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Biological response in organisms collected near the discharge point of the wastewater treatment plant of Stavanger

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Abstract

The effluent from the IVAR SNJ wastewater treatment plant is discharged into the marine recipient Håsteinsfjord in the North Sea, 1.6 kilometers from the coast of the Stavanger peninsula. The treated discharged wastewater can still contain a small amount of certain pollutants known as emerging contaminants (ECs), that can have negative effects on the living organisms. A biomonitoring study was performed using three different species: the common periwinkle (*Littorina littorea*), the Norway king crab (*Lithodes maja*), and the Norway lobster (*Nephrops norvegicus*), as bioindicators for evaluation of the biological effects in these organisms living near the effluent discharge point. Biomarkers used in this research study included condition index (CI) for all three species, lysosomal membrane stability (LMS), acetylcholinesterase assay (AChE), and micronucleus assay (MN) for periwinkles. Morphological measurements from all three species were also taken. Snail specimens were collected in September 2019 from three coastal locations, two near the discharge point: Sandestranda and Randabergbukta, and one reference location: Solastranda. Lobster and crab specimens are collected in October 2019 from two marine locations, one in the immediate vicinity of the discharge point: Håsteinsfjord, and one reference location: Boknafjord.

The total data obtained from the biomarkers selected for this study showed that there was no significant difference between organisms from the reference locations and organisms from locations near the wastewater discharge point. The only significant lower value was observed with LMS in *L. littorea* species, sampled from the site near the effluent point, Sandestranda, indicating that organisms from this location are subjected to a general environmental stress, that can be a consequence of wastewater discharge from IVAR SNJ WWTP, or a result of other sources of contaminations or stress. CI values for all three species were not significantly different, indicating that there was not any significant negative effect on the conditions needed for growth and reproduction in all three species. AChE activity in tissues of sampled snails did not exhibit any significant difference between sampling locations, indicating that there are no neurotoxic pollutants capable to induce inhibiting of AChE in *L. littorea*, or their concentration is too low. Two different procedures have been used for MN assay, but due to the technical failures sufficiently reliable results could not be obtained so they are not included in this study.

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Finally, I would like to thank my closest and dearest people. To my family, especially to my mother and brother, thank you for the endless moral support, there are no words to express my love and gratitude. To my closest friends for their continuing encouragement and support. And at the end to You my love, to you who have lived through my every stressful moment, who always believed in me, even when I didn't, and you who gave me the most beautiful gift possible, I thank you with all my being.

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List of Acronyms

ACh	Acetylcholine
AChE	Acetylcholinesterase
ATC	Acetylthiocholine
BOD	Biological Oxygen Demand
ChE	Cholinesterase
CI	Condition Index
COD	Chemical Oxygen Demand
DREAM	Dose-related Risk and Effect Assessment Model
DTNB	5,5-DiThiobis-2-NitroBenzoic acid
EBRP	Enhanced Biological Phosphorus Removal
ECs	Emerging Contaminants
EDCs	Endocrine Disrupting Compounds
IVAR	Interkommunalt, Vann, Avløp og Renovasjon
LMS	Lysosomal membrane stability
MN	Micronucleus/Micronuclei
NRF	South Africa's National Research Foundation
NRRT	Neutral Red Retention Time
OPs	Organophosphorus compounds
PAHs	Polycyclic Aromatic Hydrocarbons
PCPs	Personal Care Products
PhACs	Pharmaceuticals
RCN	Norwegian Research Council
RO	Reverse Osmosis
SANOCEAN	South Africa-Norway Research Cooperation on Blue Economy, Climate Change, the Environment and Sustainable Energy
SNJ	Sentralrenseanlegget Nord-Jæren
TNB	5-Thio-2-NitroBenzoic acid
TOC	Total Organic Carbon
UiS	University of Stavanger
WWTP	Wastewater Treatment Plant

1 Introduction

1.1 Aim of the Study

This thesis aims to evaluate the potential biological effects in organisms collected in areas close to the discharge point of IVARs Regional Wastewater Treatment Plant ("Sentralrenseanlegget Nord-Jæren" - SNJ), which serves the city of Stavanger. This research activity is part of the project entitled "Marine Sewage Outfalls – Environmental Impact Evaluation", funded by the Research Council of Norway within the SANOCEAN program.

The study areas included coastal and marine stations near the discharge point and included three different species: the common periwinkle (*Littorina littorea*), the Norway king crab (*Lithodes maja*), and the Norway lobster (*Nephrops norvegicus*). The biological evaluation of *L. littorea* combined several biomarkers, which are considered to provide insight about the health condition of this omnivorous grazer species. The battery of biomarkers included lysosomal membrane stability (LMS), condition index (CI), acetylcholinesterase assay (AChE), and micronucleus assay (MN). While for the biological evaluation of *L. maja* and *N. norvegicus* CI was used.

1.2 Wastewater characteristics

The treatment requirements, design, and operation of treatment facilities are established based on the composition of the incoming wastewater, the amount of discharge, and the conditions of receiving water as compared to costs and environmental protection interests in the area (Tchobanoglous *et al.* 2014; Miljødepartementet 2004).

All wastewaters are characterized by their quality and quantity, which depends on factors such as climate, human activity, level of development, culture, population habits, etc. The quality of the wastewater is described through its physical (temperature, turbidity, color, odor), chemical (total solids, total phosphorus, total nitrogen, pH, alkalinity, chlorides, heavy metals, organic matter: chemical oxygen demand - COD, biological oxygen demand - BOD_5, total organic carbon - TOC) and organisms present (microorganism content) characteristics (Von Sperling 2007).

Sources of wastewater

The municipal wastewater sources are usually: domestic or sanitary wastewater, industrial wastewater, infiltration/inflow (I/I), and stormwater (Tchobanoglous*, et al.* 2014).

Domestic or *sanitary wastewater* originates from institutions, residential, commercial, and public facilities (Tchobanoglous*, et al.* 2014). They contain substances in the form of real solutions, colloidal solutions - non-precipitating substances, suspended and floating substances. They are also defined by the

presence of pathogenic microorganisms, primarily of human origin (feces, urine, mucus), disinfectants, pharmaceuticals (metabolized pharmaceuticals), and surfactants (personal care and cleaning products) (Povrenović & Knežević 2013).

Industrial wastewater derives from industrial processes and is diverse in its characteristics, depending on the industry. They are much more polluted than sanitary wastewater, which depends on the type of industry and the amount of wastewater it produces. Wastewater from industries must be treated within the industrial plant, to the prescribed level, before discharged directly into natural water intakes or into the city sewage system (Povrenović & Knežević 2013).

Infiltration/inflow (I/I) water is water that can enter the sewerage network through damaged pipes, fault connections, and damaged manholes (infiltration). On the other hand, it can enter the sewerage network intentionally, in the case of a combined sewage system (inflow) (Sola *et al.* 2018). In a combined (unitary) sewage system stormwater enters the system through storm drain connections (Tchobanoglous*, et al.* 2014) from where it is transported together with wastewater to wastewater facilities. Wastewater from combined systems, in areas with heavy rainfall, is far more diluted, and stormwater significantly contributes to the total load arriving at the wastewater treatment plant (WWTP) (Von Sperling 2007). Sources of infiltration/inflow water are rainfall, groundwater, and leakages from the water supply system (Sola*, et al.* 2018).

Stormwater includes runoff water from rainfalls and snowmelt (Tchobanoglous*, et al.* 2014).

Approximately 99.9 % of domestic wastewater is water, the remainder consists of organic and inorganic, suspended and dissolved solids, together with microorganisms. Because of this 0.1 %, wastewater needs to be treated (Von Sperling 2007). Due to its geographical position with the high amount of precipitation and the fact that wastewater and stormwater are mostly transported in a common system, wastewater in Norway is generally cold and diluted, with low nutrient content (Ødegaard 1999). This is especially true for Western Norway, where a marine climate prevails, with mean annual precipitation of around 2,250 mm (Weibull 2020). In a combined system during excessive rainfall, effluent can run directly into a recipient water body through the spillways.

1.2.1 Emerging contaminants

In recent years, researchers have focused on new pollutants collectively referred to as emerging contaminants (ECs) also identified in wastewater. Sources of ECs can be industrial, from households, agricultural, aquacultural, hospital, or laboratory wastewater. Conventical wastewater treatment plants are not designed to remove most of these chemicals, which can sometimes be partially removed. Besides, ECs are mobile, persistent, bioaccumulate, and can reach the environment as well by leaching from landfills,

arable land, illegal waste discharges, etc. As such, ECs have been found in several environmental matrices, in different concentrations, depending on their physicochemical properties, the amount of use, and disposal. It includes marine biological organisms, sediments, and water bodies (Bilal *et al.* 2019; Tijani *et al.* 2015).

ECs can be divided into three main categories: pharmaceuticals (PhACs), personal care products (PCPs), and endocrine disrupting compounds (EDCs). Furthermore, here should also be included pesticides components, flame retardants, nanomaterials, metabolites of ECs, etc. (Gogoi *et al.* 2018).

Pharmaceuticals

PhACs are natural or synthetic chemical compounds designed to treat various diseases, inflammations, pains, and symptoms, both in humans and in animals. They can be categorized as antidepressants, analgesics, antibiotics, beta-blockers, stimulants, anticancer drugs, etc. (Tijani*, et al.* 2015). The main route for PhACs to the environment is through municipal wastewaters. As they are not completely degraded inside the body, partially metabolized drugs are excreted and end up in wastewater treatment plants. The other way they end up in the environment is by improper disposal of expired medications in toilets. Non-biodegradable PhAC products constantly enter the receiving water as dissolved contaminants at very low concentrations (in a range from ng/L to µg/L) by discharge from the wastewater treatment plant (Ternes *et al.* 2004).

The persistence of PhACs in the environment depends on the predetermined action for which they are designed for. Some are hydrophilic, easily degradable in water, short-living, while others can take up to several years to decompose (Tijani*, et al.* 2015).

The main concern is not about the acute toxicity of PhACs but rather about their chronic toxicity on aquatic organisms, as they are constantly, over their entire life, exposed to PhAC metabolites from wastewater discharges (Gogoi*, et al.* 2018; Jiang *et al.* 2013).

Personal care products

PPCs include cosmetic products, perfumes, shampoos, etc., whose components enter the wastewater by washing, bathing, and showering. During the treatment process in WWTPs, most of PCPs components are sorb onto sludge and sediments due to their lipophilic characteristics (Ternes*, et al.* 2004). Synthetic detergents are considered one of the major pollutants in the aquatic environment. Although, they are non-accumulative due to good biodegradability, in some zones they occur in increased concentrations due to constant input. The anion surfactant sodium dodecylbenzene (SDBS), which is a raw material in various detergents, because of its electric attraction, can be combined with the AChE enzyme. This will result in enhanced affinity between enzyme and substrate, and change the enzyme activity (Lionetto *et al.* 2012).

Endocrine disruptors

EDCs can be natural or artificial chemicals that, when ingested into the body, affect the proper functioning of endocrine systems. Inside the body, they can disrupt endocrine glands that secrete hormones, by coping or obstructing hormones, or by binding directly to the receptors. Thus, disabling the message-sending mechanism inside the body that regulates homeostasis, development, reproduction, and behavior. EDCs can cause adverse effects on aquatic organisms at exposure levels that are far lower than carcinogen exposure levels of concern (Gogoi*, et al.* 2018; Tijani*, et al.* 2015).

WWTPs are not designed for the elimination of mixtures of ECs, which occur in very small concentrations (µg/L), as their main focus is on the removal of phosphorus and nitrogenous compounds, biodegradable carbon, and microorganisms. Due to insufficient knowledge of their physical, chemical, and biochemical characteristics, it is difficult to fully predict their behavior in the process of wastewater treatment (Von Sperling 2007). It is also possible that processes that are an integral part of wastewater treatment, such as biological and chemical degradation, transform pollutants into more toxic forms than their parent compounds. An example is the generation of transformation products, as a result of partial oxidation of pharmaceuticals (Gogoi*, et al.* 2018).

1.3 The IVAR SNJ wastewater treatment plant

IVAR is a company owned by eleven municipalities in Rogaland county in southwestern Norway. The main focus of the company is the construction and operation of municipal facilities for handling water, wastewater, and solid waste. IVARs Regional Wastewater Treatment Plant (SNJ) (Figure 1.1), which has been in operation since 1992, receives wastewater from five municipalities: Stavanger, Sandnes, Sola, Gjesdal, and Randaberg.

Figure 1.1 IVAR SNJ WWTP (Photo: ivar.no).

In 2018 the capacity of the treatment plant was increased and the main purification process was changed from chemical precipitation to enhanced biological phosphorus removal (EBPR) based on an activated sludge system (Danielsen 2018). The plant expansion and rebuild of the purification process have been done in order to keep up with the population growth as well as with discharge requirements regarding organic material.

Today IVAR SNJ receives a wastewater load of more than 300,000 pe (population equivalents). The current designed capacity is for 400,000 pe, which, based on the expected population growth, should meet the need for the next fifteen years, until 2035. In the last step of the rebuild, the capacity of the plant can be increased to 500,000 pe (IVAR 2020).

According to the Norwegian Pollution Control Regulation (2004), wastewater discharge requirements in Norway are related to the conditions of receiving water bodies (Tornes 2001). The discharge outfall for treated wastewater from IVAR SNJ is located at 80 meters depth and 1.6 kilometers from the coast into the Håsteinsfjord fjord in the North Sea. The area that is characterized as less sensitive (Miljødepartementet 2004), as it is coastal area, with good water exchange and it is not exposed to oxygen loss or is in danger to become eutrophic as a result of discharge of treated wastewater.

1.3.1 Wastewater Treatment Technology and Discharge

As the coastal area of the discharge from the IVAR SNJ WWTP is characterized as a less sensitive area, there is a requirement for the removal of 70 % of all biodegradable organic matter before discharge and no requirement for phosphorus removal (Table 1.1).

Table 1.1 Requirements for discharges from wastewater treatment plants from urban areas according to the Norwegian Pollution Control Regulations (2004).

Parameters		Concentration	Minimum percentage reduction
Biochemical oxygen demand (BOD_5 at 20°C) without nitrification		25 mg/L O_2	70 - 90
Chemical oxygen demand (COD)		125 mg/L O_2	75
Total amount of suspended solids	(for >10,000 pe)	35 mg/L	90
	(for 2,000 – 10,000 pe)	60 mg/L	70

WWTPs that are using biological treatment technology are more efficient than chemical WWTPs in the removal of organic material. Today IVAR SNJ WWTP achieves up to 80 % removal of organic material (IVAR 2020), which is above the requirement. In addition, even though there is no requirement for phosphorous removal, IVAR SNJ WWTP applies biological recovery of phosphorus from the produced sludge and reuses it in the production of fertilizer, Minorga.

There are several processing steps in the wastewater treatment line. Traditionally wastewater treatment includes primary, secondary, tertiary, and sometimes advanced treatment (Tchobanoglous*, et al.* 2014). There are 3 process lines at IVAR SNJ WWTP, each consisting of 1 bioreactor and 4 settling tanks. The bioreactors contain 3 anaerobic tanks (A1, A2, and A3) and 1 aerobic tank (IVAR 2020). The wastewater treatment process at IVAR SNJ WWTP is presented in Figure 1.2.

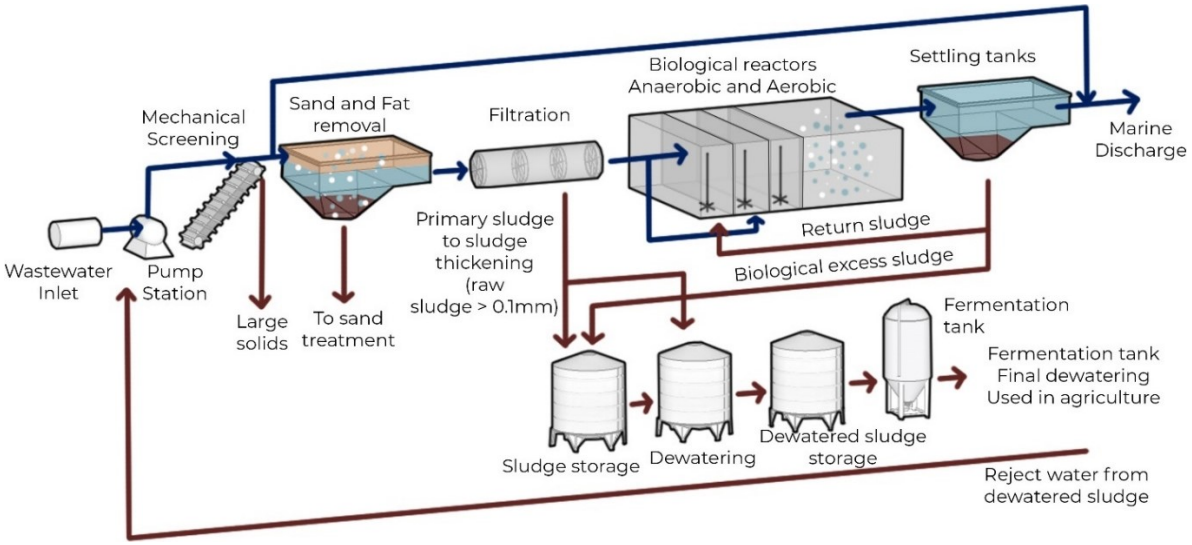


Figure 1.2 Schematic of the wastewater treatment process at IVAR SNJ WWTP

Primary treatment

In the first process of the primary treatment at IVAR SNJ WWTP, coarse material is separated from the rest of the inlet flow by mechanical screening. Screens have a 6 mm opening. It removes insoluble solid material such as branches, rags, leaves, rocks, paper, etc. Removal of large solids prevents damage and/or clogging downstream in the treatment plant (Tchobanoglous*, et al.* 2014).

The second process in the primary treatment is an aerated sedimentation tank, in which fats and sand are removed. Fats and oil will, with the help of rising air bubbles, float at the surface and form a scum layer where it is scraped off and sent to the sludge treatment. Sand is collected at the bottom of the tank, from where it is removed, washed, and deposited.

Drum filters are the next process on the wastewater treatment line. They have a filter opening of 0.1 mm and are used for the removal of suspended particulate material from the rest of the flow.

Secondary treatment

The biological reactor for the activated sludge process is divided into two parts. The first part contains three anaerobic reactors (A1, A2, A3) with mixers, while the second part is one aerobic reactor where the dissolved oxygen concentration is maintained with constant aeration. The anaerobic environment in the first three reactors supports the development of added phosphorus-accumulating bacteria. Next in the aerobic reactor phosphorus uptake occurs, and removal from the wastewater. In this way, not only

phosphorus is removed but organic matter as well through aerobic degradation. As stated earlier, the increased removal of organic matter is the reason for the reconstruction of IVAR SNJ WWTP.

In the final sedimentation tank, bacteria are removed. From here the part of created sludge is recycled to the first anaerobic reactor (A1) in order to maintain a suitable concentration of microorganisms. After bacterial removal treated wastewater is discharged into the Håsteinsfjord fjord in the North Sea.

But this kind of treatment alone (Enhanced Biological Phosphorus Removal - EBPR) does not ensure complete removal of the ECs (Gogoi*, et al.* 2018).

According to the Norwegian Pollution Control Regulation (2004), part 4, chapter 11, in addition to monitoring the biological and chemical oxygen consumption, shown in Table 1.1, large WWTPs, greater than or equal to 50,000 pe, such as the IVAR SNJ WWTP, are required to monitor other chemicals of concern, described in the regulation. Monitoring is carried out by taking six inlet and outlet samples per year from sewage systems greater than or equal to 20,000 pe and analyzing parameters from Table 1.2, and three inlet and outlet samples per year for systems greater or equal to 50,000 pe and analyzing parameters from Table 1.3.

Table 1.2 Analysis parameters for drainage systems greater than or equal to 20,000 pe from the Norwegian Pollution Control Regulations (2004).

Parameters	Detection limit
Heavy metals	
As, Cr, Cu, Ni, Zn and Pb	≤ 1 µg/L
Cd and Hg	≤ 0.1 µg/L

Table 1.3 Analysis parameters for drainage systems greater than or equal to 50,000 pe from the Norwegian Pollution Control Regulations (2004).

Parameters	Detection limit
Brominated flame retardants (BFH)	
Tetrabromodiphenyl ether (BDE-47), Pentabromodiphenyl ether (BDE-99 and BDE-100), Octabromodiphenyl ether (BDE-183) and Deca-bromodiphenyl ether (BDE-209), Tetrabromobisphenol A (TBBPA) and Hexabromocyclode.	≤ 10 ng/L
Polycyclic Aromatic Hydrocarbons (Σ PAH), sum of the following PAH compounds	
phenanthrene, anthracene, pyrene, fluorants, benzo (a) fluorene, benzo (b) fluorene, cross / triphenylene, benzo (a) anthracene, benzo (b) fluorants, benzo (k) fluorotants, benzo (e) pyrene, benzo (a) pyrene, dibenzo (a, h) anthracene, indeno (1,2,3-c, d) pyrene and benzo (g, h, i) perylene, dibenzo (a, e) pyrene, dibenzo (a, h) pyrene, dibenzo (a, i) pyrene.	≤ 0.2µg/L
Polychlorinated biphenyls (Σ PCB_7), sum of the 7 individual compounds	
No. 28, 52, 101, 118, 138, 153 and 180.	≤ 10 ng/L
Diethylhexyl phthalate (DEHP)	≤ 0.1µg/L
Nonylphenol (NP): 4-nonylphenol	≤ 0.1 µg/L

1.4 Environmental monitoring

Today there is a global awareness that our everyday activities lead to the release of pollutants into the environment which poses, to a greater or lesser extent, a risk to the ecosystems. Even so, the concentration of chemical pollutants in environmental matrices is constantly increasing as a consequence of anthropogenic activities, generating harmful conditions for biological life (Lionetto *et al.* 2019).

