UNIVERSIDADE FEDERAL DO PARANÁ

LEONARDO SANDRINI NETO

AVALIAÇÃO DA CONTAMINAÇÃO POR HIDROCARBONETOS EM DISTINTOS NÍVEIS DE ORGANIZAÇÃO BIOLÓGICA

CURITIBA
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Tese apresentada como requisito parcial à obtenção do grau de Doutor em Zoologia, Curso de Pós-Graduação em Zoologia, Setor de Ciências Biológicas da Universidade Federal do Paraná.

Orientador: Dr. Paulo da Cunha Lana

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Leonardo Sandrini Neto

"Avaliação da contaminação por hidrocarbonetos em distintos níveis de organização biológica"

Tese aprovada como requisito parcial para obtenção do grau de Doutor em Zoologia, do Setor de Ciências Biológicas da Universidade Federal do Paraná, pela seguinte Comissão Examinadora:

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“To call in the statistician after the experiment is done may be no more than asking him to perform a post-mortem examination: he may be able to say what the experiment died of.”

Ronald Fisher

“An approximate answer to the right problem is worth a good deal more than an exact answer to an approximate problem.”

John Tukey

RESUMO

Avaliar os impactos da poluição marinha é uma tarefa complexa que demanda a aplicação de abordagens capazes de distinguir a perturbação antrópica da variabilidade espaço-temporal intrínseca aos ambientes costeiros e oceânicos. Distúrbios antropogênicos provocam respostas em distintos níveis de organização biológica, desde alterações bioquímicas até mudanças na estrutura de comunidades, em múltiplas escalas espaciais e temporais. Os efeitos deletérios de contaminantes têm sido historicamente investigados através de testes toxicológicos. Estes ensaios são conduzidos em condições laboratoriais controladas, que limitam o seu potencial preditivo e a sua capacidade de generalização. Avanços recentes na ecologia experimental de campo, com o desenvolvimento de delineamentos experimentais robustos e procedimentos analíticos associados, aumentaram a habilidade de detectar impactos decorrentes de distúrbios induzidos pelas atividades humanas. Neste sentido, abordagens de laboratório e de campo devem ser vistas como necessariamente complementares para a avaliação da poluição marinha. Atividades humanas desenvolvidas nas regiões costeiras têm aumentado a intensidade e frequência de eventos de exposição discreta ou aguda a contaminantes, tais como derrames acidentais de óleo. No entanto, a aplicação de experimentos manipulativos de campo para testar seus efeitos ainda é incipiente. Esta tese teve o objetivo geral de examinar como os efeitos da exposição a hidrocarbonetos de petróleo condicionam respostas biológicas em diversos níveis de organização, por meio de experimentos manipulativos de campo e de laboratório. Para tal, uma ampla variedade de técnicas e abordagens metodológicas considerando diferentes espécies-alvo foi adotada. A tese está estruturada em quatro capítulos que avaliaram: (i) os efeitos de um derrame experimental *in situ* de óleo diesel sobre associações de nematoides marinhos de vida livre utilizando um delineamento MBACI (Multiple Before–After–Control–Impact); (ii) os efeitos de repetidos derrames experimentais de óleo diesel sobre a estrutura das associações macrofaunais através da comparação de três frequências de derrames (a cada 2, 4 e 8 semanas) com duas dosagens de diesel (2,5 e 5 L m^{-2}) em um experimento fatorial com controles assimétricos; (iii) os efeitos de repetidos derrames experimentais de óleo diesel na resposta de biomarcadores de estresse oxidativo no bivalve *Anomalocardia brasiliana*, no gastrópode *Neritina virginea* e no poliqueta *Laeonereis culveri* através da comparação de três frequências de derrames (a cada 1, 2 e 4 dias) com duas dosagens de diesel (1 e 2 L m^{-2}) em um experimento fatorial com controles assimétricos; e (iv) os efeitos da dispersão mecânica (agitação) e química (uso de dispersantes) do óleo cru na resposta de diferentes biomarcadores e nas taxas de crescimento de juvenis do peixe-lobo *Anarhichas denticulatus* após exposição experimental em laboratório por 48 h. Não foram observadas diferenças significativas na diversidade, densidade total e estrutura das associações de nematoides entre os tratamentos controle e impacto, antes e depois do derrame experimental. Embora sejam frequentemente considerados animais sensíveis, nematoides marinhos de vida livre mostraram-se resilientes à contaminação por hidrocarbonetos de petróleo. Repetidos eventos de exposição ao óleo diesel, por sua vez, afetaram drasticamente a estrutura das associações macrofaunais e reduziram a densidade total e densidade dos táxons dominantes. Geralmente, derrames frequentes de baixa dosagem foram mais deletérios do que derrames menos frequentes de alta dosagem. Aumentos significativos na densidade populacional de espécies oportunistas foram observados em resposta a derrames pouco frequentes. Repetidos eventos de exposição ao óleo diesel também foram responsáveis pela indução das enzimas superóxido dismutase (SOD) e glutationa *S*-transferase (GST), incremento nos níveis de peroxidação lipídica (LPO) e depleção na concentração de glutationa reduzida (GSH) em *A. flexuosa* e *L. culveri*. O gastrópode *N. virginea* exibiu apenas uma depleção significativa nos níveis de GSH em derrames frequentes de alta dosagem. O sistema de defesa enzimático contra espécies reativas de

oxigênio não foi ativado em *N. virginea* e dano oxidativo em termos de LPO não foi observado. De maneira geral, atividades enzimáticas e dano oxidativo em *A. flexuosa* e *L. culveri* foram induzidos por derrames frequentes de baixa dosagem quando comparados com derrames menos frequentes de alta dosagem. Contudo, um padrão inverso foi observado nas respostas antioxidantes de *N. virginea*. Estes resultados enfatizam a importância de diferentes regimes de exposição na determinação da magnitude dos impactos por óleo. O bivalve *A. flexuosa* e o poliqueta *L. culveri* foram considerados sentinelas adequados para monitoramento da poluição por hidrocarbonetos de petróleo em áreas costeiras. Finalmente, a concentração relativa de metabólitos biliares de HPA e a atividade da etoxiresorufina-*O*-deetilase (EROD) do peixe-lobo *A. denticulatus* experimentalmente exposta a óleo disperso (tanto mecanicamente como quimicamente) foram significativamente maiores que no tratamento controle. Foi também observada uma inibição da atividade de acetilcolinesterase (AChE) em peixes expostos a óleo disperso. Além disso, o crescimento do peixe-lobo, tanto em biomassa quanto em comprimento, foi significativamente superior no controle que nos tratamentos de exposição. No entanto, a resposta de diferentes biomarcadores enzimáticos juntamente com estimativas de crescimento do peixe-lobo *A. denticulatus* indicaram uma toxicidade semelhante entre a dispersão química e mecânica do óleo cru. A detecção de respostas no nível suborganísmico (biomarcadores) juntamente com reduções no crescimento de juvenis do peixe-lobo alertam para potenciais efeitos tardios em populações afetadas por derrames de óleo. A demonstração destas respostas subletais em animais expostos destacam a toxicidade muitas vezes não aparente de derrames agudos, que podem afetar populações a médio e longo prazo, mesmo na ausência de mortalidade massiva.

Palavras-chave: experimento de campo; hidrocarbonetos de petróleo; biomarcadores; nematoides; macrofauna; peixe-lobo

ABSTRACT

The assessment of contaminant impacts is a complex task that demands a wide range of approaches to distinguish the anthropogenic disturbances from the inherent spatio-temporal variability of costal environments. In this thesis, the effects of exposure to petroleum hydrocarbons were assessed at multiple levels of biological organization, from biochemical alterations to changes in community structure. To achieve this goal, a variety of research approaches including laboratory-based experiments and manipulative field experiments with different target-species were used. The thesis is composed by four chapters that assessed: (i) the effects of a diesel oil spill on the structure of free-living marine nematode assemblages through a multiple before-after-control-impact (MBACI) design; (ii) the effects of the frequency (every 2, 4 and 8 weeks) and dosage (2.5 and 5 L m^{-2}) of experimental diesel spills on the structure of intertidal macrofaunal assemblages in a factorial experiment with asymmetrical controls; (iii) the effects of the frequency (every 1, 2 and 4 days) and dosage (1 and 2 L m^{-2}) of experimental diesel spills on oxidative stress biomarkers in the bivalve *Anomalocardia flexuosa*, the gastropod *Neritina virginea* and the polychaete *Laeonereis culveri* in a factorial experiment with asymmetrical controls; and (iv) the effects of mechanically dispersed and chemically dispersed oil on biomarkers response and growth of the wolfish *Anarhichas denticulatus* after 48 h laboratory exposure. No significant differences were observed in nematode total density, number of taxa and the overall assemblage structure between the control and impact treatments from before to after the experimental spill. Despite being considered good indicators of environmental impacts, free-living marine nematodes were considered resilient to contamination by petroleum hydrocarbons. Repeated oil spills dramatically altered the overall structure of assemblages and reduced the total density of macrofauna and densities of dominant taxa. Increasing the frequency of oil spills negatively affected macrofauna. In general, frequent low-dosage oil spills were more deleterious than infrequent high-dosage ones. The main direct effect of frequent diesel spills on the bivalve *A. flexuosa* and the polychaete *L. culveri* was the induction of superoxide dismutase (SOD) and glutathione *S*-Transferase (GST) activities, a significant increase in lipid peroxide levels (LPO) and a decrease in reduced glutathione (GSH) concentration. The gastropod *N. virginea* only exhibited a significant GSH depletion when exposed to frequent high-dosage spills. *N. virginea* did not activate enzymatic defense system against ROS and oxidative damage to lipids was not observed. Overall, enzymatic activities and oxidative damage in *A. flexuosa* and *L. culveri* were induced by frequent low-dosage spills compared to infrequent high-dosage spills, although the opposite pattern was observed for *N. virginea* antioxidant responses. These results highlight the importance of different exposure regimes in determining the extent of oil impacts. The bivalve *A. flexuosa* and the polychaete *L. culveri* were considered suitable sentinels for petroleum pollution monitoring in nearshore environments. At last, the relative concentration of biliary PAH metabolites and the activity ethoxyresorufin-O-deethylase (EROD) in *A. denticulatus* were significantly higher in dispersed oil (both mechanically and chemically) compared to control. Also, a significant inhibition of acetylcholinesterase (AChE) activity was detected in exposure treatments. Growth rate was significantly higher in control compared to mechanically and chemically dispersed oil. The lack of differences between chemically and mechanically dispersed oil in biomarkers response and growth suggests that dispersant application is no more toxic than the natural oil dispersion. These results indicate the potential for population-level effects resulting from exposure to oil.

Keywords: field experiment; petroleum hydrocarbons; biomarkers; nematodes; macrofauna; wolfish

LISTA DE ARTIGOS

I. Are changes in the structure of nematode assemblages reliable indicators of moderate petroleum contamination?

Leite, D.S., Sandrini-Neto, L., Camargo, M.Z., Thomas, M.C., Lana, P.C., 2014. Mar. Pollut. Bull. 83, 38–47.

II. Are intertidal soft sediment assemblages affected by repeated oil spill events? A field-based experimental approach

Sandrini-Neto, L., Martins, C.C., Lana, P.C. Manuscrito formatado para submissão segundo as normas da revista Environmental Pollution.

III. Antioxidant responses in estuarine invertebrates exposed to repeated oil spills: Effects of frequency and dosage in a field manipulative experiment

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IV. Effects of dispersed oil exposure on biomarker responses and growth in juvenile wolfish *Anarhichas denticulatus*

Sandrini-Neto, L., Geraudie, P., Santana, M.S., Camus, L. Manuscrito formatado para submissão segundo as normas da revista Marine Pollution Bulletin.

PUBLICAÇÕES QUE NÃO CONSTAM NA TESE

Os artigos e livro abaixo foram publicados durante o período do doutorado. Apesar de não serem incluídos como capítulos, sua menção é válida, uma vez que possuem relação direta com escopo geral desta tese.

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SUMÁRIO

INTRODUÇÃO GERAL

Os ambientes aquáticos marinhos têm enfrentado nas últimas décadas um aumento significativo das pressões humanas, como o despejo de efluentes domésticos e industriais, exploração de recursos biológicos e minerais, tráfego marítimo, atividades de dragagem, turismo, entre outros (Borja et al., 2008; Borja et al., 2012; Muniz et al., 2013). Consequentemente, os oceanos, estuários e ambientes costeiros vêm sofrendo perda de habitats, sobrexploração de recursos e redução da biodiversidade (Halpern et al., 2007; Halpern et al. 2008; Sauco et al., 2013).

Ambientes costeiros e estuarinos são frequentemente o repositório final de contaminantes provenientes das atividades humanas. Detectar e compreender os efeitos causados pela entrada de contaminantes nestes sistemas tornou-se um problema central da ecologia aplicada (Underwood, 2000; Terlizzi et al., 2005), principalmente diante da necessidade de mitigar os potenciais impactos gerados. Contudo, avaliar os impactos ecológicos da poluição marinha é uma tarefa complexa. Ela requer o desenvolvimento e aplicação de procedimentos analíticos capazes de distinguir a perturbação antrópica da grande variabilidade espaço-temporal intrínseca aos ambientes costeiros e oceânicos (Underwood, 2000).

Abordagens correntes para a investigação da poluição marinha

Distúrbios antropogênicos têm o potencial de provocar respostas em diferentes níveis de organização biológica, desde as alterações bioquímicas até mudanças na abundância populacional e composição de comunidades, em distintas escalas espaciais e temporais. Sistemas biológicos, contudo, são altamente dinâmicos e naturalmente variáveis no espaço e no tempo (Underwood, 2000; Chollett e Bone, 2007). Distúrbios induzidos pelo homem são apenas um dos muitos processos que podem influenciar seus

padrões de variabilidade. Assim, um passo crucial na detecção dos efeitos adversos causados pelas atividades humanas consiste no desenvolvimento de um conjunto confiável, rápido e eficiente de técnicas analíticas e procedimentos experimentais capazes de distinguir mudanças nas respostas biológicas decorrentes de processos naturais daquelas induzidas pelo homem (Underwood, 2000; Terlizzi et al., 2005).

Tradicionalmente, os efeitos deletérios de contaminantes têm sido investigados através de ensaios toxicológicos, frequentemente utilizados por instrumentos de gestão ambiental para monitoramento e regulação da entrada de contaminantes nos ambientes marinhos. Estes testes procuram isolar o efeito dos contaminantes de outros fatores e possuem a função primária de definir relações de dose-resposta em condições laboratoriais controladas (Johnston e Keough, 2005, Goodsell et al., 2008). São altamente vantajosos pela sua rapidez e elevada reprodutibilidade. No entanto, diante da enorme quantidade de novos compostos químicos sintetizados a cada ano, testes toxicológicos vêm passando por um processo de excessiva padronização e consequente simplificação, sendo frequentemente restritos a ensaios de curta duração com apenas uma única espécie sob condições laboratoriais altamente controladas (Wilding e Maltby, 2006).

Ambientes controlados de laboratório não refletem a elevada variabilidade natural dos ambientes marinhos e as interações dos organismos com o contaminante, que podem afetar os mecanismos de incorporação, bioacumulação e até mesmo de detoxificação (Reid e MacFarlane, 2003; Goodsell et al., 2008; Marques et al., 2014). Assim, quando utilizados isoladamente, ensaios toxicológicos de laboratório são insuficientes para a adequada predição das respostas biológicas aos contaminantes. Neste contexto, têm um potencial limitado para generalizações ecológicas (Underwood, 1995; Johnston e Keough, 2002; Reid e MacFarlane, 2003; Goodsell et al., 2009; Marques et al., 2014).

Muitos dos avanços recentes da ecologia experimental de campo têm sido aplicados na pesquisa e na solução dos problemas decorrentes da poluição marinha (Underwood, 1996; Reid e MacFarlane, 2003; Johnston e Keough, 2005; Egres et al., 2012; Díaz-Jaramillo et al., 2013; Leite et al., 2014; Marques et al., 2014). O desenvolvimento de delineamentos experimentais robustos, apropriadamente replicados no espaço e no tempo, aumentou consideravelmente a habilidade de detectar impactos decorrentes de distúrbios induzidos pelas atividades humanas. Em especial, os delineamentos que seguem a lógica BACI (*Before–After–Control–Impact*) originalmente proposta por Green (1979), com suas necessárias derivações para incorporar múltiplas escalas de tempo e espaço, têm sido amplamente empregados na investigação de impactos ambientais. O princípio lógico destes modelos analíticos é de que distúrbios antrópicos em áreas "impacto" causarão padrões de mudança distintos de antes para depois do seu início, quando comparadas a mudanças naturais em áreas "controle" (Green, 1979; Underwood, 1992; Underwood, 2000). O impacto causa uma interação entre espaço e tempo, detectada estatisticamente como uma mudança entre áreas controle e impacto de antes para depois do distúrbio ter ocorrido (Underwood, 2000; Downes et al., 2002; Terlizzi et al., 2005).

É importante ressaltar que estes delineamentos logicamente estruturados podem ser aplicados na detecção de impactos reais (originados por acidentes, construções, etc.) ou experimentais (com manipulação direta dos contaminantes em campo). Além disso, experimentos de campo, sejam estes mensurativos ou manipulativos (Underwood et al., 2000), devem ser necessariamente combinados com experimentos laboratoriais. Abordagens em laboratório e em campo devem ser complementares no processo de investigação da poluição marinha, uma vez que juntas permitem compreender padrões das respostas biológicas e os mecanismos responsáveis pelos efeitos observados (Underwood et al., 2000; Reid e MacFarlane, 2003).

Natureza dos distúrbios antrópicos

Distúrbios induzidos pelo homem são muito variáveis, sobretudo quando consideradas as atividades potencialmente impactantes desenvolvidas nas regiões costeiras. Distúrbios antrópicos nestes ambientes são tipicamente categorizados como sendo do tipo *press* (persistente) e *pulse* (transicional) (Glasby e Underwood, 1996). Apesar de conceitualmente distintos, distúrbios *press* e *pulse* são frequentemente utilizados de maneira indiscriminada na literatura científica. Distúrbios tipo *press* são mais ou menos contínuos ao longo do tempo, como o despejo de efluentes domésticos e resíduos industriais. Distúrbios dessa natureza podem acarretar cenários de contaminação crônica, de grande persistência no ambiente. Efeitos gerados pelos distúrbios *press* são relativamente previsíveis, como a sequência de estágios de sucessão macrobêntica em relação ao enriquecimento orgânico proposta por Pearson e Rosenberg (1978).

Em contraste, distúrbios tipo *pulse* são eventos de exposição discretos e de curta duração, mas possuem magnitude variável e podem ser repetidos em frequências distintas. Derrames acidentais de petróleo, despejos periódicos licenciados de contaminantes e algumas atividades sazonais potencialmente impactantes (e.g. pesca, turismo, etc.) são exemplos deste tipo de distúrbio. Em geral, assume-se que distúrbios *pulse* geram impactos de curta duração (Underwood, 2000), porém suas consequências podem se manifestar a longo prazo e persistirem por muito tempo após a exposição aos contaminantes (Glasby e Underwood, 1996). Atividades humanas nas regiões costeiras têm aumentado a intensidade e frequência de eventos de exposição discreta a contaminantes (Johnston e Keough, 2005), mas o desenvolvimento de experimentos manipulativos de campo para testar seus efeitos ainda é incipiente. Estes experimentos possibilitarão fazer predições mais seguras sobre a persistência e extensão dos eventos de poluição marinha (Underwood, 1996; Goodsell et al., 2009).

A poluição por hidrocarbonetos de petróleo

A contaminação por hidrocarbonetos alifáticos (HAs) e policíclicos aromáticos (HPAs) é particularmente preocupante nos ambientes estuarinos, devido à sua distribuição ubíqua nos sedimentos e, principalmente, em razão do potencial genotóxico, mutagênico e carcinogênico de alguns HPAs (Wang et al., 2011). Os hidrocarbonetos alifáticos podem ser sintetizados naturalmente por organismos como plantas superiores, bactérias, fitoplâncton e zooplâncton, além de fazerem parte da composição do petróleo (Wang et al*.,* 2009). Os HAs correspondem a um amplo grupo de compostos orgânicos, que inclui os alcanos e cicloalcanos (de cadeia normal e ramificada), alcenos e cicloalcenos (de cadeia normal e ramificada), alcinos, terpanos, hopanos, esteranos, entre outras classes (Dauner et al., 2015).

Os HPAs, por outro lado, são oriundos principalmente de fontes antrópicas, formados da combustão incompleta de combustíveis fósseis e biomassa vegetal, além de fazerem parte do petróleo bruto e de seus derivados (Liu et al., 2009). HPAs são moléculas com pelo menos dois anéis aromáticos, sendo o naftaleno o representante mais simples desta classe de hidrocarbonetos (Dauner et al., 2015). Os HPAs podem ser categorizados em dois grupos, quanto a sua origem, estrutura e propriedades químicas: 1) os HPAs de menor massa molecular contém dois a três anéis aromáticos, frequentemente apresentam homólogos alquilados, e são principalmente associados a fontes petrogênicas, como derrames de petróleo ou de seus derivados; 2) os HPAs de maior massa molecular contém mais de três anéis aromáticos, sendo comumente associados a fontes pirolíticas, como a combustão incompleta de material orgânico além de emissões veiculares e industriais (Notar et al., 2001; Dauner et al., 2015).

Derrames acidentais de óleo constituem uma das principais fontes de hidrocarbonetos de petróleo nos sistemas marinhos e podem acarretar efeitos deletérios à biota em distintos níveis de organização (NRC, 2003, Egres et al., 2012). Apesar do

significativo declínio no número de acidentes desde dos anos 1970, ambientes marinhos e costeiros ainda são severamente ameaçados por estes impactos (Stevens et al., 2012). Derrames de óleo no ambiente marinho podem ocorrer através das atividades de exploração, produção e transporte de petróleo e de seus subprodutos (NRC, 2003; Gong et al., 2014). Além disso, óleo pode ser introduzido no ambiente marinho através de descargas operacionais, que mesmo ilegais, ocorrem regularmente (Stout e Wang, 2010). Como destacado acima, derrames de óleo são caracterizados como perturbações discretas do tipo *pulse*, contudo, constituintes do petróleo como os HPAs podem acumular nos sedimentos e causar impactos ambientais crônicos e de longo-prazo (Kingston, 2002).

A avaliação dos impactos por hidrocarbonetos de petróleo nos ambientes marinhos é rotineiramente descritiva e executada após derrames acidentais de óleo. Tais avaliações contemplam indicadores biológicos em distintos níveis de organização, desde respostas suborganísmicas como a atividade de enzimas antioxidantes e dano celular (Jewett et al., 2002; Katsumiti et al., 2009; Tim-Tim et al., 2009; Morales-Caselles et al., 2009; Sureda et al., 2011), até mudanças na estrutura das comunidades de animais e plantas (Jewett et al., 1999; Ocon et al., 2008; Payne et al., 2008). No entanto, avaliações de impacto após derrames de oportunidade frequentemente produzem resultados ambíguos e de difícil interpretação, uma vez que não apresentam controles experimentais confiáveis e os padrões de variabilidade das respostas biológicas são raramente conhecidos antes dos derrames acontecerem. Estas limitações impossibilitam o estabelecimento de relações de causalidade entre a presença do óleo e a manifestação das respostas biológicas. Consequentemente, as diferenças encontradas, seja qual for o nível de organização biológica adotado, podem simplesmente refletir a variabilidade natural intrínseca aos ambientes marinhos e costeiros.

Estudos envolvendo o transplante de organismos para áreas contaminadas têm se mostrado uma alternativa mais robusta àqueles conduzidos após derrames de

oportunidade, sendo particularmente úteis em programas de monitoramento costeiro (Díaz-Jaramillo et al., 2013; Turja et al., 2013; Turja et al., 2014; Vidal-Liñán et al., 2014). Tais estudos estão focados na avaliação das respostas no nível suborganísmico, como a concentração de contaminantes (metais, PCBs e HPAs) nos tecidos animais e atividade de biomarcadores enzimáticos (Turja et al., 2013; Vidal-Liñán et al., 2014). Apesar de permitirem uma avaliação integrada com vistas ao monitoramento da poluição em áreas costeiras, a aplicação de tais ferramentas não permite estabelecer uma relação causal, direta e previsível entre a exposição de determinado contaminante e as respostas biológicas observadas. Além disso, muitos dos biomarcadores utilizados nestes monitoramentos não são específicos, sendo fortemente influenciados por variações sazonais, como já demonstrado para a atividade das enzimas antioxidantes glutationa peroxidase e catalase (Vidal-Liñán et al. 2010; Vidal Liñán et al., 2014).

Mais recentemente, experimentos manipulativos com simulações de derrames *in situ* têm sido conduzidos para avaliar as respostas biológicas à contaminação por hidrocarbonetos em vários níveis de organização (Egres et al., 2012; Leite et al., 2014; Marques et al., 2014). Trabalhos com esta abordagem são mais apropriados para o estabelecimento de uma relação de causalidade entre o despejo experimental de óleo e as subsequentes respostas biológicas (Glasby e Underwood, 1996). Contudo, estes estudos ainda são escassos na literatura científica, principalmente se considerado que os eventos de exposição a hidrocarbonetos de petróleo são de natureza discreta (i.e. tipo *pulse*) e ocorrem em distintas frequências e intensidades.

Finalmente, a avaliação dos efeitos da poluição por petróleo no ambiente marinho deve considerar respostas biológicas em diferentes níveis de organização, pois estas medidas atendem diferentes propósitos. Medidas nos níveis organísmicos e suborganísmicos fornecem sinais prévios de contaminação e possível deterioração ambiental, sendo em geral consideradas as medidas mais sensíveis de poluição (Underwood e Petersen, 1988; Hansen, 2003; Pereira et al., 2014). Por outro lado,

mudanças na estrutura das comunidades biológicas fornecem uma boa indicação das consequências da poluição no nível dos ecossistemas. Estas diferentes medidas em conjunto com experimentos manipulativos de campo e laboratório auxiliarão a detecção, interpretação e previsão dos impactos causados pelos hidrocarbonetos de petróleo no ambiente marinho.

Estruturação da tese

Nesta tese, os efeitos da contaminação por hidrocarbonetos de petróleo foi investigada em distintos níveis de organização biológica através de experimentos manipulativos de campo e de laboratório. O trabalho procurou reforçar a ideia de que a detecção e a interpretação dos impactos de óleo sobre a biota marinha devem necessariamente envolver a aplicação de distintas disciplinas e abordagens metodológicas.

A tese está estruturada em quatro capítulos, redigidos em inglês, no formato de manuscritos publicados ou preparados para submissão em revistas científicas. O primeiro capítulo avaliou os efeitos de um derrame experimental *in situ* de óleo diesel sobre a estrutura de associações de nematoides marinhos de vida livre utilizando um delineamento MBACI (Multiple Before–After–Control–Impact). Para garantir a uniformidade na formatação geral da tese, este capítulo é apresentado na forma de manuscrito, mas a separata do artigo publicado na *Marine Pollution Bulletin* encontra-se no Anexo. No segundo capítulo, foram investigados os efeitos de repetidos derrames experimentais de óleo diesel sobre a estrutura das associações macrofaunais através da comparação de três frequências de derrames com duas dosagens de óleo diesel em um experimento fatorial com controles assimétricos. No terceiro capítulo foram avaliadas as respostas de biomarcadores de estresse oxidativo no bivalve *Anomalocardia brasiliana*, no gastrópode *Neritina virginea* e no poliqueta *Laeonereis culveri* após exposição

experimental a óleo diesel. Assim como no segundo capítulo, três frequências de derrames foram comparadas com duas dosagens de óleo em um experimento fatorial com controles assimétricos. Finalmente, o quarto capítulo avaliou a sensibilidade de juvenis do peixe-lobo (*Anarhichas denticulatus*) expostos a óleo cru, comparando os efeitos da dispersão mecânica (agitação) e química (uso de dispersantes) do óleo na resposta de biomarcadores e no crescimento.

Capítulo I

Are changes in the structure of nematode assemblages reliable indicators of moderate petroleum contamination?

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Are changes in the structure of nematode assemblages reliable indicators of moderate petroleum contamination?

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Abstract

This study assesses through a multiple before-after-control-impact (MBACI) design the effects of diesel oil on the structure of nematode assemblages in unvegetated tidal flats of a subtropical estuary. Oil-exposed treatments were contrasted with controls for a duration of four successive days before and after an experimental spill in three distinct areas of the Paranaguá Estuarine Complex (Southern Brazil). No significant differences were observed in nematode total density, number of taxa and the overall assemblage structure between the control and impact treatments from before to after the experimental spill. This reinforces the idea that, despite being good indicators of environmental stress, free-living marine nematodes are able to tolerate low concentrations of hydrocarbons and to survive

in moderately contaminated areas. We also show that robust experimental designs are useful to avoid confounding expected natural variability with the effects of a mild impact.

