MARINHA DO BRASIL
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PROGRAMA ASSOCIADO DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA MARINHA

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AVALIAÇÃO DA CONTAMINAÇÃO DE OSTRAS POR ÓLEO DIESEL ATRAVÉS DE ANÁLISES IN SITU E IN VITRO UTILIZANDO HIDROCARBONETOS POLICÍCLICOS AROMÁTICOS E ISÓTOPOS ESTÁVEIS COMO MARCADORES

Arraial do Cabo

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia Marinha do Instituto de Estudos do Mar Almirante Paulo Moreira (IEAPM)/Universidade Federal Fluminense (UFF) como requisito parcial para obtenção do título de Doutor em Biotecnologia Marinha. Linha de Pesquisa: Biotecnologia Ambiental.

Orientador: Profa. Dra. Louisi Souza de Oliveira

Coorientadores: Profa. Dra. Cássia de Oliveira Farias

Prof. Dr. Marcelo Costa Muniz

Prof. Dr. Roberto Meigikos dos Anjos

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Eu tenho um barco, de luz e madeira,
e a Lua cheia, para me guiar
Eu tenho o mapa das ilhas do mundo
e estrelas todas, para navegar
Não tenho pressa, nem frio nem calor,
tenho o sol nascente, para me levar
Eu tenho a brisa que vem lá do mar,
e a vida inteira, para navegar

Fernando Corona / Leo Henkin (adaptação)

ARTIGOS CIENTÍFICOS PRODUZIDOS DURANTE A TESE DE DOUTORADO

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Artigos em construção

1 - Polycyclic aromatic hydrocarbons and stable isotopes analysis of oysters *Crassostrea brasiliana* following exposure to diesel fuel oil-water dispersion (Capítulo 3).

- Neste experimento foram realizadas coletas de ostras e análises de expressão gênica, enzimas antioxidantes e avaliação histológica por grupos de trabalho parceiros. Estes dados e análises serão incluídos no artigo a ser submetido. Previsão de submissão: dezembro de 2024.

2 - PAHs accumulation analysis of oyster *Crassostrea brasiliana* following exposure to different concentrations of diesel fuel oil-water dispersion (Capítulo 4).

- Neste experimento foram realizadas coletas de ostras e análises de expressão gênica e microbiológicas por grupos de trabalho parceiros. Estes dados e análises serão incluídos no artigo a ser submetido. Previsão de submissão: dezembro de 2024.

RESUMO

O petróleo é importante fonte energética e base para a produção de inúmeros itens utilizados pela sociedade humana. Ele representa risco ambiental constante, gerando prejuízos às comunidades biológicas marinhas. Como é um composto tóxico com características físicas e químicas peculiares, podem ocasionar efeitos letais, subletais e alterações bioquímicas nos organismos marinhos. O estudo destas alterações, através de bioindicadores e biomarcadores, pode trazer informações valiosas para a avaliação das consequências da introdução do óleo no ambiente. Ostras são ótimos bioindicadores, uma vez que são filtradores sésseis. O presente estudo tem como objetivo utilizar técnicas de quantificação de hidrocarbonetos policíclicos aromáticos e isótopos estáveis (^{13}C e ^{15}N) em tecidos de ostras para investigar os impactos do óleo sobre estes organismos. Procurando atingir as metas estabelecidas, foram realizadas amostragens em campo, além de bioensaios em laboratório. Em um primeiro momento (capítulo 1), foram coletadas ostras em dois pontos de Arraial do Cabo com impactos antrópicos diferenciados. As assinaturas isotópicas de ^{13}C e ^{15}N nos tecidos de ostras não apresentaram diferenças significativas entre os pontos de amostragem. Por outro lado, o seston apresentou diferenças, indicando um maior aporte terrígeno na estação Praia do Forno, não sendo possível identificar influência de fontes petrogênicas. Estes resultados reforçaram a necessidade da realização de bioensaios para testar os efeitos diretos do óleo sobre estes bivalves. Visando o desenvolvimento destes bioensaios, no capítulo 2 foi efetuado então teste de comparação entre duas técnicas de dispersão de óleo em água: “oil-in-water dispersion” (OWD) e “water accomodated fraction” (WAF). Neste teste, as duas técnicas se mostraram eficientes quanto à dispersão do óleo, com uma maior concentração de HPAs atingida pela técnica WAF e maior homogeneidade das réplicas produzidas com OWD. Com isso foi escolhida a OWD para os bioensaios, pois, além de ter se mostrado eficiente, possui logística mais simples. No capítulo 3 foi efetuado experimento submetendo a ostra *Crassostrea brasiliana* à exposição a óleo diesel disperso em água através da técnica OWD. Foram quantificados então HPAs na água e nos tecidos totais das ostras, além de isótopos estáveis $\delta^{13}C$ e $\delta^{15}N$, percentual de nitrogênio e carbono em brânquias, hepatopâncreas e músculos em um estudo de sete dias. Como resultados principais, foi possível verificar que a concentração de HPAs na água caiu rapidamente, havendo bioacumulação nas ostras nas primeiras 48h. Após sete dias as concentrações nos tecidos também reduziram, demonstrando que houve depuração quanto aos HPAs testados. Estes registros confirmaram o óleo diesel como um óleo extremamente volátil e a capacidade de *Crassostrea brasiliana* como

bioindicador. Quanto aos isótopos estáveis, não houve diferenças significativas entre amostras controle e tratamento, o que indica que a taxa de reciclagem lenta e a rápida redução dos contaminantes na água impossibilitaram uma alteração isotópica detectável. Por fim, no capítulo 4, foi realizada nova exposição das ostras a OWDs produzidos com 5 concentrações de óleo diesel (1:5, 1:10, 1:100, 1:1.000 e 1:10.000), com o objetivo de avaliar a bioacumulação e mortalidade das ostras nestes diferentes contextos. Em geral os resultados demonstraram uma baixíssima mortalidade em todas os casos, não diferenciando o controle. A bioacumulação registrada não foi muito diferente entre as 3 maiores concentrações, o que provavelmente ocorreu devido à rápida redução dos HPAs mais abundantes (os mais leves, especialmente o naftaleno). Com isso a espécie *Crassostrea brasiliana* se mostrou mais uma vez um bom bioindicador, não só pelas características inerentes a filtrador semi-séssil, mas também pela sua resiliência e capacidade de bioacumulação.

Palavras-chave: Avaliação ambiental; Bioacumulação; Bioensaio; Bioindicador; *Crassostrea*; Derramamento de óleo; Monitoramento; Poluição marinha; Toxicidade.

ABSTRACT

Oil is a crucial energy source and serves as the foundation for the production of countless items utilized by human society. However, it poses a persistent environmental risk, inflicting harm on marine biological communities. As a toxic compound with unique physical and chemical properties, oil can induce both lethal and sublethal effects, as well as biochemical changes in marine organisms. Investigating these changes through bioindicators and biomarkers can yield valuable insights for assessing the consequences of oil introduction into the environment. Oysters are excellent bioindicators due to their filter-feeding behavior and sessile nature. The present study aims to employ techniques for quantifying polycyclic aromatic hydrocarbons (PAHs) and stable isotopes (^{13}C and ^{15}N) in oyster tissues to investigate the impacts of oil on these organisms. To achieve the established objectives, field sampling was conducted alongside laboratory bioassays. In the first stage (Chapter 1), oysters were collected from two locations in Arraial do Cabo, each exhibiting different levels of anthropogenic impact. The stable isotopes ^{13}C and ^{15}N in their tissues were analyzed. The isotopic signatures in the oyster tissues did not reveal significant differences between the sampling sites. Conversely, the seston displayed variations, indicating a greater terrigenous contribution at the Praia do Forno station, although the influence of petrogenic sources could not be identified. These findings underscored the necessity of conducting bioassays to evaluate the direct effects of oil on these bivalves. Aiming at developing these bioassays, Chapter 2 presents a comparative test between two oil-in-water dispersion techniques: “oil-in-water dispersion” (OWD) and “water accommodated fraction” (WAF). This test demonstrated that both techniques were effective in terms of oil dispersion, with the WAF technique achieving a higher concentration of PAHs, while the OWD method produced greater homogeneity among the replicates. Consequently, OWD was selected for the bioassays due to its efficiency and simpler logistical requirements. In Chapter 3, an experiment was conducted exposing the oyster *Crassostrea brasiliana* to diesel oil dispersed in water using the OWD technique. PAHs were quantified in both the water and the total tissues of the oysters, along with stable isotopes $\delta^{13}C$ and $\delta^{15}N$, and the nitrogen and carbon percentages in the gills, hepatopancreas, and muscles over a seven-day period. The primary results indicated that the concentration of PAHs in the water decreased rapidly, with bioaccumulation observed in the oysters within the first 48 hours. After seven days, the concentrations in the tissues also diminished, demonstrating the depuration of the tested PAHs. These findings confirmed diesel oil as an extremely volatile substance and highlighted the capacity of *Crassostrea brasiliana* as a bioindicator. Regarding the stable isotopes, no significant differences were observed between control and treatment

samples, suggesting that the slow recycling rate and rapid reduction of contaminants in the water hindered the detection of any isotopic changes. In Chapter 4, oysters were exposed to oil-water dispersions (OWDs) created with five concentrations of diesel oil (1:5, 1:10, 1:100, 1:1.000, and 1:10.000) to evaluate bioaccumulation and mortality rates in these varying conditions. Overall, the results indicated very low mortality across all treatments, with no difference observed between the experimental groups and the control. The bioaccumulation levels recorded were similar among the three highest concentrations, likely due to the rapid degradation of the most abundant polycyclic aromatic hydrocarbons (PAHs), particularly the lighter compounds such as naphthalene. Consequently, the species *Crassostrea brasiliana* demonstrated its effectiveness as a bioindicator, attributable not only to its inherent characteristics as a semi-sessile filter feeder but also to its resilience and capacity for bioaccumulation.

Keywords: Environmental assessment; Bioaccumulation; Bioassay; Bioindicator; *Crassostrea*; Oil spill; Monitoring; Marine pollution; Toxicity.

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HPA	**Hidrocarbonetos Policíclicos Aromáticos**
µl	**Microlitro**
^{13}C	**Carbono 13**
^{15}N	**Carbono 15**
ANOVA	**Análise de Variância**
CG	**Cromatografia Gasosa**
cm	**Centímetro**
CP	**Cais da Praia dos Anjos**
EA-IRMS	**Analisador Elementar de Razão Isotópica**
EM	**Espectrômetro de Massas**
g	**Grama**
GF/F	**Filtro de Microfibra de Vidro**
HPLC	**High Performance Liquid Chromatography**
IEAPM	**Instituto de Estudos do Mar Almirante Paulo Moreira**
L	**Litro**
m	**Metro**
mbar	**Milibar**
min	**Minuto**
mL	**Microlitro**
mm	**Milímetro**
n	**Número**
ng	**Nanograma**
ºC	**Graus Celsious**
OWD	**Oil in Water Dispersion**
p	**Probabilidade de Significância**
PAH	**Policiclic Aromatic Hydrocarbons**
PF	**Praia do Forno**
TC	**Carbono Total**
TN	**Nitrogênio Total**
UERJ	**Universidade do Estado do Rio de Janeiro**
UFF	**Universidade Federal Fluminense**
WAF	**Water Associated Fraction**

1 CAPÍTULO 1 - CONTEXTUALIZAÇÃO CONCEITUAL

1.1 Introdução Geral

1.1.1 Importância social e riscos associados ao petróleo

O petróleo é uma das fontes energéticas mais importantes para a sociedade (IEA, 2021a). Em 2020, o consumo mundial deste produto foi de 14,378 milhões de m^3 por dia (IEA, 2021b). Sua extração, armazenamento e transporte representam risco potencial de poluição no mar.

Historicamente, o rompimento de dutos, acidentes em navios petroleiros e plataformas, assim como os resíduos de óleo de pequenas embarcações, impacta o ambiente marinho (Peterson et al, 2003, Christensen et al, 2010, Eichler, 2014, Rabalais & Turner, 2016, Chen *et al.*, 2019, Magris & Giarrizzo, 2020). Em março de 1989, por exemplo, o rompimento do casco do navio Exxon Valdez foi responsável pelo despejo de 42.000 m^3 de óleo pesado no Alasca. A falta de equipamentos de resposta e a proximidade da linha de costa agravaram a situação que culminou em um grande desastre ambiental, contaminando 1990 km de costões rochosos e praias e matando milhares de animais marinhos (Peterson et al, 2003). Posteriormente, em abril de 2010, a perda de controle sobre um poço submarino e subsequente explosão de uma plataforma levou ao derramamento de aproximadamente 790.000 m^3 no Golfo do México, o maior volume da história, constituindo um marco para a comunidade internacional (Rabalais & Turner, 2016). No Brasil, em janeiro de 2000, o rompimento de um duto da Petrobras na baía de Guanabara contaminou 40 km da costa, incluindo ambientes sensíveis, como manguezais e praias arenosas abrigadas (Eichler, 2014). Em 2019, um outro grande derramamento ocorreu em território brasileiro, atingindo aproximadamente 3000 km da costa e contaminando centenas de praias, estuários, recifes e manguezais (Escobar, 2019; Soares et al, 2020; Soares et al, 2021). Aproximadamente 400 localidades distribuídas do sudeste ao norte do Brasil foram expostas a centenas de toneladas de resíduos de óleo cru e distúrbios físicos e químicos associados (Magris & Giarrizzo, 2020).

Os impactos ambientais decorrentes da presença de óleo no mar provocam alterações nas estruturas das comunidades biológicas (Peterson *et al.*, 2003; Fukuyama *et al.*, 2014; Gusmão *et al.*, 2021), podendo ainda ocasionar riscos à saúde humana (D'Andrea & Reddy, 2014; Pena *et al.*, 2020).

1.1.2 Impacto por óleo nos organismos marinhos e o potencial dos bioindicadores

Diversos estudos demonstram que as características físicas e a composição química do petróleo ocasionam uma série de efeitos deletérios aos organismos marinhos (Luchmann *et al.*, 2011; Nwipie *et al.*, 2019; Gan *et al.*, 2021; Ferreira *et al.*, 2023). Quando em contato com animais, a alta viscosidade acarreta aderência à superfície de suas estruturas corporais, podendo levar à perda de mobilidade e bloqueio de órgãos responsáveis por funções como visão e respiração (IPIECA, 2015). Múltiplas substâncias presentes em sua composição apresentam toxicidade elevada levando a efeitos letais e subletais (IPIECA, *Op. Cit.*). Em organismos bentônicos, os efeitos letais são rapidamente observados, e facilmente quantificáveis (Lira et al, 2021; Craveiro et al, 2021). Após a morte, os tecidos moles são extraídos pela ação das ondas, alimentação de outros invertebrados e peixes, e biodegradação restando apenas as carapaças ou o substrato vazio.

Os efeitos subletais, por outro lado, não são tão aparentes. Para a identificação destes efeitos é necessário selecionar indicadores fisiológicos e bioquímicos ligados a funções afetadas pela poluição. Um exemplo destes indicadores são enzimas específicas que são sintetizadas quando o organismo está sob estresse oxidativo (Pampanin et al, 2005). Estas enzimas têm sido utilizadas em alguns estudos para a detecção de impacto por óleo a partir da quantificação da expressão dos genes codificantes ou do nível de atividade enzimática (Sureda et al, 2011; Sardi et al, 2017; Zamora-briseño *et al.*, 2021). Isótopos estáveis, como o ^{13}C, têm grande potencial como marcadores do contato dos organismos com o óleo, uma vez que se apresentam em quantidades específicas em fontes petrogênicas (Graham et. al., 2010). A bioacumulação de compostos provenientes do petróleo nos tecidos pode ser medida diretamente, por exemplo, através da extração e identificação de hidrocarbonetos policíclicos aromáticos (HPAs), o que é bastante útil quando se procura uma identificação direta da contaminação (Wang *et al.*, 2020; Gan *et al.*, 2021; Soliman *et al.*, 2022).

A pesquisa de impactos ambientais depende da seleção de organismos sensíveis aos poluentes a serem avaliados. Moluscos bivalves são filtradores com mobilidade reduzida ou inexistente, o que facilita a localização e o manejo das populações e proporciona a submissão integral às condições ambientais dos locais onde vivem, possibilitando sua utilização como indicadores de qualidade do ambiente (Fiori *et al.*, 2018; Phan *et al.*, 2020). Eles constituem uma importante fonte de proteína animal para o ser humano, em especial para as comunidades costeiras, sendo explorados

através da extração direta ou através de cultivos (Wijsman *et al.*, 2019). Dentre os bivalves, as ostras assumem papel especial, sendo abundantes em áreas costeiras e possuindo habilidade de bioconcentrar poluentes (Li *et al.*, 2019; Phan *et al.*, 2020; Soliman *et al.*, 2022). Em especial, as ostras são excelentes biomonitores de absorção de HPAs devido ao seu alto conteúdo lipídico (Gan et. al., 2021). Diversos pesquisadores utilizaram estes organismos com o objetivo de estudar a bioacumulação destes compostos orgânicos (Bustamante *et al.*, 2012; Aguirre-Rubí et. al., 2018; Soliman *et al.*, 2022). Oros et. al. (2005) realizaram estudo comparativo e detectaram que *Crassostrea gigas* bioacumulou quantidades de HPAs superiores a outros dois bivalves (mexilhão *Mytilus californianus* e ameijôa *Corbicula fluminea*). A espécie *Crassostrea brasiliana* é nativa da costa brasileira, com ampla distribuição geográfica, que se estende do estado do Maranhão a Santa Catarina (Amaral & Simone, 2014). Seu cultivo já é bem estabelecido no país, o que facilita a aquisição para a realização de experimentos (Souza *et al.*, 2022). Neste contexto favorável, a espécie tem sido utilizada frequentemente para o monitoramento de poluição com base em marcadores biológicos e químicos (Zacchi *et al.*, 2018; Nobre *et al.*, 2020; Campos *et al.*, 2022).

1.1.3 O impacto por óleo diesel no ambiente e suas características principais

O óleo diesel é um óleo considerado leve devido à sua composição ser predominantemente de hidrocarbonetos com baixa massa molecular, como o naftaleno (Patel *et al.*, 2021). Esta característica dá a este óleo propriedades singulares, que devem ser levadas em consideração quando estudamos seu impacto no meio ambiente marinho. Os compostos mais leves tendem a ser mais solúveis e mais voláteis (Patel *et al.*, 2021). Com isso eles são rapidamente disponibilizados no ambiente após um derramamento, ocasionando toxicidade aguda, diferentemente dos óleos mais pesados, que em um primeiro momento causam grande impacto físico (Hettithanthri *et al.*, 2024). Nesse cenário, a evaporação e dissolução espalha os compostos rapidamente no ambiente (Hettithanthri *et al.*, 2024).

1.1.4 Hidrocarbonetos policíclicos aromáticos (HPAs) e os bioindicadores

Hidrocarbonetos policíclicos aromáticos (HPAs) são compostos orgânicos que possuem em sua estrutura química pelo menos dois anéis benzênicos. Estão presentes no petróleo, possuem alta persistência no ambiente, e alta carcinogenicidade e toxicidade (Lawal, 2017). A absorção destes compostos pela biota marinha pode ocasionar efeitos deletérios, assim como para a saúde das pessoas devido ao consumo

de frutos do mar (Pinho et. al, 2022). Neste contexto, a análise de HPAs em animais marinhos, em especial animais que componham a dieta de comunidades costeiras, assume especial importância. A Agência de Proteção Ambiental dos Estados Unidos (U.S. EPA) definiu 16 HPAs como prioritários (Zelinkova e Wenzl, 2015) devido à sua alta toxicidade (tabela 1). A partir deste momento, estes 16 HPAs têm sido utilizados como referência para estudos ambientais, na avaliação direta da contaminação em frutos do mar (Pinho *et al.*, 2022) e como parâmetro de correlação com biomarcadores (Sardi et al, 2017; Knapik *et al.*, 2020). Os HPAs podem ser classificados de acordo com seu peso molecular, que aumenta de acordo com a quantidade de anéis benzênicos (tabela 1). Compostos com menor peso molecular como o naftaleno, tendem a ser mais solúveis o que favorece uma contaminação aguda, porém são relativamente menos tóxicos (Gan *et al.*, 2021; Othman, 2023). A volatilidade é outra propriedade que depende do peso molecular, sendo maior em HPAs com pesos menores (Gbeddy, *et al.*, 2022).

Tabela 1 - 16 HPAs prioritários (U.S. EPA).

HPAs	Número de Anéis Benzênicos	Faixa de peso molecular
Naftaleno	2	Baixo
Acenaftileno	2	Baixo
Acenafteno	2	Baixo
Fluoreno	2	Baixo
Fenantreno	3	Baixo
Antraceno	3	Baixo
Fluoranteno	3	Baixo
Pireno	4	Médio
Benzo(a)antranceno	4	Médio
Criseno	4	Médio
Benzo(b)fluoranteno	4	Médio
Benzo(k)fluoranteno	4	Médio
Benzo(a)pireno	5	Alto
Indeno(1,2,3-cd)pireno	5	Alto
Dibenzo(a,h)antraceno	5	Alto
Benzo(g,h,i)perileno	6	Alto

Fonte: Produzida pelo autor; Referências: Zelinkova e Wenzl, 2015; Othman, 2023.

1.1.5 Isótopos estáveis e os bioindicadores

Os isótopos estáveis são aqueles cujos núcleos permanecem com características únicas e imutáveis. Já os instáveis são radioativos, ou seja, seus núcleos perdem matéria e energia, sofrendo decaimento ao longo do tempo e transformando-se em outra espécie atômica (Michener & Lajtha 2008; De Barros Ferraz et al., 2009). Com isto o estudo dos isótopos estáveis é baseado na medição das suas massas, que permite a identificação de cada um e posterior quantificação da sua abundância em determinada amostra. Por convenção, a composição isotópica de determinado elemento é dada pela relação do isótopo mais raro com o mais abundante, o que geralmente significa a razão entre o mais pesado e o mais leve (De Barros Ferraz et al., 2009). Com isso, por exemplo, a composição relativa ao ^{13}C é dada pela razão entre a abundância deste isótopo e a de ^{12}C. Para a padronização da quantificação dos isótopos, foram estabelecidos padrões reconhecidos internacionalmente. Então uma razão isotópica é expressa sempre relativamente a estes padrões, dando origem então à notação δ conforme fórmula a seguir:

$$\delta = \frac{\text{Razão isotópica da Amostra}}{\text{Razão isotópica Padrão}} - 1$$

Esta relação é então multiplicada por 1000, o que gera a notação δ expressa em ‰ (partes por mil). Como exemplo, o padrão utilizado para o ^{13}C é a composição isotópica em um fóssil calcáreo chamado Pee Dee Belemnite (PDB), com razão isotópica de 0,0112372, que multiplicada por mil, é apresentada como 11,2372 ‰ (De Barros Ferraz et al., 2009).

Uma molécula possui as mesmas características funcionais independentemente de suas razões isotópicas (HOEF, 1997) Por outro lado, as características físico-químicas específicas de cada isótopo são responsáveis pelo fracionamento isotópico ao longo dos processos químicos e biológicos (De Barros Ferraz et al., 2009). Com isso, o estudo das diferenças quantitativas é muito útil para a investigação dos processos que deram origem à assinatura isotópica de determinada amostra (De Barros Ferraz et al., 2009). A aplicação dos isótopos estáveis para estudos ambientais reside no fato de que a razão encontrada nos diferentes compostos orgânicos se relaciona às fontes que originaram estes compostos (De Barros Ferraz et al., 2009). Quando há uma reação química, o produto resultante possui relação isotópica com as substâncias de origem, assim como os tecidos de um animal possuem relação isotópica com os alimentos que ele consome (De Barros Ferraz et al., 2009). Esta relação é baseada no princípio do fracionamento isotópico, que pode ser termodinâmico ou cinético (Michener & Lajtha

2008). O fracionamento termodinâmico está relacionado a reações químicas em equilíbrio, como a evaporação por exemplo. Neste caso, isótopos mais leves, como o ^{12}C, tendem a passar para a fase gasosa com maior facilidade, enriquecendo a fase líquida, onde a quantidade e ^{13}C aumenta (Michener & Lajtha 2008). O equilíbrio termodinâmico, e consequentemente as razões isotópicas, são dependentes da temperatura do sistema. Já o fracionamento cinético é relacionado com reações químicas unidirecionais, ou seja, que não estão em equilíbrio (Michener & Lajtha 2008). Um exemplo são as reações enzimáticas que ocorrem durante a assimilação do nitrogênio. Estas reações eliminam de forma preferencial o isótopo mais leve (^{14}N), o que acaba por enriquecer os valores de $\delta^{15}N$ de um organismo em relação às suas fontes alimentares em aproximadamente 3‰ (Fry, 2006).

A determinação do $\delta^{13}C$ vem sendo utilizada na pesquisa ambiental com o objetivo de identificar as fontes de carbono, como terrígenas ou marinhas, poluentes petrogênicos, entre outros (Wang *et al.*, 2020; Vezzone, 2020; Srinivas *et al.*, 2022). Desta forma, estes isótopos podem ser úteis na busca por marcadores que demonstrem os efeitos da contaminação nos organismos marinhos (WILSON et al, 2016; LIU et al, 2019).

Já a quantificação do $\delta^{15}N$ tem sido utilizada de forma bastante ampla em estudos a respeito da cadeia trófica (Layman *et al.*, 2012). Neste contexto, a distância entre o valor de $\delta^{15}N$ detectado entre dois organismos possibilita a inferência sobre suas posições hierárquicas na teia trófica (DeNiro, 1978; Vander Zanden & Rasmussen, 2001; Post, 2002). Esta característica pode ser útil em outros tipos de análise. A reciclagem (“turnover”) dos isótopos em diferentes tecidos não ocorre de forma similar, podendo ser mais ou menos acelerada (Deudero *et al.*, 2009; Bearham *et al.*, 2023). Com isso o valor de $\delta^{15}N$ pode ajudar a indicar mudanças na dieta devido a alterações ambientais, se a avaliação for efetuada em variados tecidos (Bearham *et al.*, 2023). Alguns estudos relacionam o isótopo estável ^{15}N com a poluição orgânica por esgoto, encontrando alterações significativas ligadas a este tipo de poluição (Rogers, 2003; Orlandi *et al.*, 2014). Com isso a utilidade do ^{15}N para estudos de impacto perpassa tanto o entendimento das características tróficas do organismo, o que ajuda a entender a sua dieta e origem dos alimentos, quanto a influência de poluentes presentes no ambiente que possam ser absorvidos direta ou indiretamente.

Nesse contexto, os isótopos estáveis são utilizados para a avaliação de impactos antropogênicos, possibilitando inclusive identificar gradientes de poluição (Kopprio et al, 2018). Como o valor de isótopos presente em determinados poluentes é característico, é possível que suas análises nos organismos auxiliem na investigação do grau de contaminação.

1.2 Objetivo geral

- Investigar o impacto do óleo diesel sobre ostras através de análises in situ e in vitro utilizando HPAs e isótopos estáveis como marcadores.

1.2.1 Objetivos específicos

- Avaliar o impacto da contaminação sobre as assinaturas isotópicas de ^{13}C e ^{15}N em tecidos de ostras em dois pontos antropicamente distintos (Capítulo 1).

- Avaliar duas das principais metodologias de dispersão de óleo diesel em água do ponto de vista da reprodutibilidade e da quantidade de HPAs presentes na mistura (Capítulo 2).

- Analisar o efeito da exposição de *Crassostrea brasiliana* à contaminação por óleo diesel em relação à bioacumulação de HPAs nos seus tecidos totais, e assinaturas isotópicas em brânquias, hepatopâncreas e músculos (Capítulo 3).

- Avaliar a mortalidade da ostra *C. brasiliana* em resposta a diferentes concentrações de óleo diesel, assim como a bioacumulação de HPAs nos seus tecidos totais (Capítulo 4).

1.3 Hipóteses

1 – É possível avaliar o impacto por óleo diesel no ambiente através da quantificação de hidrocarbonetos policíclicos aromáticos em ostras.

2 – Alterações nas assinaturas isotópicas de ^{13}C e ^{15}N em tecidos de ostras podem sinalizar contaminação por óleo.

1.4 Metodologias empregadas nas análises de HPAs e Isótopos Estáveis

Ao longo do estudo, foram efetuadas análises de HPAs em amostras de água (ou solução aquosa água:óleo diesel) e tecidos de ostras e, isótopos estáveis em tecidos de ostras e filtros contendo a fração do seston presente na água do mar. As

metodologias específicas de amostragem e análises serão detalhadas nos capítulos 2 a 5, de acordo com o foco de cada experimento.

