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MADGETT, A.S., YATES, K., WEBSTER, L., MCKENZIE, C. and MOFFAT, C.F.

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The Concentration and Biomagnification of Trace Metals and Metalloids Across Four Trophic Levels in a Marine Food Web

Alethea S. Madgett[1,2*], Kyari Yates[1], Lynda Webster[2], Craig McKenzie[3], Colin F. Moffat[1]

[1] School of Pharmacy and Life Sciences, Robert Gordon University, Aberdeen AB10 7JG, UK.
[2] Marine Scotland Science, Marine Laboratory, 375 Victoria Road, Aberdeen AB11 9DB, UK.
[3] Leverhulme Research Centre for Forensic Science, Ewing Building, University of Dundee, DD1 4HN, UK.
*Corresponding Author.

1. Highlights

- Mixed effects model analysis revealed Hg, Cd, Cu, Ni and Zn to have a significant relationship with trophic level.
- An unbalanced dataset was found to influence the calculated TMF and in some cases, the overall conclusion of the trophic transfer of metals/metalloids.
- The detected and trophic adjusted Hg concentrations in shark, fish and invertebrates were all above the EU Hg biota EQS.
- The number of factors required to ensure the calculation of a reliable TMF has raised questions about the application and worth for environmental assessment purposes.

2. Abstract

To be able to assess progress towards “Good Environmental Status” adopted across European Member States, and by the United Kingdom through its 3-stage Marine Strategy, contaminant concentrations and their biological effects need to be assessed in environmental samples by comparison to assessment criteria. This study examines the variability of concentrations (inter- and intra- species variation) of three priority heavy metals (Hg, Cd and Pb) and six additional trace metals and metalloids (As, Ni, Se, Zn, Cu and Cr) in twenty-three species across four trophic levels from different locations around Scotland. Trophic magnification factors (TMFs) were calculated using two methods for metals/metalloids possessing a significant trophic relationship (Hg, Cd, Cu, Ni and Zn) to refine and improve the application of TMFs to assess and predict biomagnification risk of metals/metalloids to biota in the environment. It was concluded that a reasonable balance in sample numbers of lower- versus higher-trophic level organisms is highly recommended when calculating TMFs and appropriate species selection is vital to ensure TMFs accurately represent the selected ecosystem.

3. Graphical abstract

4. Key words

Metals, Biomagnification, Scotland, Assessment, Trophic, Contamination

5. Introduction

Metals and metalloids are naturally present in the environment due to erosion (removal) of underlying rocks, volcanic emissions and weathering (breakdown) of rocks and soils. However, their extraction, production, use and release anthropogenically can lead to an increase in their environmental concentrations such that they may be toxic to biota (Richir and Gobert, 2016). Metals and metalloids can be divided into two categories – essential and non-essential. Essential metals and metalloids are required in organisms for normal physiological

functioning and are depleted by a variety of metabolic processes utilising energy. Examples include the cofactor zinc (Zn), which is used in over 100 enzyme reactions, chromium (Cr) which is involved in lipid metabolism and required for maintaining normal glucose metabolism and cobalt (Co), which is a key component of vitamin B12, a coenzyme in a number of cellular processes including the oxidation of fatty acids and the synthesis of deoxyribonucleic acid (DNA) (Pouil *et al*., 2017). Essential metals and metalloids are however toxic at a threshold concentration, above which adverse biological effects begin to occur (Scheuhammer *et al*., 2015). Factors affecting toxicity (e.g. metabolism) include those affecting the organism's ability to regulate and detoxify accumulated elements and those influencing metal uptake, which varies between and within species (Rainbow, Phillips and Depledge, 1990). For example, a study by Hédouin *et al*. (2010) analysed Ni accumulation in clams and oysters. Although both bivalve species were shown to efficiently assimilate Ni ingested with their food (especially clams) and retain it very efficiently (especially oysters), they displayed different bioaccumulation behaviour for Ni suggesting different environmental interactions and/or physiological ability.

Non-essential metalloids and heavy metals persist in the marine environment for millions of years and move through various biogeochemical cycles. Unlike some organic chemicals, the majority of metals cannot be easily metabolised into less toxic compounds (Morel and Price, 2003). Their persistent nature results in the bioaccumulation and biomagnification through the marine food web (Atwell, Hobson and Welch, 1998), and their potentially toxic effects pose a serious threat to marine organisms, particularly long-lived marine mammals (Ali and Khan, 2018).

Common heavy metal and metalloid pollutants include chromium (Cr), nickel (Ni), copper (Cu), Zn, cadmium (Cd), lead (Pb), mercury (Hg) and arsenic (As), with the non-essential elements Hg, Pb, Cd and As of the greatest concern because of their high degree of toxicity. Some non-essential elements are biomagnified to higher trophic levels (marine top predators) including marine mammals and seabirds, with Hg in particular reported to have an estimated biomagnification factor of 6.0 ± 3.7 for each trophic level in polar marine food webs (Lavoie *et al*., 2013) and 5.4 for each trophic level in tropical marine food webs (Kehrig *et al*., 2013). After its release into the environment (into soil and water), inorganic Hg is acted upon by bacteria, leading to its transformation to methylmercury (MeHg). Of the different chemical forms of Hg, MeHg is the most toxic and abundant in the marine food web (Maage *et al*., 2017). MeHg is lipophilic in nature and can easily permeate across biomembranes such as the blood–brain barrier into the central nervous system causing sensory and motor deficits and behavioural impairment (Zheng *et al*., 2019). Inorganic Hg follows a non-uniform pattern

of distribution and has been found to significantly accumulate in fish gills, liver, heart and muscle tissue, causing oxidative stress (Monteiro, Rantin and Kalinin, 2010).

It is currently unknown whether species at all trophic levels are at risk from specific pollutant concentrations, so data must be adjusted with the use of trophic magnification factors (TMFs). TMFs are a metric of contaminant biomagnification through the food web, indicating the average increase in concentration of chemicals per trophic level. A value >1 indicates biomagnification and a value <1 indicates trophic dilution (Hallanger *et al*., 2010). The current TMF approach assumes that diet is the major route of contaminant exposure and trophic level is the main driver of their accumulation in the food web. It was concluded in Madgett *et al.* (2019) that to conduct effective environmental assessments using TMFs, influencing factors need to be considered (besides appropriate trophic level data) to fully understand the complexity of marine systems and contaminant trophic transfer, instead of using nominal TMFs or trophic level values from databases (e.g. Fishbase) or other studies which introduce errors in the trophic level adjustment (Won *et al.,* 2018). For example, a knowledge of the accumulation pattern used by a chosen invertebrate for each metal to be monitored is essential and the particular physiology of the invertebrate controls the subsequent fate of a trace metal within that organism. The metal could be for essential metabolic purposes (Zn, Cu), excreted, stored in the body or exert a toxic effect, gaining access to a particular biomolecule (Rainbow, 2003).

There are numerous physiological and ecological factors that might affect metal contamination and bioaccumulation in the marine environment: geographic location (Frodello and Marchand, 2001; Lewis and Devereux, 2009; Xia *et al*., 2019; Mikolajczyk *et al*., 2020; Lawson *et al*., 2021), feeding patterns (Azevedo *et al*., 2020), age and size of species (Mustafa and Guluzar, 2003; Farkas, Salánki and Specziár, 2003; Le Bourg, 2019), sex (Gewurtz, Bhavsar and Fletcher, 2011; Jankovská *et al*., 2013), tissue type (Bilandžić *et al*., 2012; Al-Ansari, 2017) and metabolic rates (Caurant, Navarro and Amiard, 1996; Seco *et al*., 2021).

In this study, we examine the variability of concentrations (inter- and intra- species variation) of three priority heavy metals (Hg, Cd and Pb) and six additional trace metals and metalloids (As, Ni, Se, Zn, Cu and Cr). This was done to determine whether biomagnification occurs in the specific food web being investigated and to establish whether the application of TMFs is appropriate for the development of a consistent, trophic specific biota assessment criteria.

Samples were divided into sixteen sample categories (refer to Madgett *et al*., 2019 for the categorisation of twenty-three species using fatty acid and stable isotope ratio analysis). The samples were from different marine locations around Scotland. The samples were used to investigate the relationship between metal concentration and key influencing factors on

metal/metalloid accumulation (trophic level, region, sample categorisation and physiological features). TMFs were calculated using both traditional and balanced methods (Borgå *et al*., 2012; Brisebois, 2013) for the metals/metalloids possessing a significant trophic relationship. To the authors knowledge, this is the first time both methods have been applied and compared for the determination of metals and metalloids TMFs (Brisebois, 2013 focused on organic contaminants). This is also the first time the balanced method has been applied in a trophic level adjustment to enable an environmental assessment.

6. Experimental Procedure and Data Analysis

6.1. Sample Collection and Preparation

Sample collection and preparation is discussed in detail in Madgett *et al.* 2019. In summary, 211 samples, including seven fish species (haddock, whiting, hake, plaice, dab, herring and sprat), one shark species (small-spotted catshark) and thirteen invertebrate species (horse mussel, brittle star, hermit crab, edible crab, common starfish, swimming crab, shore crab, European lobster, *Nephrops*, whelk, sea mouse, squat lobster and veined squid) were collected from nine locations around Scotland between 2015 and 2017 during December and February (Figure 1). Predatory species such as the small-spotted catshark are good indicators of elemental contamination in the marine environment due to their high trophic level position and long lifespan (Bellante, *et al*., 2011). Sampling was opportunistic and took place as part of a marine environmental assessment cruises. The areas sampled were a mixture of urbanised and industrialised estuarine locations (Clyde: Holy Loch, Pladda, Hunterston; Forth: Tancred Bank) and more offshore locations (Moray Firth, Burra Haaf, Montrose Bank, Solway Firth, Firth of Forth; Figure 1) covering four biogeographic assessment regions. The biogeographic regions around Scotland used in this study (Irish Sea, Minches and Western Scotland, Northern North Sea and Scottish Continental Shelf) were designated based on physical and biological features (UKMMAS, 2010). The regions have been used in a variety of marine assessments (e.g. Charting Progress 2 (a comprehensive report on the state of UK seas; JNCC, 2010) and for Marine Strategy Framework Directive/UK Marine Strategy reporting purposes) to improve our understanding of the environment, managing and collecting data within a structured and co-ordinated approach.

Sample preparation resulted in five tissue types (whole animal, muscle, liver, soft body and brown meat). To ensure sufficient sample quantity for analysis, fish, catshark and invertebrates were pooled. Due to factors such as species, location and sample yield, pools

were composed of three to six individuals for fish, catshark, common starfish and squid. The remaining invertebrates ranged from twenty to one hundred individuals per pool (Madgett *et al.,* 2019). The length was recorded for all fish and catshark and whole animal weight was recorded for individuals except squat lobsters, swimming crabs, hermit crabs, shore crabs, *Nephrops* and brittle star where the overall pool was weighed (due to the small size and large quantity of individuals). For fish and catshark samples, liver and muscle were dissected and homogenised separately. Brown meat and muscle were dissected from edible crab and lobster and separately homogenised. Otoliths were removed from fish collected from one cruise in 2016 (36 individuals including haddock, whiting, plaice and dab) and stored in sealed plastic vials. Otoliths were sent to an analyst for microstructure examination to determine the age distribution of the fish. Small fish (<120 mm) were homogenised whole. Common starfish and brittle star were homogenised whole (including their exoskeleton) as was the sea mouse. Horse mussel, whelk, swimming crab and shore crab had their exoskeletons removed and the soft body was homogenised. Squat lobster, *Nephrops* and hermit crab had their muscular tails isolated and then homogenised. Squid mantle, which is composed of a muscular framework of connective tissue fibres, was dissected, homogenised and classified as muscle tissue.

Calanus spp. and *Pseudocalanus* spp. were collected from a site 3 nautical miles east of Stonehaven on the east coast of Scotland (Figure 1) in 2018 from the MRV *Temora*. A 1 m ring net, with a 350 µm mesh and a non-filtering cod end was used to minimise damage to the animals which were stored on the deck in 15 L plastic buckets out of the wind and sunlight until arrival at the Marine Laboratory. The target herbivorous species were isolated using a Zeiss Stemi-11 stereomicroscope and stored at -20˚C.

6.2. Sample Digestion

Samples were digested and analysed as reported in Robinson, *et al*. (2017). Briefly, homogenised biota (0.5 g for fish and shark muscle and whole and invertebrates soft body, whole and muscle; and 0.3 g for fish and shark liver, invertebrate brown meat and whole zooplankton (copepods)) and associated Certified Reference Material (CRM) (0.2 g)) were digested overnight using nitric acid (2.5 mL, Aristar grade) and hydrogen peroxide (3.5 mL, VWR Merck, Lutterworth, UK; Suprapure grade). The digestion programme (Berghof Speedwave Xpert Microwave Digestion System) took the temperature to 70 ºC over 2 minutes, after which the temperature was kept constant for 5 minutes, prior to ramping to 210 ºC, over 13 minutes. After 25 minutes at 210 ºC, the system was cooled to 50 ºC and maintained at that temperature for 20 minutes. Each digestion run included one procedural blank and one

CRM (NRCC Canada). The CRM was selected based on the digested tissue type and included TORT-3 (shellfish hepatopancreas), DORM-4 (fish muscle) and DOLT-5 (fish liver). After digestion, the vessels were allowed to cool to room temperature before each sample was diluted to 25.0 mL with ultra-pure water.

6.3. Determination of trace metals and metalloids by inductively coupled plasma mass spectrometry (ICP-MS)

The sample digests (see Section 6.2) were diluted a further 5-fold using a solution containing ultra-pure water, concentrated hydrochloric acid and gold (Au) to remove any memory effects in the determination of Hg (Robinson *et al*., 2017). Calibration standards containing the trace elements Hg, Fe, Zn, Cu, As, Pb, Cd, Se, and Au were prepared in 2% nitric and 1% hydrochloric acid solutions at a total concentration range of 0 – 1,000 µg/l. Calibration standards, quality assurance samples and sample digest solutions were analysed using an Agilent Technologies 7700x inductively coupled plasma mass spectrometer (ICP-MS) equipped with a peristaltic pump and AS-90/91 autosampler, Micromist nebuliser, Peltier-cooled quartz modified Scott spray chamber and quartz torch. Analytical standards and tuning solutions were obtained from Essex Laboratories (UK).

