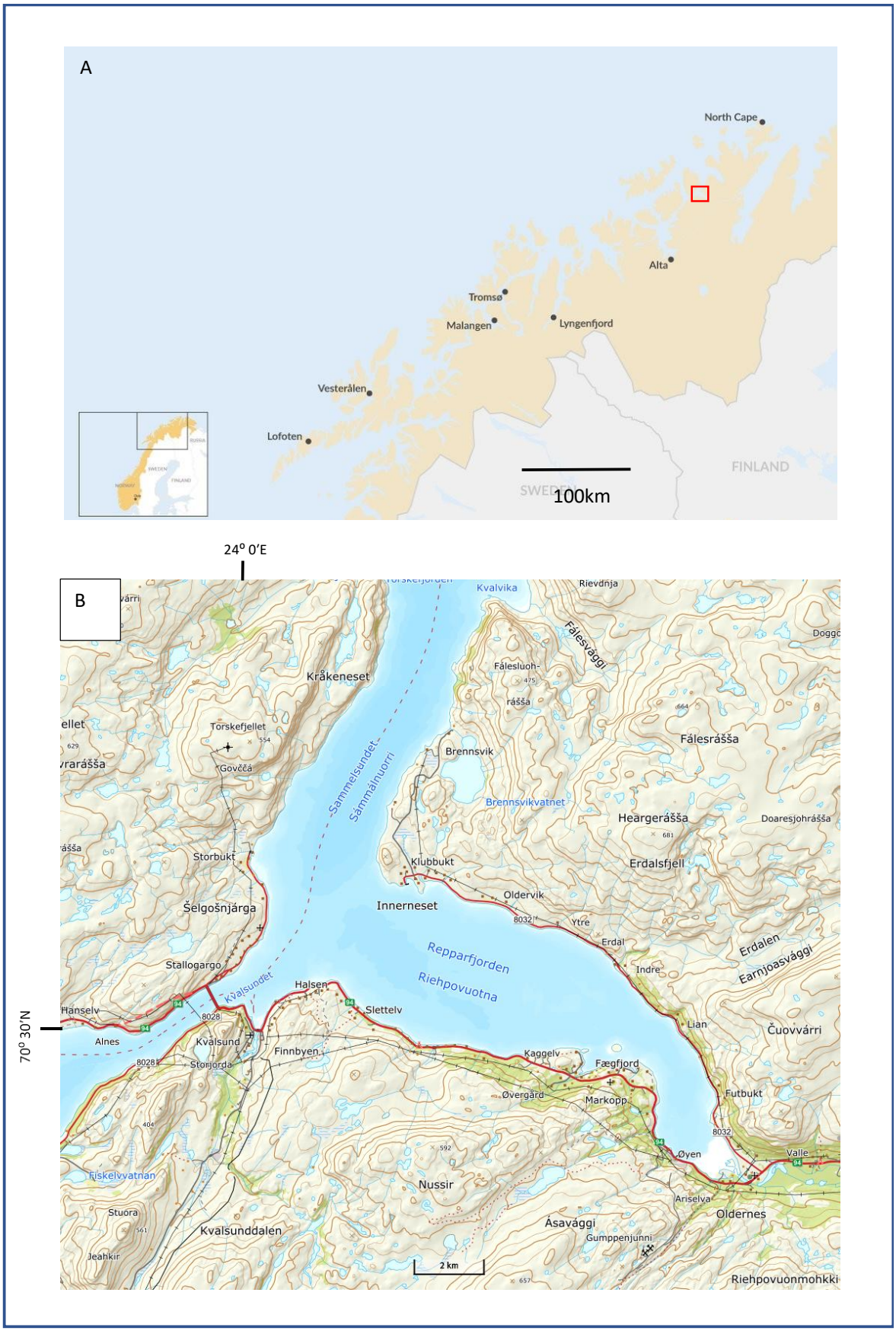


Figure 5. *A general location of the study area within Norway. B shows a detailed location of the area as indicated by the red square in Fig 5A.*

1.6 Previous studies

Although studies on the Repparfjorden are relatively scant, there exists a relatively large body of research on the biological effects and consequences of marine tailings disposal on biota in other fjords in Norway.

A toxicity study conducted by Lillicrap et al. (2011) determined a Lowest Observed Effect Concentration (LOEC) of mine tailings from Nussir ASA at 100% v/v and a No Observed Effect Concentration (NOEC) at 32% v/v (v/v: mine tailings/water). The alga *Skeletonema costatum* (primary producer), the benthic copepod *Tisbe battagliai* (primary consumer), and the polychaete worm *Arenicola marina* (deposit feeder) representing different trophic levels in the marine environment were used as bioindicators in the study.

Sedimentological and geochemical analysis of sediment cores in Repparfjorden showed a restricted distribution pattern of tailings-affected sediments to a small area of the inner fjord and to a discrete layer of the outer fjord close to the sill which separates the inner and outer fjord. The study concluded on a probable exposure of Cu to the ecological communities of the inner fjord due to the presence of the tailings-affected sediment in layers up to the sediment-water layer (Sternal et al., 2017).

A mineralogical and geochemical study conducted by Mun et al., (2020) determined high Cu concentrations (up to 747.7 ppm) in the uppermost 9 cm sediments in Repparfjorden. The high Cu concentrations in sediments were attributed to the disposal of Cu mine tailings. The low total organic carbon values obtained in the study showed the impact of submarine mine tailings disposal on benthic biota (Mun et al., 2020).

Roda et al., (2020) showed the interaction between Cu and MP. Many studies have also shown the adsorption of Cu on MP, therefore, highlighting the relevance of monitoring marine litter and heavy metals (Davarpanah & Guilhermino, 2015; Brennecke et al, 2016; Qiao et al., 2019).

2. BACKGROUND

2.1 Marine pollution

The marine environment has been subjected to anthropogenic pollution causing changes in the physical and chemical properties of the environment for a long time (Bergmann et al., 2015). The physical and chemical environmental changes, including alterations in temperature, salinity, pH, turbidity, water chemistry, color, and gas content, may, in turn, affect most aquatic biota. Anthropogenic activities such as mining (Ramirez-Llodra et al., 2015), discharge of sewage effluent (Al-Musharafi et al, 2013), indiscriminate disposal of plastic waste (Lebreton & Andrady, 2019), urbanization and industrialization (Wang et al., 2013) impact marine ecosystems at the global scale and all levels of complex life (Nogales et al., 2011). Anthropogenic activities release contaminants such as heavy metals (e.g. Cu, Hg, As, Pb, Cd, Co, and Ni) and plastic debris which may bioaccumulate and biomagnify in marine organisms and present a potential threat to the marine ecosystem (Barnes et al., 2009; Rainbow, 2002). Figure 6 shows the global distribution of different types/sources of pollutants in the marine environment.

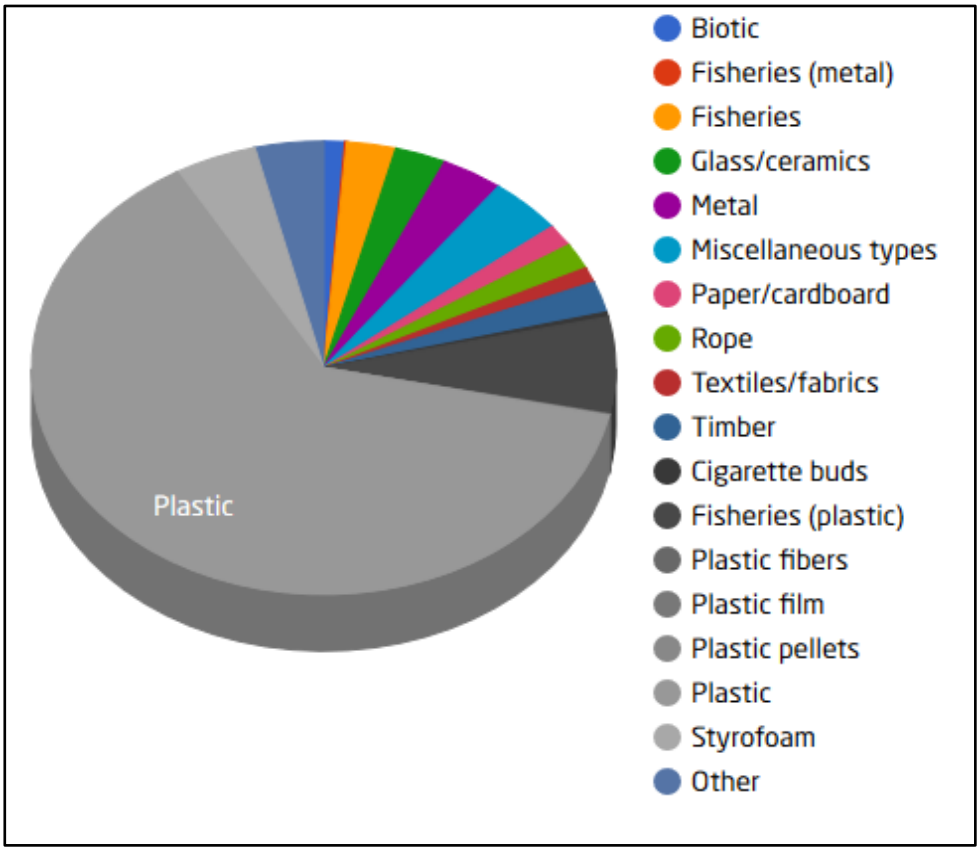


Figure 6. *Global composition of marine litter as of 2020 from the Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research (AWI-Litterbase, 2020).*

2.2 Immuno-toxicological effects of Cu

Copper is a common naturally occurring substance in the earth's crust. It is a heavy metal with a density greater than 5 g/cm³. According to the Norwegian classification system of contaminants, the background concentrations of Cu in seawater and sediments are < 0.3 µg/l and < 35 mg/kg, respectively. At concentrations of > 7.7 µg/l and > 220 mg/kg in seawater and sediments respectively, a class five (V) level of severe acute toxic effect is attained (Miljødirektorat, 2016).

Background concentrations of Cu in seawater and sediment resulting from the weathering of Cu-bearing parent rocks and the concentrations vary among different localities based on the local geology. Copper concentrations in both newly processed mine tailings and mine tailings deposited in Repparfjorden in the 1970s were discovered to be above the Norwegian background levels of Cu concentrations in sediment with the potential of causing adverse effects in the marine environment (Pedersen et al., 2017).

Non-essential heavy metals such as mercury, lead, and thallium are toxic even at low concentrations. Conversely, essential heavy metals including Cu are needed to maintain ionic balance, metabolism and form an integral part of amino acids, nucleic acids, and other structural compounds (Valko et al., 2005). Copper, an essential micronutrient is necessary for metabolic processes, physiological functions, and forms a co-factor of many important enzymes involved in several vital biological processes. For example, cytochrome c oxidase which acts as an enzymatic co-factor in cellular respiration in eukaryotes, and Cu/zinc superoxide dismutase (SOD) enzymes that provide cellular defense against reactive oxygen species (ROS) (Gaetke & Chow, 2003). Copper, however, becomes toxic at elevated levels causing acute and chronic effects. Long-term exposure and accumulation of Cu in aquatic organisms causes effects such as impairment of feeding mechanisms (Nicholson, 2003), alteration of growth rates and reproduction (Fitzpatrick et al., 2008), and oxidative stress (Lushchak, 2011; Gomes et al., 2012). Human activities such as mining, smelting, industrial and domestic waste emission, use of antifouling paints (Dafforn et al., 2011; Bighiu et al., 2017), and sewage sludge (Fjällborg & Dave, 2003) result in elevated levels of Cu in the marine environment (Blossom, 2015). High Cu concentrations in sediments are reported to have a negative correlation with species diversity of benthic fauna, such that among the 50 most frequently occurring species, 20 were missing from Cu polluted (Cu> 200 ppm) stations in Norwegian fjords (Rygg, 1985). By comparison,

the concentration of Cu in the upper 9 cm of the Repparfjorden tailing sediments has been measured to be 747.7 ppm (Mun et al., 2020). The toxicity of Cu is primarily due to the imbalance between the production of pro-oxidants and the generation of antioxidants to detoxify the ROS; termed as oxidative stress (Flora et al., 2008). Copper is a redox metal and its participation in Fenton and Haber-Weiss reactions catalyzes the formation of ROS leading to oxidative stress (Lushchak, 2011; Gomes et al., 2012).

The Fenton and Haber-Weiss reaction plays a significant role in ROS production in which Cu due to its multiple valence states, reacts with molecular oxygen to produce ROS (Das et al., 2015). Molecular oxygen (O_2) reduces or gain electrons leading to the formation of superoxide (O^{2-}) which often produces other ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\circ}OH$) and peroxynitrite ($ONOO^-$) (Lushchak, 2011). Copper shifts between cuprous (Cu^+) and cupric (Cu^{2+}) ionic forms due to their oxidative and reductive characteristics. The majority of the body's Cu is in the cupric form. During the Fenton and Haber Weiss reaction of Cu (see Equations 1-3), cupric ion reduces to cuprous ion and subsequently produces ROS causing oxidative stress (Cuypers et al., 2010).

	$Cu^{2+} + O_2^- \rightarrow Cu^+ + O_2$	---------- Eqn 1
Fenton	$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + {}^{\circ}OH + OH^-$	---------- Eqn 2
Haber Weiss	$O_2^- + H_2O_2 \rightarrow O_2 + {}^{\circ}OH + OH^-$	---------- Eqn 3

Reactive oxygen species have essential roles in cell signaling, apoptosis, gene expression, and ion transportation but excess levels cause damaging effects to tissues and cells such as lipid peroxidation, DNA lesions, and mutation (Lushchak, 2011).

Copper toxicity is dependent upon its availability and the physicochemical properties of the particular environment such as pH, water hardness, organic content, soil, and sediment type (Campbell et al., 2014).

2.3 Sediments containing Cu

Sediments act as a sink for pollutants, predominantly metals. Metals are removed from the water column into sediments by complexation, precipitation, and adsorption processes (Förstner, 1979; Flemming & Trevors, 1989). The complexation mechanism controls the form and concentration of dissolved Cu (Abbasse et al., 2003). Precipitation or dissolution of Cu is determined by the concentration of the ions and the solubility product constants (K_{sp}). Adsorption is significant in the removal of dissolved Cu from polluted surface waters ultimately into sediments, where Cu is adsorbed to living and non-living particulate matter. These processes and the resultant mobility, speciation, partitioning, and bioavailability of Cu are dependent on the natural environment (Usdhhs, 2004; Ivanina et al., 2013; Thomas, 2018). Some of these environmental factors are pH, nature of the sorbent, and concentration of organic and inorganic ligands (Violante et al., 2010). Copper has a strong affinity for complexation with organic ligands such as humic substances, dissolved organic matter, and bacterial particles (Neff, 2002; Abbasse et al., 2003). The high content of Cu found in the soft tissues of mussels collected from the Norwegian Sea off Spitsbergen and the Baltic Sea off Poland was attributed to the relatively high bioavailability of Cu due to the formation of complexes with humic substances (Pempkowiak et al., 1999). Another field study on the benthic colonization on polluted sediments in the Oslofjorden reported that Cu in experimental doses of 400-1500 mg/kg impacted the colonization of several taxa as a result of the increased bioavailability of Cu in sediment with a lower concentration of organic carbon (Trannum et al., 2004). The toxic effect of Cu increases at low pH and salinity. Decreasing salinity decreases precipitation of the metal ions thereby, increasing the concentration of the dissolved metal ions. Decreasing pH impacts biota living in a contaminated environment by increasing the bioavailability and toxicity of many pH-sensitive metals such as Cu (Ansari et al., 2004; Lewis et al., 2016).

