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An integrated biological effects assessment of the discharge water into the Sunndalsfjord from an aluminium smelter

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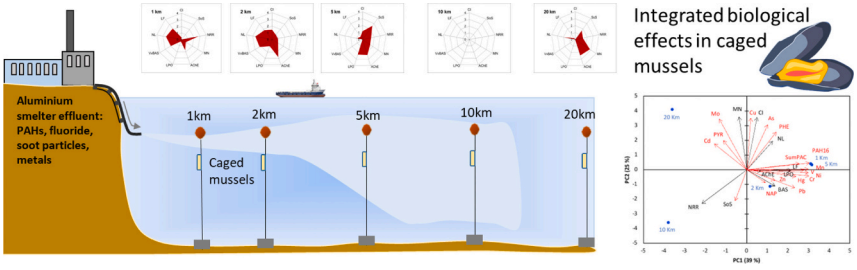
HIGHLIGHTS

- Change in PAH profile in mussels indicated exposure to aluminium smelter effluent.
- Low chemical exposure in field transplanted mussels, highest chemical concentration in T0 mussels
- Higher biological effects responses shown in mussels closer to the aluminium smelter
- IBR/n integrated biological responses with highest stress in mussels 1–5 km away.
- Overall, the mussels measured a low impact of the aluminium smelter discharge effluent in the fjord.

GRAPHICAL ABSTRACT



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ABSTRACT

An integrated biological effects study using field transplanted mussels was applied to determine the potential biological effects of an effluent discharge from an aluminium smelter into a Norwegian fjord. Chemical body burden and biological effects were measured in mussels positioned downstream (1, 2, 5, 10 and 20 km) from the aluminium smelters discharge for a period of 6 weeks. A suite of biomarkers, from whole organism to subcellular responses were measured. Chemical concentrations in mussel tissues were low; however, a change in the PAC (polyaromatic compound) profile from high to low pyrogenic influence provided evidence of exposure to the smelter's effluent. Overall, the biological responses observed where greater in the mussels positioned closest to the smelter (1–5 km). Lowest chemical accumulation and biomarker responses were observed in mussels positioned 10 km from the smelter and were considered as the reference field population. Mussels located furthest from the smelter (20 km) exhibited significant biomarker responses and suggested a different contaminant source within the fjord. The integrated biological response index (IBR) was applied and reflected the expected level of exposure to the smelters discharge, with highest IBR calculated in mussels positioned closest to the discharge (1–5 km). Principal component analysis (PCA) also differentiated among mussel groups, with the most impacted located closest to the smelter. Not one chemical factor could explain the biological responses observed in mussels, but the presence of PAH16, PAH41 and metals Mn, Ni and Cr were the main contributors measured to the higher stress seen in the mussels from the 1 and 5 km groups.

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1. Introduction

Mussels (*Mytilus* sp.) are widely used in biological effects studies, where the health status of a sub population of mussels, based on measurements of chemical bioaccumulation and biological responses, can reflect that of the environment in which they live (reviewed in Beyer et al., 2017). The present study describes an integrated biological effects assessment where field transplanted mussels have been used to assess the potential impact of an aluminium smelter that discharges effluent into a Norwegian fjord. The aluminium smelter, operated by Hydro AS since 1954, is located at Sunndal and discharges waste under licence into the Sunndalsfjord in Norway. The Sunndal plant is one of Europe's largest and most modern producer of primary aluminium with over 400,000 t manufactured annually. In addition, the plant produces 500,000 t of casthouse products and 80,000 t of anodes annually (https://www.hydro.com). The waste effluent from the smelter that enters the Sunndalsfjord originates mostly from seawater scrubbing of furnace off-gases, which contain polyaromatic compounds (PACs), fluoride, soot particles and various metals. Concentrations of PACs and metals have been previously monitored in sediment, water and biota within the fjord over the last 25 years (Bakke and Håvardstun, 2012; Næs et al., 2008; Oug et al., 1998; Næs et al., 1995), and it is these contaminants that are considered to be the main concern and threat to marine life within the fjord.

PACs 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). PAC concentrations as high as 700 mg/kg (d.w.) have been reported in sediment within the Sunndalsfjord, which is over 1000 times the expected coastal water background level (Oug et al., 1998; Næs and Oug, 1997; Næs et al., 1995; Knutzen, 1995). PAC concentrations of the water column are considered to be much lower than that of the sediment, although actual concentrations have not been reported with previous monitoring efforts focussing on the sediment compartment

This 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 within Sunndalsfjord. The mussels ability to filter large volumes of seawater and bioaccumulate contaminants such as PACs and metals from the filtered seawater make them ideal for biomonitoring. They also have a wide range of sensitive biological effects methods that can be easily measured and can be used to provide an holistic integrated assessment of organism health. The biological effects methods selected represent, whole organism responses (condition index, CI; stress on stress, SoS), subcellular responses (lysosomal membrane stability, LMS; micronuclei, MN; acetylcholine esterase, AChE; lipofuscin, LPO) and histochemical changes (Volume of basophilic cells, VvBAS; neutral lipid, NL; lipofuscin accumulation, LF). These methods measure general health responses as well as genotoxic (MN), neurotoxic (AChE) and oxidative stress (LPO) endpoints, which are known to be responsive to chemical exposure (reviewed in Beyer et al., 2017). Applying a suite of biological effects in mussels covers many of the mechanisms of toxicity that chemicals can cause, and is an approach widely used and recommended to assess the impacts of chemical discharges in the marine environment (Davies and Vethaak, 2012).

2. Method

2.1. Transplantation of field mussels

Mussels (~600) 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 (~200 L) 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 marine algal culture and seawater parameters (temperature and salinity) measured daily. 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., 2015a). 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. The length of all mussels used in the study was measured and were of a similar size (shell length 52.5 ± 4.6 mm, mean ± standard deviation).

