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Abstract

The waste water effluent from the Søderberg aluminium production plant Alcoa Lista is discharged into the marine recipient Husebybukta. The discharge contains several compounds that can have toxic effects on the living organisms. A biomonitoring study was performed using periwinkles (*Littorina littorea*) as bioindicators to assess the biological effects in the snails living in the vicinity of the production plant. The battery of biomarkers included lysosomal membrane stability (LMS), condition index (CI), micronuclei (MN), and metallothionein (MT). Morphological measurements were also taken. Snails were collected monthly from February to April 2017 from three sites in Lista: Haugestranda, Tjuvholmen, and Litlerauna. Snails were also collected from an additional reference station in Viste, Rogaland in the same sampling intervals.

The overall data from the battery of biomarkers selected for this study was capable of showing differences between sites, probably due to the differences in contaminant levels. Significantly lower LMS values were observed in snails from the two sites closest to the production plant (Haugestranda and Tjuvholmen), indicating that organisms are subjected to a general environmental stress, potentially due to the waste water discharge from Alcoa Lista. CI values were not significantly different in all organism collected at the area of Lista (both close to the production plant and in the reference location), indicating that the potential general stress registered at cellular level with the LMS assay is probably not causing physiological level effect in *L. littorea* Significantly higher MN frequencies were observed in snails collected in the vicinity of the production plant (Haugestranda and Tjuvholmen) compared to the reference sites, indicating that organisms close to the Alcoa Lista plant are subjected to a significant genotoxic stress possibly from toxic compounds discharge from the production plant. MT concentrations in tissues of snails collected at Lista were similar, indicating that the waste water discharge from Alcoa Lista does not contain pollutants capable of induction MT or their concentration is too low to induce MT, e.g. heavy metals.

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List of Acronyms

ANOVA	Analysis of variance
CI	Condition Index
ERA	Ecological Risk Assessment
EQS	Environmental Quality Standard
ICES	International Council for the Exploration of the Sea
IRIS	International Research Institute of Stavanger
LMS	Lysosomal membrane stability
MN	Micronucleus/Micronuclei
MT	Metallothionein
NPB	Nucleoplasmic bridge
NRRT	Neutral Red Retention Time
PAH	Polycyclic Aromatic Hydrocarbon
PCA	Principal component analysis
UiS	University of Stavanger

1 Introduction

1.1 Aim of the Study

The aim of this thesis was to monitor the potential biological effects in periwinkles (*Littorina littorea*) collected in an area close to a waste water effluent from the primary aluminium production plant of Alcoa Lista (Norway). The battery of biomarkers included lysosomal membrane stability (LMS), condition index (CI), micronucleus assay (MN), and metallothionein content (MT).

Biomarker results were also related to the most recent chemical body burden analyses performed in the recipient by Kroglund (2016, 2014) to assess the ecological state of the recipient.

1.2 Primary Aluminium Production

Waste water effluents from the aluminium production industry can represent a source of pollution in Norwegian fjords and coastal waters. The complex chemical composition of the effluents depends on the production and water treatment technology used in the production process. The ecological effects of these effluents in the smelter-affected fjords have been researched in environmental monitoring programs for many years (Norsk Aluminiumsindustri og Miljø, 1995).

1.2.1 Production Technology

The main industrial process of the smelting aluminium is the Hall-Héroult process. In this process, aluminium oxide (Al_2O_3) is reduced to liquid aluminium (Al) in a series of electrolysis cells. Aluminium oxide is dissolved in molten cryolite (Na_3AlF_6) in a cell, to form a molten salt bath that is electrolysed. The cryolite is used to lower the melting point of aluminium oxide from 2072 ºC to around 950-970 ºC, which is the operating temperature in the cell bath. The reduced aluminium has a higher density than the bath mixture and thus gathers at the bottom of the cell. Aluminium is regularly syphoned out, and new aluminium oxide is continuously added to the salt bath. Production of 1 kg of aluminium uses approximately 2 kg of aluminium oxide, 0.5 kg of anode coal and 13-17 kWh of electricity (Norsk Aluminiumsindustri og Miljø, 1995).

Aluminium production technology using electrolysis can be divided into two main types of production cells; the Søderberg cell and the Pre-bake cell. The cells are differentiated by the composition and use of the anode in the production process.

The Søderberg cell consists of one large carbonaceous anode mass block housed by a steel container. The anode mass is continuously baked from the bottom-up as it is lowered into the bath of the production oven to keep a constant distance to the cathode during the production process (*in situ* baking). Anode mass is refilled on top of the anode as the mass is being used. This production technology results in

large quantities of gas emissions consisting mainly of fluorides, dust particles, SO_2, CO_2, CO as well as smaller amounts of hydrocarbons (about 20% PAHs).

The pre-bake cell consists of several anode blocks that are pre-baked in separate ovens. This eliminates the hydrocarbon emissions associated with the baking of the anode, as they are collected in the respective pre-baking ovens. The separate anode blocks are used and replaced in the cell during production. (Norsk Aluminiumsindustri og Miljø, 1995).

The Søderberg technology is known to be the less efficient technology of the two, when considering emission rates, specific energy consumption and current use (Norsk Aluminiumsindustri og Miljø, 1995).

1.3 Alcoa Lista

Alcoa Lista is a Norwegian aluminium production plant located in Farsund, Vest-Agder (Figure 1.1). It was formerly known as Elkem Lista, until Alcoa Norway became the sole owner of Elkem Aluminium ANS in 2009. Production started at the plant in 1971, and the production capacity was later expanded in 1975. The Søderberg production technology has been used in the plant since the start and has since been modernised, especially in the period 1995-2005, to meet new emission legislations and to increase the overall production efficiency. Alcoa Lista is still continuously seeking to improve the efficiency of their use of Søderberg technology. Alcoa Lista is the only one of the seven aluminium production plants in Norway that still uses the Søderberg technology. Today's annual aluminium production is around 95.000 tonnes (Kroglund, 2016).

Figure 1.1 Alcoa Lista seen from North East (Photo: Private)

1.3.1 Gas Treatment

The gas emissions from the Søderberg ovens are first collected into big bag filters and dry-treated by addition of aluminium oxide. Most of the fluoride, particles and polycyclic hydrocarbons (PAHs) are removed from the gas in this step. The aluminium oxide used in the dry-treatment is re-used in the electrolysis to minimize the loss of fluoride. More of the fluorides, PAHs, particles and SO_2 are removed in

the next treatment step by sea water scrubbing. PAHs and fluorides are the most important contaminant compounds in the effluents from the gas treatment facilities.

1.3.2 Waste Water Effluent

The waste water effluent from Alcoa Lista collects waste water from several sources of use in different compartments of the plant. This includes sea water used in gas scrubbing and freshwater used for cooling purposes in the casting studio and the anode mass factory. Rainwater from the outside area, including runoff from landfills, are also collected into the effluent (Kroglund, 2016). In addition to the components originating from the aluminium production, the waste water effluent from Alcoa Lista also contains pollutants (mainly heavy metals) originating from the intake of sea water from Lundevågen used in the scrubbing process. Lundevågen is a nearby fjord with polluted sediments related to industrial and shipping activities (Kroglund, 2014; Larsen, 2015). The annual release of compounds in the waste water effluent is given in Table 1.1.

Table 1.1 Components of the waste water effluent from Alcoa Lista 2012-2016 (norskeutslipp.no)

Component	Unit	2012	2013	2014	2015	2016
PAH	kg/year	803	820	724	626	[N.A.]
Anthracene	kg/year	17	16	13	[N.A.]	12
Benzo[ghi]perylene (BghiP)	kg/year	49	43	32	29	35
Naphthalene	kg/year	48	42	30	23	20
Fluoranthene	kg/year	483	489	451	[N.A.]	405
Lead (Pb)	kg/year	95	47	62	55	37
Cyanide, free	kg/year	245	54	23	10	7
Arsenic (As)	kg/year	6,1	3,8	4	4,4	9,1
Fluorides	tonnes/year	257	221	237	257	269
Cadmium (Cd)	kg/year	2,7	1,9	2,1	2,2	9,2
Suspended Solids (SS)	tonnes/year	241	232	187	275	259
Copper (Cu)	kg/year	2,2	2	3,6	4,9	79
Chrome (Cr)	kg/year	5	1,8	2,6	8,8	17
Mercury (Hg)	kg/year	0,03	0,01	0,02	0,02	0,38
Molybdenum (Mo)	kg/year	4	2	2,4	2,5	302
Nickel (Ni)	kg/year	57	54	64	77	123
Zinc (Zn)	kg/year	14	7	11	11	374
Cobalt	kg/year	2,7	2,2	2,8	[N.A.]	4,8

The waste water effluent consists of environmentally hazardous compounds such as PAHs, heavy metals, fluorides and suspended solids. PAHs have been identified as the compounds of main concern in the effluent. The presence of PAHs in waste water effluents from aluminium production plants are especially associated with the use of the Søderberg technology. Here PAHs are originated mainly from the sea water

scrubbing of production hall ventilation air, pot fumes, and anode production (Næs & Oug, 1997a; Norsk Aluminiumsindustri og Miljø, 1995). Alcoa Lista has significantly lowered the emission rates from the production process following the modernisation of the Søderberg technology. The annual amount of PAHs released in the waste water effluent have decreased after 2005 and have stabilised at a level ~5-6 times lower than in the late 1990's and early 2000's (Figure 1.2).

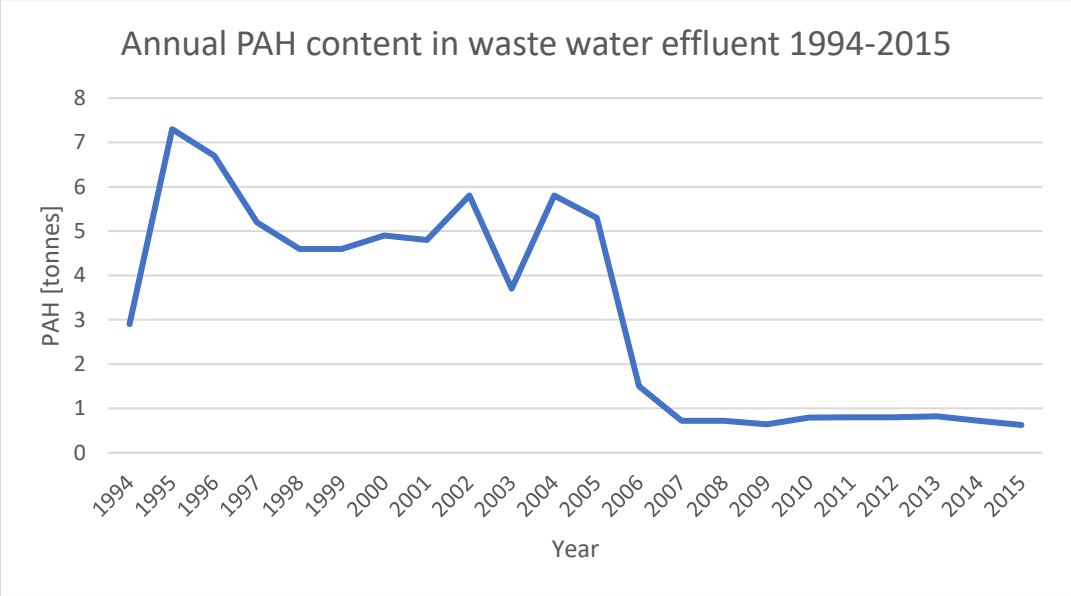


Figure 1.2 Annual PAH content released in waste water effluent from Alcoa Lista 1994-2015 (norskeutslipp.no)

PAH concentrations in the waste water effluent have been measured since 1975. However, in many cases only two annual measurements were made based on day mixed samples until 1990. There is little knowledge of the overall representativeness of these two annual measurements, given the large variations in emissions. Consequently, there are uncertainties related to the total annual PAH estimations in the period 1975-1990 (Figure 1.3) (Kroglund, 2008).

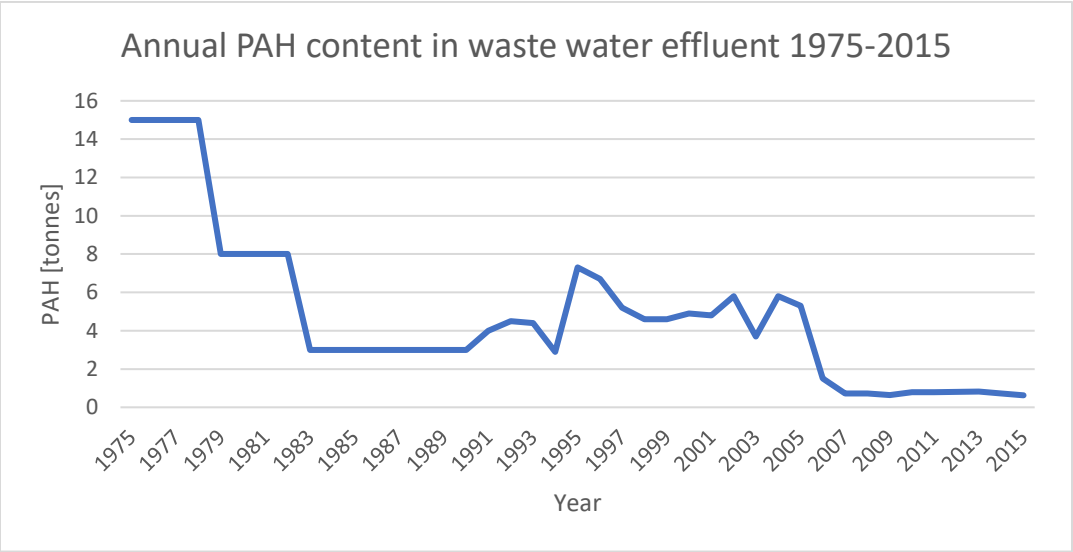


Figure 1.3 Annual PAH content released in waste water effluent from Alcoa Lista 1975-2015 (norskeutslipp.no; Kroglund, 2008)

The waste water effluent is released into the Husebybukta marine recipient in Lista. The recipient is an open, wind exposed, shallow bight with sandy sediments. From 1971 to 1995 the effluent was released into the beachside in Husebybukta, before being rerouted in December 1995.

1.4 Environmental Monitoring

Chemical pollutants released from industrial activities into environmental compartments pose risks to the ecosystems. Environmental assessments are commonly conducted to determine the magnitude of biological effects exerted on biological life by anthropogenic pollutants and to assess the ecological risk related to the pollutant stress (Walker *et al.*, 2012).

Pollution in aquatic environments is assessed per its potential to affect biological elements and impair the ecological status of water bodies. Historical approaches of conducting ecological assessments in aquatic environments have focused on determining physical and chemical variables, such as chemical pollutant concentrations in biota and water column. This was done using chemical analyses as a tool. Today it is widely recognized that the traditional use of chemical analyses is not sufficient to evaluate the pollution risk, as chemical analysis does not provide information on the toxic biological effects caused by pollutants (Galloway *et al*., 2004).

To get an understanding of the integrated environmental effects of pollution it is important to address the several interacting environmental, ecological and biological factors that influence the behaviour, bioavailability, bioaccumulation potential, and toxic potential of chemical pollutants in different

environmental compartments. Interaction between mixtures of different pollutants and the above-mentioned factors in contaminated areas can give rise to additive, synergistic and/or antagonistic effects following pollutant uptake in biota (Walker *et al*., 2012).

The individual organisms' physiological responses to a pollutant or a mixture of pollutants must be monitored to obtain an accurate assessment of the overall state of an ecosystem (Moore *et al.*, 2004). Establishing the risk related to key components in an ecosystem is a standard approach to environmental assessment. The biological responses and adverse effects are monitored on different biological organizational levels in ecosystems subjected to environmental pollution (Figure 1.4).

A biomonitoring approach to environmental assessment that accounts for the various factors interacting in the uptake and metabolism of pollutants in biota has gained momentum in ecotoxicological research over the last decades. This approach usually involves a combination of the traditional monitoring tools based on chemical analyses, and the modern tools based on biological responses known as biomarkers (Walker *et al.*, 2012).

1.4.1 Biological Responses to Pollution

Organisms exposed to harmful pollutants may start exhibiting symptoms that are indicative of an exposure and/or a biological damage. Figure 1.4 illustrates the hierarchical sequence of responses typically observed in biological systems exposed to environmental pollutant stress.

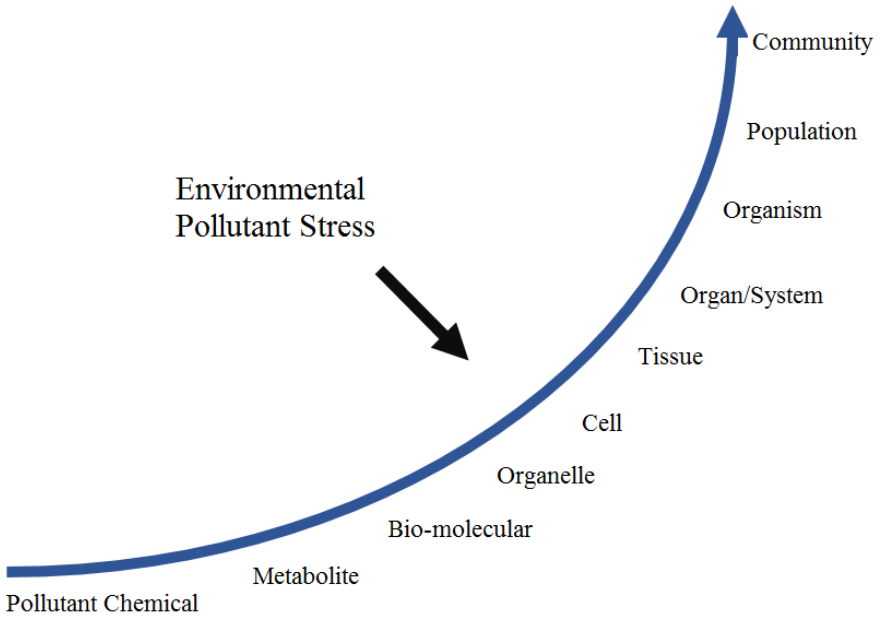


Figure 1.4 Hierarchical sequence of biological responses.

The biological responses are often categorised as protective and non-protective. Some protective mechanisms work by reducing the concentrations of free pollutants in cells, thus preventing or reducing the pollutants' ability to exert toxic effects on the cell. An example of a protective response to environmental pollution is the increased induction of metal-binding proteins known as MTs following heavy metal exposure. These proteins reduce the bioavailability of heavy metal ions in organisms as a protective measure. Non-protective biological responses to pollutants can indicate toxic effects that are already experienced in cells, such as DNA adduct formation following exposure to genotoxic pollutants.

Protective cellular mechanisms will counteract the toxic effects of pollutants to a certain capacity. Pollutant exposure exceeding the detoxifying capacity of an organism may increase the persistence of cellular damage. Sufficient pollutant exposure can cause the lower level toxic effects to develop into more adverse biological effects, such as tissue dysfunction, and may eventually accumulate into adverse effects on higher levels of biological organisation (Figure 1.4) (Moore *et al.*, 2004; UNEP/RAMOGE, 1999).

The information gathered from these sub-lethal responses can be used in environmental assessments to prevent the possibility that the observed biological responses eventually develop into adverse effects on higher organisational levels (Handy *et al.*, 2003).

1.4.2 Biomarkers

Several definitions of biomarkers have been proposed. Walker *et al.* (2012) defines a biomarker as "a biological response following the exposure of an environmental pollutant chemical at the bio-molecular, cellular, tissue/organ, and organismal level demonstrating a departure from the normal status". Biomarker analyses can provide information on physiological, biochemical, anatomical and behavioural responses in individual organisms that can be related to pollutant exposure in biomonitoring assessments.

Biomarkers are usually divided into two categories: 1) biomarkers of exposure, which indicate exposure to a specific class of pollutants, and 2) biomarkers of effects, which demonstrates adverse effects or health impairments in test organisms. Several additional classifications of biomarkers have been proposed, such as exposure and effect biomarkers (Handy *et al.*, 2003), biomarkers of susceptibility (Albertini *et al.*, 2000) and latent effect biomarkers (Depledge & Rossi, 1994). However, the most widely used biomarker categorisation divides between biomarkers of effect and biomarkers of exposure (Walker *et al.*, 2012).

1.4.2.1 Biomarkers of Exposure

Biomarkers of exposure rely on two fundamental principles of the dose-response relationship. (Handy *et al*., 2003):

i. The pollutant concentration in cells/tissues is consistent with the biomarker response

ii. The pollutant concentration in cells/tissues correlates well with environmental concentrations of the pollutant (albeit the "bioavailable" fraction)

As mentioned before, the combination of chemical analysis and biomarker analysis allows for the correlation of pollutant concentrations in the different environmental compartments and the biomarker response.

Biomarkers of exposure are "early signs" based on endpoints at low levels of biological organisation that indicate exposure to specific pollutants, sometimes without assessing the degree of adverse effects. The endpoints in exposure biomarkers are generally based on the responses (induction/inhibition) of specific enzymes involved in biotransformation and detoxification processes. An example of an exposure biomarker is MT induction following heavy metal exposure.

1.4.2.2 Biomarkers of Effects

The fundamental assumption in effect biomarker studies is that a biological process (i.e. the biomarker) that is essential to the normal function of cells, tissue or whole organism is compromised following pollutant exposure (Handy *et al.*, 2003).

Biomarkers of effects focus on pathological endpoints at all levels of biological organisation. The biological level at which an effect is detected usually reflects the current state of a continuous toxic mechanism, as well as its toxic significance and ecological relevance (Figure 1.5). Effect biomarker studies often focus on endpoints on low levels of biological organisation (e.g. molecular, cellular) that can be related to adverse effects on higher levels through pathogenic pathways (Moore *et al.*, 2004). This biomarker approach is often used in genotoxic assessments of long-term adverse biological effects. Increased values in biomarkers that are on pathogenic pathways related to disease indicate an increased risk of disease.

