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# Multivariate analysis of otolith microchemistry can discriminate the source of oil contamination in exposed fish

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## Highlights:

- Laboratory trial of fish (n=56) exposed via diet to two crude oils and three metals
- Ba and Al from crude oils absorbed into otoliths in a dose-dependent manner
- Multivariate analysis (PCA-LDA) of fish otolith metals discriminates between crude oils
- Ni, Fe and V not absorbed into the otolith *via* the dietary exposure route

## Keywords:

Otolith, vanadium, nickel, crude oil, heavy fuel oil, PCA, LDA

### 1.1. Abstract

The uptake of metals into the aragonite lattice of the fish otolith (ear-bone) has been used for decades as a historical record of exposure to metals in polluted environments. The relative abundance of two metals in particular, Ni and V, are used in forensic chemical analysis of crude oils to assist in confirming its origin. In this study we investigate the potential for metal accumulation in otoliths to act as a biomarker of exposure to crude oil.

Using a 33-day static-renewal laboratory trial design, 56 juvenile *Lates calcarifer* (commonly known as Asian seabass or barramundi) were fed diets enriched with V (20mg/kg), Ni (500mg/kg), Fe (500mg/kg), and two crude oils with distinctly different metals profiles: a heavy fuel oil (1% w/w) and a typical Australian medium crude (1% w/w).

Fish exposed to crude oils showed Ba and Al retained in otoliths in a dose-dependent manner, but fish fed V-, Ni- and Fe-enriched diets showed no metal increase in otoliths, indicating that V, Ni and Fe are not incorporated into the otolith of *L. calcarifer* via dietary exposure. For crude oils, incorporation into otolith for many metals is likely limited due to porphyrin casing reducing their bioavailability. Principal components analysis (PCA) and subsequent linear discriminatory analysis (LDA) of selected otolith metals demonstrated that, even despite large variability in the metal abundances detected in otolith between individuals within the test groups (cv = 1.00), it is possible to discriminate between fish exposed to different crude oils using multivariate analysis of their otolith microchemistry.

## 1.2. Introduction

Crude oils are ubiquitous marine pollutants. Given the dependence of the shipping industry on heavy fuel oil, the periodic unintentional release of petroleum hydrocarbons into the environment in the future is likely to match the historical record of oil spills of the past few decades. Incidents such as the *Prestige* oil spill that released 60,000 tonnes of heavy fuel oil near the Spanish coastline in 2002, the Montara well failure in Australia in 2009 that released 47,000 tonnes of crude oil into the Timor Sea, the Deepwater Horizon (DWH) spill of 650,000 tonnes of crude oil in the Gulf of Mexico in 2010 and the recent Mauritius MV *Wakashio* fuel oil spill have repeatedly demonstrated the large scale environmental impacts inevitably caused by these events.

International maritime law holds to the principal that the polluter must pay. Particularly in the case of smaller scale incidents, identifying the source of the spill is the starting point of most litigation

proceedings. Fingerprinting crude oils is complicated by the degradation of oil during weathering (loss of volatile and polar compounds; Gagnon *et al*, 1999; Scarlett *et al*, 2021) of oil released into the environment. Crude oils contain characteristic amounts of metals such as V (as a vanadyl complex) and Ni (Yasnygina *et al*, 2006; Pereira *et al*, 2010) as well as other metals such as Cu, Zn and Mn (Woltering *et al*, 2016) whose relative abundance may be used in forensic chemistry to assist in identifying different oils (Barwise, 1990; Pereira *et al*, 2010). In crude oils, these metals are predominantly incorporated in porphyrins (Dunning *et al*, 1960; Grice *et al*, 1996; Biesaga *et al*, 2000; Ali and Abbas, 2006; Woltering *et al*, 2016) found in the asphaltene fraction. Following the natural weathering process of crude oils exposed to environmental factors, porphyrin-bound metals typically end up in the tar balls that remain on the sea-floor, or wash up on beaches following an oil spill (National Research Council, 2003; Suneel *et al*, 2015; Scarlett *et al*, 2019) and become deposited in sediment (Boehm *et al*, 1987; Boehm *et al*, 2008).

Fish exposed to metals may incorporate these metals into the otolith (ear bone), where bi- and tri-valent metals can replace Ca ions in the aragonite lattice (reviewed by Campana, 1999). The mechanism for this is complicated (Thomas *et al*, 2017) and only partially understood. Prior to otolith incorporation, metals must first be absorbed into the bloodstream either via the gills in the case of waterborne metals, or via the intestine in the case of metals present in the diet. From there they must cross the otolith haemolymph barrier prior to ossification (Campana, 1999). The mechanisms by which this occurs appear to be specific to individual metals, which follow different routes to otolith incorporation (Milton and Chenery, 2001). For example, Zn can be incorporated into the otolith only via the dietary route (Ranaldi and Gagnon 2008a), whereas others such as Pb, Sr and Cu can only be incorporated via the aqueous route (Milton *et al*, 2000). Still others, such as Cd, are incorporated into the otolith via either pathway (Ranaldi and Gagnon, 2009).

