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Accepted Manuscript

Bioaccumulation of metals in juvenile rainbow trout (*oncorhynchus mykiss*) via dietary exposure to blue mussels

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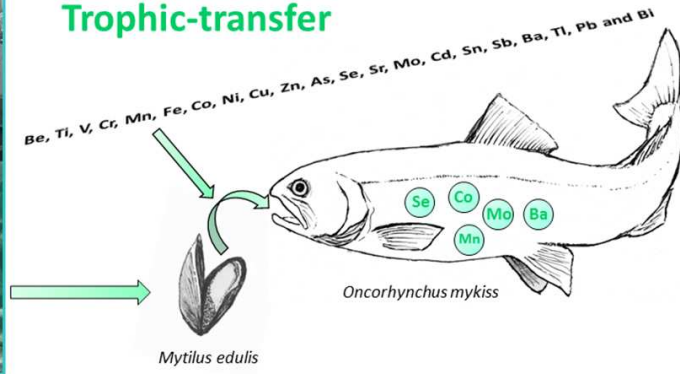
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Trophic-transfer



ACCEPTED MANUSCRIPT

1 **BIOACCUMULATION OF METALS IN JUVENILE RAINBOW TROUT**
2 **(ONCORHYNCHUS MYKISS) VIA DIETARY EXPOSURE TO BLUE MUSSELS**

3

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26 **Abstract**

27 The potential for metals to bioaccumulate in aquatic species, such as fish, via trophic level
28 transfer was investigated. An *in vivo* experiment was set up in a flow-through system in
29 which juvenile rainbow trout were fed blue mussels collected from a Class A pristine site and
30 an effluent-impacted river estuary, over a period of 28 days. Selected elements (As, Cd, Cr,
31 Co, Cu, Fe, Pb, Mn, Mo, Ni, Se, Sn, V, Zn) were determined in the mussels and fish tissues
32 (muscle and skin) collected at 0, 14 and 28 days. This study reveals the occurrence of metals
33 in mussels sampled in the Irish marine environment and highlights the bioaccumulation
34 potential of metals in fish tissues via trophic transfer. All 14 monitored metals were
35 determined in the mussels collected from both sites and mussels collected from the effluent-
36 impacted site contained three times more Co, Mo, Sn and V than the mussels collected from
37 the Class A site. Following a 28-day dietary exposure, concentrations of As and Se (fish
38 muscle), and Pb, Se and Zn (fish skin), were significantly greater in fish feeding on
39 contaminated mussels compared to those with a regular fish feed diet. The significance of
40 metal detection and bioaccumulation in the mussel and fish tissues, highlights the potential
41 for metal exposure to humans through the food chain. As fish are recommended as a healthy
42 and nutritious food source, it is important to fully understand metal bioaccumulation in
43 commercially important aquatic species and ensure the safety of human consumers.

44

45 **Keywords:** Aquatic pollutants · Metals · Trophic transfer · Bivalves · Fish · Inductively
46 coupled plasma – mass spectrometry

47

48 **1.0 Introduction**

49 Metals are naturally occurring constituents of the earth's crust that can be divided into
50 biologically essential and non-essential groups. Essential groups of metals are vital for certain

51 biochemical and physiological functions and are classed according to their concentration in
52 the body i.e. macro and micro-essential metals (Underwood, 1971; Reinhold, 1975). Other
53 natural metal components of the environment include non-essential metals such as cadmium,
54 lead, mercury and arsenic which have no known biological function but exposure to
55 excessive quantities could lead to poisoning (Naja and Volesky, 2009). These non-essential
56 metals in particular have increased in concentration in the aquatic environment over recent
57 years due to the rise in anthropogenic activities such as agriculture, mining and industrial
58 processes (Cobelo-Garcia et al., 2004; Yilmaz, 2010). Due to their stable nature, these
59 elements can accumulate and persist in water, soil, sediment and biotic matrices following
60 entry into the aquatic environment (Tudor et al., 2006). Increasing efforts in wastewater
61 treatment have resulted following the establishment of strict environmental standards and
62 laws for the regulation of industrial emissions, however, a recent study by Jones et al. showed
63 significant metal concentrations entering the Irish aquatic environment from municipal
64 wastewater treatment plants (Healy et al., 2016; Jones et al., 2016).

65 In aquatic systems, the availability of a metal to an organism depends on many
66 physico-chemical factors such as metal concentration, solubility, pH, dissolved oxygen,
67 temperature, water hardness, salinity, as well as biological factors such as species specific
68 uptake mechanism, age and feeding habits (Jeziarska and Witeska, 2006). Furthermore,
69 metals may bind to organic compounds, suspended particles and sediments present in the
70 aquatic environment therefore affecting availability to aquatic life (Dallinger et al., 1987).
71 For fish species, there are two main mechanisms by which metals may enter the body: direct
72 entry via the gills or the body surface and trophic transfer via the alimentary tract (Ciardullo
73 et al., 2008; Sauliutė and Svecevičius, 2015). For the normal metabolism of fish, essential
74 metals must be taken up from water, food or sediment, however, similar to essential metals,
75 uptake and bioaccumulation of non-essential metals can also occur (Subotic et al., 2013).

76 Direct uptake of non-essential metals and elevated levels of essential metals in aquatic biota
77 has been shown to be toxic at trace concentrations, causing severe alterations to physiological
78 functions, growth rates and reproduction and in some cases have led to mortality (Fisher and
79 Hook, 2002; Tchobanoglous et al., 2003). The Oslo-Paris Convention for the Protection of
80 the Marine Environment of the North-East Atlantic (OSPAR) monitors and regulates
81 environmental conditions to inform policymakers such as the European Community (EC)
82 about current hazardous water pollutants. Under the most recent EU Water Framework
83 Directive (WFD), cadmium, lead, nickel, mercury, and organotin compounds are listed as
84 priority substances due to the level of concern surrounding their persistence, bioaccumulation
85 and/or toxicity in the aquatic environment and for which environmental quality standards
86 (EQSs) are specified in water, sediment and biota. The presence of these compounds needs to
87 be substantially reduced in the aquatic environment and, in the case of cadmium, mercury and
88 organotin; these compounds have been identified as priority hazardous substances which
89 need to be phased out of use (EU WFD, 2013).

90 Many fish species are among the top consumers of trophic pyramids in aquatic
91 ecosystems, feeding on algae, benthic animals and plants, and as a consequence, they are
92 potentially endangered by both water-borne and diet-borne pollutants transferred along the
93 food chain (Sauliutė and Svecevičius, 2015). Dietary exposure may be a major uptake route
94 of many potentially toxic metals in aquatic biota, however, bioavailability of dietary metals is
95 still not considered in regulatory guidelines and data regarding metal bioaccumulation in
96 aquatic organisms via trophic transfer is lacking. In addition, fish is highly recommended as a
97 food source as part of a healthy and nutritious diet for humans but the presence of these
98 chemical pollutants is concerning, particularly for regular consumers of fish and a more
99 comprehensive understanding of metal bioaccumulation via dietary intake is required. For the
100 first time, this study will investigate the potential for bioaccumulation of a range of metals in

101 juvenile rainbow trout via dietary exposure to wild bivalves sourced from two locations off
102 the Irish coast. An attempt was also made to evaluate the contributions of fish feed to metal
103 uptake by fish and assess its potential impact on human health.