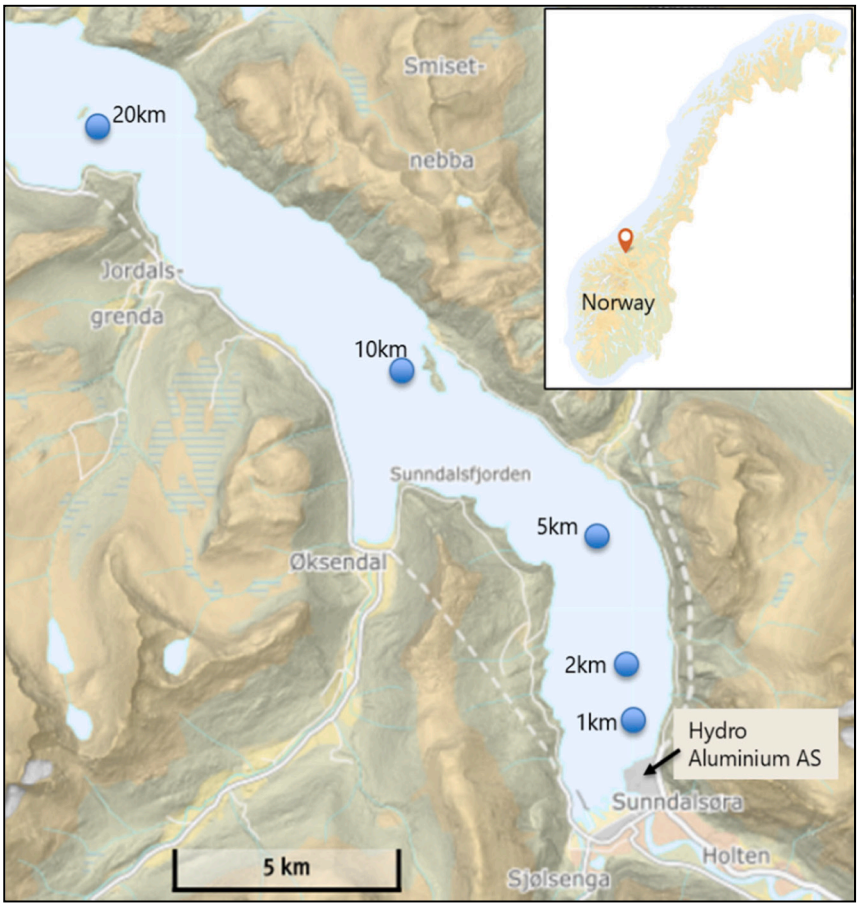
The evening before field deployment, the mussels were carefully placed in nylon mesh socks (mesh size 1.5 cm), which were knotted at intervals to create five pockets of 20 mussels. Care was taken to ensure sufficient space was provided so as not to impede gaping and filtration. The mussels were placed 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 considered to be in optimal condition prior to field deployment and no mortalities were observed.

Approximately 100 mussels were attached to five mussel rigs, positioned at known distances from the aluminium smelters discharge outlet in the Sunndalsfjord (Fig. 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. This was greater than the maximum draft expected for the large transport vessels known to frequent the harbour of the smelter. Assuming adequate food is available, mussels can handle depths of 15 m and more without any obvious adverse impacts on organism health as shown in previous studies (Pampanin et al., 2019; Brooks et al., 2023). Although the impacts of depth on biomarker responses cannot be totally ruled out, the mussels were held at the same depth at each station, and it was therefore assumed that direct comparisons between groups could be made.

The mussels were deployed on 30th April 2019, and collected 6 weeks later on 10th June 2019. Temperature sensors were deployed at the same depth as the mussels and temperatures of 8 ± 1 °C were measured in the Sunndalsfjord at all stations 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 h. The mussels were kept in the chilled cooler box overnight and processed the following day. No mussel mortalities were observed during retrieval. It should be noted that due to logistical limitations, the field transplanted mussels could not be sampled immediately upon collection. The approach taken, as described above, was considered to be the best available option. All field mussels were treated in the same way in order to reduce any confounding factors that could interfere with biomarker responses and enable suitable comparisons between the mussel groups.

2.2. Analysis of mussel samples

Length measurements were collected 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



Station	Latitude	Longitude
1km	62° 41.483 N	8° 32.967 E
2km	62° 42.167 N	8° 32.783 E
5km	62° 43.600 N	8° 21.200 E
10km	62° 45.003 N	8° 26.117 E
20km	62° 47.483 N	8° 17.283 E

Fig. 1. Location of the aluminium smelter at the end of the Sunndalsfjord, Norway. Approximate location of the mussels with distance from the smelter discharge outlet and specific coordinates.

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 (Neutral lipid (NL), volume of basophilic cells (VvBAS), and lipofuscin accumulation (LF)), 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 compounds and biological effect responses. These mussels were referred to as the day 0 (T0) group, since they reflect the condition of the mussels at the start of the field exposure. The T0 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 54 PACs and metal concentrations. Gas chromatography (GC) coupled to mass spectrometry (MS) was employed for the chemical analysis. The method employed has been previously published (Meland et al., 2019) but updated to include more PACs as described in a recent report (Grung et al., 2020). A total of 54 different PACs were analysed including the PAH16 compounds. Alkylated PACs of naphthalene (N) (C1–4), fluorene (F) (C1–3), dibenzothiophene (D) (C1–3), phenanthrene/anthracene (P/A) (C1–4), pyrene/fluoranthene (PY/FLA) (C1–2) and chrysenes (C) (C1–2) were also analysed. In addition, some alkylated compounds of heavier PACs were also included in the analysis.

The pyrogenic index (PI) was calculated according to Stogiannidis and Laane (2015) as follows:

$$PI = \frac{ACY + ACE + A + PY + FLA + BaA + BbjF + BkF + BeP + BaP + PER + BgP + IP + DahA}{N(1-4) + P(1-4) + D(1-3) + F(0-4) + C(0-4)}$$

The numbers signify the number of alkylated carbons attached to the PAC (1–4 alkyl group(s)). Some minor differences to the original PI should be noted; since the chromatographic method employed here did not separate BbF from BjF, the sum BbF and BjF (BbjF) was used instead of BbF. For dibenzothiophene (D), the C4 alkylated Ds were not quantified (in the original D1–4 should be used).

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

2.2.2. Condition index

Condition index (CI) is used to provide a simple measure of organism health status, encompassing the physiological activity such as growth, reproduction, secretion, etc., under environmental conditions. The 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.