The ability of Cu to adsorb to MP may cause synergistic effects in aquatic living organisms (Davarpanah & Guilhermino, 2015; Qiao et al., 2019). Synergism is defined as two or more chemicals exerting a larger effect than predicted (Cedergreen, 2014). In a study of population growth with microalgae *Tetraselmis chuii*, the adsorption of Cu to polyethylene MP was confirmed (Davarpanah & Guilhermino, 2015). Roda et al. (2020) concluded on the interaction between Cu and polyethylene MP and the deleterious effects (genotoxic, neurotoxic, and physiological) caused by the uptake of Cu and polyethylene MP both alone and combined on *Prochilodus lineatus*. The uptake of Cu adsorbed on MP aggravates toxicity via inhibition of

Cu-ion transport and enhanced oxidative stress. The widespread presence of MP in the marine environment significantly affects the bioaccumulation, homeostasis, and toxicity of heavy metals (Brennecke et al., 2016).

2.4 Microplastics and their toxicity

Microplastics are plastic debris or particles ranging in size from 0.1 to 5 mm in their longest dimension (Cole et al., 2011; Bråte et al., 2017). Plastic contamination in the world's oceans has emerged as a consequential issue due to their mass production and usage, indiscriminate disposal, ubiquitous distribution and long-range transport potential, persistence, and most importantly the potential threats they pose to marine organisms and ecosystems (Anbumani & Kakkar, 2018). Microplastics have been identified in many environmental matrices globally including surface water, beaches, marine biota, consumables sourced from the sea, and deep-sea sediments (Law & Thompson, 2014). The physical behavior (i.e. migration, sedimentation, and accumulation), chemical behavior (i.e. degradation and adsorption) and bio-behavior (i.e. ingestion, translocation, and biodegradation) of MP contribute to their negative impacts on the marine environment (Wang et al., 2016; Bråte et al., 2017). Microplastics leach toxic substances such as monomers and plastic additives, capable of causing carcinogenesis and endocrine disruption (Hermabessiere et al., 2017). They act as vectors for the adsorption of hydrophobic organic pollutants and heavy metal ions due to their large area to volume ratio and hydrophobic properties. The hydrophobicity of plastics favors the fouling of pollutants (Artham et al., 2009). Microplastics polymer type, shape, size, density, and chemical composition influence the fouling of pollutants. Microplastics have been shown to induce harmful effects such as oxidative damage of cellular components via the production of ROS altering antioxidant capacity, genotoxicity, neurotoxicity, biochemical, physiological, behavioral and histological responses (Anbumani & Kakkar, 2018; Roda et al., 2020; Santos et al., 2020).

2.5 Mussels

2.5.1 Biology and habitat

Blue mussels are active filter feeders pumping and filtering large volumes of water over their ciliated gills. They feed mainly on unicellular algae, bacteria, and organic materials. They spawn when food availability is high and excrete excess food as pseudofeces particles. They are attached to rocks and other hard substrates using their byssal thread. They are euryhaline organisms found in a wide variety of ecological niche, ranging from the littoral to shallow littoral. They can tolerate and adapt to varying environmental parameters (Canada Fisheries and Oceans, 2003; Beyer et al., 2017).

2.5.2 Mussels as bioindicators

Mussels are often used as bioindicators for assessing the environmental quality of seawater and for pollution monitoring (Rainbow, 2002; Beyer et al., 2017; Li et al., 2019). They are ideal bioindicators due to their availability and abundance, hardiness (easy to keep in culture and suitable for ecotoxicological laboratory exposure studies), sessile nature, filtering feeding behavior, ability to provide an integrative measure of the concentration and bioavailability of pollutants due to their ability to absorb and accumulate pollutant with limited biotransformation (Beyer et al., 2017). Mussels are good model organisms in revealing MP uptake, accumulation, and toxicity (Li et al., 2019) and show greater responses in toxicity tests with sediments (Besser et al., 2009; Lusher et al., 2017). Mussels clear MP with the same extent than food items (microalgae) of similar size (Fernández & Albentosa, 2019). A visual representation of the major internal features of mussels is shown in Fig. 7.

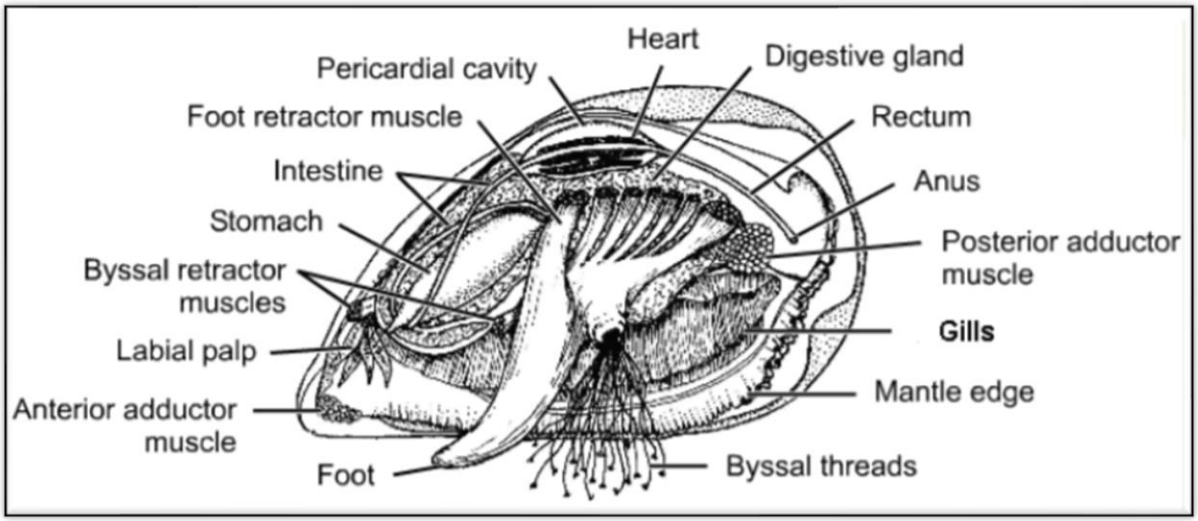


Figure 7. *A visual representation of the dorsal, posterior, and ventral part of mussels. [Figure reproduced from Canada Fisheries and Oceans, (2003)]***.**

A complex balance between contaminant uptake and depuration determines whether a contaminant at any given time will concentrate or depurate in mussels (Beyer et al., 2017). Uptake is facilitated by three mechanisms including passive diffusion, active transport or endocytosis, and the formation of mineralized granules (Beyer et al., 2017). The gill tissue is the main site of dissolved metals uptake, where metals are bound to metallothionein (MT), incorporated into lysosomes, and released basally towards the blood plasma and circulating hemocyte (Marigómez et al., 2002). Particulate metal uptake occurs predominantly by endocytosis in the digestive system of mussels (Marigómez et al., 2002). The uptake and tissue accumulation of absorbed metals by mussels depend on several factors including essentiality of the specific metal, chemical speciation duration, the concentration of the exposure, biological factors such as body size, sex, nutritional and reproductive status of mussels, and environmental factors such as salinity, temperature and organic matter concentration (Wang & Fisher, 1999; Rainbow & Luoma, 2011). Higher levels of metal concentrations were found to accumulate in the digestive gland than in other tissues (gills, mantle, and foot) in a study conducted using the green-lipped mussel, *Pema canaliculus* as a bioindicator for coastal contamination in New Zealand (Chandurvelan et al., 2015).

The digestive gland is the main tissue for metal storage in bivalves since it is consequential for the detoxification of metal; (Wallace et al., 2003; Wanick et al., 2012). Mussels detoxify metals by binding active metal ions within proteins such as MT and depositing them in insoluble forms in intracellular organelles (lysosomes). Lysosomes are membrane-bound organelles involved

in the digestion and removal of intracellular substances including organic and inorganic xenobiotics and pathogens. The lysosomal system reduces cellular toxicity leading metal ions to exocytosis and excretion from the body via feces (Viarengo et al., 1987). Most of these lysosomes are localized in the digestive glands of mussels. The digestive glands of mussels have been widely used as a target-tissue for investigating the effects of xenobiotics at the cellular, molecular, and biochemical levels due to the important role they play in processes such as immune defense, homeostasis, elimination, and detoxification of xenobiotics (Marigómez et al., 2002; Faggio et al., 2018).

2.6 Biomarkers

A biomarker is a biological response that can be measured in tissue samples, body fluids, or at the level of the whole organism which signals exposure to, or adverse effects of anthropogenic chemicals or radiation (Depledge, 1993). Biomarkers include behavioral parameters or physiological responses such as changes in reproductive, feeding, and social behavior (Monserrat et al., 2007). Biomarkers indicate the biological effects of pollutants, which are not seen in non-contaminated environments. Biomarkers can be either specific or non-specific (Chandurvelan et al., 2015). Specific biomarkers include the measure of cholinesterase activity that responds to the presence of organophosphorus, carbamate pesticides, and neurotoxins (Monserrat et al., 2007). Non-specific biomarkers include the determination of oxidative stress, DNA damage, antioxidant, and immune responses. The study of a range of parameters that comprise an integrated biological system (e.g. the immune system) provides a comprehensive measure of the health status of the organism, reflecting pollutant induced stress, disease susceptibility, and an organism's chances of survival (Anderson, 1990; Pipe et al., 1995; Morley, 2010).

2.7 General overview of the mussel immune system

The relative simplicity of the invertebrate immune system mediated by innate immune functions makes it a sensitive and accessible means of monitoring marine environmental pollution (Ellis et al., 2011). The innate immune system is non-adaptive (Tiscar & Mosca,

2004). The defense mechanisms of mussels consist firstly of the mucosal epithelial barriers, circulating hemocytes, and humoral factors that coordinate immune functions triggering a wide range of immune responses (Koutsogiannaki & Kaloyianni, 2010). Shells, cuticle, and mucous layers found in the majority of invertebrates act as the first line of defense to potential pathogens. Hemocytes play a fundamental role in the innate immune responses of mollusks including phagocytosis, respiratory burst leading to ROS production, and the synthesis of nitric oxide (NO) (Mydlarz et al., 2006). The circulating blood cells of mussels referred to as hemocytes secrete humoral factors including lectins, nitric oxide, lysosomal enzymes, and antimicrobial peptides involved in the communication and integration of immune functions (Koutsogiannaki & Kaloyianni, 2010).

The main function of the immune system is to protect an organism against infectious agents. However, many environmental contaminants and chemicals affect the immune system, leading to an increase in disease susceptibility and mortality. Diseases such as larval abnormalities, shell deformities, and tissue damage have been observed in shellfish in association with pollutant xenobiotics (Pipe & Coles, 1995; Dyrynda et al., 1997; Galloway & Depledge, 2001). Pipe & Coles, (1995) recorded increased mortality of mussels after experimental exposure to a highly concentrated Cu solution followed by subsequent exposure to a disease-causing agent. The high proportion of mussel mortality in the study was attributed to a deteriorated immune system after exposure to the Cu solution.

2.8 Phagocytosis

Phagocytosis is a cellular immunity mediated by hemocytes (Liu et al., 2020). It is an endocytic process in which foreign particles or pathogens larger than 0.5 μm are recognized, taken up, engulfed, degraded, or weakened and cleared by hydrolytic enzymes (Liu et al., 2020) (see Fig. 8). It comprises different phases namely recognition, chemotactic migration, adhesion, ingestion, destruction, and the elimination of foreign particles (Mydlarz et al., 2006) (see Fig. 8). Hemocytes are categorized into two main subpopulations namely granulocytes and hyalinocytes. Granulocytes have granules in their cytoplasm whiles hyalinocytes or agranulocytes possess no or few granules. Granulocytes are more actively phagocytic. Granulocytes consist of the basophilic (small granules) and eosinophilic (larger granules) (Wang et al., 2012). The induction of xenobiotics or stress results in alterations in total

hemocyte counts and relative proportion of blood cell type. Copper exposure was shown by Pipe et al. (1999) to increase the proportion of granulocytes in oysters and mussels and reduce the proportion of hyalinocytes.

The phagocytic activity serves as a proxy for measuring the general capacity of an organism to mount immune responses. It is measured by determining either the proportion of hemocytes that are phagocytically active (Gagnaire et al., 2006; Park et al., 2020) or the phagocytic index which refers to the number of bacteria engulfed by each hemocyte (Cameron & Heath, 1988).

Many methodologies have been used in the measuring of phagocytosis. They include the measuring of the uptake of fluorescent latex beads (Duchemin et al., 2007) and neutral red stained zymosan (Pipe et al., 1999) using flow cytometric analysis and microplate analysis respectively. Studies on phagocytosis of MP and bioparticles have also been conducted using the immune cells of aquatic animals (e.g. Park et al., 2020).

Phagocytic activity is significantly reduced by several anthropogenically induced stressors such as pollution via contaminants such as Cu (Pipe et al., 1999; Parry & Pipe, 2004). Phagocytic indices in exposure experiments are shown to increase following short-term and low-level contaminants and decrease after exposure to prolonged and high-level contaminants (Parry & Pipe, 2004).

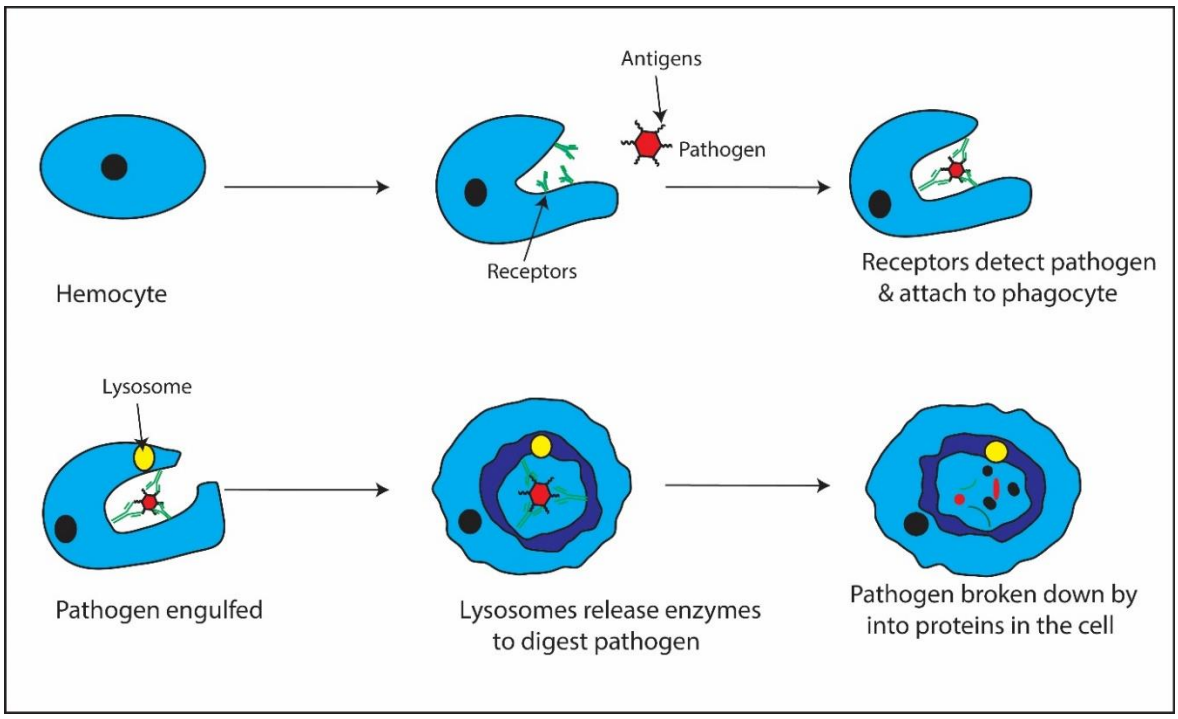


Figure 8. *Simplified representation of phases involved in phagocytosis*

Environmental parameters such as temperature, salinity, and seasonal variation, and biological parameters such as sex and age causes variations in phagocytic immune response (Parry & Pipe, 2004). These complex interactions affect phagocytic activity results during studies of toxicological effects (Parry & Pipe, 2004; Ellis et al., 2011). Parry & Pipe, (2004) observed an altering effect of Cu with environmental variables including temperature and the presence of potential pathogens.