The ICP-MS was operated in standard (axial) mode. Germanium internal standard (m/z 72) was used to monitor and correct for any instrumental drift. Plasma correction was carried out whenever any part of the sample introduction system (e.g. spray-chamber, nebuliser, torch, cones, or lens assembly) had been changed/cleaned, and at least every six months. Full instrument parameters are provided in Robinson *et al.,* (2017).

6.4. Trophic magnification factor calculation

Linear regressions of log-transformed concentrations versus trophic level based on equation 1 (Kidd *et al*., 2018) were used to determine TMF values.

$$Log_{10}[Concentration] = a + b*TL \qquad \text{Equation 1}$$

[Concentration] is the concentration of the chemical, TL is the trophic level determined from $\delta^{15}N$ for the species under analysis (Madgett *et al.,* 2019); a and b are the intercept and slope of the linear regression line, respectively. The slope (b) is then used to calculate TMF as:

$$TMF = 10^{b} \qquad \text{Equation 2}$$

6.5. Trophic level concentration adjustment

Trophic level adjusted concentrations ($Conc_{TL\text{-}adj}$) were determined using Equation 3, as recommended in OSPAR (2016b)

$$Conc_{TL\text{-}adj} = Conc_{biota} * TMF^{(4\text{-}TL(x))} \qquad \text{Equation 3}$$

$Conc_{biota}$ is the sample concentration of the element on a wet weight (ww) basis; TMF is the TMF calculated using Equation 2; and 4-TL(x) = 4 is the calculated trophic level of the sample, calculated using the $\delta^{15}N$ (Equation 1 in Madgett *et al*., 2019). A trophic level of 4 is used in Equation 3 as the latest European Commission (EC) guidance on the implementation of the biota environmental quality standard (EQS) states that the EQS is set for animals of trophic level 4. This is a theoretical trophic level assigned as metal or metalloid concentrations critical to predators (or consumers) are most likely to occur in fish (OSPAR, 2016b).

6.6. Quality Control

Analyses at Marine Scotland Science were conducted within a laboratory accredited to ISO-17025 by the UK Accreditation Service (UKAS). All analytical batches included the analysis of blanks and CRMs, with the results recorded on Shewhart control charts with warning and control limits set at two- and three-times standard deviation respectively. External quality assurance was confirmed through successful participation in the QUASIMEME proficiency testing scheme.

6.7. Data Analysis

Statistical analysis was undertaken on Minitab 17®. The normality of the data distribution for trace metal/metalloid concentrations were examined using the Ryan-Joiner test and data logarithmically transformed where appropriate. Analysis of Variance (ANOVA) at the 95% confidence level, with Tukey's pair-wise comparisons was carried out to establish significant differences in logarithmically transformed metal/metalloid concentrations (µg/kg ww) between species, categories and regions. Mixed effects model analysis was used to determine the interaction and significant relationships existing between the logarithmically transformed metal/metalloid concentrations (µg/kg ww) and investigated variables (sample category, trophic level and region). Pearson's correlation was used to measure the linear correlation between metal/metalloid concentrations with potential influencing variables such as age, length and weight. Box plots were developed to explore differences of logarithmically transformed trace metal/metalloid concentrations within and between categories, species, and

regions and to visualise data outliers. Microsoft Office Excel was used to create bar charts, pie charts, a Venn diagram and plotting the Log_{10} [metal/metalloid concentration] against trophic level for both the traditional and balanced methods.

7. Results and Discussion

The concentrations of three priority heavy metals (Hg, Cd and Pb) and additional trace metals and metalloids (metals; Cr, Cu, Ni and Zn, metalloids; As, Se) were measured in each of the sixteen sample categories (Table 1) by ICP-MS. The significance of the effects and interactions of numerous physiological and ecological variables on metal contamination (tissue type, trophic level, region, sample category, within-category species, age, weight, collection year and length) were determined using mixed effects model analysis, ANOVA Tukey and Pearson's correlation analysis. This established which metals required further analysis for trophic dilution/magnification.

The most abundant metals/metalloids are Zn, As and Cu (Table 1), with concentrations above 40,000 µg/kg ww across the sample categories, with Zn showing the highest maximum concentration (341,000 µg/kg ww in benthic invertebrate soft body). The other metals/metalloid have concentrations below 10,000 µg/kg ww. The lowest metal/metalloid concentration detected was 5.05 µg/kg ww of Hg in demersal roundfish liver. The benthic invertebrate soft body category shows a large degree of variation of Zn concentration, with a range of 23,500 µg/kg ww in swimming crab to 341,000 µg/kg ww in horse mussel (Table 1), suggesting a species-specific accumulation. In a number of cases, especially for the metals Pb and Cr, concentrations were less than the limit of detection (LoD), calculated using 4.65* standard deviation from replicate analysis of blanks. However, Cu, Se and Zn were detected in all samples (Table 1).

7.1. Tissue selection

Metal absorption by animals and the different tissues therein depends on a variety of factors, often directly relating to the physiology and metabolism of the animal concerned. Elemental concentrations within different tissues can vary significantly due to the specific tissue function and chemical affinity (Aguilar, Borrell and Pastor, 1999). In order to conduct a comprehensive analysis between sample categories and conduct mixed effects model analysis, the appropriate tissue type must be selected for each shark and fish category (where tissues have been taken from the same animal and separated) to reduce variation.

7.1.1. Shark and fish

Hg has been reported to bind to muscle tissue in fish due to its high affinity for phosphate and sulphur in proteins making it a suitable tissue for assessing Hg concentrations in fish; whilst Cd and Pb are analysed in fish liver due to the organs' storage and detoxification function (Agusa *et al*., 2005; Bat *et al*., 2015). This is supported by the findings of this study, where the concentration of Hg in demersal shark muscle (262.0 – 990.0 µg/kg ww), pelagic roundfish muscle (43.30 – 50.60 µg/kg ww), demersal roundfish muscle (25.90 – 105.0 µg/kg ww) and flatfish muscle (20.10 – 341.0 µg/kg ww) was significantly higher than the concentration in the liver tissue of the corresponding category (demersal shark liver 24.70 – 201.0 µg/kg ww; pelagic roundfish liver 16.30 – 18.10 µg/kg ww; demersal roundfish liver 5.050 – 51.00 µg/kg ww; flatfish liver 26.20 – 278.0 µg/kg ww; ($p<0.05$ ANOVA, Tukey); Table 1). A similar concentration variation was found with As, where demersal shark muscle (15,200 – 25,500 µg/kg ww), pelagic roundfish muscle (4,330 – 6,040 µg/kg ww), demersal roundfish muscle (1,540 – 42,900 µg/kg ww) and flatfish muscle (5,380 – 23,200 µg/kg ww) had significantly higher concentrations than in the liver tissues of the corresponding category (demersal shark liver 8,580 – 18,500 µg/kg ww; pelagic roundfish liver 3,260 – 4,930 µg/kg ww; demersal roundfish liver 2,410 – 21,000 µg/kg ww; flatfish liver 2,940 – 29,300 µg/kg ww; ($p<0.05$ ANOVA, Tukey; Table 1). Previous studies report mixed results on the tissue speciation of As. Gieter, *et al*., (2002) found that As concentrations were higher in fish muscle and shellfish species from the North Sea, whilst studies by Suner, *et al*., (1999) and Gao, *et al*., (2018) found that fish liver contained more As than muscle tissue, where the biotransformation of inorganic As to the organic forms (e.g. arsenobetaine) takes place. Based on the data from this study, mixed effects model analysis was conducted on the muscle tissue of shark and fish (pelagic, demersal and flat) for the assessment of Hg and As.

The concentration of Cd, Cr, Cu, Ni and Zn was significantly higher in demersal roundfish liver and flatfish liver than the corresponding muscle tissue ($p<0.05$ ANOVA, Tukey; Table 1). The concentration of Se was, however, significantly higher in demersal roundfish muscle (331.0 – 2,780 µg/kg ww) than the corresponding liver tissue (246.0 – 1,770 µg/kg ww) but significantly higher in the liver tissue of pelagic roundfish (459.0 – 523.0 µg/kg ww) than in their muscle tissue (336.0 – 348.0 µg/kg ww) ($p<0.05$ ANOVA, Tukey; Table 1). There was no significant difference in Se concentration between flatfish liver (318.0 – 1,810 µg/kg ww) and muscle (210.0 – 1,940 µg/kg ww) ($p<0.05$ ANOVA, Tukey; Table 1). There was no Pb detected in pelagic roundfish muscle and liver for a comparison to be made.

Demersal shark had a different metal/metalloid tissue profile compared to the fish categories, where the concentration of Cd, Zn and Cu was significantly higher in the liver tissue than the

corresponding muscle tissue. There was no significant difference between the muscle and liver tissue concentrations of Pb, Cr, Ni and Se ($p<0.05$ ANOVA, Tukey; Table 1). On this basis, and for consistency, mixed effects model analysis was conducted on the liver tissue of shark and fish (pelagic, demersal and flatfish) for Pb, Cd, Se, Zn, Cu, Ni and Cr.

7.1.2. Invertebrates

Demersal invertebrates muscle and benthic invertebrates soft body, muscle, whole and brown meat were assessed as the tissues were separated for sample quantity purposes and contained different species. Benthic invertebrates brown meat, extracted from European lobster (n=1; made up of 9 individuals) and edible crab (n=2) and muscle from the same species contributed to the benthic invertebrates muscle category. Brown meat is found in the shell cavity of the crab and is composed of the hepatopancreas which has long been reported to contain relatively high metal concentrations (particularly Cd and Pb), generally well above levels measured in the muscle from legs and claws (Davies, 1981; Barrento *et al*., 2009a; Barrento *et al*., 2009b; Noël *et al*., 2011; Bolam *et al*., 2016). Cd concentrations found in the meat of crustaceans represents a public health issue for many countries worldwide, and the assessment of the risks and benefits of the human consumption of brown meat remains challenging and controversial, where official legal limits of exposure are still lacking (Ervik, Lierhagen, and Asimakopoulosb, 2020). Both brown meat and muscle tissue were analysed due to the lack of tissue specific contaminant data for assessment purposes, and the physiological differences existing between the tissues discussed above.

7.2. Metal and metalloid concentrations and trophic magnification

Before the calculation of TMFs can occur, metals and metalloids with a trophic relationship need to be identified. Mixed effects model analysis was used to determine the interaction between concentration and trophic level, sample category and region (Figure S1). Mixed effects model analysis revealed that Zn concentration had a statistically significant relationship with trophic level ($p<0.05$) while Hg, Cu, Ni and Cd concentrations had a statistically significant relationship with both sample category and trophic level ($p<0.05$). Cr was found to have a statistically significant relationship with sample category while As and Se were found to have a statistically significant relationship with sample category and region (Figure S1). There was no significant relationship between Pb concentration and sample category, trophic level or region ($p>0.05$) (Figure S1).

The five metals that were found to have a significant relationship with trophic level (Zn, Hg, Cu, Ni and Cd) were considered for further analysis of trophic magnification/dilution. Metals

with ecological and physiological accumulation routes (such as region and sample category specific concentrations) need to be assessed to reduce the variation as much as possible when calculating TMFs, to improve the reliability and applicability of the calculated TMF.

A recent paper by Kidd *et al.*, (2019) provided practical guidance for selecting or determining TMFs, stating that in order to have a higher level of confidence in the TMFs and their applicability, there must be: inclusion of several lower-trophic-level taxa; reasonable balance with respect to sample numbers of lower versus higher trophic level organisms; data should only be analysed for TMFs on contaminant concentrations above the detection limit; biota must be from the same food web; and there must be a sufficient trophic level range.

Samples analysed for metals in this study were previously analysed for fatty acids to determine the feeding patterns and stable isotopes ($\delta^{15}N$) to determine the trophic level (Madgett *et al*., 2019). Thirteen lower trophic level invertebrate species were included and a sufficient trophic level range (four trophic levels) was utilised. Samples with a concentration below the LoD have not been included in the calculation of TMFs. Due to the opportunistic nature of sampling, a similar number of samples for each trophic level (and all biogeographic areas) could not be achieved, which is a common global limitation of environmental sampling.

An alternative method of TMF calculation known as the 'balanced method' is based on a regression of geometric mean concentrations and trophic levels, rather than concentrations and trophic levels of each individual organism (Brisebois, 2013). Calculating the geometric mean reduces the influence of unbalanced sampling, i.e., a larger number of samples at certain trophic levels. To the authors' knowledge, no previous studies have investigated the TMFs of trace metals using the balanced method. For metals with a trophic relationship (Figure S1), metal concentrations were plotted using both the traditional method (Log_{10} [metal concentration] against trophic level) and the balanced method (geometric mean Log_{10} [metal concentration] against geometric mean trophic level) to enable the determination of the TMF for that metal.

Calculating the TMF of trace metals is more complex than that of organic contaminants, as metals such as Zn, Cu and Ni are essential elements and are required for an organism's natural physiological processes. There will be interspecies differences (Rainbow, 2003). The sources of between and within category variance for metals shown to be associated with trophic level (Hg, Cd, Cu, Ni, and Zn) were identified.

7.2.1. Mercury

Figure 2a shows the logarithmically transformed Hg concentration in shark and fish muscle and demersal and benthic invertebrates. Catshark, being a predatory species, has a significantly higher Hg concentration in their tissues than the other sample categories ($p<0.05$, ANOVA, Tukey) (Figure 2a). Most shark species accumulate high concentrations of Hg in their muscle tissue through their diet (Branco *et al*., 2007; Rumbold *et al.,* 2014). This study suggests the trophic relationship identified by the mixed model analysis is a biomagnification effect. Demersal sharks were collected from the Irish Sea and Madgett *et al*., (2019) reported demersal sharks to have little variation in trophic level and within-species dietary pattern, which could explain the low degree of variation of Hg concentration in this category (Table 1).