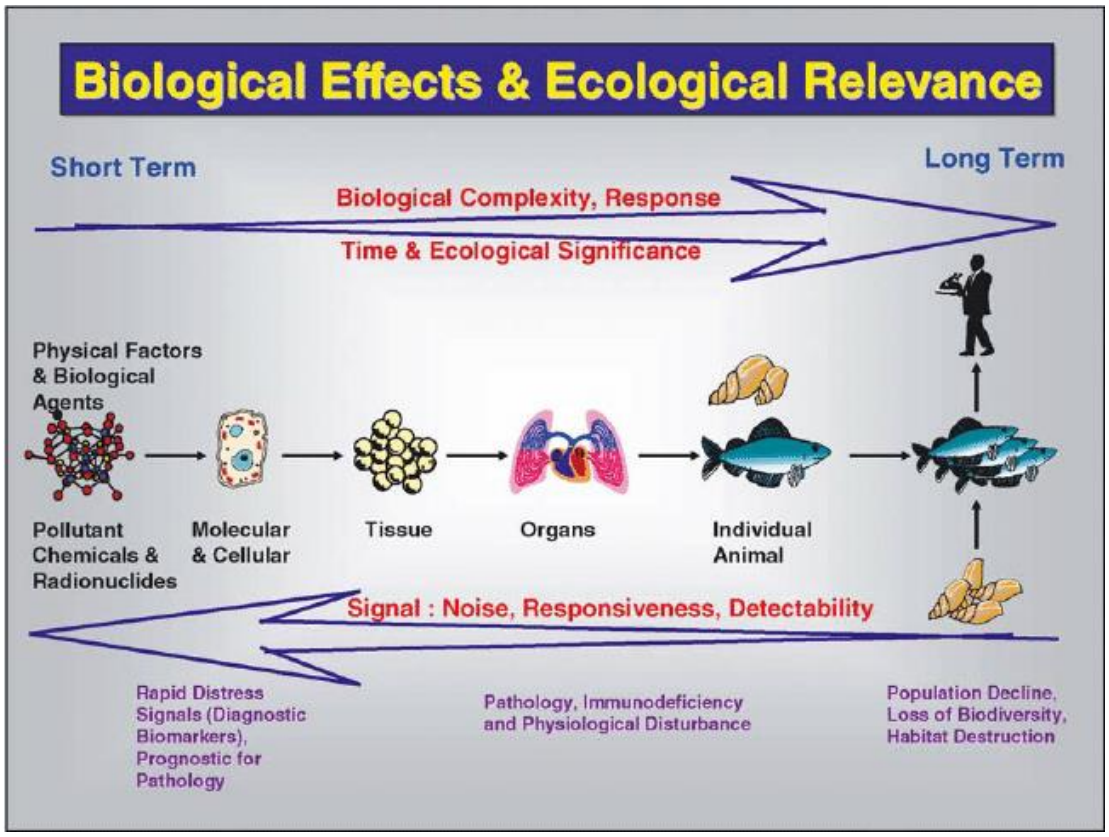


Figure 1.5 Diagram representing the relationship between environmental stress and ecological relevance (Moore *et al*., 2004)

1.4.2.3 Advantages and Limits of the Application of Biomarkers

Using biomarkers as a tool in environmental risk assessment (ERA) can be highly advantageous for several reasons (Handy *et al.*, 2003):

i. Biomarker responses may indicate the presence and bioavailability of pollutants, rather than a simple biologically inert form of pollutant.
ii. Using a suite of several individual biomarkers may reveal the presence of pollutants not suspected initially.
iii. The persistence of biomarker responses after a transient exposure to a pollutant that has since degraded and is no longer detectable may allow biomarkers to detect intermittent pollution events that routine chemical monitoring may miss.
iv. Biomarker analyses are often easier to perform and considerably less expensive than a wide range of chemical analyses.

However, there are certain limits of application in the use of biomarkers. These are often due to variability in the biomarker responses. Variability is often experienced over the change of seasons and following fluctuations in environmental (temperature, dissolved oxygen, salinity, daylight, etc.) and

biological (sexual hormones, genotype, phenotype, tolerance, plasticity, body size, age, sex, etc.) factors. Certain biomarker responses experience natural variation throughout the seasons due to physiological changes from changes in environmental factors or biological events, such as reproduction and growth phases. These annual biomarker response variations should be well understood in order to properly analyse, assess and distinguish between the effects of pollutant exposure and natural physiological changes (Petrović *et al.*, 2004).

Handy *et al.* (2003) proposed some methods to account for and minimize variability in biomarker responses. Careful selections should be made of the most suitable sentinel organisms in the reference and polluted sites within study areas. Biomonitoring programs should be carefully designed and timed to cover the different environmental seasons experienced by the population to achieve an overall assessment of the state of the population over a certain time and to account for factors related to seasonal variations.

Populations in natural ecosystems often show interindividual differences in biomarker responses that can be difficult to accurately assess in biomonitoring programs. The characteristics of the ecosystem is highly influential in these cases, as natural ecosystems often exhibit heterogeneous patterns of pollutant distribution that can cause physiochemical and biological characteristics to vary over small distances. Organisms located in different parts of an ecosystem may then show variation in biomarker responses. Interindividual variations in biomarker responses is also highly influenced by individual biological characteristics, such as age, size, genotype, etc. (Depledge & Rossi, 1994).

1.4.3 Previous Environmental Assessment in the Coastal Marine Regions of Lista

The complex mixture of compounds in the waste water effluent from Alcoa Lista exposes the marine ecosystems in Lista to a variety of different environmental stress factors. Pollutant concentrations in biological tissues and water bodies have been the focus in previous environmental monitoring programs in Husebybukta and the surrounding coastal area. Chemical analysis has been used as the main tool in these assessments (Kroglund, 2016). So far, biomarker studies have not been incorporated into previous environmental monitoring programs in this area.

PAHs have been identified as the components of main concern in this effluent. Consequently, concentrations of different PAH compounds in biological tissues and water bodies have been the focus in previous studies (Kroglund, 2016, 2008; Næs *et al*., 1998a; Næs & Oug, 1997a; Knutzen *et al.* 1995; Table 1.2). Concentrations of heavy metals such as lead (Pb), cadmium (Cd), mercury (Hg) and nickel (Ni) were permanently incorporated into the monitoring program in 2013 to fit the list of prioritised compounds in environmental monitoring programs set by the Norwegian Environment Agency. In previous monitoring surveys, heavy metal concentrations have occasionally been included on a smaller scale than in Kroglund (2016).

Table 1.2 Studies of PAH accumulation in *L. littorea* from the coastal regions of Lista

Year	Months	Comment	References
1985-1990	Sep	Annual samplings from three stations (Tjuvholmen, Havik, and Litlerauna)	Jacobsen *et al.*, 1996 (NIVA 3474-96) (Results only)
1995	June, Aug, Nov	Three samplings from four stations (Tjuvholmen, Haugestranda, Havik, and Litlerauna)	Jacobsen *et al.*, 1996 (NIVA 3474-96)
1996-1998		Up to 10 annual samplings throughout the year from four stations (Tjuvholmen, Haugestranda, Havik, and Litlerauna)	Svein-Harry Samuelsen to Statens Forurensingstilsyn, September 4th 1998 in *Resipientundersøkelse*. (NIVA 414-98)
1999-2007	July, Aug, Oct, Nov	Four annual samplings from four stations (Tjuvholmen, Haugestranda, Havik, and Litlerauna)	Moy & Kroglund, 2002 (NIVA 4549-02) Kroglund, 2004 (NIVA 4835-04) Kroglund, 2008 (NIVA 5653-08)
2009-2011	June, Aug, Oct, Nov	Four bi-annual samplings from four stations (Tjuvholmen, Haugestranda, Havik, and Litlerauna)	Kroglund, 2012 (NIVA 6419-12)
2013-2015	Aug, Oct	Two samplings in 2013 from six stations (Tjuvholmen, Haugestranda, Havik, Storevik, Kviljoodden, and Litlerauna)	Kroglund, 2014 (NIVA 0091-14) Kroglund, 2016 (NIVA 6974-16)

Samplings have predominantly been performed in the later months of the year. Seasonal variances in pollutant tissue concentrations were observed, with lower values during the summer months.

1.4.3.1 Previous Chemical monitoring at Alcoa Lista

Studies performed in the last decade have generally concluded that organisms directly exposed to the waste water effluent from Alcoa Lista in Husebybukta are "very highly polluted". PAH tissue concentrations in aquatic organisms decrease with increasing distance to the effluent release point. The most recent studies by Kroglund (2016, 2014) concluded that the sentinel organisms (*Mytilus edulis* and *L. littorea*) from the monitoring stations within three kilometres from the discharge point contained concentrations of benzo[a]pyrene (BaP), fluoranthene, PAH_{16} and benzo(a)anthracene exceeding the environmental quality standards (EQS) limits set by the EU. Significant PAH concentrations were observed in biological tissues within 3 km from the discharge point. The conclusion was that effluent pollutants affected biological organisms within 8 km from the discharge point. Organisms from the reference station in Litlerauna, 10 km west of the release point, were marked as not contaminated by PAHs.

Kroglund (2008) recorded significant seasonal variations of PAH concentrations in periwinkles (*L. littorea*) from Husebybukta in 2004-2007. Four annual samplings were performed in this study (June, August, October and November) and a seasonal pattern was found, in which periwinkles showed higher concentrations in the last months of the year. Factors affecting bioaccumulation of PAHs in the periwinkle

are both environmental (e.g. temperature, salinity, oxygen) and biological ones (e.g. growth season, reproductive activity). The seasonal variations in PAH tissue concentrations were monitored throughout the entire year in 1997 (Figure 1.6). The trend in PAH tissue concentrations showed high values drastically decreasing during the summer months before gradually rising in the autumn until reaching a similar plateau of high values in the later months of the year. It is interesting to note that the lower concentrations measured coincided with the warmer months, when *L. littorea* undergoes drastic physiological changes linked to spawning.

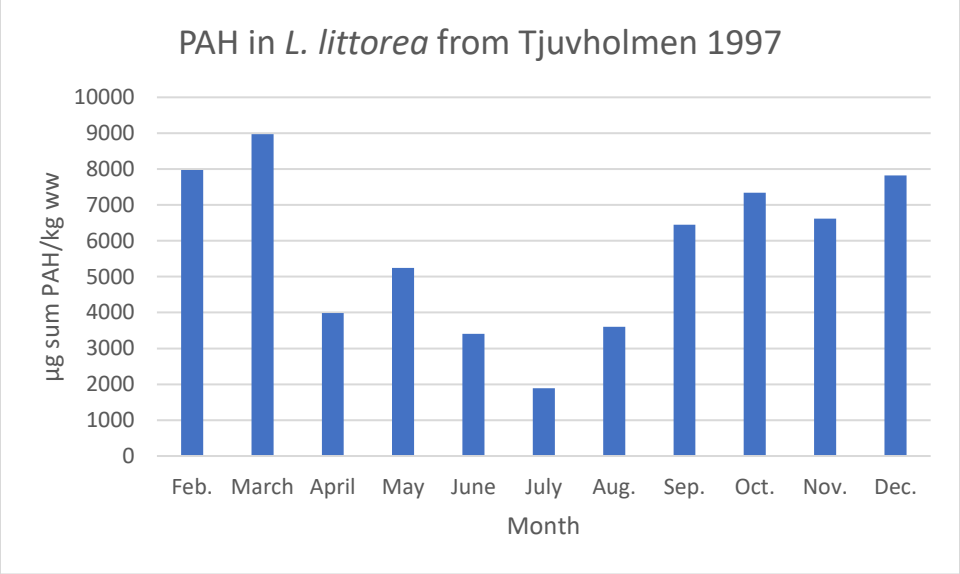


Figure 1.6 Seasonal variances in PAH tissue concentrations in L. littorea collected from Tjuvholmen in 1997 (NIVA 414-98).

BaP is a highly potent genotoxic PAH compound, whose metabolites can exert carcinogenic, immunotoxic, clastogenic, and mutagenic effects in organisms upon bioaccumulation and metabolism of the original compound (Gangar *et al.,* 2010). The risk of biological damage from exposure to BaP and other PAH compounds in the effluent is particularly interesting in biomarker studies.

Næs *et al*., (1998b, 1997b) investigated the bioavailability of the PAH present in Husebybukta. High concentrations of PAHs were observed in the sediments. However, the environmental effects in the test organisms seemed lower than anticipated. The coarse sandy bottom sediments in the recipient does not allow for much adsorption of PAHs. Black carbon soot particles were shown to contribute significantly to the adsorption of PAHs, rendering PAH compounds non-bioavailable to certain species, due to their strong permanent binding as soot particles (Ruus *et al*., 2010). Figure 1.7 shows a diagrammatic representation of the PAH pollutant benzo[a]pyrene (BaP) binding to natural particles (Moore *et al.*, 2004). Hydrophobic contaminants, such as PAHs, tend to adsorb to organic and inorganic particulates and colloidal organic

carbon and sediment. This sorption phenomenon results in PAHs seldom being truly dissolved in the water phase, thus reducing its bioavailability.

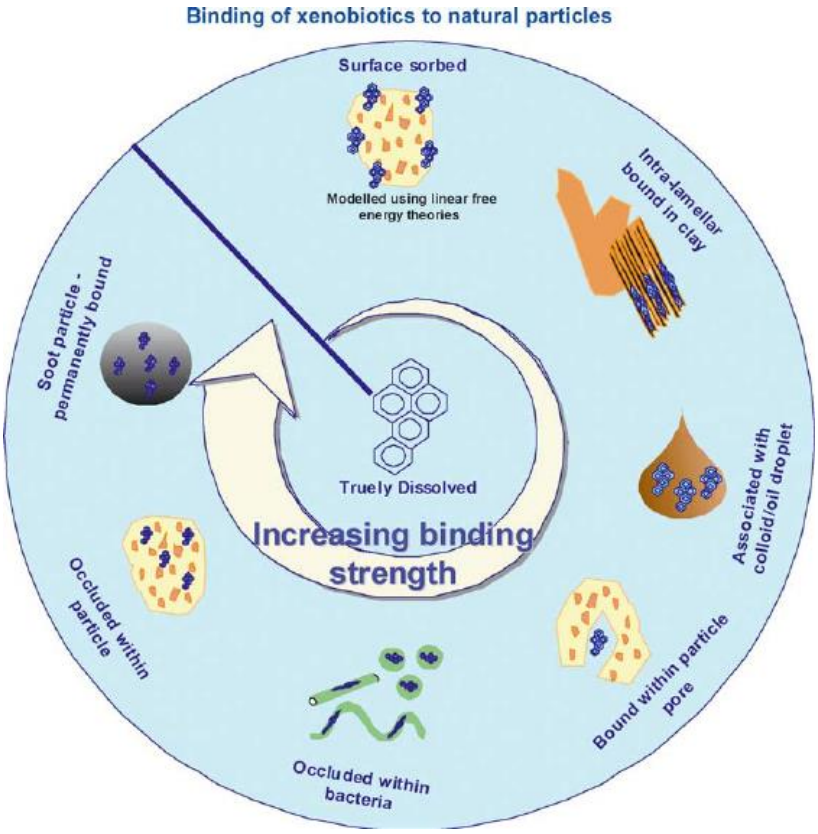


Figure 1.7 Diagrammatic representation of the PAH pollutant benzo(a)pyrene binding to natural particles (Moore *et al*., 2004).

Kroglund (2016, 2014) revealed that concentrations of Cd and Pb in the water column in Husebybukta exceeded the environmental quality standards (EQS) limits set by the EU. The indicator organisms in Husebybukta showed heavy metal concentrations below the set limits, suggesting that the risk of adverse biological effects of heavy metal exposure is considered to be low. However, a combination of the carcinogenic agents BaP, Cd, and Pb in areas close to the effluent release point can give rise to additive or synergistic effects upon uptake in biota.

1.4.3.2 Indicator Organisms in Previous Environmental Assessment

Previous environmental monitoring programs at Lista have been performed analysing the chemical content of the common periwinkle (*L. littorea*), the blue mussel (*M. edulis*), and the common limpet (*Patella vulgata*). These organisms are microphagous feeders that can ingest PAHs and other particles adhered to food particles. The periwinkle and the limpet scrape particles and microalgae from rocks and sea lettuce with raspy tongues, while the mussels are filter feeders and ingest food particles mainly from the water phase (Næs *et al.* 1998a).

The blue mussel is usually the preferred organism in similar monitoring programs, due to its filter feeding habits and their low metabolic transformation rate that allows it to bioaccumulate pollutants (Galloway *et al.*, 2006). The blue mussel has not settled in Husebybukta as a habitat, as it prefers living in intertidal littoral zones attached to rocks and other hard substrates, whereas Husebybukta is dominated by sandy bottom sediments (Kroglund, 2014). Therefore, there are not enough blue mussels naturally occurring in this area (Kroglund, 2016, 2014). Attempts have been made in conducting active biomonitoring programs using caged mussels with no success (Kroglund, 2016, 2014). Naturally occurring periwinkles were collected from the monitoring stations instead. The limpet was used as an indicator organism from 1978 to 1985. After a decline in the limpet population in the recipient, it was replaced by the periwinkle in the monitoring program in the mid-1980s (Kroglund, 2014; Næs *et al.*, 1998). The periwinkle has since been the preferred indicator organism in this area.

1.5 The Periwinkle (*Littorina littorea*)

Figure 1.8 Periwinkle (*L. littorea*) from Tjuvholmen, Lista (Photo: Private)

The periwinkle (Figure 1.8) was chosen as the sentinel species in this thesis due to its natural abundance in the recipient.

1.5.1 General Biology

The periwinkle is a common North Atlantic gastropod that is distributed from northern Spain to the White Sea (Jackson, 2002). *L. littorea* is a shallow water species that can be found from the upper shore to the sub-littoral zone in almost all kinds of shore environments. It prefers open rocky coasts as its habitat, but can also be found in sandy or muddy environments in sheltered coastal locations. *L. littorea* occupy an important trophic position and have a high influence in coastal ecosystems in the North Atlantic. Its feeding habit as a grazer have resulted in the periwinkle drastically altering the New England intertidal community by allowing slower growing algal species to dominate over the faster growing algal species that are the periwinkle's preferred food (Lubchenco, 1978). The periwinkle is a resilient organism that is considered an invading species causing ecological alterations in coastal regions in North America (Jackson, 2002).

Periwinkles breed from December to May in most regions, however, longer breeding seasons occur in the southernmost areas of distribution (Oehlmann, 2004). The females produce about 500 egg capsules, each containing 1-5 eggs, that hatch after 5-6 days as veliger larvae. Most male periwinkles shed their penis after the breeding season, leaving them with just the base of their reproductive organ for a few months until a new grows out in late summer or autumn. Periwinkles are a quite long-lived species (5-10 years in pristine environments, Jackson, 2002). Periwinkles reach sexual maturity at the age of 12-18 months. The shell height is then 10-15 mm. Male periwinkles tend to be slightly larger than females, being able to reach a shell height up to 52 mm. The large variety in shell length can often be related to the characteristics of the habitat, especially the salinity of the ambient water, as the periwinkle is fairly tolerant of brackish water (Jackson, 2002). The largest specimens have been found in locations of higher natural salinity (35 000 ppm), while the smallest specimens are usually found in brackish estuaries (< 30 000 ppm) (Oehlmann, 2004).

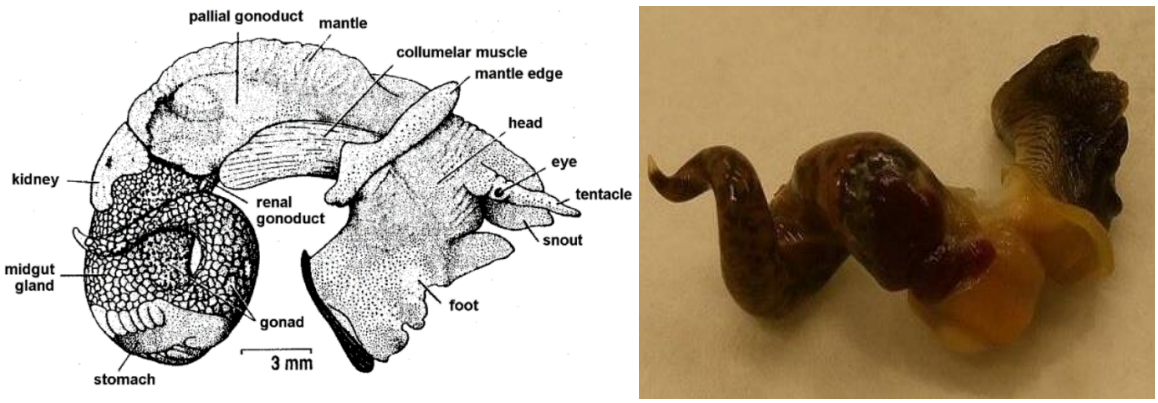


Figure 1.9 Left: Female *L. littorea* drawn anatomy (Oehlmann, 2004). Right: *L. littorea* from Lista.

It has an open circulatory system composed of haemocytes, which are mobile haemolymph defence cells responsible for the recognition and detoxification of a wide variety of pathogens (Iakovleva *et al.*, 2006). The main functions of the haemocytes are cytotoxic activities, phagocytosis, acid phosphatase

activities, and generation of reactive oxygen species (ROS) (Gorbushin & Iavkovleva, 2007). Gorbushin & Iakovleva (2006) observed three morphotypes of haemocytes when studying the haemogram of periwinkles in the White Sea. They proposed that the three morphotypes corresponds to cells in different states of maturation, i.e. juvenile, intermediate, and mature cells. The cells are mainly distinguishable by their respective glycogen deposit amount, nucleus shape and location, and nucleoplasmic ratio. The haemogram parameters showed seasonal variations in cell concentration and morphotype composition in the study, where cell concentration peaked twice during the summer months.

1.5.2 *Littorina littorea* as Sentinel Species in Environmental Monitoring

A fundamental criteria of a good indicator organism in environmental assessment is its representativeness of an ecosystem. Good correlation between tissue pollutant concentrations, biological responses and environmental pollutant concentrations is required for sentinel species in environmental assessment. Lowe *et al*. (2006) encourages the use of *L. littorea* as a model of grazers in environmental assessment, as its tissue concentrations, biological responses, and general health condition have been shown to be a good reflection of the state of its surrounding environment. Its feeding habits allows it to take up particulate organic and inorganic matter as well as colloidal organic compounds, which hydrophobic pollutants tend to absorb (Moore *et al*., 2004). Periwinkles are also exposed to dissolved and re-suspended contaminants in the water phase. The different routes of exposure and uptake of contaminants and the periwinkle's ability to bioaccumulate contaminants usually makes *L. littorea* a good biological indicator of its surrounding environment. The abundance of periwinkles and their strong resistance to environmental contamination and poor conditions enables them to occupy wide and diverse geographical areas of different contamination levels. Periwinkles also have a satisfactory sensitivity towards pollution to obtain environmental quality gradients in biomonitoring programs. This allows for the use of the periwinkle use as sentinel species in biomonitoring of both pristine and polluted sites (Noventa, 2010).

It has been widely applied as a bioindicator for endocrine disruption. Periwinkles exposed to tributyltin (TBT) contamination in marine waters have shown the characteristic development of intersex (Van den Broeck *et al.*, 2007; Oehlmann, 2004; Galloway *et al.*, 2004; Bauer *et al.*, 1997; Bauer *et al*., 1995).

The haemolymph constitutes the sample matrix in a variety of biomarker assays, such as the neutral red retention time (NRRT) assay (Lowe *et al.*, 2006), the comet assay (Noventa, 2010), the MN assay (UNEP/RAMOGE, 1999) and the phagocytosis assay (Iakovleva *et al.*, 2006).

The periwinkle is regarded as a good bioindicator of metal exposure and contamination. Studies based on chemical analysis have found good correlations between the metal levels in periwinkle soft tissue (e.g. kidney, gill and digestive gland) and the surrounding environmental abiotic concentrations (Amiard *et*

al., 2006; Leung & Furness, 1999; Bebianno & Langston, 1998; Langston & Zhou, 1986). MT induction in *L. littorea* soft tissue is regarded as a good biomarker of exposure to heavy metals, particularly Cd (Amiard *et al.*, 2006).

1.6 Assessment of Pollution Related Stresses in Biomonitoring

1.6.1 General Health Status – Lysosomal Membrane Stability

Assessment of the general health status of organisms is very valuable when performing biomonitoring studies. General toxic effect endpoints are not always easy to address in complex organisms, and the factors leading to endpoints are not always easy to assess accurately. Implementing non-specific effect biomarkers in the biomonitoring can give a general picture of the overall health status in a population. The LMS assay ticks all the boxes of a good non-specific biomarker of effects and it is often used in biomonitoring programs as an indicator of the general health (ICES, 2015).