1.4.1 Análise de HPAs

Descontaminação do material a ser empregado

Toda a vidraria utilizada ao longo das análises foi descontaminada com o objetivo de retirar toda a carga orgânica e evitar interferência nos resultados. O procedimento consistiu em: lavagem com água corrente; limpeza com detergente neutro a 5% (Extran®); lavagem com água corrente; lavagem com água destilada; secagem natural ou com acetona HPLC (LICHROSOLV®); e lavagem com diclorometano HPLC (LICHROSOLV®). No caso de vidrarias não volumétricas, a lavagem com diclorometano foi substituída por descontaminação em mufla a 450 ºC por 6 horas.

Extração em amostras de água

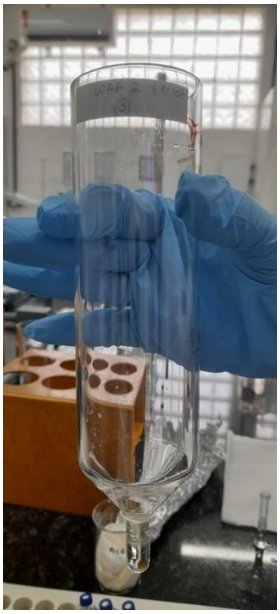
Em todas as análises de HPAs na água, alíquotas de 1L foram coletadas de cada unidade amostral com ajuda de garrafas descontaminadas e proveta volumétrica. As amostras foram armazenadas e resfriadas a 8°C até o momento da extração dos HPAs, não excedendo 7 dias. O processo de extração líquido-líquido seguiu o método EPA-3510 (EPA, 1986a) e foi efetuado no Laboratório de Geoquímica Ambiental Forense do Instituto de Estudos do Mar Almirante Paulo Moreira (LGAF/IEAPM). Cada alíquota foi transferida para funis de separação com capacidade para 1,5 L, aos quais foram adicionados 30 mL de diclorometano HPLC (LICHROSOLV®) (fig. 1).

Figura 1 – Amostras de água em funis de separação para extração de HPAs.

Fonte: Produzida pelo autor; Laboratório da Divisão de Química e Geoquímica Ambiental / IEAPM.

Para validação do procedimento de extração foram adicionados 25 µL de solução do padrão sub-rogado p-terfenil-d14 (4 ng.µL^{-1}) totalizando a inserção de 100 ng de padrão na amostra. Os funis com a solução foram submetidos então a agitação vigorosa por 3 minutos, seguida de repouso de 10 minutos, para a completa separação das fases. O solvente foi então transferido para frasco TurboVap® e todo o procedimento repetido por mais duas vezes, totalizando o uso de 90 mL de solvente. Os extratos foram então reduzidos em TurboVap® sob fluxo de nitrogênio (temperatura de banho 38ºC, fluxo de 6 L/ min e pressão de 14 PSI) a volumes inferiores a 1mL e adicionado diclorometano à até completar-se o volume de 1 mL. Cada extrato foi transferido para vials, aos quais foram adicionados 100 ng de mistura de padrões internos (compostos deuterados naftaleno-d8, acenafteno-d10, fenantreno-d10, criseno-d12 e perileno-d12) e armazenados em freezer até a análise por cromatografia gasosa acoplada à espectrometria de massas (figura 2).

Figura 2 – Amostra já extraída e reduzida em frasco TurboVap@.



Fonte: Produzida pelo autor; Laboratório da Divisão de Química e Geoquímica Ambiental / IEAPM.

As etapas seguintes, de clean up e fracionamento foram realizadas apenas para as amostras de tecido, uma vez que as amostras de água já se apresentaram bastante limpas.

Extração em tecidos de ostras

Atualmente são utilizados diversos métodos para a extração de HPAs em tecidos de animais, sendo que a escolha influencia a quantidade de solventes utilizados, o tempo despendido e a eficiência da extração (Guimarães *et al.*, 2022). No presente estudo, a escolha pela extração por Soxhlet visou garantir uma recuperação relativamente alta dos compostos pretendidos, a partir da utilização de um método consagrado (Guimarães *et al.*, *Op. Cit.*). O processo de extração, assim como a posterior análise de HPAs, foi efetuado no Laboratório de Geoquímica Orgânica Marinha da Faculdade de Oceanografia da Universidade do Estado do Rio de Janeiro (LAGOM/UERJ), Rio de Janeiro, RJ.

Preparação das amostras em tecidos de ostras e extração

As conchas de cada ostra foram higienizadas com escovas e água ultrapura para evitar a contaminação dos tecidos com óleo aderido à superfície externa das conchas. Os tecidos moles totais dos indivíduos foram retirados de suas conchas com a utilização de espátulas e bisturis. As amostras foram homogeneizadas com a ajuda de equipamento Ultra Turrax (Ika Labortechnik). Alíquotas de aproximadamente 3 g misturadas com sulfato de sódio foram então inseridas em cadinhos de vidro, aos quais foram adicionados 100 ng de padrão sub-rogado (p-terfenil-d14). Foi realizada, então, a extração em Soxhlet por 24h, através da mistura de solventes diclorometano e acetona (9:1) (figura 3). Cada rodada de extrações de tecidos de ostras foi acompanhada de um branco, representado apenas por sulfato de sódio puro. A extração foi realizada ainda em amostras de 3g de material de referência de tecido de bivalve certificado IAEA-451 (IAEA, 2013) com o objetivo de avaliar a qualidade do processo.

Figura 3 – Extração de amostras de ostra por sistema Soxhlet.

Fonte: Produzida pelo autor; Laboratório da Divisão de Química e Geoquímica Ambiental / IEAPM.

Em seguida, os extratos foram concentrados através de evaporador rotativo a vácuo (Tecnal®) (vácuo de 290 mbar, temperatura de banho 35 ºC) até o volume de aproximadamente 1mL (figura 4) e transferidos para vial de 10 mL. Quando necessária evaporação extra para atingir o volume requerido foi realizada redução sob fluxo de nitrogênio no próprio vial. Após este procedimento as amostras foram armazenadas sob refrigeração até realização da etapa de clean up.

Figura 4 – Concentração da amostra com evaporador rotativo a vácuo.

Fonte: Produzida pelo autor; Laboratório de Geoquímica Orgânica Marinha da UERJ (LAGOM).

Clean up e fracionamento

A etapa de clean up é realizada com objetivo de reduzir a carga orgânica das amostras extraídas. Para isso, foram utilizadas colunas de vidro com 30 cm de comprimento e 1,8 cm de diâmetro previamente descontaminadas. Foram inseridos, então, 20 g de alumina desativada a 2% acomodada sobre pequena camada de lã de vidro e preenchida com diclorometano HPLC (LICHROSOLV®). Após a adição da amostra, foi realizada a eluição com mais 100 mL de diclorometano. O extrato foi recolhido em frasco TurboVap@. No mesmo frasco, foi feita a troca de solventes para n-hexano, através da evaporação do diclorometano e adição de n-hexano em sequência, sem, no entanto, permitir que o extrato seque. Após a redução a aproximadamente 1 mL o extrato foi transferido para vial e armazenado em refrigeração até a etapa de fracionamento. Quando necessária evaporação extra para atingir o volume requerido foi realizada redução sob fluxo de nitrogênio no próprio vial. O fracionamento foi realizado para separar as frações de hidrocarbonetos alifáticos e aromáticos, segundo método EPA-3630C (EPA, 1996). As amostras foram aplicadas em coluna de 30 cm de comprimento com 1,3 cm de diâmetro interno preenchidas com 7 g de alumina (Óxido de aluminio 90; SHELF LIFE®) desativada a 2%, 10g de sílica gel (60; 0,063-0,200 mm; SHELF LIFE®) desativada a 5% e 1 g de sulfato de sódio (Sulfato de sódio anidro para análise; EMSURE®) descontaminado em mufla. Com a coluna montada, as amostras foram então eluídas com solventes de polaridade crescente: para os alifáticos (descartados após a extração) 30 mL de n-hexano e para os aromáticos 75 mL de mistura de n-hexano e diclorometano (1:1) (figura 5). Os extratos obtidos foram então reduzidos em frascos TurboVap® para pouco menos de 1 mL e então avolumados para exatos 1 mL com n-hexano. Após esta etapa, foram transferidos para vials de injeção onde foram adicionados 100 ng de mistura de padrões internos (compostos deuterados naftaleno-d8, acenafteno-d10, 1,4-diclorobenzeno-d4, fenantreno--d10, criseno-d12 e perileno-d12; AccuStandard®, 4mg/mL) e armazenados em freezer até a análise em cromatógrafo acoplado a espectrômetro de massas.

Figura 5– Fracionamento das amostras em coluna aberta.

Fonte: Produzida pelo autor; Laboratório de Geoquímica Orgânica Marinha da UERJ (LAGOM).

Quantificação dos HPAs

Para a identificação e quantificação dos HPAs, tanto para as amostras de água quanto de tecidos de ostras, foi utilizada cromatografia gasosa acoplada à espectrometria de massas (CG/EM) seguindo método EPA 8270D (EPA, 1986b) de acordo com as condições descritas na tabela 2. O equipamento utilizado, da marca Thermo Scientific®, modelo ISQ, foi calibrado através de dez soluções padrão (1, 2, 5, 10, 20, 50, 100, 200, 400 e 1000 $ng.mL^{-1}$; AccuStandard®, 4 $mg.mL^{-1}$) contendo os 16 HPAs estudados, naftaleno, acenafteno, acenaftileno, fluoreno, fenantreno, antraceno, pireno, fluoranteno, benzo(a)antraceno, criseno, benzo(b)fluoranteno, benzo(k)fluoranteno, benzo(a)pireno, indeno(1,2,3-cd)pireno, dibenzo(a,h)antraceno e benzo(ghi)pireno, e os padrões internos deuterados op. cit. (cada um na concentração de 100 ng L^{-1}). O limite de quantificação foi calculado como a razão entre a menor concentração da curva de calibração (1ng.mL-1) e o volume ou peso da amostra extraída. Os limites de detecção foram calculados a partir da injeção de um padrão em

baixa concentração para cada HPA analisado. Este processo foi repetido oito vezes e dos valores obtidos, calculado o desvio padrão e multiplicado por 3. O resultado é dividido pelo volume de água ou massa de tecido de ostra extraído (Araújo, 2008).

Tabela 2 - Condições instrumentais para determinação de HPAs individuais.

Equipamento		Thermo Scientific® modelo ISQ
Coluna		Agilent DB-5MS (30m, 0,25 mm de DI e 0,25 µm de filme)
Programa de temperatura		50 ºC durante 5 min
		50 ºC min^{-1} até 80 ºC
		6 ºC min^{-1} de 80 ºC a 280 ºC
		12 ºC min^{-1} de 280 ºC a 305 ºC
		305 ºC min^{-1} por 7 min
Gás de arraste		hélio 1,2 min^{-1}
Volume de injeção		2 µL

Fonte: Produzida pelo autor.

1.4.2 Análise de isótopos estáveis e percentuais de carbono e nitrogênio

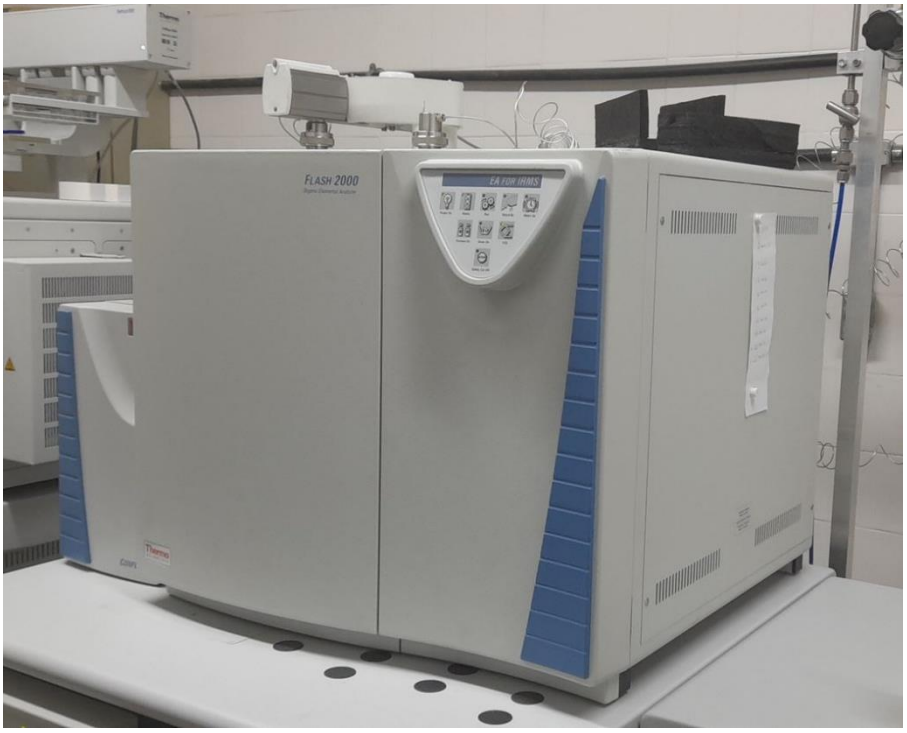
Os tecidos músculo, hepatopâncreas e brânquias das ostras foram analisados separadamente para as determinações elementares. Para as análises isotópica e elementar, cada amostra precisou ser seca e reduzida a massas adequadas. No caso das amostras de tecidos de ostras a secagem foi realizada através de liofilização (liofilizador Christ®/Beta 1-8 LSCPlus; temperatura inicial da amostra: -15ºC; tempo mínimo; 48h; pressão: 0,1mBar; temperatura da prateleira: -15ºC), com posterior maceração e pesagem (figura 6).

Figura 6 – Maceração e pesagem das amostras de tecidos de ostras.

Fonte: Produzida pelo autor; Laboratório de Radioecologia e Alterações Ambientais (LARA) / UFF.

As análises de isótopos estáveis foram efetuadas no Laboratório de Radioecologia e Alterações Ambientais da Universidade Federal Fluminense (UFF), Niterói, RJ. Antes do início da análise das amostras, foi realizado teste da massa mínima necessária para a quantificação elementar. Para isso, foram pesadas em balança de precisão (Mettler Toledo; resolução de 1 µg) porções de teste das amostras pretendidas com massas variadas (dentro da faixa de 0,4 e 1 mg). Para efetuar a pesagem as amostras já foram introduzidas em cápsulas e estanho próprias para as análises seguintes. As amostras a serem testadas foram então inseridas em analisador elementar acoplado em espectrômetro de massa de razão isotópica (EA-IRMS;Thermo Electron Corp., Bremen, Germany) (figura 7), buscando-se então verificar, em cada uma, se os sinais obtidos de carbono e nitrogênio eram ideais para a quantificação. Para a calibração da análise foram utilizados padrões de proteína (B2155 PROTEIN: $\delta^{13}C = -26,98 \pm 0.13$, $\delta^{15}N = 5,94 \pm 0.08$, glicina ($\delta^{13}C = -20,29 \pm 0.04$, $\delta^{15}N = -20,68 \pm 0.06$) e uréia (IVA33802174 UREA: $\delta^{13}C = -41.3 \pm 0.04$, $\delta^{15}N = -0.32 \pm 0.02$), além de cápsulas de estanho vazias. Após definição da massa a ser utilizada, prosseguiu-se com as análises das amostras pretendidas. Nos capítulos 1 e 3 serão especificados os quantitativos específicos de amostras e réplicas para cada estudo.

Figura 7 – Analisador elementar acoplado em espectrômetro de massa de razão isotópica (EA-IRMS).



Fonte: Produzida pelo autor; Laboratório de Radioecologia e Alterações Ambientais (LARA) / UFF.

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2 CAPÍTULO 2 – Artigo submetido à revista Aquatic Conservation: Marine and Freshwater Ecosystems: Stable isotopes analysis of oysters as a tool for environmental monitoring in a Marine Extractive Reserve.

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Abstract

The stable isotopes $\delta^{13}C$ and $\delta^{15}N$ are considered valuable biomarkers to analyze trophic links and nutrient sources, but few studies evaluated their effectiveness for environmental monitoring. ^{13}C and ^{15}N have distinct signatures in organic compounds, which can be utilized to identify potential carbon and nitrogen sources. Marine bivalves are often employed in environmental studies as efficient bioindicators because sessile filter feeders tend to bioaccumulate pollutants. The present study analyzed $\delta^{13}C$ and $\delta^{15}N$ in seston and oysters inhabiting two areas with different environmental conditions in a Marine Extractive Reserve. Anjos Beach Pier (AB) is situated near a pier designated for fishing and tourism vessels, while Forno Beach is located in the rocky shoreline adjacent to this beach. The isotopic values were compared for two trophic levels and three oyster tissues, resulting in a broad view of local dynamics. Seston samples from Forno Beach exhibited depleted $\delta^{13}C$ values, possibly reflecting a terrigenous carbon contribution in this area. Considering oyster tissues, $\delta^{13}C$ and $\delta^{15}N$ values in hepatopancreas were similar to seston, possibly due to oysters' role as filter feeders, supporting the use of

digestive tissues for assessing short-term changes of environmental conditions. Moreover, isotope values for oyster gills and muscles suggest long-term homogeneous conditions for AB and FB, with a predominance of marine carbon and nitrogen sources. Our results underline the relevance of analyzing bivalve tissues separately since they display different turnover rates and depict variable time frames of environmental conditions. This article provides valuable information on the variables that must be considered when applying stable isotope analysis in coastal environmental monitoring, highlights knowledge gaps, and recommends best practices for future work in this area.

Keywords: carbon isotopes; nitrogen isotopes; tracer; bioindicator; environmental impact; biomarker.

2.1 Introduction

The uncontrolled population growth along the coast represents a constant challenge to environmental management (Zhai et al, 2020; Pena et al, 2020). Marine pollution monitoring often relies on indicator organisms, which experience physiological, biochemical, or molecular changes in response to seawater contamination (Ferreira *et al.*, 2023). Bivalve mollusks are often selected as bioindicators because they are sessile filter feeders that absorb and accumulate seawater pollutants (Fiori *et al.*, 2018; Phan *et al.*, 2019). These motivated the continuous search for pollution biochemical markers in bivalve tissues (Sardi *et al.*, 2017; López-Landavery *et al.*, 2019; Araújo *et al.*, 2021). Recently, several studies have applied stable isotopes ^{13}C and ^{15}N as pollution biomarkers due to their particular signature in a variety of organic compounds, which can be used to identify the type and potential sources of contamination in marine organisms (Wang *et al.*, 2020; Vezzone *et al.*, 2021; Felizardo *et al.*, 2021; Srinivas *et al.*, 2022). Besides, the $\delta^{13}C$ isotopic signatures in pollutants are expected to be transferred from producers to higher trophic levels (Zanden and Rasmussen, 2001; Post, 2002). Graham *et al.* (2010) employed the $\delta^{13}C$ signature as a marker of oil pollution and observed that the lighter $\delta^{13}C$ of petrogenic sources led to the decrease of values in planktonic communities. Regarding the ^{15}N signature, several studies reported that a high input of urban effluents in coastal environments resulted in the reduction of $\delta^{15}N$ values in suspended particulate organic material (Ke *et al.* 2020) and in fish tissues (Gaston and Suthers, 2004).

Moreover, stable isotope analyses are broadly used to infer potential sources of dissolved and particulate organic matter (DOM/ POM) in coastal environments. Indeed, multiple origins were associated with organic matter in coastal waters, including local

primary production and microbial decomposition of algal biomass; terrestrial sources by decomposition of soil and plant matter; and anthropogenic sources, such as industrial and domestic sewage (Lee *et al.*, 2020). Another important factor to consider is that the isotopic composition in a consumer's tissue reflects their current diet and consumption in the preceding weeks and months, allowing for medium-term evaluations (Buchheister and Latour, 2010). $\delta^{13}C$ and $\delta^{15}N$ fractioning also provide valuable information for the study of ecological niches and animal interactions in wild communities. In that sense Shipley and Matich (2020) reviewed the important factors that interfere on data interpretation, such as physiological conditions, sampling and storage procedure and statistical analysis.

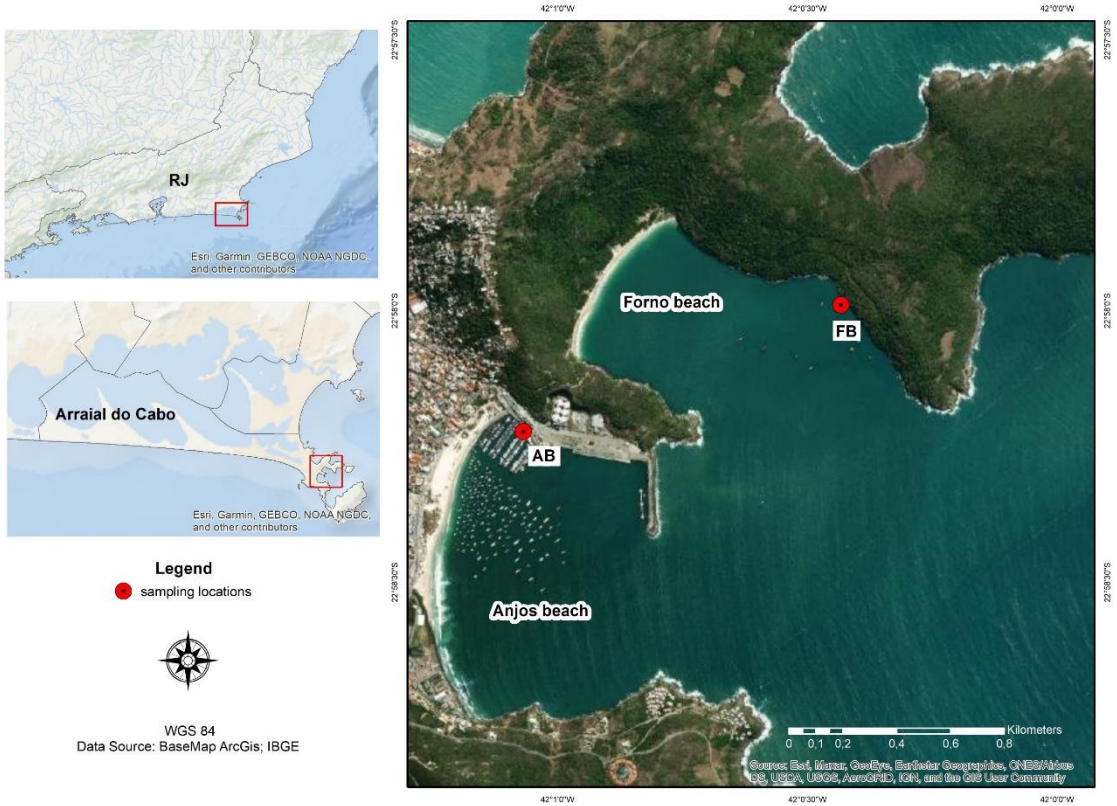
Stable isotopes appear as promising tools for environmental monitoring, although the current literature on the subject remains scarce. The present study applies $\delta^{13}C$ and $\delta^{15}N$ stable isotopes analysis of seston and bivalve tissues to assess the environmental conditions of the coastal region in Arraial do Cabo, a tourist destination situated within a Marine Extractive Reserve (RESEXmar-AC).

2.2 Methodology

2.2.1 Study area

Samples were collected from two sites with varying environmental conditions in Arraial do Cabo, a marine extractive reserve characterized by multiple uses of the sea, including boat traffic, diving, swimming, and artisanal fishery (De Melo *et al.*, 2009; Sarmento *et al.*, 2020; ICMBIO, 2020). Site one is located at the Anjos Beach pier (AB) (Fig. 1), the primary nautical support point for Arraial do Cabo, housing the Fisher's Marina, the floating pier of Anjos Beach, and a commercial harbor (ICMBIO, 2020). The frequent transit and docking of hundreds of boats could be a source of chronic release of oil at this location (Warnken and Byrnes, 2004; Lin *et al.*, 2007). This area also experiences occasional sewage discharge events triggered by heavy rainfall (ICMBIO, 2020). Site two is located on the rocky shore of Forno Beach (FB), which is subject to a low degree of anthropogenic disturbance, restricted to the presence of tourists and a small bivalve mollusk farm (Galvao *et al.*, 2012; ICMBIO, 2020).

Figure 1. Location of sampling sites at Arraial do Cabo, RJ, Brazil (AB = Anjos Beach; FB = Forno Beach).



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2.2.2 Sample collection and preparation

Seawater was collected in 4 L triplicate samples at each site to analyze stable isotopes in the seston. Samples were pre-filtered using a 200 µm mesh to exclude large organisms and limit the size of the seston to be analyzed, and then vacuum filtered using 0.7 µm GF/F filters (Schoo *et al.*, 2018). The filters were previously dried and decontaminated in a muffle furnace (8 hours at 450 ºC), and individually weighed on a precision scale. After filtration, the filters were dried at 60°C until they reached a constant weight and then stored for isotopic analysis. Six individuals from the Ostreidae family were collected at each sampling point and identified as *Saccostrea cucullata* and *Crassostrea brasiliana*. The specimens were transported in a refrigerated container to the laboratory and then frozen for later dissection. Each specimen's gills, hepatopancreas, and muscle tissues were separated, weighed, placed in 2 mL Eppendorf® tubes, and individually frozen at -20 ºC. The tissues were then freeze-dried and macerated in an agate mortar and pestle. All samples were collected during the low spring tide in December 2021.

2.2.3 Stable isotope analysis

The quantification of stable isotopes ($\delta^{13}C$ and $\delta^{15}N$), total carbon (TC) and total nitrogen (TN) was performed on aliquots of dry filters (12 mg) and of each freeze-dried oyster tissue (0.7 mg), weighed using tin capsules and a precision scale. A blank analysis was conducted to determine the percentage of potential residues of inorganic carbon and nitrogen present in the filter fabrication material. TC, TN, $\delta^{13}C$, and $\delta^{15}N$ levels were quantified using an elemental analyzer coupled to an isotope ratio mass spectrometer (EA Flash 2000 coupled to an IRMS Delta Advantage; Thermo Electron Corp., Bremen, Germany), as described by Vezzone *et al.* (2021). To calculate the analysis error, reference materials were used, along with empty tin capsules: B2155 PROTEIN ($\delta^{13}C$ = −26,98 ± 0.13, $\delta^{15}N$ = 5,94 ± 0,08), USGS65 GLYCINE ($\delta^{13}C$ = −20.29 ± 0,04, $\delta^{15}N$ = −20,68 ± 0.06), and IVA33802174 UREA ($\delta^{13}C$ = −41,3 ± 0.04, $\delta^{15}N$ = −0,32 ± 0,02). The measured analytical errors were ± 0,04 ‰ for $\delta^{13}C$, ± 0,04 ‰ for $\delta^{15}N$, ± 0,5 % for TC and ± 0,6 % for TN.

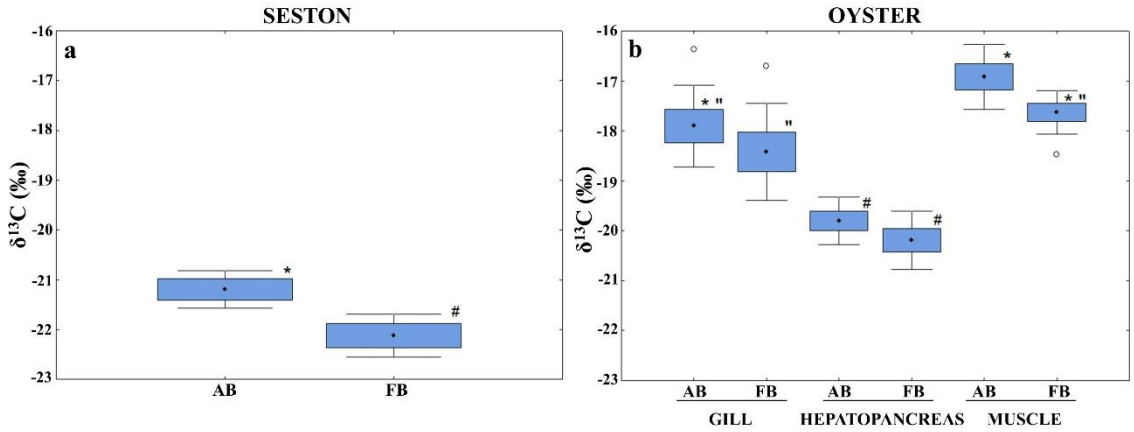
2.2.4 Statistical analysis

The data were determined to be normally distributed through Kolmogorov-Smirnov and Shapiro-Wilk tests and compared through analysis of variance (ANOVA), followed by Tukey multiple comparison tests (Zar, 1996). Significant differences were identified by a coefficient $p \leq 0.05$. The results were represented in boxplot graphics depicting the mean ± standard deviation. All statistical analyses were performed with the software Excel® and Statistica 7.0®.

2.3 Results

Stable isotope analyses in seston samples revealed a significant decrease of $\delta^{13}C$ at Forno Beach (FB) compared to the Anjos Beach (AB) pier (Fig. 2a, $p = 0,048$). $\delta^{13}C$ in oyster tissues showed the same trend observed for seston (Fig. 2b, $p > 0,05$). When comparing oyster tissues, the hepatopancreas exhibits $\delta^{13}C$ values significantly lower than gills and muscle, and closer to the values observed for seston (Fig. 2b).