Keywords: Nematodes; Experimental oil spill; Diesel; MBACI; Paranaguá Bay

1. Introduction

Accidents involving oil spills such as the Torrey Canyon in England, Tampico Maru in the United States, Amoco Cadiz in France (Botello and Macko, 1982) and, more recently, the largest spill in history in the Gulf of Mexico, between April and July of 2010 (Mariano et al., 2011), have attracted the interest of the general public and scientists towards oil contamination of the oceans. Previous oil-spilling accidents in Brazil, such as those caused by the ships Norma and Vicuña, which released naphtha, methanol, diesel, and bunker in the Paranaguá Bay in 2001 and 2004, emphasize the need to assess the intensity and extent of damage caused by oil spills, as a first basis for monitoring and control measures.

Estuaries act as sinks for sediment and the associated pollutants from numerous human activities (Yang et al., 2006; Wang et al., 2012). Estuarine habitats are also considered more vulnerable to the impacts of oil spills because the confinement can favour the accumulation of hydrocarbons, mainly in intertidal vegetated areas (Sanz-Lázaro and Marín, 2009).

Oil effects on the benthic macrofauna have been extensively investigated through descriptive (Gómez Gesteira and Dauvin, 2000; Edgar et al., 2003; Zenetos et al., 2004; Andersen et al., 2008; Morales-Caselles et al., 2008; Ocon et al., 2008) and experimental approaches, both in the field (Faraco and Lana, 2003; Schratzberger et al., 2003; Lu and Wu, 2006; Egres et al., 2012) and laboratory (Bhattacharyya et al., 2003). However, few

studies have experimentally investigated the effects of exposure to hydrocarbons on meiofaunal organisms (Fleeger and Chandler, 1983; Ansari and Ingole, 2002; Mahmoudi et al., 2005; Ansari et al., 2010; Beyrem et al., 2010; Boufahja et al., 2011).

Meiofaunal organisms in general and nematodes in particular are considered good indicators of contamination for their high abundance and diversity, short generation time, and direct benthic development (Fleeger and Chandler, 1983; Kennedy and Jacoby, 1999; Ansari et al., 2010). In addition, they are present in different sediment types, hydrodynamic conditions, and environments (Bongers and Ferris, 1999). Furthermore, their predominantly benthic life allows for direct contact with components dissolved in the interstitial water through their permeable cuticle (Warwick, 1981; Heip et al., 1985; Vranken and Heip, 1986; Bongers et al., 1991; Bongers and Ferris, 1999). Another advantage of using nematodes in environmental impact studies is the small sample volume necessary for routine studies, thereby allowing a large number of samples to be collected and, thus, ensuring statistical significance (Bongers and Ferris, 1999). In this context, the responses from nematode assemblages to environmental changes might provide stronger evidence of oil contamination than those obtained from other animals.

Assessments of the effects of oil spills on marine meiofauna are often contradictory and inconsistent. Overall responses seem to be dependent on the amount of oil spilled, environmental characteristics and target taxonomic groups (Fleeger and Chandler, 1983). Decreases in meiofaunal density and taxonomic richness have been repeatedly reported after experimental oil spills (Boucher, 1980; Danovaro et al., 1995; Mahmoudi et al., 2005) and exposure to sediments contaminated by mineral and synthetic lubricating oils (Beyrem et al., 2010). However, some meiofaunal taxa can be highly tolerant to contamination by hydrocarbons and positively respond to the experimental exposure (Fleeger and Chandler, 1983; Mahmoudi et al., 2005).

The analyses of impacts involving oil spills are often carried out after accidents and include descriptions of biological responses from plant and animal communities.

Rarely, the pre-disturbance context is adequately known and inferences on the disturbance are made using a simple comparison between previously impacted locations and undisturbed control sites. Consequently, differences in the composition and structure of assemblages might simply reflect background variability preceding the spill (Underwood, 2000). Micro- and meso-scale experimental approaches are, therefore, more appropriate to establish a causal relationship between oil exposure and the biological responses (Glasby and Underwood, 1996). In this study, we investigated the effects of an experimental marine diesel spill on nematode assemblages using a multiple before–after control–impact (MBACI) design (Keough and Mapstone, 1997; Downes et al., 2002). We hypothesized that total density, number of taxa and overall structure of nematode assemblages living in oil-exposed areas would be significantly different from those in control areas, from before and after the experimental spill.

2. Materials and methods

2.1. Study area

The Paranaguá Estuarine Complex on the coast of Paraná State (48°25'W, 25°30'S) is formed by two main axes, the Paranaguá and Antonina Bays (east–west oriented) and the Laranjeiras and Pinheiros Bays (north–south oriented). This system comprises a diversity of estuarine and coastal ecosystems including coastal dunes, mangroves, salt marshes, rocky shores, and extensive tidal flats (Lana et al., 2001).

The Cotinga Channel (Fig. 1) is about 15 km long and receives freshwater input from the Maciel, Guaraguaçú, Correias, Almeidas, and Itiberê Rivers. Noernberg et al. (2006) classified this region as a sub-estuary based on its hydrographic and morphological features. This sub-estuary is composed of many meandering rivers with extensive floodplains, which favours the formation of unvegetated flats mainly through

sediment delivery from tidal flows from east to west. Domestic effluents from the city of Paranaguá, where the municipal sewage is still discharged *in natura* in the estuary, reach the Cotinga Channel through the Itiberê River.

The tidal flats used in the experiment are located along the Cotinga Channel; the most internal area is near the mouth of the Guaraguaçú River, the intermediate area is near Rasa Island**,** and the most external area is near the mouth of the Maciel River (Fig. 1).

2.2. Experimental design and field procedures

We carried out an acute non-cumulative field experiment with the simulation of a single oil spill. Impacted treatments were contrasted with controls in three distinct areas over four successive sampling times, two before and two after the spill (Fig. 2). The MBACI design was used because it is logically capable of separating the effects of the experimental spill from the background environmental variation by using multiple controls and impacted areas (Keough and Mapstone, 1997). The temporal samplings, equally replicated at the times before and after the experimental spill, ensured the correct interpretation of interactions between time and space. The appropriate spatial and temporal replication ensures that the resulting estimates are reliable (Glasby and Underwood, 1996).

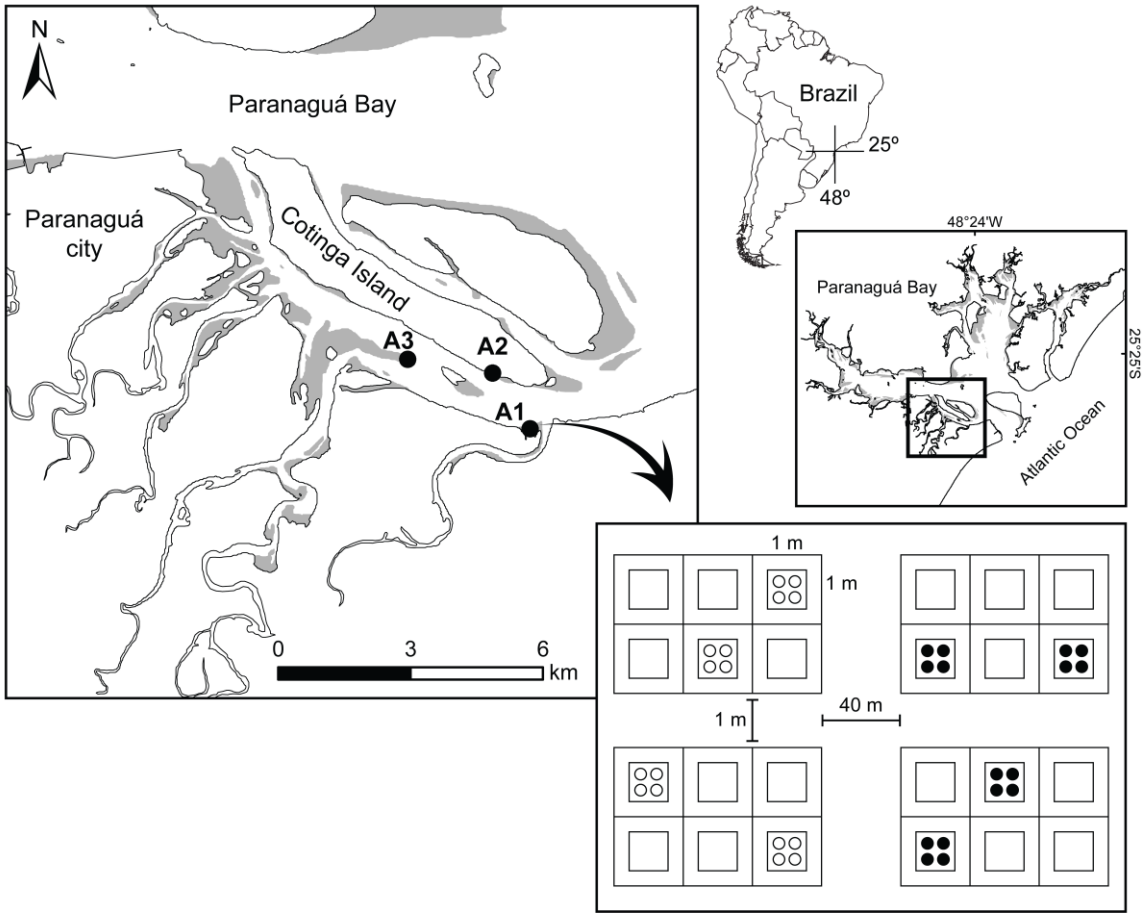

Fig. 1. Map of the Paranaguá Estuarine Complex with the location of intertidal flats and schematic representation of plots with control (○) and impact (●) experimental units.

127

128 Experimental blocks were established in three areas along the Cotinga Channel.
129 Each area included one experimental block corresponding to the impact treatment with
130 the diesel spill and an undisturbed control. The control and impact blocks were
131 established 40 m apart in each area and were positioned at similar tidal levels. Each block
132 consisted of 12 1-m^2 plots with centralized experimental units of 0.35 × 0.35 m (Fig. 1).
133 Plots were arranged in rows with a delimited pathway to avoid trampling and additional
134 disturbances during sampling. Four of these 12 1-m^2 plots in each block were randomly
135 assigned and actually used for sampling (Fig. 1).

136

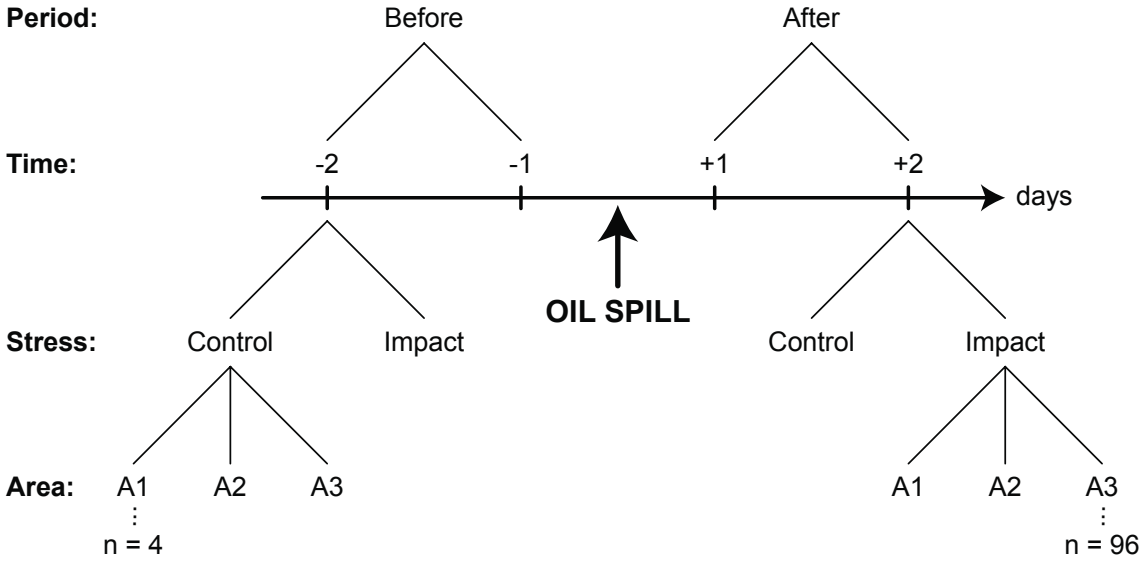

Fig. 2. Multiple before–after control–impact (MBACI) experimental design used in this study.

137

138 The experiment was conducted during low tide, with the simulation of a single spill
139 in early 2010, followed by the monitoring of biological responses between control and
140 impact treatments in pre-established temporal scales two days before and two days after
141 the oil exposure. In each centralized experimental unit of the impact treatment, 2500 ml of
142 marine fuel oil, commercially named Marine Diesel Oil (MDO), was uniformly poured using
143 a garden watering can. Maritime diesel oil is largely used as a fuel by small and medium
144 vessels and in the auxiliary engines of large vessels. Marine fuel oil is produced by mixing
145 of heavy oil fractions obtained by atmospheric distillation with fractions from secondary
146 crude oil processing. The spilled oil was contained by wooden square artifacts properly
147 allocated to prevent its dispersion and cross-contamination of the control treatments.

148

149 *2.3. Biological sampling and processing*

150

151 Four replicated cores were sampled for meiofaunal analyses from each randomly
152 assigned treatment plot (control and impact), in the three unvegetated tidal flats during
153 each of the four sampling times (1 and 2 days before and 1 and 2 days after the

154 experimental oil spill) (Fig. 2). Meiofauna samples were taken using a corer 2.5 cm in
155 diameter and 5 cm in height. Samples were processed according to the procedure
156 proposed by Somerfield and Warwick (1996). Samples were first fixed in 4%
157 formaldehyde and then were sieved through a 63-μm mesh. The retained material was
158 separated using colloidal silica (Ludox TM 50) diluted to a specific gravity of 1.15 g cm^{-3};
159 this procedure was repeated three times. The final supernatant sample was transferred to
160 a Dollfus plate, and 100 individuals (or all individuals if the total number was less than
161 100) were removed and diaphanized according to De Grisse (1969). Subsequently,
162 permanent slides with approximately 10 individuals were assembled, and nematodes
163 were counted and identified at the genus level under a stereomicroscope. The
164 identification keys by Platt and Warwick (1983, 1988) and Warwick et al. (1998) were
165 used. Finally, the total abundance of each species was calculated from the ratio between
166 the frequency of each species among the 100 individuals and the total number in each
167 sample.
168 Three sediment-replicated cores were collected from each treatment at each
169 sampling time for chlorophyll-*a* and phaeopigment analyses; these samples were kept
170 frozen until the analysis. Pigments were extracted from sediment samples with 10 ml of
171 100% acetone (Strickland and Parsons, 1972). The chlorophyll-*a* and phaeopigment
172 concentrations were estimated using the equation described by Lorenzen (1967).
173
174 *2.4. Sampling and processing of physicochemical variables*
175
176 Sediment samples were collected before and after oil exposure from both control
177 and impact treatments for grain size analysis and determination of organic matter content.
178 Grain size analysis was conducted by pipetting and sieving (Suguio, 1973) and
179 granulometric parameters (i.e., sediment grain size in phi, sorting and clay percentage)
180 obtained using SysGran software, version 3.0 (Camargo, 2006). The organic matter

content was determined by differences between the initial and final weights after burning 5 g of sediment at 550 °C for 1 h.

Additional sediment samples were collected before and after the experimental oil spill from both treatments to determine the aliphatic hydrocarbon concentrations. The analytical procedures for sample preparation and determination of aliphatic hydrocarbons were performed according to the methods described by UNEP (1991) and Martins et al. (2004). The levels of hydrocarbon contamination in the sediments were estimated using the concentration of total aliphatics, concentration of unresolved complex mixture (UCM), and association between even and odd chain alkanes (CPI).

Water salinity and temperature (both in sediment and water) were measured *in situ* on all sampling days using a precision digital thermometer and a portable refractometer.

2.5. Data analysis

The total density of nematodes, number of taxa, and density of dominant (three taxa comprising 57% of total density) and constant taxa (three taxa comprising only 15% of total density but present in nearly all samples) were analyzed separately using analysis of variance. ANOVA was also applied to test for differences in the concentration of chlorophyll-*a* and phaeopigments. The linear model consisted of four factors: Stress (two levels, fixed and crossed – control and impact), Period (two levels, fixed and crossed – before and after), Areas (three levels, random and nested within Stress) and Times (two levels, fixed and nested within Period). In such a design, the impact is identified as the interaction Stress × Period indicating an overall difference between the impacted areas compared to controls from before to after the experimental oil spill.

Degrees of freedom, mean square estimates and *F*-ratios for the MBACI model were calculated according to Keough and Mapstone (1997) and Downes et al. (2002) in the R environment (R Core Tem, 2012). The homogeneity of variances was verified with

Cochran's test, and data were transformed when necessary. To meet the homoscedasticity assumption, *Parodontophora* densities were ln(x+1) transformed; densities of *Pseudolella* and the concentration of phaeopigments were transformed to square-root.

Differences among nematode assemblages were tested by permutational multivariate analysis of variance (Anderson, 2001) using the same linear model from the univariate analyses through the PERMANOVA software, version 1.6 (Anderson, 2005). A non-metric, multidimensional scaling analysis (nMDS) was performed to visualize the main variation trends of nematode assemblages between treatments and periods. All multivariate analyses used the dissimilarity coefficient of Bray-Curtis with ln(x+1) transformed data.

3. Results

3.1. Environmental variables and photosynthetic pigments

The sediment of the experimental areas was mainly composed of fine and very fine sand with a low organic matter content (1.2–4.7%). The sediment composition varied slightly among the areas; Area 1 showed a higher percentage of fine sand than the other areas (Table 1).

The water and sediment temperatures remained relatively uniform throughout the study period, ranging from 28 to 29 °C in the water and from 19 to 27 °C in the sediment. A slight salinity gradient was observed from the most external Area 1 (salinity ranging from 26 to 30) to the most internal Area 3 (salinity ranging from 22.5 to 25).

No significant differences were observed in the concentrations of chlorophyll-*a* and phaeopigments in any term of the MBACI model ($P > 0.05$ in all cases). The concentrations of phaeopigments were higher than the chlorophyll-*a* concentration in all

235 cases, ranging from an undetectable amount (hereafter referred as 0) to 157.34 μg g^{-1} in
236 the control, and from 0 to 301.81 μg g^{-1} in the impact treatment. Chlorophyll-*a*
237 concentrations were often below the detection limit and varied from 0 to 65.91 μg g^{-1} in
238 the control, and from 0 to 65.59 μg g^{-1} in oil-exposed sediments.
239

Table 1. Percentages of granulometric fractions and organic matter content in sediment samples of the studied areas (A1, A2, A3) between control and oil-exposed treatments, both before and after the experimental spill.

	Before						After					
	Control			Impact			Control			Impact		
	A1	A2	A3	A1	A2	A3	A1	A2	A3	A1	A2	A3
Coarse sand	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Medium sand	0.2	0.0	0.1	0.1	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0
Fine sand	28.9	6.2	1.1	26.2	6.2	14.2	35.7	6.3	0.8	8.3	14.2	20.3
Very fine sand	57.6	75.7	78.7	54.8	84.8	77.3	55.0	82.1	87.6	82.2	73.4	74.9
Silt	9.5	12.9	7.6	6.9	5.8	3.8	7.1	7.6	8.8	3.1	4.3	2.9
Clay	3.6	4.5	12.2	11.8	2.6	4.5	1.8	3.5	2.6	6.2	7.9	1.8
Organic matter	3.6	4.7	2.9	2.9	3.4	4.6	2.6	2.1	1.2	4.2	2.7	2.1

240

241 *3.2. Aliphatic hydrocarbons*

242

243 The sum of total aliphatics ranged from 1.42 to 2.77 μg g^{-1} of dry sediment in the
244 control treatment, and from 2.31 to 30.8 μg g^{-1} in the impact treatment (Table 2). The
245 presence of UCM (unresolved complex mixture), with values exceeding 60% of total
246 aliphatics, were only recorded in the oil-exposed areas one day after the experimental spill
247 (Table 2).
248 The CPI (Carbon Preferential Index) varied from 5.81 to 6.31 in the control
249 treatment, and from 5.64 to 6.40 in the impact treatment before the spill (Table 2),
250 indicating the predominance of n-alkanes in higher plants. However, one day after the

experimental spill the CPI value approached 1.0 in the impact treatment in Areas 2 and 3, which indicates an oil-genic influence (Table 2).

Table 2. Concentrations and evaluation parameters applied to aliphatic hydrocarbons in sediment samples of the studied areas (A1, A2, A3) between control and oil-exposed treatments, both before and after the experimental spill. AHs, total aliphatic hydrocarbons (μg g^{-1} dry weight); UCM, unresolved complex mixture (μg g^{-1} dry weight); CPI, Carbon Preferential Index.

	Before						After					
	Control			Impact			Control			Impact		
	A1	A2	A3	A1	A2	A3	A1	A2	A3	A1	A2	A3
AHs	1.88	2.77	1.42	3.98	2.31	2.67	1.93	2.55	1.84	10.69	30.88	16.42
UCM	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.34	20.9	10.7
CPI	5.81	6.31	5.91	5.64	6.40	5.87	5.84	6.12	5.79	4.90	2.38	2.62

n.d. = not detected

3.3. Nematodes

A total of 166,980 individuals were counted, belonging to 33 different genera numerically dominated by *Terschellingia*, *Spirinia* and *Sabatieria*. Overall, no significant differences were detected in total density of nematodes, number of taxa, and density of dominant and constant genera between the impacted and control treatments from before to after the experimental spill. No patterns of decreasing total density and the total number of taxa were observed (Fig. 3). However, a sharp decrease in the total density of nematodes was evident after the impact, only in Area 2, followed by a fast recovery two days after the spill (Fig. 3).

The total density and number of taxa showed no significant differences for the main effects. The main source of variability was associated with the spatial heterogeneity, with significant differences observed among areas (Table 3).

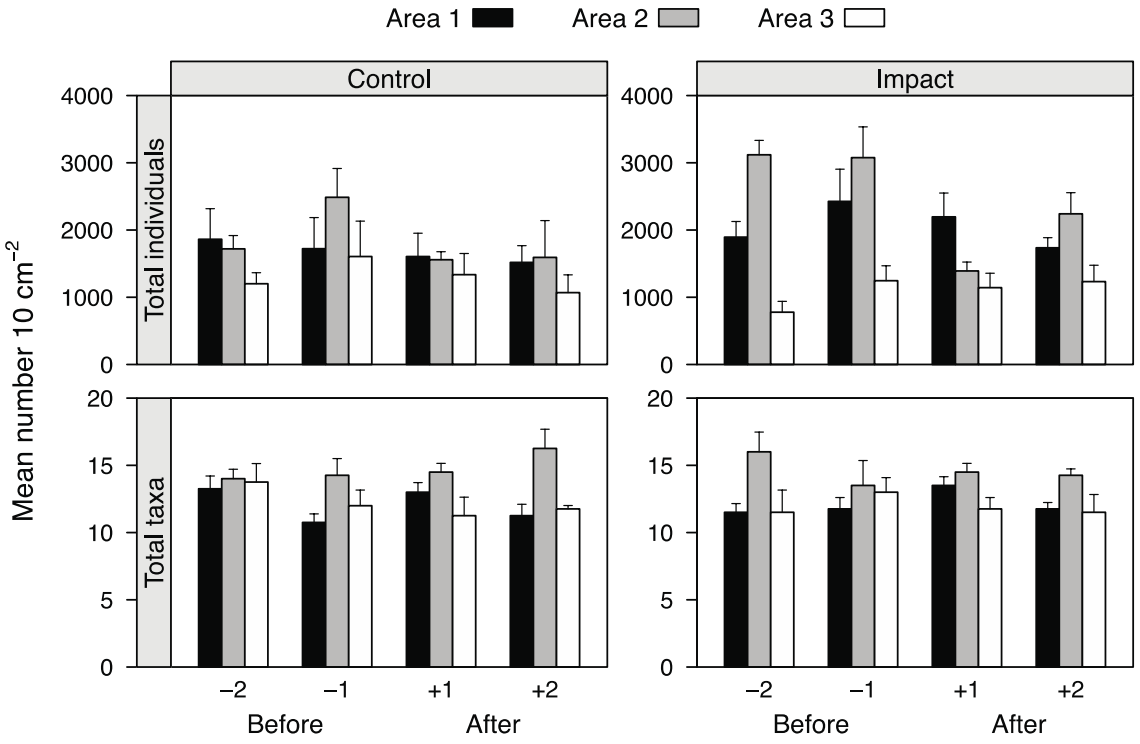

Fig. 3. Mean (SE, n = 4) density of nematodes, and number of taxa in the three areas of control and impact treatments, from before (–2, –1 days) to after (+1, +2 days) the experimental oil spill.

268

269 No significant variation was recorded in the density of the dominant taxa (i.e.,
270 *Terschellingia*, *Spirinia* and *Sabatieria*) that could be unequivocally attributed to the
271 experimental spill (Fig. 4). *Terschellingia* density varied only between periods, regardless
272 of the effects of treatments or areas (Table 3). Significant differences were detected in the
273 density of *Sabatieria* among areas and sampling times, whereas densities of *Spirinia*
274 differed only among areas (Table 3).

275 The three more constant genera (i.e., *Parodontophora*, *Metachromadora* and
276 *Pseudolella*) showed responses similar to the patterns from the numerically dominant
277 taxa, without significant reductions or variations that could be attributed to the
278 experimental spill (Fig. 5; Table 3). Densities varied mainly among areas, especially for
279 *Pseudolella*, which presented a relatively high and constant density in Areas 1 and 2, and
280 extremely low in Area 3.

281 Similar to the patterns observed in the univariate analyses, the nMDS ordination
282 diagram and PERMANOVA results showed no evidence of differences related to the
283 experimental spill, but revealed a remarkable difference in the structure of nematode
284 assemblages among the studied areas (Fig. 6; Table 4).

Table 3. Summary of analysis of variance (n = 4 replicate cores) of the MBACI model for total density of nematodes (a), total number of taxa (b) and densities of dominant (c–e) and constant taxa (f–h).