Phagocytosis and nitric oxide synthase activation enhance the production of ROS such as NO (Smith et al., 2000; Gourdon et al., 2001; Wang et al., 2012).

2.9 Nitric oxide synthesis

Nitric oxide is a major secretory product of the enzymatic activity of NO synthase (NOS) that initiates host defense and homeostasis at the presence of xenobiotics or stress (Rodríguez-Ramos et al., 2010). It is not cytotoxic itself but plays an important role as a signal molecule in organisms and the elimination of pathogens (Hetrick & Schoenfisch, 2009). Nitric oxide and other ROS represent some of the main innate immune mechanisms in invertebrates. Metals and MP have been demonstrated to increase ROS in organisms (Davarpanah & Guilhermino, 2015). Nitric oxide is highly reactive and rapidly scavenged by endogenous compounds including molecular oxygen (O_2), thiols (e.g., MT, glutathione), and other free radicals such as hydroxide radicals. Nitric oxide is measured by measuring the stable end metabolite of NO which are nitrite (NO_2^-) and nitrate (NO_3^-) (Tsikas, 2007). Nitrite and nitrate in biological fluids can be detected and measured using methods such as absorbance, fluorescence, and chemiluminescence based approaches, electrochemistry, and electron paramagnetic resonance (Hetrick & Schoenfisch, 2009). A diazotization assay (Griess reaction) using a spectrometric absorbance-based method is used in this study. The uptake of Cu and MP induces oxidative stress which leads to failure of the antioxidant defense system (Al-Subiai et al., 2011; Lushchak, 2011). The antioxidant system includes both specific (superoxide dismutase (SOD), catalases (CAT), Selenium-dependent glutathione peroxidases), and non-specific (MT and ferritin) antioxidant proteins (Lushchak, 2011). The non-specific antioxidants are mediated by proteins that bind to transition metal ions mainly iron and Cu (Lushchak, 2011).

3. MATERIALS AND METHODS

3.1 Sampling

3.1.1 Animal collection

Mussels (*Mytilus edulis*) (n = 168) were collected at low tide from an unpolluted site in Bodø, Norway (67º12´01´´ N, 14 º 37´56´´ E) in January 2020. After collection, the mussels were transported from the collection site to the laboratory (Mørkvedbukta Research Station). The average shell length and weight of mussels were (4.14 ± 0.36 cm) and (10.71 ± 2.66 g) respectively. The mussels were acclimatized for three days by being placed in a large flow-through tank containing unfiltered seawater with a light and dark cycle of 12h:12h. The mussels were not fed during the acclimatization and exposure period. Seawater temperature (14.03 ± 2.15 °C) and dissolved oxygen (101.41 ± 2.42 %) were measured during the acclimatization and exposure experiment. No spawning or mortality was observed during acclimatization and the batch of stock mussels was used for the exposure experiment. The collection, handling, transfer, and acclimatization was carried out appropriately to minimize animal suffering or stress.

3.1.2 Sediment sampling

The tailing sediment sample used in this study was provided from a previous study by Sternal et al., (2017). One core (core 1087) from the seventeen sediment cores sampled by Sternal et al (2017) in their study was provided. The sampling methodology is fully described in Sternal et al., (2017) and references therein, and is briefly presented here. The location of the provided core is shown in Fig. 9.

Sediment core 1087 was retrieved with a box corer, from the outer fjord, at a water depth of 65m, and 14cm long of recovered sediment. Core sediment was sliced into samples of 1cm thickness and frozen immediately after retrieval. Core sediment from 0-5cm was used for this experiment (treatment with old mine tailing). Measurement of metal concentrations in sediment was done as described in Sternal et al., (2017). The concentrations of the different metals in sediment core 1087 are shown in [Appendix 1 as provided by Sternal et al. (2017)]. The fresh mine tailings (FT) used for the experiment were provided by Nussir ASA and was made up of ore (drill core) from the Nussir mine and processed at SGS Mineral Services, Canada, to simulate the commercial mining metal extraction processes as described in Pedersen et al., (2017). The fresh mine tailings used had about 1000 mg/kg Cu concentration.

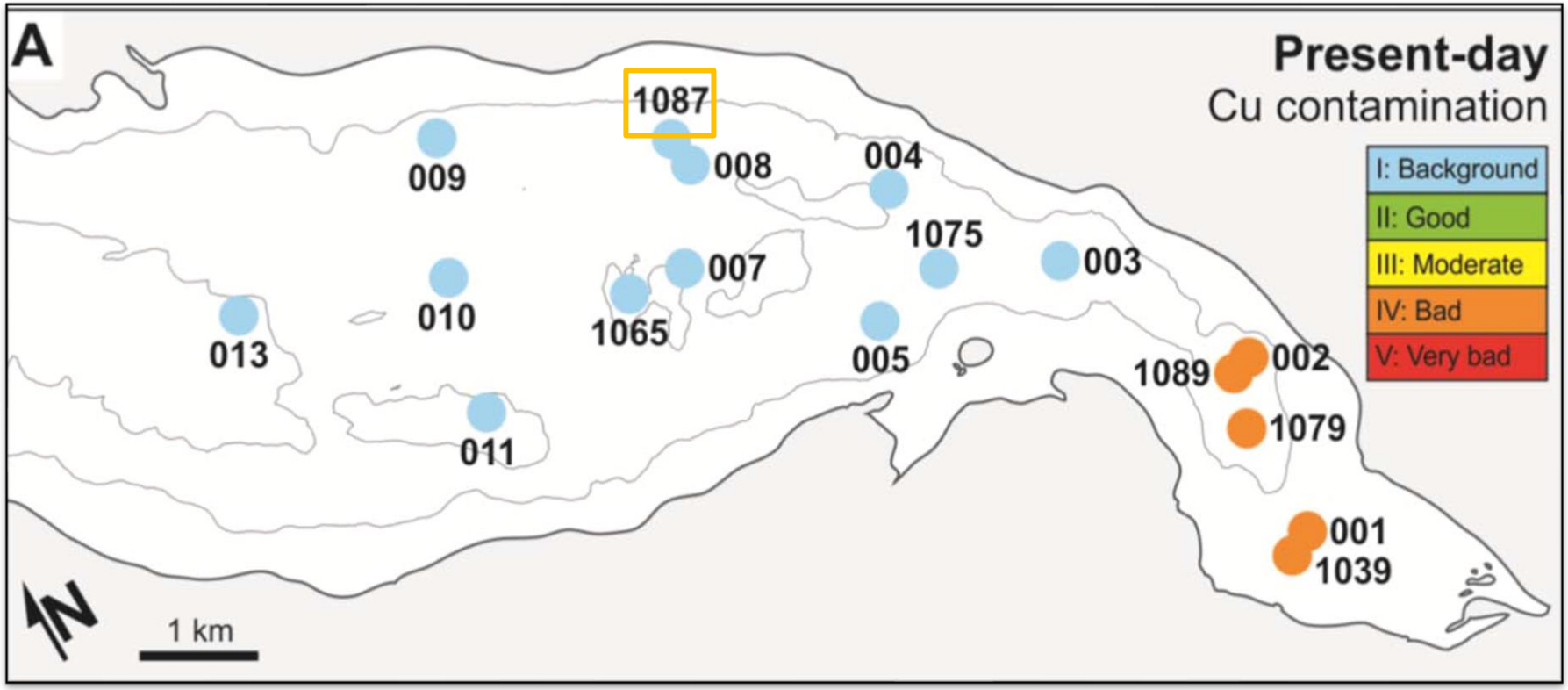


Figure 9. *A map showing the location of sampled cores in the study by Sternal et al. (2017). The provided core (1087) used in this study is indicated by the orange box. The map is reproduced from Sternal et al. (2017).*

3.2 Experimental design

The experimental and exposure designs are presented in Figures 10 and 11, respectively. All laboratory glassware and equipment were autoclaved before use. Mussels (n=7 animals per beaker) were exposed to 8 sets of different treatments in 2-liter glass beakers with each treatment being carried out in triplicate (i.e. in total 21 mussels were analyzed for each exposure treatment). Seawater filtered through a 10 µm mesh and sterilized through ultraviolet radiation was used for the exposure treatments. The treatment tanks were randomized for each replicate and the same volume of autoclaved millipore water was added to all treatment tanks to ensure equal salinity levels in all tanks.

After acclimatization, mussels were exposed for 3 days without feeding and change of water during exposure treatment. The 8 sets of exposures included exposure to a high (H) and low (L) concentration of dissolved Cu, MP alone (M), sediment from Repparfjorden alone (mine tailings deposited in Repparfjorden from the 1970s, here referred as old mine tailings) (T), a mixture of old mine tailings and MP (TM), a mixture of MP and a high concentration of dissolved Cu (MH), newly processed mine tailings (ground core drill, referred to as fresh tailings) (FT) and a control (C).

The low (20 µg/l) and high (40 µg/l) concentrations of dissolved Cu were prepared from two different primary stock solutions of Cu in distilled water using Cu chloride ($CuCl_2$) (97% purity) (Sigma Aldrich). Polyethylene (PE) (50 mg/l) MP (Clear Polyethylene Microsphere 0.96 g/cc 10-45 µm) were used for the MP exposure. The Cu-MP complex was prepared by mixing 50 mg of MP with a 50 ml Cu solution (40 µg/l) in conical centrifuge tubes. The tubes were subsequently set in 360° rotation for 72 hours at 20 rpm. This was done to allow uniform adsorption of metal on the MP. The plastic pellets were filtered out after rotation using a bottled-top vacuum filtration system with polyethersulphone (PES) membrane (pore size 0.2 µm).

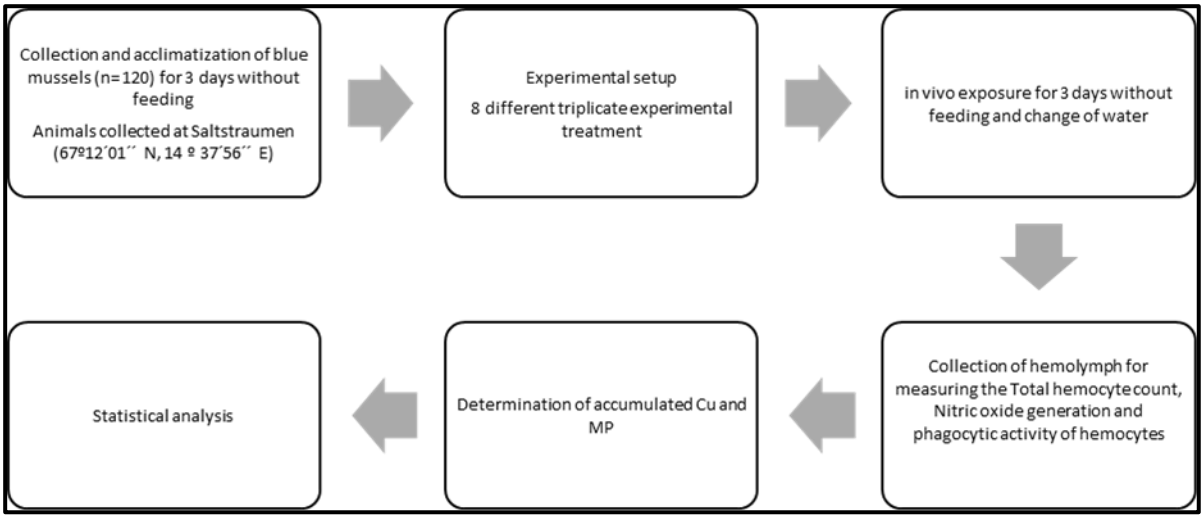


Figure 10. *Flow chart illustrating the experimental design to assess the biological effect of mussels (Mytilus edulis) after exposure to xenobiotics (Cu, mine tailing sediment of a Cu mine and MP)*

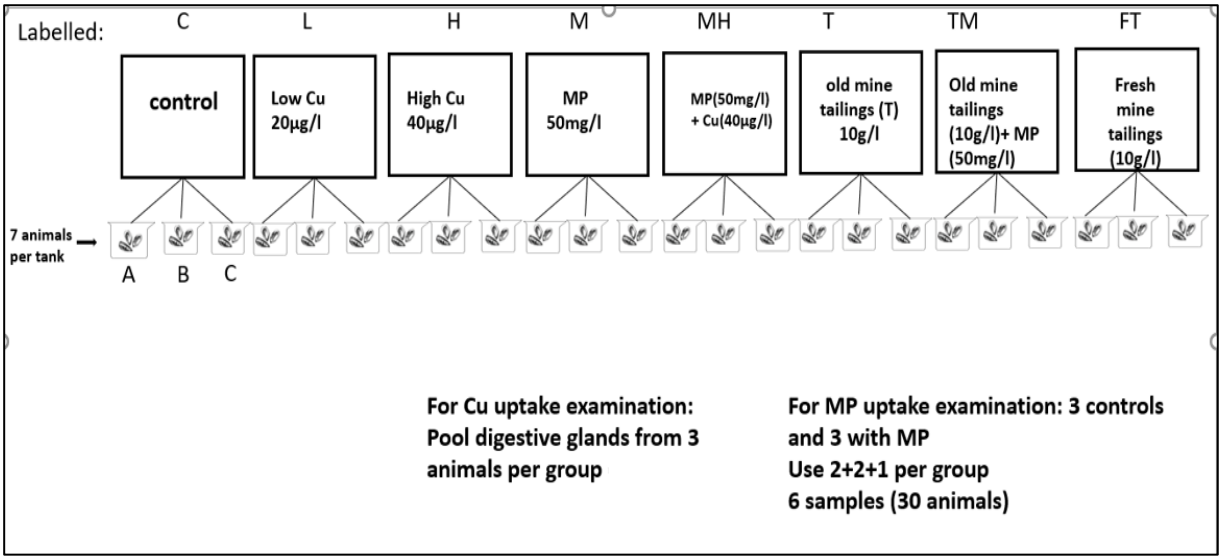


Figure 11. *Presentation of the exposure treatment types, labeling of treatment type, and sampling for Cu uptake, and MP uptake examination.*

3.3 Experimental methodologies

3.3.1 Collection of hemolymphs

After 3 days of exposure, 3 mussels from each exposure treatment (n= 3×3 for each treatment type) were collected, and with the use of a 1 ml hypodermic syringe fitted with a 21-gauge needle, hemolymph was withdrawn from the posterior adductor muscles as described in Martínez et al. (2015). The posterior adductor muscle of mussels was assessed by prying the

valves apart approximately midway towards the posterior from the byssus using a fixed scalpel blade. Water retained within the shell was drained out before attempting to withdraw hemolymph. Hemolymph samples were diluted in equal volumes of filtered seawater (FSW) (1v:1v). The seawater was filtered through a 0.2 µm bottled-top vacuum filtration system with polyethersulphone (PES) membrane to avoid clumping of hemocytes. Hemolymph was dispensed in 1.5 ml Eppendorf tubes and placed on ice until hemocyte count to avoid the mortality of hemocytes. Digestive glands were excised from the same 3 mussels from which hemolymph samples were withdrawn and from 2 other mussels of the same treatment group. Excised digestive glands were placed in labeled cryotubes and cryopreserved in liquid nitrogen (LN) during sampling.

3.3.2 Hemocyte count

A hemocytometer, an improved microscope slide, having two identical wells into which a small volume of cell suspension is pipetted; was used to measure the number of hemocytes in 10 µl of resuspended hemolymph/FSW mixture. The hemocyte concentration is expressed as cells/ml. Cell concentrations were adjusted to 2×10^5 cells/ml with FSW to avoid the coagulation of hemocytes (Antoun, 2011) before the measurement of immune parameters; the NO production and the phagocytic hemocytes. Hemolymph volumes of 100 µl and 250 µl were used for the NO and phagocytic activity of the hemocytes respectively after adjustment of hemocyte.

3.3.3 Nitric oxide assay

Nitric oxide is an unstable, volatile, and transient molecule oxidizing to nitrite and nitrate, and the concentration of these anions has been used as a quantitative measure of NO production in hemolymph supernatant (Tsikas, 2007).