Flatfish muscle had a significantly higher Hg concentration ($p<0.05$, ANOVA, Tukey) than the other fish muscle and invertebrates categories (Figure 2a), ranging from 20.1 – 341.0 µg/kg ww; Table 1). This is to be expected as flatfish are directly exposed to near-shore seabed sediments which act as a sink for heavy metals from inputs such as rivers and atmospheric depositions (Fergusson, 1990; Förstner and Müller, 1981; Glasby and Szefer, 1998; Szefer, 2002; França *et al.,* 2005). On further investigation, it was found that flatfish from the Irish Sea Biogeographic Region (Solway Firth; Figure 1) had a significantly higher concentration of Hg ($p<0.05$ ANOVA, Tukey) in their muscle (158.0 – 341.0 µg/kg ww; n=2), than those from the Northern North Sea (Moray Firth; Figure 1) (89.80 – 131.0 µg/kg ww; n=3) and the Scottish Continental Shelf (Burra Haaf; Figure 1) (20.10 – 138.0 µg/kg ww; n=7). Although the sample size is small, this provides an indication of a localised regional influence on Hg concentration in flatfish. Physiological features such as average pool age, weight and length were not found to significantly influence the Hg concentration in the separate flatfish muscle and demersal roundfish muscle categories ($p>0.05$).

The Irish Sea Biogeographic Region (particularly the Clyde) is a contaminated region due to nearby industrial and wastewater discharges (UKMMAS, 2010). OSPAR's Intermediate Assessment 2017 (OSPAR, 2017) found that Hg concentrations in biota were at or above Background Assessment Concentrations (BAC: 35 µg/kg ww in fish muscle) in all of the assessment areas (304 monitoring sites across 12 assessment areas), with the highest concentrations found in the Norwegian Trench, Northern North Sea, Southern North Sea and Irish Sea Biogeographic Region, at around twice the BAC. Scotland's Marine Assessment 2020 (Marine Scotland, 2020) found that Hg concentrations in biota were similar in all three biogeographic regions (Irish Sea, Northern North Sea and Minches and Western Scotland) and were consistently above the BAC. Only one flatfish sample pool collected from the Scottish Continental Shelf in this study had a Hg concentration below the BAC.

The EU biota EQS for Hg represents a concentration in fish at which birds and mammals are protected against the effects of Hg via secondary poisoning i.e. the uptake of contaminants from prey. A maximum recommended concentration for Hg in whole fish of 20 µg/kg ww, expressed as total Hg, was set in Directive 2013/39/EU. The mean Hg concentration (ww) in the muscle tissue of demersal roundfish, pelagic roundfish and flatfish, the whole tissue of demersal roundfish and pelagic roundfish and liver tissue of demersal roundfish and flatfish reported in this study exceed the EQS concentration (Figure 3). It is recognised that the EQS for Hg refers only to whole fish and an accepted tissue-whole organism conversion factor is not currently available.

When shark, fish and invertebrates were analysed, three outliers were identified (Figure 2a) within the benthic invertebrates muscle category ($p<0.05$). Pooled edible crab (n=1; made up of 14 individuals) and pooled European lobster (n=1; made up of 9 individuals) had a significantly higher concentration of Hg in their muscle and pooled hermit crab (n=1; made up of 10 individuals) had a significantly lower concentration of Hg in their muscle than the other benthic invertebrate muscle category species ($p<0.05$, ANOVA, Tukey; Figure 4). Noël, *et al*., (2011) determined the Hg, Cd and Pb concentrations in white meat (muscle) and brown meat of crustaceans collected in France and found the concentration of Hg in three lobster brown meat and muscle samples to range from 43.00 – 65.00 µg/kg ww and 115.0 – 148.0 µg/kg ww, respectively. In this study, the concentration of Hg in the one pooled lobster sample (62.00 µg/kg ww) was comparable with the concentration of Hg in three lobster brown meat samples (43.00 – 65.00 µg/kg ww) collected in France (Noël *et al*., 2011).

Mixed effects model analysis revealed a statistically significant relationship between Hg concentration, trophic level and sample category. Pooled demersal shark samples had a significantly higher muscle concentration of Hg than the fish and invertebrate species (Figure 2a; $p<0.05$, ANOVA, Tukey). On this basis, the TMF of Hg was calculated including (Figure 5) and excluding (Figure 6) demersal shark muscle using both the traditional (a) and balanced (b) methods (summarised in Table 2).

Hg was found to biomagnify when demersal shark, fish and invertebrates were included, using the traditional method and balanced method (Table 2). When upper trophic level demersal shark was excluded in the analysis, the calculated TMF changed from indicating trophic magnification (traditional: 1.4; balanced: 3.3) to trophic dilution (traditional: 0.9; balanced: 0.6) (Table 2). This demonstrates the importance of the presence of higher trophic level predators when calculating the Hg TMF. Flatfish and benthic invertebrates were shown to have a much higher concentration of Hg in relation to trophic level in comparison to the other categories (Figure 6b). A similar finding was reported by Kasper *et al*., (2009) where inorganic Hg

concentrations decreased with the increase of trophic level (across four trophic levels), due to the feeding habits of detritivores being closely associated with the bottom sediment.

Although the overall biomagnification trend is the same using both methods (Table 2), the balanced method TMFs were higher for Hg when shark, fish and invertebrates were analysed. There was also a larger difference between trophic magnification and dilution using the balanced method (Table 2), showing the importance of a balanced dataset when calculating TMFs, which from these findings is recommended when calculating the TMF of Hg in food webs composed of higher trophic level predators.

The correlation was however not significant ($p>0.05$) when using the balanced method for both calculated TMFs (Table 2), suggesting that a larger dataset is required to sufficiently test significance using a balanced dataset than presented here.

The TMFs reported for Hg in this study using the traditional and balanced methods are lower than previously calculated in other studies, where an average TMF of 7.0 ± 4.9 was reported for a number of sites and ecosystems in Lavoie *et al*, (2013). A study in Mason Bay, Korea (Kim *et al*., 2012) reported a TMF of 2.5 for the magnification of Hg using fish species, polychaete, bivalves, crustacean and cephalopod. A TMF of 2.8 was reported in a study on the continental shelf for a food chain composed of zooplankton, crustaceans, all bony fish and squid groups (Pethybridge *et al*., 2012).

The latest EC guidance on the implementation of the biota EQS states that the EQS is set for animals of trophic level 4 and that concentration data should be corrected using TMFs before comparison (OSPAR, 2016b). The Hg EQS used presents difficulties as it is set at a level for whole fish (20 µg/kg ww) which is close to or below the BAC set by the OSPAR Commission for blue mussels (BAC: 18 µg/kg ww) and fish muscle (BAC: 35 µg/kg ww) (Robinson *et al*., 2017). The Hg concentration of shark and fish muscle and invertebrates was adjusted (Equation 3; OSPAR, 2016b) using the calculated TMF (all trophic levels to represent the food web) obtained by both methods for a comparison to the Hg EQS (Table 3).

The detected wet weight concentrations and the adjusted concentrations using the TMFs obtained by both methods are all above the Hg biota EQS (20 µg/kg ww). The TMF calculated using the traditional method has only slightly adjusted the original ww concentrations but the balanced dataset is more influential, halving the original ww concentration of demersal shark and doubling the original ww concentration of benthic invertebrates brown meat. This data has shown that trophic adjustment using a balanced dataset TMF has an influence on the adjusted concentration and is highly recommended for a true indication of secondary poisoning by biomagnification.

7.2.2. Cadmium

Figure 2b shows the boxplots of the logarithmically transformed Cd concentration in shark and fish liver, demersal and benthic invertebrates and zooplankton. Benthic invertebrates brown meat, soft body and zooplankton had a significantly higher Cd concentration than the other sample categories ($p<0.05$, ANOVA, Tukey). This suggests a species-specific accumulation of Cd in the food web rather than as a result of biomagnification.

A large degree of variation of Cd concentration was found in benthic invertebrates soft body in comparison to the fish, invertebrates and zooplankton categories, ranging from 31.70 µg/kg ww in pooled swimming crab to 6,220 µg/kg ww in pooled horse mussel (Table 1).

A regional comparison on the benthic invertebrates soft category found there was no significant regional influence within this category on both a biogeographic or localised regional level ($p<0.05$, ANOVA, Tukey). A larger dataset is required for a more comprehensive analysis. There was a within category species influence on Cd concentration where horse mussel had a significantly higher concentration of Cd ($p<0.05$, ANOVA, Tukey) in its tissues (3,960 ± 2,961 µg/kg ww; n (number of pools) =2) than whelk (530.3 ± 246.9 µg/kg ww; n=7), swimming crab (376.3 ± 356.6 µg/kg ww; n=6) and shore crab (130.5 ± 0.500 µg/kg ww; n=2).

Horse mussels usually live part-buried in soft to coarse sediments and are larger in size than blue mussels, which are known to accumulate a high concentration of Cd. Blue mussels are considered a suitable indicator species of metal contamination (Andersen, Maage and Johannessen, 1996). Many horse mussels live for more than 25 years and some survive for up to 50 years. This compares with a lifespan of 2-3 years for blue mussels (SNH, 2019). Young horse mussels are predated on by crabs and starfish until over 6 cm long. Their long-term exposure to sediment, greater lifespan and carnivorous behaviour appears to result in greater accumulation of Cd, Ni and Zn compared to the other species in this study. Due to predators feeding on smaller and younger animals with a lower concentration of trace metals, the trophic transfer of these metals would not be as high.

Overnell and Trewhella, (1979) reported Cd concentrations of up to 50,000 µg/kg ww in edible crab from Orkney and Shetland, whilst Falconer *et al*., (1986) reported concentrations of up to 61,300 µg/kg ww in edible crab from sixteen areas around the Scottish coast. Similarly, Noël *et al*., (2011) reported concentrations of up to 61,600 µg/kg ww with a mean of 12,800 µg/kg ww in 40 edible crabs originating from France, the UK and Ireland. The concentrations of Cd in edible crabs in this study are much lower than these reported values (Figure 2b).

Mixed effects model analysis indicated a statistically significant relationship between Cd concentration, sample category and trophic level ($p<0.05$). Pooled benthic invertebrates

brown meat, soft body and zooplankton had a significantly higher concentration of Cd ($p<0.05$, ANOVA, Tukey) in their tissues than the other categories (Figure 2b). On this basis, the TMF of Cd was calculated including (Figure S2) and excluding (Figure S3) benthic invertebrates brown meat, soft body and zooplankton using both the traditional (a) and balanced (b) methods and given in Table 2.

Cd was found to trophic dilute when all trophic levels (shark, fish, invertebrates and zooplankton) were analysed and when excluding benthic invertebrates brown meat, soft body and zooplankton using both the traditional and balanced methods (Table 2). The calculated TMFs using the balanced method were the same (0.7) when the categories containing species with a potential specific physiological basis for Cd (benthic invertebrates brown meat, soft body and zooplankton) were not included (Table 2). Although the degree of trophic dilution is marginally lower using the balanced method (closer to 1), overall, the calculated trophic dilution is the same for both methods (traditional and balanced). This suggests that in the case of Cd, an unbalanced dataset does not influence the calculated TMF. Similar to Hg, the correlation was not significant ($p>0.05$) when using the balanced method for both calculated TMFs (Table 2).

These findings are in agreement with previously reported studies. A study in the South China Sea on Cd and Pb in twelve marine organisms of differing trophic levels showed that these metals did not biomagnify (Gu *et al*, 2018) and a study in the Western Patagonia and Antarctic Pensinsula found that although Cd biomagnified in macroinvertebrates, significant trophic dilution occurred when higher trophic level organisms were assessed (Espejo *et al*., 2018).

7.2.3. Copper

Benthic invertebrates soft body and brown meat have a significantly higher concentration of Cu in their tissues than the other shark, fish and invertebrate categories ($p<0.05$, ANOVA, Tukey; Figure 2c; Table 1). Similar to Cd, this suggests a species-specific accumulation of Cu in the food web rather than biomagnification.

The hepatopancreas (making up the brown meat) has been found to contain raised Cu concentrations in comparison to muscle tissue, varying with moult cycle and associated physiological changes and blood concentration of the oxygen binding haemolymph pigment, haemocyanin (Rainbow, 2018). This could explain the significantly higher Cu concentration in brown meat observed in this study. Pooled whelk had the highest variability, ranging from 21,000 - 65,900 µg/kg ww (n=7 pools). It has been reported that molluscs can store Cu in granules, leading to elevated Cu concentrations (Marigómez, *et al*., 2002; Cheung and Wang,

2008). Higher trophic level organisms are however able to better regulate Cu to avoid lethal concentrations (Neff, 2002).

Two horse mussel sample pools (circled on Figure 2c) have a significantly lower concentration of Cu in their tissue than the other benthic invertebrate soft body species ($p<0.05$, ANOVA, Tukey). Unlike Cd and Zn, Cu uptake is regulated by bivalves, but with different efficiency from species to species (Pan and Wang, 2012). This could explain the low concentration of Cu in horse mussel noted in this study in comparison to the other benthic invertebrate soft body species, but a larger dataset is required with a higher number of bivalve samples for a comprehensive assessment.

There are no assessment criteria for the detrimental effects of Cu available for fish or shellfish as Cu is an essential element. Many organisms can regulate the uptake and release of Cu (OSPAR, 2016c) which can limit the observed concentrations.

Mixed effects model analysis identified a statistically significant relationship between Cu concentration, sample category and trophic level ($p<0.05$; Figure S1). Pooled benthic invertebrates soft body and brown meat had a significantly higher concentration of Cu in their tissues than the other categories (Figure 2c) ($p<0.05$, ANOVA, Tukey). Therefore, the TMF of Cu was calculated both including (Figure S4) and excluding (Figure S5) benthic invertebrates soft body and brown meat using both the traditional (a) and balanced (b) methods with the TMF values presented in Table 2.