104

105 **2.0 Materials and Methods**

106 *2.1 Experimental setup*

107 The facilities at Shannon Aquatic Toxicology Laboratory, Ireland were used for this exposure
108 experiment. Juvenile rainbow trout, (*Oncorhynchus mykiss*, Walbaum, 1752, Salmoniformes,
109 Actinopterygii, approximate weight 50 ± 15 g), were sourced from a pond system fish farm
110 facility (Roscrea, Ireland) and acclimatised for 13 days in one large tank of carbon filtered
111 municipal supply water. A flow-through system was established for nine 70 L aerated, glass
112 covered tanks, using the same water supply set at a flow rate of 0.2 L min^{-1} . Organisation for
113 Economic Co-operation and Development (OECD) Guideline No. 305 was followed for the
114 set-up and duration of this exposure with any exceptions noted (OECD, 2012). Tanks were
115 organised randomly, as shown in Figure S1 of the supplementary material. Six fish were
116 weighed (see Table S1 of the supplemental data for individual weights) and transferred into
117 each tank to acclimatise for a further 24 h to reduce stress levels before exposure initiation.

118

119 *2.2 Feeding*

120 During acclimatisation, fish were fed Nutra Parr 1.8 fish feed pellets (Skretting UK,
121 Northwich) daily at 1-2% of total fish weight. The experimental design included nine
122 exposure tanks i.e. three control tanks (EXP1) in which fish continued to feed on the
123 commercial Nutra Parr 1.8 pellets, three mussel control tanks (EXP2) in which fish were fed
124 wild blue mussels (*Mytilus edulis*) sourced from a Class A shellfish production area under EC
125 Regulation 854/2004 (<230 *E. coli* per 100 g of bivalve mollusc flesh and intra-valvular

126 fluid), off the west coast of Ireland, and three exposed mussel tanks (EXP3) in which fish
127 were fed wild blue mussels (*Mytilus edulis*) collected from an effluent wastewater exposure
128 site on the east coast of Ireland. The mussels chosen for this study were of the same size class
129 (4-6 cm) and were collected at the end of August (2012), before the spawning period in
130 September. After collection, mussels (n=100 for each site) were transported back to the
131 laboratory in a cooler box, rinsed free of debris with ultra-pure water, de-shelled, pooled,
132 chopped into small fragments, weighed into feed bags for each day of exposure and stored at
133 -80 °C until laboratory analysis. Bagged mussel feed was removed from the freezer, cut into
134 small frozen pellets and fed to the corresponding tanks. All tanks were fed daily at 2% of the
135 total fish weight present in the tank. For the control tanks, fish were fed commercial fish feed
136 pellets at the same quantities fed to the fish in the mussel control and exposure tanks.
137 Commercial fish feed can contain both macro- (sodium, chloride, potassium and phosphorus)
138 and micro- (copper, chromium, iodine, zinc and selenium) minerals so it was important to
139 sample fish post-acclimatisation to assess initial levels of metals in the fish pre-exposure.
140 Fish faeces were removed approximately 6 h after feeding by siphoning from the base of the
141 tank system.

142

143 2.3 Sampling

144 Fish (n=3) were sampled from the acclimation tank before the first day of exposure (0 d) as a
145 control and from each of the nine exposure tanks after 14 days (14 d) and 28 days (28 d) of
146 feeding (n=9 per exposure). Fish were individually caught with a net, sacrificed and length
147 and weight measurements were recorded. Fish fillets (average weight of 12 g×2) were
148 collected at each sampling time point, dissected and placed in labelled plastic bags. All
149 samples were transported back to the laboratory on dry ice and frozen at -80 °C for
150 subsequent analysis.

151

152 *2.4 Sample preparation and analysis of fish and blue mussel tissue*

153 Mussel and fish samples were washed with Milli-Q water [18.3 M Ω -cm, Millipore, Bedford,
154 USA] to remove debris and any adhering particulate material and all samples were freeze-
155 dried at -52 °C [FreeZone 12, Labconco, Missouri, USA]. Fish samples were separated into
156 muscle and skin tissues and pulverised in an agate ball mill (Fritsch™ Pulverisette 6
157 Planetary Mono Mill). Aliquots of tissue (approximately 0.25 g) were decomposed and
158 mineralised using closed vessel microwave digestion (Multiwave 3000, Anton Paar, Graz,
159 Austria (Ratcliff et al., 2016)) in a class 10,000 (ISO class 7) clean room using 3 mL of 67-
160 69% HNO₃ [SpA grade, Romil™, Cambridge, UK] and 3 mL of 30% H₂O₂ [TraceSelect®
161 Ultra, Sigma-Aldrich, St. Louis, USA].

162 Metal concentrations in the samples (As, Cd, Cr, Co, Cu, Fe, Pb, Mn, Mo, Ni, Se, Sn,
163 V, Zn) were determined using a Perkin Elmer ELAN, DRC-e (Waltham, USA) inductively
164 coupled plasma mass spectrometer (ICP-MS) in standard mode and equipped with a flow
165 injection autosampler (FIAS 93 plus) in a class 1,000 clean room (ISO class 6). The
166 determination of Cr, Fe, Zn, Ni and Se was carried out in dynamic reaction cell (DRC) mode
167 with methane as the reaction gas and for As with oxygen as the reaction gas (Staunton et al.,
168 2014; Healy et al., 2016). Calibration standard solutions were prepared from a customized
169 multi-element standard (Inorganic Ventures, 1000 $\mu\text{g mL}^{-1}$) prepared in Milli-Q™ water and
170 rhodium (¹⁰³Rh) and indium (¹¹⁵In) were used as internal standards to account for
171 instrumental drift and matrix effects.

172

173 *2.5 Quality control*

174 Certified reference materials (CRMs) of NIES No. 6 (*Mytilus edulis*; National Institute for
175 Environmental Studies, Japan), ERM® – BB422 (Fish Muscle – *Pollachius virens*, European

176 Reference Materials, Joint Research Centre, Institute for Reference Materials and
177 Measurements, Belgium) and DOLT-4 (Dogfish liver certified reference material for trace
178 metals, National Research Council of Canada [NRC-CNRC]) were used for standardisation
179 and method validation. Procedural blanks and CRMs were included in each analytical batch
180 and the precision of the technique was evaluated by the incorporation and assessment of
181 duplicate samples and calibration check standards throughout the multi-element
182 determination.

183

184 *2.6 Data processing and statistical analysis*

185 Statistical analyses were performed using IBM SPSS Statistics software (Version 22.0,
186 Released 2013, IBM Corp., Armonk, NY, USA.). To test whether the dataset was of
187 Gaussian distribution, a Shapiro-Wilk normality test was used. Since most of the data set was
188 not normally distributed, with non-homogeneous variances, nonparametric tests were applied.
189 For the comparison of metal concentrations between exposures at defined time points and
190 metal concentrations over time for each exposure experiment, a Kruskal–Wallis test with
191 Dunns post-test were used. The statistical significance level was set to $p < 0.05$.

