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Fish Fingerprints
Signatures of Oil Contamination

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This thesis is presented for the Degree of
Doctor of Philosophy
of
Curtin University

January 2022

Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Animal Ethics (For projects involving animal use) The research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes 8th edition (2013). The proposed research study received animalethics approval from the Curtin University Animal Ethics Committee, Approval Number **#ARE2019/11**

Acknowledgements

To Parvane, thanks for coming on the adventure! You have survived big kangaroos, bigger snakes, enormous lizards, spiders as big as your hand… and a sometimes preoccupied scientist, and done it all with a sparkle.

A debt of gratitude to Professor Marthe Monique Gagnon, whose endless optimism taught me to have fun with the process and enjoy the ride!

Thanks to Distinguished Professor Kliti Grice for encouragement and support.

Many thanks to Professor Emeritus Steven Rowland, whose wealth of knowledge and suggestion of where to look ultimately led to the success of the current work.

Thanks to Rowan Kleindienst for advice in tank setup, all things plumbing, and inexhaustible good humour.

Many thanks to Dr. Md. Reaz Chaklader, the barramundi whisperer, whose quick thinking, decisive action and animal husbandry expertise saved the day!

And finally, this work could not have been possible without the infinite patience of Dr. Alan Scarlett, who showed this humble biochemist the precise elegance of analytical chemistry. Your attention to detail made me spaced out! Thank you for all your support during the endless re-writes of my prose.

Table of Contents

Tables and Figures

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

Chapter 6:

Abbreviations

8-oxo-dG	8-oxo-7,8-dihydro-2'-deoxyguanosine
ACO	Australian crude oil (Montara)
ASV	amplicon sequence variant
ANOVA	analysis of variance
ANODIS	analysis of dissimilarity
AChE	acetylcholinesterase
BSA-J	bicyclic sesquiterpanes A-J
BTEX	benzene, toluene, ethylene and xylene
CAL	Calliance well platform
CAS	Caswell well platform
CAT	catalase
CF	Fulton's condition factor
CRX	Crux-3 well platform
DCM	dichloromethane
DWH	Deepwater Horizon
ELW	Eland-West well platform
EROD	ethoxyresorufin-O-deethylase
GAB	Great Australian Bight
GC-MS	gas chromatography mass spectrometry
GCxGC-MS	two-dimensional gas chromatography mass spectrometry
GPX	glutathione peroxidase
GST	glutathione S-transferase
HFO	heavy fuel oil
HSP70	heat shock protein 70
HIS	hepatosomatic index
IL-1	interleukin 1
IL-7	interleukin 7
IL-8	interleukin 8
IL-10	interleukin 10

ICP-AES	inductively coupled atomic electron spectrometry
ICP-MS	inductively coupled plasma mass spectrometry
KEGG	Kyoto Encyclopaedia of Genes and Genomes
KOH	potassium hydroxide
LA-ICP-MS	laser ablative inductively coupled plasma mass spectrometry
LDA	linear discriminatory analysis
LOD	limit of detection
LSO	very low sulfur oil
MCO	Montara crude oil
NW Shelf	Northwest Shelf, Australia
OCP	organochlorine pesticide
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate buffered saline
PCA	principal components analysis
ROS	reactive oxygen species
SE	standard error
SIM	selected ion monitoring
SOD	superoxide dismutase
TNF-α	tumour necrosis factor α
TPH	total petroleum hydrocarbons
Tukey's HSD	Tukey's honestly significant difference
UCM	unresolved complex mixture
WAF	water accommodated fraction

Thesis Abstract

Crude oils are highly complicated mixtures which vary in composition depending on their geographical location. In the case of an oil spill, international maritime law holds to the "polluter pays" principle. The first step in litigation or impact assessment proceedings is to identify the source oil, which in forensic chemistry is commonly achieved by comparison of the relative abundance (ratios) of key "fingerprinting" compounds present in petroleum hydrocarbon fluids in an environmental sample with those in a reference sample of the suspected source oil. This process depends on the availability of both environmental and reference oil samples for comparison, and is complicated by environmental degradation (weathering) that changes the chemical composition of oils immediately upon release into the environment.

A core tenet of ecotoxicology is that organisms exposed to toxigenic chemicals in the environment exhibit measurable alterations in behaviour and metabolism – biomarkers that indicate exposure to, and the subsequent adverse effects of harmful compounds in the environment, such as petroleum hydrocarbons. The hypothesis of the current work is that fish exposed to crude oils or derived petroleum products such as heavy fuel oils exhibit sufficiently distinctive biochemical and geochemical biomarker responses such that the source oil to which they were exposed can be identified.

A 35-day laboratory trial with a static renewal design was conducted, exposing *Lates calcarifer* (barramundi, or Asian seabass; n= 56) via diet (1% w/w) to Montara (MCO; a medium crude oil from the Australian NW Shelf), a heavy fuel oil (HFO), or to low dose mixtures of selected petroleum hydrocarbons plus one of three metals commonly found in crude oil: V, Ni or Fe. Following euthanasia, serum and a wide range of tissue samples were taken and analysed for biochemical biomarkers, otolith metal microchemistry, gut microbiome phylogeny, and geochemical fingerprinting compounds sequestered in adipose tissue and white muscle.

Analyses of ecotoxicological biomarkers showed that biochemical responses such as hepatic Cyp1a enzyme activity (EROD), brain acetylcholinesterase (AChE) and biliary metabolites of polycyclic aromatic hydrocarbons (PAHs) significantly differed between fish in the MCO and HFO exposure test groups such that fish from either test group could be differentiated by principal components analysis (PCA). Other biomarkers, such as liver somatic index (LSI), heat shock protein (HSP70) and 8-oxo-dG (DNA damage), were not useful in discriminating between test groups. Baseline values indicating healthy ranges for unexposed *L.calcarifer* were also established for 11 biochemical biomarkers.

Metals in the aquatic environment can be absorbed into the annular growth rings of the otolith (earbone) of exposed fish such that a permanent historical record of exposure is formed. Laser ablative inductively coupled plasma mass spectrometry (LA-ICP-MS) analysis of fish otoliths showed no deposition of V, Ni or Fe, indicating that these metals are not able to be absorbed *via* the dietary route of exposure. However, other metals present in crude oils such as Al and Ba were found in otoliths in fish exposed to MCO and HFO in concentrations proportional to those in the respective exposure oils. Fish exposed to oils were able to be discriminated by linear discriminatory analysis (LDA) of a PCA output (PCA-LDA) of selected metals detected in otoliths, with correct identification of the source oil in 32 out of 36 fish (88.9%).

Microbiome 16S rRNA analysis of barramundi intestinal contents showed that the normal core taxa of the fish gut microbiome exhibit significant changes in response to dietary toxicant exposure, enabling the identification of novel potential biomarkers that indicate exposure to crude oil or metals. Microbial phyla Firmicutes, Bacteriodetes and Proteobacteria are enriched in the gut microbiome of barramundi exposed via diet to V, Fe and Ni respectively. At the genus level, *Photobacterium* is enriched in a dose-dependent manner in response to dietary exposure to PAHs. Reductions in *Lactobacillus* were observed in fish exposed to dietary metals. Concurrently, the up-regulation of pro-inflammatory cytokines IL-1, IL-10 and TNF-α in the fish was observed in barramundi exposed to metals and HFO, but not to MCO.

Fish sequester geochemical biomarkers in their tissue following chronic exposure to dietary petroleum hydrocarbons, with 95% of the compounds found in the brown adipose tissue adjacent to the intestine. Most classes of compounds commonly used for forensic fingerprinting, such as PAHs, regular isoprenoids and the *n*-alkanes bioaccumulate at such varying rates that their diagnostic ratios are not conserved. However, gas chromatography mass spectrometry (GC-MS) analysis of bicyclic sesquiterpanes (bicyclanes) in adipose tissue of fish exposed to MCO or HFO enabled characteristic ratios of these compounds to be calculated, showing a high correlation ($r^2 > 0.98$) with those in the respective exposure oils. Further, a LDA model trained using a tailored dataset of bicyclane profiles of five crude oils from the NW Shelf, three heavy fuel oils (including a very low sulfur oil) and eight coastal asphaltites from the Great Australian Bight was able to correctly identify the bicyclane profile in adipose tissue extracts from all fish exposed to either MCO (n=9) or HFO (n=9) with a posterior probability exceeding 95%. This proof of concept work demonstrates how multivariate analysis of bicyclane profiles in the adipose tissue of oil-exposed fish (i.e., a "fish fingerprint") can be used to non-subjectively identify the oil of exposure, even when challenged with similar oils from sources in close geographical proximity.

This work has application to impact assessment and litigation proceedings following environmental oil spills, for exposure durations of 35 days or less. Further research is needed to determine minimum exposure durations that result in detectable concentrations of adipose tissue bicyclanes, and to establish depuration rates of bicyclanes, which may not be readily eliminated by fish cellular metabolism, and hence may be able to identify a source oil even after other signs of a spill have dissipated in the environment. "Fish fingerprinting" is of particular use in smaller scale incidents when the source of an oil spill is unclear, or when environmental samples of a spilled oil are unavailable.

Chapter 1: Introduction

1.1. Oils and the environment

Oil spills are an unfortunate and inevitable result of the extraction and transport of fossil fuels (Paine et al. 1996). The production and distribution of petroleum hydrocarbons fluids entails inherent risks that result in the periodic unintended release of hydrocarbons into the environment. International maritime law states that "the polluter must pay" for environmental damage and remediation efforts following an oil spill (Allen 2011; Caballero and Soto-Oñate 2017; Schwartz 2010). However legal proceedings to compel polluters to meet their obligations can be lengthy, and excessively expensive (Caballero-Miguez and Fernández-González 2015). One example is the 2009 West Atlas/Montara oil spill, where 4750 tonnes of crude oil were released into the Timor Sea (Burns and Jones 2016; Gagnon and Rawson 2012; Spies et al. 2017) over a period of 74 days (Gullett 2021; Hunter 2010). A class action commenced in the Australian Federal Court in 2016 by aquaculture farmers in Indonesia (Ryan 2018; Ryan and Parry 2021b) against PPTEP Australia Pty Ltd sought $200 million for damages incurred between 2009 and 2014, and spent five years in court before resolution in favour of the plaintiffs (Gullett 2021; Ryan and Parry 2021a). The first step in such litigation proceedings is connecting environmental harm to a specific oil, which can be challenging in cases where multiple candidates for the source oil exist.

Petroleum hydrocarbons found in the environment may come from a variety of sources including natural seeps (Burns et al. 2010; Jernelöv 2010; King et al. 2021), or anthropogenic sources including oil-well failures like the Macondo/Deepwater Horizon (DWH) release of 420 million tonnes of crude oil (Beyer et al. 2016; Kujawinski et al. 2020; Passow and Overton 2021) and shipping accidents such as the Spanish *Prestige* wreck in 2002 (Albaigés Riera et al. 2006) or the more recent grounding of the *M.V. Wakashio* in 2020 in Mauritius (Scarlett et al. 2021) which spilled 17,000 and 1,000 tonnes of heavy fuel oil respectively.

The first step in environmental impact assessment or in litigation proceedings following a spill, particularly in smaller scale incidents where the origins of the pollution may be unclear, is

identification of the source of the oil. In order to make the polluter pay, it is necessary to determine the origin of the source oil, which can be found in the environment as freshly spilt oil, as heavily weathered oil or as metabolites in organisms exposed to the oil. A critical aspect of the scientific evidence for assessing the impact of a spill is the ability to link weathered oil products, or metabolites found in marine organisms, with that of the spilled oil.

Crude oils are highly complex mixtures comprised of several thousand compounds (Yang et al. 2017); the result of the diagenesis and categenesis of organic matter over millions of years. The forensic identification of crude oils (commonly referred to as "fingerprinting") is normally performed by comparing the relative abundance of key geochemical biomarker compounds which are common to most oils, such as steranes and hopanes, polycyclic aromatic hydrocarbons (PAHs) or regular isoprenoids such as pristane, phytane and the *n*-alkane series (Goto et al. 2021; Stout et al. 2016; Yang et al. 2017). Metals are also present in crude oils, typically complexed within porphyrins (Ali and Abbas 2006; Biesaga et al. 2000; Dunning et al. 1960; Grice et al. 1996; Woltering et al. 2016), and diagnostic ratios of the relative abundances of metals, in particular vanadium (V as vanadyl VO) and nickel (Ni) (Sugiyama and Williams-Jones 2018), are also used as a line of evidence to identify crude oils (Pereira et al. 2010; Yasnygina et al. 2006). Depending on the origin of the oil, the relative abundances of these and other chemical geochemical biomarkers may be sufficiently different to allow discrimination between oils.

Heavy fuel oils (HFO, also commonly referred to as bunker oils or heavy diesel oils) are typically blends of the residual left-over products of distilling crude oils (Fritt-Rasmussen et al. 2018; Uhler et al. 2016) that vary in their physical and chemical properties. Depending on the crude oil from which they were derived, HFOs also have distinctive chemical profiles that can be distinguished by the comparison of the relative abundances of geochemical biomarker compounds (Stout et al. 2016; Uhler et al. 2016).

Fingerprinting of crude oils and fuel oils that have been released into the environment is further complicated by weathering – the partial degradation of oils that alter its chemical profile by a variety

of means (NRC 2003; Wang et al. 2021). Compounds in crude oils that are sufficiently polar to dissolve in water enter the water column (dissolution), microbes can utilise some of the compounds as an important source of energy leading to biodegradation of an oil, UV-facilitated transformation of compounds (photo-degradation) and the loss of low molecular weight aromatic compounds through evaporation all commence immediately upon release of oil into the environment. For this reason, the chemical fingerprinting of oils released in the environment tends to focus on those classes of compounds which are resistant to weathering processes, and ignores those which are subject to environmental loss or biodegradation.

Once released into the environment, compounds from oils can travel long distances and persist for long periods of time (Barron et al. 2020). Environmental surveys after the Alaskan *Exxon Valez* incident in 1989 found that although the volatiles like benzene, ethylbenzene, toluene and xylene (BTEX) fraction of oil had almost completely evaporated within two weeks (Short 2003; Wolfe et al. 1994), mass balance analyses estimated that three years later 15% of spilled oil residues remained in inter-or sub-tidal sediments (Nixon and Michel 2018; Wolfe et al. 1994), and these residues of weathered oil were still present in detectable concentrations 16 years after the initial spill (Short et al. 2007). Similarly, Macondo oil from the 2010 DWH incident persisted in coastal sediments for a year following the oil spill (Liu et al. 2012; Mahmoudi et al. 2013; Passow and Stout 2020), even though the well platform was located 66 km offshore, and the majority of the released hydrocarbons were largely deposited on the sea-floor of the Gulf of Mexico due to the extreme depth of the breach (Valentine et al. 2014).

Once in the environment, compounds from crude oils can enter the food chain (Zabbey et al. 2017) through uptake by aquatic plants (Buskey et al. 2016) and filter feeders such as bivalve molluscs (Donkin et al. 2003; Pérez-Cadahía et al. 2004). Lipophilic compounds ($LogK_{OW}$ <4.5) tend to bioaccumulate in organisms exposed to the oil (Gissi et al. 2015; Hellou et al. 2002; Lombardo et al. 2010; Veith et al. 1979). Transfer of crude oil compounds occurs between successive trophic levels (Scarlett et al. 2009), leading to the bioconcentration of petroleum hydrocarbons in predatory

carnivorous species (D'Costa et al. 2017; Snyder et al. 2015). Hence biomagnification increases the exposure of predatory species to the toxigenic compounds in crude oil such as PAHs. Following the DWH incident, total petroleum hydrocarbons (TPH) in various commercial species of fish in the Gulf of Mexico averaged 0.4% w/w, with a maximum of 2.2% w/w (Sammarco et al. 2013). The toxicokinetics in oil-exposed fish of some crude oil compounds such as *n*-alkanes has been described (Cravedi, 1983), but bioaccumulation rates and metabolic fates of many crude oil compounds in fish have not been investigated, including those compounds of interest for forensic identification purposes such as steranes, hopanes and bicyclic sesquiterpanes (bicyclanes).

1.2. Mechanisms of toxicity and biochemical biomarkers

When exposed to environmental toxicants, fish exhibit biochemical and physiological changes (biochemical biomarkers[1]), as opposed to the geochemical biomarkers mentioned previously), which can be measured and used to quantify an adverse effect on the organism (Depledge 2020; Lomartire et al. 2021; Van der Oost et al. 2003). There are numerous ecotoxicological biomarkers indicative of exposure to a wide variety of toxicants (Kroon et al. 2017), but here only those indicators relevant to the current study are described.

1.2.1. Liver detoxification enzymes

One of the classes of compounds in the highly complex mixture of any crude oil is the polycyclic aromatic hydrocarbons (PAHs), known to have a number of toxigenic effects in fish and other organisms (Logan 2007; Santana et al. 2018). Comprised of two to five conjoined benzene rings, PAHs are a large group of compounds with many alkylated species of the respective parent compounds (Pirsaheb et al. 2020). The high molecular weight phenanthrenes, pyrenes and benzo(*a*)pyrenes with three, four and five rings respectively, induce the AhR-mediated expression of

[1] This thesis covers multiple scientific disciplines, which have different meanings for the word "biomarker". In geochemistry, biomarkers are the compounds used to identify, date and otherwise characterise an oil (i.e. "geochemical biomarker"). In ecotoxicology, biomarkers are defined as molecules, genes or physiological parameters by which the exposure to a pollutant, and the subsequent toxicological effects in an organism, can be measured. In this chapter, "biomarker" is used in the ecotoxicological sense.

hepatic Cyp1a enzymes such as ethoxyresorufin-O-deethylase (EROD) (Baali and Yahyaoui 2019). The removal of lipophilic PAHs and other xenobiotics such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and dioxins (Hampel et al. 2016; Van der Oost et al. 2003) is a two-step biotransformation process carried out in the liver. Initially a -OH, $-NH_2$ or -SH functional group is added onto one of the rings by a Cyp1a enzyme (Phase I), followed by a conjugation step (Phase II) facilitated by the glutathione S-transferase (GST) family of enzymes which covalently bond a glutathione amino acid to the activated PAH (Hampel et al. 2016; Incardona 2017).

After biotransformation and conjugation, the polar metabolite produced by Cyp1a enzymes in the liver are transferred to the bile for elimination. The subsequent presence of these PAH metabolites in fish bile as an indicator for exposure to crude oil (Beyer et al. 2020; Dearnley et al. 2020) have been demonstrated in laboratory studies (Aas et al. 2000; Gagnon and Holdway 2000; Nahrgang et al. 2010) and in environmental impact assessments of oil spill-affected regions (Aas and Klungsøyr 1998; Pulster et al. 2020; Silva et al. 2021; Snyder et al. 2015).

During AhR-mediated xenobiotic removal, reactive oxidative species (ROS) of molecules can be generated (Santana et al. 2018), which have the potential to cause structural damage to a large range of macromolecules such as DNA, high molecular weight carbohydrates and proteins (Hampel et al. 2016). This leads to oxidative stress, which in turn leads to increased expression of enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX) which convert ROS such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) into water, plus other non-toxic side products.

This same metabolic pathway is also used to breakdown estrogenic compounds, thereby maintaining the hormone balance of the fish. Exposure to Cyp1a inducing toxicants such as the PAHs in crude oil can thereby also cause endocrine disrupting effects (Kar et al. 2021; Whyte et al. 2000) by an associated increase in the degradation rates of estrogenic compounds.

Other forms of crude oil toxicity include the exacerbation of hypoxia, heat stress and behavioural changes due to stress effects (Khursigara et al. 2019). Independently of the AhR-mediated xenobiotic

biotransformation and removal pathway, exposure to PAHs in crude oils cause increased rates of developmental defects in fish embryos including heart malformation (Incardona et al. 2005), as demonstrated in trials exposing zebrafish to both weathered and unweathered oil from the *Exxon Valdez* and DWH oil spills (Incardona et al. 2013).

1.2.2. DNA damage (8-oxo-dG)

The ROS produced by AhR-mediated PAH removal interacts with DNA to form adducts (Van der Oost et al. 2003) by reducing guanosine to 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) (Valavanidis et al. 2009). Repairing DNA damage by the replacement of lesion-affected nucleotides results in the excision and release of 8-oxo-dG (Gorokhova et al. 2020; Machella et al. 2005). When detected in serum, the presence of extracellular 8-oxo-dG is a biochemical biomarker indicative of increased rates of DNA damage (Gagnon and Rawson 2016).

1.2.3. Acetylcholinesterase

Acetylcholinesterase (AChE) is an enzyme that facilitates the conversion of the nerve synapse transmission chemical acetylcholine to choline and acetate, terminating nerve activation (Colovic et al. 2013). Inhibition of AChE in fish is caused by a number of anthropogenic compounds including pesticides (Butcherine et al. 2022; Fu et al. 2018; Olson and Christensen 1980) and the high molecular weight PAHs (>170 g/mol) in crude oils such as pyrene and benzo(*a*)pyrene (Aguilar et al. 2020; Olivares-Rubio and Espinosa-Aguirre 2021). Inhibition effects are more pronounced in the brain tissue of fish exposed to xenobiotics compared to other tissues such as the liver (Tenji et al. 2020). Fish exposed to AChE inhibitors exhibit muscle twitching, loss of equilibrium and may display a variety of altered behaviours (Sandahl et al. 2005) that are likely caused by interferences in brain function (Scott and Sloman 2004).

AChE inhibition following exposure to PAHs has been associated with fish behavioural changes such as reduced swimming speed (Oliveira et al. 2012) and decreased predation rates (Torreiro-Melo et al. 2015), but such effects are not specifically indicators of PAH exposure and may be a result of non-specific narcosis caused by other compounds in crude oils (Kasumyan 2001).

1.2.4. Physiological indices

General assessments of fish physiological parameters are useful, albeit non-specific, indicators of exposure to toxicants. Fulton's condition factor (CF) is a body mass index for fish based on a simple weight: length calculation, which can reflect a range of environmental factors including exposure to toxicants such crude oils (Snyder et al. 2019), increased energy demand for detoxification processes, or nutritional deficit as a flow-on effect caused by pollutants in the environment adversely affecting food sources. The hepatosomatic index (HSI) is the ratio of liver weight compared to overall body weight (less viscera). As the main detoxification organ, the liver increases in size following chronic exposure to a large variety of xenobiotic compounds (Tenji et al. 2020; Van der Oost et al. 2003), and thereby can indicate the long-term exposure. However, such indices vary greatly between species of fish, are not useful for environmental impact assessments without baseline values from unexposed fish for comparison.

1.3. Metals in crude oils and fish otolith deposition

Metals present in crude oils vary greatly, depending on the petrogenic source material. Ni and V (as vanadyl), for example, have been reported in various light and heavy crude oils at concentrations ranging from 0.01 to 20 mg/kg (Pereira et al. 2010; Yasnygina et al. 2006). In small quantities, some metals in the aquatic environment, including Ni, V, Fe , Zn and Cr can be viewed as micronutrients rather than toxicants (Hodson 1988).

Otoliths are calcified structures located in the inner ear of teleost fish. Otoliths consist of alternating layers of aragonite and protein deposited continuously as annular rings throughout the lifetime of the fish, similar to the growth rings in the trunk of a tree. Trace metals present in the ambient water and/or food may be incorporated in the growing otolith through substitution for Ca in the aragonite crystalline matrix or through co-precipitation of another carbonate (Campana 1999). Of central importance, measurements of metals found in the otolith annular rings of fish reflect historical environmental concentrations, forming a record of exposure (Ranaldi and Gagnon 2008b).

Deposition of metals into the otolith is a complex process (Campana 1999; Thomas et al. 2017; Thomas and Swearer 2019), involving first uptake by the waterborne route *via* the gills, or by the dietary route *via* the gastrointestinal tract, and then transfer across the blood-haemolymph barrier. The route by which metals can be incorporated into otolith varies between metals (Milton et al. 2000; Ranaldi and Gagnon 2008a; Ranaldi and Gagnon 2010). However, the incorporation routes for the two metals most important for crude oil identification, V and Ni, have not yet been explored.

In *Lates calcarifer* metals have been shown to be incorporated into otolith aragonite in fish found in rivers polluted by mine runoff (Milton et al. 2000). Similarly, ratios of metals detected in the otolith of fish have also been used to establish a timeline of historical exposure to environmental contaminants, reflecting seasonal migration routes and pollution exposure patterns (Daros et al. 2022; Friedrich and Halden 2010; Nelson et al. 2015; Rolls 2014). The elemental composition of the otolith's successive annular rings can provide a temporal record of the historical exposure of fish to bioavailable metals (Arai et al. 2007; Daros et al. 2022; Friedrich and Halden 2010; Long et al. 2014). Following the DHW spill, attempts to use otolith microchemistry as a biomarker of crude oil exposure were unsuccessful, partially because of the low metal content of Macondo oil (Lopez-Duarte et al. 2016; Nelson et al. 2015). Laboratory studies using water accommodated fractions of oil from the *Prestige* spill, however, demonstrate deposition of several metals in fish otolith, but not those used typically in crude oil forensic identification, Ni and V. The routes by which these two metals may be incorporated into fish otolith have also not yet been investigated under controlled laboratory conditions.

1.4. Gut microbiome

The development of rapid sequencing techniques (e.g. Illumina MiSeq) has allowed simultaneous characterisation of entire microbial communities (microbiomes), such as those found in the gastrointestinal tract of fish, based on the sequences of the 16S rRNA gene (Ghanbari et al. 2015).

279 The gut microbiome of fish is established early in the life of the juvenile fish, and is typically
280 comprised of core phyla Proteobacteria, Firmicutes and Bacteroidetes (Egerton et al. 2018; Talwar et
281 al. 2018) which vary between species (Egerton et al. 2018). The relative abundance of core taxa in
282 the gut microbiome is influenced predominantly by diet, but also by a range of environmental
283 factors such as pH and salinity (Givens et al. 2015). In the euryhaline barramundi, the core taxa of
284 the gut microbiome are similar for freshwater or marine-reared fish (Zheng et al. 2019), and dietary
285 studies have established a core taxa typically dominated by the phylum Proteobacteria (Gupta et al.
286 2020).

287 A healthy gut microbiome decreases the rate of diseases caused by invasive pathogenic species of
288 bacteria by spatial exclusion (Ringø et al. 2010) and host immune system cross-talk (Ringø et al.
289 2015). In aquaculture, the use of probiotic dietary supplements (e.g. *Lactobacillus*) are used
290 prophylactically to promote fish health (Ringø et al. 2015). A variety of toxigenic compounds alter
291 the relative abundance of taxa in the gut microbiome of exposed fish (Ringø et al. 2015), including
292 crude oil (Brown-Peterson et al. 2015; DeBofsky et al. 2020b; Walter et al. 2019), phenanthrene
293 (Hano et al. 2021) and benzo(*a*)pyrene (DeBofsky et al. 2020a; Quintanilla-Mena et al. 2021). Hence
294 enrichment of specific taxa in the gut microbiome has potential as a biomarker of exposure to crude
295 oil, although this has yet to be fully elucidated, and has not been investigated in relation to exposure
296 to crude oil.

297 Once the phylogenic composition of a microbiome is known, libraries of genetic and metabolic
298 pathways such as the Kyoto Encyclopaedia of Genes and Genomes (KEGG) (Kanehisa and Goto 2000;
299 Kanehisa et al. 2017) can be used to make predictions of the metabolic potential of the microbiome
300 to perform various functions, including the degradation of toxigenic chemicals such as the PAHs in
301 crude oil (DeBofsky et al. 2020a). Metabolic products of the gut microbiome influence the immune
302 system of the host fish (Bruce and Brown 2017; Sakai et al. 2020), interacting *via* activation of AhR-
303 mediated responses in the host fish and the stimulation of immune system agonists (Ringø et al.

2015). Cytokines are one such group of immune system signalling chemicals that regulate aspects of the immune response, such as inflammation. A number of pro- and anti-inflammatory cytokines have been detected in fish (Sakai et al. 2020), and have been used as biomarkers indicating fish exposure to a variety of xenobiotics such as pesticides, microplastics and bisphenol-A (BPA) (Montero et al. 2022; Torrealba et al. 2019). It seems likely that cytokine responses could also indicate exposure to other toxicants as well such as metals and PAHs in crude oils.

1.5. Environmental impact assessment

Following an oil spill, on-going environmental impacts can be measured for long periods afterwards. Remediation efforts aim to return an environment to pre-spill conditions, which in the majority of cases, such as the Gulf of Mexico prior to the DWH incident, are not scientifically defined (Murawski et al. 2016). Estimating the extent of environmental damage after the fact in the unfortunate event of an oil spill is made much more difficult in the absence of experimentally obtained data describing pre-accident environmental baseline conditions (Soto et al. 2014). Indeed, without baseline data, it would be impossible to ascertain the point at which any remedial measures have returned a contaminated site back to its original pre-accident condition (Murawski et al. 2016; Pulster et al. 2020), and a polluter can be said to have met their obligations under the “polluter pays” principle. Biomarker studies in fish have been used to describe baseline environmental conditions and fish health parameters before any potentially polluting incidents (Nunes et al. 2015; Pulster et al. 2020). Fish biomarkers are also a tool to monitor environmental recovery and remediation efficacy post-incident (Martínez-Gómez et al. 2009; Smeltz et al. 2017), such as the aftermath of the Montara well failure in Australia (Gagnon and Rawson 2012), the *Prestige* incident in Spain (Martínez-Gómez et al. 2009), and the Deepwater Horizon accident in the Gulf of Mexico (Snyder et al. 2015; Snyder et al. 2019).

Biomarker profiles in fish vary between species (Kroon et al. 2017; Van der Oost et al. 2003); hence published baseline studies are not necessarily indicative for other species. A recent and

comprehensive baseline study in the Gulf of Mexico describes baseline ecotoxicological biomarker data such as Fulton's condition factor and biliary PAH metabolites for 91 fish species (Pulster et al. 2020). However species not endemic to this region such as *Lates calcarifer* (barramundi, or Asian sea-bass) have not been included. In the future event of an oil spill, baseline data describing pre-accident conditions of species endemic to the affected area would be needed to assess the environmental impact and subsequent progress of remediation activities. Barramundi have potential as a sentinel species able to provide data for environmental impact assessment. Barramundi have been used as a test species to describe the effects of agricultural pesticide run-off (Kroon et al. 2015), estuarine flow rates (Staunton-Smith et al. 2004) and the combination effects of microplastics and pyrene (Guven et al. 2018). Comprehensive baseline data defining the biochemical and physiological biomarker profiles of healthy, unexposed fish have not previously been described.

1.6. Laboratory Trial Design

Laboratory studies seeking to simulate the toxicological effects of crude oil spills on fish facilitate petroleum hydrocarbon exposure by either the dietary route (Bautista et al. 2019; Nahrgang et al. 2010; Vieweg et al. 2018; Vignet et al. 2014), or the waterborne route via the gills (Aas et al. 2000; Amendola-Pimenta et al. 2020; Esteban-Sánchez et al. 2021; Heintz et al. 1999). As crude oils and their derivatives such as fuel oils are complex mixtures, estimation of the ecotoxicological effects should be considered holistically using whole oils. Single substance studies (e.g particular well-studied PAHs such as phenanthrene, pyrene or benzo(*a)*pyrene) fail to take into account synergistic or antagonistic effects, and a large proportion of the potentially toxigenic hydrocarbons in crude oils are often present in an unresolved complex mixture (UCM), unable to be individually identified (Booth et al. 2007).

Some of the compounds of interest for fingerprinting and ecotoxicological purposes in crude oils, such as PAHs, dissolve into the water column forming part of the water-accommodated fraction

(WAF), whereas many others such as *n*-alkanes, isoprenoids and bicyclanes are not particularly water-soluble. Even using established standard methods (Adams et al. 2017), generation of WAF in a controlled laboratory setting yields highly variable concentrations of compounds (Hodson et al. 2019), making accurate dosing and experimental repetition challenging (Barron et al. 2004; Singer et al. 2000). Hence, for fingerprinting studies in fish, dietary exposure is preferable.

The present works deal with dietary exposure of barramundi to crude oil and HFO. Typically, field and laboratory studies investigating the effects of exposure to petroleum hydrocarbons describe the effects of exposure to a singular oil. Conversely, in this study the effects caused by exposure to identical doses of two different oils are compared, using barramundi as the test species. Barramundi are a carnivorous teleost fish that has a wide global distribution, ranging east from the Persian Gulf throughout Asia to Australia, and extending north to China and Japan (Boonyaratpalin 2017; Hardin and Hill 2012). Barramundi is a commercially important aquaculture species (Mathew 2009; Siddik et al. 2016), and in Florida, U.S.A. it is regarded as a pest species (Hardin and Hill 2012). Able to tolerate a wide range of saline, pH, and temperature conditions (Jerry 2013) barramundi are found in both marine and freshwater riverine environments in tropical and sub-tropical regions. Barramundi is a suitable test species to investigate the toxicological effects, biomarker responses and fingerprinting potential of fish exposed to petroleum hydrocarbons because its hardiness makes it relatively easy to keep in a laboratory environment, and due to its commercial uses, nursery-reared barramundi stock were readily available from a local supplier. Its wide geographical dispersal makes it a potential bioindicator species in a variety of environments where unintended releases of petroleum hydrocarbon fluids may occur.

1.7. Aim and objectives

The overall aim of the current work was to determine whether a specific crude oil can be fingerprinted and forensically identified using the biochemical and geochemical biomarkers in fish exposed to the oil.

To this end a wide range of biochemical and geochemical biomarkers were measured in various tissues of fish exposed via diet to two chemically different oils, examples of which have previously been spilled in the marine environment, and multivariate analysis was used to discriminate between exposure groups.

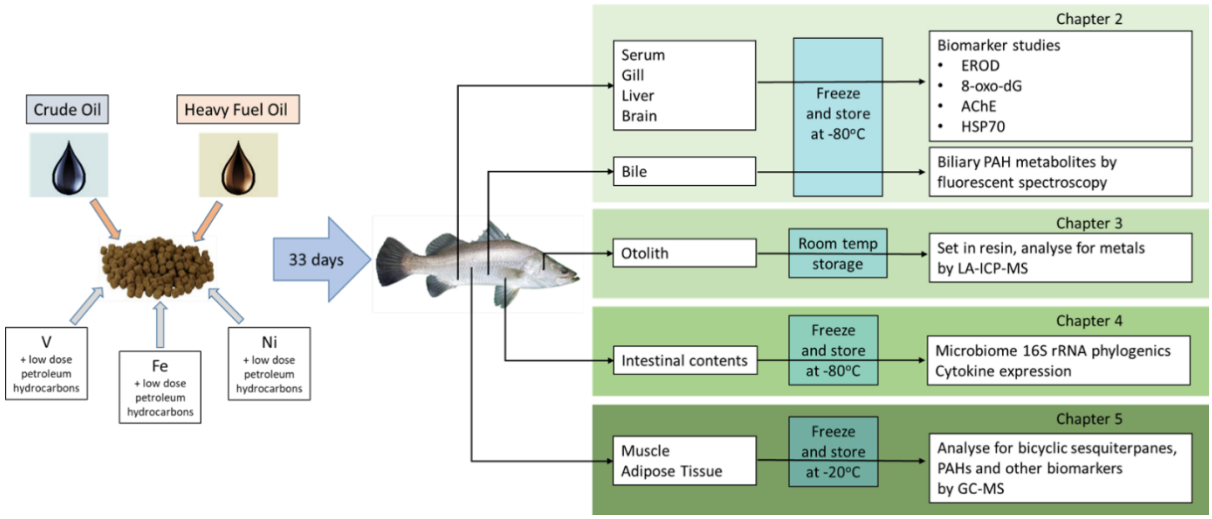

Figure 1: Schematic of sampling regime

In these laboratory exposure trials, a static-renewal design was employed with 100L aquaria containing natural Indian Ocean seawater with four fish per tank. Barramundi were fed commercial fish meal (control) or fish meal spiked with Montara crude oil, HFO, or low dose mixtures of petroleum hydrocarbons plus one of three metals: Ni, V or Fe. Dietary exposure to the toxicants was maintained for 33 days followed by a two-day depuration period. Following euthanasia, samples of serum, liver, brain, gill, bile, adipose tissue, white muscle, otolith and intestinal contents were taken (Figure 1). Biochemical and geochemical analyses of the various fish tissue samples, and subsequent

multivariate statistics are described in detail in the five manuscripts which form the data chapters of this thesis:

Chapter 2: Discriminating source of oil contamination in teleost fish, *Lates calcarifer*, using multivariate analysis of a suite of physiological and behavioral biomarkers

(Submitted to *Marine Pollution Bulletin*: 10th July 2021, Revised 16 August 2021, Accepted 20 August 2021, Published: 1st November 2021)

Chapter 3: Multivariate analysis of otolith microchemistry can discriminate the source of oil contamination in exposed fish

(Submitted to *Comparative Biochemistry and Physiology, Part C*: 11th October 2021; Revised 26 November 2021, Accepted 18 December 2021, Published: 29th December 2021)

Chapter 4: Gut microbiome as a potential biomarker in fish – dietary exposure to petroleum hydrocarbons and metals, metabolic functions and cytokine expression in juvenile *Lates calcarifer*

(Submitted to *Frontiers in Microbiology:* 1st December 2021)

Chapter 5: Fish Fingerprinting: Identifying crude oil pollutants using bicyclic sesquiterpanes (bicyclanes) in the tissues of exposed fish

(Submitted to *Environmental Toxicology and Chemistry*: 6th December 2021)

Chapter 6: Crude oil identification using linear discriminatory analysis (LDA) of bicyclic sesquiterpanes (bicyclanes) in the adipose tissue of exposed fish

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Chapter 2: Discriminating source of oil contamination in teleost fish, *Lates calcarifer*, using multivariate analysis of a suite of physiological and behavioural biomarkers

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This article was published in the peer reviewed journal *Marine Pollution Bulletin*, Volume 172, p.112898. Francis Spilsbury, Alan Scarlett, Kliti Grice, Marthe Monique Gagnon. Discriminating source of oil contamination in teleost fish, *Lates calcarifer*, using multivariate analysis of a suite of physiological and behavioural biomarkers. Copyright Elsevier 2021.

Submitted to *Marine Pollution Bulletin*: 10th July 2021, Revised 16 August 2021, Accepted 20 August 2021, Published: 1st November 2021.

https://doi.org/10.1016/j.marpolbul.2021.112898

Keywords

Crude oil, biomarkers, barramundi, bunker C, Montara, PCA

Highlights

- Fish exposed to crude and heavy fuel oils via dietary exposure for 33 days
- Distinctive profiles of 12 biomarkers produced for oil-exposed fish
- Individual biomarker responses dependent on characteristics of exposure oil
- PCA analyses able to discriminate between crude and heavy fuel oil exposure
- Biomarker profiles inform on the oil characteristics biota is exposed to

2.1. Abstract

The release of petroleum hydrocarbons into the environment from natural seeps, well blowouts, pipeline leaks, shipping accidents and deliberate tank washing poses an ongoing threat to marine ecosystems. Distinguishing the source of oil contamination in exposed biota can be relatively straightforward if samples of the oil are available but, in their absence, such discrimination in fish

poses a major challenge. The use of physiological and behavioral biomarker analysis provides a useful tool to describe sub-lethal effects of toxicant exposure.