		(a) Total individuals		(b) Total taxa		(c) *Spirinia*	
Source	df	MS	*F*	MS	*F*	MS	*F*
Stress = S	1	1708800.67	0.380	0.38	0.011	64325.26	0.223
Period = P	1	3400548.17	2.809	0.00	0.000	204333.76	1.605
Areas(Stress) = A(S)	4	4500681.21	10.379***	35.56	8.002***	287923.08	3.498*
Times(Period) = T(P)	2	662541.33	1.740	4.27	0.845	115620.05	2.523
S × P	1	77520.67	0.064	0.00	0.000	6256.51	0.049
S × T(P)	2	107506.83	0.282	3.02	0.598	5682.89	0.124
A(S) × P	4	1210545.04	3.179	5.00	0.990	127317.17	2.779
A(S) × T(P)	8	380769.96	0.878	5.05	1.137	45820.38	0.557
Residual	72	433621.83		4.44		82302.06	
		(d) *Terschellingia*		(e) *Sabatieria*		(f) *Metachromadora*	
Source	df	MS	*F*	MS	*F*	MS	*F*
Stress = S	1	34352.67	0.353	4959.37	0.048	6936.00	0.048
Period = P	1	348968.17	11.256*	39366.00	2.501	2301.04	0.100
Areas(Stress) = A(S)	4	97387.42	2.399	103005.34	3.093*	144438.74	23.049***
Times(Period) = T(P)	2	89347.08	3.923	128509.69	5.243*	5271.04	0.370
S × P	1	6337.50	0.204	39528.17	2.511	9841.50	0.426
S × T(P)	2	3408.33	0.150	8195.85	0.334	22095.08	1.553
A(S) × P	4	31003.96	1.361	15739.30	0.642	23122.86	1.625
A(S) × T(P)	8	22777.83	0.561	24512.24	0.736	14228.66	2.271*
Residual	72	40594.78		33298.53		6266.62	
		(g) *Parodontophora*		(h) *Pseudollela*			
Source	df	MS	*F*	MS	*F*		
Stress = S	1	2.65	0.231	7.22	0.015		
Period = P	1	3.76	4.464	5.99	1.153		
Areas(Stress) = A(S)	4	11.48	5.558***	468.16	40.520***		
Times(Period) = T(P)	2	0.63	0.250	6.80	0.475		
S × P	1	2.67	3.178	7.62	1.467		
S × T(P)	2	1.46	0.584	5.35	0.374		
A(S) × P	4	0.84	0.337	5.20	0.363		
A(S) × T(P)	8	2.50	1.211	14.31	1.239		
Residual	72	2.06		11.55			

Significance codes: *$P < 0.05$; ***$P < 0.001$

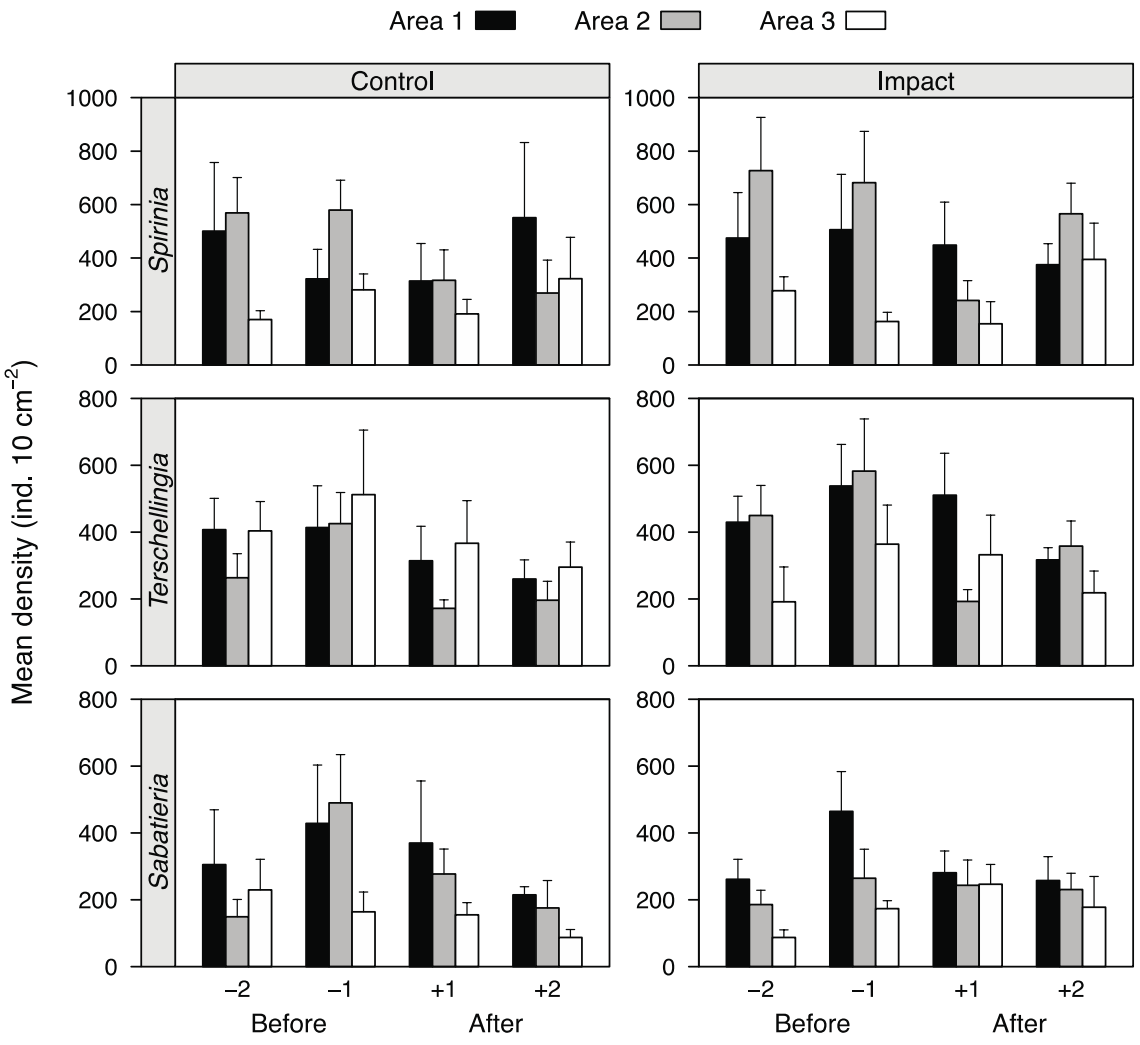

Fig. 4. Mean (SE, n = 4) density of dominant nematode taxa in the three areas of control and impact treatments, from before (–2, –1 days) to after (+1, +2 days) the experimental oil spill.

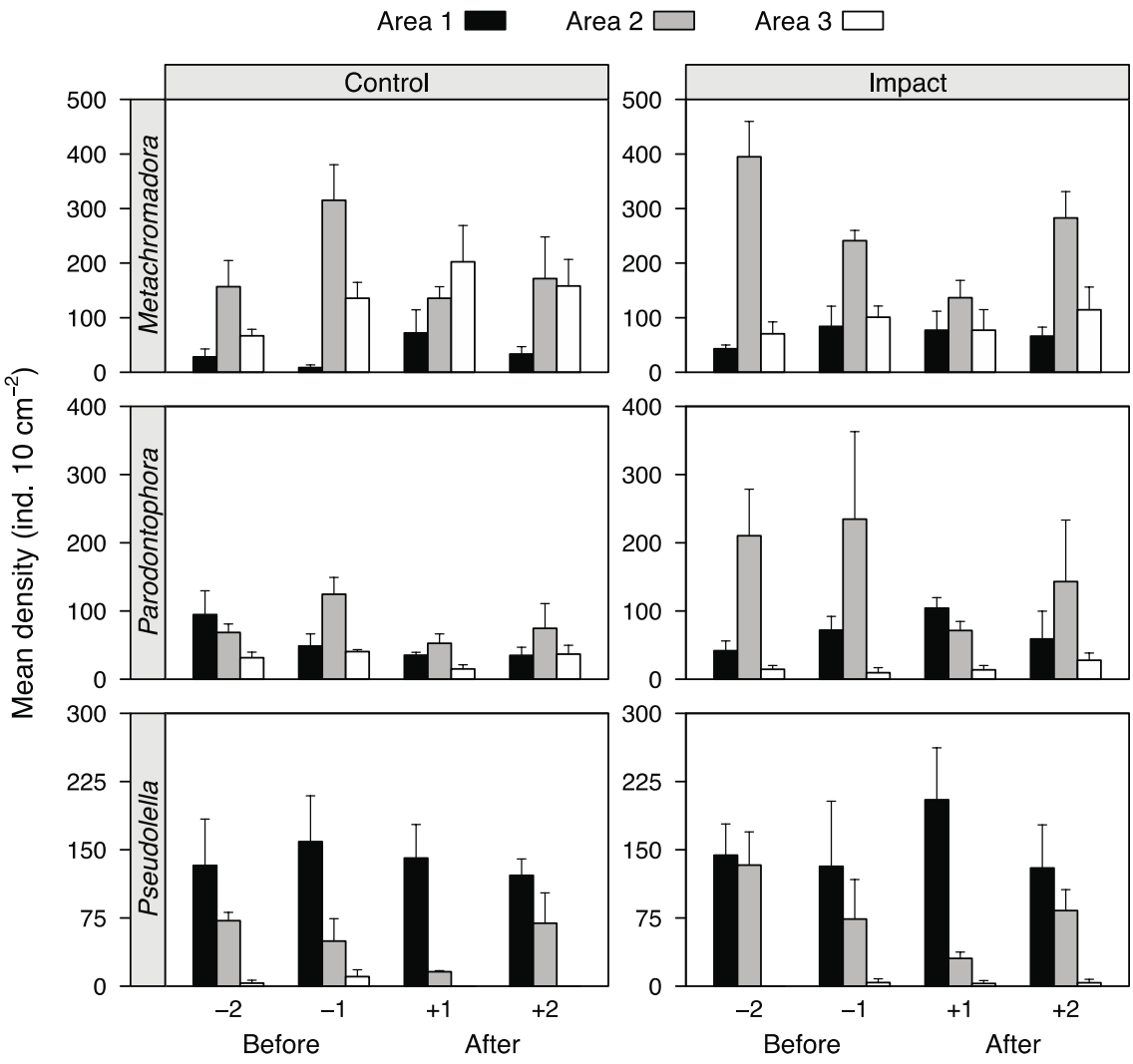

Fig. 5. Mean (SE, n = 4) density of the constant nematode taxa in the three areas of control and impact treatments, from before (–2, –1 days) to after (+1, +2 days) the experimental oil spill.

Table 4. Summary of PERMANOVA (9999 permutations, n = 4 replicate cores) of the MBACI model based on Bray-Curtis dissimilarities of ln(x+1) transformed nematode densities.

Source	df	MS	*Pseudo-F*
Stress = S	1	698.17	0.141
Period = P	1	1203.20	3.624*
Areas(Stress) = A(S)	4	4948.90	10.706***
Times(Period) = T(P)	2	1149.80	1.990
S × P	1	558.71	1.683
S × T(P)	2	567.36	0.982
A(S) × P	4	331.98	0.718
A(S) × T(P)	8	577.96	1.250
Residual	72	462.26	

Significance codes: *$P < 0.05$; ***$P < 0.001$

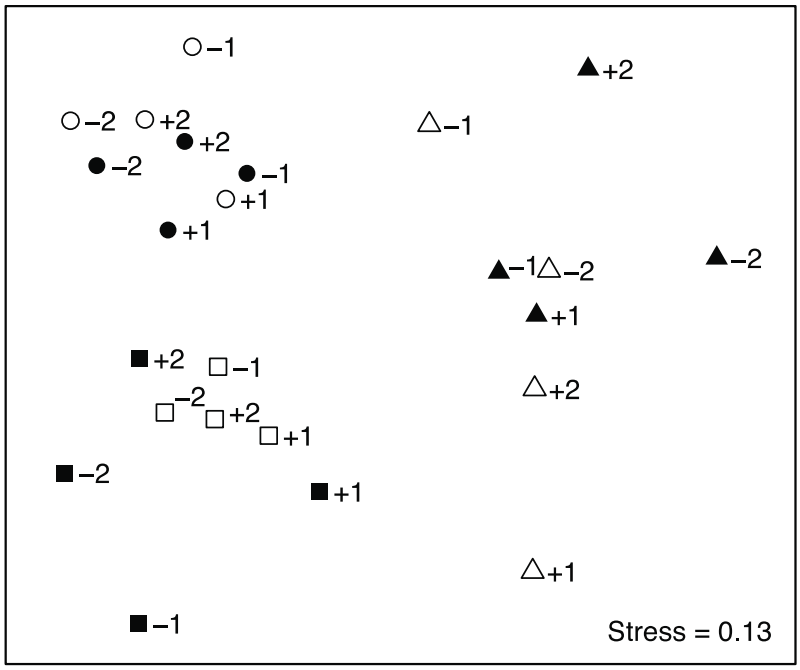

Fig. 6. Non-metric multidimensional scaling (nMDS) of nematode assemblages based on a Bray-Curtis similarity matrix of ln(x+1) transformed data comparing the control (Area 1 = ○; Area 2 = □; Area 3 = △) and impact (Area 1 = ●; Area 2 = ■; Area 3 = ▲) treatments from before (–2, –1 days) to after (+1, +2 days) the experimental oil spill.

4. Discussion

We rejected the hypothesis that total density, number of taxa and overall structure of nematode assemblages in oil-exposed areas would be significantly different from those in control areas, from before to after an experimental spill. No alterations in the structure of nematode assemblages were identified that could be unequivocally attributed to the experimental contamination by diesel fuel, at least in the spatial and temporal scales adopted.

The manipulative MBACI model (Keough and Mapstone, 1997; Downes et al., 2002), applied both in the univariate and multivariate analyses, showed that differences were due more to the spatial variability and heterogeneity among areas than to the experimental spill itself. No significant reductions or increases in the densities of the dominant and constant taxa were recorded after the experimental spill, except in Area 2. In this area, a decrease in the overall density of nematodes was observed reflected by the responses from the *Parodontophora*, *Terschellingia*, and *Spirinia* genera after the impact, yet detected on day 1 and followed by a fast recovery on day 2. This type of response is defined by Underwood (2000) as a "pulse disturbance", i.e., a short-term effect with a sudden drop in the density of organisms followed by a fast recovery.

The rapid recovery of benthic communities after small-scale disturbances is well known in the local literature and has been reported in previous experiments (Faraco and Lana, 2003; Egres et al., 2012; Gern and Lana, 2013; Sandrini-Neto and Lana, 2014). This rapid recovery is usually associated with the active migration of juvenile and adult animals from adjacent areas to the experimental plots (Negrello Filho et al., 2006; Sandrini-Neto and Lana, 2014), larval recruitment (Carman et al., 2000), or tolerance to toxic compounds by recolonizing species (Schratzberger et al., 2003; Beyrem et al., 2010). In the case of small animals devoid of larval development such as nematodes, the possibility of active/passive transport of resuspended adults and juveniles by currents

312 from nearby areas should also be considered as a relevant recolonization mechanism
313 (Thomas and Lana, 2011).
314 The time scales used in our experiment were short and, therefore, there was not
315 enough time for the development and subsequent direct meiofaunal recruitment. In this
316 context, the persistence or rapid recolonization of affected assemblages certainly
317 occurred because the dominant and constant species were tolerant to small
318 concentrations of oil, rapidly migrating, or being passively transported from adjacent
319 areas.
320 Many factors can explain the broad variability of species-specific responses to
321 contaminants. For instance, sediment texture and seawater properties can affect
322 contaminant bioavailability (Langston and Spence, 1994), which in turn depends on
323 partitioning between the sediment, pore water and overlying water (Austen and McEvoy,
324 1997) and also of the sediment organic carbon content (Di Toro et al., 1991).
325 Total density and the number of nematode taxa only differed among experimental
326 areas. These differences highlight the importance of physical, chemical, and biological
327 gradients that create great spatial and temporal heterogeneity for the resident fauna (Rodil
328 et al., 2006; Gingold et al., 2010). Nematode assemblage structure varies greatly at
329 distinct spatial and temporal scales as a response to the scale-dependent nature of most
330 ecological processes (Blome et al., 1999). Overall, density and diversity of nematodes are
331 regulated by sediment-related variables, particularly the grain size (Steyaert et al., 1999).
332 Coarser sediments promote more diverse nematode assemblages, whereas finer
333 sediments are characterized by low diversity but generally high densities (Coull and
334 Chandler, 1992; Steyaert et al., 1999). There were, however, no clear relationships
335 between nematode patterns and grain-size characteristics of the sediments in our study,
336 despite a slight difference in granulometry among experimental areas.
337 Similar to the patterns described for density and number of nematode taxa, the
338 densities of the dominant taxa (i.e., *Terschellingia*, *Spirinia* and *Sabatieria*) did not suffer

339 significant changes that could be attributed to the experimental oil spill. *Terschellingia*
340 densities varied significantly between sampling times regardless of treatment or area
341 suggesting some environmental change affecting all treatments and areas in a similar
342 way. Similarly, no significant variations were detected in the densities of the constant
343 genera *Pseudolella*, *Parodontophora* and *Metachromadora* because of the experimental
344 oil spill. Again, other sources of environmental variability proved to be more important
345 because heterogeneity was observed among the experimental areas.
346 The non-metric multidimensional scaling (nMDS) showed a clear separation of the
347 areas, indicating that the spatial variability in the structure of assemblages was more
348 important than variation putatively introduced by the oil spill. The multivariate analysis
349 reinforces the pattern found in the univariate analyses, which in almost all cases showed
350 significant differences among the studied areas.
351 Hydrocarbon analyses indicated that oil-exposed plots were effectively
352 contaminated, although the persistence of contaminants also varied significantly between
353 areas. According to Volkman et al. (1992), concentrations of total aliphatic hydrocarbons
354 below 10 $\mu g\ g^{-1}$ indicate that sediments are not impacted by hydrocarbons, whereas
355 values greater than 100 $\mu g\ g^{-1}$, along with the presence of UCM, indicate oil
356 contamination. All control treatments presented values below 10 $\mu g\ g^{-1}$ indicating no
357 hydrocarbon contamination. However, the impact treatments in all areas presented
358 intermediate values between 10 and 100 $\mu g\ g^{-1}$, which indicate small alterations in the
359 environment.
360 The presence of UCM is usually associated with degraded or weathered petroleum
361 residues and provides strong evidence for petroleum contamination in sediment samples
362 (Readman et al., 2002; Azimi et al., 2005; Maioli et al., 2011). In this study, the presence
363 of UCM with values exceeding 60% of total aliphatics were only recorded in the impact
364 treatments one day after the experimental spill, indicating the contamination caused by
365 petroleum hydrocarbons.

366 The CPI is used to determine the origin of compounds taking into account the
367 concentrations of hydrocarbons with odd carbon chains over the even carbon chains in *n*-
368 alkanes of greater molecular mass (C_{25}–C_{34}) (Wang et al., 1999). Values around 1.0 and
369 high concentrations of total aliphatics indicate an anthropogenic origin of *n*-alkanes from
370 petrogenic contamination (Wang et al., 1999), whereas values greater than 4.0 indicate a
371 biogenic origin of *n*-alkanes associated with terrigenous input (Hostettler et al., 1999). This
372 index ranged from 5.81 to 6.31 before the spill in the control treatments and from 5.64 to
373 6.40 in the impact treatments, indicating that in all cases the hydrocarbons were
374 essentially of terrigenous biogenic origin before the spill. The value approached 1.0 after
375 the spill in the impact treatments, particularly in Areas 2 and 3, indicating the influence of
376 the oil spill in these areas.
377 Analyses of the effects of oil spills on meiofauna have generated contradictory or
378 inconsistent results due to different approaches applied to different environments.
379 According to Coull and Chandler (1992), hydrocarbon effects on meiofauna depend on
380 the oil type, crude oils being less toxic than refined oil. Results are also affected whether
381 or not meiofauna are exposed in the field or laboratory conditions. Toxicant dosage should
382 be higher in field exposure than *in vitro* in order to detect toxic effects (Coull and
383 Chandler, 1992). Finally, pollutant effects on meiofauna depend on taxon sensitivity.
384 Generally, the response of nematodes to pollution is not uniform and relatively weak when
385 compared to other meiofauna groups, especially copepods (Coull and Chandler, 1992).
386 Some studies have reported decreases in the density and diversity of taxa while
387 others reported no significant effects or even increases in the densities of organisms.
388 Fleeger and Chandler (1983) carried out an experiment of crude oil spillage over a bank of
389 *Spartina alterniflora* in Louisiana and recorded increased densities in the dominant
390 meiofaunal groups. In a microcosm experiment, Mahmoudi et al. (2005) demonstrated
391 that responses of nematode species to diesel exposure were varied. They observed that
392 some taxa (e.g., *Chaetonema* sp.) are extremely sensitive to the effects of diesel oil,

393 whereas others (e.g., *Daptonema* spp.) showed increased densities, which could suggest
394 opportunistic behaviour. Danovaro et al. (1995) recorded a decrease in the densities of
395 the majority of taxa and defined the meiofauna as being extremely sensitive to oil impact.
396 The use of different contaminants in manipulative experiments can also lead to
397 contradictory results. Nematode assemblages seemed to be more affected by synthetic
398 lubricant oils that contain highly toxic additives (Thompson et al., 2007), which are often
399 more recalcitrant to biodegradation than the base oil (Powell et al., 2005). A series of
400 laboratory microcosm experiments showed that *Daptonema trabeculosum* and *Spirinia*
401 *gerlachi* were eliminated with synthetic lubricant oils. However, densities of *Spirinia*
402 *gerlachi* only increased in synthetic lubricant oils “used” and ‘‘clean” and *Terschellingia*
403 *longicaudata* increased in “pure” synthetic lubricant. Therefore, these species were
404 categorized as “intolerant” or “resistant”, depending on the type of contaminant.
405 The experimental areas are subjected to constant tidal influence and are located
406 near the mouth of rivers, which can accelerate the dispersion and dilution of oil. These
407 conditions can also favour rapid recolonization of organisms moving from nearby areas.
408 Thomas and Lana (2011) showed that nematodes from the same region can disperse up
409 to 2 metres when carried over by currents in the water column during a single tidal event.
410 This distance would be enough for organisms from other areas to quickly move towards
411 the impact treatment areas and recolonize them in a few hours.
412 In this sense, the experimental analysis showed that the local nematode
413 assemblages displayed a resilient behaviour, being able to withstand small concentrations
414 of hydrocarbons or rapidly recolonize impacted areas. The rapid recovery of the impacted
415 areas is probably associated with the dynamics of the studied areas, which favours the
416 dispersion of pollutants through intense tidal currents and enables a rapid meiofaunal
417 recolonization.
418

5. Conclusions

By comparing oil-exposed treatments and controls through an MBACI design, we showed that marine free-living nematodes in unvegetated tidal flats of a subtropical estuary are resilient to oil disturbance. Despite being considered good indicators of environmental impacts, these organisms were able to tolerate low concentrations of hydrocarbons in sediments and to survive in moderately petroleum-contaminated areas. There were no significant changes in overall abundance and number of taxa between control and impacted treatments from before to after the oil spill. Significant differences in the structure of nematode assemblages were more related to the spatial and temporal variability than to the presence of oil contaminants in the sediment. However, it should be stressed that observed patterns are potentially scale-dependent, both in space and over time.

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Capítulo II

Are intertidal soft sediment assemblages affected by repeated oil spill events? A field-based experimental approach

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Are intertidal soft sediment assemblages affected by repeated oil spill events? A field-based experimental approach

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Abstract

This study investigates the impact of repeated diesel spills on the structure of intertidal macrofaunal assemblages of a subtropical estuary. Three frequencies of exposure events were compared against two dosages of oil in a factorial experiment with asymmetrical controls. Hypotheses were tested to distinguish between (i) the overall effect of oil spills, (ii) the effect of diesel dosage via different exposure regimes, and (iii) the effect of time since last spill. Repeated oil spills dramatically altered the overall structure of assemblages and reduced the total density of macrofauna and densities of dominant taxa. Increasing the frequency of oil spills negatively affected macrofauna. In general, frequent low-dosage oil spills were more deleterious than infrequent high-dosage ones. However, increases in densities of some taxa, mainly the gastropod *Heleobia australis*, were

observed in response to infrequent spills. Our results highlight the importance of repeated exposure events in determining the extent of oil impacts.

Keywords: Macrofauna; Field experiment; Oil exposure; Frequency; Diesel; Paranaguá Bay

1. Introduction

Marine and estuarine macrofaunal assemblages are subject to abiotic and biotic disturbance, which include both natural (e.g. bioturbation, waves, currents and storms) and human induced events (e.g. bottom trawling, dredging, sewage discharges and oil spills). Disturbance is recognized as an important factor structuring soft-sediment assemblages (Zajac et al., 1998) and despite the large range of potential causes they can all in principle be characterized by their frequency, intensity and scale (Whomersley et al., 2010).

Oil spills are among the main sources of anthropogenic disturbances in marine environments (Gong et al., 2014; Tansel, 2014). Despite a significant decline in the number of accidents since the 1970s, marine and coastal ecosystems are still threatened by oil impacts (Stevens et al., 2012). Oil may be released into the marine environment from routine or accidental discharges as a result of exploration, production, and transport activities (NRC, 2003; Gong et al., 2014). Once oil is released into the environment, it undergoes several processes (e.g. spreading, evaporation, dissolution, emulsification, and sedimentation), which may differ in terms of persistence and transformation profiles between marine and coastal environments (Tansel, 2014).

Effects of crude and refined petroleum hydrocarbons on benthic assemblages have been extensively investigated through descriptive (Gómez Gesteira and Dauvin,

2000; Edgar et al., 2003; Zenetos et al., 2004; Andersen et al., 2008; Ocon et al., 2008; Yu et al., 2013) and manipulative experimental approaches (Carman et al., 2000; Faraco and Lana, 2003; Schratzberger et al., 2003; Lu and Wu, 2006; Egres et al., 2012, Leite et al., 2014). Marine benthic organisms are often considered good indicators of pollution due to their ecological importance, numerical abundance and close association with the sediments, where contaminants tend to accumulate (Hyland et al., 2005). However, descriptive or experimental assessments of the effects of oil spills on benthic organisms have often produced contradictory results, even in similar habitats and with the same taxonomic groups (see examples in Carman et al., 2000).

Most information on the effects of petroleum hydrocarbons on macrobenthic species originates from acute, non-cumulative, single-dosage oil spills. This is unfortunate, particularly in coastal and estuarine habitats, where intense traffic of small and mid-size ships, together with fishing and recreational boats are often responsible for the release of petroleum products at a range of frequencies and intensities. Most of these vessels use marine diesel oil as fuel, which is less persistent than crude oil but highly toxic (Lytle and Peckarsky, 2001). Little is known of how repeated oil exposure events at varying frequencies and intensities affect assemblage structure, especially in the field. Many field-based experimental studies have related the effects of physical disturbance on assemblage structure to their frequency and intensity, which is not a widespread approach in the study of pollutants (Johnston and Keough, 2005). However, both frequency and intensity of any disturbance presumably play an important role in determining the extent of an impact (Johnston and Keough, 2005; Goodsell et al., 2008).

In this study, we assess the effects of the frequency and intensity of experimental diesel spills on intertidal macrofaunal assemblages. By comparing the effects of three frequencies of exposure events against two dosages of oil in a factorial experiment with asymmetrical controls, we tested the following hypotheses: 1) if macrofaunal abundance and structure are overall affected by repeated oil spill events, then assemblages exposed

to frequent high-dosage spills will significantly differ from those in the control treatment; 2) if different exposure regimes are determinant causes of variability, then assemblages exposed to frequent low-dosage spills will be significantly different from those exposed to infrequent high-dosage spills; 3) if the time elapsed since the last oil spill is determinant, then assemblages exposed to the same dosage of oil under the same frequency, but for which the timing of exposure differed, will be significantly different from each other.

2. Materials and methods

2.1. Study area

Experimental oil spills were carried out at an unvegetated tidal flat in the Cotinga sub-estuary (25°32'24.2"S, 48°27'20.1"W), a 20-km channel located in the polyhaline sector of Paranaguá Bay, southern Brazil. The Cotinga sub-estuary receives sewage discharges from Paranaguá city (Barboza et al. 2013). The waste of nearly 50% of the city's population of about 150,000 undergoes treatment, while the rest is directly discarded without any treatment (Souza et al., 2013). Other potential impact sources include industrial activities related to an oil terminal, which may contribute to a progressive increase in the disposal of petroleum products (Abreu-Mota et al., 2014).

Despite the existence of a fecal contamination gradient along the channel, from the vicinity of Paranaguá port towards the open sea, local sediments are not contaminated by petroleum hydrocarbons (Abreu-Mota, 2014). Intertidal sediments are very similar along the sub-estuary and are predominantly composed by moderately to well-sorted very fine sands (Souza et al., 2013). Tides are semi-diurnal with diurnal inequalities and may reach up to 1.7 m in the sub-estuary during spring tides (Lana et al., 2001; Marone et al., 2005).

2.2. Experimental design and field procedures

We carried out a 15-week field experiment simulating repeated oil spill events, which occurred at varying frequencies and intensities. The design comprised an undisturbed control and seven oil exposure treatments. All of these consisted of the interaction between two dosages of oil (2.5 and 5 L m^{-2}) spilled at three distinct frequencies (every 2 weeks, 4 weeks or 8 weeks) starting from day one, except for the staggered treatment. This particular high-dosage/low-frequency treatment was 4-week delayed and specifically designed to evaluate the effect of time since last spill. Hereafter the treatments are referred to as Control, 2w2.5, 2w5.0, 4w2.5, 4w5.0, 8w2.5, 8w5.0 and 8w5.0-st, respectively (Table 1). Our design and analysis were based on the experiment conducted by Johnston and Keough (2005), which investigated the impact of varying frequencies and intensities of copper pulses on estuarine sessile invertebrates.

Treatments were assigned randomly to sixteen 1-m^2 plots positioned at similar tidal levels; i.e. two replicated plots were assigned for each undisturbed control and oil exposure treatments. Experimental spills were done during the low tide when the tidal flat was emerged, thus optimizing the time for the oil to percolate into the sediment. In each plot of the impact treatments, 2.5 or 5 L of marine diesel oil were uniformly poured using a garden watering can according to the schedule in Table 1. Marine diesel oil is largely used as a fuel by small and medium vessels and in the auxiliary engines of large vessels (Leite et al., 2014). The spilled oil was contained by zinc square artifacts pushed into the sediment in order to prevent its dispersion and cross-contamination of the control treatment.

Five replicate cores from each exposure treatment and undisturbed control plots were sampled a day after the last oil spill event. Macrofauna samples were taken using a corer 15 cm in diameter and 10 cm in height. Each sediment sample was sieved through a

133 0.5 mm mesh and fixed in 10% formalin; animals were counted and identified to the
134 lowest taxonomic level with a stereo-microscope.
135

Table 1. Schedule of repeated oil spill events with the indication of different treatments in the experimental design and overall volume of diesel.

