Incidence of persistent contaminants through Blue mussels biomonitoring from Flekkefjord fjord and their relevance on food safety

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ABSTRACT

Dredging activities can lead to the resuspension of contaminated sediments, resulting in a potential hazard for the whole ecosystem and also for human health. A six-months active biomonitoring was performed in order to monitor the trends of different classes of both legacy (organochlorine – OCPs - and organophosphate (OPs) compounds and polychlorinated biphenyls - PCBs) and emerging (polybromodiphenyl ethers – PBDE - and per- and polyfluoroalkyl substances – PFASs) organohalogen compounds, as well as polycyclic aromatic hydrocarbons (PAHs), in blue mussel (*Mytilus edulis* spp.) specimens transplanted at different depths in the Flekkefjord fjord. Such biomonitoring was performed to evaluate the efficacy of sediment restoration activities and to check for the potential environmental risk for the biota and food safety for human seafood. A negligible contamination by OCPs, OPs, PBDEs and PFASs was noted in mussels over the six-months biomonitoring, while a notable increase of the concentrations of PCBs and PAHs occurred in mussels transplanted at 15 m depth in three sampling sites within the fjord, as a consequence of an undersea landslide occurred during restoration activities. Levels of PCBs and PAHs suggested a potential risk for mussel predators and also for the human health, as they exceeded the limit set by European Commission for the consumption of bivalve mollusks. These results confirm the reliability of active biomonitoring to flank dredging activities aimed at ecosystem restoration in order to monitor the trend of contaminants and to estimate the potential risk for the aquatic communities and human health.

Keywords: biomonitoring; blue mussel; organohalogen compounds; PAHs; food safety

1. Introduction

Bottom sediments are sinks for several organic chemicals contaminating the marine environment. Such contaminants are often associated with sediment particles and/or to particulate organic matter, other organic molecules and colloids in sediments (Cornelissen et al. 2005). However, the link between contaminants and sediments is not permanent. In fact, variation in physical and chemical characteristics (e.g., pH, salinity, redox potential), natural resuspension phenomena caused by waves, currents and bioturbation and/or and anthropic disturbances, including boat wash, dredging and disposal actions or bottom trawling, can lead to the resuspension of these particle-associated contaminants into the overlying water (Hedman et al. 2009; Jonas and Millward 2010; Juwarkar et al. 2010). Particle-associated and dissolved contaminants that are suspended or released from contaminated sediments returned as available for the uptake by organisms, either *via* particle uptake or *via* transport across biological membranes (e.g., Storelli and Marcotrigiano, 2000; Eggleton and Thomas 2004; Conte et al., 2016; Çulha et al., 2016), representing a serious hazard for the health of living organisms and, potentially, of humans eating contaminated organisms. For instance, field studies have demonstrated that dredging operations of contaminated sediments enhanced the uptake of polycyclic aromatic hydrocarbons (PAHs) and heavy metals (e.g., Bocchetti et al. 2008), as well as of polychlorinated biphenyl (PCBs; Bellas et al. 2007), in mussel species. Despite these findings, periodical dredging activities are necessary for the preservation of navigation depths in ports, as well as for restoration purposes of contaminated ecosystems, leading to a potential resuspension of contaminated sediments and/or the necessity to correctly manage the huge amount of removed sediments. Biomonitoring represents a valuable approach to flank restoration activities because it allows to evaluate the effectiveness of such interventions in reducing

chemical exposure and effects and to assess the effects of a particular restoration activity before, during, and after its conclusion. In fact, biomonitoring returns useful information to establish the baseline levels and the changes over time of environmental contamination, and simultaneously can provide an early warning signal of potential environmental and human health impacts due to release of hazardous substances (NRC 1991). In particular, active biomonitoring method relying on the transplantation of mussels from an unpolluted site and exposing them to different sites (e.g., Kljaković-Gašpić et al. 2006; Milun et al. 2016), represents an excellent approach to monitor the levels and spatial–temporal trends of contaminants in marine ecosystems. Indeed, such approach allows to control some confounding factors (i.e., mussel age, sexual maturity stage and background concentration of contaminants) which can complicate data interpretation in comparison with resident mussels.

Flekkefjord is a municipality located in the Vest-Agder county (Southern Norway; Figure 1) that owes its name by the local fjord called Flekkefjorden, one of the 24 high priority polluted fjords in Norway (https://www.miljodirektoratet.no). Previous industrial activity and municipal waste contributed to its local contamination, so that diverse monitoring studies revealed the presence of PCBs and heavy metals in seawater, sediments and biota sampled in different sites within the fjord (Haker 2011; Misrund 2012). For these reasons, the municipality has decided to perform a recovery action of Flekkefjord fjord by dredging bottom contaminated sediments and to cover the seabed with sand in order to isolate any residual of contamination.