Figure 2. $\delta^{13}C$ levels in (a) seston (n = 3) and (b) oyster tissues (n = 6) at sampling sites in Arraial do Cabo. Mean ± standard deviation; boxes indicate standard errors. AB = Anjos Beach Pier; FB = Forno Beach. Equal symbols (*, ", #) indicate no significant differences.



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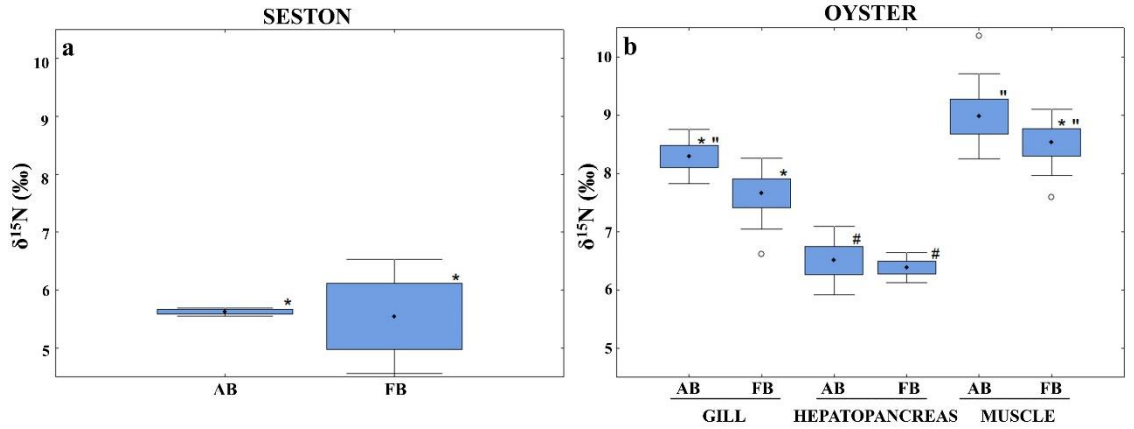
The $\delta^{15}N$ mean values were similar between AB and FB, considering both seston and oyster samples (Fig. 3). When comparing different oyster tissues, the hepatopancreas had the lowest $\delta^{15}N$ values (Fig. 3b), closely resembling those observed in the seston, whereas the values for muscles and gills exceed the seston by more than 2‰.

Figure 3. $\delta^{15}N$ levels in (a) seston (n = 3) and (b) oyster tissues (n = 6) at sampling sites in Arraial do Cabo. Mean ± standard deviation; boxes indicate standard errors. AB = Anjos Beach Pier; FB = Forno Beach. Different symbols (*, ", #) indicate statistically significant differences.



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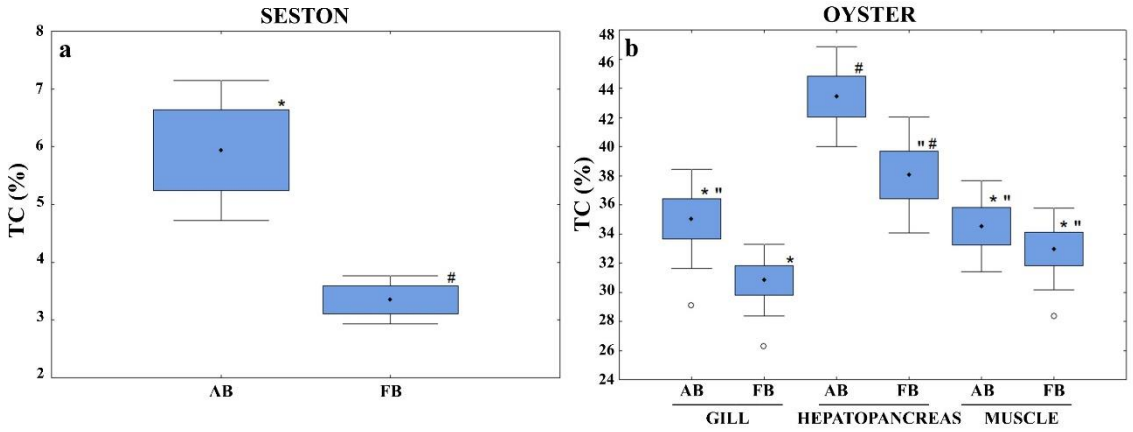
The mean total carbon (TC) percentages were significantly higher for seston in AB than in FB (Fig. 4a, p = 0,025). Oyster tissues did not exhibit significant variations in TC values between sampling sites (Fig. 4b).

Figure 4. Total carbon percentage (TC%) in (a) seston (n = 3) and (b) oyster tissues (n = 6) at sampling sites in Arraial do Cabo. Mean ± standard deviation; boxes indicate standard errors. AB = Anjos Beach Pier; FB = Forno Beach. Different symbols (*, ", #) indicate statistically significant differences.



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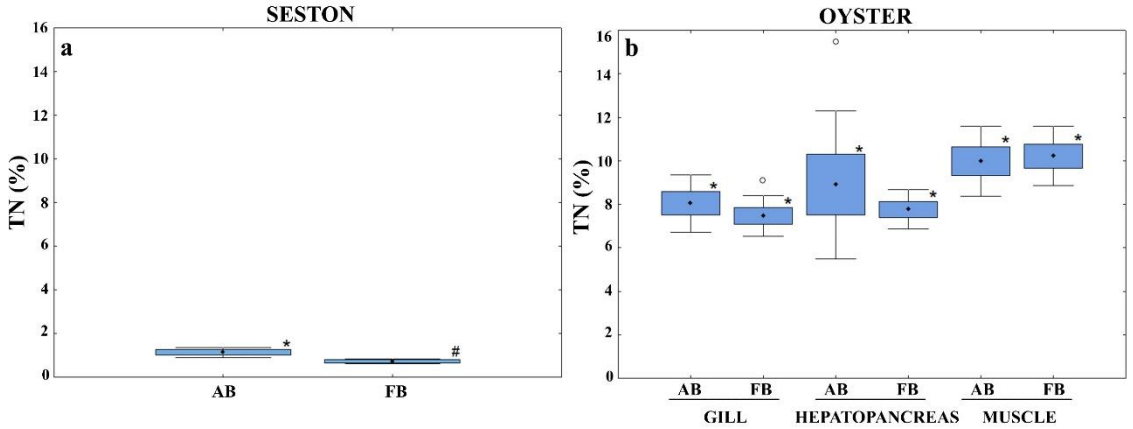
A significant difference was also observed between sampling sites for seston TN levels, with higher values in AB than in FB (Fig. 5a, p = 0,047). TN levels for oyster tissues were similar between sampling sites and among tissue types (Fig. 5b).

Figure 5. Total nitrogen percentage (TN%) in (a) seston (n = 3) and (b) oyster tissues (n = 6) at sampling sites in Arraial do Cabo. Mean ± standard deviation; boxes indicate standard errors. AB = Anjos Beach Pier; FB = Forno Beach. Different symbols (*, ", #) indicate statistically significant differences.



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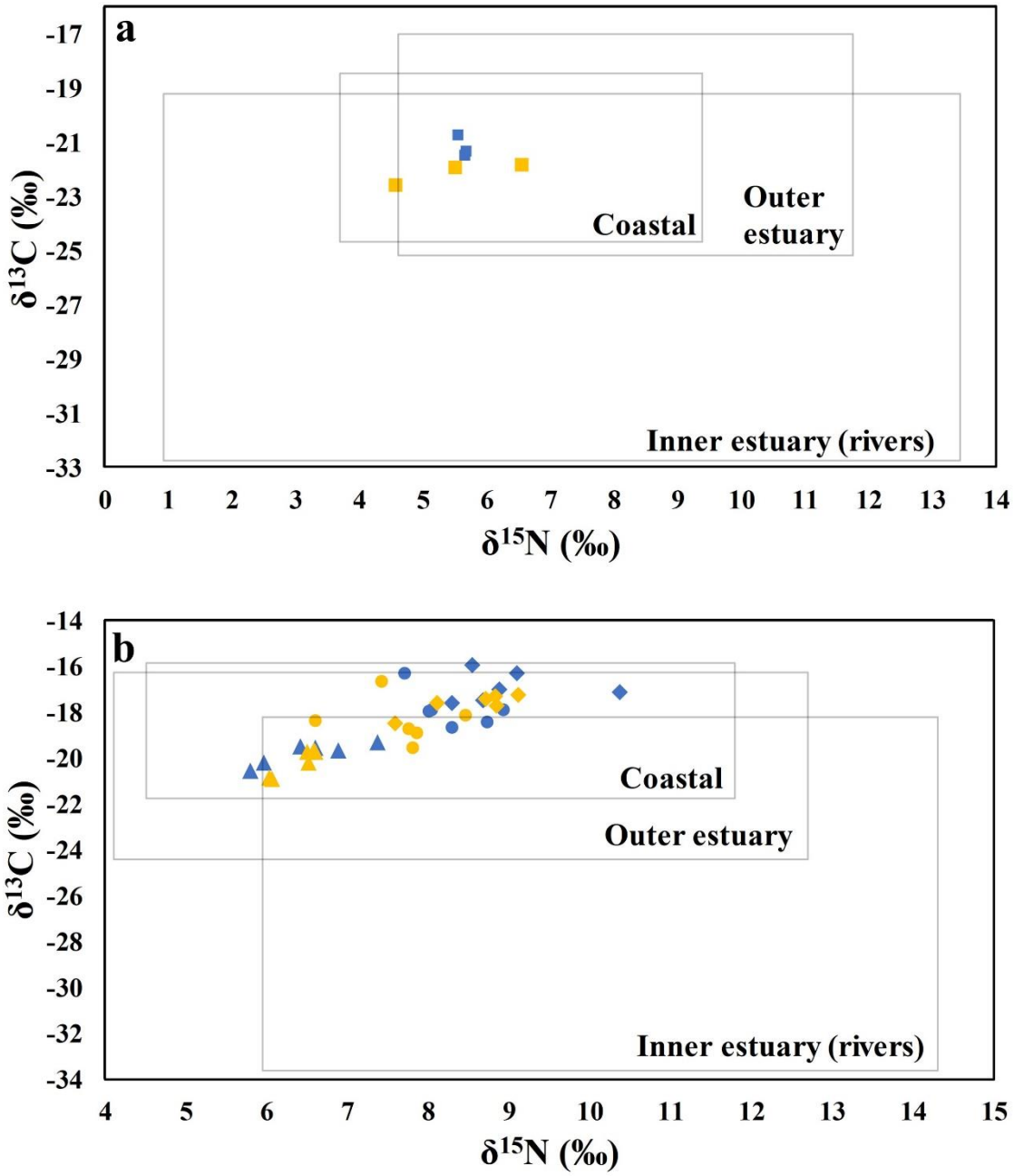
In general, when compared with previous studies (Table A.2-A.3), the isotopic signatures obtained for seston and oysters were similar to the values found in coastal regions (Fig. 6). Moreover, seston samples from FB showed variable $\delta^{15}N$ levels, compatible to coastal and estuary signatures (Fig. 6a). In contrast, $\delta^{13}C$ values for oyster muscle tissues are out of the range observed for inner estuary samples and similar to the observed for coastal and outer estuary samples (Fig. 6b).

Figure 6. $\delta^{13}C$ and $\delta^{15}N$ in seston (a) and oyster tissues (b) were compared with those reported in previous studies for coastal areas, including external (deltas, lagoons, and bays) and internal (rivers) estuarine areas. Symbols: ■ = seston; ▲= hepatopancreas; ● = gills; ♦ = muscle. Blue = Anjos Beach Pier; Yellow = Forno Beach. Gray rectangles represent values obtained from previous studies (References at Tables A.2 and A.3).



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2.4 Discussion

$\delta^{13}C$ and TC values for seston suggest different environmental conditions between the AB and FB stations during collection (Roth *et al.*, 2016; Srinivas *et al.*, 2022). However, the enrichment of $\delta^{13}C$ at station AB is contrary to the trend of $\delta^{13}C$ decrease

observed in areas with a significant input of urban effluents (Rogers, 2003; Gaston and Suthers, 2004) or subjected to oil spill incidents (Graham *et al.* 2010). Indeed, local dynamics may favor the influence of enriched carbon sources of marine origin at AB, supplanting the effects of occasional oil or sewage pollution (Bhardan *et al.*, 2015; Kopprio *et al.*, 2018).

Moreover, the decrease of $\delta^{13}C$ at FB could be attributed to terrigenous carbon input, since this station is located next to rocky shores in a cove surrounded by terrestrial vegetation. A similar trend in ^{13}C signature was observed by Bearham *et al.* (2023) and Bardhan *et al.* (2015), which demonstrated that depleted $\delta^{13}C$ values in particulate organic matter are characteristics of environments where terrestrial carbon sources predominate. In contrast, the $\delta^{13}C$ values in oyster tissues were similar between sampling sites, potentially reflecting longer-term environmental conditions when compared to seston. The $\delta^{13}C$ values for oysters are directly linked to their dietary intake and indicate feeding patterns over weeks or months (Zanden and Rasmussen, 2001; Post, 2002). These results suggest the contribution of terrigenous carbon sources at FB during sampling and the trend to long-term homogenization of carbon sources between FB and AB.

The nitrogen isotopic ratio observed in both seston and oyster tissues was similar across the sampling stations, indicating similar nitrogen sources, at least on a scale of days to months preceding the collections. However, the higher standard deviation observed for seston $\delta^{15}N$ values in FB suggests a greater variability of nitrogen sources in this location, when compared to AB. Indeed, $\delta^{15}N$ values for seston at FB are compatible with marine and terrestrial sources (Table A.2). The present results do not reflect the occurrence of urban effluents at AB, possibly because sewage discharge is occasional in this area, and restricted to periods of heavy rainfall. Besides, studies using $\delta^{15}N$ values to assess the effects of anthropogenic effluents in coastal areas show contrasting results. Ke *et al.* (2020), for example, attributed a decrease of recorded nitrogen isotopic signatures (below 2‰) to the impact of urban sewage, while Rožič *et al.* (2015) observed the opposite result (enriched $\delta^{15}N$ signatures above 5‰). These contrasting results reinforce the need to standardize methods in studies that apply $\delta^{15}N$ analysis for environmental monitoring.

The comparison between oyster tissues showed that the values of $\delta^{13}C$ and $\delta^{15}N$ were lower in the hepatopancreas than in the other tissues, and closer to those recorded for seston. This difference in isotopic signatures of digestive tissues is partly attributed to their higher recycling rate, which reflects recent feeding behavior (Raikow and Hamilton, 2001; Cabanellas-Reboredo *et al.*, 2009; Özdilek *et al.*, 2019). On the other hand, gill and muscle tissues exhibit a distinct isotopic fractionation than

hepatopancreas, leading to higher values for the studied isotopes (Yokohama *et al.*, 2008; de Barros Ferraz *et al.*, 2009). The $\delta^{15}N$ values for oyster muscles and gills are more than 2‰ higher than those of seston, reflecting the higher trophic position of oysters in the food chain in relation to seston (Zanden and Rasmussen, 2001; Post, 2002; Layman *et al.*, 2012). Similarly, previous studies demonstrated that the isotopic fractionation of $\delta^{13}C$ between muscles and their sources varies on average around 1 ± 1 ‰, while variations in $\delta^{15}N$ along the food chain show that consumers have isotopic signatures higher than those of their diets, with an average value of about 3.0 ± 1.0 ‰ per trophic level, considering only the feeding factor (Post, 2002; McCutchan *et al.*, 2003). Recent studies emphasize that each environment must be carefully evaluated because trophic structures are unique and intrinsically linked to environmental conditions (Shipley & Matich, 2020; Kjeldgaard *et al.*, 2021).

Considering the variations of isotope fractioning among different ecological niches and consumer tissues, the decrease in $\delta^{13}C$ in the seston and hepatopancreas at FB reflects the short-term environmental conditions, which might indicate the influence of low $\delta^{13}C$ sources at the sampling moment, such as terrigenous material (Riera & Richard, 1996; Bardham *et al.*, 2015; Bearham *et al.*, 2023). In contrast, $\delta^{13}C$ values for oyster muscle tissue are higher than the hepatopancreas and out of the range observed for inner estuary samples (Table A.3), which could indicate the long-term predominance of marine sources. The difference in isotopic signature between fast and slow turnover tissues was considered by Bearham *et al.* (2023) as an important tool to evaluate environmental variability. The authors noticed that investigating dietary links based on isotopes is more challenging in dynamic than stable environments.

Indeed, the interpretation of environmental isotopic data is complex and challenging, because of the myriad of biotic and abiotic factors influencing isotopic turnover (Liu *et al.*, 2018; Bauer *et al.*, 2021; Yang *et al.*, 2021). The isotopic signature in indicator organisms is influenced not only by the current environmental conditions but also by their diet and the recycling rate for carbon and nitrogen in the analyzed tissues. The recycling speed is influenced by life stage, metabolism (Herzka and Holt, 2000), and abiotic factors, such as temperature (Dattagupta *et al.* 2004). The present study contributes to the understanding of isotope fractioning and turnover in natural marine environments and in bivalve tissues. Further research is necessary to evaluate the effects of physicochemical and biological factors on isotope signature and to expand the application of this tool for environmental monitoring.

2.5 Conclusion

The $\delta^{13}C$ signature was successfully applied to differentiate two areas with particular environmental characteristics in the RESEXmar-AC, and to identify a possible influence of terrigenous carbon sources at Forno Beach. The values obtained for $\delta^{13}C$ and $\delta^{15}N$ from seston and oyster hepatopancreas reflect this short-term difference. Still, the isotopic signature of oyster gills and muscles depicts a long-term scenario of homogeneous environmental conditions for AB and FB. The present results reinforce the importance of analyzing bivalve tissues separately since the different turnover rates could lead to misinterpretation of isotopic values.

2.6 Acknowledgements

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2.7 Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

2.8 Funding statement

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2.9 Conflict of interest disclosure

The authors declare no conflicts of interest.

2.10 References

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2.11 Appendix A. Supplementary data

Table A.1 – Average values of total carbon (TC), total nitrogen (TN), $\delta^{13}C$, and $\delta^{15}N$ in tissue samples of oysters and seston from the two sampling stations.

Sampling Site	Analysis Matrix	TC (wt%) (mean ± sd)	TN (wt%) (mean ± sd)	$\delta^{13}C$ (‰) (mean ± sd)	$\delta^{15}N$ (‰) (mean ± sd)
Anjos Beach	Gills	35,04 ± (3,42)	8,04 ± (1,31)	-17,9 ± (0,82)	8,29 ± (0,46)
	Hepatopancreas	43,44 ± (3,43)	8,9 ± (3,41)	-19,8 ± (0,48)	6,5 ± (0,59)
	Muscle	34,53 ± (3,13)	9,98 ± (1,62)	-16,92 ± (0,65)	8,98 ± (0,73)
	Seston	5,94 ± (1,21)	1,13 ± (0,22)	-21,2 ± (0,38)	5,62 ± (0,07)
Forno Beach	Gills	30,82 ± (2,46)	7,48 ± (0,94)	-18,42 ± (0,97)	7,65 ± (0,61)
	Hepatopancreas	38,05 ± (3,96)	7,77 ± (0,9)	-20,19 ± (0,58)	6,38 ± (0,26)
	Muscle	32,96 ± (2,8)	10,22 ± (1,37)	-17,63 ± (0,44)	8,53 ± (0,57)
	Seston	3,35 ± (0,42)	0,72 ± (0,11)	-22,12 ± (0,43)	5,54 ± (0,99)

Source: Produced by the author.

Table A.2 - Values of $\delta^{13}C$ and $\delta^{15}N$ in seston or suspended particulate organic material for coastal areas, including external (deltas, lagoons and bays) and internal (rivers) estuary regions in previous studies.

Ecosystem type	$\delta^{13}C$	$\delta^{15}N$	Reference
Coastal	-21,8±0,5 to -19,5±0,6		(Riera & Richards, 1996)
	-24,8		(Malet *et al.*, 2007)
	-20,7±0,2	6,2±0,2	(Kolasinski *et al.*, 2011)
	-24,3±0,4	6,1±0,3	(Carmichael et al 2012)

	-23±0,3 to -18±4,2	7,0±1,3 to 7,6±0,3	(Marchais *et al.*, 2013)
	-22,8 to -18,4	3,7 to 9,3	(Han *et al.*, 2015)
	-24,7 to -23,6		(Bearham *et al.*, 2023)
Outer estuary			
	-22,6±0,7 to -21,7		(Riera & Richards, 1996)
		4,56±0,5 to 6,74±1,34	(Bucci *et al.*, 2007)
	-25,3±0,2	4,9±0,2	(Carmichael *et al.*, 2012)
	-21,1±1,4 to -20,5±5,2	7,2±0,6	(Marchais 2013)
	-22,75±1,62 to -21,48±1,26	3,88±2,15 to 5,2±2,35	(Ke *et al.*, 2020)
	-24 to -17	6,3 to 11,7	(Schoo *et al.*, 2021)
	-20,8	5,4±0,9 to 7,8±1,2	(Park *et al.*, 2022)
	-25,7 to -20,68	4,59 to 5,41	(Bearham *et al.*, 2023)
Inner estuary (rivers)			
	-29,2±0,4 to -22,6±0,5		(Riera & Richards, 1996)
	-32,8 to -26,5		(Riera & Richards, 1997)
	-31,5±0,7 to -23,3±1,9	4,0±0,2 to 9,9±0,2	(Piola *et al.*, 2006)
		4,56±0,5 to 6,74±1,34	(Bucci *et al.*, 2007)
	-19,2		(Malet *et al.*, 2007)
		5,3 to 13,4	(Fertig *et al.*, 2010)
	-22,2±0,1 to -21,9±0,5	6,8±0,3 to 7,4±0,5	(Marchais *et al.*, 2013)
	-29,72 to -24.47	0.87 to 4,57	(Bardhan *et al.*, 2015)
	-26,2 to -24,8		(Bearham *et al.*, 2023)

Source: Produced by the author.

Table A.3 – Values of $\delta^{13}C$ and $\delta^{15}N$ in oyster tissues for coastal areas, including external (deltas, lagoons and bays) and internal (rivers) estuary regions in previous studies.

Ecosystem	Species	$\delta^{13}C$	$\delta^{15}N$	Reference
Coastal	*Crassostrea virginica*	-21,9		(Wade *et al.*, 1989)
	Crassostrea gigas	-21,8 ± 0,5 to -19,5 ± 0,6		(Riera & Richards 1996)
	Crassostrea gigas	-19,09 ± 0,06	8,12 ± 0,01	(Dubois *et al.*, 2007)
	Crassostrea gigas	-21,52 ± 0,9		(Xu & Yang, 2007)
	Crassostrea gigas	-20 ± 0,4 to -18,6 ± 0,3	9,4 ± 0,5 to 9,6 ± 0,4	(Yang & Shin, 2009)
	Crassostrea gigas	-19,7 ± 0,12 to -17,53 ± 0,17	8,28 ± 0,32 to 11,79 ± 0,23	(Lefebvre *et al.*, 2009)
	Crassostrea gigas	-17,6 ± 0,3 to -17,3 ± 0,2	9.7± 0,3 to 10,4 ± 0,5	(Marchais *et al.*, 2013)
	Crassostrea gigas	-19,4 ± 0,9 to -17,9 ± 0,3	4,5 to 8,6	(Han *et al.*, 2015)
	Crassostrea gasar	-15,9 ± 1,17	7,59 ± 1,0	(Vinagre *et al.*, 2018)
	Crassostrea gigas	-20,87 ± 0,61	8,18 ± 0,5	(Mello, 2019)
	Crassostrea gigas	-19,57 ± 0,52 to -19,54 ± 0,56	8,24 ± 0,46 to 8,31 ± 0,48	(Park *et al.*, 2021)
	Saccostrea cucullata	-21,41 ± 1,74	4,4 ± 0,14	(Bearham *et al.*, 2023)
Outer estuary				
	Crassostrea gigas	-22,6 ± 0,8 to -21,7		(Riera & Richards, 1996)
	Crassostrea gigas	-20,2 to -16,2		(Paulet *et al.*, 2006)
	Crassostrea gasar	-17,7 ± 0,6	11,5 to 12,7 ± 0,1	(Bodin *et al.*, 2011)
	Crassostrea gigas	-17,3 ± 0,3 to -16,9 ± 0,2	10.0 ± 0,3 to 10,8 ± 0,3	(Marchais *et al.*, 2013)

	Saccostrea cucullata	-24,4 ± 0,58	4,11 ± 0,21	(Bearham *et al.*, 2023)
Inner estuary (rivers)				
	Crassostrea gigas	-29,2 ± 0,4 to -23,2 ± 0,6		(Riera & Richards, 1996)
	Crassostrea virginica		12,2 ± 0,3 to 14,3 ± 0,1	(Fertig *et al.*, 2010)
	Crassostrea gasar	-19,6 ± 0,1 to -18,8 ± 0,2	8,0 ± 0,1 to 8,2 ± 0,1	(Bodin *et al.*, 2011)
	Crassostrea gigas	-18,2	11 ± 0,7 to 12,1 ± 0,4	(Marchais *et al.*, 2013)
	Crassostrea gasar	-33,6 ± 0,45	5,9 ± 1,04	(Vilhena *et al.*, 2021)
	Saccostrea cucullata	-24,84 ± 0,29	5,15 ± 0,15	(Bearham *et al.*, 2023)

Source: Produced by the author.

2.12 Supplementary data references

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3 Capítulo 3 – Assessment of efficiency and reproducibility in the preparation of oil-water dispersion for bioassays: An alternative to water accommodated fraction (WAF) limitations.

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All authors contributed to the study conception and design. Material preparation and data collection were performed by Pedro Sant'Anna Cortez, Nícollas Menezes Ferreira, Dayana Maia Montalvão, Ana Paula de França Leal, Luciana Altvater, and Louisi Souza de Oliveira. The analyses were performed by Pedro Sant' Anna Cortez, Dayana Maia Montalvão, Ana Paula de França Leal, Louisi Souza de Oliveira and Cássia de Oliveira Farias. Ricardo Coutinho, Louisi Souza de Oliveira, Giselle Pinto de Faria Lopes, Márcio Martins Lobão, and Roberto Meigikos dos Anjos contributed to funding acquisition. The first draft of the manuscript was written by Pedro Sant' Anna Cortez and all authors

commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Abstract

Bioassays are often employed to simulate the introduction of oil pollutants in marine environments. However, the current lack of scientific consensus poses the need for standardization to accomplish efficient and reproducible oil-water dispersions, especially when large volumes are necessary. This study aimed to quantitatively compare the frequently used methods Water-Accommodated Fraction (WAF) and Oil-in-Water Dispersion (OWD) regarding polycyclic aromatic hydrocarbon (PAH) final concentration and reproducibility among replicates. Both methods achieved concentrations of PAHs consistent with those observed in seawater after oil spill incidents, although there were notably higher concentrations in samples generated by WAF. In contrast, this method resulted in higher variability among replicates for all sixteen analyzed PAHs, compared to OWD. The differences in techniques were more prominent for low and medium-molecular weight PAHs. The volatility of diesel oil components, combined with the high agitation speed used for OWD, is the probable cause of the differences observed between the methods. In this study, the production of OWD proved to be more effective than WAF, considering simpler logistics, reduced steps, and lower variability between replicates. By quantitatively comparing the most frequently used oil dispersion methods and discussing their advantages and limitations, we contribute to the proposal of a bioassay framework, which should increase the comparability and reliability of future studies in the area.

Keywords: Polycyclic aromatic hydrocarbons (PAHs); Oil-in-Water Dispersion (OWD); oil spill; oil dispersion; ecotoxicology; bioassay.

3.1 Introduction

Oil spill incidents reduce marine biodiversity, impact multiple users in coastal areas, and are a concern for the scientific community, environmental organizations, and governments (Asif *et al.*, 2022). Bioassays are often employed to analyze the direct biological effects and evaluate oil absorption by marine organisms (Ferreira *et al.*, 2023). However, simulating the introduction of these pollutants in laboratory experiments is a challenge because the hydrophobic nature of oil hampers the preparation of consistent oil-water dispersions. The Chemical Response to Oil Spills: Ecological Effects Research

Forum (CROSERF) was formed in 1994 as an attempt to develop standardized methods for oil toxicity studies. Since then, several modifications have been proposed to address new questions from the scientific community as the resolution of analytical methods has improved. The main oil dispersion methods currently used for bioassays are the Water-Accommodated Fraction (WAF) and Oil-in-Water Dispersion (OWD) (Wade *et al.*, 2022).