192

193 **3.0 Results and Discussion**

194 *3.1 Quality control*

195 Both fish and mussel CRMs were utilised for method validation and quality control. All
196 experimental values are shown in Table S2 in the supplementary data and agree well with the
197 certified reference values given.

198

199 *3.2 Metal concentrations in fish feed and blue mussels collected from the Irish coastline*

200 Nutra Parr 1.8 fish feed is a typical fingerling diet for trout weighing between 5-15 g and was
201 administered during the depuration phase and the 28 d EXP1 experiment. As this batch of
202 feed was not directly analysed for minerals, theoretical levels of metal content (Cu, Fe, Mn,
203 Se, Zn) have been provided by the manufacturer and are shown in Table 1. None of the other
204 metals studied were added to the feed during the production process, however, the presence
205 of these metals cannot be ruled out as raw materials used in the production of this feed e.g.
206 fishmeal and fish oil, can be potential sources of agricultural chemical residues and metals
207 (FAO, 2002). Metal concentrations (As, Cd, Cr, Co, Cu, Fe, Pb, Mn, Mo, Ni, Se, Sn, V, Zn)
208 were determined in marine mussel tissue collected from a Class A shellfish production site
209 off the west coast of Ireland (used in EXP2) and effluent exposed marine mussels from the
210 highly contaminated site off the east coast of Ireland (used in EXP3). Mussels from both sites
211 were found to contain all of the selected metals with tin measuring lowest at $<0.1 \mu\text{g g}^{-1}$ dry
212 weight and iron and zinc measuring highest at $304 \mu\text{g g}^{-1}$ dry weight (EXP3) and $121 \mu\text{g g}^{-1}$
213 dry weight (EXP2), respectively (Table 1). As mussels can be consumed directly by humans
214 it is important to note that all of the metal residues measured in the mussel tissues collected
215 from both sites were below specified MRL values (European Commission Regulation
216 1881/2006) and deemed fit for human consumption.

217

Metal	Average concentration ($\mu\text{g g}^{-1}$ dry weight) \pm S.D.		
	Fish feed (EXP1)	Mussels (EXP2)	Mussels (EXP3)
As	-	26.749 \pm 14.303	16.314 \pm 0.019
Cd	-	0.558 \pm 0.141	0.646 \pm 0.004
Cr	-	1.045 \pm 0.051	2.124 \pm 0.060
Co	-	1.498 \pm 0.643	5.096 \pm 0.657
Cu	11.9	5.893 \pm 0.582	7.902 \pm 0.682
Fe	105.7	129.282 \pm 4.936	303.627 \pm 23.027
Pb	-	2.755 \pm 2.441	3.094 \pm 0.043
Mn	15.7	2.906 \pm 0.288	6.931 \pm 0.108
Mo	-	0.647 \pm 0.127	3.972 \pm 0.275
Ni	-	1.789 \pm 0.537	4.052 \pm 0.050
Se	0.65	3.246 \pm 0.935	5.124 \pm 0.146
Sn	-	0.005 \pm 0.002	0.085 \pm 0.007
V	-	0.440 \pm 0.032	1.389 \pm 0.033
Zn	140.5	121.110 \pm 51.838	102.360 \pm 8.634

218 **Table 1.** Metal concentrations in fish feed (theoretical value) administered during the
 219 depuration phase and to fish in EXP1 experiment, and mussel tissues (measured average
 220 value and standard deviation) fed to fish in EXP2 (Class A site) and EXP3 (contaminated
 221 site) experiments. Mussel tissues analysed were a pooled and homogenised sample (n=2).

222
 223
 224 The monitoring of metals in Irish marine waters, sediments, fish and shellfish tissues
 225 is carried out to meet the requirements of the EU Water Framework Directive (WFD) and the
 226 EC Quality of Shellfish Waters Regulation and, to contribute to the Co-ordinated
 227 Environmental Monitoring Programme (CEMP) and Joint Assessment and Monitoring
 228 Programme (JAMP) of the OSPAR Convention. These national water monitoring studies aim
 229 to provide tested methodologies to enable comparable maritime data for assessment. As well
 230 as priority metals, such as Cd, Hg and Pb, several other essential micro-elements, such as Zn,
 231 Cu, Cr, As, Ni and Ag are also regularly monitored for and assessed in the aquatic
 232 environment. As highlighted by previous studies, aquatic species at lower trophic levels may

233 not possess a metabolic system as efficient or complex as their predators, increasing their
234 susceptibility to contaminant bioaccumulation and more markedly reflecting contamination in
235 the marine environment (Kainz and Fisk, 2009). In particular, bivalve molluscs are widely
236 used in marine monitoring programmes as they can reside in areas where metals and other
237 contaminants may be abundant and feed on the surrounding water and sediment (Fung et al.,
238 2004; Hunt and Slone, 2010). Metal concentrations detected in mussel and fish tissues are
239 measured against several assessment criteria, shown in Table 2, namely environmental
240 quality standards (EQSs) set by the EU WFD, background assessment concentrations (BACs)
241 set by the OSPAR CEMP and guide values set by the EC Regulation for shellfish tissues.
242 Using these values to assess the metal concentrations detected in the mussels collected for
243 this study (shown in Table 1), copper, lead and zinc concentrations determined in both mussel
244 samples exceed the BACs outlined by the OSPAR CEMP. BAC values are used by OSPAR
245 to highlight metal concentrations higher than background levels but particularly for metals in
246 biological systems where a more in depth assessment criteria is required, the current risk of
247 effects associated with specified BAC values are unknown. The data yielded correlates to
248 information provided by the annual CEMP reports that show upwards trends in copper and
249 lead concentrations in mussels residing in the Irish Sea and more recently, concentrations of
250 Cu and Zn exceeding the stated BAC values in blue mussels collected around the Irish coast
251 (OSPAR, 2013, 2014). Other metals measured in the sampled mussels close to CEMP
252 background assessment concentrations included Cd, As (west coast only) and Ni (east coast
253 only).

254 As stated above, annual reports by the OSPAR CEMP show clear upwards trends in
255 copper concentrations in mussels in the Irish Sea which was also the case for Cd, Hg and Pb.
256 Concentrations of Hg in sediment are at levels giving rise to risk of pollution effects in the
257 Irish Sea, but, levels in fish and shellfish remain generally below EU maximum food residue

258 limits ($<0.5 \mu\text{g g}^{-1}$ wet weight) (EU WFD, 2013). As temporal trends in concentrations can
259 only be determined using data collected systematically over relatively long periods, relatively
260 few significant trends could be discerned for trace metals in Irish waters due to limited data
261 series, although, a significant upward trend was detected particularly for Cd, Cu and Zn at the
262 North Bull Island site (Co. Dublin) in recent years (McGarrigle, 2010). This finding is
263 supported by a recent monitoring study which showed elevated levels of Pb, Cu and Zn in
264 surface waters sourced from inner city and industrial locations such as Dublin City
265 Docklands and in some cases, EQS values for these metals in surface waters were exceeded
266 (Jones et al., 2016). Shellfish sampled by the Marine Institute from the Irish coastline has
267 been previously shown to exceed the guide values given in the EC Quality of Shellfish
268 Waters Regulation for Cd, As, Ni and Pb in shellfish tissue (McGarrigle, 2010).

