An integrative biological effects assessment of a mine discharge into a Norwegian fjord using field transplanted mussels

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Abstract

The blue mussel (*Mytilus* sp.) has been used to assess the potential biological effects of the discharge effluent from the Omya Hustadmarmor mine, which releases its tailings into the Frænfjord near Molde, Norway. Chemical body burden and a suite of biological effects markers were measured in mussels positioned for 8 weeks at known distances from the discharge outlet. The biomarkers used included: condition index (CI); stress on stress (SoS); micronuclei formation (MN); acetylcholine esterase (AChE) inhibition, lipid peroxidation (LPO) and Neutral lipid (NL) accumulation. Methyl triethanol ammonium (MTA), a chemical marker for the esterquat based flotation chemical (FLOT2015), known to be used at the mine, was detected in mussels positioned 1500 m and 2000 m downstream from the discharge outlet. Overall the biological responses indicated an increased level of stress in mussels located closest to the discharge outlet. The same biomarkers (MN, SoS, NL) were responsible for the integrated biological response (IBR/n) of the two closest stations and indicates a response to a common point source. The integrated biological response index (IBR/n) reflected the expected level of exposure to the mine effluent, with the highest IBR/n calculated in mussels positioned closest to the discharge. Principal component analysis (PCA) also showed a clear separation between the mussel groups, with the most stressed mussels located closest to the mine tailing outlet. Although not one chemical factor could explain the increased stress on the mussels, highest metal (As, Co, Ni, Cd, Zn, Ag, Cu, Fe) and MTA concentrations were detected in the mussel group located closest to the mine discharge.

41 1. Introduction

42 The discharge of mine tailings into a marine recipient is relatively commonplace in Norway
43 where vast quantities of processed mine tailings are discharged into coastal fjords. In certain
44 instances, the discharged tailings have been used to create land bodies and new harbour
45 areas (e.g. Sydvaranger in Kirkenes, Norway). However, the deposition of mine tailings into
46 Norwegian fjords is a controversial topic with concerns over the effects on marine life,
47 particularly benthic communities but also local fish populations and their potential impact on
48 human health.

49 Omya Hustadmarmor located on the West coast of Norway releases fine particulates from the
50 processing of limestone into the Frænfjord on the west coast of Norway (Figure 1). The tailings
51 consist of approximately 50% limestone ($CaCO_3$), which mostly comprises of small particles
52 (<20 µm diameter). The remaining proportion consist of quarts, feldspar, mica and iron
53 sulphides in addition to production chemicals such as the flotation chemical, known
54 commercially as FLOT2015. The tailings have been discharged into the Frænfjord through an
55 underwater pipeline since 1978 and an impacted zone around the discharge outlet of 1 to 2
56 km is evident, with low abundance of benthic marine life (Brooks et al., 2015a).

57 The main physical effect of the tailings is from the physical smothering of the benthic
58 community in the vicinity or within the impacted zone adjacent to the discharge area. Effects
59 of the tailings on the benthic communities near to and moving away from the outlet within the
60 Frænfjord have been monitored over the last 20 to 30 years and is summarised in Brooks et
61 al. (2015a). However, the effects on organisms within the water column have received
62 relatively little attention and to the authors knowledge this is the first time a biological effects
63 monitoring study using field transplanted mussels has been employed within the fjord to
64 investigate possible biological effects of exposure to the tailing plume.

A suite of sensitive health biomarkers in mussels have been measured to provide a holistic assessment of organism health status as a result of exposure to the plume compared to a reference source. Similar approaches have been used on other mine discharges where significant health effects have been observed (Brooks et al., 2015b; Zorita et al., 2006; Grout and Levings, 2001). The biomarkers selected provide information on the general health status and/or specific (genotoxic, neurotoxic) responses at whole organism, tissue and subcellular level following exposure to environmental stress such as contaminant exposure.

The condition index (CI) provides a measure of the overall health of the mussel, summarising the mussels physiological status (e.g. growth, reproduction, metabolism) under given environmental conditions. Stress on stress (SoS) also provides a general assessment of mussel health, measuring the tolerance of the mussel to survive in air. SoS has been responsive to point source discharges (Brooks et al. 2015b) as well as providing an indication of environmental health (Hellou and Law, 2003). Micronuclei (MN) formation is a sensitive biomarker of genotoxicity (Bolognesi et al. 1996). MN are chromatin-containing structures that are surrounded by a membrane with no detectable link to the cell nucleus. The frequency of MN provides evidence of DNA breakage and spindle dysfunction and reveals a time integrated response to complex mixtures of contaminants (e.g. MacGregor, 1991; Zoll-Moreux, 1999). The measure of neutral lipids in the mussel digestive cells has been found to be a useful marker of change in the physiology of cells (Viarengo et al. 2007). Exposure to organic contaminants has been linked with changes in the metabolism of neutral lipids resulting in accumulation inside lysosomes of the digestive gland (Moore, 1988). Acetylcholine esterase (AChE) is an essential neurotransmission enzyme, that when inhibited, can result in a variety of effects on the central nervous system (Costa, 2006). AChE inhibition assay has been used to assess the neurotoxicity of environmental samples (Froment et al. 2016; Bocquené et al. 1990). Lipid peroxidation (LPO) is characterized by the oxidative deterioration

90 of polyunsaturated fatty acids present in cellular membranes, which can alter membrane
91 fluidity and permeability or attack other intracellular molecules (Halliwell and Gutteridge,
92 2007). The formation of lipid peroxides is characterized by the presence of by-products as
93 malondialdehyde (MDA) and hydroxyalkenals, that have been routinely measured in bivalve
94 species to reflect contaminant-induced oxidative damage (e.g. Pereira et al., 2013).