WAF preparation consists of dispersing oil in water using a low energy mixing with a magnetic stirrer. The CROSERF established strict protocols for WAF, which are adequate for preparing between 2 and 20 L solutions (Singer *et al.*, 2000; Loughery *et al.*, 2023). To fully understand the impact of oil spills in aquatic and marine environments, it is necessary to perform mesocosm experiments with the affected organisms (i.e. mollusks, crustaceans, fish), which demand the preparation of hundreds of liters of WAF for toxicity tests. Several studies modified the original WAF protocol to address the demands for large-scale production (Wade *et al.*, 2017; Parkerton *et al.*, 2023). However, serial dilution of WAF is not recommended as the absolute and relative hydrocarbon concentrations may not change linearly due to the variable solubility of the oil components, making it difficult to obtain the expected values (Singer *et al.*, 2000). On the other hand, if diluting the WAF is not recommended, producing large volumes of an aqueous solution through this method requires a robust and rarely available infrastructure (Wade *et al.*, 2017; Wade *et al.*, 2022; Lee *et al.*, 2023).

In contrast, OWD involves the direct and vigorous mixing of oil-water mixtures. The more vigorous the agitation, the closer the dispersion is to the original oil, while only a fraction of the components are detected in the dissolved phase (Wade *et al.*, 2022). The main advantage of this method is the possibility to produce the oil-water mixtures with a simple infrastructure. However, little is known about the factors that could influence the loss of volatile compounds, such as agitation speed and headspace volume.

In the last decades, diverse modifications to the standard CROSERF protocols were applied to toxicology studies to meet specific experiment requirements (Bejarano *et al.*, 2006; Lüchmann *et al.*, 2011; Müller *et al.*, 2018; Wheeler *et al.*, 2020; Loughery *et al.*, 2023). The selection of the most suitable technique depends on tests and the research question to be addressed (Lee *et al.*, 2023; Parkerton *et al.*, 2023). Each method has its specificity and generally does not meet all the researcher's needs, which motivates constant scientific discussion (Anderson *et al.*, 1974; Echols *et al.*, 2015; Wade *et al.*, 2022; Lee *et al.*, 2023; Parkerton *et al.*, 2023). Nonetheless, the efficiency and reproductivity of adapted protocols were rarely evaluated. The present study compared OWD and WAF regarding the efficiency of dispersing diesel oil in seawater and the capacity to produce replicates with uniform polycyclic aromatic hydrocarbon (PAH) concentrations. By comparing the main oil dispersion methods currently used, we

contribute to the definition of a framework for bioassays, increasing the comparability between studies and the reliability of the obtained results to support government decisions during oil spill incidents.

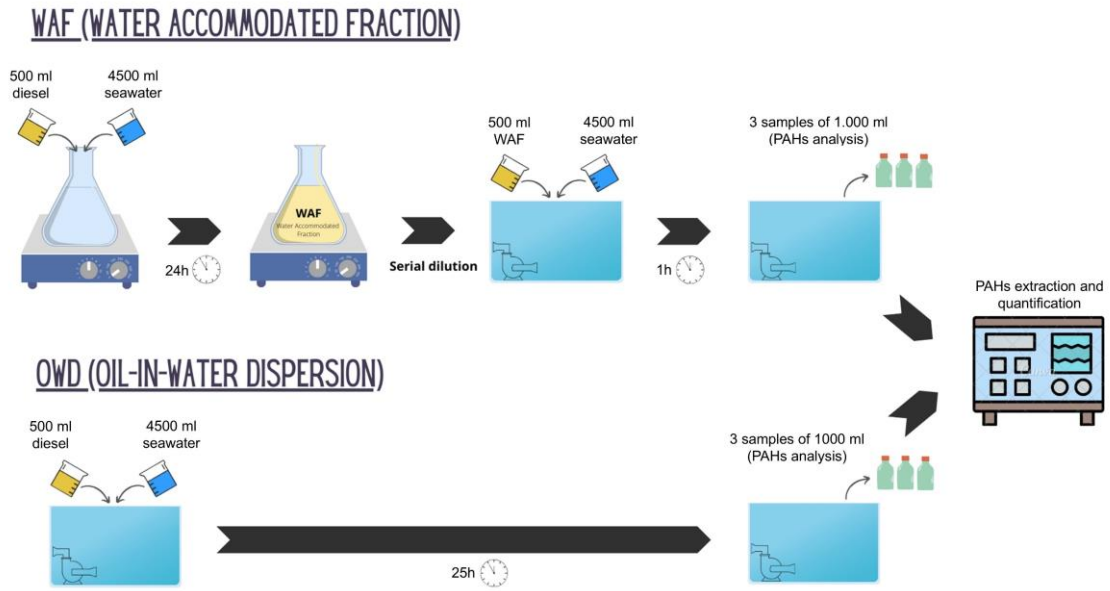
3.2 Materials and Methods

3.2.1 WAF and OWD preparation

Oil dispersions were prepared by using modified WAF and OWD protocols to a final ratio of 1:100 diesel oil: seawater (Fig. 1). WAF preparation was conducted by adapting the protocol established by the CROSERF (Aurand & Coelho, 2005). In the first dilution step, 500 mL of diesel oil was mixed with 4.5 L of artificial seawater (Fauna Marin® Professional Sea Salt / salinity 35) in a glass container, which was then sealed and protected from light. The oil: seawater mixture was mixed using a magnetic stirrer for 24 hours in the dark at a temperature of 25 ºC and a speed of 1,080 rpm. After this period, 0,5 L of the prepared fraction was collected by siphoning and diluted in 4.5 L of artificial saltwater in a glass container. The diluted solution was homogenized by stirring with an oil free immersion pump (Sarlobetter® pump / 1000 $L.h^{-1}$) for 1 hour. Three replicates of the WAF solution (1L each) were then collected for PAH analysis. At this stage, we aimed to test an adaptation of the WAF technique using serial dilution.

OWD preparation was conducted following the protocol described by Echols *et al.* (2015) with modifications. 50 mL of diesel oil was added to 4,95 L of artificial seawater (Fauna Marin® Professional Sea Salt / salinity 35) in a sealed container protected from light, to reduce volatilization and photodegradation. The mixture was stirred for 25 hours using an oil free immersion pump (Sarlobetter® pump / 1000 $L.h^{-1}$), in the dark and at 25 ºC. After this period, three replicates of the OWD solution (1 L each) were collected through siphoning and stored at 8 ºC for the analysis of PAH concentration.

Figure 1 - Methodological scheme adopted for oil dispersion and comparison of Water-Accommodated Fraction (WAF) and Oil-in-Water Dispersion (OWD) techniques.



3.2.2 PAH analysis

The extraction of PAHs was performed using the EPA method 3510C (EPA, 1996a). Aliquots of 1 L of seawater and 30 mL of dichloromethane (HPLC-grade, Merck) were transferred to separatory funnels. The surrogate standard p-terphenyl-d14 (100 ng, Sigma-Aldrich®) was added to validate the procedure. The PAH extraction method is considered appropriate if the recovery of the surrogate standard p-terphenyl-d14 is between 40% and 125% (Sauer & Boehm, 1995). The extraction was conducted by vigorously shaking for 3 minutes, followed by resting for 10 minutes to allow for complete separation of the phases. The solvent was transferred to decontaminated bottles, and the entire procedure was repeated two more times, using a total of 90 mL of solvent. The extracts were then reduced in TurboVap™ under a nitrogen flow. After that, 100 ng of internal standard mixtures were added, including deuterated compounds naphthalene-d8, acenaphthene-d10, 1,4-dichlorobenzene-d4, phenanthrene-d10, chrysene-d12, and perylene-d12 from AccuStandard® diluted to a concentration of 4 ng μL^{-1}. The reduced volumes were increased to 1 mL using dichloromethane (HPLC, Merck), transferred to injection vials, and stored at -20 ºC. Qualitative and quantitative analyses of PAHs were performed using a gas chromatograph coupled to a mass spectrometer (GC/MS - Thermo Scientific®, model ISQ), following the EPA method 8270D (EPA, 1996b). Analyses were performed under the following chromatographic conditions: injection volume of 2 µL; Agilent DB-5MS column (30 m; 0.25 mm internal diameter; 0.25 µm film);

carrier gas helium (flow rate of 1.2 mL min^{-1} and purity of 99.999%). The temperature program used was as follows: 50 ºC for 5 min; 50 ºC.min^{-1} up to 80 ºC; 60 ºC.min^{-1} from 80 ºC to 280 ºC; 12 ºC min^{-1} from 280 ºC to 305 ºC; and finally, maintaining a temperature of 305 ºC for 7 min. For quantitative analysis, a curve was prepared by injecting ten standard solutions (1, 2, 5, 10, 20, 50, 100, 200, 400 and 1000 ng mL^{-1}) containing all non-alkylated PAHs to be analyzed (Naphthalene, Acenaphthylene, Acenaphthene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benzo(a)anthracene, Chrysene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(a)pyrene, Indene(1,2,3 -cd)pyrene, Dibenzo(a,h)anthracene and Benzo(g,h,i)perylene) and the deuterated internal standards already mentioned (each at a concentration of 100 ng L^{-1}). The quantification limit was calculated as the ratio between the concentration of the lowest standard solution (1ng mL^{-1}) on each calibration curve and the volume of the extracted sample.

3.2.3 Statistical analysis

Softwares Excel® and Statistica® 7.0 were used for statistical analyses. The data from these groups were found to be normally distributed according to Kolmogorov-Smirnov and Shapiro-Wilk tests. The values were compared through T-tests and significant differences were represented by a coefficient $p < 0.05$. The similarity analysis based on Euclidean distances was also performed for comparison between samples.

3.3 Results

The method's quantitation limit was 1 ng L^{-1}. The average concentration of the ∑16 PAHs was 135.502,51 ng L^{-1} (± 26.688,79 ng L^{-1}) for OWD and 410.302,37 ng L^{-1} (± 246.650,29 ng L^{-1}) for WAF (Table 1). Naphthalene was the most abundant PAH in OWD and WAF diesel oil dispersions, representing 98.56% of the PAHs in OWD samples and 98.89% in WAF samples.

Table 1 – Concentration of the 16 polycyclic aromatic hydrocarbons (PAHs) analyzed in Water-Accommodated Fraction (WAF) and Oil-in-Water Dispersion (OWD). Values expressed in ng L^{-1}. Mean ± standard deviation. Limit of quantification = 1 ng L^{-1}.

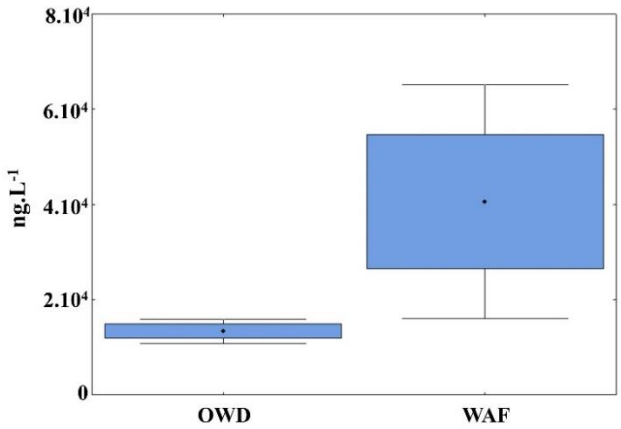
PAHs	OWD (mean ± sd)	WAF (mean ± sd)	Benzene rings	Molecular weight range
Naphthalene	133552,9 ± (25887,75)	405735,13 ± (245057,46)	2	Low
Acenaphthylene	324,89 ± (159,38)	728,23 ± (285,55)	2	Low
Acenaphthene	419,83 ± (225,78)	877,04 ± (341,76)	2	Low
Fluorene	376,01 ± (203,34)	868,77 ± (328,99)	2	Low
Phenanthrene	457,74 ± (217,37)	1279,89 ± (455,97)	3	Low

Anthracene	33,67 ± (16,1)	93,9 ± (33,16)	3	Low
Fluoranthene	55,6 ± (20,44)	94,54 ± (63,58)	3	Low
Pyrene	162,22 ± (75,57)	469,26 ± (156,88)	4	Medium
Benzo(a)anthracene	10,79 ± (4,47)	16,29 ± (6,45)	4	Medium
Chrysene	30,26 ± (11,21)	54 ± (18,58)	4	Medium
Benzo(b)fluoranthene	22,07 ± (12,4)	22,59 ± (9,64)	4	Medium
Benzo(k)fluoranthene	3,79 ± (1,89)	3,68 ± (1,22)	4	Medium
Benzo(a)pyrene	28,57 ± (14,35)	27,55 ± (10,88)	5	High
Indene(1,2,3 -cd)pyrene	2,82 ± (0,23)	4,44 ± (2,42)	5	High
Dibenzo(a,h)anthracene	4,62 ± (1,87)	7,02 ± (2,38)	5	High
Benzo[ghi]perylene	17,67 ± (9,16)	21,51 ± (8,16)	6	High
∑16 PAHs	135502,51 ± (26688,79)	410302,37 ± (246650,29)	NA	NA

Source: Produced by the autor. NA: Not applicable. PAHs molecular weight References: Peng et al,, 2016; Alexandrino *et al.*, 2024.

Although the average of the sum of the ∑16 PAHs concentration values was higher in WAF than in OWD, no significant differences were detected between the two methods (Paired t test, t4 = -1.91, P = 0.13). The standard deviation for WAF was larger than for OWD, indicating greater variability between replicates (Fig. 2).

Figure 2 - Mean, standard errors (boxes), and standard deviation (bars) of 16 polycyclic aromatic hydrocarbons (PAHs) in Water- Accommodated Fraction (WAF) and Oil-in-Water Dispersion (OWD) samples.

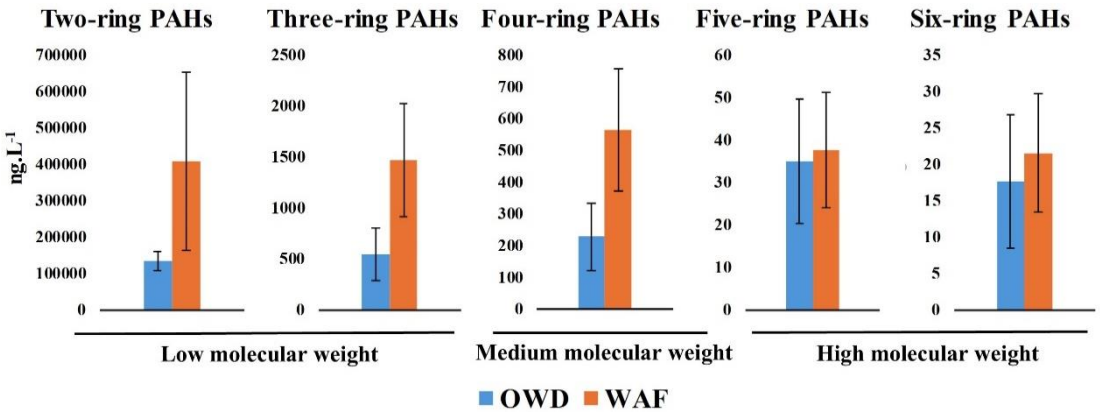


Source: Produced by the author.

PAHs were analyzed considering the number of benzene rings and the molecular weight to understand whether different classes of PAH would behave differently on the evaluated dispersion methods. PAHs with two benzene rings closely resembled the findings for ∑16 PAHs, because naphthalene, the most abundant PAH in the oil used, belongs to this group. Low and medium molecular weight PAHs were generally more concentrated in WAF than in OWD, with larger variability among sample concentrations

in the former method (Table 1, Fig. 3). For high molecular weight PAHs, with five and six benzene rings, the concentrations were similar for OWD and WAF, with high standard deviations for both methods. When applying the Paired t-test to compare PAH concentrations for each class of molecular weight, as represented by the number of benzene rings, no significant differences were found between WAF and OWD (two rings: t4 = -1.91, P = 0.13; three rings: t4 = -2.62, P = 0.06; four rings: t4 = -2.66, P = 0.05; five rings: t4 = -0.21, P = 0.84; six rings: t4 = -0.54, P = 0.61).

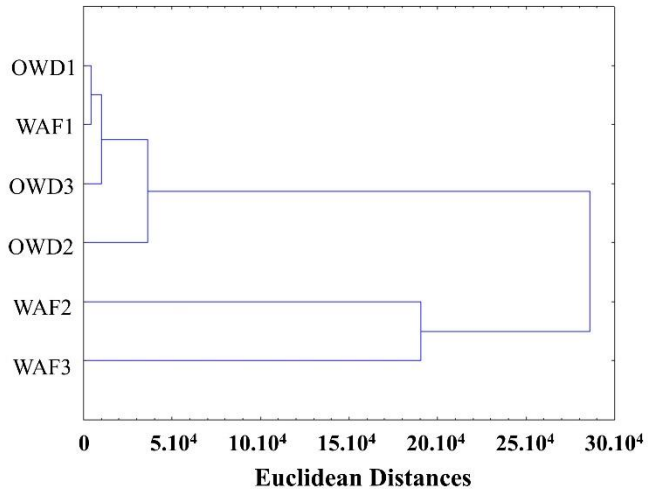
Figure 3 - Mean and standard deviation of polycyclic aromatic hydrocarbons (PAHs) separated by number of benzene rings and molecular weight in Water- Accommodated Fraction (WAF) and Oil-in-Water Dispersion (OWD) samples.



Source: Produced by the author.

Cluster analysis based on Euclidean distances (Fig. 4) for PAH values revealed two large groups. The first group consists of all OWD replicates and one WAF replicate (WAF1), with a Euclidean distance index of 36.044. The second group, with lower similarity, formed by the remaining WAF replicates, has an index of 190.462. The Euclidian distance between groups 1 and 2 is 285.858. These results indicate that the OWD replicates are closer to each other compared to the WAF replicates.

Figure 4 - Cluster analysis (Euclidean distances) of Water- Accommodated Fraction (WAF) and Oil-in-Water Dispersion (OWD)samples including values of 16 individual polycyclic aromatic hydrocarbons (PAHs).



Source: Produced by the author.

3.4 Discussion

The OWD and WAF adapted protocols were efficient in producing oil in water dispersions and yielded average PAH levels ranging between 135.502 ng L^{-1} and 410.302 ng L^{-1}, respectively, which are comparable to the observed in natural environments following oil spill incidents. For comparison, Cripps and Shears (1997) recorded total PAH values ranging from 11.300 to 222.000 ng L^{-1} in seawater after a large diesel oil spill in Antarctica. In a long-term study, Fernández-Tajes *et al.* (2011) analyzed water samples collected four years after the oil tanker Prestige oil spill in Spain. They recorded total PAHs ranging from 200 to 800 ng L^{-1}, which were significantly higher than the levels found at the non-impacted reference station.

Although the same dilution factor was applied to both techniques, higher concentrations of PAHs were obtained through WAF than OWD protocol. In bioassays, the depletion of PAHs was previously attributed to evaporation and adhesion to the containers' walls during dispersion preparation and over experiments (Carrasco-Navarro *et al.*, 2015; Weinnig *et al.*, 2020). Since OWD preparation involves a higher energy agitation than WAF, evaporation tends to be more relevant, leading to lower PAH concentrations. Some studies indicate that wind speed, a factor that promotes agitation on the sea surface, is one of the most significant factors for the evaporation of PAHs after oil spills (Stiver & MacCay, 1984; Ramírez *et al.*, 2017; Wang *et al.*, 2021).

The lighter fractions of oil are more volatile and tend to be the first to evaporate (Mishra & Kumar 2015; Pimsee *et al.*, 2014). This should explain why low and medium

molecular weight PAH, such as the two-benzene ring naphthalene, were less abundant in OWD than in WAF. In contrast, the concentrations obtained for the heaviest compounds, with five and six benzene rings, were very similar between OWD and WAF samples, suggesting that evaporation of low molecular weight volatile compounds may be the main factor driving PAH depletion in OWD dispersion. According to Wolska *et al.* (2005), PAHs with higher boiling points, and consequently higher molecular weights (Achten & Andersson, 2015), are more prone to adhere to the walls of experimental units. In the present study, the concentrations of PAHs with five and six benzene rings were similar between the OWD and WAF methods, suggesting that adhesion to the container's walls occurred at an approximately equivalent level.

Contrasting results were observed by Echols *et al.*, (2015), which obtained higher levels of PAHs and more acute toxicity in OWD than WAF. However, in this study, OWD was prepared through manual agitation of crude oil: water dispersion for 2 minutes only. The less vigorous agitation of a heavy crude oil may have reduced the evaporation of volatile PAHs and was only possible because the authors prepared small volumes of OWD for toxicity tests using bacteria and small-bodied mysid shrimps. The production of large volumes of oil in water dispersions poses new challenges and is needed for ecotoxicological studies using large organisms, such as fishes and crustaceans.

Regarding the reproducibility of the tested dispersion methods, the present study showed higher standard deviation values for PAH concentrations in WAF than OWD, especially considering low and medium molecular weight PAHs. The serial dilution of WAF dispersion may result in higher variability among replicates observed for this method compared to OWD. Since the magnetic stirring of large volumes requires laboratory infrastructure often unavailable for researchers, WAF serial dilution was performed to evaluate the effectiveness and reproducibility of the method when it is unfeasible to magnetically stir the entire required volume of water-oil dispersion. However, our results confirm that the serial dilution of WAF is not recommended, because the variable solubility of individual hydrocarbons makes it difficult to achieve homogenization (Aurand & Coelho, 2005).

Considering that the adapted WAF and OWD protocols resulted in PAH concentrations comparable to those observed in oil spill incidents, both methods could be applied to simulation bioassays. The proposed OWD-adapted protocol is a simple technique to produce diesel oil: water dispersion with homogeneous replicates. Future studies should compare the efficiency and reproducibility of OWD protocols with different agitation speeds and durations. Also, it is advisable to implement contingency measures to minimize variability among replicates, for example, fractionally collecting aliquots from the initial stock and randomly distributing them among replicates. Finally, monitoring

each experimental unit through PAH analysis is recommended to have full control of the experiment, regardless of the chosen dispersion method.

3.5 Conclusion

The toxicity studies involving large organisms require the preparation of large volumes of oil in water dispersions. In the absence of updated standardized protocols, researchers may adapt the original methods according to their needs, sometimes without proper evaluation of their effectiveness and reproducibility. The present study compared two adapted methods used to disperse diesel oil in water, and both proved to be efficient. OWD-adapted protocol resulted in lower PAH values, particularly for compounds with low and medium molecular weights. This may be attributed to increased evaporation caused by the higher energy associated with this technique. Despite producing higher PAH values, WAF adapted protocol displayed higher variability among replicates, possibly because of serial dilution. Considering the challenges of preparing large volumes of oil in water dispersions, we suggest OWD is a better fit, but further studies should be realized to improve the method. Further, we recommend implementing PAH analysis for each replicate, independently of the method of choice, as a good practice to reduce uncertainty regarding the oil in water dispersions used in scientific studies. Considering that the results obtained were related to diesel, a light oil, it would be interesting to conduct similar tests with other types of oils that have different compositions.

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3.8 Data availability statement

Data will be made available on a reasonable request to the corresponding author.

3.9 Financial interests

The authors declare they have no financial interests.

3.10 Conflict of interest disclosure

The authors declare no conflicts of interest.

3.11 Ethics approval

The ethics approval does not apply to this study.

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4 Capítulo 4 - Polycyclic aromatic hydrocarbons and stable isotopes analysis of oysters *Crassostrea brasiliana* following exposure to diesel fuel oil-water dispersion.

Abstract

The physical characteristics and chemical composition of petroleum can have harmful effects on marine organisms. To assess and measure contamination in surviving organisms, it is essential to choose appropriate indicators. The quantitative analysis of PAHs found in the tissues of marine organisms is commonly employed to detect marine oil contamination. Additionally, stable isotopes have distinct signatures in oil and can be utilized to investigate pollution at a physiological level. However, interpreting this information can often be challenging due to the numerous variables in the environment. In this context, bioassays provide the opportunity to quantitatively examine contaminants in organisms in a controlled setting. The current study aims to assess the presence of diesel oil in the oyster *Crassostrea brasiliana* by analyzing PAHs and stable isotopes. The oysters were exposed to oil-water dispersion (OWD) diesel oil for 24 hours, 48 hours, and 7 days. The concentrations of PAHs in the OWD decreased rapidly, resulting in a reduction of oyster exposure to 10% of PAHs after 48 hours from the commencement of the experiment. *Crassostrea brasiliana* was able to bioaccumulate PAHs until 48 hours and eliminated these components by the end of the experiment. Significant differences in the stable ^{13}C and ^{15}N isotopes contents were not observed between the “control” and “treatment” oysters, which could be attributed to a short exposure time. Future bioassays should consider medium-term oil exposure effects on oysters' stable isotope signatures.

Keywords: Environmental assessment; bioindicator; bioaccumulation; bioassay.

4.1 Introduction

Petroleum is an organic compound with harmful characteristics for the marine biological community. The effects caused by petroleum and its derivatives largely depend on their composition. Heavier oils, such as crude oil, tend to cause more physical damage due to their high viscosity, in addition to having a greater carcinogenic potential (Serkovskaya, 1996; Pimsee *et al.*, 2014; IPIECA, 2015; Patel *et al.*, 2020). Lighter oils, such as diesel oil, are more soluble and volatile, and have acute toxicity (Neff *et al.*, 2000; Hansen *et al.*, 2013; Pimsee *et al.*, 2014; IPIECA, 2015; Patel *et al.*, 2020). Adherence to body surfaces can cause loss of mobility, block sensory organs, and lead

to death, while high toxicity results in lethal and sublethal effects (IPIECA, 2015). In benthic organisms, lethal effects cause population reduction and can be directly quantified (Lira *et al.*, 2021; Craveiro *et al.*, 2021; da Rosa, 2022; Yaghmour *et al.*, 2022). On the other hand, marine organisms in a region impacted by oil do not always die due to multiple factors (Filho *et al.*, 2024), and the sublethal effects are not always clear. Therefore, it is interesting to select biochemical indicators that allow the quantification of contamination. A classic method for identifying and quantifying oil contamination is the analysis of Polycyclic Aromatic Hydrocarbons (PAHs) present in the tissues of marine organisms (Hamacher *et al.*, 2022; Soliman *et al.*, 2022; Al-Shamary *et al.*, 2023).

Another potentially useful technique is the quantification of stable isotopes, as they are linked to the sources carbon and nitrogen consumed by a given organism (Wang *et al.*, 2020; Vezzone, 2020; Srinivas *et al.*, 2022). These isotopes have been utilized as markers to identify sublethal oil impacts on marine organisms (Botello *et al.*, 1982; Graham *et al.*, 2010; Park *et al.*, 2022). As an example, ^{13}C rates were previously used as a biochemical indicator by Graham *et al.* (2010) to assess the impact of the Deepwater Horizon oil spill on phytoplankton. When conducting studies using biochemical markers, it is crucial to select effective bioindicator organisms.

Bivalves, benthic filter feeders, have been employed as bioindicators for their capacity to accumulate PAHs in their tissues (Idowu *et al.*, 2020; Gan *et al.*, 2021; Soliman *et al.*, 2022). Studies of oil spills using bioindicators have been frequently employed, generating a large amount of information (Gesteira & Dauvin, 2000; Bolognesi *et al.*, 2006; Ferreira *et al.*, 2023). Thus, to verify the effects of contaminants directly on the organism, an extremely efficient way is to carry out bioassays, since it is possible to control the variable factors in the environment, reaching a direct response (Briand, 2009; Podlesińska & Dąbrowska, 2019; Luan *et al.*, 2020; Rakaj *et al.*, 2021). The present study aims toevaluate the contamination of diesel oil in the oyster *Crassostrea brasiliana* through a bioassay, analyzing PAHs and stable isotopes.

4.2 Material and methods

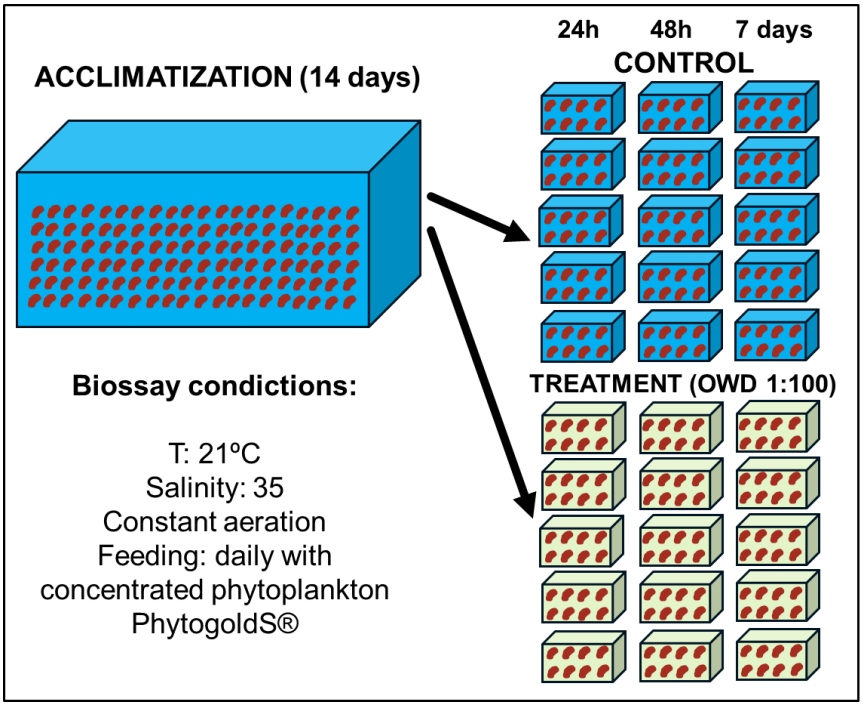
4.2.1 Experimental design

The experimental design is illustrated in Figure 1. *Crassostrea brasiliana* adult specimens of similar size were obtained from an oyster farming area at the Marine Mollusks Laboratory (Santa Catarina Federal University) in Florianópolis, Brazil. The oysters were screened to remove associated epibiont organisms, acclimatized for 14 days in a tank with artificial seawater (Fauna Marin® Professional Sea Salt) at constant

temperature (21ºC) and salinity (35) and fed with suspension phytoplankton (Brightwell PhytogoldS®) once a day.