269

270

Metal	EQS, BACs and guide values for metal residues in biota ($\mu\text{g g}^{-1}$ dry weight)					MRLs for metals in foodstuffs ^a ($\mu\text{g g}^{-1}$ dry weight)	
	EU WFD (2000)		OSPAR (2014)		EC Regulation in ISI (2006)	EU Commission (2006)	
	Environmental quality standard (EQS) ^a		Background assessment concentrations (BACs)		Guide values for metal concentrations		
	<i>Mussels</i>	<i>Fish</i>	<i>Mussels</i>	<i>Fish</i> ^a	<i>Shellfish</i>	<i>Mussels</i>	<i>Fish</i>
Cd	-	-	0.96	0.13	5	5.0	0.25-0.5
Cu	-	-	6	-	400	-	-
Pb	-	-	1.3	0.13	7.5	7.5	1.5
Zn	-	-	63	-	4000	-	-
As	-	-	-	-	30	-	-
Cr	-	-	-	-	6	-	-
Ni	-	-	-	-	5	-	-
Ag	-	-	-	-	15	-	-
Sn	-	-	-	-	-	1000*	1000*

271 *For tinned food

272 ^a Converted value from wet weight to dry weight using a factor of 5 (Law et al., 2010).

273

274 **Table 2.** List of metals and their environmental quality standard in biota as set out in the EU Water Framework Directive (WFD), the OSPAR
 275 Coordinated Environmental Monitoring Programme (CEMP) and the EC Regulations on the Quality of Shellfish Waters as well as the maximum
 276 residue limits (MRLs) for metals in mussels and fish as foodstuffs, as set by the EU Commission.

277

278 3.3 The effect of diet on the accumulation of metals in fish

279 The experimental design of this study was based on an organism's ability to graze on lower
280 trophic species more susceptible to metal bioaccumulation and aimed to assess the potential
281 for metal exposure and bioaccumulation within fish and up a trophic level potentially leading
282 to human exposure. Rainbow trout are carnivores that feed on small insects, fish and
283 invertebrates. Blue mussels have been used in previous rainbow trout diet studies (Berge and
284 Austreng, 1989). More recently, Arneson et al. (2015) recommended blue mussels as a
285 'sustainable and environmentally friendly' fish feed additive due to their high production
286 rates and high protein and amino acid content. However, due to the effective accumulation of
287 metals in mussels, metal monitoring is required for this fishmeal alternative.

288 Previously established methods were applied for the identification of metals in fish
289 tissues following a 28-day *in vivo* bioaccumulation experiment. Fish were sampled from the
290 acclimation tank pre-exposure and from each of the nine tanks at 14 d and 28 d to evaluate
291 bioaccumulation of selected metals in rainbow trout feeding on contaminated mussel tissue.
292 As fish skin often remains attached to the muscle when consumed by humans, metals were
293 also determined in the skin to highlight all potential routes of human dietary exposure to
294 metals. Fish muscle and skin were collected from each fish sample and analysed via triplicate
295 injection on the ICP-MS. Water pH, temperature and dissolved oxygen content were
296 measured throughout the 28-day *in vivo* experiment on the days marked in Table S3 (a)-(c) in
297 the supplemental data.

298 The data presented within this paper is a reflection of the exact experimental
299 conditions described and attempts to depict a worst-case (using wastewater effluent exposed
300 mussels in EXP3) and best-case (mussels deemed suitable for human consumption in EXP2)
301 scenario, thus representing two different extremes of dietary exposure. Using mussels from a
302 site where these fish thrive naturally may yield results similar to those achieved in EXP2 and

303 EXP3, however, as previous national monitoring studies (Jones et al., 2016) have shown, the
304 spatial occurrence of metals and their concentrations can vary from site to site. From the
305 results shown in Table 1, it can be accepted that juvenile rainbow trout in EXP2 and EXP3
306 were exposed to varied concentrations of metals through a diet of wild marine mussels. The
307 average metal concentrations measured in the fish muscle and skin sampled over the 28-day
308 exposure are shown in Tables 3 and 4, respectively. Boxplots were used to clearly depict the
309 spread of data points (interquartile range or IQR), the median value, errors in the form of
310 whiskers (Tukey style i.e. no more than $1.5 \times \text{IQR}$) and outliers for each dataset shown as an
311 asterisk. Statistical results for all metals can be found in Tables S4 (a) and (b) and S5 (a) and
312 (b) in the supplemental data. Those metals showing statistically significant differences
313 ($p < 0.05$) in fish muscle and skin tissue concentrations across timepoints and exposures are
314 shown in Figure 1 for priority metals and in Figure 2 for all other unregulated

315

Metal	Average concentration in fish muscle ($\mu\text{g g}^{-1}$ dry weight) \pm S.D.						
	0 d	14 d			28 d		
	(n=8)	EXP1 (n=9)	EXP2 (n=9)	EXP3 (n=9)	EXP1 (n=9)	EXP2 (n=9)	EXP3 (n=9)
As	3.773 \pm 1.013	3.900 \pm 0.595	4.869 \pm 0.717	4.454 \pm 0.639	4.242 \pm 1.343	5.241 \pm 0.674	5.360 \pm 0.543
Cd	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cr	0.046 \pm 0.023	0.031 \pm 0.017	0.027 \pm 0.008	0.029 \pm 0.009	0.025 \pm 0.010	0.022 \pm 0.008	0.027 \pm 0.019
Co	n.d.	n.d.	0.010 \pm 0.008	0.027 \pm 0.032	0.010 \pm 0.003	0.011 \pm 0.002	0.020 \pm 0.004
Cu	1.471 \pm 0.181	1.599 \pm 0.207	1.763 \pm 0.127	1.809 \pm 0.170	1.528 \pm 0.201	1.691 \pm 0.157	1.666 \pm 0.223
Fe	16.311 \pm 3.374	13.261 \pm 3.752	14.686 \pm 2.559	11.123 \pm 0.972	14.586 \pm 5.272	14.469 \pm 3.083	11.801 \pm 1.454
Pb	0.018 \pm 0.004	0.007 \pm 0.005	0.015 \pm 0.007	0.013 \pm 0.004	0.007 \pm 0.003	0.009 \pm 0.003	0.008 \pm 0.003
Mn	1.491 \pm 0.339	1.002 \pm 0.466	1.091 \pm 0.314	0.789 \pm 0.196	1.192 \pm 0.620	1.005 \pm 0.465	0.816 \pm 0.138
Mo	0.022 \pm 0.017	0.011 \pm 0.002	0.017 \pm 0.011	0.021 \pm 0.005	0.017 \pm 0.003	0.013 \pm 0.002	0.025 \pm 0.007
Ni	0.059 \pm 0.016	0.079 \pm 0.025	0.080 \pm 0.021	0.063 \pm 0.016	0.045 \pm 0.008	0.044 \pm 0.010	0.050 \pm 0.012
Se	0.833 \pm 0.077	0.866 \pm 0.105	0.822 \pm 0.139	0.921 \pm 0.117	0.817 \pm 0.050	0.960 \pm 0.082	0.931 \pm 0.057
Sn	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
V	0.024 \pm 0.007	0.022 \pm 0.006	0.024 \pm 0.006	0.019 \pm 0.002	0.023 \pm 0.011	0.022 \pm 0.007	0.020 \pm 0.004
Zn	24.523 \pm 3.167	25.177 \pm 3.346	23.290 \pm 2.987	23.269 \pm 3.124	25.835 \pm 5.081	22.506 \pm 2.097	22.188 \pm 2.421

316 n.d. = Not detected

317 **Table 3.** Metal concentrations measured in fish muscle sampled from each exposure (EXP1, EXP2 and EXP3) at all sampling time points (0, 14
318 and 28 days).