Cu was found to trophic dilute (0.9) in a food web composed of shark, fish, invertebrates and zooplankton when using the traditional method, but biomagnify when calculated using the balanced method (1.5) (Table 2). Once the dataset was balanced, this difference in trophic transfer was found to be due to zooplankton having a lower concentration of Cu in relation to its trophic level than the other sample categories. The calculated TMF was higher using both methods once benthic invertebrates soft body and brown meat were removed from the dataset (Table 2) which was expected, as the sample categories with a tendency to accumulate high concentrations of Cu have been removed from the analysis. The correlation was only significant ($p<0.05$) when using the balanced method and traditional method for the food web excluding benthic invertebrates soft body and brown meat from the analysis, suggesting that in this case, removing categories containing species with a specific physiological need for Cu has significantly reduced the variation associated with the calculated TMF. This highlights the importance of species selection and a balanced dataset when calculating the TMF of Cu, suggesting the different utilisation rates of Cu between trophic levels significantly influences the regression ($p<0.05$).

In contrast to this study, trophic dilution of Cu has been reported in other studies. This is likely due to the higher number of species at lower trophic levels incorporated into the TMF calculation in this study. A study by Schneider *et al*., (2018) ranked mean Cu concentrations in the order: herbivores-suspension feeders > detritivores > autotrophs > carnivores, with calculated trophic dilution. Another study by Barwick and Maher (2003) found that Cu did not biomagnify in a temperate seagrass ecosystem, where lower trophic organisms such as molluscs and crustaceans accumulated high concentrations of Cu due to the essential nature of this trace metal.

7.2.4. Nickel

Benthic invertebrates soft body, brown meat, whole and zooplankton have significantly higher concentrations of Ni than the other shark, fish and invertebrate categories ($p<0.05$, ANOVA, Tukey; Figure 2d). As noted for Cd and Cu, this suggests a species-specific accumulation of Ni rather than biomagnification.

Demersal roundfish liver concentrations were more variable than the shark and other fish categories (Figure 2d). In demersal roundfish liver, pooled whiting has the minimum category concentration of <13.00 µg/kg ww (LoD) and the maximum category concentration of 458.0 µg/kg ww. Samples collected from the Irish Sea Biogeographic Region (98.29 ± 140.1 µg/kg ww; n=8 pools) had a higher concentration of Ni in their liver than those from the Northern North Sea (24.00 ± 4.500 µg/kg ww; n=2 pools) and Scottish Continental Shelf (17.48 ± 9.165 µg/kg ww; n=5 pools), but this difference was not significant ($p>0.05$). Although the sample size is small, it provides an indication of a localised regional influence on Ni concentration in whiting. A larger dataset will however be required for a comprehensive analysis. Features such as average pool age, weight, length and trophic level were not found to significantly influence the Ni concentration in whiting liver ($p>0.05$).

There were three outliers identified (Figure 2d) - one sea mouse sample pool and two edible crab muscle sample pools ($p<0.05$). There is only one sea mouse sample in this study (made up of 33 individuals), but this provides an indication of the accumulation ability of this species for Ni which requires further study. It is also important to note that sea mouse could be differentiated from the benthic invertebrates whole category during fatty acid analysis (Madgett *et al*., 2019), suggesting a different dietary pattern from common starfish and brittle star. A study by Danje and Manoj, (2015) analysed the concentration of Cu, Ni, Zn and Cd in polychaete worms (*Nereididae*), mud skipper and mud crab from three sites in the Purna river estuary, India. It was reported that polychaete worms accumulated a higher concentration of

Ni in their tissues than the Ni concentration in sediment, with samples collected from one site having five times the Ni concentration in their tissues than the sediment.

The two edible crab muscle samples were collected from different sites: the Irish Sea Biogeographic Region (Solway Firth; Figure 1) and Northern North Sea (Montrose bank; Figure 1), and the equivalent brown meat tissue from both sample pools had a higher concentration of Ni than in the muscle tissue (brown meat: 322.0 – 1,140 µg/kg ww; muscle: 9.36 – 110.0 µg/kg ww). A higher concentration of metals is expected from the brown meat of crab, as it is taken from the soft body of the crab which is mainly composed of the gonads and hepatopancreas, known to contain high concentrations of trace metals due to the detoxifying nature of the hepatopancreas (Bolam and Bersuder, 2013a and Bolam and Bersuder, 2013b).

There was no significant regional influence ($p>0.05$) for the benthic invertebrates muscle category, but this is likely due to the number of different species present in this category (5) each with differing accumulation rates depending on physiological requirements. Further study is required on a species level with a larger dataset.

Benthic invertebrates soft body had a Ni concentration range of 183.0 µg/kg ww in pooled whelk to 3,660 µg/kg ww in pooled horse mussel. As with Cd, this accumulation in horse mussel is likely due to their constant exposure to sediment and lifespan of 25-50 years.

The higher concentration and species-specific variation of Ni concentration in benthic invertebrates has been previously reported. Hédouin *et al*., (2010) analysed Ni accumulation in clams and oysters and although both bivalve species were shown to efficiently assimilate Ni ingested with their food, they displayed different bioaccumulation behaviour for Ni suggesting different environmental interactions and/or physiological ability. The majority of studies have focussed on smaller, lower trophic level mussels (several species) as target organisms worldwide, concluding this species is an effective bio-indicator of Ni concentrations in sea water (Lu and Wang, 2018; Mejdoub *et al*., 2018; Azizi *et al*., 2018).

Mixed effects model analysis identified a statistically significant relationship between Ni concentration, sample category and trophic level (Figure S1; $p<0.05$). Pooled benthic invertebrates soft body, brown meat, whole and zooplankton had a significantly higher concentration of Ni ($p<0.05$, ANOVA, Tukey) than the other categories (Figure 2d). There are too few lower trophic level samples available to calculate TMF when pooled benthic invertebrates soft body, brown meat, whole and zooplankton are not included in the analysis following the guidance by Kidd *et al*., (2019). On this basis, the TMF of Ni was determined (Table 2) for all trophic levels including benthic invertebrates soft body, brown meat, whole

and zooplankton (Figure S6) using both the traditional (a) and balanced (b) methods to determine whether Ni biomagnifies in the marine food web in this study.

The calculated TMFs using the traditional method (0.4) and balanced method (0.5) found Ni to trophic dilute in the marine food web, suggesting a balanced data set does not influence the calculated TMF. The correlation was however not significant ($p>0.05$) when analysing all trophic levels using both methods, suggesting that a larger dataset is required to establish significance when analysing the trophic relationship of Ni. There is relatively limited data available for the biomagnification/trophic dilution of Ni, but a study by Cardwell *et al* (2013) found that Ni generally does not biomagnify in food chains consisting of organisms occupying trophic level 3 and over. A study by Blewett and Leonard, (2017) found that organism physiology appears to be the main driver of the toxic impact of Ni rather than bioaccumulation but concluded that mechanisms of Ni toxicity in the marine environment are still not well understood.

7.2.5. Zinc

Benthic invertebrates soft body, whole and brown meat have significantly higher concentration of Zn than the other sample categories ($p<0.05$, ANOVA, Tukey; Figure 2e). Similar to Cd, Cu and Ni, this suggests a species-specific accumulation of Zn in the food web rather than a result of biomagnification.

Benthic invertebrates soft body concentrations are more variable compared to the other demersal shark, fish and invertebrate sample categories, with a range of 23,500 µg/kg ww in swimming crab to 341,000 µg/kg ww in pooled horse mussel (Table 1; Figure 2e). This was also found by Chou *et al* (2003) where the uptake of Zn by horse mussel was extremely high, suggesting that this element may play a biological role in this species and correlates with the findings for Cd and Ni.

Edible crab (n=2 pools) had a significantly higher concentration of Zn in their muscle tissue than the other benthic invertebrate muscle species (Table 1). Unlike Ni, the equivalent edible crab brown meat had a much lower concentration of Zn, suggesting a tissue specific accumulation. A study by Zhang *et al* (2019) investigated the concentration of Cu, Zn, Mn, Cd and Cr in three crab species from mangrove wetlands in China and compared their findings to those reported in eighteen species of crab from twelve studies worldwide. Significant differences between tissue types were found in all three species, where concentrations of Cu and Cd were significantly higher in the hepatopancreas than in the muscle and carapace and Zn tended to be accumulated within the muscle. They found metal concentrations in muscle

tissues followed a trend of Zn > Mn ≥ Cu > Cd > Cr in all the species, while in the hepatopancreas, the trend varied with species as reported in this study.

Mixed effects model analysis revealed that there was a statistically significant relationship between Zn concentration and trophic level ($p<0.05$). Pooled benthic invertebrates soft body, whole and brown meat have a significantly higher concentration of Zn ($p<0.05$, ANOVA, Tukey) than the other sample categories (Figure 2e). Similar to Ni, there are too few lower trophic level samples available to calculate TMF when pooled benthic invertebrates soft body, brown meat and whole are not included in the analysis (following the guidance by Kidd *et al.,* (2019)). On this basis, the TMF of Zn was determined for all trophic levels including these categories (Figure S7) using both the traditional (a) and balanced (b) methods and are shown in Table 2.

Zn was found to trophic dilute in the marine food web using the traditional method (0.9), but biomagnify when using the balanced method (2.1). Similar to Cu, this shows the importance of a balanced dataset when calculating the TMF of Zn, particularly when different utilisation rates of Zn exist between trophic levels (a higher utilisation rate when a dataset has more benthic invertebrates at a particular trophic level will influence the regression). Similar to Ni, the correlation was not significant ($p>0.05$) when analysing all trophic levels, suggesting that a larger dataset is required to establish significance when analysing the trophic relationship of Zn.

Using the traditional method, Cardwell *et al* (2013) found that Zn generally does not biomagnify through food chains consisting of primary producers, macroinvertebrate consumers, and fish occupying trophic level 3 and higher, which is similar to the food web in this study. The lack of trophic magnification of Zn is supported by other studies (Barwick and Maher, 2003; Mathews and Fisher, 2008; Guo *et al*., 2016). Further studies are required to analyse the biomagnification of Zn using a balanced dataset.

7.3. Application of TMFs

TMFs have been suggested as a reliable tool for the assessment of the bioaccumulation of organic and inorganic contaminants in environmental samples (Borgå *et al*., 2012). Refinement and application of the TMF technique should improve the assessment and predict the biomagnification and risk of hazardous substances to the environment.

There are a range of variable inputs of chemicals into the marine environment that are likely to affect the calculation of contaminant accumulation in food webs; including the spatial variation of contamination within and across ecosystems, the characterisation of food webs,

chemical properties of contaminants, species selection, seasonal variation and analytical considerations. This study aimed to follow the guidance suggested by Kidd *et al*., (2018) to minimise the variation of each of these variable inputs on the calculated TMF.

The guidance (Kidd *et al*., 2018) suggests that an unbalanced design, with a variable number of samples representing each trophic level, will influence TMF values. Previous research has focussed on using a traditional method of TMF calculation across trophic levels of variable sample numbers (Kidd *et al*., 2018). Sufficient sample numbers in all areas representing the four trophic levels could not be achieved for this study, resulting in an unbalanced dataset. Due to the opportunistic nature of environmental sampling, this is a challenge for all such studies being heavily influenced by species availability and selection at different sampling locations. This study included fourteen benthic invertebrate species, revealing the extent of species-specific accumulation of Cd, Cu, Ni and Zn in the food web. There was a considerable degree of variation associated with the calculated TMFs, resulting in uncertainty for a trophic level adjustment to compare to assessment criteria. A method for adapting data handling for calculating TMFs includes the "balanced method" (Brisebois, 2013) which was investigated alongside the traditional method in this study.

When shark, fish, invertebrates and zooplankton were analysed, covering four trophic levels, biomagnification was found to occur in the food web for Hg using both the traditional and balanced methods and Cu and Zn using the balanced method. This suggests that for Cu and Zn, the different utilisation rate of invertebrate species does influence the calculated TMF in an unbalanced dataset. When sample categories with a significantly different concentration of a metal were removed from the analysis, biomagnification was found to occur for Cu using the traditional method and balanced method. Removing these categories resulted in a change from biomagnification to trophic dilution for Hg, suggesting that biomagnification does not occur in a food web composed of lower trophic level organisms (invertebrates, small fish (sprat) and flatfish. This also changed the outcome for Cu using the traditional method from trophic dilution to biomagnification, but as this is an unbalanced dataset, it could be a result of a lower number of invertebrates (benthic invertebrates soft body and brown meat were removed) accumulating a higher concentration of this metal in relation to their trophic level than the higher trophic level species. Cd and Ni were the only metals found to trophic dilute using both methods.

The concentration-trophic level correlation was significant ($p<0.05$) for Hg and Cd using the traditional method but not significant ($p>0.05$) when using the balanced method, suggesting that a larger dataset is required to sufficiently test significance using the balanced method. The correlation was significant ($p<0.05$) for Cu when categories with a significantly higher

concentration in their tissues (benthic invertebrates soft body and brown meat) were removed from the analysis using the traditional method and balanced method, suggesting that these categories were significantly influencing the trophic transfer of Cu in the marine food web. The correlation was not significant ($p>0.05$) for Ni and Zn in all the associated figures.

An unbalanced dataset has been found to influence the calculated TMF and in some cases, the overall conclusion of the trophic transfer of metals/metalloids. This balanced method has proven particularly useful when the dataset contains significant outliers and concentrations <LoD and is recommended for calculating TMFs; to ensure the TMF is a true indication of biomagnification potential and overcome the issues encountered of an unbalanced design. Although TMFs provide valuable information regarding bioaccumulation potential and should be incorporated into regulatory decision making, species selection and data treatment must be carefully considered and kept consistent in future studies.

8. Conclusions

This study examined the variability of concentrations (inter- and intra- species variation) of three priority heavy metals (Hg, Cd and Pb) and six additional trace metals and metalloids (As, Ni, Se, Zn, Cu and Cr) in the sixteen sample categories from nine different locations around Scotland. Mixed effects model analysis was used to determine the metals/metalloids with a trophic relationship and the factors influencing TMF variability were identified.