The present study aimed at monitoring the trends of different classes of organic chemicals accumulated in blue mussel (*Mytilus edulis* spp.) specimens transplanted in the Flekkefjord fjord in order to 1) evaluate the efficacy of sediment restoration activities and simultaneously; 2) check for the potential environmental risk for the biota and 3) assess the food safety of seafood

for human consumption. Because of their peculiar biological and ecological characteristics, as well as for their commercial value as food, blue mussels were used as sentinels of anthropogenic contamination trends in coastal waters for a long time (e.g., Farrington et al. 2016; Beyer et al. 2017). Accordingly, monitoring activities using the blue mussel have been a part of the Norwegian coastal environmental monitoring program (MILKYS) since 1981 (Green et al., 2015). For these reasons, an active biomonitoring survey, using transplanted mussels in sites where indigenous conspecifics are scarce or absent, represents a valid and valuable approach to monitor the spatial and temporal trend of contamination in marine ecosystems, as well as to assess environmental risk by comparing measured levels with quality standards or regulatory benchmarks (Beyer et al. 2017). Moreover, as blue mussels represent an important seafood for humans, active biomonitoring data can be also useful to assess potential risk to human health due to consumption of mussels, through the comparison of measured levels of specific contaminants with consumer safety thresholds, such as maximum acceptable toxicant concentrations, which have been established within the environmental legislation of many coastal countries (Beyer et al. 2017). Blue mussel specimens were caged at different depths (5 and 15 m depth) in five sites within the fjord; the four sites inside the fjord were expected to be influenced by sediment restoration activities, while a single site outside the fjord was chosen as a putative reference site with little to no expected perturbation due to the restoration efforts. The tissue concentration of both legacy, namely fifteen organochlorine compounds (OCPs), six organophosphate compounds (OPs), six target polychlorinated biphenyl congeners (PCBs) and four polycyclic aromatic hydrocarbons (PAHs), and emerging contaminants, namely seven polybromodiphenyl ether congeners (PBDEs), fluorobromodiphenyl ether (FBDE) and seventeen per- and polyfluoroalkyl substances (PFASs), were measured in blue mussels over a six-month period of time to depict

113 the trend of contamination by organic chemicals within the fjord and to assess the potential risk
114 for biota and humans.
115

116 ## 2. Materials and methods

117 *2.1 Study design and field work*

118 The field work was performed in the Flekkefjord fjord during the period June the 27th and
119 December the 15th of 2018. A suitable number of blue mussels (size range 3-5 cm in length) was
120 purchased from the mussel farm located in Kaldvellfjord (Lillesand, Norway), which is far from
121 point sources of contamination (Schøyen et al. 2017). Mussels were transported to Flekkefjord in
122 a cooling box within ~ 2 hours. Five caging sites (S1 - S5; Figure 1) were previously identified
123 on the basis of the levels of organic chemicals and heavy metals measured in fjord sediments
124 (Haker 2011; Misrund 2012). The caging site 1 (S1; 58° 16' 30.0" N - 6° 39' 12.9" E) was located
125 in the outer part of Flekkefjord fjord and was chosen as a reference site, while the caging sites
126 S2-S5 were close to the planned sediment restoration activities. In detail, S2 (58° 17' 02.7" N - 6°
127 39' 15.6" E) was placed nearby an old ship industry, S3 (58° 17' 23.0" N - 6° 39' 30.9" E) was
128 located nearby the old industrial area called ‘Slippen’, whereas S4 (58° 17' 33.8" N - 6° 39' 41.3"
129 E) and S5 (58° 17' 43.3" N - 6° 39' 12.5" E) were located close to an old landfill and an
130 abandoned tannery, respectively. Two cages were prepared for each site containing
131 approximatively 300 mussels each. The cages were placed from boat and checked by a scuba
132 diver in each site at two different depths, namely 5 and 15 m depth, using buoys, ropes and
133 weights. The biomonitoring started on June the 27th (t = 0 days), soon after their placement in
134 water, and then on July the 27th (t = 30 days). These samplings allowed to define the background
135 levels of contamination characterizing the fjord before the beginning of dredging operations,

which started on August 2018. Later, other three samplings were performed on October the 10th (t = 135 days), November the 15th (t = 166 days) and December the 15th (t = 196 days) to follow the trend of the contaminant levels. About 50 mussels were collected from each cage at both the selected depths in a single day for each sampling site. After collection, mussels were transported in the lab within one hour, where they were frozen and stored at – 20 °C until chemical analyses. The mussels were not depurated before freezing. Unfortunately, we could not collect mussels placed in cages of S1 and S2 after t = 166 days because coastal storms wiped out the cages. For this reason, data on bioaccumulation in mussels from S1 and S2 at t = 166 days and t = 196 days are missing. Moreover, we could not collect a sample at t = 4 in S5 because all the mussels had died possibly due to the landslide in close vicinity. The soft tissue was separated from the shells and pools of about 50 individuals were prepared for each site, depth (5 and 15 m) and time of sampling. After homogenization, the samples were stored at -20 °C until chemical analyses.