Metal analysis of otoliths *in situ* by laser ablative inductively coupled plasma mass spectrometry (LA-ICP-MS) (Woodhead *et al*, 2007) has been used to establish a historical record of fish migratory

patterns as they move through areas of varying metal contamination (Rolls, 2014; Milton *et al*, 2000; Long *et al*, 2014), and as a biomarker for exposure to crude oils (Morales-Nin *et al*, 2007; Nelson *et al*, 2015; López-Duarte *et al*, 2016) and other anthropogenic sources of metals in the environment (Arslan and Secor, 2005; Friedrich and Halden, 2010; Ranaldi and Gagnon, 2008b, 2010). Field studies show that metals found in the otoliths of exposed fish reflect environmental concentrations for some metals such as Cu but other metals such as Zn, Pb and Mn do not appear to be correlated to environmental concentrations (Milton *et al*, 2000; Andronis *et al*, 2017).

In environments polluted with petroleum hydrocarbons, crude oil compounds can accumulate in tissues of exposed aquatic organisms (Khan *et al*, 1995; Rabalais and Turner, 2016; D'Costa *et al*, 2017; Ahmed *et al*, 2019). In heavily industrialised areas, total petroleum hydrocarbon (TPH) levels have been reported in fish tissue at concentrations ranging from 10 to 1,500 mg/kg (Ansari *et al*, 2012; Ahmed *et al*, 2019; Enuneku *et al*, 2015; Jisr *et al*, 2020). Following a spill, compounds from crude oils enter food webs (Buskey *et al*, 2016), become biomagnified in successive trophic levels, and may reach high levels in carnivorous fish species. This is well illustrated by field studies after DWH where TPH in tissues of exposed commercial fish species were as high as 21,575 mg/kg (2.2% w/w) with a mean concentration of 3,968 mg/kg (0.4% w/w) (Sammarco *et al*, 2013). In the field, the authors have observed fish feeding on oil particles mistaking them for food, and in a laboratory setting copepods have been reported directly ingesting emulsified oil particles (Gyllenberg, 1981).

In order to investigate the suitability of otolith microchemistry as a prospective biomarker tool for discriminating exposure to various crude oils, we conducted a 33-day dietary exposure study in juvenile *Lates calcarifer*. This pelagic carnivorous teleost fish is a common aquaculture species and popular sports-fish found in tropical and sub-tropical environments ranging from the Persian Gulf to northern Australia (Boonyaratpalin 2017; Grey 1987; Mathew 2009). Its globally widespread marine and riverine dispersal, and hardy tolerance of a range of temperature, pH and saline conditions (Jerry 2013), make it a suitable test species to investigate the potential effects of oil spills which may

occur in a wide variety of environmental conditions. We hypothesised that metals in crude oils, including those classically used in crude oil fingerprinting such as V and Ni would be incorporated in otoliths of exposed fish in characteristic concentrations to facilitate identification of the respective crude oil they were exposed to.

### 1.3. Methods

All fish were handled in accordance with Curtin University animal ethics approval number ARE2019/11.

#### 1.3.1. In-vivo exposure of *L. calcarifer*

A total of 56 juvenile *L. calcarifer* (10-15cm in length) were purchased from a commercial hatchery. Fish were kept in tanks containing 100L of natural Indian Ocean seawater with four fish per tank. The trial was a static renewal design using external canister biofilters with a flow rate of approximately 5L/min. Experimental conditions were maintained at  $28 \pm 2$  °C, dissolved oxygen  $> 5.0$  mg/L, pH  $7.6 \pm 0.6$ , salinity of  $32 \pm 2$  ppt and a 12-hour light/dark cycle. Water exchanges of 10-60% total tank volume were performed as indicated by daily water quality testing.

Fish were fed either commercial fishmeal (Nova FF 3mm, Skretting Pty Ltd, Perth, Australia) as the control (n = 12 fish), fishmeal enriched with 20 mg/kg V (as  $V_2O_5$ ) (n = 4 fish), fishmeal enriched with 500 mg/kg Ni (as  $NiSO_4$ ) (n = 8 fish), fishmeal enriched with 500 mg/kg Fe (as  $FeSO_4$ ) (n = 8), fishmeal spiked with 1% w/w HFO (A.P.I. 11.1) (n = 12 fish), or fishmeal spiked with 1% w/w MCO (A.P.I. 31.0) (n = 12 fish).

