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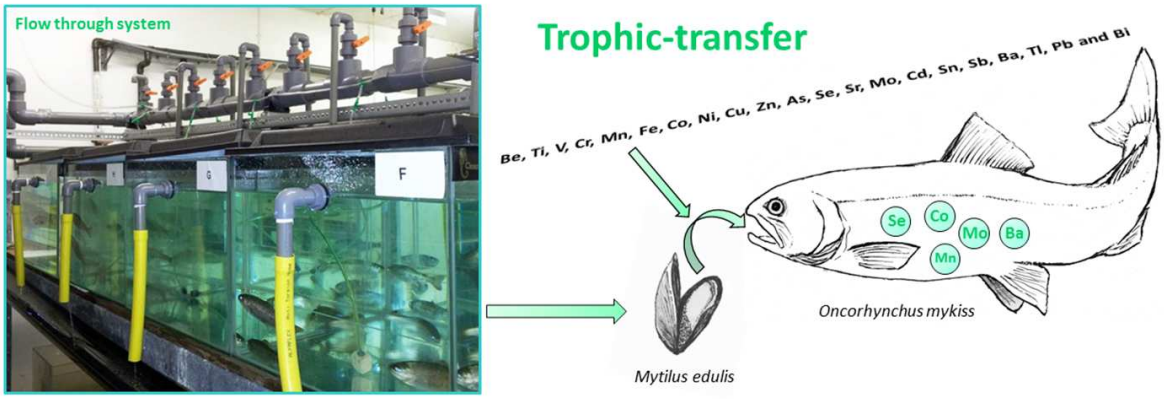

BIOACCUMULATION OF METALS IN JUVENILE RAINBOW TROUT (ONCORHYNCHUS MYKISS) VIA DIETARY EXPOSURE TO BLUE MUSSELS

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Abstract

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

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

1.0 Introduction

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

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

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

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

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

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

2.0 Materials and Methods

2.1 Experimental setup

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

2.2 Feeding

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

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

2.3 Sampling

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

2.4 Sample preparation and analysis of fish and blue mussel tissue

Mussel and fish samples were washed with Milli-Q water [18.3 MΩ·cm, Millipore, Bedford, USA] to remove debris and any adhering particulate material and all samples were freeze-dried at -52 °C [FreeZone 12, Labconco, Missouri, USA]. Fish samples were separated into muscle and skin tissues and pulverised in an agate ball mill (Fritsch™ Pulverisette 6 Planetary Mono Mill). Aliquots of tissue (approximately 0.25 g) were decomposed and mineralised using closed vessel microwave digestion (Multiwave 3000, Anton Paar, Graz, Austria (Ratcliff et al., 2016)) in a class 10,000 (ISO class 7) clean room using 3 mL of 67-69% HNO_3 [SpA grade, Romil™, Cambridge, UK] and 3 mL of 30% H_2O_2 [TraceSelect® Ultra, Sigma-Aldrich, St. Louis, USA].

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

2.5 Quality control

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

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

2.6 Data processing and statistical analysis

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

3.0 Results and Discussion

3.1 Quality control

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

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

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

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

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

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

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

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

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

Metal	EQS, BACs and guide values for metal residues in biota ($\mu g\ g^{-1}$ dry weight)					MRLs for metals in foodstuffs[a] ($\mu g\ g^{-1}$ 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		
	Mussels	*Fish*	*Mussels*	*Fish*[a]	*Shellfish*	*Mussels*	*Fish*
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*