Mixed effects model analysis revealed Hg, Cd, Cu, Ni and Zn to have a significant relationship with trophic level. None of the metals with a trophic relationship (Hg, Cd, Cu, Ni and Zn) had a significant relationship with biogeographic region. The findings from this study show that benthic invertebrates have a species-specific accumulation of Cd, Cu, Ni and Zn in the food web rather than concentrations as a result of biomagnification, which can reduce the reliability of the calculated TMFs.

TMFs were calculated on those categories possessing a significant trophic relationship and TMFs calculated using two methods: traditional and balanced methods (to determine whether biomagnification occurs in the food web and to establish whether the application of TMFs is appropriate for the calculation of TMFs). It was established that a large amount of sampling, stable isotope analysis and contaminant analysis would be required to calculate reliable TMFs. This would be time consuming, require a lot of resources (expensive) and deplete species populations on a regional scale which is not realistic, questioning the application and worth of TMFs for marine environmental assessment of metals and metalloids.

The data in this study contributes ecosystem specific contaminant data for the wider development of TMFs. Although TMFs provide valuable information regarding bioaccumulation potential and could be incorporated into regulatory decision making, species selection and data treatment must be kept consistent in future studies. Due to the essential nature of some trace metals, appropriate species selection is vital to ensure TMFs represent the selected ecosystem, which is difficult to achieve. This study has shown that for Cu and Zn in particular, a balanced dataset has reduced the variation that species with a potential specific physiological basis have on the TMF and has changed the outcome of the trophic relationship from trophic dilution to biomagnification. On this basis, the balanced method is recommended for future studies of contaminant biomagnification.

9. Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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12. List of Tables

Table 1: Mean concentration range (µg/kg wet weight) of the priority heavy metals mercury (Hg), lead (Pb) and cadmium (Cd) and additional trace metals and metalloids arsenic (As), chromium (Cr), copper (Cu), nickel (Ni), selenium (Se) and zinc (Zn) in the muscle, liver, homogenised whole, brown meat and soft body analysed across the sixteen categories. The number of individuals per pool is presented in Madgett *et al.* (2019). Not all the LoD values (<) are to four significant figures to account for instrumental precision.

Sample Category	Number of pools	Hg µg/kg ww	Pb µg/kg ww	Cd µg/kg ww	As µg/kg ww	Ni µg/kg ww
Pelagic Roundfish Whole	3	37.20 – 42.10	<13.6 – 15.20	9.040 – 10.90	5,660 – 6,010	14.00 – 18.00
Pelagic Roundfish Muscle	2	43.30 – 50.60	<13.6	<4.76	4,330 – 6,040	<8.93
Pelagic Roundfish Liver	2	16.30 – 18.10	<13.6	32.80 – 39.20	3,260 – 4,930	16.20 – 21.60
Demersal Shark Muscle	12	262.0 – 990.0	<13.6 – 86.20	<4.76 – 16.10	15,200 – 25,500	<8.93 – 35.80
Demersal Shark Liver	12	24.70 – 201.0	<13.6 – 46.70	55.10 – 569.0	8,580 – 18,500	<8.93 – 20.70
Demersal Roundfish Whole	6	18.90 – 58.20	65.20 – 225.0	20.00 – 251.0	5,510 – 7,640	122.0 – 799.0
Demersal Roundfish Muscle	30	25.90 – 105.0	<13.6 – 50.70	<4.76 – 14.80	1,540 – 42,900	<8.93 – 86.10
Demersal Roundfish Liver	30	5.050 – 51.00	<13.6 – 329.0	4.830 – 179.0	2,410 – 21,000	9.99 – 458.0
Flatfish Muscle	12	20.10 – 341.0	<13.6 – 19.80	<4.76 – 9.460	5,380 – 23,200	<13.00 – 27.50
Flatfish Liver	12	26.20 – 278.0	15.40 – 627.0	63.00 – 645.0	2,940 – 29,300	19.20 – 101.0
Demersal Invertebrates Muscle	2	25.50 – 26.30	<13.6	17.00 ± 25.10	8,070 – 9,260	<8.93 – 20.70
Benthic Invertebrates Whole	11	22.20 – 127.0	194.0 – 8,870	100.0 – 486.0	2,280 – 17,000	131.0 – 1,760
Benthic Invertebrates Muscle	13	23.00 – 273.0	<13.6 – 183.0	24.80 – 300.0	4,590 – 26,200	<8.93 – 299.0
Benthic Invertebrates Brown Meat	3	62.00 – 80.40	<13.6 – 144.0	713.0 – 3,340	8,990 – 11,700	191.0 – 1,140
Benthic Invertebrates Soft Body	17	35.20 – 129.0	157.0 – 7,580	31.70 – 6,920	1,910 – 35,300	183.0 – 3,660
Zooplankton Whole	3	<3.16	63.31 – 112.4	99.76 – 248.7	530.7 – 630.3	107.7 – 148.8

Table 1 (continued): Mean concentration range (µg/kg wet weight) of the priority heavy metals mercury (Hg), lead (Pb) and cadmium (Cd) and additional trace metals and metalloids arsenic (As), chromium (Cr), copper (Cu), nickel (Ni), selenium (Se) and zinc (Zn) in the muscle, liver, homogenised whole, brown meat and soft body analysed across the sixteen categories. The number of individuals per pool is presented in Madgett *et al* (2019). Not all the LoD values (<) are to four significant figures to account for instrumental precision.

Sample Category	Number of samples	Cr µg/kg ww	Cu µg/kg ww	Se µg/kg ww	Zn µg/kg ww
Pelagic Roundfish Whole	3	<30.6	478.0 – 625.0	379.0 – 388.0	15,100 – 17,600
Pelagic Roundfish Muscle	2	<30.6	416.0 – 500.0	336.0 – 348.0	2,070 - 2,110
Pelagic Roundfish Liver	2	<30.6	863.0 – 991.0	459.0 – 523.0	5,500 – 8,740
Demersal Shark Muscle	12	<30.6 – 109.0	213.0 – 469.0	315.0 – 747.0	6,160 – 13,100
Demersal Shark Liver	12	<30.6 – 306.0	805.0 – 6,660	352.0 – 903.0	3,550 – 11,100
Demersal Roundfish Whole	6	55.80 – 271.0	724.0 – 5,370	504.0 – 867.0	10,200 – 38,000
Demersal Roundfish Muscle	30	<30.6 – 115.0	147.0 – 501.0	331.0 – 2,780	2,940 – 14,100
Demersal Roundfish Liver	30	<30.6 – 462.0	864.0 – 4,790	246.0 – 1,770	10,100 – 36,500
Flatfish Muscle	12	<30.6 – 112.0	122.0 – 1,200	210.0 – 1,940	5,070 – 11,100
Flatfish Liver	12	<30.6 – 95.40	826.0 – 6,730	318.0 – 1,810	22,700 – 52,100
Demersal Invertebrates Muscle	2	<30.6	2,310 – 3,060	303.0 – 392.0	11,800 – 14,100
Benthic Invertebrates Whole	11	145.0 – 1,390	917.0 – 4,860	321.0 – 1,440	38,000 – 105,000
Benthic Invertebrates Muscle	13	<30.6 – 368.0	5,980 – 16,900	491.0 – 1,230	12,200 – 79,200
Benthic Invertebrates Brown Meat	3	<30.6 – 111.0	19,100 – 68,400	1,150 – 2,280	16,600 – 55,800
Benthic Invertebrates Soft Body	17	60.60 – 408.0	4,090 – 65,900	475.0 – 2,090	23,500 – 341,000
Zooplankton Whole	3	<30.6 – 42.53	106.2 – 858.84	240.7 – 357.2	4,429 – 11,946

Table 2: Calculated TMFs of Hg, Cd, Cu, Ni and Zn using the traditional and balanced methods in the study food web composed of demersal shark, fish: pelagic, demersal and flatfish, invertebrates and zooplankton (all trophic levels) and the food web excluding categories identified in section 7.2. There were too few lower trophic level samples available to calculate TMF when significantly different sample categories were excluded from the analysis for Ni and Zn.

Metal	Traditional method		Balanced method	
	All trophic levels	Excluding selected categories [1]	All trophic levels	Excluding selected categories
Hg	1.4 ($p<0.05$)	0.9 ($p<0.05$)	3.3 ($p>0.05$)	0.6 ($p>0.05$)
Cd	0.4 ($p<0.05$)	0.5 ($p<0.05$)	0.7 ($p>0.05$)	0.7 ($p>0.05$)
Cu	0.9 ($p>0.05$)	1.5 ($p<0.05$)	1.8 ($p>0.05$)	2.0 ($p<0.05$)
Ni	0.4 ($p>0.05$)	-	0.5 ($p>0.05$)	-
Zn	0.9 ($p>0.05$)	-	2.1 ($p>0.05$)	-

Table 3: The trophic adjusted Hg concentrations using the TMFs calculated using the traditional method (TMF, 1.4) and balanced method (TMF, 3.3) analysing shark, fish and invertebrates.

Category	Sample number	Mean trophic level (Madgett *et al.*, 2019)	Mean Hg concentration µg/kg ww	Trophic adjusted Hg concentration µg/kg ww using TMF 1.4	Trophic adjusted Hg concentration µg/kg ww using TMF 3.3
Demersal Shark Muscle	12	4.55	486.5	**403.8**	253.0
Pelagic Roundfish Muscle	2	3.75	46.95	51.18	63.38
Demersal Roundfish Muscle	30	4.40	53.02	46.13	32.87
Flatfish Muscle	12	3.64	110.3	124.6	169.9
Demersal Invertebrates Muscle	2	3.87	25.90	26.94	30.30
Benthic Invertebrates Whole	11	3.42	69.85	85.22	139.7
Benthic Invertebrates Muscle	13	3.68	69.08	76.68	101.5
Benthic Invertebrates Brown Meat	3	3.24	72.87	94.00	180.7
Benthic Invertebrates Soft Body	17	3.53	71.35	83.48	124.9

[1] Categories excluded for analysis using the traditional and balanced methods include demersal shark muscle for Hg, benthic invertebrates brown meat, soft body and zooplankton for Cd and benthic invertebrates brown meat and soft body for Cu.

13. List of Figures

Figure 1: Sample Sites: Fish, catshark and marine invertebrate samples were collected by the MRV *Scotia* and MRV *Alba na Mara* between 2015 and 2017 from Tancred Bank, Montrose Bank, Moray Firth, Burra Haaf, Holy Loch, Hunterston, Pladda, Outer Firth of Forth (North East (NE) Dunbar) and Solway Firth (black circles). Zooplankton were collected from the Scottish Observatory site off Stonehaven from the RV *Temora* in 2017 (red circle).

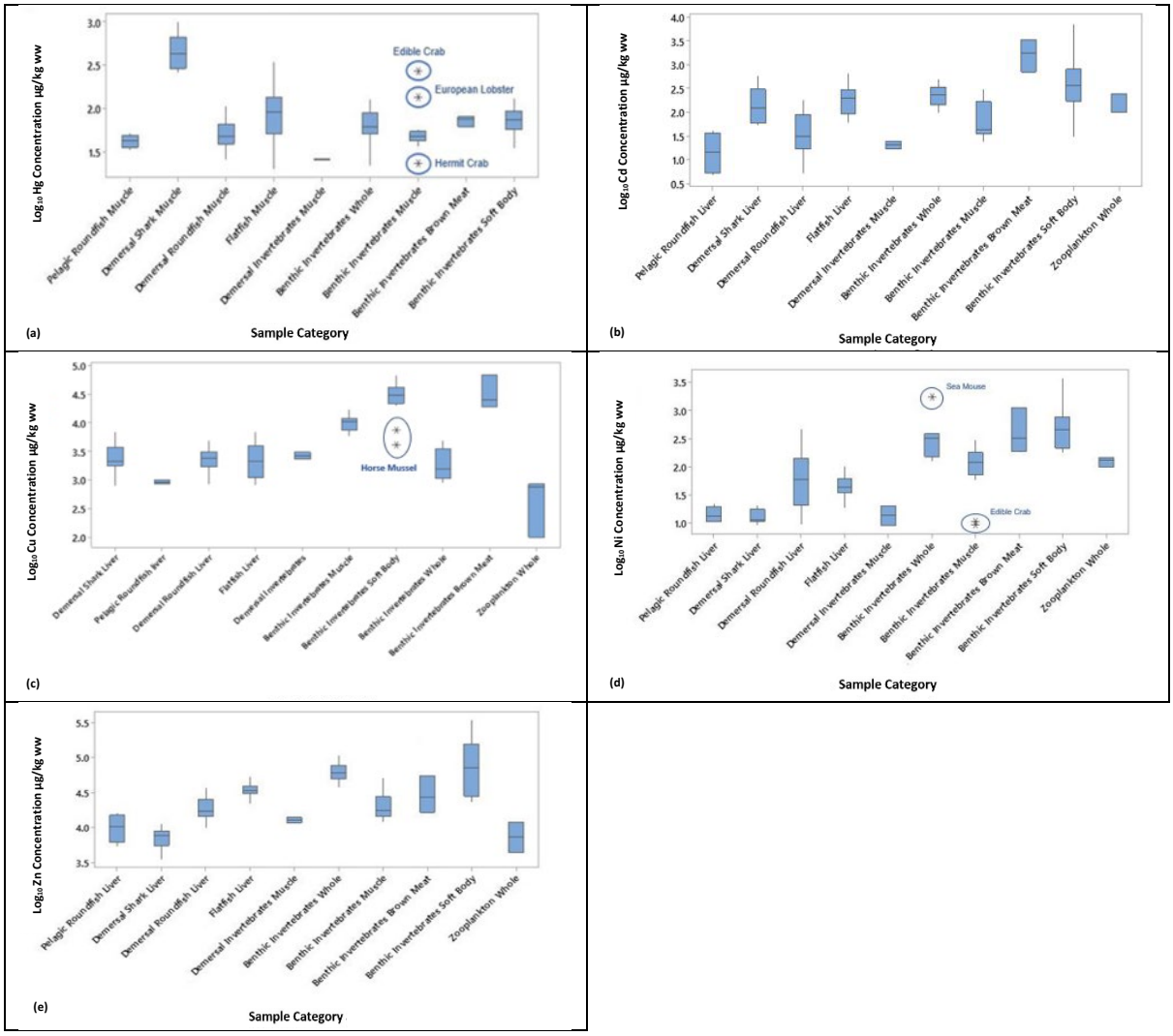

Figure 2: Box plots of (a) Log_{10} Hg concentration (µg/kg ww) in nine sample categories (demersal shark and fish muscle, demersal invertebrates and benthic invertebrates). Zooplankton was not included in the analysis as the Hg concentration was below the LoD. One edible crab, one European lobster and one hermit crab sample pool were identified as data outliers ($p<0.05$); (b) Log_{10} Cd concentration (µg/kg ww) in ten sample categories (demersal shark, fish muscle, demersal invertebrates, benthic invertebrates and zooplankton); (c) Log_{10} Cu concentration (µg/kg ww) in ten sample categories (demersal shark, fish muscle, demersal invertebrates, benthic invertebrates and zooplankton). Two horse mussel sample pools were identified as data outliers ($p<0.05$); (d) Log_{10} Ni concentration (µg/kg ww) in ten sample categories (demersal shark, fish muscle, demersal invertebrates, benthic invertebrates and zooplankton). One sea mouse and two edible crab sample pools were identified as data outliers ($p<0.05$). (e) Log_{10} Zn concentration (µg/kg ww) in ten sample categories (demersal shark and fish muscle, demersal invertebrates, benthic invertebrates and zooplankton). Error bars are to one standard deviation.

