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ESPIAL

Integrated biological effects assessment of the discharge water into the Sunndals fjord from an aluminium smelter

Photo source: Steven Brooks NIVA

Norwegian Institute for Water Research

REPORT

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Summary

The following study describes an integrated biological effects monitoring programme using field transplanted mussels to determine the potential biological effects of the effluent discharge from Hydro's aluminium smelter in the Sunndals fjord. Chemical body burden (PAH and metals) and a suite of biological effects markers were measured in mussels positioned at known distances (1, 2, 5, 10 and 20 km) from the aluminium smelter for 6 weeks. Overall, the biological responses observed where greater in the mussels positioned closest to the smelters discharge (1 – 5 km), although the chemical concentrations in mussel tissues were low and below the expected threshold levels. The lowest chemical accumulation and biomarker responses were observed in mussels positioned 10 km from the smelter. Mussels located furthest from the smelter exhibited significant biomarker responses, probably associated with a different contaminant source. The integrated biological response index (IBR) reflected the expected level of exposure to the smelters discharge and the Principal component analysis (PCA) differentiated between the mussel groups, with the most impacted located closest to the smelters discharge. Not one chemical factor explained the biological responses in mussels, correlations were found between certain measured contaminants (i.e. PAH, Mn, Ni and Cr) and distance from the smelter, although the concentrations of these contaminants were low and unlikely to have caused the biological effects observed in the mussels (1 and 5 km).

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ESPIAL

Integrated biological effects assessment of the discharge water into the Sunndals fjord from an aluminium smelter

Preface

The Work described in this report is part of the ESPIAL project. ESPIAL (=Ensuring the Environmental Sustainability of production of PrImary ALuminium) is a multidisciplinary study on the local environmental effects around aluminium smelters in the Nordic countries. The study is initiated and sponsored by "Aluminiumsindustriens Miljøsekretariat" (AMS), and is building on a similar, but more extensive study of the Norwegian smelters from 1991-94, called the "Effect Study". ESPIAL involves new field studies conducted in 2018 – 2020 on the marine environment, air quality, effects on vegetation and on wildlife, in addition to a review of studies conducted through the period after the Effect Study on these subjects. The report at hand is a contribution to ESPIAL and aimed to determine if the discharge water from the aluminium smelter at Sunndalsøra was potentially harmful to marine life within the Sunndalsfjord seawater recipient. Mussels were placed within the fjord for 6-weeks at five distances (1 to 20 km) from the Al smelter with the help of the boat and crew of G. Øye AS. The deployment and retrieval of the mussels was performed by Dr Steven Brooks together with G. Øye AS. Processing of the mussels and laboratory analyses were performed by NIVA Scientists, Dr Tânia Gomes, Dr Karina Petersen, Maria Elisabetta Michelangeli and Dr Steven Brooks. The report was written by Dr Steven Brooks and Dr Tânia Gomes. The project was managed by Dr Ailbhe Macken.

Oslo, October 2022

Dr Ailbhe Macken (project manager)

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Summary

The following study describes an integrated biological effects monitoring programme using field transplanted mussels to determine the potential biological effects of the effluent discharge from the aluminium smelter operated by Hydro AS in the Sunndals fjord in Norway. Chemical body burden (41 PAH and metal concentrations) and a suite of biological effects markers were measured in mussels positioned at known distances (1, 2, 5, 10 and 20 km) from the aluminium smelters discharge for a period of 6 weeks. The biomarkers used included: condition index (CI); stress on stress (SoS); neutral red retention (NRR); micronuclei formation (MN); acetylcholine esterase (AChE) inhibition; lipid peroxidation (LPO); volume density of basophilic cells (VvBAS); Neutral lipid (NL); and lipofuscin (LF) accumulation.

Overall, the biological responses observed where greater in the mussels positioned closest to the smelters discharge (1 – 5 km), although the chemical concentrations measured in mussel tissues were low and below the expected threshold levels where biological responses would be expected. The lowest chemical accumulation and biomarker responses were observed in mussels positioned 10 km from the smelters effluent and could be considered as the field reference population. Mussels located furthest from the smelter (20 km) exhibited significant biomarker responses, probably associated with a different contaminant source within the fjord.

The integrated biological response index (IBR) also reflected the expected level of exposure to the smelters discharge, with the highest IBR calculated in mussels positioned closest to the discharge (1 – 5 km), followed by the 20 km group. The Lowest IBR was measured in the 10 km mussel group. The Principal component analysis (PCA) also differentiated among mussel groups, with the most impacted located closest to the smelters discharge. Not one chemical factor explained the observed biological responses in mussels, correlations were found between certain measured contaminants (i.e. PAH, Mn, Ni and Cr) and distance from the smelter, although the concentrations of these contaminants were low and unlikely to have caused the biological effects observed in the mussels from the 1 and 5 km groups.

Sammendrag

Tittel: Integrated biological effects assessment of the discharge water into the Sunndals fjord from an aluminium smelter
År: 2020
Forfatter(e): Steven Brooks, Tânia Gomes
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Den følgende studien beskriver et integrert overvåkningsprogram for biologiske effekter ved bruk av felttransplanterte blåskjell for å bestemme de potensielle biologiske effektene av utslipp fra aluminiumssmelteverket som drives av Hydro AS i Sunndalsfjorden i Norge. Kjemisk kroppsbyrde (41 PAH- og metallkonsentrasjoner) og en rekke markører for biologiske effekter ble målt i blåskjell plassert på kjente avstander (1, 2, 5, 10 og 20 km) fra utslippet fra aluminiumsmelteverket i en periode på 6 uker. Biomarkørene som ble brukt inkluderer: kondisjonsindeks (CI); stress på stress (SoS); nøytral rød retensjon (NRR); dannelse av mikrokjerner (MN); inhibering av acetylkolinesterase (AChE); lipidperoksidasjon (LPO); volumtetthet av basofile celler (VvBAS); Nøytral lipid (NL); og lipofuscin (LF) akkumulering.

Samlet sett var de observerte biologiske responsene større i blåskjellene som var plassert nærmest smelteverkets utslipp (1-5 km), selv om de kjemiske konsentrasjonene målt i blåskjellvev var lave og under de forventede terskelnivåene der biologiske responser kunne forventes. De laveste kjemiske akkumuleringene og biomarkørresponsene ble observert i blåskjell plassert 10 km fra smelteverkets utslipp og kan betraktes som referansefeltpopulasjonen. Blåskjell som var plassert lengst fra utslippet (20 km) viste betydelige biomarkørresponser, sannsynligvis knyttet til en annen forurensningskilde i fjorden.

Den integrerte biologiske responsindeksen (IBR) reflekterte også forventet eksponeringsnivå for smelteverkets utslipp, med høyest IBR beregnet i blåskjell plassert nærmest utslippet (1-5 km), etterfulgt av 20 km-gruppen. Laveste IBR ble målt i blåskjell plassert 10 km fra utslippet. Prinsipal komponent analyse (PCA) differensierte også mellom blåskjellgrupper, med de mest berørte lokalisert nærmest smelteverkets utslipp. Det var ingen enkelt kjemisk faktor som kunne forklare de biologiske responsene i blåskjell, men det ble funnet sammenhenger mellom visse målte forurensninger (dvs. PAH, Mn, Ni og Cr) og avstand fra smelteverket, selv om konsentrasjonene av disse forurensningene var lave og sannsynligvis ikke kan ha forårsaket de biologiske effektene observert i blåskjellene fra 1- og 5 km-gruppene.

1 Introduction

The following study describes an integrated biological effects monitoring programme using field transplanted mussels to determine the potential biological effects of the effluent discharge from an aluminium smelter. The aluminium smelter, operated by Hydro AS since 1954, is located at Sunndal and discharges waste under licence into the Sunndals fjord in Norway. The Sunndal plant is Europe's largest and most modern producer of primary aluminium with over 400 000 tonnes annually. In addition, the plant produces 500 000 tonnes of casthouse products and 80 000 tonnes of anodes annually (https://www.hydro.com).

The waste effluent from the smelter originates mostly from seawater scrubbing of furnace off-gases, which contain PAHs, fluoride, soot particles and metals. Concentrations of PAHs and metals have been previously monitored in sediment, water and biota within the fjord over the last 36 years (e.g. Oug et al 1998), with the first baseline surveys taking place in 1986. It is these contaminants that are considered to be the main concern and threat to marine life. PAHs have been found to accumulate significantly in both sediment and biota near the effluent discharge and although concentrations have been expected to decrease sharply with distance from the discharge outlet, elevated concentrations have been measured several kilometers away (Næs et al. 1995). PAH concentrations of the sediment as high as 700 mg/kg (d.w.) have been reported within the Sunndals fjord, which is over 1000 times the expected coastal sediment background level (Næs et al., 1995; Knutzen, 1995).

The main sources of PAH discharge from the smelter were formerly the Søderberg potlines, until 2002-2004 when they were replaced with a modern prebake potline. The remaining discharges of PAH have since then mainly come from the anode plant seawater scrubber. While the potline scrubbers have a deepwater discharge, the anode plant discharges into a pond at the riverside. From here, the effluent percolates through the pond wall into the mouth of the river Driva. Further dispersion of this effluent will therefore mainly be in the brackish surface water of the fjord.

As a consequence of these production changes, more recent studies have identified some improvement of chemical concentrations within the fjord. For example, in 2015, PAH and metal concentrations in sediment samples, although found to exceed the EQS values and consequently deemed as poor chemical status, were overall showing a downward trend in chemical concentrations from previous years (Borgersen & Berge, 2016). Sediment concentrations of PAH16 ranged from 29.3

at the closest station to the smelter down to 2.6 mg/kg (d.w.) approximately 10 km away. These can be compared to a sediment EQS values of 2 mg/kg (d.w.). Furthermore, and even more recently, analysis of PAH and metals in wild populations of mussels (*Mytilus* sp.) collected from within the Sunndals fjord, were indicative of good chemical status with no concentration exceeding the environmental quality standards (EQS) (Øxnevad & Håvardstum , 2018). These latest studies indicate an improvement of environmental conditions within the fjord.

The current investigation focusses on the water column and the potential effects of chemicals from the aluminium smelter on pelagic organisms. This is the first time that the biological effects of mussels exposed within the water column to the discharge plume have been determined. Mussels are used widely in biological effects monitoring programmes, they are sessile and easily transplanted into different environments, they can filter large volumes of seawater, bioaccumulate contaminants from the filtered seawater, and have a wide range of sensitive biological effects methods that can be easily measured. Many of the biological effects measurements have internationally recognised background and environmental assessment criteria (BAC and EAC, Davies and Vethaak, 2012), which enables the biological response(s) to be placed into context to other similar studies. A brief description of the biological effects measurements used is provided.

Condition index is used to provide a simple measure of organism health status, encompassing the physiological activity such as growth, reproduction, secretion, etc., under environmental conditions. Condition index is a general health parameter that can be influenced by both biotic and abiotic factors that can cause physiological stress on the organism.

The **stress on stress** (SoS) biomarker, is a simple and low-cost whole organism response that can show pollutant induced alterations in mussel physiology. The ability of mussels to keep their shells closed and resist air exposure is related to the amount of energy (adenosine triphosphate, ATP) available to fuel their adductor muscle (De Zwaan and Mathiew, 1992). If metabolic energy is spent on detoxification processes in mussels exposed to contaminants, less energy is available for other physiological processes.

The **neutral red retention** (NRR) assay is a method to detect the resilience of lysosome membranes in mussel haemocytes and provides a measure of the functional integrity of cells. The membrane integrity

of lysosomes has been found to be affected by a range of environmental stressors, including metals and organic chemicals (Lowe et al. 1995).

Micronuclei (MN) are chromatin-containing structures that are surrounded by a membrane and have no detectable link to the cell nucleus. The MN test provides an indication of chromosomal damage, and has been found to show a time-integrated response to complex mixtures of pollutants (Baršienė et al. 2006). The frequency of MN is regarded as an important tool for *in situ* monitoring of DNA damage.

The **acetylcholine esterase** (AChE) assay has been used to assess the neurotoxicity of environmental samples and exposure to neurotoxic compounds. AChE is an essential enzyme involved in neurotransmission. An inhibition of AChE causes various effects on the central nervous system. Different types of compounds have been described as AChE inhibitors, such as organophosphorus and carbamate pesticides and PAHs.

Lipid peroxidation (LPO) is characterized by the oxidative deterioration of polyunsaturated fatty acids present in cellular membranes, which can alter membrane fluidity and permeability. The formation of lipid peroxides is characterized by the presence of by-products as malondialdehyde (MDA) and hydroxyalkenals, that have been routinely measured in bivalve species to reflect contaminant-induced oxidative damage.

The **volume of basophilic cells** (VvBAS), provides a sensitive indication of sub lethal damage due to contaminant exposure. The epithelium of the digestive gland is comprised of two cell types: digestive and basophilic cells. Under normal physiological conditions the digestive cells outnumber the basophilic cells, but under different stress situations, including exposure to pollutants, the number of basophilic cells increases. These changes in the cell type composition are due to digestive cell loss resulting from environmental stress.

Elevated levels of **neutral lipid** (NL) within the lysosomes of digestive glands of mussels have been linked with organism stress and reduced health status. Lipophilic contaminants can alter the metabolism of neutral lipids leading to abnormal accumulation of that lipid class inside lysosomes, which is measurable.

Lipofuscin (LF) granule accumulation is a measure of oxidative stress. Oxidative stress leads to peroxidation of cellular membranes, these peroxidation end-products accumulate in lysosomes as insoluble granules referred to as lipofuscin. Since lipofuscin is not degradable they provide a measurable level of oxidative stress experienced by the mussel.

2 Methods

2.1 Transplantation of field mussels

Mussels were collected in early April 2019 from the lower intertidal shore region of the outer Oslo fjord (59°36'55.5"N 10°39'04.2"E), near the NIVA marine research station in Solbergstrand, Norway. The mussels were held in flow-through tanks of filtered seawater at a flow rate of approximately 20 L/ min at a temperature of 8 ± 1°C for approximately two weeks prior to field deployment. During the acclimation phase mussels were fed daily with a live algal culture. Species identification was not performed on individual mussels, however, the mussels sampled from this location have all previously been identified as *M. edulis* (Brooks and Farmen, 2013; Brooks et al. 2015). Therefore, it was assumed that most if not all individuals were *M*. *edulis* and species differences in biomarker response and chemical bioaccumulation were not a confounding factor in this study. All mussels used in the study were measured and were of a similar size (Shell length 52.5 ± 4.6 mm, mean ± standard deviation).

The evening before field deployment, the mussels were carefully placed in nylon mesh socks. The mesh socks were knotted at intervals to create five pockets of 20 mussels, ensuring sufficient space was provided so as not to impede gaping and filtration. The mussels were placed in a polystyrene fish box with ice packs and fresh kelp to ensure conditions were cold and moist during transport. The mussels were transported by airfreight to the field site ready for deployment the following morning. Mussels were in optimal condition prior to deployment with no mortalities observed.