2.2. Chemicals and reagents

A mixed solution of PCB congeners (CB-28; CB-52; CB-101; CB-138; CB-153 and CB-180), CB-209 (internal standard [IS] for PCBs and PAHs), a mixed solution of PBDEs (BDE-28; BDE-33; BDE-47; BDE-99; BDE-100; BDE-153 and BDE-154 numbered according to the IUPAC nomenclature) and fluorobromodiphenyl ether (FBDE), as well as the internal standard (IS) for flame retardants, were purchased from AccuStandard (New Haven, USA). A standard solution of 15 organochlorine compounds (OCPs) and their metabolites (α–HCH; hexachlorobenzene; β-HCH; lindane; heptachlor; aldrin; heptachlor epoxide; trans chlordane; 4,4′-dichlorodiphenyldichloroethylene [4,4′-DDE]; endosulfan I; endosulfan II, endosulfan sulfate; endrin, 4,4′-dichlorodiphenyldichloroethane [4,4′-DDD], 2,4′-

dichlorodiphenyltrichloroethane [2,4′-DDT]), six organophosphate compounds (OPs – i.e., demeton, disulfoton, diazinon, phorate, mevinphos, ethoprophos) and a standard solution of four polycyclic aromatic hydrocarbons (i.e., chrysene, benzo(α)anthracene, benzo(β)fluoranthene and benzo(α)pyrene) were purchased from Restek (Bellefonte, PA, USA). The 17 per- and polyfluoroalkyl substances (PFASs) examined were perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluorobutane sulphonic acid (PFBS), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorohexane sulphonate (PFHxS), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluorooctane sulfonic acid (PFOS), perfluorododecanoic acid (PFDoA), perfluoroundecanoic acid (PFUnDA), sodium perfluoro-1-decanesulfonate (PFDS), perfluorotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA), perfluorohexadecanoic acid (PFHxDA) and perfluorooctadecanoic acid (PFODA). All of these compounds and the two 13C-labeled internal standards (ISs) MPFNA and MPFOS were purchased from Fluka (Sigma Aldrich, St. Louis, MO, USA).

2.3. Analytical standards

Stock solutions (10 µg/mL in hexane) of OCPs, OPs, PCBs, PBDEs and PAHs were used to prepare the working solutions by serial dilutions. Mixed compound calibration solution, in hexane, was prepared daily and the proper volume was used as a spiking solution as well. Stock solutions of PFASs (1 mg/mL) were dissolved in methanol, from which working solutions at the concentrations of 10 and 100 ng/mL were prepared during each analytical session. All the standard solution were stored at -20 °C.

182 *2.4. Extraction procedure for OCPs, OPs, PCBs, PBDEs and PAHs*

183 The extraction of PCBs, PBDEs, OCPs, OPs and PAHs form mussels was performed using the
184 QuEChERS approach according to the validated method described by Chiesa et al. (2018). A 1 g
185 aliquot of sample was homogenized and transferred to a QuEChERS extraction tube, and then
186 the ISs were added. A mixture (4:1 v/v) of hexane/acetone (10 mL) was added as extraction
187 solvent; the tube was shaken for 1 min using a vortex and centrifuged for 10 min at 5,000 × g at
188 4 °C. Then, the supernatant was transferred to a QuEChERS extraction tube, shaken and
189 centrifuged at the same conditions. The supernatant was transferred into clean-up tube (Z-Sep) to
190 eliminate interference as possible. The extract was transferred into a flask and evaporated under
191 vacuum in a centrifugal evaporator at 35 °C. The residue was dissolved in 1mL of hexane and
192 analyzed by GC/MS-MS.

193

194 *2.5. Extraction procedure for PFASs*

195 The analysis of PFASs in mussel tissues was performed according to a validated method
196 described in Chiesa et al. (2018). Briefly, 2 g of sample were spiked with the 2 internal standards
197 at the concentration of 5 ng/mL and 10 mL of acetonitrile were added for extraction and protein
198 precipitation; the sample was vortexed and sonicated for 15 min. After centrifugation (2,500 × g,
199 4 °C for 10 min), the supernatant was evaporated in a rotary vacuum evaporator at 40 °C. The
200 extract was suspended in 10 mL of water and purified by SPE Oasis WAX Cartridges under
201 vacuum. The SPE cartridges were preconditioned with 3 mL of 0.5% ammonium hydroxide in
202 methanol, 3 mL of methanol, and 3 mL of Milli-Q water. After sample loading, the cartridges
203 were washed with 3 mL of 25 mM acetate buffer pH 4.5 to minimize interferences, followed by
204 2 mL of methanol. The elution was done with 3 mL of 0.5% ammonium hydroxide in methanol

and the eluate was dried and then suspended in 100 μL of methanol:ammonium formate 20 mM (10:90 v/v).