95 The main chemicals of concern within the tailing discharge include the naturally occurring
96 metal ions and the added production chemicals. The production chemicals include the
97 esterquat flotation chemical known commercially as FLOT2015. This product is formed mainly
98 by a mixture of unsaturated fatty acids mono-, di- and/or triesterified with a methyl triethanol
99 ammonium (MTA) moiety that belongs to the family of the esterquats tensioactives. In contact
100 with water, the product undergoes hydrolytic reactions that break the ester linkage and yield
101 a mixture of fatty acids and MTA (Figure 2). The MTA compound is measurable and provides
102 an indication of the presence of the flotation chemical (FLOT2015) within the recipient and/ or
103 that which has accumulated in exposed mussels.

104 The aim of the study was to determine the potential biological effects of the Hustadmarmor
105 discharge tailings on mussels living in the water column at known distances from the
106 discharge outlet. The placement of the mussels, away from the immediate vicinity of the
107 discharge outlet, was designed in order to measure the sub-lethal effects of the tailings on
108 the mussels and thereby minimising the physical effects of the tailing particles. Correlation
109 of the chemical measurements with the biological effects will be used to indicate potential
110 candidate compounds causing the biological responses.

111 ## 2. Methods

112 ### 2.1. Transplantation of field mussels

113 Mussels were collected in early April 2016 from the lower intertidal shore region of the outer
114 Oslo fjord (59°36'55.5"N 10°39'04.2"E), near the NIVA marine research station in
115 Solbergstrand, Norway. The mussels were taken to the marine research station and placed
116 in flow-through tanks of filtered seawater. The seawater flow rate was approximately 20 L/
117 min at a temperature of 8 ± 1°C for approximately two weeks prior to field deployment. During
118 this time the mussels were fed daily with a live algal culture. Although species identification
119 was not performed on individual mussels during this study, the mussels sampled from this
120 location were all previously identified as *M. edulis* (Brooks and Farmen, 2013). Therefore, it
121 was assumed that most if not all individuals were *M. edulis* and species differences in
122 biomarker response and chemical bioaccumulation were not a confounding factor in this
123 study. All mussels used were of a similar size (44.6 ± 3.4 mm, mean ± standard deviation).

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

132 Approximately 100 mussels were attached to three moorings, positioned at known distances
133 from the Hustadmarmor discharge outlet in the Frænfjord (Figure 2). The moorings, standing
134 vertically in the water column, consisted of an anchor, rope and two 8 kg buoys with no surface
135 marker buoy. They were positioned at 1500, 2000 and 6000 m from the discharge outlet with
136 the aid of a small boat (MS Emilie). The mussels were secured to the rope with cable ties and

were 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 27th and collected after 8 weeks on June 21st. Mussels were collected with divers from the MS Emilie in the morning, placed in a cooler box with wet kelp and cooling blocks, and transported via overnight courier to the NIVA laboratory in Oslo. No mussel mortalities were observed during inspection, although a film of fine sediment was evident on the mussels from the stations 1500 m and 2000 m from the discharge outlet, indicating exposure to the discharge plume. The surface seawater temperature on deployment was approximately 10 ± 1 °C, with a salinity of 33 ± 1 ‰.

2.2. Analysis of mussel samples

The mussels were processed on the day of arrival at the NIVA laboratory in Oslo. Biometry measurements (length, width, breadth, wet weight) were taken from all mussels sampled. Haemolymph was taken for micronuclei (MN) assessment, whilst in the same individual mussels the gill tissue was removed and snap frozen in liquid nitrogen for lipid peroxidation (LPO) and acetylcholine esterase inhibition (AChE). Digestive gland samples were also excised from the same individuals and snap frozen in liquid nitrogen for analysis of neutral lipid (NL) accumulation. The snap frozen tissues were stored at -80°C until required for analysis. 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 briefly 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 suspect substances such as the industrial residue MTA (Figure 2). Ultra-high performance chromatography (UPLC) coupled to high resolution mass spectrometry (HRMS) was employed for the chemical analysis.

Extraction procedure

Sample preparation for mussels was performed with ultrasound assisted solid-liquid extraction (Bransonic® ultrasonic bath, 5510), based on previous validated analytical methodologies to analyse cationic surfactants. Recoveries of control samples (in triplicate) were performed by spiking unexposed mussels with the esterquat core Tris(2-hydroxyethyl) methylammonium at concentration levels of 1, 10 and 100 ng/ g. The reference standard (as methylsulfate salt) was purchased from Sigma-Aldrich (Germany).