Fish were fed twice per day to a total of 2% bodyweight per day for 33 days, followed by a 2-day depuration period. Fish were euthanized by ike-jime, weighed, and their otoliths were surgically removed, weighed, dried and stored at room temperature.

Otoliths were mounted in resin, with several otoliths per mount, and the mount face abraded with 2000-grit wet and dry sandpaper. Due to the concave otolith shape, grinding was halted once

sufficient material was exposed for LA-ICP-MS analysis in order to preserve the integrity of the distal edge containing the most recent growth (Dehghani *et al*, 2015; Kerambrun *et al*, 2012) (Figure 1).

### 1.3.2. LA-ICP-MS Analysis

Analysis was undertaken using a RESOLution M-50A-LR incorporating a Compex 102 excimer laser, coupled to an Agilent 8900x QQQ ICP-MS at the GeoHistory Facility, John de Laeter Centre, Curtin University. Following a 30s period of background analysis and two cleaning pulses (to remove surface contamination), samples were spot ablated for 40 s at a 10Hz repetition rate, using a 50  $\mu\text{m}$  beam and laser energy of 3.0 J  $\text{cm}^{-2}$ . Oxide polyatomic interferences were minimized by tuning flow rates for a ThO/Th of < 0.5%. The sample cell was flushed with ultrahigh purity He (320 mL  $\text{min}^{-1}$ ) and N<sub>2</sub> (1.2 mL  $\text{min}^{-1}$ ) and high purity Ar was employed as the plasma carrier gas. International glass standard NIST 612 was used as the primary reference material, to calculate elemental concentrations (using stoichiometric aragonite <sup>43</sup>Ca as the internal standard element and assuming 40.04% Ca in otoliths) and to correct for instrument drift on all elements. Secondary standards (NIST 610 glass and MACS-3B pressed calcium carbonate powder) yielded results within 5% of the recommended values, except Mg (22%), Ti (12%), and Bi (12%) for secondary standard NIST610, and <10% and B, Zn, As, Nb, Mo, Ag, Cd, Sb, Tl, Pb, Bi which yielded errors of 10-50% for secondary standard MACS-3. The higher errors on the latter standard are attributed to the more heterogeneous nature of a pressed powder pellet when compared to a silicate glass such as those in the NIST 61x series standards (Wilson *et al*, 2008; Jochum *et al*, 2016). Standard blocks were run every 15 unknowns.