	Low-dosage spills (2.5 L m^{-2})			**High-dosage spills (5 L m^{-2})**			
	2w2.5	4w2.5	8w2.5	2w5.0	4w5.0	8w5.0	8w5.0-st
Week 1	oil spill	oil spill	oil spill	oil spill	oil spill	oil spill	
Week 3	oil spill			oil spill			
Week 5	oil spill	oil spill		oil spill	oil spill		oil spill
Week 7	oil spill			oil spill			
Week 9	oil spill	oil spill	oil spill	oil spill	oil spill	oil spill	
Week 11	oil spill			oil spill			
Week 13	oil spill	oil spill		oil spill	oil spill		oil spill
Week 15	oil spill			oil spill			
Overall volume	20 L	10 L	5 L	40 L	20 L	10 L	10 L

136

137 A sediment sample was also collected from each plot a day after the last oil spill
138 event to determine the concentration of polycyclic aromatic hydrocarbons (PAHs). The top
139 2 cm of surface sediment was collected with a spoon and placed in pre-cleaned aluminum
140 foil and stored at –20 °C. The material was freeze-dried, carefully homogenized with a
141 mortar, and stored in clean glass bottles at room temperature prior to PAHs analysis.
142

143 *2.3. PAHs analysis*

144

145 The analytical procedures for sample extraction and determination of PAHs were
146 performed according to the methods described by UNEP (1992) and Martins et al. (2011),
147 respectively. Briefly, 15 g of sediment samples from each plot was extracted for 8 h using
148 80 mL of a mixture (1:1) of hexanes/dichloromethane. A mixture of surrogates

149 (naphthalene-d_8, acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12} and perylene-d_{12})
150 was added to each sample. The extract was purified by column chromatography using 5%
151 deactivated alumina and silica. The organic proxies were eluted in three fractions using 10
152 mL of hexanes (fraction 1 - aliphatic hydrocarbons, not presented in this study) and 15 mL
153 of 30% dichloromethane/ hexanes (fraction 2 - PAHs). Fractions 1 and 2 were
154 concentrated to 1 mL in hexanes. An aliquot of 1 μL of each extract was injected for gas
155 chromatographic analysis.
156 The analyses were performed with an Agilent GC (model 6890) coupled to an
157 Agilent mass spectrometer detector (Agilent 5975C inert MSD with Triple-Axis Detector)
158 and an Agilent 19091J-433 capillary fused silica column. Helium was used as the carrier
159 gas. Compounds were identified by matching retention times and ion mass fragments with
160 results from standard mixtures of PAHs from the National Institute of Standards and
161 Technology, USA (NIST 2260 – Aromatic Hydrocarbons Standard Reference Material).
162
163 *2.4. Data analysis*
164
165 Analyses of variance with a series of planned contrasts were used to test
166 hypotheses about differences in total density of macrofauna and densities of dominant
167 taxa (eight taxa present in nearly all samples comprising 90% of total abundance). We
168 followed the procedures described by Underwood (1997) for the analysis of an
169 asymmetrical experiment. The cause of asymmetry in the design is that there can only be
170 a single group of replicated controls, although Frequency and Dosage are orthogonal
171 factors to one another. Asymmetry is also caused by the staggered treatment, which
172 cannot be used as a factor level in the factorial analysis.
173 Hence, differences among all eight treatments were initially tested by a single-
174 factor analysis of variance. The mean square error and residual degrees of freedom from
175 this analysis provided the error term for any subsequent test, including all planned

176 contrasts (Quinn and Keough, 2002; Johnston and Keough, 2005). Then, a two-factor
177 analysis of variance with Frequency (3 levels, fixed) and Dosage (2 levels, fixed, crossed
178 with Frequency) as factors was conducted. This factorial ANOVA excluded the Control
179 and 8w5.0-st treatments. A series of planned contrasts were then performed to test
180 specific comparisons of means:
181
182 (i) A first planned comparison was conducted to test the difference between
183 Control and 2w5.0 (frequent high-dosage oil spills).
184 (ii) If there was a significant Frequency × Dosage interaction in the factorial
185 ANOVA, then three planned contrasts were done to test the effect of dosage
186 at each frequency (i.e. 2w2.5 vs. 2w5.0, 4w2.5 vs. 4w5.0 and 8w2.5 vs.
187 8w5.0). We skipped this step if the interaction was not significant.
188 (iii) Three planned contrasts were used to test the differences between treatments
189 that received the same overall amount of oil, but according to distinct
190 exposure regimes (i.e. 2w2.5 vs. 4w5.0, 4w2.5 vs. 8w5.0 and 4w2.5 vs.
191 8w5.0-st).
192 (iv) Finally, to evaluate the effect of time since last oil spill, a comparison was
193 conducted of 8w5.0 against 8w5.0-st.
194
195 Homogeneity of variances was checked using Cochran's test and data were
196 transformed to square-root, fourth-root or ln(x + 1) when necessary. The number of
197 contrasts did not exceed 7 degrees of freedom, except for the analyses of the polychaete
198 *Sigambra grubii* and the bivalve *Anomalocardia flexuosa*, for which comparisons were
199 tested using the Dunn-Sidák adjusted significance level of 0.006. Univariate data analysis
200 and graphs were generated using R programming language (R Core Team, 2013)
201 combined with GAD (Sandrini-Neto and Camargo, 2012) and sciplot (Morales, 2012)
202 packages.

Differences among macrofaunal assemblages were tested by a permutational multivariate analysis of variance (Anderson, 2001) through the PERMANOVA+ add-on package for PRIMER v6 (Clarke and Gorley, 2006; Anderson et al., 2008) using the same sequence of planned contrasts from the univariate analyses. A principal coordinates analysis (PCO) was used to visualize differences in overall assemblage structure among treatments. Each treatment in the PCO plot was shown as a single object representing the distances among centroids. This provides a suitable visual complement to PERMANOVA output (Anderson et al., 2008). All multivariate analyses were performed on the basis of the Bray-Curtis similarity measure of untransformed macrofauna densities.

3. Results

3.1. Polycyclic aromatic hydrocarbons

Total PAHs concentration (ΣPAHs), excluding perylene, which can be associated with diagenetic sources, ranged from 0.5 to 3.7 ng g^{-1} in the control treatment, and from 2.2 to 112.0 ng g^{-1} in exposure treatments (Table 2). Concentrations of low molecular weight (LMW – 2 and 3 rings) PAHs varied from an undetectable amount to 0.9 ng g^{-1} in the control treatment, and from 1.4 to 99.3 ng g^{-1} in oil-exposed sediments (Table 2). High molecular weight (HMW – 4 to 6 rings) PAHs were not detected in control plots, while in oil-exposed sediments HMW PAHs ranged from an undetectable amount to 78.3 ng g^{-1} (Table 2). Despite the low ΣPAHs concentrations, the dominance of LMW PAHs in oil-exposed sediments suggests a petrogenic source, i.e., experimental oil spills.

227 *3.2. Macrofauna*

228

229 A total of 16,228 individuals belonging to 69 different taxa were recorded.
230 Macrofaunal assemblages were numerically dominated by ostracods (which accounted for
231 37% of total abundance) and by the gastropod *Heleobia australis* (36% of total
232 abundance). The polychaetes *Glycinde multidens* and *Sigambra grubii*, a tubificin
233 oligochaete, the bivalves *Anomalocardia flexuosa* and *Tellina versicolor* and the
234 gastropod *Bulla strita* altogether contributed to only 17% of total abundance, although
235 they were found in at least 68% of the samples.

236

237 *3.3. Effects of frequency and intensity of oil spills on total number of individuals and*
238 *dominant taxa*

239

240 Compared to the control plots, frequent high-dosage oil spills (2w5.0) significantly
241 reduced the total density of macrofauna and densities of dominant taxa, with the
242 exception of the polychaete *S. grubii*, which was not affected by these exposure events
243 (Table 3; Fig. 1). The major differences between treatments were caused by the
244 frequency of oil spills (Table 3). Different dosages of oil had no effect on most taxa,
245 except via a Frequency and Dosage interaction in the analysis of *S. grubii* and *A. flexuosa*
246 (Table 3). Planned contrasts revealed that higher dosages of oil reduced the densities of
247 this bivalve, but this was only observed at the highest frequency of spills (i.e. every 2
248 weeks). *S. grubii* densities, however, were increased by higher dosages of oil at spills
249 occurring every 8 weeks (Table 3; Fig. 1).

Table 2. Polycyclic aromatic hydrocarbons (PAH) concentrations and related parameters in control and oil-exposed sediments. ΣPAHs, total polycyclic aromatic hydrocarbons (ng g^{-1} dry weight); 2–3 rings, total PAHs with two to three aromatic rings (ng g^{-1} dry weight); 4–6 rings, total PAHs with four to six aromatic rings (ng g^{-1} dry weight).

	Low-dosage spills (2.5 L m^{-2})						**High-dosage spills (5.0 L m^{-2})**								**Control**	
	2w2.5		4w2.5		8w2.5		2w5.0		4w5.0		8w5.0		8w5.0-st			
	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2
ΣPAHs	53.22	2.17	4.42	2.77	6.26	112.02	11.27	2.83	4.52	96.06	32.64	14.48	80.84	3.82	3.72	0.51
2–3 rings	47.98	1.40	2.64	1.41	2.84	99.25	2.75	1.44	2.42	85.94	26.44	9.07	72.22	1.80	0.90	n.d.
4–6 rings	0.55	n.d.	n.d.	n.d.	n.d.	6.01	n.d.	n.d.	n.d.	3.30	n.d.	n.d.	2.89	n.d.	n.d.	n.d.

n.d. = not detected

Conversely, densities of the bivalve *T. versicolor* and the gastropod *B. strita* were not affected by either dosage or frequency of exposure events (Table 3; Fig. 1). These species were overall sensitive to oil spills, independently of their frequency, intensity and timing. Densities of *T. versicolor* and *B. striata* decreased by a factor of 2.7 and 4.7, respectively, in impact treatments (Fig. 1). A similar pattern was observed for ostracods, despite a significant effect of frequency (Table 3). Planned comparisons shown that Ostracoda densities did not differ between treatments that received an equivalent overall volume of oil at distinct frequencies (Table 3; Fig. 1).

Frequent low-dosage oil spills decreased the total density of macrofauna and densities of *G. multidens*, Oligochaeta and *H. australis* compared to infrequent high-dosage exposures (Frequency vs. Dosage planned comparisons in Table 3; Fig. 1). Total density of macrofauna together with *H. australis* densities were reduced by exposure to a low-dosage oil spill every 2 weeks rather than a high-dosage spill every 4 weeks. Similarly, a low-dosage oil spill every 4 weeks decreased the densities of *G. multidens* and oligochaetes compared to a high-dosage oil spill every 8 weeks (Table 3; Fig. 1). Conversely, *S. grubii* densities were increased by a high-dosage oil spill every 8 weeks compared to low-dosage oil spill every 4 weeks (Table 3; Fig. 1).

There were also significant differences between treatments that received the same dosage of oil under the same frequency of exposure, but for which the timing of exposure differed by 4 weeks. The later oil spills (8w5.0-st treatment) reduced the total density of macrofauna together with *S. grubii*, *G. multidens*, *H. australis* and oligochaete densities (Table 3; Fig. 1).

Table 3. Asymmetrical analysis of variance including the main test and subsequent planned comparisons for total macrofaunal density and densities of dominant taxa.

							Planned comparisons				
	Main test			**Factorial**			Exposure	Frequency vs. Dosage			Timing
	All treatments			Excludes Control and 8w5.0-st			Control vs. 2w5.0	2w2.5 vs. 4w5.0	4w2.5 vs. 8w5.0	4w2.5 vs. 8w5.0-st	8w5.0 vs. 8w5.0-st
	df	MS	*F*		*df*	*F*	*F*	*F*	*F*	*F*	*F*
Total individuals (log)											
Among all treatments	7	1.936	**9.227*****	Frequency	2	**25.844*****	**34.965*****	**5.429***	3.336	0.017	**3.834***
Error	72	0.210		Dosage	1	0.964					
				F × D	2	0.497					
Glycinde multidens											
Among all treatments	7	15.555	**3.499****	Frequency	2	**9.211*****	**6.478***	0.281	**4.960***	0.551	**8.817****
Error	72	4.446		Dosage	1	0.034					
				F × D	2	2.171					
Sigambra grubii											
Among all treatments	7	54.427	**4.595*****	Frequency	2	**9.754*****	0.422	0.068	**16.753*****	0.827	**10.134****
Error	72	11.846		Dosage	1	3.242					
				F × D	2	**4.335***		2w: 2.5 vs. 5.0	4w: 2.5 vs. 5.0	8w: 2.5 vs. 5.0	
								0.017	0.038	**11.856*****	
Oligochaeta (√)											
Among all treatments	7	7.267	**5.229*****	Frequency	2	**9.716*****	**15.217*****	0.164	**11.170****	0.008	**10.582****
Error	72	1.390		Dosage	1	0.024					
				F × D	2	0.726					
Ostracoda (√)											
Among all treatments	7	40.702	**5.620*****	Frequency	2	**5.437****	**32.039*****	0.297	0.240	0.009	0.157
Error	72	7.242		Dosage	1	3.754					
				F × D	2	0.574					

(continued on next page)

Table 3 (*continued*)

	Main test			**Factorial**			**Planned comparisons**				
							Exposure	Frequency vs. Dosage			Timing
	All treatments			Excludes Control and 8w5.0-st			Control vs. 2w5.0	2w2.5 vs. 4w5.0	4w2.5 vs. 8w5.0	4w2.5 vs. 8w5.0-st	8w5.0 vs. 8w5.0-st
	df	MS	*F*		*df*	*F*	*F*	*F*	*F*	*F*	*F*
Anomalocardia flexuosa (√√)											
Among all treatments	7	0.561	**6.080*** **	Frequency	2	**13.642*** **	**11.566** **	2.235	0.869	2.630	0.476
Error	72	0.092		Dosage	1	3.572					
				F × D	2	**3.553***		2w: 2.5 vs. 5.0	4w: 2.5 vs. 5.0	8w: 2.5 vs. 5.0	
								8.109 **	0.811	1.759	
Tellina versicolor (√)											
Among all treatments	7	1.866	**2.293***	Frequency	2	1.510	**9.845** **	0.893	0.042	0.007	0.082
Error	72	0.814		Dosage	1	0.012					
				F × D	2	0.166					
Bulla striata (√)											
Among all treatments	7	6.028	**5.405*** **	Frequency	2	0.669	**20.738*** **	2.362	0.339	0.371	0.001
Error	72	1.115		Dosage	1	1.236					
				F × D	2	0.442					
Heleobia australis (log)											
All treatments	7	6.316	**12.250*** **	Frequency	2	**39.383*** **	**27.592*** **	**13.012*** **	2.963	2.058	**9.959** **
Error	72	0.516		Dosage	1	1.251					
				F × D	2	1.915					

All planned comparisons had 1,72 degrees of freedom and were tested against the mean square error from the main test among all treatments. For all taxa, significance of planned comparisons was assessed at α=0.05, except for *Sigambra grubii* and *Anomalocardia flexuosa* for which comparisons were tested using the Dunn-Sidák adjusted significance level of 0.006. Significant *F* values are highlighted in bold. Type of data transform is given in brackets.
Significant codes: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

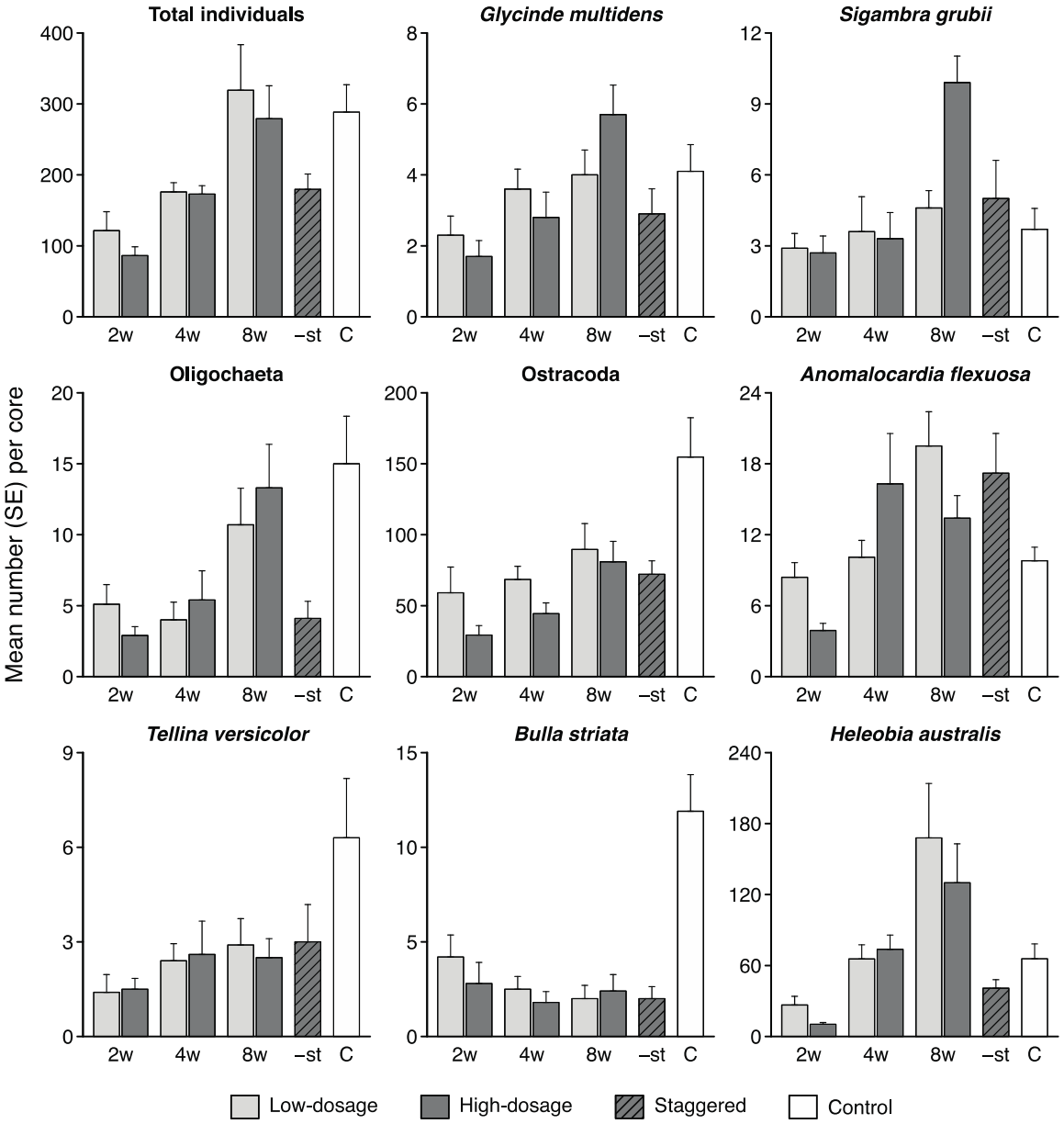

Fig. 1. Total number of individuals and densities of dominant taxa (+ S.E.) in response to varying frequencies and intensities of repeated exposure events. Oil spills occurred every 2 weeks (2w), 4 weeks (4w) or 8 weeks (8w). Undisturbed controls (C) are shown in white, low-dosage (2.5 L m^{-2}) spills are shown in light gray and high-dosage (5 L m^{-2}) spills are shown in dark gray. Hatched dark gray bars indicate that the timing of the high-dosage spills was staggered.

272 *3.4. Effects of frequency and intensity of oil spills on macrofaunal assemblage structure*

273

274 The structure of macrofaunal assemblages was strongly affected by frequent high-
275 dosage oil spills, as indicated by the significant difference between control and 2w5.0
276 treatments (Table 4). This pattern was clearly confirmed by the PCO ordination (Fig. 2),
277 which also showed that assemblages in control plots differed from those in oil-exposure
278 treatments.

279

Table 4. Asymmetrical PERMANOVA (9999 permutations) including the main test and subsequent planned comparisons of macrofaunal assemblages among treatments.

	Main test			**Factorial**		
	All treatments			Excludes Control and 8w5.0-st		
	df	MS	Pseudo-*F*		*df*	Pseudo-*F*
Among all treatments	7	5000.713	**4.916*****	Frequency	2	**10.119*****
Error	72	1017.342		Dosage	1	**2.610***
				F × D	2	1.442

Planned comparisons

Exposure	Frequency vs. Dosage			Timing
Control vs. 2w5.0	2w2.5 vs. 4w5.0	4w2.5 vs. 8w5.0	4w2.5 vs. 8w5.0-st	8w5.0 vs. 8w5.0-st
Pseudo-*F*	Pseudo-*F*	Pseudo-*F*	Pseudo-*F*	Pseudo-*F*
13.070***	**4.539****	1.728	1.971	**3.036***

All planned comparisons had 1,72 degrees of freedom and were tested against the mean square error from the main test among all treatments. Significant Pseudo-*F* values are highlighted in bold. Significant codes: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

280

281 In contrast to univariate results, where most differences were caused only by the
282 frequency of spills, macrofaunal assemblages were affected both by frequency and
283 dosage of spills independently (factorial analysis in Table 4). Assemblages impacted with
284 the same overall volume of diesel, but to different exposure regimes, only differed
285 between 2w2.5 and 4w5.0 treatments (Table 4; Fig. 2). Both treatments received an
286 overall amount of 20 L of diesel per plot in exposure events occurring every 2 or 4 weeks.

287 Assemblages were not affected by different frequencies of spills when exposed to half of
288 the overall oil volume (i.e. 10 L of diesel per plot in total), as indicated by non-significant
289 4w2.5 vs. 8w5.0 and 4w2.5 vs. 8w5.0-st comparisons (Table 4).
290

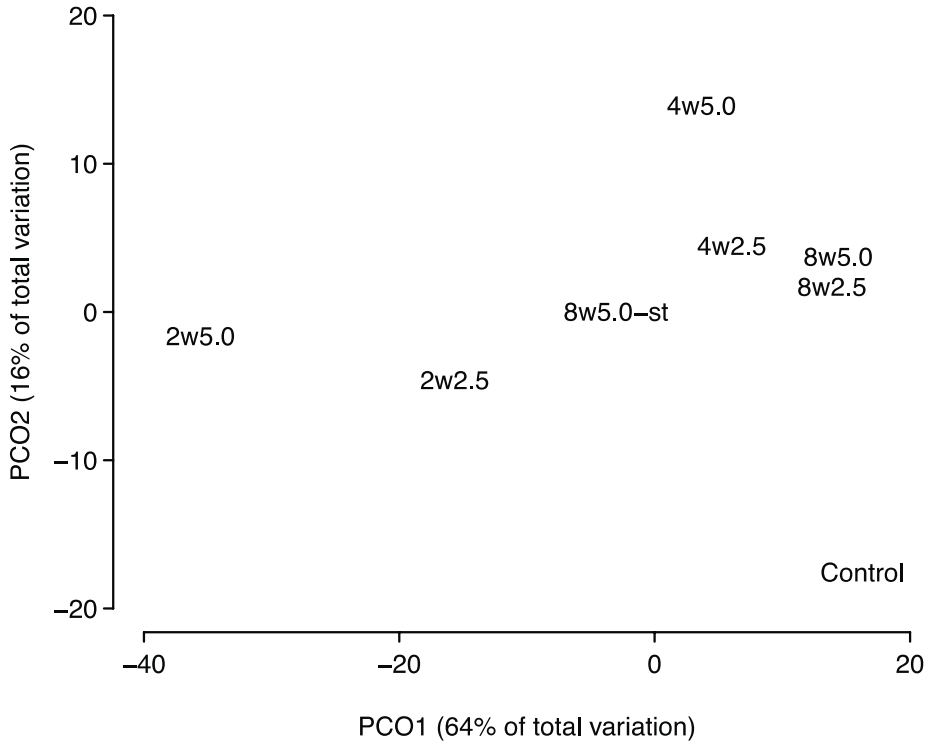

Fig. 2. Principal coordinates analysis (PCO) of distances among centroids on the basis of the Bray-Curtis similarity measure of untransformed data. Treatments are undisturbed Control and exposure events, consisting of the interaction between 2.5 L m^{-2} and 5 L m^{-2} of oil spilled every 2 weeks, 4 weeks or 8 weeks (2w2.5, 2w5.0, 4w2.5, 4w5.0, 8w2.5 and 8w5.0, respectively). A further 8 weeks high-dosage treatment (8w5.0-st) was staggered by 4 weeks in order to evaluate the effect of time since last spill. See Methods section for more details.

291

292 Assemblages were also affected by the timing of exposure events (8w5.0 vs.
293 8w5.0-st). There was a significant difference between treatments that received the same
294 amount of diesel (5 L m^{-2}) over the same frequency (every 8 weeks), but for which the
295 timing of exposure differed by 4 weeks (Table 4; Fig. 2).
296

4. Discussion

Repeated oil spill events occurring at different frequencies and concentrations dramatically altered the overall structure of macrofaunal assemblages. The main direct effect of diesel spills was the decrease in overall density of macrofauna and densities of numerically dominant taxa. In general, increasing the frequency of oil exposure events also negatively affected macrofaunal taxa. Moreover, assemblages exposed to exposure regimes with equivalent frequency and dosage of diesel, but with different timing of spills, exhibit negative responses to more recent spill events. Based on these findings, none of the three working hypotheses were rejected.

However, there were important species-specific departures from the general patterns outlined above. The gastropod *B. striata* and the bivalve *T. versicolor* were both very sensitive to diesel exposure, independently of their frequency, intensity and timing. Despite being considered pollutant-sensitive taxa (Choueri et al., 2009), previous studies reported that *Tellina* species survived the Amoco Cadiz oil spill (Teal and Howarth, 1984), whereas *Bulla* responses to oil exposure were rather inconsistent (Egres et al., 2012). Whether these two species are good indicators or sentinels of oil impacts remains unclear.

Ostracods were also overall sensitive to oil exposure events, despite a significant effect of frequency of spills. The high sensitivity of ostracods to oil contamination is known from several field and laboratory studies (Carman et al., 2000; Donavaro, 2000; Mostafawi, 2001; Stark et al., 2003; Millward et al., 2004; Ruiz et al., 2005, Egres et al., 2012). Frequent oil spills may cause their disappearance or a strong reduction in the numbers of individuals in a relatively short time period (Ruiz et al., 2005). However, most of these findings are referred to meiofaunal groups and relatively little is known about sensitivity of macrofaunal ostracods to contaminants. In an experimental *in situ* diesel spill, Egres et al. (2012) reported that macrofaunal ostracods were absent in the oil-

exposure treatment shortly after the spill, but high densities were again recorded two days later.

The introduction of petroleum hydrocarbons into the marine environment can result in the elimination of more sensitive species due to the high toxicity of some compounds, while promoting increases in the abundance of some tolerant species (Peterson et al., 1996; Venturini and Tommasi, 2004). In general, our results indicate that increasing the frequency of spills reduced the total density of macrofauna and densities of dominant taxa, particularly *H. australis*, *G. multidens* and oligochaetes. However, increases in densities of some taxa (notably *H. australis*) were observed in response to infrequent spills compared to undisturbed controls. We interpret this positive response as an indirect effect of exposure due to the opportunistic strategies of these species, which were able to tolerate intermediate levels of diesel contamination. The sensitive taxa found in our study (i.e. *B. striata*, *T. versicolor* and ostracods) were not able to tolerate repeated oil spills at any frequency, while opportunistic species could benefit from such conditions and rapidly colonize moderately petroleum-contaminated plots.

Distinct responses of a same species to varying exposure regimes can also explain the inconsistencies found in some studies. For example, the carnivorous polychaete *G. multidens* may be initially sensitive to diesel oil spills (Faraco and Lana, 2003; Egres et al., 2012), however high densities of this species can be found in sediments severely contaminated by polycyclic aromatic hydrocarbons (Venturini and Tommasi, 2004; Venturini et al., 2008). Thus, varying exposure regimes and background petroleum contamination at different areas may be responsible for these apparent contradictory responses to oil contamination.

Our results also showed that *S. grubii* is extremely resilient to oil disturbance. Densities of this polychaete in the control plots did not differ from those in plots frequently impacted by high-dosage spills. Also, *S. grubii* was the only taxa that positively responded to higher dosages of oil, with increasing densities in plots exposed to 5 L of diesel every 8

weeks compared to plots exposed to 2.5 L of diesel every 4 weeks. This polychaete species is commonly reported for estuarine sediments heavily impacted by petroleum hydrocarbons (Peso-Aguiar et al., 2000; Venturini and Tommasi, 2004; Venturini et al., 2008).

Polychaetes accumulate PAHs from both particulate and dissolved phases, but little is known about the elimination of these compounds through biotransformation (Jørgensen et al., 2008). The ability of polychaetes to metabolize PAHs largely differs among species, which may account for some of the reasons the potential hazards tend to differ (Oug et al., 1998). Low assimilation efficiency and high biotransformation capacity are expected to be found in organisms at high trophic levels (Wan et al., 2007). Thus, high concentrations of PAHs are found in organisms at low trophic levels (such as deposit feeders), while at high trophic levels low PAHs concentrations occur due to trophic dilution in the marine food webs (Wan et al., 2007; Jørgensen et al., 2008). *S. grubii* is a predator and its tolerance to oil exposure might be explained by its capability to metabolize PAHs compared to other taxa in this study, although species-specific differences in biotransformation efficiency has not been explained (Jørgensen et al., 2008). Therefore, further research is needed to estimate the efficacy of *S. grubii* to metabolize PAHs into more hydrophilic metabolites.