Mussels were freeze dried for 24 h using a lyophilizer (Lyovac GT2, Leyvold-Heraeus). The dried material was milled using an agate mortar to obtain a fine powder. 250 mg of the mussel powder was transferred into a polypropylene tube (VWR Collection) and 3 extractions using 4 ml acetonitrile/ formic acid 3% were performed. After each extraction, the tube was centrifuged at 3000 rpm for 10 minutes and the supernatant was taken and transferred into another polypropylene tube. The extract was evaporated to dryness under a nitrogen stream

and reconstituted in 1 ml acetonitrile/formic acid 3%. 300 µl volumes were transferred into a chromatographic vial with insert and analysed through UPLC-HRMS.

UPLC-HRMS Method

The analysis was performed using UPLC (Ultimate 3000 chromatograph with autosampler) coupled to QExactive detector from Thermo Scientific. The MS analysis was performed with an electrospray ionization (ESI) interface in positive ionization mode. Chromatographic separation was performed using an Acquity BEH C18 column (150 mm x 2.1 mm i.d. 1.7 µm particle size; Waters Corp. Mildford, MA, USA) where the separation was performed in 28 minutes using a binary mobile phase of formic acid 0.1 % (solvent A) and acetonitrile (solvent B) at 0.4 ml/ min. The gradient elution starts with 98% A and then increasing B to 100% in 28 min: Solvent A, held for 5 min; 5–10 linear rate to 100% B, 10–26 linear rate to 100% B, held for 0.5 min; reconditioning with a linear rate to 98% A, 26–28 min. Due to the chemical structure of suspect substances (cationic surfactants with large straight-chain and branched alkanes), the gradient was performed using a ramp in the flow rate (0.4 ml/ min from 0 to 5 min, 0.5 ml/ min from 5 to 10 min, 0.5 ml/ min held for 15 min and coming back to 0.4 ml/ min). 5 µl of extracted sample was directly injected in the system.

For the MS detection, an acquisition method based on full scan mode at 70 000 resolution power was performed using wide range of masses (100-1000 Da) in order to acquire the maximum amount of data. Parallel to full-scan MS acquisition, data-dependent acquisition was used where the threshold of intensity (1000 counts) was used for triggering the ion masses to a MS/ MS experiment (35 000 resolution power). This MS/ MS method also included a list of m/ z ions which were suspected of being present in this area. Chromatograms obtained were compared with blank extraction samples in order to identify the chromatographic peaks from suspected substances as well as unknown substances. As a first

approach, only those peaks that were not detected in the day zero samples were considered as substance candidates for further evaluation. In a second approach based on metabolomics aspects of the mussels, other peaks with significant increment of the signal compared with mussels no-exposed were also explored. The MS/ MS spectra obtained were carefully studied in order to propose a chemical structure. The assignment of a fragmentation profile detected in the MS/ MS spectra of each candidate was supported by Mass Frontier (software from Thermo Science), which has enabled the theoretical generation of mass fragments based on a proposed chemical structure. Also the m/z Cloud library from Thermo Science ® was consulted for the confirmation.

Metal analysis

Metal concentrations (Al, Ag, As, Cd, Co, Cr, Cu, Fe, Ni, Pb, Sn, 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 (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.

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

2.2.3. Stress on stress

229 The stress on stress (SoS) assessment was measured with twenty mussels from each group.
230 Mussels were placed in a humid chamber at 15 ± 0.5°C with a 16 h: 8 h light dark cycle. The
231 mussels were checked every 24 ± 2 h and mortalities were recorded and removed from the
232 incubator. Mussels were considered deceased if their shells were gaping and showed no sign
233 of movement after gentle tapping on their shells.

234

235 2.2.4. Micronuclei formation

236 The micronuclei (MN) assessment deployed in this study has been described in more detail
237 elsewhere (Brooks et al., 2015b). Therefore, only a brief description is provided herein.
238 Approximately 0.1 ml of haemolymph was removed from the posterior adductor muscle of the
239 mussel with a syringe and needle (0.6 ml) containing 0.1 ml of PBS buffer (100 mM PBS, 10
240 mM EDTA). The haemolymph and PBS buffer mix was placed on a microscope slide in a humid
241 chamber for 15 min to enable the haemocytes to adhere. The adhered haemocytes were fixed
242 in 1% glutaraldehyde for 5 min. rinsed in PBS buffer and left to air-dry in the dark overnight.

243 Slides were stained with 1 µg/ ml bisbenzimide 33258 (Hoechst) solution for 5 min, rinsed with
244 distilled water and mounted in glycerol McIlvaine buffer (1:1). The frequency of MN was
245 measured on coded slides without knowledge of the exposure status of the samples to
246 eliminate bias. The frequency of micronuclei in haemocytes was determined microscopically
247 (×100 objective) on a minimum of 2500 cells per exposure group. Micronuclei were scored in
248 cells with intact cellular and nuclear membranes when: 1) nucleus and micronuclei have a
249 common cytoplasm, 2) colour intensity and texture of micronuclei is similar to the nucleus, 3)
250 the size of the micronuclei is equal or smaller than 1/3 of the nucleus, 4) MN are apparent as
251 spherical structures with a sharp contour.