In this study we describe the responses of 12 biomarkers in *Lates calcarifer* (Asian seabass) following a 33-day dietary exposure (1% w/w) to heavy fuel oil (HFO) and to Montara, a typical Australian medium crude oil (MCO). Principal components analysis was used to differentiate between fish exposed to HFO from those exposed to MCO. Inferences can be made about the composition of an oil from the biomarker profiles produced in exposed fish.

2.2. Introduction

The introduction of petroleum hydrocarbons into the environment can occur from natural processes such as marine seeps (Burns *et al.*, 2010) or due to human activities. Large-scale anthropogenic releases such as the Deepwater Horizon oil spill are extreme events with long-term environmental consequences (Nunes *et al*., 2015; Snyder *et al*., 2017; Smeltz *et al*., 2017). Although on smaller scale, the blowout from the West Atlas (Montara) well platform in Northwestern Australia in 2009 resulted in the unintentional release of an estimated 4,750 tonnes of medium-light crude oil (Gagnon and Rawson, 2012; Burns and Jones, 2016; Spies *et al.,* 2017) over a period of 74 days (Hunter, 2010). Shipping accidents periodically occur, resulting in highly publicized released of petroleum hydrocarbons into the environment such as the 2002 Prestige spill of 17,000 tonnes of heavy fuel oil (HFO) off the coast of Spain (Albaigés Riera *et al*., 2006; Gonzales *et al*., 2006), and the recent grounding of the *M.V.* Wakashio in Mauritius in 2020 where an estimated 1000 tonnes of a new type of low sulfur fuel oil was spilled (Seveso *et al*., 2021).

Crude oils are highly complex mixtures of several thousand compounds with chemical biomarker profiles that differ greatly depending on the source. In heavily developed industrial areas, petroleum hydrocarbon pollutants found in the environment may originate from several sources, each with a distinctive chemical fingerprint (Elfadly *et al*., 2017). Heavy fuel oil (HFO) (also termed bunker oil or

heavy diesel oil) refers to blended residual products from the distillation of crude oil commonly used in merchant vessels (Fritt-Rasmussen *et al.*, 2018). HFO produced from different crude oils are distinguishable by specific chemical biomarkers (Uhler *et al.*, 2016). Their universal use in shipping has led to their frequent release either intentionally (e.g. tank washing) or accidentally, and hence an understanding of the environmental effects of HFO discharges is important. On exposure to the environment, the composition of crude and fuel oils changes rapidly as lower molecular weight volatile compounds evaporate, water-soluble compounds enter the water column, microbial metabolism and UV-degradation all combine to weather crude oil until eventually the asphaltene-rich residue fraction remains, often washing up on beaches as tar balls (Scarlett *et al.*, 2019).

Laboratory-based ecotoxicological studies seek to simulate a complicated environmental picture where sub-lethal effects play a significant role (Whitehead, 2013) in the impacts to organisms in a spill-affected area. The various biomarkers measured in such studies can show evidence of exposure to a class of toxicants, or provide quantitation of the effects of this exposure (van der Oost *et al*., 1993). Many previous toxicity studies have concentrated on the Water-Accommodated Fraction (WAF) of crude oil, and have sought to simulate the complex, partition-driven adverse environmental effects of oil spills by using flow-through systems over contaminated gravel (e.g. Heintz *et al.*, 1999) or mechanical methods (e.g. Aas *et al.*, 2000) to generate WAF from crude oils. Laboratory methods to generate WAF often result in highly variable concentrations of the compounds of interest which makes replication difficult (Singer *et al.*, 2000; Barron *et al.*, 2003). Studies using dietary exposures are possibly more repeatable, but there is limited data available. Exploring the sub-lethal toxigenic effects of crude oil compounds via the dietary route has shown behavioral changes in Siamese fighting fish (*Betta splendends*) (Bautista *et al*., 2019) and zebrafish (*Dario rario*) (Vignet *et al.*, 2014b), activation of Cyp1a mediated responses (Narghang *et al.*, 2010) and changes in serum biochemistry (Vieweg *et al.*, 2018) in polar cod (*Boreogadus saida*), and growth inhibition in zebrafish (Vignet *et al.*, 2014a). Hence, dietary exposure has the potential to produce reproducible sublethal effects using well-characterised whole oils.

906 Fish present in a spill-affected site may be exposed to toxicants from crude oils via dietary intake, or
907 water-borne via the gills. Various species of fish from sites with high sediment petroleum
908 hydrocarbon concentrations show absorption and retention of crude oil compounds in muscle tissue
909 (Ahmed *et al.*, 2019). Lipophilic compounds (i.e. with an octanol-water partition coefficient $LogK_{OW}$ >
910 4) have previously been shown to be taken up by fish via the dietary route (McKim, 1994; Law and
911 Hellou, 1999), but there is a paucity of data on this. Anecdotally, the authors have observed fish in
912 oil spill affected areas feeding on floating wax residues coated with oil, mistaking them for food.
913 Bioconcentration and biomagnification may enhance the impacts of crude oil toxicogenuic
914 compounds to marine organisms (Varanasi *,* 1989; Hellou *et al.*, 2004). Compounds with $LogK_{OW}$ >
915 4.5 are likely to bioaccumulate (Veith *et al.*, 1979; Hellou *et al.* 2002; Lombardo *et al.,* 2010; Gissi *et*
916 *al.*, 2015; ECHA, 2017) and biomagnify in food webs (Voutsas *et al.,* 2002).

917 The classical toxicogenesis of petrogenic compounds such as PAHs has been well-described
918 elsewhere (reviewed by Renaud and Deschaux, 2006). Likewise, the adverse effects of metals on fish
919 physiology and behavior are well-established (Atchison *et al.*, 1987; Wood, 2011). However, given
920 the enormous number of compounds present in crude oils, it is exceedingly difficult to describe the
921 toxic effects of the individual constituent compounds contributing to observed adverse effects.
922 Hence to fully describe the toxicity of a crude oil, it is necessary to study its effects *in toto*, rather
923 than selectively choosing groups of known toxicogenic compounds and applying classic mixture
924 toxicity models.

925 *Lates calcarifer,* commonly known as Asian seabass, barramundi or Australian seabass is a predatory
926 teleost fish found in both freshwater, estuarine and marine environments. A popular sportsfish and
927 important for aquaculture (Mathew, 2019), it is raised in commercial operations throughout south-
928 east Asia (Boonyaratpalin, 2017) and elsewhere (Hardin and Hill, 2012). It has a wide global natural
929 distribution in temperate and tropical waters with genetically distinct natural populations (Yue *et al.*,
930 2009) ranging from the eastern tip of Papua New Guinea to the Persian Gulf (Grey, 1987). Its wide

distribution and hardy ability to tolerate a range of environmental conditions make it a suitable test species for laboratory-based studies concerned with the ecotoxicological effects of crude oil spills.

Following an oil spill, the ability to distinguish whether fish have been exposed to a medium crude oil or a heavy fuel oil could be of benefit in terms of assessing the impact on ecosystem health and litigation proceedings. In this study, we aim to ascertain if exposure to two different petroleum products, a heavy fuel oil and a medium crude oil, produce significantly distinct effects in a common teleost fish. In addition to individual biomarker responses, we aim to establish if the integrated set of biomarkers has the potential to discriminate between the biomarker responses in such a way as could be predicted based on the character of the oils. Overall, we aim to test the hypothesis that the source of the binary exposure could be differentiated based on a suite of physiological and behavioral biomarkers as measured in *L. calcarifer*.

2.3. Materials and Methods

2.3.1. Characterization of Oils

The HFO, a typical bunker C fuel oil (API 11.4) was supplied by the BP Kwinana Oil Refinery (Western Australia). The Australian MCO (API 31.0) was supplied by PTTEP Pty Ltd. As highly volatile components within oils are usually rapidly lost on exposure to the environment, the medium crude oil (MCO) was weathered for 5 days using a published method (Smith *et al.*, 2006) to simulate post-spill conditions. Heavy fuel oils are typically blends of residual post-refinement products (Lewis, 2002; Fritt-Rasmussen *et al.*, 2018), and have already undergone treatments exceeding the weathering protocol used for MCO. The HFO was analysed as received.

2.3.2. Metals Analysis

A sample of crude oil was accurately weighed and repeatedly digested in nitric acid, and finally in a mixture of nitric/perchloric acids. The digestate was taken to incipient dryness and the residue was dissolved in high purity nitric (0.7 mL), hydrochloric (0.2 mL) acids and high purity water (25 mL). Samples were analysed in triplicate, and quantified by inductively coupled plasma atomic emission

957 spectroscopy (ICP-AES) and inductively coupled plasma mass spectrometry (ICP-MS) against a
958 commercial standard (AccuTrace High Purity multi-element standards, Choice Analytical).

959 ### 2.3.3. Preparation of Spiked Fish Feeds

960 Dry fishmeal (Nova FF 3mm, Skretting Pty Ltd, Perth, Australia) was powderized in a food processor,
961 and then 180g samples were spiked with either 3.4 g of HFO or weathered MCO and mixed
962 thoroughly in a stainless steel benchtop mixer before 200 mL of warmed 10% w/v gelatin solution
963 was added. The mixture was uniformly spread on a stainless steel tray, covered in aluminum foil and
964 placed in an air-tight container at 4^{o}C for 12 h. On setting, the resultant fish feed was manually sliced
965 into approximately 2 mm cubes, weighed and stored at -20^{o}C until used.

966 All stainless steel mixing and cutting apparatus was thoroughly cleaned, and double-rinsed with
967 methanol followed by dichloromethane (DCM) between preparations.

968 ### 2.3.4. Polycyclic Aromatic Hydrocarbons

969 The MCO, HFO and fish feeds spiked with the respective oils were analysed for a suite of 40 PAHs
970 using standard published methods (Forth *et al.*, 2017). Oils were diluted in DCM, and an internal
971 standard added to a 1ml aliquot of the extract. Fish feeds (10g) were extracted by sonication in
972 acetone/DCM, and chemically dried using sodium sulphate.

973 Oils and fish feed extracts were analysed for PAHs using GC mass spectrometry (GC-MS) selected ion
974 monitoring (SIM). PAHs were quantitated by comparison to external standards (Accustandard,
975 Connecticut, U.S.A.). Alkylated-PAHs were quantitated using the response factors of the appropriate
976 parent PAH using the protocol of Forth *et al.* (2017). All extractions and analyses were performed in
977 triplicate.

2.3.5. Fish Exposure and Sampling

Juvenile barramundi (10-15 cm) were obtained from a commercial hatchery. Originally raised in freshwater, the fish were gradually acclimatized to 34 ppt salinity seawater over 5 days before being transferred to 100 L tanks. Natural Indian Ocean seawater was collected from a coastal region 100 km north of Perth, Western Australia. Fish were handled and maintained in accordance with Curtin University animal ethics approval ARE2019/11. Each of the three exposure groups (negative control, MCO and HFO) were tested in triplicate, with 4 fish per tank (n=12 per treatment). A closed recirculating system via an external canister biofilter was used with a flow rate of approximately 5L/min. Water was maintained at 28 ^{o}C (±2 ^{o}C) using in-tank submersible heaters, and was aerated to achieve dissolved oxygen of not less than 5.0 mg/L. Fish health was maintained by daily monitoring of total ammonia, dissolved oxygen, pH, salinity and temperature with partial water exchanges of 10 - 60% of the 100 L tank volume performed daily as required.

Fish were fed twice per day to a total of 2% body weight per day, with either commercial fish meal (negative control) or commercial fish meal spiked with 1 % w/w MCO or 1 %w/w HFO. Post-feeding, excrement and any fish feed not consumed was removed one hour after feeding.

Fish were exposed for 33 days, followed by 2 days without feeding to ensure sufficient contents of the bile duct for sampling. Fish were euthanized by ike-jime, a blood sample was immediately taken from the caudal vein using an un-heparinized syringe. Haematocrit was measured by the capillary method using heparinized tubes, and blood was allowed to clot for 45 minutes on ice before centrifugation at 5000xG for 5 minutes followed by removal of serum into 2 mL cryovials which were snap frozen in liquid nitrogen before being stored at -80 ^{o}C until analysis. Physiological parameters of standard and fork length, whole wet weight and carcass weight (body weight without viscera) were recorded.

The liver was excised and weighed, the brain was surgically removed, samples of gill tissue were excised, and bile was collected directly from the bile duct using a 1.0 mL syringe and 22-gauge needle. All tissue samples were divided among several separate 2 mL cryovials which were immediately snap frozen in liquid nitrogen and stored at -80°C until analysis.

2.3.6. Physiological Parameters

Fulton's condition factor (CF) was calculated as:

$$\text{CF} = \left[\frac{W_c}{{L_f}^3}\right] \times 10^6$$

where L_f is the fork length (in mm) of the fish and W_c is the carcass weight (g).

The hepatosomatic index (HSI) was calculated as:

$$\text{HSI} = \left[\frac{W_l}{W_c}\right] \times 100$$

where W_c is the carcass weight (g) and W_l is the liver weight (g).

2.3.7. Biochemical Analyses

DNA damage was estimated by quantifying 8-oxo-dG in serum using a commercially available ELISA kit (StressMarq Biosciences, Vancouver, Canada, catalog number SKT-120-965) as per manufacturer's instructions.

Acetylcholinetserase (AChE) in brain tissue was quantified using a commercially available ELISA kit (Cusabio Biotech, Houston, U.S.A., catalog number CSB-E17001Fh). Samples were thawed on ice, surface rinsed with chilled phosphate buffered saline, pH 7.4 (PBS) and a 10% w/v homogenate prepared in PBS. Samples were not diluted prior to analysis.

Heat shock protein 70 (HSP70) in gill tissue was similarly quantified using a commercially available ELISA kit (Cusabio Biotech, Houston, U.S.A., catalog number CSB-E16327Fh). Samples were thawed on ice, surface rinsed with PBS, and a 10% w/v homogenate of excised lamellae prepared in PBS.

Ethoxyresorufin deethylase (EROD) activity was quantified in liver tissue using a published spectrofluorimetric method (Hodson *et al.*, 1991). Liver samples were thawed on ice and a 20% w/v homogenate prepared in chilled HEPES buffer, pH 7.5. Homogenates were centrifuged at 12000xg for 20 minutes at 4^{o}C, and the microsome-rich S9 fraction of the supernatant was collected for analysis. EROD activity was reported as pmol of substrate converted to product per minute.

Biliary PAH metabolites were estimated using the method of Lin *et al.,* 1996. As standards, naphthol (excitation/emission wavelengths of 290/335nm), a phenanthrol standard (Torreira-Melo, 2015) (excitation/emission wavelengths of 260/380nm) and pyrenol (excitation/emission wavelengths of 340/380nm and 380/430nm for pyrene-type and benzo(*a*)pyrene-type metabolites respectively) were used. Sample fluorescence was measured using a Perkin–Elmer LS-5 Luminescence Spectrometer, and reported as μg of equivalent fluorescence of the relevant standard-type.

All biochemical biomarkers were normalised to total protein in the sample, measured using the Bradford method (Bradford, 1976; Bio-Rad, 1979) with bovine serum albumin (BSA) as a standard and a BioRad iMark Microplate Absorbance Reader to measure absorbance at 595nm.

2.3.8. Behavioural Effects

Impacts on foraging behaviour was estimated via the rate of food consumption. Daily feed was weighed, and the time taken for each tank of four fish to consume their allotment of approximately 5g of food was measured and averaged by the number of fish in the tank (i.e. 4 fish). Feeding rate was reported in grams of food ingested per minute per fish (g/min/fish).

2.3.9. Liver Histomorphology

Four liver samples from each treatment group were randomly selected for histomorphological analysis. Samples were sectioned, mounted and stained by the Western Australian Government Department of Primary Industries and Regional Development (DPIRD) and interpreted by a veterinary pathologist.

2.3.10. Data Handling

All data analyses were conducted using R statistical software, version 4.02. Significant difference between means of exposure groups for the various biomarkers was established using Tukey's HSD. Differences in biomarker profiles between exposure groups were characterised by principal components analysis (PCA) (Le *et al.*, 2008). Individuals missing values for any particular biomarker were included in the PCA analysis by substituting missing values with the mean of the respective exposure group for that biomarker (Husson *et al*., 2016).

2.4. Results and Discussion

All confidence intervals provided are standard error.

2.4.1. Characterization of Oils

The HFO was found to be highly sulfurous (10200 ± 850 mg sulfur/kg), with higher levels of iron (37.90 ± 1.47 mg/kg), nickel (12.23 ± 0.71 mg/kg) and vanadium (15.27 ± 081 mg/kg) relative to MCO but differences between other element concentrations were less pronounced (Table 1).

MCO contained higher concentrations of naphthalenes (29800 ± 1180 mg/kg) and phenanthrenes (6370 ± 210 mg/kg) than the HFO (11900 ± 124 mg/kg and 4830 ± 39 mg/kg respectively). Conversely, the HFO contained higher concentrations of the larger 4-ring pyrenes (2550 ± 49 mg/kg) than MCO (910 ± 22 mg/kg). Of particular ecotoxicological interest, the HFO contained 891 ± 29 mg/kg benzopyrenes, which were absent in MCO. Total PAH concentration (a sum of 40 measured PAH compounds) measured in fish feed used in this study averaged 600 mg/kg (MCO) and 425

mg/kg (HFO) fish food respectively (Table S1). These are environmentally relevant concentrations: in spill-affected zones after the Deepwater Horizon incident, total PAH concentrations in sediments were as high as 355mg/kg (Turner *et al.*, 2014) and 856mg/kg (Wang *et al.*, 2014).

Table 1: Selected Metals and Total PAHs measured in MCO and HFO.

	Compound	MCO (mg/kg)	HFO (mg/kg)
Total PAHs*	Naphthalenes (C1-C4)	29800 ± 1180	11900 ± 124
	Phenanthrenes (C1-C4)	6370 ± 210	4830 ± 39
	Pyrenes/Fluoranthenes	910 ± 22	2550 ± 49
	Benzopyrenes/Benzofluoranthenes	0 ± 0	891 ± 29
	Dibenzothiophenes	1270 ± 46	3530 ± 69
	Chrysenes	61 ± 2	2970 ± 29
Metals	Aluminium	30.70 ± 17.7	15.44 ± 8.91
	Arsenic	< 0.03	0.04 ± 0.02
	Barium	0.11 ± 0.06	1.32 ± 0.76
	Chromium	0.89 ± 0.52	0.24 ± 0.14
	Cobalt	< 0.46	2.15 ± 1.24
	Copper	0.45 ± 0.26	< 0.31
	Iron	4.73 ± 2.73	37.90 ± 0.22
	Lead	0.08 ± 0.05	0.04 ± 0.02
	Molybdenum	< 0.01	0.05 ± 0.03
	Nickel	0.11 ± 0.06	12.23 ± 0.71
	Silver	< 0.01	< 0.01
	Sulfur	394 ± 227	10200 ± 850
	Tin	0.18 ± 0.1	0.13 ± 0.07
	Titanium	< 0.24	3.24 ± 1.87
	Vanadium	< 0.03	15.27 ± 0.81
	Zinc	1.47 ± 0.85	1.19 ± 0.69

*Total PAH is defined as the sum of parent compounds plus all alkylated C1, C2, C3 and C4 homologues.
Values denoted with "<" were below the stated limit of reporting (see Tables S1, S2 and S3).
A full list of all PAHs and metals included in the analytical suites, and the analysis of fish food spiked with oil, is provided in the supplementary information.

2.4.2. Physiological Parameters

Mean CF was significantly lower ($p = 0.015$) in HFO exposed fish (14.38 ± 0.44) compared to negative controls (16.09 ± 0.25) (Figure 1a). Mean CF in MCO (14.73 ± 0.48) exposed fish were also comparably lower than negative control fish, but not significantly so ($p = 0.060$). Toxicant exposure

carries with it an associated energy burden on the organism (Marchand *et al.*, 2004) as it both metabolizes and excretes xenobiotic compounds, and repairs any associated damage that may occur, for example by reactive oxidative species (ROS) generated through the Cyp1a mediated metabolism of PAHs. The CF of fish exposed to MCO was not significantly different from that of fish exposed to HFO ($p = 0.819$), suggesting that the specific composition of the oil does not affect the energy burden required by the organism to deal with ingested toxicants.

Hepatosomatic Index was not significantly different between any of the treatment groups ($p = 0.093$) (Figure 2b). Faster growing juvenile fish tend to show higher rates of liver hyperplasia than slower growing adult fish (van der Oost *et al.,* 2002), but as the liver has both storage and detoxifying functions, the enlargement of the liver in response to exposure to a toxicant can be reduced to the point of no-net increase by poor nutrition (Schlenk and Benson, 2017). It may also be that the duration of our trial at 35 days was insufficient for an increase to be seen in the liver size of fish exposed to petroleum hydrocarbons. Significant HSI responses to hydrocarbons from crude oil were not found in other laboratory exposure studies in Atlantic cod (*Gadus morhua*) (Aas *et al.*, 2000) or Atlantic salmon (*Salmo salar)* (Gagnon and Holdway, 2002). Field studies following the 2009 Montara oil spill similarly showed no significant changes to HSI (Gagnon and Rawson, 2012) in either red emperor (*Lutjanus sebae*) or goldband snapper (*Pristipomoides multidens*) despite the 74-day duration of the petroleum release (Hunter, 2010, Burns *et al.* 2010).

Haematocrit varied between treatment groups (Figure 1c). HFO exposed fish had a significantly lower ($p = 0.001$) mean haematocrit (0.199 ± 0.014) compared to negative control fish (0.290 ± 0.018). Repeating the pattern found with CF, MCO-exposed fish also had a lower haematocrit (0.238 ± 0.012) which approached significance ($p = 0.069$). Lower haematocrit implies a reduction in blood oxygenation, which has metabolic consequences that are possibly a contributing factor in the lower CF evident in fish exposed to crude oils.

2.4.3. Biomarkers of Exposure

Biliary PAH metabolites in each treatment varied generally proportionately to the relative abundance of the parent compounds in the respective crude oils (Figure 1(i), (j), (k) and (l)). Mean biliary metabolite concentrations in MCO- and HFO-exposed fish were significantly different from negative control fish ($p < 0.001$), with the exception of benzo(*a)*pyrene type metabolites in MCO-exposed fish (15.96 ng/mg protein ± 0.67) which were non-significantly higher ($p = 0.120$) than negative control fish (7.69 ng/mg protein ± 0.39), reflecting the paucity of larger molecular weight PAHs found in the MCO used in this study.

Compared to negative controls, EROD activity in fish exposed to MCO showed no significant increase compared to negative controls ($p = 0.995$). EROD activity was clearly induced in fish exposed to HFO (2.08 ± 0.39 pmol/min/mg protein), which was significantly higher than both the negative control group (0.96 ± 0.08 pmol/min/mg protein, $p = 0.012$) and fish exposed to MCO (0.99 ± 0.18 pmol/min/mg protein, $p = 0.026$) (Figure 1f).

Although the ability of petroleum hydrocarbons to induce EROD activity varies greatly between fish species (White *et al.*, 2000), *L.calcarifer* exhibits significant EROD induction following intra-peritoneal injection of petroleum oils (Mercurio *et al.*, 2004; Gagnon and Rawson, 2017). Lower molecular weight PAHs such as naphthalenes (two rings) and phenanthrenes (three rings) that are present in relatively high abundance in MCO have a lower CYP1a induction potential than the larger PAHs with four or five ring structures (Whyte *et al.*, 2000). The lack of EROD induction in MCO-exposed fish is likely due to the paucity of higher molecular weight compounds in the PAH profile of MCO.

Gill tissue HSP70 concentration was elevated in HFO-exposed fish (4.71 ± 0.64 pg/mg protein) and in MCO-exposed fish (4.86 ± 0.51 pg/mg protein) compared to negative control fish (3.87 ± 0.38 pg/mg protein) (Figure 1g), but this was not statistically significant (t-test, $p \geq 0.10$). HSP70 induction is a complex biological process (Morimoto, 1998), is not specific to petroleum hydrocarbons (Whyte *et al.*, 2000), and can be induced by several classes of compounds (e.g. PCBs).

2.4.4. Biomarkers of Effect

The levels of DNA damage (as 8-oxo-dG) did not change between test groups, with serum concentrations of 0.880 ± 0.047 ng/mg protein, 0.889 ± 0.043 ng/mg protein and 0.850 ± 0.039 ng/mg protein detected in negative control, MCO- and HFO-exposed fish respectively. PAHs and metals are among the causes of elevated serum 8-oxo-dG (Valvanidis *et al.*, 2009). The bioavailability of metals is a crucial factor in the mechanism of oxidative DNA damage from coal fly ash, rich in vanadium and nickel (Prahalad *et al.*, 2000). Although vanadium and nickel are present in HFO in small amounts (15.3 μg/g and 12.2 μg/g respectively), metals in crude oils are generally found in the asphaltene fraction complexed inside porphyrins (Biesaga *et al.*, 2000) and other metal porphyrins can exist (Woltering *et al*., 2016), and may not be bioavailable via the dietary route. This could explain the observed lack of difference in 8-oxo-dG between groups in our experiment.

AChE concentration in brain tissue homogenate decreased significantly (t-test, $p \leq 0.025$) in fish exposed to HFO (0.69 ± 0.05 ng/mg protein) and MCO (0.86 ± 0.05 ng/mg protein) compared to fish in the negative control group (0.90 ± 0.04 ng/mg protein) (Figure 1h).

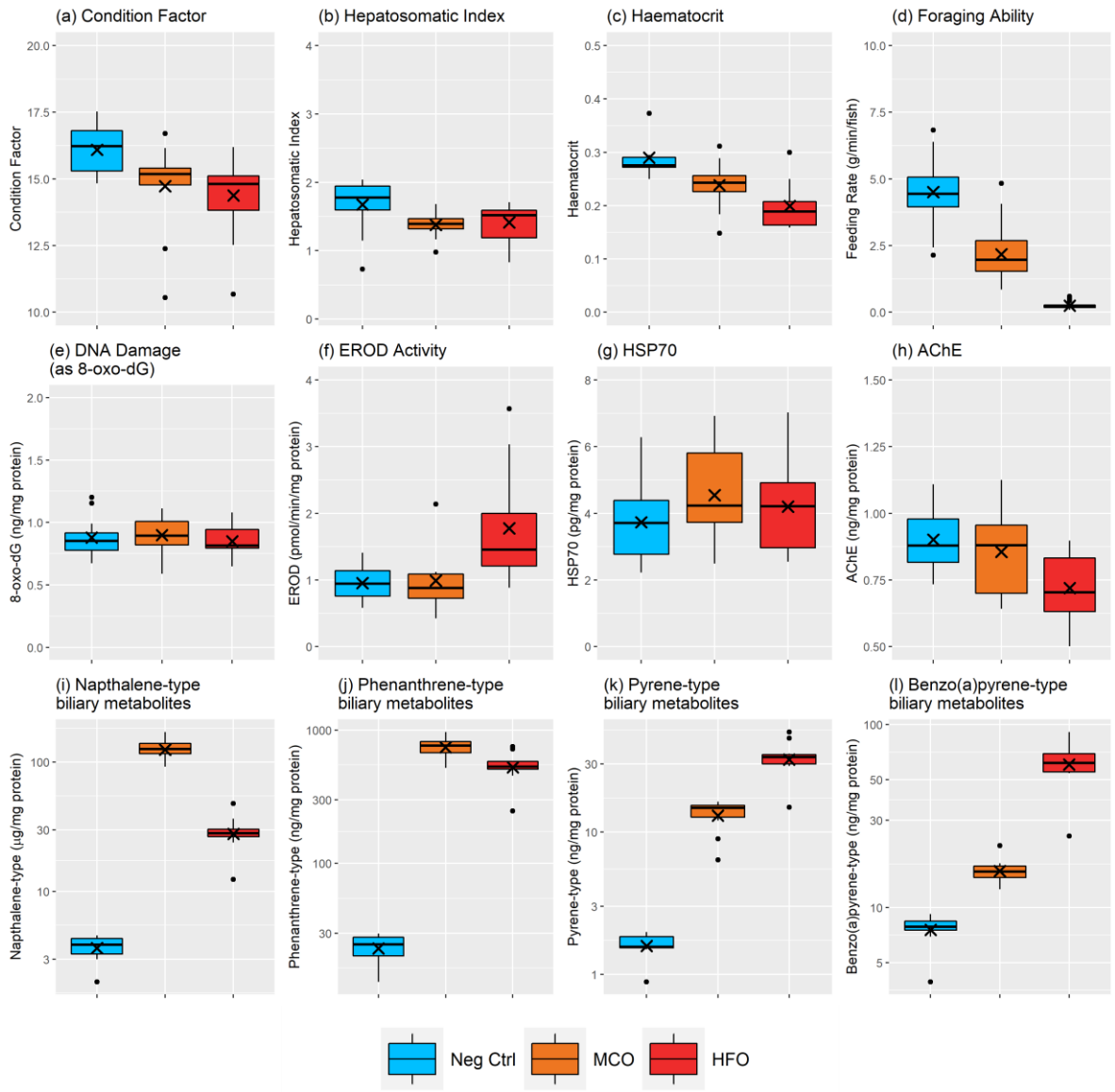


Figure 1: Boxplots of 12 biomarker responses of *Lates calcarifer* exposed to petroleum hydrocarbons. Lines are the median, the means are denoted by 'x', and dots are outliers.

* Indicates result statistically significantly different from negative control ($p \leq 0.05$).

2.4.5. Behavioral Changes

L. calcarifer are a known sportfish, and aggressively compete for food even in captivity. Fish in the negative control group had a mean feeding rate of 4.51 ± 0.10 g of food ingested/min/fish. Fish exposed to petroleum hydrocarbons exhibited significantly lower ($p < 0.001$) feeding rates of 2.17 ± 0.09 g/min/fish and 0.244 ± 0.01 g/min/fish for MCO- and HFO-exposed fish respectively (Figure 1d).

Anecdotally, fish exposed to HFO visually appeared intoxicated, slow swimming or immobile, and were slow to respond to stimuli. Similar observations have been reported in other fish species exposed to petroleum hydrocarbons (reviewed by Kasumayan, 2001; Weiss and Candelmo, 2012). Exposure to the WAF of fuel oils was reported to impair the ability of rainbow trout (*Oncorhynchus mykiss*) to successfully predate (Folmar *et al*., 1982), and greatly reduced the feeding rate of gobies (*Gobionellus boleosoma) (Greg et al., 1997). The present study demonstrates that dietary exposure also produces typical narcosis effects in barramundi.*

Among the various drivers of adverse behavioral impacts in fish, cholinesterase inhibition is an important mechanism driving behavioral pathology (Scott and Sloman, 2004). In the present study, an association appears to be present between lowered AChE and decreased feeding rate. This agrees with findings in other studies that suggest lowered AChE activity in response to toxicant exposure in mosquitofish (*Gambusia affinis*) is associated with decreased swimming speed (Rao *et al.*, 2005). In a laboratory setting, exposure to phenanthrene has been shown to cause reduced swimming speed and alter swimming patterns in guppies (*Poecilia vivipara)* (Torreira-Melo *et al.*, 2015). In the field, brown trout (*Salmo trutta)* swim slower in streams highly polluted with a complex mixture of toxicants including PAHs than in more mildly polluted streams (Triebskorn *et al.*, 1997).

There also appears to be a relationship in the present study between decreased haematocrit and reduced feeding rates. This agrees with other findings that decreased haematocrit and red blood cell count is associated with decreased swimming speed and predation activity in *L. calcarifer* (Satheeshkumar *et al., 2012)*, providing a second measure of a biological impact which might translate into reduced foraging ability in PAH-exposed fish.

2.4.6. Liver Histomorphology

Histomorphological analysis showed only very minor qualitative differences between test groups (Fig S1). Adipocytes were generally plump and clear in appearance, except for MCO-exposed fish which

were mildly collapsed. Hepatocytes were slightly smaller in size in MCO- and HFO-exposed fish compared to negative control fish. In both MCO and HFO test groups, zymogen granules were observed in 50% of the exocrine pancreas cytoplasm, compared to 50-70% in the negative control group.

If dietary exposure to crude oils caused hyperplasia (i.e. enlarged hepatocytes), a higher HIS would be expected, however in the current study the lack of variation in hepatocyte size was mirrored by the lack of variation in HSI. General indications of long-term toxicant exposure include toxicopathic liver lesions, and elevated macrophage immigration and the resulting macrophage aggregates (Guilio and Hilton, 2008). Toxicopathic liver lesions in English sole (*Pleuronectes vetulus)* were associated in a dose-dependent manner with sediment PAH concentrations and biliary PAH metabolites in a field survey of the Vancouver Harbour, Canada (Stehr *et al.*, 2004). It is possible that the trial exposure duration of 33 days was insufficient to cause significant histological changes.

2.4.7. Biomarker Baseline

The normal, or baseline, ranges for the measured suite of biomarkers, in healthy *L. calcarifer* not exposed to oils are presented in Table 2. The baseline ranges were defined as 2× the standard error of the mean value from the negative control group (OSPAR, 2013). Data on pre-exposure values for biomarkers are of critical importance in environmental impact studies attempting to estimate the adverse effects of an oil spill (Nunes *et al.,* 2015). In the aftermath of the Deepwater Horizon oil spill, the absence of pre-incident baseline data of fish health was an obstacle to fully assessing the long-term environmental impacts of the incident (e.g. Shigenaka, 2014; Murawski *et al.*, 2014).

A PCA of 11 of the biomarkers included in the study using Bray-Curtis distancing shows two principal components which represent 50.7% of the total variability of the combined biomarker dataset (Figure 2).

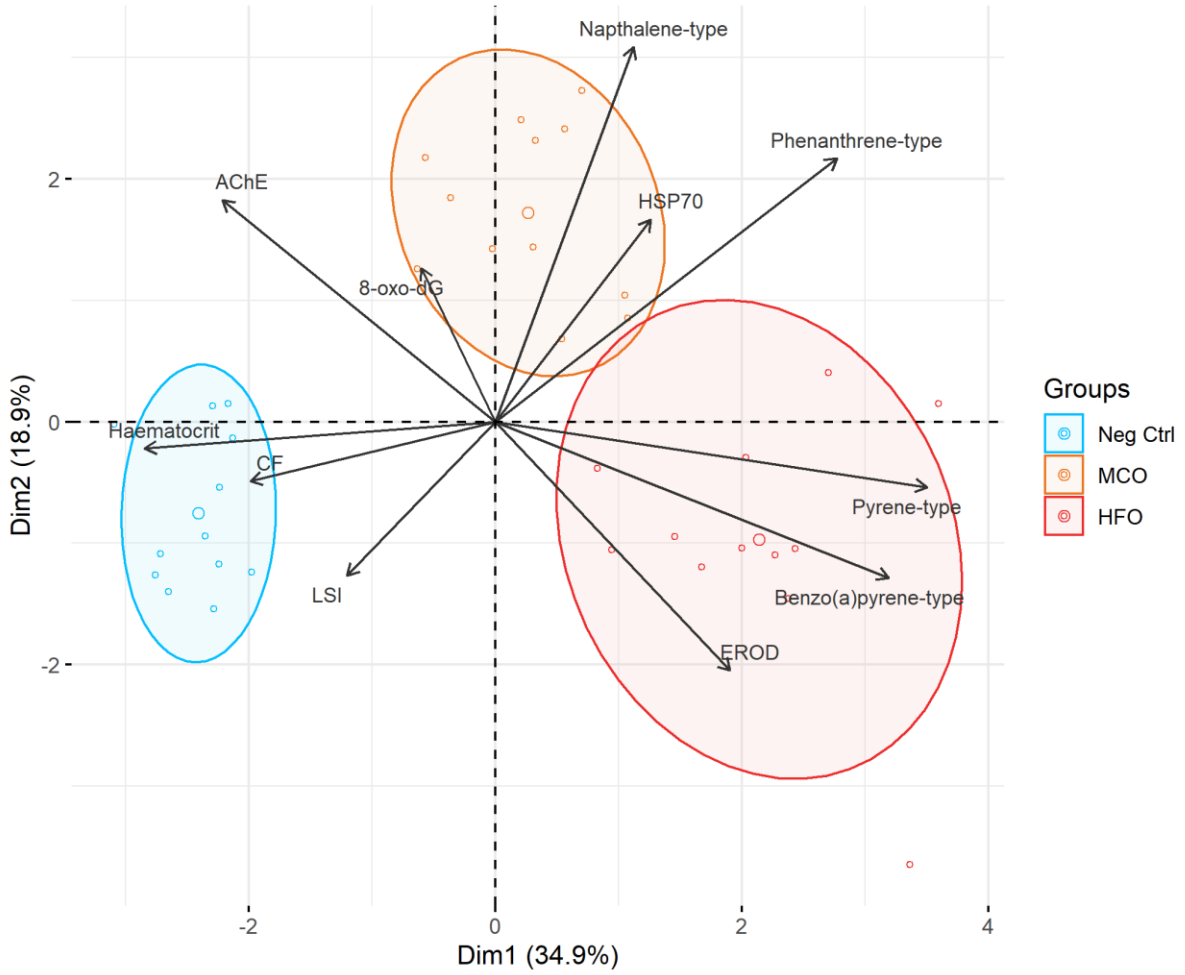
Table 2: Baseline values of 11 Biomarkers for healthy juvenile *Lates calcarifer.*

Biomarker	***Lates calcarifer* Baseline Range**
Condition Factor	15.58 - 16.61
Hepatosomatic Index	1.45 - 1.90
Haematocrit	0.25 - 0.33
Naphthalene-type Biliary Metabolites (μg/mg protein)	3.30 - 4.18
Phenanthrene-type Biliary Metabolites (μg/mg protein)	20.69 - 26.55
Pyrene-type Biliary Metabolites (μg/mg protein)	1.45 - 1.78
Benzo(*a*)pyrene-type Biliary Metabolites (μg/mg protein)	6.91 - 8.48
DNA Damage (as 8-oxo-dG) ng/mg protein)	0.78 - 0.97
AChE (ng/mg protein)	0.82 - 0.98
HSP70 (pg/mg protein)	2.95 - 4.51
EROD (pmol/min/mg protein)	0.80 - 1.11

2.4.8. Multivariate Analysis

The three treatment groups are significantly separated from each other (Tukey's HSD, $p < 0.05$), and their positions on the principal component axes are driven by different biomarkers. The positions of HFO-exposed fish are influenced by the presence of pyrene-type and benzo(a)pyrene type biliary metabolites, increased EROD activity, and lower AChE in brain tissue. MCO-exposed fish are influenced by naphthalene-type and phenanthrene type biliary metabolites, and by HSP70 concentration in gill tissue. The position of negative control fish is largely determined by higher condition factor and haematocrit, and an absence of other elevated biomarkers.

Biliary PAH metabolites, AChE concentration and EROD activation had the highest discriminatory power in describing the exposure and effects of petroleum hydrocarbon exposure in *L. calcarifer.* DNA damage (as serum 8-oxo-dG), HSI and HSP70 had the least. This agrees with findings in similar dietary petroleum hydrocarbon exposure studies in other species (Nahrang *et al. 2009).*



1227

1228 Figure 2: PCA biplot of biomarker profiles of L. calcarifer exposed to petroleum hydrocarbons.

1229

1230 The separation on the principal component axes is in accordance with the respective composition of
1231 the crude oils to which the fish were exposed. MCO has higher concentrations of naphthalenes and
1232 phenanthrenes and virtually no pyrenes or benzo(*a*)pyrenes, implying that it will be a poor inducer
1233 of Cyp1a enzymes such as EROD. In contrast, HFO has relatively low concentrations of two and three
1234 ring aromatics and greater concentrations of larger structures. In the absence of actual chemical
1235 analyses of an oil, general inferences can be made about the composition of the crude oils to which
1236 *L. calcarifer* were exposed, given the signature differences in biomarker responses of exposed fish.
1237 Following an oil spill it can be assumed that fish ill-health is related to the oil known to be spilled, but

it is possible that fish have been exposed to a different petroleum hydrocarbon source or other stressors. The integration of all biomarkers in a single PCA biplot may help to confirm or reject certain oils as sources for observed adverse effects on fish in an oil spill zone, and reinforce evidence that fish have been exposed to, and affected by, exposure to petroleum hydrocarbons.

