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Do distributions of diamondoid hydrocarbons accumulated in oil-contaminated fish tissues help to identify the sources of oil?

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ABSTRACT

Identifying the sources of environmental oil contamination can be challenging, especially for oil in motile organisms such as fish. Lipophilic hydrocarbons from oil can bioaccumulate in fish adipose tissue and potentially provide a forensic "fingerprint" of the original oil. Herein, diamondoid hydrocarbon distributions were employed to provide such fingerprints. Indices produced from diamondoids were used to compare extracts from fish adipose tissues and the crude and fuel oils to which the fish were exposed under laboratory conditions. A suite of 20 diamondoids was found to have bioaccumulated in the dietary-exposed fish. Cross-plots of indices between fish and exposure oils were close to the ideal 1:1 relationship. Comparisons with diamondoid distributions of non-exposure oils produced overall, but not exclusively, weaker correlations. Linear Discriminatory Analysis on a combined set of 15 diamondoid and bicyclane molecular ratios was able to identify the exposure oils, so a use of both compound classes is preferable.

1. Introduction

The frequency of large crude oil spills from tankers and the corresponding quantity of oil spilled has declined over the past few decades (ITOPF, 2023). However, large oil spills (>700 t) still occur, such as that caused when the bulk carrier CF *Crystal* collided with the oil tanker *Sanchi* in the East China sea in 2018, releasing 113,000 t of condensate (Chen et al., 2020; ITOPF, 2023). During 2022, three large crude oil spills and four medium spills (7–700 t) were recorded, but most of the oil released was from small spills of <7 t (ITOPF, 2023). Identifying the source of oil contamination is often straight forward, but this is not always the case and legal disputes can last for many years (e.g., the case of the Montara well blowout in 2009 in the Timor Sea has only been settled quite recently (Burns and Jones, 2016; Gagnon and Rawson, 2012; Parry and Ryan, 2021)).

The initial step in assessment and litigation proceedings, is often the defensible forensic identification (commonly referred to as "fingerprinting"), of the source oil(s) (Stout et al., 2001). Forensic identification of spilled oils typically involves analyses of the relative abundances of key chemical biomarker compounds, including *n*-alkanes and acyclic and polycyclic isoprenoids, for which there are well established methods for the analysis of water, sediments and sessile organisms (Stout et al., 2001; Wang and Fingas, 2003; Yang et al., 2017). Identifying the sources of oil contamination in motile species, such as fish, has proved much more challenging.

Recently, we reported a method for fingerprinting oil bioaccumulated in fish lipid tissue (Spilsbury et al., 2023). In the latter laboratory study, ratios of bicylanes (bicyclic sesquiterpanes) in two oils were found to be conserved in the lipid tissues of the fish species barramundi, *Lates calcarifer,* fed on oil-enriched diets. Six diagnostic bicyclane ratios showed high linear correlations ($r^2 > 0.98$) with those of each of the two source oils, which were a light-medium crude oil and a heavy fuel oil.

One drawback of the latter method is that it relies on data from a single suite of chemical biomarkers. Although bicyclanes are ubiquitous in crude oils, they may be only present at low concentrations (Stout et al., 2016; Wang et al., 2013; Wang et al., 2005). Acquisition of data for ratios of additional chemical biomarkers could potentially extend the

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capacity of the method. Indeed, in a methodology for oil spill fingerprinting, Daling et al. (2002) stated that acquiring a broad range of target compounds allows flexibility in the selection of diagnostic ratios appropriate to each oil spill. It was also noted by Spilsbury et al. (2023) that some diamondoid hydrocarbons appeared to have been bioaccumulated in the fish adipose tissues for which co-elution in the GC–MS analyses made identification and quantitation unreliable. It was suggested that comprehensive two-dimensional gas chromatography with time of flight mass spectrometry (GC × GC-TOFMS) may be better suited to diamondoid analysis as this has been demonstrated previously (Gaines et al., 1999; Li et al., 2012; Reddy et al., 2002; Scarlett et al., 2019a; Silva et al., 2013; Spaak et al., 2020; Tran et al., 2010). In GC × GC, the separating power of the first dimension column is multiplied by that of the second column; thus much greater chromatographic separation of complex mixtures is achieved (Dimandja, 2003; Phillips and Beens, 1999).

Diamondoids are compounds composed of saturated hydrocarbons arranged in cage-like structures resembling diamonds and they have been widely detected in crude oils. Diagnostics diamondoids distributions are widely used in organic geochemistry, principally as oil maturity and source indicators, due to the high thermal stability and resistance to biodegradation of the hydrocarbons (Chen et al., 1996; Dahl et al., 1999; Grice et al., 2000; Mansoori, 2007; Marchand, 2003; Moldowan et al., 2015; Stout and Douglas, 2004; Wang et al., 2006; Wingert, 1992). They are particularly useful in the analyses of oils where the abundances of sterane and hopane biomarkers is low or nil (e.g. in many condensates (Spaak et al., 2020)). A number of diamondoid indices (DIs), based on tricyclic (adamantanes) and pentacyclic (diamantanes), have been developed (Chen et al., 1998; Chen et al., 1996; Wingert, 1992) and, more recently, indices based on the tetracyclic caged structure ethanoadamantane has been explored (Scarlett et al., 2019c). The molecular weights of the diamondoids used in the above indices are similar to that of the C_{15} bicyclanes used by Spilsbury et al. (2023) i.e. 202 Da for methyldiamantane compared to 208 Da for C_{15} bicyclanes. Small hydrocarbons tend to be readily bioavailable but may also be more prone to weathering processes. The highly branched nature of the bicyclanes provides resistance to weathering but clearly did not impede uptake into the fish adipose tissues (Spilsbury et al., 2023). In comparison, the cage structure of the diamondoids provides them with some protection from weathering processes, but how this might affect their bioavailibility is unknown. To our knowledge, the accumulation of diamondoids in the tissues of organisms has not been studied previously.

In the current study we used GC × GC-TOFMS to analyse two exposure oils and corresponding extracts of fish adipose tissues from oil exposed fish, as in a previous study (Spilsbury et al., 2023). We aimed to establish if commonly used DIs were conserved in the fish adipose tissue and to test statistically whether such ratios were sufficient alone to identify the oil in the food fed to the fish. Finally, we wished to test whether the fingerprint of DIs obtained from the fish adipose tissues could be used to differentiate between a much wider suite of crude oils, condensates and fuel oils. To achieve this, we tested whether Linear Discriminatory Analysis (LDA) of the data was able to identify the sources of oil contamination using a combination of diamondoid indices and bicyclane ratios derived from fish tissue extracts and thus could produce a more robust fingerprinting method than by either the diamondoids or bicyclanes alone.

2. Materials and methods

2.1. Oils used in fish exposure trials

Montara crude oil (MCO) (API 31.0) was provided by PTTEP Pty Ltd. A heavy fuel oil (HFO) (API 11.4) was supplied by the BP Kwinana Oil Refinery (Western Australia). Spiked recoveries of adamantane, 1,3-dimethyladamantane and diamantane were 57 %, 64 % and 101 % respectively. The study design and characteristics of the oils plus additional spiked recovery data for a range of standards are described in full elsewhere (Spilsbury et al., 2021; Spilsbury et al., 2023).