Our experiment was not specifically designed to assess the recovery rate of assemblages following oil spills. Despite the relatively large experimental unit (1-m^2 plots) used in our study in comparison to other field oil-exposure experiments, the repeated collection of samples on the same plots several times after the final exposure would certainly generate non-independent data. Still, the inclusion of the staggered treatment allowed us to compare the effects of the same intensity (5 L m^{-2}) and frequency of spills (every 8 weeks), but with different times for recovery from the final exposure event.

The timing of oil spills was significant to the impact in total numbers of macrofaunal taxa and overall assemblage structure. Total density of macrofauna and densities of

individual taxa in plots that received the final oil spill 6 weeks before sampling did not differ from those in controls, whereas taxa subjected to a final spill just 2 weeks before sampling still not recovered from impact. Very fast recovery of benthic assemblages after small-scale disturbances, when most species reach background abundances within a few weeks, is a well-known pattern in soft sediment habitats (Bolam et al., 2004; Negrello Filho et al., 2006; Sandrini-Neto and Lana, 2014). However, recovery time found in this study was substantially longer than typically reported after single diesel spills in Paranaguá Bay (Faraco and Lana, 2003; Egres et al., 2012). We attributed this to the larger experimental units used in our study and to the fact that assemblages were exposed to repeated spills.

The location used for experimental field exposures is subjected to constant tidal influence and is situated near the mouth of rivers (particularly the Guaraguaçu river), which can accelerate the dispersion and dilution of oil (Egres et al., 2012; Leite et al., 2014). The low PAHs concentrations in sediments of experimental plots were probably associated with the dynamics of the studied area, which favors the dispersion of pollutants through intense tidal currents. According to Notar et al. (2001), ΣPAH concentrations higher than 500 ng g^{-1} are indicative of highly contaminated sediments. This threshold value was not exceeded in any of the impacted plots and larger concentrations of PAHs had no relation to either frequency or intensity of oil spills. In fact, the largest ΣPAH concentration (i.e. 112 ng g^{-1}) was found in an experimental plot exposed to infrequent low-dosage oil spills (8w2.5).

Moreover, low PAHs concentrations in the present study can also be explained by the sampling procedure used to collect the sediment; i.e., only a thin layer (top 2 cm) of surface sediments was sampled for PAH analysis. However, signs of diesel oil were immediately visible after removing sediment cores when sampling for macrofauna (personal observation), indicating that oil was accumulated in sub-superficial layers (5–10 cm deep). Therefore, we suggest that the low persistence of diesel in superficial

sediments, as indicated by the chemical analysis, can be attributed to local hydrodynamics and percolation of oil through sub-superficial layers, directly affecting the infauna.

5. Conclusions

Understanding the impacts of petroleum hydrocarbons on marine and estuarine systems using macrobenthic invertebrates as indicators is a complex, yet necessary task. Experimental *in situ* simulations of oil exposure events with different frequencies and dosages provide a useful tool for detecting and quantifying environmental impacts. We have shown that intertidal macrofaunal assemblages exposed to the same overall diesel release, but at distinct exposure regimes, are strongly affected by the frequency of oil spills. In general, frequent small exposures are more deleterious than infrequent large ones. This has direct implications for monitoring protocols and mitigating actions, since tracking frequent small oil spills is difficult, still they are potentially widespread in estuarine environments.

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Capítulo III

Antioxidant responses in estuarine invertebrates exposed to repeated oil spills: Effects of frequency and dosage in a field manipulative experiment

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Antioxidant responses in estuarine invertebrates exposed to repeated oil spills: Effects of frequency and dosage in a field manipulative experiment

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Abstract

We have experimentally investigated the effects of repeated diesel spills on the bivalve *Anomalocardia flexuosa*, the gastropod *Neritina virginea* and the polychaete *Laeonereis culveri*, by monitoring the responses of oxidative stress biomarkers in a subtropical estuary. Three frequencies of exposure events were compared against two dosages of oil in a factorial experiment with asymmetrical controls. Hypotheses were tested to distinguish between (i) the overall effect of oil spills, (ii) the effect of diesel dosage via different exposure regimes, and (iii) the effect of time since last spill. Antioxidant defense responses and oxidative damage in the bivalve *A. flexuosa* and the polychaete *L. culveri*

were overall significantly affected by frequent oil spills compared to undisturbed controls. The main effects of diesel spills on both species were the induction of SOD and GST activities, a significant increase in LPO levels and a decrease in GSH concentration. *N. virginea* was particularly tolerant to oil exposure, with the exception of a significant GSH depletion. Overall, enzymatic activities and oxidative damage in *A. flexuosa* and *L. culveri* were induced by frequent low-dosage spills compared to infrequent high-dosage spills, although the opposite pattern was observed for *N. virginea* antioxidant responses. Antioxidant responses in *A. flexuosa* and *L. culveri* were not affected by timing of exposure events. However, our results revealed that *N. virginea* might have a belated response to acute high-dosage exposure. Experimental *in situ* simulations of oil exposure events with varying frequencies and intensities provide a useful tool for detecting and quantifying environmental impacts. In general, antioxidant biomarkers were induced by frequent low-dosage exposures compared to infrequent high-dosage ones. The bivalve *A. flexuosa* and the polychaete *L. culveri* are more suitable sentinels due to their greater responsiveness to oil and also to their wider geographical distribution.

Keywords: Oxidative stress; Biomarkers; Field experiment; Polycyclic aromatic hydrocarbons; Diesel; Paranaguá Bay

1. Introduction

The production of reactive oxygen species (ROS) occurs naturally during cellular aerobic respiration processes (Vidal-Liñán and Bellas, 2013), but can also be highly affected by environmental factors, such as salinity and temperature (Lushchak, 2011), or exposure to contaminants (Monserrat et al., 2007; Lüchman et al., 2011; Marques et al., 2014). Increased ROS levels may induce lipid, protein and DNA oxidation, leading to

several deleterious effects at cellular level (Monserrat et al., 2007; Vidal-Liñán and Bellas, 2013). Cells are protected against the deleterious effects of oxyradical generation by maintaining ROS at low levels through several antioxidant defenses, which include both enzymatic and non-enzymatic antioxidants (Kaloyianni et al., 2009; Lüchman et al., 2011; Turja et al., 2013; Zanette et al., 2015).

Changes in antioxidant defenses can be used as indicators of contaminant exposure. The antioxidant system involves enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Among the non-enzymatic defenses, glutathione (GSH) participates in many important biological processes including protection against toxic compounds (Lüchman et al., 2011). Moreover, enzymes involved in the elimination of ROS byproducts, such as glutathione S-transferase (GST), play an important role as indirect antioxidant (Boutet et al., 2004; Lüchman et al., 2011; Zanette et al., 2015). Eventually, deficiency in the antioxidant system of cells can increase the lipid peroxide levels (LPO), a major mechanism by which oxyradicals can damage the cellular membrane lipids (Turja et al., 2013; Zanette et al., 2015).

Polycyclic aromatic hydrocarbons (PAHs) are a common source of contamination in the aquatic environment, mostly as a result of petroleum-related activities (Lüchman et al., 2014). PAHs are primarily associated with anthropogenic sources, particularly fossil fuels and their derivatives. The process of partial combustion, accidental oils spills and the disposal of domestic and industrial effluents are the major sources of PAHs to coastal systems (Martins et al., 2011; Abreu-Mota, 2014). PAHs may affect aquatic organisms in many ways and the oxidative stress is one of the key elements of their toxicity (Lushchak, 2011). PAHs are primarily metabolized via hydroxylation (phase-I reactions) and detoxified by enzymes in the cytochrome P450 system (Lushchak, 2011; Lüchman et al., 2014).

Many studies have reported changes in oxidative stress biomarkers as a response to PAHs exposure in marine invertebrates, particularly in bivalves (Turja et al., 2013;

Marques et al., 2014; Lüchman et al., 2014; Turja et al., 2014; Vidal-Liñán et al., 2014; Won et al., 2013), but also in polychaetes (Nesto et al., 2010; Ramos-Gómez et al., 2011; Won et al., 2013). Filter-feeding mollusks are often used as sentinels in pollution monitoring due to their significant ability to bioaccumulate pollutants as well as to respond to their presence (Solé et al., 2009; Lüchman et al., 2011). Polychaete worms are also good sentinels because they can adapt to stressful environmental conditions, are distributed worldwide and present a sedentary lifestyle (Solé et al., 2009; Díaz-Jaramillo et al., 2011). However, few studies have investigated the effects of PAHs on oxidative stress biomarkers in other marine invertebrates, such as crabs (Martín-Díaz et al., 2007; Morales-Caselles et al., 2008; Ricciardi et al., 2010) and gastropod mollusks (Reid and MacFarlane, 2003; Sarkar et al., 2006; Tim-Tim et al., 2009).

Experiments evaluating biomarker responses have often been done under laboratory conditions (Silva et al., 2005; Luchman et al., 2011; Luna-Acosta et al., 2011) to isolate the putative effects of PAH exposure from other factors. Such experiments, however, do not include the full set of naturally occurring abiotic and biotic variables (Goodsell et al., 2009), which can affect the persistence of contaminants and, ultimately, the response of selected biomarkers. Thus, results from laboratory studies should be compared to robust field experiments in order to generate ecologically relevant information (Reid and MacFarlane, 2003; Nesto et al., 2010; Díaz-Jaramillo et al., 2013; Marques et al., 2014). Field experiments can be conducted whether by transplanting organisms to polluted areas (e.g. Díaz-Jaramillo et al., 2013; Turja et al., 2014) or by experimentally adding contaminants to natural sites (e.g. Marques et al., 2014).

Particularly in coastal and estuarine habitats, the intense traffic of small and mid size ships, together with fishing and recreational boats are often responsible for the release of petroleum products at a range of frequencies and intensities. Most of these vessels use marine diesel oil as fuel, which is less persistent than crude oil although it is highly toxic (Lytle and Peckarsky, 2001). Nonetheless, biomarker responses to PAH

exposure in marine invertebrates are often evaluated from acute, non-cumulative, single-dosage oil spills. Impact assessments are commonly carried out after accidents through descriptive approaches (Tim-Tim et al., 2009; Morales-Caselles et al., 2008; Sureda et al., 2011), but also by the use of field manipulative experiments (Marques et al., 2014). Consequently, little is known of how repeated oil spills at varying frequencies and intensities can affect biomarkers responses, especially in the field.

In this study, we examined the effects of the frequency and intensity of experimental diesel spills on enzyme activities (SOD, CAT, GST and GPx) and levels of reduced glutathione (GSH) and lipid peroxidation (LPO) in three macrofaunal species: the bivalve *Anomalocardia flexuosa* (formerly identified as *Anomalocardia brasiliana*), the gastropod *Neritina virginea* and the polychaete *Laeonereis culveri* (formerly identified as *Laeonereis acuta*). These species were chosen because they are adapted to stressful environmental conditions, relatively sessile, widely distributed and occupy different trophic levels. *A. flexuosa* is a filter feeder that feeds mostly on plankton; *N. virginea* is a grazer that feeds mainly on epiphytic algae, and *L. culveri* is a deposit feeder that forages within the sediment column.

By comparing the effects of three frequencies of exposure events against two dosages of oil in a factorial experiment with asymmetrical controls, we tested the following hypotheses: 1) if selected biomarkers are affected by repeated oil spill events, then biomarker responses in organisms exposed to frequent spills will be significantly different from those in the control treatment; 2) if different exposure regimes are determinant causes of variability, then biomarker responses in organisms exposed to frequent low-dosage spills will be significantly different from those exposed to infrequent high-dosage spills; 3) if the time elapsed since the last oil spill is determinant, then biomarker responses in organisms exposed to the same dosage of oil under the same frequency, but for which the timing of exposure differed, will vary significantly.

2. Materials and methods

2.1. Study area

Experimental oil spills were conducted on an intertidal flat at Papagaios Island in the polyhaline Cotinga sub-estuary (Fig. 1), a 20-km channel located in Paranaguá Bay (southern Brazil). Local tidal flats are mainly composed by moderately to well-sorted very fine sands (Souza et al., 2013) and are often covered with seaweeds such as *Ulva* and *Enteromorpha* (Ulvaceae, Chlorophyta) or diatom biofilms. The tidal regime is mainly semidiurnal, with diurnal inequalities, and may reach up to 1.7 m in the sub-estuary during spring tides (Lana et al., 2001; Marone et al., 2005).

The Cotinga sub-estuary receives a considerable amount of domestic effluents from the city of Paranaguá, where the municipal sewage is still discharged *in natura* (Leite et al., 2014). The waste of nearly 50% of the city's population of about 150,000 undergoes treatment, while the rest is directly discarded without any treatment (Souza et al., 2013). Other potential impact sources include the presence of an oil terminal, a grain port and tourism, which may contribute to a progressive increase in the disposal of domestic and industrial sewage, petroleum hydrocarbons, heavy metals and organic pollutants such as polychlorinated biphenyls (Barboza et al., 2013; Abreu-Mota et al., 2014).

Despite the existence of many man-induced impacts in the Cotinga sub-estuary, local sediments are not considered contaminated by oil (Abreu-Mota et al., 2014), although a gradient of fecal contamination from the vicinity of Paranaguá port towards the open sea is detected along the channel (Barboza et al., 2013).

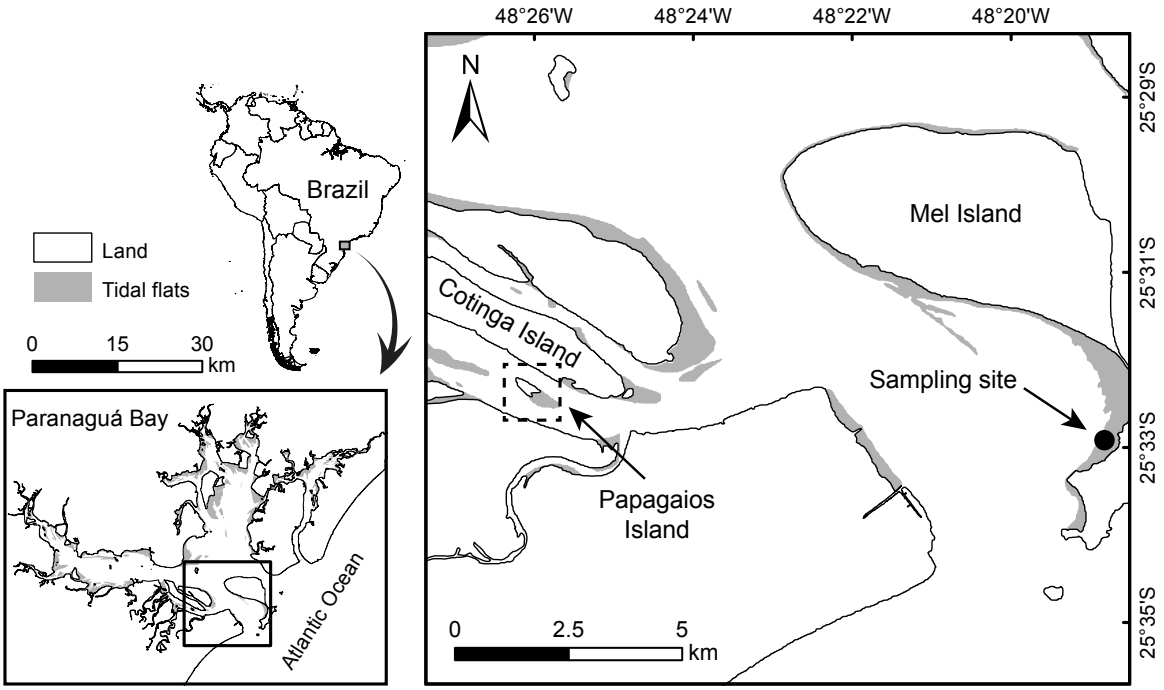

Fig. 1. Map of the Paranaguá Bay showing the location of the sampling site and the intertidal flat used for experimental exposures.

159

160 *2.2. Sampling of selected species*

161

162 Selected species of macrofauna were collected at an intertidal flat located on the
163 southwest margin of Mel Island (Fig. 1), at the entrance to Paranaguá Bay (25°33'25.5"S,
164 48°18'42.4"W). This area is approximately 12 km away from the Papagaios Island, where
165 the experimental exposures took place, and it is also considered not contaminated by
166 petroleum hydrocarbons (Martins et al., 2009). Sediments are predominantly composed of
167 very fine sand with low silt–clay content; see Sandrini-Neto and Lana (2014) for details.
168 This area was selected because large high-density patches of the polychaete *L. culveri*
169 are easily identified on sediment surface. The bivalve *A. flexuosa* and the gastropod *N.*
170 *virginea* also occur in high densities and can be easily collected by hand.
171 Forty-eight sediment samples were collected in a *L. culveri* patch of 5 × 5 m using
172 a cylindrical corer (10 cm in diameter) pushed to the depth of 10 cm. Pilot sampling

revealed that *L. culveri* densities in this patch were around 30 individuals per core, with specimens weighting between 50 and 120 mg. Each sediment sample was immediately transferred to a 1-L glass beaker. This procedure preserved the vertical stratification of the sediment, considering that the internal diameter of a 1-L beaker is 10 cm. In each beaker, three individuals of *A. flexuosa* and five *N. virginea* were introduced. Prior to field deployment, beakers were covered with a 0.5 mm mesh, which prevented organisms from escaping, while allowing water renewal and aeration.

2.3. Experimental design and field procedures

A 4-day field experiment simulating repeated oil spill events at varying frequencies and intensities was conducted to evaluate the biomarker responses in selected macrofaunal species. The design comprised an undisturbed control and seven oil exposure treatments. All of these consisted of the interaction between two dosages of oil (250 and 500 mL/0.25 m^2) spilled at three distinct frequencies (every 1 day, 2 days or 4 days) starting from day one, except for the staggered treatment. This particular high-dosage/low-frequency treatment was 2 days delayed and was designed to evaluate the effect of time since last spill. Hereafter the treatments are referred to as Control, 1d250, 1d500, 2d250, 2d500, 4d250, 4d500 and 4d500-st, respectively (Table 1). Our design and analysis were based on the experiment conducted by Johnston and Keough (2005), which investigated the impact of varying frequencies and intensities of copper pulses on estuarine sessile invertebrates.

Treatments were assigned randomly to sixteen plots of 0.5 $\times$ 0.5 m demarcated at similar tidal levels; i.e. two replicated plots were assigned for each undisturbed control and oil exposure treatments. In each plot, three 1-L glass beakers filled with sediment and containing the selected species were deployed approximately 20 cm apart at low tide. Beakers were pushed into a hole dug into the sediment by a cylindrical corer (10 cm in

diameter) to a depth of 10 cm. Experimental spills were done when the tidal flat was emerged, thus optimizing the time for the oil to percolate into the sediment. In each plot of the impact treatments, 250 or 500 mL of marine diesel oil were uniformly poured using a garden watering can according to the schedule in Table 1. Marine diesel oil is largely used as a fuel by small and medium vessels and in the auxiliary engines of large vessels (Leite et al., 2014). The spilled oil was contained by zinc square artifacts pushed into the sediment in order to prevent its dispersion and cross-contamination of the control treatment.

Table 1. Schedule of repeated oil spill events with the indication of different exposure treatments in the experimental design and overall volume of diesel.

	Low-dosage spills (250 mL/0.25 m^2)			**High-dosage spills (500 mL/0.25 m^2)**			
	1d250	2d250	4d250	1d500	2d500	4d500	4d500-st
Day 1	oil spill	oil spill	oil spill	oil spill	oil spill	oil spill	
Day 2	oil spill			oil spill			
Day 3	oil spill	oil spill		oil spill	oil spill		oil spill
Day 4	oil spill			oil spill			
Overall volume	1000 mL	500 mL	250 mL	2000 mL	1000 mL	500 mL	500 mL

A day after the last oil spill event, beakers from each exposure treatment and undisturbed control plots were retrieved during low tide and transported to the laboratory. At the same day, the sediment of each beaker was carefully washed through a 1-mm mesh sieve and individuals of selected species were retrieved. Digestive glands of *A. flexuosa*, the soft-tissue of *N. virginea* and the whole body of *L. culveri* were frozen in liquid nitrogen and stored at –80 °C until biomarker analysis.

A sediment sample was also collected from each plot a day after the last oil spill event to determine the concentration of polycyclic aromatic hydrocarbons (PAHs). The top 2 cm of surface sediment was collected with a spoon and placed in pre-cleaned aluminum

foil and stored at –20 °C. The material was freeze-dried, carefully homogenized with a mortar, and stored in clean glass bottles at room temperature prior to PAHs analysis.

2.4. Laboratory procedures

2.4.1. Biomarkers

Pooled digestive glands of *A. flexuosa* (n = 2–3 per sample), soft-tissue of *N. virginea* (n = 3–5 per sample) and a pool of *L. culveri* (n = 5–10 per sample) were used. The tissue was homogenized (1:10 w/v) in 0.1 M potassium phosphate buffer, pH 7.0, using an IKA T10 basic Ultra-Turrax homogenizer. Homogenates were centrifuged at 15,000*g* for 30 min (4 °C) and the supernatants were collected and stored at –80 °C until analysis. The supernatants were used to estimate the protein content, activities of SOD, CAT, GST, GPx and levels of GSH and LPO.

Total protein content in the homogenate was measured at 595 nm following Bradford's method (Bradford, 1976), with bovine serum albumin as standard; SOD activity was measured at 440 nm using the method described by Gao et al. (1998); CAT activity was measured at 240 nm, based on procedures described by Aebi (1984); GST activity was measured at 340 nm according to Keen et al. (1976); GPx activity was determined at 340 nm as described in Hafeman et al. (1974); GSH concentration was determined at 415 nm according to Sedlak and Lindsay (1968); LPO levels were measured using the ferrous oxidation–xylenol assay at 570 nm (Jiang et al., 1992).

The TECAN Sunrise microplate spectrophotometer was used for protein, SOD, GST, GPx, GSH and LPO measurements; The Biotek Synergy HT multimode microplate reader was used for CAT activity measurements.

2.4.2. PAHs analysis

The analytical procedures for sample extraction and determination of PAHs were performed according to the methods described by UNEP (1992) and Martins et al. (2011), respectively. Briefly, 15 g of sediment samples from each plot was extracted for 8 h using 80 mL of a mixture (1:1) of hexanes/dichloromethane. A mixture of surrogates (naphthalene-d_8, acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12} and perylene-d_{12}) was added to each sample. The extract was purified by column chromatography using 5% deactivated alumina and silica. The organic proxies were eluted in three fractions using 10 mL of hexanes (fraction 1 - aliphatic hydrocarbons, not presented in this study) and 15 mL of 30% dichloromethane/ hexanes (fraction 2 - PAHs). Fractions 1 and 2 were concentrated to 1 mL in hexanes. An aliquot of 1 μL of each extract was injected for gas chromatographic analysis.

The analyses were performed with an Agilent GC (model 6890) coupled to an Agilent mass spectrometer detector (Agilent 5975C inert MSD with Triple-Axis Detector) and an Agilent 19091J-433 capillary fused silica column. Helium was used as the carrier gas. Compounds were identified by matching retention times and ion mass fragments with results from standard mixtures of PAHs from the National Institute of Standards and Technology, USA (NIST 2260 – Aromatic Hydrocarbons Standard Reference Material).

2.5. Data analysis

Analyses of variance with a series of planned contrasts were used to test hypotheses about differences for each of the biomarkers in *A. flexuosa*, *N. virginea* and *L. culveri*. We followed the procedures described by Underwood (1997) for the analysis of an asymmetrical experiment. The cause of asymmetry in the design is that there can only be a single group of replicated controls although Frequency and Dosage are orthogonal

factors to one another. Asymmetry is also caused by the staggered treatment, which cannot be used as a factor level in the factorial analysis.

Hence, differences among all eight treatments were initially tested by a single-factor analysis of variance. The mean square error and residual degrees of freedom from this analysis provided the error term for any subsequent test, including all contrasts (Quinn and Keough, 2002; Johnston and Keough, 2005). Then, a two-factor analysis of variance with Frequency (3 levels, fixed) and Dosage (2 levels, fixed, crossed with Frequency) as factors was conducted. This factorial ANOVA excluded the Control and 4d500-st treatments. A series of planned contrasts were then performed to test specific comparisons of means:

(i) A first planned comparison was conducted to test the difference between Control and 1d500 (frequent high-dosage spills). If this particular treatment was missing due to mortality of organisms, then comparisons with control were performed using 1d250 (frequent low-dosage spills).

(ii) If there was a significant Frequency $\times$ Dosage interaction in the factorial ANOVA, then three planned contrasts were done to test the effect of dosage at each frequency (i.e. 1d250 vs. 1d500, 2d250 vs. 2d500, and 4d250 vs. 4d500). We skipped this step if the interaction was not significant.

(iii) Two planned contrasts were used to test the differences between treatments that received the same overall dosage of oil, but according to distinct exposure regimes (i.e. 1d250 vs. 2d500, and 2d250 vs. 4d500).

(iv) Finally, to evaluate the effect of time since last oil spill, a comparison was conducted of 4d500 vs. 4d500-st.

Homogeneity of variances was verified using Cochran's test and data were transformed to $\ln(x + 1)$ when necessary. The number of contrasts did not exceed the

297 experimental degrees of freedom. Thus the experiment-wise error rate was maintained at
298 α=0.05. Data analysis and graphs were generated using R programming language (R
299 Core Team, 2013) combined with GAD (Sandrini-Neto and Camargo, 2012) and sciplot
300 (Morales, 2012) packages.
301
302 **3. Results**
303
304 *3.1. Biomarker responses in the bivalve A. flexuosa*
305
306 The activities of SOD, GST and levels of LPO were significantly increased by
307 frequent high-dosage oil spills (1d500) compared to the control treatment. Moreover, GSH
308 concentration was significantly reduced in bivalves exposed to frequent high-dosage
309 spills. However, CAT and GPx activities were not affected by this exposure regime
310 (Control vs. 1d500 comparison in Table 2; Fig. 2).
311 The major differences in biomarker responses between treatments were caused
312 by the frequency of oil spills (Table 2). Most biomarkers were not affected by different
313 dosages of oil, except for a significant dosage effect in GST activity and by the interaction
314 between frequency and dosage in the analysis of GPx (Table 2). However, planned
315 comparisons revealed inconsistent patterns regarding the effect of dosage. For example,
316 GPx activity was inhibited by higher dosages of oil in daily spills, but this pattern was the
317 opposite in treatments that were exposed every 2 days.
318 Activities and levels of most biomarkers were significantly higher in frequent low-
319 dosage oil spills compared to infrequent high-dosage oil spills (Frequency vs. Dosage
320 planned comparisons in Table 2; Fig. 2). The activities of SOD and GST together with
321 levels of GSH were increased by exposure to a low-dosage oil spill every one day rather
322 than a high-dosage spill every 2 days (1d250 vs. 2d500 comparison in Table 2; Fig. 2).
323 Similarly, a low-dosage oil spill every 2 days significantly increased the CAT activity and

Table 2. Asymmetrical analysis of variance including the main test and subsequent planned comparisons for activities of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and levels of reduced glutathione (GSH) and lipid peroxidation (LPO) in *Anomalocardia flexuosa*.