**For tinned food*

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

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

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

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

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

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

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

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

n.d. = Not detected

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

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

n.d. = Not detected

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

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

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

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

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

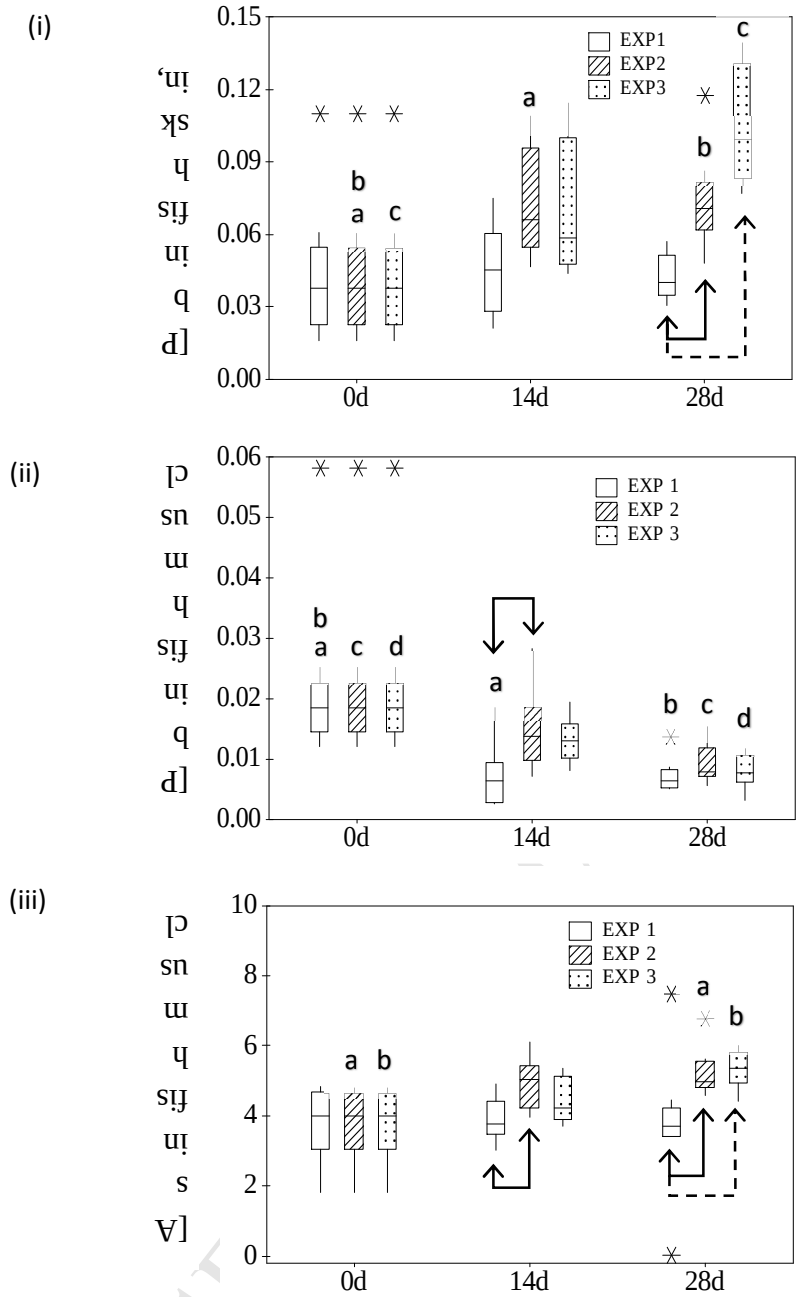
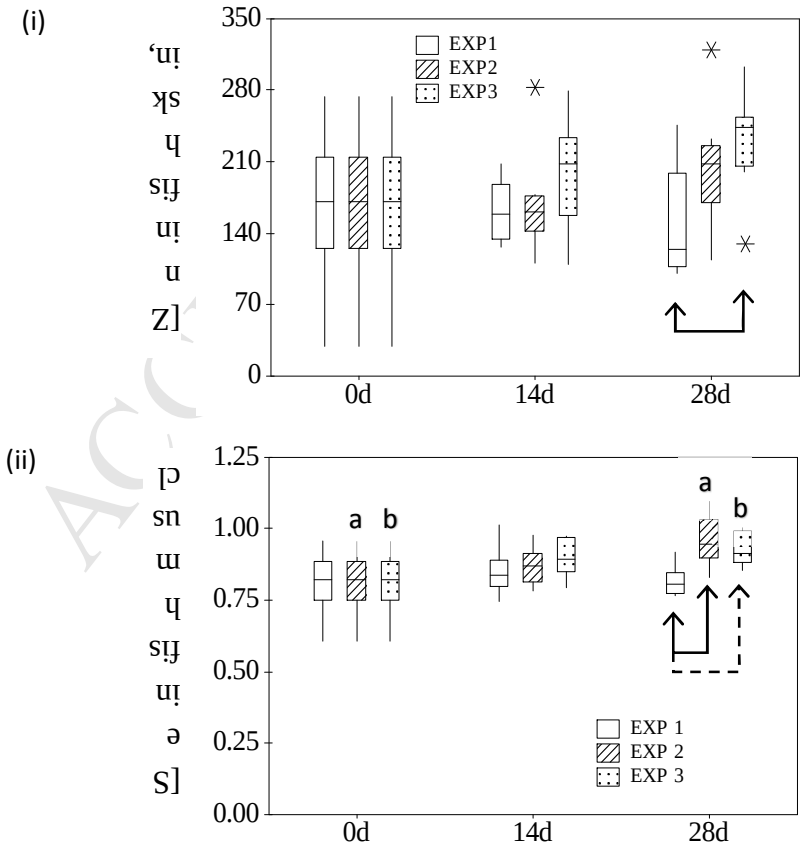


Figure 1. Boxplots depicting the change in priority metal concentrations (µg g^{-1}) determined in fish muscle and skin tissues across time points (0, 14 and 28 d) and show lead concentrations measured in (i) fish skin and (ii) fish muscle; and (iii) arsenic concentrations measured in fish muscle. Boxplots display the interquartile range, median value, error bars (≤1.5×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.