Environmental monitoring combines mechanisms that allow understanding and assessment of the ecological risk, and determine the degree of impact and degradation of the environment by anthropogenic pollutants (Walker *et al.* 2012; Galloway *et al.* 2006). In the past, environmental risk assessment relied solely on the identification of physical and chemical variables in an ecosystem. In recent times, it has become clear that chemical data on pollutant concentrations in biota and environmental matrices alone are not sufficient to provide us with information on potential toxic biological effects caused by pollutants, as contamination by itself is not an indication of toxicity (Lionetto*, et al.* 2019; Galloway *et al.* 2004; Long & Chapman 1985).

When conducting a risk assessment, it must be taken into account that organisms and communities in any natural environment are exposed to a complex mixture of chemical compounds (Connon *et al.* 2012). For example, marine ecosystems are difficult to analyze because of the vastness of the system and the difficulty of relating any effects seen to specific chemicals (Walker*, et al.* 2012). Every year, several thousand newly designed chemicals are released into the aquatic environment, where for most of them there is no toxicological information (Galloway*, et al.* 2006). As stated earlier, there is a continuous income of organic chemicals which include pharmaceuticals, detergents, hormones, and pesticides from treated municipal wastewater. Their simultaneous presence, diversity in chemical nature, and toxicity can cause additive/synergic effects on the organisms. In addition, the bioavailability of pollutants is affected by environmental factors, like temperature, pH, salinity, etc. Organisms have different sensitivity to pollutant exposure and effects, as well. These are all factors that need to be considered in order to fully comprehend the integrated environmental effects of pollution in different environmental compartments (Connon*, et al.* 2012).

All of the above has led to the development of measurable effects of chemical pollution on living organisms by combining chemical and biological approaches in pollution monitoring (Lionetto*, et al.* 2019).

1.4.1 Biomarkers

A biomarker or biological endpoint marker is defined as any biological response to an environmental chemical at the individual level, level of the organization of the whole organism, or at lower levels of biological organization, at organ – tissue, cellular, and molecular level, demonstrating a departure

from the normal status. That response could be physiological, biochemical, morphological, histological, and/or behavioral (Walker*, et al.* 2012).

The development and use of biomarkers in ecotoxicology arose from the need for early warning tools for the detection of exposure and adverse biological responses to pollutants (Forbes *et al.* 2006). Before they found application in ecotoxicology, biomarkers have been used in human toxicology in which they have proved to be very useful as measures of human exposure to chemicals as well as in providing early warning signals for specific diseases or syndromes (Timbrell 1998). Biomarkers allow the obtaining of information on exposure to a particular group of chemicals based on monitoring the mechanism of the action itself, instead of monitoring all chemicals that have that particular mechanism of action. In this way, they provide us with a certain shortcut where one single measure can replace many chemical measurements (Hanson *et al.* 2013). Accordingly, biomarkers range from very specific, for example, an enzyme aminolevulinic acid dehydrates (ALAD), that is inhibited only by lead or metallothionein induction by heavy metals, to non-specific like DNA damage or immune system deterioration that can be caused by a wide variety of chemicals (Lionetto*, et al.* 2019; Walker*, et al.* 2012; Gil & Pla 2000). The general rule is that the biomarkers measured at higher levels of the biological organization have higher ecological relevance and lower specificity (Figure 1.3). The primary effect of chemicals is manifested at lower levels of biological organization. Measurements at this level can be assumed to represent early warning signals for effects at higher levels (Hanson*, et al.* 2013).

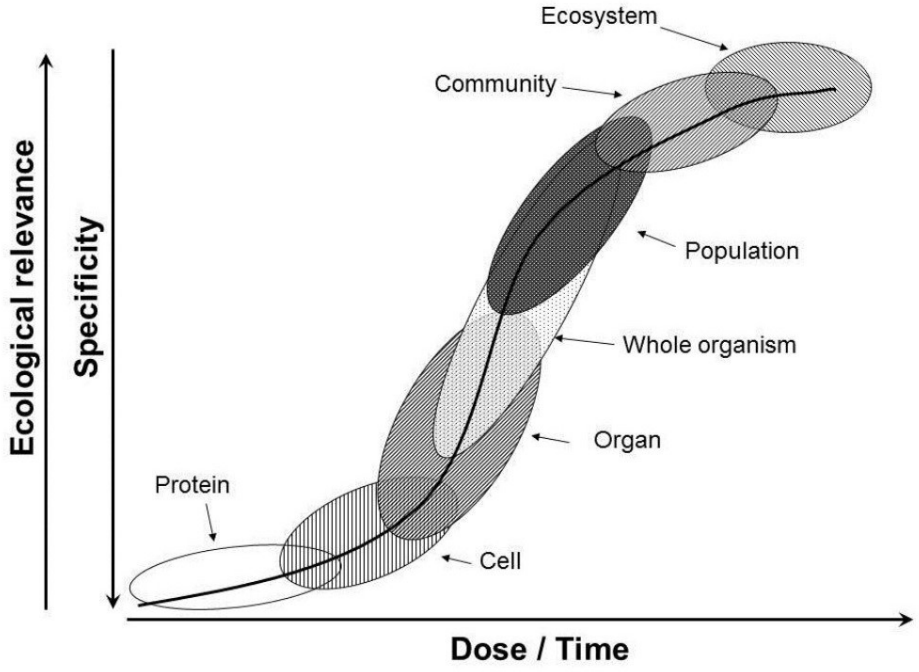


Figure 1.3 Ecological relevance and specificity relative to dose and/or time (Hanson*, et al.* 2013).

Biomarkers are usually classified into biomarkers of exposure and biomarkers of effect (Walker*, et al.* 2012). Biomarkers of exposure indicate exposure that is a reflection of the internal concentration of chemicals or metabolites (dose-response relationship), and can be applied as screening tools for specific chemical groups (Connon*, et al.* 2012; Handy *et al.* 2003). Biomarkers of effect indicate adverse effects or functional changes in an organism at all levels of biological organization. The best biomarkers from this group are those that are crucial for the normal function of cells, tissues, or organisms. A good example of an effect biomarker is the inhibition of AChE which relates directly to an adverse effect on a biological organism (Handy*, et al.* 2003).

1.4.1.1 Advantages and limitations of biomarkers

The use of biomarkers as an environmental monitoring tool in ecotoxicology and ecological risk assessment has its advantages and limitations. According to Handy, *et al*. (2003) the advantages of using the biomarkers are:

- Their responses can indicate the presence of biologically available contaminants;
- By using a suite of several individual biomarkers the presence of contaminants that were not initially suspected to be found could be revealed;
- They can detect sporadic exposure to pollutants that could be missed by routine chemical analysis. The reason is that biomarker responses persist long after cessation of exposure to a pollutant that has degraded and is no longer detectable by chemical analysis;
- Compared to chemical analyses biomarker analyses are generally less expensive and easier to perform.

Limitations in biomarker application are mainly related to variability in their responses. It is likely that the different responses are caused by the ecosystem complexity, seasonal cycles, fluctuation of biological and/or environmental factors, and multiple stressors (Hook *et al.* 2014). Differences in biomarker responses among individuals of the same species collected at relatively close locations are a result of uneven distribution of contaminants inside environmental compartments and individual biological characteristics such as age, sex, size, etc. (Handy*, et al.* 2003).

With knowledge regarding which species are being exposed or impacted, which toxicants are present, and exposure history, biomarker responses have a better chance of being interpreted correctly (Forbes*, et al.* 2006). Also, to fully understand the nonchemical factors such as geographical influences, habitat parameters (temperature, salinity, dissolved oxygen, nutritional state, etc.) and their seasonal variation, as well as variation in reproduction and growth phases, increases the ability to differentiate between nonchemical effects from those involving chemical stressors (Almeida *et al.* 2013). Careful selections of reference and contaminated sites, with similar general hydrology and geochemistry, within the study area as well as the selection of most suitable sentinel organisms, are just some of the choices that

should be made in order to minimized biomarker response variability in biomonitoring programs (Handy, *et al.* 2003).

1.4.2 DREAM

The DREAM (Dose-related Risk and Effect Assessment Model) is a software tool that is designed to support environmental risk assessment of discharge of complex chemical mixtures, such as those associated with offshore industry, to the marine environment. It is a three-dimensional numerical simulation model that calculates multi-chemical transport, fate, and concentration (up to 200 different chemicals), based on their physical, chemical, and toxicological parameters within a certain time frame. Simultaneously the model can also calculate exposure, dose, and effects in the marine environment. The model is driven by winds and currents that are created by other numerical models or measured as time series in the study location, and information about the depth of discharge (Reed & Rye 2011).

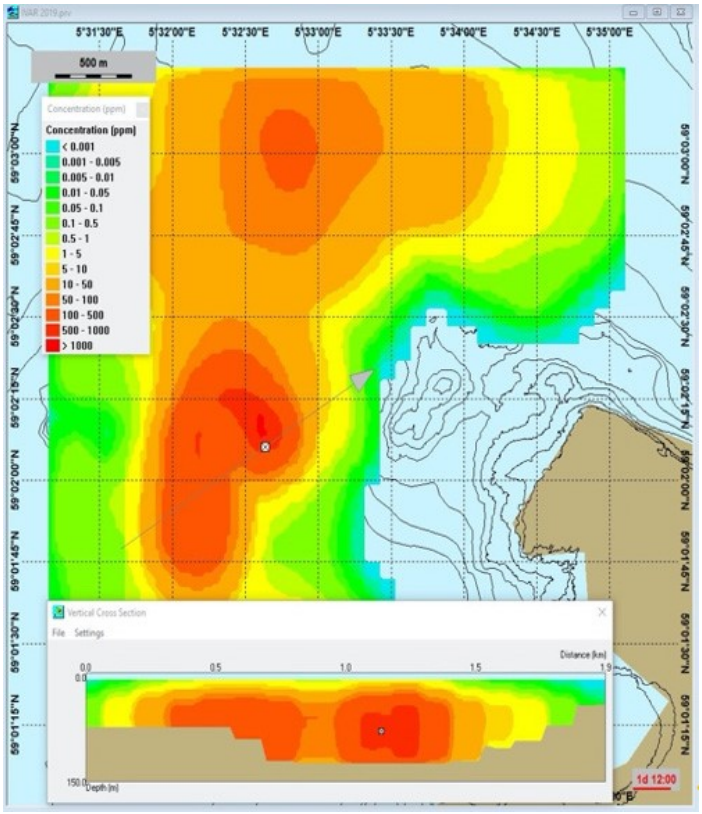


Figure 1.4 Snapshot of IVAR SNJ WWTP discharge from the DREAM model, showing both the bird's-eye view and the vertical cross-section.

In Marine Sewage Outfalls – Environmental Impact Evaluation project the DREAM was used to assess the recipient area of the IVAR SNJ WWTP discharge. The plume discharge rate model with predicted concentrations was computed, based on the average flow rates during dry weather (Figure 1.4). This model was used as a guide for selecting the sampling sites.

1.5 Organisms used in the environmental study

Invertebrate species were chosen for the study as they are abundant components of most aquatic ecosystems and representatives of different trophic levels that can be readily identified (Galloway, *et al.* 2004). The animals selected are the Norway king crab (*Lithodes maja*), an omnivore, the Norway lobster (*Nephrops norvegicus*), and the common periwinkle (*Littorina littorea*), a grazer of microorganisms, detritus, and algae, inhabiting the upper to sublittoral shore (Galloway, *et al.* 2004). The testing regime included both biomarkers of exposure and effect.

1.5.1 The periwinkle (*Littorina littorea*)

Figure 1.5 Periwinkle (*L. littorea*), location Randabergbukta (Photo: Private).

The periwinkle is one of the most common North Atlantic gastropods, distributed from southern Portugal to southern Spitzbergen in the eastern Atlantic, and in the western Atlantic from Virginia to Greenland (Cummins*, et al.* 2002). It is an inter-tidal shallow-water species that can be found in almost all kinds of shores, from the upper shore into the sublittoral, up to 15 m (Noventa *et al.* 2011), while in the colder and more northerly parts even up to 60 m depth (Oehlmann 2004). It can occupy sheltered sandy or muddy habitats like estuaries, and mudflats, but it prefers less exposed rocky coasts (Jackson 2008). Its feeding habit as an omnivorous grazer (green seaweeds, detritus, and microorganisms), where occasionally it may feed on the dead animal matter (Bauer *et al.* 1995), leads to ingestion of contaminants adsorbed to algae, and microorganisms (Noventa*, et al.* 2011). Seasonal variations and tidal cycles influence the feeding activity of the periwinkle. The feeding activity is higher during damp conditions and when the animal is immersed by the tide, during which they can modify shore habitat (Cummins*, et al.* 2002).

There are five stages of development which go from immature to fully mature and spawning and to spent. Maturation, breeding, and spawning time are dependent on local food availability and exposure, while egg release is impacted by the tidal cycle. Fertilization is internal, after which, the females release about 500 planktonic egg capsules (Cummins*, et al.* 2002). Each egg capsule contains 1-5 eggs, that hatches into a free-swimming veliger larva after 5-6 days, and remains in this planktonic stage 6-7 weeks. The

breeding period is usually from December to May in most regions. After breeding season male periwinkles shed their reproductive organ until a new one is build up upon the penis base, before the next breeding season, in late summer or autumn (Oehlmann 2004). During that period of reproduction inactivity, the growth rate of the periwinkles increases and it decreases when gonad maturation begins again the following November. In the period of the maximum reproductive activity, growth is completely stopped (Cummins, *et al.* 2002).

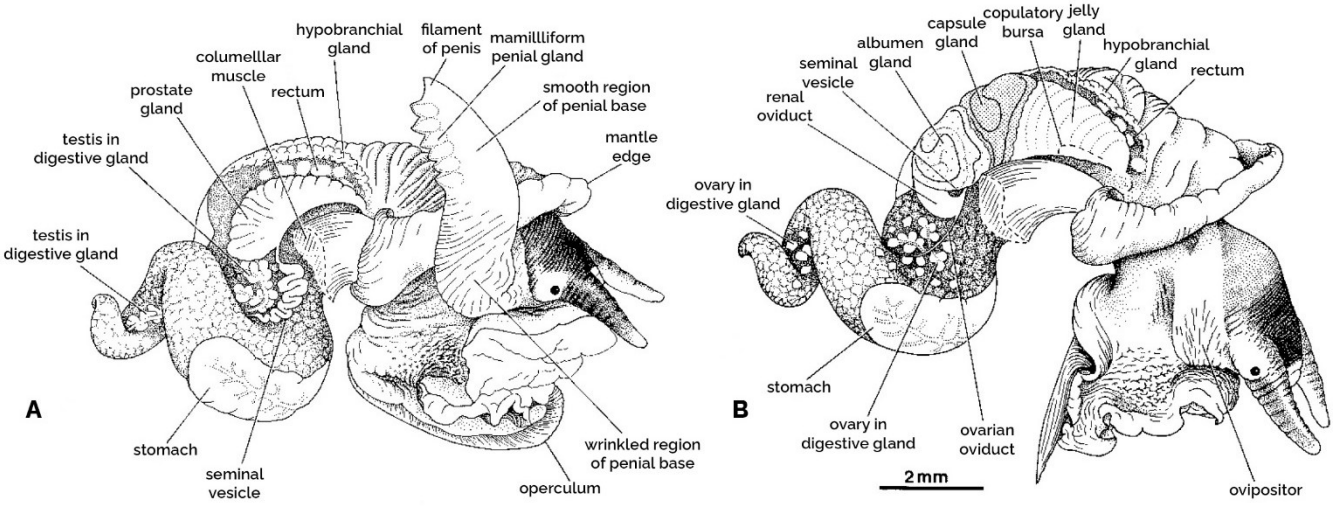


Figure 1.6 A: Male and B: Female *Littorina compressa* drawn anatomy. The anatomy of which closely resembles that of *L. littorea* (Cummins *et al.* 2002).

The shell of the *L. littorea* is sharply conical with a pointed apex and surface sculpturing. Its color is generally dark grey-brown or black, with an ear-shaped aperture (Jackson 2008). They can live longer than 9 years, depending on the environmental conditions. At a maturity age of 12-18 months, the shell reaches the height of 10-12 mm, while adults usually reach 40 mm shell height (Bauer, *et al.* 1995). Male specimens have a tendency to be slightly larger, with a shell that can be up to 52 mm (Jackson 2008). Parasite infection, predation, food availability, and habitat, affect growth and survival, as well as population density where competition for recourses act as a growth limit (Cummins, *et al.* 2002). The salinity of the ambient water particularly can affect the species growth, where the larger specimens can be found in locations of higher natural salinity of 35,000 ppm, while smaller individuals normally occur in brackish estuaries (Oehlmann 2004).

Humoral and cell-mediated are two types of immunity in the internal defense system of the Mollusca. The main role in both defense processes is performed by the circulating haemolymph cells (haemocytes) that are responsible for the recognition and elimination of a wide variety of pathogens. In the study conducted by Gorbushin and Iakovleva (2006) was found, through monitoring of the periwinkle

haemogram, that minimal haemocyte concentration in haemolymph is during the breeding period while there are two maximum concentration peaks during the summer months.

The use of *L. littorea* as a sentinel species in environmental assessment is well justified as they are resilient to environmental contamination and pore conditions, which allows them to be widespread at different geographical areas and at different contamination levels. There is also a detailed knowledge of the anatomy and biology of the periwinkles. This, in combination with a long life span, and feeding habits give this species the potential to bioaccumulate considerable quantities of hydrophobic contaminants, in addition to exposure to dissolved contaminants from the water phase (Noventa*, et al.* 2011).

In environmental monitoring studies, periwinkles have been used mainly as a bioindicator for the endocrine disruption caused by exposure to tributyltin (TBT) pollution in a marine environment, which can lead to the development of intersex, and reducing reproductive ability in this species (Noventa*, et al.* 2011; Oehlmann 2004; Galloway*, et al.* 2004; Bauer*, et al.* 1995). Other conducted biological studies, beside intersex development analysis, includes hemolymph sample assays, such as the neutral red retention time (NRRT) assay (Noventa*, et al.* 2011; Lowe *et al.* 2006), the comet assay (Noventa*, et al.* 2011), and the micronucleus (MN) assay (UNEP/RAMOGE 1999). Chemical analysis of periwinkle soft tissue as a good environmental indicator of PAHs and heavy metal contamination is also documented (Ololade *et al.* 2012; Noventa*, et al.* 2011).

1.5.2 The Norway King crab (*Lithodes maja*)

The Norway King crab or commonly called the northern stone crab is a member of the King crab (*Lithodidae*) family. In Norway, it is known by the name of Trollkrabbe. *L. maja* has a unique look with the pear-shaped shell and long, strong spines, longest around the perimeter of the carapace. Its coloration, from bright orange-red to brown, long legs and a highly developed branchial chamber are characteristic for the deep-sea crabs. The abdomen is formed by calcified plates which are symmetric in males but unsymmetric in females (Stevens & Lovrich 2014; DFO 1998).

The northern stone crab has a global distribution from far north as Svalbard, to the Barents and White Seas, along the coast of Norway and the North Sea, alongside Iceland, the British Isles, and the Netherlands in the northeast Atlantic. In the northwest Atlantic, it has been located from the Davis Strait, alongside the west and east coasts of Greenland, and the North American coast to Newfoundland (Stevens & Lovrich 2014; DFO 1998). It has been found on the open coast and offshore, where it mostly inhabits sandy and clay sea beds, between 10 and 1000 meters in depth (Wilson 2006) with the greatest concentration usually from 400 to 500 m. The temperature range on which they occur is normally 0°C–11°C (Stevens & Lovrich 2014).

Figure 1.7 Norway King Crab (*L. maja*) (Photo adopted from Mbakwe (2016)).

The Norway King crab is, as well as all the other crabs, decapod, it has five pairs of legs. The fifth pair is atrophied and hidden under the shell. Also, like all the other crustaceans, *L. maja* grows by molting (DFO 1998). Molting is the process that allows crustaceans expansion of the body by periodic replacement of the complex multilayered, hard outer, exoskeleton. The period between molting, or intermolt period, could last a couple of months, while molt increment, during which physical expansion occurs, lasts a couple of minutes. Molting begins when the shell reaches its maximum hardness. Several weeks prior to the change, the exoskeleton becomes dark brown. During the molting process, the crab rapidly absorbs water and expands, leading to a split of exoskeleton along the rear margin, which allows the crab to back up out of the shell. The new exoskeleton, which is extremely soft in the first couple of hours, hardens over time until the next

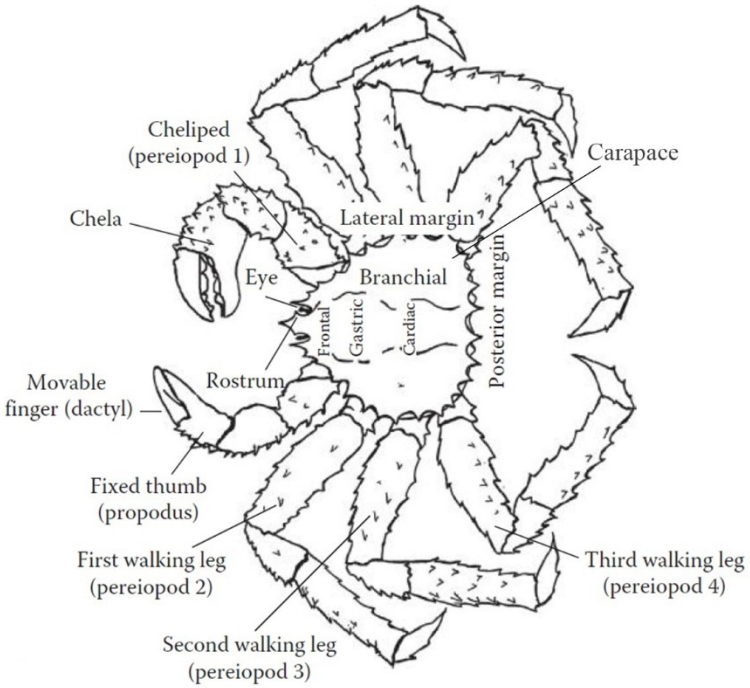


Figure 1.8 External dorsal anatomy of the King crab exoskeleton (Donaldson & Byersdorfer 2014).

molting. In the first years of life, molting occurs more than five times per year. With age frequency of molting decreases (Stevens & Jewett 2014).