1.6.1.1 Lysosomal System

Lysosomes are cytoplasmic organelles containing over 40 different classes of hydrolytic enzymes (e.g. proteases, nucleases, carboxylases, lipases, etc.) enclosed by a single-layer semi-permeable membrane. Lysosomes have a remarkable capability to accumulate and hydrolyse a wide variety of biological compounds, such as proteins, DNA, RNA, polysaccharides and lipids. The detoxifying abilities of lysosomes enable them to function as the digestive system of the cell by degrading xenobiotics taken up in the cell and by degrading obsolete cell components in the process known as autophagy (Figure 1.10) (Moore *et al.*, 2008).

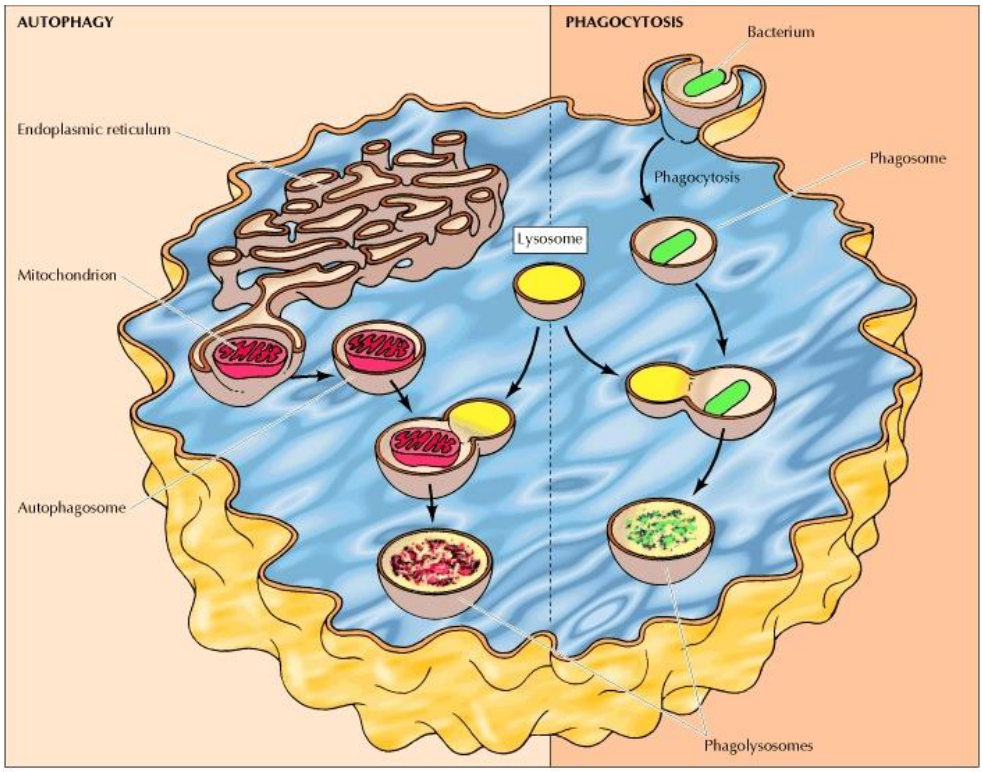


Figure 1.10 Lysosomes in autophagy and phagocytosis (Cooper & Hausman, 2009)

The lysosomal system is also involved in regulating catabolic rate of different cellular macromolecules and they are especially useful for detoxifying purposes. Lysosomes ideally operate at more acidic conditions (pH ≈ 4.5-5.0) than the cytosol (pH ≈ 7.2).

The ATP-dependent membrane proton pumping system protects the cytosol and the rest of the cell from the degradative enzymes contained within the lysosomal membrane. The pump is a V-class H^+ ATPase pump, and its main function is to acidify the lumen of the lysosome by pumping H^+ ions from the cytosol into the lysosome. Impairment of the ability of this system will cause the acidic lysosomal contents to leak into the cytosol, with following negative consequences on the functionality of the cell (ICES, 2015).

Lysosomal Impairment

Overloading of the lysosomes' detoxifying capacity results in lysosomal impairment, which can lead to leaking of the acidic lysosomal contents into the cytosol, thus causing cell damage and tissue dysfunction (ICES, 2004). Lysosomes can be impaired by several chemical and non-chemical stressors, such as metals, organic pollutants, osmotic shock, hyperthermia, dietary depletion, etc. that may act separately or simultaneously. Lysosomal impairments usually fall under three categories, which are changes in membrane permeability, changes in lysosomal contents or changes in fusion events (ICES, 2004). The

impairment of lysosomes reflects the organism's weakened ability to detoxify xenobiotics. It can also be linked to growth inhibition, proteins metabolism and immunocompetence (Moore *et al.*, 2004).

1.6.1.2 Neutral Red Retention Time Assay

A range of bioassays have been developed to assess lysosomal integrity and the different types of lysosomal impairment. The NRRT assay is widely applied in monitoring LMS in aquatic organisms. The NRRT assay takes advantage of the lysosomes' accumulating abilities and, in the case of stressed organisms, the lack thereof. The NRRT assay measures the uptake and lysosomal retention time of a neutral red dye quantitatively by visualisation using a microscope.

The NRRT assay is an *in vivo* cytochemical analysis that measures the LMS. The NRRT assay is based on the ability of the lysosomes to trap, sequester and accumulate the lipophilic neutral red dye by protonation. This ability depends on the status of the lysosome and its membrane proton pump, both which can be subjected to impairment following lysosomal damage. The rationale is that the lysosomes of a healthy, non-stressed organism will have a better ability to accumulate the neutral red dye and contain it by protonation for a longer time than the impaired lysosomes of an organism that has been exposed to certain stress factors. The assay measures the amount of time taking for the neutral red dye to start leaking into the cytosol. The NRRT reflects the state of the membrane proton pump, and the tendency of acidic enzyme release from the lysosomes into the cytosol (ICES, 2004, 2015).

1.6.1.3 Advantages and Limits of Application

LMS represents one of the simplest, cheapest, and most sensitive biomarker to evaluate the general physiological status of marine organisms. The NRRT assay is easy to perform and is highly recommended for assessing LMS in marine biomonitoring programs (ICES, 2004, 2015). The test has been thoroughly and carefully assessed through laboratory and field projects. The NRRT assay is non-destructive, meaning the organisms are not unduly stressed during extraction, and it can be performed on small quantities of haemolymph (ICES, 2015; Gorbushin & Iavkovleva, 2006).

Seasonal fluctuations in environmental abiotic factors (e.g. salinity, pH, temperature, food, oxygen) are often addressed as a major concern in biomarker studies. However, they seem to not be of notable influence in the NRRT assay. The same can be said about size differences in test organisms (ICES, 2004, 2015). Nevertheless, organisms can exhibit decreasing lysosomal stability when undergoing significant physiological changes during the growth and reproductive seasons (Noventa, 2010; Petrovic *et al.*, 2004).

The wide variety of environmental pollutants in the waste water effluent from Alcoa Lista can cause many toxic mechanisms upon uptake in the organisms present in the recipient. The role of lysosomes as the

major detoxifying mechanism, as well as the variability in lysosomal stress inducers, makes the LMS assay a good addition to biomarker suites in general.

Several studies have performed the NRRT assay on the periwinkle (*L. littorea*) using haemolymph as sampling matrix, with results showing good correlations to general stress exerted on the organism (Noventa & Pavoni, 2011; Noventa, 2010; Lowe *et al.*, 2006; Moore & Lowe, 2004).

1.6.2 General Health Status - The Condition Index

The CI is a measurement of individual physiological health status, commonly used in biomonitoring programs. It is often used to quantify the fat reserves of individuals. Fat reserves are important sources of energy for organisms when undergoing biological activities such as reproduction and migration that are highly energy demanding. It is also important for survival during longer periods of scarcity.

CIs are generally based on some combination of either the ratio of body mass and linear dimensions of an organism, such as total body length. CIs can also be pure weight ratios of dry/wet weight values in tissues or ratios of whole organism and certain tissues weight. Higher values of CIs usually indicate that an individual have greater energy reserves.

In *L. littorea*, CIs are generally measured as dry/wet weight ratios of soft tissues. If the tissues of individuals are to be used in further biological testing, a CI measured by the ratio of total wet body weight and total wet tissue weight can be used (Amiard *et al.*, 2004).

1.6.3 Genotoxicity

Genotoxic agents are normally characterised as chemical (e.g. PAH metabolites, heavy metal ions) or physical (e.g. UV, X-ray) agents. Genotoxic agents are known for their ability to cause chromosomal damage by altering DNA sequences. Cells have DNA repair mechanisms that usually inhibit the toxic effects of genotoxic agents efficiently. However, failure to properly repair DNA lesions can lead to adverse effects. Genotoxic lesions usually appear as either DNA adducts, DNA strand breaks, modified bases, or DNA crosslinks (Walker *et al.*, 2012).

Several PAHs, including BaP and dibenzo(a,h)anthracene, are known as genotoxic pollutants that have highly reactive metabolites that are activated through oxidation by cytochrome P_{450} during phase I metabolism. The original PAH compounds are generally stable and unreactive, however, their short-lived, highly reactive and electrophilic metabolites have a high affinity for the nucleophilic sites on cellular macromolecules, such as DNA (UNEP/RAMOGE, 1999). PAH metabolites covalently bound to DNA are a type of DNA adducts that can generate a variety of DNA lesions that pose problems for later DNA replication (Walker *et al.*, 2012; Fenech, 2000).

Organisms exposed to chemical genotoxic agents can show an increased MN formation as an effect (Gangar *et al*., 2010). An increased frequency of MN in cells is a biomarker of genotoxic effects reflecting both exposure to clastogenic and aneugenic agents (Albertini *et al.*, 2000).

1.6.3.1 The Micronucleus Assay

MN are small, extranuclear bodies that can be present near the cell nucleus. MN formation arise from either clastogenic (chromosome breaking) or aneugenic (whole chromosome lagging) failure events during mitosis. The nature of the failure event decides the structural characteristics of the MN. MN containing chromosomal fragments can result from several clastogenic events, such as direct DNA breakage, replication of damaged DNA templates, or inhibition of DNA synthesis. MN containing whole chromosomes generated from aneugenic events generally arise from failure of the mitotic apparatus, alterations in cellular physiology, or mechanical disruption (Albertini *et al.*, 2000).

Acentric chromosomal fragments or whole lagging chromosomes that fail to properly attach to the mitotic spindle poles during anaphase is excluded from both daughter nuclei, however, is still part of one of the daughter cells. The part of the genome excluded from the primary daughter nuclei will develop its own nuclear membrane and form one or several smaller nuclei relative to the primary daughter nuclei, thus referred to as a micronucleus (Figure 1.11) (Gangar *et al*., 2010).

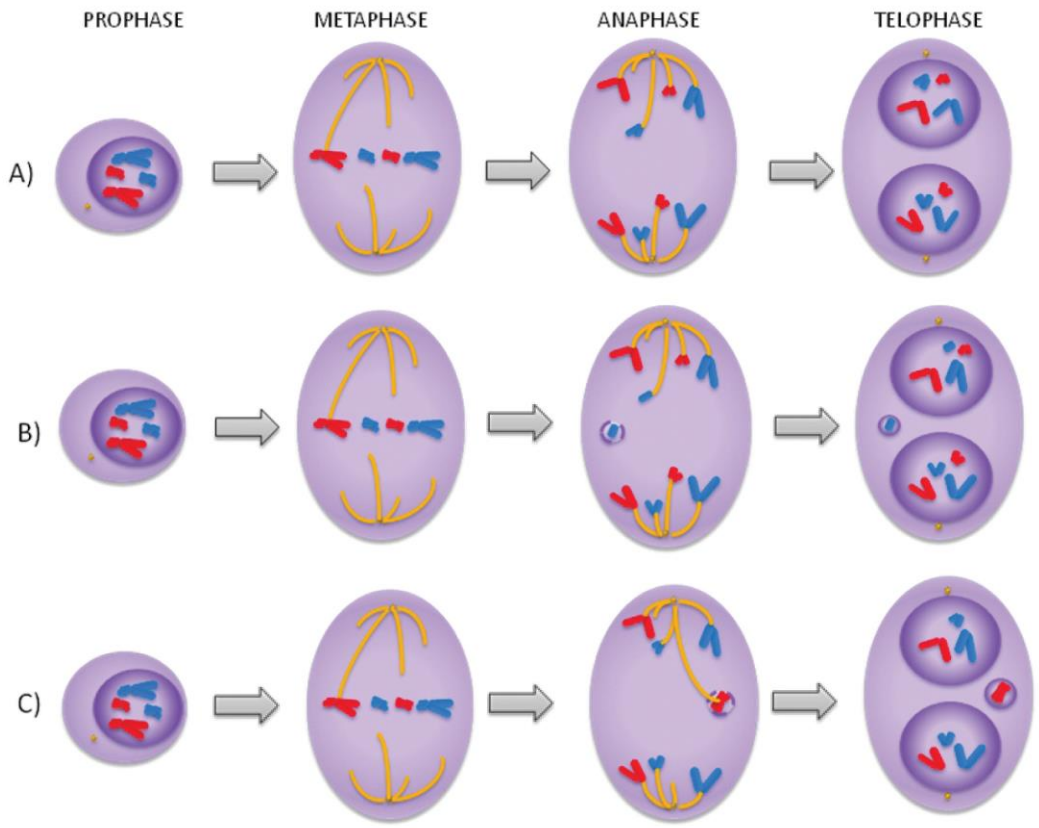


Figure 1.11 Schematic illustration of MN formation following B) clastogenic events or C) aneugenic events. Proper mitosis shown in A). (Zelazna *et al*., 2011)

The *in vivo* MN assay, developed independently by Heddle and Schmid in the late 1970s, is widely used in biomonitoring for measuring chromosomal damage and detecting DNA abnormalities. The MN assay provides information on the accumulated genetic damage over the lifespan of the cells.

MN frequency is estimated and scored visually from fixed suspensions of 1000-2000 cells smeared and stained on microscope slides under light microscope. All cells in the sample are analysed for MN. Schmid (1975) defined a widely-used observation criteria of MN evaluation, in which:

- MN shape are oval or round
- MN diameter is less than one-third of the main nucleus
- MN staining intensity is the same as the main nucleus
- Only intact MN clearly separated from the main nucleus is counted

The MN frequency is expressed as either the percentage or thousandth number of micronucleated cells to cells with normal sized nuclei. The MN frequency can be related to chromosome loss and breakage, which can cause long-term consequences, such as mutagenesis and carcinogenesis (Fenech, 2000).

1.6.3.2 Use of the Micronucleus Assay in this Study

The wastewater effluent from Alcoa Lista contains several genotoxic compounds, such as BaP, benzo(a,h)anthracene, and heavy metals. The MN assay was incorporated into this biomarker study to account for possible genotoxic effects from the wastewater compounds in an easy, repeatable and cost-efficient process.

1.6.3.3 Advantages and Limits of Application

The MN assay is simplistic and cost-efficient compared to other assays used for measuring chromosomal damage. MN assays can give reliable measurements of both chromosome loss and chromosome breakage, making them among the preferred methods of assessing overall chromosome damage (Fenech, 2000).

MN formation in wild organisms have been found to be influenced by a variety of environmental and physiological factors, such as age, reproduction and growth phases, season, oxygen levels, salinity, temperature, mitosis frequency, and growth period (Burgeot *et al.*, 1996).

Fenech (2000) states that the MN assay results are only viable of it can be proven that the MN formation is a direct result of exposure to genotoxic agents.

1.6.4 Metallothionein

1.6.4.1 Structure and Properties

The MT protein was first discovered by Margoshes & Valee (1957) when they isolated a Cd-binding, cysteine-rich protein from the kidney cortex of horses. MTs are heat stable, cysteine-rich, non-enzymatic cluster proteins with low molecular weight (6-7 kDa) that belongs to a superfamily of intracellular metal-binding proteins. MT proteins have a characteristic amino acid composition; they completely lack aromatic amino acids in the primary sequence, and cysteines make up one third of its amino acid residues.

The high cysteine content from thiol groups (-SH) is an important characteristic of MT. The electrophilic properties of the sulphur in these groups gives MT a remarkable ability to chelate a variety of heavy metals (e.g. Ag (I), Au (II), Cd (II), Co (II), Cu (II), Fe (II), Hg (II), Pb (II), Pt (II), Tc (II), and Zn (II) (Stillman, 1995)). The location and sequence of cysteine show a highly conservative pattern in the amino acid composition in MT. The pattern of the Cys-Cys, Cys-X-Cys and Cys-X-Y-Cys sequences, where X and Y denote amino acids other than cysteine, largely determines the behaviour of MT isoforms. It is also the criterion that allows the distinction between different structural MT classes (Amiard *et al*., 2006).

Heavy metals sharing similar stoichiometric characteristics as the divalent essential metals Cu and Zn will be able to bind to the thiol groups of the protein. MTs can be saturated with several different metals simultaneously, depending on its amino acid characteristics and affinity for metal ions. MTs *in vitro* affinity

for different metal ions varies with its cysteine residue characteristics, however, it generally decreases in the hierarchical sequence Hg > Cu, Ag, Bi >> Cd > Pb > Zn > Co = Ni (Amiard *et al*., 2006). Heavy metals with a high affinity are able to displace heavy metals of lower affinity bound to MT.

Littorina littorea MTs

MTs in *L. littorea* (Ll-MT) are larger than MTs found in most species, having a predicted molecular mass of 10 kDa. Ll-MT consists of 99 residues, in which 27 are cysteine (Cys) residues. 20 of the cysteine residues in Ll-MT fall in the Cys-X-Cys sequencing pattern. The Cys-Cys pattern in lacking in Ll-MT, similarly to MTs of other mollusc species (English & Storey, 2003). Baumann *et al.* (2017) suggested that the protein is formed in a three-domain structure (α1, α2 and β), similarly to MTs in other marine gastropods (Figure 1.12).

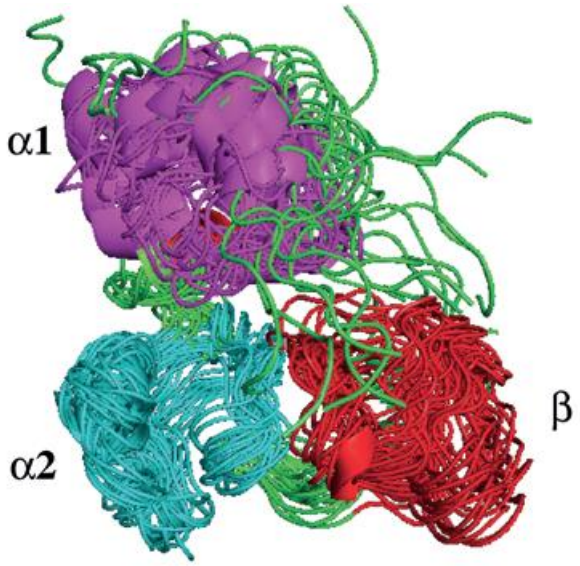


Figure 1.12 *L. littorea* MT structure model (Baumann *et al*., 2017)

Assuming a similar stoichiometric ratio in Ll-MTs as in other marine gastropods (9 Cys residues per 3 equivalents of divalent heavy metal ions) would suggest that the 27 Cys residues in the Ll-MT protein is capable of binding 9 divalent heavy metal ions.

1.6.4.2 Metallothionein Induction and Functions

MTs are readily induced by a variety of physiologic and toxicologic factors, and the proteins are naturally present in different tissues of a variety of organisms. MT induction has been proven to be influenced by exposure to inducing agents such as heavy metals, hormones, thermal stress, pharmaceuticals, steroids, organic solvents, alcohols, cytokines, alkylating agents, radiation, infections, and ROS (Ruttkay-Nedeckt *et al*., 2013; Mao *et al*., 2012; Viarengo *et al*., 2000). The amino acid composition of MT influences the behaviour of the protein, thus different isoforms of MT show varying sensitivity to different inducing

agents. The most effective inducing agent of MT is the essential metal zinc (Zn), and several functions of MT are due to Zn ion interactions.

The high number of stimulating factors in the induction of MT makes it difficult to identify its specific physiological functions. It is a protein whose physiological functions are not yet fully understood. However, most authors agree that MTs are multifunctional proteins playing a dominant role in metal-regulating and detoxifying processes. Given their remarkable metal-binding ability, the primary roles of MTs are generally considered to involve 1) homeostatic regulation of the essential metals Zn and Cu, and 2) bioaccumulation and detoxification of excess amounts of essential and non-essential metals (e.g. Cd, Hg) (Amiard *et al*., 2006). MTs are, to a smaller degree, involved in other pathophysiological processes, such as protection against oxidative and free radical stress (Ruttkay-Nedeckt *et al*., 2013; Viarengo *et al*., 2000), cell proliferation and apoptosis, chemo-resistance, and radiotherapy-resistance (Shariati & Shariati, 2011; Florianczyk, 2007; Klaassen *et al*., 1999). MTs can generally be considered as stress-related proteins.

MT induction following heavy metal pollutant exposure is regarded as a protective sub-lethal detoxification response. Heavy metal cations accumulated in the cells stimulate MT synthesis as a protective measure. Their high affinity for heavy metals appear to make MTs the preferred intercellular ligands for bioaccumulated heavy metals (Amiard *et al.*, 2006). Zn is the most effective inducing agent of MT. However, it is also one of the heavy metals of a lesser affinity to MT, making it likely to be replaced by other heavy metals of higher affinity. Roesijadi (1996) proposed a model of the coupled MT induction and repair of a ligand exposed to heavy metals (Figure 1.13) (Amiard *et al.*, 2006).

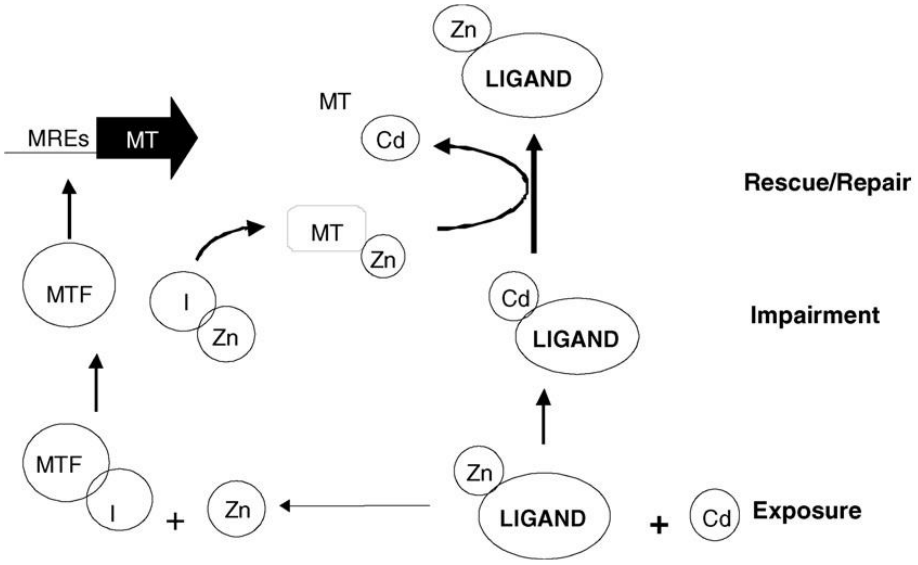


Figure 1.13 Coupled model of MT induction (by Zn) and repair of ligands targeted by heavy metals (Cd). MT: metallothionein; MRE: metal regulatory element; MTF: metal transcription factor; MTI: metal transcription inhibitor (Amiard *et al*. (2006), after Roesijadi (1996))

MT expression level is sensitive to heavy metal exposure and dose dependent up to a specific threshold, after which the MT expression is not positively correlated (Mao *et al*., 2012). Brown & Parson (1978) proposed that if the heavy metal uptake in an organism exceeds the detoxifying metal-binding capacity of its inducible MT proteins it can result in the excess heavy metals starting to "spill over" into sites of toxic action, i.e. the cytosolic enzyme pool. Non-essential heavy metals "spilled" are then available to interact at sites of adverse action by binding to other less-preferred cellular ligands, and toxic effects are first observed.