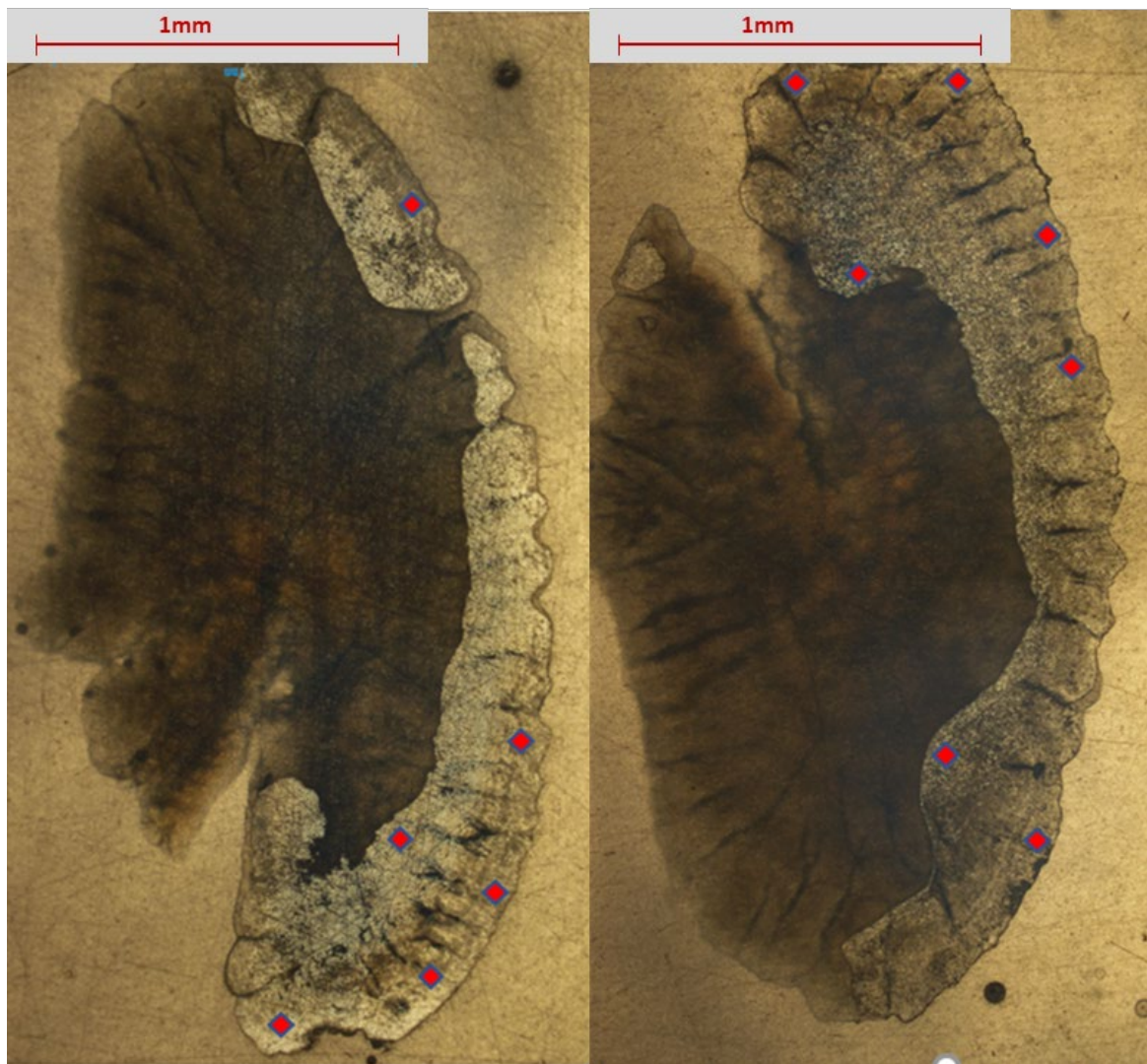


Figure 1: Light microscope (x40 objective) images of resin-mounted otoliths. Light areas are the distal otolith edge (most recent growth) exposed by grinding, the dark areas are those still embedded in resin. Red markings are targeting points for LA-ICP-MS.

The mass spectra were reduced using the Trace Elements data reduction scheme in Iolite (Paton *et al*, 2011 and references therein). Data were collected on the following 34 isotopes:  $^{11}\text{B}$ ,  $^{25}\text{Mg}$ ,  $^{27}\text{Al}$ ,  $^{29}\text{Si}$ ,  $^{34}\text{S}$ ,  $^{47}\text{Ti}$ ,  $^{51}\text{V}$ ,  $^{52}\text{Cr}$ ,  $^{55}\text{Mn}$ ,  $^{57}\text{Fe}$ ,  $^{59}\text{Co}$ ,  $^{60}\text{Ni}$ ,  $^{61}\text{Ni}$ ,  $^{63}\text{Cu}$ ,  $^{66}\text{Zn}$ ,  $^{75}\text{As}$ ,  $^{77}\text{Se}$ ,  $^{88}\text{Sr}$ ,  $^{89}\text{Y}$ ,  $^{90}\text{Zr}$ ,  $^{93}\text{Nb}$ ,  $^{95}\text{Mo}$ ,  $^{107}\text{Ag}$ ,  $^{111}\text{Cd}$ ,  $^{118}\text{Sn}$ ,  $^{121}\text{Sb}$ ,  $^{133}\text{Cs}$ ,  $^{137}\text{Ba}$ ,  $^{197}\text{Au}$ ,  $^{205}\text{Tl}$ ,  $^{208}\text{Pb}$ ,  $^{209}\text{Bi}$ ,  $^{232}\text{Th}$  and  $^{238}\text{U}$ . Results are provided in Table 1. Uncertainties are given as standard error (SE), and limit of detection (LOD) calculated using the Howell method (Howell *et al*, 2013). Between five to eight points were sampled per otolith,

predominantly on the distal edge (Figure 1), and an average calculated for each otolith, for each metal detected.

### 1.3.3. Metal Analysis of Crude Oils

A sample of each oil was accurately weighed and then repeatedly digested in nitric acid, followed by a final digestion in nitric and perchloric acid. Taken to incipient dryness, the sample was redissolved in high purity nitric acid (0.7mL), hydrochloric acid (0.2mL) and double distilled water (25mL), before quantitation for a suite of 61 metals by ICP-AES and ICP-MS using AccuTrace multi element standards (Choice Analytical, Australia).

### 1.3.4. Data Handling

Data was analysed using R statistical software (v 1.4).

Significant differences ( $p < 0.05$ ) between test group means was determined by one-way ANOVA, followed by pair-wise application of Tukey's honestly significant difference (Tukey's HSD) ( $p < 0.05$ ).

Principal components analysis (PCA) was conducted using the FactoMiner R package (Lê *et al*, 2008).

The PCA analysis was constrained to the metals detected on average in otolith at concentrations greater than twice their respective limits of reporting. Subsequent linear discriminatory analysis (LDA) was conducted using the MASS R package (Venables and Ripley 2002).