The largest male specimen recorded had 1.075 kg and carapace length (CL), measured from the eye to the center of the rear part of the cephalothorax, around 114 mm. Although the female specimens are somewhat smaller with the highest recorded weight of 0.429 kg and 93 mm CL, on average, there is no significant difference in size between females and males. The majority of male northern stone crabs reach gonadal maturity at approximately 85 mm CL, while females at approximately 60 mm CL (DFO 1998). According to the model developed by Brown and Thatje (2019), which gives the ratio of size to age for the northern stone crab, sexual maturity for male specimens is at around 18 years while for females at around 16 years. Each female carries from 1,250 to 5,000 eggs, from which crabs hatch in the form of the larva. Before taking the adult form and migrate to the sea bed, crabs in the first three months live in surface water (Brown & Thatje 2019; DFO 1998).

The biological tendency of king crabs to gather in aggregations can significantly alter the abundance and diversity of local benthic fauna, by predation, reducing soft-bottom communities (gastropods, bivalves, echinoderms, and polychaetes), as well as physical habitat itself by removing infaunal organisms (Stevens & Jewett 2014).

Crustaceans are ecologically sensitive organisms and have been used as bioindicators to detect environmental changes in biochemical composition of sediment, organic content, and pollution in coastal and marine environments (*e.g.*, ocean acidification). Environmental stress can alter the distribution, abundance, and development of crustaceans (Naser 2013; Long*, et al.* 2013).

1.5.3 The Norway lobster (*Nephrops norvegicus*)

The Norway lobster is also a crustacean decapod and is a member of the *Nephropidae* family. It has a typical clawed lobster physiognomy, with a slenderer body shape and longer claws with spiny ridges, compared to other lobster species. Its color is generally from pale orange to reddish-orange, with darker markings on the carpopodites (Figure 1.9). Distinguishing characteristics of *N. norvegicus* also include non-segmented carapace, large, black, moveable kidney-shaped eyes, rostrum (eye protection) that has three pairs of lateral spines, and segmented abdomen with broad tail (Figure 1.10) (Sabatini & Hill 2008; Bell *et al.* 2006; Farmer 1975).

Geographically, the Norway lobster is widespread throughout the northeastern Atlantic, where it occupies the continental shelves and the upper continental slopes, from Iceland and Norway in the north to Morocco in the south, and Egypt in the southeast, including western and central Mediterranean, and the Adriatic Sea (Sabatini & Hill 2008; Farmer 1975). Its distribution is highly discontinuous as it has a burrowing behavior and prefers a particular type of muddy seabed sediments with > 40 % of silt and clay, that can support their unlined burrows (Bell*, et al.* 2006; Chapman 1980). *N. norvegicus* depth range is between 15 and 800 m (Farmer 1975), but it mostly can be found between 100 and 300 m, at temperatures between 6 and 10 °C. On the seabed surface they are constructing extensive, branching burrows, that can be 100 mm in diameter and from 200 to 300 mm in depth. There is a general assumption that the main purpose of the burrow is to provide shelter and protection from predators, so *Nephrops* exit their burrows only to feed and mate, thus reducing the risk of predation. Especially at the stage when they are particularly vulnerable, when juvenile, newly molted, or ovigerous. On the other hand, larger individuals are spending more time out of their shelters in order to obtain enough food (Chapman 1980). *N. norvegicus* is largely omnivorous and scavengers, and it feeds mostly on other benthic animals. While the main predators are fish, usually cod in the northern parts (Farmer 1975).

Figure 1.9 Norway Lobster (*N. norvegicus*) (Photo by Chris Inge Reiersen Espeland).

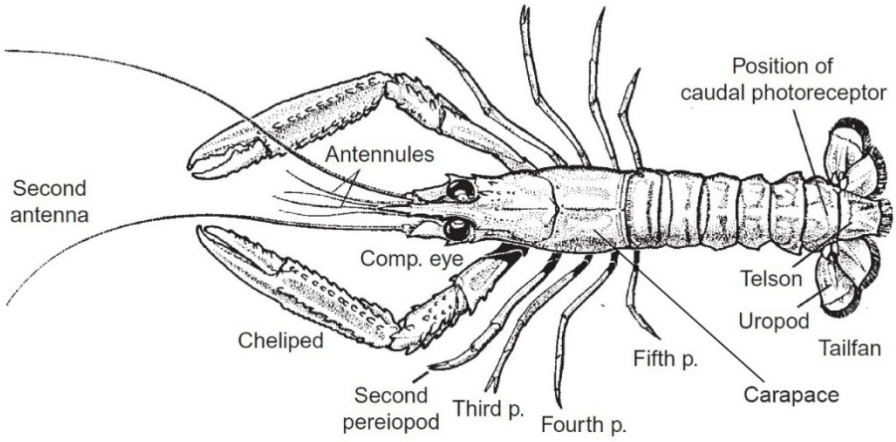


Figure 1.10 *N. norvegicus* drawn anatomy (Katoh *et al.* 2013).

N. norvegicus is a very sedentary species, it moves more by crawling than swimming, so rarely migrates over distances longer than a few hundred meters (Sabatini & Hill 2008; Bell*, et al.* 2006). It covers the greatest distance during the larva stage as they are planktonic and free-swimming (Farmer 1975). Females usually produce from 250 to 2000 pelagic larvae during each breeding season (Chapman 1980). *N. norvegicus* grows also by a discontinuous process of molting. The process is similar to that already described for the Norway king crabs. In the beginning frequency of molting is once a month. Later upon sexual maturity growth slows down to a couple of molts per year. After reaching sexual maturity, when carapace reaches approximately 40 mm length, the intermolt period becomes longer and the frequency decreases to 0-1 molt a year for females, and 1-2 molts for males (Chapman 1980). The life span of *N. norvegicus* is around 10 years. Because of its wide distribution, the size at sexual maturity, and spawning periods vary between locations. The usual age of sexual maturity for males is between 4 and 4.5 years, and carapace length from 29 to 46 mm, while for females between 3 to 3.5 years and carapace length from 29 to 34 mm (Sabatini & Hill 2008).

The Norway lobster is commercially the most important crustacean in Europe, it is fished wherever it is found in exploitable quantities (Bell*, et al.* 2006). As a result of uneven molting, male individuals are growing faster and bigger and consequently constitute a greater proportion of the catches (Farmer 1975). Environmental variations, fishing pressure, and genetic factors affect growth, development, and population density, which can vary significantly between areas (Queiros *et al.* 2012).

1.6 The selected biomarkers

1.6.1 General health status – Lysosomal membrane stability

Evaluation of the general health status of an organism is an important part of most biomonitoring studies, which can provide information about the population's overall health. The LMS has been used as a general health indicator within the framework of several pollution biomonitoring programs (Martínez-Gómez *et al.* 2015).

Lysosomes are cytoplasmic organelles with a single layer semipermeable membrane. These organelles contain over 40 different classes of hydrolytic enzymes (proteases, nucleases, lipases, etc.), that allow them to accumulate and hydrolyze biological compounds such as nucleic acids, proteins, lipids, and polysaccharides (Martínez-Gómez*, et al.* 2015). The universal role of lysosomes as a digestive system of the cell, by degradation of damaged and longer-lived cell material in the cytoplasm through autophagy process and by degradation of xenobiotics taken up into the cell by endocytosis, gives them detoxifying abilities (Moore *et al.* 2008). In addition to being part of the cellular digestive system, lysosomes are also

involved in the regulation of the catabolic rate of different cellular macromolecules, especially proteins, which is in support of the degradation process.

Lysosomal matrix is more acidic from its cytosol surrounding. Such acidic environment, with a pH value 4.5-5, is maintained with ATPase proton-pumping system across the lysosomal membrane, energetically ATP-dependent, that pumps H^+ ions inside the lysosomal cellular compartment and with acidic enzymes within the lysosomal matrix (Martínez-Gómez*, et al.* 2015). Any impairment of this system will cause an outpouring of the lysosomal acidic content into the cytosol, which can result in disruption of cell function that can lead to tissue damage and eventually to the reduction in the organism's general health status. There are essentially three categories of lysosomal impairments, which are changes in fusion events, changes in membrane permeability, and changes in lysosomal content (Martínez-Gómez*, et al.* 2015; Moore *et al.* 2004). These impairments of the lysosomal system can be caused by a combination or single action of several chemical and non-chemical stressors, like hypoxia, dietary depletion, organic compounds, metals, etc. (Moore*, et al.* 2004).

1.6.1.1 The neutral red retention time assay

Lysosomal capability for accumulating a wide range of xenobiotics has been used for the development of *in vivo* cytochemical neutral red retention time (NRRT) method for determining lysosomal membrane damage (Moore*, et al.* 2008; Lowe *et al.* 1992).

The NRRT assay is based on measuring the uptake and retention time of an amphiphilic, weak cationic neutral red dye inside the lysosomal organelles. Neutral red dye is trapped by protonation, sequestered, and accumulates within the lysosomal matrix. The ability and capacity of cells to accumulate and retain the neutral red dye reflects the level of lysosomal integrity and thus the organism's overall health. Healthier cells of organisms not exposed to any contaminant stress can retain longer and accumulate larger quantities of the dye compared to the damaged cells of exposed organisms. Lysosomal alterations caused by the uptake of neutral red, and in the case of the impaired membrane, leakage back into the cytosol, are monitored and quantified by visualization using a light microscope (Martínez-Gómez*, et al.* 2015; Moore*, et al.* 2004).

The method is rapid, inexpensive, and easy to perform. It has been adapted to be used on blood/hemolymph cells, and it can be conducted on a very small amount of sample, making it non-destructive to the host organism (Martínez-Gómez*, et al.* 2015). LMS is one of the most robust and sensitive effect biomarkers of non-specific physiological stress. Through various studies, it has been shown not to be affected by fluctuations of environmental factors, such as pH, salinity, temperature, oxygen, and food, neither by physical characteristics of an organism itself, like the size for example. On the other hand, it has

been observed that during the periods of organism's development and reproductive seasons, lysosomal stability has been decreased (Noventa 2010; Moore*, et al.* 2004). This method also has its limitations. Visualization and quantification could be subjective, and due to the time factor, limited to a smaller number of individuals per analysis session (Weeks & Svendsen 1996).

Wastewater effluents have a wide range of pollutants that, even in small concentrations, can increase the fragility and affect lysosomal membrane stability, making it a good biomarker of choice for this study. The NRRT assay is widely applied in monitoring LMS in aquatic organisms, and it has been performed with *L. littorea* as species of interest in several studies (Noventa*, et al.* 2011; Noventa 2010; Lowe*, et al.* 2006).

1.6.2 General health status - The condition index

The CI is one of the most commonly used biomarkers of biological effect in biomonitoring programs. It is used as a measure of energetic status, which reflects the nutritional and physiological health condition of an individual organism. CI is a physiological determination of the energy available for growth, migration, and reproduction (Moore*, et al.* 2004), generally expressed as fat reserves of individuals.

CIs are usually based on the ratio of body mass and different linear morphometric variables of an organism, such as body length, depending on the species. CIs can also be based on the weight/weight relationship, for example, a decrease in organ weight relative to whole body weight can reflect organ toxicity or disease (Hook*, et al.* 2014), or it can be the ratio of dry/wet weight in tissues.

Many factors, in addition to contaminants, like population density, food availability, environmental conditions, and their seasonal variations, could affect the condition index of an organism (Martínez-Gómez*, et al.* 2015).

For crustaceans, CI is normally measured as the relationship of total weight to carapace length, or to total length (Farmer 1975), while for the molluscs as a dry/wet weight relationship. In the case that the tissue of the examined molluscs individuals is used for further biological analysis, the condition index can be measured as the ratio of total wet body weight and total wet tissue weight (Amiard *et al.* 2004).

1.6.3 Genotoxicity

Chemical compounds and physical agents, such as ionizing radiation, that can induce alteration of DNA replication and genetic transmission are known as genotoxic agents. Usually, cellular enzymes are able to repair these alterations unless they are inhibited by some contaminants, like heavy metals. If the cell's defense mechanism fails to repair the disrupted DNA structure of the cell, caused by the action of genotoxic agents, damage genetic material can be transferred further by cell division producing mutant

cells, resulting in adverse effects at the level of the whole organism, and later at the population level (Walker*, et al.* 2012; Noventa 2010).

Chemical genotoxic compounds can be divided according to the mechanism of interaction with DNA into those with direct and those with indirect interaction. Genotoxicants that interact directly with genetic material include active compounds like hydrogen peroxide, herbicides, alkylating agents, etc. (Noventa 2010). On the other side, bioaccumulated genotoxic pollutants such as certain PAHs (e.g. benzo(a)pyrene, dibenzo(a,h)anthracene), aflatoxin, etc., which are relatively stable compounds, do not interact with DNA directly but indirectly through their metabolites. These highly reactive, short-lived metabolites are usually a by-product of oxidative metabolism by cytochrome P_{450}, with an affinity toward nucleophilic sites on cellular macromolecules, such as DNA (Walker*, et al.* 2012; UNEP/RAMOGE 1999).

There are several biomarkers for assessing genotoxicity *in situ*. Frequent occurrence of micronuclei (MN) formations is one of the biomarkers of the effect, that are detecting the presence of abnormal DNA structures (Noventa 2010). It has been routinely used in the monitoring of environmental pollution, and in combination with other physiological and biochemical biomarkers to fully assess the pollution status of affected areas (D'Costa *et al.* 2019).

1.6.3.1 Micronucleus assay

The micronucleus (MN) assay is initially developed by Schmid and Heddle in the late 1970s primarily for screening chemicals for chromosome damage effects in bone marrow cells of hamsters. Since then, the method has been successfully adopted to study genotoxicity in aquatic organisms as an "early warning" signal tool. It is a simple, sensitive, and rapid *in vivo* assay that quantifies MN formations, and provides information about genetic damage of the cells (D'Costa*, et al.* 2019; Noventa 2010).

Micronuclei are small round-shaped extranuclear bodies that are visible in the cytoplasm of cells. Formations of the micronuclei arise during mitosis, and originate from either chromosomal fragments (clastogenic events) or from whole chromosomal lagging (aneugenic events) in the anaphase mitosis stage which leads to loss of the chromosome from daughter nuclei (Luzhna *et al.* 2013). MN containing chromosomal fragments as their genetic structural material are a result of clastogenic events that can be a direct DNA breakage, replication on a damaged DNA template, or inhibition of DNA synthesis. Contrarily, MN containing a whole chromosome are a result of aneugenic events associated with defects that are preventing the chromosome to move toward the spindle poles. These defects usually can be a failure of the mitotic spindle, damaged kinetochore, centromeric DNA hypomethylation, and disruption in the cell cycle control system (Figure 1.11) (Luzhna*, et al.* 2013; Noventa 2010; Albertini *et al.* 2000).

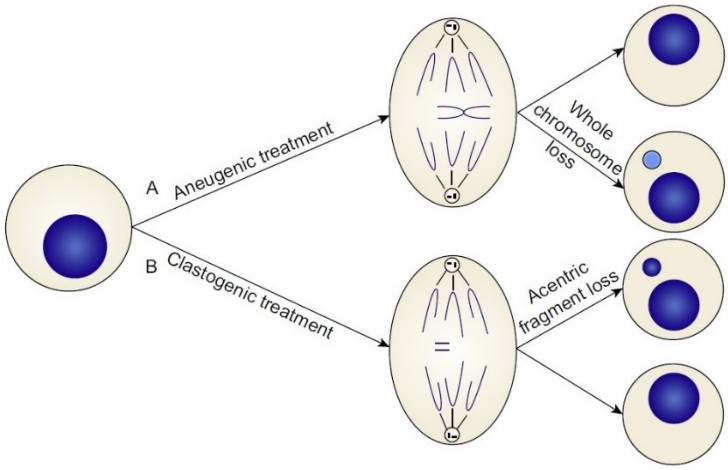


Figure 1.11 Mechanism of micronuclei formation (D'Costa, *et al.* 2019).

Quantification of micronuclei frequency is performed by visual observation under a light microscope. About 1000-2000 cells are usually scored from the smeared and fixed cell suspension on a microscope slide (Albertini, *et al.* 2000). Observation criteria that nucleus should meet in order to be considered as a micronucleus is defined by Schmid (1975):

- Size is less or equal to 1/3 of the main nucleus
- Completely separated from the main nucleus
- The shape is oval or round
- Staining intensity is similar to the main nucleus
- Located on the same optical plane as the main nucleus

The frequency of the micronuclei formations can be expressed as a number of MN per 1000 cells scored or as the percentage. DNA damage and MN frequency can be a reflection of exposure and genotoxic effects, which can lead to long-term consequences, such as mutagenesis and carcinogenesis (Noventa 2010).

Domestic wastewaters can contain a wide range of genotoxic substances, whose bioaccumulation in exposed organisms can potentially bring to genotoxic effects. In order to account for them, the MN assay was included in this study.

The technical limitation of this method is, as with the NRRT assay, due to the visual micronuclei scoring, subjectivity. Additionally, it has been found that physiological and environmental factors, as growth period, age, bioaccumulation capacity, reproduction, mitosis frequency, season variation, temperature, oxygen availability, and salinity, are influencing the micronuclei formation in wild organisms. The presence of micronuclei must be associated with clastogenic and aneugenic events as a result of exposure to genotoxic agents in order for the results to be applicable (Noventa 2010).

1.6.4 Neurotoxicity

The nervous system, which is an integral part of all animals (except sponges), has a vital role in regulating the functions of organisms, enabling communication between receptors and effectors, that converts the impulse into action. Information passes through the nervous system along the axons of neurons (nerve cells) as electrical impulses, and from one neuron to another across synapses (nerve junctions) by chemical messengers (neurotransmitters) (Walker*, et al.* 2012).

Toxic chemicals affect the nervous system of both vertebrates and invertebrates, by disturbing the normal transmission of impulses along nerves and/or across synapses. Neurotoxins can occur naturally or can be manufactured. There are five major groups of insecticides among anthropogenic neurotoxins, which are usually emphasized – organophosphorus, organochlorine, carbamates, neonicotinoid, and pyrethroid (Walker*, et al.* 2012).

1.6.4.1 Acetylcholinesterase mechanism of action

Acetylcholine (ACh) is a neurotransmitter that released from nerve endings diffuse across the synaptic cleft by a presynaptic impulse and interacts with receptors on the postsynaptic membranes of adjacent neurons, generating a signal so that the impulse is carried on (Walker*, et al.* 2012; Emson & Kerkut 1971). Reducing ACh concentration regulates nervous transmission. Therefore, it is important that this signal be rapidly terminated after its initial reaction.

Acetylcholinesterase (AChE, EC 3.1.1.7) is a serine hydrolase mainly found at neuromuscular junctions and cholinergic brain synapses (Colović *et al.* 2013). It is part of the cholinesterase (ChE) enzyme family present both in vertebrates and invertebrates (Bocquené *et al.* 1997; Emson & Kerkut 1971). In invertebrates, cholinesterase enzymes are often highly polymorphic (Kim & Lee 2017; Gaitonde *et al.* 2006). The main role of AChE is to quickly terminate the impulse transmission by breaking down the neurotransmitter acetylcholine (ACh) at cholinergic synapses. AChE enzyme catalyzes rapid hydrolysis of the ACh into inactive choline and acetic acid, a reaction that is necessary to allow a cholinergic neuron to return to its resting state after activation. The inactive choline that is released by the breakdown of ACh is picked up again by the presynaptic nerve, and by combining it with acetyl-CoA, through acetyltransferase, synthesizes a new neurotransmitter (Figure 1.12) (Colović*, et al.* 2013; Massoulié *et al.* 1993). AChE is one of the fastest enzymes, it has high catalytic power, where each molecule of AChE degrades about 25,000 molecules of ACh per second, close to the rate of a diffusion-controlled reaction (Colović*, et al.* 2013; Quinn 1987).

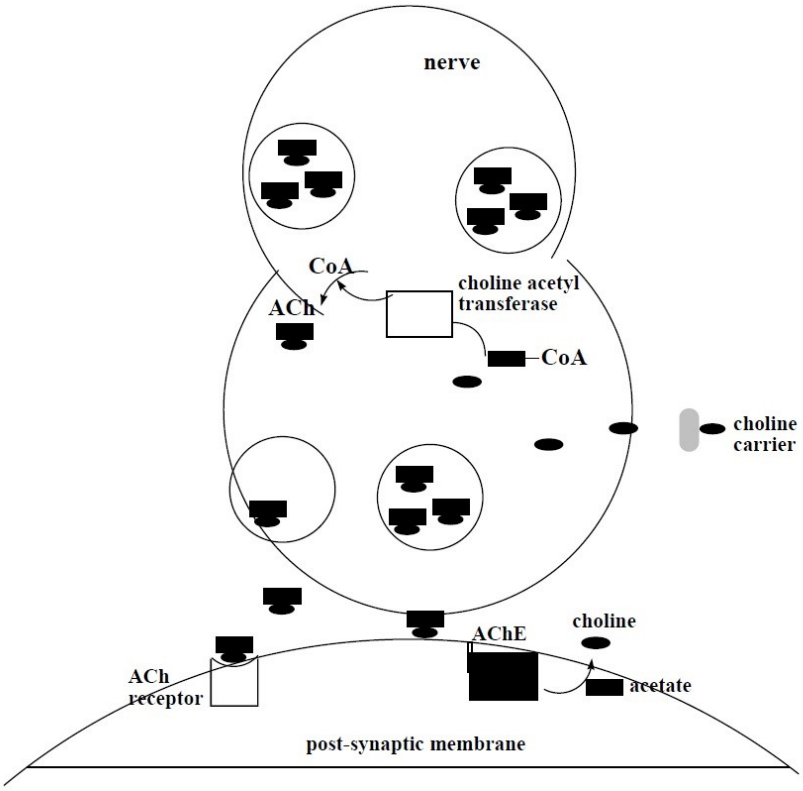


Figure 1.12 Mechanism of AChE action in neurotransmission (Colović, *et al.* 2013).