2.2. Additional oils used to test discrimination

Data from a range of crude oils, condensates and fuel oils were used to determine if indices calculated from diamondoids bioaccumulated in the fish adipose tissues could be statistically discriminated from those of a wider range of oils. The oils were chosen to be highly challenging to the method i.e., oils closely related geographically, geochemically or geologically, to the oils used in the fish exposure trials. These oils were divided into two groups. In Group-A, the oils were analyzed by GC × GC-TOFMS in triplicate to obtain data for statistical testing. In Group-B, data were available from previous studies (Scarlett et al., 2019c; Spaak et al., 2020). Group-A: two oils from the Carnarvon and Gippsland basins, Eaglehawk (EGH) and Lakes Entrance (LKE) respectively, were available from a previous study (Grice et al., 2000). A very low sulfur fuel oil, referred to as VLSFO, compliant with the International Maritime Organization directive (IMO, 2020) containing <0.5 % sulfur from the MV *Wakashio* (LSO), and an ultra low sulfur fuel oil (USO) containing 0.1 % sulfur, (referred to as ULSFO) compliant with the more restrictive sulfur emission control areas, were available from previous studies (Nelson et al., 2022; Scarlett et al., 2021). A traditional non-IMO-2020 compliant heavy fuel oil, Bunker C (BNC) was obtained from a ship docked in Fremantle Port, Western Australia. From the NW shelf of Australia, condensates from the Crux-3 (CRX) and Calliance (CAL), plus a light crude oil from Elang West (ELW) wells, were available from a previous study (Scarlett et al., 2019c). Group-B: from the NW shelf of Australia, condensates from Dinichthys-1 (DN-1), Dinichthys North (DNN), Concerto (CCO), Scott Reef (SCR), and Ichthys-1A (IC-1) wells plus Gwydion (GWN), Focus (FCS) and Cornea (CNA) crude oils were available from a previous study (Scarlett et al., 2019c). Further details of the oils are given in Table 1.

2.3. Fractionation of oils

Condensates and light oils were analyzed as whole oils dissolved in *n*-hexane. For heavier oils (including MCO), fractionation was performed to isolate the saturated hydrocarbon fractions. Small silica columns were prepared in glass Pasteur pipettes with 0.5 g of silica gel *n*-hexane slurry. Approximately 10 mg of oil was applied to the column, and the saturated hydrocarbon fraction eluted with 3 mL of *n*-hexane was then evaporated under a gentle nitrogen stream to the required concentration.

2.4. Fish exposure and sampling

All fish were handled in accordance with Curtin University animal ethics approval ARE2019/11. The method is described by Spilsbury et al. (2021, 2023). Juvenile barramundi (10–15 cm in length) were obtained from a local commercial hatchery and 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.

Fish were fed commercial fishmeal (3 mm Nova FF, Skretting Pty Ltd., Perth, Australia) twice daily to a total of approximately 2 % body weight per day (Hellou et al., 2002). Fish were fed either plain fishmeal (negative control), fish meal spiked with 1 % *w*/w HFO or fish meal spiked with 1 % w/w MCO. Fish were exposed to the oils via diet continuously for 33 days, followed by a 2-day depuration period. Following sacrifice by ike-jime, samples of brown adipose tissue (2–5 g) adjacent to the intestine were removed and stored at −20 °C prior to analysis. Recovery trials for a range of authentic standards were performed by spiking into methanol followed by digestion, extraction and fractionation as described in Section 2.5 (Spilsbury et al., 2023).

Table 1
Condensates, crude oils and fuel oils analyzed for diamondoids by GCxGC-TOFMS.

Oil/well	Acronym	Group[a]	Type	Basin	Biodeg.	Family[b]
Heavy fuel oil	HFO	Study	Heavy fuel oil	n/a	n/a	n/a
Montara	MCO	Study	Light/medium crude oil	Bonaparte	No	I
Eaglehawk	EGH	A	Crude oil	Carnarvon	Mixed	n/a
Lakes entrance	LKE	A	Crude oil	Gippsland	Severe 7	n/a
Very low sulfur fuel oil	LSO	A	Heavy fuel oil	n/a	n/a	n/a
Ultra low sulfur fuel oil	USO	A	Heavy fuel oil	n/a	n/a	n/a
Bunker C	BNC	A	Heavy fuel oil	n/a	n/a	n/a
Crux-3	CRX	A	Condensate	Browse	No	I
Calliance	CAL	A	Condensate	Browse	No	II
Elang west	ELW	A	Light crude oil	Bonaparte	No	IV
Dinichthys north	DNN	B	Condensate	Browse	No	II
Dinichthys-1	DN-1	B	Condensate	Browse	No	III
Ichthys-1	IC-1	B	Condensate	Browse	No	III
Concerto	CCO	B	Condensate	Browse	No	III
Scott reef	SCR	B	Condensate	Browse	No	II
Focus	FCS	B	Crude oil	Browse	No	IV
Gwydion	GWN	B	Crude oil	Browse	No	IV
Cornea	CNA	B	Crude oil	Browse	No	IV

[a] Group refers to oils used for fish exposure trial ('Study', $n = 5$ analyzed), additional oils for statistical comparison ('Group A', $n = 3$) and further additional oils for visual inspection only of correlation plots (Group-B, $n = 1$).
[b] Family refers to groupings based on a dendrogram comparing Browse Basin fluid families with those of selected samples from the Bonaparte Basin (Spaak et al., 2020).

2.5. Extraction and chromatography of fish adipose tissues

The method is described in Spilsbury et al. (2021, 2023). Briefly, known quantities of frozen adipose tissue samples were thawed and digested in methanol under reflux for 2 h. Cool digests were filtered and extracted using 3 × 25 mL *n*-hexane, followed by a 25 mL *n*-hexane glassware rinse. Extracts were reduced in volume to a few mL via rotary evaporation 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 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 with 40 mL of *n*-hexane. This fraction was reduced in volume by rotary evaporation and a gentle nitrogen stream.

2.6. GC×GC-TOFMS

The *n*-hexane-soluble fractions of whole oils and condensates plus saturate fractions were analyzed using a Leco Pegasus IV system equipped with dual stage cryogenic modulator (Leco, Saint Joseph, MI, USA). The primary column was a 60 m × 0.25 mm × 0.25 μm Rxi 5Sil MS (Restek, Bellefonte, PA, USA). The secondary column was a 1.2 m × 0.25 mm × 0.15 μm BPX50 (SGE, Ringwood, Australia). The carrier gas was ultrahigh purity helium with constant flow of 1 mL min^{-1}. The inlet temperature was 310 °C with 1 μL injection. The GC conditions were: 80 °C (12.5 min isothermal), then 1.25 °C min^{-1} to 180 °C and then 20 °C min^{-1} to 320 °C, (5 min isothermal). The secondary oven temperature was offset by 5 °C and modulator temperature was offset 15 °C. The modulation period was 6 s. The mass spectrometer electron ionisation was 70 *eV*; the ion source temperature was 250 °C and the transfer line temperature 320 °C. The scan speed was 100 Hz with a range of 45–580 Da. ChromaTOF (LECO) software package was used for instrument control and data analysis. Minimum acceptable signal to noise ratio was 30:1. Coefficient of variation for triplicate analyses of the lowest concentrations of standards was <3 %. Six-point calibration curves of adamantane and diamantane standards over the range 0, 0.25, 0.5, 1.0, 5.0 and 10.0 ng on column was linear with r^2 values >0.998. Diamondoids were identified by their chromatographic elution times in two dimensions and their mass spectra were compared with reference standards and in-house TOFMS libraries spectra plus that of National Institute of Standards & Technology (NIST, Gaithersburg, MD, USA) libraries. Ions used for quantitation of peak volumes are provided in Table 2.