							Planned comparisons			
	Main test			**Factorial**			Oil effect	Frequency vs. Dosage		Timing
	All treatments			Excludes Control and 4d500-st			Control vs. 1d500	1d250 vs. 2d500	2d250 vs. 4d500	4d500 vs. 4d500-st
	df	MS	*F*		*df*	*F*	*F*	*F*	*F*	*F*
SOD										
Treat	7	6499.14	**4.94*****	Frequency	2	**6.19****	**5.30***	**3.81***	3.52	0.20
Error	40	1316.74		Dosage	1	3.20				
				F × D	2	2.03				
CAT										
Treat	7	66.64	**2.23***	Frequency	2	1.59	0.16	0.46	**3.99***	0.35
Error	40	29.90		Dosage	1	0.12				
				F × D	2	1.82				
GST										
Treat	7	6635.69	**5.93*****	Frequency	2	**3.31***	**5.13***	**6.13***	0.01	**4.48***
Error	40	1119.38		Dosage	1	**5.34***				
				F × D	2	2.39				
GPx (log)										
Treat	7	0.55	**8.38*****	Frequency	2	22.75***	1.47	0.91	**39.57*****	**13.69*****
Error	40	0.07		Dosage	1	0.51				
				F × D	2	**5.74****	Dosage (250 vs. 500)			
							1d	2d	4d	
							5.12*	**5.55***	1.31	
GSH (log)										
Treat	7	1.60	**19.11*****	Frequency	2	**11.07*****	**48.06*****	**17.78*****	0.68	0.15
Error	40	0.08		Dosage	1	4.75				
				F × D	2	1.39				
LPO (log)										
Treat	7	0.77	**8.14*****	Frequency	2	**14.97*****	**11.70*****	0.14	**13.20*****	0.01
Error	40	0.09		Dosage	1	0.55				
				F × D	2	2.21				

All planned comparisons had 1,40 degrees of freedom and were tested against the mean square error from the main test among all treatments. For all biomarkers, significance of planned comparisons was assessed at α=0.05. Significant *F* values are highlighted in bold. Type of data transform is given in brackets.
Significant codes: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

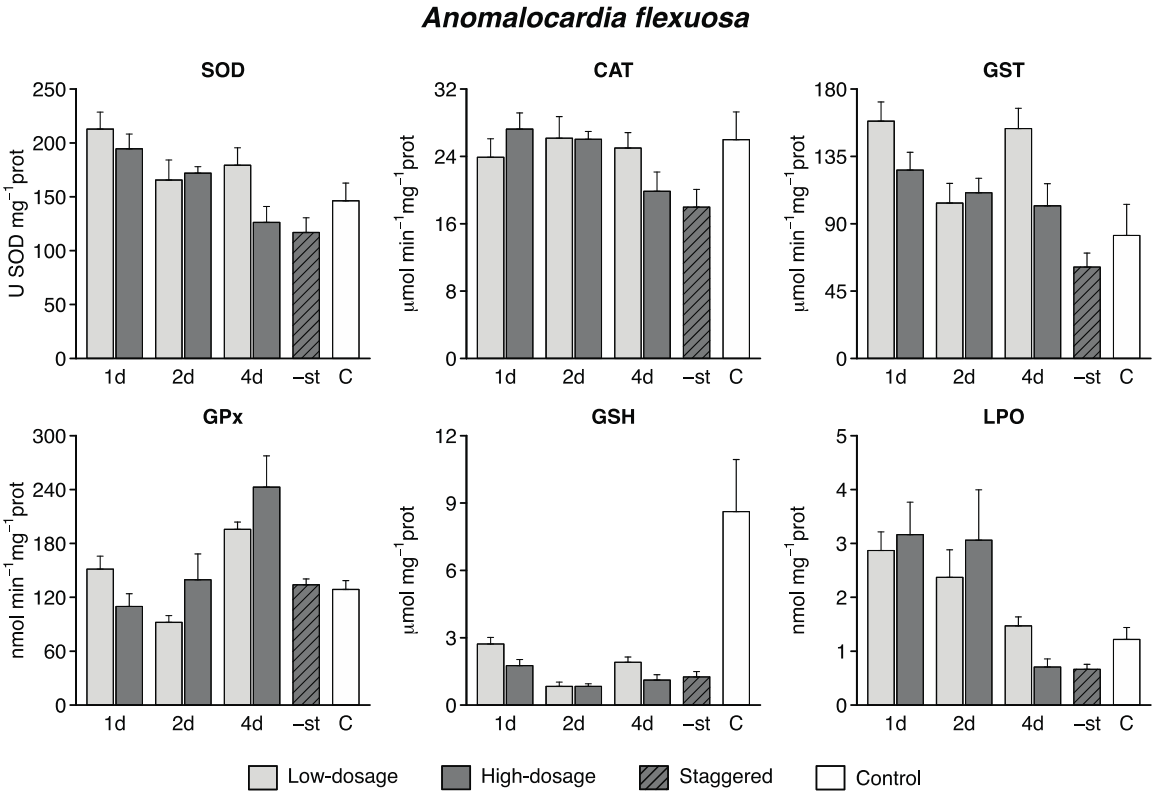

Fig. 2. Activities of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and levels of reduced glutathione (GSH) and lipid peroxidation (LPO) in the bivalve *Anomalocardia flexuosa* in response to varying frequencies and intensities of repeated exposure events. Oil spills occurred every 1-day (1d), 2 days (2d) or 4 days (4d). Undisturbed controls (C) are shown in white, low-dosage (250 mL/0.25 m^2) spills are shown in light gray and high-dosage (500 mL/0.25 m^2) spills are shown in dark gray. Hatched dark gray bars indicate that the timing of the high-dosage spills was staggered.

326 LPO levels compared to a high-dosage oil spill every 4 days (2d250 vs. 4d500
327 comparisons in Table 2; Fig. 2). However, GPx activity was inhibited by frequent low-
328 dosage oil spills compared to infrequent high-dosage ones (2d250 vs. 4d500 comparison
329 in Table 2; Fig. 2).

330 Generally, there was no significant difference between treatments that received
331 the same dosage and frequency of exposure, but for which the timing of exposure differed
332 (4d500 vs. 4d500-st comparison in Table 2; Fig. 2). The exception was the activities of
333 GST and GPx, which were both reduced by a high-dosage oil spill occurring 1 day before
334 the end of the experiment (4d500-st), rather than 3 days before the end (4d500).

335 *3.2. Biomarker responses in the gastropod N. virginea*

336

337 Overall, biomarker responses were not affected in gastropods exposed to frequent
338 high-dosage oil spills compared to the control treatment, except for a significant decrease
339 in GSH levels (Control vs. 1d500 comparison in Table 3; Fig. 3).

340 There were major differences between treatments caused by the combined effect
341 of frequency and dosage, as detected by significant statistical interactions in the factorial
342 ANOVA (Table 3). The activities of SOD, CAT and levels of LPO increased significantly
343 with higher dosages of oil, but this was observed only in treatments that were exposed
344 every 2 days (Table 3; Fig. 3).

345 In contrast to the patterns described for *A. flexuosa*, activities of most biomarkers
346 in *N. virginea* were significantly induced by infrequent high-dosage oil spills compared to
347 frequent low-dosage oil spills (Frequency vs. Dosage planned comparisons in Table 3;
348 Fig. 3). SOD activity was induced by exposure to a high-dosage spill every 2 days
349 compared to a low-dosage oil spill every day (1d250 vs. 2d500 comparison in Table 2;
350 Fig. 2). Likewise, a high-dosage oil spill every 4 days significantly increased the GPx
351 activity and levels of GSH compared to a low-dosage oil spill every 2 days (2d250 vs.
352 4d500 comparison in Table 3; Fig. 3). Finally, the activities of CAT and GST were
353 increased by infrequent high-dosage spills compared to frequent low-dosage spills in both
354 1d250 vs. 2d500 and 2d250 vs. 4d5.0 planned comparisons (Table 3; Fig. 3).

355 Biomarker responses in *N. virginea* were also affected by the timing of exposure
356 events (4d500 vs. 4d500-st comparison in Table 3; Fig. 3). The activities of SOD, CAT,
357 GST and GPx together with GSH levels were reduced by a later oil spill (4d500-st).

358

Table 3. Asymmetrical analysis of variance including the main test and subsequent planned comparisons for activities of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and levels of reduced glutathione (GSH) and lipid peroxidation (LPO) in *Neritina virginea*.

							Planned comparisons			
	Main test			**Factorial**			Oil effect	Frequency vs. Dosage		Timing
	All treatments			Excludes Control and 4d500-st			Control vs. 1d500	1d250 vs. 2d500	2d250 vs. 4d500	4d500 vs. 4d500-st
	df	MS	*F*		*df*	*F*	*F*	*F*	*F*	*F*
SOD										
Treat	7	24551.18	**5.11*****	Frequency	2	2.32	0.14	**13.17*****	2.99	**9.89****
Error	40	4802.12		Dosage	1	7.13**				
				F × D	2	**5.15****	Dosage (250 vs. 500)			
							1d	2d	4d	
							0.03	**17.30*****	0.09	
CAT (log)										
Treat	7	0.47	**4.60*****	Frequency	2	1.75	0.36	**6.75****	**4.36***	**12.26*****
Error	40	0.10		Dosage	1	3.96				
				F × D	2	**4.21***	Dosage (250 vs. 500)			
							1d	2d	4d	
							0.15	**12.12*****	0.12	
GST (log)										
Treat	7	0.37	**6.69*****	Frequency	2	**18.73*****	3.16	**10.23****	**11.28****	**15.01*****
Error	40	0.06		Dosage	1	2.35				
				F × D	2	0.96				
GPx										
Treat	7	5119.17	**2.39***	Frequency	2	**5.82****	3.53	0.53	**10.98****	**4.02***
Error	40	2139.59		Dosage	1	0.22				
				F × D	2	1.46				
GSH (log)										
Treat	7	1.35	**6.21*****	Frequency	2	**18.09*****	**4.38***	2.03	**29.89*****	**9.28****
Error	40	0.22		Dosage	1	0.95				
				F × D	2	1.41				
LPO										
Treat	7	39.23	**2.87***	Frequency	2	2.95	1.49	0.27	0.57	0.01
Error	40	13.66		Dosage	1	0.01				
				F × D	2	**4.22***	Dosage (250 vs. 500)			
							1d	2d	4d	
							1.73	**5.79***	0.93	

All planned comparisons had 1,40 degrees of freedom and were tested against the mean square error from the main test among all treatments. For all biomarkers, significance of planned comparisons was assessed at α=0.05. Significant *F* values are highlighted in bold. Type of data transform is given in brackets.
Significant codes: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

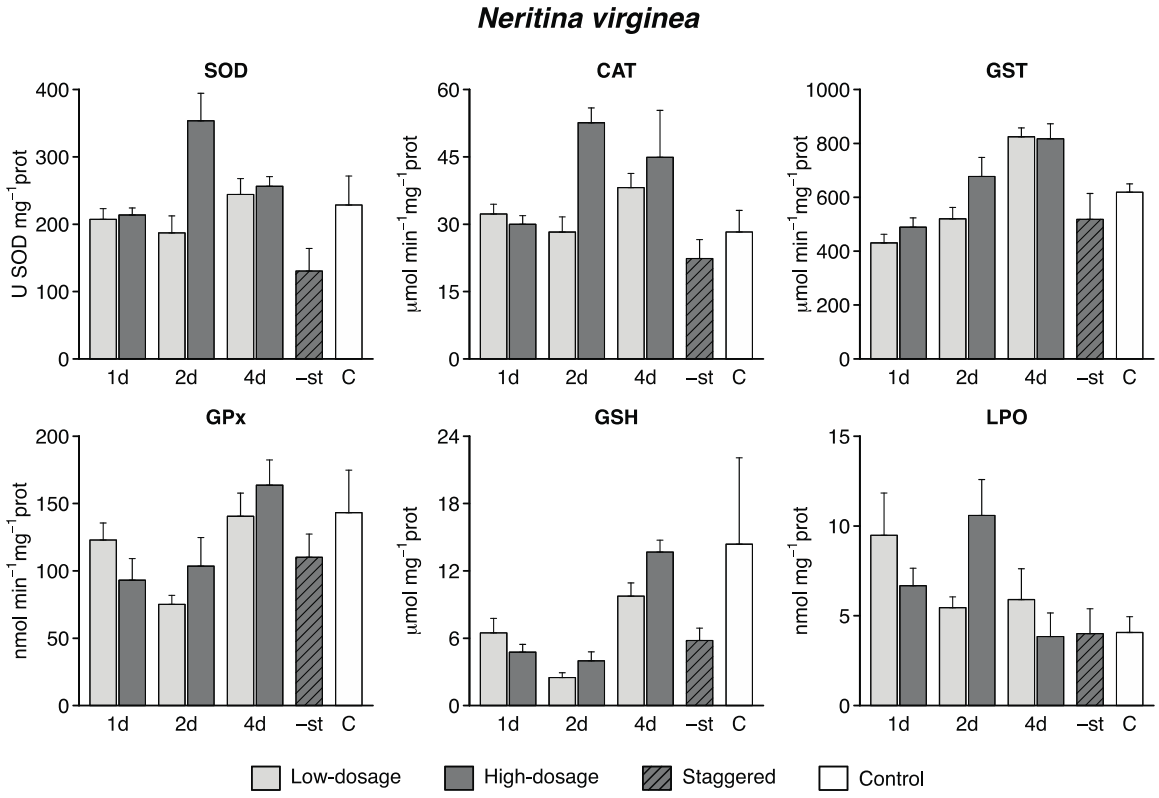

Fig. 3. Activities of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and levels of reduced glutathione (GSH) and lipid peroxidation (LPO) in the gastropod *Neritina virginea* in response to varying frequencies and intensities of repeated exposure events. Oil spills occurred every 1-day (1d), 2 days (2d) or 4 days (4d). Undisturbed controls (C) are shown in white, low-dosage (250 mL/0.25 m^2) spills are shown in light gray and high-dosage (500 mL/0.25 m^2) spills are shown in dark gray. Hatched dark gray bars indicate that the timing of the high-dosage spills was staggered.

3.3. Biomarker responses in the polychaete L. culveri

The polychaete *L. culveri* was strongly affected by the experimental oil spills. In contrast to *A. flexuosa* and *N. virginea*, *L. culveri* displayed massive mortality in frequent high-dosage oil spills (1d500). Therefore, this particular treatment was missing and planned comparisons with control were performed using 1d250 treatment instead. Nevertheless, losing 1d500 treatments had no severe implications for the general interpretation of the statistical output and the hypothesis being tested.

The activities of SOD, CAT, GST and GPx and levels of LPO were significantly increased by frequent low-dosage oil spills (1d250) compared to the control treatment. Furthermore, the concentration of GSH was significantly reduced in polychaetes exposed to frequent low-dosage spills (Control vs. 1d250 comparison in Table 4; Fig. 4).

Differences in GST activity and LPO levels were caused by the frequency of oil spills while differences in GPx activity and GSH concentration were caused by the interaction between frequency and dosage. SOD and CAT activities did not differ between frequency, dosage or their interaction (Table 4). The high-dosage spills induced the GPx activity and increased GSH concentrations, but this was observed only at treatments that were exposed once (4d comparisons in Table 4; Fig. 4).

Overall, patterns of biomarker responses in the polychaete *L. culveri* were similar to those described for the bivalve *A. flexuosa*. Enzymatic activities were significantly induced by frequent low-dosage oil spills compared to infrequent high-dosage oil spills (Frequency vs. Dosage planned comparisons in Table 4; Fig. 4), except for CAT activity and GSH levels that did not differ significantly. A low-dosage oil spill every 2 days significantly increased the levels of LPO compared to a high-dosage oil spill every 4 days (2d250 vs. 4d500 comparison in Table 4; Fig. 4). Similarly, the activities of SOD and GST were increased by frequent low-dosage spills compared to infrequent high-dosage spills in both 1d250 vs. 2d500 and 2d250 vs. 4d5.0 planned comparisons (Table 4; Fig. 4).

GPx activity exhibited inconsistent patterns in treatments exposed to the same overall volume of diesel, but according to different exposure regimes. Despite being significantly induced by frequent low-dosage spills compared to infrequent high-dosage spills in 1d250 vs. 2d500 comparison, GPx activity was inhibited in 2d250 vs. 4d5.0 comparison (Table 4; Fig. 4).

Biomarker responses in *L. culveri* were not affected by the timing of exposure events, with the exception of GPx activity, which was inhibited by the later oil spill (4d500 vs. 4d500-st comparison in Table 4; Fig. 4).

Table 4. Asymmetrical analysis of variance including the main test and subsequent planned comparisons for activities of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and levels of reduced glutathione (GSH) and lipid peroxidation (LPO) in *Laeonereis culveri*.

							Planned comparisons			
	Main test			**Factorial**			Oil effect	Frequency vs. Dosage		Timing
	All treatments			Excludes Control and 4d500-st			Control vs. 1d250	1d250 vs. 2d500	2d250 vs. 4d500	4d500 vs. 4d500-st
	df	MS	*F*		*df*	*F*	*F*	*F*	*F*	*F*
SOD										
Treat	6	20275.03	**2.78***	Frequency	1	1.27	**5.16***	**4.12***	**4.26***	1.25
Error	35	7297.00		Dosage	1	3.22				
				F × D	1	2.20				
CAT										
Treat	6	30.75	1.46	Frequency	1	0.01	**4.49***	2.68	1.15	0.28
Error	35	21.10		Dosage	1	1.95				
				F × D	1	0.004				
GST										
Treat	6	425.26	**11.34*****	Frequency	1	**14.99*****	**46.63*****	**13.97*****	**16.85*****	1.95
Error	35	37.51		Dosage	1	3.74				
				F × D	1	1.46				
GPx (log)										
Treat	6	0.51	**6.78*****	Frequency	1	13.32***	**10.10****	**7.86****	**22.31*****	**8.53****
Error	35	0.08		Dosage	1	9.19**				
				F × D	1	**7.79****	Dosage (250 vs. 500)			
							2d	4d		
							0.03	**16.95*****		
GSH (log)										
Treat	6	0.82	**3.60****	Frequency	1	0.27	**4.61***	0.45	1.81	0.16
Error	35	0.23		Dosage	1	1.91				
				F × D	1	**6.90***	Dosage (250 vs. 500)			
							2d	4d		
							0.78	**8.03****		
LPO (log)										
Treat	6	4.48	**17.66*****	Frequency	1	**43.34*****	**53.94*****	0.30	**21.67*****	0.001
Error	35	0.25		Dosage	1	0.00				
				F × D	1	0.32				

All planned comparisons had 1,35 degrees of freedom and were tested against the mean square error from the main test among all treatments. For all biomarkers, significance of planned comparisons was assessed at α=0.05. Significant *F* values are highlighted in bold. Type of data transform is given in brackets.
Significant codes: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

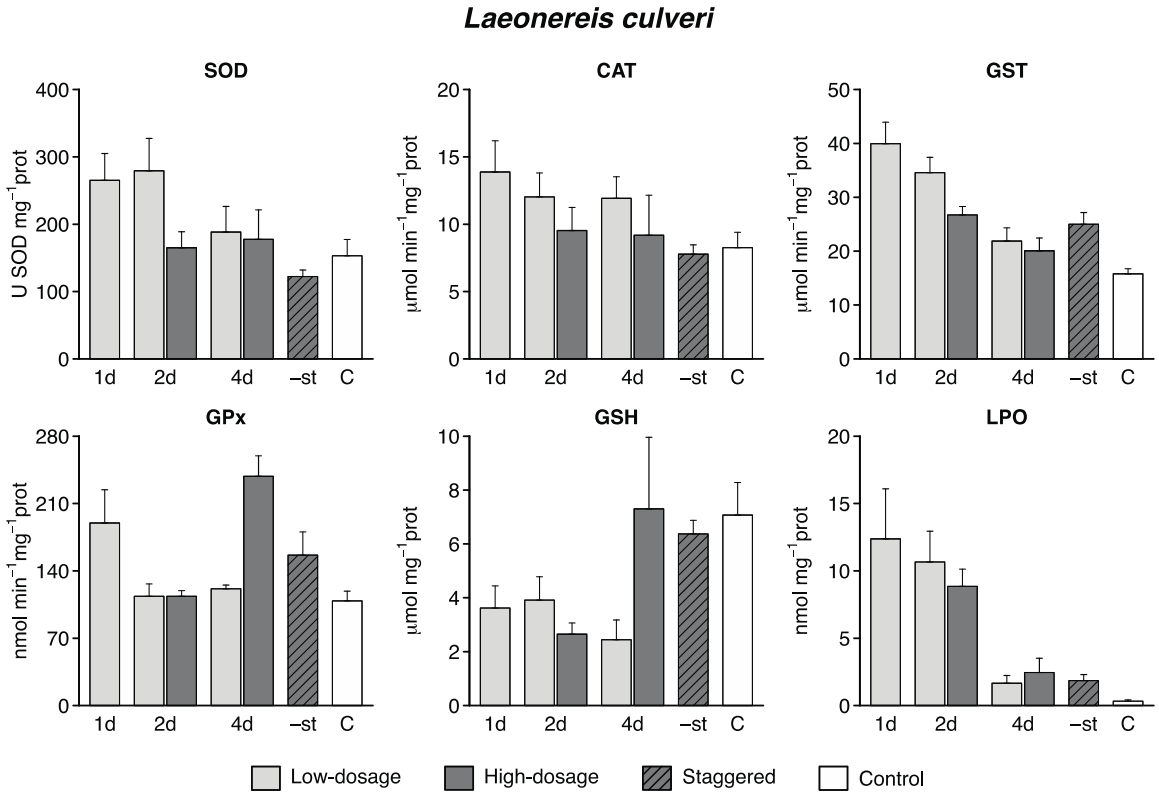

Fig. 4. Activities of superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and levels of reduced glutathione (GSH) and lipid peroxidation (LPO) in the polychaete *Laeonereis culveri* in response to varying frequencies and intensities of repeated exposure events. Oil spills occurred every 1-day (1d), 2 days (2d) or 4 days (4d). Undisturbed controls (C) are shown in white, low-dosage (250 mL/0.25 m^2) spills are shown in light gray and high-dosage (500 mL/0.25 m^2) spills are shown in dark gray. Hatched dark gray bars indicate that the timing of the high-dosage spills was staggered.

3.4. Polycyclic aromatic hydrocarbons

Concentrations of PAHs and related parameters are given in Table 5. Total PAHs concentration (ΣPAHs), excluding perylene, which can be associated with diagenetic sources, ranged from 13.1 to 26.2 ng g^{-1} in the control treatment, and from 219.4 to 3027.5 ng g^{-1} in exposure treatments. Higher ΣPAHs concentrations were observed in plots exposed to frequent high-dosage oil spills.

Table 5. Polycyclic aromatic hydrocarbons (PAH) concentrations and related parameters in control and oil-exposed sediments. ΣPAHs, total polycyclic aromatic hydrocarbons (ng g^{-1} dry weight); 2–3 rings, total PAHs with two to three aromatic rings (ng g^{-1} dry weight); 4–6 rings, total PAHs with four to six aromatic rings (ng g^{-1} dry weight); LMW/HMW, ratio between the low molecular weight (2–3 rings) and high molecular weight (4–6 rings) PAHs and; Fl/(Fl+Py), fluoranthene/fluoranthene + pyrene isomer pair ratio.

	Low-dosage spills (250 mL/0.25 m^2)						**High-dosage spills (500 mL/0.25 m^2)**								**Control**	
	1d250		2d250		4d250		1d500		2d500		4d500		4d500-st			
	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2	Plot 1	Plot 2
ΣPAHs	445.92	280.47	1524.79	655.01	236.88	219.38	2153.74	3027.46	989.53	764.99	504.77	275.64	1492.56	277.43	26.15	13.09
2–3 rings	163.32	43.95	156.38	124.26	48.35	32.54	223.54	328.07	139.18	107.50	49.46	41.82	456.33	82.18	9.62	4.50
4–6 rings	32.07	12.77	24.68	22.20	19.77	21.19	45.87	53.29	27.60	17.99	17.35	24.19	78.32	9.11	9.46	3.97
LMW/HMW	5.09	3.44	6.34	5.60	2.45	1.54	4.87	6.16	5.04	5.98	2.85	1.73	5.83	9.02	1.02	1.13
Fl/(Fl+Py)	0.47	0.39	0.33	0.42	0.44	0.37	0.30	0.28	0.40	0.15	0.39	0.44	0.45	0.43	0.54	0.54

Concentrations of low molecular weight (LMW – 2 and 3 rings) PAHs varied from 4.5 to 9.6 ng g^{-1} in the control treatment, and from 32.5 to 456.3 ng g^{-1} in oil-exposed sediments. High molecular weight (HMW – 4 to 6 rings) PAHs ranged from 3.97 to 9.46 ng g^{-1} in control plots, and from 9.1 to 78.3 ng g^{-1} in exposure plots. The clear dominance of LMW PAHs in all samples from diesel-exposed plots indicates a petrogenic source; i.e., experimental oil spills (LMW/HMW > 1). On the other hand, the predominance of HMW PAHs in control plots may be indicative of pyrolytic sources (LMW/HMW = 1), such as fossil fuel and biomass combustion (Martins et al., 2010; Dauner et al., 2015) that are not related to the experimental diesel spills.

Isomer pair ratios of PAHs can also be used to identify PAH sources in estuarine sediments (Martins et al., 2011). We used the fluoranthene/fluoranthene + pyrene (Fl/Fl + Py) ratio that is indicative of the following sources: Fl/Fl + Py < 0.40 is dominance of petroleum, 0.40–0.50 is dominance of petroleum combustion, and >0.50 is dominance of coal and biomass (grass and wood) burning. The Fl/Fl + Py isomer pair ratios revealed PAHs associated with the direct introduction of petroleum in most diesel-exposed plots. Only in control plots the Fl/Fl + Py ratios were larger than 0.50 (Table 5), which suggests biomass combustion.

4. Discussion

4.1. PAHs

The concentration of PAHs in the sediment indicated that oil-exposed plots were effectively contaminated by diesel, reaching levels close to those reported for polluted regions (Venturini et al., 2008; Martins et al., 2011). According to Notar et al. (2001) ΣPAH concentrations higher than 500 ng g^{-1} are indicative of highly contaminated sediments. This threshold value was exceeded in most of the impacted plots, particularly those

428 exposed to high dosages of diesel. Moreover, none of the control plots were contaminated
429 by diesel exposure, as indicated by the low ΣPAH concentration (between 13 and 26 ng
430 g^{-1}).

431 The composition of individual PAHs is known to indicate the petrogenic or
432 pyrogenic sources in aquatic systems, usually by the use of several diagnostic ratios
433 (Martins et al., 2011). Natural sources of pyrogenic hydrocarbons include forest and grass
434 fires, while anthropogenic sources include vehicular and industrial emissions (Yunker et
435 al., 2002). Natural petrogenic hydrocarbon sources include crude oil seeps and coal and
436 shale deposits, while anthropogenic sources include oil spills, chronic discharges and coal
437 (Harris et al., 2011). Polycyclic aromatic hydrocarbons of pyrogenic origin were already
438 present in local sediments, as indicated by the predominance of HMW PAHs in control
439 plots. The clear dominance of LMW PAHs in all samples from diesel-exposed plots
440 indicates a petrogenic source, which could be unequivocally related to the experimental oil
441 spills.

442 The Fl/Fl + Py isomer pair ratios calculated for impacted plots showed PAHs
443 associated with the direct introduction of petroleum and derivatives in most cases (Fl/Fl +
444 Py < 0.40), although petroleum combustion sources were also detected (Fl/Fl + Py
445 between 0.40 and 0.50). Such intermediate Fl/Fl + Py values, however, may be indicative
446 of PAHs degradation in the sediment. Control plots presented Fl/Fl + Py ratios > 0.50,
447 which according to Yunker et al. (2002) are values characteristic of grass, wood or coal
448 combustion.

449

450 *4.2. Oil exposure effects*

451

452 Antioxidant defense responses and oxidative damage measured in the bivalve *A.*
453 *flexuosa* and the polychaete *L. culveri* were overall strongly affected by frequent oil spills
454 compared to undisturbed controls. The main direct effect of frequent diesel spills on both

455 species was the induction of SOD and GST activities, a significant increase in LPO levels
456 and a decrease in GSH concentration. *L. culveri* was more sensitive to oil impact than *A.*
457 *flexuosa*, since it exhibited massive mortality when exposed to frequent high-dosage spills
458 (1d500) and responded in terms of induction of CAT and GPx by frequent low-dosage oil
459 spills (1d250).

460 Our results also showed that the gastropod *N. virginea* did not activate enzymatic
461 defense system against ROS. Most biomarker responses in *N. virginea* exposed to
462 frequent high-dosage spills did not differ from those in control, except for a significant
463 depletion in GSH levels. Significant changes in GSH levels may be an important indicator
464 of the detoxification ability of an organism, particularly under severe exposure to pollutants
465 (Stara et al., 2012). Low levels of oxidative stress may increase the GSH synthesis and
466 detoxifying enzymes activities, while severe oxidative stress may cause the oxidation of
467 GSH to GSSG, and the lowering of antioxidant enzymes (Elia et al., 2006). Therefore, the
468 hypothesis that biomarker responses are overall affected by repeated oil spills was not
469 rejected for *A. flexuosa* and *L. culveri*, although partially refuted for *N. virginea*.

470 Overall, the antioxidant parameters measured in this study have previously
471 demonstrated suitable for evaluating the effects of PAHs exposure on bivalves, both in the
472 field (Torres et al., 2002; Box et al., 2007; Vidal-Liñán et al., 2010; Sureda et al., 2011;
473 Turja et al., 2013) and in the laboratory (Silva et al., 2005; Richardson et al., 2008;
474 Lüchman et al., 2011; Luna-Acosta et al., 2011; Turja et al., 2014). Nereid polychaetes
475 are also known to activate such antioxidant defenses, particularly when exposed to metals
476 (Ventura-Lima et al., 2007; Ferreira-Cravo et al., 2009; Díaz-Jaramillo et al., 2013), but
477 also to PAHs (Sun and Zhou, 2008; Sun et al., 2009).