AChE inhibitors or anticholinesterases prevent or reduce the hydrolyses of acetylcholine, by binding to the catalytic site of the AChE enzyme. This results in an increase of ACh concentration in synapses, which leads to overstimulation of the receptors. The continuous stimulation of nerve fibers can result in a synaptic block, and acetylcholine will no longer be able to carry signals across the synapse. In the case of neuromuscular junctions in vertebrates, inhibition of AChE can lead to muscular spasms, paralysis of, for example, the diaphragm muscle, and finally death. In other cases, it can cause tremors and twitches, and coordinate disturbance (Walker, *et al.* 2012; Cajaraville *et al.* 2000; Bocquené & Galgani 1998).

1.6.4.2 Acetylcholinesterase as a biomarker

AChE activity measurement represents a well-known biomarker of both exposure and effect, for monitoring of environmental contamination caused by neurotoxic substances. It has been used frequently in marine environment biomonitoring on both fish and invertebrates (Gaitonde, *et al.* 2006; Lionetto *et al.* 2003; Dailianis *et al.* 2003; Cajaraville, *et al.* 2000; Sturm *et al.* 1999; Cerón *et al.* 1996; Galgani *et al.* 1992). Inhibition of the AChE enzyme has been directly linked to the toxic mechanism of organophosphorus and carbamates pesticides. However, in addition to them, evidence indicates the inhibition of AChE from other environmental contaminants including persistent organic pollutants (POPs),

heavy metals (Hg^{2+}, Cd^{2+}, Cu^{2+}, Pb^{2+}), polycyclic aromatic hydrocarbons (PAHs), detergents (SDBS), and components of complex chemical mixtures (Fu *et al.* 2018; Lionetto*, et al.* 2012). It has been shown that the AChE enzyme is present in different tissues of marine organisms. In the muscles and brain of fish, where the highest activity was found, in adductor muscle, digestive gland and gills of shellfish, and abdominal muscle of crustaceans. While mollusks show the lowest activity (Bocquené & Galgani 1998).

Environmental factors, such as temperature, salinity, trophic conditions, and dissolved oxygen content influence acetylcholinesterase activity in aquatic invertebrates, while salinity also has an effect on the uptake (and thus on toxicity) of pollutants (Lehtonen *et al.* 2006). As in all environmental monitoring studies, local abiotic factors and seasonal differences at the studied location have to be considered. The selected species must be well distributed, its biology well known, it must show a detectable AChE activity, and to have a limited range of migration. All the samples from one location, or ideally from all locations, should be analysed under the same conditions at the same time. The recommended number of samples required is a minimum of six to ten animals per site (Bocquené & Galgani 1998).

As recent studies have shown, AChE enzyme inhibition is affected by other pollutants as well and not only by organophosphorus and carbamates pesticides, in order to avoid wrong conclusions in biomonitoring studies, it should not be viewed solely as biomarkers of specific exposure but more as a general biomarker of neurotoxicity, at least in cases where the pollutant is unknown and chemical monitoring has not been performed (Lionetto*, et al.* 2012).

The most common assay for the determination of acetylcholinesterase activity is based on Ellman's method (1961). Where using an acetylthiocholine (ATC) and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) as substrate leads to a reaction that results in the production of 5-thio-2-nitrobenzoic acid (TNB), that has a yellow color due to the shift of electrons to the sulfur atom (Pohanka *et al.* 2011).

2 Materials and Methods

2.1 Sampling Sites

Samplings were performed on coastal beaches and marine areas located along the southwest coast of Norway (Figure 2.1). Periwinkles were sampled from two coastal stations in relative proximity of the discharge point, Sande beach and Randabergbukta, and from one reference station, Sola beach. Crabs and lobsters were sampled at four marine stations in Håsteinsfjord, all within 1 km radius of the effluent discharged point from IVAR SNJ WWTP. In addition, Boknafjord was chosen as a reference area, where crabs and lobsters were sampled from four stations as well, all within 500 m distance from each other (Table 2.1).

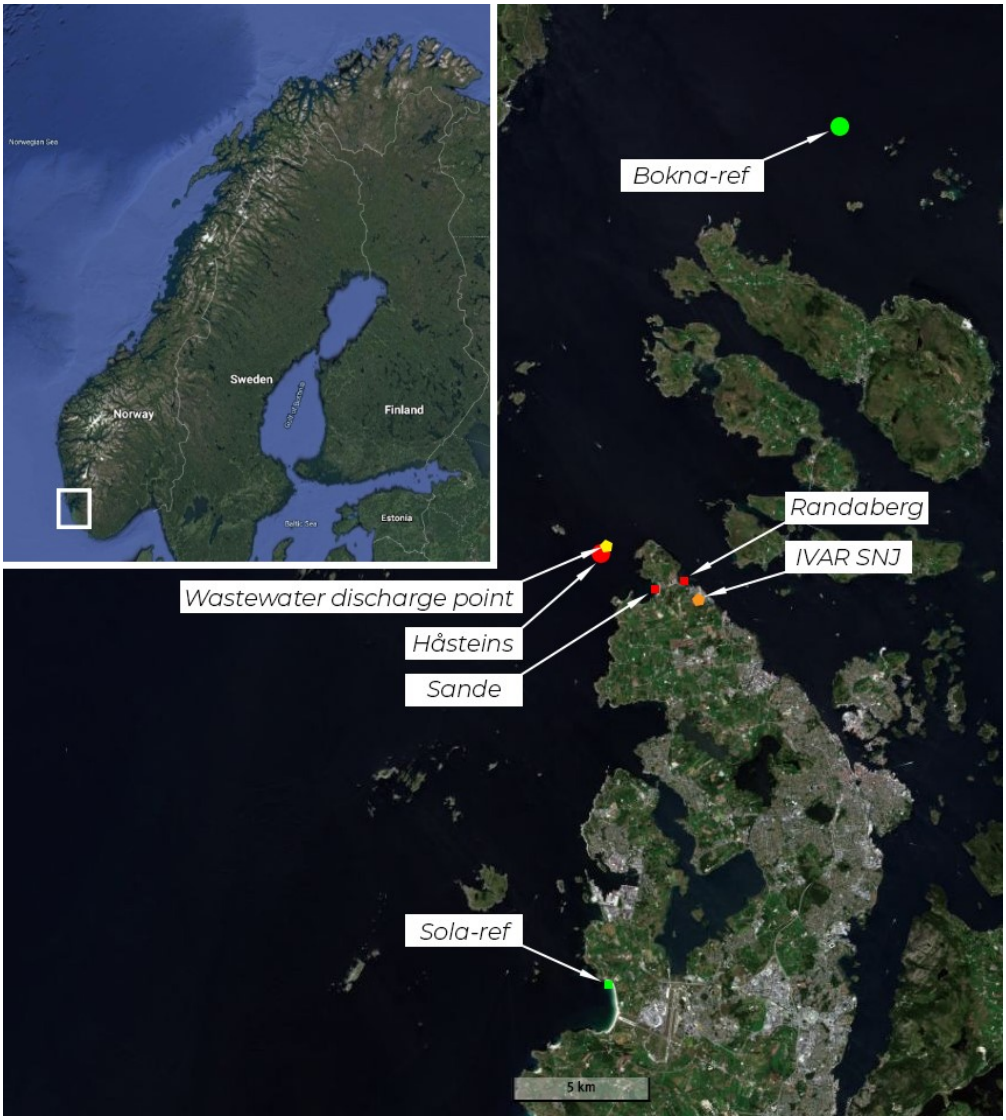


Figure 2.1 Map of Southwest Norway with overview of all sampling locations (©Google; ©Kartverket).

Table 2.1 Overview of all sampling locations.

Species	Location	Station Code		Coordinates
Common periwinkle (*Littorina littorea*)	Sandestranda	Sande		N 59°01.160, E 5°35.488
	Randabergbukta	Randaberg		N 59°01.415, E 5°36.397
	Solastranda	Sola-ref		N 58°53.587, E 5°35.578
Norway lobster (*Nephrops norvegicus*) and Troll crab (*Lithodes maja*)	Håsteinsfjord	Håsteins	Hå 1	N 59°01.836, E 5°33.075
			Hå 2	N 59°01.874, E 5°32.652
			Hå 3	N 59°01.419, E 5°32.809
			Hå 4	N 59°01.743, E 5°32.666
	Boknafjord	Bokna-ref	Bo-ref 1	N 59°10.624, E5°39.604
			Bo-ref 2	N 59°10.456, E 5°39.905
			Bo-ref 3	N 59°10.459, E 5°39.657
			Bo-ref 4	N 59°10.714, E 5°39.779

2.1.1 Coastal Sites

Sandestranda and Randabergbukta are two coastal sites that were selected based on the DREAM model of the wastewater discharge plume, according to which the potential impact of the effluent on the given locations could be expected. Solastranda, a referent site, was chosen based on its position, which is far from the effluent wastewater discharge zone and other known potential sources of anthropogenic pollution.

2.1.1.1 Sandestranda and Randabergbukta

Both sites are at the northern end of the Stavanger peninsula, in the rural municipality Randaberg (Figure 2.2). It is one of the municipalities in Rogaland county, in southwestern Norway, with sizable agricultural areas. Beach sediments dominate along the coast, consisting of sand with varying gravel content with boulders in the outer regions of the bays. It has, as well as the whole Stavanger peninsula, a typical Atlantic climate with a lot of precipitation and mild winters, and an average temperature of around 7 ^{O}C (Nyborg & Ulfeng 2017).

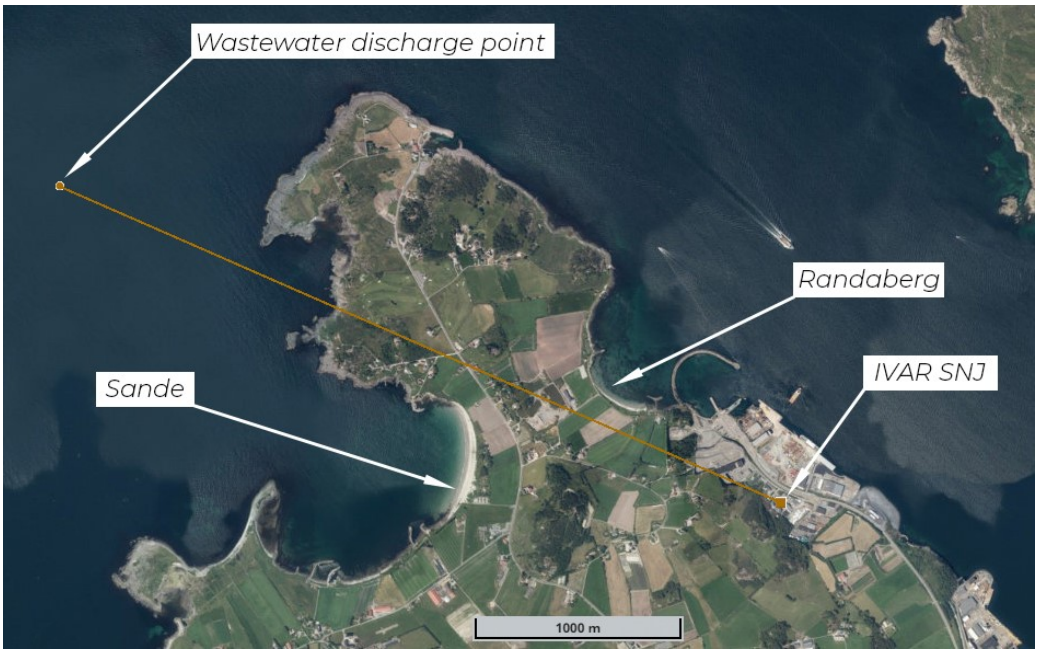


Figure 2.2 Overview of sampling stations in Randaberg (©Temakart-Rogaland).

Sandestranda is a wind-exposed, sandy beach interspersed with large boulders concentrated in certain parts. It is located in Sandeviga bay on the northwest side of the Stavanger peninsula, 4 km north of the Randaberg city center. The surrounding countryside has agricultural activities, from which, due to heavy rainfall, runoff descends directly to the beach. Sandestranda is open towards the Håsteinsfjord and is approximately 2.4 kilometers from the IVAR SNJ WWTP wastewater effluent release point. The sampling station for this site is located on the southern part of the beach (Figure 2.3).

Figure 2.3 Sandestranda sampling station (Photo: Private).

Randabergbukta bay is located on the opposite, northeast side of the Stavanger peninsula, about 1 km north from the IVAR SNJ facilities and approximately 3 km in aerial distance from the wastewater discharge point. There are industrial and shipping activities south of the bay. The topography of the beach is similar to the rest of the peninsula coast, sandy with varying gravel content, and there are also agricultural runoffs. There is a small dockage located in the southern part of the bay, which was the sampling station for this location (Figure 2.4).

Figure 2.4 Randabergbukta sampling station (Photo: Private).

2.1.1.2 Solastranda

Solastranda is around 3 kilometers long, open, sandy beach. It is located on the west side of the Stavanger peninsula in Sola municipality, south of Stavanger city, 2 km from the Stavanger Airport and approximately 16 km in aerial distance from the wastewater discharge point (Figure 2.5). The periwinkles from Solastranda were sampled from the northern part of the beach, on a rocky terrain by the Strandleiren chapel (Figure 2.6).

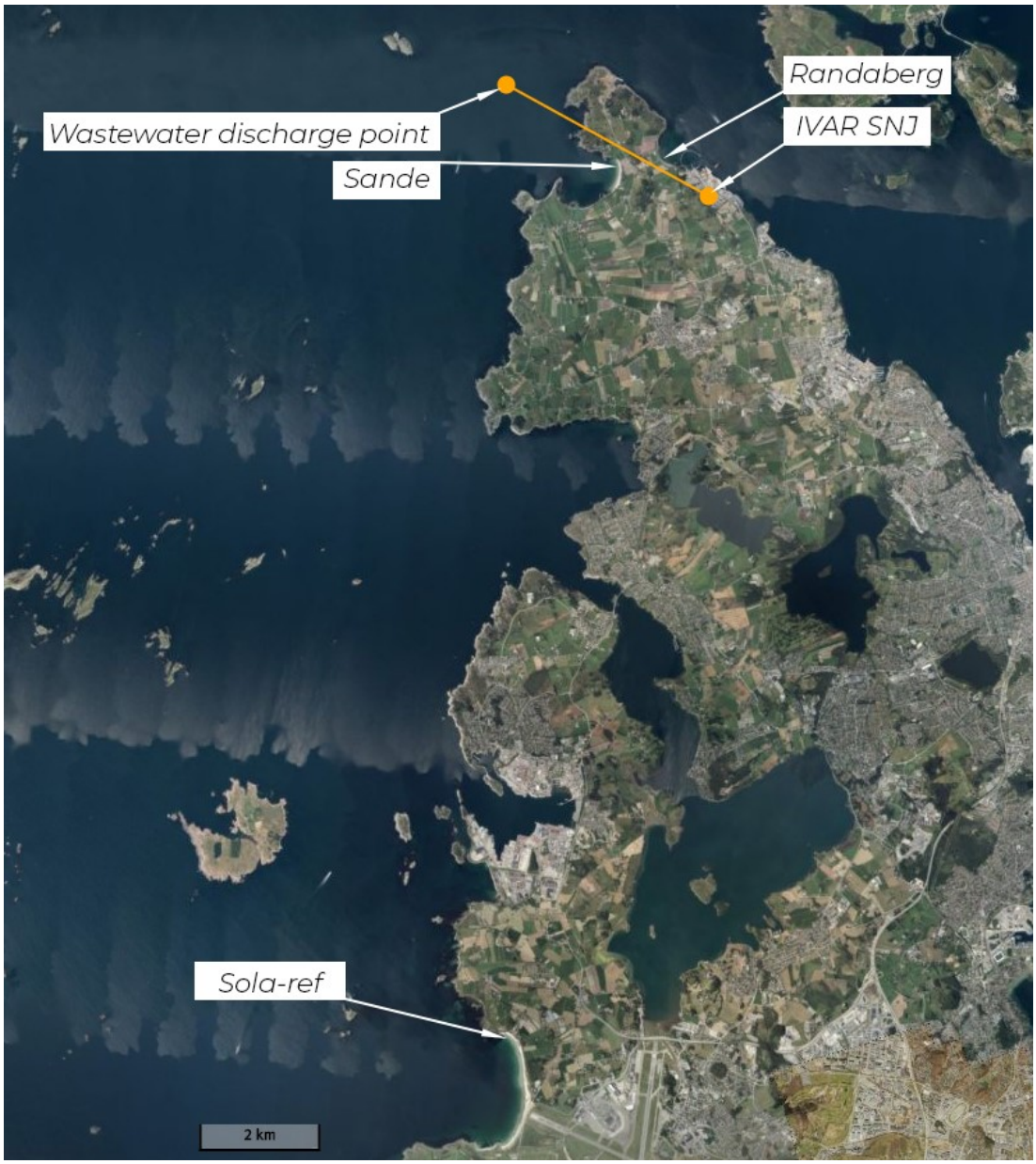


Figure 2.5 Overview of coastal sampling stations (©Kartverket).

Figure 2.6 Solastranda sampling station (Photo: Private).

2.1.2 Marine area

Sampling was conducted at two locations relatively far from the coast, using a boat. One in the immediate vicinity of the outflow and the other at the approximately 17 km aerial distance north of the outflow (Figure 2.7).

2.1.2.1 Håsteinsfjord

Håsteinsfjord is the main recipient of wastewater from the Stavanger peninsula. It is from 100-300 meters deep. In the ’90s, several preliminary studies have shown that the fjord has good flow conditions and water exchange, thus it is able to receive, quickly mix and dilute large amounts of wastewater (Tvedten *et al.* 2003).

The outfall from the IVAR SNJ WWTP is 1.6 km west from the Stavanger peninsula at 80 m depth in the Håsteinsfjord. Sampling was performed within a radius of one kilometer of the discharge point, the area that is directly exposed to the wastewater effluent. Within that radius, biological organisms were collected from four sampling stations. Station codes and coordinates are given in Table 2.1. As the sampling stations are close to each other, it was assumed that the organisms were exposed to the same concentration of the effluent, which was confirmed with the DREAM plume model. Thus, the classification of the samples was not done by the stations but by the area.

2.1.2.2 Boknafjord

Boknafjord is a large, 96 km long, open fjord in the northeast part of Rogaland county, that separates Jæren-Stavanger and Karmøy-Haugesund (Thorsnæs 2020). Here sampling was done in the open, deep part of the fjord, away from all known sources of pollution. It was carried out in the same way as in the Håsteinsfjord, from four sampling stations without separating the specimens.

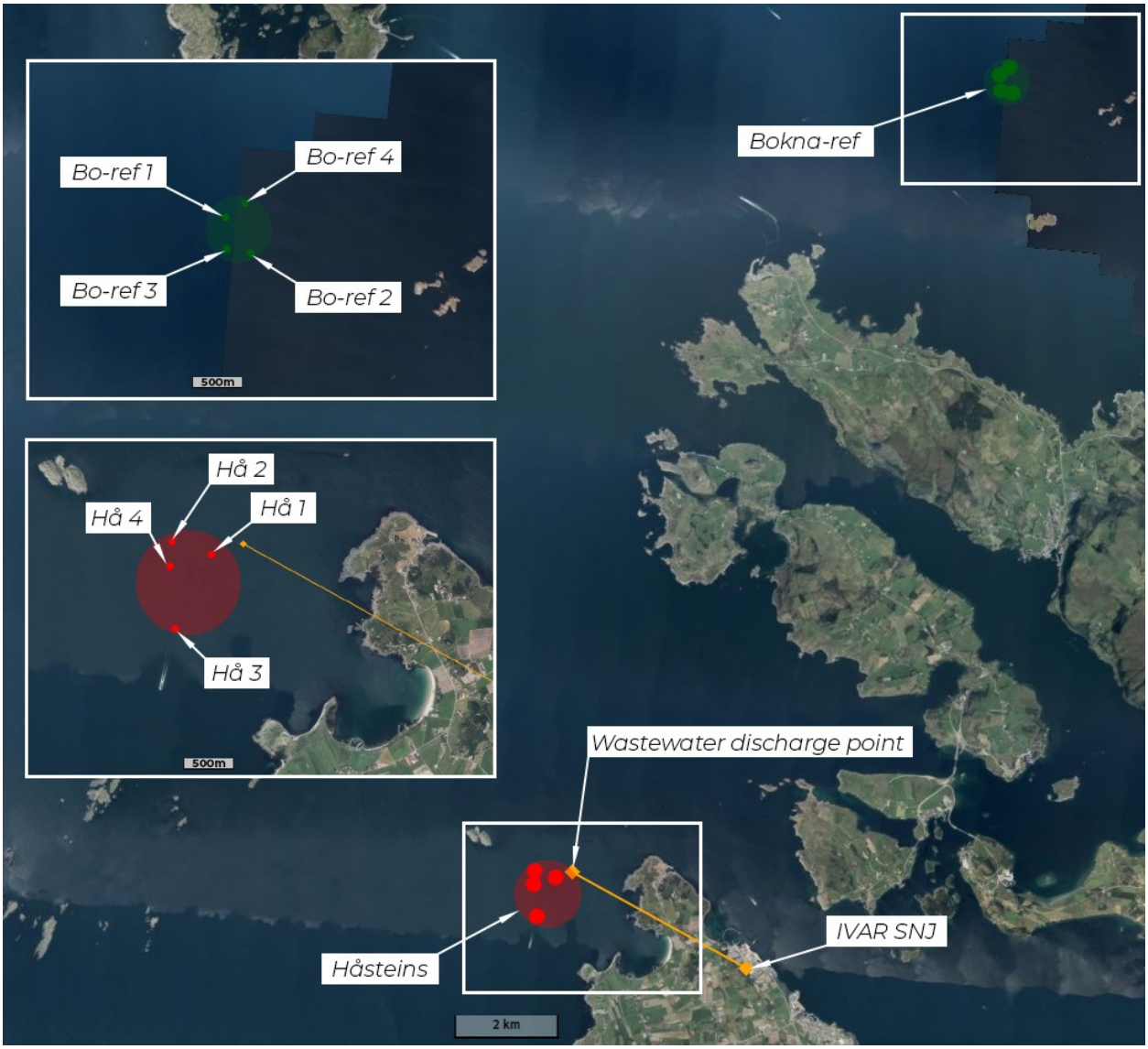


Figure 2.7 Overview of coastal sampling stations (©Kartverket).