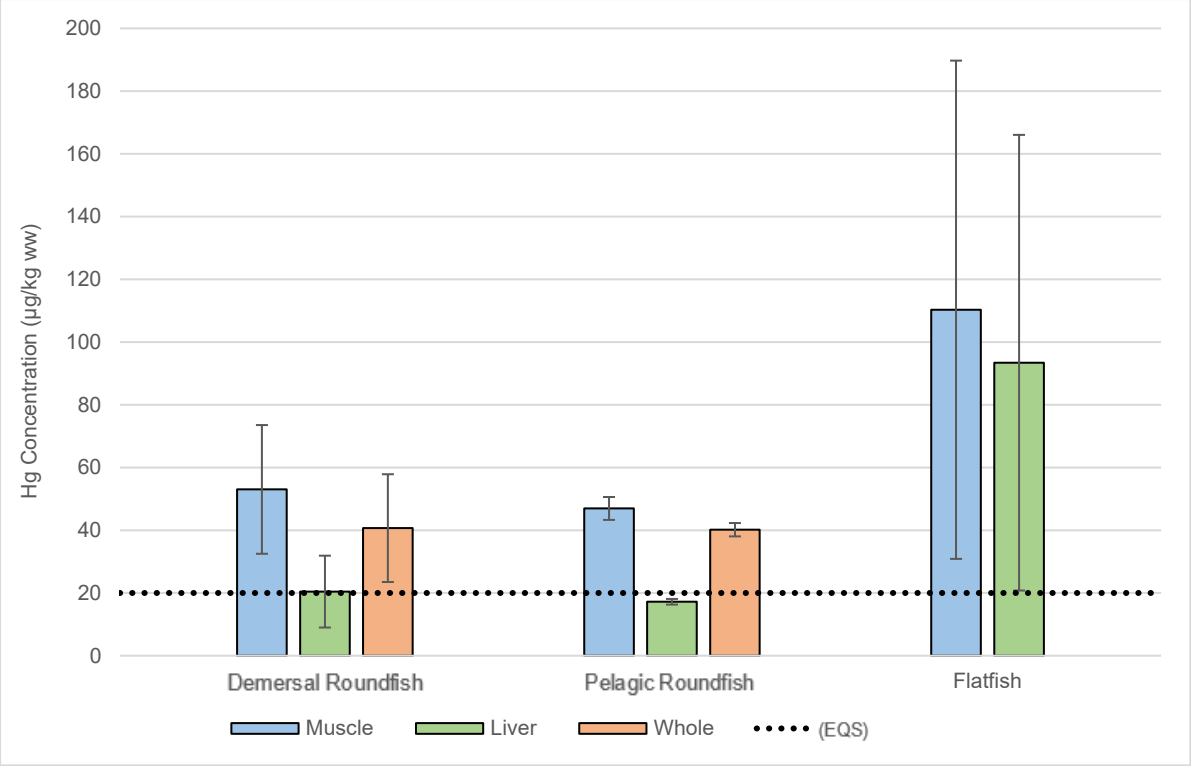

Figure 3: Bar graph showing the Hg concentration (µg/kg ww) in the tissue of each fish category (demersal roundfish, pelagic roundfish and flatfish) in comparison to the $EQS_{(biota)}$ (horizontal dashed line). Error bars are to one standard deviation. Flatfish were analysed using their muscle and liver tissues only as whole tissue was not available for this category.

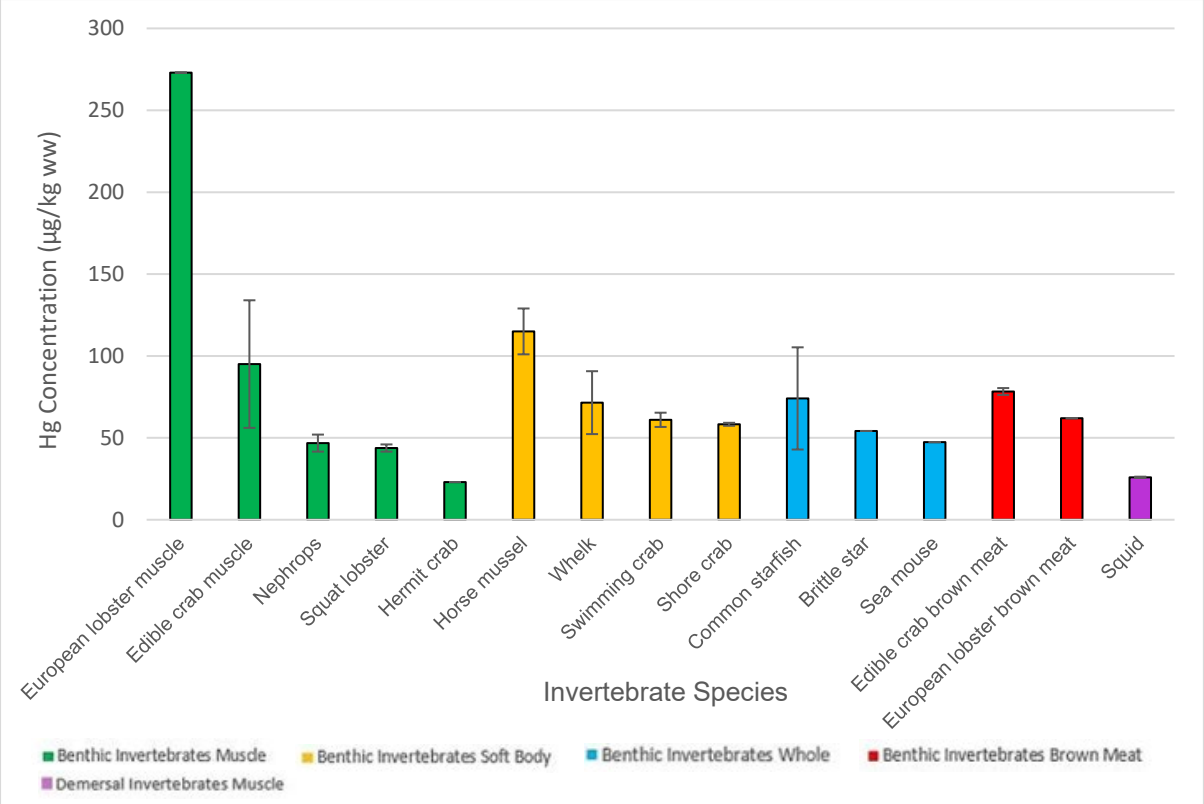

Figure 4: Bar graph showing the Hg concentration (µg/kg ww) in the tissue of each invertebrate species (benthic and demersal). Error bars are to one standard deviation. Lobster muscle, hermit crab, brittle star, sea mouse and lobster brown meat do not have error bars as there was only one sample pool available.

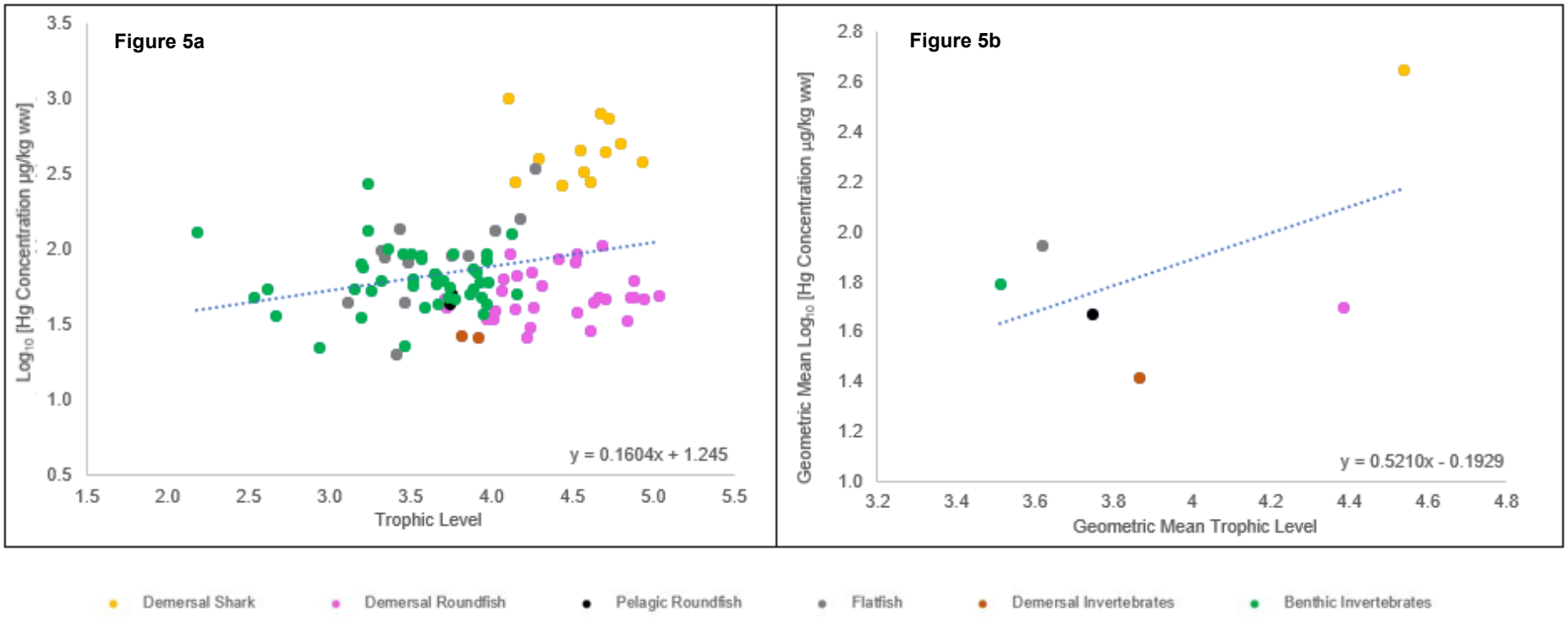

Figure 5: (a) Relationship between trophic level and logarithmically transformed Hg concentration (µg/kg ww) in demersal shark muscle (yellow), fish muscle: demersal (pink), flatfish (grey), pelagic (black) and invertebrates: demersal invertebrate muscle (brown), and benthic invertebrate whole, muscle, brown meat, soft body (green). **(b)** Relationship between geometric mean trophic level and logarithmically transformed geometric mean Hg concentration (µg/kg ww) in demersal shark muscle (yellow), fish muscle: demersal (pink), flatfish (grey), pelagic (black) and invertebrates: demersal invertebrate muscle (brown) and benthic invertebrate whole, muscle, brown meat, soft body (green).