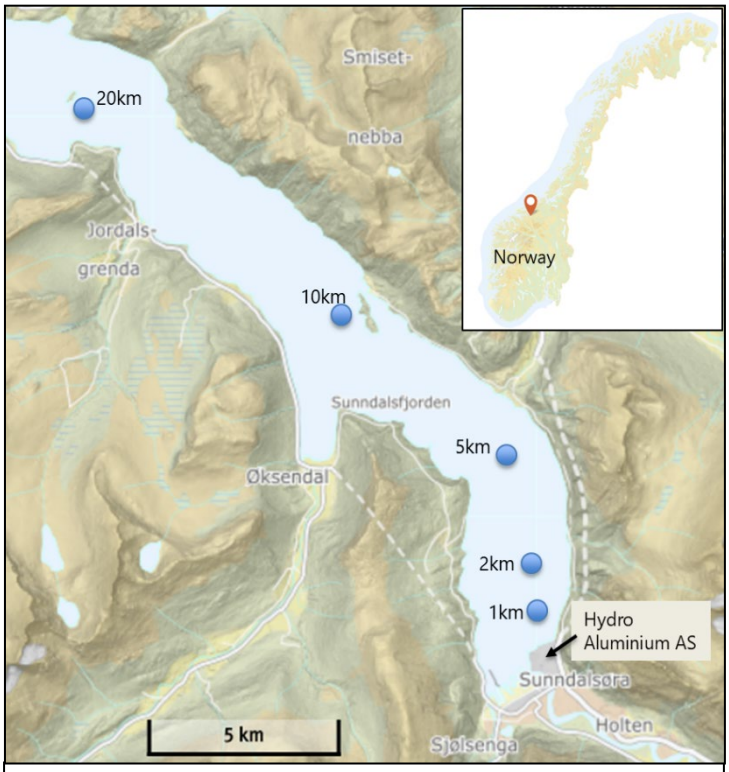

Figure 1. Location of the aluminium smelter at the end of the Sunndals fjord, Norway and the approximate location of the mussels with distance from the smelter discharge outlet.

Approximately 100 mussels were attached to five mussel rigs, positioned at known distances from the aluminium smelters discharge outlet in the Sunndals fjord (Figure 1). The moorings, standing vertically

in the water column, consisted of a concrete anchor, rope, acoustic release and two 8 kg buoys with no surface marker buoy. They were positioned at 1, 2, 5, 10 and 20 km from the discharge outlet with the aid of a small boat and crew (G. Øye AS). The mussels were secured to the rope with cable ties and positioned at a depth of 18-20 m. To avoid contact with shipping vessels in the area, the top buoy of each mooring was held beneath the water line at a depth of 15 m.

The mussels were deployed on April 30th, 2019 and collected 6 weeks later on June 10th, 2019. Temperature sensors were deployed with the mussels and measured 8 ± 1˚C in the Sunndals fjord at all stations measured during the 6-week exposure. Mussels were retrieved by sending an individual release code to the acoustic release transponders, which detached from the anchor and enabled the mussels and rig to be collected from the surface of the water. The mussels were placed in a cooler box with wet kelp and cooling blocks and transported by airfreight to the NIVA laboratory in Oslo within 4 hours. The mussels were kept in the cooler box overnight and processed the following day. No mussel mortalities were observed during inspection.

2.2 Analysis of mussel samples

Length measurements were taken from all mussels sampled. Haemolymph was taken from 10 mussels for micronuclei (MN) assessment. In the same individuals, gill and digestive gland were removed and snap frozen in liquid nitrogen then stored at -80°C until used for measurements of lipid peroxidation (LPO). Haemolymph was taken from another 15 mussels for neutral red retention (NRR). The gills and digestive gland of these mussels were removed and snap frozen in liquid nitrogen then stored at -80°C until used for acetylcholine esterase (AChE) inhibition and histochemistry, respectively. Additional mussels were used for stress on stress (SoS) and for condition index (CI). Furthermore, three replicates of five mussels were pooled for chemical analysis. Details of each biological effects measurement and chemical analyses are provided below.

The remaining mussels that were held in filtered seawater at the marine research station for two weeks but were not used in the field exposure were sampled for the same chemical analysis and biological effect responses. These mussels were referred to as the Day 0 group, since they reflect the condition of the mussels at the start of the field exposure. The Day 0 mussels were sampled on the day after the field mussels were placed in the fjord.

2.2.1 Tissue chemistry

Mussels were opened by cutting through their posterior adductor muscle with a sterile scalpel, excess water was drained, and the soft tissue removed and placed in a high temperature treated (550°C) glass container. For each exposure group, triplicate samples of five mussels per sample were collected for analysis of 40 PAH and metal concentrations. Ultra-high-performance chromatography (UPLC) coupled to high resolution mass spectrometry (HRMS) was employed for the chemical analysis.

Metal analysis

Metal concentrations (As, Cd, Cr, Cu, Pb, Mn, Hg, Mo, Ni, V, Zn) were determined in homogenised whole soft tissue samples using inductively coupled plasma-mass spectrometer (ICP-MS, Perkin-Elmer Sciex ELAN 6000).

2.2.2 Condition index

The condition index (CI) was measured in fifteen mussels from each group by determining the ratio of the dry weight of the soft tissue divided by the valve dry weight multiplied by 100 (Moschino and Marin, 2006; Orban et al., 2002). The dry weight values were recorded after oven drying the shell and the soft tissue at 80°C for 24 h.

$$CI = \left(\frac{soft\ tissue\ dry\ weight\ (g)}{shell\ dry\ weight\ (g)}\right) x\ 100$$

2.2.3 Stress on stress

The stress on stress (SoS) assessment was measured with fifteen mussels from each group. Mussels were placed in a humid chamber at 15 ± 0.5°C with a 16 h: 8 h light dark cycle. The mussels were checked every 24 ± 4 h and mortalities were recorded and removed from the incubator. Mussels were considered deceased if their shells were gaping and showed no sign of movement after gentle tapping on their shells.

2.2.4 Neutral red retention

Lysosomal stability was measured in mussel haemocytes using the neutral red retention (NRR) procedure adapted from Lowe and Pipe (1994). Approximately 0.1 ml of haemolymph was removed from the adductor muscle of the mussel with a syringe containing approximately 0.1 ml of filtered (0.2 µm) seawater. The haemolymph/saline solution was placed in a microcentrifuge tube, from which a 40 µl sample was removed and pipetted onto the centre of a microscope slide. The slide was left in a

dark humid chamber for 15 min to allow the cells to adhere to the slide. Excess liquid was removed from the slide after this time and 40 µl of neutral red solution added. The neutral red solution was taken up inside the haemocytes and stored within the lysosome of the mussel. The ability of the lysosome to retain the neutral red solution was checked every 15 min by light microscopy (x400 magnification). The test was terminated and the time recorded when greater than 50% of the haemocytes leaked the neutral red dye out of the lysosome into the cytosol.

2.2.5 Micronuclei formation

Approximately 0.1 ml of haemolymph was removed from the posterior adductor muscle of the mussel with a syringe and needle (0.6 ml) containing 0.1 ml of PBS buffer (100 mM PBS, 10 mM EDTA). The haemolymph and PBS buffer mixed solution was placed on a microscope slide in a humid chamber for 15 min to enable the haemocytes to adhere. The adhered haemocytes were fixed with 1% glutaraldehyde in 100 mM PBS for 5 min, rinsed in PBS buffer and left to air-dry in the dark overnight. Slides were stained with 1 µg/ ml bisbenzimide 33258 (Hoechst) solution for 5 min, rinsed with distilled water and mounted in glycerol McIlvaine buffer (1:1). The frequency of MN was measured on coded slides without knowledge of the exposure status of the samples to eliminate bias. The frequency of micronuclei in haemocytes was determined microscopically (×100 objective) on a minimum of 2500 cells per exposure group. Micronuclei were scored in cells with intact cellular and nuclear membranes when: 1) nucleus and micronuclei have a common cytoplasm, 2) colour intensity and texture of micronuclei is similar to the nucleus, 3) the size of the micronuclei is equal or smaller than 1/3 of the nucleus, 4) MN are apparent as spherical structures with a sharp contour.

2.2.6 Acetylcholine esterase inhibition

Acetylcholine esterase (AChE) activity was determined in the gills of fifteen mussels. Gills were homogenized on ice in five volumes of Tris–HCl buffer (100 mM, pH 8.0) containing 10% Triton and the resulting homogenate was centrifuged at 12,000 g for 30 minutes at 4°C. Measurements of AChE activity were performed following the method described by Bocquené and Galgani (1998). This method is based on the coupled enzyme reaction of acetylthiocholine (ATC) as the specific substrate for AChE and 5,50-dithio-bis-2-nitrobenzoate as an indicator for the enzyme reaction at 405 nm using a molar extinction coefficient of 13.6 mM/cm. AChE activity was expressed in nmol of ATC per min per mg of total protein.

2.2.7 Lipid peroxidation

Lipid peroxidation (LPO) was evaluated by determining malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE), both by-products of polyunsaturated fatty acid peroxidation, following the method described by Erdelmeier et al. (1998). Briefly, the gills of 10 mussels were homogenized in 3 volumes of 0.02 M Tris-HCl containing 0.5 M BHT (pH 7.4) at 4ºC. The resulting homogenate was centrifuged at 15,000 g for 20 minutes at 4ºC and the supernatant used for total protein determination and LPO analysis. LPO analysis was based on the reaction of two moles of N-methyl-2-phenylindole (3:1 mixture of acetonitrile/methanol), a chromogenic reagent, with one mole of either MDA or 4-HNE under acidic conditions (methanesulfonic acid) at 45°C for 60 min to yield a stable chromophore. Malondialdehyde bis-(1,1,3,3-tetrametoxypropane) was used as a standard at a maximal absorbance of 586 nm. LPO levels were expressed as nmol MDA + 4-HNE per gram of total protein.

2.2.8 Total protein concentration

Total protein concentration was measured in the cytosolic fractions of the gill samples used for AChE activity and LPO levels according to the Lowry method (Lowry, 1951) using Bovine Immunoglobulin G (IgG) as a standard.

2.2.9 Histochemical methods

Frozen sections (10 µm) of digestive gland tissue were prepared on a cryostat (Leica CM1860), with object and knife temperatures set at -20 and -18°C respectively. The freshly cut sections were placed on labelled microscope slides with duplicate sections prepared for each mussel. Separate slides were prepared for the three histochemical endpoints: 1) Volume of basophilic cells to digestive cells (VvBAS); 2) neutral lipid (NL); and 3) lipofuscin (LF) accumulation. The slides were kept frozen (-20°C) for 24 h before they were fixed and stained for the different endpoints.

VvBAS: The sections were fixed in Baker's calcium formol for 5 min, rinsed briefly in distilled water and stained with Gills haematoxylin for 15 seconds. The sections were then rinsed in flowing tap water for 20 min and stained with eosin-phloxin solution for 30 seconds, before being rinsed in 80% ethanol and allowed to air dry for a few hours before mounted with Euparal.

The volume density of basophilic cells in the digestive gland of mussels was determined microscopically by means of stereology using a Weibel graticule eye piece (M-168; Weibel, 1979). Counts were made

in 10 fields of view for each mussel (400 x magnification). The volume density of basophilic cells (VvBAS) was calculated using the equation:

$$VvBAS = (X1 + X2 + \ldots + Xn)/ (m \times n)$$

Where X = number of segment edges (from Weibel graticule) falling on basophilic cells; m = total number of segment edges falling on digestive tissue; n = number of counts (10 for each mussel).

NL: The sections were fixed in Baker's calcium formol for 15 min, rinsed briefly in distilled water and placed in 60% triethyl phosphate for 1 min before staining with oil red O solution for 15 min. The sections were then rinsed with 60% triethyl phosphate and distilled water and air-dried overnight before mounting in glycerol gelatin. The accumulation of neutral lipid was evaluated microscopically (x400 magnification). The percentage area of tissue section covered by neutral lipids was assessed in 15 randomly selected fields of view for each mussel by semi-quantitative grading, with 10 mussels analysed per group.

LF: The digestive gland sections were fixed in Baker's calcium formol for 15 min, rinsed in distilled water and incubated for 5 min in Schmorl's solution (1% ferric chloride, 1% potassium ferricyanide ratio 3:1). The slides were washed in 1% acetic acid for 1 min, rinsed in distilled water and mounted in UV-free mounting media. The percentage accumulation of lipofuscin in 15 microscope fields of view (x 400 magnification) was assessed using computer assisted image analysis (Cell-D, Olympus).

2.3 Statistical analysis

Statistical analyses and graphs for all individual biomarkers (except SoS) were performed using Statistica 13 (Dell). All data were tested for normality and homogeneity of variance with a Levene's test. Since homogeneity of variance was achieved, significant differences between groups were detected using one-way analysis of variance (ANOVA) and the Tukey post-hoc test, with the level of significance set at p=0.05.

2.3.1 Integrated assessment

The Integrative Biological Response (IBR) index was developed to systematically combine a suite of biomarker responses in order to provide a holistic evaluation of organism health status following chemical exposure (Beliaeff and Burgeot, 2002). The IBR/n, which accounts for the number of biomarkers in the data set, was used to integrate the biomarker data (Broeg and Lehtonen, 2006). In the present study CI, SoS, NRR, MN, AChE, LPO, VvBAS, NL and LF were selected for the IBR calculation.

The inverse values of CI, SoS, NRR, and AChE were used since a decrease was reflective of an adverse impact. The IBR index was calculated by summing-up triangular star plot areas for each two neighbouring biomarkers in a data set.

2.3.2 Principle component analysis (PCA)

A Principal component analysis (PCA) was performed using XLStat2019® (Addinsoft, Paris, France) to highlight the main variables responsible for the variance of data obtained for all groups. A Pearson's correlation analysis was also performed to evaluate the strength of association between chemical body burden and biological responses of mussels. The level of significance was set to p=0.05.

3 Results

3.1 Chemical concentrations in mussels

3.1.1 PAH

The concentration of PAH in mussels from the day zero group (T0) and in mussels located at specific distances downstream from the aluminium smelter are shown in Figure 2 to Figure 5. Highest concentrations of PAH16 were found in mussels from the T0 group (Figure 2). For the field transplanted mussels, highest concentrations were measured in those mussels positioned within 5 km of the smelter, whilst lowest concentrations were measured in mussels 10 and 20 km away. A very similar profile to PAH16 was also shown when adding up the measured concentrations of 41 PAH compounds in the mussel groups (Figure 3).