252

253 2.2.5. Neutral lipid accumulation

254 Frozen sections (10 µm) of digestive gland tissue were prepared on a cryostat (Leica CM1860),
255 with duplicate sections prepared for each mussel. The sections were placed on to glass
256 microscope slides and fixed in Baker's calcium formal for 15 min. The fixed sections were
257 briefly rinsed in distilled water before placed in 60% triethyl phosphate for 1 min, stained in
258 oil red O solution for 15 min at room temperature, rinsed again in 60% triethyl phosphate and
259 finally in distilled water before air-drying overnight in the dark and mounting in glycerol
260 gelatin.

261 The accumulation of neutral lipid was evaluated microscopically (40x objective). The
262 percentage area of tissue section covered by neutral lipids was assessed in 8 randomly
263 selected fields of view for each mussel, with 10 mussels analysed per group.

264

265 2.2.6. Acetylcholine esterase inhibition

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

274

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 15 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 minutes at 4°C 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.

2.3. Integrated assessment (IBR and/or principal component)

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

297 status following chemical exposure (Beliaeff and Burgeot, 2002). The IBR/n, which accounts
298 for the number of biomarkers in the data set, was used to integrate the biomarker data (Broeg
299 and Lehtonen, 2006). In the present study CI, SoS, MN, AChE inhibition, LPO and NL
300 accumulation were selected for the IBR calculation. The inverse values of CI, SS, and AChE
301 were used since a decrease was reflective of an adverse impact. The IBR index was calculated
302 by summing-up triangular star plot areas for each two neighbouring biomarkers in a data set.
303 For more information, the exact procedure has been previous described in Brooks et al.,
304 (2015b).

305 A Principal component analysis (PCA) was performed using XLStat2017® (Addinsoft, Paris,
306 France) to highlight the main variables responsible for the variance of data obtained for all
307 groups. A Pearson's correlation analysis was also performed to evaluate the strength of
308 association between chemical body burden and biological responses of mussels. The level of
309 significance was set to p=0.05.

310

311 2.4. Statistical analysis

312 Analysis of variance (ANOVA) followed by a Tukey post-hoc test was performed on the
313 biological effects data to determine statistical differences between groups. Homogeneity of
314 variance was determined with a Levene's test prior to testing, and where necessary, data were
315 log transformed to achieve homogeneity. However, in cases where homogeneity of variances
316 was not achieved a Kruskal-Wallis non-parametric analysis was used.

317 3. Results

318 3.1. Chemical analysis

Methyl triethanol ammonium (m/z 164.12794) was found in exposed mussels. The identification of MTA core in extracts obtained from mussels exposed to the tailings are presented in figure 3. Methyl triethanol ammonium was only detected in mussels from the two closest stations (Table 1). Highest concentrations were found in mussels located 1500 m from the discharge outlet (0.30 ± 0.05 µg/ g) compared to those positioned at 2000 m (0.14 ± 0.06 µg/ g). To the authors knowledge this is the first time MTA has been found in mussels, either wild or field transplanted.

Other industrial substances used in mining activities, such as the esters related to certain technical products (Figure 2), were also screened for but were not detected in the field exposed mussels. However, natural substances relating to the metabolism of the mussel, which can become elevated under stress conditions, were found with significant increments in field exposed mussels. This was the case for arachidonic acid, which is an anti-inflammatory substance naturally metabolized by blue mussel under stress conditions (e.g. rapid temperature changes, Fokina et al., 2015). Arachidonic acid was identified in mussels (non-exposed and exposed) by using HRMS (Figure 4) and confirmed by the m/z Cloud library (Figure 5). The value of the chromatographic area of arachidonic acid was found up to seven-fold higher in field exposed mussels than the day 0 mussels (Figure 6). The arachidonic acid was highest in mussels from the station closest to the discharge (1500 m), slightly lower in mussels at 2000 m and even lower in mussels 6000 m away. This increase in the arachidonic acid in mussels located closer to the mine discharge would suggest that there was an external source provoking a stress response in the mussels. Despite this, there was no direct relationship between the presence of the MTA and the increase in arachidonic acid in mussels.

A total of 12 metals were measured in the homogenised soft tissue of mussels from the different groups (Table 1). Of the 12 metals measured, As, Cd, Co, Fe, Zn and Ni had higher

concentrations in the exposed mussels compared to the pre-exposed group (Day 0). These six metals were highest in the mussels located closest to the discharge outlet. This suggests the contribution of these metals from the mine tailing point source. The concentration of metals measured in the soft tissue of transplanted mussel, based on the Norwegian Environmental agencies guidelines (Molvær et al., 1997), indicated either unpolluted or low levels of pollution (Class I). Of the metals analysed, only As had a classification of moderately polluted (Class II). Overall, the metal body burden concentrations were not considered a concern for organism health.