2.2 Sampling

2.2.1 Coastal sampling

Periwinkles from all three coastal stations around the Stavanger peninsula were sampled by hand from the shore during low tide. About 30 individuals per site were collected and put into separate glass

bottles containing seawater from the corresponding locations (Appendix B). The glass bottles with the specimens were put into cold boxes and transported to the UiS laboratory. In the laboratory, periwinkles were transferred into three separated aerated plastic boxes filled with seawater from the location from where they were collected (Appendix B). Laboratory analysis was carried out after a two-hour adaptation period to the new environment, upon arrival.

Table 2.2 Overview of *L. littorea* sampling.

Location	Station Code	Sampling date	
		Sampling 1	Sampling 2
Solastranda	Sola-ref	17.09.19	28.09.19
Randabergbukta	Randaberg	18.09.19	28.09.19
Sandestranda	Sande	18.09.19	28.09.19

2.2.2 Marine sampling

Norway lobster and Troll crab were sampled by a boat using bottom trawl, which sailed out of Tungevika marina in Randaberg (Figure 2.8). A total of seven specimens of crab and eleven specimens of lobster from both locations were caught. Upon return to the marina, specimens were put into cold boxes, separated by location, and transferred to the UiS. The assays were performed shortly upon arrival at the laboratory.

Figure 2.8 Sampling boat, Tungevika marina, Randaberg.

Table 2.3 Overview of *N. norvegicus* and *L. maja* sampling.

Location	Station Code	Sampling date
Håsteinsfjord	Håsteins	31.10.19
Boknafjord	Bokna-ref	31.10.19

2.3 Biomarker analyses

All raw data of morphological measurements and biological test results for NRRT assay and CI are given in Appendix A, while the results for AChE assay are given in Appendix C, with a correlation to sample codes from Appendix A.

2.3.1 Sample Preparation

2.3.1.1 Haemolymph extraction

Haemolymph extraction from *L. littorea* was a part of NRRT and MN assays. The procedure was adapted and modified after Gorbushin & Iakovleva (2006), and Noventa et al. (2011). Between 200-300 µL of haemolymph was withdrawn from 15 specimens from each site by inserting a hypodermic syringe with a 21-gauge hypodermic needle behind the ocular tentacles. Extracted haemolymph samples were put into Eppendorf tubes and kept on ice for less than 30 min until use in the assays. For the NRRT assay, 30 µL of extracted haemolymph was used, while the remaining was used in the MN assay.

2.3.1.2 Soft tissue preparation

Soft tissue preparation of *L. littorea* was conducted as a part of the CI and AChE assays. During morphological measurements of snails, in order to determine the weight of soft tissue of the individual specimens, shells were carefully cracked open and removed by gently detaching, using pincette, columellar muscle from its attachment area to the shell. After the weighing, tissues of ten individuals from each site were put into the cryotubes and stored in the freezer at -80^{O}C, for later determination of the cholinesterase activity.

2.3.2 Neutral Red Retention Time Assay

NRRT assay was performed only on haemocytes of the periwinkles by using the *in vivo* cytochemical method described by Lowe *et al.* (1992), with minor modifications. Before starting the assay, Neutral Red Stock Solution and Neutral Red Working Solution were prepared. The stock solution was prepared one day in advance by dissolving 20 mg of Neutral Red dye powder in 1 mL of Dimethyl sulfoxide (DMSO) and stored in the refrigerator in a light-proof vial. Because stock solution solidifies at the low temperatures, on the day of the assay, shortly before use, it was kept outside the fridge at room temperature. The working solution was prepared by adding 5 µL of stock solution to 995 µL of filtered seawater and kept in the light-proof Eppendorf tube during the experiment. After the experiment, the working solution was safely discarded and a fresh solution was made on every new day of the assay.

Volumes of 30 µL of sampled haemolymph were transferred on the center of poly-L-lysine-coated microscope slides and placed in a light-proof humidity chamber containing wet paper towels. Slides were

left inside the chamber at room temperature for 10 minutes to allow the cells to adhere to the slide. Previously prepared 30 µL of the working solution was added to the slides (Appendix B), which were then covered with a 22x22 mm coverslip, and left in the humidity chamber to allow the uptake of neutral red into cells by membrane diffusion and to be trapped within the lysosome matrix (Mamaca *et al.* 2005). Slides were systematically examined one by one, by using a digital microscope at x4, x10, and x40 magnification (Figure 3.2), first at 15 min then after half an hour at 30 min interval until the endpoint was reached. The endpoint parameter is the time at which at least 50% of the cells show dye leakage through the lysosome membrane into the cytosol or abnormalities in lysosomal shape. Each slide was examined for no more than one minute. The last noted time before a sample reaches the endpoint is recorded as the NRRT.

2.3.3 Morphological measurements and condition index

Morphological measurements were taken from all individuals, of all three species, and the condition index was calculated for each species individually.

2.3.3.1 The Norway king crab

Morphological measurement for each specimen of the Norway king crab was performed with a Vernier caliper, excluding any protruding crests or spines. Measurements included carapace length (CL), carapace width (CW), rostrum base width (RW), orbital spine width (OW), the first spine length (SL), and the total wet weight. Measurement methods are shown in Figure 2.9. The carapace length was measured from the baseline of the orbit to the posterior edge of the carapace. These morphological measurement methods for king crabs were adapted from Brown & Thatje (2019), and Long*, et al.* (2013).

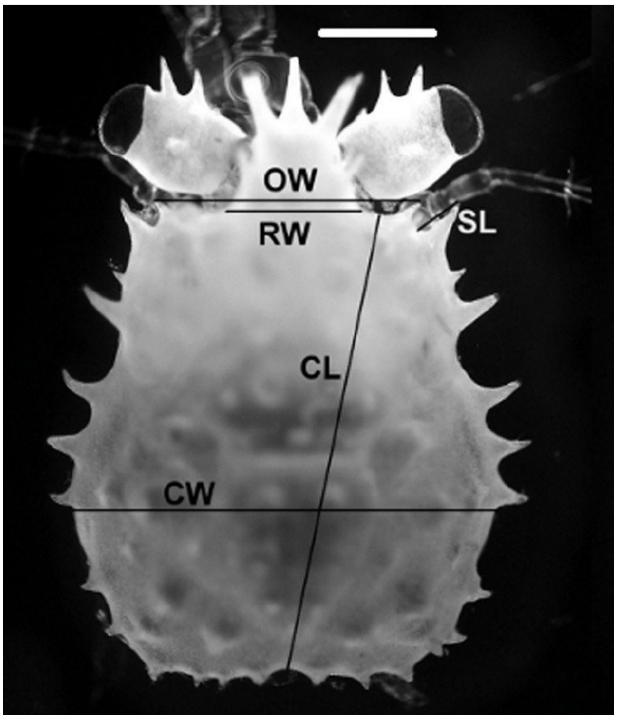


Figure 2.9 Morphological measurements of king crabs. CL: carapace length; CW: carapace width; RW: rostrum base width; OW: orbital spine width; SL: first spine length. The scale bars is 0.5 mm. (Adopted from Long *et al.* (2013)).

The weight of each crab was noted and CI was calculated as the mass in grams divided by the CL^3 in centimeters, modified from Long*, et al.* (2013):

$$CI_C = 100 * \frac{\text{Total wet weight [g]}}{\text{Carapace length}^3\text{[cm]}}$$

2.3.3.2 The Norway lobster

Standard morphological measurements for *N. norvegicus* are carapace length, tail length and width, abdominal width, width and depth of crusher and cutter claws, total length (TL), overall length (OL), and total wet weight (Mori *et al.* 1994; Farmer 1975). For this study total length (TL), overall length (OL), and total wet weight were examined. Measurement methods are shown in Figure 2.10. Total length (TL), is measured with a specimen placed on its back, so that abdominal segments are not flexed, from the tip of the rostrum to the posterior edge of the telson, excluding the setae. Overall length (OL) is measured from the tip of the claws according to the same principle CI was calculated according to Farmer (1975):

$$\mathrm{CI_L} = 100 * \frac{\text{Total wet weight [g]}}{\text{Total length}^3\text{[cm]}}$$

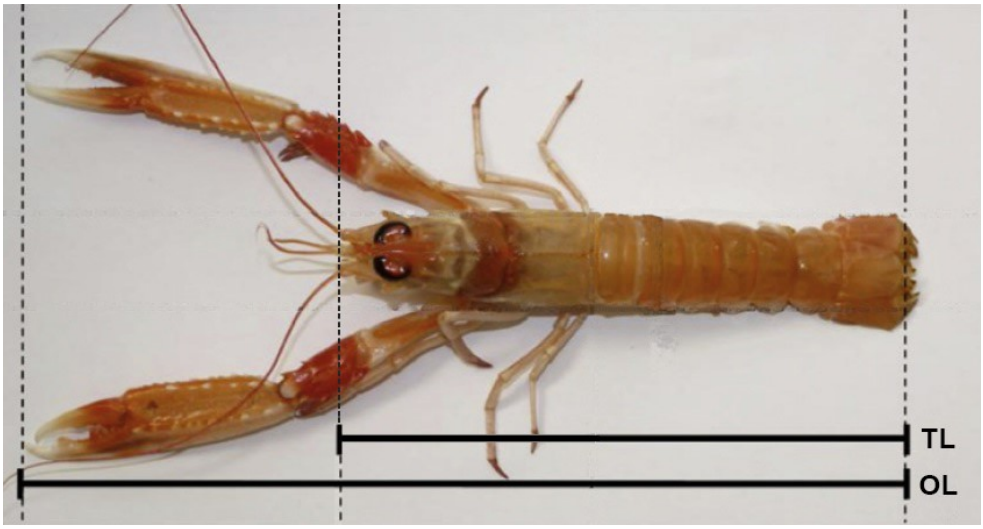


Figure 2.10 Morphological measurements of *N. norvegicus*. TL: total length; OL: overall length. (Adopted from Queiros, *et al.* (2012) and modified according to Mori, *et al.* (1994)).

2.3.3.3 The periwinkle

Morphological measurements for the periwinkles included the total weight of the individual (soft tissue + shell), and total wet weight of soft tissue, shell height and width, aperture height and width, and top height. Measuring methods are shown in Figure 2.11. After measuring the total weight of individuals, shells were carefully removed to extract soft tissue. Weight of wet soft tissue and total weight were recorded and used to determine condition index (CI). Condition index was calculated according to Amiard *et al*. (2004):

$$\mathrm{CI_P} = 100 * \frac{\text{Weight of wet soft tissue}}{\text{Total weight}}$$

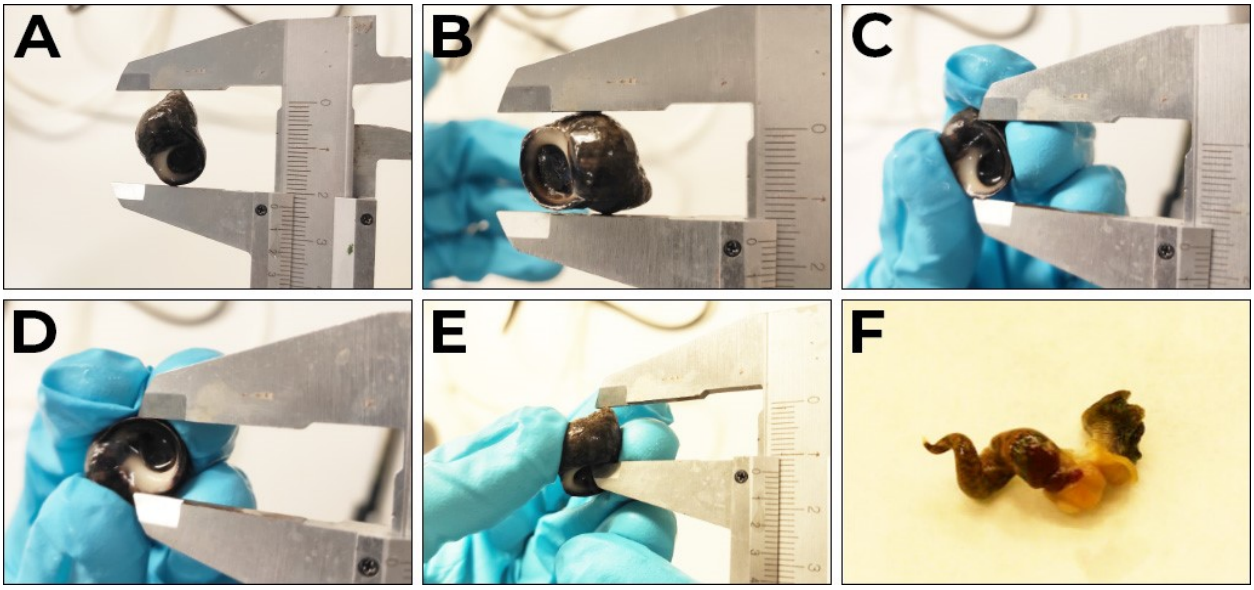


Figure 2.11 Morphological shell measurements of *L. littorea*, by Vernier caliper A: shell height; B: shell width; C: aperture height; D: aperture width, E: top height; F: soft tissue.

2.3.4 The micronucleus assay

Two MN assay protocols have been used for this analysis. A modified version of UNEP/RAMOGE (1999) protocol was followed during MN assay performed on specimens from first sampling, while protocol from Venier *et al.* (1997) was applied with specimens from second sampling (Table 2.*2*).

Sampling I

Haemolymph extracted from *L. littorea* specimens collected during first sampling was smeared on microscope slides and left to dry at room temperature. Haemocytes, generally preferred as a matrix for MN assay, were fixed by immersing the slides in Carnoy's solution, with a 3:1 methanol:acetic acid ratio, for 20 min at room temperature. Once again slides were left to dry at room temperature and afterward stored in a microscope slide box. Later, the slides were stained in the microscope slide staining dish containing a 3 % (v/v) Giemsa solution for 10 min and gently rinsed in tap water. DPX Mounting Media was used for gluing the coverslips on microscope slides.

During the examination of slides under the digital microscope (VisiScope® BL224T1), with x100 magnification, it was observed that haemocytes cells were not stained properly in order to evaluate and quantify MN formations with certainty.

The whole procedure was repeated with haemolymph samples that have been remained, but with a modified staining process. One set of replicate slides was stained with 3 % Giemsa solution, this time for 15 min, and another with 6 % Giemsa solution for 10 min (Appendix B). However, due to the failure of the Giemsa solution, haemocytes cells again were not stained properly.

Sampling II

As desired results were not obtained with specimens from the first sampling, a second sampling was necessary in order to collect more organisms for the analysis. The second sampling was conducted ten days after the first one. Before morphological measurements, 800 μL of haemolymph, from 15 new individuals per station, was extracted with the syringe containing 400 μL of seawater solution (seawater + EDTA 10 mM, at 1:1 ratio). Haemocytes were applied to glass microscope slides by a centrifuge with 3 different sample volumes (40 μL, 200 μL, and 290 μL), giving 3 replicates from each sample. They were centrifuged in Eppendorf refrigerated centrifuge 5424 R at 800 rpm for 2 min at 4 ^{O}C. For cell fixation, slides were dipped in cold methanol for 15 min. After the slides dried at room temperature and before staining, with a new Giemsa solution, haemocytes cells were examined under the digital microscope. It has been noticed that most of the haemocytes cells were not properly distributed, and even with successful staining, it would be difficult to evaluate and quantify MN formations with certainty.

In both cases, using two methods, due to technical failures, sufficiently reliable results could not have been obtained, thus they will not be further considered or be included in the results and discussion part of this study.

2.3.5 Acetylcholinesterase assay

Determination of AChE activity in *L. littorea* was conducted according to ICES TIMES No. 22 (Bocquené & Galgani 1998), adapted from Ellman *et al.* (1961), with some modifications referenced to Gaitonde *et al.* (2006). In this assay, AChE activity of tissue homogenates is evaluated utilizing a change in absorbance during time, by spectrophotometrically following the increase of yellow color produced from thiocholine when it reacts with dithiobis nitrobenzoic ion (DTNB - Ellman's Reagent) (Ellman*, et al.* 1961).

Acetylcholinesterase sample preparation

Soft tissues of ten individual snails from each sampling site, which were previously stored in the freezer at -80 ^{O}C, were used in this assay. The foot was removed, using a scalpel, from each individual, and the remainder of the soft tissue, digestive gland, gills, and muscles, were re-measured. Tissues from two individual animals from the same site were pooled together to make a composite sample of around 1g, giving five pooled samples for each location (Appendix B). The AChE enzymes were isolated from the tissues by homogenization with 0.02 M potassium phosphate buffer (pH 7-7.5) spiked with 0.1 % Triton X 100, at the ratio of 1/3 weight per volume (1 g of tissue per 3 mL of buffer) using a potter. The homogenates were centrifuged in Eppendorf refrigerated centrifuge 5424 R at 10,000 G for 20 min at 4 ^{O}C. Extracted supernatant was used for the determination of protein concentration and cholinesterase activity (cholinesterase source).

Measurement of total protein concentration

Total protein concentrations were determined according to the Lowry *et al.* (1951) method, adapted for measurement by plate reader. In order to determine the protein concentration in the samples, it was first necessary to obtain a calibration curve based on a standard sample of known concentration. This is done by using a Modified Lowry Protein Assay Kit (Thermo Fisher Scientific Inc.) with bovine serum albumin (BSA) as the standard.

Diluted albumin (BSA) standards (concentration of stock solution – 2000 µg/mL) were prepared with the same 0.02 M potassium phosphate buffer (pH 7-7.5) spiked with 0.1 % Triton X 100 buffer, with final concentrations ranging from 0 (blank) to 1500 µg/mL. 40 µL of each standard was transferred into a 96 microplate well. 200 µL of Modified Lowry Reagent was added to each well within a very short time using a repeater pipette. After 10 minutes of incubation 20 µL of prepared 1X Folin-Ciocalteu reagent was added to each well with a multichannel pipette. 1X Folin-Ciocalteu reagent was prepared by diluting the supplied 2X reagent with ultrapure water at the ratio of 1:1. Because of its instability 1X reagent was prepared on the same day of use.

In order to get the most accurate result and minimize the human error factor, the whole procedure was done in eight replicate series, at the same time, on the same microplate. After 30 minutes of incubation, absorbance was measured at 750 nm by SpectraMax Paradigm Multi-Mode Microplate Reader, average values were determined, and the calibration curve was obtained (Appendix B).

The same procedure was repeated with samples of unknown protein concentrations in six replicants from each pooled sample. The average value of absorbance was determined for each pooled sample and concentration was calculated from the standard curve. After the first measurements showed that the concentration of protein in samples was too high, serial dilution was performed until measured adsorption/concentration entered in the calibration curve range. The range was obtained at 20-fold dilution.

Measurement of total cholinesterase activity

AChE activity was determined spectrophotometrically by measuring the increase in absorbance of the sample at 412 nm, which is a consequence of the production of the yellow-colored TNB (5-thio-2-nitrobenzoic acid) (Ellman*, et al.* 1961). 10 µL of supernatant was transferred into a 48 microplate well. Subsequently, 340 µL of the same 0.02 M phosphate buffer (pH 7-7.5) spiked with 0.1 % Triton X 100, and 20 µL of 0.01 M DTNB (5,5-dithiobis-2-nitrobenzoic acid), which is prepared in 0.1 M pH 8 TRIS/HCl, were added. After 5 minutes and just before reading, 10 µL of 0.1 M acetylthiocholine substrate (ATC in distilled water) was added. Enzyme activity - the change in absorbance (kinetics), was measured by SpectraMax Paradigm Multi-Mode Microplate Reader at 412 nm at room temperature, every 15 s for a total of 10 min.

Change in absorbance during enzyme activity is the product of reactions:

Acetylthiocholine (ATC) → thiocholine + acetate

Thiocholine + dithiobisnitrobenzoate (DTNB) → 5-thio-2-nitrobenzoic acid (TNB)

AChE inhibitors are affecting the production of Acetylthiocholine (ATC) that leads to decreasing the production of TNB, which results in lower absorbance (Ellman*, et al.* 1961).

Acetylcholinesterase activity calculation

Obtained concentration values of proteins from 20-fold diluted samples were corrected for this diluted factor. The change in absorbance (OD) per minute is determined by subtracting the measured absorbance of substrate hydrolysis without the enzyme, from the absorbance increase per minute measured for the sample. AChE activity is expressed as micromole of ATC hydrolysed per minute per milligram of protein (Bocquené & Galgani 1998), and it is calculated by the formula:

$$\text{AChE activity } (\mu\text{mol ATC min}^{-1}\text{mg protein}^{-1}) = \frac{\Delta A_{412} * \text{Vol}_T * 1000}{1.36 * 10^4 * \text{lightpath} * \text{Vol}_s * [\text{protein}]}$$

ΔA_{412} = change in absorbance (OD) per min, corrected for spontaneous hydrolysis;

Vol_T = total assay volume (0.38 mL);

1.36 x 10^4 = extinction coefficient of TNB (M^{-1} cm^{-1});

Lightpath = microplate well depth (1 cm);

Vol_S = sample volume (in mL);

[protein] = concentration of protein in the enzymatic extract, corrected for dilution factor (mg mL^{-1}).

2.4 Statistical analysis

Results were analysed using the statistical packages Minitab (Minitab® Version 20.2 for Windows) and SPSS (IBM® SPSS® Statistics® Version 26 for Windows). Statistical differences between the groups of biological data were assessed with analysis of variance using one-way ANOVA. Where there were only two categorical groups the independent t-test was used.

Homogeneity of variance for the different categorical groups was checked using the Levene's test. Where the assumption of homogeneity was violated the Welch ANOVA analysis of variance was performed, with the Games-Howell post-hoc test to compare significant differences from the reference group. Where homogeneity of variance was achieved the Scheffé F-test was used within the one-way ANOVA test. Differences at the $p \leq 0.05$ level were considered significant.

Analysis of the measured variables, for the periwinkle, included lysosomal membrane stability-NRRT assay, CI, and AChE assay. For the NRRT assay, Levene's test showed heterogeneity of variance within groups, so p-values were calculated by using Games-Howell post-hoc test within the Welch ANOVA test. For the periwinkle CI, normal distribution and homogeneity of variance were verified by Shapiro-Wilk normality test ($p > 0.05$), before statistical analysis, hence one-way ANOVA was used, with Scheffé F-test. For the AChE assay, p-values were calculated by using Scheffé F-test within one-way ANOVA.