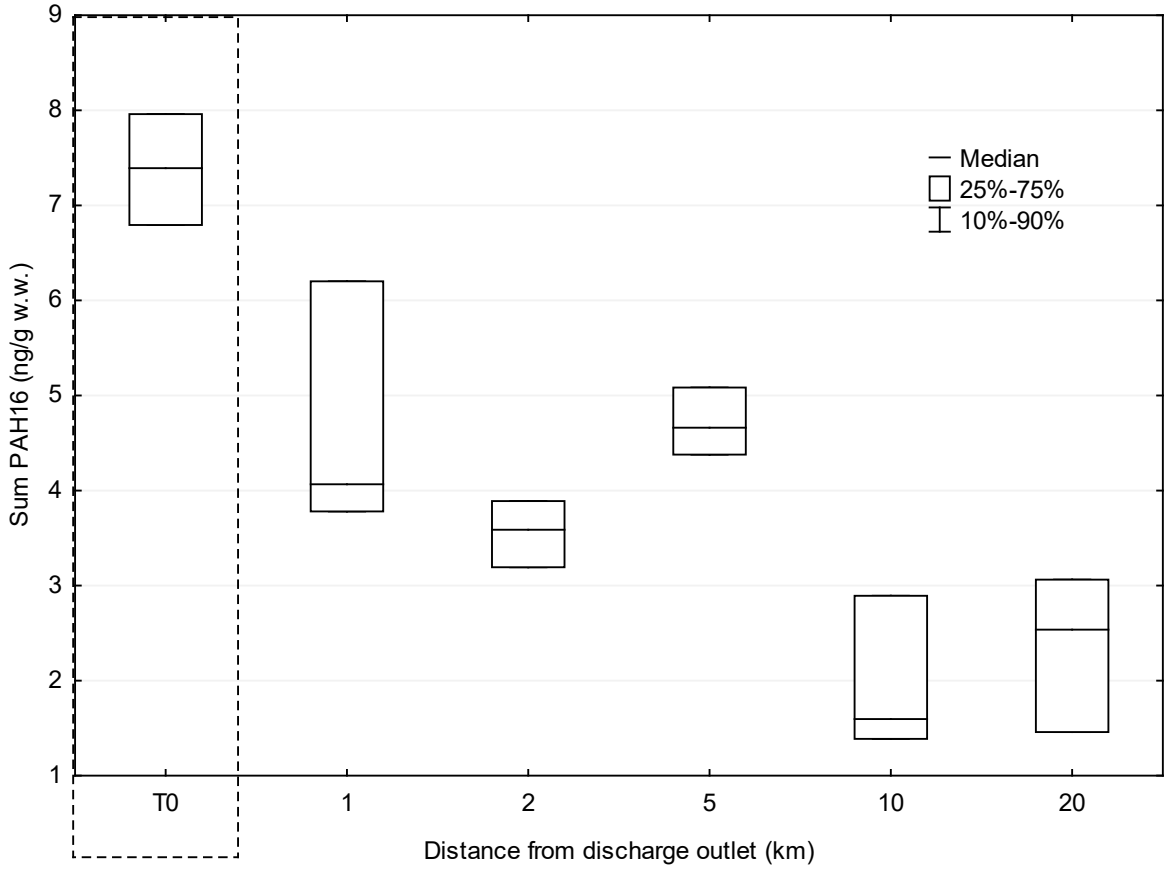

Figure 2. Sum of PAH16 in mussels from the day zero group (T0) and in mussels located at different distances from the Sunndals aluminium smelters discharge outlet (median, quartiles, 10/90 percentiles, n=3).

The sum of 41 PAH only marginally increased the PAH concentration in the mussels above that of PAH16, which indicated that the PAH16 contributed most to the measured PAH accumulation in the

mussels. For both the sum of naphthalenes (Figure 4) and the sum of phenanthrenes (Figure 5), the T0 group measured higher PAH concentration than the field exposed mussels. Equally low concentrations of naphthalenes and phenanthrenes were measured in all field exposed groups.

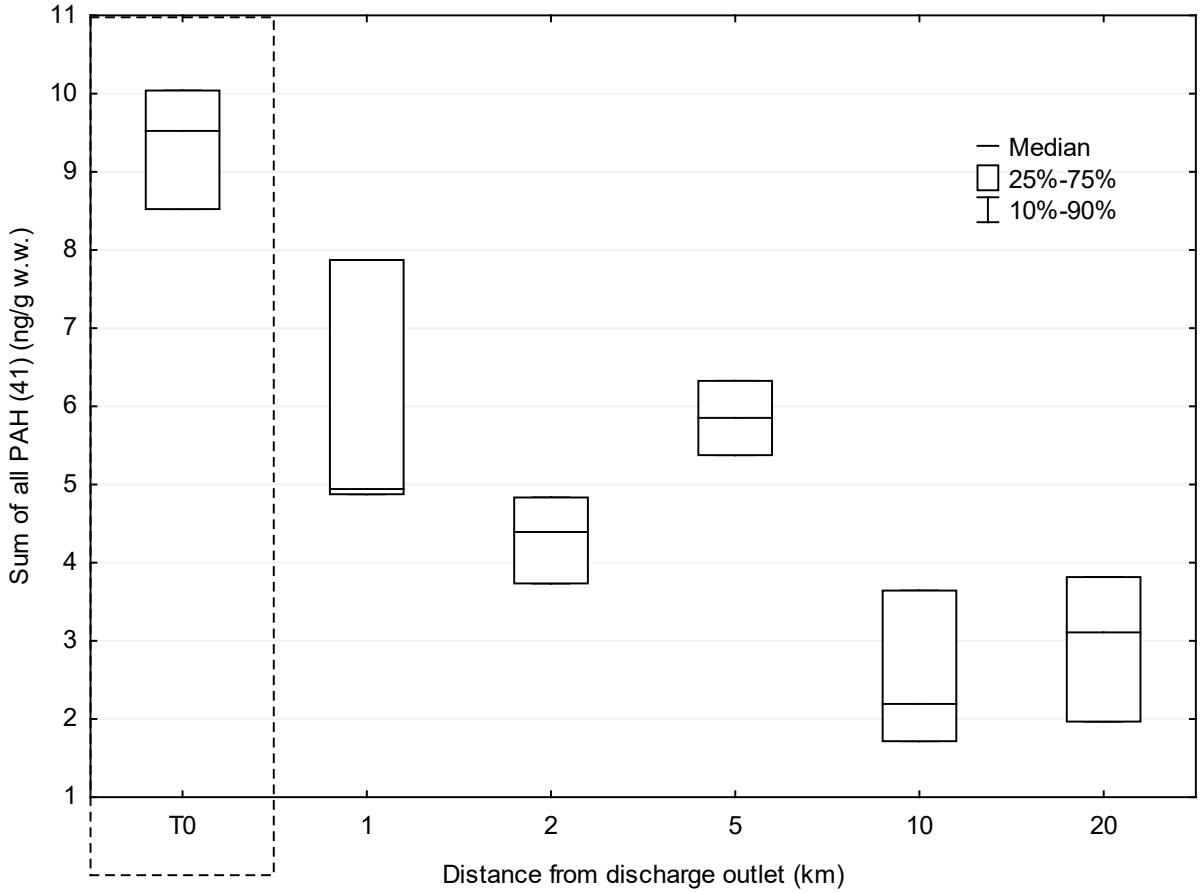

Figure 3. Sum of all PAH compounds measured (41) in mussels from the day zero group (T0) and in mussels located at different distances from the Sunndals aluminium smelters discharge outlet (median, quartiles, 10/90 percentiles, n=3).

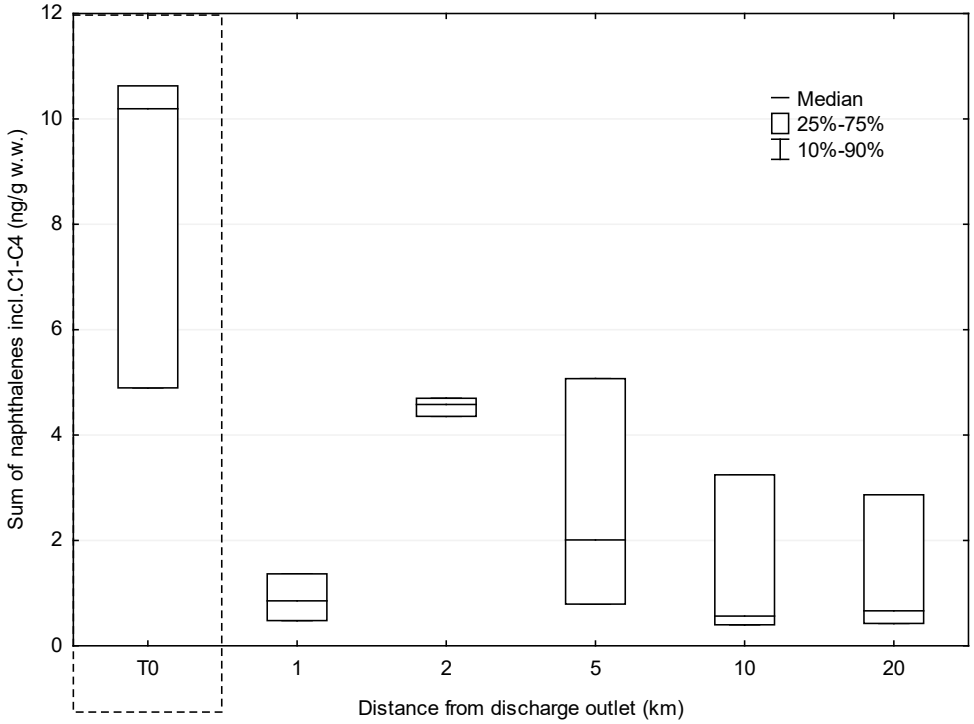

Figure 4. Sum of naphthalenes including C_1 to C_4 in mussels from the day zero group (T0) and in mussels located at different distances from the Sunndals aluminium smelters discharge outlet (median, quartiles, 10/90 percentiles, n=3).

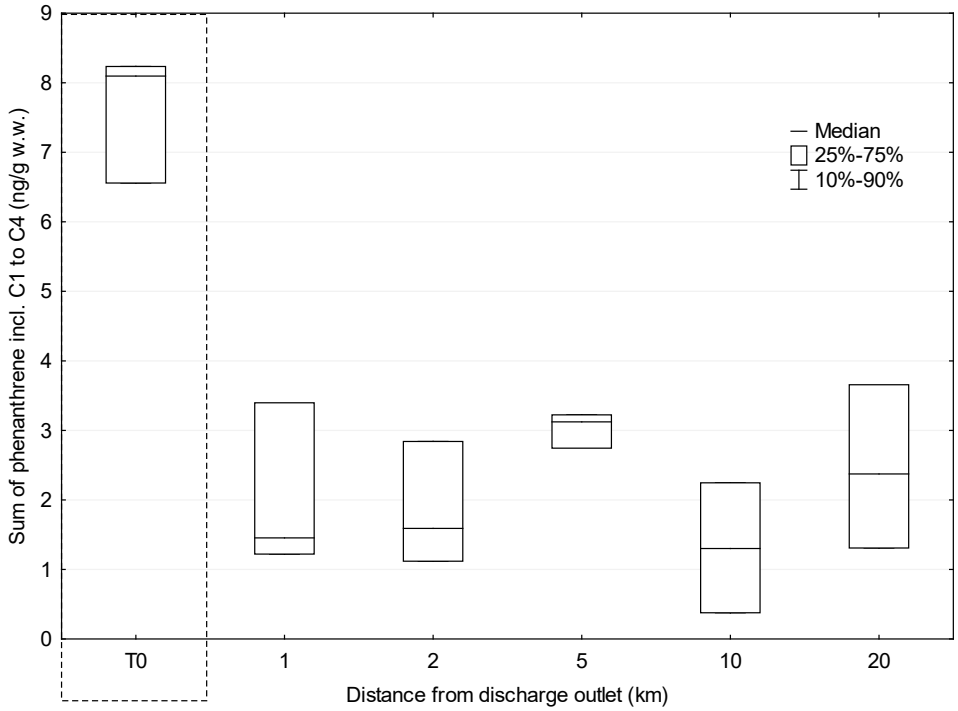

Figure 5. Sum of phenanthrenes including C_1 to C_4 in mussels from the day zero group (T0) and in mussels located at different distances from the Sunndals aluminium smelters discharge outlet (median, quartiles, 10/90 percentiles, n=3).

The PAH concentrations in mussel tissue with respect to the number of aromatic rings (2 to 6) are shown in Figure 6. The total PAH includes also the alkylated PAH and appear therefore higher than the total PAH shown in Figure 3. In general, a larger number of aromatic rings indicates a heavier PAH. The data shows that 2-4 ring PAHs are desorbed during the 6-week deployment period from the starting concentration in the day zero (T0) mussel group. However, 5-6 ring PAHs are not desorbed in the mussels positioned 1 km from the smelter, partly desorbed in mussels 2 and 5 km away and desorbed in mussels 10 and 20 km away. No 6 ring PAHs were detected in mussels 20 km away from the smelter.

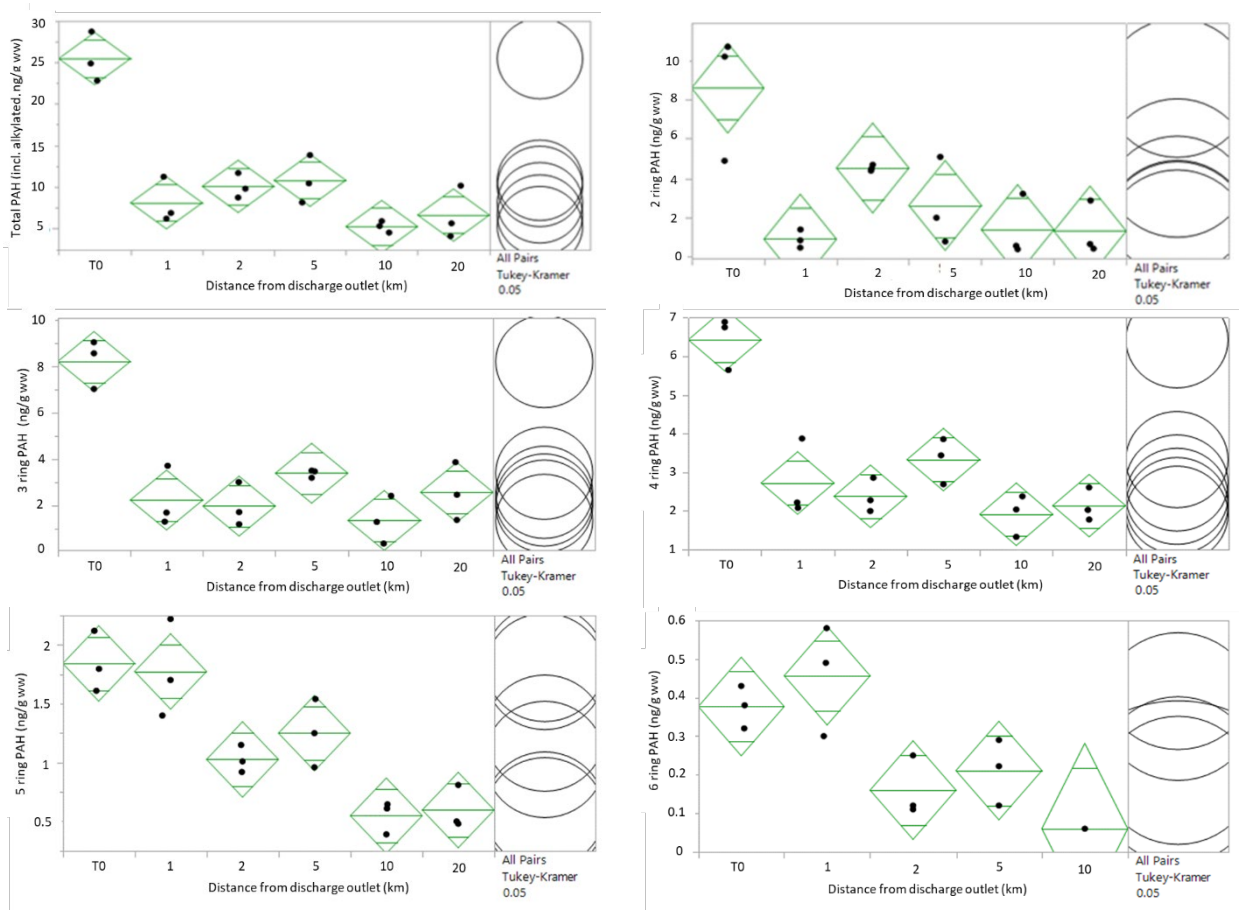

Figure 6. PAH concentrations in mussels from the day zero group (T0) and in mussels located at different distances from the Sunndals aluminium smelters discharge outlet. The results are grouped with respect to the number of aromatic rings, from lighter PAH (2-3 rings) to heavier PAH (5-6 rings).