For metals Pb, Cr and particularly Al, concentrations in the Day 0 group were markedly higher than the field transplanted mussels, which would suggest the mussels were exposed to these metals whilst held in flowing seawater at the Marine research station for two weeks prior to deployment. However, based on the Norwegian authorities' guidelines, the concentrations were indicative of unpolluted or low levels of pollution (Molvær et al., 1997).

3.2. Biological effects measurements

There were no significant differences in mussel length between the different groups. It was therefore assumed that mussel size and age had no significant confounding effects on biomarker responses or chemical bioaccumulation. For all mussels analysed in this study, the median length was 43.5 mm with a 10^{th} and 90^{th} percentiles of 40.7 and 49.0 mm respectively.

3.2.1. Condition index

Condition index was calculated as the ratio of the meat dry weight over the valve dry weight multiplied by 100, with a lower CI indicative of a relatively poorer health status. Comparison

366 between groups showed significantly lower CI values in mussels from Day 0 and 1500 m from
367 the discharge outlet compared to those mussels from 2000 m and 6000 m (Figure 7A). This
368 would indicate that mussels had a lower CI when deployed into the field (Day 0), which
369 increased in groups 2000 m and 6000 m from the outlet during the 8-week exposure.

370 3.2.2. Stress on stress

371 The ability of mussels to survive in air provides an additional stress to the possible chemical
372 stress on the mussels within the field exposure. Typical stress on stress curves were found
373 for mussels in all groups (Figure 7B). However, the exponential decrease in survival occurred
374 at different times for the different mussel groups. Lowest 50% lethal threshold (LT_{50}) values
375 were recorded in mussels from the closest site to the discharge outlet (LT_{50} = 4.4 d) narrowly
376 followed by mussels from 2000 m away (LT_{50} = 5 d) and then those mussels furthest away (6000
377 m, LT_{50} = 6 d). The mussels which survived longest out of water and were therefore considered
378 to be in the best general health were those from the Day 0 group with an LT_{50} of 7.5 d.
379 Statistical analysis was not performed on the stress on stress data.

380 3.2.3. Micronuclei formation

381 Micronuclei formation in the haemocytes of mussels is a well validated method that provides
382 a sensitive measure of a genotoxic re sponse of an organism following exposure to
383 environmental mixtures (Baršiene et al., 2008). The frequency of micronuclei in the
384 haemolymph samples of mussels from the different groups is shown in figure 7C. Highest
385 frequencies of micronuclei were recorded in mussels located closest (1500 m) to the
386 discharge (9.9/ 1000 cells) as well as those from 2000 m (8.5/ 1000 cells). These values were
387 significantly higher than those from the pre-exposure group (day 0, 3.6/ 1000 cells) and the
388 field reference groups (6000 m, 1.8/ 1000 cells) (ANOVA, Tukey, $p<0.05$).

3.2.4. Acetylcholine esterase inhibition

Acetylcholine esterase measured in gill homogenates of mussels from the different exposure groups is shown in figure 7D. Acetylcholine esterase activity in all groups was measured between 6.4 and 6.9 nmol ATC/ min/ mg protein, with no significant differences between the mussel groups.

3.2.5. Lipid peroxidation

Lipid peroxidation provides a simple measure of oxidative stress experienced at the sub-cellular level. In the field exposed mussels, no significant difference in lipid peroxidation was observed despite a slight increase in the mussels closest to the discharge outlet (Figure 7E). However, highest levels of lipid peroxidation where measured in the gill cells of pre-exposed mussels, which were significantly higher than those from the 2000 m and 6000 m groups

3.2.6. Neutral lipid accumulation

Histochemical examination of the digestive cells of the mussels were performed in order to determine the relative proportion of neutral lipid present (Figure 7F). A relationship between increased neutral lipid and proximity to the discharge outlet was found, with highest neutral lipids (as %) found in mussels from the closest station to the discharge (1500 m). Statistically significant differences were only found between the two closest stations and the day 0 mussel group.

3.3. Integration of the biological effects response

Integration of the biological effects were performed using the IBR/n (Fig. 8). The positioning of the biomarkers around the star plot can influence the IBR/n, therefore care was taken to positon the biomarkers based on their similarity in either cellular and / or physiological

412 function as recommended (Broeg & Lehtonen, 2006). Several combinations were tested and
413 the one shown in figure 8 was considered to provide the best representation of the integrated
414 biomarker data. The highest IBR/n response was clearly identified in the mussels from the
415 closest station. Most of the biomarkers (except lipid peroxidation) contributed equally to the
416 IBR/n calculation. The mussels located 2000 m from the discharge had the next highest IBR/n
417 with main contributions from stress on stress, micronuclei and AChE inhibition. At 6000 m
418 from the discharge outlet the biomarker responses were at background levels, the star plot
419 revealed small contributions from neutral lipid and stress on stress. However, since these
420 biomarkers were not positioned adjacent to each other the IBR/n was not calculated, although
421 the IBR/n would have been low in any case. The mussels from the day 0 group did show an
422 IBR/n response with contributions from biomarkers lipid peroxidation and AChE inhibition.
423 Although condition index was also elevated in day 0 mussels it did not contribute towards the
424 IBR/n.