## 1.4. Results and Discussion

### 1.4.1. Metals in Crude Oils

The two oils used as dietary supplements in this study have very different metal profiles. The MCO is generally poor in metals compared to the HFO (Table 1). The HFO is highly sulfurous and contains relatively high amounts of Fe ( $37.9 \pm 1.47$  mg/kg), Ni ( $12.23 \pm 0.71$  mg/kg) and V ( $15.3 \pm 0.9$  mg/kg) compared to MCO ( $4.73 \pm 1.85$  mg/kg,  $0.07 \pm 0.06$ mg/kg and  $<0.03$  mg/kg for Fe, Ni and V



respectively). The two oils contain similar small quantities of Zn, Cr, Pb and Sn. Of particular interest, HFO contains higher amounts of Al ( $15.44 \pm 8.98$  mg/kg) and Ba ( $1.32 \pm 0.08$  mg/kg) compared to MCO ( $10.23$  mg/kg and  $0.11$ mg/kg respectively).

#### 1.4.2. Metals in Otolith

Few metal species were detected above the limit of detection (LOD) in any of the 56 otoliths analysed by LA-ICP-MS. Only 11 of the 34 metals were detected on average more than twice their LOD: Al, Ba, Cr, Co, Cu, Pb, Fe, Mo, Mg, Ni, and Zn (Table 1).

Table 1: Selected metals analysis of crude oils, and of otoliths of *L. calcarifer* exposed to dietary crude oil or metal-enriched diets.

Metal	Metals in Crude Oils (mg/kg)*		Metals in otolith (mg/kg) <sup>§</sup>		
	MCO	HFO	Control	MCO	HFO
Al Aluminium	$10.23 \pm 10.23$	$15.44 \pm 8.98$	$0.004 \pm 0.003$	$0.057 \pm 0.040$	$0.170 \pm 0.085$
Ag Silver	0.000	0.000	0.000	0.000	0.000
As Arsenic	0.000	$0.041 \pm 0.008$	$0.120 \pm 0.012$	$0.231 \pm 0.007$	$0.090 \pm 0.008$
Ba Barium	$0.113 \pm 0.072$	$1.311 \pm 0.078$	$10.26 \pm 0.29$	$11.63 \pm 0.38$	$13.93 \pm 0.86$
Cd Cadmium	$0.004 \pm 0.003$	0.000	0.000	0.000	0.000
Co Cobalt	0.000	$1.430 \pm 1.116$	$0.001 \pm 0.000$	$0.002 \pm 0.001$	$0.001 \pm 0.000$
Cr Chromium	$0.298 \pm 0.290$	$0.243 \pm 0.131$	$1.006 \pm 0.010$	$0.949 \pm 0.008$	$0.928 \pm 0.015$
Cu Copper	$0.150 \pm 0.150$	0.000	$0.371 \pm 0.146$	$0.196 \pm 0.016$	$0.189 \pm 0.023$
Fe Iron	$4.730 \pm 1.854$	$37.90 \pm 1.47$	$14.29 \pm 0.24$	$11.82 \pm 0.20$	$10.28 \pm 0.11$
Mg Magnesium	$1.197 \pm 0.944$	$1.800 \pm 0.468$	$24.63 \pm 1.21$	$23.69 \pm 1.18$	$30.85 \pm 2.71$
Mo Molybdenum	0.000	$0.052 \pm 0.003$	0.000	0.000	0.000
Ni Nickel	$0.070 \pm 0.039$	$12.23 \pm 0.71$	$1.284 \pm 0.030$	$1.111 \pm 0.045$	$0.939 \pm 0.031$
Pb Lead	$0.083 \pm 0.03$	$0.042 \pm 0.17$	$0.032 \pm 0.031$	$0.001 \pm 0.000$	$0.001 \pm 0.001$
S Sulfur	$393.6 \pm 36.3$	$10250 \pm 850$	$277.8 \pm 11.8$	$235.9 \pm 7.0$	$198.3 \pm 6.1$
Sb Antimony	0.000	$0.459 \pm 0.19$	$0.001 \pm 0.000$	0.000	0.000
Se Selenium	$0.061 \pm 0.036$	$0.007 \pm 0.007$	$0.016 \pm 0.007$	$0.008 \pm 0.004$	$0.022 \pm 0.013$
Sn Tin	$0.117 \pm 0.103$	$0.128 \pm 0.057$	$0.004 \pm 0.003$	$0.001 \pm 0.000$	$0.003 \pm 0.002$
Sr Strontium	$0.226 \pm 0.191$	$0.432 \pm 0.152$	$1533 \pm 54$	$1585 \pm 44$	$1548 \pm 46$
Ti Titanium	0.000	$3.240 \pm 0.127$	0.000	$0.004 \pm 0.004$	$0.002 \pm 0.002$
V Vanadium	0.000	$15.27 \pm 0.87$	0.000	0.000	0.000
Zn Zinc	$1.473 \pm 0.117$	$1.194 \pm 0.126$	$0.379 \pm 0.053$	$0.320 \pm 0.021$	$0.376 \pm 0.023$

For the calculation of means, analyses below the limit of reporting were assumed to be zero.