2.7. Calculation of diamondoid indices and statistical analyses

Diamondoids were identified by elution order and mass spectra based on authentic standards and published data (Table 2). Two-dimensional elution positions are provided in Fig. 1. Indices were

Table 2
Diamondoids identified in oils and fish adipose tissue extracts analyzed by GCxGC-TOFMS.

Peak no.	Compound	Acronym	Chemical formula	Mol. wt. (Da)	Quant. ion (m/z)
1	Adamantane	A	$C_{10}H_{16}$	136	136
2	1-Methyladamantane	1-MA	$C_{11}H_{18}$	150	135
3	2-Methyladamantane	2-MA	$C_{11}H_{18}$	150	135
4	1,3-Dimethyladamantane	1,3-DMA	$C_{12}H_{20}$	164	149
5	1,4-Dimethyladamantane (*cis*)	1,4-DMA	$C_{12}H_{20}$	164	149
6	1,4-Dimethyladamantane (*trans*)	1,4-DMA	$C_{12}H_{20}$	164	149
7	1,2-Dimethyladamantane	1,2-DMA	$C_{12}H_{20}$	164	149
8	1,3,5-Trimethyladamantane	1,3,5-TMA	$C_{14}H_{22}$	178	163
9	1,3,6-Trimethyladamantane	1,3,6-TMA	$C_{14}H_{22}$	178	163
10	1,3,4-Trimethyladamantane (*cis*)	1,3,4-TMA	$C_{14}H_{22}$	178	163
11	1,3,4-Trimethyladamantane (*trans*)	1,3,4-TMA	$C_{14}H_{22}$	178	163
12	1,2,3-Trimethyladamantane	1,2,3-TMA	$C_{14}H_{22}$	178	163
13	Ethanoadamantane	E	$C_{12}H_{18}$	162	162
14	1-Methylethanoadamantane[a]	1-ME	$C_{13}H_{20}$	176	161
15	6-Methylethanoadamantane[a]	6-ME	$C_{13}H_{20}$	176	161
16	2-Methylethanoadamantane[a]	2-ME	$C_{13}H_{20}$	176	161
17	Diamantane	D	$C_{14}H_{20}$	188	188
18	4-Methyldiamantane	4-MD	$C_{15}H_{22}$	202	187
19	1-Methyldiamantane	1-MD	$C_{15}H_{22}$	202	187
20	3-Methyldiamantane	3-MD	$C_{15}H_{22}$	202	187

[a] Tentative identification (Scarlett et al., 2019).

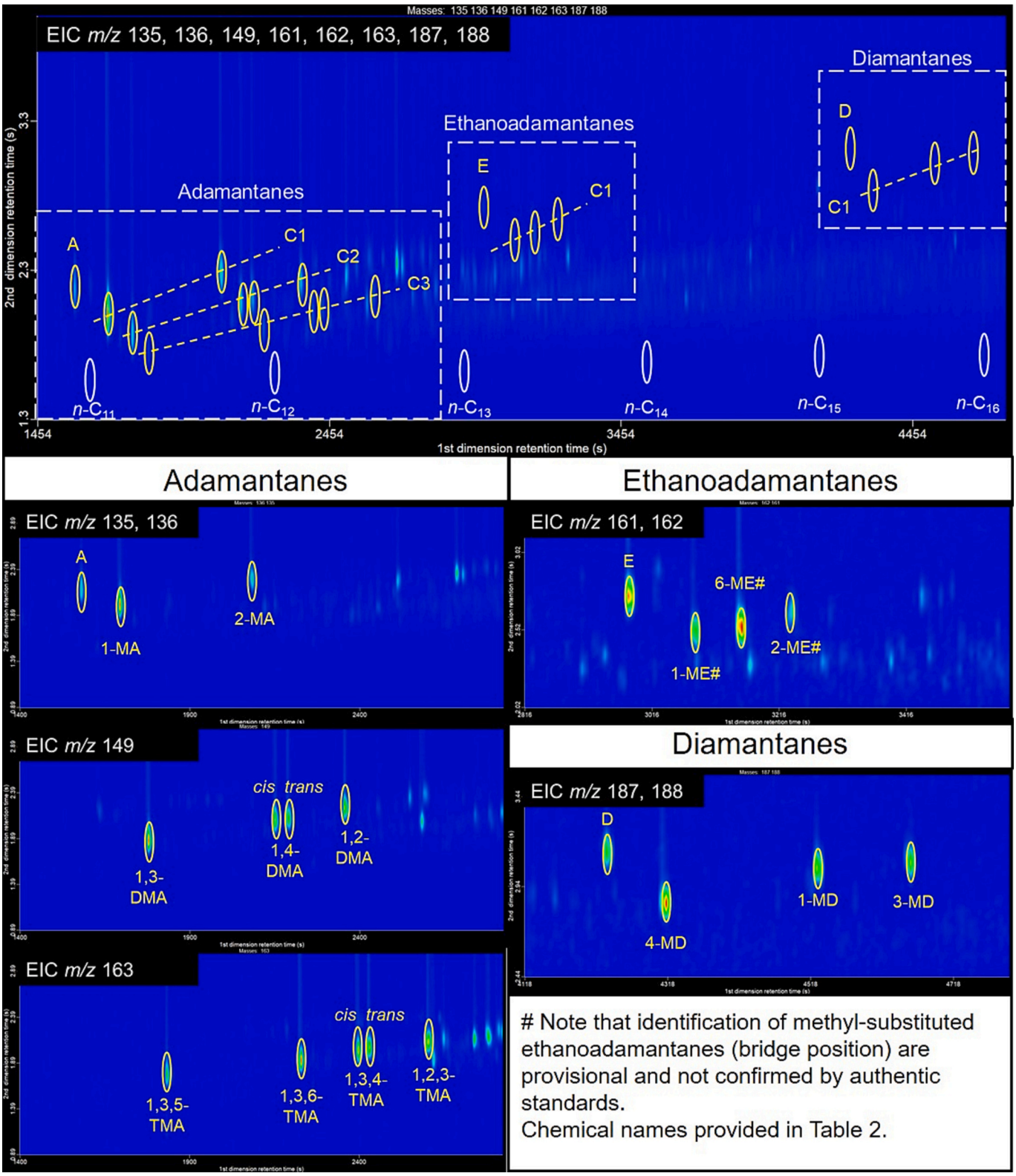

Fig. 1. GCxGC-TOFMS Extracted Ion Chromatograms (EIC) showing elution positions of adamantanes, ethanoadamantanes and diamantanes present in oils and fish adipose tissue. Top panel provides an overview with elution positions relative to n-alkanes and lower panels showing positions of individual homologues.

calculated from the relative abundances of diamondoids using peak volume comparisons of major ions (typically the base ion) for each class of diamondoid e.g., for methyl adamantanes this was *m/z* 135. Formulae for indices are provided in Table 3. The majority of the indices are ratios of for, example, peaks A and B,that take the form used by petroleum geochemists i.e. based on the formula (1):

$$DI = A/(A+B) \quad (1)$$

This formula constrains the ratios to values between 0 and 1. However, petroleum geochemists typically use Σmethyl diamantanes (MD) to

Table 3
Indices calculated from GCxGC-TOFMS analyses of diamondoids present in oils and fish adipose tissue extracts.