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

2.2.3. Stress on stress

The stress-on-stress (SoS) test provides a measure of the mussel's ability to keep their shells closed and resist air exposure. It is related to the amount of energy (adenosine triphosphate, ATP) available to fuel their adductor muscle (De Zwaan and Mathiew, 1992). Therefore, if metabolic energy is spent on detoxification processes in mussels exposed to contaminants, less energy is available for other physiological processes. The SoS assessment was measured in 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 of 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). The NRR assay detects the resilience of lysosome membranes in mussel haemocytes and provides a measure of the functional integrity of cells. 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 (×400 magnification). The test was terminated, and the time recorded when >50 % of the haemocytes leaked the neutral red dye out of the lysosome into the cytosol.

2.2.5. Micronuclei formation

Micronuclei (MN) are chromatin-containing structures that are surrounded by a membrane and have no detectable link to the cell nucleus. The frequency of MN in mussel haemocytes provides an indication of genotoxicty through chromosomal damage, which is known to be induced by chemical exposure (Baršienė et al., 2006). The frequency of MN in mussel haemocytes was determined in 10 individuals per group. 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 with 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) is an essential enzyme involved in neuronal transmission in animal cells. The inhibition of AChE causes various effects on the central nervous system and several compounds including pesticides and PAHs have been found to be AChE inhibitors (Bocquené et al., 1995; Froment et al., 2016). 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 min 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 (ACT, final concentration 2.63 mM) 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 ACT per min per mg of total protein.

2.2.7. Lipid peroxidation

Lipid peroxidation (LPO) provides a measure of the oxidative deterioration of polyunsaturated fatty acids present in cellular membranes, which can alter membrane fluidity and permeability (Erdelmeier et al., 1998). By measuring the by-products of polyunsaturated fatty acid peroxidation (i.e., malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE)) in mussel gill samples, an assessment of oxidative damage in the gills of mussels can be obtained. 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 4 °C. The resulting homogenate was centrifuged at 15,000*g* for 20 min at 4 °C and the supernatant used for total protein determination and LPO analysis. LPO analysis was based on the reaction of 2 mol of *N*-methyl-2-phenylindole (3:1 mixture of acetonitrile/methanol), a chromogenic reagent, with 1 mol 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 concentrations

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 et al., 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.

2.2.9.1. VvBAS. An increase in the cell density of basophilic cells to digestive cells is known to be due to digestive cell loss and is induced by chemical exposure (Zaldibar et al., 2007). To determine 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 s. The sections were then rinsed in flowing tap water for 20 min and stained with eosin-phloxin solution for 30 s, before being rinsed in 80 % ethanol and allowed to air dry for a few hours before being mounted with Euparal.

The VvBAS of mussels was determined microscopically with a Weibel graticule eye piece (M-168; Weibel, 1979). Counts were made in 10 fields of view for each mussel (400× magnification). The volume density of basophilic cells (VvBAS) was calculated using the equation:

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

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).

2.2.9.2. NL. Elevated levels of NL within the lysosomes of mussel digestive glands have been linked with exposure to lipophilic contaminants and reduced health status. Measuring NL accumulation can therefore indicate organism exposure and general health of the mussel. For the NL assessment, 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 (×400 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.

2.2.9.3. LF. Granules of LF can accumulate in lysosomes as a result of peroxidation of cellular membranes. Since lipofuscin is not degradable they provide a measurable level of oxidative stress experienced by the mussel. To determine LF accumulation 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 (×400 magnification) was assessed using computer assisted image analysis (Cell-D, Olympus).

2.3. Statistical assessment

Statistical analyses were performed using Statistica 13 (Dell). All data were tested for normality and homogeneity of variance with a Levene's test. When homogeneity of variance was achieved, significant differences between groups were determined using one-way analysis of variance (ANOVA) and the Tukey post-hoc test, with the level of significance set at $p = 0.05$. The non-parametric Kruskal Wallis test was performed when homogeneity of variance was not achieved.

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 as described in Brooks et al. (2015b).

A Principal component analysis (PCA) was performed using XLStat2020® (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 PAH16 and total PACs in mussels from the day zero group (T0) and in mussels located at specific distances downstream from the aluminium smelter are shown in Fig. 2. Highest concentrations of PAH16 were found in mussels from the T0 group (Fig. 2a). 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 54 PAH compounds (sum PAC) in the mussel groups (Fig. 2b). The percentage of PAH16 of the total PAC varied between 61 and 26 %, and higher percentages were observed in the mussels positioned 1 km from the aluminium smelter (55–61 % vs 26–53 %).

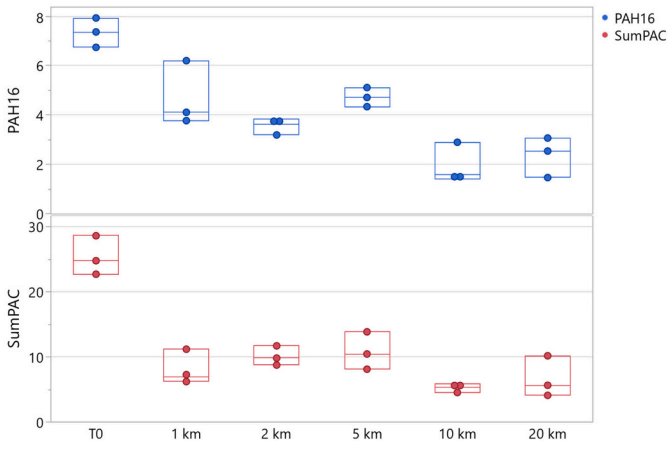


Fig. 2. Sum of PAH16 and sum of PACs in mussels from the day zero group (T0) and in mussels located at different distances from the Sunndals aluminium smelters discharge (ng/g w.w., n = 3).