Acknowledgements

Many thanks to Professor Steve Rowland for advice and insight in the writing of this manuscript, and to Rowan Kliendienst for trial exposure tank set up and sampling assistance.

Funding

This work was supported by an ARC-Linkage grant provided by the Australian Research Council (grant number LP170101000).

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Chapter 3: Multivariate analysis of otolith microchemistry can discriminate the source of oil contamination in exposed fish

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This article was published in the peer reviewed journal *Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology*, Volume 254, p. 109253. Francis Spilsbury, Bradley McDonald, Kai Rankenburg, Noreen J. Evans, Kliti Grice, Marthe Monique Gagnon. Discriminating source of oil contamination in teleost fish, *Lates calcarifer*, using multivariate analysis of a suite of physiological and behavioural biomarkers. Copyright Elsevier 2021.

Submitted to *Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology* 11th October 2021; Revised 26th November 2021, Accepted 18th December 2021, Published 29th December 2021.

https://doi.org/10.1016/j.cbpc.2021.109253

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

3.1. 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.

3.2. 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 4,750 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.

3.3. Methods

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

3.3.1. 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 ± 2 ^{o}C, dissolved oxygen > 5.0 mg/L, pH 7.6 ± 0.6, salinity of 32 ± 2ppt 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).

3.3.2. 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 µm beam and laser energy of 3.0 J 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 min^{-1}) and N_2 (1.2 mL 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 ^{43}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.

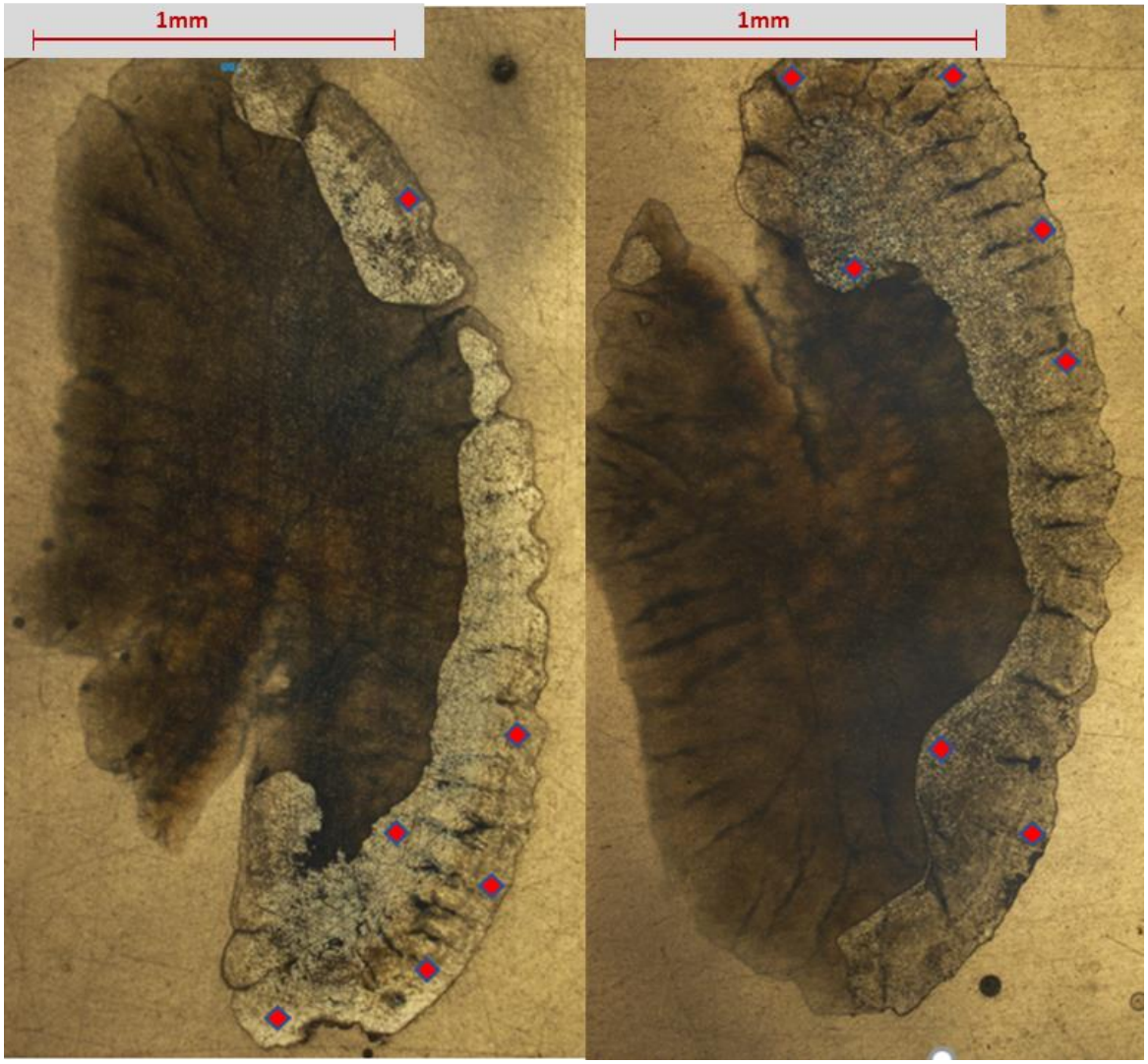

Figure 1: Light microscope (x40 objective) images of resin-mounted otoliths. Light areas are the distal otolith edge (most recent growth) exposed by grinding, the dark areas are those still embedded in resin. Red markings are targeting points for LA-ICP-MS.

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}B, ^{25}Mg, ^{27}Al, ^{29}Si, ^{34}S, ^{47}Ti, ^{51}V, ^{52}Cr, ^{55}Mn, ^{57}Fe, ^{59}Co, ^{60}Ni, ^{61}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{77}Se, ^{88}Sr, ^{89}Y, ^{90}Zr, ^{93}Nb, ^{95}Mo, ^{107}Ag, ^{111}Cd, ^{118}Sn, ^{121}Sb, ^{133}Cs, ^{137}Ba, ^{197}Au, ^{205}Tl, ^{208}Pb, ^{209}Bi, ^{232}Th and ^{238}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.

3.3.3. Metal Analysis of Crude Oils

A sample of each oil was accurately weighed and then repeatedly digested in nictric 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).

3.3.4. 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 conducting 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).

3.4. Results and Discussion

3.4.1. 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 ± 1.47 mg/kg), Ni (12.23 ± 0.71 mg/kg) and V (15.3 ± 0.9 mg/kg) compared to MCO (4.73 ± 1.85 mg/kg, 0.07 ± 0.06mg/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 ± 8.98 mg/kg) and Ba (1.32 ± 0.08 mg/kg) compared to MCO (10.23 mg/kg and 0.11mg/kg respectively).

3.4.2. 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*.

		Metals in Crude Oils (mg/kg)*		Metals in otolith (mg/kg) §		
	Metal	**MCO**	**HFO**	**Control**	**MCO**	**HFO**
Al	Aluminium	10.23 ± 10.23	15.44 ± 8.98	0.004 ± 0.003	0.057 ± 0.040	0.170 ± 0.085
Ag	Silver	0.000	0.000	0.000	0.000	0.000
As	Arsenic	0.000	0.041 ± 0.008	0.120 ± 0.012	0.231 ± 0.007	0.090 ± 0.008
Ba	Barium	0.113 ± 0.072	1.311 ± 0.078	10.26 ± 0.29	11.63 ± 0.38	13.93 ± 0.86
Cd	Cadmium	0.004 ± 0.003	0.000	0.000	0.000	0.000
Co	Cobalt	0.000	1.430 ± 1.116	0.001 ± 0.000	0.002 ± 0.001	0.001 ± 0.000
Cr	Chromium	0.298 ± 0.290	0.243 ± 0.131	1.006 ± 0.010	0.949 ± 0.008	0.928 ± 0.015
Cu	Copper	0.150 ± 0.150	0.000	0.371 ± 0.146	0.196 ± 0.016	0.189 ± 0.023
Fe	Iron	4.730 ± 1.854	37.90 ± 1.47	14.29 ± 0.24	11.82 ± 0.20	10.28 ± 0.11
Mg	Magnesium	1.197 ± 0.944	1.800 ± 0.468	24.63 ± 1.21	23.69 ± 1.18	30.85 ± 2.71
Mo	Molybdenum	0.000	0.052 ± 0.003	0.000	0.000	0.000
Ni	Nickel	0.070 ± 0.039	12.23 ± 0.71	1.284 ± 0.030	1.111 ± 0.045	0.939 ± 0.031
Pb	Lead	0.083 ± 0.03	0.042 ± 0.17	0.032 ± 0.031	0.001 ± 0.000	0.001 ± 0.001
S	Sulfur	393.6 ± 36.3	10250 ± 850	277.8 ± 11.8	235.9 ± 7.0	198.3 ± 6.1
Sb	Antinomy	0.000	0.459 ± 0.19	0.001 ± 0.000	0.000	0.000
Se	Selenium	0.061 ± 0.036	0.007 ± 0.007	0.016 ± 0.007	0.008 ± 0.004	0.022 ± 0.013
Sn	Tin	0.117 ± 0.103	0.128 ± 0.057	0.004 ± 0.003	0.001 ± 0.000	0.003 ± 0.002
Sr	Strontium	0.226 ± 0.191	0.432 ± 0.152	1533 ± 54	1585 ± 44	1548 ± 46
Ti	Titanium	0.000	3.240 ± 0.127	0.000	0.004 ± 0.004	0.002 ± 0.002
V	Vanadium	0.000	15.27 ± 0.87	0.000	0.000	0.000
Zn	Zinc	1.473 ± 0.117	1.194 ± 0.126	0.379 ± 0.053	0.320 ± 0.021	0.376 ± 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
§ 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 ± 0.12mg/kg and 1.19 ± 0.13 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 ± 0.08 mg/kg in HFO-exposed fish, which was higher than in control fish at 0.003 ± 0.003 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 ± 0.04 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 ± 0.38 mg/kg and 13.93 ± 0.86 mg/kg respectively, significantly higher (ANOVA, $p < 0.009$) than control fish with 10.26 ± 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 μg/kg in Indian Ocean surface seawater (Jeandal *et al,* 1996) may reasonably account for the high Ba concentration detected in otoliths of control fish.

Porphryin-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 porphyrin-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 ± 1.18 mg/kg) and HFO (30.85 ± 2.71 mg/kg), which although high, were

not significantly different (ANOVA, $p < 0.020$) from Mg detected in control fish (24.63 ± 1.21 mg/kg). Although Mg is present in both MCO (1.20 ± 0.94 mg/kg) and HFO (1.80 ± 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, $p = 0.61$).

3.4.3. 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 12th 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).

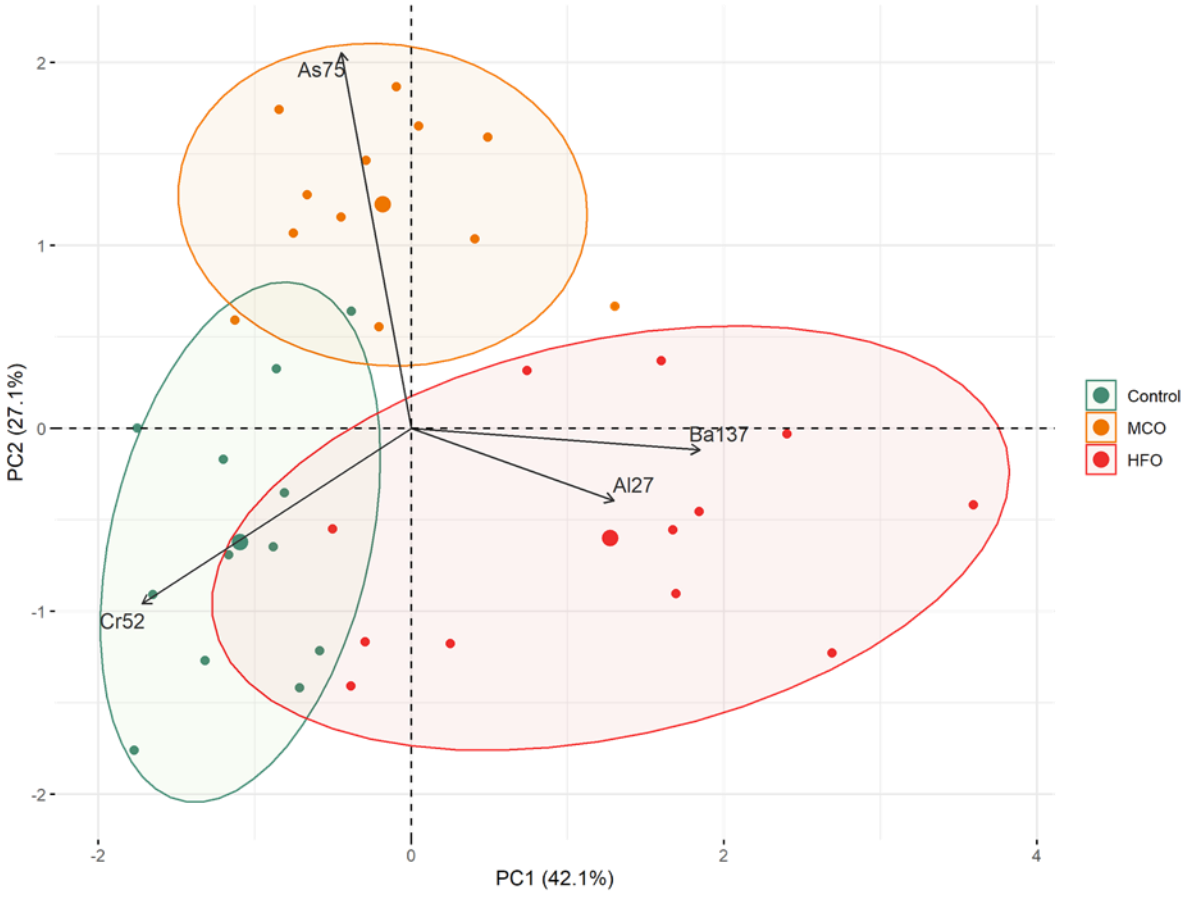

Figure 2: Principal components analysis (PCA) of four otolith metal levels in L. calcarifer exposed via the dietary route to Montara crude oil (MCO), or to heavy fuel oil (HFO).

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 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.

3.5. 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.

Acknowledgements

Many thanks to Rowan Kliendienst for trial tank set up and sampling assistance.

PPTEPAA Pty Ltd greatly assisted in the procurement of the crude oils used in this study.

Research in the John de Laeter Centre GeoHistory Facility is enabled by AuScope (auscope.org.au) and the Australian Government via the National Collaborative Research Infrastructure Strategy (NCRIS).

Funding

This work was supported by an ARC-Linkage grant provided by the Australian Research Council (grant number LP170101000).

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Chapter 4: Gut microbiome as a potential biomarker in fish – dietary exposure to petroleum hydrocarbons and metals, metabolic functions and cytokine expression in juvenile *Lates calcarifer*

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In review. Submitted to *Frontiers in Microbiology:* 1st December 2021

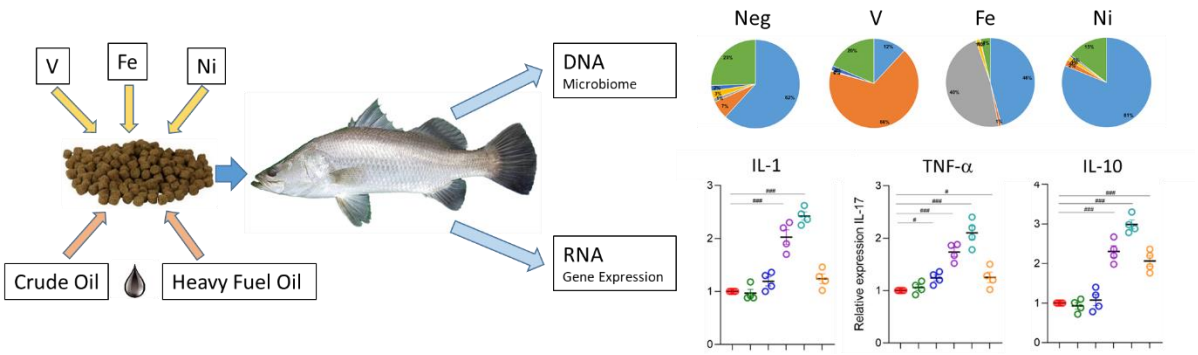
Keywords

Barramundi, ecotoxicology, metals, crude oil, gut microbiome, cytokines, bioinformatics.

Highlights

- Fish exposed to dietary metals and crude oils exhibit changes in the gut microbiome
- *Photobacterium* identified as potential biomarker genus for high m.w. PAH exposure
- V, Ni and Fe enriches phyla Firmicutes, Bacteroidetes and Proteobacteria
- Microbiome diversity reduced by dietary metals, but not by petroleum hydrocarbons
- IL-1, IL-10 and TNF-α expression increased after exposure to metals, heavy fuel oil

Graphical Abstract



4.1. Abstract

The gut microbiome of fish contains core taxa whose relative abundances are modulated in response to diet, environmental factors, and exposure to toxicogenic chemicals, influencing the health of the host fish. Recent advances in genomics and metabolomics have suggested the potential of microbiome analysis to be a biomarker for exposure to toxicogenic compounds.

In this 35-day laboratory study, 16S RNA sequencing and multivariate analysis was used to explore changes in the microbiome of juvenile *Lates calcarifer* exposed to dietary sub-lethal doses of three metals: vanadium (20mg/kg), nickel (480mg/kg) and iron (470mg/kg); and to two oils : bunker C heavy fuel oil (1%w/w), and Montara, a typical Australian medium crude oil (1%w/w).

Diversity of the gut microbiome was significantly reduced compared to negative controls in fish exposed to metals, but not petroleum hydrocarbons. The core taxa in the microbiome of negative control fish was comprised of phyla Proteobacteria (62%), Firmicutes (7%), Plantomycetes (3%), Actinobacteria (2%), Bacteroidetes (1%) and others (25%). Differences in the relative abundances of bacterial phyla of metals exposed fish were pronounced, with the microbiome of Ni-, V- and Fe-exposed fish dominated by Proteobacteria (81%), Firmicutes (68%) and Bacteroidetes (48%) respectively. The genus *Photobacterium* was enriched proportionally to the concentration of polycyclic aromatic hydrocarbons (PAHs) in oil-exposed fish. The probiotic lactic acid bacteria, *Lactobacillus* was significantly reduced in the microbiota of fish exposed to metals.

Transcription of cytokines IL-1, IL-10 and TNF-α were significantly up-regulated in fish exposed to metals, but unchanged in oil exposed fish compared to negative controls. However, IL-7 was significantly down-regulated in fish exposed to V, Ni, Fe and heavy fuel oil.

Fish gut microbiome exhibits distinctive changes in response to specific toxicants, and shows potential for use as biomarkers of exposure to V, Ni, Fe and to PAHs present in crude oil.

4.2. Introduction

The microbiome of the gastrointestinal tract in plays an important role in maintaining the overall health of fish (Hoseinifar *et al*, 2019), including bi-directional biochemical interactions that influence the immune system (Gomez *et al*, 2008; Xia *et al*, 2014; *Adomovsky et al. 2018).* The "typical" makeup of the fish gut microbiome is comprised of core taxa of bacteria predominantly from the phyla *Proteobacteria*, *Firmicutes, Actinobacteria,* Fusobacteria and *Bacteroidetes* (Cahill, 1990; Gomez *et al*, 2008; Roeselers *et al, 2011;* Ganbari *et al*, 2015; Adomovsky *et al, 2018*). The relative abundance of genera present in the gut microbiome varies greatly however between species of fish (Givens *et al*, 2015; Edgerton *et al*, 2018; Nikouli *et al*, 2021), and between individuals within a species (Burke *et al*, 2011).

Trophic level, and thereby diet, is the predominant factor influencing the relative abundances of phyla present in the microbiome of fish (Estruch *et al*, 2015; Talwar *et al*, 2018; Edgerton *et al*, 2018; reviewed by Legrand *et al, 2020*). *Lates calcarifer* (Barramundi or Asian-seabass) is a popular sports-fish and is a common aquaculture species farmed throughout Asia. Dietary studies have established the relative prevalence of taxa in the microbiome of *Lates calcarifer* (Gupta *et al*, 2020; Xia *et al,* 2014*)*, which is generally similar to that found in other comparable species of carnivorous fish (Edgerton *et al*, 2018). Changes in diet result in a change in the relative abundance of dominant genera in the gut microbiome in *L. calcarifer* (Gupta *et al,* 2020) and other fish species (Edgerton *et al,* 2018; Ringø *et al, 2016;* Estruch *et al*, 2015; Xia *et al*, 2014).

Exposure to anthropogenic toxicants such as metals and petroleum hydrocarbons also alter the gut microbiome in fish, as illustrated by field studies following the DWH spill (Brown-Peterson *et al*, 2015) and a riverine oil spill in Saskatchewan, Canada (DeBofsky *et al*, 2020). Laboratory studies have shown that red bream (*Pagrus major*) exposed to phenanthrene produce significant changes in gut microbiome (Hano *et al*, 2021). Similarly, benthic microbial communities exhibit a profile shift

following exposure to mixtures of benzo(*a*)pyrene and fluorene (Kahla *et al*, 2021). Perhaps more ecologically relevant, exposing flounder (*Paralichthys dentatus*) to WAF generated from crude oil from the DWH spill has similarly shown to produce significant changes in the relative abundances of bacterial genera of the gut microbiome (Amendola-Pimenta *et al*, 2020).

The gut microbiome plays a significant role in the overall metabolic outcomes of a host organism challenged by environmental toxicants. For example, bacterial metabolism assists the host fish in the detoxification of ammonia (Turner and Bucking, 2019). The gut microbiome of farmed *Scophthalmus maximus* (turbot) contain genes for heavy metal resistance, and exhibit a functional emphasis of iron uptake and metabolism (Xing *et al*, 2013). Vanadium nitrogenase facilitates an alternative pathway for nitrogen fixation (Gustafsson, 2019), utilized by *Pseudomonas* and *Cyanobacter* species among others (Lyalkova and Yurkova, 1992; Pessoa *et al*, 2015). *Lactobacillus*, a lactic acid producing bacteria used as a probiotic in aquaculture, is associated with improved resilience against bacterial and viral pathogens (Collins, 2019; He *et al*, 2017) and moderates the effects of lead (Giri *et al*, 2018) and cadmium (Zhai *et al*, 2017) exposure.

Crude oils are highly complex mixtures of compounds which may enter food webs in the event of a spill (Buskey, 2016), and subsequently biomagnify in species of exposed fish to levels as high as 2.2% w/w (Sammarco, 2013). Persisting in the environment for several years post-release (Boehm, 2008), petroleum hydrocarbons are retained in the tissues of fish for months after exposure has ceased (Cravedi and Tuillez, 1986), and ecotoxicological biomarkers indicating continued exposure remain elevated months after an oil spill (Smeltz et al, 2017).

In contaminated environments, bacterial communities shift towards those resistant taxa that are able to metabolize or sequester toxicants. For example, microbial communities in oil-contaminated soils contain PAH-metabolizing bacteria (Zafra *et al,* 2014; Lee *et al,* 2018; Haritash, 2020), and bacterial communities in vanadium contaminated soils were found to be dominated by Bacteroidetes, Proteobacteria, Actinobacteria and Firmicutes (Zhang *et al*, 2018, 2019; Lu *et al*,

2019), all of which are core taxa found in abundance in the gut microbiome of many species of fish. It seems likely that the gut microbiome of fish exposed to toxicants such as PAHs or metals may become dominated by those taxa able to metabolise those contaminants, and thereby reduce the toxic burden on the host organism by co-metabolisation.

Changes to the gut microbiome in response to toxicants may reduce the community complexity (deBofsky *et al*, 2021), alter the metabolic outcomes of the bacterial communities present (Adomovsky *et al*, 2018), and may have a use in ecotoxicological fingerprinting to identify classes of anthropogenic toxicants to which organisms are exposed (Adomovsky *et al*, 2018; Walter *et al,* 2019).

Here we present the analysis of the gut microbiome of *L. calcarifer* exposed via diet to a bunker C heavy fuel oil (HFO), to a typical Australian medium crude oil (ACO), and to three mixtures of a selection of petroleum hydrocarbons enriched with sub-lethal doses of vanadium, nickel and iron respectively. Non-metric multidimensional scaling (nMDS) analysis was used to differentiate the microbiome community profiles of the various exposure groups, and comparative analyses of dominant phyla and genera were used alongside cytokine gene expression in the gut microbiome to ascertain the suitability of fish gut genomics as a potential ecotoxicological biomarker.

4.3. Materials and Methods

4.3.1. In-vivo Fish Exposure and Sampling

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

Juvenile fish (n = 56; 10-15 cm in length; mean weight 85 ± 2 g) were obtained from a local commercial hatchery. Following a 5-day acclimatization to 32 ppt saline conditions, fish were placed in tanks containing 100 L of natural Indian Ocean seawater sourced north of Perth, Australia with four fish per tank. A static-renewal design was used with a 12-hour light/dark interval. Water quality

2129 was maintained at 28 ± 2°C, 32 ± 4 ppt salinity, pH 7.6 ± 0.6, dissolved oxygen > 5.0 mg/L and total
2130 ammonia < 2.0 mg/L, assisted by Astro 2212 external canister biofilters with a flow rate of
2131 approximately 5 L/min and up to 50% water exchanges as required.

2132 Fish were fed 2% bodyweight per day commercial fishmeal (Nova FF, Skretting Pty Ltd, Perth,
2133 Australia), in-line with similar exposure trials (e.g. Hellou and Leonard, 2004). Due to a paucity of
2134 ecotoxicological data specifically for *L. calcarifer*, the sub-lethal dosage of metals and individual
2135 petroleum hydrocarbons was estimated using published NOEC (no observed effect concentration)
2136 data for mortality of other fish species (Hilton and Bettger, 1988; Ptashynski *et al*, 2002; Craig *et al*,
2137 2009; US EPA, 2019).

2138 Fish in the negative control group were fed unaltered fishmeal (n=12). Fish in the petroleum
2139 hydrocarbon test groups were fed fishmeal spiked with 1% w/w ACO (n=12), or fishmeal spiked with
2140 1% w/w HFO (n=12). An additional three groups were fed fishmeal enriched with a small amount of
2141 a mixture of aromatic and saturated petroleum hydrocarbons (total petroleum hydrocarbons
2142 approximately 25mg/kg) and either 20 mg/kg vanadium (V) (n=4), 470 mg/kg iron (Fe) (n=8) or
2143 480mg /kg nickel (Ni) (n=8). The detailed composition of fishmeal given to each treatment group is
2144 summarized in Tables 1, S1, S2 and S3.

2145 Fish were exposed for a total of 33 days, followed by a 2-day depuration period before euthanasia
2146 using the ike-jime technique. The intestinal tract was removed, stripped using Teflon tweezers, and
2147 whole gut contents collected in 2mL cryovials that were immediately frozen in liquid nitrogen and
2148 then stored at -80°C until analysis.

2149 An outline of study design is presented in Figure S1.

2150

4.3.2. PAH and Metals Analysis of Fish Feed

4.3.2.1. Polycyclic Aromatic Hydrocarbons

Fish feeds used in the trial were analysed for a suite of 38 PAHs by a commercial consultant laboratory (ChemCentre, Perth, Australia) using standard published methods (Forth *et al*, 2017). Analyses were performed in triplicate.

Briefly, an internal standard was added to precisely weighed samples of fish feeds, and an extraction was performed by sonication in acetone/dichloromethane, followed by chemical drying with sodium sulphate. Quantitation by GC-MS (SIM) was against a commercially available standard (AccuStandard, Connecticut, U.S.A.). The response factors of the respective parent PAH were used to quantitate alkylated-PAHs (Forth *et al*, 2017).

4.3.2.2. Metals Analysis

Metals in crude oils were quantified by ICP-AES and ICP-MS by a commercial laboratory (TSW Analytical, Perth, Australia). Analyses for a suite of 61 metals were performed in triplicate. Briefly, an accurately weighed sample of oil was digested in nitric acid repeatedly, and then finally in a mixture of nitric and perchloric acid. Once taken to incipient dryness, the digestate was re-dissolved in nitric acid, hydrochloric acid and high purity water. Quantification was performed against a commercially available standard (AccuTrace High Purity multi-element standards, Choice Analytical).

4.3.3. Microbiome Analysis

4.3.3.1. Collection and processing of samples

Intestinal contents samples for microbiome analysis were taken from fish from each test group: negative control (n=12), ACO (n=12), HFO (n=12), V-enriched (n=4), Fe-enriched (n=8) and Ni-enriched (n=8) diets. Concurrently, samples of feed (n=4) were collected randomly from each of the six dietary test groups, and seawater samples (n = 6) were collected before the start of the trail from the marine water supply chain. The gut samples of fish were collected inside a biosafety cabinet. Precisely 200 mg of gut and feed samples with 100 µl of DEPC-treated water were homogenized using a tissue lyser (Qiagen, Hilden, Germany) with beads. The water samples were concentrated first by centrifugation at 8,000*g* for 10 minutes in 50 ml fresh falcon tubes, the process being

repeated four times before filtration in 0.2-µm polycarbonate filters. The filters were then cut into small pieces (~1 mm) inside a biosafety cabinet and transferred into 2-ml Eppendorf tubes.

4.3.3.2. DNA extraction and PCR amplification of 16S rRNA gene

Bacterial DNA from 86 processed samples (56 gut, 24 feed and 6 water) was extracted using DNeasy Power Soil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quantity and quality of DNA were checked using a NanoDrop Spectrophotometer 2000c (Thermo Fisher Scientific, Waltham, MA, USA). A final DNA concentration of 50 ng/µl was achieved by dilution. PCR amplification of V3V4 hypervariable regions of bacteria was performed according to the Illumina 16S metagenomic sequencing protocol (Part # 15044223 Rev. B). The 50 µl of PCR master mix was prepared by mixing 2 µl of template DNA (50 ng/µl), 1 µl each of forward and reverse primers, 25 µl of Hot Start Taq 2X Master Mix (New England BioLab Inc., USA), and 21 µl DEPC-treated water. A total of 35 cycles of amplification were performed in a S1000 Gradient Thermal Cycler (Bio-Rad Laboratories, Inc., Foster City, California, USA). Beads purification, amplicon barcoding, and pooling were performed according to the Illumina 16S standard protocol (Part # 15044223 Rev. B). Sequencing was performed with Illumina MiSeq platforms (Illumina Inc., San Diego, California, USA) using a MiSeq reagent kit (600 cycles, Part # MS-102-3003).

4.3.3.3. Processing of Illumina reads

The initial quality of raw fastq sequences was checked in FastQC (Andrews 2010), multiQC (Ewels et al. 2016) and Micca stat (Albanese et al. 2015). Trimming of low quality reads and removal of adapter sequences were performed using BBduk (Bushnell 2014) with the following parameters: qtrim=r, trimq=20, ktrim=r, k=23, mink=11, hdist=1, minlen=200, tpe, tbo. The merging, filtering, de-duplicating (fastq-uniques) and picking of amplicon sequence variants (ASVs) was performed in a USEARCH pipeline by implementing UPARSE and UNOISE3 (Edgar 2010, 2013, 2016a). The final set of ASVs was filtered for chimeras using UCHIME2 (Edgar 2016b). UNOISE3 flow was used to map all the merge reads to a non-chimeric ASVs table. Each representative ASV was assigned to different taxa levels against the SILVA 132 release (Quast et al. 2013). Multiple sequence alignment was performed

using micca_msa followed by a rooted phylogenetic tree was construction in micca_rooted_tree (v1.7.0) (Albanese et al. 2015). Each sample of gut, water and detritus was set to a uniform depth of 7495 bp for the calculation of alpha-beta diversity and microbial community composition.

4.3.3.4. Downstream bioinformatics

Alpha diversity regarding richness, Fisher Alpha, Shannon and Simpson indices were calculated in microbiomeSeq (https://github.com/umerijaz/microbiomeSeq). Non-metric multidimensional scaling (nMDS) was used to display beta-ordination in terms of Bray-Curtis dissimilarity of relative abundance. Relative abundance of bacteria at phylum and genus level were calculated in phyloseq (McMurdie and Holmes 2013), vegan (Dixon, 2003) and ampvis2 (Andersen et al. 2018) R packages. Metagenome prediction from the 16S rRNA AVS dataset was performed using Picrust2 algorithm in support of KEGG pathways descriptions (Douglas et al. 2020).

4.3.4. Gene expression analysis

Based on recent studies on genes expression analysis (Chaklader et al. 2021; Gupta et al. 2020; Siddik et al. 2020) of *L. calcarifer* after feeding trials and immune-related transcriptome analysis, four pro- and anti-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukins IL-1, IL-8, IL-10 and IL-17 were tested for their relative expression in real-time PCR. For the gene expression, gut samples (n = 4) from each group were preserved in RNA-later according to manufacturer's instructions and stored at -80^{o}C until processing. Samples were thawed on ice followed by RNA extraction using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) after following manufacturer's instructions for tissue samples. Digestion of DNA and removal of enzymes was performed using TURBO DNA-free^{TM} kit (Thermo Fisher Scientific, USA). RNeasy MiniElute Clean Up kit (Qiagen, Hilden, Germany) was used for the purification of RNA. Quality of extracted RNA was checked using 1% agarose gel. The RNA concentration was measured in a Qubit 4 fluorometer (Thermo Fisher Scientific, USA) and NanoDrop Spectrophotometer 2000c (Thermo Fisher Scientific, Waltham, MA, USA). The presence of any DNA inhibitors was checked further with PCR amplification of bacterial universal 16S, 27F and 1492R. The first strand cDNA was synthesized using SuperScript^{TM}

IV First-Strand Synthesis System (Thermo Fisher Scientific, USA). Quantitative real-time PCR was performed using PowerUp™ SYBR Master Mix (Thermo Scientific, USA) and gene primers with CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Inc., USA). The relative expression level of each gene was calculated using the $2^{-\Delta\Delta CT}$ method, following normalisation against the β-actin reference gene (Livak and Schmittgen, 2001).

4.3.5. Statistical analysis

One-way ANOVA followed by Tukey's HSD was used to compare alpha diversity among the groups. Non-parametric statistical analysis of the distance metric was performed using ANODIS with 1000 permutations. Differential abundance of microbial communities at genus level was calculated using the Kuskall-Wallis test followed by a Dunn post-hoc with Bonferroni adjustment.

Significantly altered metabolic pathways were identified by linear discriminant analysis (LDA) with stringent LDA cut-off value of ≥ 4.0 used to compare the functional features of microbial compositions. At all stages, p-value of < 0.05 was considered statistically significant. The "Pearson" correlation coefficient of taxa abundance and dietary variables were calculated using the microbiomeSeq R package.

4.4. Results and Discussion

4.4.1. Characterization of Oils

The two oils we have chosen for this study are chemically very different. HFO is highly sulfurous (102 mg/kg) compared to ACO (3.9 mg/kg) (Tables 1, S2). The PAH profiles of the two oils are also dissimilar: ACO has higher levels of bicyclic aromatics (491 mg/kg) compared to HFO (245 mg/kg), similar levels of tricyclic aromatics (160 mg/kg and 150 mg/kg respectively), and lower levels of the higher molecular weight tetracyclic (4.5 mg/kg and 29 mg/kg respectively) and pentacyclic aromatic compounds (0.87 and 19 mg/kg respectively). In all crude oils, the concentration of metals varies greatly (Yasnygina *et al*, 2006; Pereira *et al*, 2010). Metals of note present in HFO are iron (37.9 mg/kg), vanadium (15.7 mg/kg), nickel (12.2 mg/kg), cobalt (2.15 mg/kg) and zinc (1.19 mg/kg). ACO

contains less iron (4.73 mg/kg), nickel (0.11 mg/kg) and no vanadium or cobalt. ACO contains slightly lower amounts of aluminum (10.2 mg/kg) than HFO (15.4 mg/kg) and similar low levels of tin (0.18 mg/kg and 0.13 mg/kg respectively). The dosage of PAHs and metals in the fish feeds used in the study are summarized in Table 1.

Table 1: Toxicant additives in fish feed[§]

	Compound	Neg Control Fish Feed (mg/kg)	ACO Fish Feed (mg/kg)	HFO Fish Feed (mg/kg)	V-Enriched Fish Feed (mg/kg)	Fe-Enriched Fish Feed (mg/kg)	Ni-Enriched Fish Feed (mg/kg)
PAHs	Total bicyclic aromatics*	0.00	491	245	3.00	2.17	2.47
	Total tricyclic aromatics*	0.00	160	150	4.90	3.37	0.57
	Total tetracyclic aromatics*	0.00	4.50	29.0	3.37	0.00	0.00
	Total pentacyclic aromatics*	0.00	0.87	19.0	0.33	0.00	0.00
	Total PAH	**0.00**	**656**	**443**	**11.6**	**5.50**	**3.00**
Metals	Sulfur	0.00	3.90	102	0.00	270	260
	Vanadium	0.00	0.00	0.15	19.4	0.00	0.00
	Iron	0.00	0.00	0.38	0.00	470	0.00
	Nickel	0.00	0.00	0.12	0.00	0.00	480
	Aluminium	0.00	0.31	0.15	0.00	0.00	0.00
	Tin	0.00	0.18	0.13	0.00	0.00	0.00

*Total bicyclic aromatics include naphthalene, dibenzothiophene and any alkylated versions thereof.
*Total tricyclic aromatics include phenanthrene, retene. fluoranthene and any alkylated versions thereof.
*Total tetracyclic aromatics include pyrene, chrysene, benz(*a*)fluoranthene, benzo(b,k)anthracene and any alkylated versions thereof.
*Total pentacyclic aromatics include benzo(*a*)pyrene, indenopyrene and dibenzoanthracene and any alkylated versions thereof.
[§]See Tables S1, S2 and S3 for full details.

4.4.2. Sequence statistics and alpha-beta diversity measurements

A total of 5.6 million quality reads were obtained from 86 samples of fish feed, tank water and gut samples. For gut samples alone, 4.2 million quality reads were obtained. The reads were classified into 6104 ASVs, 14 phyla and 478 genera. In the gut, 6104 ASVs were classified into 14 phyla and 336 genera. The average number of reads (52,486.2 ± 14,592.8), Good's coverage index (0.997 ± 0.001), and rarefaction curve (Figure S1) indicated that each sample was sequenced at high depth to capture maximum bacteria at various taxa levels.

The water and feed had significantly higher bacterial diversity (Figure 1A) and composed of completely different bacterial groups compared to the gut microbiome, as revealed in beta-ordination (Figure 1B). Rearing water harbor diverse and different groups of bacteria (Qin et al. 2016), suggesting a little or no correlation to the fish gut bacterial communities (Giatsis et al. 2015; Zeng et al. 2020). Microbiome bacteria are determined early in the life of fish (Talwar *et al.* 2018), and gut bacteria are influenced by diet (Nguyen *et al.* 2021; Parata *et al.* 2020; Serra *et al.* 2021). Hence the shift of microbial communities in the gut of fish in this study primarily arise from the diets used to feed juvenile *L. calcarifer*.