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Metal	Average concentration in fish skin ($\mu\text{g g}^{-1}$ dry weight) \pm S.D.						
	0 d	14 d			28 d		
	(n=8)	EXP1 (n=9)	EXP2 (n=9)	EXP3 (n=9)	EXP1 (n=9)	EXP2 (n=9)	EXP3 (n=9)
As	2.088 \pm 0.798	1.986 \pm 0.322	2.179 \pm 0.386	1.837 \pm 0.337	1.867 \pm 0.543	1.817 \pm 0.254	1.717 \pm 0.398
Cd	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cr	0.059 \pm 0.020	0.063 \pm 0.052	0.050 \pm 0.015	0.048 \pm 0.024	0.042 \pm 0.014	0.115 \pm 0.207	0.053 \pm 0.017
Co	n.d.	0.030 \pm 0.009	0.031 \pm 0.013	0.039 \pm 0.012	0.039 \pm 0.008	0.041 \pm 0.005	0.043 \pm 0.010
Cu	1.751 \pm 0.340	2.032 \pm 0.255	1.785 \pm 0.472	1.413 \pm 0.226	1.683 \pm 0.512	1.415 \pm 0.407	1.410 \pm 0.192
Fe	49.490 \pm 18.658	46.530 \pm 12.857	52.203 \pm 15.568	54.904 \pm 14.180	57.761 \pm 14.112	60.339 \pm 10.030	64.943 \pm 10.908
Pb	0.043 \pm 0.029	0.045 \pm 0.019	0.074 \pm 0.022	0.072 \pm 0.027	0.043 \pm 0.009	0.074 \pm 0.020	0.106 \pm 0.024
Mn	6.620 \pm 3.171	5.752 \pm 1.956	5.460 \pm 1.778	6.371 \pm 2.208	7.039 \pm 1.795	5.816 \pm 1.803	7.731 \pm 2.294
Mo	0.034 \pm 0.013	0.046 \pm 0.007	0.026 \pm 0.008	0.042 \pm 0.009	0.038 \pm 0.008	0.025 \pm 0.005	0.067 \pm 0.021
Ni	0.103 \pm 0.026	0.212 \pm 0.082	0.234 \pm 0.103	0.152 \pm 0.051	0.105 \pm 0.034	0.168 \pm 0.137	0.126 \pm 0.024
Se	0.756 \pm 0.063	0.651 \pm 0.093	0.625 \pm 0.058	0.762 \pm 0.186	0.714 \pm 0.065	0.873 \pm 0.144	0.970 \pm 0.163
Sn	0.018 \pm 0.014	0.011 \pm 0.004	0.030 \pm 0.025	0.012 \pm 0.003	0.013 \pm 0.006	0.012 \pm 0.006	0.012 \pm 0.006
V	0.095 \pm 0.054	0.062 \pm 0.017	0.094 \pm 0.041	0.107 \pm 0.028	0.097 \pm 0.043	0.086 \pm 0.027	0.157 \pm 0.055
Zn	167.422 \pm 70.713	161.034 \pm 28.970	167.303 \pm 47.803	196.963 \pm 51.603	149.767 \pm 52.333	202.861 \pm 56.186	228.043 \pm 47.865

324 n.d. = Not detected

325 **Table 4.** Metal concentrations measured in fish skin sampled from each exposure (EXP1, EXP2 and EXP3) at all sampling time points (0, 14
326 and 28 days).

327

328 metals. Significant differences in metal concentrations across timepoints for each exposure
329 are highlighted with lower-case letters and significant differences between exposures at
330 specific timepoints are shown with solid and dashed lines. Although every attempt was made
331 to select fish of similar size, there was still considerable variability in the metal
332 concentrations determined in these fish populations prior to *in vivo* exposure, mainly
333 attributable to intra-species and size variations which may explain why there were large non-
334 parametric variances observed across the data. Fish growth was measured across all
335 exposures over the 28-day exposure time. For the controlled fish feed study (EXP1), growth
336 measured highest at 37-50%. In comparison, the mussel control study (EXP2) measured
337 growth between 0-16% and the mussel exposure study (EXP3) measured growth between 0-
338 4%. Similar to results shown by Berge and Austreng (1989), where rainbow trout were fed
339 diets of blue mussel tissue, poorer fish growth was observed with increased levels of blue
340 mussel in the diet. Growth performance was also previously monitored in a Nordic study in
341 2015 in which rainbow trout fed fishmeal and mussel meal based diets were compared.
342 Poorer growth was observed in the mussel meal based diet but only when the fish were fed in
343 a restrictive manner with controlled portions. When fed '*ad libidum*', the lower methionine
344 level in the restricted mussel meal diet was not limited and resulted in the same growth
345 performance as the fishmeal due to the greater feed and protein intake (Arneson et al., 2015).

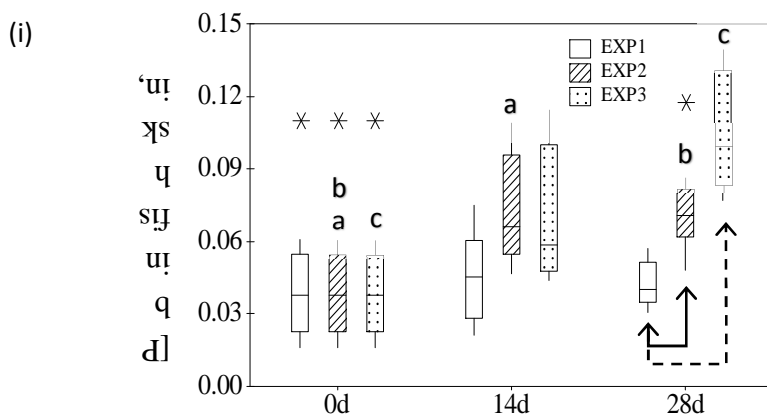
346

347 3.3.1 Temporal accumulation of priority and regulated metals (Pb, Cd, Ni, As, Sn)