Index	Formulae
Methyl Adamantane Index (MAI)	1-MA/(1-MA + 2-MA)
Dimethyl Adamantane Index 1 (DMAI 1)	1,3-DMA/(1,2-DMA + 1,3-DMA)
Dimethyl Adamantane Index 2 (DMAI 2)	1,3-DMA/(1,3-DMA + 1,4-DMA)
Trimethyl Adamantane Index 1 (TMAI 1)	1,3,5-TMA/(1,3,5-TMA + 1,3,4-TMA
Trimethyl Adamantane Index 2 (TMAI 2)	1,3,5-TMA/(1,3,5-TMA + 1,3,6-TMA
Trimethyl Adamantane Index 3 (TMAI 3)	1,3,5-TMA/(1,3,5-TMA + 1,2,3-TMA)
Methyl Ethanoadamantane Index 2 (MEI-2)	6-ME/(1-ME +6-ME +2-ME)
Methyl Diamantane Index (MDI)	4-MD/(4-MD + 1-MD + 3-MD)
Methylsubstituted to parent diamantanes (MD/D)	(4-MD + 1-MD + 3-MD)/D
Alternative form of MD/D constrained to values 0–1	(4-MD + 1-MD + 3-MD)/(D + 4-MD + 1-MD + 3-MD)

diamantane (D) in its unconstrained form. Herein, for comparisons of DIs we adapted the latter ratio to comply with formula (1) by using ΣMD/(D + ΣMD) (Table 3).

As part of the 'Nord test', Daling et al. (2002) suggested that for correlations between spilled and source oils, if the 95 % Confidence Intervals (CI) of all key diagnostic ratios resistant to weathering (mostly sterane and hopane biomarkers) cross the ideal 1:1 relationship, this should be classed as a positive match. If all fall within the 98 % CI, this should be classed as a probable match, but if any key diagnostic ratio fall outside, this should be classed as a non-match. These criteria were applied to correlations between DIs derived from (i) the fish adipose tissues and that of the oil to which they were exposed, (ii) to those from the exposure study oils to which fish were not exposed, and (iii) to a range of additional oils not part of the fish exposure trials. The 95 % and 98 % confidence intervals were calculated based on Student's *t* distribution corresponding to the degrees of freedom for the number of analyses performed i.e. $N-1$. The Relative Difference ($DIFF_R$) between the DIs derived from fish extracts and oils was calculated as:

$$DIFFR = [Abs(DIfish - DIoil)]/[(DIfish + DIoil)/2] \times 100 \quad (2)$$

If the $DIFF_R$ was greater than the 95 % CI for all DIs derived from the fish extracts, the oil was considered a non-match. The data were also subject to the dimensionality reduction technique, LDA.

2.8. Linear discriminatory analysis (LDA)

Statistical analyses were conducted using R, version 4.0 R-Core Team (2021). Correlations between combined diamondoid and bicyclane ratios in adipose tissue of exposed fish and their respective exposure oils and the respective adipose tissue of exposed fish were established using the lm: Fitting Linear Models function from the R stats package.

LDA was performed using the MASS R package (W. N. Venables and Ripley, 2002). An LDA model was defined using a "training" data set consisting of nine DIs (Table 3) plus six bicyclane ratios (Spilsbury et al., 2023) in the two exposure oils, plus five other petroleum products, each analyzed in triplicate: HFO, BNC, LSO, MCO, CRX, CAL, and ELW. Exposure oil predictions from the LDA model were then obtained using a "test" data set of the same 15 diamondoid and bicyclane ratios from adipose tissue extracts from fish exposed to MCO ($n = 8$) or to HFO (n = 8).

3. Results and discussion

This study builds upon a previous investigation (Spilsbury et al., 2023) in which a limited number of ratios derived from bicyclane hydrocarbon distributions extracted from fish tissues were used to identify one of two possible dietary exposure oils, simulating a scenario which may occur during, or after, an oil spill. In the current study, our primary aim was to test if diamondoid hydrocarbons were bioaccumulated in fish and, if so, whether they could also be used to help identify the source oils, or to add certainty to the identifications based on bicyclanes alone.

3.1. Diamondoids in exposure oils and additional crude oils, condensates and fuel oils

Both MCO and HFO contained a suite of diamondoids ranging from adamantane (136 Da) to methyldiamantanes (202 Da) (Fig. 1, Table 2). In addition to these diamondoids, the tetracyclic caged alkane ethanoadamantane (162 Da) and some methylethanoadamantanes were present in both oils. The identification of the latter is tentative as no authentic standards were available for the methyl-substituted homologues (Scarlett et al., 2019c). Peak volumes were low in both oils, especially for trimethyladamantanes and methylethanoadamantanes. This provides a robust test of the method. In contrast, the condensate contained relatively large concentrations of diamondoids i.e., analysis produced orders of magnitude higher peak volumes at the corresponding analysis concentration. The diamondoids were also present in the VLSFO spilled from the MV *Wakashio*, but were absent from two other VLSFOs analyzed by Nelson et al. (2022) and so could not be included in the current investigation. An ULSFO analyzed in the latter study also contained diamondoids, so these data were included in the current study.

3.2. Diamondoids in fish tissues

The lowest molecular weight diamondoid, adamantane, was confirmed to be present (by comparison to data for an authentic standard) in tissues of fish exposed to MCO and to HFO. Similarly, the pentacyclic diamantane was also confirmed to be present in all fish. To our knowledge this is the first confirmation of diamondoids bioaccumulated in the tissues of fish, or indeed any other species. Ethanoadamantane was also confirmed to be present in the fish tissues. As this compound is probably less common in oils than some of the other caged hydrocarbons, the presence of abundant ethanoadamantane in an oil, but absence in biota, may be useful as a means of eliminating an oil as the source of a contamination event. Suites of alkylated homologues were also identified in the fish tissues. The ratios of alkylated to parent compounds, for both adamantane and ethanoadamantane, were quite variable. This could be due to differential uptake into the tissues, metabolism, or more likely, evaporative processes during extraction and clean-up of the fish tissues. Spiked recoveries of adamantane and 1,3-dimethyladamantane were 57 % and 64 % respectively, somewhat less than for diamantane (101 %) and for higher molecular weight alkanes, tridecane (85 %) and heptadecane (101 %) (Spilsbury et al., 2023). Hence, only indices based on diamondoids of identical molecular weight were used in this study e.g. ratios of methyladamantanes rather than methyladamantanes/adamantane. An exception to this was the methyldiamantane/diamantane (used in the form ΣMD/(D + ΣMD) index which had coefficient of variations of <2 %. Hence, this index was included in the study (Table 3). Of the 20 diamondoids identified in both oils and fish tissue extracts (Table 2), all but adamantane and ethanoadamantane were used to produce indices to compare fish tissue extracts with oils.

