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Fish Fingerprinting: Identifying Crude Oil Pollutants using Bicyclic Sesquiterpanes (Bicyclanes) in the Tissues of Exposed Fish

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Abstract: In the present study, we investigated the possibility of identifying the source oils of exposed fish using ratios of bicyclic sesquiterpane (bicyclane) chemical biomarkers. In the event of an oil spill, identification of source oil(s) for assessment, or for 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, so far as we are aware this has never been successfully demonstrated for oil-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 or to Montara, an Australian medium crude oil. Two-dimensional gas chromatography with high-resolution mass spectrometry and gas chromatography–mass spectrometry were then used to measure selected ratios of the bicyclanes to examine whether the ratios were statistically reproducibly conserved in the fish tissues. Six diagnostic bicyclane ratios showed high correlation ($r^2 > 0.98$) with those of each of the two source oils. A linear discriminatory analysis model showed that nine different petroleum products could be reproducibly discriminated using these bicyclane ratios. The model was then used to correctly identify the bicyclane profiles of each of the two exposure oils in the adipose tissue extracts of each of the 18 fish fed oil-enriched diets. From our initial study, bicyclane biomarkers appear to show good potential for providing reliable forensic fingerprints of the sources of oil contamination of exposed fish. Further research is needed to investigate the minimum exposure times required for bicyclane bioaccumulation to achieve detectable concentrations in fish adipose tissues and to determine bicyclane depuration rates once exposure to oil has ceased. *Environ Toxicol Chem* 2023;42:7–18. © 2022 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Crude oil; ecotoxicology; fingerprinting; heavy fuel oil; linear discriminatory analysis; Montara

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(s) (Stout et al., 2001). Methods for the forensic identification of spilled oils in water, sediments, and sessile filtering organisms are quite well established (Stout et al., 2001; Wang & Fingas, 2003; Yang et al., 2017) and typically involve analyses of the relative abundances of key chemical biomarker compounds such as acyclic isoprenoids, *n*-alkanes, and polycyclic isoprenoids such as steranes and hopanes. The latter compounds are also relatively unaffected by weathering processes such as evaporation and biodegradation, and may then provide a

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unique fingerprint which remains valid even after prolonged periods of environmental exposure.

However, there is little evidence that such relatively high molecular weight compounds as steranes and hopanes are accumulated by motile organisms, such as fish. A single previous study attempting crude oil identification in exposed fish using steranes and hopanes was successful in identifying the oil in only two of 12 fish tissue samples tested (Manan et al., 2011). Nonetheless, oils may certainly be consumed by fish, as witnessed following the oil spill resulting from the Montara Well blowout in 2009 in the Timor Sea (Burns & Jones, 2016; Gagnon & Rawson, 2012), where wild fish were observed to feed on waxy oil residues. Indeed, in field studies following the 2010 Macondo Well, *Deepwater Horizon* incident in the Gulf of Mexico, total petroleum hydrocarbon (TPH) concentrations in the tissues of exposed species of commercial fish were as high as 2.2% w/w (Sammarco et al., 2013) and in industrial areas, such as the Gulf of Suez, TPH levels in white muscle of fish were up to 0.15% w/w (Ahmed et al., 2019). Certainly, some relatively nontoxic saturated petroleum hydrocarbons, such as some *n*-alkanes and pristane, are bioaccumulated and may remain in fish adipose tissues for up to 5 months following exposure (Cravedi & Tulliez, 1986).

Bicyclanes meet many of the criteria necessary for oil identification. For instance, they are ubiquitous in crude oils (Stout et al., 2016; Wang et al., 2005, 2013), including in oils from a wide range of geological periods. A review of current literature failed to find any occurrence of bicyclanes other than in ancient sediments and crude oils, so their presence in fish tissues would be unlikely to result from natural non-oil sources. Importantly, bicyclanes are often present in differing proportions in different oils, such that it is possible to characterize oils from different sources (Alexander et al., 1984; Nelson et al., 2022b; Noble et al., 1987). Indeed, for some fuel oils and light crude oils lacking significant proportions of steranes and hopanes, bicyclanes allow the differentiation of a variety of petroleum products from one another (Wang et al., 2005; Yang et al., 2012). Bicyclanes are also resistant to weathering (Wang et al., 2005; Yang et al., 2012) and are not known to be toxic to fish or to other marine species (Jansen & De Groot, 2004), yet bicyclanes have a sufficiently low molecular weight (typically ~200 g/mol) and lipophilicity ($\log K_{OW}$ 6.36; ECOSAR; US Environmental Protection Agency [USEPA], 2021) to suggest that passive uptake across cell membranes would occur (Streit, 1998). Although they may be able to be degraded by bacterial consortia (Maier, 2019), bicyclanes are not known to be subject to cellular metabolic processes in fish. They would therefore appear to be good candidate biomarkers for forensic fingerprinting analyses of oils in the tissues of exposed fish. To our knowledge, this has yet to be shown experimentally or in the field.

Any forensic use of biomarkers, including bicyclanes, to fingerprint oils in fish will require statistical verification of the reproducibility of the methods. Computerized multivariate statistical analysis comparisons of biomarker ratios provide an objective approach. Linear discriminatory analysis (LDA) is a form of multivariate analysis commonly reported in literature in relation to machine learning applications, such as facial recognition systems (Kaur et al., 2020). Unlike principal component analysis (PCA), which seeks to reduce the dimensionality of complex datasets by establishing principal components that retain as much of the variability in a data set as possible, LDA instead optimizes for the greatest differences between specified categorical variables to maximize discrimination between sample groups (Skrobot et al., 2007; Sparks et al., 1999). Once a 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).

Lates calcarifer (barramundi or Asian sea bass) is a carnivorous teleost fish widely used in aquaculture (Boonyaratpalin, 2017; Mathew, 2009), with a wide-ranging global distribution in both marine and freshwater environments (Grey, 1987). Laboratory studies seeking to simulate the toxicological effects of crude oil exposure on various species of fish have used either a waterborne route via the gills (Aas et al., 2000; Heintz et al., 1999) or a dietary route (Bautista et al., 2019; Nahrgang et al., 2010; Spilsbury et al., 2021; Vieweg et al., 2018; Vignet et al., 2014). Both are realistic and relevant. For instance, dietary exposure of *L. calcarifer* to sublethal doses of Montara crude oil from the Timor Sea (off northern Australia) and a heavy fuel oil led to a range of behavioral, physiological, and biochemical responses (Spilsbury et al., 2021). These included decreased foraging ability, decreased brain acetyl-cholinesterase concentration, increased hepatic 7-ethoxyresorufin o-dealkylase (EROD) activity, and decreased condition factor (Spilsbury et al., 2021).