\*Means of triplicate ICP-MS analysis of crude oil

<sup>§</sup> Means of *in-situ* LA-ICP-MS analysis of otoliths from all fish in each respective test group.

Abbreviations: MCO = Montara crude oil, HFO = heavy fuel oil

Fish fed any of the three diets enriched with metals did not show increased otolith concentrations of V, Ni or Fe compared to controls (Table 1). Given the high concentration of these metals in the enriched feeds, this implies that these metals are not incorporated via the dietary route of exposure into *L. calcarifer* otoliths.

Between all test groups, there was no significant difference in otolith Zn concentrations (ANOVA,  $p = 0.47$ ), a metal known to be incorporated into fish otolith via the dietary route (Ranaldi and Gagnon 2008a), even though it is present in both MCO and HFO ( $1.47 \pm 0.12 \text{ mg/kg}$  and  $1.19 \pm 0.13 \text{ mg/kg}$  respectively). This may be due to a lack of bioavailability of some porphyrin-bound metals in crude oils, which have a very low water solubility due to their planar hydrophobic structure (Mitchell, 2016). Hence, porphyrin-secluded metals do not dissolve in the water-accommodated fraction (WAF) of spilled oils, and consequently are not available for absorption via the gills. Minimal absorption via the gastrointestinal tract would subsequently result in the elimination of porphyrin-embedded metals via faeces. Evidences are available from studies conducted by Lopez-Duarte *et al* (2016) who reported that fish exposed to the Gulf of Mexico 2010 oil spill had levels of Ni and V in their otoliths comparable to those of reference fish. Metals from crude oils are also not retained in the muscle tissue of exposed fish. Grosser *et al* (2012) used ICP-MS analysis of the muscle tissue of post-spill Gulf of Mexico tuna to show no significant difference between metals concentration in muscle tissue of unexposed fish to compared to fish exposed to crude oil following the DWH incident.

Seemingly, in fish exposed to crude oils Al was incorporated into otolith in a dose dependent manner ( $r^2 = 0.85$ , using test group averages). Aluminium was detected in otolith at a mean concentration of  $0.17 \pm 0.08 \text{ mg/kg}$  in HFO-exposed fish, which was higher than in control fish at  $0.003 \pm 0.003 \text{ mg/kg}$ , approaching significance (ANOVA,  $p = 0.06$ ). Elevated mean concentrations of Al in otolith was also detected in MCO exposed fish at  $0.06 \pm 0.04 \text{ mg/kg}$ , but this was not significantly different to Al levels in control fish (ANOVA,  $p = 0.20$ ). Aluminium is not widely studied due to its comparatively low

toxicity (Crichton, 2012), and this is the first time to our knowledge that Al uptake into otoliths has been reported.

Likewise, Ba also appeared to be incorporated into the otolith of oil-exposed fish in levels proportional to those present in oil-spiked feeds ( $r^2 = 0.91$ , using test group averages). MCO- and HFO-exposed fish had mean distal otolith Ba concentrations of  $11.63 \pm 0.38$  mg/kg and  $13.93 \pm 0.86$  mg/kg respectively, significantly higher (ANOVA,  $p < 0.009$ ) than control fish with  $10.26 \pm 0.29$  mg/kg. This agrees with field studies in the Gulf of Mexico, where fish exposed to Macondo Oil showed a five-fold increase in otolith Ba concentration compared to unexposed fish (Lopez-Duarte *et al*, 2016). Natural background Ba concentrations of  $5.4 \mu\text{g/kg}$  in Indian Ocean surface seawater (Jeandal *et al*, 1996) may reasonably account for the high Ba concentration detected in otoliths of control fish.

Porphyryin-bound metals found in crude oils such as Ni, V, Mg, Zn, Fe, Mn, Co, and Cu (Scheer and Katz, 1975; Beisaga *et al*, 2000; Woltering *et al*, 2016) are the end-result of diagenesis and catagenesis of metalloproteins and other complex biologically active molecules in organic material. Chlorophyll and haemoglobin can be considered the most classic textbook examples, with atoms of Mg and Fe positioned in their respective active sites (Waldron and Robinson, 2009). Situated in the centre of a large molecular structure may shield Ni (Hausinger, 1997; Boer *et al*, 2014), V (Lyalkova and Yurkova, 1992; Pessoa *et al*, 2015; Gustafsson, 2019) and other porphyryin-bound metals in crude oils from interacting with other biological molecules. The accumulation of transition metals into otolith may also be complicated by the competition for these metals by other biologically active metalloproteins in the endolymph (Thomas *et al*, 2017). Other metals such as Ag, Al, Ba, Se and Sn however, are not known to have a functional role in metalloproteins, and are not generally incorporated into complicated, biochemically active molecular structures (Crichton 2012; Briffa *et al*, 2020). This may explain why some metals were found in otolith while others were absent - Al and Ba

might not be sequestered inside large molecular structures in crude oils and are hence more biologically available.