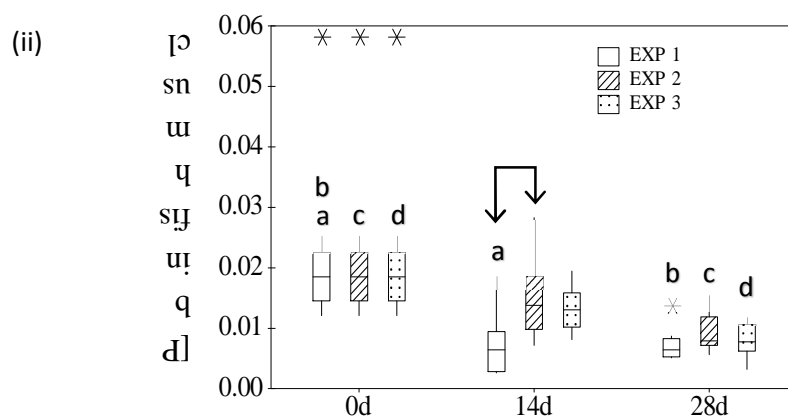
348 Lead was the only priority metal measured in all mussel samples at concentrations exceeding
349 the BAC values for mussels and in addition to this, lead was also found in fish skin pre-
350 exposure at almost double that of the BAC value for fish. Significant increases in lead
351 concentrations were observed in fish skin across time for EXP2 (represented by *a* and *b*) and
352 EXP3 (represented by *c*) following the consumption of lead-contaminated mussel tissues over

353 28 days, as shown in Figure 1 (i). Significant differences were shown between these mussel-
354 fed exposures and EXP1 at 28 d (solid and dashed lines) as EXP1 showed no significant
355 change in lead concentrations in the fish skin over the same period (see Table S4 (b)).
356 Interestingly, lead concentrations showed a statistically significant decrease in fish muscle
357 collected from all three exposures over the 28-day period (Figure 1 (ii)) resulting in no
358 significant difference observed between exposures at 28 d. In contrast, arsenic concentrations
359 significantly increased in fish muscle collected from both EXP2 (represented by *a*) and EXP3
360 (represented by *b*) with no significant change observed in EXP1 over the same 28-day period.
361 This resulted in significant differences between the mussel-fed exposures and EXP1 at 28 d
362 as shown in Figure 1 (iii) (solid and dashed lines). No significant changes in arsenic
363 concentrations in fish skin were recorded (see Table S4 (b)). Nickel and tin concentrations
364 did not change significantly at 28 d but instead displayed significant differences for
365 concentrations measured in muscle (nickel only as tin was not detected in fish muscle) and
366 skin tissues, at 14 d across exposure types and over the first and latter half of the 28-day
367 period within each exposure (see Tables S4 (a) and (b) and S5 (a) and (b) in the supplemental
368 information). For metals where significant differences were observed at or between 14 d, it
369 has been suggested that the accumulation of metals in fish at sub-lethal exposure is time
370 dependent and during the initial period of exposure, the metal is absorbed and accumulated at
371 a high rate, but then the level stabilises when an equilibrium of metal uptake and excretion
372 rates is attained. This may be true to a greater extent for low level non-essential potentially
373 toxic metals than more essential metals (Dallinger et al., 1987; Jezierska and Witeska, 2006).
374 Cadmium was not present in the fish tissues at quantifiable levels and thus any changes in
375 concentration over time could not be determined.

376

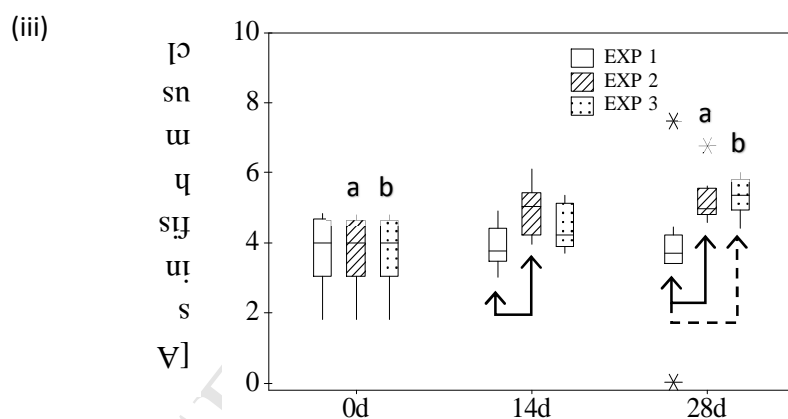


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Figure 1. Boxplots depicting the change in priority metal concentrations ($\mu\text{g g}^{-1}$) determined in fish muscle and skin tissues across time points (0, 14 and 28 d) and show lead concentrations measured in (i) fish skin and (ii) fish muscle; and (iii) arsenic concentrations measured in fish muscle. Boxplots display the interquartile range, median value, error bars ($\leq 1.5 \times \text{IQR}$) and outliers (*) for each dataset. Letters denote significant differences measured within exposures over time, solid and dashed lines denote significant differences measured between exposures at certain time points.

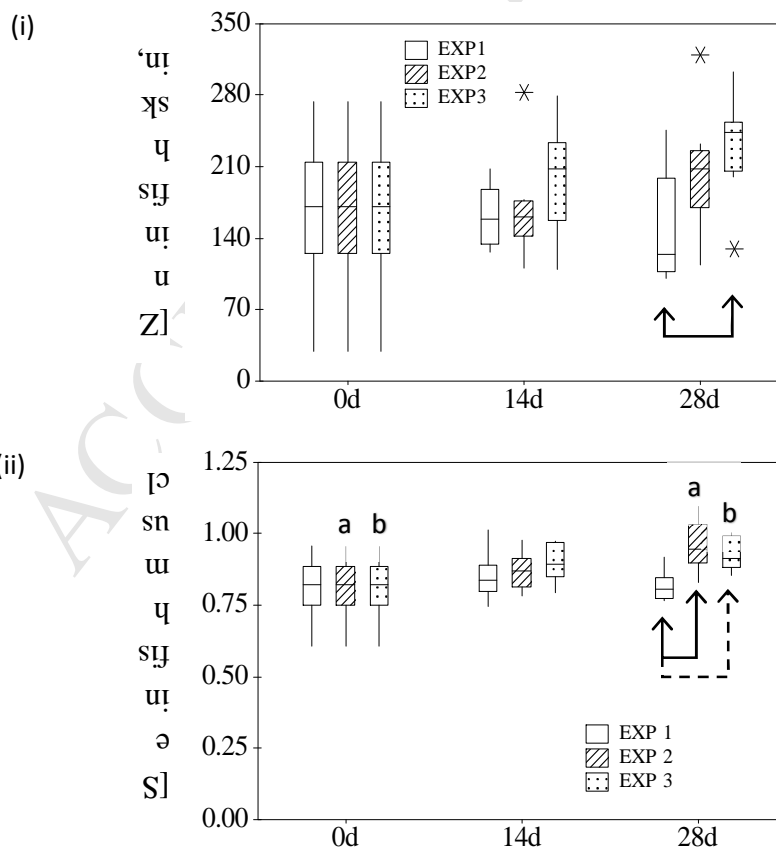
386 3.3.2 Temporal accumulation of essential (Cu, Fe, Mn, Mo, Se, V, Zn) and non-essential (Cr,
387 Co) unregulated metals