425

426 3.4. Relationship between chemical measurements and biological effects

427 A PCA was applied to all data to help discriminate the main variables responsible for the
428 variance of chemical body burden and biological effects detected in mussels (Fig.9). Overall,
429 the PCA showed a clear spatial differentiation between mussel groups, highlighting the
430 different responses obtained in relation to proximity to the discharge outlet. PC1 accounted
431 for 63.1% of variance and showed a clear separation between non-exposed mussels and those
432 deployed at the Hustadmarmor discharge outlet in the Frænfjord. Mussels collected at day 0
433 presented higher stress on stress and LPO levels, closely associated with maximum
434 concentrations of Cr, Al and Pb, while the remaining chemical levels and biomarker responses
435 were higher in the exposed mussel groups. PC2 explained 32.1% of the variance and

differentiated mussels located closest to the discharge outlet (1500 m) from those positioned further away (2000 m and 6000 m). The PCA confirmed that mussels located 1500 m from the discharge outlet are the most environmentally stressed, followed by mussels 2000 m away. Mussels from these stations presented the highest concentrations of Fe, Cu, Zn, Ni, Cd, As, Co, and Ag, as well as arachidonic acid and MTA, associated with stronger responses in micronuclei formation and neutral lipids. In opposition, the mussel group positioned furthest away from the discharge outlet presented the highest condition index and AChE activity, being the less impacted group of mussels.

Correlation analysis showed several statistical significant associations between the biological endpoints and the chemical measurements determined in mussel tissues (Table S1 in Supplementary information). Neutral lipids were positively correlated with Co, Ag and arachidonic acid ($p<0.05$), while lipid peroxidation was positively correlated with Cr. On the other hand, stress on stress showed a negative significant correlation with As, Cd, Ni, arachidonic acid and neutral lipids, while arachidonic acid was positively correlated with As.

4. Discussion

4.1. Chemical uptake and biomarker responses

The field exposure was designed to investigate the sub-lethal effects of the tailings, with the closest station positioned approximately 1500 m from the discharge outlet. This was to reduce the acute impact of particle smothering on the mussels, but still ensuring the mussels were exposed to the mine discharge through either suspended fine particles and/ or dissolved chemicals in the recipient. The presence of MTA in the soft tissue of mussels from the two closest stations to the discharge outlet confirmed exposure of the mussels to the mine tailing

459 effluent. This was also confirmed by visual inspection of the mussels during collection, since
460 a white film of fine particles, was found coating the external shells. Although links between
461 chemical exposure and effect will be discussed, physical effects of the particles on the
462 mussels should also be considered. Recent electron microscopic observation revealed that
463 Hustadmarmor tailings mostly consisted of fine grained particles (Trannum et al., 2018). Such
464 fine-grained particles can cause clogging and damage of feeding and respiratory organs such
465 as the gills. This is particularly important in the filter feeding mussel. Consequently, particle
466 interactions could be expected to intensify the observed effects of chemical exposure.

467

468 Overall, the biomarker results appeared to agree with exposure intensity with higher stress
469 responses in mussels from the closest stations, however individual biomarkers in some cases
470 were found to have varying responses. The stress hormone arachidonic acid showed a
471 relationship with proximity to the discharge outlet, with highest values in mussels from the
472 two closest stations, significantly lower at the furthest field transplanted station, and almost
473 absent in the Day 0 group. Similar increases in arachidonic acid and/ or the enzyme
474 arachidonic acid cyclooxygenase (COX), as a stress marker, have been reported previously in
475 freshwater mussels positioned downstream from a sewage treatment plant (Gagné et al.,
476 2007a). Elevated COX activities were also reported in freshwater mussels exposed to an
477 aeration lagoon containing domestic waste water (Gagné et al 2007b). These studies highlight
478 the potential of this stress biomarker in biological effects studies.

479

480 Information on the general physiological status of the mussels, as a result of contaminant
481 exposure, can be obtained from the CI measurements. In the case of the field transplanted
482 mussels, the CI showed a very clear relationship with distance from the Hustadmarmor

discharge outlet, indicating that those mussels closest to the discharge, were in a lower physiological condition. This may be due to the increased proximity to the discharge outlet, where mussels were experiencing exposure to higher particulate loads and elevated chemical concentrations. However, the CI profile is clouded slightly by the low physiological status of the Day 0 mussels, which had a CI comparable to that of the mussels from the closest group, and suggests that the mussel population used in the study was not in optimal physiological condition at the start of the field exposure. What appears likely is that the transplanted mussels experienced warmer seawater temperatures and higher food availability than those mussels held within the Solbergstrand seawater system. The seawater temperature of the Frænfjord was approximately 10 ± 1 °C, compared to 8 ± 1 °C at Solbergstrand. Food availability and temperature is known to influence biological effects measurements in both field (Brenner et al., 2014; Leiniö and Lehtonen, 2005; Orban et al., 2002) and laboratory studies (Múgica et al., 2015). The 8-week exposure was sufficient time for the mussels at the two furthest stations (2000 m and 6000 m), to increase their CI. However, this was not achieved in the mussels from the closest group, which were more influenced by the tailing discharge.