Seawater typically contains Mg at very high concentrations around 1200 mg/kg (Bruland *et al*, 2013; Mewes *et al*, 2014). This likely caused interference for results of Mg found abundantly in otoliths of fish exposed to MCO ( $23.69 \pm 1.18$  mg/kg) and HFO ( $30.85 \pm 2.71$  mg/kg), which although high, were not significantly different (ANOVA,  $p < 0.020$ ) from Mg detected in control fish ( $24.63 \pm 1.21$  mg/kg). Although Mg is present in both MCO ( $1.20 \pm 0.94$  mg/kg) and HFO ( $1.80 \pm 0.47$  mg/kg), it cannot be excluded that Mg found in analysed otoliths largely originated from seawater and was absorbed via the gills rather than from crude oils spiked into fish feed (Limburg *et al*, 2018). Similarly, B is present in seawater at an approximate concentration of 5 mg/kg (Kabay *et al*, 2010; Wolska and Bryjak, 2013; Bruland *et al*, 2013) and was detected in all otoliths of fish exposed to crude oils at concentrations around 1mg/kg, not significantly different from controls (ANOVA,  $p = 0.61$ ).

#### 1.4.3. Multivariate Analysis

Four otolith metals were selected for inclusion in the multivariate analysis (Al, As, Ba, and Cr) based on the following criteria: they are metals not present in seawater in concentrations above 0.1mM (e.g. B, Mg and Sr), may be incorporated into otolith via the dietary exposure route (unlike Pb, Sr or Cu), and they are not known to be commonly found in porphyrins or other metalloproteins (e.g. Fe, Mg, Co, Zn, Mn, Ni, and V) and are hence more likely to be bioavailable in crude oils. The four selected metals conformed to these characteristics, are also present in the crude oils used in this study, and were detected in otoliths of oil-exposed fish at levels (on average) at least double their respective analytical LOD. Other metals such as Ag, Se and Sn were notably excluded from this analysis as they were not on average detected in otolith of exposed fish in the current study at more than double their respective LOD, even though they meet all the other criteria.

The PCA of the otolith concentrations of the four included metals (Al, As, Ba, and Cr) produces two principal component factors (PC1 and PC2) which together retain 69.2% of the total variability of the dataset (Figure 2). Individuals within test groups displayed a large degree of variation in otolith metals composition, with a mean coefficient of variation for all metals concentrations of 1.00 within each test group. Despite this high degree of variation, the PCA plot shows a separation of the MCO, HFO and control test groups, which was confirmed by the application of Tukeys's HSD to the derived Cartesian coordinates for each test group ( $p < 0.046$ ). The position of individual fish on the PCA axes is driven predominantly by their respective concentrations of Al and Ba (Figure 2), which are higher in HFO compared to MCO (Table 1), and hence result in HFO-exposed fish positioned further in the direction of these respective variables along the x-axis (PC1) within the ordination plot (Figure 2). A subsequent LDA of the PCA output with leave-one-out cross validation (11 out of the 12 replicates from each test group used as a training set, followed by a prediction of the exposure test group for the 12<sup>th</sup> fish) showed a 88.9% success rate for the correct prediction of exposure test group for each fish based on their respective otolith microchemistry profiles of Al, Ba, Cr and As (Figure S1).