3.1.2 Metals

The metal concentrations measured in the whole mussel homogenates of day zero and field transplanted mussels are shown in Table 1. For the 11 metals measured, there were no clear differences between metal concentration and proximity to the aluminium smelter. Elevated

concentrations of manganese were measured in T0 mussels (median 11 mg/kg w.w.) compared to the field transplanted mussels (median 0.011 to 0.022 mg/kg w.w.).

Table 1. Accumulation of metals in the soft tissue of mussels from the day zero group (T0) and in mussels located at different distances from the Sunndals aluminium smelters discharge outlet (n=3, median value in bold for each group, mg/kg w.w.)

Mussel group (km from smelter)	As	Cd	Cr	Cu	Pb	Mn	Hg	Mo	Ni	V	Zn
1	3.2	0.23	**0.34**	1.3	**0.09**	1.8	0.015	0.3	**0.4**	**0.3**	**20**
	2.5	**0.16**	0.52	**1.2**	0.1	2.1	**0.017**	**0.3**	0.5	0.4	15
	2.7	0.15	0.37	1.1	0.08	**1.9**	0.024	0.2	0.4	0.3	35
2	2.8	0.17	0.22	1.1	0.09	1.1	0.014	0.3	**0.4**	**0.3**	**17**
	2.7	0.2	0.38	**1.1**	**0.09**	**1.7**	**0.011**	**0.3**	0.5	0.3	13
	2.9	**0.18**	**0.29**	1.1	0.08	1.7	0.01	0.2	0.4	0.2	17
5	2.9	**0.18**	**0.35**	1.1	**0.08**	**1.6**	0.028	**0.3**	**0.5**	**0.3**	**32**
	3.2	0.14	0.25	**1.1**	0.07	1.4	**0.022**	0.2	0.3	0.2	42
	2.8	0.18	0.53	1.2	0.08	1.9	0.01	0.3	0.6	0.3	20
10	2.4	**0.18**	0.15	**1**	**0.07**	0.8	0.006	0.2	0.3	**0.2**	**19**
	2.9	0.16	**0.15**	1.2	0.06	**0.9**	**0.009**	**0.2**	**0.3**	0.2	17
	2.7	0.19	0.24	1	0.09	0.9	0.025	0.3	0.4	0.2	34
20	3.2	0.21	0.17	1.3	0.08	**0.9**	**0.011**	0.5	0.4	**0.2**	21
	2.7	**0.18**	0.12	1.1	**0.07**	0.8	0.009	**0.4**	**0.3**	0.2	14
	2.9	0.18	**0.13**	**1.3**	0.05	1	0.011	0.4	0.3	0.2	**17**
T0	2.8	0.18	**0.24**	1.1	0.12	**11**	0.008	0.1	**0.4**	**0.3**	15
	3	0.16	0.16	**1.1**	**0.14**	8.8	**0.013**	**0.1**	0.2	0.3	**19**
	2.8	**0.18**	0.39	1.2	0.16	14	0.015	0.1	0.5	0.4	25

3.2 Biomarker responses in mussels

3.2.1 Condition index

The condition index calculated by dividing the dry weight of the mussel soft tissue by the dry weight of the shell multiplied by 100, is shown in Figure 7. There were no significant differences found in condition index between the mussel groups (ANOVA, Tukey, p>0.05).

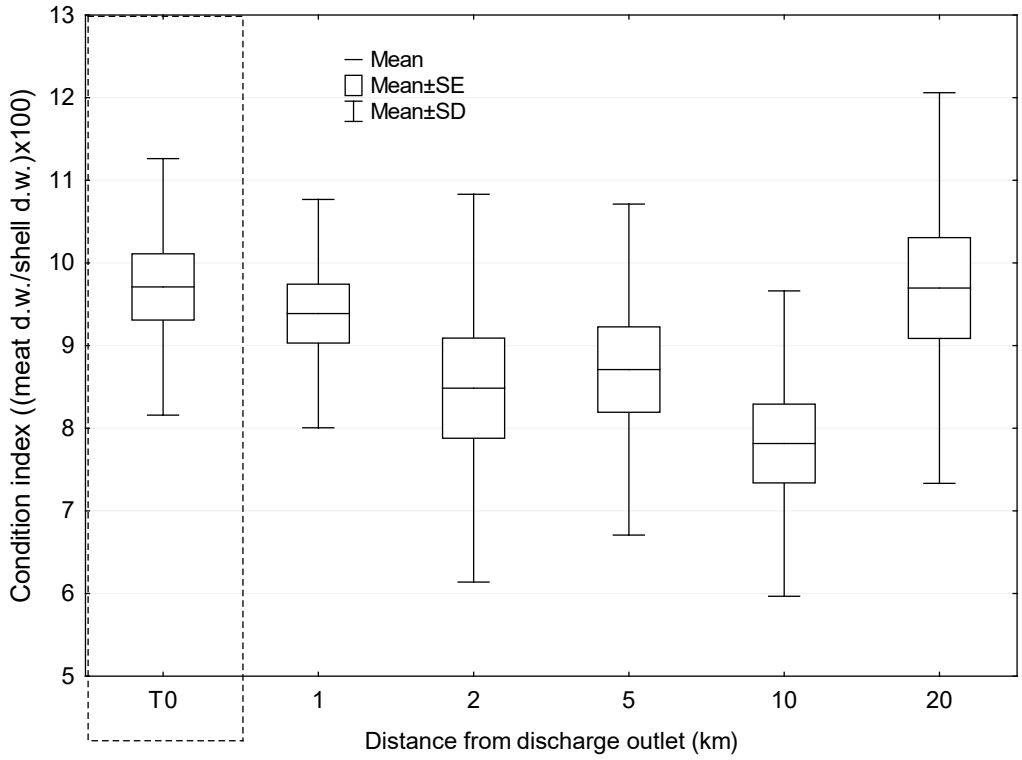

Figure 7. Condition index in mussels from the day zero group (T0) and in mussels located at different distance from the Sunndals aluminium smelters discharge outlet. Mean ± SD, n=15). No significant differences between the groups (ANOVA, Tukey p>0.05).

3.2.2 Stress on stress

The stress on stress test, which measures the duration of time that a mussel can survive out of water and provides an indication of the general fitness of the mussel, is shown in Figure 8. The survival curves of the different mussel groups were similar and LT_{50} values, which is the time needed to cause 50% mortality of the population, with a narrow range between 7 and 8.2 days. The lowest LT_{50} was observed in mussels 5 km from the smelter, whilst the highest were in mussels from 10 km and 1 km. Mussels from the T0 group did survive longer (14 days) than the other mussel groups (11 days).

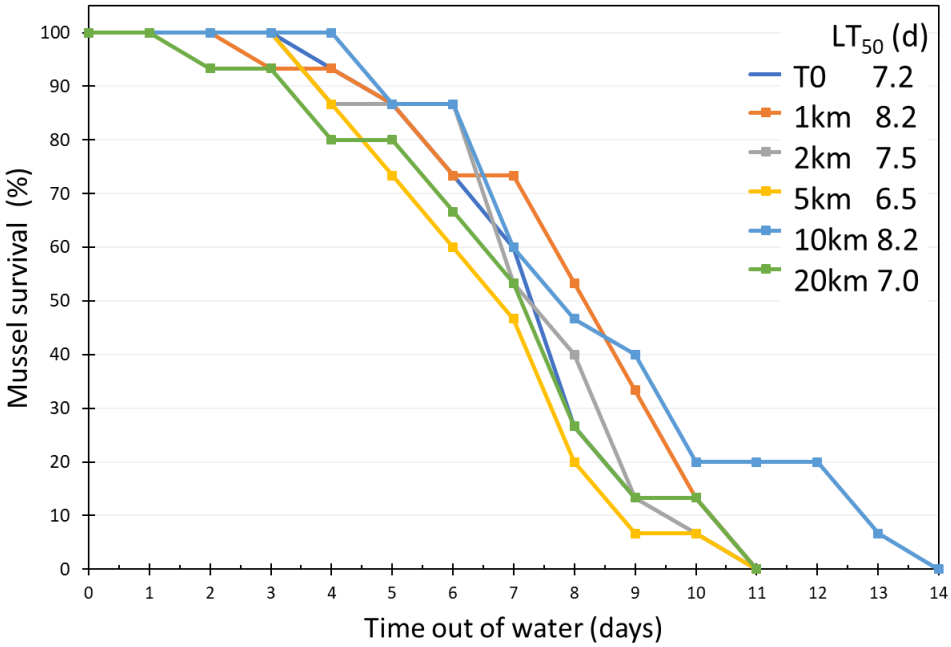

Figure 8. Stress on stress in mussels from the day zero group (T0) and in mussels located at different distances from the Sunndals aluminium smelters discharge outlet (n=15).

3.2.3 Neutral red retention

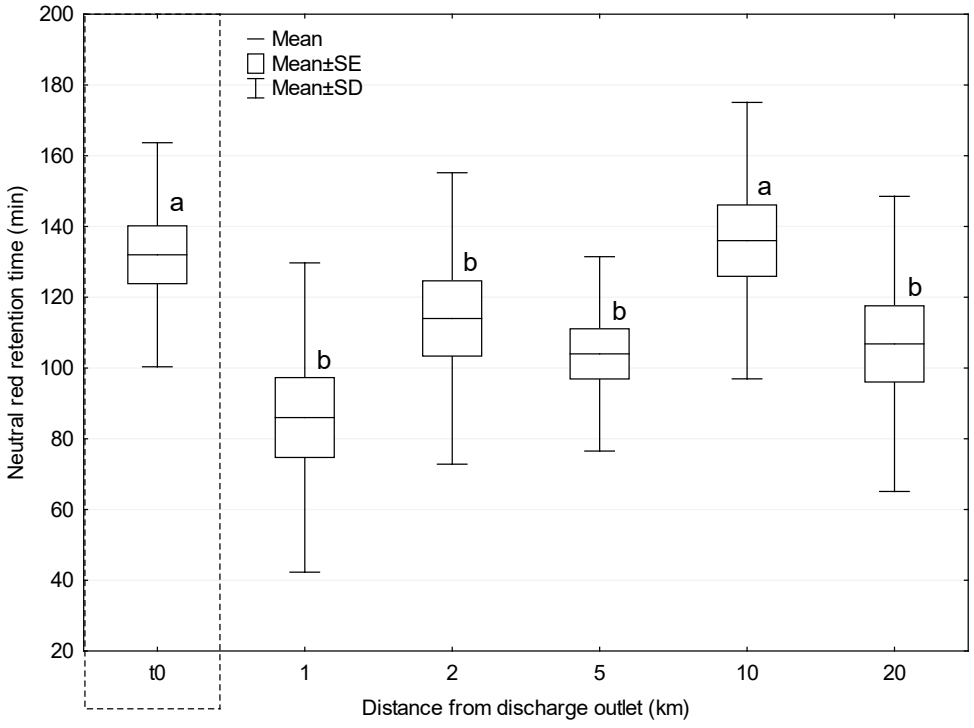

Figure 9. Neutral red retention (NRR) in mussels from the day zero group (T0) and in mussels located at different distances from the Sunndals aluminium smelters discharge outlet (Mean ± SD, n=15). Letters denote significant differences between groups (ANOVA, Tukey p<0.05).

Significant differences in NRR were found between the different mussel groups with highest values, indicating a better overall fitness of the mussel, in the time zero group and in the mussels positioned 10 km from the discharge outlet (Figure 9). The lower retention times were observed in the mussels located closest to the discharge outlet, with lowest values in mussel from 1 km away. However, significantly reduced lysosomal retention times were observed at the furthest location (20 km), indicating a stress response.

3.2.4 Micronuclei formation

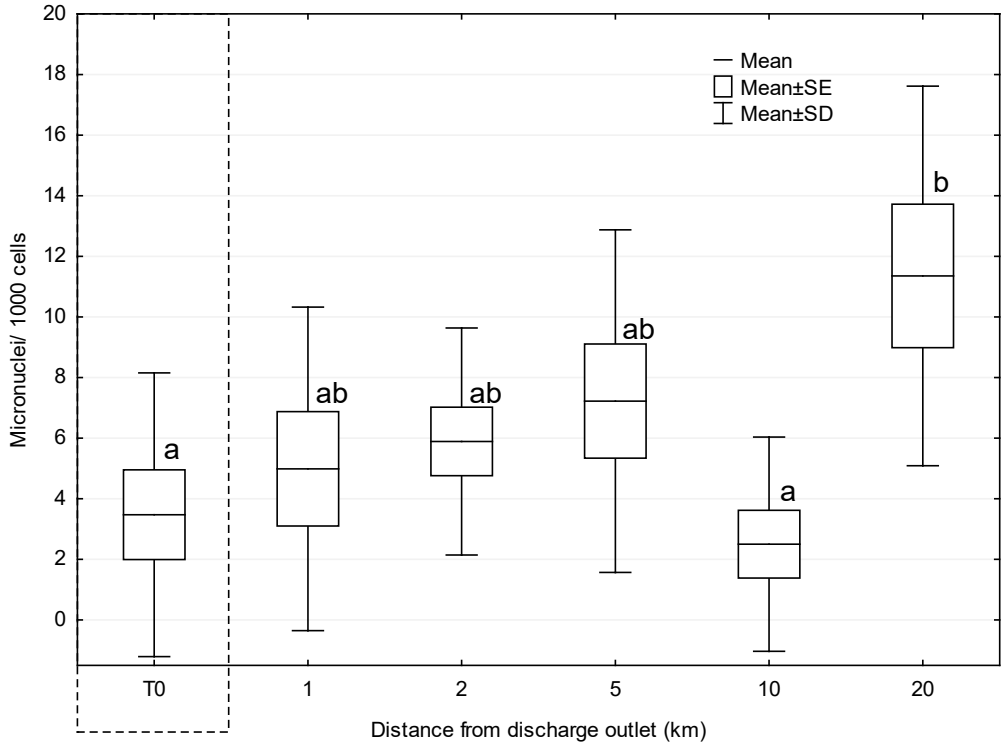

Figure 10. Frequency of micronuclei in mussels from the day zero group (T0) and in mussels located at different distances from the Sunndals aluminium smelters discharge outlet (Mean ± SD). Letters denote significant differences between groups (ANOVA, Tukey $p<0.05$).

Genotoxicity measured by the frequency of micronuclei in mussel haemocytes indicated significant differences between the mussel groups (Figure 10). Lowest micronuclei numbers were found in the time zero mussels and those located 10 km from the discharge. Although slightly elevated in mussel located 1 to 5 km from the discharge, these were not significantly elevated above the time zero and 10 km group. Highest and statistically significant MN numbers were observed in mussels located furthest from the discharge at 20 km.

Results showed a good agreement between the NRR and MN, with healthy mussels from time zero and those located 10 km from the discharge outlet, whilst impacts on mussels at 1 to 5 km and in the 20 km group.