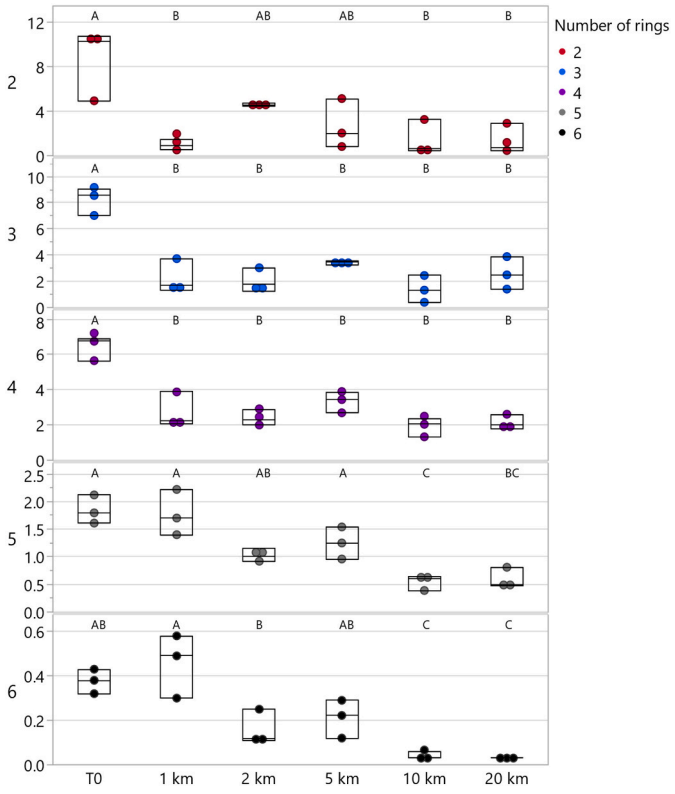


Fig. 3. PAC concentrations (ng/g w.w., n = 3) in mussels from the day zero group (T0) and in mussels located at different distances from the Sunndals aluminium smelters discharge outlet. The results are grouped with respect to the number of rings, from lighter PAH (2–3 rings) to heavier PAH (5–6 rings). See Table S1 for information about PACs and number of rings. Different letters denote significant differences between groups (ANOVA, Tukey $p < 0.05$).

The PAC concentrations in mussel tissue with respect to the number of aromatic rings (2 to 6) are shown in Fig. 3. In general, a larger number of rings indicate a heavier PAC. The data shows that 2–4 ring PACs are desorbed during the 6-week deployment period from the starting concentration in the day zero (T0) mussel group. However, 5–6 ring PACs 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 PACs were detected in mussels 20 km away from the smelter. In addition, the pyrogenic index (PI) (Stogiannidis and Laane, 2015) of the PAC composition in the mussels was calculated and are shown in Fig. 4, indicating that the PACs at the closest station were of pyrogenic origin, whilst the PACs at the rest of the stations were of mainly petrogenic origin.

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.).

3.2. Biomarker responses in mussels

3.2.1. Whole organism - general health

The CI calculated by dividing the dry weight of the mussel soft tissue by the dry weight of the shell multiplied by 100, is shown in Fig. 5a. There were no significant differences found in condition index between the mussel groups (ANOVA, Tukey, $p > 0.05$).

The SoS tests, 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 Fig. 5b. 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, were within 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 away. Mussels from the T0 group did survive longer (14 days) than the other mussel groups (11 days).

3.2.2. Histochemistry

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 (Cajaraville et al., 1990) is shown in Fig. 5c. 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).

The accumulation of NL in the digestive gland cells of mussels from the different groups are shown in Fig. 5d. As described for VvBAS,

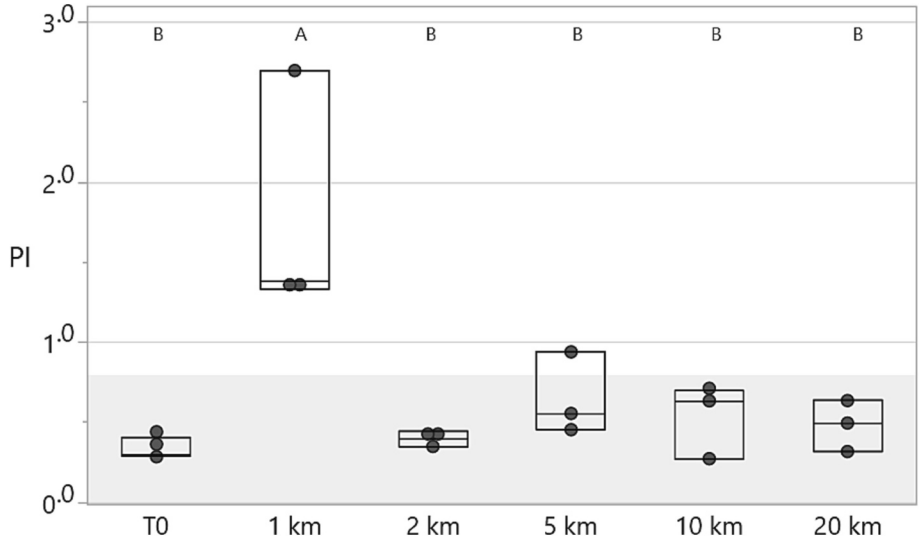


Fig. 4. Pyrogenic index (PI) of PACs in different mussels. The petrogenic index area is indicated with grey background, whilst pyrogenic index area is white. Different letters denote significant differences between groups (ANOVA, Tukey $p < 0.05$).

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.) Arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), vanadium (V), zinc (Zn).

Mussel group	As	Cd	Cr	Cu	Pb	Mn	Hg	Mo	Ni	V	Zn
	3.2	0.23	**0.34**	1.3	**0.09**	1.8	0.015	0.3	**0.4**	**0.3**	**20**
1	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
	2.8	0.17	**0.29**	1.1	**0.09**	1.1	0.014	0.3	**0.4**	**0.3**	**17**
2	2.9	**0.18**	0.22	**1.1**	0.09	1.7	**0.011**	**0.3**	0.5	0.3	13
	2.7	0.20	0.38	1.1	0.09	**1.7**	0.010	0.2	0.4	0.2	17
	2.9	0.14	**0.35**	1.1	**0.08**	1.4	0.028	0.3	**0.5**	**0.3**	**32**
5	3.2	**0.18**	0.25	**1.1**	0.07	1.9	**0.022**	**0.3**	0.3	0.3	42
	2.8	0.18	0.53	1.2	0.08	**1.6**	0.010	0.2	0.6	0.2	20
	2.4	0.16	**0.15**	1.0	**0.07**	0.8	0.006	0.2	**0.3**	**0.2**	**19**
10	2.9	**0.18**	0.15	**1.0**	0.06	0.9	**0.009**	**0.2**	0.3	0.2	17
	2.7	0.19	0.24	1.2	0.09	**0.9**	0.025	0.3	0.4	0.2	34
	3.2	0.21	**0.13**	1.3	**0.07**	0.8	0.009	0.5	**0.3**	**0.2**	**17**
20	2.7	**0.18**	0.17	**1.3**	0.08	1.0	**0.011**	**0.4**	0.3	0.2	14
	2.9	0.18	0.12	1.1	0.05	**0.9**	0.011	0.4	0.4	0.2	21
	2.8	0.16	**0.24**	1.2	**0.14**	8.8	0.008	0.1	**0.4**	**0.3**	**19**
T0	3.0	**0.18**	0.16	**1.1**	0.12	14.0	**0.013**	**0.1**	0.2	0.3	15
	2.8	0.18	0.39	1.1	0.16	**11.0**	0.015	0.1	0.5	0.4	25

significantly higher NL accumulation was shown in mussels located closest to the discharge outlet (1 and 2 km). Significantly lower NL levels were found in mussels 10 km from the discharge outlet. However, mussel located at the furthest location from the discharge (20 km) had NL concentrations that were not significantly different from the two closest stations and indicates a different source of stress exposure impacting these mussels.