2.6. GC-MS/MS analyses

Triple quadrupole mass spectrometry (QqQ) in electronic impact (EI) mode was used for the simultaneous detection and quantification of compounds. The mass condition was the same of our previous work (Chiesa et al., 2018). A GC Trace 1310 chromatograph coupled to a TSQ8000 triple quadrupole mass detector (Thermo Fisher Scientific, Palo Alto, CA, USA) was used to confirm and quantify residues by using a fused-silica capillary column Rt-5MS Crossbond-5% diphenyl 95% dimethylpolysiloxane (35 m x 0.25 mm i.d., 0.25 μm film thickness, Restek, Bellefonte, PA, USA). The oven temperature program and all operation parameters are reported in the work mentioned before. The QqQ mass spectrometer was operated in selected reaction monitoring mode (SRM) detecting two-three transitions per analyte. Identification of POPs was carried out by comparing sample peak relative retention times with those obtained for standards under the same conditions and the MS/MS fragmentation spectra obtained for each compound. The XcaliburTM processing and instrument control software program and Trace Finder 3.0 for data analysis and reporting (Thermo Fisher Scientific) were used.

2.7. LC-HRMS analyses

The HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) was coupled to a QExactive Orbitrap (Thermo Scientific, San Jose,CA, USA), equipped with a heated electrospray ionization (HESI) source operating in negative mode. A Synergi Hydro-RP reversephase HPLC column (150 × 2.0 mm, 4 μm particle size), with a C18 guard column (4 × 3.0 mm) (Phenomenex,

Torrance, CA, USA) was used for the chromatographic separation. Stainless steel capillary tubes were used for minimizing PFAS background contamination in the system. Moreover, since PFOA and PFOS were always present in the chromatographic system, we introduced a small Megabond WR C18 column (5 cm × 4.6 mm, i.d. 10 μm) between pump and injector, allowing us to delay elution of the contaminants of the system by 2 min relative to the analytes present in the samples. The mobile phases were: Solvents A (aqueous ammonium formate, 20 mM) and B (MeOH). The gradient and all the mass parameters are well described in Chiesa et al. (2018). Xcalibur™ 3.0 was the software (Thermo Fisher Scientific, San Jose, CA, USA) used to control the HPLC-HRMS system and elaborate data.

2.8 Statistical analysis

General linear models (GLM) including the site, the depth and the time of sampling as factors, and their two-way interactions, were run for the sum of PCBs and PAHs. Statistical analyses were performed only on PCBs and PAHs because other organohalogen compounds were detected only occasionally during the 6-months biomonitoring. When chemical analyses returned PCB or PAH level below the limit of quantification (<LOQ) we used the half of the LOQ as a value for statistical analysis. Two-way interactions were removed from the final models in a single step because they were all non-significant. All the analyses were performed using SPSS 21.0 statistical software.

3. Results

The survival rate of caged mussels during the six-months biomonitoring of the Flekkefjord fjord was high, with only a limited mortality observed within the cages placed at both 5 m and 15 m

depth. We could not monitor the health status of mussels in S1 and S2 after the third sampling (t = 166 days) because the cages disappeared. The cage placed at 15 m depth in S3 was plundered by crabs after the third sampling (t = 166 days), so we collected less than 50 mussels at the fourth and fifth sampling. Full mortality of mussels was noted at t = 166 days in the cage placed at 15 m depth in S5, precluding the sampling of organisms at t = 196 days. However, although the mussels were died, the soft tissues were inside the shells were collected for chemical analyses in order to assess if the cause of death was due to the uptake of contaminants or other causes. Levels of contaminants measured in blue mussels before their placement in the Flekkefjord fjord (t = 0 day) were very low. Only levels of CB-52 (range 2.67-2.80 ng/g fresh weight - f.w.), benzo(β)fluoranthene (range 3.09-3.54 ng/g wet weight) and benzo(α)pyrene (range 3.12-3.39 ng/g f.w.) and pentafluorobenzoic acid (PFBA; range 3.28-6.77 ng/g f.w.) were detected and quantified in most of samples, while CB-101, -138 and -153, hexachlorobenzene, p,p'-DDE and phorate were detected in few samples at concentrations below the limit of quantification. No other compounds were not detected in the mussels at t = 0 day. Overall, OCPs (Table S1), OPs (Table S2) and PBDEs (Table S3) were not detected in mussels collected from t = 30 days and t = 196 days, with the exception for p,p'-DDE and HCB, which were detected at concentrations over the LOQ in 55% and 32% of samples respectively, and p,p'-DDD (10% of samples >LOQ), which was measured in concentration ranging between 12.4 and 15.4 ng/g f.w. (Table S1). Similarly, PFASs were not detected after their placement in the fjord, with the exception for PFBA and perfluorooctanesulfonic acid (PFOS), whose concentrations resulted over the LOQ in the 19% and 10% of samples, respectively and ranged between 2.13-6.01 ng/g f.w. for PFBA and 0.11-0.42 ng/g f.w. for PFOS (Table S4). In contrast, PCBs and PAHs were detected respectively in 90% to 100% of samples collected during t = 30 days and t = 196 days period. The ΣPCB