Although bicyclanes meet many of the criteria necessary to be used for forensic fingerprinting it is unknown if they are adequately bioavailable and bioaccumulate in sufficient concentrations to be readily extractable for analyses. The crucial question is: are the characteristic bicyclane ratios observed in the oil maintained in the tissues even after extraction and processing? The present study aims to answer these questions by exposing juvenile *L. calcarifer* to two types of oil, a medium crude oil and a heavy fuel oil, then extracting the tissues and analyzing them using gas chromatography–mass spectrometry (GC-MS). To verify peak identification, two-dimensional gas chromatography with high-resolution mass spectrometry (GC × GC-HRMS) was applied. This also provided an opportunity to assess if the enhanced resolution of two-dimensional chromatography would provide a greater ability to differentiate oils. Finally, we determined if the ratios of bicyclanes bioaccumulated in oil-exposed fish are sufficiently conserved and discriminatory to be differentiated from a wider set of oils using multivariate analysis. We demonstrated that an LDA model trained using six diagnostic bicyclane ratios from a reference set of petroleum products including crude oils, condensates, fuel oils, and asphaltites can be used to successfully identify the oil to which individual fish have been exposed.

MATERIALS AND METHODS

Oils used in fish exposure trials

A heavy fuel oil (API 11.4) was supplied by the BP Kwinana Oil Refinery and Montara crude oil (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).

Oils used for LDA

In addition to the two exposure oils described above, seven other petroleum products (crude oils, condensates, fuel oils, and asphaltites) were used for statistical analysis to determine if any bicyclanes bioaccumulated in the fish tissues could be discriminated from a wider range of oils. From the northwest shelf of Australia, two light crudes from the Caswell and Eland West wells and condensates from the Crux and Calliance wells were available from a previous study (Spaak et al., 2020). An additional heavy fuel oil, Bunker C, was obtained from a ship docked in Fremantle Port, Western Australia, and a very low sulfur oil from the MV *Wakashio* was available from previous studies (Nelson et al., 2022b; Scarlett et al., 2021). Coastal asphaltites collected from the Great Australian Bight were also available from a previous study (Scarlett et al., 2019a). Weathering of the asphaltites ranged from mild to heavy (denoted W1–W5, respectively).

Fractionation of oils for GC-MS analysis

Small silica columns were prepared in glass Pasteur pipettes with 0.5 g of silica gel hexane slurry. Approximately 10 mg of oil was applied to the column, and the saturates fraction eluted with 3 ml of hexane then evaporated under a gentle nitrogen stream to approximately 0.5 ml.

GC-MS

Saturate samples (1 µl) were injected (splitless) into an Agilent GC 7890B fitted with a DB-1MS column (Agilent), coupled to an Agilent MS 5977B. The temperature was initially held at 40 °C for 1 min before being increased at a ramp of 6 °C/min to 320 °C and then held isothermally for 28 min.

Sterane and hopane biomarkers were analyzed by GC-MS selected ion monitoring analysis (*m/z* 123, 191, 205, 217, 218, 358, 370, 372, 384, 386, 398, 400, 412, 414, 426, 428, 440, 442, 454, 456). Bicyclanes were analyzed by GC-MS full-scan extracted ion chromatography (EIC; *m/z* 179, 193, 208).

Data were analyzed by Agilent ChemStation software, with compound identification using National Institute of Standards and Technology library searches. Kovats retention indices (temperature programmed) were calculated using ASTM International Method D6730 (ASTM International, 2021). All confidence intervals provided are 2× standard error (2SE).

GC × GC-HRMS

The method is described in full by Nelson et al. (2022b). In brief: GC × GC-HRMS utilized a LECO Pegasus GC × GC-high resolution time of flight (HRT) 4D instrument consisting of an Agilent 7890B GC configured with a LECO LPAL3 split/splitless auto-injector and a dual stage cryogenic modulator. The carrier gas was He at a flow rate of 1 ml/min in constant flow mode. Samples were injected in splitless mode, either as whole oils diluted in dichloromethane or saturate fractions of fish tissue extracts in hexane (identical to those analyzed by GC-MS). The hot jet temperature offset was 15 °C above the secondary oven and the inlet temperature was isothermal at 300 °C. The first-dimension column was a Restek Rxi-1ms, (60 m × 0.25 mm × 0.25 µm) and second dimension separations were performed on a 50% phenyl polysilphenylene-siloxane column (SGE BPX50, 1.2 m × 0.10 mm × 0.1 µm). The main oven temperature was held isothermal at 75 °C (12.5 min) and then ramped to 275 °C at 1.25 °C/min. The second-dimension oven was held isothermal at 80 °C (12.5 min) and then ramped from 80 °C to 280 °C at 1.25 °C/min. The hot jet pulse width was 1.5 s, and the modulation period between stages was 5 s with a cooling period of 1 s between stages. HRT data were sampled at an acquisition rate of 200 spectra/s. The ionization method was electron ionization with an electron energy of 70 eV and an extraction frequency of 1.5 kHz. Bicyclanes were detected and identified using extracted ions *m/z* 123.1168, 137.1325, 179.1794, 193.1951, 207.2107, 208.2186, 222.2342 an 236.2499 amu.

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 °C, salinity 32 ppt, dissolved O_2 >5 mg/L before transfer 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 recirculating 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) twice daily to a total of approximately 2% bodyweight per day (Hellou et al., 2002). Fish were fed plain fishmeal (negative control), fish meal spiked with 1% w/w heavy fuel oil or fish meal spiked with 1% w/w Montara crude oil. Fish food was stored at −20 °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 feed. Fish were exposed to crude oils via diet continuously for 33 days, followed by a 2-day depuration period. Following euthanization by ike-jime, samples of brown adipose tissue (typically 2–5 g) adjacent to the intestine were removed and stored at −20 °C prior to analysis.