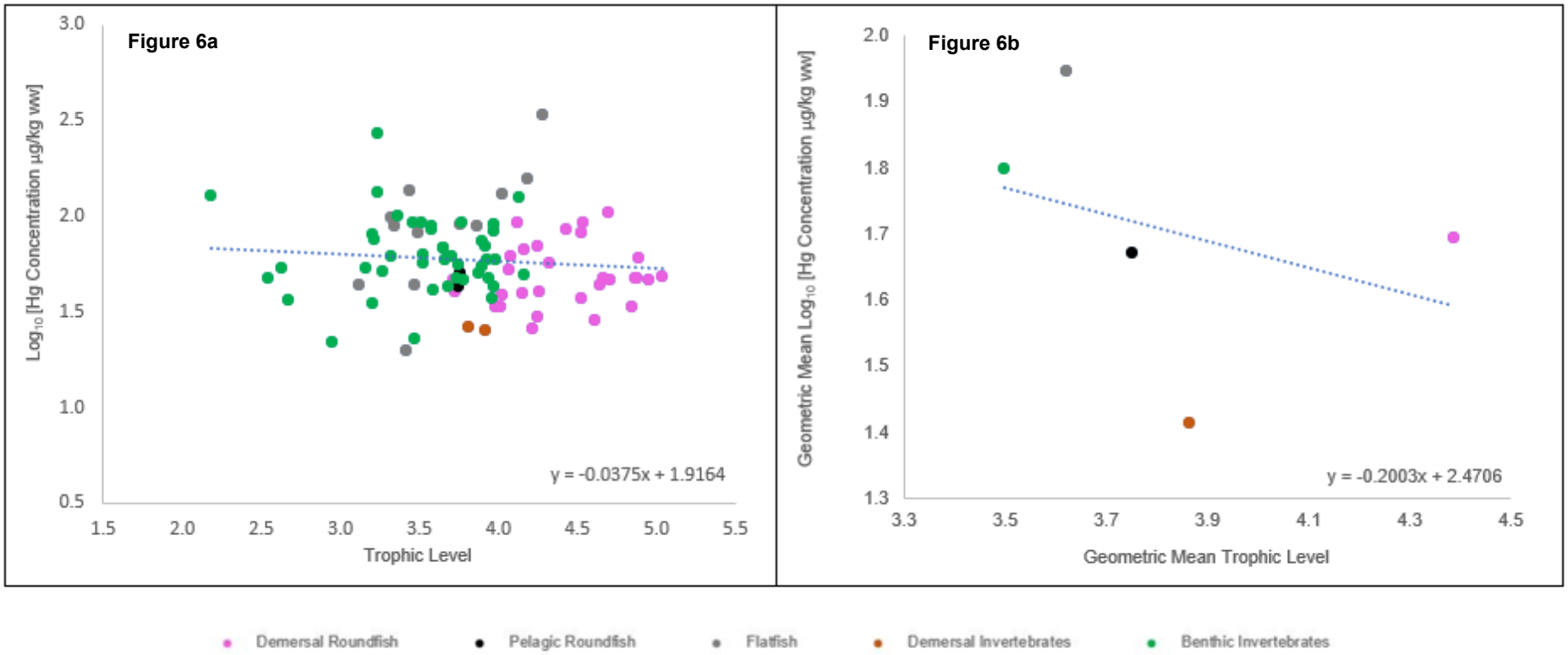

Figure 6 (a) Relationship between trophic level and logarithmically transformed Hg concentration (µg/kg ww) in fish muscle: demersal (pink), flatfish (grey), pelagic (black) and Invertebrates: demersal invertebrate muscle (brown) and benthic invertebrate whole, muscle, brown meat, soft body (green). **(b)** Relationship between geometric mean trophic level and logarithmically transformed geometric mean Hg concentration (µg/kg ww) in fish muscle: demersal (pink), flatfish (grey), pelagic (black) and Invertebrates: demersal invertebrate muscle (brown), and benthic invertebrate whole, muscle, brown meat, soft body (green).

The Concentration and Biomagnification of Trace Metals and Metalloids Across Four Trophic Levels in a Marine Food Web

Alethea S. Madgett[1,2*], Kyari Yates[1], Lynda Webster[2], Craig McKenzie[3], Colin F. Moffat[1]

[1] School of Pharmacy and Life Sciences, Robert Gordon University, Aberdeen AB10 7GJ, UK.
[2] Marine Scotland Science, Marine Laboratory, 375 Victoria Road, Aberdeen AB11 9DB, UK.
[3] Leverhulme Research Centre for Forensic Science, Ewing Building, University of Dundee, DD1 4HN, UK.
*Corresponding Author.

Supplementary Data

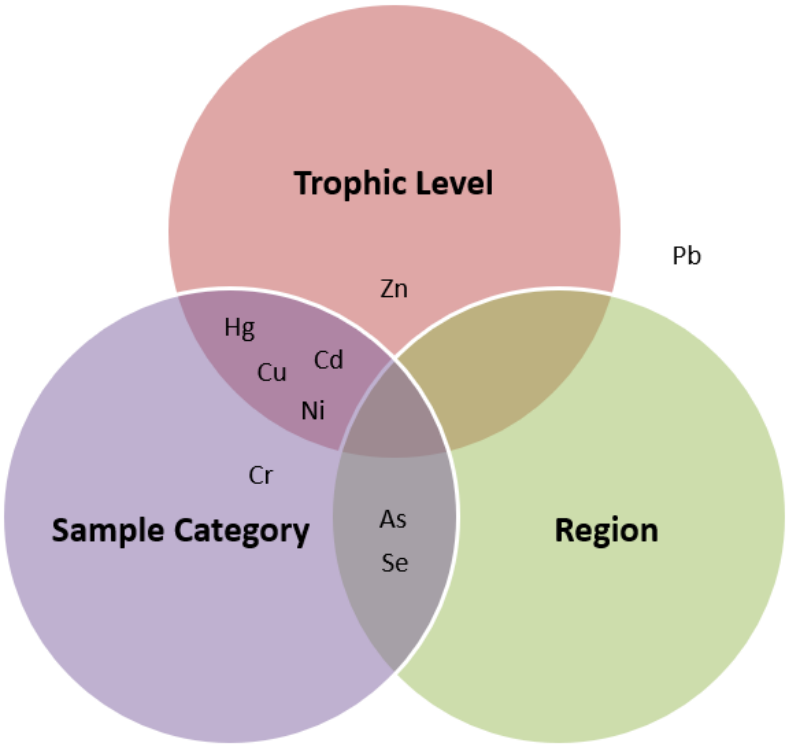

Figure S1: Venn diagram showing the output of mixed effects model analysis conducted on demersal shark, fish (demersal, pelagic, flatfish), invertebrates (benthic and demersal) and zooplankton, where the concentration of eight metals and metalloids (Zn, Hg, Cd, Cu, Ni, Cr, As and Se) (µg/kg ww) have a relationship with the three variables (trophic level, sample category and region). Pb sits outside all categories showing no relationship to the three variables. The greatest overlap was between Trophic Level and Sample Category.

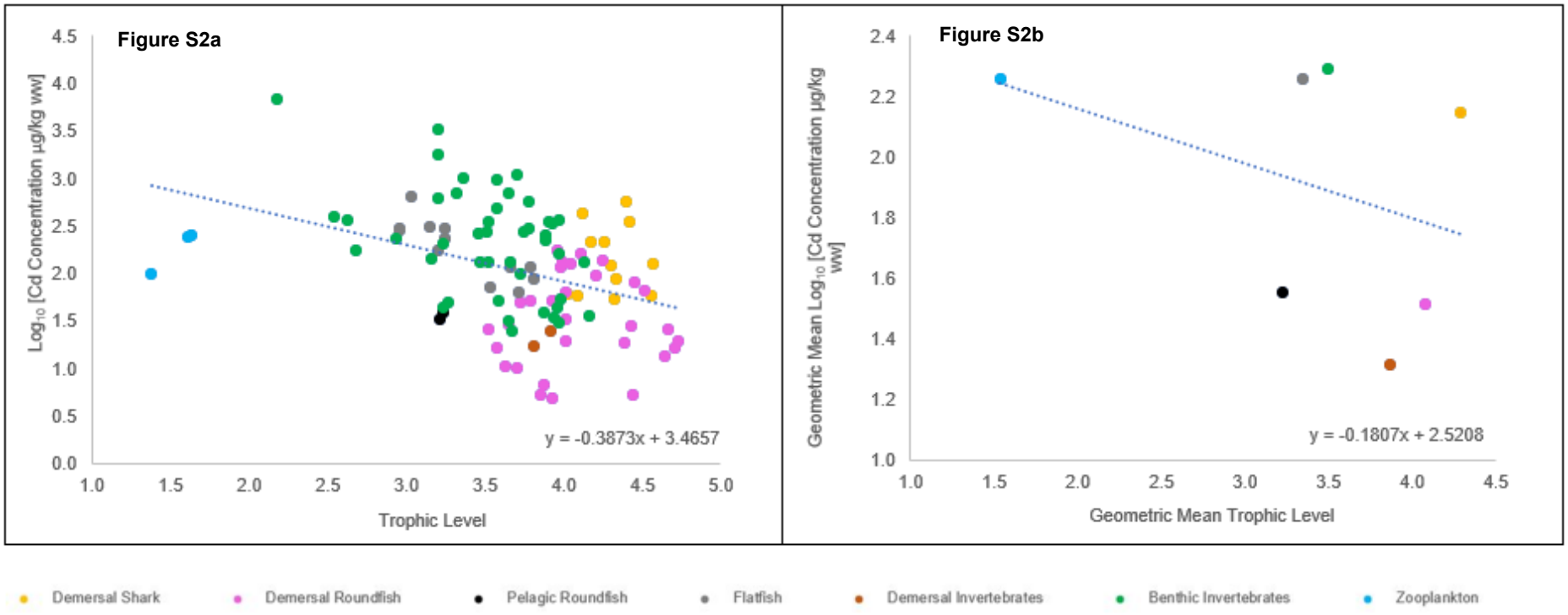

Figure S2: (a) Relationship between trophic level and logarithmically transformed Cd concentration (µg/kg ww) in demersal shark liver (yellow), fish liver: demersal (pink), flatfish (grey), pelagic (black), Invertebrates: demersal invertebrate muscle (brown) and benthic invertebrate whole, muscle, brown meat, soft body (green) and zooplankton (blue). **(b)** Relationship between geometric mean trophic level and logarithmically transformed geometric mean Cd concentration (µg/kg ww) in demersal shark liver (yellow), fish liver: demersal (pink), flatfish (grey), pelagic (black), Invertebrates: demersal invertebrate muscle (brown), and benthic invertebrate whole, muscle, brown meat, soft body (green) and zooplankton (blue).

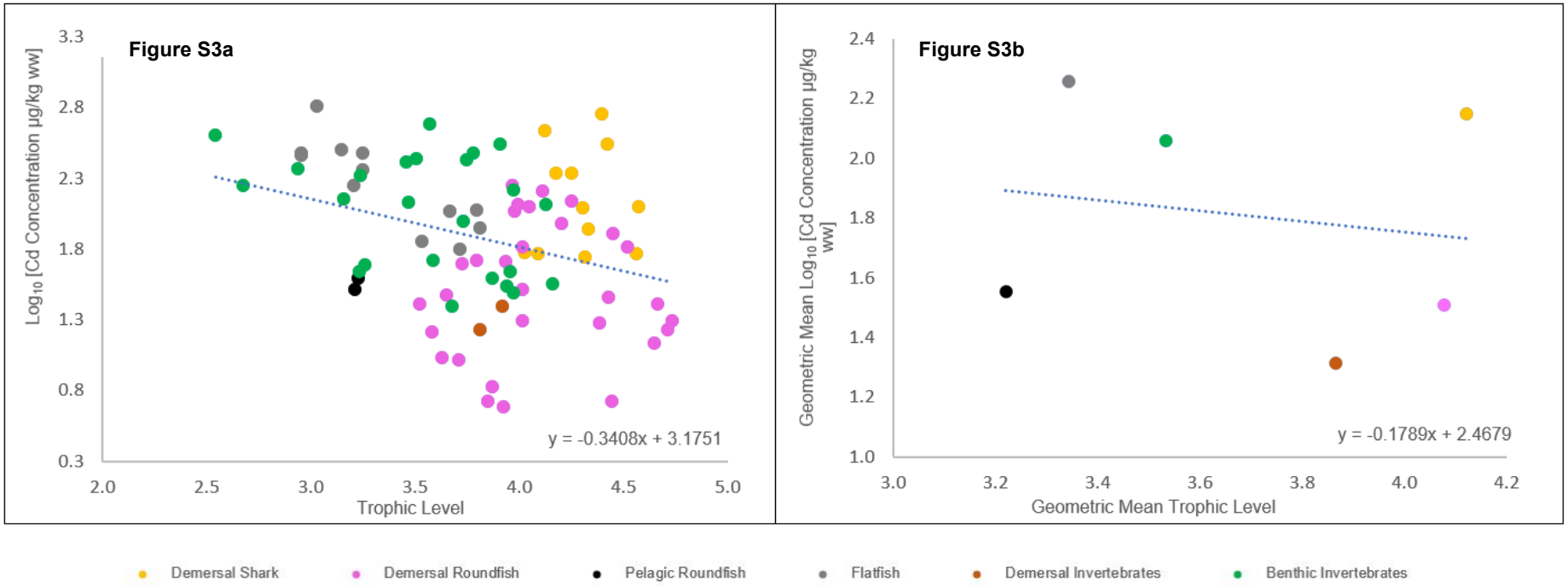

Figure S3: (a) Relationship between trophic level and logarithmically transformed Cd concentration (µg/kg ww) in demersal shark liver: fish: demersal (pink), flatfish (grey), pelagic (black), Invertebrates: demersal invertebrate muscle (brown), and benthic invertebrate whole, muscle (green). **(b)** Relationship between geometric mean trophic level and logarithmically transformed geometric mean Cd concentration (µg/kg ww) in demersal shark liver, fish liver: demersal (pink), flatfish (grey), pelagic (black), Invertebrates: demersal invertebrate muscle (brown), and benthic invertebrate whole, muscle (green).