Having confirmed the presence of diamondoids in the fish tissues, the next question was: are the ratios in the oils conserved within the fish tissues? To test this, means and the 95 % CIs of DIs derived from the exposure oils, MCO and HFO, were plotted against those derived from the fish adipose tissue extracts (Fig. 2 A and B). A perfect linear correlation would be a 1:1 relationship between the indices derived from the fish tissues and the corresponding oil to which they were exposed. For both MCO-exposed fish and HFO-exposed fish, very good linear correlations were obtained ($y = 1.038x - 0.000$ $R^2 = 0.977$ and $y = 0.937x + 0.053$ $R^2 = 0.952$; Fig. 2A and B respectively). This indicates that the diamondoid ratios are very well conserved in the fish tissues and in a similar manner as that found for bicyclanes (Spilsbury et al., 2023).

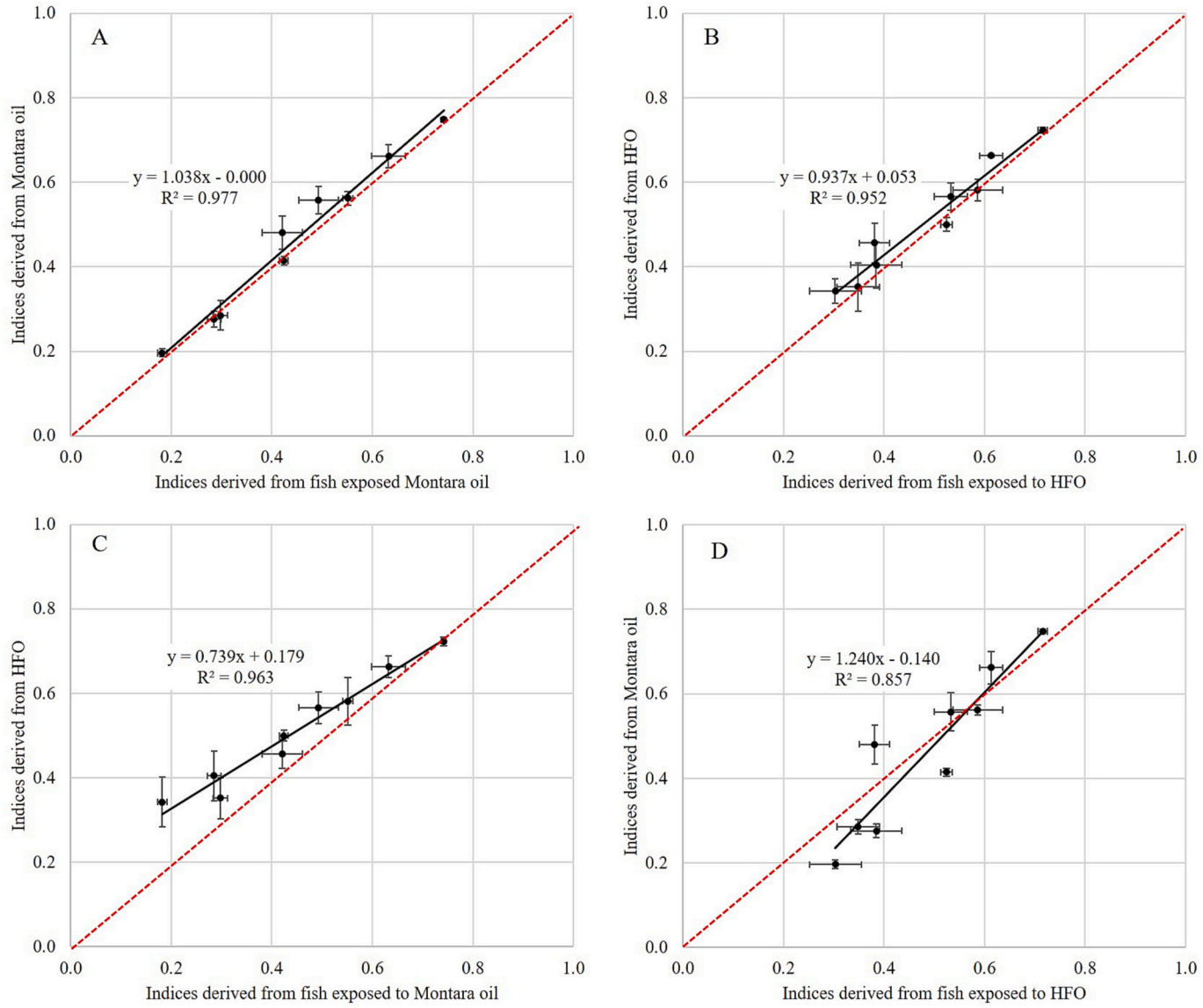

Fig. 2. Cross-plots of diamondoid indices derived from fish-adipose tissue and the oils to which the fish were exposed (A and B), and the study oils to which the fish were not exposed (C and D). Solid black lines correspond to the linear regressions of the x – y plots. Red dotted lines are the ideal 1:1 relationship. Error bars are 95 % CI.

However, based on the suggestions of Daling et al. (2002), the 95 % CI of all key diagnostic ratios should cross the 1:1 correlation line to be classed as a positive match. The majority of the individual indices plotted very close to the 1:1 line (Fig. 2 A and B) but DIs derived from the fish extracts were not classified as matching those from the oils to which the fish were exposed (Supplementary Information Tables S1 and S2). Of the six bicyclane ratios used by Spilsbury et al. (2023), the 95 % CI of one from each of oil – fish tissue correlations did not cross the 1:1 fit line: nevertheless, the bicyclane ratios proved to be excellent at identifying the oils to which the fish were exposed. Thus, the application of DIs for the identification of sources of oil contamination appears promising, although at this stage, it was unclear just which indices would be key and which might prove to be of more limited use.

3.3. *Discrimination between exposure oils and additional oils*

In order for a method to be used to identify the source of a contamination, it must not only show a good correlation between the bioaccumulated hydrocarbons and the oil to which the organism has been exposed, it must also be able to allow discrimination between the source oil and other potential candidate polluting oils. To test this, we plotted the DIs derived from the tissues of fish exposed to MCO against those from the HFO used in the parallel exposure study (Fig. 2C). Similarly, indices derived from tissues of HFO-exposed fish and MCO were plotted (Fig. 2D). The slopes of the regression lines were, as expected, now further from the ideal 1:1 and the 95 % CIs of many of the indices more distant from the 1:1 line. However, although the latter cross-plots were non-matching, in the absence of comparison with the actual oil that the fish were exposed, the correlations might appear to be potential matches. The ability to clearly differentiate between multiple possible sources is critical if DIs are to contribute to the detection of oil contamination in fish.