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

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

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



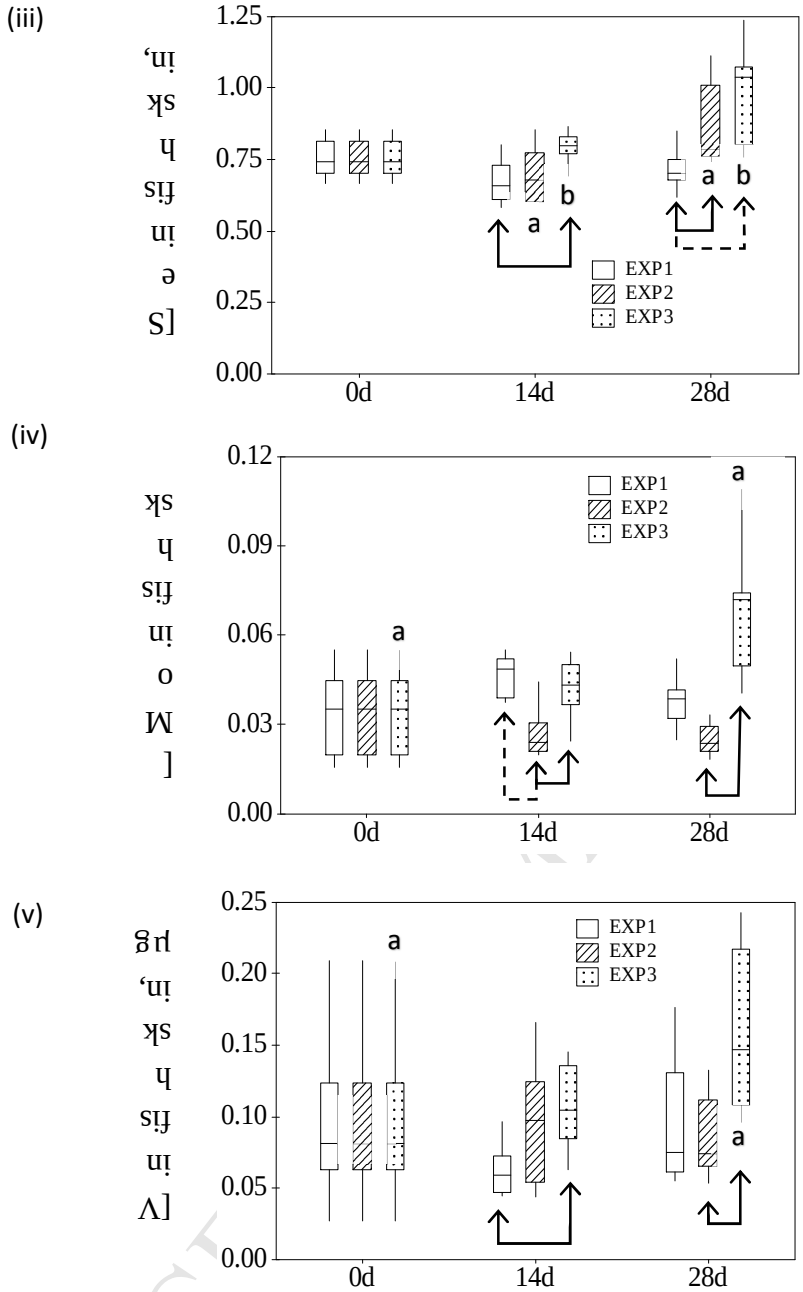


Figure 2. Boxplots depicting the change in essential and non-essential metal concentrations (µg g^{-1}) determined across time points (0, 14 and 28 d) for (i) zinc measured in fish skin; selenium measured in (ii) fish muscle and (iii) fish skin; (iv) molybdenum measured in fish skin; and (v) vanadium in fish skin. Boxplots display the interquartile range, median value, error bars (≤1.5×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.

3.3.3 The propensity of select metals to accumulate in fish muscle and skin tissues

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

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

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

3.4 Potential for human exposure via seafood consumption

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

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

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

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

4.0 Conclusions

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

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

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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.