LF 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 (Fig. 5e).

3.2.3. Subcellular responses

Significant differences in NRR were found between the different mussel groups with highest values, indicating a better overall fitness of the mussel, in the T0 group and in the mussels positioned 10 km from the discharge outlet (Fig. 6a). 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.

Genotoxicity measured by the frequency of MN in mussel haemocytes indicated significant differences between the mussel groups (Fig. 6b). Lowest MN numbers were found in T0 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 T0 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.

The exposure to neurotoxic compounds measured by the inhibition of the AChE enzyme is shown in Fig. 6c. Compared to the T0 group all mussels had significantly lower levels of AChE activity. For the mussels located in the Sunndalsfjord there was no significant difference in AChE activity between the mussel groups.

Lipid peroxidation measured as the quantity of MDA and 4-HNE in gill tissue samples of mussels is shown in Fig. 6d. 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.

3.2.4. Integrated biological response

The 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 (Fig. 7). The star plots indicate the contribution of the different biomarkers to the overall IBR/n value. Higher IBR/n was 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.

3.2.5. Principal component analysis

Th PCA was used to discriminate the main variables responsible for the variance of chemical body burden and biological effects measured in transplanted mussels (Fig. 8). Mussels from the T0 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 Sunndalsfjord. 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 39 % of variance and showed a separation between the 3 groups located closest to the discharge outlet (1, 2 and 5 km) and those furthest away (10 and 20 km). PC2 explained 25 % 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, SUM PACs, 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 Sunndal 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 (supplementary information). As for

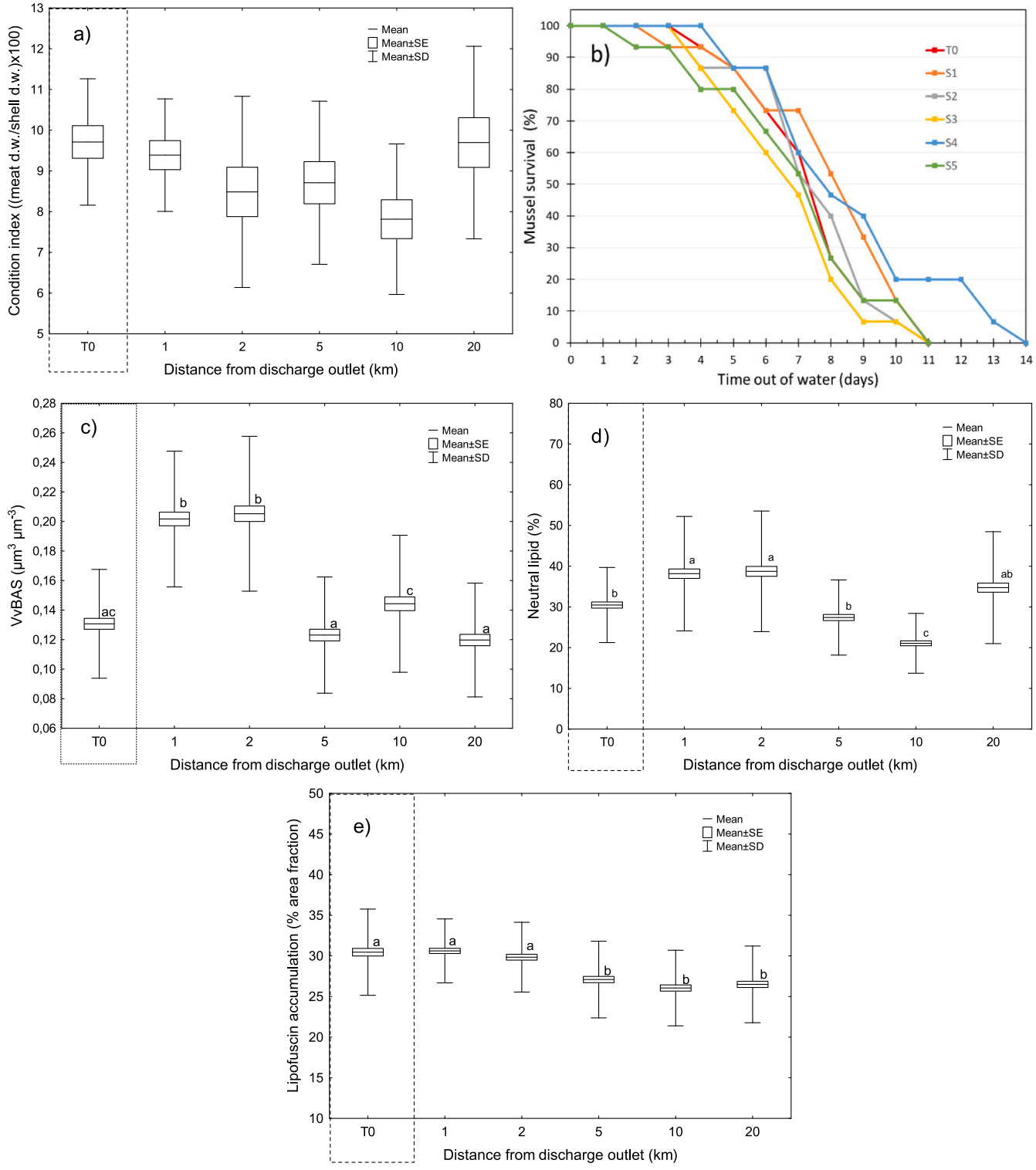


Fig. 5. Whole organism and histochemical biomarker responses from the day zero group (T0) and in field transplanted mussels. Mussels placed for 6-weeks in Sunndalsfjord recipient at known distances from the aluminium smelters discharge outlet. Different letters denote significant differences between groups (ANOVA, Tukey $p < 0.05$).