The CI results for the Norway king crab and the Norway lobster were analysed with the independent t-test, as there are only two categorical groups.

3 Results and discussion

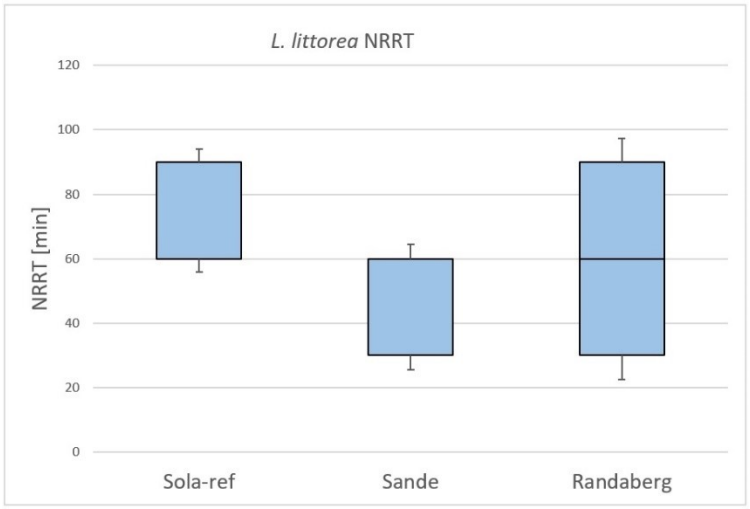
All raw data of morphological measurements and biological test results for NRRT assay and CI are provided in Appendix A, while Appendix C shows the results for the AChE assay. Each biomarker has been discussed separately, and Pearson correlation coefficient multiparametric analysis was performed on biological test results obtained from *L. littorea* species analysis.

3.1 The neutral red retention time assay

The NRRT assay was performed on *L. littorea*, a species that is not often used as a bioindicator and it has been used only in a few studies. The assay results are summarized in Figure 3.1 and observations of colored hemolymph cells observed at two different times are shown in Figure 3.2. The median value of the NRRT assay conducted on haemocytes blood cells of organisms collected from all three sampling stations was 60 min. For specimens collected at the reference station, Sola-ref, the time between individuals varied from 60 to 90 min, while for specimens from Sande and Randaberg stations varied between 30 and 90 min. No statistically significant differences between values recorded were found, except between those sampled at Sola-ref and Sande stations (Welch ANOVA, Games-Howell $p \leq 0.05$).

The LMS assay results have been used as an indicator of general health conditions. This analysis has shown that only *L. littorea* collected from the Sande station, 2.4 kilometers from the IVAR SNJ WWTP wastewater effluent release point, has poor health condition compared with organisms from the reference station, Sola-ref. The statistically significant difference indicates that collected organisms close to the wastewater outfall are subjected to the general environmental stress, that can be due to the wastewater discharge from IVAR SNJ WWTP, but it can also be a result of agricultural runoff from surrounding arable land, or due to some other sources.

However, it has been observed, by comparing the obtained results with the results from the study performed by Noventa (2010), on the same species along the British south and south-west coast, that most of the specimens recorded from reference stations in both studies have values higher than 60 min, but the recorded values of exposed organisms are around 15 min, drastically lower than in this study, indicating a lower level of exposure here.



NRRT	Randaberg	Sande	Sola-ref
Sola-ref	n.s.	*	
Sande	n.s.		
Randaberg			

Figure 3.1 Box and whisker diagram of NRRT results for *L. littorea*. Boxes indicate 95 % of values; horizontal lines in boxes indicate median values; whiskers are standard error bars. Statistical comparisons were done using the post hoc Games-Howell test and results are reported on the right side of the figure, *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; n.s.: not significant.

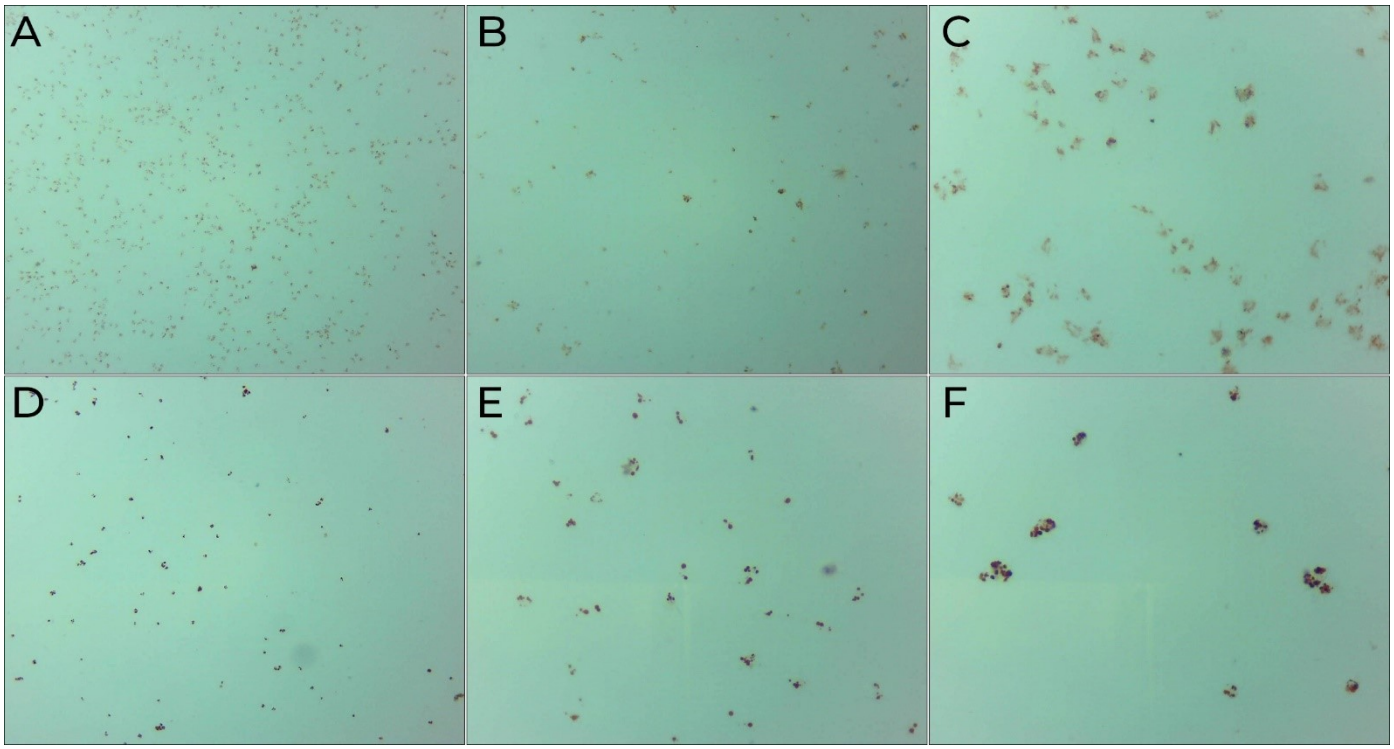


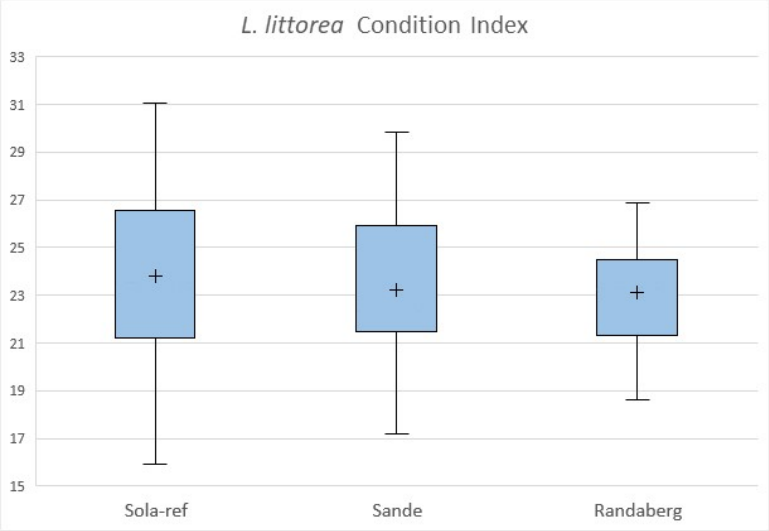
Figure 3.2 Colored hemolymph cells from the periwinkle (*L. littorea*). Examination at: A-C: 30 min; D-F: 90 min (endpoint), with digital microscope (VisiScope® BL224T1- Camera-X3N) at: A and D: x4; B and E: x10; C and F: x40 magnification.

3.2 Condition index

Common periwinkle

CI results for the periwinkle are summarized in Figure 3.3. Mean values in *L. littorea* collected from the sampling stations around the Stavanger peninsula varied in the very close range between 23.1, for organisms collected at Randaberg station, and 23.8 for organisms collected at Sola-ref station. For specimens collected at Sande station, the CI mean value was 23.2. Values recorded from sampled snails were not significantly different (ANOVA, Scheffé $p \leq 0.05$).

Considering the CI as a measure of energetic status, which reflects the nutritional and physiological health condition of an individual organism (Moore*, et al.* 2004), and as there was no significant difference between sampled organisms from all three stations, indicates that exposure to the wastewater effluent from IVAR SNJ WWTP does not have any significant negative effect on the conditions needed for growth and reproduction of this species.



CI	Randaberg	Sande	Sola-ref
Sola-ref	n.s.	n.s.	
Sande	n.s.		
Randaberg			

Figure 3.3 Box and whisker diagram of CI results for *L. littorea*. Boxes indicate 25% - 75% of values; plus signs (+) in boxes indicate mean values; whiskers indicate 10% - 90% of values. Statistical comparisons were done using the post hoc Scheffé test and results are reported on the right side of the figure, *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; n.s.: not significant.

The Norway king crab and the Norway lobster

CI results for the Norway king crab and the Norway lobster are summarized in Figure 3.4 and Figure 3.5 respectively. The mean CI values in *L. maja* collected from marine sampling stations showed that organisms sampled at reference station, Boka-ref, had a lower 48 mean value, compared to organisms collected within a 1 km radius from effluent discharge point at Håsteins station with 53.5 mean value.

The same was observed with the mean CI values in *N. norvegicus*. The mean value in organisms collected at the Boka-ref reference station was 2, while the mean CI value in organisms collected at the Håsteins station was 2.5. However, for both species, a significant statistical difference has not been shown (independent t-test $p \leq 0.05$).

Although there is no significant difference between individuals sampled at two different locations of both species, a difference in the distribution of CI values and mean values were noticeable, indicating slightly better conditions needed for growth and reproduction of both species around wastewater discharge point compared to the reference station. The finding indicates that exposure to the wastewater effluent from IVAR SNJ WWTP does not have any significant negative effect on these species.

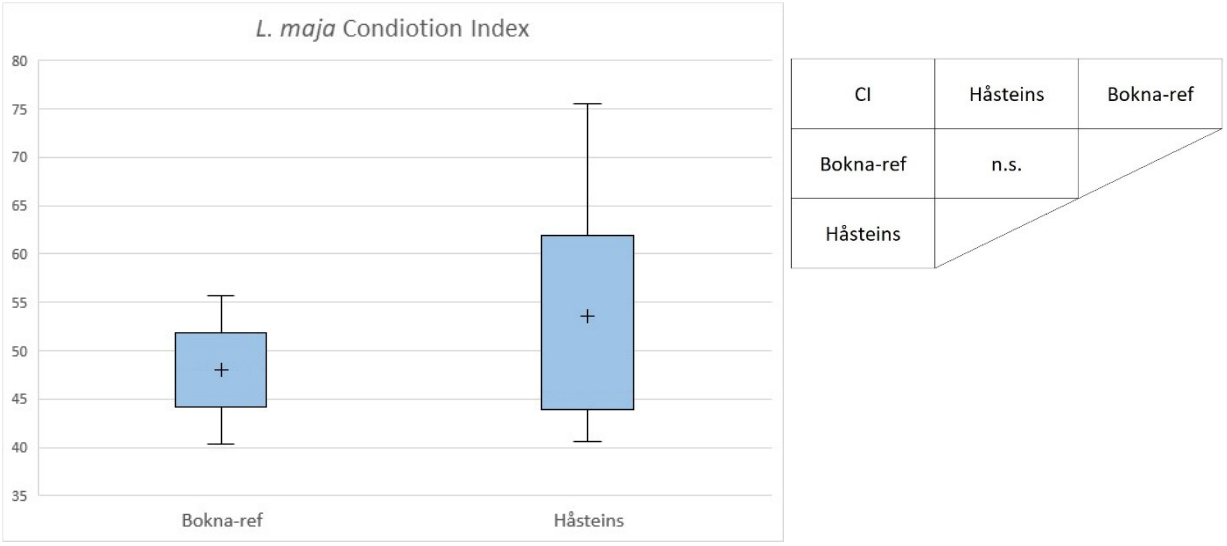


Figure 3.4 Box and whisker diagram of CI results for *L. maja*. Boxes indicate 25% - 75% of values; plus signs (+) in boxes indicate mean values; whiskers indicate lowest and highest data point excluding outliers. Statistical comparisons were done using the independent t-test and results are reported on the right side of the figure, *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; n.s.: not significant.

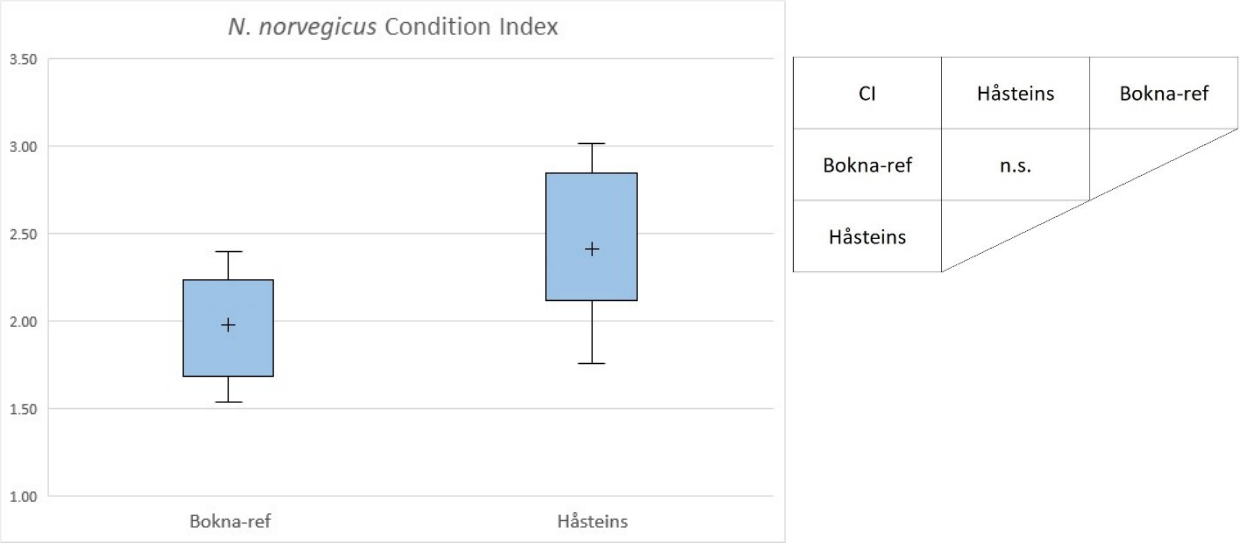


Figure 3.5 Box and whisker diagram of CI results for *N. norvegicus*. Boxes indicate 25% - 75% of values; plus signs (+) in boxes indicate mean values; whiskers indicate lowest and highest data point excluding outliers. Statistical comparisons were done using the independent t-test and results are reported on the right side of the figure, *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; n.s.: not significant.

3.3 Morphological measurement

Morphological measurement results for each species are reported in Figure 3.6 for the Norway king crab, Figure 3.7 for the Norway lobster, and Figure 3.8 for the periwinkle. Measurement methods for each species are described in Figure 2.9, Figure 2.10, and Figure 2.11 respectively. From the figures, it can be noticed that the organisms from the different sites were of comparable sizes.

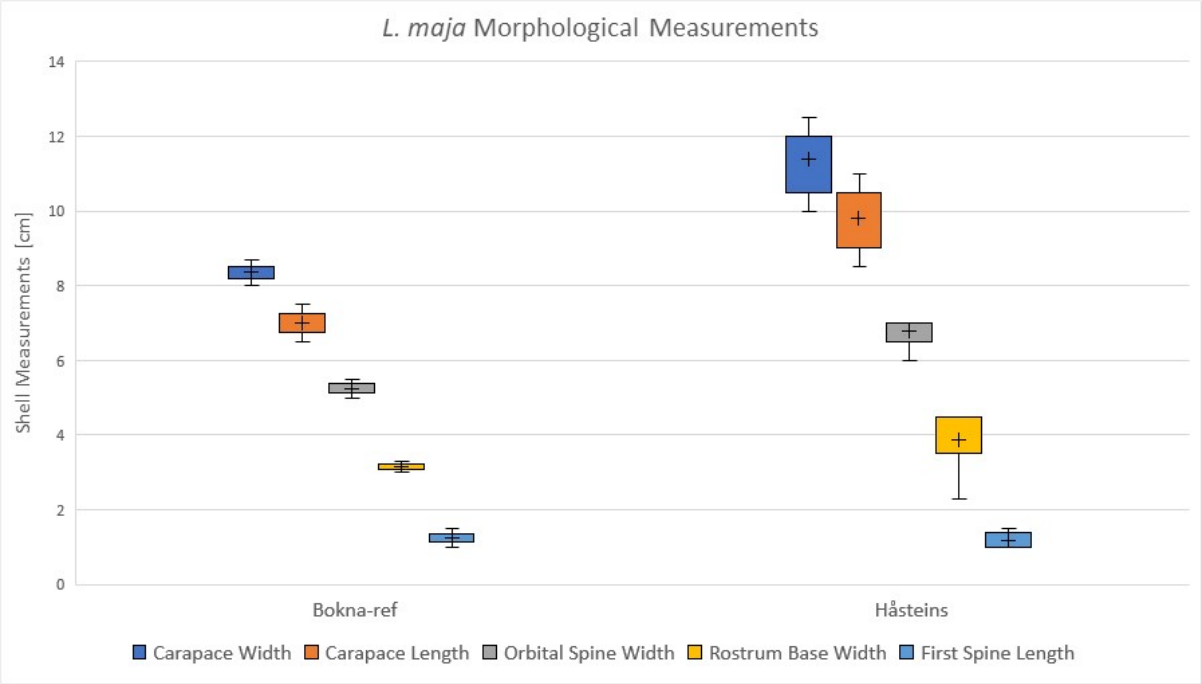


Figure 3.6 Box and whisker diagram of morphological shell measurements for *L. maja*. Boxes indicate 25% - 75% of values; plus signs (+) in boxes indicate mean values; whiskers indicate lowest and highest data point excluding outliers.

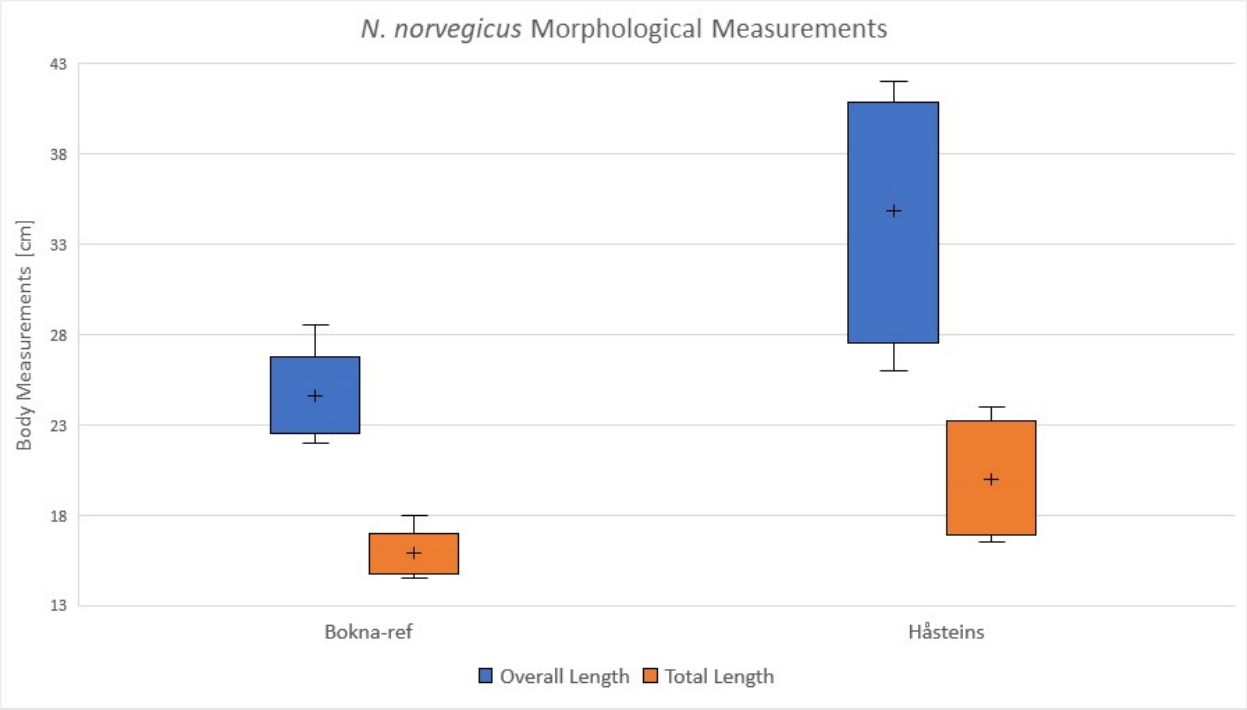


Figure 3.7 Box and whisker diagram of morphological measurements for *N. norvegicus*. Boxes indicate 25% - 75% of values; plus signs (+) in boxes indicate mean values; whiskers indicate lowest and highest data point excluding outliers.