Oil-water dispersed (OWD) mixture was prepared by adding diesel oil to artificial seawater (1:100), following the method proposed by Echols *et al.*, (2015) with modifications. Two hundred liters of OWD were agitated for 24 hours using three underwater pumps (Sarlobetter® pump / 1000 L h^{-1}) in a sealed container, protected from light to minimize volatilization and photochemical degradation of fuel components. The mixture was introduced into aquariums at a final volume of 9 liters. Oysters were exposed to OWD (Treatment) or artificial seawater (Control) for 24 hours, 48 hours, and 7 days. Phytoplankton suspension (Brightwell PhytogoldS®) was added to aquariums once a day. Five replicates, each containing eight oysters, were utilized for each experimental period, resulting in a total of 30 aquariums. One-liter samples of control artificial water and OWD solution were collected at each replicate at the beginning of the experiment (0 hours), and after 24 hours, 48 hours, and 7 days through siphoning Samples were stored at 8 ºC for the analysis of PAH concentration. The oysters were dissected, and three oysters were used to evaluate PAHs, one oyster was used for stable isotopes analysis and four oyster used for analyses of gene expression, antioxidant enzymes and histologic evaluation (data not presented here). To PAHs analysis the total tissue of each oyster was utilized. For stable isotope analysis, the gill, hepatopancreas, and muscle were separated, and the samples were subsequently frozen at -20 ºC.

Figure 1 – Experimental design.



Source: Produced by the author.

4.2.2 PAHs analysis

4.2.2.1 Seawater PAH extraction

The extraction of PAHs was performed using the EPA method 3510C (EPA, 1986a). Aliquots of 1 L of seawater and 30 mL of HPLC-grade dichloromethane (LICHROSOLV®) were transferred to separatory funnels. To validate the procedure, 100 ng of surrogate standard p-terphenyl-d14 (25 µL of a 4 ng μL^{-1} solution; Sigma-Aldrich®) were added. The PAH extraction method is considered appropriate if the recovery of the surrogate standard p-terphenyl-d14 is between 40% and 120% (Sauer & Boehm, 1995). The extraction was carried out by vigorously shaking for 3 minutes, followed by resting for 10 minutes for complete separation of the phases. The solvent was transferred to TurboVap® tubes, and the entire procedure was repeated twice, using a total of 90 mL of solvent. The extracts were then reduced in TurboVap™ under a nitrogen flow. After that, 100 ng of internal standard (25 µL of a 4 ng μL^{-1} solution; AccuStandard®) mixtures were added, including deuterated compounds naphthalene-d8, acenaphthene-d10, 1,4-dichlorobenzene-d4, phenanthrene-d10, chrysene-d12, and perylene-d12. After this step, the extract volumes were increased to 1 mL using dichloromethane, transferred to sealed injection vials, and stored at -20 ºC.

4.2.2.2 Oyster extraction, clean-up and extract fractioning

Tissue samples were separately homogenized with an Ultra Turrax grinder (IKA Works®). About 3 g of each tissue sample were weighed, sodium sulfate was added to remove water, and then 100 ng of p-terphenyl-d14 subrogated standard was added (25 µL of a 4 ng μL^{-1} solution; AccuStandard®). Then, each sample was extracted in Soxhlet for 24 hours with a mixture of 200 mL of dichloromethane and acetone (Supelco®) (9:1) according to EPA 3540C (EPA, 1996a). In addition to the samples, four blanks and three replicates of biological material certified for PAHs (3 g of bivalve reference material IAEA-451) were extracted. The extracts were reduced in a vacuum rotary evaporator (Tecval®) to approximately 1 mL. To reduce the organic load, a cleanup step was performed in a glass column (1.8 cm diameter) filled with n-hexane (LICHROSOLV®) and 20 g of deactivated alumina at 2% water (aluminum oxide 90; SHELF LIFE®). Each sample was added and eluted with 100 mL of dichloromethane. The extract was collected in a TurboVap® flask, reduced under a nitrogen flow. The solvent was changed to n-hexane and reduced to approximately 1 mL. After the cleanup step, fractionation was performed to separate the aliphatic and aromatic hydrocarbons, according to EPA 3630C (EPA,

1996b), by open column chromatography (columns with 30 cm in length and 1.3 cm in internal diameter were used, filled with 10 g of silica 60 (0,063-0,200 mm; SHELF LIFE®), 7 g of deactivated alumina at 2% water and 1 g of deactivated sodium sulfate at 5% water (EMSURE®). For the removal of aliphatic hydrocarbons (subsequently discarded), elution was performed with 30 mL of n-hexane and for aromatics 75 mL of solution with dichloromethane and n-hexane (1:1). The extract fractions containing PAHs were collected directly in TurboVap flasks, and reduced under nitrogen flow and transferred to injection vials after the addition of 100 ng of internal standards mix (as cited in the extraction of PAHs in water) and HPLC-grade n-hexane to a final volume of 1 mL.

4.2.2.3 Gas Chromatography - Mass Spectrometry Analysis

Qualitative and quantitative analyses of PAHs were conducted at the Laboratory of Organic and Marine Geochemistry of the Faculty of Oceanography at the State University of Rio de Janeiro (LAGOM/UERJ). The analyses were performed using a gas chromatograph coupled to a mass spectrometer (GC/MS - Thermo Scientific®, model ISQ), following the EPA method 8270D (EPA, 1986b). Analyses were performed under the following chromatographic conditions: injection volume of 2 µL; Agilent DB-5MS column (30 m; 0.25 mm internal diameter; 0.25 µm film); carrier gas helium (flow rate of 1.2 mL min^{-1} and purity of 99.999%). The temperature program used was as follows: 50 ºC for 5 min; 50 ºC min^{-1} up to 80 ºC; 60 ºC min^{-1} from 80 ºC to 280 ºC; 12 ºC min^{-1} from 280 ºC to 305 ºC; and finally, maintaining a temperature of 305 ºC for 7 min. For quantitative analysis, a curve was prepared by injecting ten standard solutions (1, 2, 5, 10, 20, 50, 100, 200, 400 and 1000 ng mL^{-1}) containing all non-alkylated PAHs to be analyzed (Naphthalene, Acenaphthylene, Acenaphthene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benzo(a)anthracene, Chrysene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(a)pyrene, Indene(1,2,3 -cd)pyrene, Dibenzo(a,h)anthracene and Benzo(g,h,i)perylene). The limit of quantification was calculated as the ratio between the concentration of the lowest standard solution on the calibration curve and the volume of the extracted sample (1 ng mL^{-1}). The limit of detection was determined by constructing a calibration curve for each of the 16 PAHs and identifying the first point on this curve.

4.2.3 Stable isotopes analysis

For the quantification of stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) and percentages of organic carbon (C) and nitrogen (N), 0.6 mg of each sample of freeze-dried oyster tissue (gill, hepatopancreas and muscle) were weighed on a precision scale and placed in tin capsules. The quantification of TC, TN and $\delta^{13}C$ and $\delta^{15}N$ values was performed using an elemental analyzer coupled to an isotopic ratio mass spectrometer (EA-IRMS; Thermo Electron Corp., Bremen, Germany). Vezzone et. al., (2020) describe the operation of the equipment. To calculate the analysis error, protein standards (B2155 PROTEIN: $\delta^{13}C = -26.98 \pm 0.13$, $\delta^{15}N = 5.94 \pm 0.08$, glycine ($\delta^{13}C = -20.29 \pm 0.04$, $\delta^{15}N = -20.68 \pm 0.06$) and urea standards (IVA33802174 UREA: $\delta^{13}C = -41.3 \pm 0.04$, $\delta^{15}N = -0.32 \pm 0.02$) were used in addition to empty tin capsules (blank). The measured analytical error was then ± 0.4 ‰ for $\delta^{13}C$, ± 0.4 ‰ for $\delta^{15}N$, ± 0.5 % for TC and ± 0.6 % for TN.

4.2.4 Data analysis

The softwares Excell®, Statistica 7.0® and R version 4.2.2. were used for statistical analyses. Data were considered normal after Kolmorogov-Smirnov and Shapiro Wilk tests. Analyses of variance (One-Way ANOVA) were performed for PAHs. When significant differences were found, represented by a coefficient $p < 0.05$, the multiple comparisons test (Tukey) was applied, aiming to identify where the differences are (ZAR, 1996). A t-test was applied to stable isotopes to focus on the differences between the control and treatment groups at each experimental time point. The bioaccumulation of PAHs in oyster tissues was demonstrated through a regression analysis, which included a correlation coefficient. To compare PAHs with stable isotopes, a Spearman correlation was conducted.

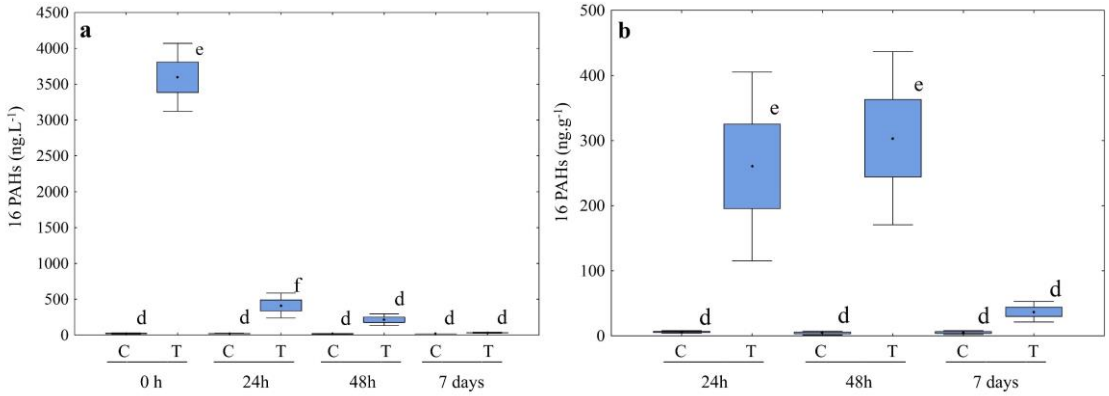
4.3 Results

4.3.1 Seawater and tissue PAHs concentration

The surrogate standard p-terphenyl-d14 recovery was between 40% and 120% in all samples, which is considered adequate (Sauer & Boehm, 1995). The water samples from the “treatment” tanks showed the highest concentrations of PAHs at the beginning of the experiment (0 h; ∑16 PAHs mean = 3.595ng L^{-1}). Naphthalene was the major quantified compound through the experiment on both oyster tissue and OWD

samples (Supplementary Material Table 1 and 2). After 24 hours, the mean for the ∑16 PAHs concentration in water was approximately ten times lower when compared to the initial time, even though it remained significantly higher than the control samples (fig. 2a). The 16 PAH average concentration continued to decrease 48 h and 7 days after the beginning of the experiment, reaching values similar to the control at the end (fig. 2a). Oyster tissues accumulated PAHs 24h and 48h after OWD addition to the tanks (∑16 PAHs mean = 260 ng g^{-1} and 303 ng g^{-1} respectively) (table 2). The values obtained for treatment samples at 24 h and 48 h were significantly higher than the control ($p < 0,05$), but after seven days this difference did not occur (fig. 2b).

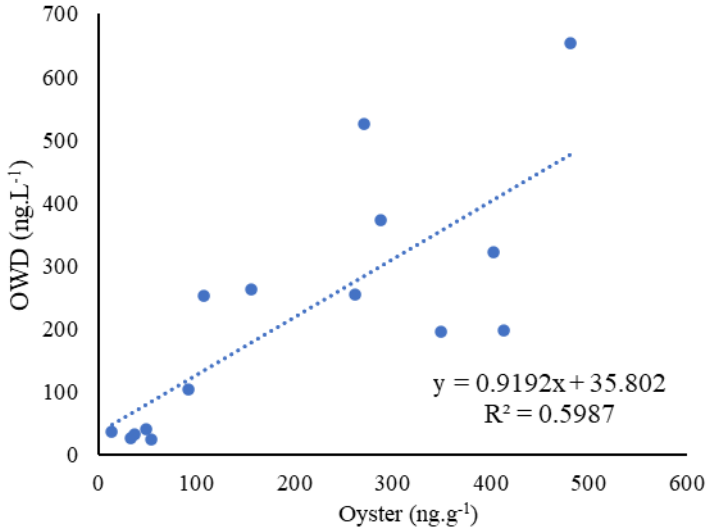
Figure 2 – Means, standard error and standard deviation of the ∑16 PAHs in the water (a) and oyster samples (b) during the study period (0 h, 24 h, 48 h, and 7 days). Different letters above the boxes indicate statistically significant differences (One-way Anova: water: $F(7, 31) = 227.46$, $p = 0,000001$; Oyster: at: $F(5, 24) = 15.09$, $p = 0,000001$). Significant differences in Tukey test: $p < 0.05$. C = control; T = treatment.



Source: Produced by the author.

Analyzing only the oysters exposed to OWD through a regression analysis, a positive correlation between PAH values for OWD and oyster tissues can be identified (Coefficient of Determination = 0.60; fig. 3).

Figure 3 – Regression analysis of ∑16 PAHs between oyster and OWD on treatment samples at 24 h, 48 h and 7 days, including the coefficient of determination.

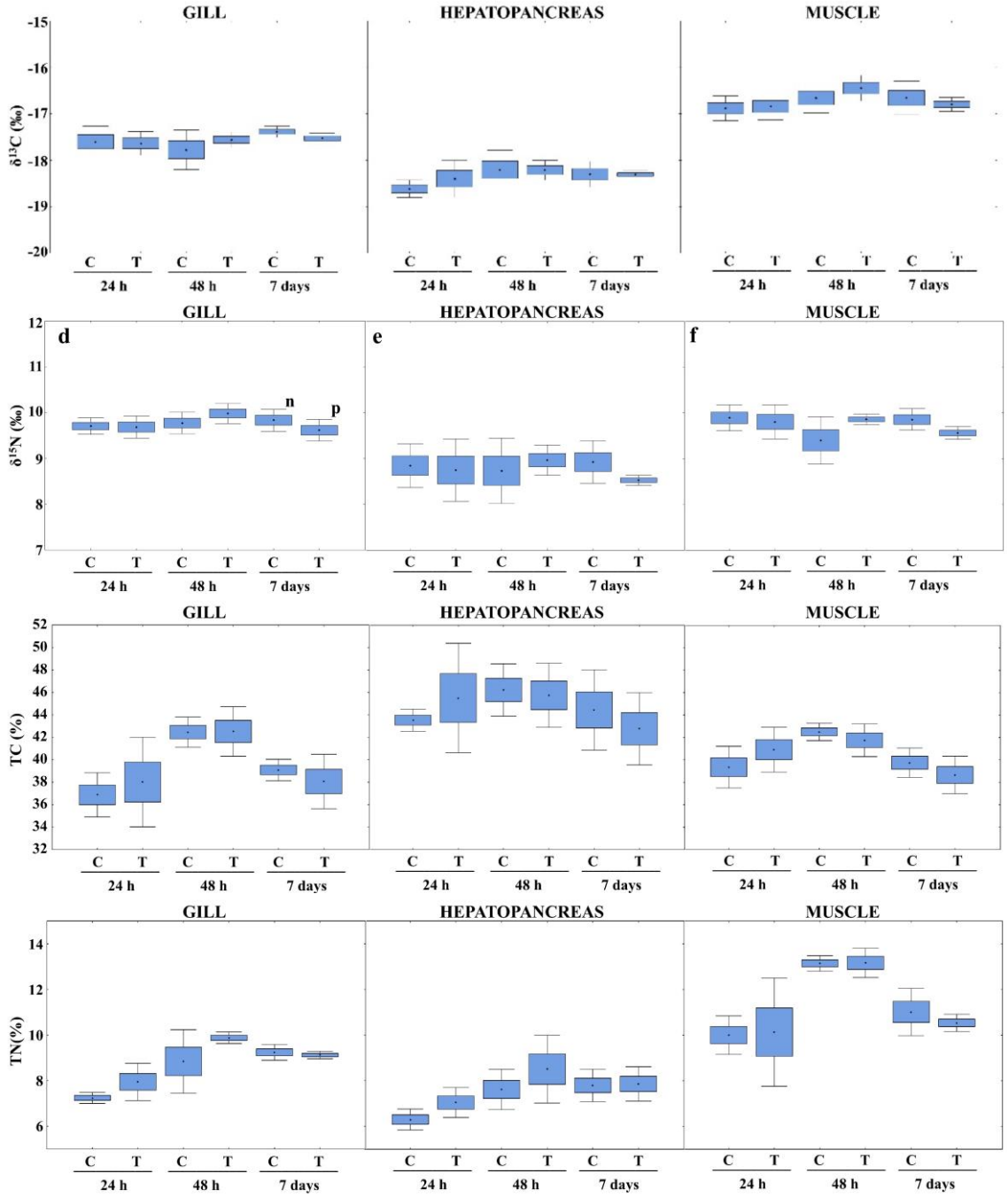


Source: Produced by the author.

4.3.2 Stable isotopes, TN and TC

In general, the values obtained for stable isotopes, TN and TC were similar between the control and treatment groups across all analyzed tissues and experimental time points, with no significant differences observed (n = 5; p varied between 0.07 and 0.97) (Fig. 4; Table 3). The exception was noted for $\delta^{15}N$ in the gills after 7 days of exposure, where very similar average values were observed; however, a significant difference was found between the treatment and control groups (n = 5; p = 0,04).

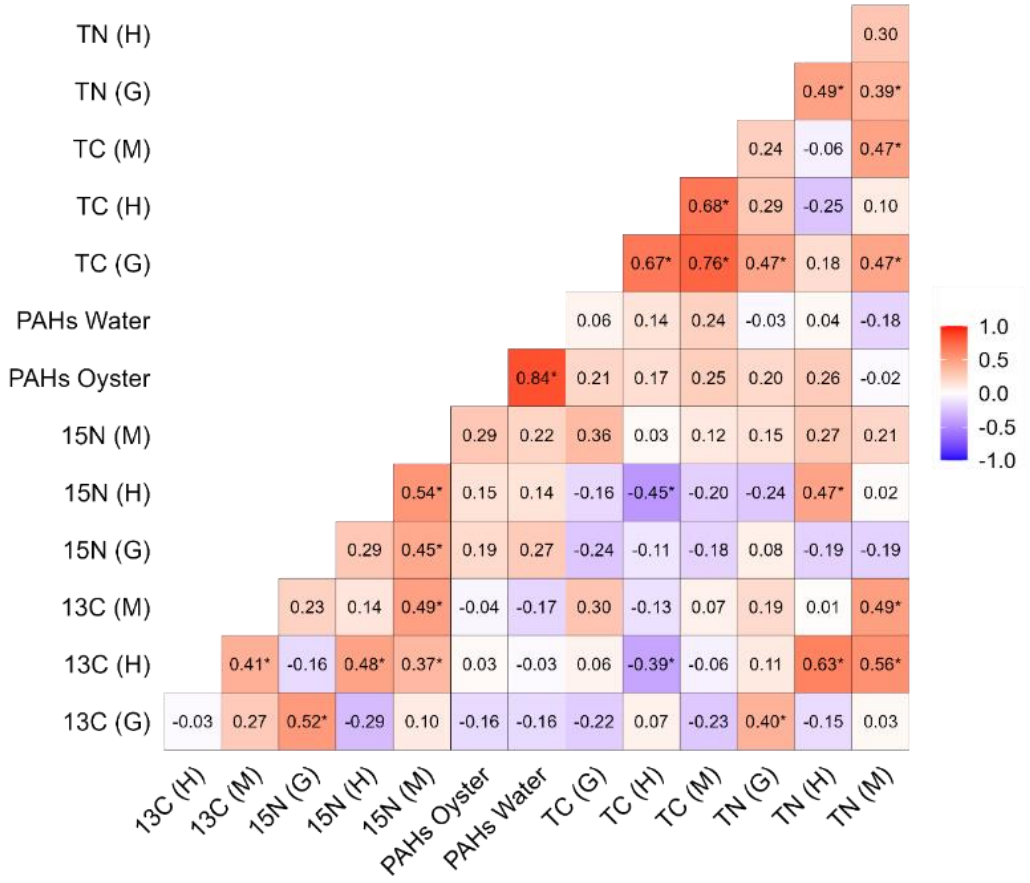
Figure 4 – Means, standard errors and standard deviation of $\delta^{13}C$, $\delta^{15}N$, TC and TN, in oyster tissues during the study period (24 h, 48 h, and 7 days). Different letters above the boxes indicate statistically significant differences. T-tests for each group: df = 8; n = 5; significant differences when p < 0,05. C = control; T = Treatment.



Source: Produced by the author.

Regarding the correlation matrix, the high correlation between the values obtained for PAHs in OWD and oysters stands out (fig. 5). Also noteworthy is the low correlations of PAHs values with all indicators linked to elemental and isotopic variations.

Figure 5 – Correlation matrix between ∑16 PAHs, stable isotopes, percentage of carbon and nitrogen in oyster samples (Pearson's Rank). The PAHs were measured in all oyster tissues, while the other indicators were measured in the gills, (G) hepatopancreas (H) and muscle (M); $^{13}C = \delta^{13}C$; $^{15}N = \delta^{15}N$; TN = percentage of nitrogen; TC = percentage of organic carbon. Symbol "*" indicates significative correlation.



Source: Produced by the author.

4.4 Discussion

4.4.1 Polycyclic aromatic hydrocarbons

The present study revealed an accelerated decrease in the PAH values in the treatment units during the first two days of the experiment. In the same way, depletions were previously reported in other bioassays (Couillard *et al.*, 2005, de Soysa *et al.*, 2012, Weinnig *et al.*, 2020; Delunardo *et al.*, 2020). These reductions were attributed both to the adhesion of organic compounds to the aquarium walls and to their volatilization (Carrasco-Navarro *et al.*, 2015, Weinnig *et al.*, 2020). Cripps & Shears (1996) found a similar pattern in a large diesel oil spill in a natural environment, with rapid depletion of total PAHs in the first 24 hours, possibly due to volatilization. These physical-chemical

processes could partially explain the percentage of PAH reduction observed in our experiments. We also observed oyster's PAH bioaccumulation, as evidenced by the progressive increase in PAH values for oyster tissues in the first 48 hours of the experiment. Similarly, Lüchmann, *et al.* (2014) analyzed sample units contaminated with phenanthrene and observed an exponential reduction where oysters were present, which did not occur in experimental units without these organisms. It is important to highlight that the volatility of phenanthrene is not as high as that of naphthalene, which is the main PAH of the OWD analyzed. This factor contributes to a greater availability of this PAH for the oysters analyzed by Lüchmann, *et al.* (2014).

Naphthalene was the major PAH in OWD, as expected for diesel oil-water solutions (Lüchmann *et al.*, 2011; Delunardo *et al.*, 2020) (tables 1 and 2 of supplementary material). This pattern was also observed in oyster tissue samples. Naphtalene is a low molecular weight compound, with only two benzene rings, which enhances its solubility and bioaccumulation in bivalve tissues (Patel *et al.*, 2020; Gan *et al.*, 2021). The high initial concentrations of this compound recorded in the OWD and the subsequent bioaccumulation in oysters confirm this pattern. Wang *et al.* (2017) recorded approximately 750 ng g^{-1} of benzo[a]pyrene in the oyster *Pinctada martensii* tissues after a 24h exposure to 3640 ng L^{-1} benzo[a]pyrene aqueous solution. Our values for naphthalene in oysters and OWD were 246 ng g^{-1} and 3518 ng L^{-1} respectively (tables 1 and 2 in the supplementary material). These values were comparable to those reported by Wang *et al.* (2017), despite differences in the contaminant matrices used (mechanically solubilized diesel oil versus chemically solubilized benzo[a]pyrene). It is worth noting that benzo[a]pyrene is a high molecular weight PAH. Wang *et al.* (2017) further documented the purification capacity of oysters after removing contaminants. A similar outcome was observed in the current study. By the end of the experiment, with decreased concentrations of PAHs in the water, the PAH values in oysters were similar in treatment and control aquariums. The purification process may be a result of the biotransformation capabilities of oysters, which convert PAHs into excretable compounds (Gan *et al.*, 2021; Ferreira *et al.*, 2023). The bioaccumulation of PAHs in oyster tissues during the first 48 hours, followed by purification after 7 days, may provide significant temporal insights for the management of oil spills at sea.

Oysters have been used as bioindicators in a series of studies regarding the presence of PAHs in water, both through bioassays (Lüchmann *et al.*, 2011; Wang *et al.*, 2017; López-Landavery *et al.*, 2019) and field studies (Idowu *et al.*, 2020; Wang *et al.*, 2020; Soliman *et al.*, 2022). The strong positive correlation found in the present study between PAHs values in OWD and oyster tissues reinforces the ability of this bivalve to bioaccumulate these compounds when they are available in the environment.

4.4.2 Stable isotopes

The absence of significant differences in $\delta^{13}C$, $\delta^{15}N$, TC and TN between control and treatment samples (fig. 4) indicates that the exposure to diesel oil dispersed in water did not alter the isotopic and elemental signatures of *Crassostrea brasiliana* during a seven-day monitoring period. This could be explained by a slow turnover of bivalves. Carmichael *et al.* (2012) found similar $\delta^{13}C$ values 60 days after transplanting *Crassostrea virginica* from a pristine to an oil-contaminated area. Fukumori *et al.*, (2008) estimated the turnover of $\delta^{13}C$ and $\delta^{15}N$ in *Pinctada fucata martensii* to be above 120 days, while Riera and Richards (1997) reached a lower time interval for *Crassostrea gigas*, between 30 and 60 days for a complete change. However, in the context of a bioassay, the absence of an isotopic shift in the tissues of oysters exposed to diesel oil in the present study is quite revealing. Most in situ stable isotopes studies face challenges in establishing a cause-and-effect relationship due to the multiple sources contributing to the final isotopic signature found in the tissues of the analyzed organisms (Carmichael *et al.*, 2012; Wilson *et al.*, 2016; Peterson *et al.*, 2017).

4.5 Conclusion

The oyster *Crassostrea brasiliana* was able to bioaccumulate PAHs from diesel oil while it was present in aquariums, confirming its ability as a bioindicator. In addition, after seven days of experiment, the average concentrations of PAHs in the tissues of oysters exposed to oil, which in 48 hours had reached 303 ng g^{-1}, were similar to those of the control oysters, measuring only 37 ng g^{-1}. This finding is relevant since, in the event of an oil spill, it indicates the duration required for oysters to be purified and can serve as a parameter for managing environmental cleanup efforts. Despite being exposed to high concentrations of diesel oil, *Crassostrea brasiliana* was able to survive and eliminate PAH, suggesting a resilience mechanism. Besides, elemental and isotopic signature were conserved in oil-exposed oysters, possibly due to a high turnover time in bivalves. Future studies should consider long-term oil exposure and OWD exchange to maintain high PAHs concentrations throughout the experiment.

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4.7 Appendix A. Supplementary material

Table 1 – PAH means and standard deviation in water samples over the study period. C = control; T = treatment; and d = days.