To further explore whether DIs have sufficient discriminating power to distinguish between alternative sources of exposure oils, indices derived from the fish tissues data were plotted against those derived from a wider range of crude oils, condensates and fuel oils (Group-A, Table 1). The cross-plots for DIs derived from MCO-exposed fish and those from condensates from the NW of Australia were further from the

ideal 1:1 line than that observed for MCO (Fig. 3 A-C). This is encouraging since a previous study (Spaak et al., 2020) showed that these condensates are closely related to MCO, and CRX even shared the same dendrogram Family grouping (Table 1). However, the cross-plot of indices derived from MCO-exposed fish and LKE crude oil was close to the 1:1 line (Fig. 3D). This crude oil is severely biodegraded (Grice et al., 2000) and perhaps could be considered a surrogate for spilled oils that have spent extended time in the ocean. Oil that spends prolonged time in the environment is subject to weathering processes, including biodegradation, and may ultimately become floating tar balls entrapped in material that is subsequently consumed by fish. In the previous study (Spilsbury et al., 2023), bicyclane ratios derived from a type of tar ball known as asphaltite were compared to the fish tissue extracts. Although some diamondoids were also present in the asphaltites (Scarlett et al., 2019b), these were insufficient to provide the necessary indices and were therefore not included in the current study. The effect of severe weathering on DIs was not part of the current study but should be taken into consideration if diamondoids are to be used to help identify sources of oil contamination. Indices derived from the additional heavy marine fuel oils tested, including two IMO-2020 compliant oils, very low and ultra low sulfur fuel oils, LSO and USO respectively, and a traditional marine fuel oil, BNC, not compliant with IMO-2020, did not closely match with those derived from fish exposed to HFO (Fig. 4).

Of the Group-B Browse basin condensates and crude oils, for which data were available from a previous study (Spaak et al., 2020) but without replication, most could be readily differentiated from the exposure oils (Fig. S1). However, DNN, DN-1 and IC-1 plotted close to the 1:1 line with MCO-exposed fish (Fig. S1). These condensates were placed by Spaak et al. (2020) in separate dendrogram Family groupings to MCO (Table 1). This highlights the potential for false positives when solely relying on DIs, especially for very closely related oils.

3.4. *Linear discriminatory analysis using combination of diamondoid and bicyclane ratios*

It was noted in our previous study (Spilsbury et al., 2023), that only six ratios were able to be calculated from the bicyclanes present in both MCO and HFO. This is potentially a limiting factor for a wider application of the method. By combining the six previously employed bicyclane ratios with the nine DIs reported herein, a total of 15 ratios was

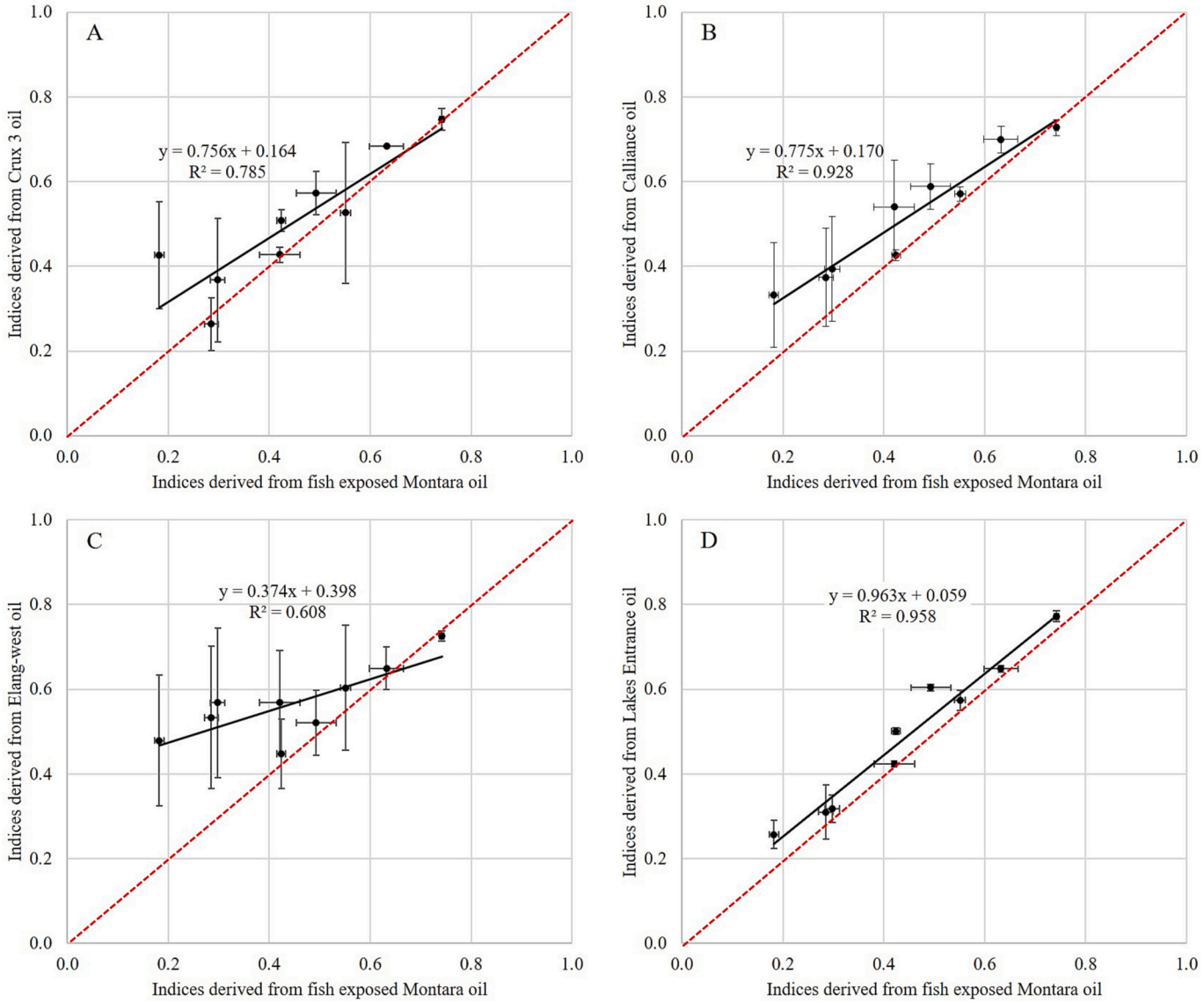

Fig. 3. Cross-plots of diamondoid indices derived from adipose tissue of fish exposed to Montara oil and Australian oils from the Browse basin (A and B), Bonaparte Basin (C), and a severely weathered oil from Gippsland basin (D). Solid black lines correspond to the linear regressions of the x – y plots. Red dotted lines are the ideal 1:1 relationship. Error bars are 95 % confidence limits.