388 Zinc concentrations in fish skin sampled from EXP1 and EXP3 at 28 d were shown to be
389 significantly different, with measured concentrations higher in the EXP3 exposure (Figure 2
390 (i)), surprising considering the mussel feed administered for this exposure contained the
391 lowest concentration of zinc (Table 1). No significant changes in zinc concentrations were
392 measured in skin sampled from EXP3, however, the slight decrease in zinc concentrations in
393 fish skin from EXP1 over the 28-day period (Table 4), although not a statistically significant
394 decrease, resulted in the significance measured between these two exposures at 28 d. No
395 significant changes in zinc concentrations in the fish muscle were measured. As shown in
396 Figure 2 (ii) and (iii), selenium concentrations in fish muscle and skin, respectively, were
397 significantly different for EXP2 and EXP3 when compared to EXP1 at 28 d. Selenium
398 concentrations measured significantly higher in fish muscle from EXP2 (represented by *a* in
399 Figure 2 (ii)) and EXP3 (represented by *b* in Figure 2 (ii)) after the 28 day exposure period,
400 however, selenium concentrations measured in fish skin at 28 days for EXP2 (represented by
401 *a* in Figure 2 (iii)) and EXP3 (represented by *b* in Figure 2 (iii)) were only significantly
402 different to those collected at 14 d but not to those concentrations measured at 0 d. The
403 sizable bioaccumulation capacity and bioavailability of selenium in rainbow trout has
404 previously highlighted its potential as a good source of selenium in the human diet (Ciardullo
405 et al., 2008). However, dietary selenium levels of $\geq 5 \mu\text{g g}^{-1}$ in foodstuffs may be considered
406 toxic which is concerning considering the selenium levels detected in the mussel feed (Table
407 1) measured up to $5 \mu\text{g g}^{-1}$ dry weight (Sciortino and Ravikumar, 1999). Molybdenum and
408 vanadium showed significant increases in concentration in fish skin sampled from the EXP3
409 exposure across the 28-day period, shown in Figure 2 (iv) and (v), respectively. This resulted
410 in significant differences measured for these two compounds in fish skin samples collected at

411 28 d between EXP2 and EXP3, but interestingly not between EXP1 and EXP3 (only for
 412 vanadium at 14 d) most likely due to the wider spread of data points for EXP1. Chromium
 413 was measured in fish skin and muscle tissues at low-level concentrations ($<0.05 \mu\text{g g}^{-1}$ dry
 414 weight) and thus any significant differences observed may be a result of the high variation
 415 between sample replicates (Tables 3 and 4). Cobalt could not be detected in fish muscle and
 416 skin tissues at 0 d due to the method sensitivity but was quantified in both tissues at 14 d and
 417 28 d which suggests an increase in cobalt concentrations, however, as the 0 d timepoint was
 418 not measured it is unknown if these results are significantly different to any original
 419 concentrations present. Copper, magnesium and manganese did not show significant changes
 420 at 28 d but, similarly to nickel and tin, displayed significant differences for concentrations
 421 measured in tissues at 14 d across exposure types.

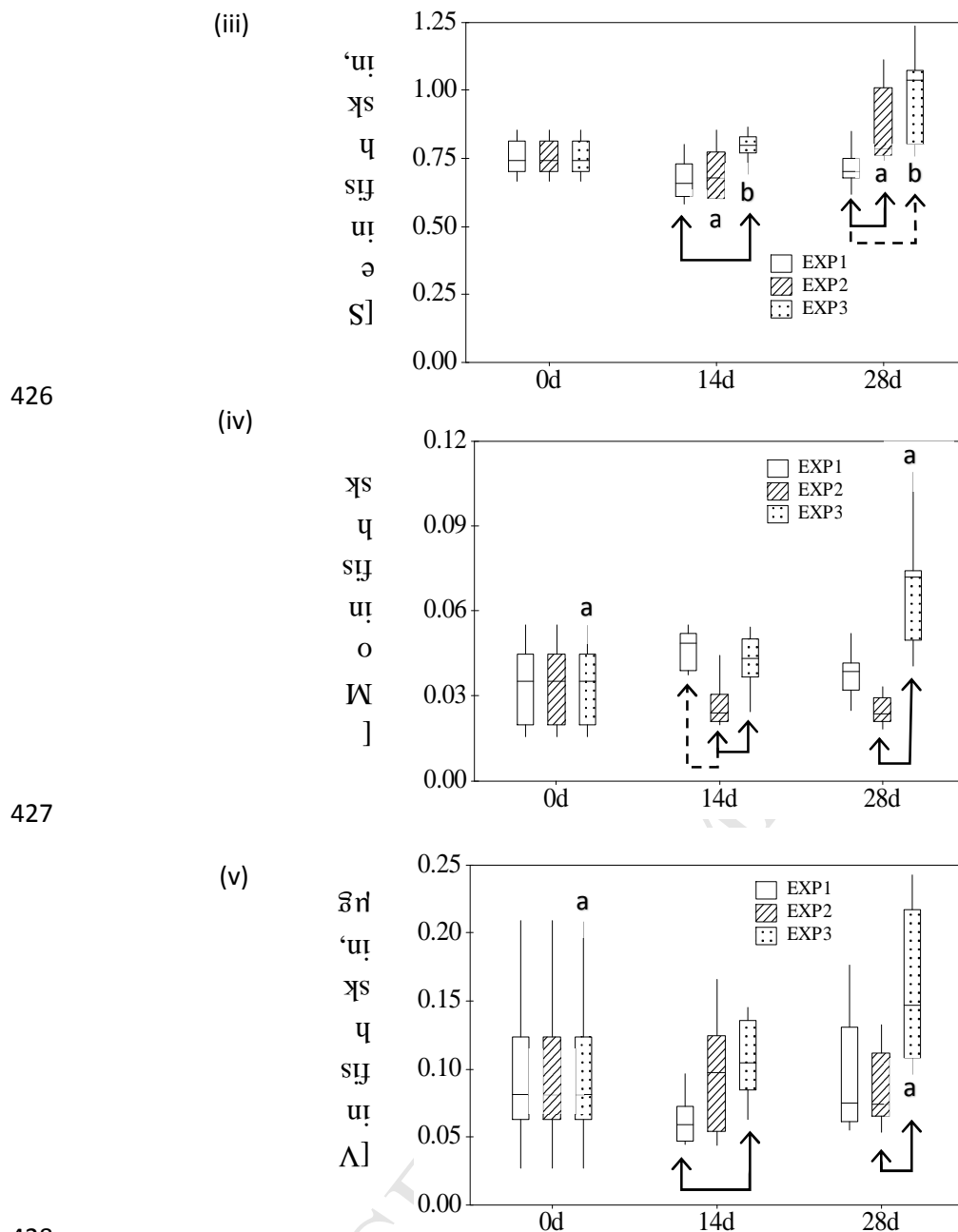
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428 **Figure 2.** Boxplots depicting the change in essential and non-essential metal concentrations
 429 ($\mu\text{g g}^{-1}$) determined across time points (0, 14 and 28 d) for (i) zinc measured in fish skin;
 430 selenium measured in (ii) fish muscle and (iii) fish skin; (iv) molybdenum measured in fish
 431 skin; and (v) vanadium in fish skin. Boxplots display the interquartile range, median value,
 432 error bars ($\leq 1.5 \times \text{IQR}$) and outliers (*) for each dataset. Letters denote significant differences
 433 measured within exposures over time, solid and dashed lines denote significant differences
 434 measured between exposures at certain time points.
 435