	0hC (mean ± dp)	0hT (mean ± dp)	24hC (mean ± sd)	24hT (mean ± sd)	48hC (mean ± sd)	48hT (mean ± sd)	7dC (mean ± sd)	7dT (mean ± sd)	Detection Limit	Quantification limit
Naphthalene	16.95 ± (6.53)	3518.87 ± (470.14)	17.29 ± (4.01)	402.8 ± (172.78)	16.08 ± (4.54)	205.42 ± (80.83)	14.87 ± (1.06)	23.55 ± (6.5)	0,20	1,00
Acenaphthylene	Nd	6.79 ± (1.31)	Nd	Nd	Nd	Nd	Nd	Nd	0,20	1,00
Acenaphthene	Nd	24.67 ± (4.69)	Nd	9.17 ± (3.77)	Nd	6.68 ± (1.32)	Nd	8.23 ± (1.51)	0,16	1,00
Fluorene	Nd	16.87 ± (2.67)	Nd	Nd	Nd	Nd	Nd	0.04 ± (0.09)	0,20	1,00
Phenanthrene	2.31 ± (0.7)	12.89 ± (2.15)	1.7 ± (0.57)	Nd	1.34 ± (0.08)	1.08 ± (2.42)	1.32 ± (0.18)	0.01 ± (0.02)	0,20	1,00
Anthracene	Nd	8.03 ± (1.85)	Nd	Nd	Nd	Nd	Nd	Nd	0,10	1,00
Fluoranthene	1.52 ± (0.3)	3.37 ± (0.91)	1.36 ± (0.67)	Nd	0.85 ± (0.57)	Nd	0.65 ± (0.6)	Nd	0,09	1,00
Pyrene	1.44 ± (0.22)	3.93 ± (0.64)	1.32 ± (0.61)	1.06 ± (1.02)	0.63 ± (0.73)	0.99 ± (0.97)	1.03 ± (0.58)	0.72 ± (0.66)	0,09	1,00
Benzo(a)anthracene	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0,07	1,00
Chrysene	0.62 ± (0.57)	Nd	Nd	Nd	Nd	0.2 ± (0.45)	Nd	Nd	0,18	1,00
Benzo[b]fluoranthene	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0,24	1,00
Benzo[k]fluoranthene	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0,21	1,00
Benzo[a]pyrene	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0,31	1,00
Indeno(1,2,3-cd)pyrene	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0,43	1,00
Dibenz[a,h]anthracene	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0,36	1,00

Benzo[g,h,i]perylene	Nd	Nd	0.43 ± (0.96)	Nd	Nd	Nd	Nd	Nd	0,29	1,00
16 PAHs	22.85 ± (7.15)	3595.41 ± (473.9)	22.1 ± (3.37)	413.03 ± (173.47)	18.9 ± (4.88)	214.37 ± (80.98)	17.88 ± (0.84)	32.55 ± (6.49)	NA	NA
p-Terfenil_d14	63.36 ± (7.67)	64.08 ± (26.94)	75.52 ± (67.64)	103.79 ± (6.83)	68.4 ± (16.91)	80.14 ± (12.97)	74.63 ± (11.82)	62.26 ± (30.13)	-	1,00

Source: Produced by the author.

Table 2 – PAH means and standard deviation in oyster samples over the study period. C = control; T = treatment; and d = days.

PAHs	24hC (mean ± sd)	24hT (mean ± sd)	48hC (mean ± sd)	48hT (mean ± sd)	7dC (mean ± sd)	7dT (mean ± sd)	Clam Standard	Detection Limit	Quantification limit
Naphthalene	0,71 ± (0,79)	246,13 ± (145,95)	0,41 ± (0,4)	276,65 ± (125,36)	1,13 ± (2,54)	24,17 ± (15,38)	0.37 ± (0.35)	0.15	0,31
Acenaphthylene	0,06 ± (0,14)	0,59 ± (0,2)	0 ± (0)	0,82 ± (0,39)	0 ± (0)	0,28 ± (0,19)	1.19 ± (0.18)	0.04	0,31
Acenaphthene	0 ± (0)	1,37 ± (0,32)	0 ± (0)	1,96 ± (0,69)	0 ± (0)	0,56 ± (0,22)	0 ± (0)	0.02	0,31
Fluorene	0,95 ± (0,58)	3,12 ± (0,62)	0,45 ± (0,46)	5,18 ± (2,04)	0,38 ± (0,45)	1,99 ± (0,49)	1.01 ± (0.26)	0.06	0,31
Phenanthrene	2,74 ± (1,25)	4,94 ± (1,29)	1,69 ± (1,1)	9,45 ± (4,99)	1,33 ± (1,03)	4,46 ± (1,13)	7.88 ± (0.67)	0.05	0,31
Anthracene	0,17 ± (0,39)	0,33 ± (0,49)	0 ± (0)	0,81 ± (0,45)	0 ± (0)	0,54 ± (0,38)	0.48 ± (0.3)	0.07	0,31
Fluoranthene	0,34 ± (0,22)	0,64 ± (0,19)	0 ± (0)	1,11 ± (0,54)	0,17 ± (0,24)	0,56 ± (0,4)	33.31 ± (4.97)	0.04	0,31
Pyrene	0,35 ± (0,23)	1,37 ± (0,51)	0,24 ± (0,35)	4,6 ± (4,87)	0,44 ± (0,15)	1,67 ± (1,15)	34.04 ± (6.38)	0.04	0,31
Benzo(a)anthracene	0 ± (0)	0 ± (0)	0 ± (0)	0,06 ± (0,13)	0 ± (0)	0 ± (0)	7.79 ± (0.31)	0.14	0,31
Chrysene	0,24 ± (0,22)	0,62 ± (0,13)	0,41 ± (0,25)	0,68 ± (0,41)	0,1 ± (0,22)	0,54 ± (0,59)	15.08 ± (13.07)	0.08	0,31
Benzo[b]fluoranthene	0,07 ± (0,15)	0,23 ± (0,22)	0,08 ± (0,18)	0,29 ± (0,28)	0,29 ± (0,29)	0,14 ± (0,19)	23.69 ± (2.8)	0.18	0,31
Benzo[k]fluoranthene	0,2 ± (0,18)	0,41 ± (0,65)	0,51 ± (0,37)	0,33 ± (0,4)	0,74 ± (0,79)	0,37 ± (0,24)	13.05 ± (1.31)	0.12	0,31
Benzo[a]pyrene	0,09 ± (0,21)	0 ± (0)	0,09 ± (0,2)	0,08 ± (0,18)	0,08 ± (0,17)	0,11 ± (0,24)	12.05 ± (0.54)	0.04	0,31
Indeno(1,2,3-cd)pyrene	0,15 ± (0,33)	0,53 ± (1,01)	0,47 ± (1,05)	1,5 ± (2,07)	0,48 ± (0,59)	1,36 ± (1,36)	4.8 ± (3.47)	0.05	0,31
Dibenz[a,h]anthracene	0 ± (0)	0,08 ± (0,17)	0,06 ± (0,14)	0,08 ± (0,17)	0 ± (0)	0,31 ± (0,43)	3.58 ± (0.99)	0.07	0,31
Benzo[g,h,i]perylene	0 ± (0)	0,07 ± (0,16)	0 ± (0)	0,08 ± (0,17)	0,06 ± (0,14)	0 ± (0)	11.19 ± (2.37)	0.03	0,31
16 PAHs	6,08 ± (1,83)	260,43 ± (145,06)	4,4 ± (2,87)	303,67 ± (132,97)	5,11 ± (2,87)	37,05 ± (15,82)	169.51 ± (9.47)	NA	NA
p-Terfenil_d14	87,78 ± (7,47)	94,19 ± (17,59)	72,66 ± (10,51)	87,19 ± (14,14)	94,71 ± (19,94)	71 ± (11,06)	86.07 ± (15.33)	-	0,31

Source: Produced by the author.

Table 3 – $\delta^{13}C$, $\delta^{15}N$, TC, TN and TC/TN, means and standard deviation in oyster tissue samples over the study period.

		24 hours		48 hours		7 days	
		Control	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
GILLS	**$\delta^{13}C$**	-17.6 ± (0.34)	-17.64 ± (0.26)	-17.77 ± (0.43)	-17.56 ± (0.16)	-17.39 ± (0.12)	-17.53 ± (0.12)
	$\delta^{15}N$	9.89 ± (0.28)	9.81 ± (0.37)	9.4 ± (0.52)	9.86 ± (0.12)	9.86 ± (0.23)	9.56 ± (0.14)
	TC (wt%)	36.87 ± (3.99)	38.01 ± (3.99)	42.46 ± (1.34)	42.52 ± (2.21)	39.09 ± (0.95)	38.06 ± (2.43)
	TN (wt%)	7.24 ± (0.24)	7.94 ± (0.82)	8.84 ± (1.39)	9.88 ± (0.25)	9.24 ± (0.35)	9.12 ± (0.16)
	TC/TN (atomic)	5.95 ± (0.43)	5.6 ± (0.47)	5.75 ± (1.17)	5.03 ± (0.33)	4.94 ± (0.19)	4.87 ± (0.34)
		24 hours		**48 hours**		**7 days**	
		Control	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
HEPATOPANCREAS	**$\delta^{13}C$**	-18.62 ± (0.19)	-18.4 ± (0.4)	-18.21 ± (0.42)	-18.21 ± (0.21)	-18.3 ± (0.28)	-18.31 ± (0.09)
	$\delta^{15}N$	8.85 ± (0.48)	8.75 ± (0.68)	8.74 ± (0.71)	8.97 ± (0.33)	8.93 ± (0.46)	8.53 ± (0.11)
	TC (wt%)	43.54 ± (4.88)	45.51 ± (4.88)	46.23 ± (2.34)	45.75 ± (2.86)	44.45 ± (3.59)	42.77 ± (3.22)

	TN (wt%)	6.3 ± (0.46)	7.04 ± (0.65)	7.62 ± (0.88)	8.51 ± (1.49)	7.8 ± (0.71)	7.86 ± (0.75)
	TC/TN (atomic)	8.1 ± (0.71)	7.6 ± (1.14)	7.18 ± (1.04)	6.46 ± (1.32)	6.68 ± (0.65)	6.42 ± (0.99)
MUSCLE		**24 hours**		**48 hours**		**7 days**	
		Control	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
	$\delta^{13}C$	-16.88 ± (0.27)	-16.84 ± (0.29)	-16.66 ± (0.32)	-16.45 ± (0.28)	-16.66 ± (0.36)	-16.8 ± (0.15)
	$\delta^{15}N$	9.71 ± (0.18)	9.68 ± (0.24)	9.77 ± (0.23)	9.98 ± (0.22)	9.83 ± (0.24)	9.62 ± (0.23)
	TC (wt%)	39.34 ± (2)	40.91 ± (2)	42.5 ± (0.77)	41.75 ± (1.46)	39.75 ± (1.31)	38.65 ± (1.68)
	TN (wt%)	10 ± (0.84)	10.13 ± (2.37)	13.14 ± (0.34)	13.16 ± (0.64)	11.01 ± (1.04)	10.54 ± (0.38)
	TC/TN (atomic)	4.6 ± (0.22)	5.05 ± (1.79)	3.78 ± (0.16)	3.7 ± (0.1)	4.23 ± (0.27)	4.28 ± (0.22)

Source: Produced by the author.

5 CAPÍTULO 5 - PAHs accumulation analysis of oysters *Crassostrea brasiliana* following exposure to different concentrations of diesel fuel oil-water dispersion.

ABSTRACT

Oil spills have detrimental effects on marine biological communities due to the high toxicity and physicochemical characteristics of these compounds. Efficient biomarkers and bioindicators can be employed to identify these disturbances and promote environmental monitoring. In this context, the implementation of bioassays is particularly useful for defining research strategies. The present study aimed to evaluate the lethal and effect concentrations of polycyclic aromatic hydrocarbons (PAHs) on the oyster species *Crassostrea brasiliana* when exposed to different concentrations of diesel oil.The oysters were exposed to five oil-to-water concentrations (1:5, 1:10, 1:100, 1:1,000 and 1:10.000) for 48 hours. The concentrations of PAHs in both the water and oyster tissues were subsequently analyzed to assess bioaccumulation. Mortality rates were very low, with no major differences between the treatments and controls. The predominant PAHs in the water were the lighter compounds, with naphthalene being particularly notable. Between the initial quantification at the beginning of the experiment and the final measurement after 48 hours, a rapid reduction in water PAH concentrations was observed. Bioaccumulation was detected in the oysters; however, there were no significant differences among the three OWDs with the highest concentrations. This phenomenon is likely attributable to the evaporation of the main PAHs, which resulted in similar outcomes. The heavier PAHs exhibited a smaller reduction in concentration in the water by the end of the experiment, and the lighter PAHs were not prominent in the oyster tissues. The oyster species studied proved to be an effective bioindicator, and based on the data, the PAHs naphthalene, fluorene, phenanthrene, and pyrene should be closely monitored in an event of a diesel oil spill.

5.1 Introduction

Petroleum is an organic compound with harmful characteristics to the marine biological community. Adherence to body surfaces can cause loss of mobility and blockage of sensory organs, while high toxicity leads to lethal and sublethal effects (IPIECA, 2015). Lethal effects are easily quantified (Lira *et al.*, 2021; Craveiro *et al.*, 2021). On the other hand, organisms that remain alive continue to be affected by the oil dispersed in seawater. To identify sublethal disturbances of oil contamination, it is necessary to select physiological and biochemical indicators. Oil spills and their

consequences have been frequently studied and monitored using bioindicators and biomarkers (Gesteira & Dauvin, 2000; Bolognesi *et al.*, 2006; Ferreira *et al.*, 2023; Mulik *et al.*, 2023). However, in order to master the techniques that enable the effective use of these organisms and generate reliable results, it is necessary to carry out tests to recognize all the tools involved (French-McCay *et al.*, 2023).

Bioassays are an excellent way to study an organism's response to contamination because they allow the management of certain environmental variables, enabling a focused analysis on specific markers of interest (Briand, 2009; Podlesińska & Dąbrowska, 2019; Luan *et al.*, 2020; Rakaj *et al.*, 2021; Colvin *et al.*, 2020; French-McCay *et al.*, 2023). PAH analysis is an efficient method for identifying and quantifying the presence of oil in marine organisms (Hamacher *et al.*, 2022; Soliman *et al.*, 2022; Al-Shamary *et al.*, 2023).

Bivalves, benthic filter feeders, have been used as bioindicators for their ability to accumulate PAHs in their tissues (Idowu *et al.*, 2020; Gan *et al.*, 2021; Soliman *et al.*, 2022; Ferreira *et al.*, 2023). Diesel oil is widely used as boat fuel, which means that there is a constant risk of spills and waste disposal in coastal areas (Soares-Gomes *et al.*, 2010; Guerin, 2015; Sardi *et al.*, 2017). While heavier oils are recognized for their greater physical impact, lighter oils, such as diesel, have more acute toxicity (IPIECA, 2015; Pannetier *et al.*, 2024). On the other hand, the composition of the oil is complex, presenting a series of compounds with specific solubilities and toxicities (IPIECA, 2015; Patel *et al.*, 2020). In this line, the different petrogenic PAHs, recognized as toxic, carcinogenic, mutagenic, genotoxic and teratogenic compounds, are also heterogeneous in their properties (Lourenço *et al.*, 2023). Fluorene, for example, is relatively more soluble in water and more volatile, but is not classified as carcinogenic (Patel *et al.*, 2020). Benzo(a)pyrene, one of the most carcinogenic PAHs, is poorly soluble, becoming less bioavailable in the marine environment in oil spills (Patel *et al.*, 2020; Lourenço *et al.*, 2023). In this context, knowledge about the impact of these compounds on marine organisms becomes extremely important. The present study aims to evaluate, through a bioassay, the bioaccumulation of PAHs and the mortality of *Crassostrea brasiliana* exposed to different concentrations of diesel oil: water dispersions.

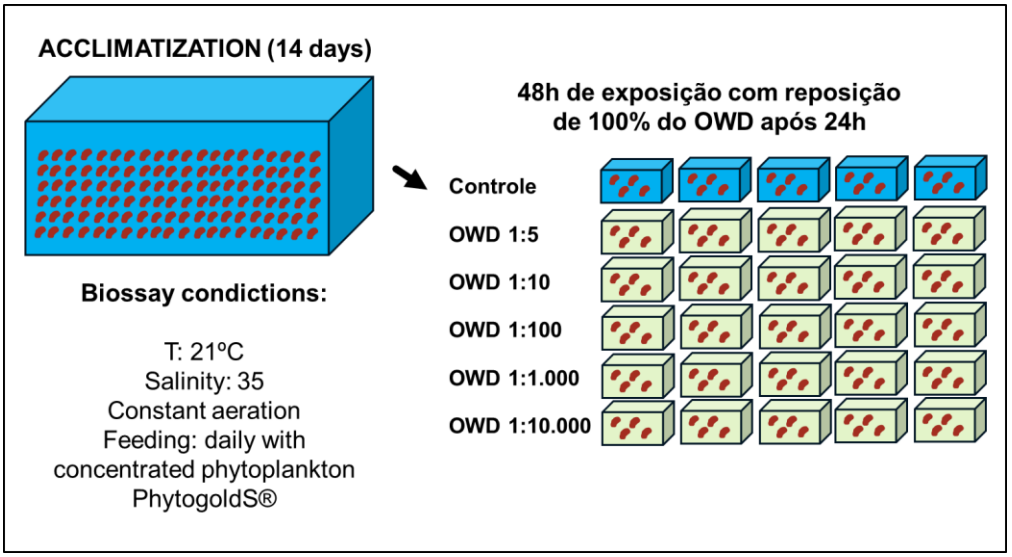
5.2 Methodology

5.2.1 Experimental design

The experimental design is illustrated in Figure 1. *Crassostrea brasiliana* specimens of similar size (approximately 8 cm) were obtained from an oyster farming area at the Marine Mollusks Laboratory (UFSC) in Florianópolis, Brazil. The oysters were cleaned to remove associated epibiont organisms, acclimatized for 14 days in a tank with artificial seawater (Fauna Marin® Professional Sea Salt) at constant temperature (21ºC) and salinity (35) and fed with phytoplankton suspension (Brightwell PhytogoldS®) once a day.

Oil-water dispersed (OWD) mixture was prepared by adding diesel oil to artificial seawater in five crescent concentrations (1:10.000; 1:1.000; 1:100; 1:10; 1:5), following the method proposed by Echols *et al.*, (2015) with modifications. OWD was agitated for 12h using underwater pumps (Sarlobetter® pump / 1000 $L.h^{-1}$) in individual sealed containers for each concentration, protected from light to avoid volatilization and photochemical degradation of fuel components. The mixture was introduced into aquariums at a final volume of 3,5 L. Oysters were exposed to OWD (Treatment) or artificial seawater (Control) for 48h. Five replicates and four oysters per replicate were used for each OWD concentration. The OWD was renewed after 24 hours with a complete 100% replacement to minimize the loss of PAHs due to evaporation and adhesion to the aquarium walls. OWD and control water samples with 1 L were collected from each aquarium for PAH analyzes immediately after preparation (0h), after renewal (24h) and in the end of the experiment (48h). The oysters were dissected and used to evaluate PAHs (2 oysters), gene expression and antioxidant enzyme activity (2 oyster in total, data not presented here). Oyster mortality was assessed after 6, 12, 18, 24 and 48 hours of exposure. Tissues for PAH analysis were frozen at -20 ºC.

Figure 1 – Experimental design.



Source: Produced by the author.

5.2.2 PAHs analysis

5.2.2.1 Seawater PAH extraction

The extraction of PAHs was conducted at the Forensic Environmental Geochemistry Laboratory of the Sea Studies Institute Admiral Paulo Moreira (LGAF/IEAPM) using the protocol based on the EPA method 3510C (EPA, 1986a). Aliquots of 1 L of seawater were transferred to separatory funnels and added 30 mL of HPLC-grade dichloromethane (LICHROSOLV®). To validate the procedure, 100 ng of surrogate standard p-terphenyl-d14 (Sigma-Aldrich®) from 25 µL of a 4 ng μL^{-1} solution were added. The PAH extraction methodology is considered appropriate if the recovery of the surrogate standard p-terphenyl-d14 is between 40% and 125% (Sauer & Boehm, 1995). The extraction was carried out by vigorously shaking for 3 minutes, followed by resting for 10 minutes to allow for complete separation of the phases. The solvent was then transferred to decontaminated bottles, and the entire procedure was repeated two more times, using a total of 90 mL of solvent. The extracts were then reduced in TurboVap™ under a nitrogen flow. After that, 100 ng of internal standard from 25 µL of a 4 ng.μL^{-1} solution mixtures were added, including deuterated compounds naphthalene-d8, cenaphthene-d10, 1,4-dichlorobenzene-d4, phenanthrene-d10, chrysene-d12, and perylene-d12 from AccuStandard®. After this step, the extracts were increased to 1 mL using dichloromethane (HPLC), transferred to injection vials, and stored at -20 ºC.

5.2.2.2 Oyster extraction, clean-up and extract fractioning

Tissue samples were separately homogenized with an Ultra Turrax grinder (IKA Works®). About 3 g of each tissue sample were weighed, sodium sulfate was added to remove water, and then 100 ng of p-terphenyl-d14 subrogated standard was added. Then, each sample was extracted in Soxhlet for 24 hours with a mixture of 200 mL of dichloromethane and acetone (Supelco®) (9:1) according to EPA 3540C (EPA, 1996a). In addition to the samples, four blanks and three replicates of biological material certified for PAHs (3g of bivalve reference material IAEA-451) were extracted. The extracts were reduced in a vacuum rotary evaporator (Tecval®) to approximately 1 ml. To reduce the organic load, a cleanup step was performed in a glass column (1,8 cm diameter) filled with n-hexane (LICHROSOLV®) and 20 g of deactivated alumina at 2 % water (aluminum oxide 90; SHELF LIFE®). Each sample was added and eluted with 100 mL of dichloromethane. The extract was collected in a TurboVap® flask, reduced under a

nitrogen flow. The solvent was changed to n-hexane and reduced to approximately 1 mL. After the cleanup step, fractionation was performed to separate the aliphatic and aromatic hydrocarbons, according to EPA 3630C (EPA, 1996b), by open column chromatography (columns with 30 cm in length and 1.3 cm in internal diameter were used, filled with 10 g of silica 60 (0,063-0,200 mm; SHELF LIFE®), 7 g of deactivated alumina at 2 % water and 1 g of deactivated sodium sulfate at 5% water (EMSURE®). For the removal of aliphatic hydrocarbons (subsequently discarded), elution was performed with 30 ml of n-hexane and for aromatics 75 ml of solution with dichloromethane and n-hexane (1:1). The extract fractions containing PAHs were collected directly in TurboVap flasks, reduced under nitrogen flow and transferred to injection vials after the addition of 100 ng of internal standards mix and HPLC-grade n-hexane to a final volume of 1 ml.

5.2.2.3 Gas Chromatography - Mass Spectrometry Analysis

Qualitative and quantitative analyses of PAHs were conducted at the Laboratory of Organic and Marine Geochemistry of the Faculty of Oceanography at the State University of Rio de Janeiro (LAGOM/UERJ). The analyses were performed using a gas chromatograph coupled to a mass spectrometer (GC/MS - Thermo Scientific®, model ISQ), following the EPA method 8270D (EPA, 1986b). Analyses were performed under the following chromatographic conditions: injection volume of 2 µL; Agilent DB-5MS column (30 m; 0.25 mm internal diameter; 0.25 µm film); carrier gas helium (flow rate of 1.2 mL min^{-1} and purity of 99.999%). The temperature program used was as follows: 50 ºC for 5 min; 50 ºC min^{-1} up to 80 ºC; 60 ºC min^{-1} from 80 ºC to 280 ºC; 12 ºC min^{-1} from 280 ºC to 305 ºC; and finally, maintaining a temperature of 305 ºC for 7 min. For quantitative analysis, a curve was prepared by injecting ten standard solutions (1, 2, 5, 10, 20, 50, 100, 200, 400 and 1000 ng mL^{-1}) containing all non-alkylated PAHs to be analyzed (Naphthalene, Acenaphthylene, Acenaphthene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benzo(a)anthracene, Chrysene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(a)pyrene, Indene(1,2,3 -cd)pyrene, Dibenzo(a,h)anthracene and Benzo(g,h,i)perylene) and the deuterated internal standards already mentioned (each at a concentration of 100 ng L^{-1}). The limit of quantification was calculated as the ratio between the concentration of the lowest standard solution on the calibration curve and the volume of the extracted sample (1 ng mL^{-1}).

5.2.4 Data analysis

The softwares Excell® and Statistica 7.0® were used for statistical analyses. To perform the analyses and enable comparisons between the experiment times, the PAH values called "Initial PAHs in water" refer to the averages between the concentrations obtained in the water samples at time 0h (immediately before the introduction of the oysters into the aquariums) and 24h (immediately after the total water change and OWDs). Data were considered normal after Kolmorogov-Smirnov and Shapiro Wilk tests, allowing the performance of analyzes of variance (ANOVA). The few outliers, defined as discrepant data points that fall outside the normal range, were excluded from the analysis since they were solely responsible for generating this deviation in the dataset. When significant differences were found, represented by a coefficient $p < 0.05$, the multiple comparisons test (Tukey) was applied.

5.3 Results

5.3.1 Oyster mortality

Oyster mortality was very low and apparently random among the OWDs produced with different concentrations of diesel oil. The concentration with the highest mortality rate was 10%, resulting in the death of two oysters, while in the other groups, only one oyster died, including the control (Table 1).

Table 1 – Oyster mortality throughout the experiment

	6h	12h	18h	24h	48h	Total
Control	1	0	0	0	0	1
20 % Oil:Water OWD	0	0	0	0	1	1
10 % Oil:Water OWD	0	0	0	0	2	2
1 % Oil:Water OWD	0	0	0	0	1	1
0,1 % Oil:Water OWD	0	0	1	0	0	1
0,01 % Oil:Water OWD	1	0	1	0	1	2

5.3.2 Polycyclic aromatic hydrocarbons in water and oyster tissues

In general, among all water and oyster tissue samples analyzed, the recovery measured through the p-terphenyl-d14 standard was satisfactory, except for 3 samples, which were disregarded. At the beginning of the experiment, Naphthalene stood out with the highest concentration in all OWDs produced, with the highest average recorded of

1708 ng L^{-1} in OWD 20 %. Other PAHs with relatively high concentrations were Fluorene, Phenanthrene, and Pyrene (maximum averages of 162 ng L^{-1}, 124 ng L^{-1} and 100 ng L^{-1} respectively, referring to OWD 20 %). Considering the different OWDs produced, the highest concentration of PAHs was observed in OWD 20 %, with a sum of the 16 quantified PAHs of 2273 ng L^{-1}. The lowest PAH values were observed in OWD 0.01 %, with 225.91 ng L^{-1}. In general, the pattern remained regarding the main PAHs identified, with Naphthalene, Fluorene, Phenanthrene, and Pyrene standing out. Fluoranthene can also be mentioned, with intermediate concentrations (33 ng L^{-1} for OWD 20 %). Heavier PAHs, such as Benzo[b]fluoranthene, Benzo[k]fluoranthene, Benzo[a]pyrene, and Indeno(1,2,3-cd)pyrene were recorded, but with low concentrations, not exceeding 6 ng L^{-1} in any of the OWDs analyzed.

Table 2 – Means and standard deviation of the PAHs in the initial water samples (ng L^{-1})(produced with 20 %, 10 %, 1 %, 0,1 % and 0,01 % diesel oil: water ratios).

INITIAL WATER PAHs	Control (média ± dp)	20% OWD (méan ± dp)	10% OWD (mean ± sd)	1% OWD (mean ± sd)	0,1% OWD (mean ± sd)	0,01% OWD (mean ± sd)	Benzene Rings	Molecular weight range
Naphthalene	10.5 ± (1.08)	1708.51 ± (298.95)	840.86 ± (150.78)	933.08 ± (214.69)	448.88 ± (53.36)	128.86 ± (18.91)	2	Low
Acenaphthylene	0 ± (0)	44.75 ± (10.4)	24.52 ± (6.62)	17.66 ± (5.21)	13.73 ± (2.06)	6.47 ± (1.21)	2	Low
Acenaphthene	0.82 ± (0.5)	58.48 ± (19.39)	29.68 ± (7.25)	30.67 ± (4.23)	21.39 ± (2.17)	8.17 ± (1.42)	2	Low
Fluorene	2.68 ± (1.08)	162.7 ± (34.05)	85.26 ± (18.33)	95.04 ± (10.56)	72.03 ± (9.63)	30.28 ± (7.43)	2	Low
Phenanthrene	4.12 ± (1.13)	124.56 ± (17.11)	57.32 ± (15.69)	48.95 ± (11.48)	35.66 ± (5.28)	24.84 ± (7.8)	3	Low
Anthracene	1.96 ± (0.15)	19.67 ± (6.51)	10.23 ± (5.02)	6.71 ± (1.25)	0 ± (0)	0 ± (0)	3	Low
Fluoranthene	2.92 ± (1.38)	33.08 ± (19.13)	43.65 ± (30.76)	10.52 ± (6.81)	24.59 ± (23.42)	4.21 ± (1.19)	3	Low
Pyrene	3.57 ± (1.87)	100.2 ± (25.56)	84.69 ± (37.19)	50.99 ± (20.52)	51.62 ± (34.38)	18.98 ± (6.54)	4	Medium
Benzo(a)anthracene	0 ± (0)	0.21 ± (0.42)	1.99 ± (2.14)	0.43 ± (0.97)	0.75 ± (0.71)	0.4 ± (0.48)	4	Medium
Chrysene	0 ± (0)	2.14 ± (2.95)	1.16 ± (0.89)	0.34 ± (0.77)	0.93 ± (1.29)	0 ± (0)	4	Medium
Benzo[b]fluoranthene	0 ± (0)	5.94 ± (4.9)	2.35 ± (2.74)	0.26 ± (0.58)	1.75 ± (2.41)	0 ± (0)	4	Medium
Benzo[k]fluoranthene	0 ± (0)	1.13 ± (2.27)	0.88 ± (1.06)	0 ± (0)	0.58 ± (0.64)	0 ± (0)	4	Medium
Benzo[a]pyrene	0 ± (0)	2.75 ± (0.95)	2.42 ± (1.31)	0.33 ± (0.74)	1.5 ± (1.66)	0.21 ± (0.42)	5	High
Indeno(1,2,3-cd)pyrene	0.84 ± (0.4)	3.58 ± (2.4)	1.76 ± (1.14)	1.7 ± (0.75)	1.08 ± (1.13)	1.74 ± (0.23)	5	High
Dibenz[a,h]anthracene	0.43 ± (0.27)	1.71 ± (0.81)	1 ± (0.4)	0.43 ± (0.26)	0 ± (0)	0 ± (0)	5	High
Benzo[g,h,i]perylene	2.31 ± (0.43)	4.56 ± (2.25)	2.31 ± (1.22)	2.94 ± (1.71)	2.47 ± (0.94)	2.01 ± (0.35)	6	High
∑16 PAHs	30.14 ± (4.01)	2273.96 ± (399.45)	1190.09 ± (176.51)	1199.96 ± (267.77)	676.96 ± (115.01)	225.91 ± (42.03)	NA	NA

Source: Produced by the author.