Extraction and chromatography of fish adipose tissues

Extraction of petroleum hydrocarbons was performed using published methods (Kelly et al., 2000; Scarlett et al., 2011). Briefly, frozen adipose tissue samples were accurately weighed, and between 2 and 5 g of tissue was transferred to a 250-ml round-bottomed flask containing 100 ml of HPLC grade methanol, 5 g of potassium hydroxide, and spiked with 1 µg of

n-decane-D_{22} and *n*-tetracosane-D_{50}. Samples were digested under reflux for 2 h. Cool digests were passed through a Whatman 113 v filter paper into a 500 ml separating funnel and extracted using 3 × 25 ml of hexane, followed by a 25-ml hexane glassware rinse. Extracts were transferred to a 500-ml round-bottomed flask and reduced in volume to approximately 2 ml via rotary evaporation before being transferred to 4-ml vials and dried by the addition of a small quantity of $MgSO_4$. Four procedural blanks were performed.

Columns were prepared in 50-ml burettes containing 6 g of activated silica and washed with 50 ml of 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 into a 50-ml round-bottomed flask with 40 ml of hexane. This fraction was reduced in volume to 1.0 ml by rotary evaporation followed by a gentle nitrogen stream.

Statistical analyses

All analyses were performed using R statistical software, Ver 4.1.0.

Bicyclanes were identified by elution order and mass spectra. Diagnostic ratios were calculated from the relative abundances of bicyclanes using direct peak area comparisons.

Correlations between bicyclane ratios in oils and the respective adipose tissue of exposed fish were established using the lm: Fitting Linear Models function from the R stats package.

Linear discriminatory analysis was performed using the MASS R package (Venables & Ripley, 2002). An LDA model was defined using a "training" data set consisting of six bicyclane ratios in the two exposure oils plus seven other petroleum products, each studied in replicate: heavy fuel oil ($n = 3$), Bunker C ($n = 3$), low sulfur oil ($n = 3$), Montara crude oil ($n = 3$), Crux ($n = 3$), Calliance ($n = 3$), Caswell ($n = 2$), Eland West ($n = 3$), and Great Australian Bight ($n = 8$). 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 Montara crude oil ($n = 9$) or heavy fuel oil ($n = 9$).

RESULTS AND DISCUSSION

Our aim in the present study was to test the feasibility of using the hydrocarbon distributions extracted from fish tissues to identify one of two possible dietary exposure oils, simulating a scenario which may occur during, or after, an oil spill.

Observations and implications from exposure study

The 33-day dietary exposure, plus 2 days' depuration, of juvenile *L. calcarifer* to the two oils resulted in 100% survivorship (Spilsbury et al., 2021). Feeding rates differed between test groups with 4.51 ± 0.10, 2.17 ± 0.09, and 0.244 ± 0.01 g/min for negative control, Montara crude oil, and heavy fuel oil fish, respectively (Spilsbury et al., 2021). Differences in behavioral and physiological biomarkers were consistent with exposure to the aromatic compounds measured in the oils previously (Spilsbury et al., 2021). From this it can be inferred that consumption of small quantities of oil (1% w/w fish food used in our study) would be unlikely to cause acute toxicity resulting in morbidity or to produce sufficient physiological/behavioral disruption to prevent wild fish from swimming away from the source of oil contamination (Burns & Jones, 2016; Gagnon & Rawson, 2012). Wild fish sampled in the absence of an obvious oil slick may therefore still carry information of past exposure.

Non-bicyclane hydrocarbons bioaccumulated in fish tissues

The saturated hydrocarbon fractions of adipose tissue extracts contained series of *n*-alkanes (Supporting Information, Figure S1). Spiked recoveries of tridecane (*n*-C_{13}), heptadecane (*n*-C_{17}), octadecane (*n*-C_{18}), and pristane (branched C_{19}) were 85% ± 7%, 100% ± 13%, 103% ± 7%, and 99% ± 8%, respectively, indicating that the distributions of the C_{15+} alkanes were probably recovered reproducibly, as is typical for the extraction and fractionation methods used. Decane (*n*-C_{10}), undecane (*n*-C_{11}), heptadecane, and pristane were present in the fish food and correspondingly in the adipose tissue of negative control fish (Supporting Information, Figure S2).

In the oils, the *n*-alkane series ranged from C_{10} to C_{29} and the most abundant *n*-alkanes were C_{22} or C_{18} for Montara crude oil and heavy fuel oil, respectively (Supporting Information, Figure S2). Fish adipose extracts of both Montara crude oil- and heavy fuel oil-exposed fish showed similar distributions but maxima for *n*-alkanes with slightly lower carbon numbers. This suggests a decrease in uptake of larger *n*-alkanes across the cell membrane of fish adipocytes similar to that observed in mussels, where uptake is 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 and pristane in the fish adipose tissues is due to the combined inputs of *n*-alkanes from the exposure oils and from the fish food (Supporting Information, Figures S2 and S3). The differences in uptake of *n*-alkanes and the presence of these and pristane in the food means that *n*-alkane and acyclic isoprenoid alkane distributions (e.g., Powell & McKirdy, 1973; Wang et al., 2007; Yang et al., 2017), which are sometimes used for fingerprinting oils in water and sediments, are not useful for fingerprinting oil residues in the tissues of fish.

Forensic identification of oils is also 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 Montara crude oil and heavy fuel oil oils at forensically useful concentrations and were not detected in the adipose tissues of the exposed fish. Similarly, a prominent suite of forensically useful bicadinanes present in heavy fuel oil was not detected by GC-MS in fish adipose tissue.

Bicyclane profiles in fish tissues

Low molecular weight bicyclic alkanes, such as decalin and C_1-decalins, were found in both exposure oils and 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. Recovery of spiked decalin was only 61%. However, using GC-MS and GC × GC-HRMS, higher molecular weight bicyclanes were also detected in the adipose tissues of the exposed fish (Figures 1 and 2), but not in the negative controls (Supporting Information, Figure S3). No authentic bicyclanes were available for spiked recovery assessments, but recoveries of C_{13} *n*-tridecane and C_{17} *n*-heptadecane (i.e., compounds with GC retention times similar to $C_{14\text{-}16}$ bicyclanes) were 85% ± 13% and 100% ± 9%, respectively. Using GC-MS extracted ion mass chromatograms (*m/z* 179 + 193 + 208), 10 bicyclanes (BSA - BSJ) were detected in the heavy fuel oil or Montara crude oil oils (Table 1 and Figure 2), although some co-elution was evident for peaks BSF, BSG, BSH, and BSI (Figure 2).