congeners ranged between 2.74 and 82.64 ng/g f.w. (Table 1). The CB-52 and CB-153 were found in more than 70% of the samples, followed by CB-138 and CB-101, which were detected in more than 55% of samples. Grouping the PCB congeners according to their chlorination grade, the fingerprint of mussels caged in Flekkefjord fjord was mainly composed of hexa- (48%), tetra- (22%) and hepta-CB (20%) congeners, whereby the CB-138 (27%) and CB-101 (21%) were the predominant congeners, followed by CB-180 (20%), CB-28 (12%), CB-153 and CB-52 (10%), independently of the depth, site and time of sampling. A significant difference in PCB concentrations accumulated in mussels caged at 5 m and 15 m depth was noted ($F = 21.463$; $P = 0.001$), with estimated mean concentrations measured in mussels caged at 15 m depth (27.203 ± 3.182 SE ng/g f.w.) about 4-fold higher than those found at 5 m depth (7.328 ± 2.931 SE ng/g f.w.), independently of the sampling site and time. This would indicate that PCB primarily has its uptake at depth and not at the surface. A significant increase of ΣPCBs was noted over the six-moths biomonitoring ($F = 6.118$; $P = 0.008$), with estimated mean concentrations measured at t = 166 days (39.785 ± 5.484 SE ng/g f.w.) and t = 196 days (35.688 ± 6.990 SE ng/g f.w.), with were about 10-fold and significantly higher than those measured at t = 0 day (3.538 ± 4.248 SE ng/g f.w.), independently of sampling depth and site. However, these data need to be considered with caution since we could not measure the concentrations of PCBs in the putatively reference sites S1 and S2 at t = 166 days and t = 196 days (see *Materials and methods section*). For the same reason, we did not observe significant differences ($F = 1.568$; $P = 0.251$) in PCB contamination among sites, although the mean levels of PCBs measured in mussels from the sites located within the fjord were about 10-fold higher than those recorded in mussels caged outside the fjord, independently of sampling depth and time. A similar pattern was also observed for PAHs, whose concentrations in mussels ranged between 3.40 and 17.20 ng/g

297 f.w. (Table 2). Benzo(β)fluoranthene and benzo(α)pyrene were measured in more than 90% of
298 the samples and were the most abundant compounds characterizing the PAH fingerprint,
299 accounting on average for the 87% of the contamination measured in mussels, independently of
300 the depth, site and time of sampling. In contrast to PCBs, levels of PAHs measured in mussels
301 caged at 5 m depth did not differ from those measured at 15 m depth ($F = 0.666$; $P = 0.432$),
302 independently of the sampling site and time. Whilst no significant differences among sites were
303 noted ($F = 2.588$; $P = 0.096$), the PAHs levels showed a significant ~2-fold increase ($F = 8.530$;
304 $P = 0.002$) at t = 166 days (estimated marginal means 10.695 ± 0.783 SE ng/g f.w.) and t = 196
305 days (estimated marginal means 12.788 ± 0.998 SE ng/g f.w.) with respect to t = 0 day
306 (estimated marginal means 6.374 ± 0.607 SE ng/g f.w.), independently of sampling depth and
307 site.

308 ## 4. Discussion

309 The present study shows that active biomonitoring using caged blue mussels represents a suitable
310 approach to monitor levels and trends of different organohalogen compounds and PAHs in the
311 Flekkefjord fjord, and to observe the efficacy and safety of ecosystem restoration activities.
312 Levels of OCPs (Table S1), OPs (Table S2), PBDEs (Table S3) and PFASs (Table S4) were not
313 detected or their concentrations were below the analytical limit of quantification in mussels
314 caged in the five sampling sites at both the depths over the six-moths biomonitoring, indicating a
315 negligible contamination of Flekkefjord fjord by these compounds. In contrast, PCBs (Table 1)
316 and PAHs (Table 2) were measured in all the sampling sites and at both the depths, showing an
317 increase of tissue concentrations over the six-month biomonitoring. In detail, after one month
318 from cages deployment (t = 30 days), the levels and the fingerprint of PCB contamination (Table
319 1 and Figure 2) measured in mussels caged at 5 m depth remained similar to those observed at

the beginning of the biomonitoring operation (t = 0 day) in all the sampling sites (average concentration 3.2 ng/g f.w.). Such levels were similar to those recorded by a six-months biomonitoring in native and transplanted blue mussels from the city harbor of Kristiansand (Norway), a moderately to severely polluted area by a mixture of inorganic and organic contaminants (Schøyen et al., 2017). An increase in PCB concentrations was observed only in S5 at the end of the biomonitoring, while in other sampling sites no variations over time were noted. In contrast, a different pattern of contamination was observed in mussels caged at 15 m depth, whereby PCBs concentrations where higher than those measured in mussels transplanted at 5 m depth and notably increased already after one-month from the beginning of the biomonitoring in mussel tissues from two sampling sites located within the fjord (S3-S4), showing higher concentrations (range 9.19 – 39.15 ng/g f.w.) compared to the Kristiansand harbor (Schøyen et al., 2017). These results were not unexpected because previous monitoring studies of sediment contamination showed high levels of PCBs and heavy metals in correspondence with these specific areas (Haker 2011; Misrund 2012), where a naval industry (S3) and a landfill (S4) were placed. Interestingly, also the PCB fingerprint differed between sampling depths and among sites. In fact, the fingerprint observed in mussels caged at 5 m depth, independently of the sampling site, was characterized only by low-chlorinated congeners (CB-28 and CB-52, the less hydrophobic), while mussels caged at 15 m depth showed high concentrations of high-chlorinated ones (penta- to hepta-CB), which are the main congeners occurring in sediments (e.g., Binelli et al. 2009; Parolini et al. 2010). These results suggest that high-chlorinated PCBs trapped in the sediments from S3 and S4 (Haker 2011; Misrund 2012) might return bioavailable for mussels living near to the bottom of the fjord as consequence of sediment resuspension, while least hydrophobic ones are present within the whole water column and can be accumulated also