At the end of the experiment, a strong reduction in the identified and quantified PAHs was observed. The most notable decrease was observed in naphthalene, with levels of 19 ng L^{-1} for OWD 20 %, which were two orders of magnitude lower compared to the initial values. Fluorene, phenanthrene, and pyrene also showed reductions but remained detectable, with maximum averages of 26 ng L^{-1}, 21 ng L^{-1}, and 24 ng L^{-1}, respectively, for OWD 20%. The trend was consistent across different OWDs, with similar

values observed for OWDs 20 %, 10 %, and 1 %. Heavier PAHs like Benzo[b]fluoranthene, Benzo[k]fluoranthene, Benzo[a]pyrene, and Indeno(1,2,3-cd)pyrene were still present, maintaining low concentrations, without a decrease compared to the beginning of the experiment.

Table 3 – Means and standard deviation of the PAHs in the final water samples (ng L^{-1}) (produced with 20 %, 10 %, 1 %, 0,1 % and 0,01 % diesel oil: water ratios).

FINAL WATER PAHs	Control (média ± dp)	20% OWD (méan ± dp)	10% OWD (mean ± sd)	1% OWD (mean ± sd)	0,1% OWD (mean ± sd)	0,01% OWD (mean ± sd)	Benzene Rings	Molecular weight range
Naphthalene	14.45 ± (1.22)	19.19 ± (10)	29.65 ± (20.76)	64.19 ± (50.09)	12.64 ± (2.28)	12.17 ± (2.2)	2	Low
Acenaphthylene	0.2 ± (0)	1.66 ± (2.93)	2.12 ± (0.1)	2.72 ± (0.45)	0.2 ± (0)	0.2 ± (0)	2	Low
Acenaphthene	0.98 ± (0.16)	6.13 ± (3.57)	2.8 ± (1.16)	5.35 ± (2.13)	0.47 ± (0.63)	0.8 ± (0.43)	2	Low
Fluorene	2.2 ± (0.2)	26.09 ± (10.3)	11.06 ± (3.88)	18.3 ± (10.86)	3.68 ± (0.84)	2.55 ± (0.27)	2	Low
Phenanthrene	3.22 ± (0.64)	20.88 ± (4.29)	11.3 ± (5.35)	14.3 ± (6.53)	3.88 ± (1.2)	3.35 ± (0.61)	3	Low
Anthracene	1.93 ± (0.14)	5.72 ± (1.12)	2.4 ± (0.35)	3.09 ± (0.78)	2.05 ± (0.17)	1.99 ± (0.07)	3	Low
Fluoranthene	4.22 ± (5.16)	6.31 ± (0.34)	6.75 ± (7.07)	4.2 ± (2.17)	2.88 ± (0.92)	2.13 ± (0.32)	3	Low
Pyrene	4.84 ± (5.35)	23.77 ± (5.19)	14.21 ± (8.67)	13.75 ± (7.64)	4.38 ± (2.16)	2.32 ± (0.77)	4	Medium
Benzo(a)anthracene	0.07 ± (0)	1.35 ± (1.49)	0.07 ± (0)	0.07 ± (0)	1.22 ± (0.78)	0.67 ± (0.7)	4	Medium
Chrysene	0.18 ± (0)	0.66 ± (0.97)	0.18 ± (0)	0.18 ± (0)	0.18 ± (0)	0.18 ± (0)	4	Medium
Benzo[b]fluoranthene	0.72 ± (0.95)	9.65 ± (3.82)	1.09 ± (1.46)	0.24 ± (0)	0.24 ± (0)	0.24 ± (0)	4	Medium
Benzo[k]fluoranthene	0.21 ± (0)	4.24 ± (1.7)	0.61 ± (0.69)	0.21 ± (0)	0.21 ± (0)	0.37 ± (0.32)	4	Medium
Benzo[a]pyrene	0.31 ± (0)	2.01 ± (0.45)	1.18 ± (1.51)	0.31 ± (0)	1.01 ± (0.82)	0.31 ± (0)	5	High
Indeno(1,2,3-cd)pyrene	0.67 ± (0.42)	4.33 ± (1.66)	1.37 ± (0.64)	1.69 ± (0.31)	1.95 ± (1.24)	1.02 ± (0.3)	5	High
Dibenz[a,h]anthracene	0.36 ± (0)	2.96 ± (1.24)	1.08 ± (0.14)	1.22 ± (0.19)	0.36 ± (0)	0.36 ± (0)	5	High
Benzo[g,h,i]perylene	1.72 ± (0.23)	5.17 ± (1.55)	1.91 ± (1.49)	3.4 ± (0.53)	3.47 ± (1.63)	1.99 ± (0.52)	6	High
∑16 PAHs	36.28 ± (11.25)	140.15 ± (27.14)	87.78 ± (50.1)	130.76 ± (71.21)	38.82 ± (9.72)	30.64 ± (2.74)	NA	NA

Source: Produced by the author.

Regarding the bioaccumulation of oysters, Phenanthrene, a PAH with 3 benzene rings, stands out with the highest values recorded in individuals exposed to the analyzed OWDs (34 ng L^{-1}, 38 ng L^{-1}, 19 ng L^{-1}, 12 ng L^{-1}, 10 ng L^{-1}, and 12 ng L^{-1} respectively for the OWDs 20 %, 10 %, 1 %, 0.01 %, and 0.01 %. Other important PAHs were Naphthalene, Fluorene, and Pyrene with values generally ranging between 14 and 30 ng L^{-1} for oysters from the OWDs treatments 20 %, 10 %, and 1 %. Among the heavier PAHs, the bioaccumulated values were quite low, with the highest recorded being Benzo[a]pyrene (3.3 ng L^{-1}) in the oysters exposed to OWD 20 %.

Table 4 – Means and standard deviation of the PAHs in the oyster samples (ng g^{-1}) (exposed to OWDs produced with 20 %, 10 %, 1 %, 0,1 % and 0,01 % diesel oil: water ratios for 48 hours).

OYSTER TISSUE PAHs	Control (média ± dp)	20% OWD (méan ± dp)	10% OWD (mean ± sd)	1% OWD (mean ± sd)	0,1% OWD (mean ± sd)	0,01% OWD (mean ± sd)	Standard bivalve tissue (mean ± sd)	Benzene Rings	Molecular weight range

Naphthalene	3.43 ± (1.92)	27.06 ± (23.33)	20.11 ± (15.78)	14.24 ± (13.26)	11.02 ± (1.87)	3.82 ± (3.08)	1.47 ± (2.55)	2	Low
Acenaphthylene	0 ± (0)	2.3 ± (1.76)	1.51 ± (0.73)	0.71 ± (1.01)	0 ± (0)	0.09 ± (0.19)	0.91 ± (0.17)	2	Low
Acenaphthene	0 ± (0)	6.1 ± (7.7)	4.19 ± (1.71)	1.56 ± (2.17)	0.14 ± (0.25)	0 ± (0)	0 ± (0)	2	Low
Fluorene	0.78 ± (0.18)	28.45 ± (14.01)	30.1 ± (7.83)	13.76 ± (13.35)	9.24 ± (3.11)	4.4 ± (2.68)	0.68 ± (0.09)	2	Low
Phenanthrene	3.8 ± (0.96)	34.03 ± (19.43)	38.29 ± (12.31)	19.22 ± (15.61)	11.76 ± (3.06)	9.9 ± (5.14)	12.29 ± (2.1)	3	Low
Anthracene	0 ± (0)	2.56 ± (2.42)	2.48 ± (1.65)	1.17 ± (1.61)	0 ± (0)	0.09 ± (0.18)	0.18 ± (0.26)	3	Low
Fluoranthene	0 ± (0)	0 ± (0)	0 ± (0)	0 ± (0)	0 ± (0)	0 ± (0)	39.18 ± (8.37)	3	Low
Pyrene	0 ± (0)	16.03 ± (15.28)	20.9 ± (12.63)	8.1 ± (13.38)	0 ± (0)	0 ± (0)	43.98 ± (33.32)	4	Medium
Benzo(a)anthracene	0.21 ± (0.48)	0.84 ± (0.77)	0 ± (0)	0.43 ± (0.63)	0 ± (0)	0.09 ± (0.17)	23.21 ± (8.95)	4	Medium
Chrysene	0.07 ± (0.16)	0.91 ± (1.19)	0.97 ± (1.64)	0.11 ± (0.24)	0.13 ± (0.23)	0 ± (0)	33.26 ± (3.93)	4	Medium
Benzo[b]fluoranthene	1.72 ± (0.67)	1.65 ± (1.29)	0.38 ± (0.36)	0.65 ± (0.26)	0 ± (0)	1.09 ± (0.8)	54.55 ± (17.78)	4	Medium
Benzo[k]fluoranthene	1.19 ± (0.64)	0.64 ± (0.15)	0.49 ± (0.33)	0.13 ± (0.29)	0 ± (0)	0.22 ± (0.27)	32.59 ± (27.73)	4	Medium
Benzo[a]pyrene	3.57 ± (0.78)	3.3 ± (1.07)	2.14 ± (0.29)	1.95 ± (0.54)	1.43 ± (0.05)	2.38 ± (0.83)	32.58 ± (37.7)	5	High
Indeno(1,2,3-cd)pyrene	0 ± (0)	0 ± (0)	0 ± (0)	0 ± (0)	0 ± (0)	0 ± (0)	33.53 ± (3.94)	5	High
Dibenz[a,h]anthracene	0 ± (0)	0 ± (0)	0 ± (0)	0 ± (0)	0 ± (0)	0 ± (0)	7.79 ± (2.66)	5	High
Benzo[g,h,i]perylene	0.1 ± (0.23)	0 ± (0)	0 ± (0)	0 ± (0)	0 ± (0)	0 ± (0)	28.15 ± (10.01)	6	High
∑16 PAHs	14.88 ± (3.32)	123.88 ± (68.44)	121.55 ± (22)	62.01 ± (53.98)	33.72 ± (8.18)	22.07 ± (12.21)	344.29 ± (64.35)	NA	NA

Source: Produced by the author.

5.3.3 Comparative analysis between initial and final OWDs and oyster tissues

When comparing the concentrations of PAHs obtained from the analysis of samples related to the OWD and control within the sampling units, some particularities can be identified. Among the ∑16 PAHs, the replicates with OWDs produced with 20 % diesel oil stand out significantly from the others (average of approximately 2300 ng L^{-1}; $p = 0.001$), including the replicates with 10 % diesel oil, which had the closest concentration (figure 2, a). The replicates with 10 % and 1 % oil showed similar concentrations (averages of approximately 1200 ng L^{-1}), with no significant differences between them, but with significant variances compared to the other OWDs ($p < 0.02$ and $p < 0.009$, respectively) (figure 2, a). The subsequent OWD, 0.1% oil, was significantly different from all others (approximately 650 ng L^{-1}; $p < 0.05$), while the lowest concentration, 0.01% oil, did not differ from the control samples (figure 2, a).

By fragmenting the analysis and considering the PAH groups based on the number of benzene rings, specific patterns can be identified. For PAHs with two benzene rings, the patterns are very similar to those identified when analyzing the sum of the ∑16 PAHs. This similarity is due to the fact that this group of PAHs is the most abundant in the sample (figure 2, d). For the groups of PAHs with 3 and 4 rings, the replicates with OWDs produced with 20 % oil approached the 10 % concentration, but still showed significant differences in the case of the first group ($p = 0.015$) and no differences for the second group (figure 2, g and j). The 1 % and 0.1 % OWDs presented concentrations of

3-ring PAHs below those above mentioned, significantly different from the control ($p = 0.02$ and $p = 0.04$, respectively). However, the concentration of 4-ring PAHs was similar among control and treatments with 1 % and 0.1 % OWD (figure 2, g and j). The groups of PAHs with 5 and 6 rings, in general, did not show significant differences between the OWDs and control samples (figure 2, m and p). The exception was in the 5-ring group, where the 20 % OWD differed from the others ($p < 0.03$), being significantly similar only to the 10 % OWD.

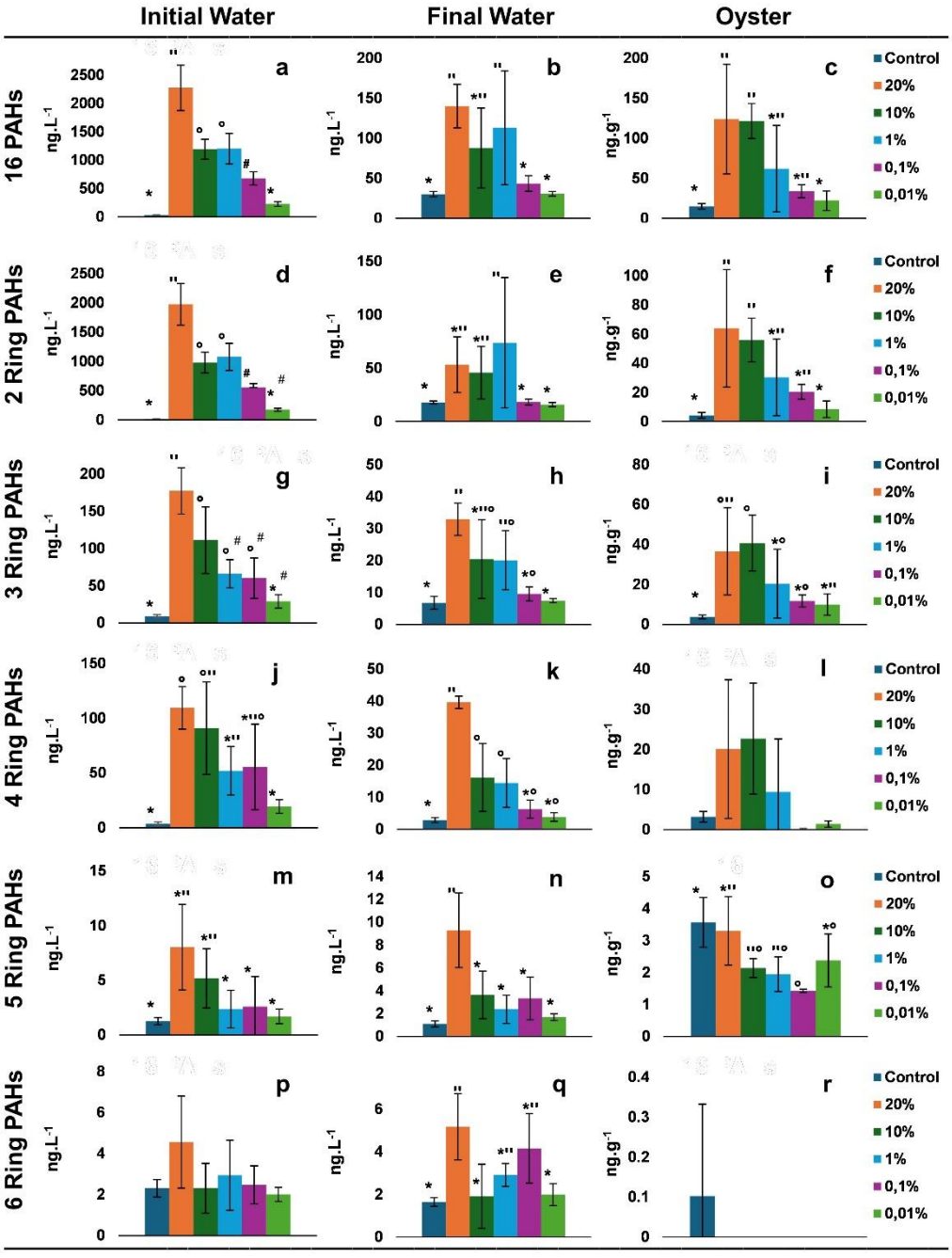
At the end of the experiment, a strong reduction in PAH concentrations was observed (figures 2 and 3). When comparing the sum of the ∑16 PAHs in OWDs at 20 %, 10 %, and 1 %, the values were one order of magnitude lower than the initial concentrations (approximately 140, 87, and 130 ng L^{-1} respectively) (figure 2a and b), with no significant differences between them. This reduction trend was consistent in the replicates with OWDs at 0.1 % and 0.01 % oil, showing no difference from the control but differing from the other OWDs at 20 % and 1 % ($p < 0.03$ and $p < 0.02$ respectively) (figures 2 and 3).

The decrease in the observed values was more pronounced for PAHs with 2 benzene rings (figure 3, b), which initially had an average of approximately 2000 ng L^{-1} in the replicates with 20 % OWD and 1000 ng L^{-1} in 10 % and 1 % OWDs (figure 2d), decreasing to 53, 45, and 88 ng L^{-1} respectively (figure 2b). By the end of the experiment, the 0.1 % and 0.01 % OWDs maintained low concentrations, similar to the control samples, with this pattern being consistent across all benzene ring groups. For PAHs with 3 and 4 rings, the reduction was relatively smaller (figure 3c and d). Initially, the average concentrations of PAHs ranged between 90 and 190 ng L^{-1} in the OWD samples with 20 % and 10 % oil, but by the end of the experiment, they ranged from 20 to 40 ng L^{-1} (figure 2g, h, j, and k). Notably, the 20 % OWD had a higher average in the group of 4 rings, significantly differing from the others ($p = 0.0001$). All OWD preparations showed very low concentrations of PAHs with 5 and 6 benzene rings, with the 20 % OWD being the only one significantly different from the control ($p = 0.0001$ and $p = 0.003$ respectively), with concentrations of 9 and 5 ng L^{-1} respectively (figure 2n and q).

Regarding bioaccumulation in oysters, an increasing trend in the average concentrations of PAHs was observed between the OWD produced with the lowest oil volume percentage (0.01 %) and the highest (20 %) (figure 2c). However, this trend did not necessarily result in significant differences. The average concentrations of the ∑16 PAHs quantified after exposure to OWD 20% and 10 % diesel oil were approximately 120 ng g^{-1} (figure 2c). Despite the higher average, they did not differ significantly from the oysters exposed to OWD 1 % (61 ng g^{-1}) and 0.1 % (33 ng g^{-1}), due to the high variability among replicates. The latter two treatments did not differ even from the control

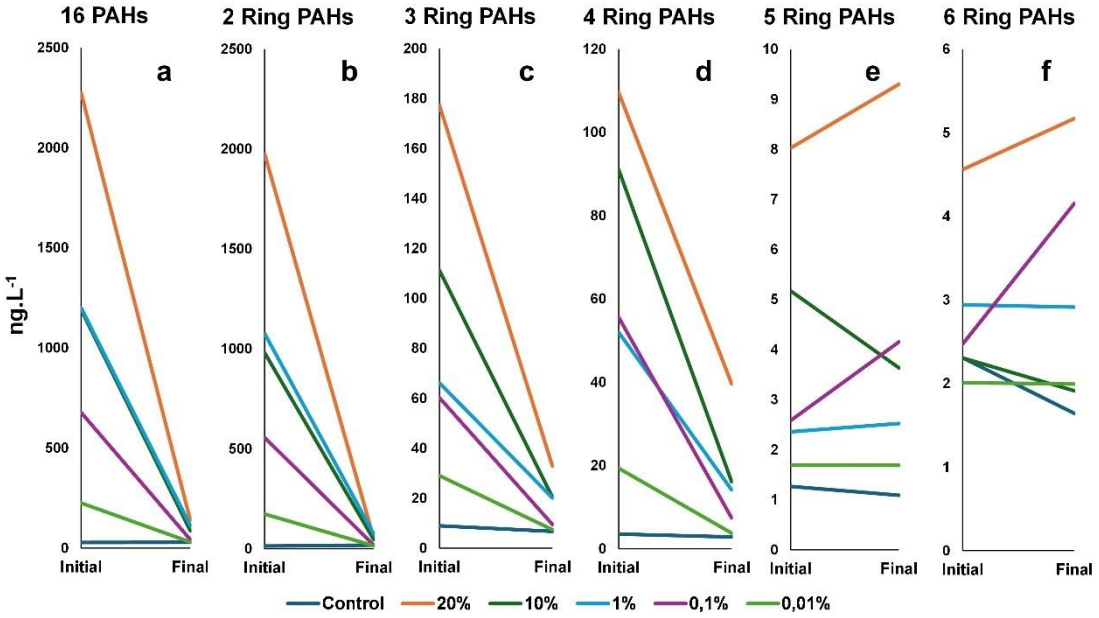
samples, as well as the samples of oysters exposed to OWD 0.01 %. In general, even with the fragmentation of the analysis of the PAHs bioaccumulated by the oysters according to the benzene rings, the patterns observed are quite similar to those found for the ∑16 PAHs. The groups of PAHs with 5 and 6 rings appear with very low concentrations, the former even below the control samples.

Figure 2 - Means and standard deviation of the PAHs in the Initial (a) and Final (b) water samples and oyster tissues (c). The figure illustrates ∑16 PAHs, two, three, four, five and six ring PAH groups in the OWD (produced with 20 %, 10 %, 1 %, 0,1 % and 0,01 % diesel oil: water ratios), Control solutions and oyster tissues. Different symbols above bars indicates significant differences.



Source: Produced by the author.

Figure 3: - Means of the PAHs in the Initial and Final water samples. The figure illustrates ∑16 PAHs, two, three, four, five and six ring PAH group in the OWD (produced with 20 %, 10 %, 1 %, 0,1 % and 0,01 % diesel oil: water ratios) and Control solutions.

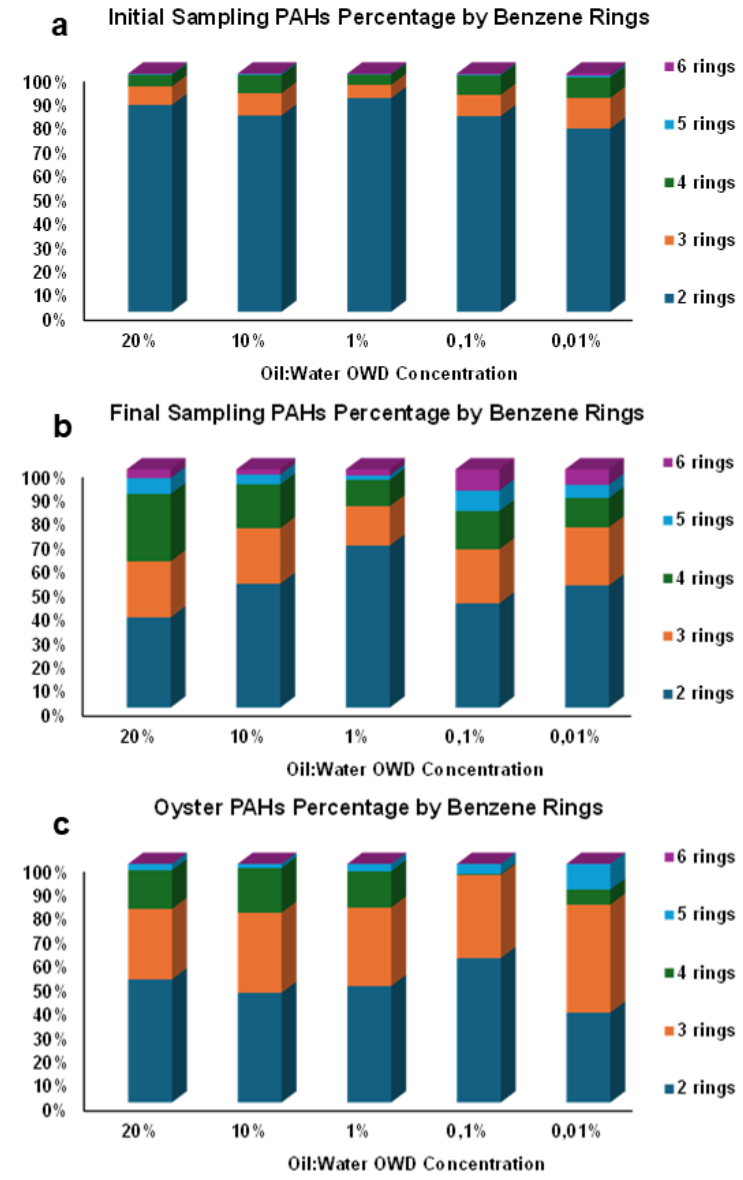


Source: Produced by the author.

5.3.4 Percentage of PAHs by groups of benzene rings in the OWDs used at the beginning and end of the experiment and in the oyster tissues

Initially, all the OWDs produced and sampled in the aquariums where the oysters were introduced presented more than 80 % of PAHs with 2 benzene rings (figure 4, a). At the end of the experiment, it is interesting to note that the percentage of PAHs according to their molecular weight (number of rings) no longer follows this pattern (figure 4b). The OWD 20 % appears with 38 % of PAHs with 2 benzene rings, the OWD 10 % with 52 %, and the OWD 1 % with 66 %. The oyster tissue samples showed great similarity in the proportion of PAHs according to the group of benzene rings when comparing the exposures to OWDs produced with 20, 10, and 1 % of diesel oil (figure 4c). The group of 2 rings appeared to vary between 45 and 50 %, the group of 3 rings between 30 and 33 %, and the group of 4 rings between 15 and 19 %.

Figure 4 – PAHs percentage by benzene rings in the OWD (produced with 20 %, 10 %, 1 %, 0.1 %, and 0.01 % diesel oil: water ratios) and oyster tissues. a: Initial sampling PAHs in OWD; b: Final sampling PAHs in OWD; c: PAHs in Oyster tissues.

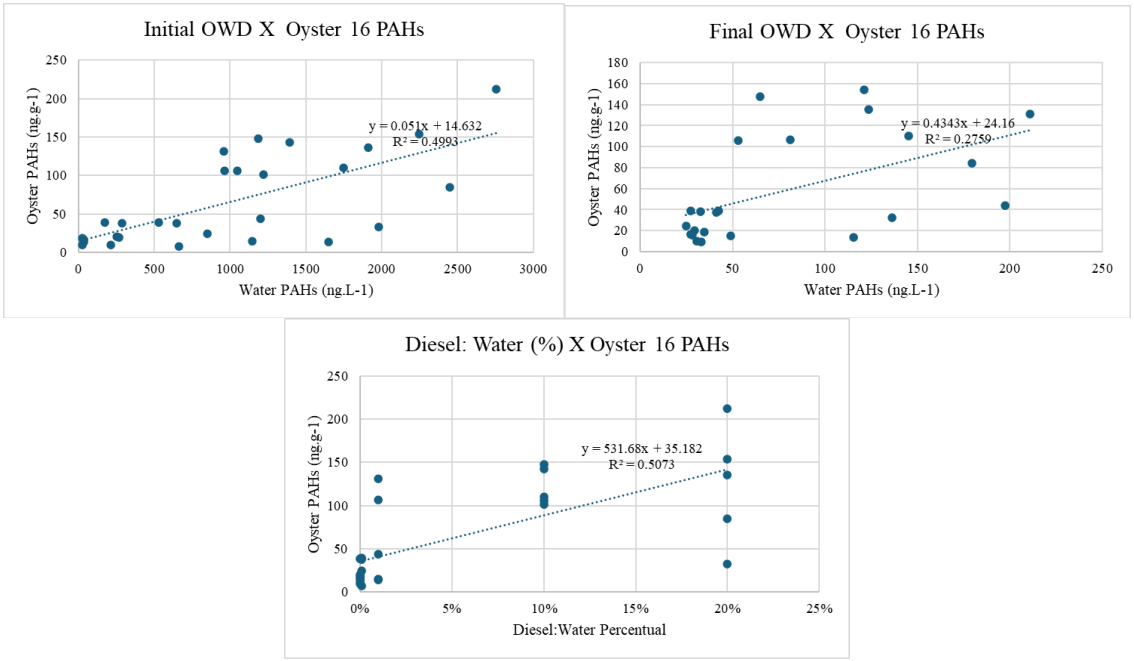


Source: Produced by the author.