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 Sum PAC levels. Lipofuscin accumulation was positively correlated with Pb and V, whilst MN formation and the volume density of basophilic cells showed positive correlation with Mo and Pb, respectively. On the other hand, stress on stress showed a negative correlation with As and phenanthrene.

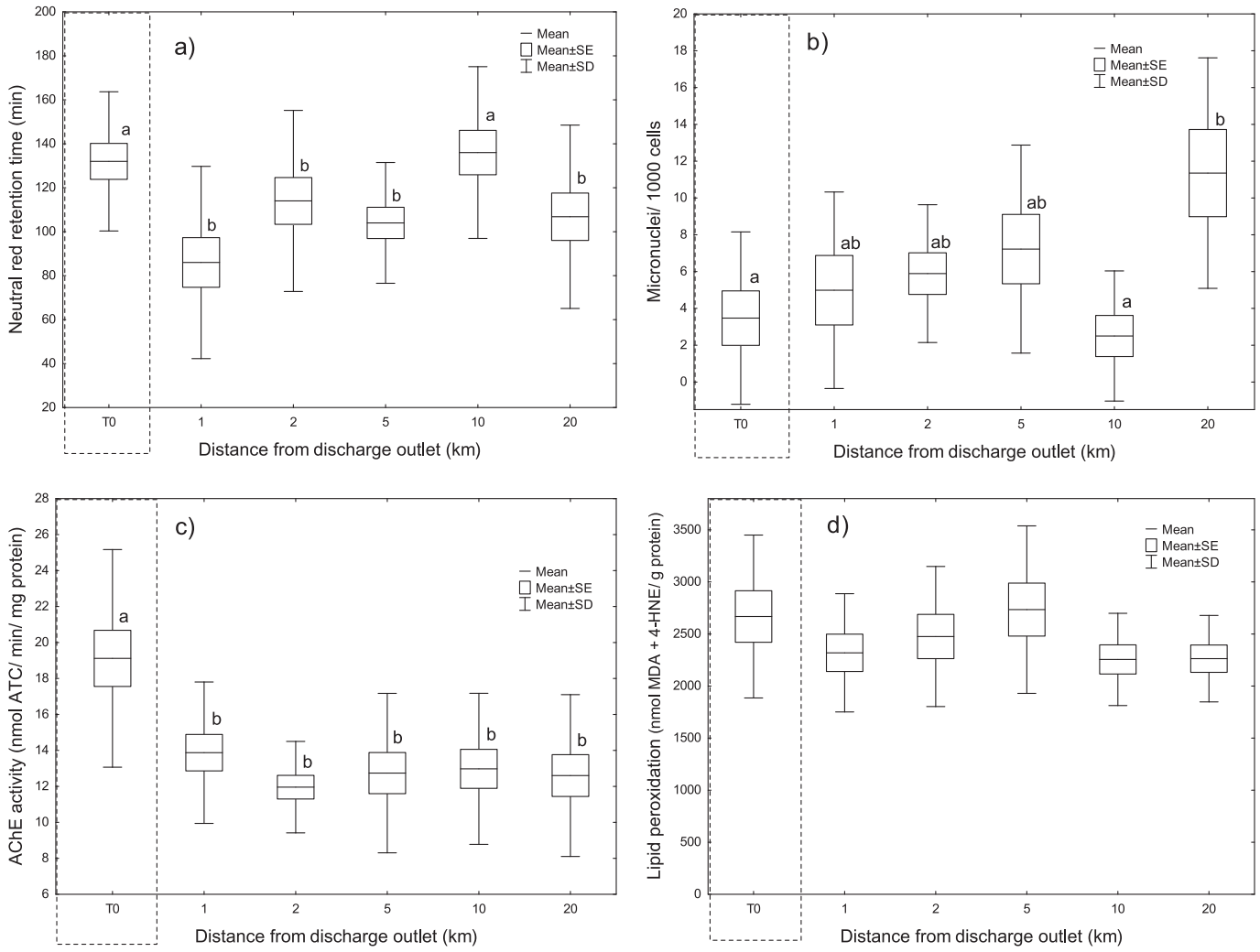


Fig. 6. Subcellular biomarker responses from the day zero group (T0) and in field transplanted mussels. Mussels placed for 6-weeks in Sunndalsfjord recipient at known distances from the aluminium smelters discharge outlet. Different letters denote significant differences between groups (ANOVA, Tukey $p > 0.05$).

4. Discussion

4.1. Contaminant concentrations in mussel tissue

The Norwegian classification system, which indicates the concentration of concern for the different 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.

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 5 km group has concentrations classified as moderate (II). However, in comparison to the PROREF 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.

PAH concentrations were elevated in the T0 mussels compared to the mussels transplanted in the Sunndalsfjord 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. However, the concentration of PAH in the mussels were overall not particularly elevated with PAH16 concentrations between 9 and 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 scheme of 34 ng/g w.w.

For comparison with other field exposures using mussels, the Sum PAC 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., 2019). Furthermore, mussels positioned 10 km from Statfjord B had sum PAC concentrations at 53 ng/g w.w., which were almost double the concentrations measured in T0 mussels. However, despite the low concentrations measured in field transplanted mussels, there was a relationship between PAC concentration in mussel tissue and their proximity to the Al smelter, with higher concentrations of PAH16 and sum PACs in mussels positioned 1 to 5 km from the discharge compared to those at 10 and 20 km away. Additionally, when the PAC accumulation in mussel tissue was grouped in relation to the number of aromatic rings, a clear desorption of 2–4 ring PAC was observed, whilst 5–6 ring PAC did not desorb in the closest mussels. This group of heavy PAC