The presence of bicyclanes in the oils up to $C_{17}H_{32}$ was confirmed by GC × GC-HRMS, which was more sensitive and specific due to the increased resolving powers of both the GC and MS systems (Nelson et al., 2022a; Figure 1). These bicyclanes were even more prominent in the tissue extracts of oil-exposed fish due to bioconcentration (Figure 1C,D), allowing a total of 21 peaks to be assigned with high mass accuracies, typically ~1 ppm in heavy fuel oil-exposed fish (Supporting Information, Figure S5a–u). In addition to C_{15} 8β(H)-drimane and C_{16} 8β(H)-homodrimane (Supporting Information, Figures S4i and S5t), which are well known in oils (Wang et al., 2007), the 2α-methyl analogs were also present in the heavy fuel oil and in heavy fuel oil-exposed fish. Both the 2α-methyl-8b(H)-drimane and 2α-methyl-8b(H)-homodrimane (Supporting Information, Figures S4q and S5u) were identified by the presence of a mass spectral ion assigned to $C_{10}H_{17}$ in the HRMS spectra, rather than the C_9H_{15} fragment ion which is characteristic of the spectra of the nor-methyl 8β isomers. The presence of these 2α-methyl analogs is potentially forensically diagnostic and may be applied in future studies (Nelson et al., 2022a). The 2α-methyl analogs were not detected in the Montara crude oil or tissue extracts from fish exposure to Montara crude oil (Figure 1A,C), so were not used in the present study.

Importantly, the distributions of bicyclanes in the two oils used in the present study were also different in other major respects: Montara crude oil contained bicyclanes BSA, BSB, BSD, BSE, BSI, and BSJ, whereas heavy fuel oil contained BSA, BSC, BSD, BSE–BSJ (Figure 2). Thus, selected ratios of different bicyclanes allowed the two oils to be differentiated from one another and in adipose tissues from fish in both oil exposure test groups.

Direct comparison of six peak area ratios (EIC *m/z* 123 + 179 + 193) of the four bicyclanes common to both oils which were well-resolved by GC-MS (Table 1) showed a good linear correlation between the relative abundances of bicyclanes in the oils and in the respective adipose tissue extracts of fish exposed to either Montara crude oil ($r^2 = 0.9819$) or heavy fuel oil ($r^2 = 0.9817$; Figure 3). Hence, even using a fairly limited number of biomarker ratios, the bicyclane distributions of the fish could be positively correlated with the oil to which they had been exposed.

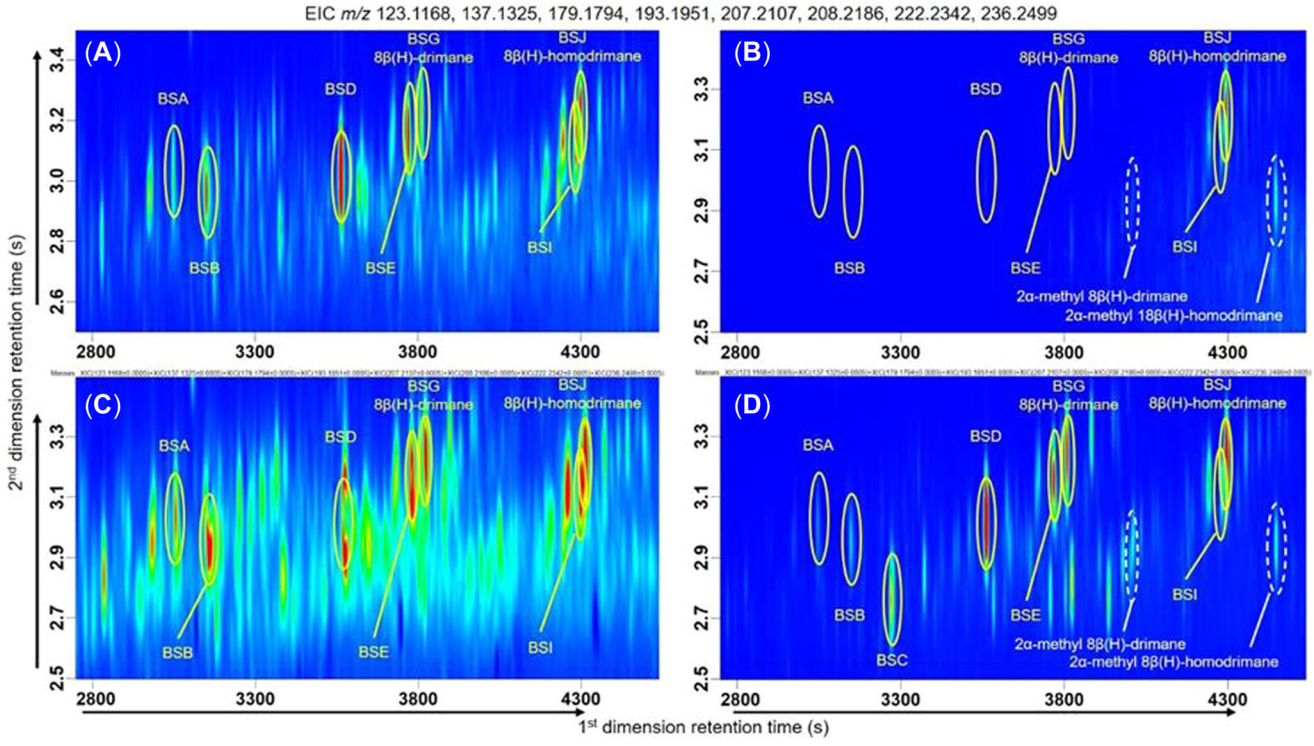

FIGURE 1: Two dimensional gas chromatography—high-resolution mass spectrometry extracted ion chromatograms (EIC) showing elution positions of bicyclanes present in fish food spiked with Montara crude oil (**A**) or heavy fuel oil (**B**), and those present in extracts of lipid tissues from fish exposed to fish food spiked with Montara crude oil (**C**) and heavy fuel oil (**D**). Table 1 shows peak identification information.