Based on the weighted (relative abundance) UniFrac metric, analysis of gut bacteria from the six different test groups revealed significant reduction of bacterial diversity in the gut of fish exposed to V, Ni and Fe, whereas no differences were observed in the groups fed crude oil compared to negative control fish fed the unaltered diet (Figure 1C). The alpha–diversity including microbial richness or species diversity and Fisher-alpha in the gut of fish fed the V-enriched diet was significantly lower compared to the negative control group ($p < 0.001$). The Fisher alpha diversity index of V-enriched diet fish was also significantly less than negative controls. The Shannon diversity index, generally a better predictor when the sample size is a large proportion of the whole population (Beck and Schwanghart, 2010), showed that the microbiome of fish fed the Ni- and Fe-enriched diets were also significantly less diverse relative to the negative control group ($p < 0.005$). Centroid analysis of beta-dispersion showed distinctly different bacteria in Fe-enriched diet groups in both weighted and unweighted UniFrac metrics compared to other groups in present study (Figure 1D).

Legrand *et al.* (2020) showed that the fish microbiome diversity decreases with the progression of gut enteritis. The decrease of diversity and dissimilar microbes in the present study (Figure 1C, Table S4) may indicate reductions in the overall gut health of fish challenged with metal-enriched diets.

Further research on histological changes experiences by the intestinal tissues would be required to confirm this.

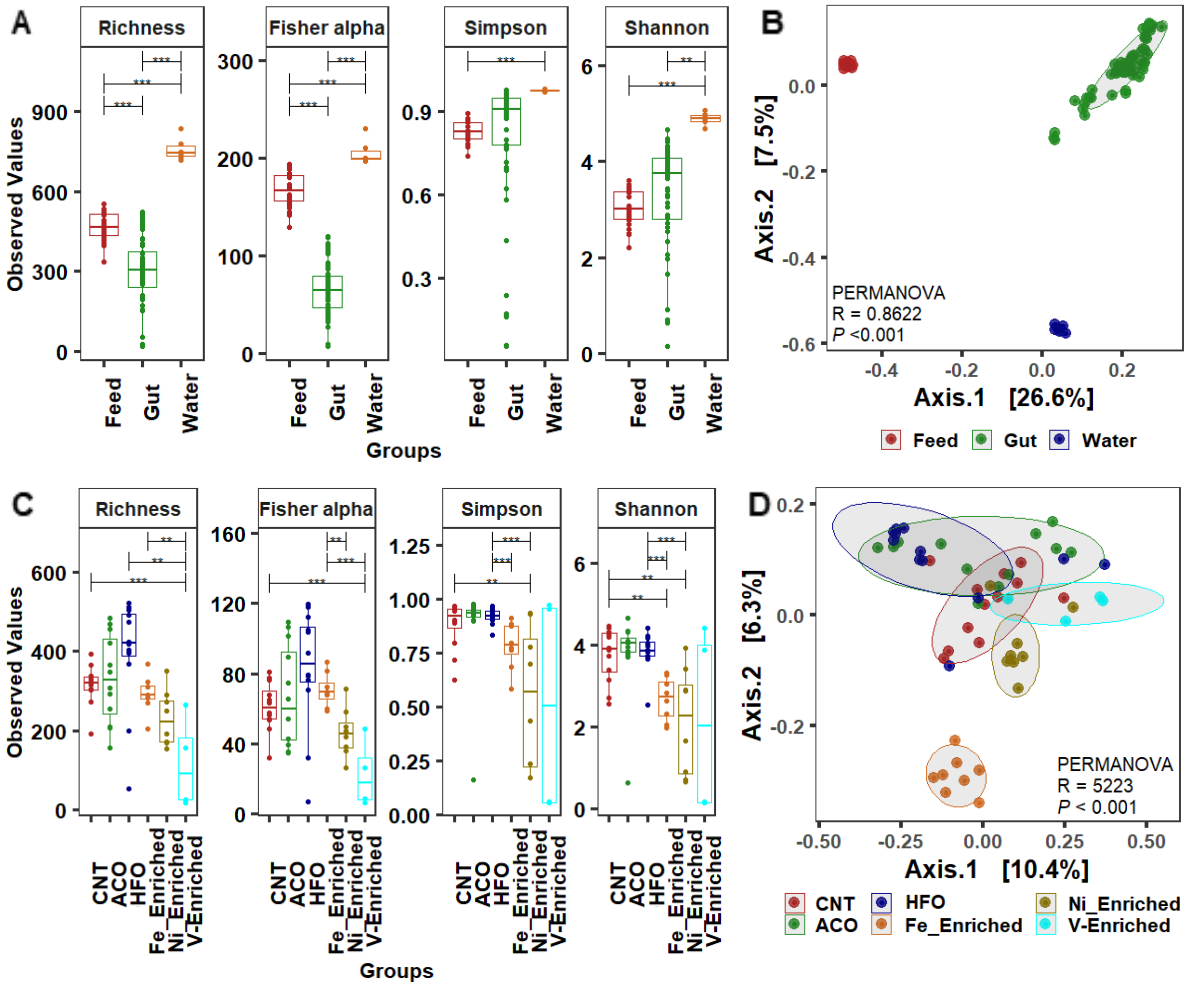


Figure 1. Alpha-beta diversity of bacterial community.
(A) Alpha-diversity measurements of bacterial communities in the gut, water and feed.
(B) Beta ordination showing clustering of bacterial ASVs in the gut, water and feed.
(C) Alpha-diversity measurements of bacterial communities in the gut with six different dietary treatments.
(D) Beta ordination showing clustering of bacterial ASVs in the gut of barramundi fed six different diets.
Abbreviations: Neg, Negative control; ACO, Australian Crude Oil; HFO, Heavy Fuel Oil.
*Significant at α-level of 0.05. **Significant at α-level of 0.005. ***Significant at α-level of 0.001.

4.4.3. Microbial composition in the water, feed and gut

4.4.3.1. Phyla

The negative control test group showed that the normal microbiome of *L. calcarifer* on a commercial fishmeal diet contains core taxa comprised of phyla Proteobacteria (62%), Firmicutes (7%),

Plantomycetes (3%), Actinobacteria (2%) and Bacteroidetes (1%). This is typical, and agrees with other microbiome studies in *L. calcarifer* (Gupta *et al*, 2019; Zheng *et al*, 2019; Chaklader *et al.* 2021a) and other species (Ghanbari *et al*, 2015; Adomovsky *et al*, 2018; DeBofsky *et al*, 2020).

All metal-enriched feeds produced highly significant changes in the fish microbiome through alteration of bacterial richness for Proteobacteria, Firmicutes and Bacteroidetes. These three bacterial phyla are mainly associated with the metabolism, nutrient assimilation and immunity of fishes. Previous reports have shown that fish exposed to metals demonstrate significantly altered bacterial abundance with complete disruption of Proteobacteria and Bacteroidetes following long-term exposure (Kakade *et al.* 2020; Meng *et al.* 2018). Fish fed a Ni-enriched diet had a microbiome dominated by Proteobacteria (81.1%), with no other individual phyla comprising more than 2% of the microbiome. Besides fish, this striking effect of dietary Ni in altering the relative abundance of Firmicutes and Bacteroidetes has also been shown in rats (Richardson *et al*, 2018).

Firmicutes comprised 67.9% of bacteria in the microbiome of V-exposed fish. V is generally the most toxic of the metals included in this study, and Firmicutes is the most resistant taxa in the fish gut that can survive in extreme environment with higher concentration of metals (Kakade *et al.* 2020; Xia *et al.* 2018). This increase in the abundance of Firmicutes bacteria may be due to vanadium resistance. Firmicutes are mainly carbohydrate-metabolizing and butyrate-producing bacteria linked to the nutrition and energy of epithelial and gastrointestinal cells, assisting in reducing the carcinogenic and inflammatory effect of metals (Collinder *et al.* 2003; Kakade *et al.* 2020). Higher Firmicutes abundance suggests restoration of the intestinal barrier function by the fish to maintain its health and immune performance.

Bacteroidetes and Proteobacteria comprised 47.7% and 46% of total ASVs respectively in the Fe-enriched group, where no other group had more 1% read abundance (Figure 2). This exposure shows complete dysbiosis of Firmicutes, a phyla that links metabolism and immunity in aquatic species (Foysal et al. 2020; Gaudioso et al. 2021). Bacteroidetes are involved in nutrient absorption and

epithelial cell maturation of fish (Evariste et al. 2019). Other reports indicate that exposure to cadmium results in a similar dominance by Bacteroidetes and Proteobacteria in the microbiome of Nile tilapia (*Oreochromis niloticus*) (Meng et al. 2018; Zhai et al. 2017).

Unlike the metal enriched diets, petroleum hydrocarbons had no pronounced effects on gut phyla with similar relative abundances of Proteobacteria, Firmicutes and Bacteroidetes in the negative control, ACO and HFO test groups.

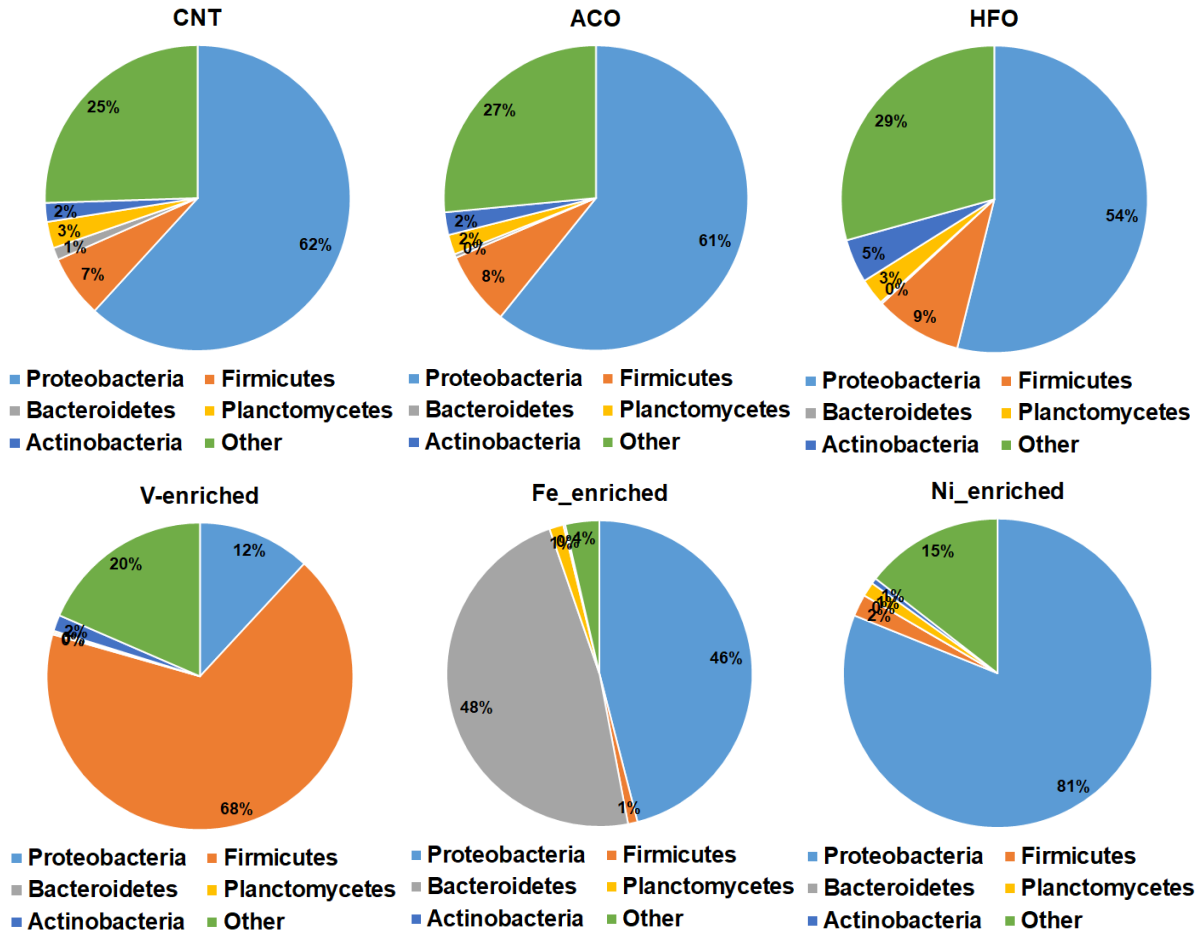


Figure 2. Relative abundance of bacteria at phylum level in the gut of barramundi with six different diets.
Abbreviations: ACO, Australian Crude Oil; HFO, Heavy Fuel Oil.

4.4.3.2. Genera

In the gut microbiome of the negative control group *Ruegeria* and *Escherichia-Shigella* were the most abundant bacteria genera, whereas both crude oil test groups favored the growth of *Photobacterium*, and to a lesser extent *Bifidobacterium* (Figures 3, S3). Among metal-exposed fish, *Phaeocystidibacter* was enriched exclusively in the Fe-enriched group, and *Enterococcus* was enriched solely in the V-enriched group (although there was variation in the distribution of reads for the genus *Enterococcus* within the V-enriched group). Also in the gut of fish fed the Fe-enriched diet, *Phaeocystidibacter*, *NS3 marine*, *Devosia*, *Illumatobacter*, *Loktanella* and *Woeseia* had significantly higher abundance compared to the microbiomes of negative control and crude oil-exposed fish. A Ni-enriched diet increased the abundance of *Coxiella*, *Escherichia-Shigella, Thalassobius* and *Cohaesibacter*.

Compared to negative control fish, *Lactobacillus* and *Legionella* were significantly reduced in the microbiomes of fish exposed to Ni, V and Fe, but not to petroleum hydrocarbons. (Figures 3, S3).

A recent study by Hano *et al* (2021) showed that *Photobacterium* is enriched in the gut of red sea bream (*Pagrus major*) following exposure to phenanthrene (a tricyclic PAH), and proposed microbiome analysis as a possible biomarker for phenanthrene exposure. However, our results indicate that an increase in the relative abundance of *Photobacterium* is likely not specific to phenanthrene, but also other higher molecular weight PAHs such as pyrenes and benzo(*a*)pyrenes as well, given the higher concentrations of these 4- and 5-ring compounds in HFO compared to ACO. *Photobacterium* increased in relative abundance in a dose-dependent manner relative to the combined total tri-, tetra-, and penta-cyclic PAHs.

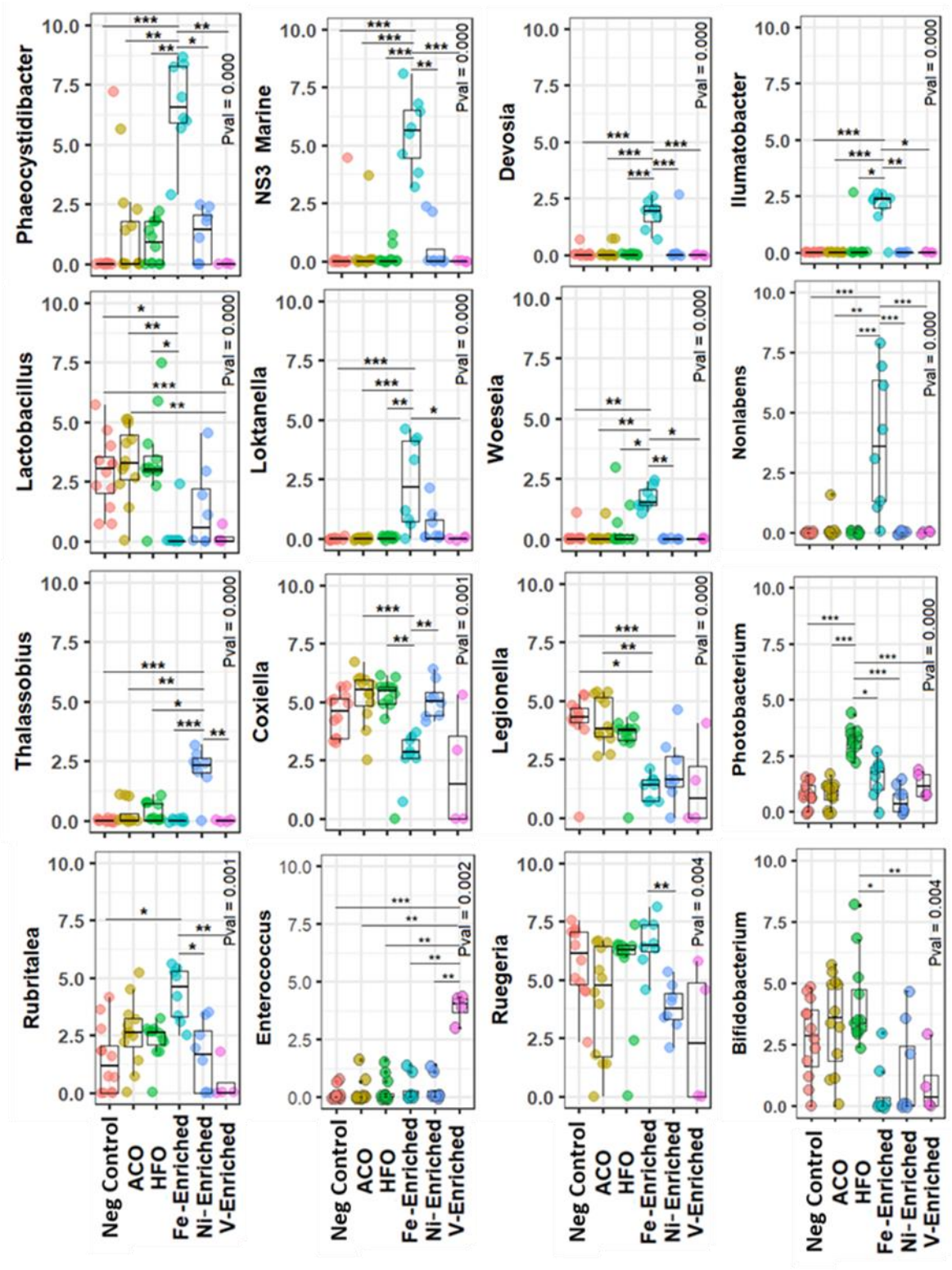


Figure 3. Differential abundance of bacteria at genus level in the gut of juvenile barramundi exposed to petroleum hydrocarbon and heavy metals.

Rarefied abundances were log1p-transformed for generating plots. Kruskal-Wallis with post-hoc Dunn test. The P-value was adjusted with Bonferroni correction. *Significant at α-level of 0.05. **Significant at α-level of 0.005. ***Significant at α-level of 0.001. Abbreviations: ACO, Australian Crude Oil; HFO, Heavy Fuel Oil.

Some genera known to be able to metabolize PAHs as their only energy source such as *Vibrio* (Walter *et al*, 2019) were enriched in fish fed petroleum hydrocarbon-enriched diets, but curiously other oil-metabolizing bacteria such as *Mycobacterium* (Walter *et al*, 2019) were not (Figure S3). Other genera capable of PAH degradation such as *Sphingomones* (Pinkayong *et al*, 2003; Milan *et al*, *2018;* Walter *et al*, *2019*), reported in the microbiome of wild fish populations exposed to petroleum hydrocarbons (Walter *et al*, 2019) were not detected in our study, probably because this genera are never introduced to the microbiome of nursery-raised fish.

Phaeocystidibacter has been found to be enriched in microbial communities exposed to fluorene and benzo(*a*)pyrene (Kahla *et al*, 2021). These compounds are present in the HFO, Ni-enriched and V-enriched test groups, none of which exhibited increases in the relative abundance of *Phaeocystidibacter.* Conversely, this genus was notably increased in the Fe-enriched test group, which was the only group in the present study to contain neither of these large molecular weight PAHs. This highlights the challenge presented by the inherent variability of microbiome analysis at the genus level.

4.4.4. Metagenome predictions

Alterations in predicted metabolic pathways were observed amongst the different treatment groups Most of the significant changes found in functional features were linked to exposure to metals. A diet containing ACO and HFO was linked to only three of 18 significantly enriched metabolic pathways. While fish exposed to Fe and Ni-enriched diet responded mostly with perturbations in the metabolism and degradation of amino acids, fish fed a V-enriched diet showed metabolic changes linked to the biosynthesis of bile acid and peptidoglycan. Other upregulated metabolic pathways are flavonoid biosynthesis in HFO, and thiamine metabolism and ribosome biogenesis in the ACO group (Figure 4).

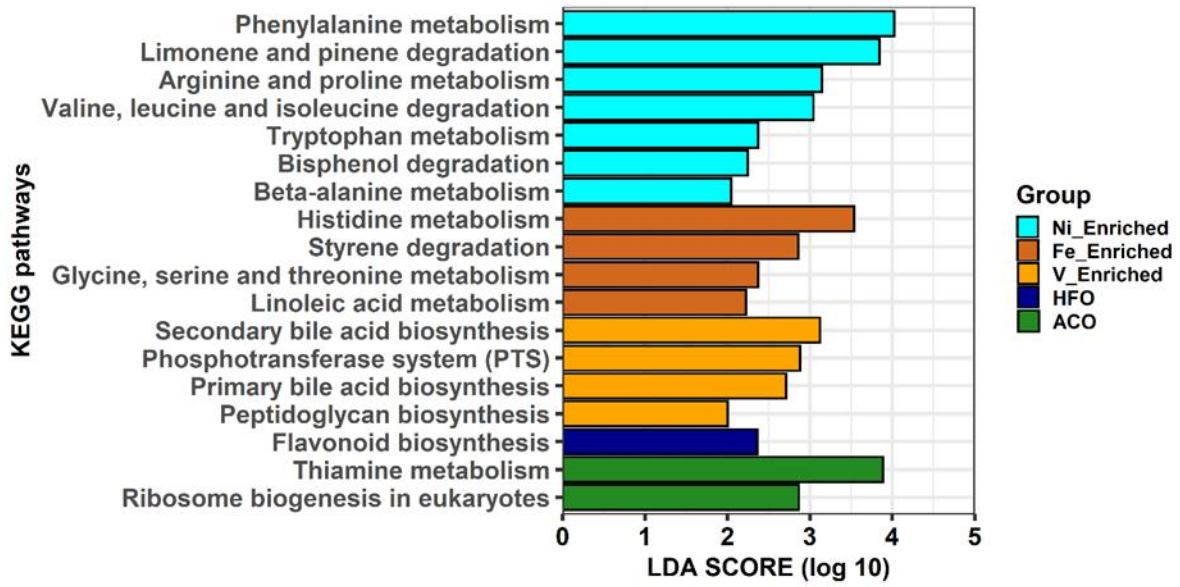


Figure 4. Predicted functional features of 16S rRNA metagenomic data using Picrust 2.
Abbreviations: ACO, Australian Crude Oil; HFO, Heavy Fuel Oil.

Although Ni is a necessary co-factor for many enzymes (Boer *et al*, 2014; Alfano and Cavazza, 2020), it appears to have no part in any of the amino acid metabolism which would explain the functional changes predicted in the microbiome of the Ni-enriched test group. Likewise, Fe and V have numerous roles in cellular biochemistry (reviewed by Beard *et al*, 1996; Gustafsson, 2019), but how they might influence for example amino acid metabolism, the breakdown of styenes or the formation of the peptidoglycan sheath on the bacterial cell wall is not mentioned in current literature. While these links are reported for the first time, a causal relationship could not at this point be established between dietary exposure to petroleum hydrocarbons or to metals.

4.4.5. Taxa-environmental correlations

A total of 27 genera in the gut were found to be influenced by various petroleum hydrocarbons and metals in the diet (Figure S4). Out of these, only 11 genera namely *Staphylococcus*, *Rubritalea*, *Phaeocystidibacter*, NS3 marine bacteria, *Nonlabens*, *Nautella*, *Lactobacillus*, *Escherichia-Shigella*, *Enterococcus*, *Cetobacterium*, and *Bifidobacterium* had more than 1% of read abundance in at least one of the group in the trial. The correlation plot shows *Phaeocystidibacter*, *NS3 marine*, and

Nonlabens preferred higher concentration of Fe for their growth and multiplication whereas an inverse association was observed between *Lactobacillus* and Fe-concentration (Figure S4). A positive association was also identified between *Thalassobius* and Ni-concentration.

4.4.6. Gene expression

Cytokines are important markers to analyse fish health and immunity. The gene expression data showed up-regulation of pro-inflammatory cytokines IL-1, IL-10 and TNF-α in fish fed V- and Fe-enriched diets. Up-regulation of IL-10 and TNF-α was also seen in the Ni-enriched test group, and TNF-α alone was up-regulated in fish exposed to dietary HFO. Downregulation of the anti-inflammatory IL-17 cytokine relative to negative control fish was observed in all test groups except in the ACO-fed treatment group. Compared to the negative control group, no changes in the relative expression level of IL-8 was observed in any of the test groups (Figure 5).

In aquaculture, the use of probiotic dietary supplements is intended to improve fish health. Changes in cytokine expression in response to probiotic supplements in diets (mainly *Lactobacillus*) has been reported in zebrafish (*Danio rerio*) (He *et al.* 2017; Perry *et al.* 2020), carp (*Cyprinus carpio)* (Giri *et al,* 2018), rainbow trout (*Oncorhynchus mykiss*) (Nikoskelainen *et al.* 2003), and crayfish marron (*Cherax cainii*) (Foysal et al. 2020). However, the Ni- and Fe-enriched dietary groups that showed the largest changes in cytokine expression also evidenced an associated significant reduction of *Lactobacillus* abundance ($p < 0.05$) in response to dietary metals exposure (Figure S3). Such a trend was also present (non-significantly) in the V-enriched dietary group. This pattern of elevated expression of pro-inflammatory cytokines and an associated reduction in the relative abundance of gut microbiome *Lactobacillus* has been reported in carp exposed to trichlorfan, an organophosphorus pesticide used for parasite control in aquaculture (Chang *et al.* 2020).

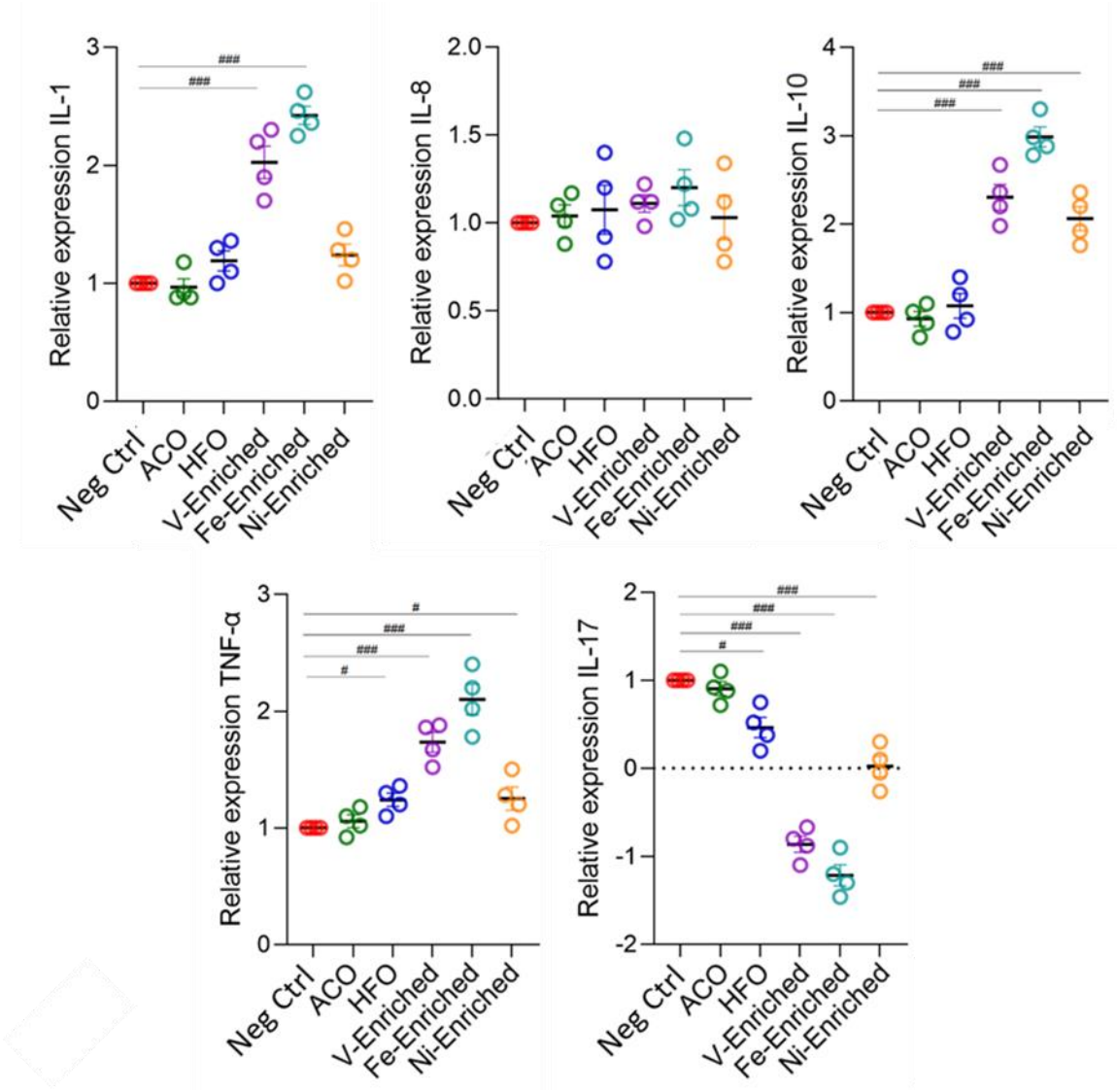


Figure 5. Expression of cytokine genes (fold changes relative to negative control) in the gut of juvenile Lates calcarifer exposed to petroleum hydrocarbons and metals.
One-way ANOVA with Dunnett post-hoc Dunn test. #Significant at α-level of 0.05. ##Significant at α-level of 0.005. ###Significant at α-level of 0.001. Abbreviations: ACO, Australian Crude Oil; HFO, heavy fuel oil.

Part of the normal microbiome of healthy fish (Ringø and Gatesoupe, 1998; Balcázar *et al,* 2007; Gómez and Balcázar, 2008), *Lactobacillus* has been shown to reduce the pathogenic effects of lead (Giri *et al*, 2018) and cadmium (Zhai *et al*, 2017), and inhibit pathogenic bacterial species (Collins, 2019; He *et al*, 2017). It may be that absence of *Lactobacillus* in the microbiome of fish may be useful as a biomarker of exposure to some specific toxicants such as metals and some pesticides, but not petroleum hydrocarbons. Conversely, another of the widely-studied lactic-acid bacteria,

Bifidobacterium, positively correlated with petroleum hydrocarbon exposure (Figure 3), and may also be a biomarker candidate worthy of future study alongside *Photobacterium*.

4.5. Conclusion

We have demonstrated that the gut microbiome of fish exposed via diet to crude oils and V, Ni and Fe undergo significant changes. In general, dietary metals exposure produced a greater reduction in diversity and elevated immune response than petroleum hydrocarbons.

Analysis for the microbiome at the phyla level provides clear indications of exposure to V, Ni and Fe, less so at the genus level where the picture is more complicated. The phyla Firmicutes is greatly emphasized in the microbiome of fish exposed to V, and Protobacteria are enriched in response to Ni. Exposure to Fe increases the abundance of Bacteroidetes, but decreases Protobacteria. At the genus level, enhanced *Photobacterium* in the fish microbiome shows potential as a biomarker of exposure to PAHs, increasing proportionately to the dietary concentration of higher molecular weight PAHs. Reductions in *Lactobacillus* may also be a candidate as a biomarker for exposure to metals, and possibly other toxicants.

Future studies are needed to further explore the potential of gut microbiome analysis as a biomarker for petroleum hydrocarbons, metals, other toxicants, or as a general indicator of fish health.

Acknowledgements

Many thanks to R. Kliendienst and M.R. Chaklader for trial tank set up and sampling assistance.

Funding

This work was supported by an ARC-Linkage grant provided by the Australian Research Council (grant number LP170101000).

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Chapter 5: Fish Fingerprinting: Identifying crude oil pollutants using bicyclic sesquiterpanes (bicyclanes) in the tissues of exposed fish

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In review. Submitted to *Environmental Toxicology and Chemistry*: 6th December 2021

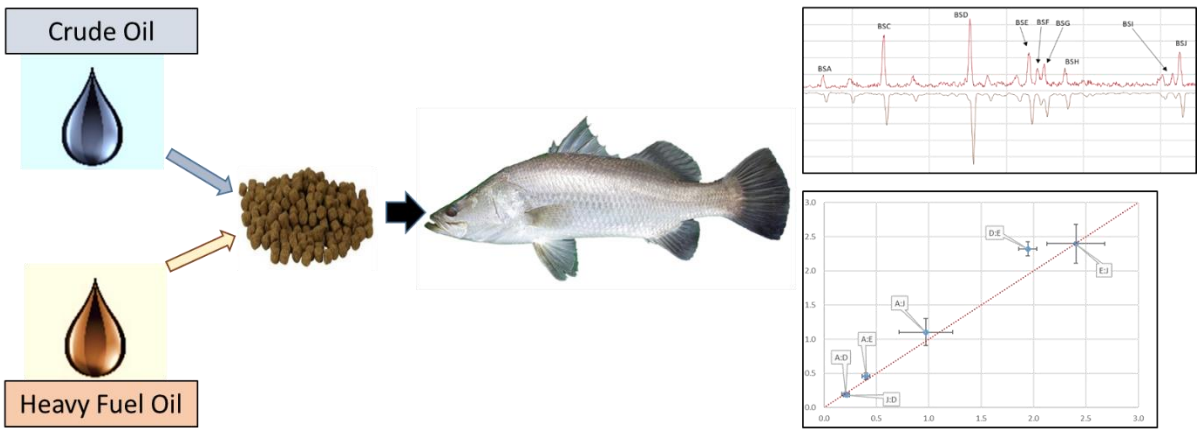
Keywords

Crude oil, fingerprinting, ecotoxicology, heavy fuel oil, Montara, bicyclic sesquiterpanes

Highlights

- *Lates calcarifer* (n=27) exposed to heavy fuel oil and a medium crude oil
- Laboratory trial with 33 day dietary exposure, 2 day depuration
- Bicyclanes concentrated in adipose tissue of oil-exposed fish in diagnostic ratios
- High correlations (r^2 >0.98) between crude oil and fish adipose tissue bicyclanes

Graphical Abstract



5.1. Abstract

In the event of a spill, identification of the source oil for assessment or litigation purposes typically uses diagnostic ratios of chemical biomarkers to produce characteristic oil 'fingerprints'. Although this has been applied in identifying oil residues in sediments, water and sessile filtering organisms, previous attempts to fingerprint crude oils using sterane and hopane ratios in exposed fish have shown only limited success

In this study, we investigated the possibility of biomarker fingerprinting of two different oils in exposed fish. In a 35-day laboratory trial, juvenile *Lates calcarifer* (barramundi, or Asian seabass) were exposed via the diet (1% w/w) to either a heavy fuel oil (HFO) or to Montara, an Australian medium crude oil (MCO), examples of which have been spilled into the marine environment previously. The usefulness of the relative abundances of groups of biomarkers, particularly the bicyclic sesquiterpanes (bicyclanes), for identifying the original oil sources, was then investigated. Polycyclic isoprenoids commonly used in oil fingerprinting, such as steranes and hopanes, were not abundant in the source oils, and were undetectable in the tissues of fish. Other groups of hydrocarbons were found to be less suitable for fingerprinting, including some polycyclic aromatic hydrocarbons (PAH) and acyclic isoprenoid hydrocarbons, which were more susceptible to metabolism, water-washing and other degradation mechanisms, or were non-specific to the source oils.

Bicyclane distributions were conserved in fish adipose tissue, with six diagnostic ratios reproducibly showing high correlation ($r^2 > 0.98$) with those in the two source oils. Further research is needed to investigate the minimum exposure times required for bioaccumulation of bicyclanes to reach detectable concentrations and to determine depuration rates once exposure to oil has ceased.

5.2. Introduction

International maritime law holds to the principle that "the polluter must pay" (Schwartz 2010). The first step in assessment and litigation proceedings, particularly for smaller scale incidents, is often

the defensible forensic identification (commonly referred to as fingerprinting) of the source oil (Stout et al. 2001). Methods for the forensic identification of spilled oils are well established (Stout et al. 2001; Wang and Fingas 2003; Yang et al. 2017). Typically, such methods involve analyses of the relative abundance of key chemical biomarker compounds such as saturated hydrocarbons including *n*-alkanes, acyclic isoprenoids (e.g. pristane and phytane), polycyclic isoprenoids such as steranes and hopanes, plus polycyclic aromatic hydrocarbons (PAHs) (e.g. naphthalene, phenanthrene, chrysene, dibenzothiophene and their alkylated homologues (Yang et al. 2017)). Ultimately, the choice of biomarker ratios for forensic identification of a spilled oil is case-specific, depending on the composition of the oil. Light crude condensates, plus some refined products, may lack the high molecular weight steranes and hopanes of heavier crude oils, for instance (Spaak et al. 2020).

Weathering is the degradation of crude oils after environmental exposure due to evaporation, dissolution into the water column, UV photo-degradation and microbial metabolism (Wang et al. 1999; Wang and Fingas 2003). Commencing immediately following release, weathering further complicates the forensic identification of spilled oil as some of the diagnostic ratios of biomarker compounds may be altered beyond use for fingerprinting purposes. Lighter molecular weight compounds such as benzene, toluene, ethylbenzene, xylene (BTEX), smaller *n*-alkanes and naphthalenes may be reduced greatly, whereas other compounds such as the polycyclic isoprenoids (Wang and Fingas 2003) and bicyclic sesquiterpanes (Wang et al. 2005) often remain relatively unaffected by environmental processes.

Petroleum hydrocarbons from oil spills also enter food webs *via* diffusion into plants (Buskey et al. 2016) and via filter-feeding species such as bivalve molluscs (Donkin et al. 2003) and then may be bioaccumulated in predatory carnivorous species (D'Costa et al. 2017; Scarlett et al. 2009; Snyder et al. 2015). In field studies following the 2010 Macondo Well, Deepwater Horizon (DWH), incident in the Gulf of Mexico, total petroleum hydrocarbon (TPH) concentrations in the tissues of exposed species of commercial fish were found to be as high as 2.2% (w/w) (Sammarco et al. 2013). In

industrial areas such as the Gulf of Suez, TPH levels in white muscle of fish have been found to be as high as 0.15% (w/w) (Ahmed et al. 2019). Once ingested by exposed fish, toxic PAHs from crude oils are predominantly metabolized in the liver by AhR-mediated processes (Reynaud and Deschaux 2006; Tuvikene 1995) and excreted via the bile, whereas some non-toxic saturated compounds such as *n*-alkanes and pristane are bioaccumulated and may remain in fish adipose tissues for up to 5 months following exposure (Cravedi and Tulliez 1986). A previous study attempting crude oil identification using petroleum hydrocarbons accumulated in the tissues of exposed fish used ratios of polycyclic isoprenoids (steranes and hopanes) (Manan et al. 2014), successfully identifying the exposure oil in only 2 out of 12 fish tissue samples tested.