Like all proteins, MTs have limited lives. Viarengo & Nott (1993) proposed that MTs are transported to lysosomes after performing one or several of its many functions. In the lysosomes, MTs are degraded similarly to other proteins. The turnover rate of Ll-MT is slow in comparison to other marine invertebrates. Bebianno & Langston (1998) found the turnover of Ll-MT to be 69 days in the gills, 160 days in the kidney and >160 days in the digestive gland. In comparison, *M. edulis* (Bebianno & Langston, 1993) and *C. virginica* (Roesijadi, 1994) have shown MT turnover rates of 25 and 4-20 days, respectively. The turnover rates are affected by the activity undergone by the proteins upon induction.

1.6.4.3 Metallothionein Biomarker Studies

The use of MT as a biomarker tool for biomonitoring the environmental metal impact has been thoroughly tested and validated over the recent decades, and is now recognized among a suite of "core biomarkers" in the European framework (UNEP/RAMOGE, 1999).

Increased MT induction or concentration in an organism is regarded as a good biomarker of exposure to heavy metals at the biochemical level of biological organisation.

The use of MT as a biomarker can be coupled with the traditional chemical monitoring of tissue metal concentrations. This will give a more reliable result in a biomonitoring by integrating pollutant concentrations obtained from chemical analyses with an integrated biomarker index demonstrating bioavailability and interactive and combined effects of the pollutants with respect to numerous environmental factors characterising the location (Amiard *et al.*, 2006; UNEP/RAMOGE, 1999). This also allows for MT biomarker studies to be extended to incorporate the "spill over" phenomenon, where adverse effects caused by "spilled" heavy metals can be regarded as the biological endpoint in the assay. The amount of heavy metals in tissues exceeding that bound to MT can be correlated with some measure of adverse effect in effect biomarker studies (Florianczyk, 2007). It is important to note that MT can only be interpreted as a biomarker of effect in conjunction with other measurements, such as tissue metal concentration or metal buffering capacity (Handy *et al.*, 2003).

Several methods have been developed to assess levels of MTs in tissues. The methods most widely used in biomonitoring are utilizing electrochemical (Olafson & Sim, 1979), radioimmunological (Roesijadi *et al*., 1988), spectrophotometric (Viarengo *et al.*, 1997) and metal saturation methods of MT quantification

(e.g. Scheuhammer & Cherian, 1986). The metal saturation methods utilize MTs' high affinity for certain heavy metals in determining MT concentrations. The binding sites of MTs are saturated with a specific metal of high affinity, e.g. Ag or Cd, in the saturation assays. The excess metals are removed before atomic absorption spectrophotometric measurement and MT calculation can be performed (Van den Broeck *et al.*, 2010; Scheuhammer & Cherian, 1986).

MT induction in *L. littorea* soft tissue is regarded as a good biomarker of exposure to heavy metals, particularly Cd (Amiard *et al.*, 2006). MT concentration in the digestive gland, kidney, and gills of periwinkles have been shown to increase at high exposure levels of heavy metals (English & Storey, 2003; Bebianno & Langston, 1998; Bebianno & Langston, 1992; Langston & Zhou, 1986; Bryan *et al.*, 1983). The digestive gland is an important site for heavy metal storage and sequestering in *L. littorea*. It contains two thirds of the total Ll-MT content, as well as other high molecular thiolic proteins, which may interfere in MT determination techniques. The kidney can also accumulate high concentrations of heavy metals and Ll-MT. MT determination in the kidney is usually not interfered by other high molecular thiolic proteins (Bebianno & Langston, 1997). Van den Broeck *et al.* (2010) found heavy metal detoxification by MTs in *L. littorea* exposed to heavy metal contaminants along the Scheldt estuary in the Netherlands to be metabolically and energetically costly, resulting in a lowered fitness condition for the organism. Leung & Furness (1999) found tissue concentrations of MTs and metals like Cd and Zn in *L. littorea* to generally decrease with increasing animal size, while total MT content per individual is a linear function of the organism's size.

1.6.4.4 Limits of Application

MT concentrations are affected by seasonal changes in environmental parameters, such as temperature, salinity, and day length. Biological factors related to individual characteristics, such as size (Leung & Furness, 1999), age, and sexual maturation, must also be considered. Seasonal effects may show significant variations in MT concentrations from combination of environmental and individual biological parameters. The overall concentration of MT depends on both inductive and degradative processes. The increased activity of the physiological processes of the protein from heavy metal exposure in organisms may be reflected as increased turnover rates of MT, rather than as an increase in its concentration.

MT induction is incredibly sensitive towards heavy metal pollutants, however, the numerous MT inducing agents unrelated to heavy metals must be carefully reviewed when applying MT as a biomarker of exposure to heavy metal pollution. MT is required to be used as only one of a suite of biomarkers due to the presence of varying inducing agents (Amiard *et al.*, 2006).

2 Materials and Methods

2.1 Sampling Sites

Periwinkles were sampled from four stations in two sampling areas located along the south-west coast of Norway (Figure 2.1). The three stations in Lista are all within 10 km of the release point of the waste water effluent from Alcoa Lista (Table 2.1, Figure 2.2). Viste in Stavanger, Rogaland was chosen as an additional reference station.

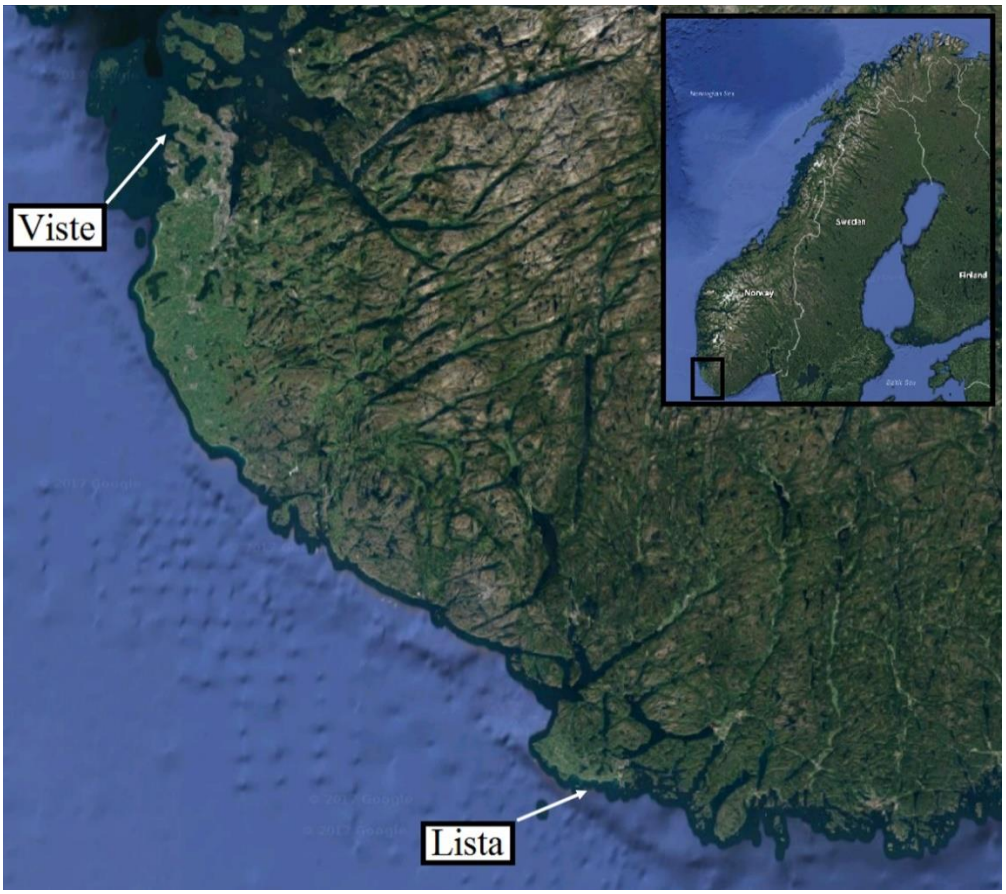


Figure 2.1 Map of South West Norway (©Google)

Table 2.1 Overview of all sampling stations.

Station Code	Location	Coordinates	Distance to release point
Alcoa 1	Haugestranda	58.06º N, 6.766º E	650 m
Alcoa 2	Tjuvholmen	58.07º N, 6.775º E	500 m
Alcoa Reference	Litlerauna	58.08º N, 6.617º E	~10 km
Viste	Viste strand	58.99º N, 5.607º E	~140 km

2.1.1 Lista

Figure 2.2 Overview of sampling stations in Lista. (©Kartverket).

Lista is a countryside dominated by agricultural activities in the municipality of Farsund in Vest-Agder county, Norway. The population of Farsund municipality is close to 10 000 people (2016). Alcoa Lista is located about 2 km in aerial distance from the town of Farsund, which is the administrative centre of the municipality. The waste water effluent from Alcoa Lista is released into Husebybukta, which is an open coastal bight. The three sampling stations in Lista included two stations directly exposed to the waste water effluent located in Husebybukta and one reference station located in Litlerauna, about 10 km west of the release point (Figure 2.2,

Table *2.1*). The selection of sampling locations in Lista was based on results from previously performed chemical analysis (Kroglund, 2016, 2014, 2008).

Husebybukta

The two exposed stations (Alcoa 1 & Alcoa 2) are located in Husebybukta. Husebybukta is an open, wind exposed, shallow bight with sandy sediments. The surrounding area is dominated by countryside with agricultural activities. The facilities of Alcoa Lista AS is located a few hundred meters north-east of Husebybukta. The waste water effluent from Alcoa Lista is released through a pipe at 2-3 meter's depth about 60 meters from the shore (Figure 2.3). There is a small dockage with a few mooring berths located in the western part of the bay.

Figure 2.3 Husebybukta overview (©Kartverket)

Figure 2.4 Husebybukta seen from West (Photo: Private).

Litlerauna

Litlerauna is a land-tied island dominated by pebble sediments in the tombolo and larger boulders in the outer region. A small building stands on the island and a couple of mooring berths are dug in the pebble and sand sediment. Litlerauna is located approximately 10 km west from the Alcoa Lista waste water

effluent release point. A larger peninsula about 200 m SE of the station is the location of a shooting range. Farsund Airport Lista is located about 2 km NE of the station.

Figure 2.5 Litlerauna (Alcoa Reference) sampling station (Photo: Private).

Climate

Lista has a sub-oceanic climate with mild winters and relatively warm summers. The normal temperature is 0,8 °C for the coldest month (February), and 15,3 °C for the warmest month (July). The average annual temperature is 7,6 °C. The growth period (number of days with an average temperature above 6 °C) is about 195 days. Normal annual precipitation is 1049 mm, with dominated precipitation in the autumn (Norske Meterologiske Institutt, 1989).

2.1.2 Viste

Visteviga is an open, wind exposed bay located in Rogaland. It was chosen as an additional reference station. There are no known sources of anthropogenic pollution in the bay. The periwinkles from Visteviga were sampled from the innermost beach located by the former hotel, Viste Strandhotell (Figure 2.6).

Figure 2.6 Viste and Stavanger overview (©Kartverket)

Figure 2.7 Viste sampling station (Photo: Private)

2.2 Sampling and pre-treatment

2.2.1 Lista

Periwinkles from the stations in Husebybukta (Haugestranda and Tjuvholmen) were sampled by hand during low tide. Periwinkles from the station in Haugestranda were sampled from shore. A boat was used to access the station on Tjuvholmen, where periwinkles were sampled from the southern side of the outer island. The sampling station in the reference site Litlerauna is located on the south-western shore of the land-tied island. Periwinkles were sampled by hand from the shore during low tide.

Temperatures and tidal measurements from the individual samplings are given in Appendix B.

Sampled periwinkles from the three stations were put into separate glass bottles containing filtered seawater. The glass bottles were put in a cold box for transportation. After the sampling, the periwinkles were transported to the UiS. The periwinkles were put in three separate plastic boxes under identical conditions. The boxes contained filtered seawater under constant aeration at a constant temperature of about 4 ºC. The specimens were kept overnight and the sampling was performed the next morning.

Table 2.2 Overview of sampling of *L. littorea* from the stations in Lista, Vest-Agder.

Station Code	Name	Sampling dates		
		Sampling 1	Sampling 2	Sampling 3
Alcoa 1	Haugestranda	02.02.17	09.03.17	10.04.17
Alcoa 2	Tjuvholmen	09.02.17	09.03.17	10.04.17
Alcoa Reference	Litlerauna	02.02.17	09.03.17	10.04.17

2.2.2 Viste, Rogaland

Sampling of periwinkles from the reference site were done during low tide. About 50 specimens were sampled by hand and immediately transferred to the laboratory in UiS where they were put in a plastic box containing clean seawater under constant aeration at a constant temperature of about 4 ºC. The specimens were purged overnight and the assays were performed the next morning.

Table 2.3 Overview of sampling of *L. littorea* from the station in Viste, Rogaland.

Station Code	Name	Sampling dates		
		Sampling 1	Sampling 2	Sampling 3
Viste	Visteviga	24.01.17	01.03.17	05.04.17

2.3 Biological Assays

All raw data of morphological measurements and biological test results are given in Appendix A.

2.3.1 Sample Preparation

2.3.1.1 Haemolymph Extraction

L. littorea haemolymph constitute the sampling matrix in the NRRT and MN assays. About 200 µL of haemolymph were extracted from the specimens by insertion of hypodermic syringes (21-gauge hypodermic needle) behind the ocular tentacles. Samples were then put in eppendorf tubes and kept on ice until the NRRT assay was performed (less than 20 min). Thirty µL of haemolymph were used in the NRRT assay. The remaining haemolymph was used in the MN assay.

2.3.1.2 Soft Tissue Preparation

Snails were dissected using a scalpel to extract pure soft tissue samples of the individual snails. Samples were put in eppendorf tubes and snap-frozen using liquid nitrogen before being stored in a freezer at -80 °C. These soft tissue samples were used for the evaluation of MT in the periwinkles.

2.3.1.3 Neutral Red Retention Time Assay

The amphiphilic and weak cationic neutral red dye was prepared for the NRRT assay by making 1) Neutral Red Stock Solution and 2) Neutral Red Working Solution:

1) Neutral Red Stock Solution. The stock solution was prepared by dissolving 20 mg of dye powder in 1 ml of Dimethyl sulfoxide (DMSO). This stock solution can be stored in a fridge in a light proof bottle for up to 3 weeks, and for a longer period if stored in a freezer.
2) Neutral Red Working Solution. The working solution was prepared by adding 5 µL of the previously prepared Neutral Red Stock Solution to 995 µL of filtered sea water. A fresh Working Solution was made on the day of use and was stored in a light proof bottle or vial.

The NRRT assay was performed using a slightly modified version of the *in vivo* cytochemical method as described by Lowe *et al*. (1992).

Thirty µL of haemolymph sample were smeared on poly-L-Lysine-coated microscope slides and incubated in a light proof humidity chamber for 10 minutes to allow the cells to attach to the slide. Thrity µL of the neutral red working solution were then added to the samples and left in the light proof humidity chamber for 10 minutes to allow for the neutral red solution to penetrate the lysosomal membrane into the lysosome matrix by protonation. After some time, depending on the state of the lysosomes, the dye will either start leaking through the membrane into the cytosol, or the lysosomes will change their normal shape. These lysosomal alterations were monitored at five minute intervals using light microscope at x40 objective. The condition of the lysosomes in each sample was observed for approximately 1 minute. The endpoint in a sample was stated when at least 50 percent of the lysosomes showed either leakages or abnormalities. The NRRT corresponded to the last time of reading before reaching the endpoint.

2.3.2 Morphological Measurements and Condition Index

Morphological measurements were taken of the different shell dimensions of all individuals. These measurements included total weight (soft tissue + shell), drained weight of whole soft tissue, height of shell (HS), width of shell (WS), height of shell top (HT), height of aperture (HA), and width of aperture (WA). Shell measurement methods are shown in Figure 2.8. Shells were carefully opened without inflicting any damage on the soft tissue by the use of a hammer. Soft tissues were gently washed with deionized water and

dried on a paper towel before being weighed. The dry/wet weight ratio (dw/ww) of the soft tissue could not be calculated, as the tissues were to be used in subsequent MT analysis.

Condition Index (CI) was calculated according to Amiard *et al.* (2004):

$$\mathrm{CI} = 100\times \frac{\text{Drained weight of soft tissues}}{\text{Total weight}}$$

This measurement of CI is recommended by the French Association for Standardization (AFNOR, NF V45-056, Sept. 1985).

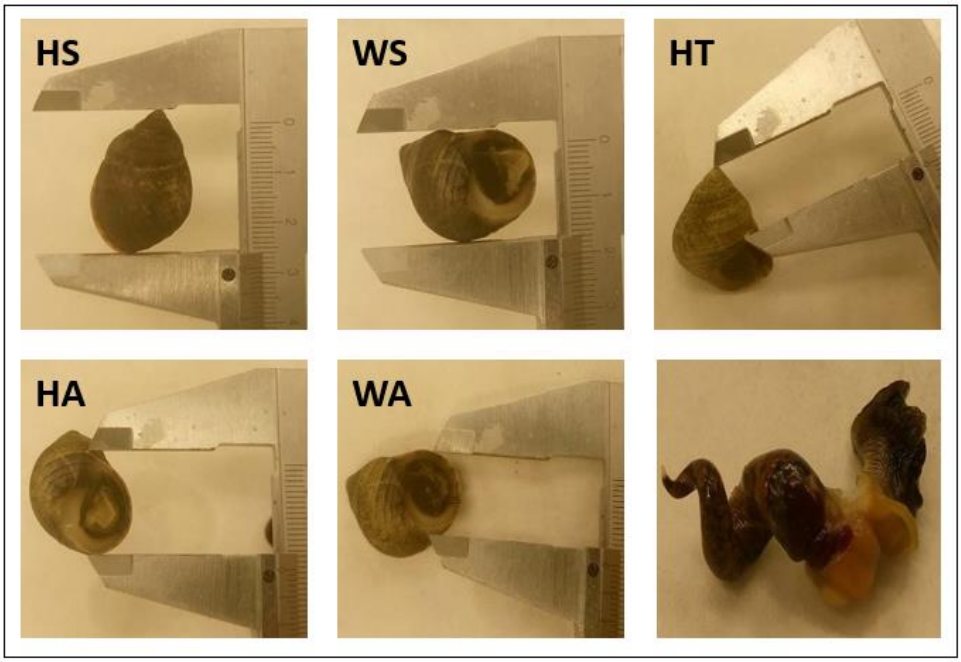


Figure 2.8 Morphological shell measurements. HS: height of shell; WS: width of shell; HT: height of shell top; HA: height of aperture; WA: width of aperture. Whole soft tissue shown in bottom right picture.

2.3.3 Micronucleus Assay

The MN assay was performed using a modified version of the protocol developed by UNEP/RAMOGE (1999).

Haemolymph from *L. littorea* was smeared on microscope slides and left to dry at room temperature. Haemocytes were fixed by dipping the slides in Carnoy's solution (methanol:acetic acid, 3:1) for 20 minutes at room temperature. The slides were left to air dry at room temperature and stored in a microscope slide box afterwards. The slides were later stained with a 3 % (v/v) Giemsa solution for 10 min and rinsed twice in tap water. Cover slips were then glued to the slides using DPX Mounting Media.

Between 1000 and 2000 haemocytes were scored under light microscope observation (x100 magnification) for each sample. Observation criteria of MN evaluation were adapted and slightly modified after Schmid (1975):

- MN shape are oval or round;
- MN diameter is less than one-third of the main nucleus;
- MN staining intensity is the same as the main nucleus;
- Only intact MN clearly separated from the main nucleus is counted;
- MN is in the same optical plane as the main nucleus.

In this case, the inclusion of the cytokinesis-block micronucleus (CBMN) technique is not necessary to obtain a precise biomarker result. Since this is a passive biomonitoring of chronic pollution exposure, all observed MN can be regarded as effects of the genotoxic insult from the waste water effluent pollutants (Fenech, 2000).

2.3.4 Metallothionein Assay

The MT assay was performed using a minor modification of the spectrophotometric method introduced by Viarengo *et al*. (1997). In this assay, MT concentration is evaluated utilizing a partially purified metalloprotein fraction obtained by acidic ethanol/chloroform fractionation of the tissue homogenate. Precautions are taken to ensure complete MT precipitation and to avoid the oxidation of sulfhydryl groups (SH), the contamination by soluble low molecular weight thiols, and enzymatic protein degradation which can occur during sample preparation. In the extracts, the concentration of MT, denatured by low pH and high ionic strength, is quantified spectrophotometrically utilizing the Ellman's SH reagent. The spectrophotometric method is a simple, repeatable, and low-cost method of detecting minimal concentrations (nmol) of MT in biological samples. It is widely suggested as a tool for MT quantification in biomonitoring programs.

Metallothionein sample preparation

For each MT sample, dissected gills and digestive glands of 3-5 individuals were homogenized in three volumes of 0.5 M sucrose, 20 mM Tris-HCl buffer, pH 8.6, with added 0.006 mM leupeptine, 0.5 mM PMSF as antiproteolytic agents and 0.01 % β-mercaptoethanol as a reducing agent. The homogenate was centrifuged at 20.000 x G for 20 min at 4 ºC in 15 ml Falcon tubes to obtain a supernatant containing MTs. One ml of the supernatant was extracted by pipette and added to 1 ml of cold (-20 ºC) absolute ethanol and 80 µl chloroform in a new 15 ml Falcon tube and vortexed for a few seconds. The sample was then centrifuged at 6000 x G for 10 min at 4 ºC. The resulting supernatant was extracted and added to 3 volumes of cold (-20 ºC) absolute ethanol and 40 µl 37 % HCl in a new 15 ml Falcon tube. The sample was stored

at -20 ºC for 1 hour and then split into three eppendorf tubes before being re-centrifuged at 6000 x G for 10 min at 4 ºC. The supernatant was removed and the pellets were run in a speed vacuum at 30 ºC for 10 min to remove moisture.

Spectrophotometric assay (Ellman's reaction)

The three pellets were each resuspended in 50 µl 0.25 M NaCl and subsequently gathered together in a single 15 ml Falcon tube. 150 µl 1 M HCl containing 4 mM EDTA was added to the sample. A volume of 4.2 ml 0.2 M Na-phosphate buffer containing 2 M NaCl and 0.43 mM DTNB (5,5-dithiobis-2-nitrobenzoic acid) at pH 8 was then added to the sample. One ml of the sample was evaluated in a spectrophotometer at 412 nm. MT concentration was estimated using reduced glutathione (GSH) as a reference standard.

GSH reference standard preparations

A standard curve was plotted by evaluating the absorbance of GSH reference concentrations of 15 µM, 30 µM, 60 µM, and 90 µM at 412 nm. One mole GSH yields 1 mole of sulfhydryl (-SH) groups.