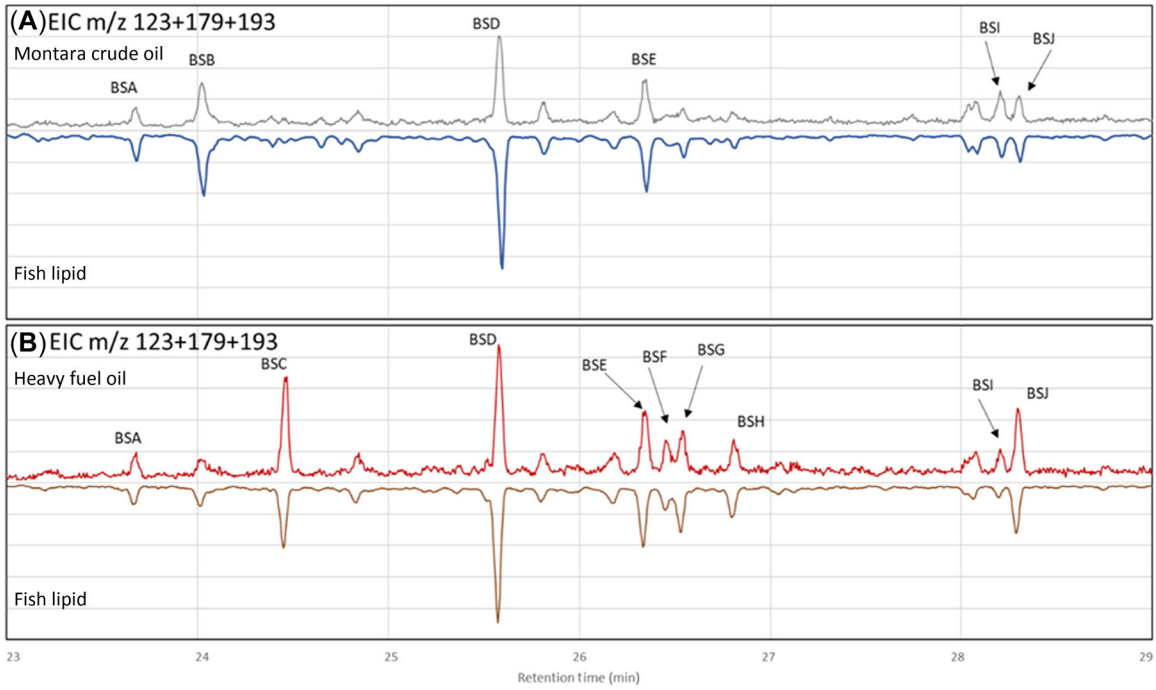

FIGURE 2: Mirrored partial gas chromatography-mass spectrometry extracted ion chromatograms (*m/z* 123 + 179 + 193) of oils (top) and fish adipose tissue extracts (bottom) for Montara crude oil (**A**) and heavy fuel oil (**B**).

LDA

Having established that ratios of bicyclanes bioaccumulated in the adipose tissue of dietarily exposed fish could be used to discriminate between two different source oils, further statistical testing was made to determine if the data were sufficiently robust to allow the differentiation of a larger number of oils. For this, multivariate LDA of bicyclane profiles was applied to identify the specific oil to which individual fish had been exposed from nine possible candidates, including crude oils and condensates originating from close geographical locations and asphaltites with varying degrees of weathering.

The reference crudes, condensates, and fuel oils for the LDA training dataset were chosen either because of their similarity to the test oils used in the dietary exposures or because they allowed investigation of weathering on bicyclane fingerprinting. A group of coastal asphaltites collected between 1990 and 2005 from a variety of locations in the Great Australian Bight had, despite the diversity in their collection, been shown to have originated from the same natural seep source but with some differences in properties attributed to weathering (Hall et al., 2014; Scarlett et al., 2019a). Bicyclanes in these samples would therefore be expected to group closely together. Differences in weathering provide the opportunity to determine if bicyclane ratios are largely unaffected by environmental degradation processes, as reported in other studies using bicyclanes as fingerprinting biomarkers (Wang et al., 2005; Yang et al., 2012).

Relationships between several oils and condensates from wells in the Browse and Bonaparte basins in the northwest shelf of Australia have been previously established using a range of techniques, such as diamondoid ratios (Spaak et al., 2020), and

TABLE 1: Bicyclanes identified by gas chromatography-mass spectrometry in the heavy fuel and Montara crude exposure oils used for fingerprinting the oils in fish adipose tissues

Bicyclane	Abbreviation	Molecular ion (*m/z*)	Base peak ion (*m/z*)	Kovats retention index
C_{14} sesquiterpane	BSA	194	179	1351
C_{14} sesquiterpane	BSB	194	179	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

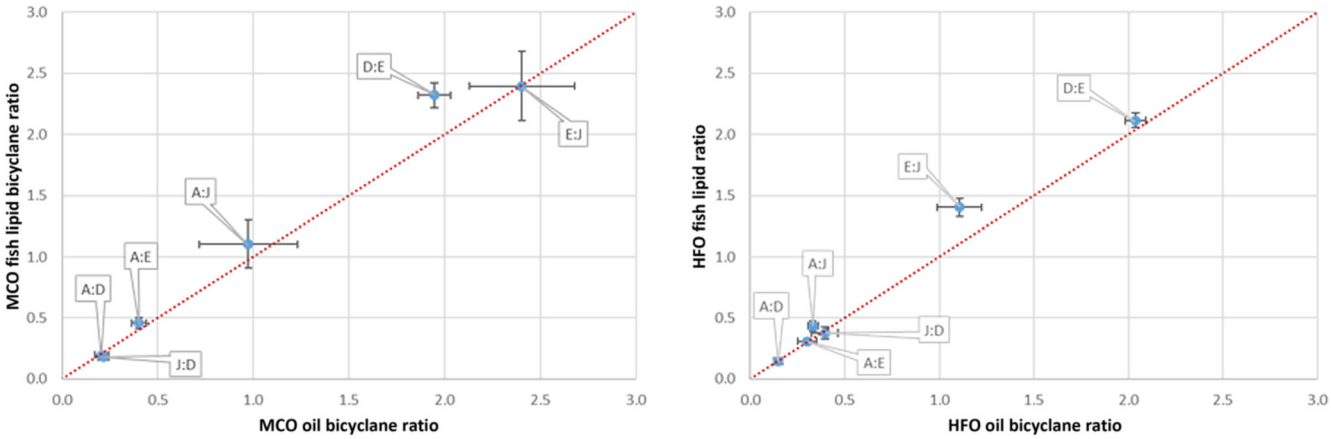

FIGURE 3: Correlation of bicyclane ratios in exposure oils with those in the respective exposed fish adipose tissue extracts. Red dotted line shows 1:1 fit. Error bars are 2 × standard error for oils (horizontal, $n = 3$) and fish adipose tissue (vertical, $n = 9$).