Previous laboratory studies seeking to simulate the toxicological effects of crude oil exposure on various species of fish have facilitated petroleum hydrocarbon exposures by either the waterborne route *via* the gills (Aas et al. 2000; Heintz et al. 1999) or *via* the dietary route (Bautista et al. 2019; Nahrgang et al. 2010; Vieweg et al. 2018; Vignet et al. 2014). Dietary exposure to sub-lethal doses of oils in the test species used in the present study, *Lates calcarifer* (barramundi or Asian sea-bass), which is a carnivorous teleost fish widely used in aquaculture (Boonyaratpalin 2017; Mathew 2009), led to a range of behavioral, physiological and biochemical responses (Spilsbury et al. 2021; Chapter 2). These included decreased foraging ability, decreased brain acetyl-cholinesterase concentration, increased hepatic EROD activity, decreased condition factor, and biliary PAH metabolite profiles which matched the source oils (Spilsbury et al. 2021; Chapter 2). Drimane-like bicyclic sesquiterpanes are ubiquitous in crude oils (Stout et al. 2016; Wang et al. 2013; Wang et al. 2005), and are likely the result of diagenetic degradation of algae and bacteria (Alexander et al. 1984; Noble et al. 1987). Their presence in Cambrian-Ordovician samples rules out the possibility that they are derived from higher plants (Alexander et al. 1984). A review of the available literature failed to find an occurrence of these bicyclanes outside of their distribution in ancient sediments and crude oils. Importantly, they are often present in differing proportions in different oils such that it is possible to characterize oils from different sources (Alexander et al. 1984; Noble et al. 1987). For some fuel oils

and lighter crude oils which lack the high molecular weight steranes and hopanes normally used for forensic identification purposes, bicyclanes have been shown to allow discrimination of a variety of petroleum products (Wang et al. 2005; Yang et al. 2012). Importantly, bicyclanes are not known to be toxic to fish or to other marine species (Jansen and De Groot 2004), but are sufficiently lipophilic ($logK_{OW}$ 6.36; US EPA, 2021b) to suggest passive uptake across cell membranes (Streit 1998), and are hence a good potential candidate for forensic fingerprinting analyses in the tissues of exposed fish.

In this study, we explore the suitability of traditional diagnostic chemical biomarker ratios used in forensic crude oil fingerprinting for adaptation to studies in fish, and demonstrate a novel application to identify a source oil using bicyclanes accumulated in the adipose tissue of fish exposed to petroleum hydrocarbons.

5.3. Materials and Methods

The heavy fuel oil (HFO) (API 11.4) was supplied by the BP Kwinana Oil Refinery (Western Australia), and the Montara crude oil (MCO) (API 31.0) was provided by PTTEP Pty Ltd. The study design and characteristics of the oils are further described elsewhere (Spilsbury et al. 2021; Chapter 2).

5.3.1. Fish Exposure and Sampling

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

Juvenile *L. calcarifer* 10-15 cm in length were obtained from a local commercial hatchery. Fish were acclimatized to test conditions of 28 ^{o}C, salinity 32 ppt, dissolved O_2 > 5 mg/L before transferal to 100 L tanks containing natural Indian Ocean seawater with four fish per tank. Mean fish weight at the commencement of the trial was 85 ± 2 g. A static renewal tank set-up was employed, using closed re-circulating canister bio-filters with a flow rate of approximately 5 L/min to assist in maintaining water quality.

Fish were fed commercial fishmeal (3 mm Nova FF, Skretting Pty Ltd, Perth, Australia) twice daily to a total of approximately 2% bodyweight per day (Hellou et al. 2002). Fish were fed either plain

fishmeal (negative control), fish meal spiked with 1% w/w heavy fuel oil (HFO) or fish meal spiked with 1% w/w Montara crude oil (MCO). Fish food was stored at -20 ^{o}C and thawed immediately before use. Daily removal of feces and any uneaten food not captured by the filter was performed manually using a hand-held suction pump after each feeding. Fish were exposed to crude oils via diet continuously for 33 days, followed by a 2-day depuration period. Following ike-jime, samples of white muscle (approximately 15 g) and brown adipose tissue (typically 2-5 g) adjacent to the intestine was removed and stored at -20 ^{o}C prior to analysis.

5.3.2. Extraction/chromatography of oils

Small silica columns were prepared in Pasteur pipettes containing 0.5 g of silica, and washed with 10 mL of hexane. 10 mg of crude oil was spiked with 10 μL of a perdeuterated standard mixture containing 0.1 mg/mL tetralin-D_{12}, naphthalene-D_8 and phenanthrene-D_{10} and loaded onto the column before elution with 3.0 mL of hexane (saturates fraction) and 3.0 mL of hexane:dichloromethane (DCM) (7:3) (aromatics fraction). Volume reduction to 1.0 mL was achieved under a gentle stream of nitrogen.

5.3.3. Extraction of fish adipose tissues

Fish adipose tissues were stored at -20 ^{o}C before use. Extraction of petroleum hydrocarbons was performed using published methods (Kelly et al. 2000). Briefly, frozen adipose tissue samples were accurately weighed, and between 2-5 g of tissue was transferred to a 250 mL round bottom flask (RBF) containing 100 mL of HPLC grade methanol, 5 g of potassium hydroxide (KOH), anti-bumping granules and spiked with 10 μL of perdeuterated standards mix containing 0.1 mg/mL of naphthalene-D_8, phenanthrene-D_{10}, tetralin-D_{12}, p-terphenyl-D_{14}, *n*-decane-D_{22} and *n*-tetracosane-D_{50}. Samples were digested under reflux for two hours. Cool digests were passed through a Whatman 113v filter paper into a 500 mL separating funnel, and extracting using 3 x 25 mL hexane, followed by a 25 mL hexane glassware rinse. Extracts were transferred to a 500 mL RBF and reduced in volume to approximately 2 mL *via* rotary evaporation before transferal to 4 mL vials and dried by the addition of a small quantity of $MgSO_4$. Four procedural blanks were performed.

5.3.4. Fish adipose tissue extract chromatography

Columns were prepared in 50 mL burettes containing 6 g of activated silica and washed with 50 mL hexane. Extracts of adipose tissue were reduced in volume to approximately 0.5 mL under a gentle stream of nitrogen and loaded onto the column before elution directly into 50 mL RBF with 40 mL of hexane (F1, saturates fraction), followed by 40 mL of hexane:DCM (7:3) (F2, aromatics fraction). Volume reduction to 1.0 mL was achieved by an initial rotary evaporation, transferal to 4mL vials, and then finally blown down under a gentle nitrogen stream.

5.3.5. GC-MS analysis

For all sample extracts and procedural blanks, a 1 μL injection volume was used. All aromatic fractions were analyzed on an Agilent GC 6890 coupled to a MS 5975B. A DB-5MS column (Agilent P/N 122-5562UI) was used. The starting temperature of 40 °C was increased with an initial temperature ramp of 3 °C/min until 280 °C, followed by a 20° C/min ramp until 325 °C and then held isothermal for 20 minutes. Saturates were analysed on an Agilent GC 7890B coupled to a MS 5977B using a DB-1MS column (Agilent P/N 122-0162UI). Temperature was initially held at 40 °C for 1 min before increased at a ramp of 6 °C/min until 320 °C and then held isothermal for 28 minutes.

Quantitation was made with reference to an in-house mixture of 27 aromatic and saturated hydrocarbons (Tables S1), analysed concurrently with samples at concentrations of 0.5, 1.0, 2.0 and 5.0 μg/mL.

Sterane and hopane biomarkers were analysed by GC-MS selected ion monitoring (SIM) analysis (*m/z* 123, 191, 205, 217, 218, 358, 370, 372, 384, 386, 398, 400, 412, 414, 426, 428, 440, 442, 454, 456).

5.3.6. Data handling

Data were analysed by Agilent ChemStation software, with compound identification using NIST library searches. Kovats retention indices (temperature programmed) were calculated using ASTM Method D6730 (ASTM, 2021). All confidence intervals provided are 2x standard error (2SE).

5.4. Results and Discussion

5.4.1. Saturated hydrocarbons

The saturated fractions of fish adipose tissue extracts showed a *n*-alkane series which is also typical of many oils (Figure 1). Blank spike recoveries for tridecane (*n*-C_{13}), heptadecane (*n*-C_{17}), octadecane (*n*-C_{18}) and pristane were 85.2%, 100.7%, 102.9% and 99.2% respectively (Table S1). Some saturated petroleum hydrocarbons of potential interest for fingerprinting, such as decane (*n*-C_{10}), undecane (*n*-C_{11}), heptadecane (*n*-C_{17}) and pristane, were also found in the fish food and correspondingly in negative control fish (Figure S1). Pristane can also occur naturally in wild fish tissue due to consumption of plankton (Ackman 1971).

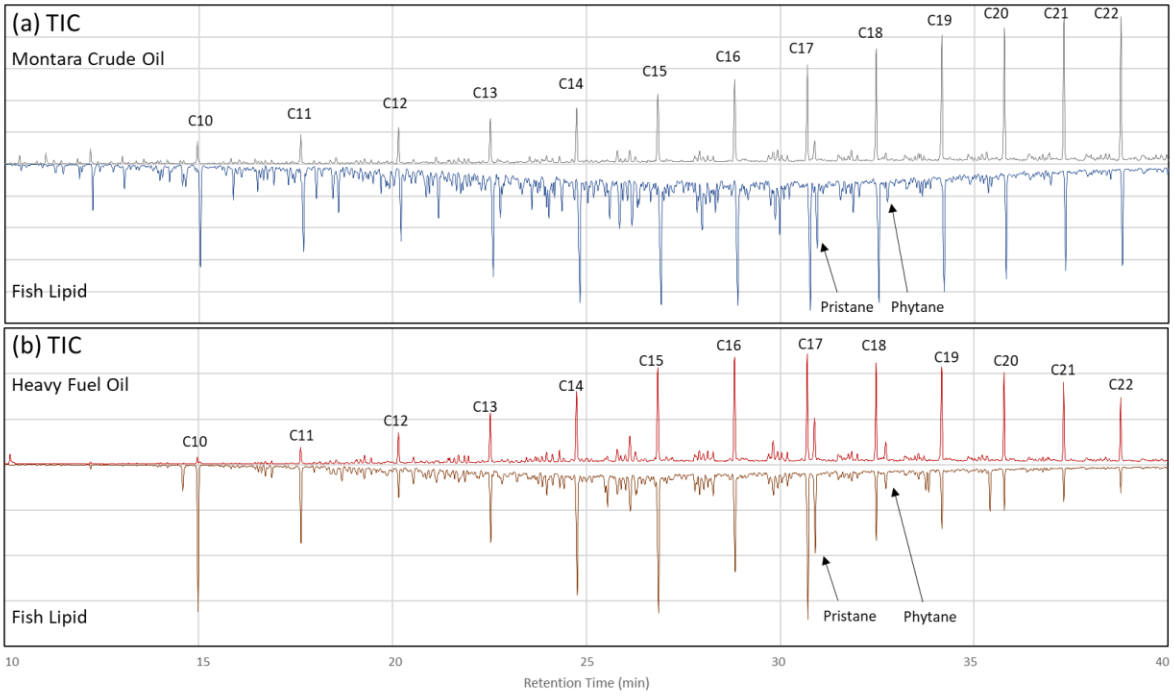


Figure 1: Mirrored partial GC-MS chromatograms (TIC) of F1 saturated fractions of oils (top) and fish adipose tissue extracts (bottom) for MCO (a) and HFO (b).

In the oils, the most abundant *n*-alkane peaks were C_{22} and C_{18} for MCO and HFO respectively (Figure 1). Interestingly, GC-MS chromatograms of fish adipose extract saturated fractions of both MCO- and HFO-exposed fish show a "left-shift" of the dominant *n*-alkane peak towards C_{15}. This suggests a decrease in uptake of larger *n*-alkanes across the cell membrane of fish adipocytes. The uptake of

petroleum hydrocarbons in mussels is also related to molar volume, decreasing above 230 cm^3/mol (Donkin et al. 1991). The observed relative increase in C_{10} and C_{11} *n*-alkanes in fish adipose tissue indicates additive effects of these hydrocarbons in fish tissue from both natural (crude oil-free food) and petrogenic sources (Figures S1, S2). The presence of pristane in the crude oil-free food means that acyclic isoprenoid ratios (Powell and McKirdy 1973; Wang et al. 2007; Yang et al. 2017) used for fingerprinting (e.g. pristane:phytane) are altered beyond use for fingerprinting purposes in the tissues of fish exposed to the oils in this study.

5.4.2. Steranes and hopanes

Forensic identification of oils is commonly performed by comparing the relative abundances and isomeric distributions of steranes and hopanes (Jones et al. 1986; Yang et al. 2017). Steranes and hopanes were not present in MCO and HFO oils at forensically useful concentrations and were not detected in the adipose tissue of exposed fish. This agrees with the results of other studies of fuel oils or other lighter crude oils and petroleum products which are sometimes not easily characterised due to a paucity of steranes and hopanes (Wang et al. 2005; Yang et al. 2012). Concordantly, Australian light/medium crude oils have previously been characterised by the relative abundances of bicyclanes instead (Alexander et al. 1984; Noble et al. 1987).

5.4.3. Bicyclic Sesquiterpanes

Ten bicyclic sesquiterpanes in total (BSA – BSJ) were found in the HFO or MCO (Figure S2). Other studies have similarly described the presence of bicyclic sesquiterpanes in fuel oils, lube oils and crude oils (Yang et al. 2009), and although the exact structures of some of these compounds are still unknown, their application in forensic fingerprinting of light refined petroleum products (Wang et al. 2005; Yang et al. 2012) and crude oils (Wang et al. 2013) has also been described. Corresponding suites of compounds were also detected in the adipose tissue of exposed fish herein (Figure 2).

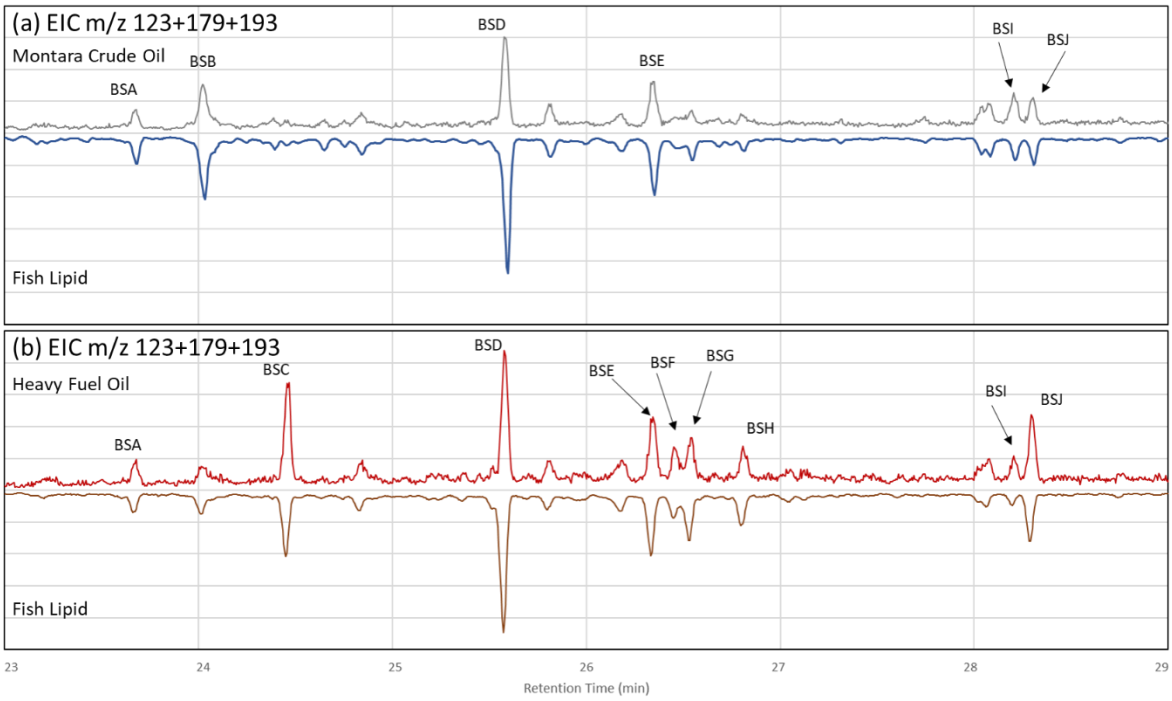


Figure 2: Mirrored partial GC-MS extracted ion chromatograms (EIC m/z 123+179+193) of oils (top) and fish adipose tissue extracts (bottom) for MCO (a) and HFO (b).

Importantly, the distributions of bicyclanes in the two oils used herein were different: MCO contained BS-A, B, D, E, I and J whereas HFO contained BS-A,C,D,E-J. Thus, ratios of the different bicyclane compounds allowed the two oils to be differentiated from one another and this was consistent for adipose tissue from fish in both oil exposure test groups (Figure 2).

Table 1: Bicyclic sesquiterpanes found in the HFO and MCO oils and used in this study for fingerprinting the oils in fish adipose tissue.

Compound	Abbreviation	Molecular Ion	Base Peak Ion	Kovats Retention Index (ASTM Method D6730)
C_{14} sesquiterpane	BSA	194	179	1351
C_{15} sesquiterpane	BSB	208	193	1366
C_{15} sesquiterpane	BSC	208	193	1385
C_{15} sesquiterpane	BSD	208	193	1438
C_{15} sesquiterpane	BSE	208	193	1475
C_{16} sesquiterpane	BSF	222	123	1480
8β(H)-drimane	BSG	208	123	1484
C_{16} sesquiterpane	BSH	222	123	1497
C_{16} sesquiterpane	BSI	222	193	1564
8β(H)-homodrimane	BSJ	222	123	1572

The bicyclanes were found in the adipose tissues of the exposed fish (Figure 2), but not in the negative controls (Figure S1). Blank spike recoveries for compounds eluting close to the $C_{14\text{-}16}$ bicyclanes were 61%, 82% and 100% respectively for the C_{10} bicyclic decalin, C_{13} *n*-tridecane and C_{17} *n*-heptadecane (Table S1). The bicyclane profiles of adipose tissue extracts from exposed fish differed characteristically, reflecting the relative abundances in the oils (Figure 3). MCO lacked BSC, BSF, BSG and BSH, whereas HFO lacked BSB. Co-elution complicated the resolution of peaks BSF, BSG, BSH and BSI. Direct comparison of the six peak area ratios (EIC *m/z* 123+179+193) of the four bicyclic sesquiterpanes common to both oils, and not impacted by co-elution (BSA, BSD, BSE and BSJ) (Table 1) showed a good linear correlation between the relative abundances of bicyclic sesquiterpanes in the oils and in the respective adipose tissue extracts of fish exposed to both MCO (r^2 = 0.9819) and HFO (r^2 = 0.9817) (Figure 3).

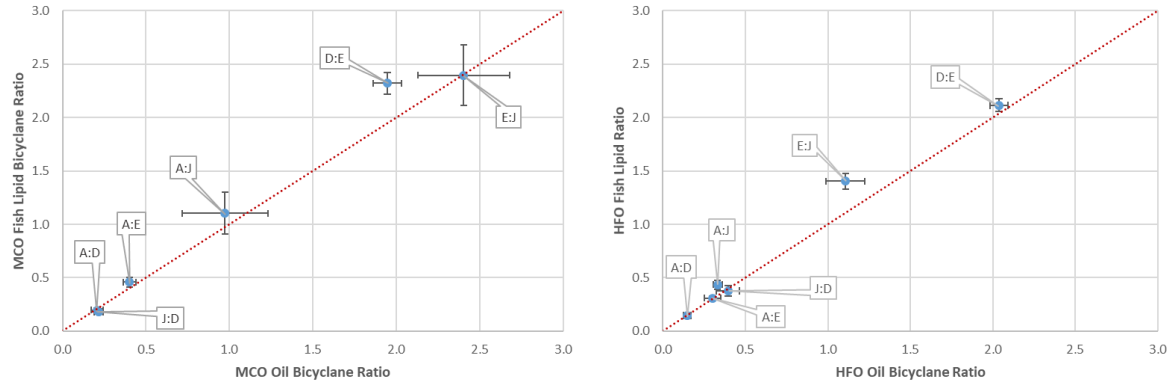


Figure 3: Correlation of bicyclic sesquiterpane ratios in exposure oils with those in the respective exposed fish adipose tissue extracts.
Red dotted line shows the 1:1 fit. Error bars are 2SE for oils (horizontal, n=3) and fish adipose tissue (vertical, n=9)

Other smaller bicyclic alkanes, such as decalin and C1-decalins were also found in both oils, and also in the adipose tissue extracts of exposed fish. However, the relative abundances of these compounds did not differ appreciably between test groups, and they were therefore not particularly

useful in differentiating between the exposure oils. One cadinane and notably a further methyldrimane (Alexander et al. 1984) present in HFO were detectable in the fish adipose tissue, these compounds were not included in fingerprinting analyses as they were not common to both oils, unlike the $C_{14\text{-}16}$ bicyclanes (Table 1). Tricyclic and pentacyclic diamondoids, i.e. adamantanes and diamantanes, have previously been used to characterize oils (Grice et al., 2000; Wang et al., 2007) and potentially could be useful for fingerprinting in oil-exposed fish. Adamantane and its alkylated homologues plus the caged tetracyclic ethanoadamantane have been reported in MCO (Scarlett et al. 2019; Spaak et al. 2020) and some alkylated adamantanes were also present in HFO. Although detectable in the tissues of oil-exposed fish tissues, and therefore forensically useful as a means of eliminating an oil as a source of contamination, the adamantanes were not common to both oils and so could not be used in the ratio correlation (Figure 3).

5.4.4. Aromatic Compounds

The oils used in this study were chosen because they are chemically very different. While MCO contains more lighter molecular weight PAHs and comparatively less of the larger four-and five-ring PAHs, HFO contains more dibenzothiophenes (Spilsbury et al. 2021; Chapter 2). MCO-exposed fish tissues contained higher total PAH concentrations than HFO-exposed fish. The adipose tissue of MCO- and HFO-exposed fish contained 67.8 ± 14.9 μg/g and 15.8 ± 2.3 μg/g total PAH respectively, whereas the white muscle contained 3.0 ± 0.9 μg/g and 0.8 ± 0.1 μg/g total PAH (Figure S3). Consistently for both exposure groups, around 95% of accumulated PAHs were found in the adipose tissue of exposed fish, with 5% sequestered in white muscle. No PAHs were detected in the tissues of negative control fish.

The PAH profiles in the oils were dissimilar to those in the respective adipose tissues of exposed fish. Alkylated (C_1-C_3) naphthalenes and phenanthrenes were present in fish adipose tissue at higher concentrations than their respective parent (C_0) compounds (Figure S4), and larger molecular weight four- and five-rings PAHs were not detected in the adipose tissue of fish exposed to either oil, suggesting a lack of sequestration.

Larger PAHs are stronger inducers of Cyp1a enzymes such as EROD (Whyte et al. 2000), and are hence largely removed via AhR-mediated metabolic processes eventually to be excreted *via* the bile (Aas et al. 1998; Beyer et al. 2010; Gagnon and Holdway 2000; Hellou and Payne 1987; Lin et al. 1996). This has also been shown specifically in *L. calcarifer,* which exhibits hepatic EROD induction in response to exposure to HFO, but not MCO (Spilsbury et al. 2021; Chapter 2). The presence of branched alkyl chains or increased alkylation on the rings of PAHs increases their lipophilicity and may also protect them from biotransformation and conjugation (Scarlett et al. 2011; Spies et al. 2017). Hence the differences in the relative proportions of PAHs sequestered in fish adipose tissue compared to the oils likely reflects different elimination rates due to fish metabolism, combined with increased uptake rates of respective increasingly alkylated compounds. In the context of fish tissue analysis, ratios of the relative abundance of PAHs for the fingerprinting of crude oils (Leeder 2010; Wang et al. 2007; Yang et al. 2017) are therefore less useful for identification of the sources of oil pollution.

Following the DWH incident, public health concerns were raised about the suitability of commercial fish species caught in oil spill zones for human consumption (Ylitalo et al. 2012). A detailed monitoring program (Ylitalo et al. 2012) showed that even when detected, the concentrations of PAHs were at least two orders of magnitude lower than the level of concern for human health risk. The adipose tissue of *L. calcarifer* is located adjoining the intestine, and is normally removed along with the viscera during gutting processes. Our findings indicate that approximately 95% of PAHs accumulated in the tissues of oil-exposed *L. calcarifer* would probably be subsequently removed during processing, further reducing possible human exposure through dietary consumption.

5.4.5. Application to Environmental Oil Spills

Species of wild fish present in an oil spill zone are often motile and may avoid spilled oil; however farmed fish may be unable to escape. The dietary exposure used in this study demonstrates that *L. calcarifer* consumed oil-tainted food, even food with an odour obvious to humans (Spilsbury et al. 2021; Chapter 2). The exposure time for this trial was 33 days, followed by a 2-day depuration

period. While it seems unlikely that wild fish would remain directly in an oil spill zone for several weeks, longitudinal field studies following oil spills have shown fish biomarker responses which indicated that petroleum hydrocarbon exposure persisted for several months after oil release cessation. For example, following the DWH spill, surveys in oil-affected areas of the Gulf of Mexico reported red snapper (*Lutjanus campechanus*) with elevated biliary PAH metabolites (Snyder et al. 2015) and increased activity of hepatic Cyp1a enzymes (Smeltz et al. 2017), indicative of petroleum hydrocarbon exposure lingering at least 12 months after cessation of petroleum hydrocarbon release.

The bioaccumulation of bicyclanes in fish adipose tissue over time probably makes detection in exposed fish easier. Two dimensional GC×GC-MS might further enhance fish tissue bicyclane fingerprinting for forensic identification of source of oil exposure by decreasing detection limits and possibly reducing co-elution (Beyer et al. 2010). Here, we have instead demonstrated the application of more traditional crude oil fingerprinting in the tissues of exposed fish using GC-MS, which is now a commonly available instrumental method in most analytical laboratories. Further research testing shorter exposure times and depuration periods would be needed to ascertain the minimum exposure duration necessary or bicyclanes to be detected in fish tissue with current analytical methods.

5.5. Conclusions

The distributions of bicyclic sesquiterpanes, known to be present in characteristically different relative abundances in many crude oils and fractionated oil products, appear to retain their diagnostic ratios, unchanged by evaporative processes and weathering, and are readily accumulated into adipocytes of fish exposed to a medium crude or a heavy fuel oil in their diets. Diagnostic ratios were conserved after ingestion and sequestration in the adipose tissues of *L. calcarifer*. In the absence of an environmental sample of spilled oil for comparison, analysis of these tissues of fish

exposed to crude oil might, in future, be useful for providing an identifying fingerprint of the source oil. Further research is needed to ascertain the minimum exposure time for bioaccumulation of bicyclic sesquiterpanes to exceed analytical limits of detection, and depuration rates once exposure has ceased.

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Chapter 6: Crude oil identification using linear discriminatory analysis (LDA) of bicyclic sesquiterpanes (bicyclanes) in the adipose tissue of oil-exposed fish

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Keywords

Crude oil, fingerprinting, ecotoxicology, heavy fuel oil, Montara, bicyclic sesquiterpanes, bunker C, LDA

Highlights:

- Laboratory exposure of *Lates calcarifer* (n=18) to Montara crude oil (MCO) or a heavy fuel oil (HFO)
- GC-MS analysis of bicyclane profiles of 16 hydrocarbons sources and adipose extracts of oil-exposed fish
- LDA analysis of fish tissue bicyclanes correctly identified MCO or HFO exposure oils (>95% proability) for 18/18 oil-exposed fish.
- Potential for use for source oil identification in environmental oil spills

6.1. Abstract

Bicyclic sesquiterpanes (bicyclanes) are apparently ubiquitous in crude oils and refined petroleum products, and upon exposure to oils in the laboratory, bicyclanes are sequestered into the adipose tissue of exposed fish. In the present proof-of-concept study, we demonstrate the application of a linear discriminatory analysis (LDA) model to identify source oils in the adipose tissue extratcs of exposed fish by a non-subjective computerized method based on bicyclane biomarker ratios.

In a laboratory trial, *Lates calcarifer* (barramundi, or Asian sea-bass) were exposed *via* diet (1% w/w) to a heavy fuel oil (n=9) or to Montara (n=9), a medium crude oil from the Australian Northwest Shelf, examples of which have both previously been spilled in the environment. An LDA model was then trained using a reference dataset of bicyclane fingerprint ratios of both exposure oils, plus four other NW Shelf crude, two fuel oils, and eight weathered asphaltites from the Great Australian Bight for comparison (16 in all). The LDA model correctly identified the corresponding bicyclane profiles of each of the two respective exposure oils from a test dataset of adipose tissue extracts of each of the 18 fish fed oil-enriched diets.

This work demonstrates the potential of using non-subjetcive computerizes comparisons of the bicyclane profiles of adipose tissue of oil-exposed fish as a forensic identification tool for impact assessment and litigation purposes in an environmental oil spill in which wild or caged fish become exposed to oil.

6.2. Introduction

In the event of an oil spill, natural weathering processes change the composition of an oil, potentially altering characteristic ratios making identification of an unknown oil more difficult (NRC 2003). Typical chemical fingerprinting of a crude oil commonly uses classes of compounds such as the steranes, hopanes and regular isoprenoids that are not greatly affected by evaporation, microbial metabolism, UV-degradation or dissolution (Stout et al. 2016; Yang et al. 2017). Bicyclic sesquiterpanes (bicyclanes) are also present in most, or possibly all, crude oils (Stout et al. 2016; Yang et al. 2017), and their characteristic ratios of different bicyclanes have been used in the forensic identification (fingerprinting) of crude oils and refined oil products, particularly in cases where other commonly used fingerprinting compounds such as steranes and hopanes are lacking (Wang et al. 2005; Yang et al. 2012). Bicyclic sesquiterpanes are also largely unaffected by weathering processes (Wang et al. 2013; Wang et al. 2005), and are not known to be subject to

cellular metabolic processes in fish, although they may be able to be degraded by bacterial consortiums (Maier 2019).

The northwest (NW) shelf of Australia is a prolifically developed oil and natural gas field and includes the Browse and Bonaparte basins (Edwards and Zumberge 2005; Le Poidevin et al. 2015; Spaak et al. 2020). These basins contain, among others, the Caswell, Calliance, Crux-3 and Montara well platforms, which are located along a 1000km stretch of the Timor Sea. Although the deposits are in relatively close geographical proximity, they originate from different source and geological periods (Spaak et al. 2020), and can be distinguished by differing characteristic geochemical biomarkers (Scarlett et al. 2019b; Spaak et al. 2020). Of particular note, the Montara and Crux-3 wells are in close proximity, access the same reservoir, and analysis of diamondoid biomarkers has shown these crudes display a high degree of similarity (Spaak et al. 2020).

Conversely, the Otway and Bight basins in the Great Australian Bight are not developed for the production of oil and natural gas, but contain a large number of natural seeps (Padley 1995) which are the likely source of the beach standings of asphaltites recorded in the area since the mid-1800s (Edwards et al. 2016). Analyses of Great Australian Bight coastal asphaltite samples collected in surveys between 1990 and 2005 suggest these originate from a common oil (likely an underwater seep), and are distinct from oils which are the source of other tar balls in the same region (Hall et al. 2014; Padley 1990; Scarlett et al. 2019a). This set of asphaltenes, although from the same source, display varying degrees of weathering (Hall et al. 2014; Scarlett et al. 2019a) and therefore provide an ideal test of the use of bicylane ratios.

Heavy fuel oils (also referred to as heavy diesel oils or bunker fuel oils) used in shipping are typically blends of residual products from crude oil refinement (Fritt-Rasmussen et al. 2018; Uhler et al. 2016), and hence their bicyclane biomarker profiles differ depending on the crude oil(s) from which they are derived. Although previously fuel oils contained up to 3.5% sulfur, recent changes to International Maritime Organisation regulations (IMO 2019) have led to the development of very

low sulfur fuel oils (LSO), an example of which was spilled during the 2020 MV *Wakashio* grounding in Mauritius (Scarlett et al. 2021).

In previous work we demonstrated that bicyclanes are sequestered in the adipose tissues of fish exposed to crude oil and heavy fuel oil (Spilsbury et al. in review; Chapter 5). Unlike other classes of hydrocarbons used for fingerprinting crude oils such as polycyclic aromatic hydrocarbons (PAHs) (Goto et al. 2021; Liu et al. 2013; Yang et al. 2017) and regular isoprenoids (Stout et al. 2016; Yang et al. 2017), the relative abundance of bicyclanes are conserved in the adipose tissues of exposed fish (Spilsbury et al. in review; Chapter 5), such that they can be used to provide a fingerprint enabling the oil of exposure to be identified.

Computerized multivariate statistical analysis comparisons of such ratios may be more objective. Linear discriminatory analysis (LDA) is a form of multivariate analysis most commonly reported in literature in relation to machine learning applications such as facial recognition systems (Kaur et al. 2020). Unlike principle components analysis (PCA) which seeks to reduce the dimensionality of complex datasets by establishing principal components that retain as much of the variability in a dataset as possible, LDA optimizes instead for the greatest differences between a specified categorical variable to maximize discrimination between sample groups (Skrobot et al. 2007; Sparks et al. 1999). Once an LDA model has been "trained" using reference data with known categorizations, predictions identifying which category an unknown sample belongs to can be made (Sparks et al. 1999).

In this proof-of-concept study, our aim is was identify from 16 possible candidates the specific oil to which a fish had been exposed, using LDA analysis of the bicyclane fingerprints from the adipose tissue. We initially trained an LDA model using a library of the bicyclane profiles of a tailored suite of 16 oil samples including heavy fuel oils (including a very low sulfur fuel oil), crude oils from the same geographical region, and coastal asphaltites from a common source with different degrees of weathering. We then applied the model to a test dataset of bicyclane profiles from the adipose

tissue of fish exposed *via* diet to either one of the heavy fuel oils, or to one of the NW shelf crudes, and demonstrate the feasibility of using fish bicyclane fingerprinting to identify an exposure oil during or after an oil spill.

6.3. Materials and Methods

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

6.3.1. Chromatography of Oils

The five crude oils used in this trial were a medium crude oil from the Montara (MCO) well, two light crudes from the Caswell (CAS) and Eland West (ELW) wells, and condensates from the Crux-3 (CRX) and Calliance (CAL) wells, as characterised in a previous study (Spaak et al. 2020). The three fuel oils included in the study were a Bunker C (BNC), a heavy fuel oil from the BP Kwinana refinery in Perth, Australia (HFO), and a very low sulfur fuel oil from the MV *Wakashio* (LSO). Eight samples of coastal asphaltites collected between 1990 and 2005 from a variety of locations in the Great Australian Bight (GAB) were also included for comparison. Weathering of the asphaltites, characterised in a previous study (Scarlett et al. 2019a), ranged from mild to heavy (denoted W1 - W5 respectively).

Small silica columns were prepared in glass Pasteur pipettes with 0.5 g of silica and rinsed with 10 mL of hexane. 10 μg of oil was applied to the column, and the F1 fraction was eluted with 3 mL of hexane into a 4 mL vial, before evaporation under a gentle nitrogen stream to approximately 0.5 mL.

6.3.2. Fish Exposure Trial

Trial design and rearing parameters are previously described in detail (Spilsbury et al. 2021; Chapter 2). Briefly, juvenile *Lates calcarifer* (10-15 cm in length, mean weight 85 ± 2 g) were obtained from a commercial hatchery, and kept in 100 L aquaria containing natural Indian Ocean seawater. For 33 days fish were fed either commercial fish-meal spiked with 1% w/w of either MCO or HFO. Following a two-day depuration period, fish were euthanized, and the brown adipose tissue adjacent to the intestine was surgically removed and immediately frozen at -20^{o}C.

6.3.3. Adipose Tissue Extraction and Chromatography

Extraction and chromatography methods are described in detail elsewhere (Spilsbury et al. in review; Chapter 5). Briefly, 2-5 g of adipose tissue was digested under reflux in a 250 mL round bottom flask (RBF) with 5 g KOH and 100 mL of methanol. Rotary evaporation reduced the volume to approximately 4mL aliquot, which was chemically dried using $MgSO_4$.

Silica chromatography columns were prepared in 50 mL burettes and rinsed with chromatography grade hexane. Adipose tissue extracts were reduced to approximately 0.5 mL under a gentle stream of nitrogen before being added directly to the column. The saturated hydrocarbon fraction (F1) was obtained by elution into a 50 mL RBF with 40 mL of hexane, and then reduced to approximately 0.5 mL by rotary evaporation, transferal to a 4mL vial and a final blowdown under gentle nitrogen. Four procedural blanks were performed, and three blank spike recoveries using an in-house standard mixture.

6.3.4. GC-MS Analysis

A 1µL injection volume was used for all samples of F1 (saturates) fractions of oils and fish adipose tissue extracts. Analyses were performed on an Agilent GC 7890B coupled to a MS 5977B using a DB-1MS column (Agilent P/N 122-0162UI; film thickness 0.25 µm, inner diameter 0.25 mm, length 60 m).

Data was analysed using Agilent ChemStation software, version F-01-03-2357. Bicyclane peaks were identified by elution order and mass spectra (Spilsbury et al. in review; Chapter 5), and peak areas were calculated from extracted ion chromatograms (EIC) using the sum of ions m/z 123, 179 and 193 (Wang et al. 2005; Yang et al. 2012; Yang et al. 2017).

6.3.5. Statistical Analysis

All analyses performed using R statistical software, version 4.1.0.

Bicyclanes were identified by elution order and mass spectra (Spilsbury et al. in review; Chapter 5). Diagnostic ratios were calculated from the relative abundances of bicyclanes common to all the oils used in this study, using direct peak area comparisons.

LDA was performed using the MASS R package (Venables and Ripley 2002). An LDA model was defined using a "training" data set consisting of six bicyclane ratios in nine petroleum products: HFO (n = 3), BNC (n = 3), LSO (n=3), MCO (n = 3), CRX (n=3), CAL (n=3), CAS (n = 2), ELW (n=3) and GAB (n = 8). Exposure oil predictions from the LDA model were then obtained using a "test" data set of the same six bicyclane ratios from adipose tissue extracts from fish exposed to MCO (n=9) or to HFO (n=9) (see Supplementary Information).

6.4. Results

In all oils and adipose tissue extracts analysed, suites of up to 10 bicyclanes were detected, eluting between C_{14} *n*-tetradecane and C_{16} *n*-hexadecane. Blank spike recoveries for saturated hydrocarbons in the same retention range range were 61%, 82% and 100% for the C_{10} bicyclic decalin, C_{13} *n*-tridecane and C_{17} *n*-heptadecane, respectively. Bicyclanes were named by elution order as BS-A through -J, consistent with previous studies (Spilsbury et al. in review; Chapter 5). Fuel oils, crudes, asphaltites and fish adipose extracts were able to be differentiated by the respective abundance of the four bicyclanes previously used for fingerprinting (Spilsbury et al. in review; Chapter 5), BSA, BSD, BSE and BSJ (8β(H)-homodrimane), plus other supplementary bicyclanes (Figure 1). The fuel oils have very similar bicyclane profiles, although they are still able to be differentiated by the relative size of the BSJ peak, as well as a prominent 8β(H)-drimane peak in the bunker C fuel oil, eluting after BSE. Likewise, the crude oils from the NW Shelf have similar bicyclane profiles, but can be distinguished by the respective size of the BSA, BSE and BSJ peaks. The asphaltites from the GAB has a distinctly different bicyclane profile with larger BSE and BSJ peaks compared to other samples, and a notable prominent bicyclane peak eluting after BSE, unique among the oils included in this study.