Nitric oxide assay was done as described in Costa et al. (2008) with minor modifications. The measuring of NO produced by hemocytes was done using the Griess reaction which quantifies nitrite (NO^-_2) content. 50 µl of hemolymph supernatant from an incubated 100 µl was withdrawn and dispensed in a well-designed 96-well plate (shown in Appendix 2). Incubation was done at room temperature for 30 min in the dark. A 50 ml of reagent A [1.465 ml H_3PO_4

(stock 85.3%) + 48.5 ml H_2Od + 0.5 g sulfonamide] and 50 ml of reagent B [(maintained in darkness) 1.465 ml H_3PO_4 (stock 85.3%) + 48.5 ml H_2Od + 0.05 g N-naphthyl-ethylenediamine] were prepared. This was followed by adding 100 µl of reagent A and 100 µl of reagent B into each well-containing hemolymph supernatant and standards. Nitrite standards were prepared for each assay to ensure accurate quantification of nitrite levels in each experimental sample. 50 µl of the $NaNO_2$ standard solutions were dispensed into the wells designated for standards. The preparation methodology is shown in Appendix 2. The micromolar concentrations of nitrite in samples were determined from a standard reference curve generated from known concentrations (100, 50, 25, 12.5, 6.25 µM) of sodium nitrite ($NaNO_2$). Blank wells were made in the design of the plate without adding any nitrite solution but autoclaved millipore water (0µM). Technical replicates were made for each sample on the same 96-well plate to test for variability. A spectrophotometer (BMG FLUOstar OPTIMA Microplate Reader) was used to determine the optical density (OD) at 520 nm. Optical density is the measure of absorbance and is defined as the ratio of the intensity of light incident upon a material and the intensity transmitted (Tsikas, 2007).

Nitrite reacts under acidic conditions with sulfanilic acid to form a diazonium cation which subsequently couples to the N-naphthyl-ethylenediamine to produce a red-violet colored azo dye of wavelength ($\lambda_{max}\approx$540nm) (Tsikas, 2007).

3.3.4 Phagocytic activity

Phagocytic activity of hemocytes was measured as described in (Park et al., 2020) using an ImageStream$^{®X}$ Mk II Imaging Flow Cytometer (Luminex Corporation, Austin, TX, USA). The multispectral imaging flow cytometry is a means of collecting information from single cells including those from fluorescent images.

1.6 µl of fluorescent bioparticles (>0.2 µm; pHrodo™ Red *Escherichia coli* Bioparticles, Thermo Fisher Scientific) were added to 250 µl of 2×10^5 cells/ml hemolymph of each sample to be measured. Two technical replicates were made for each sample. Resuspended hemolymph and bioparticles were incubated for 2 hours at 12°C. After incubation, the cell suspension was washed. The washing process involved adding 500 µl of FSW and centrifuging at (500 g, 5 min, 4°C). This washing process was to eliminate as much debris as possible, including those

particles that were not phagocyted. The supernatant was then discarded, after which 40 µl of FSW was added to the aliquot and resuspended.

1 µl of anthraquinone dye (DRAQ5 25 mM, Thermo Fisher Scientific) was added to the hemocyte samples before being analyzed in the Flow Cytometer. The fluorescent bioparticles and DRAQ5 were analyzed with a 488 nm argon-ion laser set to 10 mW and 642 nm laser set to 8 mW. The resultant images were then analyzed using IDEAS 6.1.822.0 software. All reactions were set on ice to minimize degradation.

3.3.5 Trace metals analysis in mussel digestive glands

The digestive glands of mussels from each exposure were excised in cryotubes, stored on dry ice, and sent to the Institute of Marine Research in Bergen, Norway for analysis. Trace metal analysis in the tissue samples was analyzed using the Inductively Coupled Plasma - Mass Spectrometry (ICP-MS).

Digestive gland tissue samples were stored at −80°C until further analysis. After thawing, approximately 1.2 g wet weight of tissue from each exposure replicate was washed and acid digested using 2% HNO_3. Each sample was diluted to 25 ml with 2% Ultrapure HNO_3 before being analyzed for metal content. Trace metal concentrations were expressed as mg/kg wet weight tissue.

3.3.6 Microplastic uptake measurements

Microplastic uptake was measured using the pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS) method as described in Logemann et al. (2018) and Hermabessiere et al. (2018). Mussels from the control and MP exposures (a total of 5 mussels from the three replicates of each exposure group; 6 groups) were stored at -80 °C until analysis. After thawing in the laminar airflow (LAF) bench, the soft tissue of the mussels was excised. The excised tissue from each exposure group was pooled into one sample and digested at 40 °C for 24 h using potassium hydroxide (KOH) (1.32M of KOH). Digested tissue samples were filtered with 50 ml glass filter crucibles (ROBU® Glassfilter-Geräte GmbH) with a pore size of 10 µm – 16 µm. Samples

were transferred into to Whatman GF/C glass fiber filter and packed into the pyrolysis cup for polyethylene analysis.

Quality assurance and quality control were achieved by preventing MP contamination of samples from the operators, laboratory working environment, and used solutions. Control measures such as entering the laboratory with low abrasive shoes and cotton laboratory coat, using glass or stainless-steel equipment, using perfluorinated polymer plastic equipment when necessary, and using Milli-Q water to prepare solutions were observed. Blanks without matrices were done by placing glass jars of filtered Milli-Q water in the laboratory working area and on the LAF bench per working day. This was done to check for potential contamination from the ambient air. Recovery of MP was tested on three matrices (salmon fillet, head kidney, and liver) to check whether plastic present in samples is accurately recovered during the isolation process. Limit of quantification (LOQ) was used as a detection limit.

3.4 Data Analysis

The R studio statistical software (version 1.2.5033) was used for the data analysis. The normality of data was tested by performing a Shapiro-Wilk test to determine the parametric and non-parametric data distribution (Shapiro & Martin, 1965; Karp, 1999). In the case of parametric distribution of data, Anova (analysis of variance) test was performed to determine the significant differences between treatments by comparing the means between two groups (Karp, 1999; Ferreira et al., 2014). The Kruskal-Wallis (Kruskal & Wallis, 1952) test was used for non-parametric datasets. Bartlett's test (Bartlett, 1937) was performed to check for the homogeneity of variance of data. Welch ANOVA was performed when data violates the assumptions of homogeneity of variance. Plots were done in R studio to visualize analyzed data (Karp, 1999). The reliability of the nitric oxide production assay results was analyzed by standardized measures such as the coefficient of variability (CV%). Precise and accurate results between each sample replicates were determined using CV% < 10% (Conrad, 2018). A CV% < 10% is recommended to gauge the reliability of immunoassay results during intra-assay (Conrad, 2018). A probability value <0.05 was established to determine a statistically significant difference. Multivariant data analysis was conducted using Principal Component Analysis (PCA) to identify patterns, highlighting similarities and differences in the data. The PCA aims to present the samples and variables (exposures) in a reduced number of dimensions called Principal components (PC) (Abdi & Williams, 2010).

4. RESULTS

4.1 Nitric oxide production

The intercept and gradient obtained from the standard curves (Figure 12) from each of the 3 replicates were used to determine nitrite concentrations in hemocyte samples.

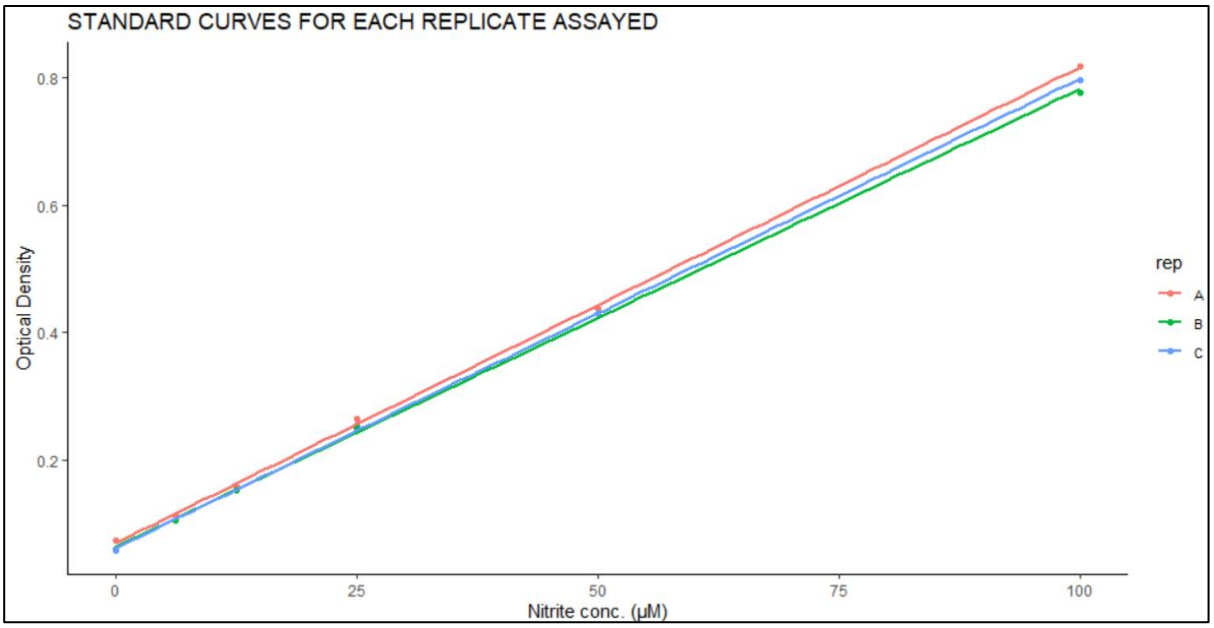


Figure 12. *Standard curves for the set of samples in each replicate. Nitric oxide concentration in each treatment sample was calculated from the standard curves using the equation (y= ax + b).*

Calculating for NO^{-}_{2} concentration in all samples in each replicate and CV%

a) Replicate A: $x = \frac{\text{(optical density value)} - (0.005125)}{(0.007471613)}$

b) Replicate B: $x = \frac{\text{(optical density value)} - (0.064257143)}{(0.007176553)}$

c) Replicate C: $x = \frac{\text{(optical density value)} - (0.062057143)}{(0.007363392)}$

d) $CV\% = \frac{\text{standard deviation}}{\text{Average (OD of technical replicate)}}$

Nitric oxide concentrations in all samples of each replicate, OD, and CV% are shown in Appendix 2. Nitric oxide concentration values with CV< 10% (marked with green color) were considered for analysis. Replicate B and C were used in the analysis.

The nitric oxide data recorded for the first replicate was unreliable due to errors in the set-up of the spectrophotometer. Fluorescence effects derived from an inaccurate experimental set-up was found to introduce a bias in absorbance measurements consistent with the findings of Laqua et al. (1988). These results were, therefore, discarded and not used for further analysis or interpretation. Negative values of NO concentrations estimated from the OD were adjusted to zero since the known concentration range of nitrite used as a standard was between 0 µM to 100 µM.

A bar graph with error bars (mean with standard deviation) was plotted to show the production of NO in hemocyte (Figure 13). Data violated the assumption of residual normality and variance homogeneity. Data on nitric oxide production of each exposure group is shown in (Table 1). The difference between H and MH was insignificant at a p-value of 0.34. Microplastic exposure and TM were also insignificantly different with a p-value of 0.083. Mussels exposed to low concentrated Cu and T recorded the highest and least concentration of NO at (5.35 ± 4.49) and (0.13 ± 0.17) respectively. Nitric oxide production between each of the eight exposures was significantly different at a p-value of 0.0072.

The PCA analysis was done on the generation of NO measured from all exposures resulted in control and TM samples being significantly correlated. Correlation coefficient values are shown in (Appendix 2). The PCA graph is shown in (Figure 14).

Table 1. *Table showing the concentrations of NO generated in mussels hemocyte after exposure treatments.*

Treatment	**Nitric levels (µM)**
Control	1.052 ± 0.939
Cu 20µg/l (L)	5.354 ± 4.346
Cu 40µg/l (H)	0.951 ± 0.591
Microplastic (M)	0.571 ± 0.829
MP + Cu (40µg/l) (MH)	2.136 ± 2.486
Repparfjord tailings (T)	0.127 ± 0.181
Repparfjord tailings + MP (TM)	2.041 ± 1.203
Drill core Nussir (FT)	1.467 ± 1.517

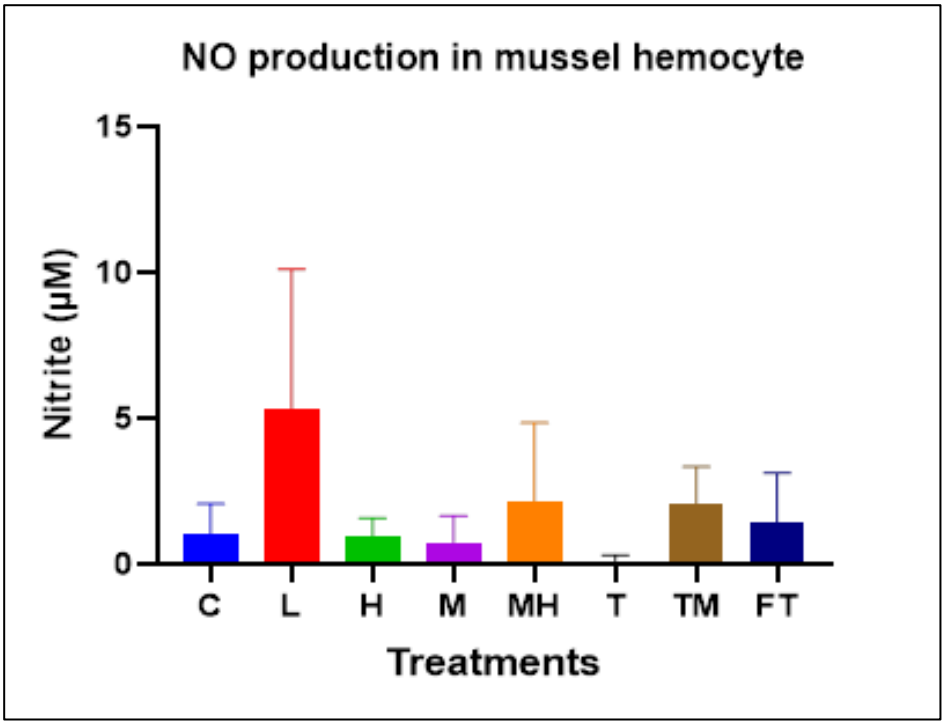


Figure 13. *Bar graph showing means of NO production of mussels after three days of exposure. (standard deviation shown as error bars).*