by mussels located at low depth. A notable increase in PCB concentration was observed in mussels caged at 15 m depth at t = 166 days in three sites within the fjord, about two months from the beginning of restoration activities, with levels ranging between 23.70 and 62.89 ng/g f.w. This dramatic increase in PCB levels might be due to a huge sediment resuspension caused by an undersea landslide in the proximity of S5. The high levels of PCBs measured at t = 166 days in S3 and S4, and not only in S5 as expected, suggest a sediment dispersion within all the fjord, leading to a homogenization of the contamination. The highest PCB levels measured at t = 166 days in all the sampling sites, whereby mussels caged in S5 were the most contaminated (82.64 ng/g f.w.), slightly decreased in t = 196 days, probably due to the sedimentation of re-suspended sediments that reduced the bioavailability of PCBs for mussels. However, we could not monitor this trend in S5 because all the mussels caged at 15 m depth died, probably as a consequence of the combined effects of accumulated chemicals and mechanical abrasion of gills, reduction in feeding rates, and increased susceptibility to diseases (e.g., Leverone 1995). In fact, a previous laboratory study of the green-lipped mussels *Perna viridis* showed that exposure to high levels of suspended solids induced ciliary damages in both the ascending and descending lamellae of the gill filaments (Cheung and Shin 2005).

In contrast to PCBs, levels of PAHs were similar in mussels caged at both the selected depths and showed a notable increased of tissue concentrations (up to 21.32 ng/g f.w.) only at the end of the biomonitoring survey compared to previous sampling times. Levels of the four monitored PAHs measured in mussels transplanted to Flekkefjord fjord were similar to those accumulated in blue mussels transplanted in the Kristiansand harbor over a six-month biomonitoring, but the maximum measured concentrations in the present study were ~ 4-fold lower than those found in native mussels from Kristiansand harbor (Schøyen et al. 2017). The higher PAH levels measured

366 in native mussels than in transplanted ones from Kristiansand harbor might be due to their longer
367 time of exposure, suggesting that steady-state conditions were not reached in deployed mussels
368 (Schøyen et al. 2017). We speculate that a similar situation occurred in mussels transplanted in
369 Flekkefjord fjord and PAHs could reach higher concentrations over a longer period of exposure.
370 The PAH increase found at t = 196 days in all the sites, except S1 and S2 where cages
371 disappeared after t = 166 days, was accompanied by a change in the PAH fingerprint. In fact, the
372 fingerprint was exclusively characterized by the presence of benzo(β)fluoranthene and
373 benzo(α)pyrene up to t = 166 days sampling, while at t = 196 days measurable concentrations of
374 chrysene and benzo(α)anthracene were found at both the selected depths. The increase of PAH
375 levels and the change in their fingerprint appears to be a consequence of sediment resuspension
376 due to undersea landslide that occurred soon after the t = 166 days sampling in S5. Alternatively,
377 the increase in tissue concentration of PAHs was found only after six months because mussels
378 needed longer time to accumulate measurable concentrations of such contaminants. In fact,
379 although a previous study demonstrated that some hydrophobic compounds, including PAHs,
380 showed linear bioaccumulation trend in the blue mussels during the first months of caging, the
381 least hydrophobic ones can follow a dissimilar trends that could be also influenced by seasonal
382 variations (Schøyen et al. 2017).

383 *4.1 Risk of secondary poisoning for blue mussel predators*

384 One of the priority task of the Water Framework Directive (WFD; 2000/60/EC) is the
385 development and the use of the so-called Environmental Quality Standards (EQSs) of prioritized
386 hazardous substances in different aquatic matrices (i.e., waters, sediments, biota) as described by
387 the EQS Directive (Directive 2013/39/EU; EC, 2013). The EQSs for biota considered by the
388 WFD are designed for fish unless other *taxa* are specified, as for example the EQS for PAHs are