therefore it might be expected that this is reflected in the bicyclane ratios. Of particular note, the Montara and Crux-3 wells are in close geographical proximity, access the same reservoir, and analysis of diamondoid biomarkers has shown these oils display a relatively high degree of similarity (Spaak et al., 2020). This provides a link between the oils used in the fish exposure trial and the additional statistical testing using LDA. Fuel oils may be mixtures and their constituents affected by distillation or catalytic processes (Peters et al., 1992). The ratios in such fuel oils are therefore unpredictable but could expected to be in low concentrations and thus analytically challenging. Our analysis makes use of a Bunker C heavy fuel oil currently in use in marine ships and a very low sulfur fuel oil spilled from the MV *Wakashio* in Mauritius in 2020 (Scarlett et al., 2021). The latter would have undergone some form of catalytic process to lower its sulfur content whereas the former is a traditional residual fuel oil.

The LDA model established using a training dataset of six bicyclane ratios from a suite of nine petroleum products (Table 2 and Figure 4A,B) produces an ordination space in which the nine petroleum products are distinctly separated (Figure 5). The fuel oils Bunker C, heavy fuel oil, and low sulfur oil were discriminated on the LD1 and LD2 cartesian axes, as were the two crude oils from the Browse Basin; Montara and Crux-. Within the ordination space, the fish adipose tissue extracts are positioned within the 95% posterior probability categorization boundaries for the respective crude oils to which the fish were exposed (Figure 5). Hence, the LDA predictions for the adipose tissue extracts from fish exposed to either Montara crude oil ($n = 9$) or heavy fuel oil ($n = 9$) correctly identified the respective oil to which each fish was exposed (see Supporting Information for R Markdown).

The position within the LDA ordination space of the Great Australian Bight asphaltites was not affected by the degree of weathering of the samples, with asphaltites with mild weathering (W1) clustered in close proximity to samples with heavy weathering (W5; Figure 5). This reinforces the supposition that bicyclanes are not greatly degraded during weathering, and their relative abundances remain consistent and conserved. Hence, even for oils that spend prolonged periods in the environment, bicyclane fingerprinting may be a viable method for identification of the source of the oil.

The 100% successful prediction rate of exposure oils of our proof-of-concept study is encouraging, given the chemical similarity of some of the oils used. Other petroleum

TABLE 2: Diagnostic ratios of four bicyclanes in crude oils, heavy fuel oils, asphaltites, and adipose tissue extracts of fish exposed to Montara crude and heavy fuel oil

Sample	*n*	BSA:BSD	BSA:BSE	BSA:BSJ	BSD:BSE	BSJ:BSD	BSE:BSJ
Bunker C	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	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	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
Great Australian Bight asphaltites	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	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	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	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	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	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

Confidence intervals are 2 × standard error.

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 each test group, as can be seen by the clustering of heavy- and Montara crude oil-exposed fish on the LDA ordination plot (Figure 5) and bicyclane ratio confidence intervals (Table 2). The conservation of the relative abundances of bicyclanes in fish adipose tissue indicates similar bioaccumulation factors among bicyclanes. This may be due in part to negligible 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.

Limitations of the present study and future research

Species of wild fish present in an oil spill zone are often motile and may avoid spilled oil. Aquaculture species such as *L. calcarifer*, however, would be unable to escape exposure should fish farming operations be affected by an oil spill. The dietary exposure used in the present study demonstrates that *L. calcarifer* consumed oil-tainted food but with a reduced feeding rate compared with negative control fish (Spilsbury et al., 2021). Drimane has an estimated water solubility of 0.043 mg/L (ECOSAR; USEPA, 2021), similar to $C_{16}H_{26}$ alkylbenzenes and $C_{16}H_{24}$ alkyltetralins, which are known to bioaccumulate in mussels via aqueous exposure (Booth et al., 2007, 2008). Future studies may include aqueous exposures.

The present study demonstrates that relative abundances of adipose bicyclanes and their corresponding fingerprint ratios