Metallothionein concentration calculation

Obtained absorbance values from MT samples were interpolated on the GSH reference curve. The corresponding values found on the X-axis represented the molar concentrations of SH groups belonging to MT present in the samples. Considering the size and residue characteristics of Ll-MT, as well as the dilution factor in the homogenizing of tissues, the concentration of Ll-MT (ng g^{-1}) can be calculated:

$$[Ll-MT]\,[\frac{ng}{g}] = \frac{Interpolated\ value\ (\frac{nmol}{g})}{27\ cys\ residues} * \ 10.000Da * \ 4{,}5 * 4$$

2.4 Statistical Analysis

Results were analysed using the statistical packages Minitab and SPSS. Data for each biomarker were analysed for comparisons of each sampling site and the sampling time using one-way ANOVA, where variances were homogeneous or by the Scheffé F-test using SPSS (Version 21 for Windows).

The inter-site and temporal variations of the measured variables (NRRT, MN, MT, and CI) were analysed using one-way ANOVA. The critical differences between the sites were assessed by the Scheffé F-test using SPSS (Version 21 for Windows). Differences at the $p \leq 0.05$ level were considered significant.

Multivariate statistical analysis was performed to summarize the pattern of variation. Principal Component Analysis (PCA) was performed to determine whether the stations were distinguishable based on the toxicological test data.

3 Results & Discussion

All raw data from biological marker analyses and morphological measurements are provided in Appendix A.

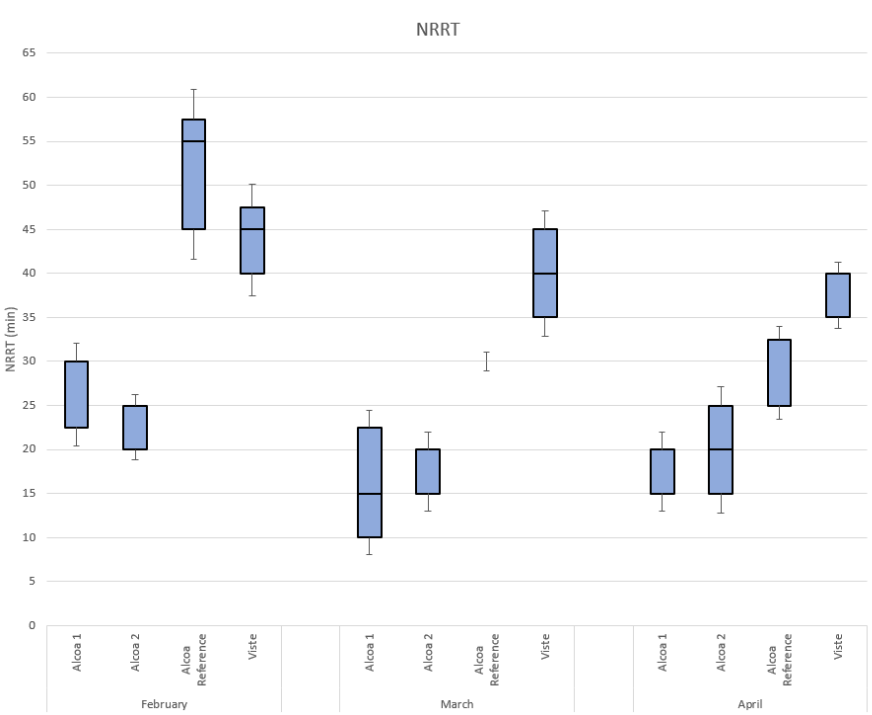
3.1 Neutral Red Retention Time assay

NRRT assay results are summarised in Figure 3.1 and Figure 3.2. Median values in the reference stations (Alcoa Reference and Viste) ranged from 25 min (Alcoa Reference, April) to 55 min (Alcoa Reference, February), with a mean value of 40 min. Organisms collected at the reference stations had similar values in February and March. A significant difference was observed in April. This could be due to the organisms in the reference station in Lista undergoing larger physiological changes over the period compared to the organisms from Viste, a feature that is highly influential in the NRRT assay. The median values in snails collected at the exposed stations Alcoa 1 and Alcoa 2 ranged from a minimum of 15 min (Alcoa 1, March) to a maximum of 30 min (Alcoa 1, February). In general, there were no significant differences between values recorded in organisms collected in the vicinity of the waste water effluent release point. Significantly lower NRRT values were recorded in snails sampled at Alcoa 1 and Alcoa 2 compared to the ones collected at the reference site in all the 3 sampling times.

Significant seasonal differences were observed in snails from Alcoa 1 (February/March: $p \leq 0.001$; February/April: $p \leq 0.01$) (Figure 3.2). A significant difference was also observed between organisms collected at Alcoa 2 in February and in March. The highest seasonal variation was observed in snails from Alcoa Reference, where values were significant different ($p \leq 0.001$) between all samplings, except for March/April.

Considering the NRRT assay results as an indicator of general physiological health conditions, *L. littorea* from the stations exposed to the waste water effluent from Alcoa Lista (Alcoa 1 and Alcoa 2) showed poor health conditions in all samplings (median NRRT < 30 min). Snails from the reference station in Lista (Alcoa Reference) presented significantly better health conditions compared to the exposed stations. The significant differences indicate that organisms close to the Alcoa Lista plant are subjected to a general environmental stress, possibly due to the waste water discharge from Alcoa Lista.

L. littorea haemolymph lysosomes showed declining integrity in all 3 stations in Lista (Alcoa 1, 2 and Reference). Declining lysosomal integrity has been observed in molluscs during breeding and spawning season in spring when both males and females undergo significant physiological changes that are highly energy consuming (Petrovic *et al.*, 2004). Significant differences were not observed between the March and April samplings in any stations, indicating that the *L. littorea* population start undergoing significant changes in lysosomal integrity and physiological parameters between February and March.



February		Reference		Exposed	
		V	A Ref	A2	A1
Exposed	A1	***	***	n.s.	
	A2	***	***		
Reference	A Ref	n.s.			
	V				

March		Reference		Exposed	
		V	A Ref	A2	A1
Exposed	A1	***	***	n.s.	
	A2	***	**		
Reference	A Ref	n.s.			
	V				

April		Reference		Exposed	
		V	A Ref	A2	A1
Exposed	A1	***	***	n.s.	
	A2	***	*		
Reference	A Ref	**			
	V				

Total		Reference		Exposed	
		V	A Ref	A2	A1
Exposed	A1	***	***	n.s.	
	A2	***	***		
Reference	A Ref	n.s.			
	V				

Figure 3.1 Box and whisker diagram of NRRT results. Boxes indicate 95 % of values; horizontal lines in boxes indicate median values; whiskers are standard error bars. A1: Alcoa 1; A2: Alcoa 2; A Ref: Alcoa Reference; V: Viste. 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.

NRRT Alcoa 1			
	S3	S2	S1
S1	**	***	
S2	n.s.		
S3			

NRRT Alcoa 2			
	S3	S2	S1
S1	n.s.	*	
S2	n.s.		
S3			

NRRT Alcoa Reference			
	S3	S2	S1
S1	***	***	
S2	n.s.		
S3			

NRRT Viste			
	S3	S2	S1
S1	n.s.	n.s.	
S2	n.s.		
S3			

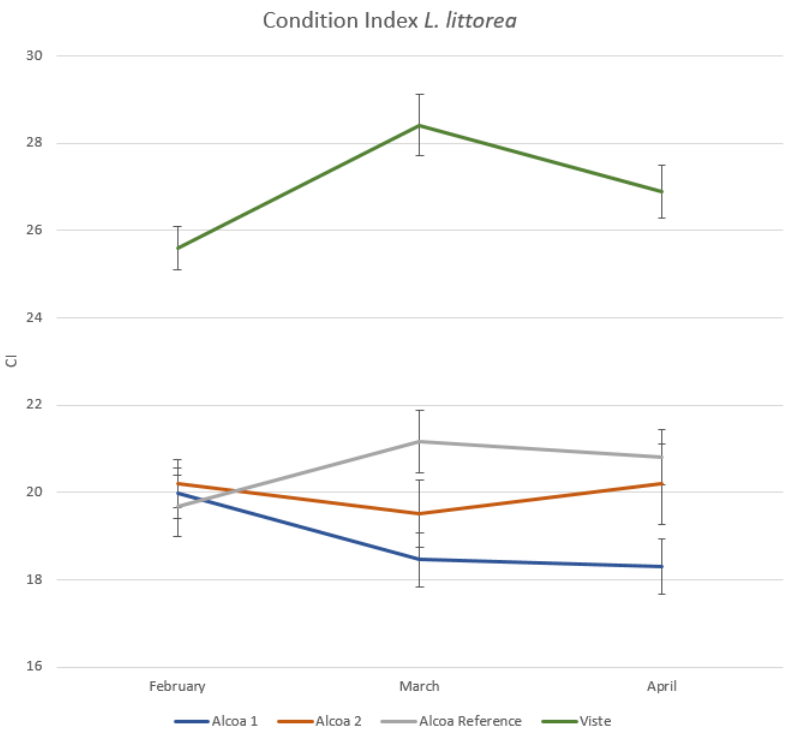
Figure 3.2 p-values for temporal variations in NRRT values in *L. littorea* from the different sampling stations calculated using the post hoc Scheffé test. S1: Sampling 1 (February); S2: Sampling 2 (March); S3: Sampling 3 (April). ***. $p \leq 0.001$; **. $p \leq 0.01$; * $p \leq 0.05$. n.s. not significant

3.2 Condition Index

CI results are summarised in Figure 3.3 and Figure 3.4. Mean CI values in *L. littorea* collected from the sampling stations in Lista ranged from 18 to 21. The lowest values were generally observed in organisms sampled from the two exposed stations Alcoa 1 and Alcoa 2, with organisms from Alcoa 1 showing the lowest ones. The highest values in Lista were observed in organisms sampled from Alcoa Reference. Values recorded in organisms sampled at Lista were not significantly different. Mean values in organisms collected from Viste ranged from 26 to 28. Significantly lower CI values were recorded in snails sampled from all the stations in Lista compared to the ones collected in Viste in all the 3 sampling times.

The stations in Lista showed limited seasonal variation. However, slightly increasing values were observed over the sampling periods in snails sampled from the reference station, in contrast to the slightly decreased values observed in snails over time in the exposed stations. Significant differences were observed between the February and March samplings in the sampling station in Viste.

Considering the CI as an indicator of an individual's general physiological health and energy reserves (Stevenson & Woods, 2006), the significant differences in CI values recorded between organisms sampled from Viste and the stations in Lista indicate that the organisms from Viste probably have better environmental conditions for growth, energy storage, and reproduction in comparison to snails from Lista. The best health conditions in Lista were observed in the Alcoa Reference station. However, all stations in Lista show similar health conditions with no significant differences. This indicates that exposure to the waste water effluent from Alcoa Lista does not have any significant negative influences on the conditions needed for growth and reproduction in *L. littorea*. No significant seasonal differences were observed in any of the stations in Lista.



February		Reference		Exposed	
		V	A Ref	A2	A1
Exposed	A1	***	n.s.	n.s.	
	A2	***	n.s.		
Reference	A Ref	***			
	V				

March		Reference		Exposed	
		V	A Ref	A2	A1
Exposed	A1	***	n.s.	n.s.	
	A2	***	n.s.		
Reference	A Ref	***			
	V				

April		Reference		Exposed	
		V	A Ref	A2	A1
Exposed	A1	***	n.s.	n.s.	
	A2	***	n.s.		
Reference	A Ref	***			
	V				

Total		Reference		Exposed	
		V	A Ref	A2	A1
Exposed	A1	***	n.s.	n.s.	
	A2	***	n.s.		
Reference	A Ref	***			
	V				

Figure 3.3 Mean CI values with standard error bars. P-values for all three samplings given in tables. A1: Alcoa 1; A2: Alcoa 2; AR: Alcoa Reference; V: Viste. 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.

CI Alcoa 1			
	S3	S2	S1
S1	n.s.	n.s.	
S2	n.s.		
S3			

CI Alcoa 2			
	S3	S2	S1
S1	n.s.	n.s.	
S2	n.s.		
S3			

CI Alcoa Reference			
	S3	S2	S1
S1	n.s.	n.s.	
S2	n.s.		
S3			

CI Viste			
	S3	S2	S1
S1	n.s.	*	
S2	n.s.		
S3			

Figure 3.4 p-values for temporal variations in CI values in *L. littorea* from the different sampling stations calculated using the post hoc Scheffé test. S1: Sampling 1 (February); S2: Sampling 2 (March); S3: Sampling 3 (April). ***. $p \leq 0.001$; **. $p \leq 0.01$; * $p \leq 0.05$. n.s. not significant

3.3 Morphological Measurements

Morphological measurements results are reported in Figure 3.5. The figure shows that organisms from the different sites were of comparable sizes. Measurement methods are described in Figure 2.8.

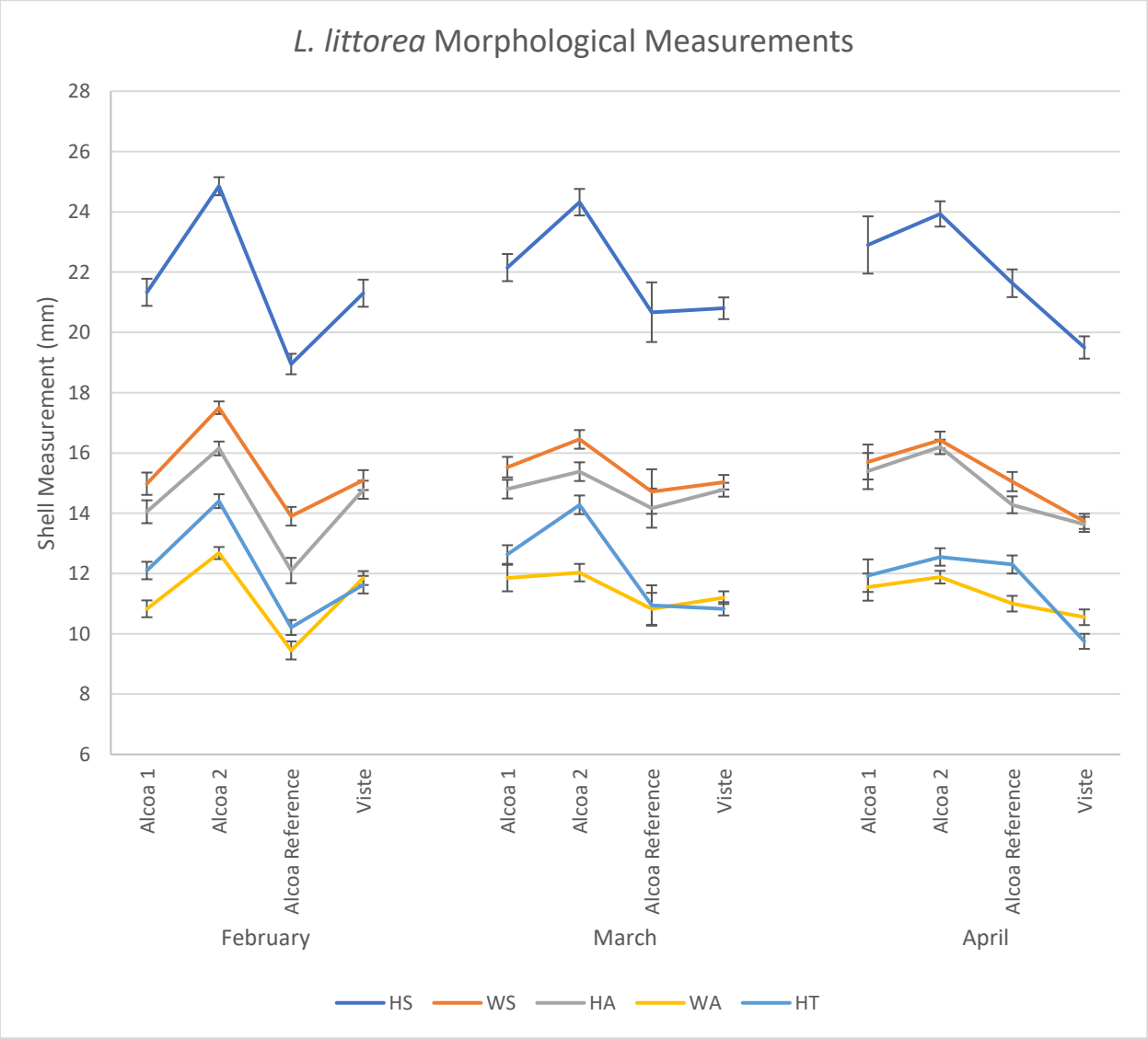


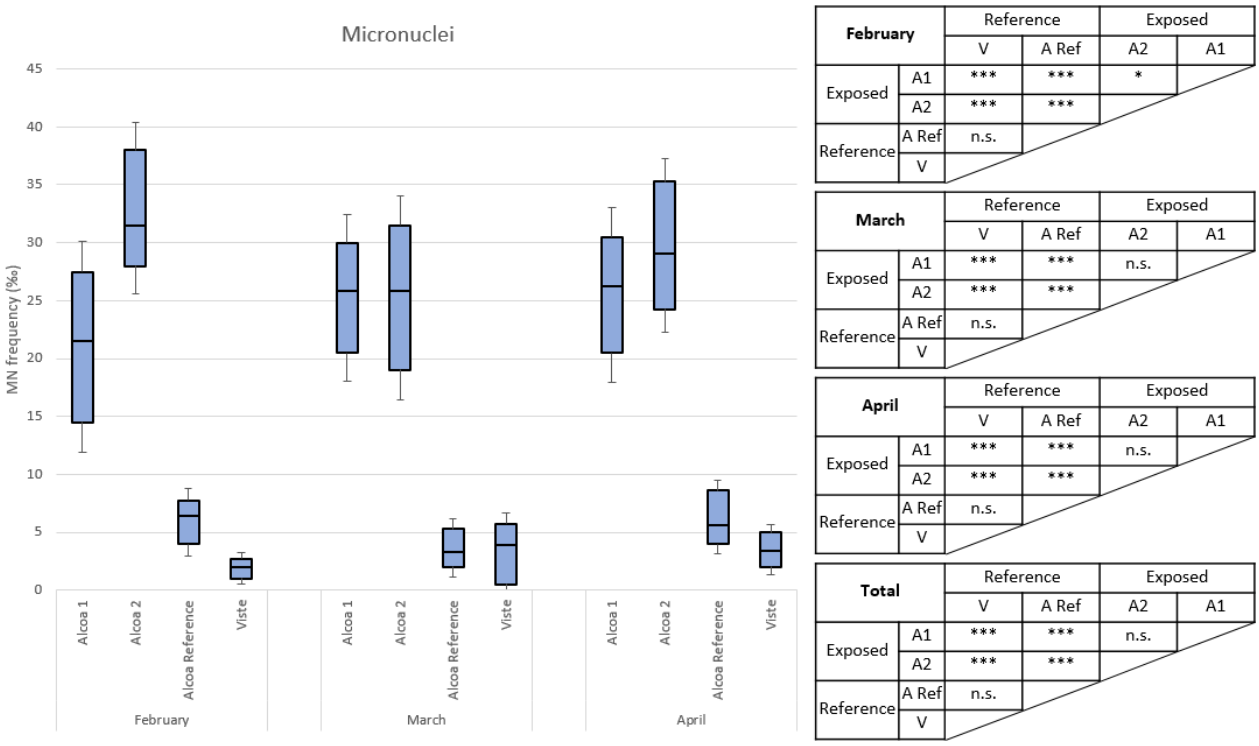
Figure 3.5 Morphological shell measurements of *L. littorea*. HS: Shell Height; WS: Shell Width; HA: Aperture Height; WA: Aperture Width; HT: Top Height.

3.4 The Micronucleus Assay

MN assay results are summarised in Figure 3.6 and Figure 3.7, and pictures of some of the various observed nuclear abnormalities are presented in Figure 3.8. Mean MN frequencies in the reference stations (Alcoa Reference and Viste) ranged from 2 ‰ (Viste, February) to 6,5 ‰ (Alcoa Reference, February). In general, there were no significant differences between values recorded in organisms collected from the reference sites. The mean values in snail collected at the exposed stations Alcoa 1 and Alcoa 2 ranged from a minimum 21 ‰ (Alcoa 1, February) to a maximum 31 ‰ (Alcoa 2, February). Organisms collected at the exposed stations had similar values in March and April. A significant difference was observed in February. Significantly higher MN values were recorded in snails sampled at the exposed sites Alcoa 1 and Alcoa 2 compared to the ones collected at the reference site in all the 3 sampling times. Seasonal differences were not considered significant in any stations.

Considering the MN assay results as an indicator of genotoxic effects, *L. littorea* from the stations exposed to the waste water effluent from Alcoa Lista (Alcoa 1 and Alcoa 2) showed signs of high genotoxic effects in all samplings. Snails from the reference station in Lista (Alcoa Reference) presented significantly lower signs of genotoxic effects compared to the exposed stations. The significant differences indicate that organisms close to the Alcoa Lista plant are subjected to a significant genotoxic stress, possibly originating from exposure to the waste water discharge from Alcoa Lista.

Previously conducted chemical analysis have concluded that *L. littorea* exposed to the waste water discharge have accumulated toxic amounts of genotoxic compounds, such as BaP, Cd, and Pb, while *L. littorea* from the reference station in Litlerauna have shown little or no accumulation of genotoxic compounds (Kroglund, 2014, 2016).



February		Reference		Exposed	
		V	A Ref	A2	A1
Exposed	A1	***	***	*	
	A2	***	***		
Reference	A Ref	n.s.			
	V				

March		Reference		Exposed	
		V	A Ref	A2	A1
Exposed	A1	***	***	n.s.	
	A2	***	***		
Reference	A Ref	n.s.			
	V				

April		Reference		Exposed	
		V	A Ref	A2	A1
Exposed	A1	***	***	n.s.	
	A2	***	***		
Reference	A Ref	n.s.			
	V				

Total		Reference		Exposed	
		V	A Ref	A2	A1
Exposed	A1	***	***	n.s.	
	A2	***	***		
Reference	A Ref	n.s.			
	V				

Figure 3.6 Box and whisker diagram of MN results. Boxes indicate 95 % of values; horizontal lines in boxes indicate mean values; whiskers are standard error bars. A1: Alcoa 1; A2: Alcoa 2; A Ref: Alcoa Reference; V: Viste. 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.