389 defined for crustacean or shellfish because fish are not considered as a suitable monitor for such
390 contaminants. Thus, the EQSs were set to depict the concentration of a specific contaminant
391 below which no chronic effects are expected to occur, including secondary poisoning and human
392 health effects (Beyer et al. 2017). EQSs were developed through a risk-based approach,
393 incorporating toxicity testing, predicted no effect concentration (PNEC) data and the use of
394 safety factors to encompass for uncertainty. In the present study, in order to assess whether the
395 mixture of contaminants measured in the blue mussels transplanted to Flekkefjord fjord might
396 pose a risk to their predators, measured concentrations (MEC) found in mussels and available
397 predicted no effect concentrations (PNEC) for secondary poisoning were used to calculate the
398 sum of MEC/PNEC ratios. The MEC/PNEC ratio obtained for each single compound was
399 summed and a potential risk was identified by a sum ≥ 1. As PNEC values we used the EQS_{biota},
400 whose goal is to protect top predators from risks of secondary poisoning via the ingestion of
401 toxic chemicals accumulated in their prey. Only the compounds we measured in blue mussels of
402 which we found the EQS_{biota} value, namely PCBs, PAHs (benzo(α)pyrene only), sum of DDT
403 homologues and PFOS (EQS directive 2013/39/EU) were included in the cumulative risk
404 assessment for secondary poisoning. The cumulative MEC/PNEC ratios calculated for the
405 mixture of contaminants measured in organisms transplanted in the Flekekfjord fjord suggests a
406 potential risk for mussel predators. In fact, whilst a negligible to low risk can be predicted for the
407 predation of mussels caged in S1 and S2 at both 5 and 15 m depth (sum of MEC/PNEC range:
408 0.03 – 4.72), a worrisome situation can occur for predators consuming mussels caged in S3, S4
409 and S5, at 5 m depth (sum of MEC/PNEC range: 3.5 – 24.78) and mainly at 15 m depth (sum of
410 MEC/PNEC range: 3.41 – 83.36). As expected, the maximum risk was calculated for mussels

411 caged in S5, whereby highest summarized MEC/PNEC values were measured at t = 196 days in
412 mussels caged at 5 m depth, and at t = 166 days in those caged at 15 m depth.
413
414 *4.2 Food safety assessment*
415 The consumption of local fishery products is considered the predominant exposure pathway to
416 persistent, bioaccumulative and toxic substances, which can represent a potential risk for human
417 health (Storelli 2008; Trocino et al. 2012; Chiesa et al. 2016; Panseri et al. 2019). The EC
418 regulation 1259/2011 set the limit for the sum of the six 'target' PCB congeners (CB-28, 52, 101,
419 138, 153 and 180) to 75 μg/kg w.w. These congeners represent approximately half of the total
420 PCBs measured in feed and food (EFSA 2012), so this value can be considered as an appropriate
421 marker of environmental contamination for occurrence and human exposure (EFSA 2006;
422 Arnich et al. 2009). In the present study, blue mussel caged at 15 m depth in S5 at t = 166 days
423 exceeded the limit of 75 μg/kg w.w. set by European Commission, while levels measured in S3
424 at t = 166 days and in S5 at t = 196 days were very close to such limit. For PAHs, the maximum
425 levels for benzo(α)pyrene and the four PAHs (chrysene, benzo(α)anthracene,
426 benzo(β)fluoranthene and benzo(α)pyrene) fixed for foodstuffs by Regulation No. 835/2011/UE
427 for bivalve molluscs were 2 μg/kg f.w. and 12 μg/kg f.w., respectively. Mussels sampled at t =
428 196 days at both the depths exceeded the threshold value for the sum of the four PAHs in S3 and
429 S4. A worrisome situation was noted for benzo(α)pyrene, whose concentrations measured in all
430 the samples (Table 2), including at t = 0 day, exceeded the threshold set by the EC Regulation. It
431 is conceivable that mussels native of the Flekkefjord fjord could reach analogue contaminant
432 concentrations of the ones measured in transplanted mussels. In 2006, the Joint FAO/WHO
433 Expert Committee on Food Additives (JECFA) used a margin of exposure (MOE) approach and

benzo(a)pyrene, as surrogate biomarker for the genotoxic and carcinogenic PAHs. In the report, the Committee concluded that even high exposition to benzo(a)pyrene (10 ng/kg body weight/day) resulted in a good MOE value of 10,000, if the BMDL of 100 µg/kg body weight/day was considered, based on a study of carcinogenicity in mice treated orally with mixtures of PAHs.

The National Institute for Public Health and the Environment (RIVM 2001) proposed a guidance value of 10 ng/kg body weight/day for the sum of the six target PCBs, derived from long-term toxicological studies on decreased specific and non-specific immune parameters as end-point in rhesus monkeys orally exposed to Aroclor 1254 and assuming that about the half of Aroclor contains indicator-PCBs . The risk for human health, as a consequence of the potential ingestion of contaminated blue mussels, was evaluated by calculating the dietary exposure (DE) for PCBs and PAHs according to the formula: EDI = (Cm × IRd)/BW (e.g., USEPA 2000; Arnich et al. 2009), where Cm represents the PCBs or PAHs concentration in blue mussels (µg/kg f.w.), IRd is the average daily ingestion rate (2.76 g/capita/day) calculated by FAOSTAT for molluscs in the Norwegian population (FAOSTAT 2015) and BW is the body weight for adults (70 kg). Dietary exposure was expressed as ng/kg/day body weight. The calculated DE did not exceed provisional tolerable daily intake for PCBs (DE range: 0.10 – 3.26 ng/kg body weight /day) and "safe" values for PAHs (DE range: 0.25 – 0.84 ng/kg body weight/day) in all the sampling, suggesting a negligible risk for human population consuming mussels from the Flekkefjord fjord. PFOS and PFBA, were found in just 10% and 19% of the samples, but, due to the recent re-evaluation of the PFOS (and PFOA) TWI by EFSA (2018) a particular attention was paid. Now, unfortunately, only the TWI for PFOS is available and, accounting for the higher concentration detected (0.41 ng/g w.w.) the estimated daily exposure, calculated as above should be 0.016