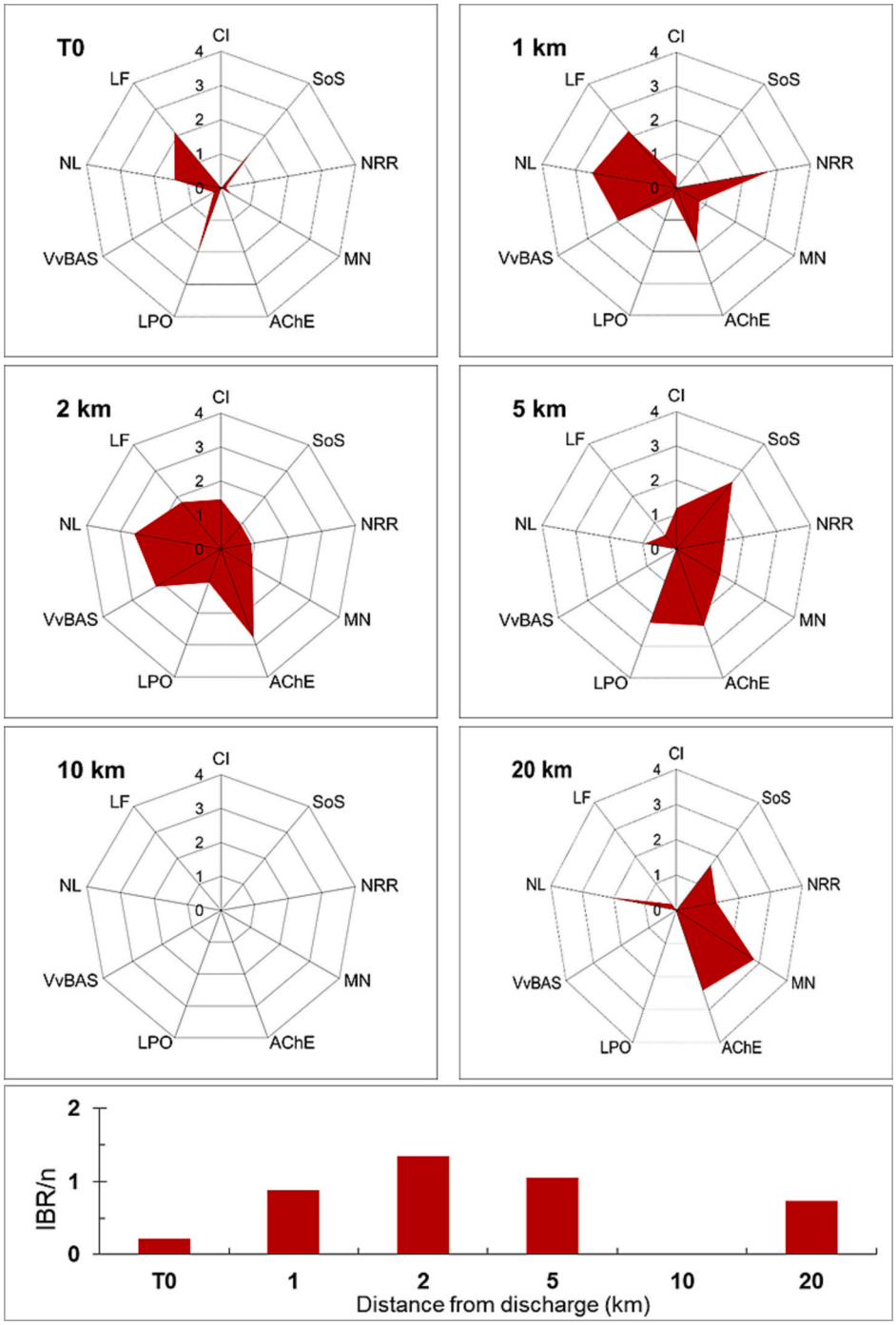


Fig. 7. Integrated biological response (IBR/n) calculated from star plots of mean normalised biomarker data in mussels located at different distances from the Sunndals aluminium smelters discharge outlet.

would include the 5 ring benzo(*a*)pyrene (BaP), benzo(*e*)pyrene, benzo (*bj*)fluoranthene (BbjF), 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 (B(*ghi*)P). The lack of desorption of these heavier PACs in the mussels closest to the Al smelter would indicate that the smelter is a source of these heavier PACs within the fjord. In support of this, the PI indicated that the source of the PACs in the mussels near the Al smelter was of pyrogenic origin, as opposed to the other mussels both at T0 and the stations further away from the smelter. Since this is the first biological effects study that has been performed using field transplanted mussels in the Sunndalsfjord, comparisons with previous studies is limited.

The low concentration of PACs in the field transplanted mussels maybe a reflection of the position of the mussels within the fjord and that they were not directly exposed to the discharge effluent from the smelter. Due to presence of large vessels in the fjord, the mussels were placed at a depth of approximately 20 m at all stations to avoid being hit, moved and/or damaged. The discharge plume from the smelter is influenced by a freshwater input into the fjord, which may result in much of the effluent remaining in the surface waters rather than mixing with the higher saline waters of the fjord. Although the change in PAC profile indicated a chronic level of exposure to the smelter effluent, higher exposures directly to the discharge effluent were unlikely to have occurred. This highlights the challenges in using fixed moorings to assess