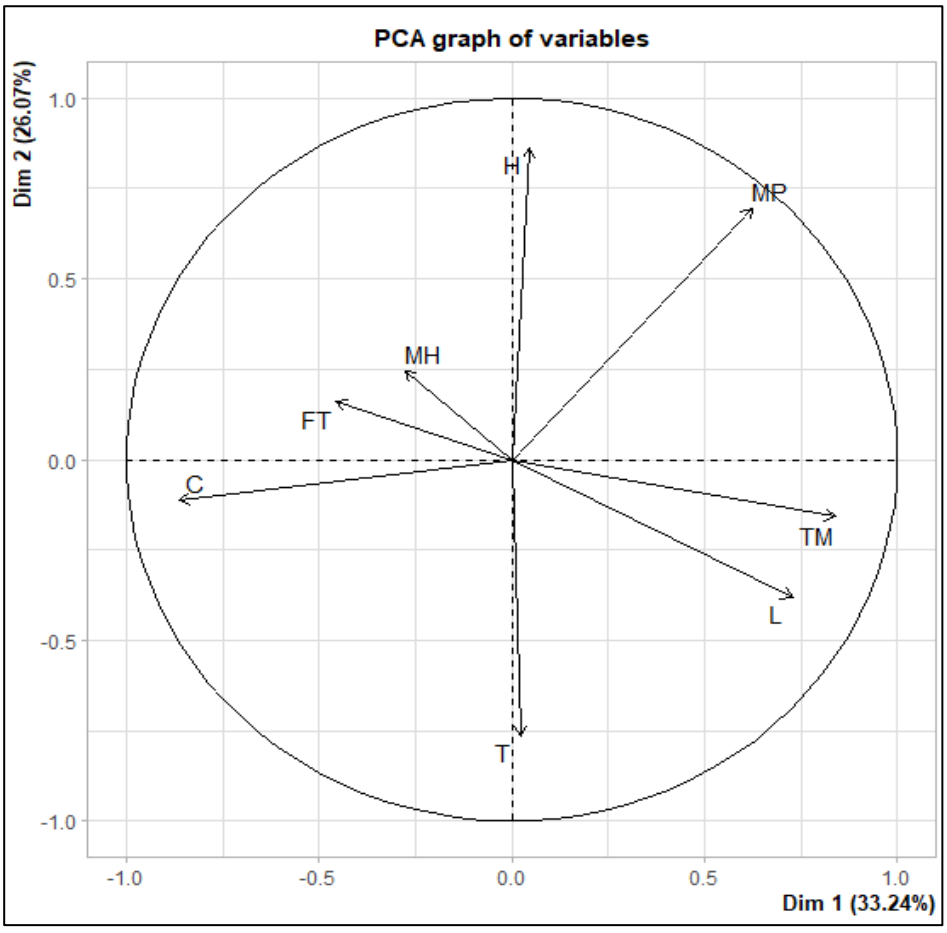
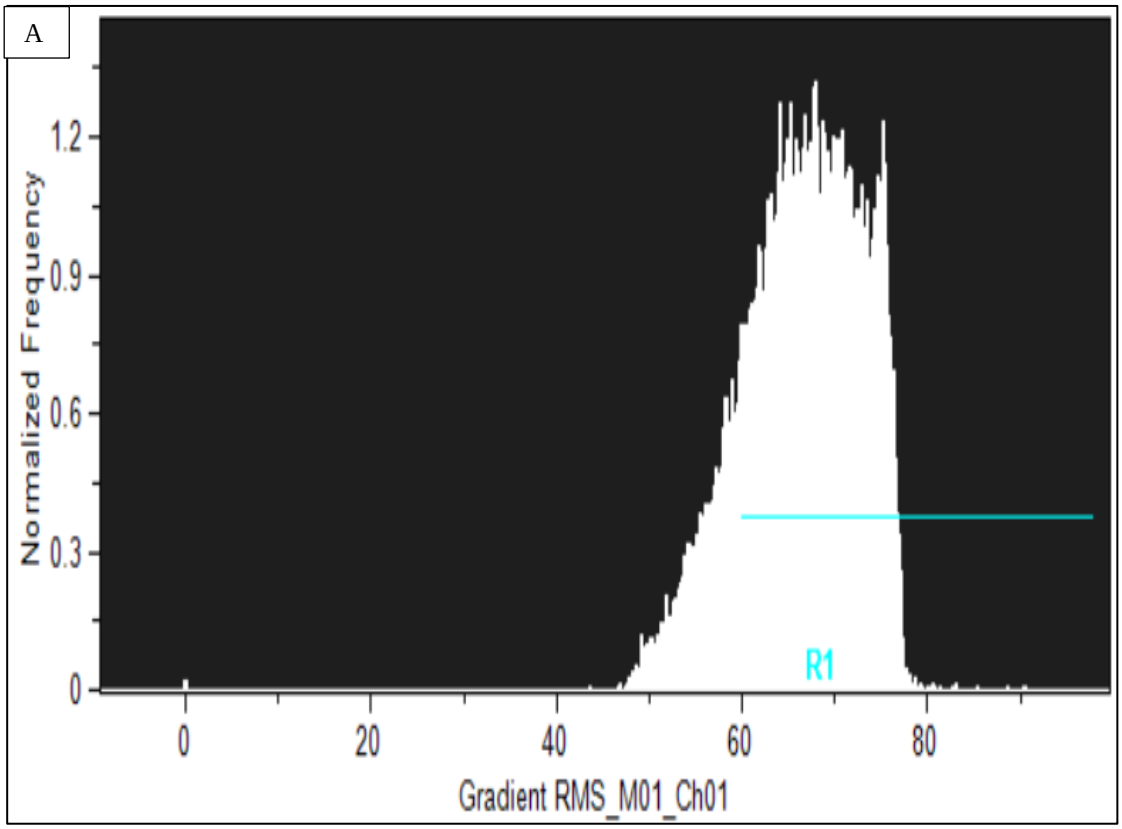


Figure 14. *PCA graph of NO generation of hemocyte in mussels exposed to all exposure treatments.*

4.2 Phagocytic activity

Gating was performed to obtain a template (modified file) to analyze all experimental files. Well-focused cells were selected using the Brightfield Gradient Root Mean Square (RMS) feature of the imaging flow cytometer. A histogram (x~y: gradient RMS ~ Normalized frequency) was plotted in channel 1 (CH01) which allowed high-quality images with RMS> 50 to be obtained. A line region covering a high Gradient RMS was drawn to gate focused cells as shown in (Figure 15A). A scatter plot (x~y: Brightfield Area ~ Brightfield Aspect Ratio) of all the hemocytes were generated. The hemocyte population was then gated by separating single cells from impurities (debris or artifacts) in channel 1 (Figure 15B). A histogram of internalization was plotted in channel 2 (CH02) to determine the number of phagocytic cells. A region to gate internalized hemocyte was drawn by viewing the imagery in selected bin mode. Phagocytic cells were gated above 0.3 normalized frequency as shown in (Figure 15C). Images in channels 1 and 2 are shown in (Figure 15D).



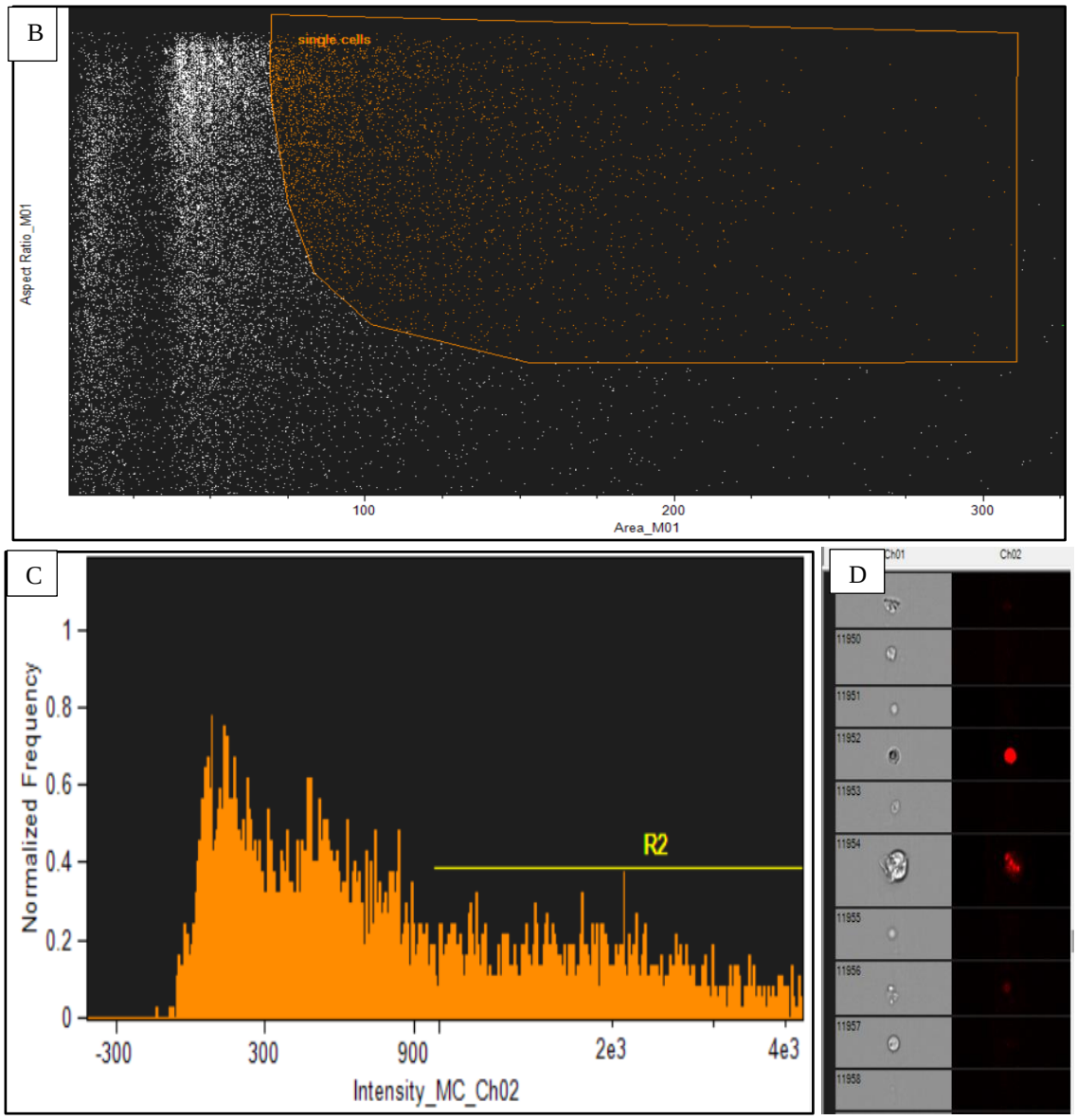


Figure 15. *(A) shows a histogram plotted in channel 1 to gate best-focused hemocytes, (B) represents a scatterplot plotted in CH02 to gate hemocyte population, (C) shows a histogram of internalization plotted in channel 2 to gate phagocytic cells and (D) represents Channel 1 and 2 show images of focused cells and phagocytic cells.*

The number of phagocytic cells measured from hemocyte samples from each replicate is shown in Appendix 3. Phagocytic cells are expressed as percentages of the total hemocyte count.

A bar plot was plotted to show the phagocytic activity of hemocytes after exposure (Figure 16). Data violated the assumption of normality and variance homogeneity. Data on the percentages of phagocytic cells in each exposure group is shown in (Table 2). Differences in the phagocytic activity of hemocytes between treatment types were insignificant. Insignificant differences were observed between (H and MH) and between (M and TM) at p-values of 0.2 and 0.067,

respectively. A high percentage of phagocytic cells was recorded for mussels exposed to L (39.77 ± 19.28 %).

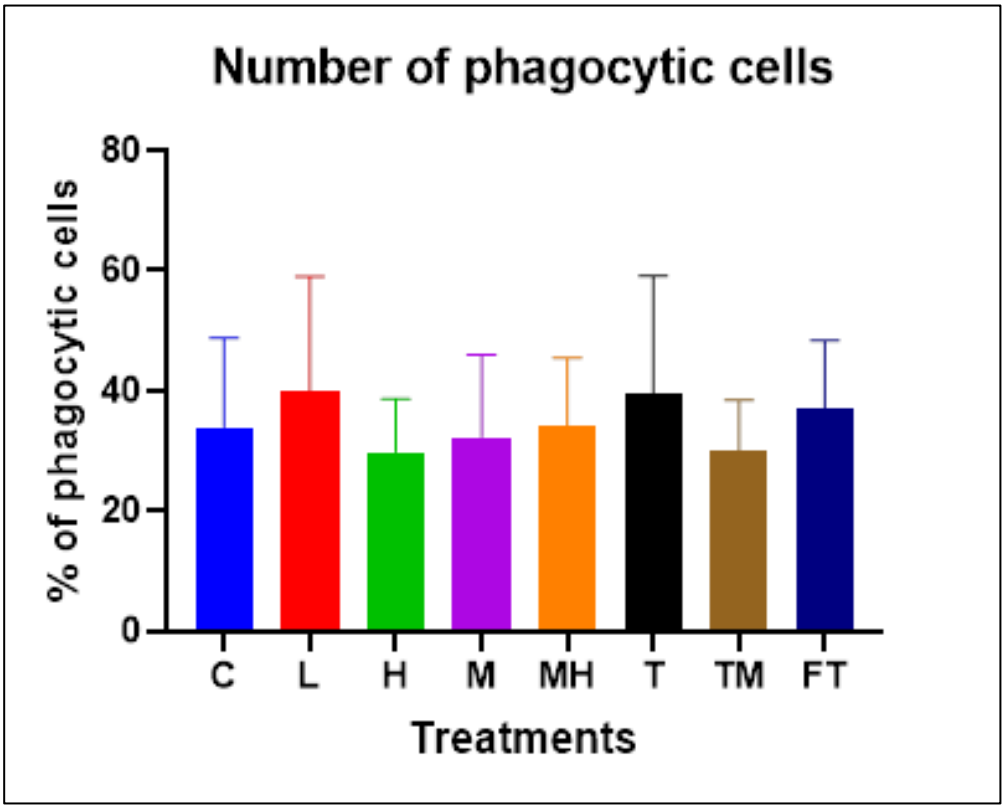


Figure 16. *Bar graph showing the phagocytic activity of hemocytes after exposure (standard deviation shown as error bars).*

Table 2. *Table showing the percentage of phagocytic hemocyte in mussels after exposure.*

Treatment	**Phagocytic hemocytes (%)**
Control	33.991 ± 14.791
Cu 20µg/l (L)	39.772 ± 19.280
Cu 40µg/l (H)	29.808 ± 8.805
Microplastic (M)	32.276 ± 13.695
MP + Cu (40µg/l) (MH)	34.262 ± 11.2666
Repparfjord tailings (T)	39.576 ± 19.526
Repparfjord tailings + MP (TM)	29.912 ± 8.538
Drill core Nussir (FT)	37.146 ± 11.222

A PCA done on the phagocytic activity of hemocytes of mussels exposed to all exposure treatments showed a cumulative variance of 77.61% for the two principal components. PC1 and PC2 accounted for 49.24 % and 28.37 % of the variability among the exposures respectively. FT contributed little to the variability of PC1. There was a significant positive correlation between the phagocytic activity of hemocytes for mussels exposed to M, H, T, MH, TM, and the control group. The correlation coefficients and p-values < 0.05 are shown in (Appendix 3).

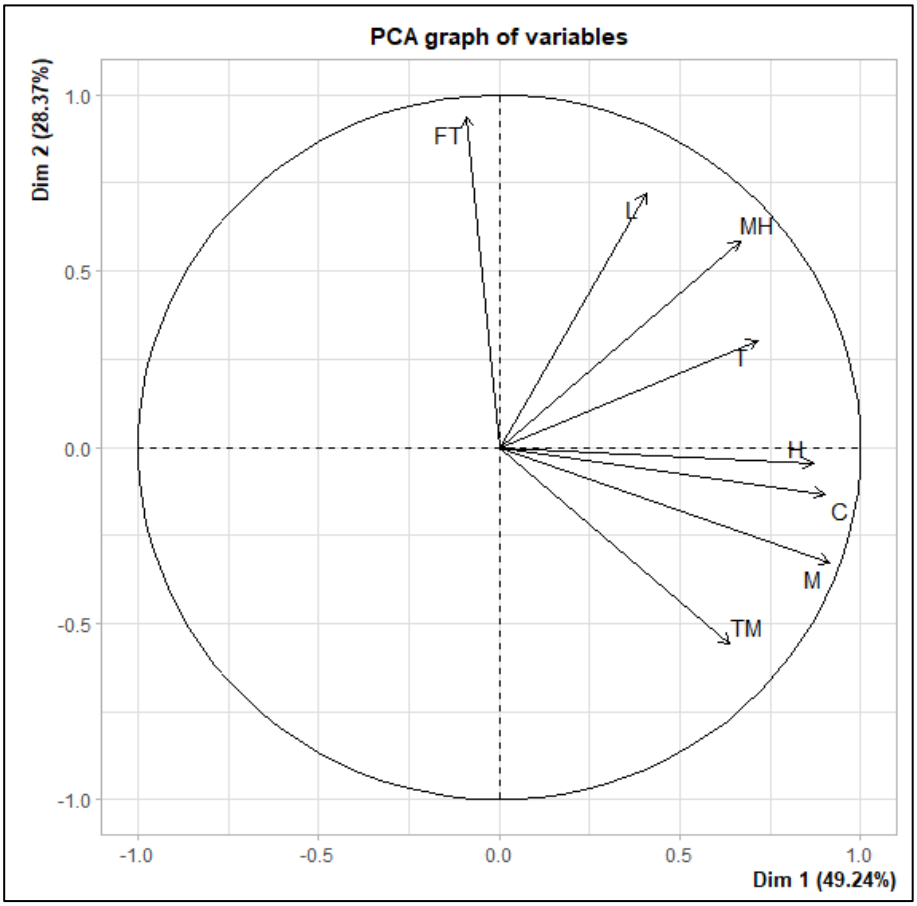


Figure 17. *PCA graph showing the phagocytic activity of hemocytes of mussels in all exposure treatments.*

4.3 Trace metals accumulation in mussel digestive glands

Iron and zinc recorded the highest concentrations in the mussel's digestive glands as shown in (Figure 18). Iron was shown to have a greater variability between the various exposures. Copper and the other trace metals measured were insignificantly different between the exposures. A high Cu concentration was, however, measured in mussels exposed to the fresh mine tailings as

shown in (Figure 19). A PCA analysis was done to evaluate the relationship between the various exposures (variables) that resulted in a positive correlation between the exposures concerning the accumulated trace metals.