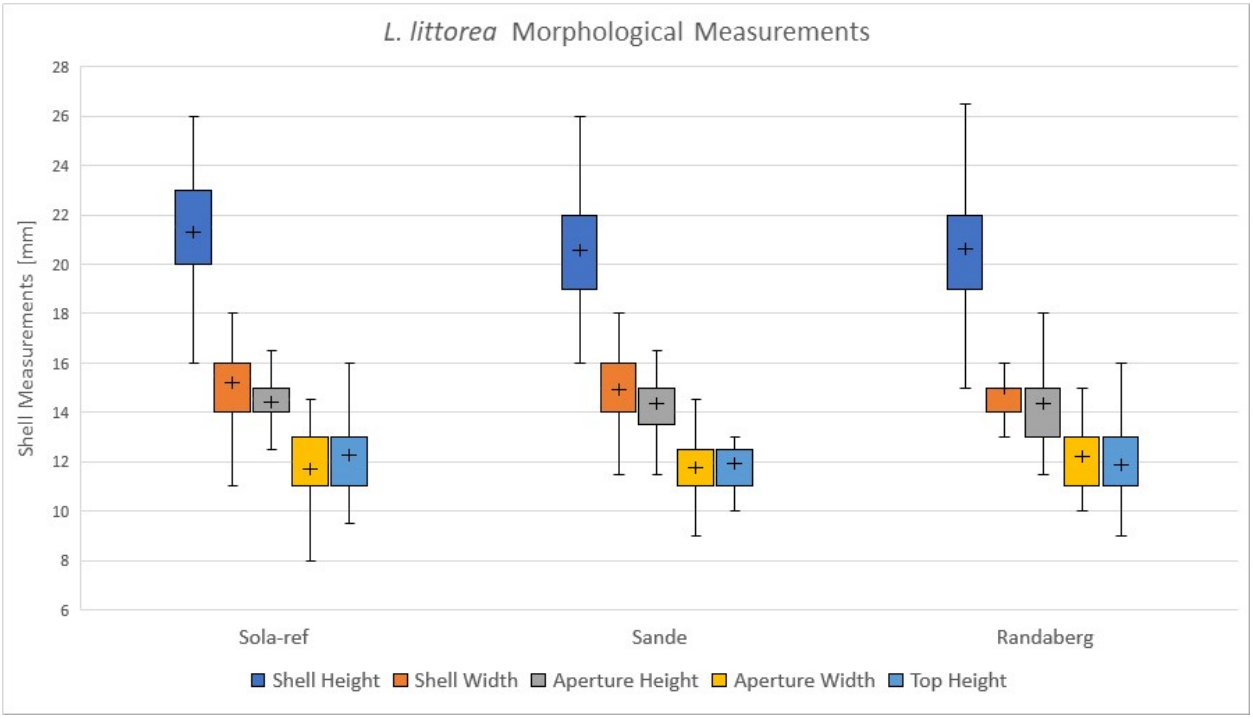
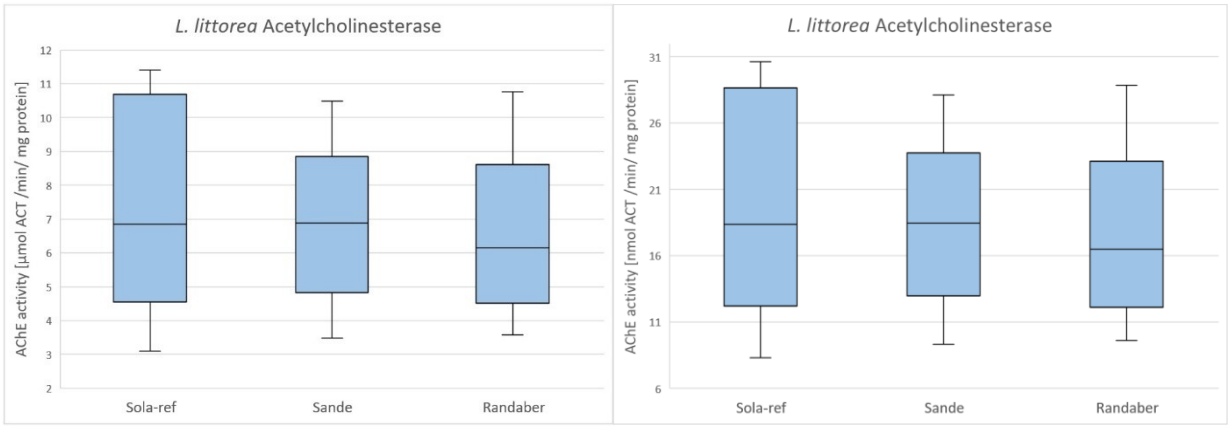


Figure 3.8 Box and whisker diagram of morphological shell measurements for *L. littorea*. Boxes indicate 25% - 75% of values; plus signs (+) in boxes indicate mean values; whiskers indicate lowest and highest data point excluding outliers.

3.4 Acetylcholinesterase assay

AChE assay results are summarized in Figure 3.9. Median AChE activity, in combined tissue of two snail individuals, ranged from 6.2 µmol ATC/min/mg protein in organisms from Randaberg station, to 6.8 and 6.9 µmol ATC/min/mg protein in organisms from Sola-ref and Sande stations respectively, all expressed as µmol ATC/min/mg protein. There were no statistically significant differences in AChE activity between values recorded in collected organisms (ANOVA, Scheffé $p \leq 0.05$).

As there were no data from any previous research of AChE activity in the tissue of *L. littorea*, obtained results are compared to ICES assessment criteria that have been reported for gill tissue of the blue mussels (*M. edulis*) from the French and Portuguese part of the Atlantic, and the Mediterranean mussel (*M. galloprovincialis*) from Mediterranean region of Spain and France (Davies & Vethaak 2012), modeled on Pampanin DM *et al.* (2019). Background assessment criteria (BAC) and environmental assessment criteria (EAC) are given in nmol ATC/min/mg protein. In order to compare the obtained results with the given criteria, additional calculations were necessary (Appendix C). Median AChE activity, expressed as nmol ATC/min/mg protein in snail tissue, ranged from 16.5 nmol ATC/min/mg protein in organisms from Randaberg station, to 18.3 and 18.5 nmol ATC/min/mg protein in organisms from Sola-ref and Sande stations respectively.



AChE	Randaberg	Sande	Sola-ref
Sola-ref	n.s.	n.s.	
Sande	n.s.		
Randaberg			

Figure 3.9 Box and whisker diagrams of AChE activity in combined tissue of *L. littorea*. In the left diagram activity expressed as µmol ATC/min/mg protein; in the right diagram activity expressed as nmol ATC/min/mg protein. Boxes indicate 25% - 75% of values; horizontal lines in boxes indicate median values; whiskers indicate lowest and highest data point excluding outliers. Statistical comparisons were done using the post hoc Scheffé test and results are reported at the bottom of the figure, *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; n.s.: not significant.

Criteria values from the *M. edulis* gill tissue for BAC between 26 and 30 nmol ATC/min/mg protein, and for EAC between 19 and 21 nmol ATC/min/mg protein have been suggested. Comparing given values with obtained results indicated that all of the periwinkle groups were below the proposed EAC and therefore suggesting a stress response, in relation to the given criteria. On the other side, criteria values from the *M. galloprovincialis* gill tissue for BAC between 15 and 29 nmol ATC/min/mg protein, and for EAC between 10 and 20 nmol ATC/min/mg protein have been suggested. Comparing with this criteria values indicated that AChE activity in snails collected around the Stavanger peninsula are inside the suggested range and therefore not showing a stress response.

However, these assessment criteria have been specified for mussels from Portuguese and French Atlantic waters and the Mediterranean region, and their comparability to *L. littorea* from the colder North Sea should be considered.

Overall, there were no significant differences between sampled organisms from all three stations around the Stavanger peninsula, indicating that exposure to the wastewater effluent from IVAR SNJ WWTP does not have any significant neurotoxicological effect on *L. littorea*.

3.5 Correlations between biomarkers evaluated in *L. littorea*

Pearson correlation coefficients are shown in Figure 3.10. The correlation was considered as significant at the $p \leq 0.05$ level. Statistically significant correlations were not observed between any of the considered biological markers.

Correlations

Total		CI	NRRT	AChE
CI	Pearson Correlation	1	-0.079	-0.225
	Sig. (2-tailed)		0.602	0.233
	N	105	46	30
NRRT	Pearson Correlation	-0.079	1	0.060
	Sig. (2-tailed)	0.602		0.754
	N	46	46	30
AChE	Pearson Correlation	-0.225	0.060	1
	Sig. (2-tailed)	0.233	0.754	
	N	30	30	30

Figure 3.10 Pearson correlation between three biological markers. Sig.: p-value; N: number of samples.

For CI values, a non-significant negative correlation was observed with both NRRT and AChE activity, indicating that *L. littorea* individuals with higher CI generally also have lower NRRT and AChE

activity values. This could be surprising since both CI and NRRT are biomarkers of the general physiological health status of an organism, where higher values indicate better health conditions. However, the negative correlation is not significant.

NRRT has shown a non-significant positive correlation with AChE activity, indicating that organisms with higher NRRT values generally have higher AChE activity. This correlation may not be surprising, since the reduced activity of the enzyme leads to the physical impairment of an organism.

4 Conclusion

In general, data obtained from the biomarkers selected for this study showed that there was no significant difference between organisms from the reference locations and organisms from locations near the wastewater discharge point. The Pearson correlation coefficients did not show statistically significant correlations between the selected biomarkers. This may be due to the relatively small sample size for certain biomarkers.

The NRRT assay results indicated that *L. littorea* individuals collected from all three coastal sites had a relatively good general health condition. Statistically significant lower general health condition was recorded in specimens collected from the coastal station closest to the wastewater effluent release point, in comparison to organisms collected from the reference station. This indicates that organisms from this sampling site are subjected to general environmental stress, which can be due to the wastewater discharge exposure, or other sources of contaminations or stress (including abiotic factors).

The CI analysis that was performed on three different species, *L. littorea,* collected from coastal stations, and *L. maja* and *N. norvegicus* specimens collected from two marine stations, one reference and one in the immediate vicinity of the wastewater discharge, did not reveal any significant differences in energy reserves and health condition between organisms of the same species from different locations. Obtained results suggested that exposure to the wastewater effluent from IVAR SNJ WWTP does not have a significant negative effect on the conditions needed for growth and reproduction in all three species, and that the potential general stress registered at the cellular level with the NRRT assay is probably not causing a physiological level effect in *L. littorea*.

The AChE assay, performed on *L. littorea*, showed that organisms collected in the vicinity of wastewater discharge point compared with those collected at the reference station do not exhibit any significant difference in AChE activity. These results are indicating that *L. littorea* specimens close to the wastewater effluent from IVAR SNJ WWTP are not subjected to any significant neurotoxic stress, meaning none or low level of exposure to pollution agents that can act as AChE inhibitors in *L. littorea*.

5 Future prospects

The results obtained and presented in this study research are subject to caution and a similar study should be repeated with a higher number of samples and an expanded list of biomarkers to confirm the findings. Viewed separately, this study is limited in terms of the short period of investigation, one month period of sampling, and relatively small sample size, and as such it is not able to provide a comprehensive picture about overall biological conditions in organisms that are exposed to the wastewater discharges from IVAR SNJ WWTP. However, this is a part of a larger multi-annual project and should be considered as such. The project entitled “Marine Sewage Outfalls – Environmental Impact Evaluation” will include in the near future analysis over a longer period of time covering seasonal variations and different periods of development in sentinel organisms, and also including more biotic and abiotic parameters, and chemical analysis, in order to provide more comprehensive information between the observed biological effects and presence of wastewater discharges.

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Appendix A – Raw data from samplings

Sampling location - Solastranda (reference site), *L. littorea*

Snail Code	Sampling Site	Sampling Date	Shell Height [mm]	Shell Width [mm]	Aperture Height [mm]	Aperture Width [mm]	Top Height [mm]	Total Wet Weight [g]	Soft tissue Wet Weight [g]	Condition Index	NRRT [min]
SR1	Sola-ref	17.09.19	21	16	14	12	12	3.25	0.52	15.91	90
SR2	Sola-ref	17.09.19	22	16	15	12	11	2.89	0.71	24.37	90
SR3	Sola-ref	17.09.19	23	18	16	13	13	3.89	0.83	21.22	90
SR4	Sola-ref	17.09.19	20	16	14	12	12	2.94	0.74	25.03	90
SR5	Sola-ref	17.09.19	23	17	15	13	14	3.66	0.74	20.25	90
SR6	Sola-ref	17.09.19	22	17	14	12	13	3.14	0.87	27.63	60
SR7	Sola-ref	17.09.19	25	17	16	13	16	4.67	0.89	19.08	60
SR8	Sola-ref	17.09.19	20	14	14	11	12	2.37	0.69	28.90	60
SR9	Sola-ref	17.09.19	20	14	13	11	12	2.19	0.51	23.39	60
SR10	Sola-ref	17.09.19	22	16.5	15	12	11	3.74	0.75	19.92	60
SR11	Sola-ref	17.09.19	21.5	15.5	14.5	11	11.5	2.98	0.59	19.64	60
SR12	Sola-ref	17.09.19	22	15.5	14.5	11	12.5	3.34	0.68	20.26	60
SR13	Sola-ref	17.09.19	20.5	15	14	12	12	2.57	0.65	25.18	90
SR14	Sola-ref	17.09.19	24	12	16	11	14	3.94	0.90	22.76	60
SR15	Sola-ref	17.09.19	21	16	14	10	11.5	3.04	0.71	23.34	90
SR16	Sola-ref	17.09.19	21	16	14	12	11	2.95	0.55	18.78	-
SR17	Sola-ref	17.09.19	20	14	12	12	11	2.76	0.62	22.30	-
SR18	Sola-ref	17.09.19	22	16	15	12	10	3.57	0.77	21.57	-
SR19	Sola-ref	17.09.19	21	15	13	12	11	3.20	0.71	22.21	-
SR20	Sola-ref	17.09.19	20	14.5	13.5	11	10.5	2.46	0.66	26.88	-
SR21	Sola-ref	28.09.19	22.5	15.5	15	13.5	14	3.25	0.79	24.15	-
SR22	Sola-ref	28.09.19	22	15.5	15	13.5	13	3.01	0.86	28.60	-
SR23	Sola-ref	28.09.19	24	16.5	16	14.5	15	3.72	0.99	26.56	-
SR24	Sola-ref	28.09.19	21	15.5	16	13	13	2.61	0.65	24.93	-
SR25	Sola-ref	28.09.19	20	14.5	15	13	12	2.65	0.70	26.28	-
SR26	Sola-ref	28.09.19	14.5	11.5	11	8	11	1.18	0.21	17.53	-
SR27	Sola-ref	28.09.19	19.5	14	14	10	11	2.12	0.64	30.06	-
SR28	Sola-ref	28.09.19	22.5	15.5	15	11	12.5	3.07	0.68	22.25	-
SR29	Sola-ref	28.09.19	24	17	16.5	13	14	3.72	0.89	24.01	-
SR30	Sola-ref	28.09.19	16	11	11.5	9	10	1.25	0.29	23.29	-
SR31	Sola-ref	28.09.19	23	15	14.5	11.5	13.5	2.60	0.80	30.77	-
SR32	Sola-ref	28.09.19	26	17	17.5	13	15.5	4.79	1.13	23.61	-
SR33	Sola-ref	28.09.19	17.5	13	12.5	10.5	9.5	1.66	0.46	27.64	-
SR34	Sola-ref	28.09.19	19	14	14	11	11	2.18	0.51	23.54	-
SR35	Sola-ref	28.09.19	23	16	14.5	11	13	2.80	0.87	31.03	-
		Mean	21.30	15.21	14.41	11.73	12.26	2.98	0.70	23.80	74.00
		SD	2.32	1.60	1.39	1.32	1.57	0.80	0.18	3.73	15.49
		SE	0.39	0.27	0.23	0.22	0.27	0.14	0.03	0.63	4.00
		Max	26.00	18.00	17.50	14.50	16.00	4.79	1.13	31.03	90.00
		Min	14.50	11.00	11.00	8.00	9.50	1.18	0.21	15.91	60.00
		Range	11.50	7.00	6.50	6.50	6.50	3.61	0.92	15.12	30.00
		Median	21.50	15.50	14.50	12.00	12.00	2.98	0.71	23.54	60.00

Sampling location - Sandestranda, *L. littorea*

Snail Code	Sampling Site	Sampling Date	Shell Height [mm]	Shell Width [mm]	Aperture Height [mm]	Aperture Width [mm]	Top Height [mm]	Total Wet Weight [g]	Soft tissue Wet Weight [g]	Condition Index	NRRT [min]
SAN1	Sande	18.09.19	23	16	15	12	13	3.34	0.87	25.90	30
SAN2	Sande	18.09.19	21	16	15	13	11	2.89	0.76	26.30	30
SAN3	Sande	18.09.19	22	15.5	16	13	12	3.41	0.78	22.87	30
SAN4	Sande	18.09.19	24	18	15	14	13	4.26	0.95	22.30	30
SAN5	Sande	18.09.19	21.5	15	14.5	11	11.9	2.94	0.68	23.18	60
SAN6	Sande	18.09.19	20	14	13.5	11.5	11.5	2.27	0.49	21.47	60
SAN7	Sande	18.09.19	21.5	17	16	14.5	12.5	3.54	0.95	26.94	60
SAN8	Sande	18.09.19	19	14	14.5	11	10	2.54	0.49	19.39	60
SAN9	Sande	18.09.19	23	16.5	16	13	13	3.83	0.84	21.80	60
SAN10	Sande	18.09.19	21	15	15	12.5	11.5	2.95	0.67	22.76	60
SAN11	Sande	18.09.19	13.5	17	16.5	12.5	13	3.74	0.99	26.46	60
SAN12	Sande	18.09.19	23	16.5	15	11	13	3.87	0.67	17.20	60
SAN13	Sande	18.09.19	22	16	15.5	13	12	3.20	0.95	29.83	60
SAN14	Sande	18.09.19	20	14	13.5	10.5	11	2.42	0.49	20.17	90
SAN15	Sande	18.09.19	20.5	14	14	11.5	11	2.48	0.64	25.65	30
SAN16	Sande	18.09.19	20	14.5	13.5	11	10.5	2.45	0.49	20.01	30
SAN17	Sande	18.09.19	21	15.5	14	11	11.5	2.91	0.86	29.35	-
SAN18	Sande	18.09.19	21.5	15	14	12	12	3.13	0.58	18.49	-
SAN19	Sande	18.09.19	22	15.5	14	12	12	3.37	0.80	23.65	-
SAN20	Sande	18.09.19	23.5	17	15	12	13	4.09	1.06	25.93	-
SAN21	Sande	28.09.19	16	11.5	11.5	10.5	10	1.17	0.28	23.99	-
SAN22	Sande	28.09.19	19.5	13.5	13.5	12	11.5	2.09	0.47	22.25	-
SAN23	Sande	28.09.19	17	13	12.5	12	11	1.83	0.39	21.33	-
SAN24	Sande	28.09.19	23	16	16	14	18	3.48	0.77	21.99	-
SAN25	Sande	28.09.19	17	13	13	11.5	11	1.81	0.50	27.60	-
SAN26	Sande	28.09.19	21.5	15	15	13	12	2.86	0.65	22.64	-
SAN27	Sande	28.09.19	19.5	14	13	11	11.5	2.06	0.45	21.70	-
SAN28	Sande	28.09.19	21	14.5	14	11.5	12	2.77	0.59	21.20	-
SAN29	Sande	28.09.19	26	17.5	16	12.5	16	4.28	0.93	21.66	-
SAN30	Sande	28.09.19	22	15.5	16	12.5	11.5	3.03	0.85	28.21	-
SAN31	Sande	28.09.19	18.5	13	13.5	10	11	1.63	0.41	24.94	-
SAN32	Sande	28.09.19	18.5	13.5	12.5	9	11	1.89	0.40	20.90	-
SAN33	Sande	28.09.19	18	13.5	13.5	10	10	2.07	0.45	21.76	-
SAN34	Sande	28.09.19	18.5	13.5	12.5	10	10	2.08	0.45	21.70	-
SAN35	Sande	28.09.19	20	14	14	11	12	2.21	0.48	21.73	-
		Mean	20.54	14.94	14.34	11.79	11.91	2.82	0.66	23.24	50.63
		SD	2.47	1.50	1.24	1.24	1.58	0.79	0.21	3.02	18.06
		SE	0.42	0.25	0.21	0.21	0.27	0.13	0.04	0.51	4.52
		Max	26.00	18.00	16.50	14.50	18.00	4.28	1.06	29.83	90.00
		Min	13.50	11.50	11.50	9.00	10.00	1.17	0.28	17.20	30.00
		Range	12.50	6.50	5.00	5.50	8.00	3.12	0.78	12.63	60.00
		Median	21.00	15.00	14.00	12.00	11.50	2.89	0.65	22.30	60.00