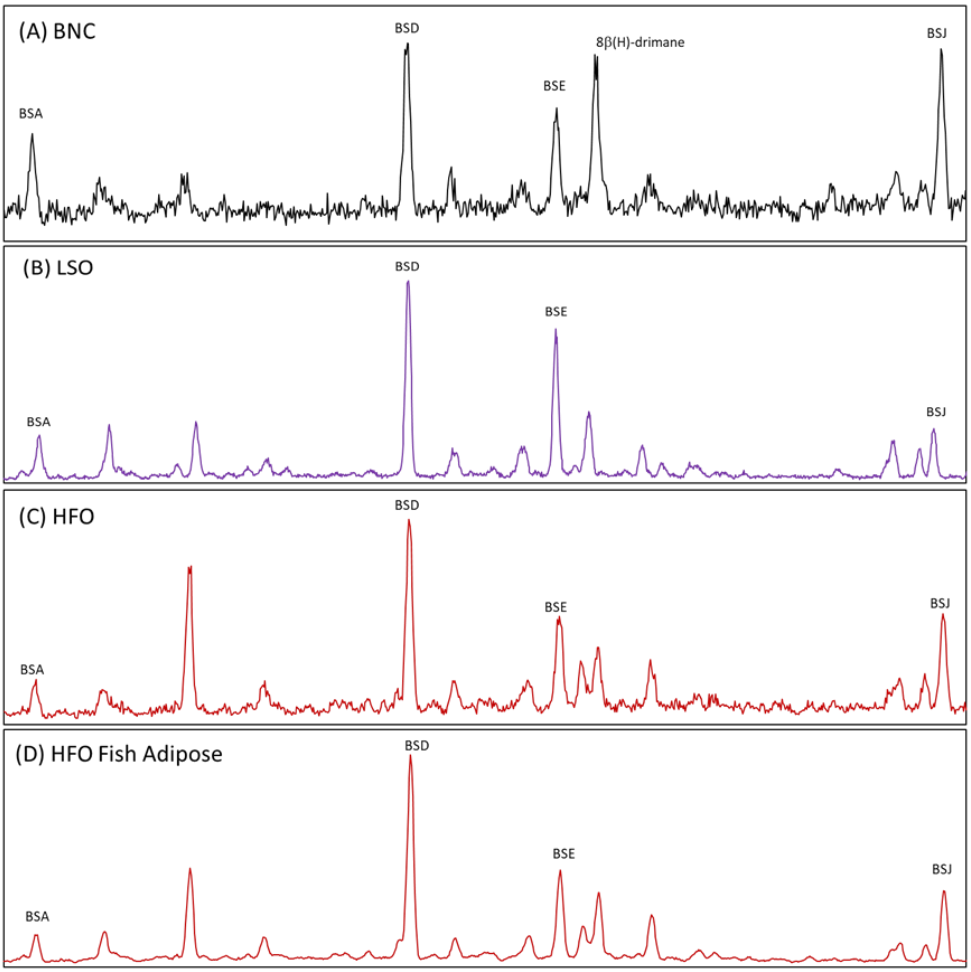

Figure 1a: Typical partial extracted ion GC-MS chromatograms (m/z 123 + 179 + 193) of fuel oils (A-C) and adipose extracts of fish exposed to fuel oil (D).

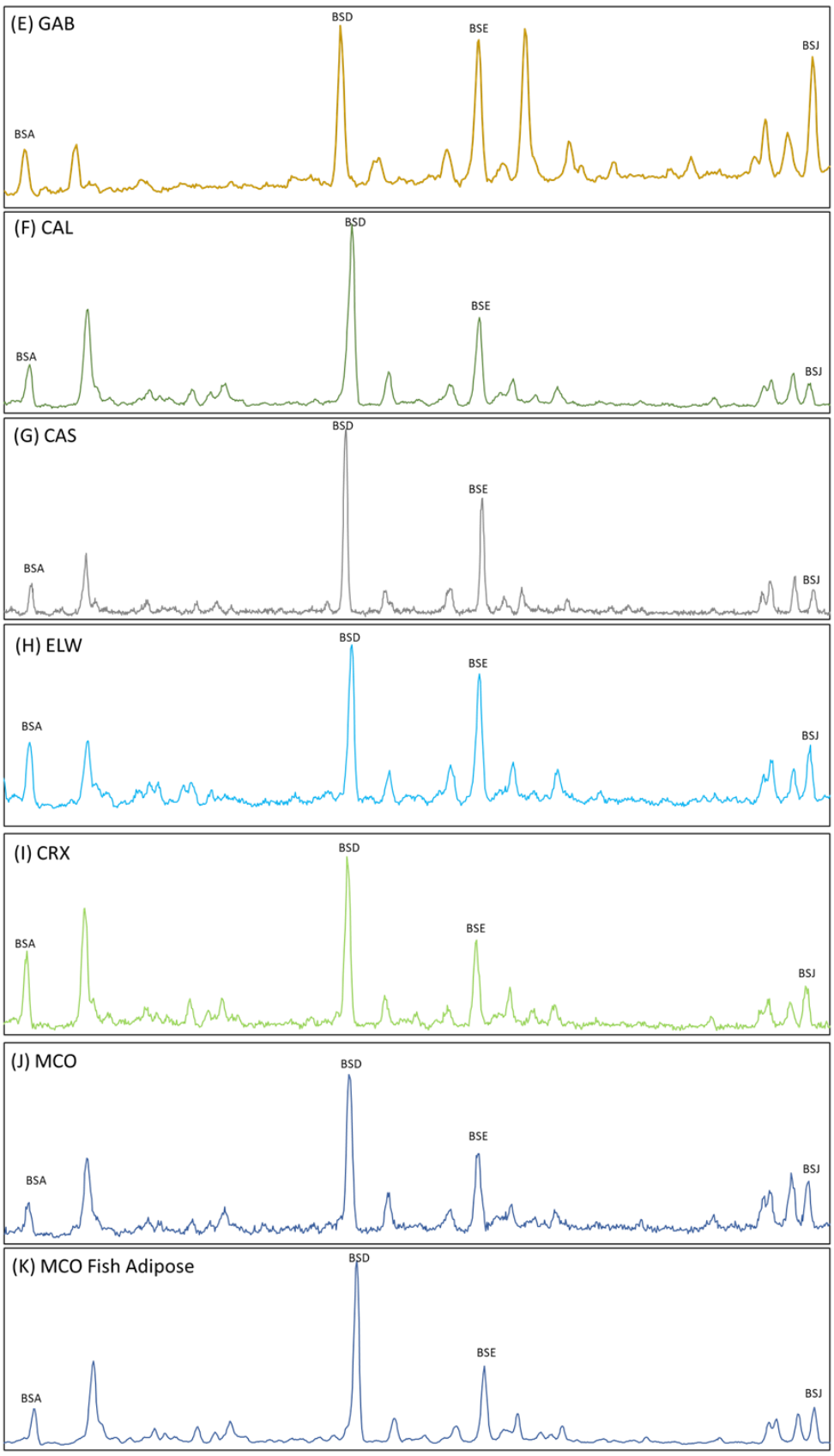

Figure 1b: Typical partial extracted ion GC-MS chromatograms (*m/z* 123 + 179 + 193) of asphaltites (E) and crude oils (F-J) and adipose extracts of fish exposed to crude oil (K).

Ratios of the four bicyclane peaks common to all samples were calculated using direct peak area comparisons (Table 1). Relative sequestration of bicyclanes in fish adipose tissue was consistent, with coefficients of variation (c.v.) for bicyclane ratios ranging from 3.8 to 11.6% for MCO-exposed fish, and 4.2 to 11.2% for HFO-exposed fish.

Table 1: Diagnostic ratios of four bicyclanes in crude oils, heavy fuel oils, asphaltites and adipose tissue extracts of fish exposed to Montara crude oil (MCO) and heavy fuel oil (HFO). Confidence intervals are 2 x standard error

Sample	*n*	BSA:BSD	BSA:BSE	BSA:BSJ	BSD:BSE	BSJ:BSD	BSE:BSJ
Bunker C (BNC)	3	0.45 ± 0.07	0.77 ± 0.15	0.55 ± 0.08	1.68 ± 0.14	0.83 ± 0.13	0.73 ± 0.16
Heavy fuel oil (HFO)	3	0.14 ± 0.02	0.30 ± 0.04	0.31 ± 0.01	2.00 ± 0.02	0.47 ± 0.07	1.07 ± 0.16
Low sulfur oil (LSO)	4	0.20 + 0.03	0.25 + 0.05	0.82 + 0.10	1.30 + 0.08	0.24 + 0.01	3.25 + 0.27
GAB asphatites (GAB)	8	0.22 ± 0.03	0.21 ± 0.03	0.23 ± 0.05	0.92 ± 0.05	0.99 ± 0.12	1.13 ± 0.13
Caswell (CAS)	2	0.23 ± 0.12	0.42 ± 0.27	1.86 ± 1.25	1.78 ± 0.24	0.13 ± 0.02	4.37 ± 0.41
Montara (MCO)	3	0.22 ± 0.04	0.43 ± 0.05	1.08 ± 0.22	1.97 ± 0.11	0.20 ± 0.01	2.52 ± 0.21
Eland West (ELW)	3	0.08 + 0.07	0.09 + 0.08	0.25 + 0.23	1.15 + 0.05	0.32 + 0.01	2.76 + 0.08
Calliance (CAL)	3	0.18 + 0.01	0.39 + 0.02	1.59 + 0.06	2.12 + 0.06	0.12 + 0.01	4.09 + 0.31
Crux-3 (CRX)	3	0.24 + 0.04	0.48 + 0.06	0.99 + 0.12	1.96 + 0.08	0.25 + 0.02	2.07 + 0.16
MCO Fish Adipose	9	0.20 ± 0.01	0.46 ± 0.03	1.10 ± 0.11	2.32 ± 0.06	0.18 ± 0.01	2.40 ± 0.16
HFO Fish Adipose	9	0.15 ± 0.01	0.31 ± 0.02	0.44 ± 0.04	2.12 ± 0.06	0.34 ± 0.01	1.40 ± 0.07

6.5. Linear Discriminatory Analysis

Using a training set of the six bicyclane ratios from the five oils produces an LDA ordination space in which the nine petroleum products are distinctly separated (Figure 2). The fuel oils BNC, HFO and LSO are able to be discriminated on the LD1 and LD2 Cartesian axes, as are MCO and CRX, two highly similar crude oils from the Browse Basin. Within the ordination space, the fish adipose tissue extracts are within the 95% posterior probability categorization boundaries for the respective crude oils that the fish were exposed to. (Figure 2). Hence, the LDA predictions for the adipose tissue extracts from fish exposed to either MCO (n=9) or HFO (n=9) correctly identified the respective oil to which each fish was exposed. (See Supplementary Information for R Markdown).

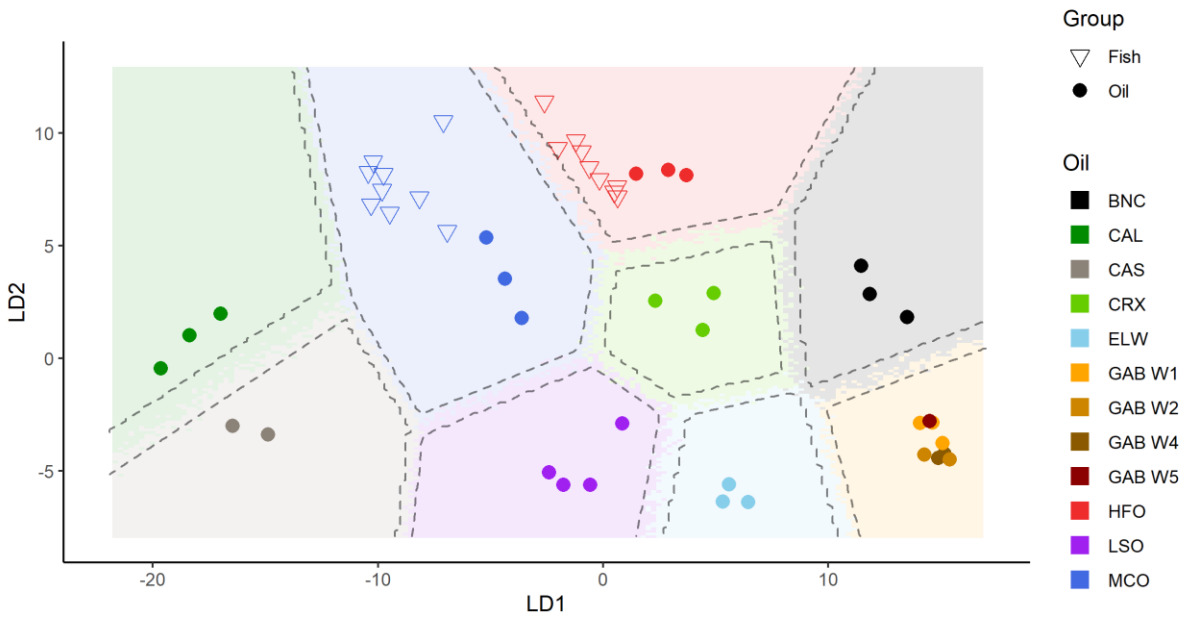

Figure 2: Linear discriminatory analysis of crude oils, heavy fuel oils, asphaltites and adipose tissue extracts of fish exposed to Montara crude oil (MCO) and heavy fuel oil (HFO). Shaded areas are the decision boundaries for the respective oils, with dotted lines indicating the 95% posterior probability demarkation.

The position within the LDA ordination space of the GAB asphaltites was not affected by the degree of weathering of the samples, with asphaltites with mild weathering (W1) clustered in close proximity, and in some cases superimposed over samples with heavy weathering (W5) (Figure 2).

6.6. Discussion

The GC-MS chromatograms of the bicyclane profiles of crude oils and heavy fuel oils used in this study correspond to other reports using bicyclanes for the forensic identification of crude oils (Wang et al. 2013) and diesel fuel oils (Stout et al. 2005; Stout et al. 2016). The likewise similar bicyclane profiles in fish adipose tissue extracts demonstrates that bicyclane deposition in the adipose tissue

of fish exposed to petroleum hydrocarbons is sufficiently discriminatory to enable source oil identification, even when challenged with similar oils from sources in close geographical proximity.

Similarly, the clustering of GAB asphaltites in the LDA ordination space indicate little change in the bicyclane profiles of asphaltites, even though they were each subject to different degrees of weathering. This reinforces the supposition that bicyclanes are not greatly degraded during weathering, and their relative abundances remain consistent. Even for oil spills that spend long periods of time in the environment exposed to weathering processes before reaching a shoreline, bicyclane fingerprinting would be a viable method for identification of the source of crude oil.

The 100% successful prediction rate of exposure oils of this proof-of-concept study is encouraging, given the chemical similarity of some of the oils used. Other petroleum fingerprinting studies using similar LDA approaches to identify solvent additives in mixtures of refined petroleum products (Skrobot et al. 2007) and to identify unknown asphalts (Ren et al. 2019) also achieved high rates of prediction success (90.0% and 96.2% respectively).

The sequestration of bicyclanes into the adipose tissues of oil-exposed fish is consistent within in each test group, as can be seen by the clustering of HFO- and MCO-exposed fish on the LDA ordination plot (Figure 2), and bicyclane ratio confidence intervals (Table 1). The conservation of the relative abundances of bicyclanes in fish adipose tissue may be due in part to only minor losses from cellular metabolic processes, and likely similar lipophilicity for various bicyclane compounds, resulting in nearly identical uptake and sequestration rates of the various individual bicyclanes in the tissues of exposed fish.

This study uses controlled laboratory exposures to crude and heavy fuel oils with a prolonged duration of several weeks. In order to field-test this method of oil identification, the future acquisition of fish exposed to petroleum hydrocarbons in an oil spill would be needed. Fish from oil spill affected aquaculture operations would be particularly suitable as this would remove doubts about oil spill avoidance by motile species, and also allow for the estimation of exposure duration,

which likely would limit the amounts of bicyclanes bioconcentrated in fish adipose tissue. This study demonstrates that relative abundances of adipose bicyclanes and their corresponding fingerprint ratios remain consistent and unchanged compared to their respective exposure oils up to the 33-day duration of the laboratory exposures, which is a realistic time-frame for application to environmental oil spills. Shorter exposure durations may result in bicyclane concentrations in fish adipose tissue that approach current limits of detection, and further research is needed to establish minimum exposure durations for detection of bicyclanes in the adipose tissue of oil-exposed fish. Application of two dimensional gas chromatography mass spectrometry (GC×GC-MS) in future studies could be beneficial due its generally lower limits of detection and superior resolution (Beyer et al. 2010). Given that bicyclanes are not readily metabolised by fish, sequestered bicyclanes in adipose tissue may remain *in-situ* for long periods post-exposure, possibly even after other signs of oil exposure have dissipated in the environment. Testing depuration rates of adipose bicyclanes in oil-exposed fish is a topic for future study.

The nine-sample library of bicyclane biomarker ratios from oils used as a training set for the LDA is a limitation of this in-principle study. To further explore the potential of bicyclane fingerprinting in fish adipose tissue for oil identification in the unfortunate event of a spill, future research is needed to expand the training set library by characterising the bicyclane profiles of a larger number of crude oils and refined petroleum products from a variety of geographical locations and sources. As the number of oils in the training data set are increased, however, more biomarkers are inevitably needed to discriminate them (Tharwat et al. 2017). Subject to confirmation that relative abundances are conserved in the long-term in the adipose tissues of exposed fish, other geochemical biomarkers such as adamanatanes, ethanoadamantanes and other diamondoids (Grice et al. 2000; Scarlett et al. 2019b; Wang et al. 2007) may also need to be included to supplement the number of biomarker ratios used to derive a fingerprint. In these cases, a subsequent reduction in dimensionality can be achieved if necessary by PCA-LDA (Skrobot et al. 2007). This approach has been used in the fingerprinting and identification of asphalts (Ren et al. 2019), and fuel oils (Sun et al. 2018), and

could be applied to analyses of adipose tissue bicyclanes of fish exposed to oils, and subsequently identify an unknown oil.

Acknowledgements

Many thanks to Gemma Spaak for providing GC-MS chromatograms for the Caswell Oils used in this study. Thanks also to Rowan Kliendienst for assistance with aquaria set up, and sampling, Md. Reaz Chaklader for animal husbandry advice and sampling assistance, and to Peter Hopper for invaluable and instrumental GC-MS assistance.

Funding

This work was supported by grants provided by the Australian Research Council (grant number LP170101000) awarded to Gagnon, Grice and Rowland.

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Chapter7: Conclusion

7.1. Biochemical Biomarkers

Crude oils are highly complex mixtures of thousands of hydrocarbons, only some of which produce toxic effects. Oils that are from varied locations, different geological periods, or are derived from discrete petrogenic source materials are chemically distinct. In some cases, such as comparing a medium crude oil from the northwest (NW) shelf of Australia like Montara, with a refined petroleum product such as a heavy fuel oil, the differences the relative abundance of key compounds such as polycyclic aromatic hydrocarbons (PAHs) give rise to measurably different biomarker profiles in fish exposed to the respective oils. Whereas previous studies have detailed biochemical biomarker responses as evidence of exposure to a specific crude oil, or to the WAF of multiple crudes, the current work is the first example of a study that compares the different biomarker responses in fish following dietary exposures to different oils in a controlled laboratory trial.

Of the 12 biochemical and physiological biomarkers included in this study, biliary PAH metabolites were the most useful for discriminating oils, and reflected the ordinal relative abundances of the corresponding two-, three-, four- and five-ring PAHs in the respective exposure oils. This finding is in agreement with impact assessment studies following the DWH incident. Although a reliable and highly sensitive short-term indicator of recent exposure to crude oil, capable of confirming exposure to very low doses of PAHs, biliary PAH metabolites do not relate quantitatively to the relative abundance of PAHs in the oil to which the fish were exposed, and hence are of limited use in crude oil identification.

When considered in conjunction with biliary PAH metabolites, EROD activity in the liver can inform on the relative amounts of naphthalene compared to the larger molecular weight PAHs. The detoxification and subsequent elimination of PAHs by hepatic AhR-mediated biotransformation pathways is not triggered by naphthalenes, but is induced more strongly by larger PAHs such as phenanthrene, pyrene and benzo(*a*)pyrene. Hence the presence of PAH metabolites in the bile

combined with a lack of EROD activation implies that fish were exposed to crude oil that has a high relative abundance of naphthalene compared to all other higher molecular weight PAHs, such as Montara. This is applicable in the forensic identification of oils by excluding a negatively matching oil based on their PAH profile.

Condition factor, haematocrit and brain AChE activity were useful indicators of crude oil exposure. However, these are not specific to crude oil exposure like biliary PAH metabolites and can also indicate exposure to a large range of other toxic compounds. Liver somatic index (LSI) and heat shock protein (HSP70) were not useful in indicating exposure to crude oil, nor discriminating to which oil a fish has been exposed. Similarly, the biomarker for DNA damage, 8-oxo-dG was not able to confirm crude oil exposure. This is unexpected, because the metabolism of metals and PAHs found in crude oils generates the oxidative stress that causes the formation of DNA adducts, and the presence of biliary PAH metabolites shows that the biotransformation of these compounds is occurring, generating reactive oxidative species (ROS) in the process. Metals exposure also induces oxidative stress and subsequent DNA damage in fish, however the metals in oils are likely not bioavailable due to porphyrin sequestration.

In this study, specific compounds causing the observed toxic effects in fish have not been identified, other than in general terms of classes of compounds such as the various PAHs or metals analysed in the respective crude oils. Toxic effects from crude oil exposure are mixture effects with a large number of potentially contributing compounds, including many not covered in the current work such those in the polar fraction, or the immeasurable alkylated hydrocarbons found in the UCM. Establishing toxic causality or describing modes of action of individual compounds that may contribute to the adverse effects of crude oil exposure is beyond the scope of this work, which selectively describes only the compounds, toxigenesis and biomarker responses applicable to the forensic identification of oils.

Previously undescribed, this study has established baseline values for biochemical and physiological biomarkers in *Lates calcarifer*. Given its wide geographical range this will undoubtedly be of use at some future point for impact assessments and environmental monitoring post-remediation should an oil spill occur anywhere where this species of fish is naturally found, or is farmed in aquaculture operations. This study has also demonstrated the measurable biochemical responses of *L. calcacifer* to environmentally relevant levels of crude oils, which also has the potential to assist in environmental impact assessments of spills in locations where this fish is found.

7.2. Otolith Microchemistry

The two metals which are commonly used in crude oil identification, Ni and V, are not incorporated into otolith *via* the dietary route, which is a novel finding previously unreported. As these metals in crude oils (Ni and VO) are also unlikely to be bioavailable for incorporation via the waterborne route due to porphyrin insolubility, the hypothesis that LA-ICP-MS analysis of these two metals in fish otoliths could be used as an historical record of crude oil exposure, and be used as a corroborating line of evidence to identify a crude oil is not supported.

Through multivariate analysis, we have shown that other selected metals can be used to discriminate between fish exposed to oils with different metals profiles, but individual fish within each respective test group displayed a large variability in the uptake and otolith deposition of metals such as Al, and Ba. Large sample numbers following chronic exposure would therefore be needed to usefully apply otolith microchemistry in an environmental oil spill scenario. It is unlikely that further research along this avenue would provide additional information useful to oil spill impact assessments.

7.3. Gut Microbiome

The gut microbiome of *L.calcarifer* is altered following dietary exposure to oils or metals. Novel potential biomarkers indicative of crude oil exposure have been identified in this work. The genus *Photobacterium* becomes enriched in the gut microbiome of *L.calcarifer* in response to dietary exposure to PAHs found in crude oils and refined oil products. As many PAHs are water soluble and thereby present in the WAF, it seems probable that this would also hold true for aqueous exposure. We have shown that the gut microbiome is not influenced by aqueous bacterial species, hence only species of bacteria that are already present can be enriched in the gut microbiome in response to xenobiotic compounds. The *Photobacterium* biomarker depends on this bacterial genus being present in the fish microbiome prior to exposure, and this study has demonstrated that *Photobacterium* is present in the gut microbiome of nursery-raised *L.calcarifer* fed commercial fishmeal, as would be the case in aquaculture operations. Further research is needed, however, to demonstrate that *Photobacterium* are also present in the gut microbiome of wild fish. In an oil spill scenario, wild fish would provide a false-negative result for gut microbiome *Photobacterium* enrichment following crude oil exposure if *Photobacterium* is absent in the pre-exposure gut microbiome.

The lactic acid bacteria *Lactobacillus* is beneficial to fish gut health, and is present in the microbiome of healthy, unexposed fish. A number of studies have demonstrated the reduction or elimination of *Lactobacillus* in the gut microbiome in response to a variety of toxic compounds. We have shown that this also occurs in the gut microbiome of *L.calcarifer* in response to chronic dietary exposure to Fe, V and Ni. A marked alteration of the relative abundance of the core phyla present in the gut microbiota also occurs following dietary metals exposure in *L.calcarifer*, with the specific enrichment of Firmicutes, Bacteroidetes and Protobacteria in response to V, Fe and Ni respectively. Although this shows promise as a novel biomarker for fish metals exposure, further research is needed to determine the minimum doses required to elicit this alteration of the gut microbiome.

The up-regulation of pro-inflammatory cytokines in the host fish in response to dietary metals and heavy fuel oil indicates an immune response, which was lacking in fish exposed to Montara. This may be linked to the relatively low abundance of the AhR-activating three to five ring PAHs in this crude oil, however a causal link has not been established and is beyond the scope of this work. Cytokine expression has been shown to be an indicator of exposure to metals and some petroleum hydrocarbons, although this is not specific to oils and is also caused by a variety of other toxicants.

7.4. Bicyclic Sesquiterpanes

Hydrocarbons from crude oils are sequestered in the tissues of exposed fish, with 95% of the compounds found in the brown adipose tissue adjoining the intestinal tract. Not all compounds commonly used to derive fingerprint ratios for forensic identification are sequestered, however. Large molecular weight PAHs (such as benzo(*a*)pyrenes and dibenzothiophenes), steranes and hopanes were not detected in the adipose tissue of fish exposed to petroleum hydrocarbons. Importantly, many of the compounds in oils used to derive fingerprint ratios for forensic identification are sequestered at different rates, likely influenced by either varying uptake rates corresponding to their respective lipophilicity, their elimination rates due to cellular metabolism, or both. Larger *n*-alkanes C_{17} to C_{24} show progressively reduced relative uptake compared to the respective relative quantities present in the respective oils. The presence of PAH metabolites in the bile (Chapter 2) demonstrates that these compounds are metabolised and eliminated by the fish via AhR-mediated biotransformation processes. Following an extended depuration period, it is likely that adipose PAH concentration would be further reduced below analytical limits of detection, although this is beyond the scope of the current work and has not been explored.

The bicyclic sesquiterpanes (bicyclanes) are a good choice of compounds for crude oil fingerprinting in the context of forensic analysis of oil-exposed fish. Importantly, bicyclanes are both ubiquitous and characteristically varied in crude oil and refined petroleum products, unlike some of the other

classes of commonly used fingerprinting compounds such as the steranes and hopanes which may be lacking in lighter crudes such as those from the Australian NW shelf. Bicyclanes in the C_{14} to C_{16} range have a high lipophilicity (e.g. 8β(H)-drimane and 8β(H)-homodrimane have a $LogK_{OW}$ of 6.2 and 6.7 respectively) that implies ready uptake into adipocytes, are not mentioned in literature as being toxic to fish or subject to substantial elimination by cellular metabolism. This results in consistent bioaccumulation factors (BAFs) among the various individual bicyclanes in the adipose tissue of fish exposed to petroleum hydrocarbons, such that the characteristic diagnostic ratios of bicyclanes are comparable to those in the source oil.

A difference in BAF would imply that disparities between the relative abundances of compounds sequestered in fish adipose tissue compared to those in a source oil would become larger with increased duration of exposure. This study has demonstrated that the fish adipose sequestration rates of bicyclanes are sufficiently similar that the relative abundances remain viable for fingerprinting up to a 33-day exposure, which is more than sufficient for an environmental oil spill scenario. Further research is needed to establish a minimum exposure time for detection of bicyclanes in adipose tissue of fish exposed to petroleum hydrocarbon fluids. Future research could also include depuration studies to establish continued bicyclane fingerprinting capability for lengths of time after exposure has ceased and environmental samples of spilled oil may no longer be available. As bicyclanes are not known to be metabolised by fish, it seems possible that they may remain detectable, and in conserved ratios permitting source oil identification for extended periods after exposure.

7.5. Environmental Applications

This study has demonstrated the novel application of bicyclane fingerprinting in identifying an oil using extracts from the adipose tissue of fish exposed to two chemically distinct oils. In the event of an oil spill, bicyclanes measured in adipose tissues of oil-exposed fish could be used to provide a

legally defensible forensic identification of a crude oil should a sample of the suspected source oil be available for comparison. In this scenario, a direct scatterplot comparison of bicyclane ratios and determination of the goodness of fit would enable the positive identification of an oil, or rejection of an oil that has a dissimilar bicyclane profile.

For cases where multiple different oils are suspected candidates for the source of an oil spill, multivariate analysis tools such as PCA and LDA are useful. In this study, a proof-of-concept demonstrating the viability of fish adipose bicyclane fingerprinting has been achieved by applying LDA, comparing bicyclane profiles in adipose tissues of fish exposed to a heavy fuel oil or a NW shelf medium crude against a tailored library of crude oils, fuel oils and asphaltites, including some crude oils that are chemically similar. Following the 2009 Montara well failure, forensic identification of Montara oil in the Timor Sea was conclusively demonstrated for the Montara Commission of Inquiry by Leeder Consulting using 12 diagnostic biomarker ratios including PAHs, steranes, hopanes and regular isoprenoids. In the current work, the identification of a source oil with a 100% success rate (n=18) was achieved using six ratios calculated from the four bicyclanes common to all the oils included in this study. This highlights the discriminatory power of fish adipose bicyclane fingerprinting, challenged with a library of nine oils for comparison.

Future studies could further stress the capabilities of fish fingerprinting by expanding the library of potentially matched oils to include from diverse geographical locations, geological periods and petrogenic source materials. More diagnostic ratios for other bicyclanes could also be included. The application of GC×GC-MS in future studies would be of benefit to resolve the co-elution problem that prevented some of the bicyclanes detected in fish adipose tissue from being used in the LDA analysis. Investigations into scenarios where multiple source oils exist (e.g. a crude oil and a diesel fuel oil) could also be conducted.

The collective current works meet the overall aim: “to determine whether a specific crude oil can be fingerprinted and forensically identified using the biochemical and chemical biomarkers in fish exposed to the oil”. The viability of “fish fingerprinting” to identify a source oil, and its potential for application to an environmental oil spill scenario has been successfully demonstrated.

Appendix A: Supplementary Information for Chapter 2

Table S1: Polycyclic Aromatic Hydrocarbon Analysis of Montara Crude Oil and Heavy Fuel Oil

Compound	Limit of Reporting (mg/kg)	Montara Crude Oil (mg/kg)	Standard Error	Heavy Fuel Oil (mg/kg)	Standard Error
Naphthalene	20	2700	100	783	9
2-Methylnaphthalene	20	7367	285	2300	0
1-methylnaphthalene	20	3700	153	1333	33
C2-alkylnaphthalenes	100	8667	338	3367	33
C3-alkylnaphthalenes	100	5433	219	2767	33
C4-alkylnaphthalenes	100	1967	88	1333	33
Total Naphthalenes	**100**	**29833**	**1179**	**11883**	**124**
Biphenyl	20	2133	67	50	1
Acenaphthylene	20	-	-	-	-
Acenaphthene	20	92	4	59	0
Dibenzofuran	20	360	15	38	0
Fluorene	20	660	25	99	1
Methylfluorenes	100	1533	67	313	3
C2-alkylfluorenes	100	1600	58	503	3
C3-alkylfluorenes	100	937	38	507	3
Anthracene	20	29	1	39	2
Phenanthrene	20	1167	33	340	6
Methylphenanthrenes	100	2133	67	1033	33
C2-alkylphenanthrenes	100	1733	67	1500	0
C3-alkylphenanthrenes	100	970	30	1300	0
C4-alkylphenanthrenes	100	367	13	660	6
Total Phenanthrenes	**-**	**6370**	**210**	**4833**	**39**
Fluoranthene	20	34	2	21	0
Pyrene	20	36	1	100	0
Methylpyrenes/fluoranthenes	100	280	10	397	3
C2-alkylpyrenes/fluoranthenes	100	283	12	753	9
C3-alkylpyrenes/fluoranthenes	100	193	7	750	21
C4-alkylpyrenes/fluoranthenes	100	107	3	550	21
Total Pyrenes/Fluoranthenes	**-**	**910**	**22**	**2550**	**49**
Dibenzothiophene	20	243	7	220	0
Methyldibenzothiophenes	100	450	15	720	6
C2-alkyldibenzothiophenes	100	380	15	1167	33
C3-alkyldibenzothiophenes	100	197	9	1033	33
C4-alkyldibenzothiophenes	100	-	-	387	7
Total Bibenzothiophenes	**-**	**1270**	**46**	**3527**	**69**
Benzo(b)fluorene	20	58	10	39	3
Benz(a)anthracene	20	-	-	72	2
Chrysene	20	61	2	150	10
Methylchrysenes	100	-	-	527	3
C2-alkylchrysenes	100	-	-	850	6
C3-alkylchrysenes	100	-	-	943	15
C4-alkylchrysenes	100	-	-	503	3

Compound	Limit of Reporting (mg/kg)	Montara Crude Oil (mg/kg)	Standard Error	Heavy Fuel Oil (mg/kg)	Standard Error
Total Chrysenes	**-**	**61**	**2**	**2973**	**29**
Benzo(b)naphtho(1,2-d)thiophene	20	54	2	129	46
Methylbenzonaphthothiophenes	100	127	3	1000	50
C2-alkylbenzonaphthothiophenes	100	110	0	1767	67
C3-alkylbenzonaphthothiophenes	100	-	-	1767	120
C4-alkylbenzonaphthothiophenes	100	-	-	857	47
Benzo(b)fluoranthene	20	-	-	29	1
Benzo(k)fluoranthene	20	-	-	-	-
Benzo(a)fluoranthene	20	-	-	-	-
Benzo(e)pyrene	20	-	-	76	5
Benzo(a)pyrene	20	-	-	69	4
Methylbenzopyrenes	100	-	-	323	12
C2-alkylbenzopyrenes	100	-	-	393	17
Total Benzopyrenes/benzofluoranthenes	**-**	**0**	**0**	**891**	**29**
Indeno(1,2,3-cd)pyrene	20	-	-	-	-
Dibenzo(a,h)anthracene	20	-	-	-	-
Benzo(g,h,i)perylene	20	-	-	21	1
Methylindenopyrenes	100	-	-	125	4
C2-alkylindenopyrenes	100	-	-	125	4

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3778 Table S2: Polycyclic Aromatic Hydrocarbon Analysis of Fish Feeds spiked with Oil

Compound	Limit of Reporting (mg/kg)	Feed + MON (mg/kg)	Standard Error	Feed + HFO (mg/kg)	Standard Error	Fish Feed (Neg Control) (mg/kg)	Standard Error
Naphthalene	0.5	41.3	23.9	12.3	7.1	<0.5	-
2-Methylnaphthalene	0.5	116.7	67.4	37.3	21.6	<0.5	-
1-methylnaphthalene	0.5	57.3	33.1	21.3	12.3	<0.5	-
Acenaphthylene	0.5	<0.5	-	<0.5	-	<0.5	-
Acenaphthene	0.5	1.5	0.8	1.0	0.6	<0.5	-
Fluorene	0.5	10.7	6.2	1.8	1.1	<0.5	-
Phenanthrene	0.5	19.0	11.0	5.3	3.1	<0.5	-
Anthracene	0.5	<0.5	-	0.7	0.4	<0.5	-
Fluoranthene	0.5	0.7	0.4	<0.5	-	<0.5	-
Pyrene	0.5	0.6	0.3	1.5	0.9	<0.5	-
Benz(a)anthracene	0.5	<0.5	-	1.1	0.7	<0.5	-
Chrysene	0.5	1.0	0.6	2.3	1.3	<0.5	-
1-methylnaphthalene	0.1	57.3	33.1	21.3	12.3	<0.1	-
2-Methylnaphthalene	0.1	116.7	67.4	37.3	21.6	<0.1	-
Benzo(b)fluoranthene	0.5	<0.5	-	0.5	0.3	<0.5	-
Benzo(k)fluoranthene	0.5	<0.5	-	<0.5	-	<0.5	-
C2-alkylnaphthalenes	0.5	136.7	78.9	52.3	30.2	<0.5	-
C3-alkylnaphthalenes	0.5	85.0	49.1	44.3	25.6	<0.5	-
C4-alkylnaphthalenes	0.5	31.0	17.9	21.7	12.5	<0.5	-
Benzo(a)pyrene	0.5	<0.5	-	1.2	0.7	<0.5	-
Methylphenanthrenes	0.5	34.7	20.0	17.0	9.8	<0.5	-
C2-alkylphenanthrenes	0.5	30.0	17.3	26.7	15.4	<0.5	-
C3-alkylphenanthrenes	0.5	46.3	26.8	52.0	30.0	<0.5	-
C4-alkylphenanthrenes	0.5	6.4	3.7	11.7	6.7	<0.5	-
Dibenzothiophene	0.5	3.9	2.2	3.4	2.0	<0.5	-
Indeno(1,2,3-cd)pyrene	0.5	<0.5	-	<0.5	-	<0.5	-
Methyldibenzothiophenes	0.5	7.2	4.1	11.7	6.7	<0.5	-
C2-alkyldibenzothiophenes	0.5	7.1	4.1	21.3	12.3	<0.5	-
Dibenzo(a,h)anthracene	0.5	<0.5	-	<0.5	-	<0.5	-
Benzo(g,h,i)perylene	0.5	<0.5	-	<0.5	-	<0.5	-
C3-alkyldibenzothiophenes	0.5	3.7	2.1	18.3	10.6	<0.5	-
Methylpyrenes/fluoranthenes	0.5	5.0	2.9	6.9	4.0	<0.5	-
C2-alkylpyrenes/fluoranthenes	0.5	4.8	2.8	13.0	7.5	<0.5	-
C3-alkylpyrenes/fluoranthenes	0.5	3.2	1.9	13.0	7.5	<0.5	-
Methylchrysenes	0.5	1.3	0.7	8.6	4.9	<0.5	-
C2-alkylchrysenes	0.5	1.7	1.0	14.7	8.5	<0.5	-
Methylbenzopyrenes	0.5	<0.5	-	5.2	3.0	<0.5	-
C2-alkylbenzopyrenes	0.5	0.6	0.3	7.2	4.2	<0.5	-
Methylindenopyrenes	0.5	0.5	0.3	2.9	1.7	<0.5	-
C2-alkylindenopyrenes	0.5	<0.5	-	2.7	1.6	<0.5	-