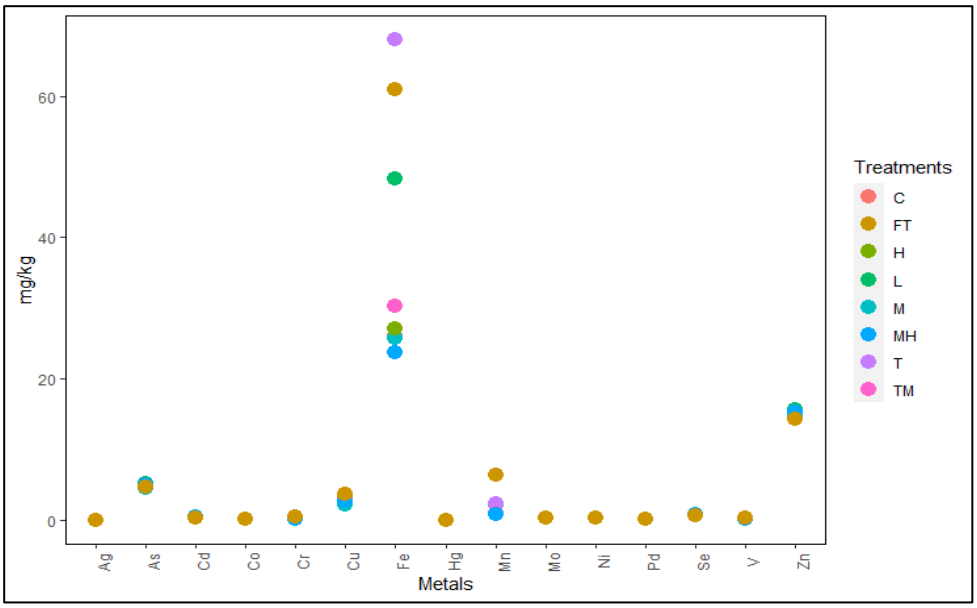


***Figure 18**. Graph showing the variability of trace metals in the digestive glands of mussels in the various treatments.*

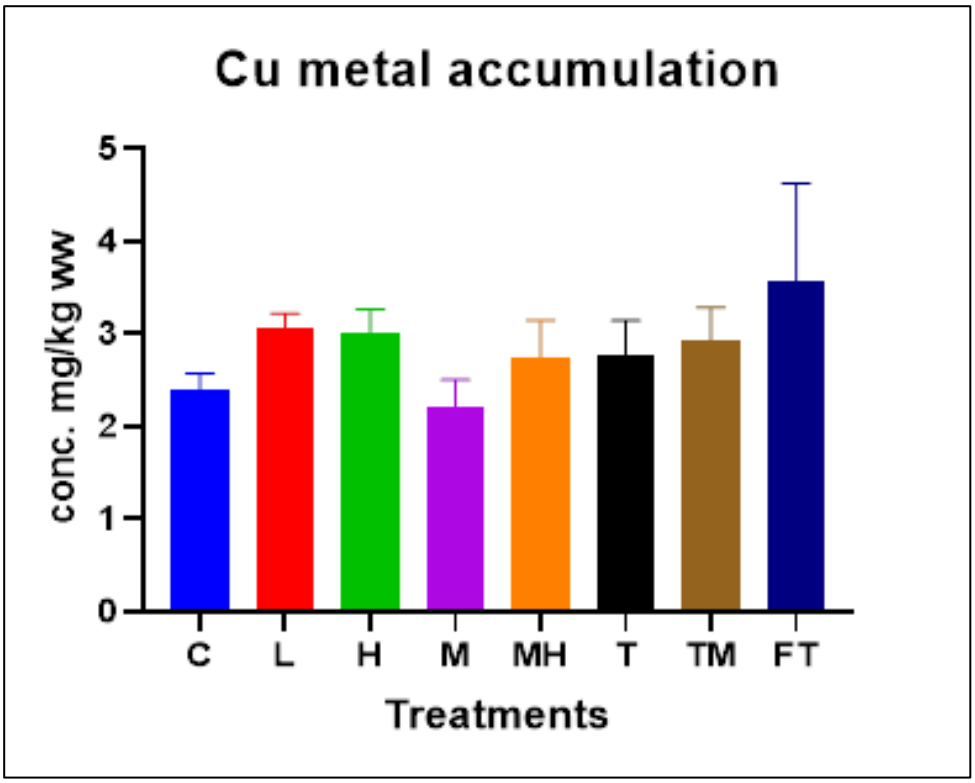


***Figure 19.** Bar graph with error bars showing the concentration of Cu in the digestive glands of exposed mussels. (mean ± stdev).*

The cumulative variance of the PCA of trace metals accumulated in the digestive glands of the blue mussels in all exposures was 99.9 % for the two principal components (Dim1 and Dim2 as shown in Figure 20). PC1 and PC2 accounted for 97.8 % and 2.1 % of the variability among the exposures, respectively. The correlation coefficients between the variables and p-values are shown in (Appendix 4).

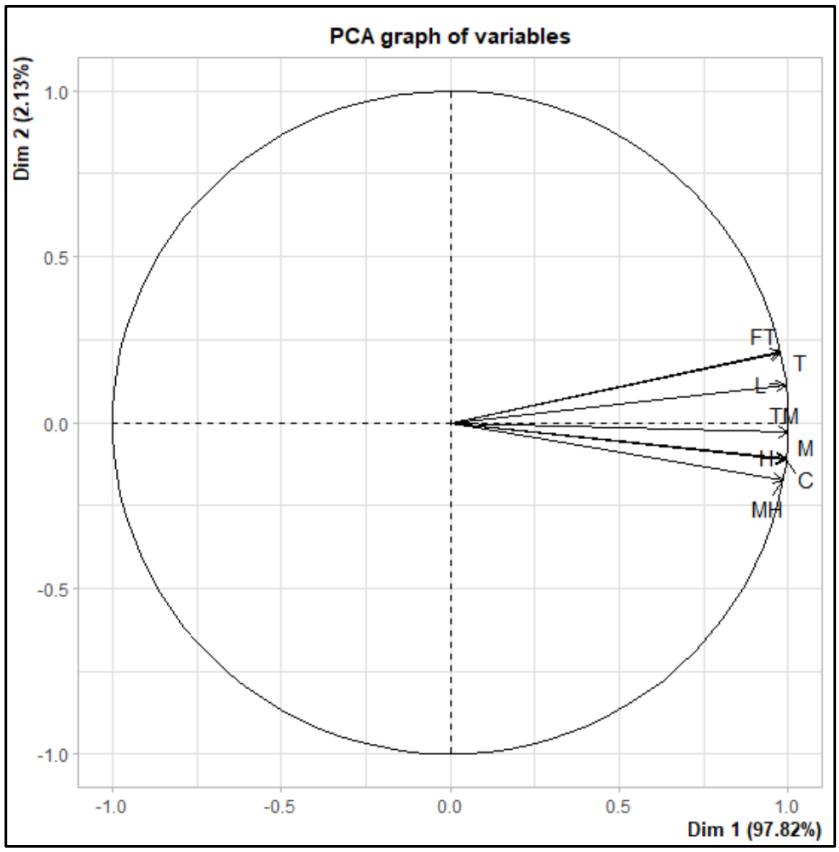


Figure 20. *PCA graph showing trace metals accumulation in mussels' digestive gland in all exposure treatment.*

The concentration of trace metals in the digestive glands of mussels is shown in (Table 3).

Table 3. *Metal concentrations were measured in the digestive gland of mussel samples from the 8 exposures.*

treatment	Metals concentration (mg /kg ww)														
	Cu	Ag	As	Cd	Co	Cr	Fe	Hg	Mn	Mo	Ni	Pb	Se	V	Zn
C	2.4 ± 0.17	0.01 ± 0.01	4.67 ± 0.38	0.41 ± 0.08	0.07 ± 0.01	0.33 ± 0.21	26 ± 2.65	0.03 ± 0.01	0.87 ± 0.08	0.32 ± 0.05	0.28 ± 0.05	0.08 ± 0.01	0.79 ± 0.08	0.19 ± 0.02	15 ± 1
M	2.2 ± 0.3	0.02 ± 0.01	4.47 ± 0.12	0.35 ± 0.04	0.07 ± 0.01	0.2 ± 0.03	25.67 ± 3.06	0.03 ± 0.001	0.84 ± 0.07	0.27 ± 0.01	0.27 ± 0.05	0.07 ± 0.002	0.71 ± 0.08	0.21 ± 0.02	14.67 ± 0.58
L	3.1 ± 0.15	0.01 ± 0.01	5.17 ± 0.25	0.34 ± 0.05	0.08 ± 0.02	0.21 ± 0.08	48.33 ± 43.5	0.03 ± 0.004	2.16 ± 1.95	0.29 ± 0.05	0.27 ± 0.08	0.11 ± 0.05	0.76 ± 0.11	0.28 ± 0.17	15.67 ± 1.15
H	3 ± 0.26	0.01 ± 0.01	5 ± 0.1	0.35 ± 0.03	0.08 ± 0.01	0.21 ± 0.05	27 ± 2.65	0.03 ± 0.003	0.92 ± 0.18	0.32 ± 0.02	0.29 ± 0.08	0.08 ± 0.009	0.75 ± 0.1	0.22 ± 0.04	15.33 ± 1.15
T	2.8 ± 0.38	0.02 ± 0.02	4.67 ± 0.31	0.32 ± 0.06	0.09 ± 0.01	0.39 ± 0.13	68 ± 34.18	0.03 ± 0.001	2.27 ± 0.91	0.29 ± 0.01	0.33 ± 0.07	0.17 ± 0.07	0.75 ± 0.06	0.37 ± 0.13	15.33 ± 1.53
TM	2.9 ± 0.35	0.03 ± 0.01	4.97 ± 0.31	0.39 ± 0.10	0.07 ± 0.02	0.26 ± 0.04	30.33 ± 4.93	0.03 ± 0.002	1.03 ± 0.06	0.3 ± 0.05	0.24 ± 0.04	0.17 ± 0.03	0.69 ± 0.07	0.27 ± 0.04	14.33 ± 1.53
MH	2.7 ± 0.42	0.01 ± 0.01	4.97 ± 0.21	0.45 ± 0.13	0.08 ± 0.01	0.22 ± 0.04	23.67 ± 2.52	0.03 ± 0.001	0.88 ± 0.06	0.28 ± 0.04	0.31 ± 0.05	0.08 ± 0.008	0.76 ± 0.04	0.22 ± 0.01	15.33 ± 1.15
FT	3.6 ± 1.06	0.02 ± 0.01	4.67 ± 0.35	0.32 ± 0.09	0.09 ± 0.01	0.4 ± 0.11	61 ± 29.51	0.03 ± 0.003	6.37 ± 6.77	0.23 ± 0.04	0.26 ± 0.06	0.12 ± 0.09	0.73 ± 0.05	0.28 ± 0.03	14.33 ± 1.15
Exposure difference	ns	Ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Trace metal pattern: Fe > Zn > As > Cu > Mn > Se > Cd > Cr > Mo > Ni > V > Pd > Co > Hg > Ag															

Data are presented as mean ± sd of the 3 replicates of each exposure with metal concentrations expressed as mg/ kg wet weight tissue. There were insignificant differences between the exposures for each metal using a one-way ANOVA test and reporting insignificance differences as "ns".

4.4 Microplastic accumulation

Polyethylene MP was measured in both the control and the MP exposed samples. Microplastic accumulation was insignificantly different between the control and exposed samples (Table 5).

Table 5. *Table showing MP accumulation in the control samples, MP exposed mussels, and blanks.*

Treatment		**PE (µg/g wet weight)**
Blank	Room	0.85
	Room	0.16
	LAF bench	<LOQ
	LAF bench	<LOQ
Control group	Control	4.03
	Cu 20µg/l (L)	2.34
	Cu 40µg/l (H)	36.4
MP exposed group	Microplastic (M)	1.5
	MP + Cu (40µg/l) (MH)	277
	Repparfjorden tailings + MP (TM)	54.9

LOQ: limit of quantitation (0.02 µg/g)

5. DISCUSSION & CONCLUSIONS

5.1 Immune response of mussels (NO production and phagocytoses)

The concentrations of nitric oxide and the percentage of phagocytic hemocyte in mussels' hemolymph varied among the exposures. There were statistically insignificant differences between the single and combined exposures for both NO generation and phagocytic hemocytes which may be attributed to several factors. These may include the lack of feeding, hemocytes' ability to regulate Cu abundance, the ability of mussels to ingest and depurate MP, the background concentration of Cu in the Repparfjorden Cu mine sediment (sediment from the outer fjord), and the decrease in Cu bioavailability due to the sediment acting as a sink for metal pollutants.

The lysosomes of hemocytes regulate the homeostasis of metals via the accumulation and detoxification of metals (Polishchuk & Polishchuk, 2016). In addition to the intralysosomal accumulation of metals, Cu is found to have a greater effect on mussels in continuous or repeated laboratory exposures than discontinuous exposures (Davenport, 1977). Mussels were discontinuously exposed in this experimental study. The ingestion and depuration of MP by mussels were not measured in this study. Some studies have observed mussels' ability to ingest and egest MP xenobiotics after uptake (Ward & Shumway, 2004; Woods et al., 2018).

A recent paper by Chae & An (2020) showed that a single exposure of mussels to MP without food resulted in rapid egestion from the mussels as compared to a slow egestion of MP by mussels exposed to algal food. They attributed this occurrence to mussels' ability to differentiate between food and other unusual suspended particles such as MP. In this project, the same polyethylene MP as used by Chae & An (2020) was used, and the mussels were not fed. The observed insignificant difference between the alone and combined exposures in this study may, therefore, be attributed to this phenomenon.

The Repparfjorden Cu mine tailings used for the experiment contained an average Cu concentration of 18 mg/kg, a concentration below the Class I or background level of Cu concentrations in sediment according to the Norwegian Environmental Quality Classification System. Core 1087 sampled from the outer fjord is shown to be natural fjord sediment containing natural background concentration levels of all heavy metals reflecting local sediment

sources (Sternal et al., 2017). This suggests that there has been very little contaminant transport from the historic disposal site to the outer fjord. The impact of the tailing deposits in the outer fjord on marine biota in general and benthic organisms such as mussels, in particular, is, therefore, likely to be minor.