436 3.3.3 The propensity of select metals to accumulate in fish muscle and skin tissues
437 Five of the fourteen monitored metals (Cu, Mn, Zn, Fe and Se) were present in all three fish
438 feeds administered. Copper, manganese and zinc were measured at lower average
439 concentrations in mussel feed, iron measured at similarly high concentrations in all samples
440 and selenium measured highest in mussel feed. In line with what was measured in the
441 different feed types, a significant increase in selenium concentrations was observed in the fish
442 tissues collected from both mussel fed exposures, highlighting the responsiveness of fish
443 muscle and skin to the dietary uptake of selenium. Certain metals such as tin, vanadium,
444 molybdenum and cobalt were measured in the effluent-exposed mussels (EXP3) at
445 concentrations at least three times those detected in the mussels collected from the Class A
446 site (EXP2) but this difference was not observed in the fish muscle or skin following dietary
447 exposure. For all other metals present in the mussel feed only, fish muscle was shown to be
448 responsive to the dietary uptake of arsenic whereas fish skin was found to be responsive to
449 the dietary uptake of lead.

450 Relatively few studies have addressed the issue of metal bioaccumulation in aquatic
451 biota via dietary intake. Nair et al. (2006) noted that metal bioaccumulation varies between
452 fish species and between metals, where the accumulation of metals was also found to be
453 greatly associated to feeding habits. Both laboratory and field experiments have shown
454 dietary intake as a major pathway of bioaccumulated metals in fish species (Spry et al., 1988;
455 Qiu et al., 2011). The majority of studies carried out to date on metal ecotoxicology also
456 focus on single element exposure to fish or invertebrate species (Pohl et al., 1997; Andrade et
457 al., 2015), however, as metals do not occur in isolation in the natural environment, further
458 study is required to assess the ecological relevance and ecotoxicological potential of
459 prevalent metal mixtures in the aquatic environment. The limited knowledge surrounding
460 metal contamination via dietary intake is of particular concern in terms of commercially

461 important species such as those examined in this study (mussels and trout), as well as other
462 threatened food webs. Closing this knowledge gap could allow for the early detection of
463 metal contamination in higher trophic levels through the examination of bioavailable metal
464 concentrations at lower trophic levels (Bonanno and Di Martino, 2016), potentially allowing
465 for the effective implementation of pre-emptive mitigation measures.

466

467 *3.4 Potential for human exposure via seafood consumption*

468 The determination of potentially harmful substances, such as metals, in aquatic organisms is
469 extremely important for human health due to the potential exposure via seafood consumption
470 (Shepherd and Bromage, 1988; Cid et al., 2001; Dadar et al., 2016). An ever increasing
471 number of studies report elevated metal concentrations in both invertebrate and fish species
472 which exceed the nationally or internationally agreed quality standards for fish meat (Elnabris
473 et al., 2013; Alkan et al., 2016). One of the main human exposure routes to toxic metals is
474 through the consumption of fish (Shepherd and Bromage, 1988; Dadar et al., 2016) but, the
475 extent to which these pollutants can travel through the food chain and ultimately pose a threat
476 to human health remains relatively unknown.

477 With regards to the metals selected as part of this study, a Spanish nature reserve was
478 severely polluted after toxic chemicals such as sulphur, lead, copper, zinc and cadmium, were
479 transported into the reserve from a burst mining dam (Grimalt et al., 1999; Lenntech, 2017).
480 The bioavailable contaminants in the environment following the Spanish ‘Doñana disaster’
481 quickly entered food chains in the affected area (Meharg et al., 1999). Elevated metal
482 concentrations were reported in many migratory and resident bird populations following the
483 incident (Taggart et al., 2006) and eight years later, elevated metal contamination were still
484 present in terrestrial food chains (Marquez-Ferrando et al., 2009). These cases highlight the
485 importance of understanding the transport, bioaccumulation and biomagnification of metals

486 along food chains. Fish species generally reside close to the top of marine food chains (Dadar
487 et al., 2016), and where metals bioaccumulate along these food webs, this could potentially
488 pose a risk to human consumers of seafood (Mathews and Fisher, 2009; Qiu et al., 2011).

489 *O. mykiss* and *M. edulis* are both commercially and socio-economically important
490 species, with an estimated global production for human consumption of 812,939 and 185,433
491 tonnes, respectfully, in 2014 (FAO, 2017). Both species represent a substantial portion of
492 global seafood production and consumption. It is therefore important to understand the
493 influence of dietary intake on the bioaccumulation and biomagnification of metals in these
494 species, and many more commercially important species, to ensure the safety of consumers
495 and the prosperity of commercial seafood production. To achieve this, more comprehensive
496 assessments are needed, in terms of dietary intake, metal ecotoxicology of metal mixtures and
497 bioaccumulation along food chains to allow for a more holistic and robust assessment of
498 bioavailable metals in commercially exploited food webs.

499 **4.0 Conclusions**

501 This study has highlighted the significance of dietary intake for the bioaccumulation of
502 metals in fish tissues and the further potential for metal exposure to human consumers of
503 commercial seafood. Mussels sourced from the contaminated exposure site contained Co,
504 Mo, Sn and V at concentrations at least three times more than those detected in the mussels
505 collected from the Class A site. Cu, Pb and Zn present in both mussel samples were found to
506 exceed the background assessment concentrations given by the OSPAR Co-Ordinated
507 Environmental Monitoring Programme (CEMP). This is particularly worrying with regards to
508 the mussels collected from the Class A shellfish site as there are no requirements for these
509 mussels to undergo depuration prior to human consumption. Pb concentrations measured in
510 fish skin were found to be high prior to the dietary experiment at almost double that of the

511 BAC value stated for fish. A significant increase in Se, Pb and As concentrations was
512 observed in the fish tissues collected from the mussel fed exposures after 28 days,
513 highlighting the responsiveness of fish muscle and/or skin to the dietary uptake of these
514 particular metals. Future research should regard dietary intake as a major source of
515 bioaccumulated metals and, where possible, metal bioaccumulation should be examined
516 across a mixture of metals for greater ecotoxicological relevance.

517

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528

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Highlights:

- 28-day *in vivo* study demonstrates metal bioaccumulation in fish via dietary intake.
- Uptake of 14 metals in rainbow trout on diets of fish feed and mussels compared.
- Effluent-impacted mussels from Irish waters contained x3 more Co, Mo, Sn and V.
- Pb, As and Se concentrations significantly greater in fish feeding on mussels.
- Highlights further potential for metal exposure to human consumers of seafood.