A second measure of physiological status and general fitness of the mussels is provided by SoS. The ability of the mussel to survive out of water has been shown to provide reliable data in biological effects studies (Brooks et al., 2015b; Wepener et al., 2008; Hellou and Law, 2003). The LT_{50} values in field exposed mussels showed a clear relationship with the distance from the discharge outlet. Lowest LT_{50} values, indicating a shorter survival time out of water, were shown in mussels from the closest station, whilst LT_{50} values increased with distance from the discharge outlet. The SoS results agree with the CI data and clearly show an impact of the

506 mine tailing discharge on mussels closer to the outlet. Unlike that described for the CI, the
507 Day 0 group showed the longest survival time out of water, with an LT_{50} of 7.5 days.

508

509 In previous biological effects studies where SoS measurements have been taken in mussels,
510 LT_{50} values between 8 and 12 were recorded for mussels held for 6 weeks in a Norwegian fjord
511 receiving tailings from an iron mine (Brooks et al., 2015b). Furthermore, an LT_{50} of 9 days was
512 reported for intertidal reference mussels from the UK, that reduced to between 5 and 7 days
513 at contaminated sites (Hellou and Law, 2003). Low LT_{50} values between 3 and 6 days were
514 found in mussels from the contaminated Scheldt estuary, which reduced even further to 2
515 days in mussels caged within this estuary (Wepener et al., 2008). The LT_{50} values of the field
516 mussels within the Frænfjord do indicate a stress response, which increases with distance to
517 the discharge outlet. However, the SoS of the Day 0 mussels were below the value expected
518 for true reference mussels.

519

520 Internationally recognised assessment criteria have been developed under ICES for many
521 biological effects measurements in mussels, including SoS (Davies and Vethaak, 2012). ICES
522 background and environmental assessment criteria (BAC and EAC) for mussel SoS have been
523 calculated as 10 and 5 days respectively. Based on these values, the mussels from the two
524 closest stations to the discharge outlet where below the EAC and thus potentially experiencing
525 detrimental effects on the exposed individuals. The two other mussel groups, including the
526 Day 0 group, were above the EAC but were still below the BAC, supporting the idea that the
527 Day 0 mussels were not in optimal condition prior to deployment.

528

529 Exposure of the mussels to genotoxic compounds within the Fraenfjord recipient is provided
530 by counting the frequency of MN within the mussel haemocytes. The ICES BAC for MN in field
531 transplanted mussels (*M. edulis*) is set at 2.5 MN per 1000 cells (Davies and Vethaak, 2012).
532 This suggests that mussels with a MN above this value are experiencing a genotoxic response
533 above typical background levels. The MN frequencies in the field exposed mussels in our study
534 showed a clear relationship between genotoxic response and distance from the discharge
535 outlet. The two closest stations showed mean MN frequencies between 8 and 10 MN/ 1000
536 cells, up to 4 times the BAC, whilst the mussels from the furthest station were below the BAC
537 value, indicating background levels. The Day 0 mussels did show MN frequencies slightly
538 above the BAC value, although significantly lower than the mussels from the two closest
539 stations. The principle component analysis revealed a close relationship between MN and
540 MTA, in addition to Cu, Sn and Zn, which may have contributed to the observed genotoxicity.
541 However, there is currently no evidence to suggest that MTA has genotoxic potential.

542

543 The AChE inhibition test provides an indication of organism exposure to neurotoxic
544 compounds. Neurotoxic compounds have the potential to interfere with important neural
545 processes of exposed organisms. ICES assessment criteria have been suggested with BAC
546 and EAC values in *M. edulis* gill tissue of 30 and 21 for French Atlantic waters, and 26 and 19
547 (nmol/ min/ mg protein) for Portuguese Atlantic waters (Davies and Vethaak, 2012). The AChE
548 activity in the present study was between 6 and 7 nmol/ min/ mg protein with no significant
549 differences between the mussel groups. These values for AChE in Norwegian waters are
550 much lower than ICES assessment criteria, described above. Whether this shows significant
551 inhibition of AChE in all mussel groups is probably unlikely. The lack of response in relation
552 to distance from the discharge outlet, would suggest it is not a stress response. Alternatively,

553 it may point to different procedures in how AChE is measured in mussel tissue and/or the
554 need to establish regional specific assessment criteria for AChE in Norwegian *M. edulis*.