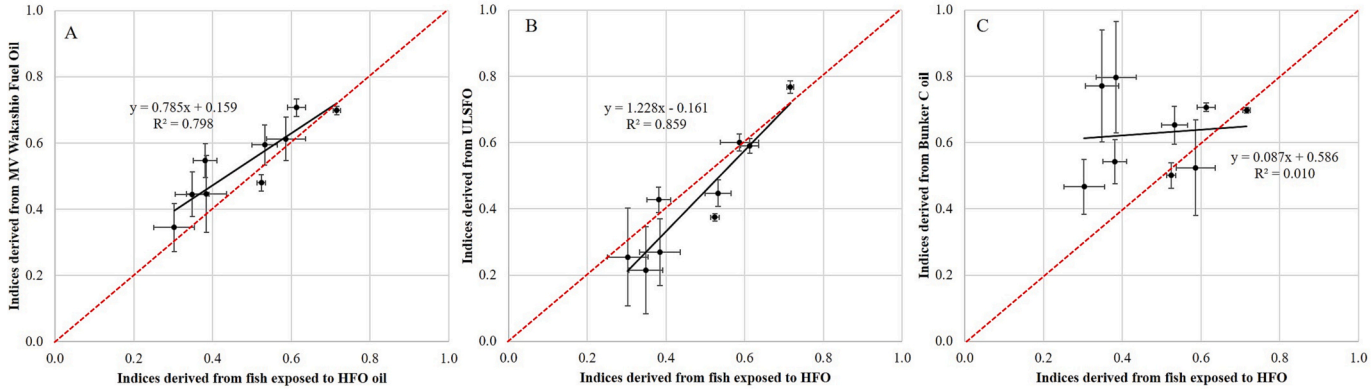

Fig. 4. Cross-plots of diamondoid indices derived from adipose tissue of fish exposed to HFO and marine heavy fuels: a Very Low Sulfur Fuel Oil (VLSFO) from the MV Wakashio (A), an Ultra Low Sulfur Fuel Oil (ULSFO) (B), and a traditional Bunker C oil (C). Solid black lines correspond to the linear regressions of the x – y plots. Red dotted lines are the ideal 1:1 relationship. Error bars are 95 % confidence limits.

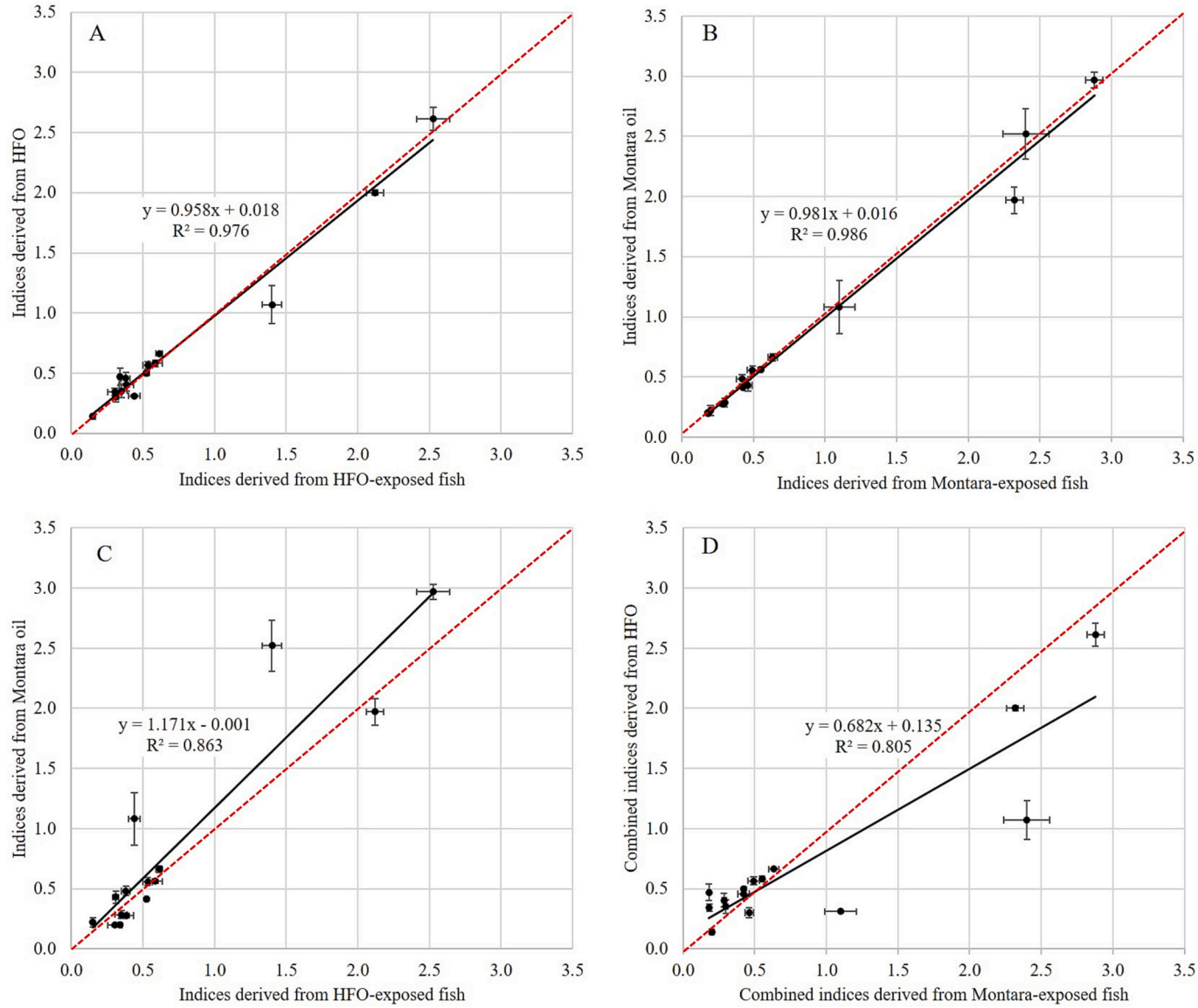

Fig. 5. Cross-plots of combined diamondoid and bicyclane ratios derived from fish-adipose tissue and the oils to which the fish were exposed (A and B), and the study oils to which the fish were not exposed (C and D). Solid black lines correspond to the linear regressions of the x – y plots. Red dotted lines are the ideal 1:1 relationship. Error bars are 95 % confidence limits.

now available. This is then analogous to what is used in typical spilled oil – source oil correlations with sterane and hopane biomarkers (Daling et al., 2002). Cross-plots of 15 combined diamondoid and bicyclane ratios derived from extracts of fish adipose tissues and the oils to which the fish were exposed produced relationships close to the ideal 1:1 (Fig. 5 A and B) whereas those for the study oils to which fish were not exposed showed weaker correlations (Fig. 5C and D). Multivariate LDA of bicyclane and diamondoid profiles was applied to identify the specific oil to which fish had been exposed from seven other candidate oils.