478 SOD induction is commonly referred as the first line of enzymatic antioxidant
479 defense in response to increasing levels of contaminant-stimulated ROS production (Lima
480 et al., 2007; Lüchman et al., 2011). SOD activity is thus considered a good biomarker of
481 pollution because of its relatively short time response to stressors. Indeed, in the present

482 study SOD activity was increased in bivalves and polychaetes exposed to frequent diesel
483 spills compared to control, but its inhibition could also be a part of the diesel toxicity
484 response, as shown for the oyster *Crassostrea brasiliana* (Lüchman et al., 2011). SOD
485 catalyses the dismutation of superoxide anion to produce hydrogen peroxide (H_2O_2),
486 which is further degraded by CAT and GPx (Halliwell and Gutteridge, 2007; Lüchman et
487 al., 2011; Turja et al., 2013). Generally, SOD activity in bivalve gills is more pronounced
488 than in the digestive glands, which could be related to the fact that gills are likely to be the
489 first organ exposed to waterborne pollutants (Luna-Acosta et al., 2011). Nonetheless, we
490 found a significant induction of SOD activity in *A. flexuosa* digestive glands, indicating that
491 this tissue may also be appropriate to evaluate oxidative stress responses in bivalves
492 exposed to repeated oil spills.

493 GPx and CAT catalyze the transformation of H_2O_2 to molecular water (H_2O); i.e.,
494 they may act on common substrates (Richardson et al., 2008). A significant induction of
495 SOD activity should, therefore, be associated with an increase in H_2O_2 production,
496 resulting in elevated CAT activity and/or GPx activity. In the present study, both
497 antioxidant enzymes were induced in *L. culveri* exposed to frequent oil spills, indicating
498 that the production of H_2O_2 by increased SOD activity was enough to prevent competition
499 between CAT and GPx for the same substrate. However, CAT and GPx activities in *A.*
500 *flexuosa* were not affected by frequent spills compared to control, despite a significant
501 induction of SOD. Absence of induction of CAT and GPx activities has previously been
502 reported in bivalve digestive glands exposed to PAHs, especially in mussels (Banni et al.,
503 2010; Lüchman et al., 2011). According to Vidal-Liñán et al. (2010, 2014) both CAT and
504 GPx activities are strongly influenced by seasonal variations in water temperature,
505 reproductive cycle and food availability, or metabolic activity and these are relevant
506 limitations for their use as reliable biomarkers of oil exposure in the field.

507 The induction of GST activity has been extensively suggested as a biomarker of
508 exposure to chemicals and detoxification of organic contaminants such as PAHs (van der

509 Oost et al., 2003; Hellou et al., 2012; Vidal-Liñán et al., 2014), although inhibitions in
510 activity caused by exposure to contaminants have also been reported (van der Oost et al.,
511 2003). PAH levels in heavily contaminated sites are also strongly correlated with GST
512 activity (Hellou et al., 2012). GST is one the most efficient phase II biotransformation
513 pathways for potentially toxic chemicals in invertebrates (Vidal-Liñán et al., 2014). GST
514 activity is less sensitive to environmental factors (such as food availability and
515 reproductive cycle) than CAT and GPx activities, being consistently higher in bivalves
516 (Vidal-Liñán et al., 2010) and nereid polychaetes (Díaz-Jaramillo et al., 2011, 2013) from
517 polluted areas. Our results suggest that GST activity is an appropriate biomarker to
518 assess the effects of repeated oil spills in *A. flexuosa* and *L. culveri* in the field, even after
519 short-term exposures (i.e. during a week).

520 Frequent diesel spills act to reduce GSH levels among all macrofaunal species
521 analyzed. GSH depletion may be explained by 1) the conjugation of glutathione to
522 oxidized PAH through the increase in GST activity (Yin et al., 2007; Milinkovitch et al.,
523 2011); 2) the decrease in GSH synthesis due to contaminant exposure (Mela et al., 2012);
524 or 3) its conversion to the oxidized form GSSG as a result of scavenging ROS (Hellou et
525 al., 2012). In addition, GSH is the substrate of glutathione peroxidase (GPx) and
526 glutathione-S-transferase (GST), which also serve in the removal of ROS and their
527 reaction products (Tausz et al., 2004). The induction of GST and GPx activities in *L.*
528 *culveri*, and GST in *A. flexuosa* exposed to frequent oil spills may be responsible for GSH
529 depletion. Since GPx and GST activities in *N. virginea* exposed to frequent high-dosage
530 spills did not differ from control, it is reasonable to infer that GSH was the main defense
531 against PAHs used by this gastropod in our experiment.

532 The effect of oxidative stress in *A. flexuosa* and *L. culveri* exposed to frequent oil
533 spills was also expressed by elevated LPO levels. The extent to which oxyradical
534 generation produces biological damage depends on the effectiveness of antioxidant
535 defenses and biotransformation enzymes (Luna-Acosta et al., 2011). Lipid peroxidation is

536 likely to be observed in the absence of sufficient antioxidant defense (Richardson et al.,
537 2008). Overall, our results indicated that significant antioxidant enzyme activity in bivalves
538 and polychaetes exposed to frequent oil spills were not sufficient to prevent oxidative
539 damage in terms of LPO. Nevertheless, increased production of ROS did not result in
540 oxidative damage to lipids in *N. virginea*.
541
542 *4.3. Equivalent overall volume of oil spilled*
543
544 Comparisons of treatments that received the same overall volume of diesel, but
545 different exposure regimes, yielded two distinct response patterns: 1) enzymatic activities
546 and oxidative damage measured in the bivalve *A. flexuosa* and the polychaete *L. culveri*
547 were induced by frequent low-dosage spills compared to infrequent high-dosage spills;
548 and 2) activities and levels of biomarkers measured in *N. virginea* were significantly
549 induced by infrequent high-dosage oil spills compared to frequent low-dosage oil spills.
550 Despite these opposite patterns, the hypothesis that different exposure regimes determine
551 variations in biomarker responses was not rejected.
552 Richardson et al. (2008) demonstrated, under laboratory conditions, that a
553 constant dosing regime of PAHs produced larger oxidative stress in green-lipped mussels
554 (*Perna viridis*) than infrequent pulsed regimes, as indicated by the induction of CAT, GSH
555 and lipid peroxidation. This pattern was clearly observed for biomarker responses in *A.*
556 *flexuosa* and *L. culveri* in our field experiment. We suggest that an increased frequency of
557 low-dosage diesel spills has a direct relationship with an increase in ROS formation,
558 followed by the induction of SOD and GST on both species. Moreover, contaminant-
559 stimulated ROS formation by higher frequencies exceeded the cellular antioxidant
560 capacity, causing the observed oxidative damage in terms of LPO. Dosage effects on both
561 *A. flexuosa* and *L. culveri* biomarkers were minor and inconsistent; e.g., GPx activity in *A.*

562 *flexuosa* was inhibit by higher dosages of oil in daily spills, but this pattern was the
563 opposite in treatments that were exposed every 2 days.
564 The fact that both *A. flexuosa* and *L. culveri* were affected by the frequency rather
565 than the dosage of diesel exposure events has important implication for pollution
566 monitoring, particularly in estuaries. Chemical pollutants in estuarine environments are
567 often discrete in time and space (i.e. pulse-like disturbance), and display marked temporal
568 and seasonal variations (Richardson et al., 2008) that are superimposed on the natural
569 dynamics of these systems (Johnston and Keough, 2005). Such variations can affect the
570 performance of biomarkers in the field and confound data interpretation in pollution
571 monitoring studies (Richardson et al., 2008; Vidal-Liñán and Bellas 2013).
572 On the other hand, the activities of SOD, CAT, GST, GPx and levels of GSH in *N.*
573 *virginea* were induced by infrequent high-dosage spills. Also, significant dosage-
574 dependent inductions were observed in SOD, CAT and LPO, but only in the intermediate
575 exposure regime (i.e. an oil spill every 2 days). These results show a direct relation
576 between the increase in PAH levels and the activation of antioxidant defenses. The
577 absence of oxidative stress in terms of lipid peroxides in *N. virginea* indicates that the
578 antioxidant responses were not overwhelmed (Geracitano et al., 2004). Marine gastropod
579 mollusks may be sensitive bioindicators of oil contamination, but few studies have
580 evaluated antioxidant responses following experimental exposure. Reid and MacFarlane
581 (2003) found a GPx dosage-dependent induction in the gastropod *Austrocochlea porcata*
582 exposed to crude oil in the laboratory, but not in the field. Further experimentation is
583 needed to evaluate antioxidant responses in *N. virginea* exposed in laboratory. Also, the
584 knowledge of seasonal baseline levels of biochemical parameters is needed for a better
585 interpretation of experimental exposure outcomes.
586

587 *4.4. Timing of oil spills*

588

589 We partially refuted the hypothesis that biomarker responses differed in organisms
590 exposed to similar oil dosages under the same exposure regime, but for which the timing
591 of exposure differed. Enzymatic activities together with GSH levels measured in *N.*
592 *virginea* were significantly reduced by a later oil spill. Biomarker responses in *A. flexuosa*
593 and *L. culveri* were not overall affected by the timing of exposure, with the exception of
594 GST and GPx activities in the bivalve and GPx activity in the polychaete, which were both
595 inhibited by the later oil spill. Lipid peroxidation was not affected by the timing of exposure
596 in any of the selected macrofaunal species.

597 Although our experiment was not specifically designed to assess the effects of
598 exposure period following oil spills, the inclusion of the staggered treatment allowed us to
599 compare the effects of single high-dosage oil spills (500 mL/0.25 m^2) with two different
600 times of exposure. The 4d500 treatment plots received a high oil dosage 4 days before
601 sampling, whereas the 4d500-st treatment plots were impacted just 2 days before
602 sampling. Extended exposure period was particularly relevant for biomarker responses in
603 the gastropod *N. virginea*. Overall, enzyme activities were significantly higher in 4d500
604 treatment compared to 4d500-st treatment. We suggest that this difference was due the
605 fact that exposure period of the later oil spill (i.e. 4d500-st) was too short to induce ROS
606 production (Milinkovitch et al., 2011). The delayed antioxidant defense responses to PAH
607 exposure was also observed in the mussel *Mytella guyanensis* exposed to the same
608 concentration of diesel in mangroves of Paranaguá Bay (Marques et al., 2014).

609 Generally, antioxidant responses in the bivalve *A. flexuosa* and the polychaete *L.*
610 *culveri* were not affected by timing of exposure events, although increased GST and GPx
611 activities in 4d500 treatment compared to 4d500-st were observed. In a laboratory
612 experiment, Richardson et al. (2008) found that induction of GST activity in green-lipped
613 mussels occurred infrequently, suggesting that GST does not immediately respond to

614 oxidative stress from short-term exposure to PAHs. Nonetheless, most of our results
615 indicate that the production of oxyradicals and activation of antioxidant defenses on both
616 species does not depend on exposure period to PAHs. However, none of antioxidant
617 responses evaluated in our study was induced by a later oil spill, indicating that long-term
618 exposures to PAHs (at least more than two days) are therefore required to evaluate
619 antioxidant biomarkers in the selected macrofaunal species.
620
621 **5. Conclusions**
622
623 Experimental *in situ* simulations of oil exposure events with different frequencies
624 and intensities provide a useful tool for detecting and quantifying environmental impacts.
625 We have shown that non-enzymatic antioxidants such as glutathione, together with
626 enzymatic antioxidants, biotransformation enzymes and lipid peroxidation in the bivalve *A.*
627 *flexuosa* and the polychaete *L. culveri* are suitable biomarkers of petroleum pollution.
628 When exposed to the same overall diesel release, but at distinct exposure regimes,
629 biomarker responses were strongly affected by the frequency of oil spills. In general,
630 biomarkers were induced by frequent small exposures compared to infrequent large ones.
631 Enzymatic defenses against ROS and oxidative damage measured in the
632 gastropod *N. virginea* were not affected by frequent oil spills. The main antioxidant
633 defense mechanism in *N. virginea* was expressed by a significant decrease in GSH levels,
634 a non-enzymatic scavenger of ROS. Our results also revealed that *N. virginea* may
635 display a belated response to acute high-dosage exposure. Further experiments are
636 therefore needed to evaluate antioxidant biomarker responses in *N. virginea*, particularly
637 involving long-term exposure periods and higher dosages of oil. Information on
638 background and seasonal variation of biomarker baseline levels for *N. virginea* is still
639 needed for a better interpretation of results from this field experiment.
640

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Capítulo IV

Effects of dispersed oil exposure on biomarker responses and growth in juvenile wolfish *Anarhichas denticulatus*

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Effects of dispersed oil exposure on biomarker responses and growth in juvenile wolfish *Anarhichas denticulatus*

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Abstract

This study evaluated the sensitivity of the wolfish *Anarhichas denticulatus* exposed to crude oil, comparing the effects of mechanically dispersed versus chemically dispersed oil using sub-lethal endpoints. To test the toxicity of this controversial technique, two experiments involving exposure of the organisms for 48 h were conducted. The first experiment assessed the effects of oil exposure on biomarker responses. The second experiment monitored the growth of juveniles over 5 weeks after exposure. Overall this study demonstrated that PAH biliary metabolites, EROD and AChE are appropriate biomarkers to assess exposure of *A. denticulatus*. Growth rate, both in length and weight,

was significantly higher in control compared to oil-exposure treatments. The lack of differences between chemically and mechanically dispersed oil in biomarkers response and growth suggests that dispersant application is no more toxic than the natural oil dispersion. The results indicate the potential for population-level effects resulting from exposure to oil.

Keywords: Crude oil; Biomarkers; Biliary metabolites; Petroleum hydrocarbons; Dispersant

1. Introduction

Some of the largest remaining oil and gas reserves are found in the Artic, causing the Barents Sea to become a major area of concern in terms of petroleum related activities (USGS, 2000). Even though this area is strictly under regulation against discharges from oil industries, accidental spills may still occur leading to disastrous scenarios if not contained. Dispersant spreading is a commonly employed method to remediate the ecological effects of petroleum on the marine environment (Milinkovitch et al., 2013). However, dispersant use may induce high concentrations of hydrocarbons in the water column, which is likely to increase exposure of aquatic organisms (Milinkovitch et al., 2011a). Therefore, there is a growing need to determine biomarkers that can be efficiently used as early warning signals of oil contamination in the Artic.

Polycyclic aromatic hydrocarbons (PAHs) are highly toxic contaminants and are regarded as primary issue for ecological risk assessment (Beyer et al., 2010). At low temperatures, the distribution, composition and physical state of PAHs are affected and therefore their bioavailability. Due to their lipophylicity and compatibility to organic materials, uptake and accumulation of PAHs in marine organisms are facilitated (Almeida

et al., 2012), with adverse consequences to biological organization such as delayed growth, reduced survival and developmental malformation (Beyer et al., 2010). Moreover, marine organisms residing in cold waters possess biological adaptions, such as higher proportion of unsaturated fatty acids in biological membranes, which may influence their susceptibility to oil-induced toxicity (Camus et al., 2002; Hannam et al., 2010).

Effects of PAHs have been largely investigated in different species from subtropical to boreal regions in an attempt to identify and determine relevant and important bioindicators and biomarkers of environmental impacts (Aas and Klungsøyr, 1998; Sturve et al., 2006; Bocchetti et al., 2008; Nahrang et al., 2010a,b; Almeida et al., 2012). Fish are recurrent bioindicators in toxicology studies mainly because they are relatively easy to obtain and play a major ecological role in the aquatic food webs (van der Oost et al., 2003). In addition, several fish species are economically important, causing them to become useful targets for the development of biomarkers and consequently risk assessment protocols (Wester et al., 1994).

Measurement of stress responses in organisms can be manifested at various levels of biological organization. The combined use of several indicators at different levels of biological organization represents a sensible strategy, although rarely applied for interpretation of the consequences of pollution (Underwood and Peterson, 1988). Organismic and sub-organismic measures potentially provide the earliest warning of possible future deterioration and may also be the most sensitive measures of pollution. The impacts of oil spills on aquatic organisms are difficult to measure, and are usually estimated from counts of mortalities observed immediately afterwards (Heintz et al., 2000). Sub-lethal effects, however, may represent a significant but hidden component to the overall toxicity of a spill and would be presumably most profound in populations exposed during early developmental stages (Rosenthal and Alderdice, 1976; Heintz et al., 2000).

Increasing oil and gas activities in the Artic has led to the development of biomarkers of PAH exposure for Artic species, such as the polar cod *Boreogadus saida* (Nahrang et al., 2010a,b) and *Gadus morhua* (Lyons et al., 2011); the Artic scallop *Chlamys islandica* (Hannam et al., 2010); and the Artic char *Salvelinus alpinus* (Jørgensen and Wolkers, 1999). The wolfish *Anarhichas denticulatus* is listed under the federal Species at Risk Act (SARA) and was afforded protection under the SARA as of June 2004. Adult northern wolffish are observed to make limited movements and are non-migratory, and reproduce in shallow waters making them highly vulnerable to oil spill. Therefore, this species may be a relevant indicator for the implementation of biomarkers.

Previous studies have shown that cytochrome P450 measured via EROD (ethoxyresorufin-*O*-deethylase) and PAH metabolites in the bile respond in a dose-dependent manner to crude oil exposure (Nahrang et al., 2010b). These biomarkers are useful and crucial to detect exposure to PAH and possible toxicity, since fish and other vertebrates do not accumulate contaminants in tissue, rather they convert PAH to molecules more suitable to excretion (Beyer et al., 2010). Antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) are critically important in detoxification mechanisms by inhibiting oxyradicals formation and are commonly applied as biomarkers of oxidative stress (van der Oost et al., 2003).

Moreover, there have been reports on changes in acetylcholinesterase (AChE) activity after chemical exposure in aquatic organisms, such as organophosphates and carbamates insecticides (Fulton et al., 2001; Kopecka et al., 2004), water-soluble fraction of crude oil (Akaishi et al., 2004) and produced water (Holth et al., 2012). This enzyme is predominantly found in the brain and muscle tissue and is responsible for maintaining normal neurotransmission by catalyzing the hydrolysis of acetylcholine. Since selective modulation of AChE activity has been observed, this enzyme may be a useful biomarker of PAH exposure.

106 This study evaluated the sensitivity of juveniles of the wolfish (*Anarhichas*
107 *denticulatus*) exposed to crude oil, comparing the biological effects of mechanically
108 dispersed versus chemically dispersed oil using sub-lethal endpoints. Dispersant
109 application is a controversial technique used in nearshore areas to clean up an oil spill
110 (Milinkovitch et al., 2012). Despite preventing the arrival of the petroleum slick in
111 ecologically sensitive habitats, dispersant use may induce high concentrations of
112 petroleum in the water column and thereby raises the toxicity for aquatic organisms. In
113 addition, the toxicity may result from the dispersant by itself or from the combined effect of
114 dispersants and oil (Ramachandran et al., 2004).
115 To test the toxicity of this controversial technique (Milinkovitch et al., 2013), two
116 experiments involving exposure of the organisms for 48 h were conducted. The first
117 experiment investigated the effects of oil on different biomarkers commonly used in oil
118 monitoring. In the second experiment, the growth of wolfish juveniles was monitored over
119 5 weeks after oil exposure. We hypothesized that biomarker responses and growth of the
120 wolfish exposed to crude oil would be significantly different from those in control
121 treatment. Also, we tested the hypothesis that exposure effects of chemically dispersed oil
122 would be significantly more toxic than mechanically dispersed oil.
123
124 **2. Materials and methods**
125
126 *2.1. Experimental design*
127
128 The design used in both experiments consisted of four treatments: control (C;
129 seawater only), mechanically dispersed oil (MD; oil only mixed thoroughly in the water),
130 chemically dispersed oil (CD; dispersant, oil and thorough agitation in the water) and
131 dispersant (D; dispersant only mixed in seawater as a internal control of CD). Slickgone

dispersant, a Type 2/Type 3 dispersant concentrate manufactured by Dasic International, was used.

The four experimental treatments were randomly assigned to twelve 105 L tanks. Each one contained a funnel at the water surface connected to a water pump. This system was set up to maintain a mixture of oil and dispersant as a homogenous solution after 24 h of homogenization. Exposure time of 48 h, with crude oil and dispersant doses of 7 g and 0.28 g, respectively, have been previously identified for their relevance and tested out on temperate organisms (Milinkovitch et al., 2012).

Physicochemical variables, such as water temperature, dissolved oxygen (in terms of saturation percentage), salinity and pH were monitored in each tank over the experimental period using a using a YSI multiparameter probe. Physicochemical parameters remained stable (Table 1) and no fish died during the all exposure.

Table 1. Physicochemical variables monitored over the experimental period. Values are the mean of three tank replicates (± SD) of control (C), dispersant only (D), mechanically dispersed oil (MD) and chemically dispersed oil (CD).

Treatment	Temperature (ºC)	Oxygen (% sat.)	Salinity	pH
C	5.07 ± 0.32	107.33 ± 0.12	35.00 ± 0.00	7.91 ± 0.01
D	5.07 ± 0.35	107.00 ± 0.10	35.33 ± 0.58	7.92 ± 0.01
MD	5.00 ± 0.17	107.67 ± 0.58	35.67 ± 0.58	7.90 ± 0.02
CD	5.27 ± 0.35	106.33 ± 0.06	35.67 ± 0.58	7.90 ± 0.00

2.2. Total petroleum hydrocarbon (TPH) seawater concentrations

The concentration of total petroleum hydrocarbons (TPH), i.e. total dissolved hydrocarbons and oil droplets in the water, was determined for all treatments at the beginning of fish exposure (i.e. after 24-h oil homogenization) and at the end of exposure (at 72 h), using the mean of three replicated measurements from each exposure tank. About 20 ml of water were collected from the middle of the tank and directly fixed with

10% of dichloromethane before being stored at 4 °C until further analysis. Water samples were extracted three times with 10 mL dichloromethane. After each extraction, the organic phase was collected in a glass bottle and filtered on anhydrous sulphate before being scanned synchronously at an arbitrary fluorescence intensity from 200 to 500 nm excitation wavelengths (UV-Vis spectrophometer, Perkin-Elmer, Deutschland) as described by Fusey and Oudot (1976). Results, expressed in mg L^{-1}, were calculated using a standard curve of marine diesel in the range of 5 to 100 mg L^{-1}.

2.3. Biomarkers

Sixty juvenile wolfish (13.8 ± 0.94 cm) bought from an aquaculture farm in Tromsø (Norway) were used for biomarker analysis (five fish were used in each tank). Animals were immediately sacrificed after 48 h exposure; the bile, liver and brain were sampled and frozen in liquid nitrogen. Bile was sampled for determination of PAH metabolites. Liver was dissected for determining the activities of ethoxyresorufin-*O*-deethylase (EROD), catalase (CAT) and glutathione peroxidase (GPx). The brain was sampled for determining the acetylcholinesterase (AChE) activity.

PAH metabolites in the bile were measured with a PerkinElmer spectrofluorometer LS55 through synchronous fluorescence scan (SFS) spectrometry. Bile extract was diluted 1:40 in distilled water and fixed wavelength fluorescence (FF) was then measured. Excitation-emission wavelength pairs 290:335, 341:383, and 380:430 were employed to detect naphthalene-derived metabolites, pyrene-derived metabolites and benzo[a]pyrene-derived metabolites, respectively (Aas et al., 2000).

Liver samples for EROD determination were homogenized with a Potter-Elvehjem type homogenizer in a 100 mM phosphate buffer (pH 7.8) containing 150 mM KCl, 1 mM dithiothreitol and 5% glycerol. Homogenates were centrifuged (10,000 *g*, 4 ºC) for 30 min. Supernatants were subsequently centrifuged (50,000 *g*, 4 ºC) during 2 h for extraction of

the microsomal fraction. Pellets (microsomes) were dissolved in phosphate buffer (pH 7.8) containing 150 mM KCl, 1 mM dithiothreitol and 20% glycerol and stored at –80 ºC until analysis. EROD activity was measured according to a modified Burke and Mayer (1974) method using a fluorometric plate reader as described by Eggens and Galgani (1992). The reaction mix consisted of 10 μL microsomal fraction in 100 mM of phosphate buffer (pH 8), ethoxyresorufin 2 μM as substrate in a final volume of 230 μL. Reaction started by adding 0.25 mM NADPH in the microwells. The resorufin production was measured in four replicates during 20 min at room temperature with a fluorimetric plate reader PerkinElmer Victor at 544/584 nm excitation/emission wavelengths, respectively. A resorufin standard curve (0–2 μM) was used for determination of the reaction rates in pmol of resorufin produced min^{-1} mg^{-1} of total microsomal protein.

Liver samples for GPx determination were homogenized (1:5 w/v) with a Potter-Elvehjem type homogenizer in a 100 mM phosphate buffer (pH 7.5) with 1 mM EDTA and 1 mM NaN_3. Homogenates were centrifuged (15,000 *g*, 4 ºC) for 30 min and the supernatants were collected and stored at –80 °C. GPx activity was measured in triplicates and expressed as μmol min^{-1} mg^{-1} of total protein (Livingstone et al., 1992). Briefly, 30 μL of homogenate was mixed with potassium phosphate buffer (100 mM, pH 7.5) containing 1 mM of EDTA, 1 mM of NaN_3 and GSH (1.5 mM) and 1 U of glutathione reductase incubated for 10 min at 20 ºC. Then, the reaction was started by addition of 25 μL of NADPH (0.12 mM) and either cumene hydroperoxyde (4 mM, total GPX activity) or hydrogen peroxide (2 mM, selenium- dependent GPX activity). The decrease of NADPH was recorded during 1min at 340nm (ε = 6.2mM^{-1} cm^{-1}) and at 20 ºC.

Live samples for CAT determination were homogenized (1:5 w/v) with a Potter-Elvehjem type homogenizer in a 50 mM phosphate buffer (pH 7.0). Homogenates were centrifuged (15,000 *g*, 4 ºC) for 15 min and the supernatants were collected and stored at –80 °C until analysis. CAT activity was measured in triplicates and expressed as

μmol min^{-1} mg^{-1} of total protein. The decrease in absorbance was recorded at 4 ºC at 240 nm (ε = 40 M^{-1} cm^{-1}) using 600 mM H_2O_2 as substrate (Claiborne, 1985).

The brain tissue for AChE determination was homogenized (1:4 w/v) with a Potter-Elvehjem type homogenizer in ice-cold 100 mM Tris-HCl buffer (pH 8.0) containing Triton X100 0.1%. Homogenates were centrifuged at 9000 *g* for 20 min (4 °C) and the supernatants were collected and stored at –80 °C until analysis. The AChE activity in the homogenate was measured according to Ellman et al. (1961) and expressed in nmoles min^{-1} mg^{-1} of brain protein.

Total protein content in microsomal and cytosolic fractions of liver and brain homogenates was measured at 595 nm following Bradford's method (Bradford, 1976), with bovine serum albumin as standard.

2.4. Growth

In order to evaluate the effects of mechanically dispersed and chemically dispersed oil on growth, wolfish juveniles (mean length: 6.39 ± 0.42 cm; and mean weight: 2.19 ± 0.36 g) bought from an aquaculture farm in Tromsø (Norway) were exposed in experimental tanks (105 L) at a density of 20 fish per tank. After 48 h exposure, animals were transferred to a raceway with isolated compartments for each experimental tank and continuous seawater flow. Fish were fed twice a day. Total length and weight were measured weekly over a five weeks period.

2.5. Data analysis

Differences in the concentration of TPH among treatments were tested for each time by a one-factor analysis of variance, using experimental tanks as replicates.

Homogeneity of variances was checked using Cochran's C test and *a posteriori* comparisons of means were performed with Student-Newman-Keuls (SNK) test.

Biomarker responses were individually analyzed with a two-factor nested analysis of variance using Treatment (Tr, four levels, fixed, C; D; MD; CD) and Tank (Ta, three levels, random and nested in Tr) as factors. Homogeneity of variances was checked using Cochran's C test and *a posteriori* comparisons of means on significant terms of interest were performed with Student-Newman-Keuls (SNK) test. Components of variance (i.e. magnitude of effects) were calculated for each source of variation (including the residual) using the residual maximum likelihood (REML) method, which is insensitive to negative estimates (Fletcher and Underwood, 2002).

A repeated-measures analysis of variance using Treatment (Tr, four levels, fixed, C; D; MD; CD) and Time (Ti, five levels, fixed, crossed with Tr) as factors was separately applied for total length and weight of wolfish. Since animals were too small for tagging, an average value for each tank was used and tanks were considered the experimental unit for the repeated measures. Mauchly's test indicated that the assumption of sphericity has been violated for total length and weight, and therefore, a Greenhouse-Geisser correction was used. *A posteriori* pairwise comparisons of means were performed using the Bonferroni adjustment.

All statistical analysis and graphs were generated using R programming language (R Core Team, 2012) combined with GAD (Sandrini-Neto and Camargo, 2012), ez (Lawrence, 2013) and sciplot (Morales, 2012) packages.

3. Results and discussion

3.1. Total petroleum hydrocarbons (TPH)

258 The concentration of TPH in water was significantly higher in oil-exposure
259 treatments compared to control and dispersant control both at 24 h and 72 h (Fig. 1a, b).
260 TPH concentration was significantly higher in the CD than in the MD at the beginning of
261 fish exposure (after 24-h oil homogenization, Fig. 1a), although they did not differ at the
262 end of the experiment (Fig. 1b).