MN Alcoa 1			
	S3	S2	S1
S1	n.s.	n.s.	
S2	n.s.		
S3			

MN Alcoa 2			
	S3	S2	S1
S1	n.s.	n.s.	
S2	n.s.		
S3			

MN Alcoa Reference			
	S3	S2	S1
S1	n.s.	n.s.	
S2	n.s.		
S3			

MN Viste			
	S3	S2	S1
S1	n.s.	n.s.	
S2	n.s.		
S3			

Figure 3.7 p-values for temporal variations in mean MN frequency in *L. littorea* from the different sampling stations calculated using the post hoc Scheffé test. S1: Sampling 1 (February); S2: Sampling 2 (March); S3: Sampling 3 (April). ***. $p \leq 0.001$; **. $p \leq 0.01$; * $p \leq 0.05$. n.s. not significant

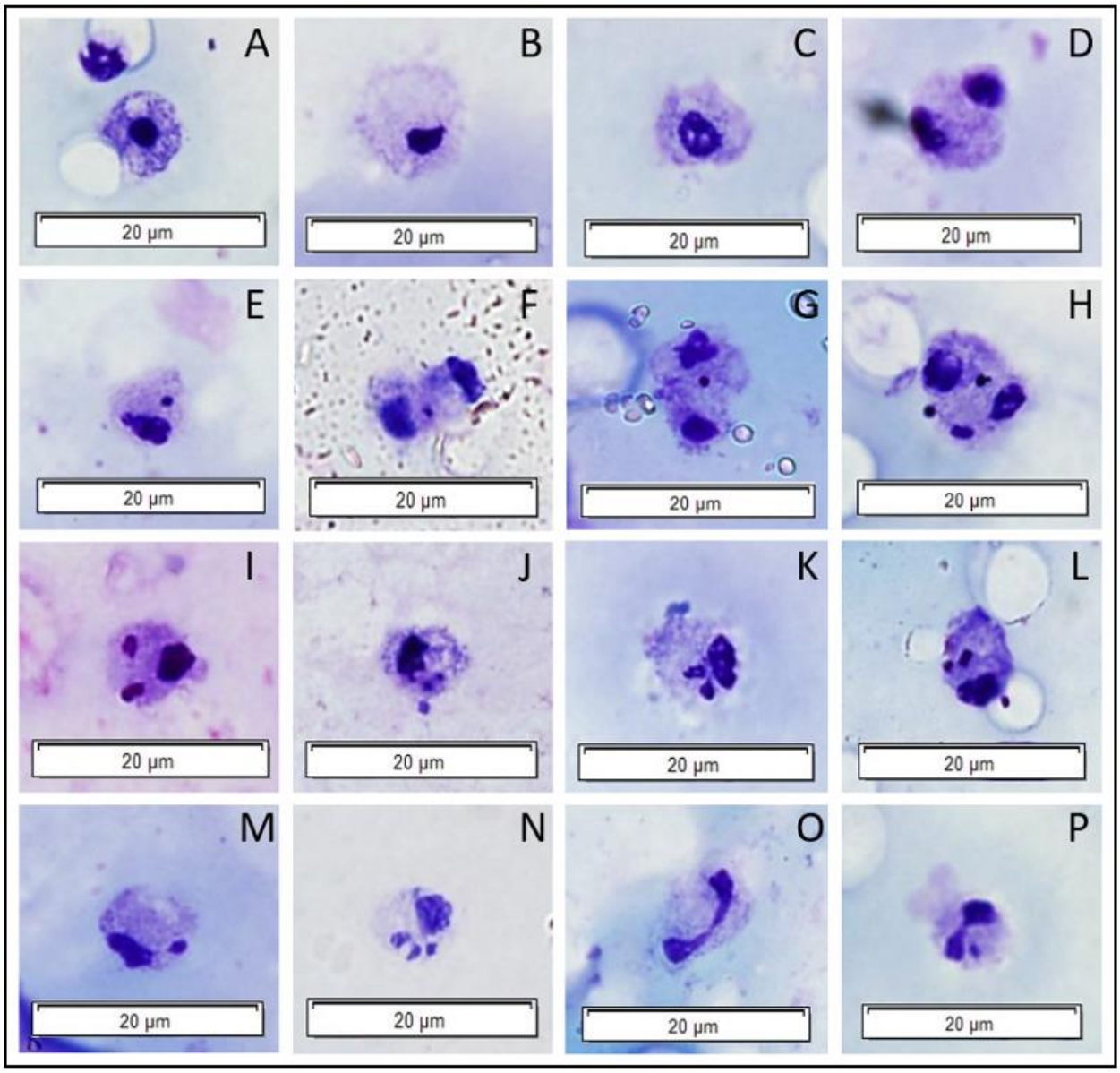


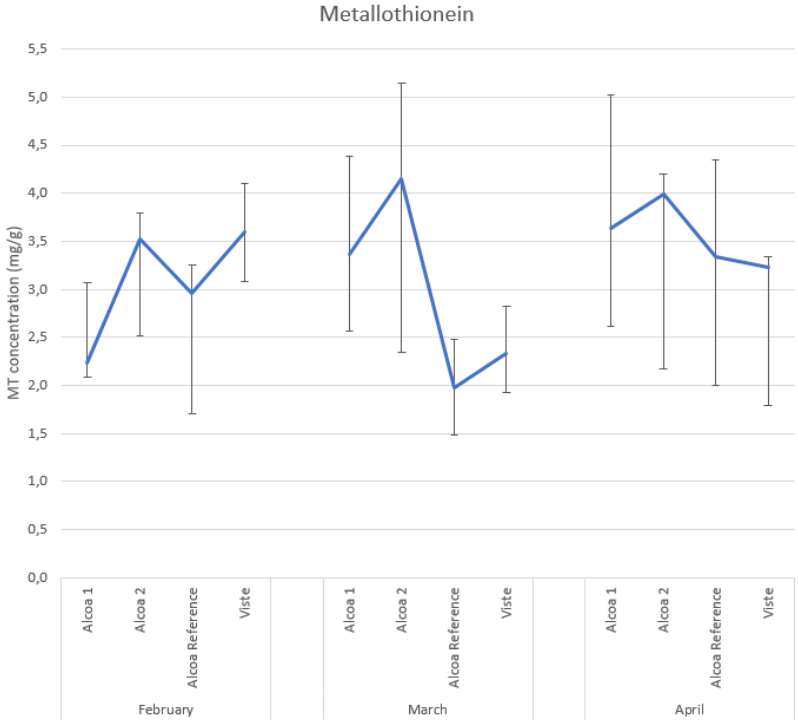
Figure 3.8 Micronuclei (MN) and nuclear abnormalities observed in the micronucleus assay. A-D: Normal nucleus; E-H: Formation of a single MN; I-L: Formation of two MN; M-P: Nuclear abnormalities (M: MN with nucleoplasmic bridge (NPB) connection to main nucleus (nuclear bud); N: Formation of three or more MN; O: Binucleated cell with a NPB connection between the two nuclei; P: Binucleated cell with NPB connection and formation of a single MN)

3.5 The Metallothionein assay

MT assay results are summarised in Figure 3.9 and Figure 3.10. Mean MT values in snails collected at the reference stations (Alcoa Reference and Viste) ranged from a minimum of 2 mg g^{-1} (Alcoa Reference, March) to a maximum of 3,6 mg g^{-1} (Viste, February). The organisms from the two reference stations did not exhibit significant differences. The mean values in snails collected at the exposed stations Alcoa 1 and Alcoa 2 ranged from a minimum of 2,24 mg g^{-1} (Alcoa 1, February) to a maximum of 4,15 mg g^{-1} (Alcoa 2,

March). There were no significant statistical differences between values recorded in organisms collected from any of the four sampling stations. Significant seasonal variance was not observed in any of the sampling stations (Figure 3.10).

Regarding MT concentrations as an indicator of heavy metal exposure, *L. littorea* collected from the sampling stations in Lista and Viste did not show any significant differences in MT tissue concentrations for any of the samplings. This indicates that the organisms from the exposed and reference stations are subjected to similar degrees of heavy metal exposure with no significant variations from February to April. The waste water discharge from Alcoa Lista does not seem to significantly influence the heavy metal uptake in the organisms within short distance of the discharge release point. The heavy metal concentrations in the discharge is relatively low, and chemical analysis previously performed in the area have concluded that neither the organisms from the direct exposure zone (~3 km of release point) or from the reference station (~10 km west of release point) had accumulated toxic amounts of heavy metals (Kroglund, 2016).



February		Reference		Exposed	
		V	A Ref	A2	A1
Exposed	A1	n.s.	n.s.	n.s.	
	A2	n.s.	n.s.		
Reference	A Ref	n.s.			
	V				

March		Reference		Exposed	
		V	A Ref	A2	A1
Exposed	A1	n.s.	n.s.	n.s.	
	A2	n.s.	n.s.		
Reference	A Ref	n.s.			
	V				

April		Reference		Exposed	
		V	A Ref	A2	A1
Exposed	A1	n.s.	n.s.	n.s.	
	A2	n.s.	n.s.		
Reference	A Ref	n.s.			
	V				

Total		Reference		Exposed	
		V	A Ref	A2	A1
Exposed	A1	n.s.	n.s.	n.s.	
	A2	n.s.	n.s.		
Reference	A Ref	n.s.			
	V				

Figure 3.9 Mean MT results with error bars. A1: Alcoa 1; A2: Alcoa 2; A Ref: Alcoa Reference; V: Viste. 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

MT Alcoa 1			
	S3	S2	S1
S1	n.s.	n.s.	
S2	n.s.		
S3			

MT Alcoa 2			
	S3	S2	S1
S1	n.s.	n.s.	
S2	n.s.		
S3			

MT Alcoa Reference			
	S3	S2	S1
S1	n.s.	n.s.	
S2	n.s.		
S3			

MT Viste			
	S3	S2	S1
S1	n.s.	n.s.	
S2	n.s.		
S3			

Figure 3.10 p-values for temporal variations in mean MT concentrations in *L. littorea* from the different sampling stations calculated using the post hoc Scheffé test. S1: Sampling 1 (February); S2: Sampling 2 (March); S3: Sampling 3 (April). ***. $p \leq 0.001$; **. $p \leq 0.01$; * $p \leq 0.05$. n.s. not significant

3.6 Correlations between the four variables MN, MT, NRRT & CI

Correlation coefficients are shown in Figure 3.11. For MN frequency, significant negative correlations ($p<0.001$) were observed with NRRT and CI, indicating that *L. littorea* individuals with higher MN frequencies generally also have lower CI and NRRT values.

NRRT values showed a significant positive correlation with CI values ($p<0.001$), indicating that organisms with higher NRRT values generally have higher CI values. The NRRT and CI parameters are both parameters indicative of general physiological health, where higher values indicate better health conditions for both parameters.

MT concentrations did not correlate with MN frequencies or NRRT. However, a significant negative correlation ($p<0.05$) was observed between MT and CI, indicating that individuals with higher MT tissue concentrations will have lower CI values.

Total		CI	NRRT	MT	MN
MN	Correlation Coefficient	-0,484***	-0,638***	0,134	
	Sig.	,000	,000	,375	
	n	165	163	46	
MT	Correlation Coefficient	-0,298*	-0,206		
	Sig.	,045	,169		
	n	46	46		
NRRT	Correlation Coefficient	0,496***			
	Sig.	,000			
	n	173			
CI	Correlation Coefficient				
	Sig.				
	n				

***. Correlation is significant at the 0.001 level.

**. Correlation is significant at the 0.01 level.

*. Correlation is significant at the 0.05 level.

Figure 3.11 Spearman rank order correlation between the four biological markers. Sig.: p-value; n: number of samples.

3.7 Multivariate Analysis – Principal Component Analysis

PCA plots were derived from the different samplings and summarised in Figure 3.12 and Figure 3.13. The PCA plot represents how the sites can be separated graphically based on raw data from biomarker analyses. The data from the two exposed stations in Lista (Alcoa 1 and Alcoa 2) are clustered together on the left-hand side of the diagrams, while the data from the reference sites in Litlerauna (Alcoa Reference) and Viste are located on the right-hand side. The Viste population is clearly distinguishable from both the exposed and the reference populations in Lista.

The overall biomarker data was capable of separating the individual organisms according to their respective sampling stations. Distinguished groups were made in the plots, clearly separating the reference stations from the exposed stations. This shows that the battery of biomarkers selected was capable of showing differences between sites, probably due to the differences in contaminant levels.

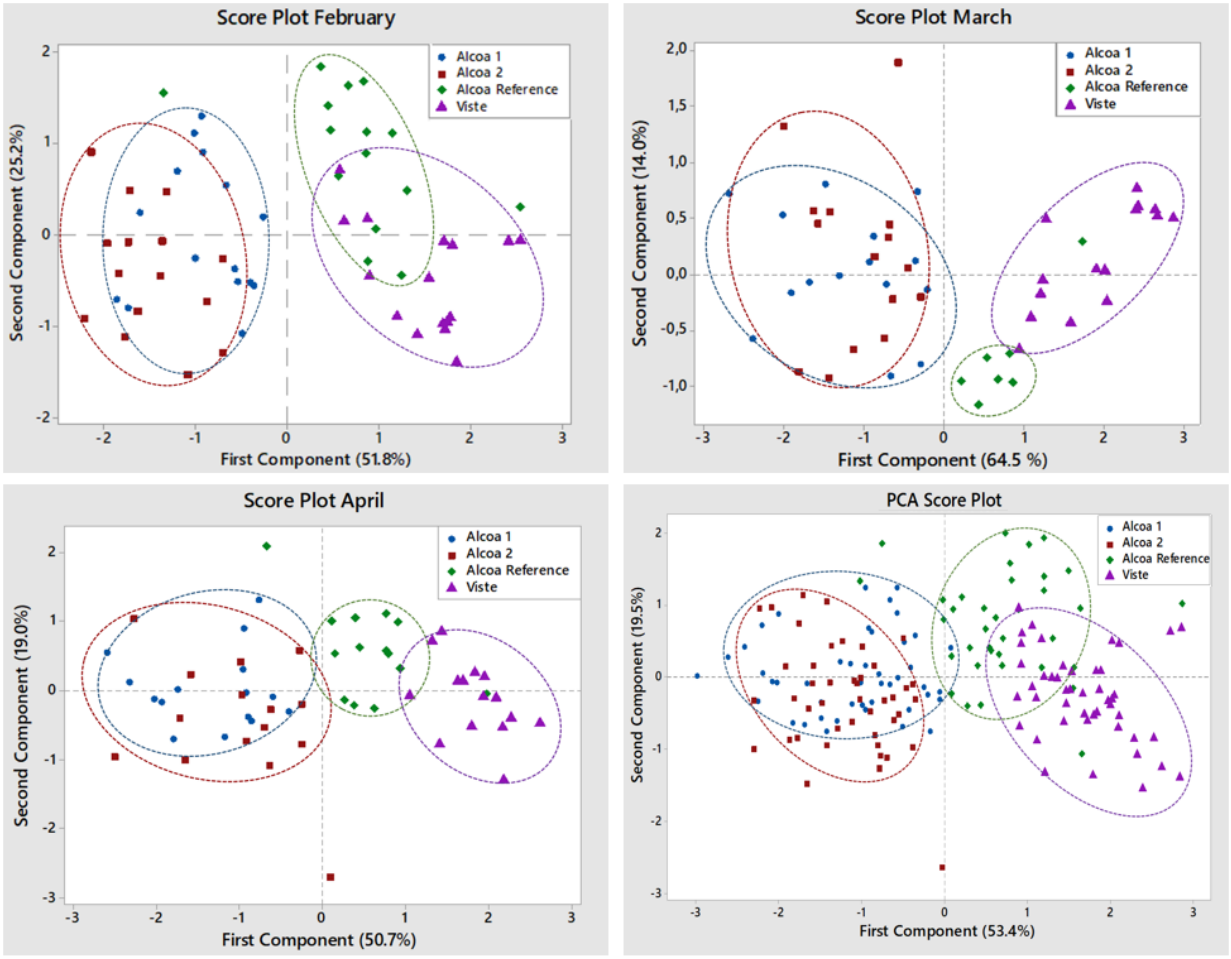


Figure 3.12 PCA plots of the monthly and total samplings. Total variances explained in the PCA: February: 77%; March: 78,5%; April: 69,7%; Total (PCA Score Plot): 72,9%

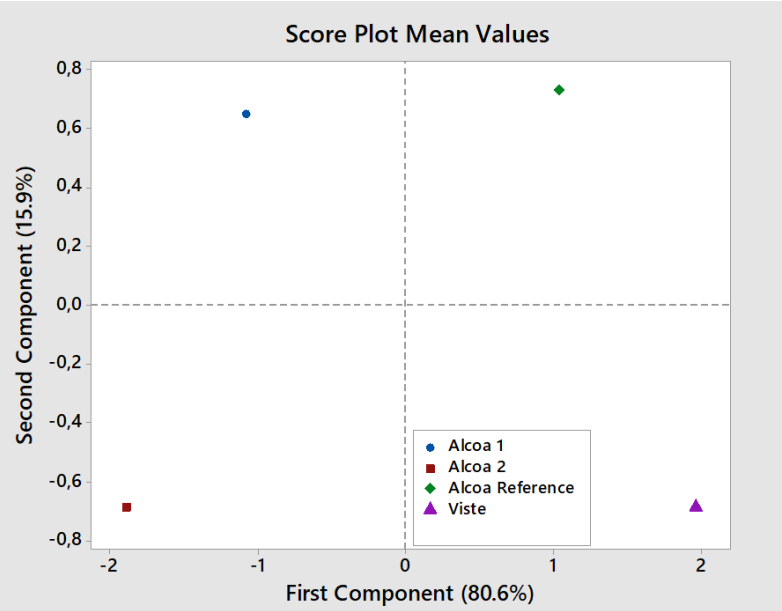


Figure 3.13 PCA score plot of the mean values of the raw biomarker data. Total variance explained in the PCA: 96.5 %

4 Conclusion

The overall data from the battery of biomarkers selected for this study was capable of showing differences between sites, probably due to the differences in contaminant levels. The PCA was able to differentiate the exposed organisms from the reference organisms.

The NRRT assay results indicated that organisms collected from stations in the vicinity of the waste water discharge from Alcoa Lista had significantly lower general health conditions in comparison to organisms collected from the reference stations, indicating that the exposed organisms are subjected to a general environmental stress, possibly due to the discharge exposure.

The CI values obtained from organisms did not reveal any significant differences in health conditions and energy reserves observed in exposed organisms compared to organisms collected from the reference stations in Lista. This indicates that exposure to the waste water discharge from Alcoa Lista does not have any significant negative influences on the conditions needed for growth and reproduction in *L. littorea*.

The MN assay results indicate that the exposed organisms exhibit significantly higher genotoxic effects compared to organisms collected from the reference stations, indicating that organisms close to the Alcoa Lista plant are subjected to a significant genotoxic stress, possibly originating from exposure to the waste water discharge from Alcoa Lista.

The MT assay results revealed no significant differences in MT concentrations in organisms from all sampling stations, indicating that the organisms from the exposed and reference stations are subjected to similar degrees of heavy metal exposure with no significant variations from February to April. The waste water discharge from Alcoa Lista does not seem to significantly influence the heavy metal uptake in the organisms within short distance of the discharge release point.

5 Future Prospects

The results from this study are subject to caution and any conclusions are hypothetical. Further research is necessary to investigate the overall biological conditions in organisms exposed to the waste water discharge from Alcoa Lista. Further studies should be performed over longer periods during different seasons and ideally not overlap with the spawning season of the sentinel organisms. More biotic and abiotic parameters should be included to get a more integrated picture of the biological effects observed in the organisms and to further assess the relation of observed biological effects to the waste water discharge.

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Appendix A – Raw data from samplings

Sampling 1 (February) - Viste

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)	MT (mg g-1)	MN (per 1000 nuclei)
81	Viste	24.jan	19,5	13,5	13,5	11	11,5	2,00	0,45	22,50	35	-	-
82	Viste	24.jan	20,5	15	12,5	12	11,5	2,54	0,58	22,83	40	-	2
83	Viste	24.jan	23	12,5	15,5	13	12,5	3,50	0,87	24,86	40	-	7
84	Viste	24.jan	21,5	15,5	15	12	12,5	2,57	0,72	28,02	35	-	0
85	Viste	24.jan	21	15	14,5	11,5	11,5	2,50	0,70	28,00	45	-	3
86	Viste	24.jan	20,5	14	13,5	11	12	2,03	0,53	26,11	45	2,710	1
87	Viste	24.jan	21,5	15,5	15	12	12,5	2,83	0,79	27,92	45		1
88	Viste	24.jan	20	14,5	13,5	12	11	2,19	0,59	26,94	35		2
89	Viste	24.jan	23,5	16	15,5	13	14	3,36	0,69	20,54	40	3,393	2
90	Viste	24.jan	21,5	15	14,5	12	13	2,49	0,74	29,72	40		0
91	Viste	24.jan	18,5	13	13	11	10,5	1,77	0,50	28,25	45		3
92	Viste	24.jan	21	15	15	11	10	2,59	0,68	26,25	70	3,812	2
93	Viste	24.jan	18,5	14	14	11	9,5	1,95	0,51	26,15	65		0
94	Viste	24.jan	22,5	16	15,5	12	12	3,39	0,95	28,02	45		-
95	Viste	24.jan	20,5	15	14,5	11	11,5	2,53	0,64	25,30	55	4,484	2
96	Viste	24.jan	25	18	17	14	13	4,25	1,06	24,94	55		3
97	Viste	24.jan	22,5	16,5	16	12	12	3,48	0,81	23,28	-	-	-
98	Viste	24.jan	26	18,5	18	14	13	4,58	1,27	27,73	-	-	-
99	Viste	24.jan	20	15,5	15,5	11,5	9	2,66	0,56	21,05	-	-	-
100	Viste	24.jan	19	14	14	10	10	2,37	0,56	23,63	-	-	-
		Mean	21,30	15,10	14,78	11,85	11,63	2,78	0,71	25,60	45,94	3,64	2,00
		SD	2,00	1,48	1,33	1,03	1,31	0,76	0,21	2,60	10,36	0,69	1,80
		SE	0,45	0,33	0,30	0,23	0,29	0,17	0,05	0,58	2,59	0,34	0,50
		Max	26,00	18,50	18,00	14,00	14,00	4,58	1,27	29,72	70,00	4,48	7,00
		Min	18,50	12,50	12,50	10,00	9,00	1,77	0,45	20,54	35,00	2,86	0,00
		Range	7,50	6,00	5,50	4,00	5,00	2,81	0,82	9,18	35,00	1,63	7,00

Sampling 1 (February) – Haugestranda (Alcoa 1)

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)	MT (mg g-1)	MN (per 1000 nuclei)
141	Alcoa 1	02.feb	21	15	15	12	12	2,79	0,53	19,00	20	-	7
142	Alcoa 1	02.feb	23	16	16,5	13,5	12,5	3,42	0,59	17,25	25	-	12
143	Alcoa 1	02.feb	21	15	14,5	11,5	11	2,96	0,68	22,97	25	-	16
144	Alcoa 1	02.feb	20,5	14,5	14,5	11	12	2,58	0,41	15,89	30	-	13
145	Alcoa 1	02.feb	21	15	14	11	11,5	2,51	0,61	24,30	30	-	27
146	Alcoa 1	02.feb	20,5	14,5	14	11	11,5	2,62	0,46	17,56	15	1,491	10
147	Alcoa 1	02.feb	26	18,5	17,5	11,5	15	5,11	1,15	22,50	40		29
148	Alcoa 1	02.feb	22	15	14,5	10	13	2,47	0,54	21,86	30		22
149	Alcoa 1	02.feb	21,5	15,5	15	10	12,5	3,06	0,63	20,59	35	3,438	16
150	Alcoa 1	02.feb	21,5	11,5	13	13	11,5	3,14	0,67	21,34	10		28
151	Alcoa 1	02.feb	19	14	11	9	11	2,39	0,39	16,32	30		16
152	Alcoa 1	02.feb	17,5	12	11	9	10	1,57	0,32	20,38	30	1,779	46
153	Alcoa 1	02.feb	19,5	14	14	10	11	2,10	0,38	18,10	20		25
154	Alcoa 1	02.feb	23	16	14	11	13	3,24	0,67	20,68	30		28
155	Alcoa 1	02.feb	23,5	15,5	13	11	14	3,05	0,68	22,30	35	-	27
156	Alcoa 1	02.feb	21,5	16	15	11	12	3,20	0,70	21,88	-	-	-
157	Alcoa 1	02.feb	19	14,5	14	10	11	2,10	0,48	22,86	-	-	-
158	Alcoa 1	02.feb	21	15	14,5	10	12	3,12	0,51	16,35	-	-	-
159	Alcoa 1	02.feb	24,5	17,5	14	11	14	4,04	0,79	19,55	-	-	-
160	Alcoa 1	02.feb	20	14,5	12	10	11,5	2,81	0,50	17,79	-	-	-
		Mean	21,33	14,98	14,05	10,83	12,10	2,91	0,58	19,97	27,00	2,26	21,47
		SD	2,03	1,65	1,71	1,27	1,28	0,78	0,20	2,59	7,97	1,02	10,03
		SE	0,45	0,37	0,38	0,28	0,29	0,17	0,04	0,58	2,06	0,59	2,59
		Max	26,00	18,50	17,50	13,50	15,00	5,11	1,15	24,30	40,00	3,44	46,00
		Min	17,50	11,50	11,00	9,00	10,00	1,57	0,32	15,89	10,00	1,57	7,00
		Range	8,50	7,00	6,50	4,50	5,00	3,54	0,83	8,41	30,00	1,87	39,00