555

556 Lipid peroxidation in the mussel gills was measured to determine the oxidative stress
557 experienced following exposure to the mine tailing discharge. Although no significant
558 difference in lipid peroxidation was found between field transplanted mussels, significantly
559 higher levels of lipid peroxidation were observed in the Day 0 group compared to two of the
560 mussel groups (2000 and 6000 m). The possible reasons for the increase in the source
561 population were unclear, but were elevated when held within the flowing seawater tanks at
562 the NIVA marine research station. ICES assessment criteria were not available for lipid
563 peroxidation in mussel tissue. When compared to previous biological effects studies, similar
564 LPO concentrations were detected in the gills of *M. galloprovincialis* collected in the Ria
565 Formosa Lagoon (900 – 5500 nmol/ g protein and 942 - 3182 nmol/ g protein) in winter and
566 summer periods (Almeida et al., 2013; Pereira et al., 2013).

567

568 Neutral lipid accumulation in the lysosomes of mussel digestive glands is a general stress
569 response to chemical exposure (Viarengo et al., 2007). Neutral lipids are mostly composed of
570 triglycerides, phospholipids, and sterol, which are the main components of cell membranes.
571 Chemical exposure is known to induce the build-up of neutral lipids in the cytoplasm, which
572 become internalised into the lysosomes through autophagic uptake. For the field exposed
573 mussels, the neutral lipid accumulation showed a relationship with distance to the discharge
574 outlet with highest percentage accumulation in mussels from the closest stations. The field
575 transplanted mussels were significantly different from the Day 0 group, with the latter having
576 much lower levels of neutral lipid present. Since neutral lipid composition is influenced by

feeding season and water temperature (Kagley et al., 2003), differences in neutral lipid between the field exposed mussels and the Day 0 group, may be explained by the differences in food availability and temperature.

4.2. Integration of the biomarker responses (IBR)

The integration of the biomarker data provides an overall assessment of the health status of the mussels from the different groups, with a higher IBR/n indicating 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/n 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/n values. As recommended for this integrative approach, biomarkers that measure similar biological responses were placed together (Broeg and Lehtonen, 2006).

The highest IBR/n (2.2) was found at the station closest to the Hustadmarmor discharge outlet. Although situated approximately 1500 m from the outlet, significant biological responses were observed. All six biomarkers contributed to the IBR/n value, and particularly NL, SoS, MN and AChE. These four biomarkers were also responsible for the lower IBR/n (0.8) of the mussels located 2000 m from the discharge, 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 Hustadmarmor discharge outlet.

General stress responses were measured with NL and SoS, whilst genotoxicity and neurotoxicity were measured with MN and AChE respectively. In contrast, an IBR/n was not calculated in the field exposed mussels positioned 6000 m from the discharge outlet: This would suggest that these mussels were not affected by the mine discharge and could be considered as a field reference population.

The Day 0 mussels had an IBR/n of 0.5, with contributions from LPO and AChE. This suggests a very different exposure profile to the two closest field transplanted mussels, with an elevated oxidative stress response (LPO) in mussels held for 2 weeks at the Marine research station.

4.3. Principle component analysis: linking chemical data with the biomarker responses

The integration of biochemical and chemical data through the PCA confirmed 'proximity to the discharge outlet' as the most important factor for spatial biomarker response, as well as the magnitude of contaminants influencing mussel response. Similar to the biomarker results obtained from the IBR/n, the PCA discriminated differences among mussels collected at the different stations, identifying mussels from the station closest to the discharge outlet (1500 m) as the most impacted and those from the station furthest away (6000 m) as the least impacted (higher AChE levels and CI).

The PCA also highlighted the presence of Fe, Cu, Zn, Ni, Cd, As, Co, Ag and MTA as the main contributors to the higher stress seen in mussels collected from the two stations closest to the discharge outlet, characterised by decreased SoS and higher NL, MN and arachidonic acid.

This association was also confirmed by the correlation analysis, which showed a significant and positive correlation between NL, Ag, Co and arachidonic acid, as well as a negative relationship between SoS and As, Cd, Ni, arachidonic acid and NL. These findings suggest a similar biological response due to a common exposure source associated with the proximity to the Hustadmarmor discharge outlet, as also seen in the IBR/n.

Finally, the PCA was also able to distinguish the Day 0 mussels from the field transplanted mussels. This distinction was characterised by higher levels of LPO in the Day 0 group, possibly associated with higher concentrations of Cr, Al and Pb in mussel tissues. In fact, the correlation analysis showed a strong positive association between LPO and Cr, which may be responsible for the oxidative stress response seen in mussels from this group. Overall, the PCA analysis improved the interpretation of the IBR results and indicated an association between the stress response seen in mussels with different levels of exposure to the mine tailing discharge.

5. Conclusions

Significant biological responses were observed in mussels positioned 1500 m downstream from the Hustadmarmor discharge outlet. The biological responses included a reduction in the general fitness of the mussel as well as increased stress and genotoxic responses. Similar but milder biological responses were observed in mussels 2000 m from the outlet. The biological responses observed are believed to be due to exposure to the suspended particles from the mine tailing discharge within the fjord. Concentrations of MTA, a chemical marker for the esterquat based flotation chemical FLOT2015 used at Hustadmarmor, was detected in

whole mussels up to 2000 m from the discharge outlet. This confirms exposure of the mussels to the mine tailing discharge and links tailing exposure (including metal concentrations) with the biological effects observed.

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