Sampling location - Randabergbukta, *L. littorea*

Snail Code	Sampling Site	Sampling Date	Shell Height [mm]	Shell Width [mm]	Aperture Height [mm]	Aperture Width [mm]	Top Height [mm]	Total Wet Weight [g]	Soft tissue Wet Weight [g]	Condition Index	NRRT [min]
RAN1	Randaberg	18.09.19	18	13	12	11	10	1.95	0.47	24.22	90
RAN2	Randaberg	18.09.19	20	15.5	15	11	11	3.43	0.64	18.60	90
RAN3	Randaberg	18.09.19	19	14	13	12	11	2.14	0.49	22.77	90
RAN4	Randaberg	18.09.19	22.5	16	15	12	13	3.46	0.93	26.86	30
RAN5	Randaberg	18.09.19	26.5	17	17.5	12.5	16	4.60	1.13	24.63	60
RAN6	Randaberg	18.09.19	21	15	15	12	12	2.90	0.64	21.98	90
RAN7	Randaberg	18.09.19	19	14	13	12	11	2.14	0.54	25.37	90
RAN8	Randaberg	18.09.19	22	15.5	15	13	13	3.27	0.79	24.13	90
RAN9	Randaberg	18.09.19	20	15	13	12	12	2.71	0.57	21.20	90
RAN10	Randaberg	18.09.19	28	27.5	18	17	15	5.70	1.14	20.00	30
RAN11	Randaberg	18.09.19	19	14	12	11	11	2.49	0.54	21.74	30
RAN12	Randaberg	18.09.19	18	13	13	11.5	10	2.08	0.49	23.52	30
RAN13	Randaberg	18.09.19	21	15	14	12	13	2.86	0.53	18.66	30
RAN14	Randaberg	18.09.19	28	19.5	19	15	16	6.32	1.29	20.44	30
RAN15	Randaberg	18.09.19	21	15	13.5	12	12	2.79	0.54	19.45	60
RAN16	Randaberg	18.09.19	20	15	14	12	11	2.68	0.67	24.93	-
RAN17	Randaberg	18.09.19	21.5	15	14	13	12	3.15	0.74	23.55	-
RAN18	Randaberg	18.09.19	21	14	13.5	13	12	2.75	0.67	24.37	-
RAN19	Randaberg	18.09.19	20	13	13	11	11	2.80	0.61	21.78	-
RAN20	Randaberg	18.09.19	20	15	14	13	11	2.96	0.68	22.89	-
RAN21	Randaberg	28.09.19	15	11	11.5	10	9	1.07	0.27	25.23	-
RAN22	Randaberg	28.09.19	17.5	13	13	12	10.5	1.82	0.41	22.54	-
RAN23	Randaberg	28.09.19	21	15	16	14	12.5	2.67	0.81	30.21	-
RAN24	Randaberg	28.09.19	20.5	14	14	13	12	2.30	0.49	21.31	-
RAN25	Randaberg	28.09.19	12	12	12	11	10	1.34	0.33	24.18	-
RAN26	Randaberg	28.09.19	19.5	14.5	19	14.5	11	2.30	0.54	23.27	-
RAN27	Randaberg	28.09.19	20.5	14.5	14.5	11.5	12	2.50	0.52	20.93	-
RAN28	Randaberg	28.09.19	24	17	17	12.5	14	3.95	0.97	24.47	-
RAN29	Randaberg	28.09.19	23	16	15.5	12.5	13	3.30	0.85	25.91	-
RAN30	Randaberg	28.09.19	19	14	13.5	11	11	2.13	0.45	20.92	-
RAN31	Randaberg	28.09.19	20.5	15	14	11	11.5	2.49	0.60	23.90	-
RAN32	Randaberg	28.09.19	22	15	14.5	11.5	12	2.57	0.58	22.73	-
RAN33	Randaberg	28.09.19	22.5	15	14.5	11.5	13.5	2.91	0.64	22.08	-
RAN34	Randaberg	28.09.19	19.5	14.5	13.5	11.5	11	2.22	0.59	26.41	-
RAN35	Randaberg	28.09.19	19	14	14	11	10.5	2.02	0.48	23.92	-
		Mean	20.60	15.01	14.37	12.19	11.90	2.82	0.65	23.12	62.00
		SD	3.07	2.63	1.87	1.35	1.60	1.05	0.23	2.43	28.83
		SE	0.52	0.45	0.32	0.23	0.27	0.18	0.04	0.41	7.45
		Max	28.00	27.50	19.00	17.00	16.00	6.32	1.29	30.21	90.00
		Min	12.00	11.00	11.50	10.00	9.00	1.07	0.27	18.60	30.00
		Range	16.00	16.50	7.50	7.00	7.00	5.25	1.02	11.61	60.00
		Median	20.50	15.00	14.00	12.00	12.00	2.68	0.59	23.27	60.00

Sampling location - Håsteinsfjord, *L. maja*

Crab Code	Sampling Site	Sampling Date	Total Wet Weight [g]	Carapace Width [cm]	Carapace Length [cm]	Rostrum Base Width [cm]	Orbital Spine Width [cm]	First Spine Length [cm]	Carapace Length (CL^3)	Condition Index
HSC1	Håsteins	31.10.2019	540	10.5	11.0	2.3	6.0	1.0	1331.00	40.57
HSC2	Håsteins	31.10.2019	320	10.0	9.0	3.5	6.5	1.5	729.00	43.90
HSC3	Håsteins	31.10.2019	755	12.5	10.0	4.5	7.0	1.4	1000.00	75.50
HSC4	Håsteins	31.10.2019	380	12.0	8.5	4.5	8.0	1.0	614.13	61.88
HSC5	Håsteins	31.10.2019	530	12.0	10.5	4.5	6.5	1.0	1157.63	45.78
		Mean	505.00	11.40	9.80	3.86	6.80	1.18	966.35	53.53
		SD	168.97	1.08	1.04	0.97	0.76	0.25	296.26	14.77
		SE	75.56	0.48	0.46	0.44	0.34	0.11	132.49	6.61
		Max	755.00	12.50	11.00	4.50	8.00	1.50	1331.00	75.50
		Min	320.00	10.00	8.50	2.30	6.00	1.00	614.13	40.57
		Range	435.00	2.50	2.50	2.20	2.00	0.50	716.88	34.93
		Median	530.00	12.00	10.00	4.50	6.50	1.00	1000.00	45.78

Sampling location - Boknafjord (reference site), *L. maja*

Crab Code	Sampling Site	Sampling Date	Total Wet Weight [g]	Carapace Width [cm]	Carapace Length [cm]	Rostrum Base Width [cm]	Orbital Spine Width [cm]	First Spine Length [cm]	Carapace Length (CL^3)	Condition Index
BRC1	Bokna-ref	31.10.2019	153	8.0	6.5	3.0	5.0	1.0	274.63	55.71
BRC2	Bokna-ref	31.10.2019	170	8.7	7.5	3.3	5.5	1.5	421.88	40.30
		Mean	161.50	8.35	7.00	3.15	5.25	1.25	348.25	48.00
		SD	12.02	0.49	0.71	0.21	0.35	0.35	104.12	10.90
		SE	8.50	0.35	0.50	0.15	0.25	0.25	73.63	7.71
		Max	170.00	8.70	7.50	3.30	5.50	1.50	421.88	55.71
		Min	153.00	8.00	6.50	3.00	5.00	1.00	274.63	40.30
		Range	17.00	0.70	1.00	0.30	0.50	0.50	147.25	15.42
		Median	161.50	8.35	7.00	3.15	5.25	1.25	348.25	48.00

Sampling location - Boknafjord (reference site), *N. norvegicus*

Lobster Code	Sampling Site	Sampling Date	Total Wet Weight [g]	Overall Length [cm]	Total Length [cm]	Total Length (CL^3)	Liver Weight [g]	Condition Index
BRL1	Bokna-ref	31.10.2019	55.8	22	14.5	3048.6	2.1	1.83
BRL2	Bokna-ref	31.10.2019	140	28.5	18	5832.0	4.6	2.40
BRL3	Bokna-ref	31.10.2019	85	25	16	4096.0	4.2	2.08
BRL4	Bokna-ref	31.10.2019	63	24.5	16	4096.0	/	1.54
BRL5	Bokna-ref	31.10.2019	70	23	15	3375.0	3.4	2.07
		Mean	82.76	22.50	18.00	4089.53	3.58	1.98
		SD	33.77	2.78	5.91	1075.91	1.10	0.32
		SE	15.10	1.24	2.64	481.16	0.55	0.14
		Max	140.00	25.00	28.50	5832.00	4.60	2.40
		Min	55.80	18.00	14.50	3048.63	2.10	1.54
		Range	84.20	7.00	14.00	2783.38	2.50	0.86
		Median	70.00	23.00	16.00	4096.00	3.80	2.07

Sampling location - Håsteinsfjord, *N. norvegicus*

Lobster Code	Sampling Site	Sampling Date	Total Wet Weight [g]	Overall Length [cm]	Total Length [cm]	Total Length (CL^3)	Liver Weight [g]	Condition Index
HSL1	Håsteins	31.10.2019	191.4	34.5	19	6859.0	5.4	2.79
HSL2	Håsteins	31.10.2019	290	40.5	23	12167.0	9.2	2.38
HSL3	Håsteins	31.10.2019	260	37.9	20.5	8615.1	9.6	3.02
HSL4	Håsteins	31.10.2019	310	42	24	13824.0	8	2.24
HSL5	Håsteins	31.10.2019	86.5	26	17	4913.0	5.1	1.76
HSL6	Håsteins	31.10.2019	102	28	16.5	4492.1	4.1	2.27
		Mean	227.58	36.18	20.70	9275.63	7.46	2.44
		SD	90.76	6.36	2.86	3686.35	2.10	0.49
		SE	40.59	2.85	1.28	1648.58	0.94	0.22
		Max	310.00	42.00	24.00	13824.00	9.60	3.02
		Min	86.50	26.00	17.00	4913.00	5.10	1.76
		Range	223.50	16.00	7.00	8911.00	4.50	1.26
		Median	260.00	37.90	20.50	8615.13	8.00	2.38

Appendix B – Sampling and laboratory procedure for *L. littorea*

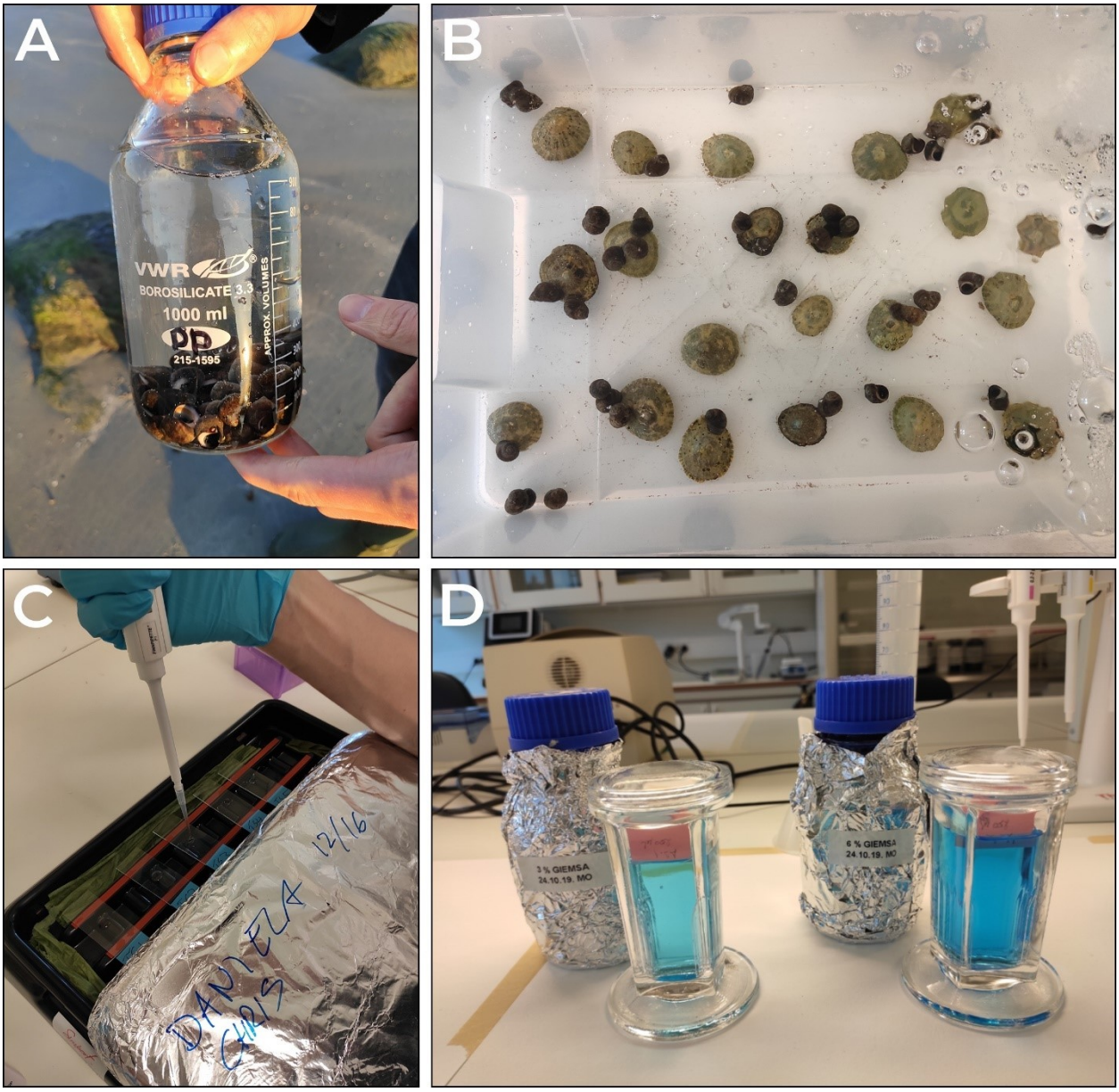
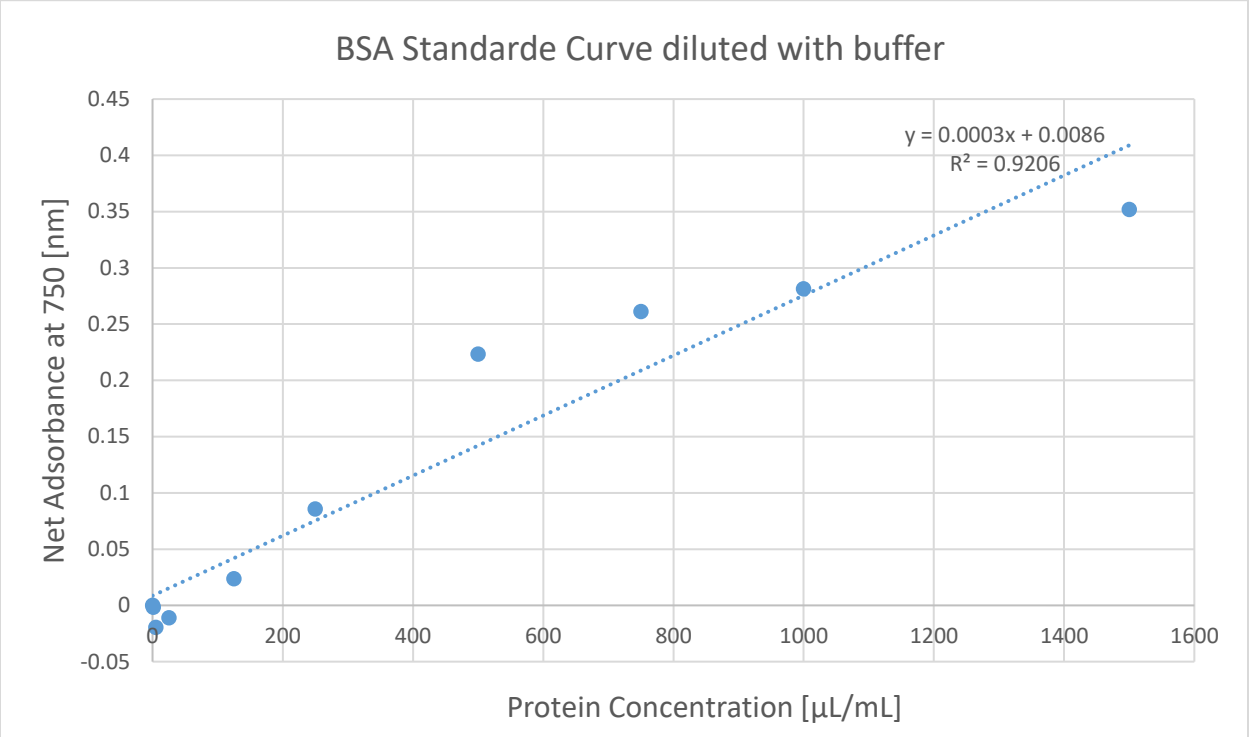


Fig. 1 A: Glass bottle with sampled *L. littorea* specimens, containing seawater from sampling location; B: Plastic tank under constant aeration, containing seawater from the sampling location of *L. littorea* specimens, with clean limpet shells used for easier access to the soft tissue of the periwinkle for haemolymph extraction; C: Adding of 30 µL working solution on the poly-L-lysine-coated microscope slides with sampled haemolymph, in a light-proof humidity chamber, as a part of NRRT assay; D: Microscope slide staining dishes with 3 % and 6 % Giemsa solution, as a part of MN assay.

Appendix C – AChE activity assay and results for *L. littorea*

Calibration curve for establishing protein concentration of AChE activity in snail (*L. littorea*) samples



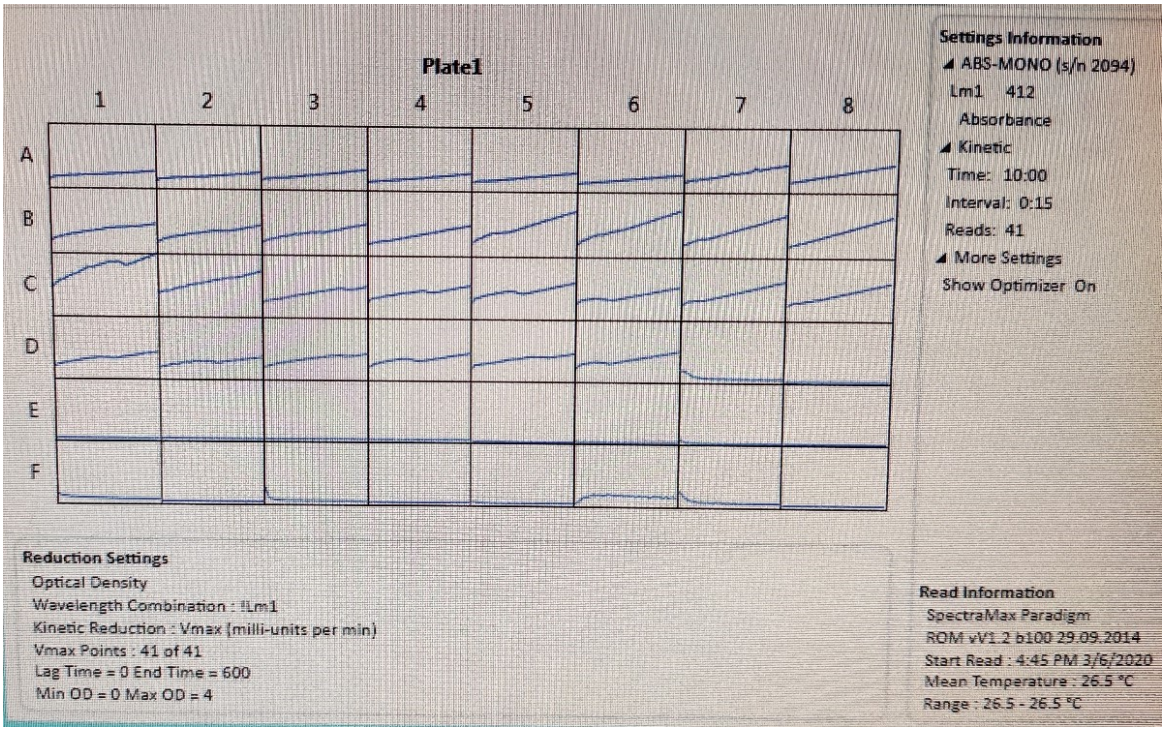


Fig. 2 Spectrophotometric measurements of change in absorbance (kinetics) in 48 microplates well, at 412 nm, at room temperature, at 15 sec for 10 min, with SpectraMax Paradigm Multi-Mode Microplate Reader, in order to determine the rate of enzymatic activity in tissue samples of *L. littorea*.

Sampling location - Solastranda (reference site)

Code of Pooled snails	Pooled snails		Combined Soft tissue Mass (without foot) [g]	AChE activity [µmol ATC/min/mg protein]	AChE activity [nmol ATC/min/mg protein]
SRP1	SR9	SR14	0.986	6.000	16.104
SRP2	SR6	SR7	1.026	3.106	8.338
SRP3	SR8	SR10	1.037	6.841	18.362
SRP4	SR11	SR13	1.018	11.410	30.626
SRP5	SR12	SR15	1.036	9.965	26.747
			Mean	7.464	20.035
			SD	3.292	8.836
			SE	1.472	3.951
			Max	11.410	30.626
			Min	3.106	8.338
			Range	8.303	22.288
			Median	6.841	18.362

Sampling location - Sandestranda

Code of Pooled snails	Pooled snails		Combined Soft tissue Mass (without foot) [g]	AChE activity [µmol ATC/min/ mg protein]	AChE activity [nmol ATC/min/mg protein]
SANP1	SAN1	SAN5	1.260	3.479	9.339
SANP2	SAN2	SAN3	1.354	7.230	19.406
SANP3	SAN4	SAN6	1.264	6.884	18.479
SANP4	SAN7	SAN8	1.210	10.485	28.144
SANP5	SAN9	SAN10	1.297	6.189	16.613
			Mean	6.853	18.396
			SD	2.508	6.732
			SE	1.122	3.010
			Max	10.485	28.144
			Min	3.479	9.339
			Range	7.006	18.805
			Median	6.884	18.479

Sampling location – Randabergbukta

Code of Pooled snails	Pooled snails		Combined Soft tissue Mass (without foot) [g]	AChE activity [µmol ATC/min/ mg protein]	AChE activity [nmol ATC/min/mg protein]
RANP1	RAN1	RAN5	1.483	3.582	9.615
RANP2	RAN2	RAN6	1.158	6.481	17.396
RANP3	RAN3	RAN10	1.445	10.758	28.877
RANP4	RAN4	RAN7	1.329	6.156	16.523
RANP5	RAN8	RAN9	1.197	5.463	14.663
			Mean	6.488	17.415
			SD	2.638	7.082
			SE	1.180	3.167
			Max	10.758	28.877
			Min	3.582	9.615
			Range	7.176	19.262
			Median	6.156	16.523

AChE activity expressed as nmol of ACT hydrolysed per minute per milligram of protein, calculated by:

$$\text{AChE activity (nmol ATC min}^{-1}\text{mg protein}^{-1}) = \frac{\Delta A_{412} * 1000}{\text{Vol}_s * \text{[protein]}} * 75$$

where 1 ΔA/min/mg corresponds to the hydrolysis of 75 nanomoles of ACT