3.2.5 Acetylcholine esterase inhibition

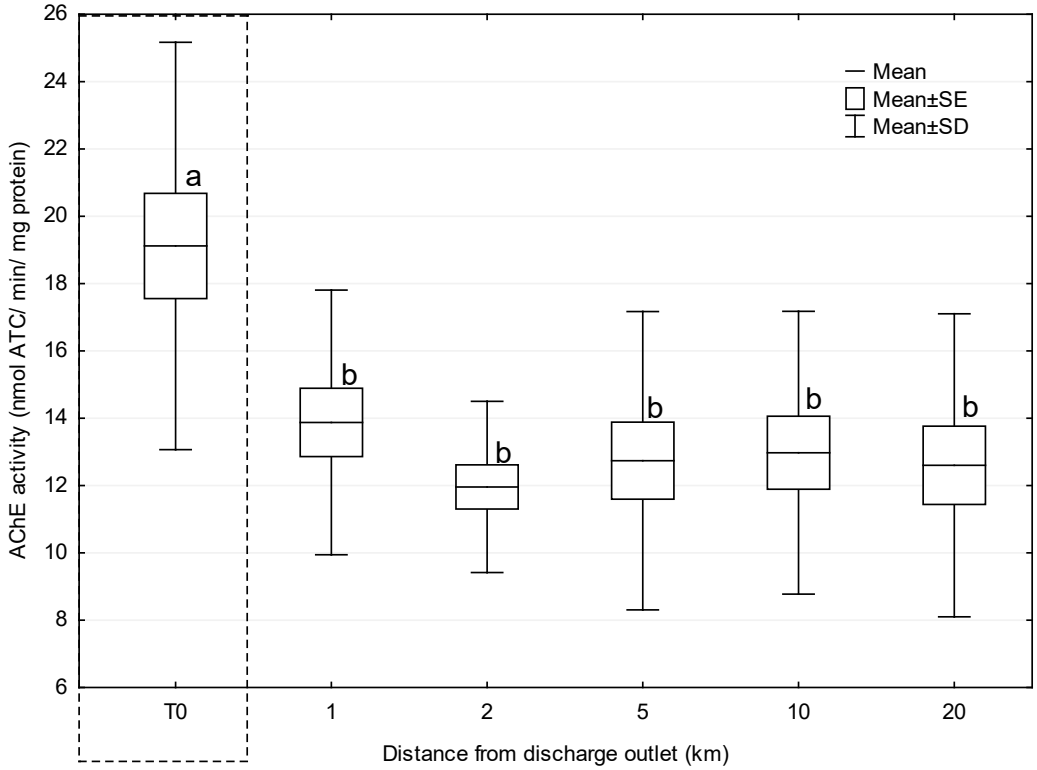

Figure 11. Acetylcholine esterase (AChE) inhibition in mussels from the day zero group (T0) and in mussels located at different distances from the Sunndals aluminium smelters discharge outlet (Mean ± SD). Letters denote significant differences between groups (ANOVA, Tukey $p<0.05$).

The exposure to neurotoxic compounds measured by the inhibition of the acetylcholine esterase (AChE) enzyme is shown in Figure 11. Compared to the time zero group all mussels had significantly lower levels of AChE activity. For the mussels located in the Sunndals fjord there was no significant difference in AChE activity between the mussel groups. BAC and EAC values are available for mussel at 26 and 19 nmol/ATC/ min/mg protein (Davies and Vethaak, 2012). Only the time zero mussels were above the EAC of 19 nmol/ATC/ min/mg protein, with all other mussel groups indicating a strong neurotoxic response.

3.2.6 Lipid peroxidation

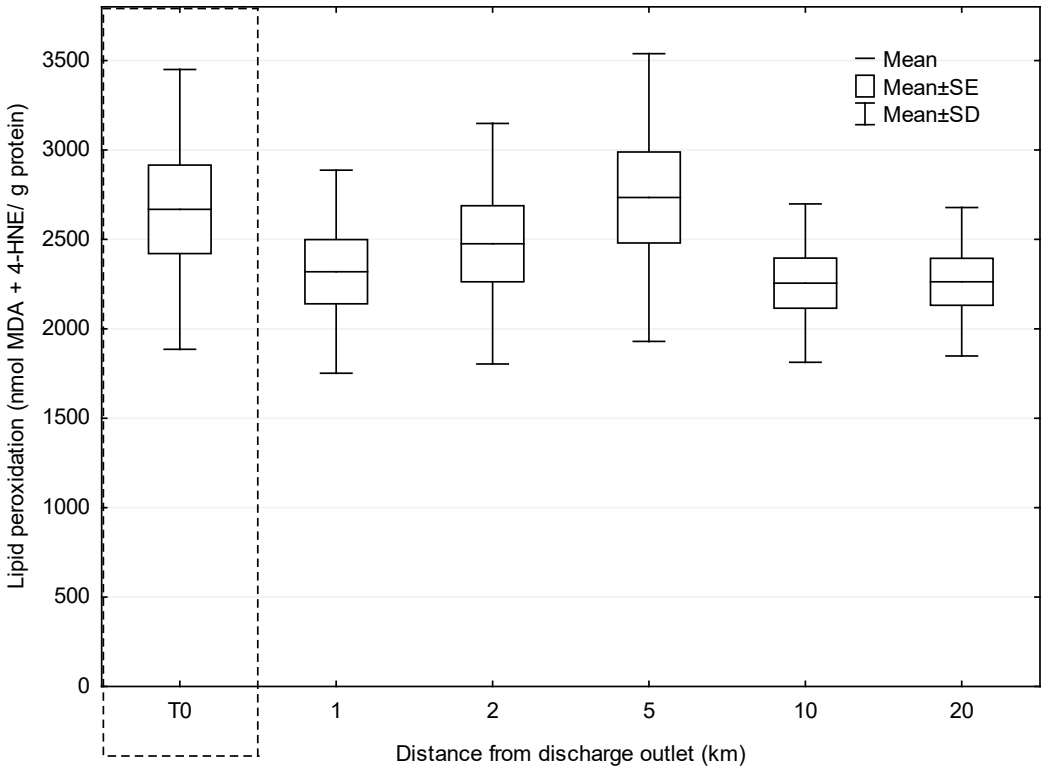

Figure 12. Lipid peroxidation in mussels from the day zero group (T0) and in mussels located at different distances from the Sunndals aluminium smelters discharge outlet (Mean ± SD). No significant differences between groups (ANOVA, Tukey p>0.05).

Lipid peroxidation measured as the quantity of MDA and 4-HNE in gill tissue samples of mussels is shown in Figure 12. Although slightly lower values were measured in the mussels furthest from the discharge (10 km and 20 km), there was no significant difference between the mussel groups. ICES assessment criteria are not currently available for LPO in mussels.

3.2.7 Volume of basophilic cells to digestive cells

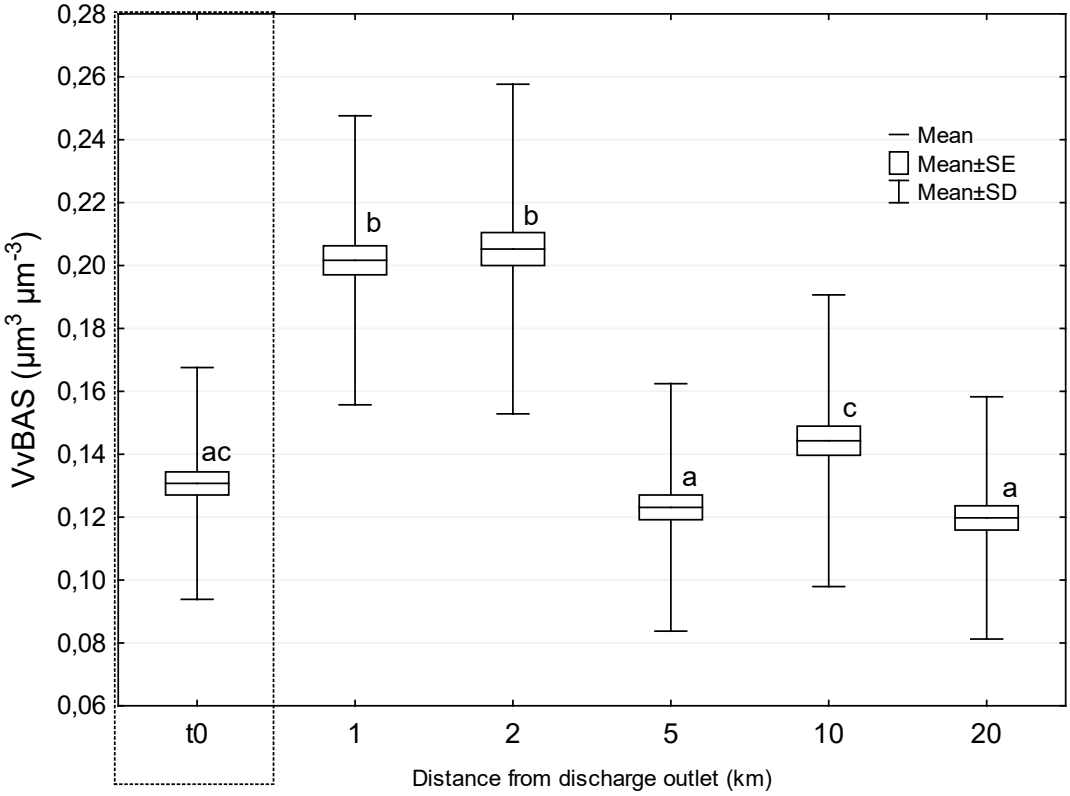

Figure 13. Volume of basophilic cells to digestive cells in the digestive gland of mussels from the day zero group (T0) and in mussels located at different distances from the Sunndals aluminium smelters discharge outlet. Mean ± SD. Letters denote significant differences between groups (ANOVA, Tukey $p<0.05$).

The change in cell composition of the mussel digestive gland, from mostly digestive cells in healthy individuals, to the increasing inclusion of basophilic cells in stressed mussels is shown in Figure 13. A significant increase in basophilic cells (VvBAS) and/ or reduction in digestive cells was found in the two mussel groups closest to the discharge outlet (1 and 2 km).

3.2.8 Neutral lipid accumulation

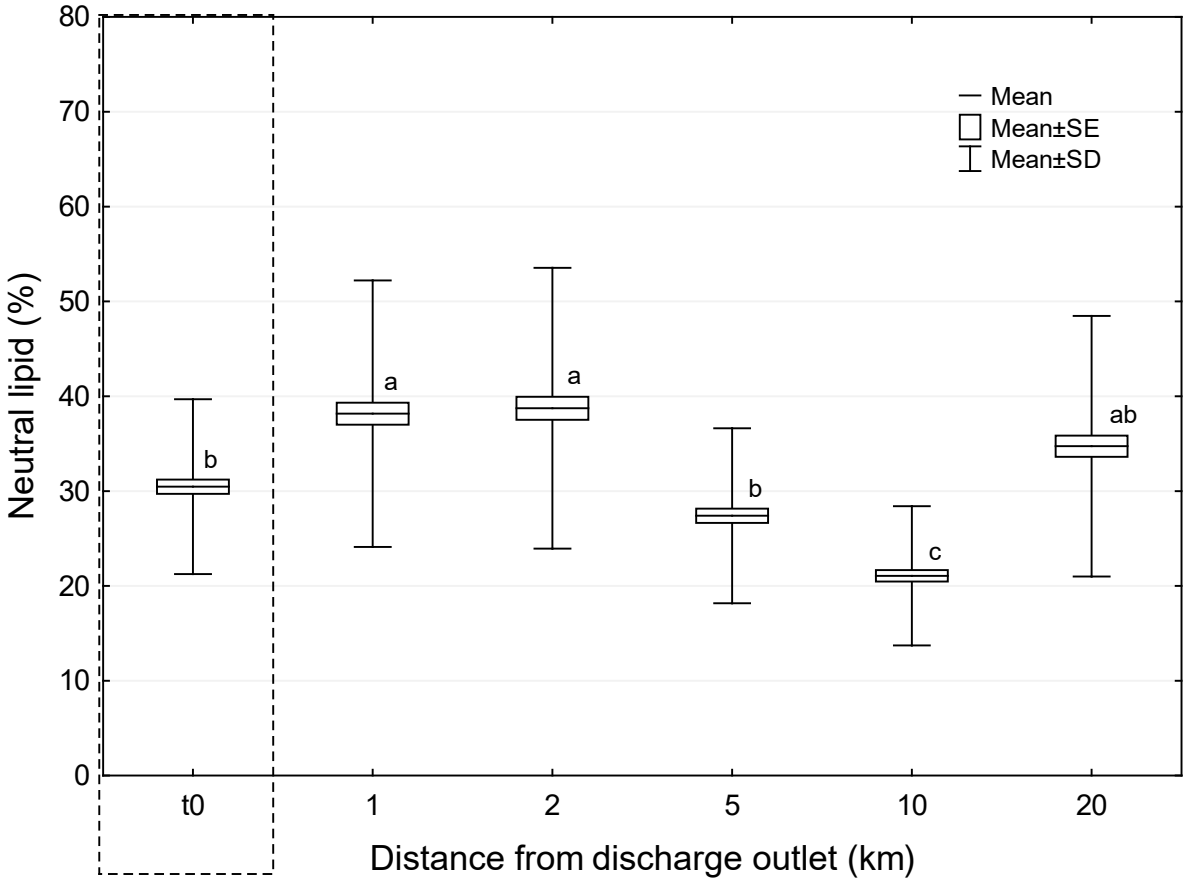

Figure 14. Neutral lipid accumulation in the digestive gland of mussels from the day zero group (T0) and in mussels located at different distances from the Sunndals aluminium smelters discharge outlet. Mean ± SD. Letters denote significant differences between groups (ANOVA, Tukey $p<0.05$).

The accumulation of neutral lipid in the digestive gland cells of mussels from the different groups are shown in Figure 14. As described for VvBAS, significantly higher neutral lipid accumulation was shown in mussels located closest to the discharge outlet (1 and 2 km). Significantly lower neutral lipid levels were found in mussels 10 km from the discharge outlet. However, mussel located at the furthest location from the discharge (20 km) had neutral lipid concentrations that were not significantly different from the two closest stations and indicates a different source of stress exposure impacting these mussels.

3.2.9 Lipofuscin accumulation

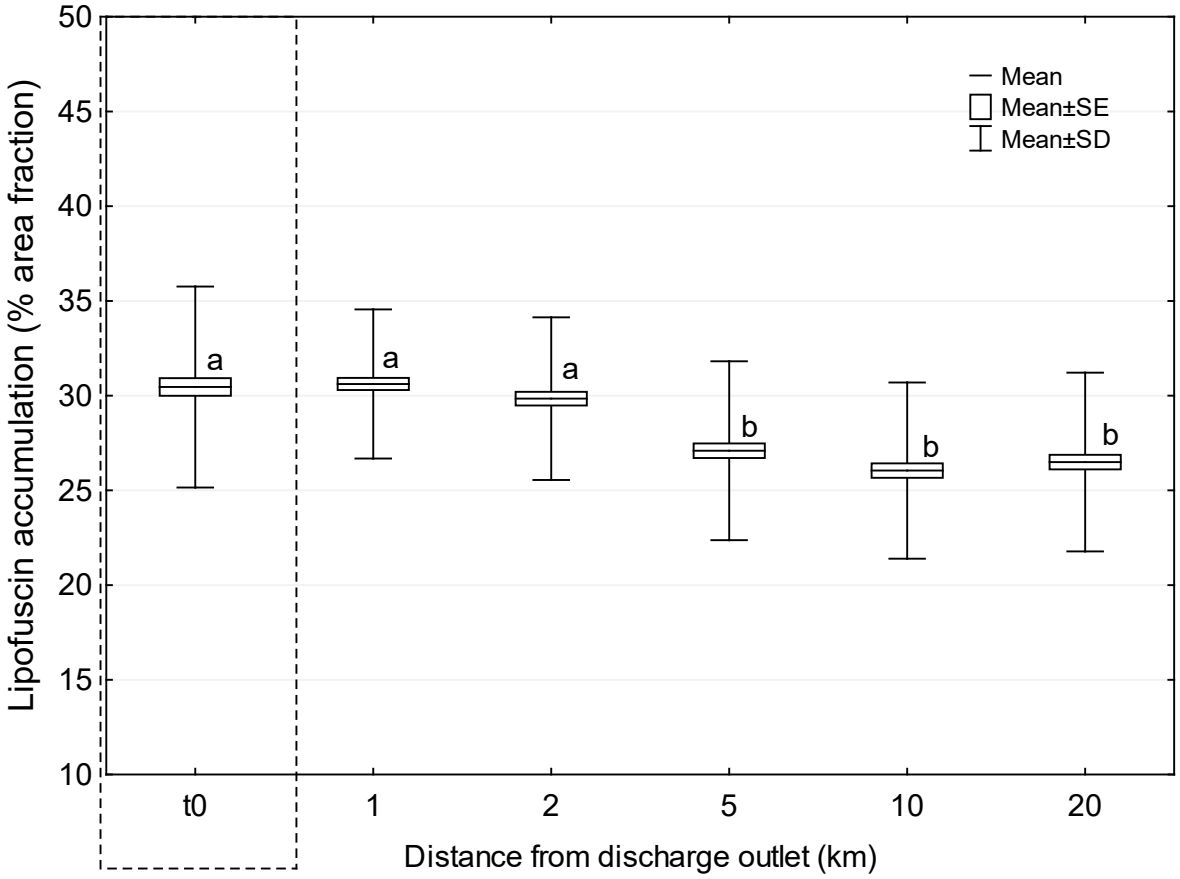

Figure 15. Lipofuscin accumulation in the digestive gland of mussels from the day zero group (T0) and in mussels located at different distances from the Sunndals aluminium smelters discharge outlet. Mean ± SD. Letters denote significant differences between groups (ANOVA, Tukey $p<0.05$).