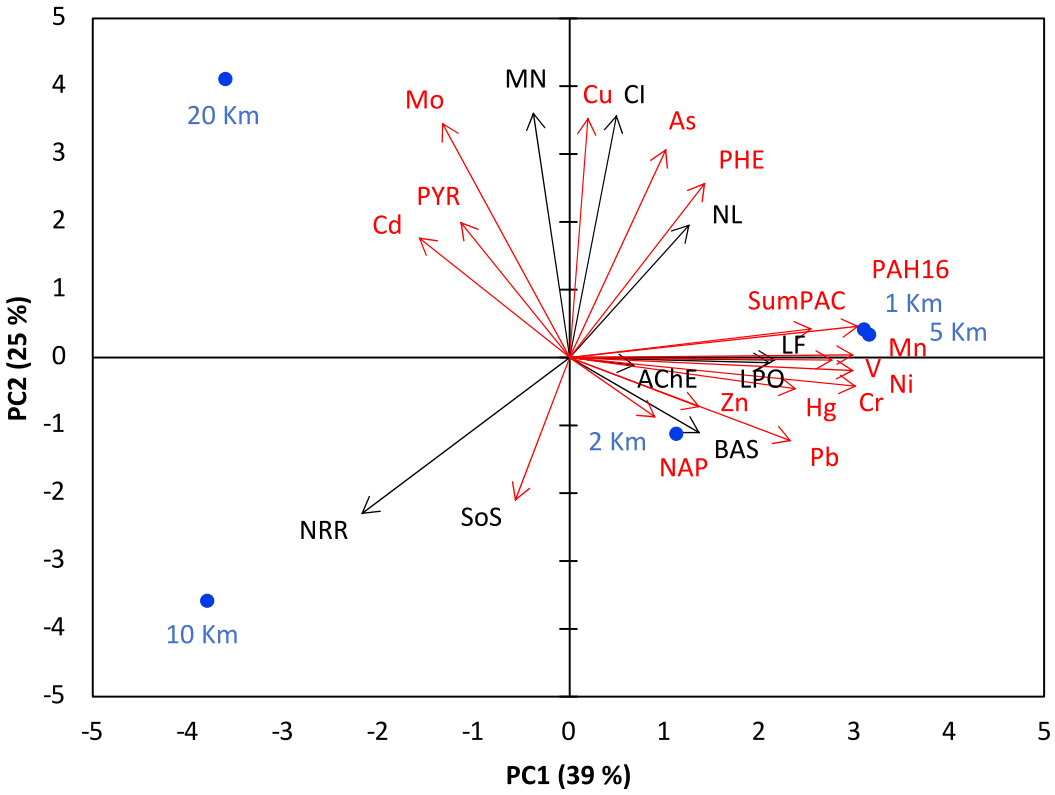


Fig. 8. Principal Component Analysis of chemical measurements (red) and biological responses (black) in mussels located at different distances from the Sunndals aluminium smelters discharge outlet (blue). PCA excludes T0 group. CI – Condition index; SoS – Stress on stress; NRR – Neutral red retention; 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; 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; SumPAC – Total polyaromatic compounds including 54 PAH compounds; AS – Arsenic; Cd – Cadmium; Cr – Chromium; Cu – Copper; Pb – Lead; Mn – Manganese; Hg – Mercury; Mo – Molybdenum; Ni – Nickel; V – Vanadium; Zn – Zinc.

Table 2
The Norwegian classification scheme for the relative risk of metal concentrations in marine mussels (mg/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.

Chemical	Classification (upper limit for class I–IV)					EQS	PROREF
	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

the impact of an effluent in receiving waters, where worst case exposures are difficult to simulate, and the plume dynamics within the recipient are so crucial to understand.

4.2. Biological effects responses

A total of nine biological effects endpoints were measured in the mussels transplanted into the Sunndalsfjord. The approach taken in using a suite of biological effects tools at different levels of biological complexity is characteristic of 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 days 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., 2015b). 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 an 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 Sunndal aluminium smelter.

The 20 km group was located within 500 m of a salmon farm, consisting of 10 large circular pens. The pens were approximately 30 m in diameter, 20–50 m deep and can hold up to 200,000 salmon. Due to the high density of fish, disease and parasites are often associated with salmon farming where veterinary medicines in the form of antibiotics and sea lice treatments are used to improve fish health. Furthermore, antifouling chemicals (e.g., copper) are used to coat the nets, and the overall high organic load from uneaten food and waste can lead to anoxic conditions beneath the nets. Considering all these potential factors, it may not be too surprising to suggest that inputs from the salmon farm may have had some impact on the mussels. In addition, approximately 5 km further out of the estuary from the 20 km mussel group, was another potential point source at Raudsand. Here industrial discharges into the fjord from a metal recycling plant that produces alloys of aluminium and magnesium may have provided an additional contaminant exposure. The main components of the discharge include metals, ammonia and elevated pH (pH 9–11). A survey conducted in 2013 reported high sediment concentrations of Pb and Zn within a few hundred meters to Raudsand, whilst high concentrations of Cu and Ni as well as polychlorinated biphenyls (PCBs) were found at all sites in the fjord within 7.5 km from Raudsand (Berge et al., 2013).

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). This suggests that mussels with a MN above this value are experiencing a genotoxic response above typical background levels. Only mussels positioned 10 km from the discharge outlet 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, near 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 Bøk fjord recipient to within 1 km of the Sydvaranger iron ore mine discharge in Finmark, Norway (Brooks et al., 2015b).

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 (Davies and Vethaak, 2012). A reduction in AChE activity may be considered to represent a neurotoxic response. Only the day zero 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 wider region of the fjord. However, previous studies using field transplanted mussels have reported AChE concentrations of 12–14 nmol/ATC/min/mg protein as indicative of normal levels (Valbonesi et al., 2003). The slight elevation in AChE activity measured in the day zero mussels compared to those placed in the field may have been caused by other factors such as oxidative stress.

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 LP 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 LP concentrations were detected in the gills of *M. edulis* placed in the Frænfjord for 6 weeks whilst 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 NL in the cytoplasm, which become internalised into the lysosomes of digestive gland cells. For the field exposed mussels, the NL 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, NL 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 provide 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 0.25, 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 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, sum PAC and metals Mn, Ni, Cr as the main contributors to the higher stress seen 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. Secondary sources from the local salmon pens and/or the industrial inputs 5 km further North at Rausand, have been suggested as possible factors.

5. Conclusion

Significant biological responses were observed in mussels 1–5 km downstream from the Al smelter in Sunndalsfjord. 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). PAC concentrations were generally low in all groups, although for field exposed groups the PAC concentrations were slightly higher in the closest groups (1–5 km) and higher concentrations of the heavier (5–6 ring) PACs were found in mussels closest to the Al smelter. However, highest PAC concentrations were observed in mussels from the T0 group, which were made up of mostly lighter 2–3 ring PACs and 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 PAC 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 and may be attributed to compounds not measured in this particular study.

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. Even though not one chemical factor explained the increased stress on mussels, the presence of PAH16, sum PACs and metals Mn, Ni, Cr were the main contributors to the higher stress seen in 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. The study highlights the advantages of using a suite of biological effects responses that can be integrated to detect the effects of chemical mixtures whose individual components are in low concentrations but through combination may cause an effect.

CRediT authorship contribution statement

S.J. Brooks: Conceptualization, Methodology, Software, Validation, Formal analysis, Writing – original draft. **T. Gomes:** Methodology, Software, Validation, Formal analysis, Writing – review & editing. **M. Grung:** Methodology, Software, Validation, Formal analysis, Writing – review & editing. **K. Petersen:** Methodology, Formal analysis, Writing – review & editing. **A. Macken:** Conceptualization, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Steven Brooks reports financial support was provided by Aluminiumindustriens Miljøsekretariat (AMS).

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.166798.

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