3779

Table S3: Metals Analysis of Heavy Fuel Oil and Montara Crude Oil

Element	Limit of Reporting (mg/kg)	MCO (1) (mg/kg)	MCO (2) (mg/kg)	MCO (3) (mg/kg)	MCO Mean (mg/kg)	Standard Error	HFO (1) (mg/kg)	HFO (2) (mg/kg)	HFO (3) (mg/kg)	HFO Mean (mg/kg)	Standard Error
Aluminium	0.01	30.70	< 0.5	< 0.5	10.23	-	8.15	33.30	4.86	15.44	8.98
Antinomy	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	0.43	0.45	0.50	0.46	0.02
Arsenic	0.03	< 0.03	< 0.03	< 0.03	< 0.03	-	0.03	0.03	0.06	0.04	0.01
Barium	0.01	0.25	0.05	0.03	0.11	0.06	1.18	1.32	1.45	1.32	0.08
Beryllium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Bismuth	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Boron	0.01	< 1.7	< 1.7	< 1.7	< 1.7	-	< 1.7	< 1.7	< 1.7	< 1.7	-
Cadmium	0.01	< 0.01	< 0.01	0.01	0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Caesium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Calcium	0.01	91.00	11.70	10.50	37.73	26.64	11.20	72.70	3.10	29.00	21.97
Cerium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	0.00	0.02	0.00	0.01	0.01
Chromium	0.01	0.89	< 0.12	< 0.12	0.89	-	0.45	0.28	0.00	0.24	0.13
Cobalt	0.01	< 0.46	< 0.46	< 0.46	< 0.46	-	< 0.46	0.66	3.63	2.15	1.48
Copper	0.03	0.45	< 0.31	< 0.31	0.45	-	< 0.31	< 0.31	< 0.31	< 0.31	-
Dysprosium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Erbium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Europium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Gadolinium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Gallium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	0.02	0.02	0.02	0.00
Germanium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Hafnium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Holmium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Indium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Iron	0.01	7.65	1.29	5.25	4.73	1.85	37.30	40.70	35.70	37.90	1.47
Lanthanum	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	0.71	0.69	0.89	0.77	0.06

Element	Limit of Reporting (mg/kg)	MCO (1) (mg/kg)	MCO (2) (mg/kg)	MCO (3) (mg/kg)	MCO Mean (mg/kg)	Standard Error	HFO (1) (mg/kg)	HFO (2) (mg/kg)	HFO (3) (mg/kg)	HFO Mean (mg/kg)	Standard Error
Lead	0.01	0.08	0.09	0.09	0.08	0.00	0.02	0.08	0.04	0.04	0.02
Lithium	0.05	< 0.05	< 0.05	< 0.05	< 0.05	-	< 0.05	0.11	< 0.05	0.11	-
Lutetium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Magnesium	0.01	3.06	0.53	< 0.15	1.80	1.04	1.43	2.73	1.24	1.80	0.47
Manganese	0.04	< 0.04	< 0.04	< 0.04	< 0.04	-	< 0.04	< 0.04	< 0.04	< 0.04	-
Mercury	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Molybdenum	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	0.05	0.06	0.06	0.05	0.00
Neodymium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Nickel	0.01	0.14	< 0.06	0.08	0.11	0.06	10.90	12.50	13.30	12.23	0.71
Niobium	0.03	< 0.03	< 0.03	< 0.03	< 0.03	-	< 0.03	< 0.03	< 0.03	< 0.03	-
Phosphorous	3.5	< 3.5	< 3.5	< 3.5	< 3.5	-	< 3.5	< 3.5	< 3.5	< 3.5	-
Potassium	0.01	38.90	35.40	36.00	36.77	1.08	4.50	12.50	7.37	8.12	2.34
Praseodymium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Rubidium	0.01	0.14	0.02	< 0.01	< 0.01	0.27	0.07	0.16	0.07	0.10	0.03
Samarium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Scandium	0.016	<0.016	<0.016	<0.016	<0.016	-	<0.016	<0.016	<0.016	<0.016	-
Selenium	0.01	0.13	< 0.01	0.06	0.09	0.03	0.00	0.02	0.00	0.01	0.01
Silicon	1	< 1	< 1	< 1	< 1	-	< 1	< 1	< 1	< 1	-
Silver	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Sodium	2.5	4.63	< 2.5	< 2.5	4.63	-	< 2.5	< 2.5	< 2.5	< 2.5	-
Strontium	0.01	0.61	0.07	< 0.01	0.34	0.27	0.26	0.74	0.30	0.43	0.15
Sulfur	0.01	335	460	386	394	36.29	11900	9800	9060	10253	850.59
Tantalum	0.04	< 0.01	< 0.01	< 0.01	< 0.04	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Terbium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Thallium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Thorium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Thulium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-

Element	Limit of Reporting (mg/kg)	MCO (1) (mg/kg)	MCO (2) (mg/kg)	MCO (3) (mg/kg)	MCO Mean (mg/kg)	Standard Error	HFO (1) (mg/kg)	HFO (2) (mg/kg)	HFO (3) (mg/kg)	HFO Mean (mg/kg)	Standard Error
Tin	0.01	0.03	< 0.01	0.32	0.18	0.10	0.19	0.02	0.18	0.13	0.06
Titanium	0.01	< 0.24	< 0.24	< 0.24	< 0.24	-	3.03	3.47	3.22	3.24	0.13
Tungsten	0.01	< 0.04	< 0.04	< 0.04	< 0.01	-	< 0.04	< 0.04	< 0.04	< 0.04	-
Uranium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Vanadium	0.03	< 0.03	< 0.03	< 0.03	< 0.03	-	14.20	14.60	17.00	15.27	0.87
Ytterbium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Yttrium	0.01	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Zinc	0.01	1.58	1.60	1.24	1.47	0.85	0.97	1.41	1.20	1.19	0.13
Zirconium	0.01	0.22	< 0.01	< 0.01	0.22	0.13	< 0.01	< 0.01	< 0.01	< 0.01	-

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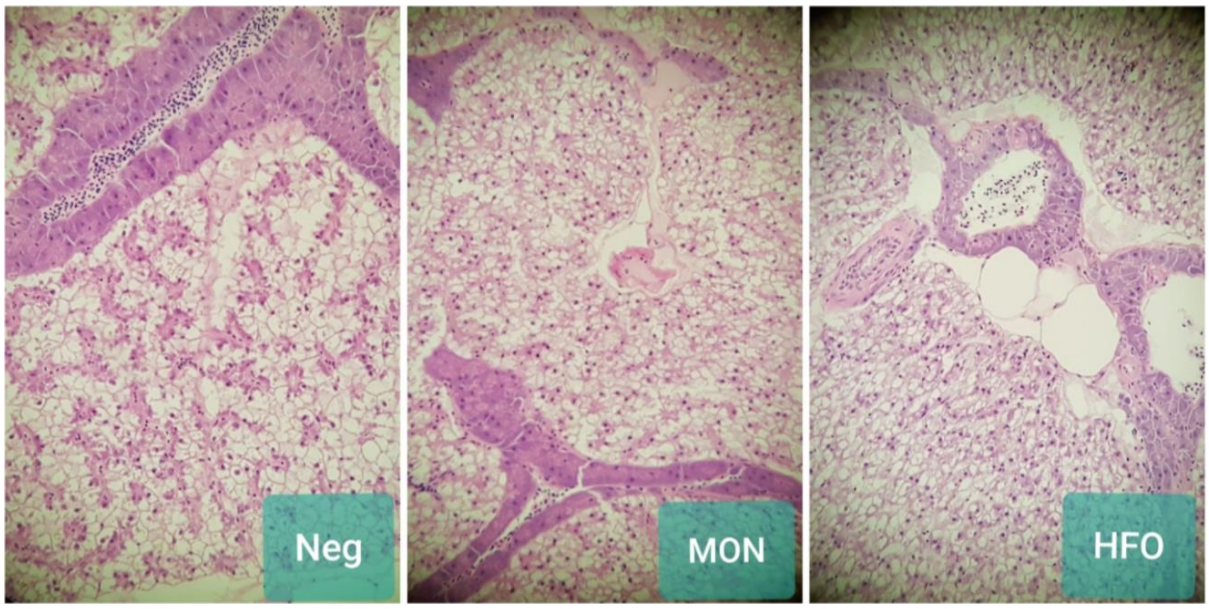

Figure S1: Liver histomorphology of *Lates calcarifer* from the three treatment groups

Appendix B: Supplementary Information for Chapter 3

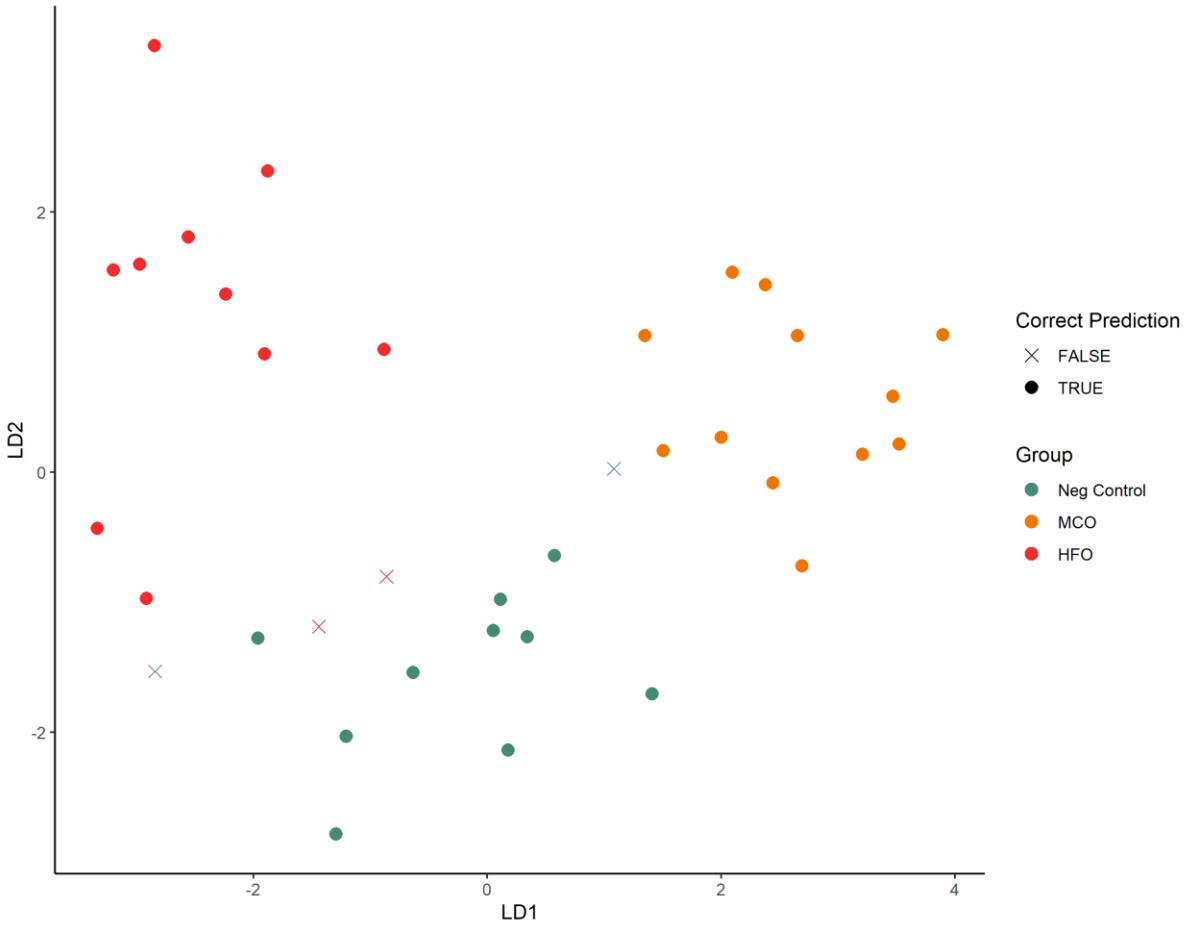

Figure S1: Linear Discriminatory Analysis (LDA) of four otolith metals (Al, As, Ba and Cr) of fish exposed to Montara (MCO) and heavy fuel oil (HFO)

Dot points are fish with correctly identified exposure test groups, crosses denote an incorrectly predicted exposure test group. Success rate is 32 out of 36 fish (88.9%)

Appendix C: Supplementary Information for Chapter 4

Table S1: Mixtures of Metal-enriched feeds plus a selection of petroleum hydrocarbons

	Compound	Concentration in Fish Feed (mg/kg)
Mix A	**Vanadium (as V_2O_5)**	**20**
	Naphthalene	1.17
	Phenanthrene	0.63
	Diphenylmethane	6.75
	Dibenzothiophene	0.63
	Fluorene	2.25
	Pyrene	1.80
	Biphenyl	3.69
	Decalin	2.43
	Adamantane	2.61
	Tridecane	4.50
Mix B	**Iron (as $FeSO_4$)**	**500**
	1-Methylnaphthalene	1.26
	3,6-Dimethylphenanthrene	0.18
	1-Pheny-dodecane	4.50
	Iso-butyl-benzene	3.09
	Retene	0.33
	1,3-Di-isopropylbenzene	3.15
	2-Methyl-indene	4.05
	Phytane	4.50
	Pristane	0.90
	Heptadecane	4.50
	Octadecane	4.50
Mix C	**Nickel (as Ni_2SO_4)**	**500**
	1, 5-Dimethynaphthalene	2.97
	Benzo(a)pyrene	1.08
	1-Methyl-fluorene	1.26
	2-Iso-propyl-naphthalene	1.53
	Indane	4.50
	Tetralin	5.13
	1,3-Dimethyladamantane	4.41
	2-methylbiphenyl	2.25

3806 Table S2: PAH Analysis of Fish Feed

	Compound	1% ACO Feed (mg/kg)	1% HFO Feed (mg/kg)	V Mix Feed (mg/kg)	Fe Mix Feed (mg/kg)	Ni Mix Feed (mg/kg)
Bicyclic Aromatics	Naphthalene	41.33	12.33	2.13	<0.1	<0.1
	2-Methylnaphthalene	116.67	37.33	<0.1	<0.1	<0.1
	1-methylnaphthalene	57.33	21.33	<0.1	2.17	<0.1
	C2-alkylnaphthalenes	136.67	52.33	<0.5	<0.5	2.47
	C3-alkylnaphthalenes	85.00	44.33	<0.5	<0.5	<0.5
	C4-alkylnaphthalenes	31.00	21.67	<0.5	<0.5	<0.5
	Dibenzothiophene	3.87	3.43	0.87	<0.5	<0.5
	Methyldibenzothiophenes	7.17	11.67	<0.5	<0.5	<0.5
	C2-alkyldibenzothiophenes	7.13	21.33	<0.5	<0.5	<0.5
	C3-alkyldibenzothiophenes	3.70	18.33	<0.5	<0.5	<0.5
	Acenaphthylene	<0.5	<0.5	<0.5	<0.5	<0.5
	Acenaphthene	1.47	0.97	<0.5	<0.5	<0.5
	Total Bicyclic aromatics	**1241**	**253**	**9.57**	**9.65**	**1477**
Tricyclic Aromatics	Phenanthrene	19.00	5.33	0.90	<0.5	<0.5
	Methylphenanthrenes	34.67	17.00	<0.5	<0.5	<0.5
	C2-alkylphenanthrenes	30.00	26.67	0.17	2.77	0.57
	C3-alkylphenanthrenes	46.33	52.00	<0.5	<0.5	<0.5
	C4-alkylphenanthrenes	6.43	11.67	0.63	0.60	<0.5
	Anthracene	<0.5	0.70	<0.5	<0.5	<0.5
	Total Tricyclic aromatics	**136**	**113**	**1.70**	**3.37**	**0.57**
Tetracyclic Aromatics	Fluoranthene	0.23	<0.5	<0.5	<0.5	<0.5
	Pyrene	0.60	1.53	2.50	<0.5	<0.5
	Benz(a)anthracene	<0.5	1.13	<0.5	<0.5	<0.5
	Chrysene	0.97	2.30	<0.5	<0.5	<0.5
	Methylpyrenes/fluoranthenes	4.97	6.93	<0.5	<0.5	<0.5
	C2-alkylpyrenes/fluoranthenes	4.77	13.00	<0.5	<0.5	<0.5
	C3-alkylpyrenes/fluoranthenes	3.23	13.00	<0.5	<0.5	<0.5
	Methylchrysenes	1.27	8.57	<0.5	<0.5	<0.5
	C2-alkylchrysenes	1.67	14.67	0.87	<0.5	<0.5
	Methylindenopyrenes	0.50	2.90	0.33	<0.5	<0.5
	C2-alkylindenopyrenes	<0.5	2.70	<0.5	<0.5	<0.5
	Fluorene	10.67	1.83	3.20	<0.5	<0.5
	Total Tetracyclic aromatics	**29**	**69**	**6.90**	**0.00**	**0.00**
Pentacyclic armoatics	Benzo(b)fluoranthene	<0.5	0.33	<0.5	<0.5	<0.5
	Benzo(k)fluoranthene	<0.5	<0.5	<0.5	<0.5	<0.5
	Benzo(a)pyrene	<0.5	1.20	<0.5	<0.5	<0.5
	Indeno(1,2,3-cd)pyrene	<0.5	<0.5	<0.5	<0.5	<0.5
	Dibenzo(a,h)anthracene	<0.5	<0.5	<0.5	<0.5	<0.5
	Benzo(g,h,i)perylene	<0.5	<0.5	<0.5	<0.5	<0.5
	Methylbenzopyrenes	<0.5	5.17	<0.5	<0.5	<0.5
	C2-alkylbenzopyrenes	0.37	7.23	<0.5	<0.5	<0.5
	Total Pentacyclic aromatics	**0.37**	**13.93**	**0.00**	**0.00**	**0.00**

Amounts reported are an average of triplicate analyses.
"<0.1" or "<0.5" denotes no amounts detected above the respective limits of reporting.

3810 Table S3: Metals Analysis of an Australian Crude Oil (ACO) and Heavy Fuel Oil (HFO)

Element	ACO (1) (mg/kg)	ACO (2) (mg/kg)	ACO (3) (mg/kg)	ACO Mean (mg/kg)	Std Err	HFO (1) (mg/kg)	HFO (2) (mg/kg)	HFO (3) (mg/kg)	HFO Mean (mg/kg)	Std Err
Aluminium	30.70	< 0.5	< 0.5	10.23	-	8.15	33.30	4.86	15.44	8.98
Antinomy	< 0.01	< 0.01	< 0.01	< 0.01	-	0.43	0.45	0.50	0.46	0.02
Arsenic	< 0.03	< 0.03	< 0.03	< 0.03	-	0.03	0.03	0.06	0.04	0.01
Barium	0.25	0.05	0.03	0.11	0.06	1.18	1.32	1.45	1.32	0.08
Beryllium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Bismuth	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Boron	< 1.7	< 1.7	< 1.7	< 1.7	-	< 1.7	< 1.7	< 1.7	< 1.7	-
Cadmium	< 0.01	< 0.01	0.01	0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Caesium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Calcium	91.00	11.70	10.50	37.73	26.64	11.20	72.70	3.10	29.00	21.9
Cerium	< 0.01	< 0.01	< 0.01	< 0.01	-	0.00	0.02	0.00	0.01	0.01
Chromium	0.89	< 0.12	< 0.12	0.89	-	0.45	0.28	0.00	0.24	0.13
Cobalt	< 0.46	< 0.46	< 0.46	< 0.46	-	< 0.46	0.66	3.63	2.15	1.48
Copper	0.45	< 0.31	< 0.31	0.45	-	< 0.31	< 0.31	< 0.31	< 0.31	-
Dysprosium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Erbium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Europium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Gadolinium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Gallium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	0.02	0.02	0.02	0.00
Germanium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Hafnium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Holmium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Indium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Iron	7.65	1.29	5.25	4.73	1.85	37.30	40.70	35.70	37.90	1.47
Lanthanum	< 0.01	< 0.01	< 0.01	< 0.01	-	0.71	0.69	0.89	0.77	0.06
Lead	0.08	0.09	0.09	0.08	0.00	0.02	0.08	0.04	0.04	0.02
Lithium	< 0.05	< 0.05	< 0.05	< 0.05	-	< 0.05	0.11	< 0.05	0.11	-
Lutetium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Magnesium	3.06	0.53	< 0.15	1.80	1.04	1.43	2.73	1.24	1.80	0.47
Manganese	< 0.04	< 0.04	< 0.04	< 0.04	-	< 0.04	< 0.04	< 0.04	< 0.04	-
Mercury	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Molybdenum	< 0.01	< 0.01	< 0.01	< 0.01	-	0.05	0.06	0.06	0.05	0.00
Neodymium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Nickel	0.14	< 0.06	0.08	0.11	0.06	10.90	12.50	13.30	12.23	0.71
Niobium	< 0.03	< 0.03	< 0.03	< 0.03	-	< 0.03	< 0.03	< 0.03	< 0.03	-
Phosphorous	< 3.5	< 3.5	< 3.5	< 3.5	-	< 3.5	< 3.5	< 3.5	< 3.5	-
Potassium	38.90	35.40	36.00	36.77	1.08	4.50	12.50	7.37	8.12	2.34
Praseodymium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Rubidium	0.14	0.02	< 0.01	< 0.01	0.27	0.07	0.16	0.07	0.10	0.03
Samarium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-

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Element	ACO (1) (mg/kg)	ACO (2) (mg/kg)	ACO (3) (mg/kg)	ACO Mean (mg/kg)	Std Err	HFO (1) (mg/kg)	HFO (2) (mg/kg)	HFO (3) (mg/kg)	HFO Mean (mg/kg)	Std Err
Selenium	0.13	< 0.01	0.06	0.09	0.03	0.00	0.02	0.00	0.01	0.01
Silicon	< 1	< 1	< 1	< 1	-	< 1	< 1	< 1	< 1	-
Silver	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Sodium	4.63	< 2.5	< 2.5	4.63	-	< 2.5	< 2.5	< 2.5	< 2.5	-
Strontium	0.61	0.07	< 0.01	0.34	0.27	0.26	0.74	0.30	0.43	0.15
Sulfur	335	460	386	394	36.2	11900	9800	9060	10253	851
Tantalum	< 0.01	< 0.01	< 0.01	< 0.04	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Terbium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Thallium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Thorium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Thulium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Tin	0.03	< 0.01	0.32	0.18	0.10	0.19	0.02	0.18	0.13	0.06
Titanium	< 0.24	< 0.24	< 0.24	< 0.24	-	3.03	3.47	3.22	3.24	0.13
Tungsten	< 0.04	< 0.04	< 0.04	< 0.01	-	< 0.04	< 0.04	< 0.04	< 0.04	-
Uranium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Vanadium	< 0.03	< 0.03	< 0.03	< 0.03	-	14.20	14.60	17.00	15.27	0.87
Ytterbium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Yttrium	< 0.01	< 0.01	< 0.01	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	-
Zinc	1.58	1.60	1.24	1.47	0.85	0.97	1.41	1.20	1.19	0.13
Zirconium	0.22	< 0.01	< 0.01	0.22	0.13	< 0.01	< 0.01	< 0.01	< 0.01	-

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Table S4. Beta-ordination PERMANOVA (Panodis) for Fe- and Ni-enriched groups

Ordination (Weighted)	P-value	Ordination (Unweighted)	P-value
Fe-enriched vs ACO	4.86E-08	Fe-enriched vs ACO	5.48E-04
Fe-enriched vs HFO	2.36E-06	Fe-enriched vs HFO	2.36E-04
Fe-enriched vs CNT	6.72E-06	Fe-enriched vs CNT	8.67E-05
Fe-enriched vs V-enriched	3.22E-05	Fe-enriched vs V-enriched	1.92E-03
Fe-enriched vs Ni-enriched	6.88E-04	Fe-enriched vs Ni-enriched	5.44E-02
Ni-enriched vs ACO	2.32E-05	Ni-enriched vs ACO	5.08E-04
Ni-enriched vs HFO	4.56E-05	Ni-enriched vs HFO	1.06E-03
Ni-enriched vs Fe-enriched	6.36E-04	Ni-enriched vs Fe-enriched	4.34E-04
Ni-enriched vs CNT	1.88E-03	Ni-enriched vs CNT	1.96E-04
Ni-enriched vs V-enriched	2.12E-03	Ni-enriched vs V-enriched	1.02E-02

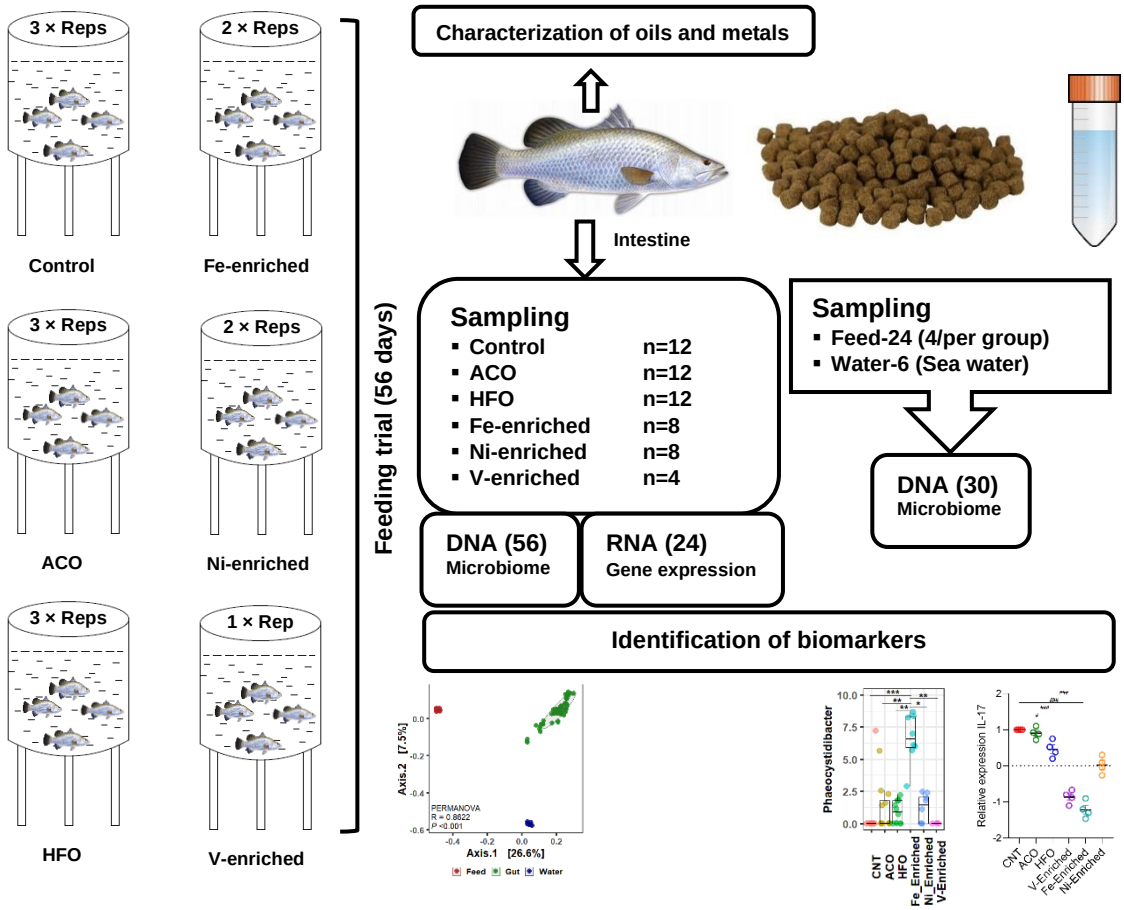


Figure S1. Flow-chart presenting the study design and sampling method

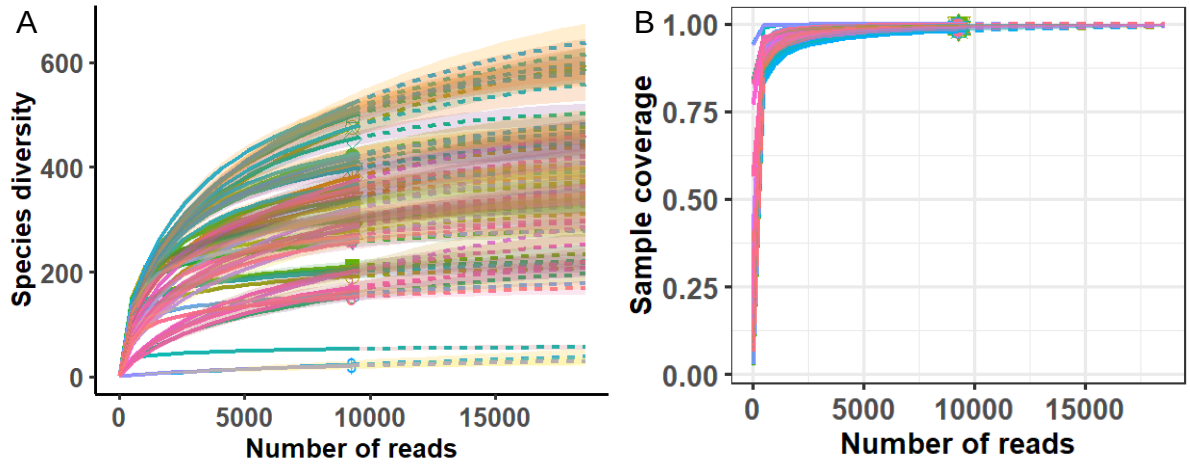


Figure S2. Rarefaction curve showing the (A) depth and (B) coverage of the sequences

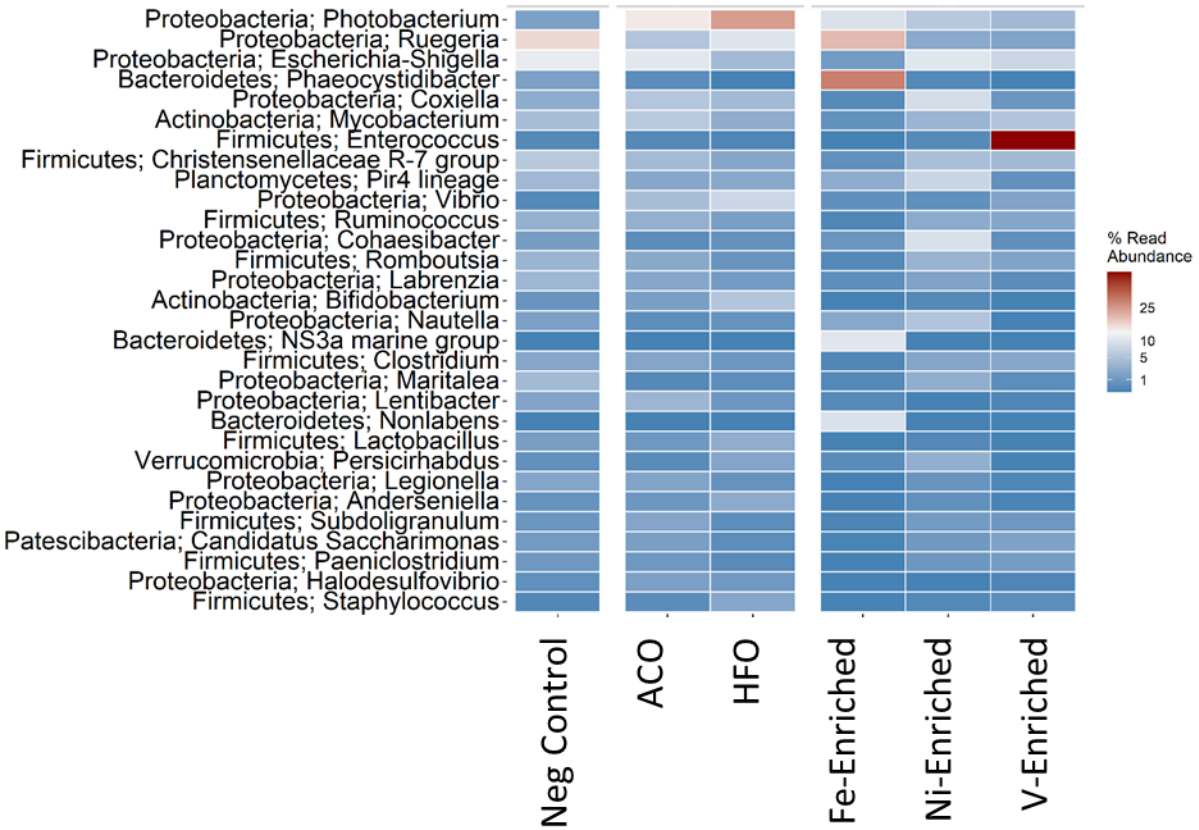


Figure S3. Relative abundance of bacteria at genus level in the gut of *Lates calcarifer* with six different diets

Abbreviations: ACO, Australian Crude Oil; HFO, Heavy Fuel Oil.

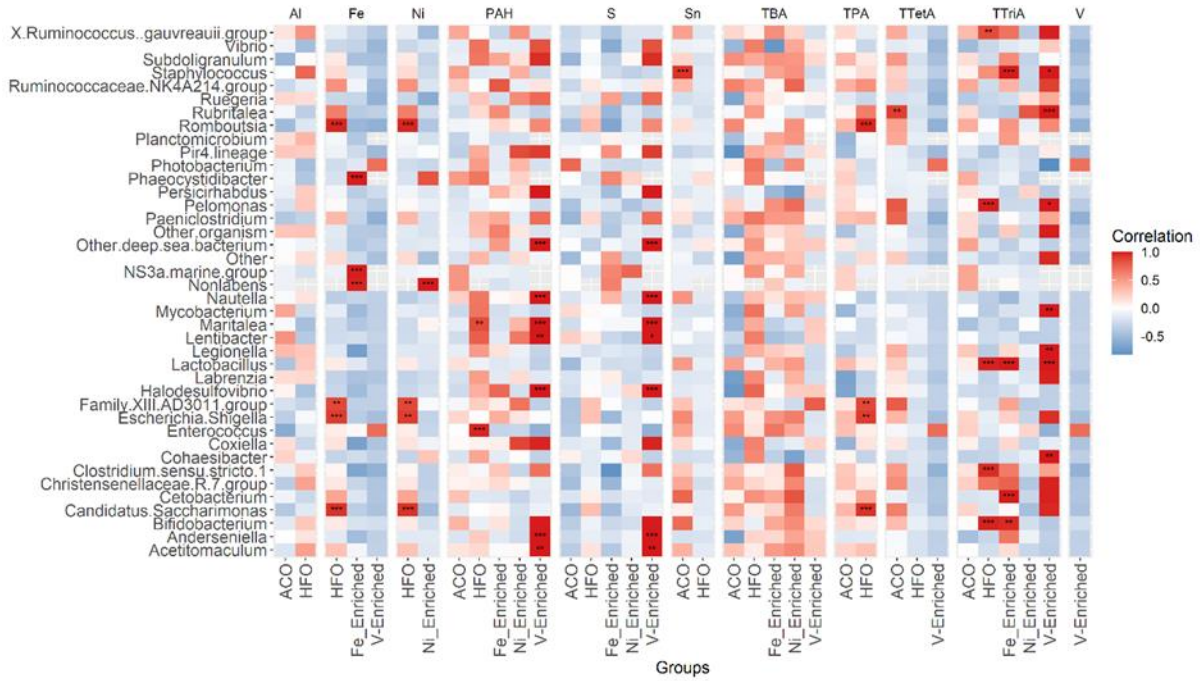


Figure S4. Pearson correlation between 40 abundant genera in fish microbiome and 5 categories of petroleum hydrocarbons and 3 metals in diets

The color code at the right indicates type and degree of correlation. *Significant at α-level of 0.05. **Significant at α-level of 0.005. ***Significant at α-level of 0.001. Abbreviations: CNT, control; ACO, Australian Crude Oil; HFO, Heavy Fuel Oil; Al, aluminium; Fe, iron; Ni, nickel; PAH, total aromatic hydrocarbon; S, sulfar; Sn, tin; TBA, total bicyclic aromatics; TPA, total penta-cyclic aromatics; TTetA, total tetra-cyclic aromatics; TTriA, total tri-cyclic aromatics; V, vanadium.

Appendix D: Supplementary Information for Chapter 5

Table S1: Blank spike recoveries for seven saturated hydrocarbons

Compound	CAS	mw	Target Ion (m/z)	Spike Amount (μg/mL)	Spike Recovery 1 Concentration (μg/mL)	Spike Recovery 2 Concentration (μg/mL)	Spike Recovery 3 Concentration (μg/mL)	Mean Spike Recovery Concentration (μg/mL ± 2SE)	% Recovery
Decalin	493-01-6	138	138	2.0	1.359	1.372	0.922	1.218 ± 0.296	**60.90**
Adamantane	281-23-2	136	136	2.0	1.285	1.288	0.849	1.140 ± 0.292	**57.00**
1,3-dimethyl-adamantane	702-79-4	164	149	2.0	1.428	1.435	0.990	1.284 ± 0.294	**64.20**
Tridecane (n-C13)	629-50-5	184	85	2.0	1.833	1.850	1.429	1.704 ± 0.276	**85.20**
Heptadecane (n-C17)	629-78-7	240	85	2.0	2.124	2.081	1.839	2.014 ± 0.178	**100.70**
Octadecane (n-C18)	593-45-3	254	85	2.0	2.150	2.104	1.920	2.058 ± 0.141	**102.90**
Pristane	1921-70-6	268	85	2.0	2.089	2.042	1.822	1.948 ± 0.165	**99.20**

Table S2: Diagnostic Ratios of bicyclic sesquiterpanes

Confidence intervals are 2 x standard error.