5.3.5 Linear regressions for PAH values in oysters and OWD

In general, the regressions relating to the ∑16 PAHs from all replicates analyzed show an increasing relationship between the PAH concentrations in the water and oysters (figure 5). The inconsistent fit of the straight lines, as indicated by the low R^2 values, is due to the high heterogeneity of the concentrations quantified in the oyster tissues.

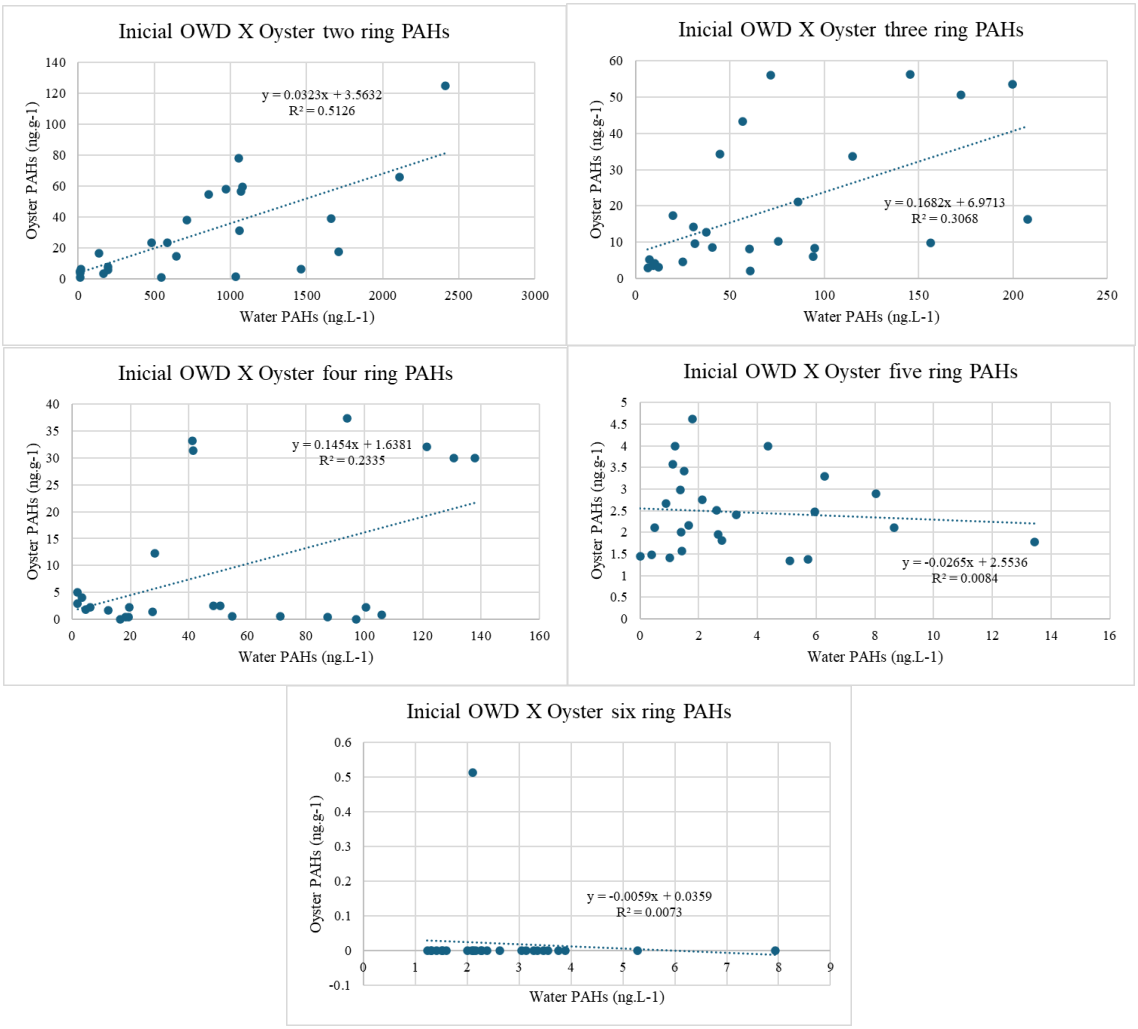
Figure 5 – Linear regressions of the ∑16 PAHs between oyster and OWD from sampling units and between oyster and the oil:water proportion of OWD.



Source: Produced by the author.

Fragmenting the analyses according to the number of benzene rings, the pattern observed for the ∑16 PAHs remains consistent, but with a decreasing alignment of the lines as the number of rings increases (figure 6). This observation suggests a higher variability in PAH concentrations with an increase in the number of rings.

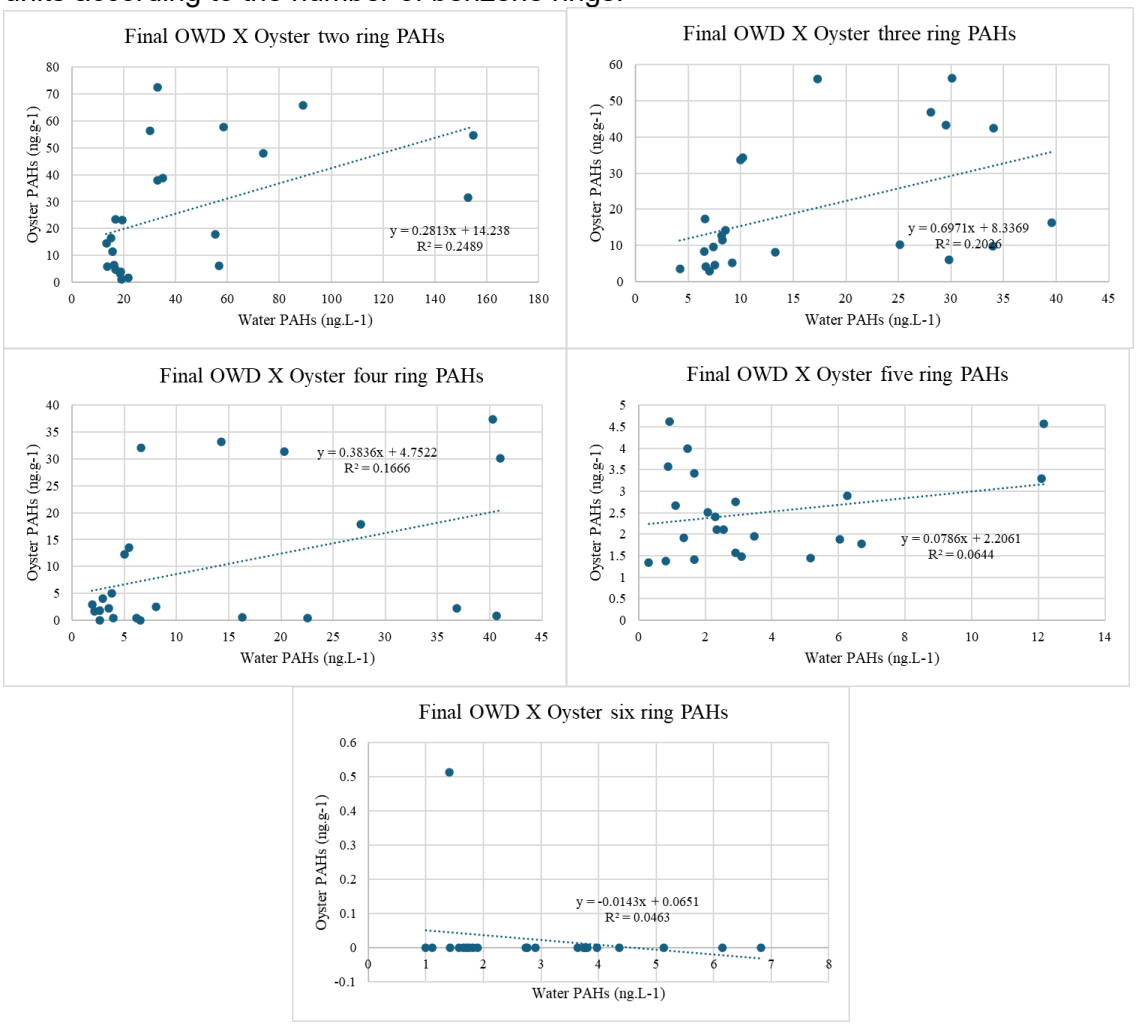
Figure 6 – Linear regression of the PAHs in oyster and OWD inicial samples from sampling units according to the number of benzene rings.



Source: Produced by the author.

At the end of the experiment, the pattern of increasing PAH values in oyster tissues in relation to the values in the water was consistent with the benzene rings. However, there was a slight deviation from a straight line, indicating a high variability in the final PAH concentrations in the sampling units (OWD) (figure 7). This suggests that both evaporation and bioaccumulation of oysters were not consistent across the replicates.

Figure 7 - Linear regression of the PAHs in oyster and OWD final samples from sampling units according to the number of benzene rings.



Source: Produced by the author.

5.4 Discussion

Very low oyster mortality was recorded in the experiment. This low mortality rate may be explained by the resilience of these animals, which have a series of physiological mechanisms that act when they come into contact with contaminants. Examples include biotransformation, which acts by converting PAHs into excretable compounds, and antioxidant defense systems, which produce a series of protective enzymes to minimize the effects on the organism (Gan *et al.*, 2021; Bastolla *et al.*, 2023; Ferreira *et al.*, 2023; Souza *et al.*, 2023). In addition, studies indicate that bivalves can alter the frequency of valve opening when exposed to toxic compounds (Redmond *et al.*, 2017; Durier *et al.*, 2021). A second reason for the low mortality rate may be the rapid reduction in the concentrations of PAHs available in the water. Factors that lead to reduced available PAH include evaporation and adhesion to aquarium walls (Carrasco-Navarro *et al.*, 2015;

Weinnig *et al.*, 2020). High heterogeneity was observed between PAH concentrations in oyster samples, as can be seen from the high standard deviations found and the scatter plots. It is possible that, for physiological reasons, such as reduced valve opening frequency, PAH bioaccumulation varied, thus influencing the recorded concentrations (Redmond *et al.*, 2017; Durier *et al.*, 2021).

In the initial analysis, the results for the ∑16 PAHs in the samples concerning the OWDs created with 20 %, 10 %, and 1 % oil-to-water ratios (concentrations at the start of the experiment) was not accompanied by the bioaccumulation in the oysters. Although the 20 % OWD had nearly double the average number of PAHs compared to the 10 % and 1 % OWDs, the oysters exposed to these solutions accumulated significantly similar values. Several factors can explain this observation. The PAHs with two benzene rings, which were the major contributors to the total of ∑16 PAHs initially, decreased rapidly by the end of the experiment, making them less available in the water. Lighter PAHs are more soluble and volatile (Patel *et al.*, 2020), leading to their removal from the water as they disperse. Consequently, there was a leveling of concentrations among the 20 %, 10 %, and 1 % OWDs, as the PAH levels decreased proportionally more in the OWDs with higher initial concentrations. The significant reduction mainly occurred in the lighter PAHs with two rings, which are more volatile (Patel *et al.*, 2020) and are efficiently accumulated by bivalves due to their lower hydrophobicity, making them more soluble and bioavailable (Skic *et al.*, 2023). When comparing the oysters exposed to the 20 %, 10 %, and 1 % OWDs, the distribution of compounds with different benzene rings was more balanced (initially, the water samples contained around 80 % of PAHs with two rings). These results, combined with the rapid decline of PAHs with lighter benzene rings in the water, approached the final concentrations of diesel oil-to-water solutions. Given the tendency for lighter PAHs to accumulate more in oysters (Skic *et al.*, 2023), it is probable that the loss of these compounds from the system resulted in lower relative absorption, leading to higher proportional values for 3 and 4 ring PAHs, which are heavier, less volatile, and more persistent in the water. Hence, it is likely that evaporation played a crucial role in reducing the concentrations of 2 ring PAHs in the water.

The main PAHs quantified in the OWD samples, including naphthalene, fluorene, phenanthrene, and pyrene, were bioaccumulated in the oyster tissues, particularly in the treatments related to OWDs at 20 %, 10 %, and 1 %. Naphthalene was identified with a high concentration in the water at the beginning of the experiment, as expected in diesel oil: water dispersions (Lüchmann *et al.*, 2011; Delunardo *et al.*, 2020). It is a compound with high toxicity (Gardiner *et al.*, 2013); however, volatility is a predominant factor that prevents its persistence in the system for long periods, yet it was still identified in *Crassostrea brasiliana*. Fluorene, the second PAH with the highest concentration in

water, was also identified with high relative concentrations in oysters, confirming expectations for a PAH with high relative solubility in water and, therefore, higher bioaccumulation (Skic *et al.*, 2023). Phenanthrene, a PAH still considered light but with three benzene rings, showed the highest bioaccumulation, despite not having very high values in the OWD. Its lower volatility (Patel *et al.*, 2021) may have contributed to this result. Pyrene, a heavier compound with four benzene rings, was recorded with slightly lower values but deserves attention, as the carcinogenicity of PAHs generally increases with the number of benzene rings, and its quinone-based metabolites are mutagenic (Saraswathy and Hallberg, 2002; Zada *et al.*, 2021). The heavier PAHs, known for being highly persistent, carcinogenic, and mutagenic (Lourenço *et al.*, 2023; Obayori *et al.*, 2024), were detected at low concentrations in the water and were barely recorded in the oyster tissues. However, it is interesting to note that these concentrations remained at the end of the experiment, unlike what occurred with the lighter PAHs, which decreased considerably. The low volatility (Patel, 2020) and lower susceptibility to accumulation by oysters probably contributed to this, due to their lower availability in the water (Skic *et al.*, 2023).

5.5 Conclusion

When exposed to high concentrations of PAHs, *Crassostrea brasiliana* demonstrated resilience, with low mortality rates. It efficiently bioaccumulated the main PAHs identified and quantified in the initial and final OWD samples. A key characteristic of a good bioindicator is the ability to reflect the environmental conditions it was exposed to over time, a trait observed in these oysters. Based on the data, the PAHs naphthalene, fluorene, phenanthrene, and pyrene are the compounds that should be closely monitored in the event of a diesel oil spill. It is also important to note that the lighter compounds quantified exhibited a rapid decrease in concentrations, providing valuable insights for a diesel oil spill scenario.

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6 DISCUSSÃO E CONCLUSÃO GERAIS

6.1 Discussão

A pesquisa bibliográfica realizada a respeito da utilização dos isótopos estáveis ^{13}C e ^{15}N sinalizou que eles podem ser de grande utilidade para a utilização como biomarcadores de contaminação, visto que as assinaturas isotópicas em certo organismo são resultado das fontes de carbono e nitrogênio disponíveis no ambiente (De Barros Ferraz et al., 2009; Wang *et al.*, 2020; Vezzone, 2020; Srinivas *et al.*, 2022). Por outro lado, o trabalho demonstrado no capítulo 1, no qual foram efetuadas análises dos tecidos de ostras em pontos da costa de Arraial do Cabo com diferentes características ambientais, ficou claro que a distinção das fontes responsáveis pelas assinaturas isotópicas não é tão simples. Analisando tecidos de ostras de diferentes naturezas, sendo os músculos com uma reciclagem mais lenta e no outro extremo hepatopâncreas com reciclagem mais rápida (Raikow and Hamilton, 2001; Yokohama *et al.*, 2008; de Barros Ferraz *et al.*, 2009; Cabanellas-Reboredo *et al.*, 2009; Özdilek *et al.*, 2019; Bearham *et al.*, 2023), não foi possível identificar diferenças significativas entre as estações de amostragem. Já o seston, que representa um retrato de curtíssimo prazo das condições ambientais (Roth *et al.*, 2016; Srinivas *et al.*, 2022), indicou a influência de fontes terrígenas na estação Praia do Forno, onde existe uma maior presença de vegetação nas encostas adjacentes. As ostras, organismos filtradores sésseis, tendem a refletir as condições ambientais a que são submetidas (Fiori *et al.*, 2018; Phan *et al.*, 2019). Porém, com uma taxa de reciclagem mais lenta, provavelmente as assinaturas isotópicas em seus tecidos retrataram a fonte principal de médio a longo prazo (de origem marinha), aproximando as estações de amostragem.

Em relação à utilização dos isótopos estáveis para a detecção da presença de óleo diesel no ambiente, a ferramenta demonstra grandes obstáculos. Segundo estudos anteriores a taxa de reciclagem dos tecidos de ostras pode chegar a 180 dias (Riera & Richards, 1997; Fukumori *et al.*, 2008; Carmichael *et al.*, 2012). Já o óleo diesel tende a se solubilizar na água do mar e evaporar para a atmosfera rapidamente, se tornando indisponível em poucos dias, mesmo quando introduzido em grandes quantidades (Cripps & Shears, 1996; Delunardo *et al.*, 2020). Levando-se em consideração apenas a taxa de reciclagem, o seston seria uma matriz mais eficaz para a análise dos isótopos estáveis e verificação quanto a contaminação por óleo diesel. Porém existe o inconveniente desta matriz não permanecer nos locais de estudo por muito tempo, e, literalmente, mudar ao sabor das ondas e marés. No caso da implementação de uma estratégia de monitoramento, este inconveniente acarreta a necessidade de redução da

resolução temporal e consequente aumento do volume de amostras a serem coletadas e analisadas, o que pode em muitos casos inviabilizar os trabalhos.

Vista a complexidade de se identificar características isotópicas provenientes de compostos característicos do petróleo no ambiente, um dos caminhos para se testar a eficiência desta ferramenta são os bioensaios, uma vez que é possível reduzir as variações ambientais em experimentos controlados (Podlesińska & Dąbrowska, 2019; Luan *et al.*, 2020; Rakaj *et al.*, 2021). Porém, no caso do petróleo e seus derivados, a natureza hidrofóbica dos compostos imprime grande dificuldade para se levar adiante os bioensaios, tornando este um campo em constante atualização (Wade *et al.*, 2017; Wade *et al.*, 2022; Lee *et al.*, 2023; Parkerton *et al.*, 2023). Existem protocolos estabelecidos para a produção de soluções óleo: água, como o estabelecido pelo "Chemical Response to Oil Spills: Ecological Effects Research Forum" (CROSERF) em 1994 (Aurand & Coelho, 2005), porém muitas vezes eles não se adequam às demandas específicas dos experimentos a serem realizados. Com isso os pesquisadores se veem obrigados a efetuar adaptações aos protocolos já existentes (Bejarano *et al.*, 2006; Lüchmann *et al.*, 2011; Müller *et al.*, 2018; Wheeler *et al.*, 2020; Loughery *et al.*, 2023). No caso dos bioensaios do presente estudo, a principal barreira a ser transposta foi a necessidade de produção de um volume grande de solução óleo: água. O protocolo existente para a produção de "water-accomodated-fraction" (WAF), utilizando agitadores magnéticos, dificulta a produção de grandes volumes de dispersão, além de não serem adaptados para estes volumes (Loughery *et al.*, 2023). Com isso foi efetuado teste (capítulo 2) para validar a técnica "oil-water-dispersion" (OWD), com a utilização de bombas de circulação, empregando assim logística mais simples e acessível para a produção de grandes volumes de dispersão em laboratório. A comparação entre as técnicas OWD e WAF resultou na ratificação da eficiência das duas para a dispersão do óleo em água, com vantagem para a primeira quanto à produção de réplicas com concentrações de HPAs menos discrepantes entre si.

O experimento conduzido no capítulo 3, com a exposição de ostras da espécie *Crassostrea brasiliana* ao OWD 1:100 produzido a partir do óleo diesel produziu uma série de resultados bastante esclarecedores. As concentrações dos 16 HPAs prioritários (Zelinkova e Wenzl, 2015) na água foi máxima no início do experimento, reduzindo rapidamente após 24 h e se igualando às amostras controle após 7 dias. Este fato ratifica o comportamento deste óleo quando diluído em água, uma vez que é considerado leve, composto predominantemente por HPAs mais voláteis e solúveis (Cripps & Shears, 1996; Lüchmann *et al.*, 2011; Delunardo *et al.*, 2020; Patel *et al.*, 2020). As ostras analisadas se mostraram eficientes quanto à bioacumulação de HPAs, conforme estudos anteriores abordando a exposição de bivalves a estes compostos (Lüchmann,

et al., 2014; Wang *et al.*, 2017). As amostras de tecidos apresentaram concentrações de HPAs crescentes nas primeiras 24 e 48 h reduzindo então até o sétimo dia e se assemelhando ao controle. A depuração de HPAs em bivalves tem sido reportada em experimentos após a suspensão da exposição aos contaminantes (Lüchmann, *et al.*, 2014; Wang *et al.*, 2017). Vista a alta volatilidade do óleo diesel e, consequentemente, sua efêmera permanência no ambiente após um derramamento (Cripps & Shears, 1996), as ostras se mostram um valioso bioindicador quanto ao restabelecimento da qualidade ambiental. Já em relação aos isótopos estáveis ^{13}C e ^{15}N, não foi possível estabelecer diferenças entre as amostras controle e tratamento. Possivelmente a reciclagem lenta destes elementos nos tecidos das ostras (Riera & Richards, 1997; Fukumori *et al.*, 2008; Carmichael *et al.*, 2012) aliada à volatilidade dos contaminantes utilizados (Delunardo *et al.*, 2020; Patel *et al.*, 2020), que se tornaram indisponíveis rapidamente, tenham impossibilitado alterações detectáveis nas assinaturas isotópicas por influência do óleo diesel. Este fato corrobora as dificuldades identificadas no capítulo 1 quanto à utilização desta ferramenta para a avaliação de impactos por óleo diesel no mar, principalmente se o derramamento for pontual, cenário testado no capítulo 3.

Conforme verificado no capítulo 4, a bioacumulação das ostras depende de forma direta da disponibilidade dos contaminantes na água. Os HPAs predominantes entre os quantificados nas amostras de água foram os mais leves, com dois anéis benzênicos (naftaleno foi o mais abundante), conforme se esperava para o óleo diesel (Lüchmann *et al.*, 2011; Delunardo *et al.*, 2020). Estes HPAs são os mais solúveis, o que facilitaria sua bioacumulação (Skic *et al.*, 2023). Porém, o que se registrou ao analisar os tecidos de ostras expostas aos OWDs produzidos com as três maiores concentrações de óleo testadas (1:5, 1:10 e 1:100) foi uma bioacumulação em níveis muito próximos. Os HPAs mais leves se tornaram indisponíveis rapidamente, provavelmente devido à volatização (Carrasco-Navarro *et al.*, 2015, Weinnig *et al.*, 2020; Patel *et al.*, 2020), aproximando as concentrações bioacumuladas. Ainda considerando os três tratamentos intermediários com concentração de OWD 1:5, 1:10 e 1:100, as proporções entre HPAs de dois, três e quatro anéis benzênicos nos tecidos das ostras foi muito mais equilibrada do que na água ao início do experimento, quando mais de 80 % dos HPAs quantificados foram de 2 anéis. Este fato reforça a observação de uma rápida redução da disponibilidade destes compostos mais leves no sistema. Conforme já citado anteriormente, a rápida redução de HPAs em experimentos com óleo diesel e derramamentos *in situ* foram registrados em alguns estudos anteriores (Cripps & Shears, 1996; Delunardo *et al.*, 2020). Por outro lado, as concentrações de HPAs medidas nos OWDs produzidos não cresceram proporcionalmente com as quantidades de óleo introduzidas na água. Com isso, apesar de, no capítulo 2, a eficiência do método

ter sido demonstrada, é possível que a dispersão efetuada através de bombas de circulação não seja tão eficiente para a comparação entre diferentes concentrações de óleo diesel. Neste contexto, o método OWD é reconhecido por produzir dispersões mais próximas do que realmente ocorreria no ambiente (Anderson *et al.*, 1974; Echols *et al.*, 2015; Wade *et al.*, 2022). Seguindo por esta linha, possivelmente em situações semelhantes ocorrendo em águas marinhas as concentrações de HPAs dispersos seguiriam padrão semelhante, com baixa homogeneidade entre amostras.

Apesar de os óleos mais leves serem reconhecidos pela sua toxicidade aguda (Anderson *et al.*, 1974; IPIECA, 2015; Pannetier *et al.*, 2024), as ostras expostas aos OWDs com 5 concentrações crescentes de diesel não experimentaram altas taxas de mortalidade. As possibilidades para esta ocorrência vão desde a resiliência destes organismos, que possuem mecanismos de proteção (Redmond *et al.*, 2017; Durier *et al.*, 2021; Gan *et al.*, 2021; Bastolla *et al.*, 2023; Ferreira *et al.*, 2023; Souza *et al.*, 2023), até o fato da possível volatização dos compostos tóxicos em tempo hábil para a manutenção da vida dos organismos testados. Esta resiliência concede à ostra *Crassostrea brasiliana* uma característica importante para enquadrá-la como um bioindicador eficiente em relação a efeitos sub-letais de poluentes, uma vez que, no caso de um derramamento de óleo diesel, espera-se que ela se mantenha sob efeito dos contaminantes sem perecer. Com isso, em tese, sua utilização para testes com marcadores químicos e biológicos poderia ser realizada para acompanhar a qualidade ambiental antes, durante e depois de um acidente. A utilização de bivalves como bioindicadores tem possibilitado o acompanhamento da bioacumulação e efeitos dos contaminantes sob diferentes concentrações e tempos de exposição (Lüchmann, *et al.*, 2014; Idowu *et al.*, 2020; Gan *et al.*, 2021; Soliman *et al.*, 2022; Ferreira *et al.*, 2023), o que reforça a indicação das ostras, especificamente *Crassostrea brasiliana*, como bioindicador de impactos ambientais no mar.

6.2 Conclusão

Os isótopos estáveis ^{13}C e ^{15}N, marcadores reconhecidos para a detecção de fontes específicas de carbono e nitrogênio, se mostraram ferramentas promissoras de avaliação de condições ambientais, uma vez que foi possível verificar diferenças significativas entre o seston dos dois pontos estudados na costa de Arraial do Cabo. Por outro lado, estas diferenças não foram detectadas nos tecidos de ostras, provavelmente devido à reciclagem lenta dos mesmos. Este resultado aponta que as assinaturas isotópicas destes organismos possivelmente refletem as condições ambientais de mais longo prazo, não sendo indicados para a avaliação de impactos agudos. Estas

conclusões foram reforçadas ao realizar-se bioensaios onde a exposição ao óleo diesel não proporcionou alterações nas assinaturas isotópicas dos tecidos das ostras. A utilização de diferentes tecidos de ostras para as análises de isótopos permitiu a verificação de que a assinatura de isótopos estáveis do hepatopâncreas se aproximou do seston, evidenciando uma reciclagem mais acelerada deste tecido quando comparado às brânquias e músculo.

A dispersão do óleo em água para a realização de bioensaios ainda é um grande desafio para os pesquisadores. Apesar de existirem protocolos, eles dificilmente se enquadram às especificidades dos experimentos e à infraestrutura disponível. O teste realizado comparando as técnicas “Water- Accommodated Fraction” (WAF) e “Oil-in-Water Dispersion” (OWD) com o óleo diesel foi bem sucedido, possibilitando a adaptação utilizada nos bioensaios.

Nos dois bioensaios realizados, o óleo diesel se mostrou um óleo extremamente volátil. Os PAHs predominantes quantificados foram os mais leves, destacando-se o Naftaleno. Em geral, as concentrações destes HPAs reduziram 90% em 24h, tornando-se rapidamente indisponíveis para as ostras expostas aos contaminantes.

Apesar do pouco tempo de exposição nos dois bioensaios realizados devido à rápida indisponibilidade do óleo diesel, *Crassostrea brasiliana* se mostrou capaz de bioacumular os HPAs analisados. Conforme registrado no capítulo 3, após 7 dias de experimento os valores de HPAs nas ostras expostas não se diferenciaram das ostras controle, demonstrando capacidade de depuração. Estas informações são valiosas quanto ao que se espera de um derramamento de óleo diesel no mar, ratificando que as ostras são bioindicadores eficientes, para a avaliação de efeitos sub-letais de impacto ambiental.

Os acidentes com derramamentos de óleo ainda são uma realidade nas áreas costeiras, causando prejuízos ao meio ambiente e demandando respostas rápidas, mas principalmente precisas à sociedade. Neste contexto, a pesquisa realizada agrega e concentra informações importantes ao conhecimento já estabelecido quanto ao estudo de isótopos estáveis e HPAs como marcadores de impacto por óleo diesel no mar, em especial utilizando-se ostras como bioindicadores.

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