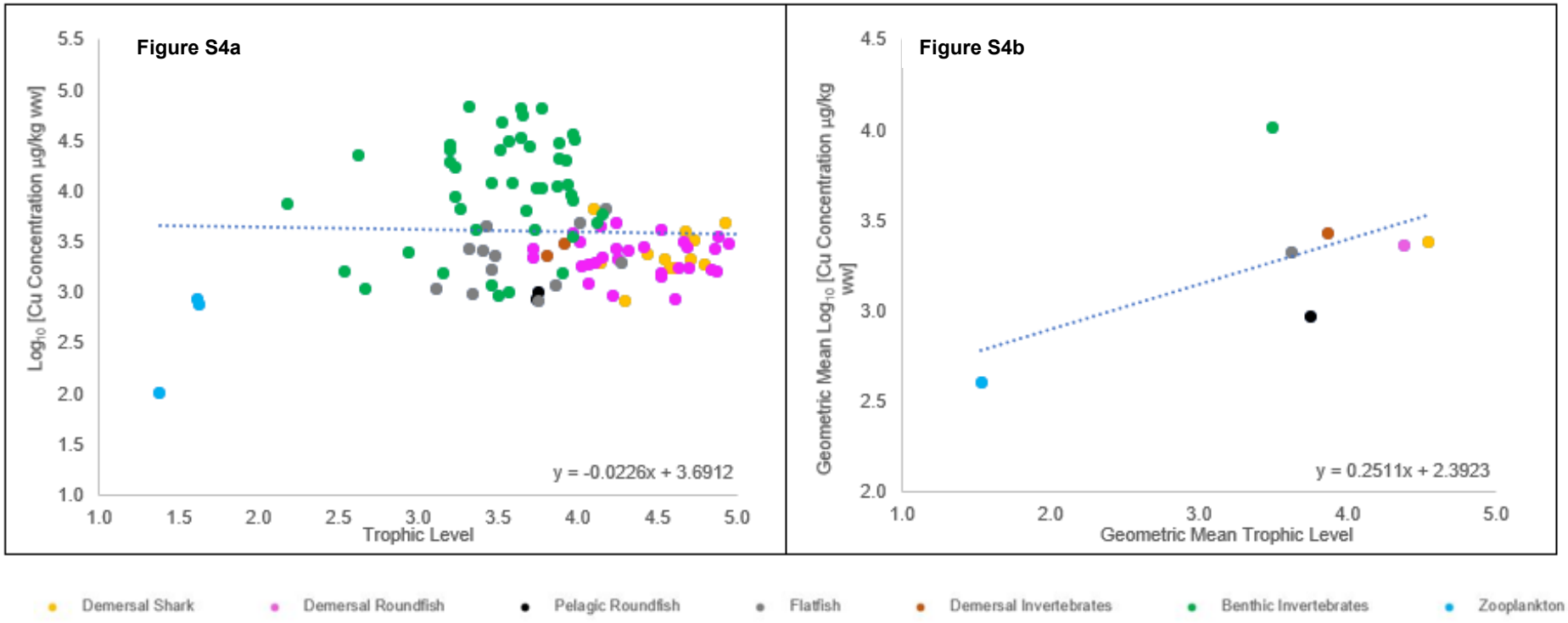

Figure S4: (a) Relationship between trophic level and logarithmically transformed Cu concentration (µg/kg ww) in shark liver (yellow), fish liver: demersal (pink), flatfish (grey), pelagic (black), Invertebrates: demersal invertebrate muscle (brown), and benthic invertebrate whole, muscle, brown meat, soft body (green) and zooplankton (blue). **(b)** Relationship between geometric mean trophic level and logarithmically transformed geometric mean Cu concentration (µg/kg ww) in shark liver (yellow), fish liver: demersal (pink), flatfish (grey), pelagic (black), Invertebrates: demersal invertebrate muscle (brown), and benthic invertebrate whole, muscle, brown meat, soft body (green) and zooplankton (blue).

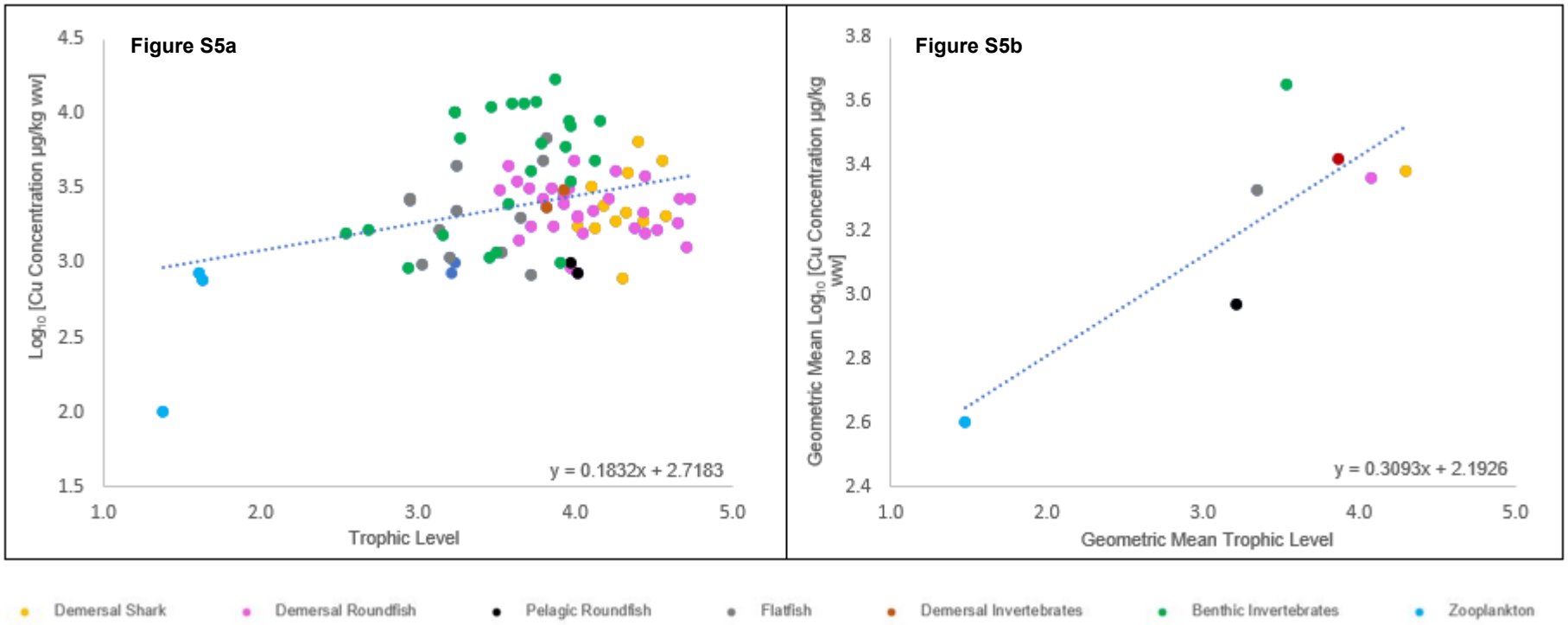

Figure S5: (a) Relationship between trophic level and logarithmically transformed Cu concentration (µg/kg ww) in demersal shark liver, fish liver: demersal (pink), flatfish (grey), pelagic (black) and Invertebrates: demersal invertebrate muscle (brown), and benthic invertebrate whole and muscle (green) and zooplankton (n=3). **(b)** Relationship between geometric mean trophic level and logarithmically transformed geometric mean Cu concentration (µg/kg ww) in fish muscle: demersal (pink), flatfish (grey), pelagic (black) and invertebrates: demersal invertebrate muscle (brown), and benthic invertebrate whole and muscle (green) and zooplankton (n=3)

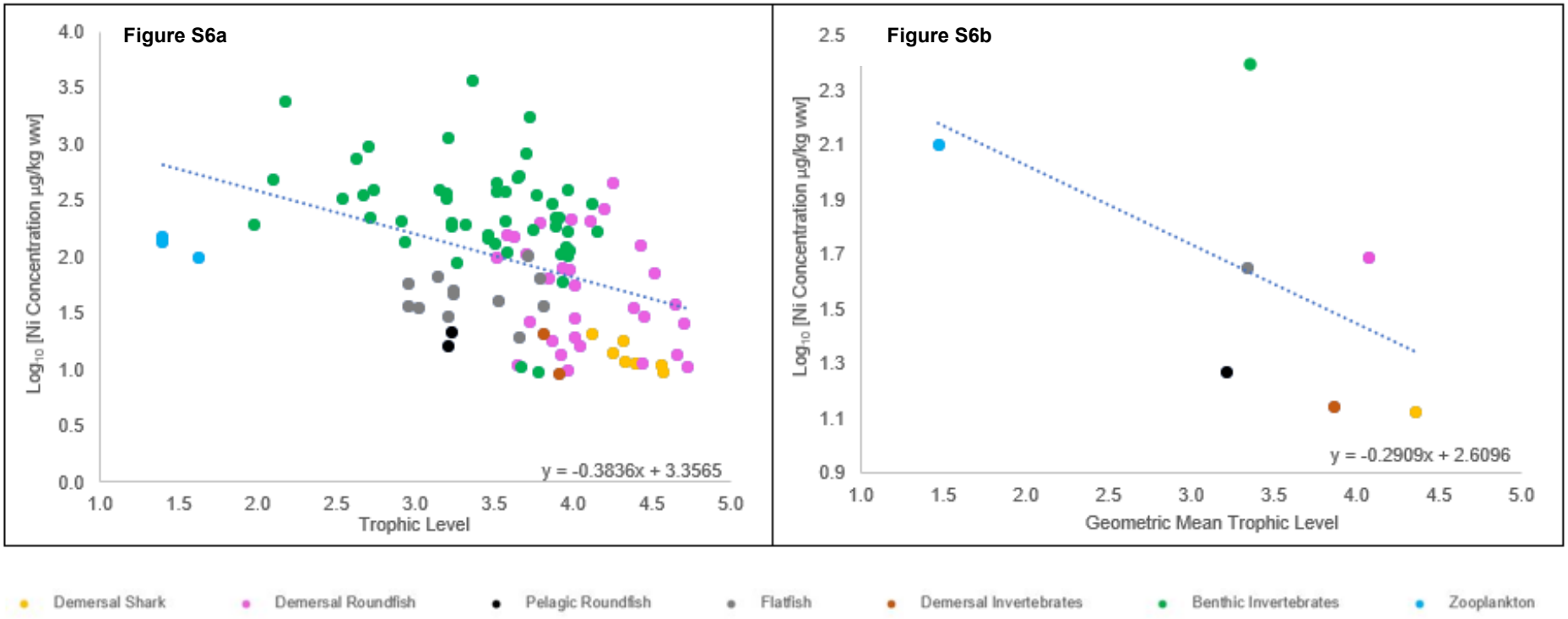

Figure S6: (a) Relationship between trophic level and logarithmically transformed Ni concentration (µg/kg ww) in demersal shark liver (yellow), fish liver: demersal (pink), flatfish (grey), pelagic (black), invertebrates: demersal invertebrate muscle (brown), and benthic invertebrate whole, muscle, brown meat, soft body (green) and zooplankton (blue). **(b)** Relationship between geometric mean trophic level and logarithmically transformed geometric mean Ni concentration (µg/kg ww) in demersal shark liver (yellow), fish liver: demersal (pink), flatfish (grey), pelagic (black), invertebrates: demersal invertebrate muscle (brown), and benthic invertebrate whole, muscle, brown meat, soft body (green) and zooplankton (blue).

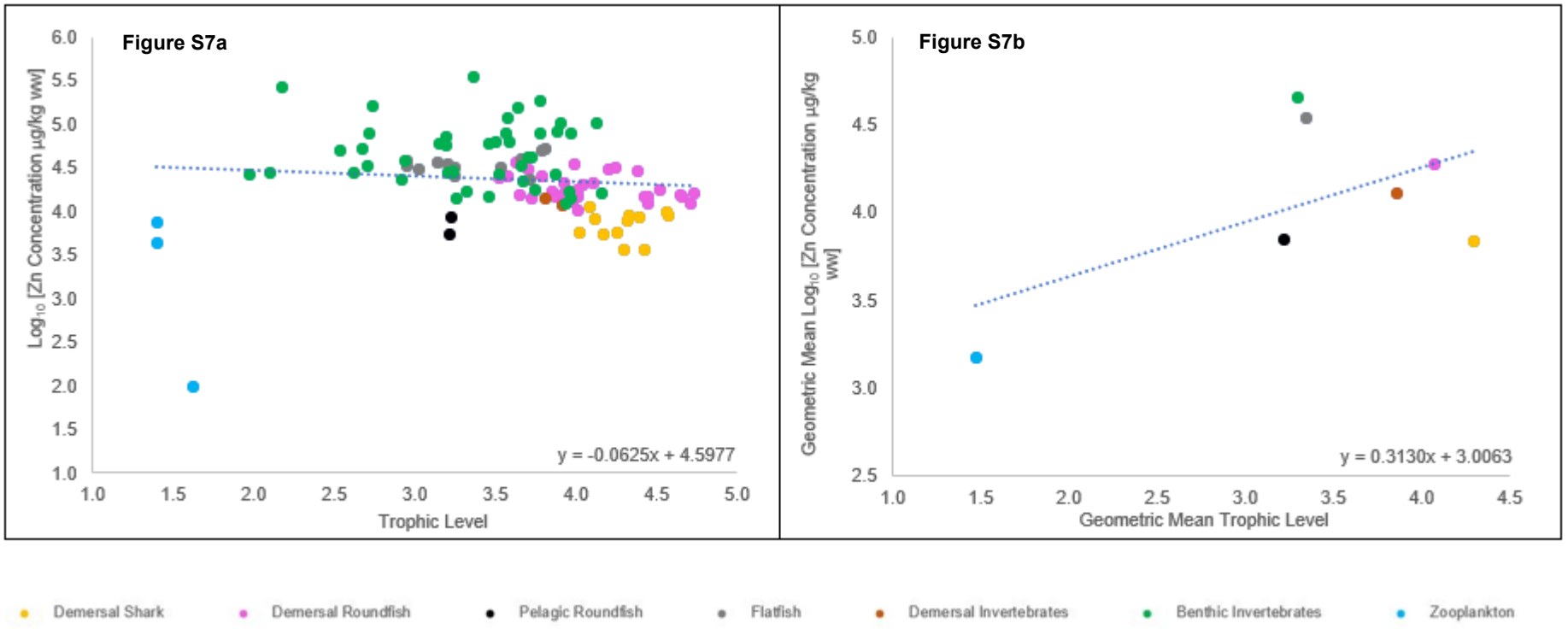

Figure S7: (a) Relationship between trophic level and logarithmically transformed Zn concentration (µg/kg ww) in demersal shark liver (yellow), fish liver: demersal (pink), flatfish (grey), pelagic (black), invertebrates: demersal invertebrate muscle (brown), and benthic invertebrate whole, muscle, brown meat, soft body (green) and zooplankton (blue). **(b)** Relationship between geometric mean trophic level and logarithmically transformed geometric mean Zn concentration (µg/kg ww) in demersal shark liver (yellow), fish liver: demersal (pink), flatfish (grey), pelagic (black), invertebrates: demersal invertebrate muscle (brown), and benthic invertebrate whole, muscle, brown meat, soft body (green) and zooplankton (blue).