Mussels exposed to 20 µg/l dissolved Cu showed the highest concentration of NO generation. Pipe et al, (1999) observed a similar result in *Mytilus edulis*, in which mussels exposed to low dissolved Cu concentration showed an increase in the generation of reactive oxygen metabolite. The high NO generation in this study is, therefore, interpreted to be a result of the increase in circulating hemocytes and is consistent with the findings of Pipe et al. (1999). Exposure to low concentration dissolved Cu results in the increase of eosinophilic hemocytes as compared to basophilic hemocytes and vice versa (Pipe et al., 1999). Eosinophilic hemocytes play a role in the generation of reactive metabolites (Pipe et al., 1997). A 100% increase (40 µg/l) in dissolved Cu concentration, therefore, resulted in lower NO generation due to the decrease in eosinophilic hemocytes.

The exposure of mussels to 40 µg/l led to a decrease in cell viability. This decrease in cell viability is observed in the lower recorded percentages of the phagocytic activity of the mussels. Reactive nitrogen species interplay with ROS causing cell death (Zhao, 2007; Orrenius et al., 2011). Nguyen et al. (2018) in an immunotoxicity study recorded increasing hemocyte mortality, production of ROS, and apoptosis of hemocytes exposed to increasing concentrations of Cu compared to a low Cu concentration. Eosinophilic hemocytes play an active role in phagocytosis (Pipe et al., 1997), which is reflected in the higher percentage of phagocytic hemocytes in mussels exposed to 20 µg/l as compared to 40 µg/l dissolved Cu. Phagocytic activity is significantly reduced by the induction of environmental stressors but this phenomenon is less generalized due to the differential stimulation of phagocytic receptors (García-García et al., 2008). As there were insignificant differences and effects on the immune response in the mussels, it suggests that the concentrations of Cu present in the area outside the historic disposal site of Repparfjorden are not significant enough to negatively impact the mussels and trigger an immune response. This agrees well with the natural background concentrations of metals in core 1087 and confirms the relatively low dispersal or transport of contaminants from the historic disposal site to the outer fjord. The likelihood of observing pristine benthic organisms and colonization of new species in areas outside the historic disposal site is, therefore, considered to be high.

The insignificant differences between the immunotoxicity of Cu concerning the production of NO and phagocytic activity of cells in the presence and absence of MP ($p > 0.05$) lead to the acceptance of the first null hypothesis in this study i.e., "the immunological effect of Cu is not affected by MP in mussels co-treated with Cu-rich mine tailings and MP (H_{01})".

5.2 Metal accumulation

As filtering organisms, bivalves readily absorb metals from their surroundings. The concentration of metals accumulated in their tissues depends on underlying factors such as essentiality, bioavailability, chemical speciation, duration, and concentration of metals (Rainbow & Luoma, 2011). Copper concentration in the blue mussels was insignificantly different between the different exposures. The insignificant difference may be due to the absence of food and the discontinuous exposure of blue mussels during this experiment as explained previously. Blue mussels filter the ambient water at a maximum rate but under suboptimal environmental conditions, including low and high algal concentrations, the filtration rate is reduced. Exposure to no or very low algae concentration leads to reduced valve gap or complete closure (Riisgård et al, 2011). The absorption of Cu on MP (Cu-MP complex) may decrease the bioavailability of the metals to the mussels, especially if the Cu-MP complex magnitude is low or not ingested by the mussels. The binding of Cu on MP may increase the bioavailability of metals to mussels when the magnitude of the Cu-MP complex is high, or uptake is high. The insignificant differences in Cu accumulation between the absence and presence of MP suggest that if the binding of Cu to the MP occurred during the preparation of the Cu-MP complex in this experiment, then the magnitude of the process was probably not high enough to influence the accumulation of Cu in the digestive glands of the mussels. Metal adsorption to MP is known to be greater in aged pellets than in new polyethylene pellets (Holmes et al., 2014). Copper concentration was, however, high in the digestive glands of mussels exposed to the fresh mine tailing depicting a high bioavailability of Cu.

The trace metal analysis shows high concentrations of essential metals and low concentrations of the non-essential metals (Fe > Zn > As > Cu > Mn > Se > Cd > Cr > Mo > Ni > V > Pd > Co > Hg > Ag). Arsenic (As), a non-essential trace metal, was high in the mussels. Most of the As accumulated in mussels are likely to be organic arsenic (As) compounds (e.g. arsenosugars and arsenobetaine) which plays an important role in osmoregulation as suggested by (Whaley-

Martin et al., 2012). This pattern of trace metal concentration (shown in Table 4) in this present study may support the view that mussels can limit and regulate the accumulation of non-essential and essential metals (e.g. Rainbow & Luoma, 2011, Chandurvelan et al., 2015). However, this cannot be conclusively determined to be the case in this study as the mine tailings and sediments used in the experiments had low concentrations of non-essential metals.

Sediment acts as a reservoir for contaminants including metals and the insignificant difference of trace metals measured between mussels exposed to mine tailing sediment and those not exposed to the mine tailings suggest a possible short exposure time. It is also conceivable that the metals were bound to the sediment particles in a way that did not make them available for uptake in the mussels.

The sediments in the outer fjord outside the historic disposal area of Repparfjorden appear to be chemically inert after acute exposure. This finding is in line with previous studies (Sternal et al., 2017), which suggested that sediments from these areas of Repparfjorden are relatively inert. This finding also supports the projected "medium to low consequences" on the far zone as anticipated by the Nussir EIA report. It may thus be speculated that the Cu-enriched sediments from Repparfjorden collected outside the historic disposal area have minor impacts on the marine habitat.

5.3 Microplastic accumulation

Microplastics were measured in both the control and the MP exposed samples. Microplastic presence in control samples could either be due to laboratory contamination (e.g. airborne contamination) (Dehaut et al., 2019) or the sampling of wild mussels. The Norwegian Institute for Water Research (NIVA) reported Bodø municipality to be polluted with MP after a monitoring program conducted along the coast of Norway using blue mussels as bioindicators (Lusher et al., 2017). The sampling location in this study was, however, further away from the location in Lusher et al. (2017) study (see Appendix 5).

Microplastics in the control samples indicates the presence of MP at the sampling site of mussels. The mussels are shown to have had varying levels of MP before the experiment. This is consistent with the findings of Lusher et al. (2017), wherein, northern sites including Bodø were observed to have higher numbers of MP per individual and grams wet weight. The control and exposed samples were insignificantly different, however, the MP measure in the control

samples was averagely lower than the MP exposed samples. Microplastics in control samples suggest the retention of MP in the soft tissues of mussels even after depuration. The retention time of MP in mussels has been observed to increase with decreasing sizes of MP (Browne et al., 2008, Van Cauwenberghe & Janssen, 2014).

Microplastics in individual mussels were not determined due to the pooled soft tissues of mussels in each treatment group. Mussels exposed to MP alone (M) and MH recorded the lowest and highest MP accumulations, respectively. The low MP accumulation in mussels exposed to MP alone may be due to the increasing depuration of MP in the absence of food (Fernández & Albentosa, 2019; Chae & An, 2020). The low MP accumulation can also be attributed to the assumption that mussel's ingestion rates decrease with decreasing food concentration. The rate of depuration of MP may affect the capacity of potentially adsorbed contaminants to be desorbed, as well as the accumulation of MP. (Fernández & Albentosa, 2019) study on the uptake, elimination, and accumulation of MP in mussels reported small MP (2 – 4 µm) to be less efficiently cleared than larger MP (> 10 µm) and larger MP (> 10 µm) to be depurated faster than smaller ones. Microplastics used in this study were > 10 µm suggesting a higher depuration. The use of the same concentration of MP throughout this experiment also may affect the significant difference in MP accumulation between the various exposures. The bioavailability of MP depends on their concentration and abundance.

5.4 Conclusions

This study is the first to investigate the simultaneous effect of Cu-mine tailings and MP on blue mussels. The insignificant difference ($p > 0.05$) in the immunological effects of Cu in the absence and presence of MP and Cu rich mine tailing sediments led to the acceptance of the first null hypothesis; "the immunological effect of Cu is not affected by MP in mussels co-treated with Cu-rich mine tailings and MP", of this study. The exposure to L and H resulted in high and low production of NO, respectively. The high NO production in mussels exposed to low concentrations of dissolved Cu indicates the induction of oxidative stress. No combined effect of Cu tailings and MP (50 mg/l) on mussels was observed after 3 days of exposure. The insignificant difference ($p > 0.05$) in the accumulation of Cu in the digestive of glands of mussels in the presence and absence of MP resulted in the acceptance of the second null hypothesis; "Cu accumulation in the digestive glands of mussels exposed to a high

concentration of dissolved Cu alone is not higher than the combined exposure of a high concentration of dissolved Cu and MP", of this study. This finding probably relies on the short exposure duration of 3 days, as well as low filtering rate due to no feeding with algae.

Outlook for further research

In this study, gene expression analysis could not be completed due to delays caused by the outbreak of the Covid-19 pandemic. Any future work on this topic should include this analysis. The study or analyses in the changes of genes expressed by an organism reveals exposure to xenobiotics, representing the cellular events contributing to the onset and progression of xenobiotics- induced adverse effects. The results from this study strongly suggest that the collected mussels already contained varying amounts of MP as evidenced in the control samples. The collection site of the mussels is likely not as unpolluted as was thought during sampling. To avoid this, future studies must use commercially purchased mussels which are unpolluted. Long term exposure of mussels to aged microplastic pellets in the presence of food should also be considered, as the lack of feeding may affect the uptake of metals.

Although the study did not find a significant combined effect of tailings and MP on the mussels, possibly due to previously discussed factors, it may be worthwhile repeating the study in a more systematic way taking into account any shortfalls in the current study. If a significant combined effect is found, it would have profound environmental implications considering the ubiquitous nature of MP in the marine environment, making it necessary to include such investigations in any EIA on submarine disposal of mine tailings.

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APPENDICES

APPENDIX 1. *Table showing the concentration of different metals in sediment core 1087 sampled from Repparfjorden. Only the first 5cm (marked green) of the core was used in this study. (Modified from Sternal et al., 2017)*

Depth (cm)	pH	Cond (mS/cm)	Eh (mV)	LOI conc (%)	TOC (%)	S (%)	Carbonate	Concentration (mg/kg)																								
								Cl	NO_3	SO_4	Al	As	Ba	Cd	Co	Cr	Cu	Fe	K	Mg	Mn	Mo	Na	Ni	P	Pb	Sb	Sr	V	Zn	Zr
0-1	8.0	14.2	95.1	3.2	0.98	0.08	2.5	11426	40	1729	5545	4.6	35	0.43	3.0	25	18	8667	2884	4635	149	3.7	10141	12	726	6.3	<0.1	7.8	17	39	0.52
1-2	8.0	12.5	95.0	4.3	0.98	0.08	1.1	11062	38	1641	5969	3.8	36	0.43	3.1	27	20	8892	3045	4717	144	3.7	8730	13	691	6.5	<0.1	5.9	18	34	0.47
2-3	8.1	10.9	94.3	2.7	0.98	0.08	0.8	8920	4	1314	5921	4.6	36	0.46	2.9	27	20	8820	2851	4548	145	4.2	7169	13	648	6.7	<0.1	4.4	19	45	0.68
3-4	8.1	9.9	93.6	2.5	0.98	0.12	0.8	8047	5	1230	5756	4.7	35	0.46	3.0	26	17	8689	2764	4368	139	4.1	6424	13	625	6.3	<0.1	4.0	18	33	0.49
4-5	8.2	8.7	91.9	4.5	0.98	0.12	1.5	7006	4	1084	5256	5.1	30	0.44	2.9	24	15	8908	2563	4084	130	4.6	5335	13	571	5.2	<0.1	4.3	17	28	0.46
5-6	8.3	7.7	90.9	2.2	0.98	0.12	3.7	5750	4	889	5628	4.9	31	0.47	3.1	24	16	8389	2810	4147	136	4.2	5500	12	581	4.6	<0.1	8.4	18	29	0.46
6-7	8.3	7.3	89.8	2.1	0.98	0.36	5.2	5318	8	857	5673	5.3	34	0.51	3.4	23	14	9866	2860	4234	142	6.1	5063	13	576	3.5	<0.1	19.4	18	29	0.48
7-8	8.2	7.4	92.2	2.1	0.74	0.18	1.7	5187	11	806	7728	4.3	41	0.62	3.9	29	16	11593	3868	5366	181	4.8	5076	15	575	3.6	<0.1	6.3	23	34	0.59
8-9	8.2	7.1	91.6	2.2	0.74	0.18	2.2	5159	5	820	8195	4.7	41	0.64	4.5	30	17	12104	4115	5667	188	5.4	5126	16	599	4.0	0.7	4.6	24	35	1.37
9-10	8.2	6.8	92.0	2.1	0.74	0.18	2.2	5602	5	894	9082	3.8	46	0.68	5.1	32	19	5622	4568	6629	219	5.1	5278	18	534	4.7	<0.1	5.5	25	36	0.99
10-11	8.2	7.9	92.7	1.9	0.52	0.18	1.3	6227	4	987	9871	5.2	49	0.79	5.4	35	22	4359	4966	6717	225	6.1	5562	18	573	5.1	0.3	5.2	28	41	0.80
11-12	8.2	8.3	92.5	1.8	0.52	0.18	1.5	5771	4	929	12521	5.7	57	0.96	6.8	39	25	5837	5948	7784	263	7.6	5979	21	567	5.4	1.3	8.0	31	46	0.81
12-13	8.1	8.8	92.3	2.3	0.52	0.18	0.9	6866	9	1093	15485	4.6	62	1.09	8.4	46	30	7207	6837	9209	299	7.6	6729	24	545	6.1	1.8	4.8	35	50	0.94
13-14	8.1	9.3	92.5	2.1	0.52	0.18	0.4	6357	5	985	15159	3.4	62	1.03	7.6	44	28	7235	6680	8800	295	6.6	6743	23	563	5.9	1.9	4.1	34	48	0.94

LOI: Loss on Ignition (standard deviation: 1-8%)
Cond: Conductivity (standard deviation: 3-5%)
Eh: Redox potential (standard deviation: <1

APPENDIX 2.

Table showing OD, CV, NO conc. values for Replicate A. Green mark shows NO with CV<10%.

Replicate A	Optical Density (nm)	Mean OD (nm)	OD Standard Deviation (σ)	Coefficient of Variability (%)	NO conc. (μM)	NO conc. Indiv. Mean (μM)
CA1	0.168	0.137	0.044	32.548	21.813	17.603
CA1	0.105				13.394	
CA2	0.111	0.144	0.047	32.530	14.144	18.574
CA2	0.177				23.004	
CA3	0.094	0.104	0.014	13.712	11.841	13.187
CA3	0.114				14.532	
LA1	0.140	0.114	0.037	31.942	19.464	16.004
LA1	0.089				12.544	
LA2	0.079	0.086	0.011	12.543	1.015	2.036
LA2	0.094				3.058	
LA3	0.122	0.119	0.004	3.632	6.783	6.376
LA3	0.116				5.969	
HA1	0.149	0.125	0.034	27.085	10.415	7.217
HA1	0.101				4.019	
HA2	0.150	0.113	0.052	46.223	10.549	5.615
HA2	0.076				0.681	
HA3	0.205	0.180	0.035	19.408	17.906	14.601
HA3	0.156				11.297	
MA1	0.179	0.150	0.041	27.605	14.434	10.529
MA1	0.121				6.623	
MA2	0.109	0.096	0.018	18.876	5.034	3.325
MA2	0.083				1.616	
MA3	0.108	0.103	0.006	6.155	4.927	4.326
MA3	0.099				3.725	
MHA1	0.120	0.104	0.022	21.626	6.476	4.360
MHA1	0.088				2.243	
MHA2	0.125	0.127	0.003	2.231	7.184	7.451
MHA2	0.129				7.718	
MHA3	0.081	0.101	0.029	28.843	1.282	4.039
MHA3	0.122				6.797	
TA1	0.077	0.069	0.012	17.549	0.801	0.401
TA1	0.060				0.000	
TA2	0.088	0.082	0.008	10.411	2.203	1.402
TA2	0.076				0.601	
TA3	0.092	0.081	0.016	19.627	2.791	1.395
TA3	0.070				0.000	
TMA1	0.132	0.129	0.004	3.233	8.145	7.751
TMA1	0.126				7.357	
TMA2	0.080	0.082	0.003	3.533	1.202	1.475
TMA2	0.084				1.749	
TMA3	0.087	0.101	0.019	19.138	2.123	3.939
TMA3	0.114				5.755	
FTA1	0.094	0.115	0.028	24.877	3.125	5.815
FTA1	0.135				8.506	
FTA2	0.087	0.082	0.008	9.428	2.163	1.435
FTA2	0.076				0.708	
FTA3	0.105	0.108	0.005	4.183	4.540	4.967
FTA3	0.111				5.395	

Table showing OD, CV, NO conc. values for Replicate B. Green mark shows NO with CV<10%.