457 ng/kg body weight/day, much lower than the TWI value of 13 ng/kg body weight/day. The data
458 on human toxicity for the most of PFAs are lacking, moreover the end-points and toxicokinetics
459 are very often different for humans and other animals (Gomis et al. 2018). A risk characterization
460 for PFBA was therefore not possible.
461

462 ## 5. Conclusion

463 The present study confirmed the active biomonitoring approach using the blue mussels as a
464 valuable tool to monitor the levels and the trends of organohalogen compounds and PAHs in
465 marine environments, as well as to check for the effectiveness and the environmental safety of
466 restoration activities of contaminated ecosystems. Our results showed that levels of OCPs, OPs,
467 PBDEs and PFASs were negligible in the Flekkefjord ecosystem, while levels of PCBs and
468 PAHs did not represent a concern before the restoration activities. However, a notable increase
469 of the contamination by PCBs and PAHs occurred as a consequence of an unexpected and huge
470 undersea landslide, which caused a resuspension of contaminated sediments. This effectively
471 masked any potential effect due to the dredging activities. Levels of PCBs accumulated in blue
472 mussels after the landslide were extremely high and could represent a serious risk of secondary
473 poisoning for blue mussel predators and also for human health, as they exceeded the thresholds
474 set by the EU for food safety. For all these reasons, the continued biomonitoring studies using
475 both transplanted and native mussels should be a priority to monitor the trend of PCB and PAH
476 contamination and, consequently, the potential risk for living organisms and human population
477 consuming seafood from Flekkefjord ecosystem. Moreover, further studies aimed at monitoring
478 the levels of such contaminants in other edible species (e.g., crustaceans and fish) living within
479 the Flekkefjord fjord and commonly consumed by the population should be encouraged in order

to estimate the transfer and potential effects over the trophic chain and to better assess the food safety and the potential risk for the consumption of fishery products.

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Table and Figure captions

Table 1: Spatial (S1-S5) and temporal (from t = 0 days to t = 196 days) variation in the concentrations of PCBs (expressed in ng/g fresh weight) measured in blue mussels transplanted at 5 and 15 m depth to Flekkefjord fjord. Blank cells indicate a missing sample; n.d. = not detected; <LOQ = below the limit of quantification.

Table 2: Spatial (S1-S5) and temporal (from t = 0 days to t = 196 days) variation in the concentrations of PAHs (expressed in ng/g fresh weight) measured in blue mussels transplanted at 5 and 15 m depth to Flekkefjord fjord. Blank cells indicate a missing sample; n.d. = not detected; <LOQ = below the limit of quantification.

Table S1: Spatial (S1-S5) and temporal (from t = 0 days to t = 196 days) variation in the concentrations of organochlorine componds (expressed in ng/g fresh weight) measured in blue mussels transplanted at 5 and 15 m depth to Flekkefjord fjord. Blank cells indicate a missing sample; n.d. = not detected; <LOQ = below the limit of quantification.

Table S2: Spatial (S1-S5) and temporal (from t = 0 days to t = 196 days) variation in the concentrations of organophosphate compounds (expressed in ng/g fresh weight) measured in blue mussels transplanted at 5 and 15 m depth to Flekkefjord fjord. Blank cells indicate a missing sample; n.d. = not detected; <LOQ = below the limit of quantification.

Table S3: spatial (S1-S5) and temporal (from t = 0 days to t = 196 days) variation in the concentrations of PBDEs measured in blue mussels transplanted at 5 and 15 m depth to Flekkefjord fjord. Blank cells indicate a missing sample; n.d. = not detected.

Table S4: spatial (S1-S5) and temporal (from t = 0 days to t = 196 days) variation in the concentrations of PFASs (expressed in ng/g fresh weight) measured in blue mussels transplanted at 5 and 15 m depth to Flekkefjord fjord. Blank cells indicate a missing sample; n.d. = not detected; <LOQ = below the limit of quantification.

Figure 1: Geographical localization of the five sites (S1 – S5) within the Flekkefjord fjord (Southern Norway) where blue mussels where caged.

Figure 2: spatial (S1-S5) and temporal (from t = 0 days to t = 196 days) variation in the concentrations sum of six target PCBs (expressed in ng/g fresh weight) measured in blue mussels transplanted at 5 and 15 m depth to Flekkefjord fjord.

Figure 3: spatial (S1-S5) and temporal (from t = 0 days to t = 196 days) variation in the concentrations sum of four target PAHs (expressed in ng/g fresh weight) measured in blue mussels transplanted at 5 and 15 m depth to Flekkefjord fjord.