Sampling 1 (February) – Tjuvholmen (Alcoa 2)

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)	MT (mg g-1)	MN (per 1000 nuclei)
201	Alcoa 2	09.feb	23,5	16	15,5	12	13	4,18	0,71	16,99	25	-	29
202	Alcoa 2	09.feb	26	18,5	17	13	14,5	4,37	0,96	21,97	25	-	44
203	Alcoa 2	09.feb	23	17	16	13	13,5	3,96	0,85	21,46	20	-	35
204	Alcoa 2	09.feb	24,5	17	17	12,5	14	4,18	1,03	24,64	25	-	28
205	Alcoa 2	09.feb	24,5	17	15	13	14,5	4,04	0,83	20,54	30	-	36
206	Alcoa 2	09.feb	23,5	16	15	12	13	3,41	0,73	21,41	25	2,005	19
207	Alcoa 2	09.feb	26	17	18	14	14	4,66	1,05	22,53	25		-
208	Alcoa 2	09.feb	23	16	15	11	14	3,50	0,66	18,86	20		30
209	Alcoa 2	09.feb	25	17	16,5	13	14	4,26	0,83	19,48	25	4,021	28
210	Alcoa 2	09.feb	27	17,5	16	12	17	4,91	0,90	18,33	25		38
211	Alcoa 2	09.feb	24,5	17,5	15,5	11,5	14	3,77	0,68	18,04	15		14
212	Alcoa 2	09.feb	26,5	18,5	16	13	16	5,35	1,05	19,63	25	4,559	39
213	Alcoa 2	09.feb	26,5	18	16,5	13,5	16	5,52	1,14	20,65	15		41
214	Alcoa 2	09.feb	27	18,5	17,5	13,5	15,5	5,72	0,85	14,86	25		-
215	Alcoa 2	09.feb	25	16,5	16,5	12	14	3,66	0,91	24,86	15	-	28
216	Alcoa 2	09.feb	23,5	16	14,5	12,5	14	3,48	0,63	18,10	-	-	-
217	Alcoa 2	09.feb	26	19	17,5	14,5	14	5,17	1,15	22,24	-	-	-
218	Alcoa 2	09.feb	23	16	14,5	12	14	3,68	0,68	18,48	-	-	-
219	Alcoa 2	09.feb	24,5	16,5	17	12	15	4,11	0,87	21,17	-	-	-
220	Alcoa 2	09.feb	24,5	17,5	16,5	13,5	14	4,68	0,92	19,66	-	-	-
		Mean	24,85	17,15	16,15	12,68	14,40	4,33	0,87	20,20	22,67	3,56	31,46
		SD	1,36	0,96	1,03	0,88	1,02	0,70	0,16	2,47	4,58	1,28	8,59
		SE	0,30	0,21	0,23	0,20	0,23	0,16	0,04	0,55	1,18	0,74	2,38
		Max	27,00	19,00	18,00	14,50	17,00	5,72	1,15	24,86	30,00	4,56	44,00
		Min	23,00	16,00	14,50	11,00	13,00	3,41	0,63	14,86	15,00	2,11	14,00
		Range	4,00	3,00	3,50	3,50	4,00	2,31	0,52	10,00	15,00	2,45	30,00

Sampling 1 (February) – Litlerauna (Alcoa Reference)

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)	MT (mg g-1)	MN (per 1000 nuclei)
181	Alcoa Reference	02.feb	21	16	15	12	11	3,70	0,65	17,57	60	-	6
182	Alcoa Reference	02.feb	19	14	12	10	11	3,10	0,69	22,26	60	-	9
183	Alcoa Reference	02.feb	17,5	12	12	8	9	1,38	0,26	18,84	45	-	4
184	Alcoa Reference	02.feb	18,5	13	13	10	9,5	1,87	0,38	20,32	50	-	4
185	Alcoa Reference	02.feb	20	15	14,5	11	10	2,65	0,53	20,00	60	-	6
186	Alcoa Reference	02.feb	21,5	15,5	15	12	11,5	2,76	0,69	25,00	75	1,093	3
187	Alcoa Reference	02.feb	21	14,5	14	10	12	2,69	0,47	17,47	55		5
188	Alcoa Reference	02.feb	19	16,5	13,5	10	10	2,04	0,47	23,04	55		14
189	Alcoa Reference	02.feb	18,5	14	10	9	10	2,04	0,40	19,61	50	4,185	2
190	Alcoa Reference	02.feb	19,5	14,5	10	9,5	10,2	2,53	0,41	16,21	55		8
191	Alcoa Reference	02.feb	20	14,5	10,5	8,5	11	2,43	0,59	24,28	40		6
192	Alcoa Reference	02.feb	18,5	13,5	9	7	11	2,06	0,30	14,56	15	3,588	-
193	Alcoa Reference	02.feb	17	12	8,5	7	10	1,39	0,35	25,18	55		14
194	Alcoa Reference	02.feb	20,5	14	12	9,5	12	2,23	0,40	17,94	45		2
195	Alcoa Reference	02.feb	20	15	11	9,5	11	2,43	0,50	20,58	45	-	7
196	Alcoa Reference	02.feb	18	13	12,5	9,5	8,5	1,63	0,36	22,09	-	-	-
197	Alcoa Reference	02.feb	16	11,5	11	8	8	1,45	0,24	16,55	-	-	-
198	Alcoa Reference	02.feb	19	13,5	13	9,5	10,5	2,19	0,42	19,18	-	-	-
199	Alcoa Reference	02.feb	17	12	12	9	9	1,58	0,26	16,46	-	-	-
200	Alcoa Reference	02.feb	17,5	14	13,5	10	9	2,25	0,37	16,44	-	-	-
		Mean	18,95	13,90	12,10	9,45	10,21	2,22	0,44	19,68	51,00	2,98	6,43
		SD	1,50	1,37	1,90	1,35	1,13	0,60	0,14	3,14	13,12	1,61	3,82
		SE	0,34	0,31	0,42	0,30	0,25	0,13	0,03	0,70	3,39	0,93	1,02
		Max	21,50	16,50	15,00	12,00	12,00	3,70	0,69	25,18	75,00	4,19	14,00
		Min	16,00	11,50	8,50	7,00	8,00	1,38	0,24	14,56	15,00	1,15	2,00
		Range	5,50	5,00	6,50	5,00	4,00	2,32	0,45	10,62	60,00	3,03	12,00

Sampling 2 (March) – Viste

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)	MT (mg g-1)	MN (per 1000 nuclei)
261	Viste	01.mar	23	16	17	13	12	3,32	1,05	31,63	45	1,762	5
262	Viste	01.mar	19,5	14	14	11	10	2,05	0,64	31,22	50		4
263	Viste	01.mar	19	14	14	10,5	10	1,98	0,52	26,26	35		0
264	Viste	01.mar	22	16	15	12	12	3,15	0,79	25,08	35	1,364	6
265	Viste	01.mar	23	17	16	12,5	12	3,38	1,01	29,88	30		11
266	Viste	01.mar	20	15	14,5	10,5	11	2,42	0,57	23,55	35		5
267	Viste	01.mar	22	15,5	15	11,5	11	2,76	0,74	26,81	20	2,392	0
268	Viste	01.mar	23,5	16,5	16	11,5	12	3,26	0,84	25,77	35		7
269	Viste	01.mar	18	13	13	9,5	9	1,63	0,50	30,67	50		8
270	Viste	01.mar	22	15	15	11,5	11,5	2,59	0,87	33,59	40	2,99	4
271	Viste	01.mar	22	16	15,5	12	11,5	3,07	0,87	28,34	40		-
272	Viste	01.mar	20	16	15,5	12	10	2,79	0,95	34,05	45		0
273	Viste	01.mar	21	15	14,5	11	10,5	2,63	0,69	26,24	45	3,139	0
274	Viste	01.mar	22	15,5	16	12,5	11	2,73	0,90	32,97	45		2
275	Viste	01.mar	19	14	14	10,5	9	2,10	0,57	27,14	45		3
276	Viste	01.mar	18,5	13,5	13	10	10	1,92	0,57	29,69	-	-	-
277	Viste	01.mar	20	14,5	14	11	11	2,31	0,57	24,68	-	-	-
278	Viste	01.mar	20,5	15	14,5	10,5	11	2,43	0,59	24,28	-	-	-
279	Viste	01.mar	21	15	15	11	12	2,84	0,79	27,82	-	-	-
280	Viste	01.mar	20	14	14	10	10	2,21	0,63	28,51	-	-	-
		Mean	20,80	15,03	14,78	11,20	10,83	2,58	0,73	28,41	39,67	2,33	3,93
		SD	1,59	1,06	1,03	0,94	0,98	0,51	0,17	3,19	8,12	0,77	3,38
		SE	0,36	0,24	0,23	0,21	0,22	0,11	0,04	0,71	2,10	0,34	0,90
		Max	23,50	17,00	17,00	13,00	12,00	3,38	1,05	34,05	50,00	3,14	11,00
		Min	18,00	13,00	13,00	9,50	9,00	1,63	0,50	23,55	20,00	1,36	0,00
		Range	5,50	4,00	4,00	3,50	3,00	1,75	0,55	10,50	30,00	1,78	11,00

Sampling 2 (March) – Haugestranda (Alcoa 1)

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)	MT (mg g-1)	MN (per 1000 nuclei)
321	Alcoa 1	09.mar	22	15,5	14	11	13	3,36	0,67	19,94	10	2,078	10
322	Alcoa 1	09.mar	23	16	15	12	13	3,45	0,68	19,71	10		25
323	Alcoa 1	09.mar	22	17	16,5	12	15	5,07	0,90	17,75	10		37
324	Alcoa 1	09.mar	23,5	16	15	16	14,5	3,83	0,56	14,62	10	2,177	45
325	Alcoa 1	09.mar	23,5	16	16	11,5	12	3,29	0,68	20,67	15		24
326	Alcoa 1	09.mar	22	15	15	11,5	12	2,82	0,52	18,44	25		28
327	Alcoa 1	09.mar	20	14	14	11	10	2,28	0,53	23,25	25	4,215	27
328	Alcoa 1	09.mar	23	15,5	15	11,5	13	3,42	0,70	20,47	25		17
329	Alcoa 1	09.mar	19,5	13	13	10	11	2,05	0,30	14,63	0		27
330	Alcoa 1	09.mar	19,5	13,5	13	10,5	11	2,10	0,29	13,81	20	3,124	30
331	Alcoa 1	09.mar	23,5	17	15	17	14	4,03	0,56	13,90	15		30
332	Alcoa 1	09.mar	21	15	14	11	12	2,76	0,56	20,29	15		36
333	Alcoa 1	09.mar	21	15	14	10,5	11,5	2,60	0,55	21,15	15	5,232	20
334	Alcoa 1	09.mar	23	16	15,5	12	12,5	3,47	0,66	19,02	20		10
335	Alcoa 1	09.mar	28,5	19,5	19	15	15,5	5,97	1,23	20,60	25		21
336	Alcoa 1	09.mar	22,5	17,5	15,5	12	12,5	3,80	0,78	20,53	-	-	-
337	Alcoa 1	09.mar	19,5	13,5	13	9,5	12	2,00	0,31	15,50	-	-	-
338	Alcoa 1	09.mar	23	15,5	15	12	13	3,59	0,65	18,11	-	-	-
339	Alcoa 1	09.mar	21,5	15	14,5	11	12	2,71	0,44	16,24	-	-	-
340	Alcoa 1	09.mar	21,5	15	14	10	13	2,67	0,55	20,60	-	-	-
		Mean	22,15	15,53	14,80	11,85	12,63	3,26	0,61	18,46	16,00	3,37	25,80
		SD	2,03	1,51	1,38	1,96	1,37	1,00	0,21	2,77	7,37	1,35	9,56
		SE	0,45	0,34	0,31	0,44	0,31	0,22	0,05	0,62	1,90	0,61	2,47
		Max	28,50	19,50	19,00	17,00	15,50	5,97	1,23	23,25	25,00	5,23	45,00
		Min	19,50	13,00	13,00	9,50	10,00	2,00	0,29	13,81	0,00	2,08	10,00
		Range	9,00	6,50	6,00	7,50	5,50	3,97	0,94	9,44	25,00	3,15	35,00

Sampling 2 (March) – Tjuvholmen (Alcoa 2)

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)	MT (mg g-1)	MN (per 1000 nuclei)
281	Alcoa 2	09.mar	24	16	15,5	12,5	14	3,82	0,90	23,56	15	2,051	25
282	Alcoa 2	09.mar	28,5	18	18,5	14	17,5	6,01	1,16	19,30	20		19
283	Alcoa 2	09.mar	24,5	17	17	13	14	4,25	1,00	23,53	0		30
284	Alcoa 2	09.mar	23	16	16	12	13	3,50	0,85	24,29	0	5,471	12
285	Alcoa 2	09.mar	23	14,5	14	10,5	14	2,93	0,39	13,31	10		21
286	Alcoa 2	09.mar	25,5	17,5	17,5	14	14	4,74	0,83	17,51	20		47
287	Alcoa 2	09.mar	23	15	14,5	11	14	3,24	0,44	13,58	15	4,305	18
288	Alcoa 2	09.mar	28	19	17	15	16,5	6,17	1,33	21,56	20		20
289	Alcoa 2	09.mar	27,8	18	15,5	12	16	4,40	1,01	22,95	15		24
290	Alcoa 2	09.mar	23,5	14,5	15	11	13	3,47	0,58	16,71	20	4,469	36
291	Alcoa 2	09.mar	27	17,5	16,5	13	15	4,78	1,18	24,69	30		42
292	Alcoa 2	09.mar	24,5	17	15,5	12	17	3,92	0,84	21,43	20		16
293	Alcoa 2	09.mar	22	14	14,5	11	13	3,06	0,60	19,61	15	4,454	33
294	Alcoa 2	09.mar	23	17	15	10	13,5	4,00	0,78	19,50	20		25
295	Alcoa 2	09.mar	23	15,5	14	13	14	3,22	0,47	14,60	20		19
296	Alcoa 2	09.mar	24,5	18	12,5	11	13,5	4,36	0,89	20,41	-	-	-
297	Alcoa 2	09.mar	23	16	14	11	13	3,41	0,63	18,48	-	-	-
298	Alcoa 2	09.mar	23	15,5	15	12	14	3,63	0,69	19,01	-	-	-
299	Alcoa 2	09.mar	22,5	15,5	15	11,5	13	3,87	0,63	16,28	-	-	-
300	Alcoa 2	09.mar	23	17,5	15	11	13,5	4,06	0,82	20,20	-	-	-
		Mean	24,32	16,45	15,38	12,03	14,28	4,04	0,80	19,52	16,00	4,15	25,80
		SD	1,99	1,39	1,40	1,31	1,39	0,87	0,25	3,44	7,84	1,26	9,95
		SE	0,44	0,31	0,31	0,29	0,31	0,20	0,06	0,77	2,02	0,56	2,57
		Max	28,50	19,00	18,50	15,00	17,50	6,17	1,33	24,69	30,00	5,47	47,00
		Min	22,00	14,00	12,50	10,00	13,00	2,93	0,39	13,31	0,00	2,05	12,00
		Range	6,50	5,00	6,00	5,00	4,50	3,24	0,94	11,38	30,00	3,42	35,00

Sampling 2 (March) – Litlerauna (Alcoa Reference)

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)	MT (mg g-1)	MN (per 1000 nuclei)
361	Alcoa Reference	09.mar	19	15	14	11,5	11	2,57	0,46	17,90	30	1,481	6
362	Alcoa Reference	09.mar	20	16	15,5	11	11	2,99	0,67	22,41	25		4
363	Alcoa Reference	09.mar	23	16,5	15	12	13	3,29	0,68	20,67	30	2,481	2
364	Alcoa Reference	09.mar	22,5	15	14	11	13	3,00	0,90	30,00	35		6
365	Alcoa Reference	09.mar	25	17	17	12	12,5	4,15	0,76	18,31	30	-	2
366	Alcoa Reference	09.mar	23,5	17	16	13	12,5	3,70	0,83	22,43	30	-	3
367	Alcoa Reference	09.mar	16	11	11	8	7,5	1,20	0,26	21,67	30	-	0
368	Alcoa Reference	09.mar	18	12	12	9	9	1,69	0,29	17,16	-	-	-
369	Alcoa Reference	09.mar	19	13	13	10	9	1,91	0,38	19,90	-	-	-
		Mean	20,67	14,72	14,17	10,83	10,94	2,72	0,58	21,16	30,00	1,98	3,29
		SD	2,97	2,22	1,94	1,58	2,02	0,97	0,24	3,84	2,89	0,71	2,21
		SE	0,99	0,74	0,65	0,53	0,67	0,32	0,08	1,28	1,09	0,50	0,84
		Max	25,00	17,00	17,00	13,00	13,00	4,15	0,90	30,00	35,00	2,48	6,00
		Min	16,00	11,00	11,00	8,00	7,50	1,20	0,26	17,16	25,00	1,48	0,00
		Range	9,00	6,00	6,00	5,00	5,50	2,95	0,64	12,84	10,00	1,00	6,00

Sampling 3 (April) – Viste

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)	MT (mg g-1)	MN (per 1000 nuclei)
370	Viste	05.apr	20	14	14	11	10	2,23	0,62	27,80	35	1,247	5
371	Viste	05.apr	16	11,5	11,5	8	7,5	1,22	0,31	25,41	35		0
372	Viste	05.apr	21,5	15,5	15	11,5	11	2,86	0,72	25,17	35		-
373	Viste	05.apr	16	12	12	8,5	8	1,35	0,34	25,19	25		1
374	Viste	05.apr	20	14	14	10	10,5	2,16	0,58	26,85	40		2
375	Viste	05.apr	17	12,5	12,5	9	8,5	1,52	0,40	26,32	40	3,162	4
376	Viste	05.apr	17	13	12,5	10	9	1,45	0,41	28,28	40		2
377	Viste	05.apr	21	15	15	11,5	11	2,50	0,70	28,00	30		8
378	Viste	05.apr	20	14	14	11	11	2,24	0,57	25,45	40	4,185	1
379	Viste	05.apr	21	15	14	11	11,5	2,45	0,61	24,90	40		2
380	Viste	05.apr	18	13	13	10	9	1,73	0,38	21,97	40		5
381	Viste	05.apr	20	15	14,5	12	9	2,20	0,64	29,09	45	4,335	3
382	Viste	05.apr	18,5	13	13	10	9,5	1,76	0,50	28,41	40		5
383	Viste	05.apr	19	13	12,5	10	11	1,92	0,42	21,88	40		2
384	Viste	05.apr	20,5	14,5	15	12	10	2,26	0,71	31,42	35	-	7
385	Viste	05.apr	21	15,5	16	13	10,5	2,67	0,76	28,46	-	-	-
386	Viste	05.apr	17,5	12,5	13	10	8	1,71	0,45	26,32	-	-	-
387	Viste	05.apr	19	14	13,5	11	10	2,09	0,55	26,32	-	-	-
388	Viste	05.apr	20	14	14	10,5	10	2,20	0,59	26,82	-	-	-
389	Viste	05.apr	20	13,5	13,5	11	10	2,02	0,68	33,66	-	-	-
		Mean	19,15	13,73	13,63	10,55	9,75	2,03	0,55	26,88	37,33	3,23	3,36
		SD	1,67	1,12	1,12	1,18	1,12	0,43	0,13	2,69	4,78	1,23	2,29
		SE	0,37	0,25	0,25	0,26	0,25	0,10	0,03	0,60	1,24	0,55	0,61
		Max	21,50	15,50	16,00	13,00	11,50	2,86	0,76	33,66	45,00	4,34	8,00
		Min	16,00	11,50	11,50	8,00	7,50	1,22	0,31	21,88	25,00	1,25	0,00
		Range	5,50	4,00	4,50	5,00	4,00	1,64	0,45	11,79	20,00	3,09	8,00

Sampling 3 (April) – Haugestranda (Alcoa 1)

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)	MT (mg g-1)	MN (per 1000 nuclei)
410	Alcoa 1	10.apr	23	14	14	11,5	13	2,85	0,49	17,19	0	2,132	22
411	Alcoa 1	10.apr	32,5	21	21	16	17,5	8,12	1,73	21,31	20		27
412	Alcoa 1	10.apr	24	17	16	12	12	3,97	0,81	20,40	25		25
413	Alcoa 1	10.apr	28	18	15	12	15	5,15	0,84	16,31	25		46
414	Alcoa 1	10.apr	19	14	14	10	10,5	2,61	0,45	17,24	15		10
415	Alcoa 1	10.apr	22	16	15,5	11,5	11	3,14	0,60	19,11	15	3,478	16
416	Alcoa 1	10.apr	19,5	14	13	10	10,5	2,16	0,49	22,69	15		14
417	Alcoa 1	10.apr	23,5	15	15	11	13	3,24	0,68	20,99	20		27
418	Alcoa 1	10.apr	19	14	14	10	9,5	1,97	0,39	19,80	20	3,528	-
419	Alcoa 1	10.apr	17,5	11,5	12	8,5	9	1,51	0,22	14,57	5		27
420	Alcoa 1	10.apr	22	14	15	10,5	11	2,52	0,50	19,84	15		38
421	Alcoa 1	10.apr	27	18,5	19	14	14	5,74	1,21	21,08	20	5,381	34
422	Alcoa 1	10.apr	23	17	16,5	13,5	12	4,17	0,72	17,27	20		40
423	Alcoa 1	10.apr	22	16	17	12	10	3,67	0,67	18,26	10		25
424	Alcoa 1	10.apr	20	13,5	13	9,5	11	2,61	0,39	14,94	30	-	19
425	Alcoa 1	10.apr	28	19	19	14	14,5	5,94	1,35	22,73	-	-	24
426	Alcoa 1	10.apr	22	16,5	16	12	10,5	3,35	0,48	14,33	-	-	-
427	Alcoa 1	10.apr	15	11	10	8	7,5	1,17	0,17	14,53	-	-	-
428	Alcoa 1	10.apr	29	19	19	14	15	6,59	0,94	14,26	-	-	-
429	Alcoa 1	10.apr	22	15	14	11	12	2,90	0,56	19,31	-	-	-
		Mean	22,90	15,70	15,40	11,55	11,93	3,67	0,68	18,31	17,00	3,63	26,27
		SD	4,27	2,59	2,67	2,02	2,40	1,80	0,38	2,83	7,75	1,33	9,92
		SE	0,95	0,58	0,60	0,45	0,54	0,40	0,09	0,63	2,00	0,67	2,56
		Max	32,50	21,00	21,00	16,00	17,50	8,12	1,73	22,73	30,00	5,38	46,00
		Min	15,00	11,00	10,00	8,00	7,50	1,17	0,17	14,26	0,00	2,13	10,00
		Range	17,50	10,00	11,00	8,00	10,00	6,95	1,56	8,46	30,00	3,25	36,00