Lipofuscin accumulation was significantly higher in the two closest stations to the Al smelter as well as the time zero group compared to the mussels at 5, 10 and 20 km away (Figure 15).

3.3 Integrated biological response

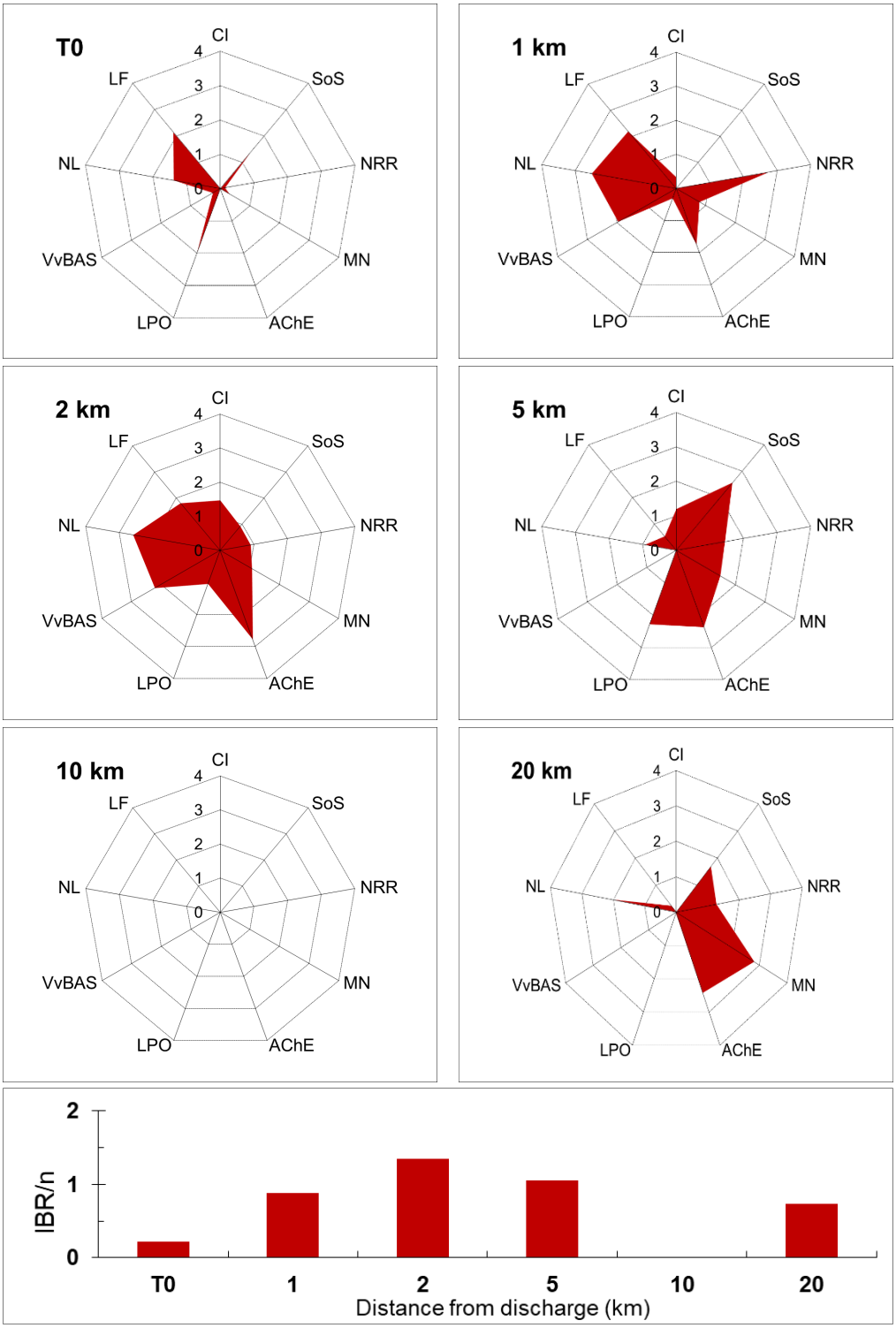

Figure 16. Integrated biological response (IBR/n) calculated from star plots of mean normalised biomarker data in mussels located at different distances from the Sunndals aluminium smelters discharge outlet.

The integrated biological response (IBR/n) was used to combine the nine individual biomarker results in order to provide an overall assessment of mussel health status from the different groups (Figure 16). The spider plots indicate the contribution of the different biomarkers to the overall IBR/n value. Higher IBR/n were observed in the mussels located at 1, 2 and 5 km from the discharge outlet, with lower IBR/n in mussels from the T0 and 10 km location. The histochemical markers, VvBAS and NL contributed most to the IBR/n score in mussels located 1 and 2 km from the discharge outlet, although for mussels at 2 km, smaller contributions were seen from all the biomarkers measured. In contrast, the 5 km mussels had contributions from AChE, MN as well as CI, SoS and NRR. The mussels at 20 km had an elevated IBR/n with contributions coming from SoS, NRR and MN. This may suggest a different source of exposure compared to those closer to the aluminium smelter discharge outlet.

3.4 Principal component analysis

Principal component analysis (PCA) was used to discriminate the main variables responsible for the variance of chemical body burden and biological effects measured in transplanted mussels (Figure 17). Mussels from the day zero group were excluded from the PCA, as the high concentrations measured for some chemicals were masking the overall contribution of variables within the mussel groups located in the Sunndals fjord. Overall, the PCA showed a clear spatial differentiation between mussel groups, highlighting the different responses obtained in relation to proximity to the discharge outlet. PC1 accounted for 41.0% of variance and showed a separation between the 3 groups located closest to the discharge outlet and those furthest away (10 and 20 Km). PC2 explained 24.2% of the variance and differentiated between the two mussel groups positioned furthest from the discharge outlet (10 and 20 km). The PCA confirmed that mussels located 1 and 5 Km from the discharge outlet are the most environmentally stressed, followed by mussels 2 km away. Mussels from these stations presented the highest concentrations of PAH 16, PAH 41, Total PAHs (including alkylated), Mn, V, Ni, Cr, Hg, Zn, Pb and Sum of naphthalene (including C_1 to C_4), associated with stronger responses in lipofuscin accumulation, lipid peroxidation, volume of basophilic cells and AChE. As expected, mussels positioned further away from the Sunndals aluminium smelters discharge were the less impacted groups. Mussels located 10 km from the discharge source presented higher SoS and NRR levels, indicative of a good health status in comparison with the other groups. Conversely, mussels at 20 Km had higher MN levels, closely associated with maximum concentrations of Mo, Cd, Cu and pyrene (including C_1 and C_2). This mussel group also presented the highest CI.

Correlation analysis showed some statistically significant associations between the chemical measurements and the biological responses in the mussel transplanted group (Tables A1 and A2 in Appendix A). As for the PCA, the data obtained for the control mussel group (T0) was not used in this analysis. The condition index was positively correlated with Cu, whilst lipid peroxidation was positively correlated with Total PAHs levels. Lipofuscin accumulation was positively correlated with Pb and V, while MN formation showed a positive correlation with Mo. On the other hand, stress on stress showed a negative correlation with As and phenanthrene.

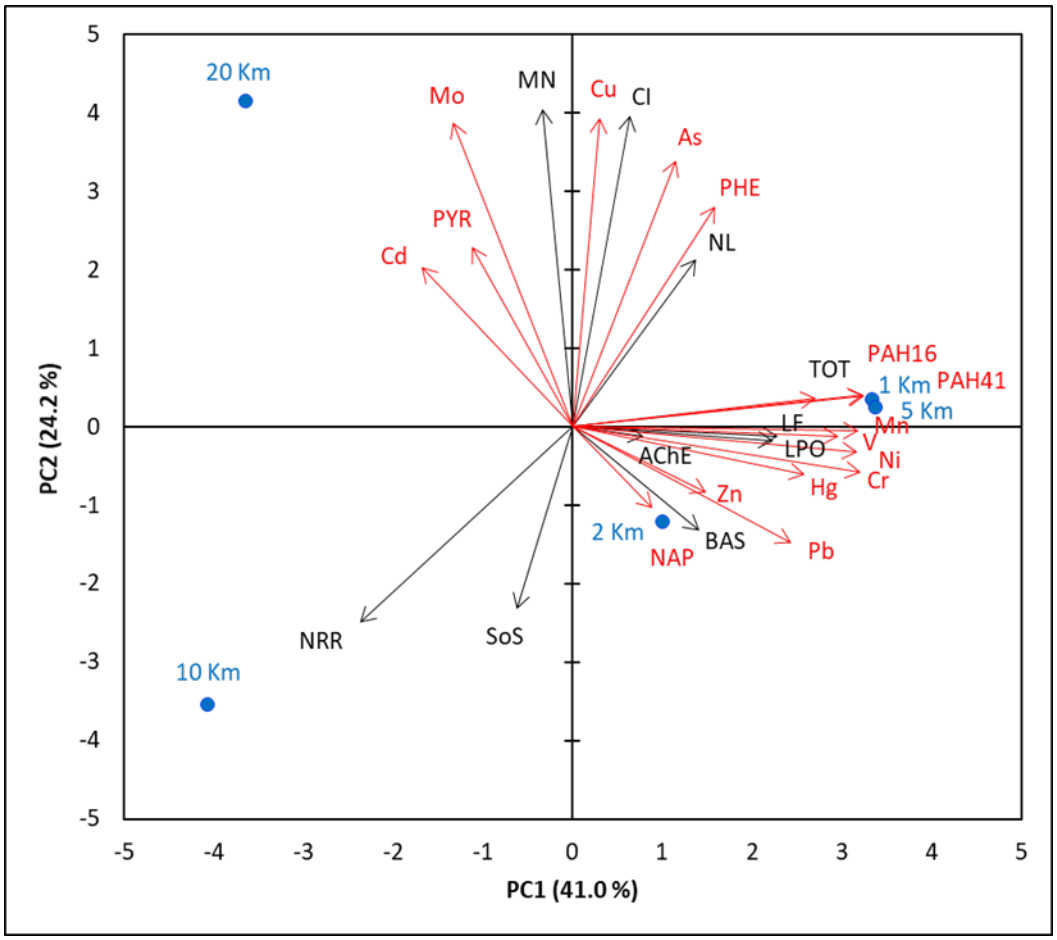

Figure 17. Principal Component Analysis of chemical measurements (red) and biological responses (black) in mussels located at different distances from the Sunndals aluminium smelters discharge outlet (blue). CI – Condition index; SoS – Stress on stress; NRR – Neutral red retention; MN – Micronuclei formation; AChE – Acetylcholine esterase activity; LPO – Lipid peroxidation; BAS – Volume of basophilic cells; NL – Neutral lipid accumulation; LF – Lipofuscin accumulation; PAH16 – Sum of PAH16; PAH 41 – Sum of PAH 41; NAP – Sum of naphthalene including C_1 to C_4; PHE – Sum of phenanthrene including C1 to C4; PYR – Sum of pyrene including C_1 and C_2; TOT – Total PAHs including alkylated; AS – Arsenic; Cd – Cadmium; Cr – Chromium; Cu – Copper; Pb – Lead; Mn – Manganese; Hg – Mercury; Mo – Molybdenum; Ni – Nickel; V – Vanadium; Zn – Zinc.

4 Discussion

4.1 Contaminant concentrations in mussel tissue

4.1.1 Metals

The Norwegian classification system indicating the different levels of concern for the concentration of metals in marine mussels are shown in Table 2. The system is based on high background concentrations derived from a plethora of monitoring programmes and investigative research. This classification system has required updating, and the Norwegian provisional high reference contaminant concentrations (PROREF) have been developed in order to provide a better safety margin of environmental risk, which are also presented in Table 2.

Table 2. The Norwegian classification scheme for the relative risk of metal concentrations in marine mussels (mg/kg w.w., PAH16 µg/kg w.w.). Including the Norwegian provisional high reference contaminant concentrations (PROREF) developed in 2018 and the Environmental Quality standard (EQS) only available for Hg.

Contaminant	classification (upper limit for class I-IV)					EQS	Proref 2018
	I insignificant	II moderate	III Marked	IV Severe	V Extreme		
As	10	30	70	140	>140		2.503
Cd	0.4	1	4	8	>8		0.18
Cu	2	6	20	40	>40		1.4
Cr	0.2	1	3	10	>10		0.361
Pb	0.6	3	8	20	>20		0.195
Hg	0.04	0.1	0.3	0.8	>0.8	0.02	0.012
Ni	1	5	10	20	>20		0.29
Zn	40	80	200	500	>500		17.66
∑PAH16	50	200	2000	5000	>5000		33.828

Based on the Norwegian classification scheme, As, Cd, Cu, Pb, Hg; and Ni concentrations in mussels were considered insignificant (level I). Only Cr at 1-5 km and T0 group as well as Zn in the 5km group have concentrations classified as moderate (II). However, in comparison to the PROREF 2018 values, As, Cd, Cr, Hg, Ni and Zn concentrations in mussels were similar or slightly above. Overall, the metal concentrations measured where at worst only slightly elevated above background concentrations and did not appear to show any clear relationship with proximity to the smelter discharge.

4.1.2 PAH

PAH concentrations were elevated in the T0 mussels compared to the mussels transplanted in the Sunndals fjord for 6 weeks. It appears therefore, that the mussels, which were depurating in the seawater system at the NIVA marine research station for 2 weeks prior to deployment had

accumulated PAHs. The day 0 mussels were sampled on the day after the deployment of the mussels in the field and were held in flowing seawater that was pumped into the station from a depth of 60 m in the outer Oslofjord. Therefore, it is unclear what was the source of PAH exposure in the day 0 group.