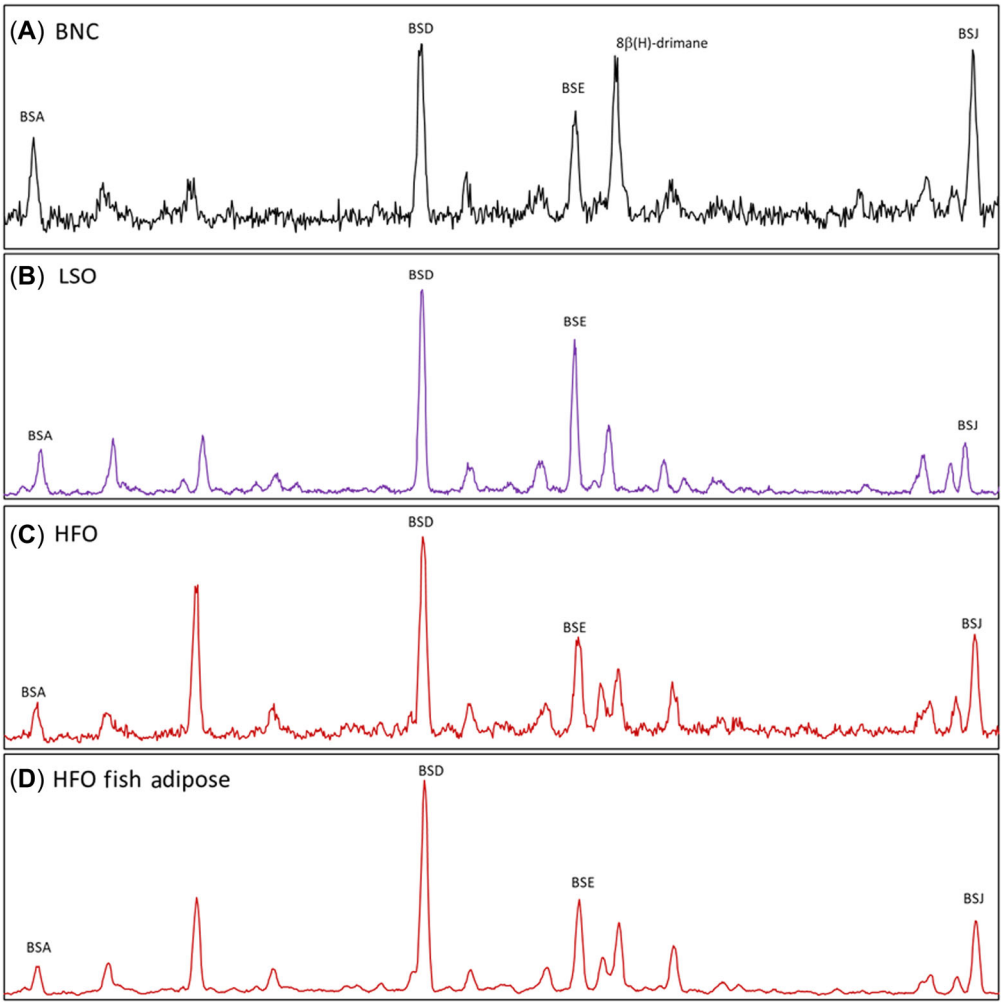

FIGURE 4: (Section 1, **A–C**) Typical partial extracted ion gas chromatography-mass spectrometry (GC-MS) chromatograms (*m/z* 123 + 179 + 193) of fuel oils (**A–C**) and adipose extracts of fish exposed to heavy fuel oil (**D**). (Section 2, **E–K**) Typical partial GC-MS chromatograms (extracted ion chromatogram: *m/z* 123 + 179 + 193) of asphaltites (**E**) and crude oils (**F–J**) and adipose extracts of fish exposed to crude oil (**K**). BNC, Bunker C; LSO, low sulfur oil; HFO, heavy fuel oil; GAB, Great Australian Bight; CAL, Calliance; CAS, Caswell; ELW, Eland West; CRX, Crux; MCO, medium crude oil.

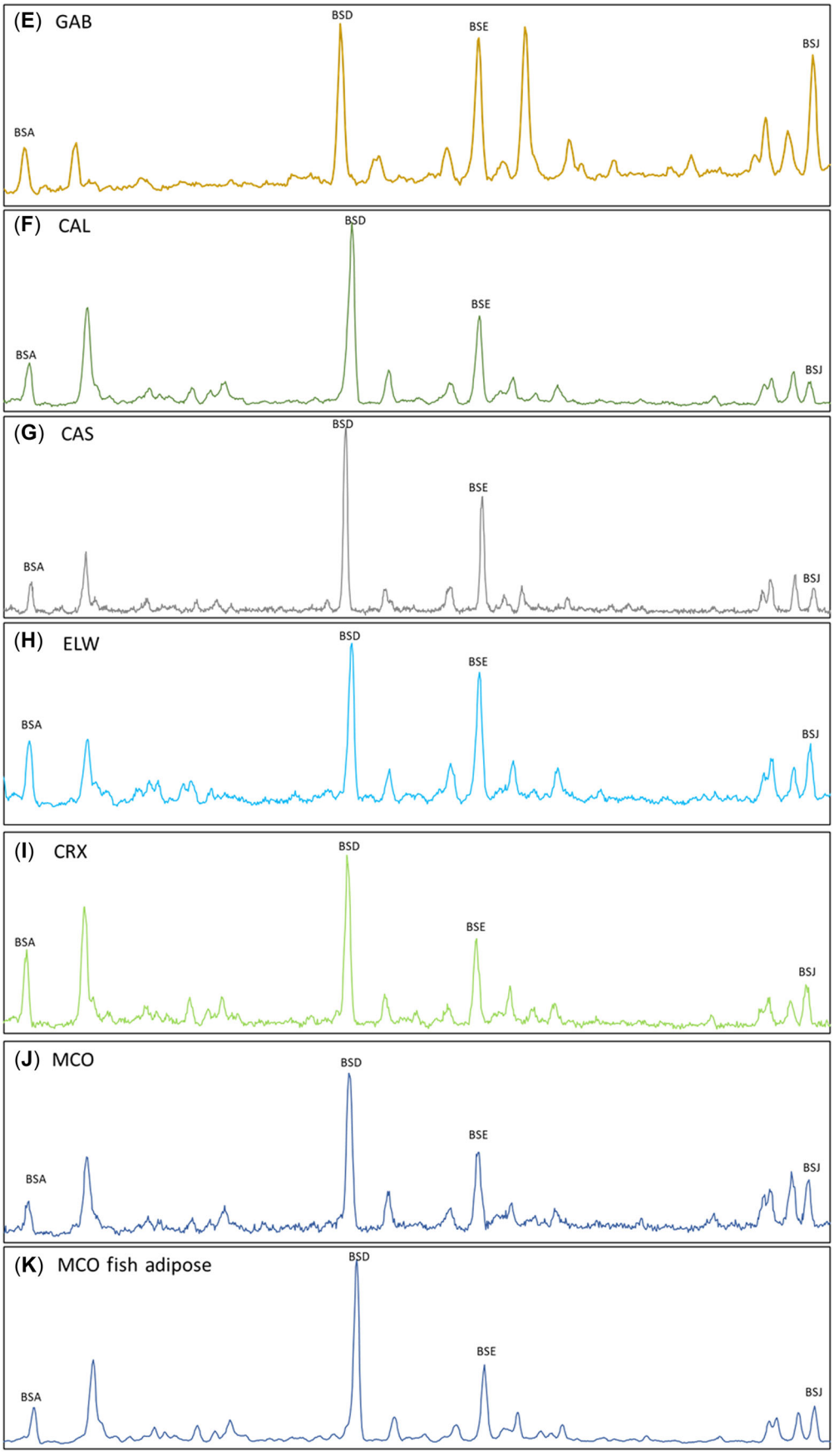

FIGURE 4: (Continued)