Sampling 3 (April) – Tjuvholmen (Alcoa 2)

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)	MT (mg g-1)	MN (per 1000 nuclei)
450	Alcoa 2	10.apr	29	19	18,5	13	15,5	6,27	1,30	20,73	20	2,06	44
451	Alcoa 2	10.apr	24	16,5	16,5	12	12	3,68	0,82	22,28	20		16
452	Alcoa 2	10.apr	22	15	15	10,5	12	2,88	0,58	20,14	0		35
453	Alcoa 2	10.apr	24,5	16,5	16	12	13,5	3,99	0,86	21,55	25		36
454	Alcoa 2	10.apr	23	16	16	11	12	3,31	0,58	17,52	15		26
455	Alcoa 2	10.apr	27	19	18,5	14	14	5,87	0,76	12,95	15	4,481	28
456	Alcoa 2	10.apr	21,5	15	15,5	12	11,5	2,94	0,72	24,49	15		25
457	Alcoa 2	10.apr	25,5	17	16	12	15	4,27	0,80	18,74	30		19
458	Alcoa 2	10.apr	24	17,5	16,5	11	11,5	4,46	0,83	18,61	20	4,843	39
459	Alcoa 2	10.apr	24,5	17	16,5	12	12,5	4,09	1,30	31,78	15		30
460	Alcoa 2	10.apr	23	15	15	11	13	2,94	0,63	21,43	20		22
461	Alcoa 2	10.apr	25	17	17	12	13	3,98	0,90	22,61	35	4,559	37
462	Alcoa 2	10.apr	24,5	16	16,5	11	12	3,70	0,73	19,73	20		25
463	Alcoa 2	10.apr	24	15,5	15,5	11	12	3,46	0,59	17,05	31		32
464	Alcoa 2	10.apr	23	17	17	13	11	3,67	0,79	21,53	25	-	31
465	Alcoa 2	10.apr	23	16	16	12	12	3,50	0,69	19,71	-	-	20
466	Alcoa 2	10.apr	24	16,5	17	13	12	4,51	0,87	19,29	-	-	-
467	Alcoa 2	10.apr	20	14	13	10	11,5	2,60	0,47	18,08	-	-	-
468	Alcoa 2	10.apr	24	17	16	13	12	4,32	0,83	19,21	-	-	-
469	Alcoa 2	10.apr	23	16	16	12	13	3,67	0,60	16,35	-	-	-
		Mean	23,93	16,43	16,20	11,88	12,55	3,91	0,78	20,19	20,40	3,99	29,06
		SD	1,89	1,27	1,06	0,96	1,31	0,98	0,22	4,13	8,42	1,29	7,75
		SE	0,42	0,28	0,24	0,21	0,29	0,22	0,05	0,92	2,18	0,65	1,94
		Max	29,00	19,00	18,50	14,00	15,50	6,27	1,30	31,78	35,00	4,84	44,00
		Min	20,00	14,00	13,00	10,00	11,00	2,60	0,47	12,95	0,00	2,06	16,00
		Range	9,00	5,00	5,50	4,00	4,50	3,67	0,83	18,84	35,00	2,78	28,00

Sampling 3 (April) – Litlerauna (Alcoa Reference)

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)	MT (mg g-1)	MN (per 1000 nuclei)
490	Alcoa Reference	10.apr	23	15,5	14,5	11	13	3,10	0,76	24,52	25	1,229	9
491	Alcoa Reference	10.apr	20	15	14	11	11	2,60	0,60	23,08	25		13
492	Alcoa Reference	10.apr	22	16	14,5	11	12	3,31	0,70	21,15	20		3
493	Alcoa Reference	10.apr	22,5	16	15	10	13	3,10	0,75	24,19	20		7
494	Alcoa Reference	10.apr	24,5	17	16,5	12,5	14	4,24	0,90	21,23	25		-
495	Alcoa Reference	10.apr	22	15,5	15,5	11,5	13	3,00	0,57	19,00	25	3,017	4
496	Alcoa Reference	10.apr	21,5	15	15	11	12	2,95	0,39	13,22	25		5
497	Alcoa Reference	10.apr	20,5	14	14	11	12	2,66	0,52	19,55	35		4
498	Alcoa Reference	10.apr	25	17	14	13	14	4,77	0,83	17,40	25	5,232	-
499	Alcoa Reference	10.apr	19	14,5	13	10	10	2,09	0,55	26,32	40		3
500	Alcoa Reference	10.apr	23,5	17	15,5	12	13,5	3,90	0,85	21,79	30		4
501	Alcoa Reference	10.apr	19,5	13	13	10,5	11	2,23	0,49	21,97	30	3,886	4
502	Alcoa Reference	10.apr	20	15	12,5	9,5	10,5	2,70	0,55	20,37	35		3
503	Alcoa Reference	10.apr	22	15,5	15	11	12	3,21	0,60	18,69	35		10
504	Alcoa Reference	10.apr	20,5	14	14	11	12	3,90	0,90	23,08	30	-	4
505	Alcoa Reference	10.apr	17	12	11	8	10	1,72	0,32	18,60	-	-	-
506	Alcoa Reference	10.apr	20	14,5	13,5	10	11,5	2,54	0,57	22,44	-	-	-
507	Alcoa Reference	10.apr	23	16	15,5	12	14	3,72	0,75	20,16	-	-	-
508	Alcoa Reference	10.apr	24,5	12,5	15	12	14,5	4,73	0,94	19,87	-	-	-
509	Alcoa Reference	10.apr	22,5	16	14,5	12	13	3,46	0,67	19,36	-	-	-
		Mean	21,63	15,05	14,28	11,00	12,30	3,20	0,66	20,80	28,33	3,34	5,62
		SD	2,06	1,42	1,25	1,15	1,36	0,83	0,17	2,88	5,88	1,68	3,18
		SE	0,46	0,32	0,28	0,26	0,30	0,19	0,04	0,64	1,52	0,84	0,82
		Max	25,00	17,00	16,50	13,00	14,50	4,77	0,94	26,32	40,00	5,23	13,00
		Min	17,00	12,00	11,00	8,00	10,00	1,72	0,32	13,22	20,00	1,23	3,00
		Range	8,00	5,00	5,50	5,00	4,50	3,05	0,62	13,10	20,00	4,00	10,00

Appendix B – Weather and tide

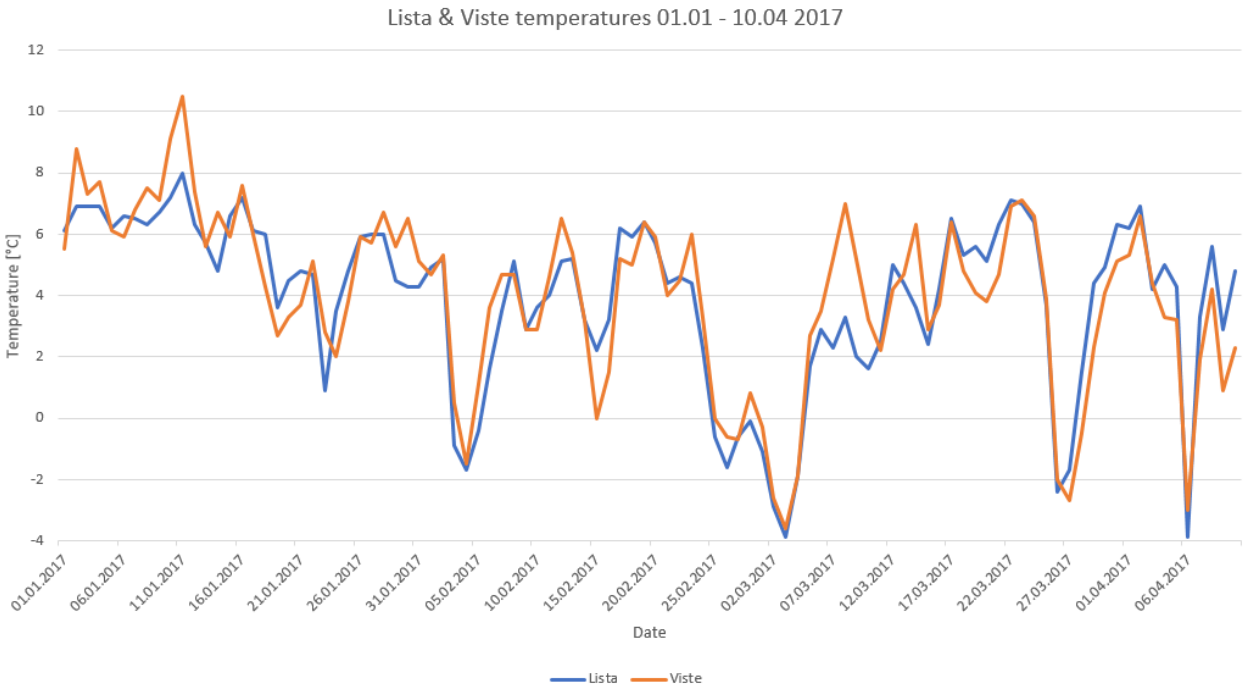
Temperatures in Lista and Viste. Green cells marks samplings.

Date	Lista	Viste
01.01.2017	6,1	5,5
02.01.2017	6,9	8,8
03.01.2017	6,9	7,3
04.01.2017	6,9	7,7
05.01.2017	6,2	6,1
06.01.2017	6,6	5,9
07.01.2017	6,5	6,8
08.01.2017	6,3	7,5
09.01.2017	6,7	7,1
10.01.2017	7,2	9,1
11.01.2017	8	10,5
12.01.2017	6,3	7,4
13.01.2017	5,7	5,6
14.01.2017	4,8	6,7
15.01.2017	6,6	5,9
16.01.2017	7,2	7,6
17.01.2017	6,1	6
18.01.2017	6	4,3
19.01.2017	3,6	2,7
20.01.2017	4,5	3,3
21.01.2017	4,8	3,7
22.01.2017	4,7	5,1
23.01.2017	0,9	2,8
24.01.2017	3,5	2
25.01.2017	4,8	3,8
26.01.2017	5,9	5,9
27.01.2017	6	5,7
28.01.2017	6	6,7
29.01.2017	4,5	5,6
30.01.2017	4,3	6,5
31.01.2017	4,3	5,1

Date	Lista	Viste
01.02.2017	4,9	4,7
09.03.2017	2	5,3
03.02.2017	-0,9	0,5
04.02.2017	-1,7	-1,5
05.02.2017	-0,4	1,1
06.02.2017	1,6	3,6
07.02.2017	3,5	4,7
08.02.2017	5,1	4,7
09.03.2017	2	2,9
10.02.2017	3,6	2,9
11.02.2017	4	4,6
12.02.2017	5,1	6,5
13.02.2017	5,2	5,4
14.02.2017	3,2	3,2
15.02.2017	2,2	0
16.02.2017	3,2	1,5
17.02.2017	6,2	5,2
18.02.2017	5,9	5
19.02.2017	6,4	6,4
20.02.2017	5,7	5,9
21.02.2017	4,4	4
22.02.2017	4,6	4,5
23.02.2017	4,4	6
24.02.2017	2,3	3,3
25.02.2017	-0,6	0
26.02.2017	-1,6	-0,6
27.02.2017	-0,6	-0,7
28.02.2017	-0,1	0,8

Date	Lista	Viste
01.03.2017	-1,1	-0,3
02.03.2017	-2,9	-2,6
03.03.2017	-3,9	-3,6
04.03.2017	-1,9	-1,9
05.03.2017	1,7	2,7
06.03.2017	2,9	3,5
07.03.2017	2,3	5,2
08.03.2017	3,3	7
09.03.2017	2	5,2
10.03.2017	1,6	3,2
11.03.2017	2,5	2,2
12.03.2017	5	4,2
13.03.2017	4,4	4,7
14.03.2017	3,6	6,3
15.03.2017	2,4	2,9
16.03.2017	4,2	3,7
17.03.2017	6,5	6,4
18.03.2017	5,3	4,8
19.03.2017	5,6	4,1
20.03.2017	5,1	3,8
21.03.2017	6,3	4,7
22.03.2017	7,1	6,9
23.03.2017	7	7,1
24.03.2017	6,4	6,6
25.03.2017	3,7	3,9
26.03.2017	-2,4	-2
27.03.2017	-1,7	-2,7
28.03.2017	1,5	-0,5
29.03.2017	4,4	2,3
30.03.2017	4,9	4,1
31.03.2017	6,3	5,1

Date	Lista	Viste
01.04.2017	6,2	5,3
02.04.2017	6,9	6,6
03.04.2017	4,2	4,4
04.04.2017	5	3,3
05.04.2017	4,3	3,2
06.04.2017	-3,9	-3
07.04.2017	3,3	1,9
08.04.2017	5,6	4,2
09.04.2017	2,9	0,9
09.03.2017	2	2,3



Temperatures in Lista and Viste

Tides in Viste on the days of sampling. Green cells mark time of sampling.

Viste

Sampling 1 - February

Tue 24.01.17

Time	Water level	Predicted tides	Weather effect
kl. 00:00	54 cm	64 cm	-11 cm
kl. 01:00	50 cm	61 cm	-11 cm
kl. 02:00	49 cm	58 cm	-9 cm
kl. 03:00	54 cm	60 cm	-6 cm
kl. 04:00	57 cm	66 cm	-9 cm
kl. 05:00	61 cm	70 cm	-9 cm
kl. 06:00	67 cm	74 cm	-6 cm
kl. 07:00	75 cm	78 cm	-4 cm
kl. 08:00	78 cm	81 cm	-3 cm
kl. 09:00	73 cm	80 cm	-7 cm
kl. 10:00	67 cm	76 cm	-8 cm
kl. 11:00	66 cm	70 cm	-5 cm
kl. 12:00	64 cm	67 cm	-3 cm
kl. 13:00	58 cm	63 cm	-6 cm
kl. 14:00	56 cm	59 cm	-3 cm
kl. 15:00	57 cm	59 cm	-2 cm
kl. 16:00	62 cm	63 cm	-1 cm
kl. 17:00	65 cm	68 cm	-3 cm
kl. 18:00	70 cm	71 cm	-1 cm
kl. 19:00	74 cm	76 cm	-2 cm
kl. 20:00	81 cm	81 cm	0 cm
kl. 21:00	79 cm	82 cm	-3 cm
kl. 22:00	72 cm	78 cm	-6 cm
kl. 23:00	66 cm	72 cm	-7 cm

Viste

Sampling 2 - March

Wed 01.03.17

Time	Water level	Predicted tides	Weather effect
kl. 00:00	115 cm	94 cm	21 cm
kl. 01:00	108 cm	88 cm	21 cm
kl. 02:00	101 cm	74 cm	27 cm
kl. 03:00	95 cm	68 cm	28 cm
kl. 04:00	83 cm	60 cm	23 cm
kl. 05:00	69 cm	44 cm	25 cm
kl. 06:00	59 cm	33 cm	26 cm
kl. 07:00	64 cm	39 cm	25 cm
kl. 08:00	80 cm	55 cm	25 cm
kl. 09:00	80 cm	63 cm	18 cm
kl. 10:00	84 cm	65 cm	18 cm
kl. 11:00	98 cm	78 cm	20 cm
kl. 12:00	114 cm	95 cm	19 cm
kl. 13:00	109 cm	95 cm	14 cm
kl. 14:00	98 cm	80 cm	18 cm
kl. 15:00	93 cm	69 cm	24 cm
kl. 16:00	84 cm	63 cm	21 cm
kl. 17:00	66 cm	49 cm	18 cm
kl. 18:00	53 cm	33 cm	20 cm
kl. 19:00	52 cm	33 cm	20 cm
kl. 20:00	67 cm	47 cm	20 cm
kl. 21:00	74 cm	57 cm	17 cm
kl. 22:00	74 cm	59 cm	15 cm
kl. 23:00	81 cm	67 cm	14 cm

Viste

Sampling 3 - April

Wed 05.04.17

Time	Water level	Predicted tides	Weather effect
kl. 00:00	69 cm	51 cm	18 cm
kl. 01:00	75 cm	55 cm	20 cm
kl. 02:00	73 cm	57 cm	15 cm
kl. 03:00	75 cm	59 cm	17 cm
kl. 04:00	81 cm	62 cm	19 cm
kl. 05:00	86 cm	68 cm	17 cm
kl. 06:00	87 cm	70 cm	18 cm
kl. 07:00	82 cm	65 cm	18 cm
kl. 08:00	81 cm	60 cm	21 cm
kl. 09:00	77 cm	57 cm	20 cm
kl. 10:00	72 cm	54 cm	18 cm
kl. 11:00	66 cm	49 cm	17 cm
kl. 12:00	61 cm	47 cm	14 cm
kl. 13:00	60 cm	48 cm	13 cm
kl. 14:00	61 cm	50 cm	10 cm
kl. 15:00	58 cm	53 cm	6 cm
kl. 16:00	58 cm	55 cm	2 cm
kl. 17:00	63 cm	62 cm	1 cm
kl. 18:00	66 cm	68 cm	-1 cm
kl. 19:00	67 cm	67 cm	0 cm
kl. 20:00	63 cm	63 cm	0 cm
kl. 21:00	59 cm	60 cm	-1 cm
kl. 22:00	53 cm	58 cm	-6 cm
kl. 23:00	49 cm	55 cm	-6 cm

Tides in Lista on the days of sampling. Green cells mark time of sampling

Lista

Sampling 1 - February

Thu 02.02.17

Time	Water level	Predicted tides	Weather effect
kl. 00:00	4 cm	32 cm	-28 cm
kl. 01:00	4 cm	31 cm	-26 cm
kl. 02:00	5 cm	31 cm	-26 cm
kl. 03:00	7 cm	32 cm	-26 cm
kl. 04:00	10 cm	35 cm	-25 cm
kl. 05:00	14 cm	38 cm	-24 cm
kl. 06:00	18 cm	41 cm	-24 cm
kl. 07:00	19 cm	43 cm	-24 cm
kl. 08:00	18 cm	43 cm	-26 cm
kl. 09:00	18 cm	42 cm	-24 cm
kl. 10:00	15 cm	40 cm	-25 cm
kl. 11:00	12 cm	37 cm	-25 cm
kl. 12:00	9 cm	33 cm	-24 cm
kl. 13:00	9 cm	31 cm	-22 cm
kl. 14:00	9 cm	30 cm	-21 cm
kl. 15:00	11 cm	31 cm	-20 cm
kl. 16:00	13 cm	32 cm	-19 cm
kl. 17:00	20 cm	35 cm	-15 cm
kl. 18:00	23 cm	39 cm	-15 cm
kl. 19:00	27 cm	41 cm	-14 cm
kl. 20:00	30 cm	42 cm	-13 cm
kl. 21:00	32 cm	42 cm	-10 cm
kl. 22:00	33 cm	41 cm	-7 cm
kl. 23:00	34 cm	38 cm	-4 cm

Lista

Sampling 1 - February

Thu 09.02.17

Time	Water level	Predicted tides	Weather effect
kl. 00:00	8 cm	39 cm	-31 cm
kl. 01:00	12 cm	43 cm	-30 cm
kl. 02:00	15 cm	44 cm	-29 cm
kl. 03:00	15 cm	44 cm	-29 cm
kl. 04:00	14 cm	42 cm	-28 cm
kl. 05:00	10 cm	38 cm	-28 cm
kl. 06:00	5 cm	34 cm	-29 cm
kl. 07:00	0 cm	29 cm	-29 cm
kl. 08:00	-2 cm	27 cm	-29 cm
kl. 09:00	-3 cm	26 cm	-29 cm
kl. 10:00	0 cm	28 cm	-28 cm
kl. 11:00	4 cm	31 cm	-27 cm
kl. 12:00	10 cm	36 cm	-25 cm
kl. 13:00	17 cm	40 cm	-23 cm
kl. 14:00	23 cm	43 cm	-20 cm
kl. 15:00	27 cm	44 cm	-18 cm
kl. 16:00	27 cm	43 cm	-17 cm
kl. 17:00	25 cm	41 cm	-15 cm
kl. 18:00	22 cm	37 cm	-15 cm
kl. 19:00	18 cm	32 cm	-15 cm
kl. 20:00	13 cm	29 cm	-16 cm
kl. 21:00	11 cm	28 cm	-17 cm
kl. 22:00	12 cm	29 cm	-18 cm
kl. 23:00	14 cm	32 cm	-18 cm

Lista

Sampling 2 - March

Thu 09.03.17

Time	Water level	Predicted tides	Weather effect
kl. 00:00	43 cm	39 cm	4 cm
kl. 01:00	47 cm	42 cm	6 cm
kl. 02:00	51 cm	42 cm	9 cm
kl. 03:00	49 cm	40 cm	8 cm
kl. 04:00	47 cm	37 cm	10 cm
kl. 05:00	42 cm	32 cm	10 cm
kl. 06:00	40 cm	27 cm	12 cm
kl. 07:00	40 cm	24 cm	16 cm
kl. 08:00	41 cm	23 cm	18 cm
kl. 09:00	44 cm	24 cm	20 cm
kl. 10:00	50 cm	27 cm	23 cm
kl. 11:00	56 cm	32 cm	25 cm
kl. 12:00	60 cm	37 cm	24 cm
kl. 13:00	64 cm	40 cm	24 cm
kl. 14:00	64 cm	42 cm	22 cm
kl. 15:00	61 cm	42 cm	20 cm
kl. 16:00	56 cm	39 cm	17 cm
kl. 17:00	49 cm	35 cm	14 cm
kl. 18:00	41 cm	31 cm	10 cm
kl. 19:00	34 cm	27 cm	7 cm
kl. 20:00	30 cm	26 cm	4 cm
kl. 21:00	28 cm	26 cm	2 cm
kl. 22:00	29 cm	28 cm	1 cm
kl. 23:00	33 cm	32 cm	1 cm

Lista

Sampling 3 - April

Mon 10.04.17

Time	Water level	Predicted tides	Weather effect
kl. 00:00	28 cm	27 cm	1 cm
kl. 01:00	30 cm	29 cm	1 cm
kl. 02:00	33 cm	33 cm	1 cm
kl. 03:00	37 cm	35 cm	2 cm
kl. 04:00	41 cm	37 cm	4 cm
kl. 05:00	40 cm	37 cm	3 cm
kl. 06:00	36 cm	36 cm	0 cm
kl. 07:00	35 cm	34 cm	1 cm
kl. 08:00	31 cm	31 cm	0 cm
kl. 09:00	30 cm	28 cm	2 cm
kl. 10:00	29 cm	26 cm	3 cm
kl. 11:00	30 cm	25 cm	5 cm
kl. 12:00	35 cm	26 cm	10 cm
kl. 13:00	39 cm	27 cm	12 cm
kl. 14:00	42 cm	30 cm	12 cm
kl. 15:00	47 cm	33 cm	14 cm
kl. 16:00	50 cm	35 cm	15 cm
kl. 17:00	49 cm	35 cm	14 cm
kl. 18:00	47 cm	35 cm	11 cm
kl. 19:00	44 cm	34 cm	10 cm
kl. 20:00	40 cm	32 cm	8 cm
kl. 21:00	36 cm	29 cm	7 cm
kl. 22:00	35 cm	27 cm	7 cm
kl. 23:00	35 cm	27 cm	8 cm