The concentrations of PAH in the mussels were overall not particularly elevated with Sum PAH41 concentrations between 9-10 ng/g. Using the Norwegian classification scheme for PAH16 in mussel tissue, all the mussels measured in the study displayed concentrations representative of the lowest class of I insignificant (Table 2). The mussel concentrations of PAH16 were also below the PROREF 2018 scheme of 34 ng/g w.w. For comparison to similar studies, mussels transplanted for 8-weeks in the seawater recipient of the Karmøy aluminium smelter accumulated PAHs between 40 to 240 ng/g w.w. (Brooks et al., 2022). The sumPAH concentration of the reference mussels used for the Karmøy study was more representative of the highest concentrations in the mussels at Sunndal. Additionally, the Sum PAH concentrations in mussel tissue exposed 500 m from the Statfjord A and B platforms for 6 weeks were above 400 ng/g w.w. (Pampanin et al., 2017). Mussels positioned 10 km from Statfjord B had Sum PAH concentrations at 53 ng/g w.w., which were above the highest concentrations in mussel tissue in the present study.

However, despite the low concentrations measured in field transplanted mussels, there was a relationship between PAH concentration in mussel tissue and their proximity to the Al smelter, with higher concentrations of PAH16 and PAH41 in mussels positioned 1 to 5km from the discharge compared to those at 10 and 20 km away. Additionally, when the PAH accumulation in mussel tissue was grouped in relation to the number of aromatic rings, a clear desorption of 2-4 ring PAH was observed, whilst 5-6 ring PAH did not desorb in the closest mussels. This group of heavy PAH would include the 5 ring benzo(a)pyrene (BaP), benzo(b)fluoranthene (B(b)F), benzo(k)fluoranthene (BkF), and dibenzo(a,h)anthracene (DahA), as well as the 6 ring indeno(1,2,3-c,d)pyrene (IcdP) and benzo(g,h,i)perylene (Bghi)P). The lack of desorption of these heavier PAHs in the mussels closest to the Al smelter would indicate that the smelter is a source of these heavier PAHs within the fjord. Since this is the first biological effects monitoring study that has been performed using field transplanted mussels in the Sunndals fjord, comparisons with previous studies is limited.

4.2 Biological effects responses

A total of nine biological effects endpoints were measured in the mussels transplanted into the Sunndals fjord. The approach taken in using a suite of biological effects tools at different levels of

biological complexity is typical in biological effects studies and can be particularly effective when exposed to mixtures of environmental chemicals. Although the integration of the biological responses in the mussels showed that the mussels closest to the Al smelter were most impacted, many of the biological effects markers were shown to respond differently between the mussel groups.

The whole organism responses of stress on stress and condition index did not significantly differentiate between the mussel groups and showed a low impact on the general health of the mussels in all groups. Comparison to previous biological effects studies where stress on stress measurements in mussels have been measured, LT_{50} values between 8 and 12 were recorded for mussels held for 6 weeks in the Sydvaranger fjord in Kirkenes, Norway that received tailings from an iron mine (Brooks et al., 2015). Furthermore, an LT_{50} of 9 days was reported for intertidal reference mussels from the UK, that reduced to between 5 and 7 days at contaminated sites (Hellou and Law, 2003).

Internationally recognised assessment criteria have been developed under ICES for many biological effects measurements in mussels, including SoS (Davies and Vethaak, 2012). ICES background and environmental assessment criteria (BAC and EAC) for mussel SoS have been calculated as 10 and 5 days respectively. Based on these values, the mussels from all stations where below the BAC but above the EAC which indicates some impact above typical background values.

Significant differences between NRR were observed between the mussel groups with significantly high values recorded for the Day 0 and 10 km mussels compared to those 1-5 km away and the 20 km mussels. The assessment criteria developed for the NRR assay include a BAC of 120 min and EAC of 50 min. Mussels with NRR above 120 min are representative of background conditions, whilst between 120 and 50 min, are considered to be under stress but compensating, and below 50 min are severely stressed and likely experiencing physiological damage. Based on these values, the time zero and 10 km group were representative of background conditions whilst mussels positioned 1, 2 and 5 km were stressed and compensating. Interestingly, the 20 km group was also within this category and may indicate a different source of exposure than chemicals from the Sunndals aluminium smelter.

The 20 km group was located within 500 m of a small salmon farm, so inputs from this activity may have had some impact on the mussels. In addition, approximately 5 km further out of the estuary was known to be a second potential point source at Raudsand where a landfill of inorganic hazardous waste is being proposed. However, at the time of the survey (2019), the landfill had not received permission

and was not expected to contribute as a new contaminant source to the fjord. However, Raudsand has a long tradition for mining as far back as the 19th century. Although mining activity was stopped in the 1980s, high productivity of iron ore was mined in the 1950s and 60s. The mining activity at Raudsand has left its mark on the local environment with monitoring programmes identifying areas of contaminated sediment in the fjord outside Raudsand. The most recent survey identified concerns over the slightly elevated concentrations of Cu, Zn, Ni and PCB7 in the sediment (Brkljacic et al., 2020).

The frequency of MN in the haemocytes of mussels is known as a sensitive biomarker of exposure to genotoxic compounds. The BAC value for MN frequency is currently 2.5 per 1000 cells (Davies and Vethaak, 2012). Based on this value only mussels positioned 10 km from the discharge outlet were below the BAC value. This suggests that mussels with a MN above this value are experiencing a genotoxic response above typical background levels. In the field transplanted mussels placed in the Sunndals fjord only the mussels positioned 10 km from the discharge had MN frequencies below the BAC. The MN in mussels from the closest stations (1-5 km) indicate an exposure resulting in genotoxic responses above background levels. However, the MN frequencies shown in mussels 1 to 5 km from the discharge were surpassed by the highest MN at the furthest station, 20 km downstream from the Al smelter. This result clearly indicates a secondary source of contaminant exposure impacting the mussels at the furthest location and supports the NRR results.

For comparison with similar biological effects studies, MN frequencies between 8 and 10 MN/1000 cells have been reported in mussels located up to 2 km from the Hustadmarmor mine in the Frænfjord, nr Molde, Norway (Brooks et al., 2018). Whilst MN frequencies between 3.6 and 4.7 MN/1000 cells have been reported in mussels held in the Bøkfjord recipient to within 1 km of the Sydvaranger iron ore mine discharge in Finmark, Norway (Brooks et al., 2015).

Neurotoxic responses were observed in all field transplanted mussels and were below the ICES BAC and EAC values of 26 and 19 nmol/ATC/min/mg protein. Since the AChE method is an inhibition assay, the lower the value the larger the neurotoxic response. Only the T0 mussels showed AChE activity above the ICES EAC, indicating low exposure to neurotoxic compounds. The lack of difference in response with distance from the Al smelter in field transplanted mussels suggests that the effects of the lower AChE activity were not related to the point discharge from the smelter. The reason for the neurotoxic responses is not clear unless it indicates a general chronic exposure to AChE inhibiting compounds in the wide region of the fjord.

Lipid peroxidation measured in the gills of the mussel is used to determine the oxidative stress experienced in the field exposed mussels. No significant difference in lipid peroxidation was found between field transplanted mussels and the T0 mussels. ICES assessment criteria were not available for lipid peroxidation in mussel tissue, which prevents comparisons. However, when compared to previous biological effects studies, similar but slightly higher LPO concentrations were detected in the gills of *M. edulis* placed in the Frænfjord for 6 weeks while exposed to the discharge from the Hustadmarmor mine (3800 – 2500 nmol MDA + 4-HNE/g protein, Brooks et al., 2018).

The histochemical markers (VvBAS, NL and LF) indicate changes in the cellular composition of mussel digestive gland cells and were found to be responsive with clear differences between mussel response and proximity to the Al smelter. Digestive cell loss, measured as VvBAS in the digestive gland, is considered a sensitive indicator of general stress in marine mussels (Zaldibar et al. 2007). ICES assessment criteria for VvBAS in mussels are available with BAC and EAC values of 0.12 and 0.18 $\mu m^3/\mu m^3$ respectively. Based on these thresholds, the two closest stations (1-2 km) were above the EAC values and clearly indicated a stress response, whilst the other mussel groups were either on or marginally above the BAC.

Neutral lipid accumulation in the lysosomes of mussel digestive glands is known as a general stress response to chemical exposure (Viarengo et al., 2007). Chemical exposure is known to induce the build-up of neutral lipids in the cytoplasm, which become internalised into the lysosomes of digestive gland cells. For the field exposed mussels, the neutral lipid accumulation showed a relationship with distance to the discharge outlet with highest percentage accumulation in mussels from the two closest stations (1-2 km) and lowest concentrations in the 10 km group. Interestingly, neutral lipid was also elevated in the 20 km mussel group and appears to support the finding of the NRR and MN that a secondary source of contaminant input is present.

Lipofuscin accumulation in field transplanted mussels did show a relationship with proximity to the Al smelter with higher LF in mussels located closest to the discharge (1 and 2 km). However, the T0 mussels had LF accumulation almost identical to the two closest groups and somewhat undermines the biological response observed. Unlike that shown for NL, LF and VvBAS did not increase in mussels from the 20 km group. ICES assessment criteria are not currently available for LF in mussels and cannot be used for comparison.

4.3 Integrated biological response (IBR)

With the integration of the biomarker data, an overall assessment of the health status of the mussels in each group can be obtained. A higher IBR is indicative of an increased stress response and lower health status. The star-plots enable a visualisation of the contribution of each of the biomarkers to the overall IBR score for each mussel group. This also highlights the importance of the positioning of the biomarkers, since different arrangements on the star-plots can often lead to different IBR values. As recommended for this integrative approach, biomarkers that measure similar biological responses were placed together (Broeg and Lehtonen, 2006).

The highest IBR values were recorded in mussels positioned between 1 and 5 km from the Al smelter, with a maximum IBR score of 1.5 for mussels 2 km away. Interestingly, the histochemical markers LF, VvBAS and NL contributed most to the IBR score of the 1 and 2 km group and provides some consensus between the effects observed at the two closest stations. Since the same biomarkers are responding in the mussels from the two closest stations at different magnitudes, it could suggest a common exposure and response to a single point source, such as the discharge from the Al smelter. In contrast, different biomarkers (SoS, NRR, MN, AChE and LPO) contributed to the IBR score of the 5 km mussels.

The IBR for both T0 and the 10 km mussel groups were low (<0.5) indicating a low level of stress in these mussels. However, mussels positioned at the furthest location from the Al smelter (20 km) did show an elevated IBR score. From the star plots, SoS, NRR, MN and AChE contributed most to the IBR of the 20 km mussel group and was not too dissimilar from the contributing biomarkers of the 5 km mussels.

The T0 mussels had an IBR of 0.5, with contributions from LF and NL. This suggests a different exposure profile to the field transplanted mussels, which were sampled after a 2-week acclimation to the 60 m water of the outer Oslo fjord at the NIVA Marine research station.

4.4 Principle component analysis

The integration of biochemical and chemical data through the PCA confirmed the proximity to the discharge outlet' as the one of the most important factors for spatial biomarker response, as well as the magnitude of contaminants influencing mussel response. Similar to the biomarker results obtained from the IBR, the PCA differentiated among mussels collected at the different stations, identifying

mussels from the stations closest to the discharge outlet (1 – 5 km) as the most impacted and those from the stations furthest away (10 – 20 km) as the least impacted. The PCA also highlights the presence of PAH16, PAH41 and metals Mn, Ni, Cr as the main contributors to the higher stress, although the concentrations of these contaminants were low and unlikely to be solely responsible for the biological effects observed in the mussels from the 1 and 5 km groups.

The PCA (PC2) also differentiated between the mussel groups furthest away from the smelter with the 20 km group having increased MN, CI and NL in addition to the contaminants Cd, pyrene, Mo, Cu, As and phenanthrene. These results indicate a different biological response of the 20 km mussels due to a possible secondary exposure source.

5 Conclusion

Significant biological responses were observed in mussels 1 – 5 km downstream from the Al smelter in Sunndals fjord. The biological responses in these mussels included a reduction in the general fitness of the mussel, with a decrease in NRR, and an increase in histochemical markers (VvBAS, NL and LF). PAH concentrations were generally low in all groups, although for field exposed groups the PAH concentrations were slightly higher in the closest groups (1 – 5 km) and higher concentrations of the heavier (5-6 ring) PAHs were found in mussels closest to the Al smelter. However, highest PAH concentrations were observed in mussels from the day zero (T0) group although they did not result in biomarker responses. Overall, the lowest chemical accumulation and biomarker responses were observed in mussels positioned 10 km from the Al smelter and could be considered as the reference field population. Interestingly, the mussels located furthest from the Al smelter at 20 km, exhibited low PAH bioaccumulation but significant biomarker responses, particularly MN formation and NL accumulation. This response was considered to be due to a different contaminant source within the fjord, such as the Salmon farm located approximately 500 m from this mussel group.

The IBR calculation was able to integrate the biological responses and showed mussels between 1 and 5 km with the highest stress response, followed by the 20 km group. Lowest stress was measured in the T0 and 10 km mussel groups. Overall, the biological responses observed where greater in the mussels positioned closest to the Al smelter, although the chemical concentrations measured in the mussel tissue were low and below the expected threshold levels where biological responses would be

expected. The PCA also differentiated among mussel groups, with the most impacted located closest to the smelters discharge. The PCA highlights the presence of PAH16, PAH41 and metals Mn, Ni, Cr as the main contributors to the higher stress, although the concentrations of these contaminants were low and unlikely to have caused the biological effects observed in the mussels from the 1 and 5 km groups. The PCA also differentiated between mussels furthest away from the smelter, with the 20 km group having increased biological responses possibly associated with a secondary exposure source.

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Appendix A.

Table A 1– p-values for the Pearson's correlation of chemical measurements and biological responses in mussels located at different distances from the Sunndals aluminium smelters discharge outlet (values in bold are different from 0 with a significance level alpha = 0.05).CI – Condition index; SoS – Stress on stress; NRR – Neutral red retention; MN – Micronuclei formation; AChE – Acetylcholine esterase activity; LPO – Lipid peroxidation; BAS – Volume of basophilic cells; NL – Neutral lipid accumulation; LF – Lipofuscin accumulation; PAH16 – Sum of PAH16; PAH 41 – Sum of PAH 41; NAP – Sum of naphthalene including C1 to C4; PHE – Sum of phenanthrene including C1 to C4; PYR – Sum of pyrene including C1 and C2; TOT – Total PAHs including alkylated; AS – Arsenic; Cd – Cadmium; Cr – Chromium; Cu – Copper; Pb – Lead; Mn – Manganese; Hg – Mercury; Mo – Molybdenum; Ni – Nickel; V – Vanadium; Zn – Zinc.