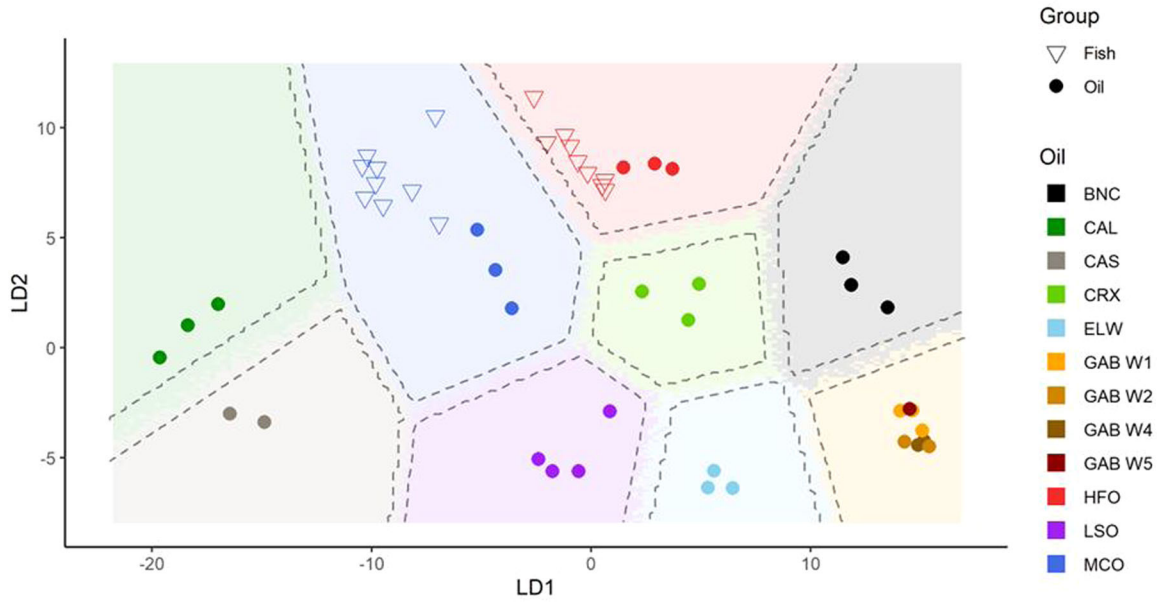

FIGURE 5: Linear discriminatory analysis of crude oils, heavy fuel oils, asphaltites, and adipose tissue extracts of fish exposed to Montara crude oil and heavy fuel oil. Shaded areas are the decision boundaries for the respective oils, with dotted lines indicating the 95% posterior probability demarcation. BNC, Bunker C; LSO, low sulfur oil; HFO, heavy fuel oil; GAB, Great Australian Bight; CAL, Calliance; CAS, Caswell; ELW, Eland West; CRX, Crux; MCO, medium crude oil.

remain consistent and unchanged compared with their respective exposure oils up to the 33-day duration plus 2-day depuration of the laboratory exposures, which is a realistic timeframe 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 and the time required to reach steady-state conditions in the adipose tissue of oil-exposed fish. Given that bicyclanes appear not to be readily metabolized by fish, sequestered bicyclanes in adipose tissue may remain in situ for long periods postexposure, possibly even after other signs of oil exposure have dissipated in the environment. The present study used a 2-day depuration period but further testing of depuration rates of adipose bicyclanes in oil-exposed fish would provide useful information when undertaking postspill sampling. To field-test this method of oil identification, the future acquisition of fish exposed to petroleum hydrocarbons in an oil spill would be needed. As an initial step, fish from oil spill-affected aquaculture operations would be particularly suitable because direct comparison between the spilled oil and the fish tissue could be made, the exposure duration known, and depuration monitored.

As demonstrated in the present study, the application of GC × GC-HRMS in future studies would be highly beneficial. However, even unit mass GC × GC-MS would provide superior chromatographic resolution and generally lower limits of detection (Beyer et al., 2010). The ideal would be identification by GC×GC-HRMS with quantitation by GC×GC-flame ionization detection (FID). Authentic standards for a suite of bicyclanes would be incredibly useful and would facilitate much greater confidence in the use of GC-MS and GC × GC-FID.

The nine-sample library of bicyclane biomarker ratios from oils used as a training set for the LDA is a limitation of the present study. To further explore the potential of bicyclane fingerprinting in fish adipose tissue for oil identification in the event of a spill, future research is needed to expand the training set library by characterizing 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 is 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 adamantanes, 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.

CONCLUSION

The distributions of bicyclanes, known to be present in characteristically different relative abundances in many crude oils and fractionated oil products, appear to retain their diagnostic ratios and are readily accumulated into adipocytes of *L. calcarifer* exposed via diet to a medium crude or a heavy fuel

oil. Multivariate statistical analysis by LDA using bicyclane data for a range of crude oils, condensates, fuel oils, and asphaltites successfully identified the exposure oils in the fish tissues. Further research is needed to ascertain the minimum exposure time for bioaccumulation of bicyclanes to exceed analytical limits of detection and depuration rates once exposure has ceased.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5489.

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Conflict of Interest—The authors declare no conflict of interest.

Disclaimer—The authors declare that they have no known competing personal relationships that could have appeared to influence the work reported in the present study.

Author Contributions Statement—**Francis D. Spilsbury:** Investigation; Data curation; Formal analysis; Methodology; Visualization; Writing—original draft; Writing—review & editing. **Alan G. Scarlett:** Conceptualization; Methodology; Supervision; Resources; Writing—review & editing; Funding acquisition. **Steven J. Rowland:** Conceptualization; Writing—review & editing; Funding acquisition. **Robert K. Nelson:** Formal analysis; Writing—review & editing. **Gemma Spaak:** Formal analysis; Writing—review & editing. **Kliti Grice:** Resources; Writing—review & editing; Funding acquisition. **Marthe Monique Gagnon:** Conceptualization; Resources; Writing—review & editing; Supervision; Resources; Funding acquisition.

Data Availability Statement—Raw data has been included in the Supplementary Materials, sufficient to allow repetition of the analyses described in the present study. Data, associated metadata, and calculation tools are available from the corresponding authors (francis.spilsbury@bioenv.gu.se; M. Gagnon@curtin.edu.au).

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