Diagnostic Ratios	MCO		HFO	
	Oil Mean (n=3)	Fish Adipose (n=9)	Oil Mean (n=3)	Fish Adipose (n=9)
BSA:BSD	0.20 ± 0.03	0.20 ± 0.02	0.15 ± 0.02	0.15 ± 0.01
BSA:BSE	0.40 ± 0.04	0.46 ± 0.05	0.30 ± 0.05	0.31 ± 0.02
BSA:BSJ	0.98 ± 0.15	1.10 ± 0.20	0.33 ± 0.03	0.43 ± 0.04
BSD:BSE	2.01 ± 0.08	2.32 ± 0.10	2.04 ± 0.05	2.11 ± 0.06
BSE:BSJ	2.43 ± 0.15	2.40 ± 0.28	1.101 ± 0.12	1.40 ± 0.07
BSJ:BSD	0.20 ± 0.005	0.18 ± 0.02	0.44 ± 0.04	0.34 ± 0.01

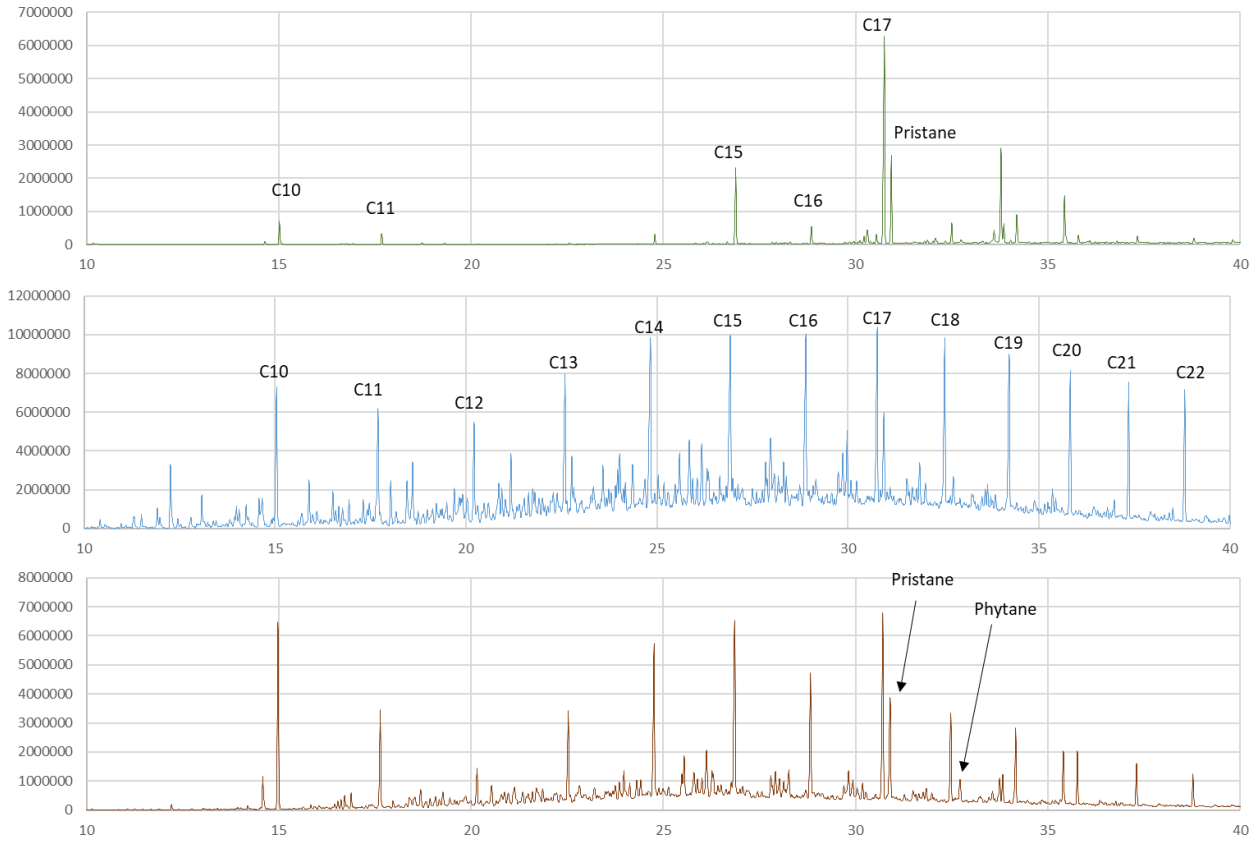


Figure S1: GC-MS partial chromatograms of fish adipose tissue extract saturated fractions (F1) of negative control fish (green, top), MCO-exposed fish (blue, middle), and HFO-exposed fish (brown, bottom)

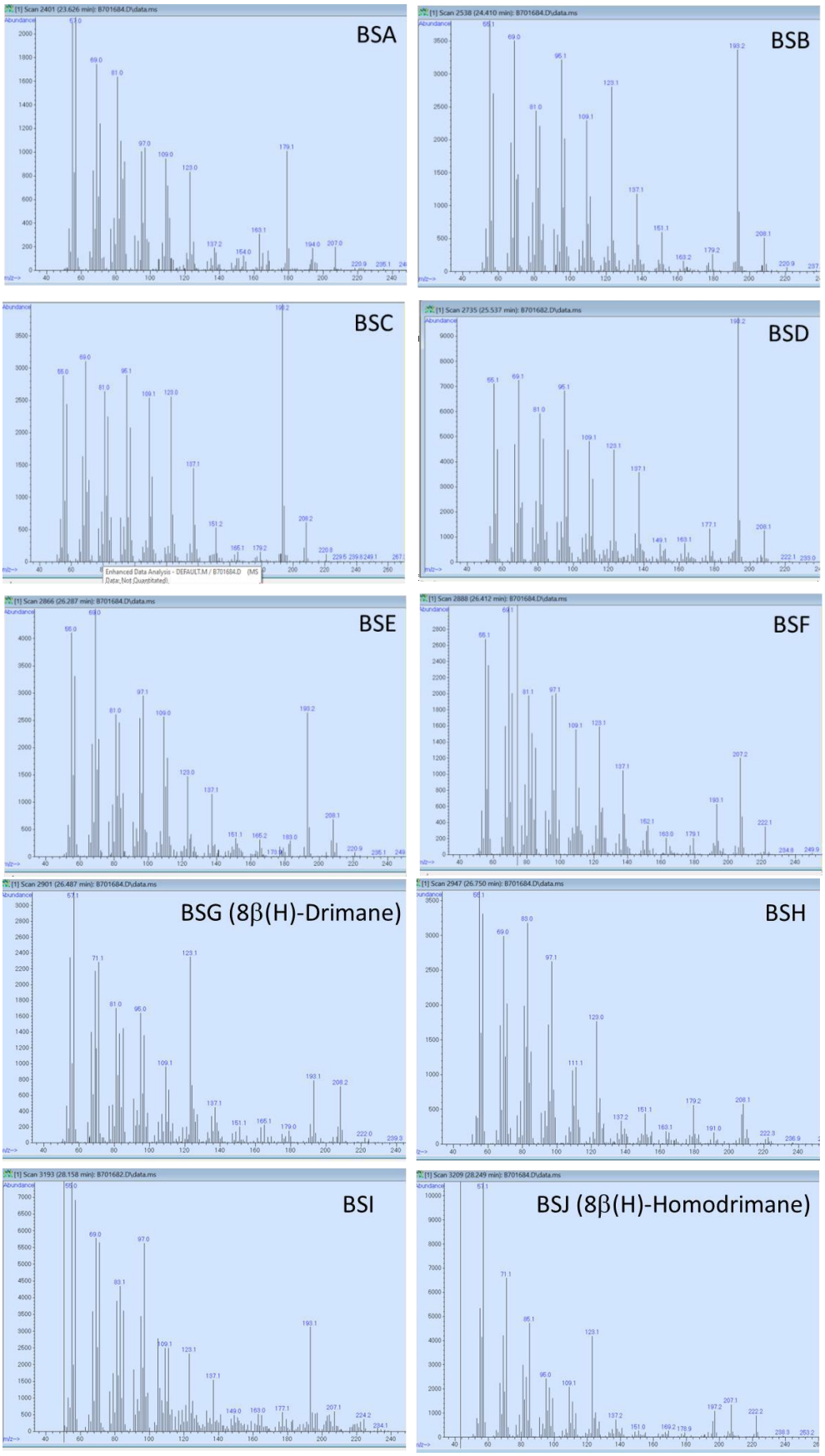


Figure S2: Mass spectrograms of bicyclic sesquiterpanes found in MCO and HFO

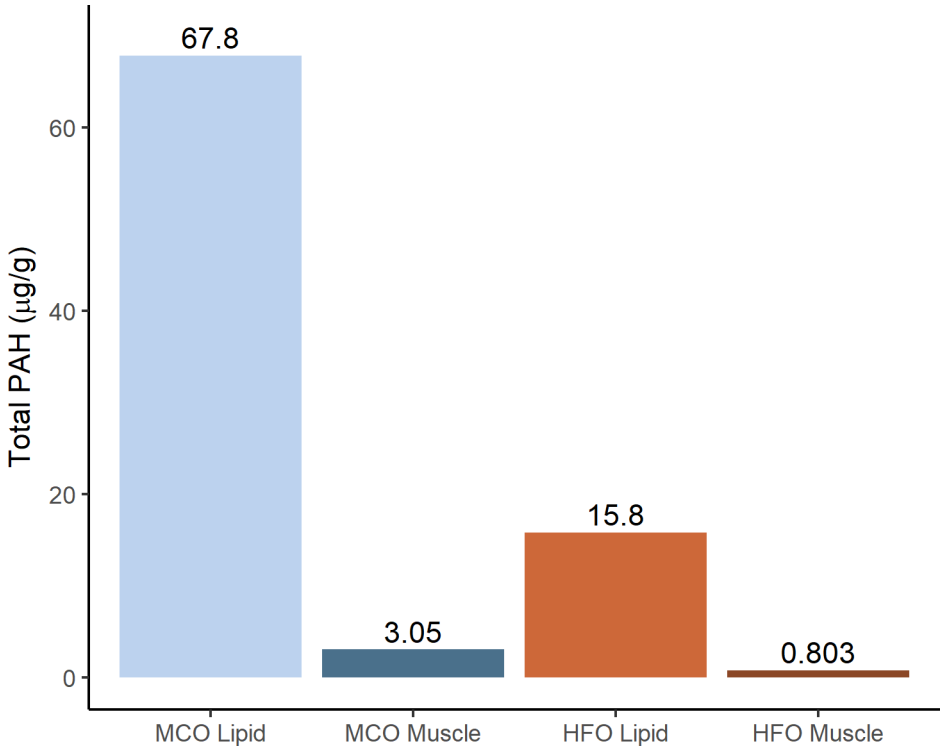


Figure S3 Concentrations of total PAH in adipose tissue and white muscle of *Lates calcarifer* exposed to 1% w/w of two crude oils via diet

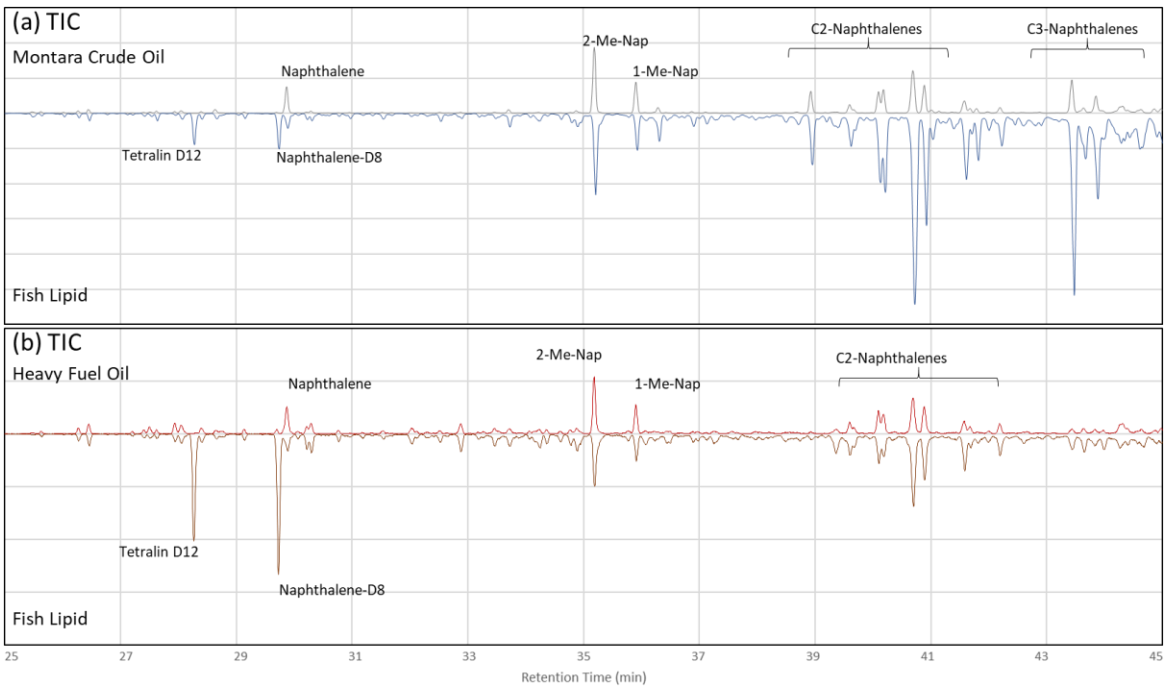


Figure S4 Mirrored GC-MS chromatograms (TIC) of F2 aromatic fractions of oils (top) and fish adipose extracts (bottom) for MCO (a) and HFO (b)

Appendix E: Supplementary Information for Chapter 6

R Markdown for LDA Analysis of Bicyclane Biomarkers

R_Bicyclane_Biomarkers_LDA

```
############
#Standard tasks at the beginning
############

rm(list=ls()) #remove ALL objects
Sys.setenv(LANG = "en") #Set language to English
Sys.setlocale("LC_ALL","English")
```

```
## [1] "LC_COLLATE=English_United States.1252;LC_CTYPE=English_United
States.1252;LC_MONETARY=English_United
States.1252;LC_NUMERIC=C;LC_TIME=English_United States.1252"
```

```
#graphics.off()

################
#install Packages
################

vers <- 4

library(tidyr)
library(readxl)
library(factoextra)
library(FactoMineR)
library(MASS)
library(reshape2)
library(scales)
library(drc)
library(ggnewscale)
library(lattice)
library(directlabels)


#Set the current workdirectory, i.e. where all files are kept
inwd ="D:/Fish Fingerprints/Paper 5 LDA/R"
outwd ="D:/Fish Fingerprints/Paper 5 LDA/R"

setwd(inwd)
```

```
####################
#IMPORT FILES:
####################
Input_Data <- data.frame(read_xlsx(paste0(inwd,"/Bicyclane Ratios mz 123 179
193 for R Import v4.xlsx"), col_names =TRUE, trim_ws = TRUE))
```

```r
#Tidy up so that it prints nicely:
Input_Data$A_D <- round(Input_Data$A_D, digits = 3)
Input_Data$A_E <- round(Input_Data$A_E, digits = 3)
Input_Data$A_J <- round(Input_Data$A_J, digits = 3)
Input_Data$D_E <- round(Input_Data$D_E, digits = 3)
Input_Data$J_D <- round(Input_Data$J_D, digits = 3)
Input_Data$E_J <- round(Input_Data$E_J, digits = 3)


#Display the raw data:
print.data.frame(Input_Data)
```

```
##     Treatment        Sample_ID Dataset   A_D   A_E   A_J   D_E   J_D   E_J
## 1     MCO_Oil        MON_Oil_1   Train 0.184 0.379 0.887 2.060 0.207 2.340
## 2     MCO_Oil        MON_Oil_2   Train 0.216 0.424 1.070 1.964 0.202 2.522
## 3     MCO_Oil        MON_Oil_3   Train 0.251 0.470 1.268 1.872 0.198 2.698
## 4     HFO_Oil        HFO_Oil_1   Train 0.127 0.255 0.313 2.008 0.407 1.225
## 5     HFO_Oil        HFO_Oil_2   Train 0.159 0.315 0.302 1.981 0.526 0.959
## 6     HFO_Oil        HFO_Oil_3   Train 0.155 0.313 0.324 2.015 0.479 1.036
## 7  GAB_Oil W1            GAB_1   Train 0.192 0.186 0.181 0.967 1.061 0.974
## 8  GAB_Oil W4            GAB_2   Train 0.272 0.228 0.232 0.838 1.172 1.018
## 9  GAB_Oil W4            GAB_3   Train 0.207 0.177 0.189 0.857 1.095 1.066
## 10 GAB_Oil W1            GAB_4   Train 0.222 0.230 0.303 1.037 0.731 1.320
## 11 GAB_Oil W2            GAB_5   Train 0.167 0.139 0.154 0.835 1.082 1.108
## 12 GAB_Oil W2            GAB_6   Train 0.275 0.260 0.382 0.945 0.718 1.473
## 13 GAB_Oil W5            GAB_7   Train 0.235 0.230 0.218 0.978 1.079 0.948
## 14 GAB_Oil W1            GAB_8   Train 0.210 0.191 0.215 0.910 0.979 1.122
## 15    BNC_Oil       Bunker_C_1   Train 0.493 0.784 0.628 1.590 0.784 0.802
## 16    BNC_Oil       Bunker_C_2   Train 0.391 0.635 0.518 1.625 0.755 0.815
## 17    BNC_Oil       Bunker_C_3   Train 0.487 0.884 0.508 1.814 0.960 0.574
## 18    CAS_Oil Browse_Caswell_1   Train 0.295 0.561 2.489 1.903 0.118 4.437
## 19    CAS_Oil Browse_Caswell_2   Train 0.173 0.288 1.237 1.661 0.140 4.294
## 20    LSO_Oil      Wakashio F1   Train 0.236 0.332 0.949 1.407 0.249 2.854
## 21    LSO_Oil  Wakashio_fuel_1   Train 0.215 0.264 0.874 1.227 0.246 3.315
## 22    LSO_Oil  Wakashio_fuel_2   Train 0.203 0.253 0.868 1.246 0.234 3.425
## 23    LSO_Oil  Wakashio_fuel_3   Train 0.216 0.283 0.967 1.309 0.224 3.416
## 24    ELW_Oil     Eland_West_1   Train 0.418 0.499 1.358 1.194 0.307 2.725
## 25    ELW_Oil     Eland_West_2   Train 0.463 0.517 1.401 1.117 0.330 2.712
## 26    ELW_Oil     Eland_West_3   Train 0.431 0.492 1.392 1.140 0.310 2.830
## 27    CAL_Oil      Calliance_1   Train 0.237 0.490 2.139 2.070 0.111 4.363
## 28    CAL_Oil      Calliance_2   Train 0.234 0.509 1.951 2.178 0.120 3.835
## 29    CAL_Oil      Calliance_3   Train 0.219 0.462 1.876 2.114 0.116 4.060
## 30    CRX_Oil          Crux3_1   Train 0.430 0.863 1.650 2.007 0.261 1.910
## 31    CRX_Oil          Crux3_2   Train 0.395 0.791 1.716 2.001 0.230 2.169
## 32    CRX_Oil          Crux3_3   Train 0.430 0.808 1.729 1.880 0.249 2.139
## 33   MCO_Fish    MCO_Adipose_1    Test 0.197 0.467 1.118 2.373 0.176 2.393
## 34   MCO_Fish    MCO_Adipose_2    Test 0.203 0.437 1.081 2.149 0.188 2.474
## 35   MCO_Fish    MCO_Adipose_3    Test 0.199 0.451 1.077 2.270 0.184 2.389
## 36   MCO_Fish    MCO_Adipose_4    Test 0.194 0.470 1.108 2.417 0.175 2.360
## 37   MCO_Fish    MCO_Adipose_5    Test 0.197 0.443 1.149 2.251 0.171 2.592
## 38   MCO_Fish    MCO_Adipose_6    Test 0.172 0.418 0.758 2.423 0.227 1.816
## 39   MCO_Fish    MCO_Adipose_7    Test 0.199 0.479 1.171 2.400 0.170 2.446
## 40   MCO_Fish    MCO_Adipose_8    Test 0.230 0.544 1.441 2.359 0.160 2.651
## 41   MCO_Fish    MCO_Adipose_9    Test 0.175 0.401 0.989 2.289 0.177 2.469
## 42   HFO_Fish    HFO_Adipose_1    Test 0.117 0.266 0.308 2.278 0.379 1.158
```

```
## 43   HFO_Fish    HFO_Adipose_2    Test 0.159 0.322 0.480 2.026 0.331 1.493
## 44   HFO_Fish    HFO_Adipose_3    Test 0.163 0.329 0.498 2.016 0.328 1.513
## 45   HFO_Fish    HFO_Adipose_4    Test 0.157 0.332 0.481 2.117 0.327 1.446
## 46   HFO_Fish    HFO_Adipose_5    Test 0.148 0.319 0.436 2.156 0.340 1.366
## 47   HFO_Fish    HFO_Adipose_6    Test 0.147 0.297 0.425 2.022 0.345 1.433
## 48   HFO_Fish    HFO_Adipose_7    Test 0.149 0.326 0.431 2.192 0.346 1.319
## 49   HFO_Fish    HFO_Adipose_8    Test 0.122 0.262 0.374 2.152 0.325 1.429
## 50   HFO_Fish    HFO_Adipose_9    Test 0.157 0.325 0.479 2.074 0.327 1.476
```

```
####################
#TIDYING UP
#MAKE A NEW DATASET THAT ONLY HAS ONE CATEGORICAL VARIABLE, AND ALL THE
BICYCLANE RATIO VARIABLES
####################

#Remove unnecessary categorical variables
#(This retains a copy of the original input file)
LDA_Data <- Input_Data[,c("Treatment","A_D", "A_E","A_J","D_E", "J_D","E_J")]



#Remove all the weathering labels from the GAB asphaltites.
#This treats all the GAB samples as though they are from the same source oil.
LDA_Data$Treatment <- gsub("GAB_Oil W1", "GAB_Oil", LDA_Data$Treatment)
LDA_Data$Treatment <- gsub("GAB_Oil W2", "GAB_Oil", LDA_Data$Treatment)
LDA_Data$Treatment <- gsub("GAB_Oil W4", "GAB_Oil", LDA_Data$Treatment)
LDA_Data$Treatment <- gsub("GAB_Oil W5", "GAB_Oil", LDA_Data$Treatment)



#House-keeping; change the class of the variable Treatment from "character" to
"factor".
LDA_Data$Treatment <- as.factor(LDA_Data$Treatment)
```

```
####################
#BEGIN LINEAR DISCRIMINATORY ANALYSIS (LDA)
####################

#Divide the dataset into two sections:
#One for training the LDA model (i.e. just the oils)
#One for testing and obtaining exposure oil predictions (i.e. just the fish
adipose tissue extracts)

Oil_Data <- subset(LDA_Data, grepl("Oil", LDA_Data$Treatment))
Fish_Data <- subset(LDA_Data, grepl("Fish", LDA_Data$Treatment))


#Remove any factor levels which are absent from the new dataframes:
Oil_Data <- as.data.frame(droplevels.data.frame(Oil_Data))
```

```r
Fish_Data <- as.data.frame(droplevels.data.frame(Fish_Data))


#Define the LDA model usign the OILs as the Training data set:
#(Prior sets the proabability of a test sample belonging to any particular
category i.e. 1/9, or 0.1111)
Model <- lda(Treatment~.,data = Oil_Data,
             prior = rep(1,9)/9)


#Apply the LDA model to the fish adipose tissue bicyclane ratios, and predict
the category:
Predict <-  Model %>% predict(LDA_Data)


#Amalgamate and tidy Up the dataframe:
Predict_Data <- cbind(Input_Data,
                      Predict$x[, c("LD1", "LD2")], Predict$class)

names(Predict_Data)[names(Predict_Data) == "Predict$class"] <- "Predicted_Oil"


#Print out a summarized version of the dataframe
#to produce a list of tested samples, plus the respective predicted exposure
oil:
print.data.frame(Predict_Data[, c("Treatment", "Sample_ID", "LD1", "LD2",
"Predicted_Oil")])
```

```
##     Treatment        Sample_ID         LD1        LD2 Predicted_Oil
## 1     MCO_Oil        MON_Oil_1  -5.1924886  5.3526283       MCO_Oil
## 2     MCO_Oil        MON_Oil_2  -4.3623356  3.5354179       MCO_Oil
## 3     MCO_Oil        MON_Oil_3  -3.6056001  1.7799003       MCO_Oil
## 4     HFO_Oil        HFO_Oil_1   1.4688317  8.1787325       HFO_Oil
## 5     HFO_Oil        HFO_Oil_2   3.6925951  8.1259935       HFO_Oil
## 6     HFO_Oil        HFO_Oil_3   2.8943547  8.3550910       HFO_Oil
## 7  GAB_Oil W1            GAB_1  14.6318778 -2.8547872       GAB_Oil
## 8  GAB_Oil W4            GAB_2  14.8758143 -4.4245547       GAB_Oil
## 9  GAB_Oil W4            GAB_3  15.1600177 -4.2241637       GAB_Oil
## 10 GAB_Oil W1            GAB_4  14.0621005 -2.8698361       GAB_Oil
## 11 GAB_Oil W2            GAB_5  15.3930371 -4.4893365       GAB_Oil
## 12 GAB_Oil W2            GAB_6  14.2634290 -4.2631540       GAB_Oil
## 13 GAB_Oil W5            GAB_7  14.4990530 -2.7898870       GAB_Oil
## 14 GAB_Oil W1            GAB_8  15.0799602 -3.7676398       GAB_Oil
## 15    BNC_Oil       Bunker_C_1  13.4912840  1.8252706       BNC_Oil
## 16    BNC_Oil       Bunker_C_2  11.8396766  2.8604152       BNC_Oil
## 17    BNC_Oil       Bunker_C_3  11.4473155  4.1120824       BNC_Oil
## 18    CAS_Oil Browse_Caswell_1 -16.4578251 -3.0027094       CAS_Oil
## 19    CAS_Oil Browse_Caswell_2 -14.8708092 -3.3691117       CAS_Oil
## 20    LSO_Oil      Wakashio F1   0.8568247 -2.8832036       LSO_Oil
## 21    LSO_Oil  Wakashio_fuel_1  -0.5802449 -5.6070466       LSO_Oil
## 22    LSO_Oil  Wakashio_fuel_2  -1.7654126 -5.6128951       LSO_Oil
## 23    LSO_Oil  Wakashio_fuel_3  -2.3959877 -5.0634554       LSO_Oil
## 24    ELW_Oil     Eland_West_1   5.5885241 -5.5918753       ELW_Oil
## 25    ELW_Oil     Eland_West_2   6.4300541 -6.3781394       ELW_Oil
```

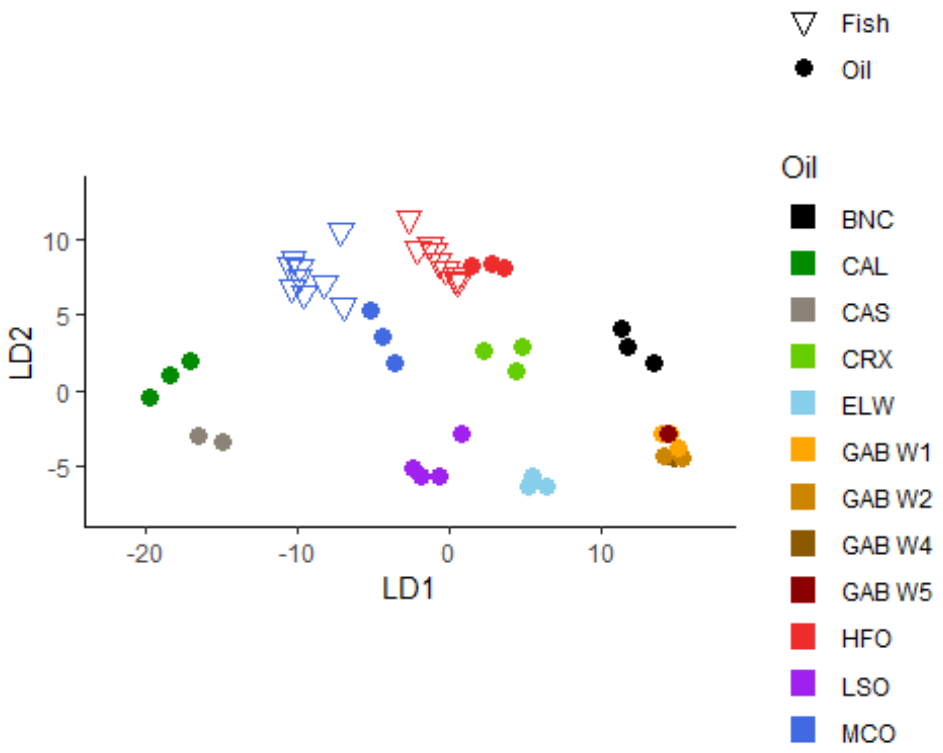
```
## 26    ELW_Oil     Eland_West_3   5.3105877 -6.3574126       ELW_Oil
## 27    CAL_Oil      Calliance_1 -19.6321404 -0.4486747       CAL_Oil
## 28    CAL_Oil      Calliance_2 -16.9825824  1.9780935       CAL_Oil
## 29    CAL_Oil      Calliance_3 -18.3663515  1.0335822       CAL_Oil
## 30    CRX_Oil          Crux3_1   4.9025021  2.8845062       CRX_Oil
## 31    CRX_Oil          Crux3_2   2.3265397  2.5569633       CRX_Oil
## 32    CRX_Oil          Crux3_3   4.4188168  1.2613669       CRX_Oil
## 33   MCO_Fish    MCO_Adipose_1  -9.7537349  8.1694640       MCO_Oil
## 34   MCO_Fish    MCO_Adipose_2  -6.9204462  5.6398839       MCO_Oil
## 35   MCO_Fish    MCO_Adipose_3  -8.1708989  7.1272388       MCO_Oil
## 36   MCO_Fish    MCO_Adipose_4 -10.2133855  8.7271471       MCO_Oil
## 37   MCO_Fish    MCO_Adipose_5  -9.4874322  6.4513835       MCO_Oil
## 38   MCO_Fish    MCO_Adipose_6  -7.1086961 10.5100781       MCO_Oil
## 39   MCO_Fish    MCO_Adipose_7 -10.4271358  8.2533684       MCO_Oil
## 40   MCO_Fish    MCO_Adipose_8 -10.3130198  6.8167534       MCO_Oil
## 41   MCO_Fish    MCO_Adipose_9  -9.8142280  7.4729919       MCO_Oil
## 42   HFO_Fish    HFO_Adipose_1  -2.6081270 11.3769447       HFO_Oil
## 43   HFO_Fish    HFO_Adipose_2   0.4919657  7.3788464       HFO_Oil
## 44   HFO_Fish    HFO_Adipose_3   0.6314198  7.1749071       HFO_Oil
## 45   HFO_Fish    HFO_Adipose_4  -0.6155808  8.4667080       HFO_Oil
## 46   HFO_Fish    HFO_Adipose_5  -0.9647621  9.1773396       HFO_Oil
## 47   HFO_Fish    HFO_Adipose_6   0.6126623  7.6207664       HFO_Oil
## 48   HFO_Fish    HFO_Adipose_7  -1.2123454  9.6585371       HFO_Oil
## 49   HFO_Fish    HFO_Adipose_8  -2.0129599  9.3263263       HFO_Oil
## 50   HFO_Fish    HFO_Adipose_9  -0.1707403  7.9490513       HFO_Oil
```

```
################################
#Generate posterior probabilities dataframe to draw the decision boundaries
################################


#Define another LDA model, using the LD1 and LD2 coordinates of the oils.
#Make a subset ƒ the predicted LD1 and LD2 coordinates that only contains the
Training data (i.e. Oils)
Oil_Data.2 <-subset(Predict_Data, grepl("Oil", LDA_Data$Treatment))

Model.2 <-lda(Predicted_Oil ~ LD1 + LD2, data = Oil_Data.2, prior = rep(1,9)/9)


#Generate new dataframe for a test series that contains
#all possible LD1 values against all possible LD2 values

#Set the upper an lower limits of LD1 and LD2:(and expand by an extra 50%)
LD1lim <- expand_range(c(min(Predict_Data$LD1), max(Predict_Data$LD1)), mul =
0.5)
LD2lim <- expand_range(c(min(Predict_Data$LD2), max(Predict_Data$LD2)), mul =
0.5)



#Make vectors (300 long) from the min and max limits of LD1 and LD2:
ld1 <- seq(LD1lim[[1]], LD1lim[[2]], length.out=300)
ld2 <- seq(LD2lim[[1]], LD2lim[[2]], length.out=300)
```

```
#Generate the giant test dataframe
Boundary_Data <- expand.grid(list(LD1=ld1,LD2=ld2))

#Apply the LDA model:
Boundary_Predict <- predict(Model.2, newdata=Boundary_Data)

#Extract the class predictions, posterior probabilties for
#each cartesian combination of LD1 and LD2
Boundary_Class <- Boundary_Predict$class
Boundary_Probs <- Boundary_Predict$posterior


#Amalgamate into a dataframe:
Boundary_DF <- data.frame(LD1=Boundary_Data$LD1,
                          LD2=Boundary_Data$LD2,
                          Boundary_Cat = Boundary_Class)


Boundary_DF <- cbind(Boundary_DF,Boundary_Probs)

#define a list of the oils:
lvls <-unique(Boundary_DF$Boundary_Cat)

#Predict from the posterior probablilities which Oil category is
#for each caretesian combination of LD1 and LD2:
Boundary_DF$Class_prob <-
apply(Boundary_DF[,as.character(lvls)],1,function(row) sample(lvls,1,prob=row))


#Melt the dataframe to make it useable for ggplot:
Boundary_Plot_Data <- melt(Boundary_DF, id.vars = c("LD1", "LD2",
"Boundary_Cat"))

#Tidy up the names in the dataframe:
names(Boundary_Plot_Data)[names(Boundary_Plot_Data) == "variable"] <-
"Predicted_Oil"
names(Boundary_Plot_Data)[names(Boundary_Plot_Data) == "value"] <-
"Posterior_Prob"

Boundary_Plot_Data$Posterior_Prob <-
as.numeric(Boundary_Plot_Data$Posterior_Prob)

#Delete the tiny probablilities less than 1% (otherwise the contour breaks
struggle)

Boundary_Plot_Data_Short <- subset(Boundary_Plot_Data,
                                   Boundary_Plot_Data$Posterior_Prob > 0.01)
```

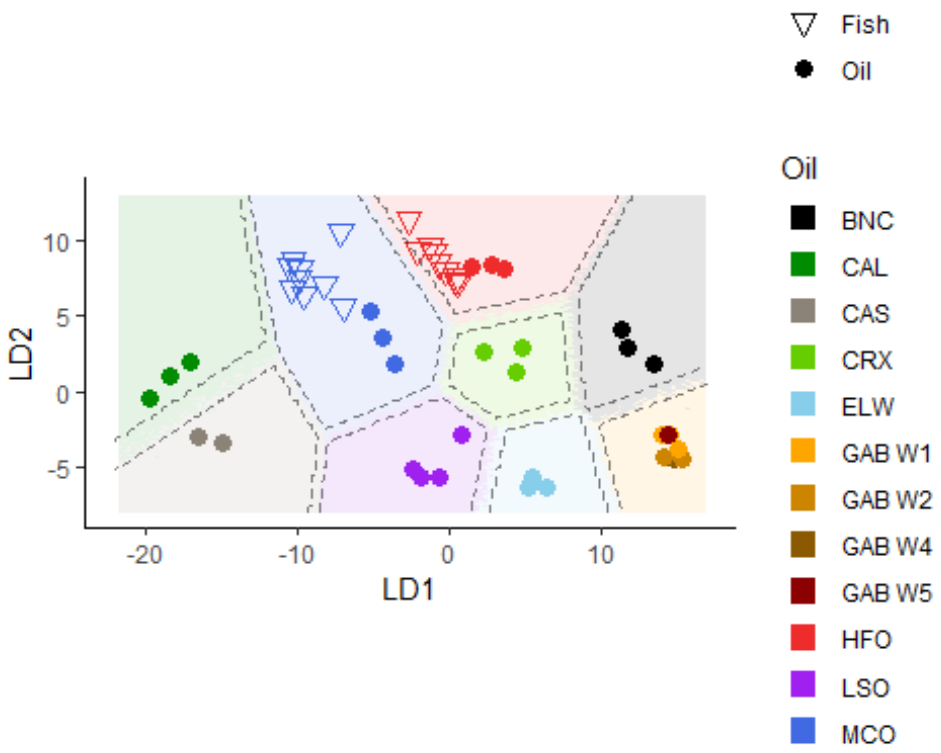
```
################
#Tidying up:
################
#Make a dataframe for the plot:
LDA_Plot_Data <- Predict_Data[c("Treatment", "Sample_ID", "LD1", "LD2",
"Predicted_Oil")]


#Make a new categorical variable called "Group" that describes whether the
sample
#is an oil, or from a fish (used for point shapes)
LDA_Plot_Data$Group <- ifelse(grepl("Oil", LDA_Plot_Data$Treatment), "Oil",
"Fish")

#Trim the category names:
LDA_Plot_Data$Treatment <- gsub("_Oil", "", LDA_Plot_Data$Treatment)
LDA_Plot_Data$Treatment <- gsub("_Fish", "", LDA_Plot_Data$Treatment)


#Define the colour schemes:
Cols_Oils_Weathered <- c("black", "green4", "antiquewhite4", "chartreuse3",
"skyblue",
                         "orange1", "orange3", "orange4", "darkred",
                         "firebrick2", "purple", "royalblue")

Cols_Oils <- c("black", "green4", "antiquewhite4", "chartreuse3", "skyblue",
               "orange1", "firebrick2", "purple", "royalblue")


###############
#Define GGPlot
###############

#First, Show the basic LDA plot:

LDA_Plot<- ggplot()+
  geom_point(data = LDA_Plot_Data, aes(x = LD1, y = LD2,
                                       color = Treatment,
                                       shape = Group),
             size = 3)+

  scale_shape_manual("Group", values = c(25, 16))+
  scale_color_manual("Oil",
                     values = Cols_Oils_Weathered)+

  coord_equal()+
  guides(color=guide_legend(override.aes = list(size=4, shape =15)))+
  theme_classic()+
  xlim(c(-22, 17))+
  ylim(c(-8,13))+

NULL
```

```
LDA_Plot
```




```
#Second, add in the decision border areas, plus the 95% probabliltiy decision
line
LDA_Plot_Boundary <- ggplot()+
  geom_raster(data=Boundary_DF, aes(x=LD1, y=LD2, fill = factor(Class_prob)),
              alpha = 0.1, show.legend = FALSE) +
  geom_point(data = LDA_Plot_Data, aes(x = LD1, y = LD2,
                                       color = Treatment,
                                       shape = Group),
             size = 3)+
  scale_shape_manual("Group", values = c(25, 16))+
  scale_color_manual("Oil",
                     values = Cols_Oils_Weathered)+
  scale_fill_manual("Decision Boundary",
                    values = Cols_Oils)+

  metR::geom_contour2(data = Boundary_Plot_Data_Short,
                      aes(x = LD1, y = LD2, z = Posterior_Prob),
                      breaks = c(0.95),    #Choose the 95% probability line to
display
                      linetype = 2,
                      alpha = 0.5) +
  coord_equal()+
  guides(color=guide_legend(override.aes = list(size=4, shape =15)))+
  theme_classic()+
  xlim(c(-22, 17))+
  ylim(c(-8,13))+
  NULL
```

```
LDA_Plot_Boundary
```




```
#Export the plot as a .pdf file (remove "#" if needed)

#ggplot2::ggsave(paste0(outwd,"/Bicycane LDA Plot (Boundaries and 0.95
Prob).png"), LDA_Plot_Boundary)
```

Appendix F: Author Attribution Statements

Author attribution statement – Chapter 2

Discriminating source of oil contamination in teleost fish, *Lates calcarifer*, using multivariate analysis of a suite of physiological and behavioural biomarkers

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Submitted to Marine Pollution Bulletin: 10th July 2021, Revised 16 August 2021, Accepted 20 August 2021, Published: 1st November 2021.
https://doi.org/10.1016/j.marpolbul.2021.112898

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

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Submitted to Comparative Biochemistry and Physiology, Part C: 11th October 2021; Revised 26th November 2021, Accepted 18th December 2021, Published: 29th December 2021.
https://doi.org/10.1016/j.cbpc.2021.109253

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Author attribution statement – Chapter 4

Gut microbiome as a potential biomarker in fish – dietary exposure to petroleum hydrocarbons and metals, metabolic functions and cytokine expression in juvenile *Lates calcarifer*

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In review. Submitted to *Frontiers in Microbiology:* 1st December 2021

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Fish Fingerprinting: Identifying crude oil pollutants using bicyclic sesquiterpanes (bicyclanes) in the tissues of exposed fish

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In review. Submitted to *Environmental Toxicology and Chemistry*: 6th December 2021

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Chapter 6: Crude oil identification using linear discriminatory analysis (LDA) of bicyclic sesquiterpanes (bicyclanes) in the adipose tissue of oil-exposed fish

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Type of Publication: Journal

Title: Marine Pollution Bulletin
Authors: Francis Spilsbury, Alan Scarlett, Kliti Grice, Marthe Monique Gagnon
Year: 2021
From page: 1
To page: 8
ISSN: 0025-326X
Volume: 172
Article title: Discriminating source of oil contamination in teleost fish, Lates calcarifer, using multivariate analysis of a suite of physiological and behavioral biomarkers

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Title: Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology
Auhtors: Francis Spilsbury, Bradley McDonald, Kai Rankenburg, Noreen J. Evans, Kliti Grice, Marthe Monique Gagnon
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