Replicate B	Optical Density (nm)	Mean OD (nm)	OD Standard Deviation (σ)	Coefficient of Variability (%)	NO conc. (μM)	NO conc. Indiv. Mean (μM)
CB1	0.067	0.077	0.014	17.724	0.382	1.720
CB1	0.086				3.058	
CB2	0.069	0.065	0.005	7.768	0.591	0.296
CB2	0.061				0.000	
CB3	0.078	0.077	0.002	2.542	1.961	1.769
CB3	0.076				1.576	
LB1	0.149	0.151	0.003	1.907	11.776	12.059
LB1	0.153				12.342	
LB2	0.094	0.095	0.001	1.442	4.126	4.261
LB2	0.096				4.395	
LB3	0.104	0.104	0.000	0.362	5.584	5.547
LB3	0.104				5.510	
HB1	0.067	0.069	0.003	4.167	0.378	0.661
HB1	0.071				0.944	
HB2	0.077	0.078	0.001	1.184	1.776	1.866
HB2	0.078				1.957	
HB3	0.069	0.071	0.003	3.898	0.633	0.905
HB3	0.073				1.176	
MB1	0.078	0.074	0.006	7.754	1.947	1.381
MB1	0.070				0.814	
MB2	0.078	0.079	0.002	2.031	1.887	2.045
MB2	0.080				2.203	
MB3	0.066	0.073	0.010	14.284	0.182	1.209
MB3	0.080				2.235	
MHB1	0.081	0.075	0.007	9.753	2.277	1.553
MHB1	0.070				0.828	
MHB2	0.064	0.065	0.002	2.463	0.000	0.135
MHB2	0.066				0.271	
MHB3	0.110	0.106	0.005	4.831	6.304	5.800
MHB3	0.102				5.296	
TB1	0.089	0.096	0.010	10.051	3.462	4.412
TB1	0.103				5.361	
TB2	0.079	0.073	0.009	12.744	2.059	1.149
TB2	0.066				0.238	
TB3	0.114	0.102	0.016	15.918	6.908	5.303
TB3	0.091				3.699	
TMB1	0.089	0.089	0.001	0.717	3.471	3.408
TMB1	0.088				3.346	
TMB2	0.082	0.087	0.007	7.900	2.468	3.144
TMB2	0.092				3.819	
TMB3	0.074	0.135	0.086	64.063	1.316	9.820
TMB3	0.196				18.325	
FTB1	0.081	0.080	0.002	2.665	2.347	2.138
FTB1	0.078				1.929	
FTB2	0.069	0.070	0.003	3.681	0.605	0.861
FTB2	0.072				1.116	
FTB3	0.073	0.090	0.024	26.760	1.176	3.541
FTB3	0.107				5.905	

Table showing OD, CV, NO conc. values for Replicate C. Green mark shows NO with CV<10%.

Replicate C	Optical Density (nm)	Mean OD (nm)	OD Standard Deviation (σ)	Coefficient of Variability (%)	NO conc. (μM)	NO conc. Indiv. Mean (μM)
CC1	0.069	0.070	0.002	3.116	0.916	1.126
CC1	0.072				1.337	
CC2	0.086	0.082	0.006	7.337	3.274	2.697
CC2	0.078				2.120	
CC3	0.068	0.065	0.003	4.951	0.739	0.429
CC3	0.063				0.119	
LC1	0.134	0.132	0.002	1.463	9.702	9.517
LC1	0.131				9.331	
LC2	0.066	0.067	0.002	2.293	0.499	0.646
LC2	0.068				0.794	
LC3	0.063	0.063	0.000	0.263	0.078	0.094
LC3	0.063				0.110	
HC1	0.062	0.084	0.031	36.590	0.028	2.980
HC1	0.106				5.932	
HC2	0.072	0.073	0.002	2.236	1.296	1.452
HC2	0.074				1.608	
HC3	0.069	0.068	0.002	2.318	0.975	0.823
HC3	0.067				0.671	
MC1	0.091	0.083	0.011	13.321	3.903	2.842
MC1	0.075				1.780	
MC2	0.076	0.070	0.009	12.758	1.884	1.031
MC2	0.063				0.178	
MC3	0.084	0.077	0.010	13.708	2.971	1.964
MC3	0.069				0.956	
MHC1	0.095	0.085	0.015	17.375	4.501	3.086
MHC1	0.074				1.672	
MHC2	0.116	0.094	0.032	33.880	7.371	4.318
MHC2	0.071				1.264	
MHC3	0.101	0.101	0.001	0.884	5.243	5.329
MHC3	0.102				5.415	
TC1	0.064	0.065	0.002	2.784	0.250	0.425
TC1	0.066				0.599	
TC2	0.074	0.068	0.009	13.047	1.595	1.595
TC2	0.061				0.000	
TC3	0.064	0.065	0.000	0.183	0.327	0.339
TC3	0.065				0.350	
TMC1	0.084	0.082	0.003	3.965	3.025	2.713
TMC1	0.080				2.401	
TMC2	0.072	0.070	0.003	3.780	1.310	1.056
TMC2	0.068				0.803	
TMC3	0.076	0.076	0.000	0.464	1.894	1.927
TMC3	0.077				1.961	
FTC1	0.201	0.220	0.026	11.833	18.888	21.382
FTC1	0.238				23.876	
FTC2	0.101	0.095	0.009	9.671	5.293	4.415
FTC2	0.088				3.537	
FTC3	0.072	0.072	0.000	0.587	1.350	1.391
FTC3	0.073				1.432	

Figure representing the design of the 96-well plate used for the NO assay. Wells shown in the orange box represent the standards used for the reference curve.

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
A	*CA1*	*LA1*	*HA1*	*MA1*	*TA1*	*TA1*	*TMA1*	*FTA1*				
B	*CA1*	*LA1*	*HA1*	*MA1*	*TA1*	*TA1*	*TMA1*	*FTA1*				
C	*CA2*	*LA2*	*HA2*	*MA2*	*TA2*	*TA2*	*TMA2*	*FTA2*				
D	*CA2*	*LA2*	*HA2*	*MA2*	*TA2*	*TA2*	*TMA2*	*FTA2*				
E	*CA3*	*LA3*	*HA3*	*MA3*	*TA3*	*TA3*	*TMA3*	*FTA3*				
F	*CA3*	*LA3*	*HA3*	*MA3*	*TA3*	*TA3*	*TMA3*	*FTA3*				
G	*B*	*100*	*50*	*25*	*12.5*	*6.25*	*B*	*100*	*50*	*25*	*12.5*	*6.25*
H	*B*	*100*	*50*	*25*	*12.5*	*6.25*	*B*	*100*	*50*	*25*	*12.5*	*6.25*

Sodium nitrite (NaNO2) standards preparation steps.

Dissolve 0.069 g of $NaNO_2$ to 1 l dH_2O to obtain 1mM $NaNO_2$ stock solution. 100 ml of the 100 mM $NaNO_2$ stock solution was dissolved in 900 mL of dH_2O to obtain 100 µM.

Prepare nitrite standards in dH_2O as shown below

***$NaNO_2$* (ml)**	**dH_2O (ml)**	**Nitrite conc (µM)**
50 ml of 100 µM (*$NaNO_2$*)	50 ml of dH_2O	50
25 ml of 50 µM (*$NaNO_2$*)	25 ml of dH_2O	25
12.5 ml of 25 µM (*$NaNO_2$*)	12.5 ml of dH_2O	12.5
6.25 ml of 12.5 µM (*$NaNO_2$*)	6.25 ml of dH_2O	6.25

Correlation coefficient values obtained from PCA done on the generated NO data.

Treatment	**correlation**	**p.value**
TM	0.8409395	0.03593821
C	-0.8626367	0.02700708

APPENDIX 3. *showing percentages of phagocytic cells in samples in Replicate A, B and C.*

Replicate A	Cell count	Phagocytic Cell Count	Phagocytic Cell Percentage	Individual Mean (%)
CA1green	3730	1312	35.174	44.118
CA1red	3168	1681	53.062	
CA2green	3372	1359	40.302	45.002
CA2red	3690	1834	49.702	
CA3green	2121	1263	59.547	59.732
CA3red	1936	1160	59.917	
LA1green	4425	1716	38.780	40.936
LA1red	4864	2096	43.092	
LA2green	2917	1078	36.956	68.806
LA2red	2588	2605	100.657	
LA3green	4025	1427	35.453	39.571
LA3red	3605	1575	43.689	
HA1green	3366	1250	37.136	39.622
HA1red	3871	1630	42.108	
HA2green	2458	904	36.778	40.834
HA2red	3043	1366	44.890	
HA3green	1787	641	35.870	38.810
HA3red	1818	759	41.749	
MA1green	2053	731	35.606	42.366
MA1red	2058	1011	49.125	
MA2green	4149	1499	36.129	40.264
MA2red	3847	1708	44.398	
MA3green	2565	1483	57.817	59.711
MA3red	2516	1550	61.606	
MHA1green	3232	864	26.733	30.830
MHA1red	3055	1067	34.926	
MHA2green	2188	1124	51.371	56.649
MHA2red	2419	1498	61.926	
MHA3green	2655	992	37.363	43.377
MHA3red	3199	1580	49.390	
TA1green	1449	1010	69.703	71.627
TA1red	1656	1218	73.551	
TA2green	1819	1330	73.117	74.074
TA2red	1606	1205	75.031	
TA3green	3622	984	27.167	32.735
TA3red	3522	1349	38.302	
TMA1green	632	178	28.165	33.655
TMA1red	889	348	39.145	
TMA2green	3169	873	27.548	29.308
TMA2red	4239	1317	31.069	
TMA3green	1486	592	39.838	42.522
TMA3red	2993	1353	45.205	
FTA1green	3638	1046	28.752	30.758
FTA1red	3342	1095	32.765	
FTA2green	3579	1723	48.142	49.449
FTA2red	3377	1714	50.755	
FTA3green	3870	1194	30.853	32.903
FTA3red	3785	1323	34.954	

Replicate B	Cell count	Phagocytic Cell Count	Phagocytic Cell Percentage	Individual Mean (%)
CB1green	2364	767	32.445	31.340
CB1red	3142	950	30.236	
CB2green	3306	644	19.480	20.479
CB2red	3138	674	21.479	
CB3green	2965	1159	39.089	39.780
CB3red	2083	843	40.470	
LB1green	1689	912	53.996	51.378
LB1red	1290	629	48.760	
LB2green	2921	991	33.927	34.276
LB2red	2862	991	34.626	
LB3green	1728	432	25.000	23.171
LB3red	2132	455	21.341	
HB1green	2317	566	24.428	23.849
HB1red	2544	592	23.270	
HB2green	1706	346	20.281	25.994
HB2red	1968	624	31.707	
HB3green	3165	658	20.790	22.517
HB3red	3407	826	24.244	
MB1green	1349	336	24.907	25.926
MB1red	1633	440	26.944	
MB2green	2216	591	26.670	28.017
MB2red	2091	614	29.364	
MB3green	3495	1057	30.243	27.187
MB3red	3771	910	24.132	
MHB1green	2031	559	27.523	32.668
MHB1red	2671	1010	37.814	
MHB2green	2781	794	28.551	24.971
MHB2red	2875	615	21.391	
MHB3green	2093	836	39.943	38.968
MHB3red	2153	818	37.993	
TB1green	1615	598	37.028	36.703
TB1red	1894	689	36.378	
TB2green	2723	865	31.766	29.656
TB2red	2534	698	27.545	
TB3green	1605	377	23.489	33.297
TB3red	1552	669	43.106	
TMB1green	2274	820	36.060	31.528
TMB1red	2304	622	26.997	
TMB2green	2237	891	39.830	35.376
TMB2red	2322	718	30.922	
TMB3green	4126	1175	28.478	24.562
TMB3red	3250	671	20.646	
FTB1green	2646	917	34.656	35.880
FTB1red	3059	1135	37.104	
FTB2green	3339	922	27.613	28.274
FTB2red	3152	912	28.934	
FTB3green	1365	542	39.707	41.523
FTB3red	3611	1565	43.340	

Replicate C	Cell count	Phagocytic Cell Count	Phagocytic Cell Percentage	Individual Mean (%)
CC1green	2619	396	15.120	11.836
CC1red	2023	173	8.552	
CC2green	852	273	32.042	31.177
CC2red	993	301	30.312	
CC3green	3025	722	23.868	22.461
CC3red	2693	567	21.055	
LC1green	529	300	56.711	54.683
LC1red	659	347	52.656	
LC2green	1799	419	23.291	21.096
LC2red	1656	313	18.901	
LC3green	1486	368	24.764	24.029
LC3red	1567	365	23.293	
HC1green	1409	281	19.943	18.902
HC1red	1271	227	17.860	
HC2green	3054	1164	38.114	32.944
HC2red	3046	846	27.774	
HC3green	2474	544	21.989	24.798
HC3red	3597	993	27.606	
MC1green	979	123	12.564	11.082
MC1red	802	77	9.601	
MC2green	3071	962	31.325	26.877
MC2red	2974	667	22.428	
MC3green	1751	535	30.554	29.057
MC3red	1680	463	27.560	
MHC1green	1924	646	33.576	33.758
MHC1red	2139	726	33.941	
MHC2green	2160	470	21.759	20.563
MHC2red	2050	397	19.366	
MHC3green	1158	325	28.066	26.571
MHC3red	1324	332	25.076	
TC1green	1703	445	26.130	22.587
TC1red	2363	450	19.044	
TC2green	2410	928	38.506	32.262
TC2red	1941	505	26.018	
TC3green	2931	851	29.034	23.239
TC3red	3130	546	17.444	
TMC1green	690	116	16.812	16.258
TMC1red	2509	394	15.703	
TMC2green	2211	560	25.328	22.733
TMC2red	1604	323	20.137	
TMC3green	3176	1238	38.980	33.263
TMC3red	2817	776	27.547	
FTC1green	881	520	59.024	59.675
FTC1red	1225	739	60.327	
FTC2green	2140	746	34.860	33.615
FTC2red	1937	627	32.370	
FTC3green	2982	695	23.307	22.233
FTC3red	3710	785	21.159	

Correlation coefficient values obtained from PCA done on the phagocytic activity of hemocyt es data.

Treatment	correlation	p-value
M	0.9156353	9.876838e-08
C	0.8994666	3.811638e-07
H	0.8684542	2.959756e-06
T	0.7153388	8.457294e-04
MH	0.6666826	2.514086e-03
TM	0.6381717	4.372868e-03

APPENDIX 4. *showing the values of the PCA. Significant correlation coefficients greater than 0.7 are bolden.*

Treatment	correlation	p-value
TM	0.9994619	1.104697e-20
M	0.9939604	7.304685e-14
H	0.9939277	7.565506e-14
C	0.9934315	1.259026e-13
L	0.9933709	1.336281e-13
MH	0.9849759	2.672021e-11
T	0.9769391	4.246554e-10
FT	0.9761105	5.331191e-10

APPENDIX 5. *Sample location of this study (yellow mark) and sample location of Lusher et al (2017) (red mark).*