Variables	CI	SoS	NRR	MN	AChE	LPO	BAS	NL	LF	PAH16	PAH41	NAP	PHE	PYR	TOT	As	Cd	Cr	Cu	Pb	Mn	Hg	Mo	Ni	V	Zn
CI	**0**	0.649	0.104	0.126	0.683	0.822	0.973	0.209	0.645	0.646	0.636	0.575	0.356	0.224	0.882	0.240	0.402	0.867	**0.002**	0.962	0.685	0.982	0.141	0.932	0.641	0.742
SoS	0.649	**0**	0.793	0.174	0.351	0.188	0.322	0.998	0.581	0.706	0.728	0.588	**0.047**	0.555	0.315	**0.037**	0.709	0.980	0.703	0.475	0.988	0.873	0.461	0.619	0.757	0.651
NRR	0.104	0.793	**0**	0.532	0.437	0.735	0.634	0.187	0.235	0.120	0.113	0.766	0.325	0.569	0.433	0.339	0.948	0.209	0.149	0.418	0.124	0.371	0.718	0.310	0.115	0.818
MN	0.126	0.174	0.532	**0**	0.589	0.895	0.495	0.499	0.768	0.966	0.955	0.979	0.204	0.610	0.796	0.077	0.450	0.658	0.142	0.412	0.788	0.637	**0.026**	0.871	0.713	0.692
AChE	0.683	0.351	0.437	0.589	**0**	0.584	0.871	0.952	0.690	0.624	0.574	0.099	0.851	0.287	0.583	0.710	0.796	0.518	0.584	0.642	0.539	0.313	0.741	0.976	0.402	0.512
LPO	0.822	0.188	0.735	0.895	0.584	**0**	0.844	0.931	0.909	0.214	0.233	0.332	0.193	0.124	**0.045**	0.319	0.165	0.293	0.713	0.766	0.391	0.307	0.623	0.096	0.611	0.290
BAS	0.973	0.322	0.634	0.495	0.871	0.844	**0**	0.226	**0.031**	0.565	0.583	0.526	0.453	0.904	0.665	0.473	0.700	0.424	0.839	**0.036**	0.309	0.997	0.492	0.509	0.218	0.460
NL	0.209	0.998	0.187	0.499	0.952	0.931	0.226	**0**	0.105	0.495	0.515	0.664	0.797	0.686	0.508	0.641	0.325	0.588	0.321	0.343	0.371	0.835	0.581	0.559	0.318	0.330
LF	0.645	0.581	0.235	0.768	0.690	0.909	**0.031**	0.105	**0**	0.232	0.242	0.653	0.900	0.989	0.421	0.893	0.828	0.183	0.785	**0.019**	0.089	0.668	0.650	0.263	**0.047**	0.703
PAH16	0.646	0.706	0.120	0.966	0.624	0.214	0.565	0.495	0.232	**0**	**< 0.0001**	0.776	0.327	0.673	0.105	0.474	0.383	**0.007**	0.756	0.206	**0.009**	0.088	0.590	**0.016**	**0.041**	0.389
PAH41	0.636	0.728	0.113	0.955	0.574	0.233	0.583	0.515	0.242	**< 0.0001**	**0**	0.833	0.331	0.711	0.126	0.484	0.374	**0.006**	0.738	0.213	**0.009**	0.075	0.589	**0.022**	**0.039**	0.366
NAP	0.575	0.588	0.766	0.979	0.099	0.332	0.526	0.664	0.653	0.776	0.833	**0**	0.921	0.095	0.203	0.882	0.897	0.794	0.412	0.604	0.773	0.749	0.693	0.420	0.914	0.672
PHE	0.356	**0.047**	0.325	0.204	0.851	0.193	0.453	0.797	0.900	0.327	0.331	0.921	**0**	0.835	0.260	**0.006**	0.606	0.567	0.396	0.763	0.582	0.454	0.519	0.390	0.765	0.428
PYR	0.224	0.555	0.569	0.610	0.287	0.124	0.904	0.686	0.989	0.673	0.711	0.095	0.835	**0**	0.232	0.965	0.267	0.632	0.144	0.739	0.765	0.749	0.306	0.338	0.961	0.633
TOT	0.882	0.315	0.433	0.796	0.583	**0.045**	0.665	0.508	0.421	0.105	0.126	0.203	0.260	0.232	**0**	0.329	0.471	0.175	0.943	0.405	0.177	0.429	0.725	**0.025**	0.319	0.657
As	0.240	**0.037**	0.339	0.077	0.710	0.319	0.473	0.641	0.893	0.474	0.484	0.882	**0.006**	0.965	0.329	**0**	0.898	0.763	0.283	0.667	0.726	0.715	0.304	0.539	0.895	0.693
Cd	0.402	0.709	0.948	0.450	0.796	0.165	0.700	0.325	0.828	0.383	0.374	0.897	0.606	0.267	0.471	0.898	**0**	0.324	0.408	0.813	0.530	0.100	0.207	0.318	0.680	**0.027**
Cr	0.867	0.980	0.209	0.658	0.518	0.293	0.424	0.588	0.183	**0.007**	**0.006**	0.794	0.567	0.632	0.175	0.763	0.324	**0**	0.971	0.106	**0.004**	0.070	0.355	**0.024**	**0.020**	0.381
Cu	**0.002**	0.703	0.149	0.142	0.584	0.713	0.839	0.321	0.785	0.756	0.738	0.412	0.396	0.144	0.943	0.283	0.408	0.971	**0**	0.830	0.804	0.986	0.122	0.918	0.745	0.800
Pb	0.962	0.475	0.418	0.412	0.642	0.766	**0.036**	0.343	**0.019**	0.206	0.213	0.604	0.763	0.739	0.405	0.667	0.813	0.106	0.830	**0**	0.071	0.478	0.289	0.192	**0.042**	0.971
Mn	0.685	0.988	0.124	0.788	0.539	0.391	0.309	0.371	0.089	**0.009**	**0.009**	0.773	0.582	0.765	0.177	0.726	0.530	**0.004**	0.804	0.071	**0**	0.151	0.493	**0.036**	**0.004**	0.578
Hg	0.982	0.873	0.371	0.637	0.313	0.307	0.997	0.835	0.668	0.088	0.075	0.749	0.454	0.749	0.429	0.715	0.100	0.070	0.986	0.478	0.151	**0**	0.382	0.175	0.205	0.055
Mo	0.141	0.461	0.718	**0.026**	0.741	0.623	0.492	0.581	0.650	0.590	0.589	0.693	0.519	0.306	0.725	0.304	0.207	0.355	0.122	0.289	0.493	0.382	**0**	0.450	0.504	0.467
Ni	0.932	0.619	0.310	0.871	0.976	0.096	0.509	0.559	0.263	**0.016**	**0.022**	0.420	0.390	0.338	**0.025**	0.539	0.318	**0.024**	0.918	0.192	**0.036**	0.175	0.450	**0**	0.105	0.458
V	0.641	0.757	0.115	0.713	0.402	0.611	0.218	0.318	**0.047**	**0.041**	**0.039**	0.914	0.765	0.961	0.319	0.895	0.680	**0.020**	0.745	**0.042**	**0.004**	0.205	0.504	0.105	**0**	0.703
Zn	0.742	0.651	0.818	0.692	0.512	0.290	0.460	0.330	0.703	0.389	0.366	0.672	0.428	0.633	0.657	0.693	**0.027**	0.381	0.800	0.971	0.578	0.055	0.467	0.458	0.703	**0**

Table A 2– r^2 values for the Pearson's correlation of chemical measurements and biological responses in mussels located at different distances from the Sunndals aluminium smelters discharge outlet (values in bold are different from 0 with a significance level alpha = 0.05).CI – Condition index; SoS – Stress on stress; NRR – Neutral red retention; MN – Micronuclei formation; AChE – Acetylcholine esterase activity; LPO – Lipid peroxidation; BAS – Volume of basophilic cells; NL – Neutral lipid accumulation; LF – Lipofuscin accumulation; PAH16 – Sum of PAH16; PAH 41 – Sum of PAH 41; NAP – Sum of naphthalene including C_1 to C_4; PHE – Sum of phenanthrene including C_1 to C_4; PYR – Sum of pyrene including C_1 and C_2; TOT – Total PAHs including alkylated; AS – Arsenic; Cd – Cadmium; Cr – Chromium; Cu – Copper; Pb – Lead; Mn – Manganese; Hg – Mercury; Mo – Molybdenum; Ni – Nickel; V – Vanadium; Zn – Zinc.

Variables	CI	SoS	NRR	MN	AChE	LPO	BAS	NL	LF	PAH16	PAH41	NAP	PHE	PYR	TOT	As	Cd	Cr	Cu	Pb	Mn	Hg	Mo	Ni	V	Zn
CI	**1**	-0.279	-0.800	0.772	0.251	-0.140	-0.021	0.678	0.283	0.282	0.290	-0.341	0.532	0.662	0.093	0.645	0.490	0.104	**0.986**	-0.030	0.250	0.014	0.754	0.053	0.286	-0.204
SoS	-0.279	**1**	0.164	-0.716	0.537	-0.700	0.564	-0.002	0.336	-0.233	-0.215	-0.329	**-0.884**	0.357	-0.570	**-0.901**	0.230	-0.016	-0.236	0.426	0.010	-0.100	-0.438	-0.304	0.192	-0.278
NRR	-0.800	0.164	**1**	-0.377	-0.459	-0.209	-0.292	-0.702	-0.651	-0.779	-0.788	0.185	-0.561	-0.346	-0.462	-0.548	-0.041	-0.677	-0.744	-0.475	-0.774	-0.518	-0.223	-0.576	-0.786	-0.143
MN	0.772	-0.716	-0.377	**1**	-0.329	0.083	-0.409	0.404	-0.183	-0.026	-0.036	-0.016	0.682	0.311	0.161	0.837	0.447	-0.272	0.753	-0.481	-0.168	-0.289	**0.922**	-0.102	-0.228	-0.244
AChE	0.251	0.537	-0.459	-0.329	**1**	-0.333	0.102	-0.038	0.246	0.300	0.341	-0.807	-0.117	0.598	-0.334	-0.229	-0.161	0.388	0.333	0.285	0.371	0.572	-0.205	0.019	0.490	0.394
LPO	-0.140	-0.700	-0.209	0.083	-0.333	**1**	-0.123	-0.054	0.071	0.672	0.653	0.554	0.694	-0.774	**0.887**	0.567	-0.726	0.592	-0.227	0.185	0.500	0.579	-0.301	0.811	0.310	0.595
BAS	-0.021	0.564	-0.292	-0.409	0.102	-0.123	**1**	0.659	**0.912**	0.348	0.333	0.381	-0.445	-0.075	0.266	-0.427	0.238	0.471	-0.127	**0.903**	0.576	-0.003	-0.411	0.396	0.668	-0.439
NL	0.678	-0.002	-0.702	0.404	-0.038	-0.054	0.659	**1**	0.798	0.409	0.391	0.267	0.160	0.249	0.397	0.286	0.561	0.329	0.565	0.544	0.518	-0.130	0.335	0.354	0.568	-0.556
LF	0.283	0.336	-0.651	-0.183	0.246	0.071	**0.912**	0.798	**1**	0.653	0.643	0.276	-0.079	0.009	0.473	-0.084	0.136	0.706	0.169	**0.937**	0.820	0.264	-0.279	0.621	**0.883**	-0.235
PAH16	0.282	-0.233	-0.779	-0.026	0.300	0.672	0.348	0.409	0.653	**1**	**0.999**	0.177	0.559	-0.260	0.799	0.426	-0.508	**0.968**	0.193	0.681	**0.962**	0.821	-0.328	**0.944**	**0.893**	0.501
PAH41	0.290	-0.215	-0.788	-0.036	0.341	0.653	0.333	0.391	0.643	**0.999**	**1**	0.132	0.555	-0.229	0.772	0.418	-0.515	**0.969**	0.207	0.673	**0.961**	0.841	-0.328	**0.930**	**0.897**	0.523
NAP	-0.341	-0.329	0.185	-0.016	-0.807	0.554	0.381	0.267	0.276	0.177	0.132	**1**	0.062	-0.812	0.684	0.093	-0.081	0.163	-0.481	0.316	0.180	-0.199	-0.244	0.474	0.068	-0.261
PHE	0.532	**-0.884**	-0.561	0.682	-0.117	0.694	-0.445	0.160	-0.079	0.559	0.555	0.062	**1**	-0.130	0.624	**0.969**	-0.314	0.347	0.495	-0.187	0.335	0.444	0.388	0.501	0.185	0.467
PYR	0.662	0.357	-0.346	0.311	0.598	-0.774	-0.075	0.249	0.009	-0.260	-0.229	-0.812	-0.130	**1**	-0.654	-0.028	0.618	-0.294	0.751	-0.207	-0.186	-0.198	0.579	-0.549	-0.031	-0.293
TOT	0.093	-0.570	-0.462	0.161	-0.334	**0.887**	0.266	0.397	0.473	0.799	0.772	0.684	0.624	-0.654	**1**	0.557	-0.429	0.715	-0.045	0.487	0.713	0.466	-0.217	**0.924**	0.567	0.272
As	0.645	**-0.901**	-0.548	0.837	-0.229	0.567	-0.427	0.286	-0.084	0.426	0.418	0.093	**0.969**	-0.028	0.557	**1**	-0.080	0.187	0.602	-0.265	0.217	0.226	0.581	0.371	0.083	0.243
Cd	0.490	0.230	-0.041	0.447	-0.161	-0.726	0.238	0.561	0.136	-0.508	-0.515	-0.081	-0.314	0.618	-0.429	-0.080	**1**	-0.562	0.484	-0.148	-0.378	-0.806	0.680	-0.568	-0.254	**-0.920**
Cr	0.104	-0.016	-0.677	-0.272	0.388	0.592	0.471	0.329	0.706	**0.968**	**0.969**	0.163	0.347	-0.294	0.715	0.187	-0.562	**1**	0.022	0.797	**0.978**	0.847	-0.533	**0.925**	**0.935**	0.509
Cu	**0.986**	-0.236	-0.744	0.753	0.333	-0.227	-0.127	0.565	0.169	0.193	0.207	-0.481	0.495	0.751	-0.045	0.602	0.484	0.022	**1**	-0.134	0.154	0.011	0.778	-0.064	0.202	-0.158
Pb	-0.030	0.426	-0.475	-0.481	0.285	0.185	**0.903**	0.544	**0.937**	0.681	0.673	0.316	-0.187	-0.207	0.487	-0.265	-0.148	0.797	-0.134	**1**	0.846	0.423	-0.596	0.696	**0.892**	-0.022
Mn	0.250	0.010	-0.774	-0.168	0.371	0.500	0.576	0.518	0.820	**0.962**	**0.961**	0.180	0.335	-0.186	0.713	0.217	-0.378	**0.978**	0.154	0.846	**1**	0.742	-0.410	**0.903**	**0.978**	0.338
Hg	0.014	-0.100	-0.518	-0.289	0.572	0.579	-0.003	-0.130	0.264	0.821	0.841	-0.199	0.444	-0.198	0.466	0.226	-0.806	0.847	0.011	0.423	0.742	**1**	-0.508	0.715	0.681	0.871
Mo	0.754	-0.438	-0.223	**0.922**	-0.205	-0.301	-0.411	0.335	-0.279	-0.328	-0.328	-0.244	0.388	0.579	-0.217	0.581	0.680	-0.533	0.778	-0.596	-0.410	-0.508	**1**	-0.448	-0.401	-0.432
Ni	0.053	-0.304	-0.576	-0.102	0.019	0.811	0.396	0.354	0.621	**0.944**	**0.930**	0.474	0.501	-0.549	**0.924**	0.371	-0.568	**0.925**	-0.064	0.696	**0.903**	0.715	-0.448	**1**	0.799	0.440
V	0.286	0.192	-0.786	-0.228	0.490	0.310	0.668	0.568	**0.883**	**0.893**	**0.897**	0.068	0.185	-0.031	0.567	0.083	-0.254	**0.935**	0.202	**0.892**	**0.978**	0.681	-0.401	0.799	**1**	0.236
Zn	-0.204	-0.278	-0.143	-0.244	0.394	0.595	-0.439	-0.556	-0.235	0.501	0.523	-0.261	0.467	-0.293	0.272	0.243	**-0.920**	0.509	-0.158	-0.022	0.338	0.871	-0.432	0.440	0.236	**1**