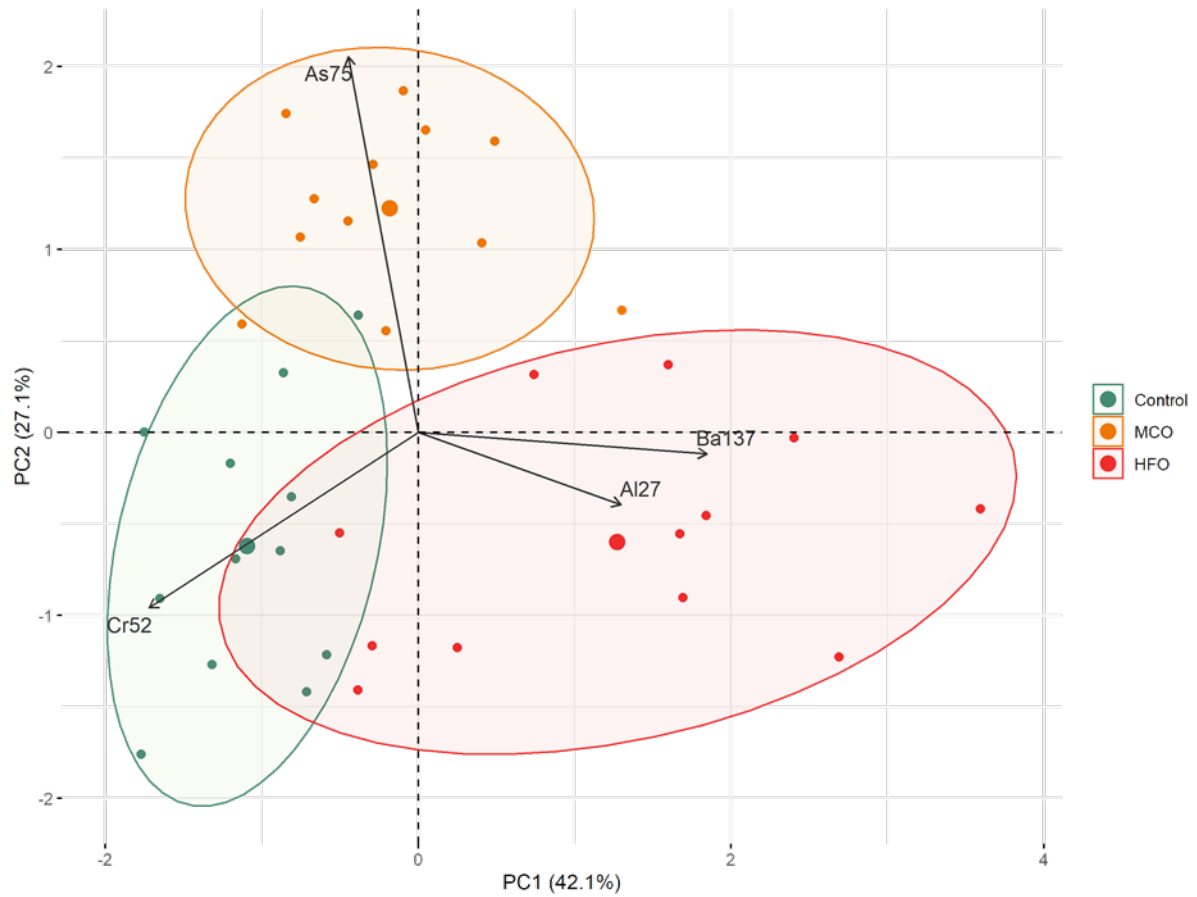


Figure 2: Principal components analysis (PCA) of four otolith metal levels in *L. calcarifer* exposed via the dietary route to Montara crude oil (MCO), or to heavy fuel oil (HFO).

Dot points are individual fish, larger circles are the respective geometric means.

The significance of this is that multivariate analysis of otolith microchemistry can provide a supplementary line of evidence to demonstrate fish exposure to crude oil. If fish suspected of having been exposed to a specific oil are available for comparison to unexposed fish, selective otolith microchemistry PCA and LDA may also be able to provide corroborating evidence to identify a specific oil in an environmental exposure scenario. Further research involving field studies (in the event of a future oil spill) would be needed to explore this idea, however.

The fish used in this study were juveniles less than a year old by the end of the exposures, and the samples of otolith analysed near the distal edge represent the most recent ear-bone growth. Spot LA-ICP-MS analysis can be targeted to a specific year in a fish's life history using otolith rings. In this way multivariate analysis of otolith microchemistry of selected metals such as Al, As, Ba and Cr (and possibly also other metals such as Ag, Se and Sn) can assist environmental managers conducting oil spill investigations or litigations to identifying historical fish exposures to crude oil even after all other

signs of exposure have dissipated in the environment. However, the permanency of metal deposition, especially Ba and Al, in otoliths would need to be demonstrated before this approach can be used in studies investigating exposure months or years after an oil spill incident.

### 1.5. Conclusions

The classical metals used in oil fingerprinting (V and Ni) are not absorbed by fish via the dietary route and consequently, are not deposited in the otolith. In crude oils, these metals are found embedded in porphyrins which likely have low bioavailability. In contrast, Al and Ba contained in crude oils are absorbed via dietary routes and deposited in significant levels in otoliths. Based on metals that accumulate in significant levels in otoliths following dietary exposure to crude oils, PCA and LDA can discriminate the oil to which fish were exposed. The rapid, low-cost analysis of otolith microchemistry combined with crude oil metal content measurement has the potential to assist oil spill investigations in identifying fish exposure to crude oil, even after all other signs of exposure have dissipated in the environment.

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