The LDA model, established using a training dataset of a total of 15 bicyclane and DIs from a suite of seven petroleum products common to both those analyzed by Spilsbury et al. (2023) and the current study, produced an ordination space in which the seven petroleum products were all separated (Fig. 6). The three fuel oils HFO, BNC, and LSO were discriminated on the LD1 and LD2 cartesian axes, as were MCO and the two Browse Basin condensates, CRX and CAL, and the Bonaparte basin ELW (Fig. 6). The fish adipose tissue extracts were within the 95 % posterior probability categorization boundaries for the respective oils to which the fish were exposed, although one of the HFO-exposed fish was very close to the border with CRX (Fig. 6). All of the MCO-exposed fish were well-separated from CRX oil, even though the oils originated from the same reservoir (Spaak et al., 2020). As with the LDA predictions for the bicyclane data alone (Spilsbury et al., 2023), the adipose tissue extracts from fish exposed to either MCO or HFO correctly identified the respective oil to which each fish was exposed. Hence, a combination of diamondoid and bicyclane ratios provides a broader range for assessing the source of contamination without diluting the predicting power of the LDA.

3.5. Limitations of the study and future research

The current study has addressed some of limitations of relying solely on bicyclane ratios for fingerprinting, as outlined by Spilsbury et al. (2023). Further studies regarding the exposure period required for the fish to bioaccumulate sufficient bicyclanes and diamondoids, and the period of depuration before such compounds may be lost, still require attention before the method can be entirely relied upon. However, oil spill responders may wish to test the method outlined herein, alongside the established methods for comparing spilled oil and potential source oils.

4. Conclusions

Diamondoid hydrocarbons are common in crude oils and can be particularly abundant in condensates that often lack the hopanoid and steroid biomarkers traditionally used for oil fingerprinting. Although diamondoids can be observed using GC–MS, the higher chromatographic resolution of GC × GC-TOFMS, especially where there are many co-eluting peaks, enables the alkylated homologues to be more readily identified with less interferences. Using GC × GC-TOFMS, 20 diamondoids were identified in fish exposed to MCO and HFO, and these were also present in sufficient abundance in the oils to be deemed suitable for the calculation of ratios. As DIs are commonly used in the field of organic geochemistry, these were employed to compare fish tissue extracts with the oils to which fish were exposed and a wider range of crude oils, condensates and fuel oils. Cross-plots revealed that DIs derived from fish tissues were strongly correlated (very close to the ideal 1:1 relationship) with the oils to which fish were exposed. However these failed the strict criteria for matching based on 95 % CI which was recommended by Daling et al. (2002) for spilled oil – source oil correlations using hopanoid and steroid biomarkers. Cross-plots between DIs derived from fish tissues and different oils showed somewhat weaker relationships, suggesting that the DIs could be used to differentiate between oils. However, a cross-plot of DIs derived from MCO-exposed fish and those from a severely biodegraded oil showed a strong correlation. Similarly, some oils from closely related oil systems produced cross-plots close to 1:1. Consequently, caution should be exercised when using DIs alone, especially when candidate spilled oils are from the same oil basin.

Combining the diamondoids with bicyclanes produced a much larger suite of 15 ratios from which forensic oil fingerprints could be derived. Applying the multivariate statistical analysis LDA to this combined suite of 15 ratios enabled all seven oils tested to be discriminated from each other, and for the exposure oils to be correctly identified from the biomarker ratios bioaccumulated in the fish adipose tissues. This suggests that a combination of ratios derived from both diamondoids and

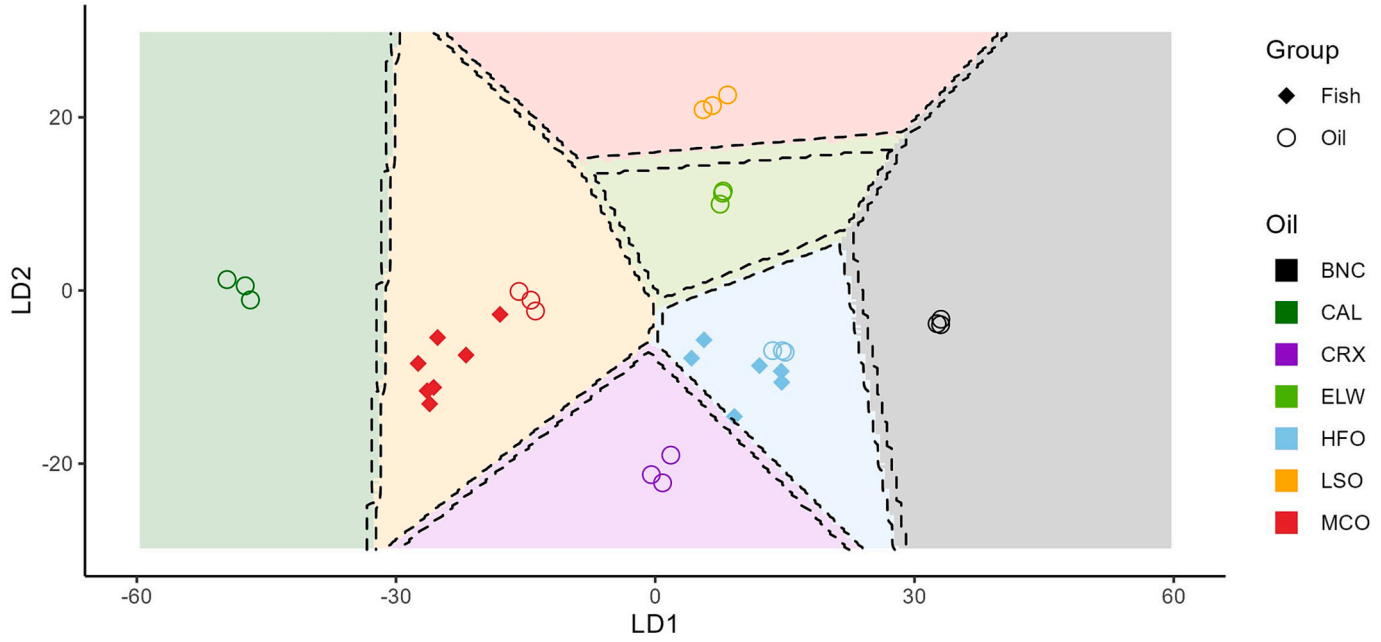

Fig. 6. Linear discriminatory analysis of crude oils, heavy fuel oils, condensates and oils from the Browse and Bonaparte basins of Australia, and adipose tissue extracts of fish exposed to Montara crude oil and heavy fuel oil. Shaded areas are the decision boundaries for the respective oils, with dotted lines indicating the 95 % posterior probability demarcation. Acronyms provided in Table 1.

bicyclanes could be employed to help identify the sources of oil contamination in fish following an oil spill.

CRediT authorship contribution statement

Alan G. Scarlett: Conceptualization, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Francis D. Spilsbury:** Investigation, Methodology, Software, Visualization, Writing – review & editing. **Steven J. Rowland:** Conceptualization, Writing – review & editing. **Marthe Monique Gagnon:** Conceptualization, Writing – review & editing, Funding acquisition, Resources. **Kliti Grice:** Funding acquisition, Resources, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supporting information consists of cross-plots of DIs derived from fish tissues and a range of Browse basin oils and condensates (Group B). A table of DIs for individual fish and tables comparing study oil DIs with those derived from fish lipid extracts. R markdown for LDA of fish lipid extracts, exposure oils and additional oils and condensates. Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2023.115836.

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