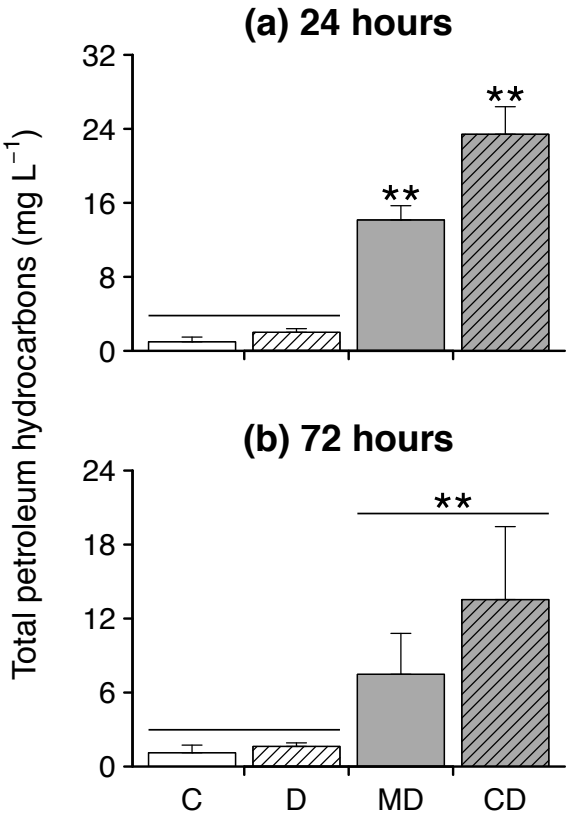

Fig. 1. Mean concentration (SE, n = 3 tanks) of total petroleum hydrocarbons (TPH) in experimental tanks after 24-h oil weathering (a) and at the end of fish exposure (b) to control (C), dispersant only (D), mechanically dispersed oil (MD) and chemically dispersed oil (CD). *denotes significant difference by SNK procedure; horizontal lines overly bars that were not different by SNK tests. Significance codes: ***P*<0.01.

263

264 The mean TPH concentrations in CD and MD observed at 24 h were decreased by
265 a factor of 2.9 and 4.8, respectively, compared to the nominal concentration used in our
266 experiment (approximately 67 mg L^{-1}). As pointed out by Milinkovitch et al. (2011a), the
267 reduction of TPH in water is probably due to the petroleum adherence to the experimental

system (particularly in the funnel) during the 24 h period of oil homogenization. Also, the fact that TPH concentration in water was found to be significantly lower in MD than in CD at 24 h can be attributed to the reduction of petroleum adherence due to dispersant (Milinkovitch et al., 2013). If extrapolated to field operations in nearshore areas, dispersing crude oil would increase the availability of TPH in the water column, while decreasing the adherence to substrates, such as intertidal sediments (Milinkovitch et al. (2011b). Nevertheless, interactions between chemically dispersed oil and sediments have not been well understood (Gong et al., 2014).

Drastic decreases in TPH concentrations are expected to be observed in offshore areas within short periods. The intensity of mixing due to wind and large air-water interface due to waves can strongly affect the persistence and transformation profiles of oil spills at sea (Tansel, 2014). In coastal habitats, however, oil contamination may persist for many years after an oil spill due to lower dilution potential in nearshore areas (Kingston, 2002). Overall, TPH concentrations in our experiment decreased by half after 72 h. This is in agreement with the results of Milinkovitch et al. (2011a, b), who have showed in a similar experimental set-up a decrease by one-third in TPH concentration over a 48 h period.

3.2. Biomarkers

No mortality was observed during exposure. Wolfish specimens showed no sign of stress, either in behavior or visible signs of changes (e.g. skin appearance). Whatever the fixed wavelength employed (Fig. 2a–c), fluorescence intensity reflecting relative concentration of biliary PAH metabolites were significantly higher in the oil treatments than control and dispersant control. Variance components showed that differences among treatments accounted for the largest proportion of total variance in naphthalene (more than 80%), pyrene (96% of total variation) and benzo[a]pyrene (93% of total variation)

295 derived type of metabolites (Table 2). The lack of significant differences between MD and
296 CD suggests that the use of oil dispersant did not increase the bioavailability of
297 hydrocarbons in crude oil.

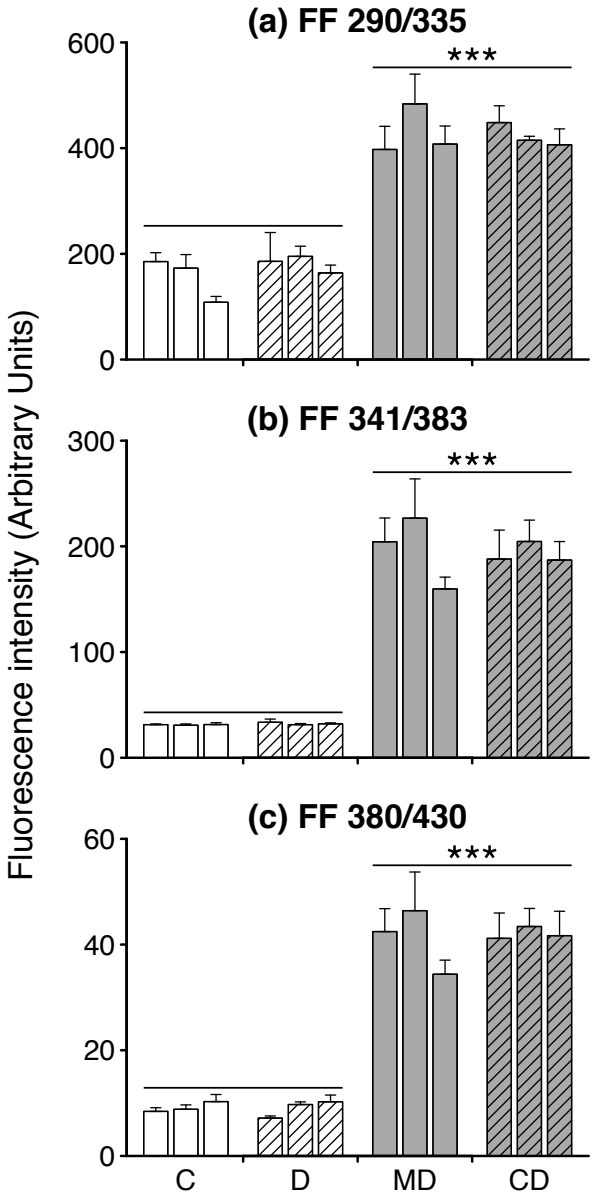

Fig. 2. Fixed wavelength fluorescence (mean+SE, n = 5 fish) of bile reflecting biliary PAHs metabolites levels after 48h exposure to control (C), dispersant only (D), mechanically dispersed oil (MD) and chemically dispersed oil (CD): (a) FF 290/335 (naphthalene derived type of metabolites); (b) FF 341/383 (benzo[a]pyrene type of metabolites); (c) FF 380/430 (pyrene derived type of metabolites). Levels expressed as fluorescence intensity. *denotes significant difference by SNK procedure; horizontal lines overly bars that were not different by SNK tests. Significance code: ****P*<0.001.

These results are explained by the highly developed enzymatic systems of aquatic vertebrates. The elevated concentration of metabolites in the bile is due to biotransformation metabolism and has been shown in previous studies (Aas et al., 2000; Aas et al., 2001; Ramachandran et al., 2004; Wessel et al., 2010). Since metabolites can bind to cellular macromolecules such as DNA, RNA and proteins, its presence and persistence may lead to mutagenesis, teratogenesis and carcinogenesis (Beyer et al., 2010). Levels of biliary metabolites are linked to biotransformation mechanisms, such as cytochromes P-450 (CYP1A) reactions, commonly measured by EROD activity (van der Oost et al., 2003).

EROD activity was significantly higher in the oil-exposure treatments compared to the control and dispersant control (Fig. 3a), however there was no significant difference between chemically and mechanically exposed treatments. Variance components indicated that 67% of total variation in EROD activity occurred among treatments (Table 2). Strong inductions of CYP1A after PAH exposure were also found in polar cod *Boreogadus saida* (Nahrgang et al., 2010a), areolated grouper *Epinephelus areolatus* (Wu et al., 2003) and European turbot *Psetta maxima* (Martin-Skilton et al., 2008). Ramachandran et al. (2004) observed increased EROD activity in chemically dispersed treatments against the water-accommodated fraction of crude oil, concluding that oil dispersants will increase the bioavailability of petroleum hydrocarbons to fish. Our results reached similar conclusions, since controls differed from oil-exposure treatments, even though there was no significant difference between mechanically and chemically dispersed oil in EROD activity. Therefore, the toxicity arises from availability of PAHs compounds in the water that is facilitated by dispersion processes, whether mechanically or chemically.

CYP1A activity also triggers oxidative stress through generation and accumulation of reactive oxygen species (ROS), which are detoxified by antioxidant enzymes to non-reactive molecules (van der Oost et al., 2003). We used CAT and GPx activities to

measure the extent of oxidative stress in liver cells after exposure. CAT and GPx catalyse transformation of H_2O_2 to molecular water (H_2O); i.e., they may act on common substrates (Richardson et al., 2008). CAT activity did not differ among experimental treatments and tanks (Fig. 3d). This enzyme activity was extremely variable among replicate fish within each tank, as shown by the high variance component calculated for the residual (approximately 87% of total variation, Table 2). Antioxidant defense parameters, such as catalase, often show contradictory responses in fish exposed to PAHs and are difficult to link to contaminant exposure (van der Oost et al., 2003).

Likewise, GPx activity is strongly influenced by seasonal variations in water temperature, reproductive cycle and food availability, and these are relevant limitations for its use as a reliable biomarker of PAH exposure (van der Oost et al., 2003; Lüchman et al., 2011). In our study, GPx activity was significantly induced by exposure to chemically dispersed oil compared to mechanically dispersed oil (Fig. 3c). Differences among experimental treatments accounted for 52% of total variance in GPx activity (Table 2). Although this might suggest that dispersant application increased antioxidant defense responses in the wolfish, GPx activity in mechanically dispersed oil did not differ from control and dispersant control. This is unexpected, since our results also shown that PAH biliary metabolite concentrations indicated that CD and MD induced similar levels of PAH incorporation. Milinkovitch et al. (2013) demonstrated that GPx activity was significantly higher in heart of juvenile golden grey mullet (*Liza aurata*) exposed to CD and MD than in the control, suggesting that chemical dispersion of the oil slick would not be more toxic than its natural dispersion. Further experiments are therefore needed to evaluate if GPx activity in the wolfish might be a useful and reliable biomarker of exposure to dispersed oil.

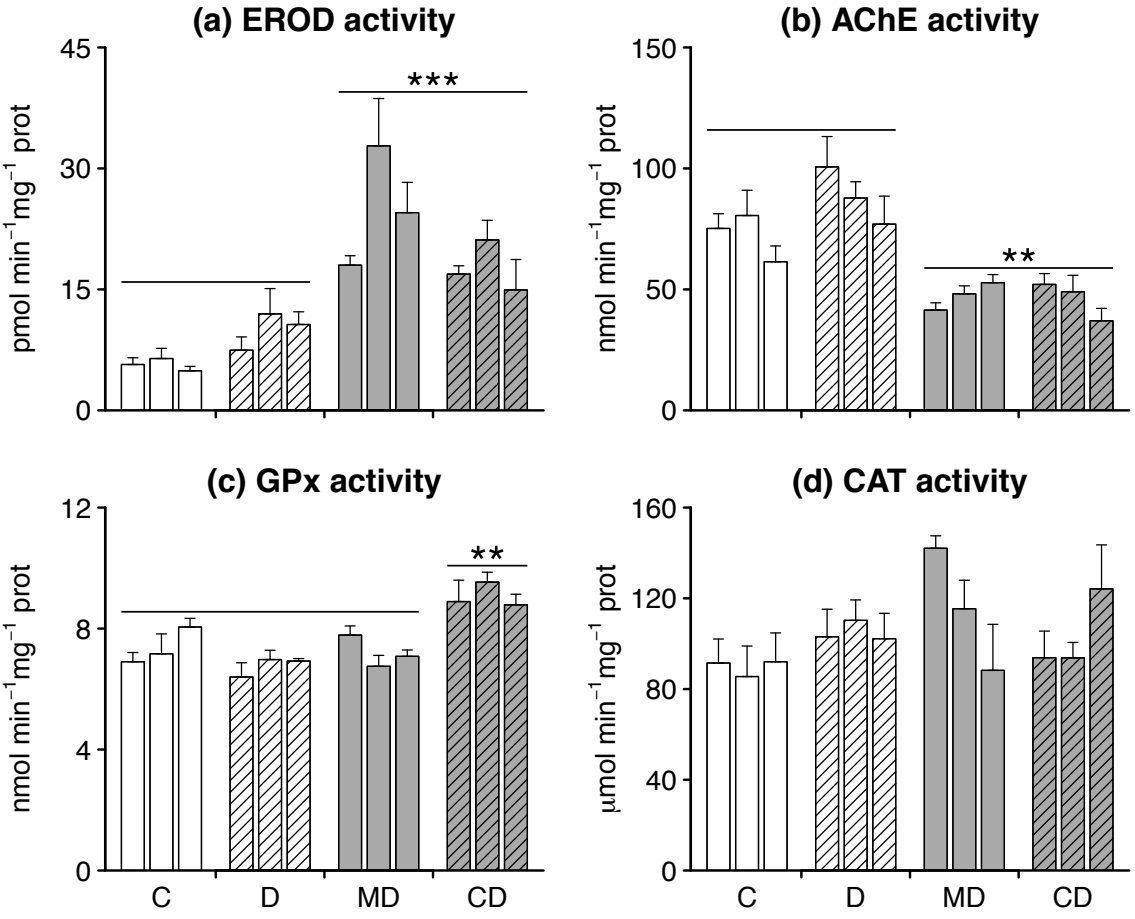

Fig. 3. Activities of selected biomarkers in the wolfish *Anarhichas denticulatus* (mean+SE, n = 5 fish) after 48h exposure to control (C), dispersant only (D), mechanically dispersed oil (MD) and chemically dispersed oil (CD): (a) EROD activity; (b) AChE activity; (c) GPx activity; (d) CAT activity. *denotes significant difference by SNK procedure; horizontal lines overly bars that were not different by SNK tests. Significance codes: ***P*<0.01; ****P*<0.001.

349

350 A significant inhibition of brain acetylcholinesterase (AChE) activity was detected
351 in the oil-exposure treatments (Fig. 3b). Variance components revealed that 57% of total
352 variation in AChE activity was due to the differences among experimental treatments
353 (Table 2). AChE is considered a specific biomarker for organophosphate and carbamate
354 insecticides (Wijeyaratne and Pathiratne, 2006), but the effects of PAHs on this enzyme is
355 still contradictory, with some studies reporting inhibition and others indicating no effects
356 (Vieira et al., 2008). AChE is crucial for the normal functioning of sensory and

357 neuromuscular systems (van der Oost et al., 2003) and its inhibition may cause
358 accumulation of acetylcholine in the synaptic cleft leading to continuous nerve firings and
359 therefore disruption of nerve impulses (Holth et al., 2012). Our findings suggest that, as
360 for pesticides, AChE may be used as a relevant biomarker for PAH exposure and
361 subsequent toxicity.
362

Table 2. Variance components (V. c.) and percent variance components (% v. c.) for selected biomarker responses in wolfish. Largest variance component for each biomarker is highlighted in bold.

	EROD activity		AChE activity		GPx activity		CAT activity	
	V. c.	% v. c.	V. c.	% v. c.	V. c.	% v. c.	V. c.	% v. c.
Treatment	1.22	**66.63**	393.54	**56.81**	0.95	**52.15**	21.90	2.32
Tank(Tr)	0.10	5.46	29.21	4.22	0.07	3.68	102.12	10.81
Residual	0.51	27.92	269.94	38.97	0.80	44.18	820.35	**86.87**
	FF 290/335		FF 341/383		FF 380/430			
	V. c.	% v. c.	V. c.	% v. c.	V. c.	% v. c.		
Treatment	21890.78	**80.07**	1.06	**96.38**	0.76	**93.01**		
Tank(Tr)	100.09	0.37	0	0	0.01	0.70		
Residual	5347.22	19.56	0.04	3.62	0.05	6.29		

363

364 *3.3. Growth*

365

366 No mortality was observed during exposure. However, some mortality occurred
367 over the five-week period after the experiment. A survival rate of 97% was observed in the
368 control, 100% in the dispersant control, 78% in mechanically dispersed oil and 92% in
369 chemically dispersed oil. The combined effect of treatment and time was statistically
370 significant, both in weight (repeated measures ANOVA, Treatment × Time interaction, df =
371 12,32; F = 21.38; P<0.001) and length of juvenile wolfish (repeated measures ANOVA,
372 Treatment × Time interaction, df = 12,32; F =13.33; P<0.001). Mean weight and length
373 immediately after exposure did not differ among treatments (Fig. 4a, b). Growth rate, both

374 in length and weight, was clearly higher in control and dispersant control compared to
375 mechanically and chemically dispersed oil (Fig. 4a, b). Fish exposed to oil treatments did
376 not show significant difference in weight (Fig. 4a), although some significant increase in
377 length was observed (Fig. 4b).

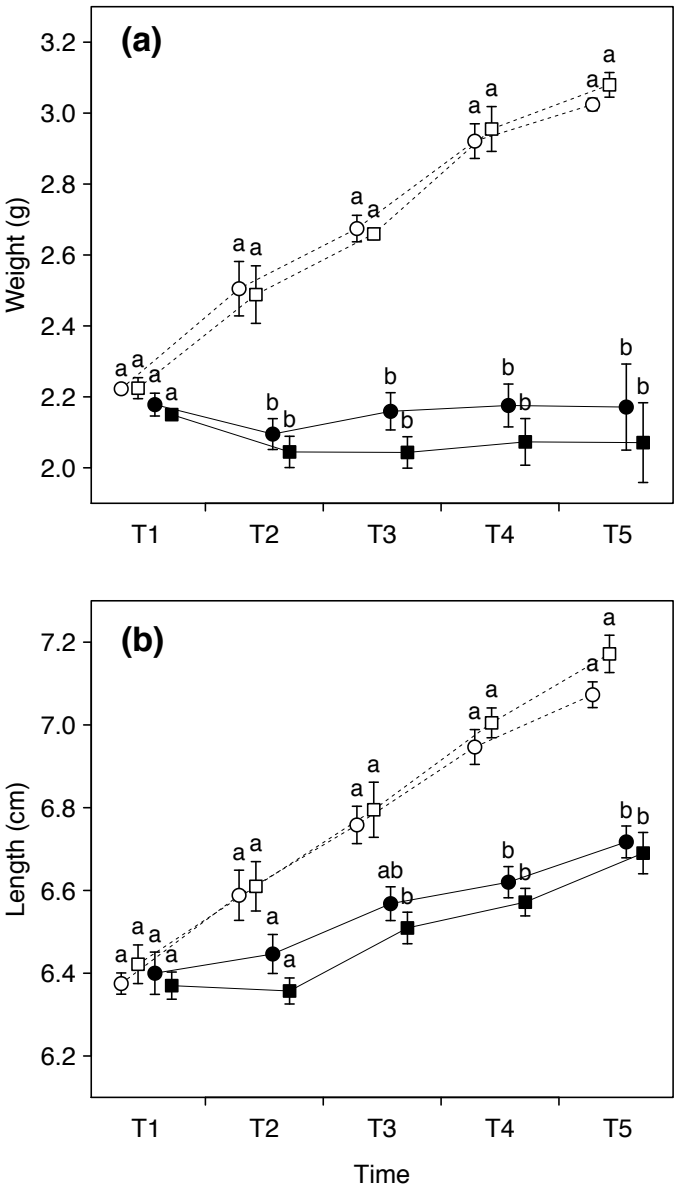

Fig. 4. Mean (SE, n = 3 tanks) weight (a) and length (b) of the wolfish juveniles during the five-week period (T1 to T5) after the exposure. Treatments are Control (○), dispersant only (□), mechanically dispersed oil (●) and chemically dispersed oil (■). At each time, different letters above error bars indicate significant differences among treatments by pairwise comparisons using the Bonferroni correction.

Several studies have shown that crude oil and its components inhibit growth of fish in a number of species, especially at earlier life stages, such as larvae and juveniles (Heintz et al., 2000; Saborido-Rey, 2007). The effects on growth of the wolfish resulting from exposure to PAHs reported here indicate that mortality levels do not necessarily reflect the overall toxicity of an oil spill. Sub-lethal effects of oil that led to reduced growth may considerably impact the recruitment strength and population dynamics, including maturation processes (Heintz et al., 2000).

4. Conclusions

Overall this study demonstrated that PAH biliary metabolites and the activities of EROD and AChE are appropriate biomarkers to assess exposure of *A. denticulatus*, a suitable bioindicator species for PAH exposure in Artic waters. There are strong correlations between CYP1A, measured by EROD activity, and PAH biliary metabolites (Jung et al., 2011) and our results confirmed and further emphasized the applicability of these biomarkers, especially when carried out simultaneously.

The deleterious effects on growth observed in our experiments can be interpreted as a late response to PAH exposure. Since biomarkers provide an early identification of change in the presence of toxic compounds (Monserrat et al., 2007), studies attempting to determine effects of contaminants must consider the connection between biochemical alterations and organismic responses. Finally, the lack of significant differences between chemically and mechanically dispersed oil in biomarker responses and growth suggest that dispersant application is no more toxic than the natural dispersion of oil.

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CONCLUSÕES GERAIS

Esta tese teve o objetivo geral de avaliar como os efeitos da exposição a hidrocarbonetos de petróleo condicionam respostas biológicas em diversos níveis de organização. Para tal, uma ampla variedade de técnicas e abordagens metodológicas considerando diferentes espécies-alvo foi adotada. Os resultados gerados forneceram uma visão mais compreensiva e integrada dos impactos de derrames de óleo em ambientes costeiros. Os experimentos realizados possibilitaram estabelecer relações causais, diretas e previsíveis entre a exposição experimental dos organismos e as subsequentes respostas biológicas. Além disso, a utilização de distintas frequências e dosagens dos derrames permitiu compreender como repetidos eventos de exposição e como a sua duração determinam a extensão dos impactos por óleo.

Muitos dos padrões de resposta aqui relatados foram previamente reportados na literatura. Decréscimos significativos na densidade de macroinvertebrados bênticos e mudanças na estrutura das associações macrofaunais são alguns dos efeitos previsíveis dos derrames de óleo, particularmente se consideradas escalas temporais de curta duração (Kingston, 2002; Ocon et al., 2008; Egres et al., 2012; Yu et al., 2013). A indução das enzimas superóxido dismutase (SOD) e glutationa *S*-transferase (GST), o incremento nos níveis de peroxidação lipídica (LPO) e a depleção na concentração de glutationa reduzida (GSH) também foram reportados em diferentes espécies de invertebrados bênticos expostos a óleo (Richardson et al., 2008; Ramos-Gómez et al., 2011; Won et al., 2013; Marques et al., 2014; Vidal-Liñán et al., 2014). Além disso, o aumento na concentração relativa de metabólitos biliares de HPA, a indução da etoxiresorufina-*O*-deetilase (EROD) e a inibição do crescimento em peixes expostos a diferentes frações e concentrações de hidrocarbonetos de petróleo já foram previamente descritos (Heintz et al., 2000; van der Oost et al., 2003; Nahrgang et al., 2010a,b; Milinkovitch et al., 2011).

No entanto, a elevada resiliência de nematoides marinhos de vida livre experimentalmente expostos a óleo diesel é de certa forma inesperada (Capítulo I). Muitos estudos reportaram padrões de resposta inconsistentes de nematoides à contaminação por óleo, mas geralmente apontam certas espécies desse grupo meiofaunal como indicadoras sensíveis (Donavaro et al., 1995; Mahmoudi et al., 2005; Beyrem et al., 2010). Nenhum dos gêneros de nematoides analisados no Capítulo I foi considerado particularmente sensível ao derrame *in situ*. Apesar do experimento MBACI descrito no Capítulo I simular um derrame agudo e não-cumulativo, nematoides foram expostos a uma elevada dosagem de óleo diesel (equivalente a 20 L m^{-2}) se comparada às dosagens utilizadas no experimento do Capítulo II (2,5 e 5 L m^{-2}) e no Capítulo III (1 e 2 L m^{-2}). Experimentos de campo envolvendo distintas dosagens e frequências de derrames, similares aos conduzidos com a macrofauna bêntica, são necessários para avaliar se nematoides marinhos de vida livre são igualmente tolerantes a distintos regimes de exposição a hidrocarbonetos de petróleo.

Embora muitos dos efeitos da poluição por hidrocarbonetos de petróleo aqui relatados já tenham sido extensivamente reportados por outros autores, a especificidade dos padrões de resposta em distintas frequências e dosagens dos derrames em campo é uma novidade na literatura. O delineamento experimental aplicado nos capítulos II e III permitiu testar não apenas o efeito da dosagem e da frequência dos derrames, mas também possibilitou contrastar diferentes regimes de exposição (i.e. derrames frequentes de baixa dosagem *versus* derrames menos frequentes de alta dosagem). Foram identificados padrões de resposta similares tanto no nível suborganísmico, como nos níveis organísmico e supraorganísmico. De maneira geral, derrames frequentes de baixa dosagem foram mais deletérios que derrames menos frequentes de alta dosagem.

Defesas antioxidantes e dano oxidativo em termos de LPO foram significativamente induzidos no bivalve *Anomalocardia flexuosa* e no poliqueta *Laeonereis culveri* quando expostos a derrames mais frequentes de baixa dosagem.

Similarmente, decréscimos significativos na densidade de macroinvertebrados e mudanças na estrutura das associações macrofaunais foram condicionados pela maior frequência dos derrames, não pela sua magnitude.

Estes resultados tem implicações diretas para o monitoramento da poluição por hidrocarbonetos de petróleo em ambientes costeiros e estuarinos. Tradicionalmente, os impactos do petróleo sobre a biota marinha são diretamente associados à magnitude dos derrames; i.e., derrames de grandes proporções são presumidamente mais deletérios que derrames de menor magnitude (Lee e Paige, 1997). Derrames de baixa dosagem são habitualmente negligenciados pelas autoridades e pela população em geral, apesar de recorrentes nos ambientes estuarinos. No entanto, derrames de grandes proporções (>100 toneladas) representam apenas 6% do volume total de óleo introduzido nos oceanos (NCR, 2003; Luna-Acosta, 2011).

A maior parte do óleo presente no mar provem de pequenos derrames (de aproximadamente 1 m^3) principalmente nas regiões próximas à costa (Luna-Acosta, 2011). Os presentes resultados enfatizam o potencial efeito de derrames de óleo pequenos, porém frequentes, sobre a biota marinha. Derrames de pequena proporção são de difícil detecção e monitoramento, mas estão presumidamente disseminados nos estuários e geram impactos em múltiplos níveis de organização biológica. Este resultado reforça a necessidade de ferramentas integradoras para o monitoramento da poluição marinha em áreas costeiras. O Capítulo III destaca o bivalve *A. flexuosa* e o poliqueta *L. culveri* como sentinelas adequadas para este propósito.

A aparente resiliência do poliqueta *Sigambra grubii* aos derrames frequentes de alta dosagem discutida no Capítulo II precisa ser mais investigada, sobretudo com o uso de enzimas de estresse oxidativo e de detoxificação, além de estimativas de dano celular. Assim como *S. grubii*, o gastrópode *Neritina virginea* foi tolerante a derrames frequentes de alta dosagem, como demonstrado pela falta de diferenças significativas na resposta dos biomarcadores analisados no Capítulo III. Estes resultados sugerem que

respostas antioxidantes em *N. virginea* foram moduladas por derrames pouco frequentes de alta dosagem, e que estas foram suficientes para prevenir danos na membrana celular como indicado pela LPO. Experimentos de campo mais longos, em conjunto com diferentes dosagens de óleo, permitirão compreender melhor a extensão dos efeitos da exposição de hidrocarbonetos de petróleo em *N. virginea*.

O Capítulo IV apresentou a primeira avaliação da sensibilidade do peixe-lobo *Anarhichas denticulatus* à contaminação por petróleo, comparando os efeitos da aplicação de dispersantes químicos e do óleo disperso mecanicamente. Dispersantes são tipicamente aplicados após derrames no oceano e permitem a transferência da mancha de óleo da superfície para a coluna d'água (Milinkovitch et al., 2011). Seu uso em ambientes costeiros, no entanto, é bastante controverso devido a baixa profundidade e ao potencial limitado de diluição do óleo nessas regiões. O uso de dispersantes também aumenta a exposição de organismos aquáticos ao petróleo (Lyons et al., 2011).

De fato, a concentração total de hidrocarbonetos de petróleo em tanques com óleo disperso quimicamente foi significativamente maior que a concentração em tanques onde o óleo foi disperso mecanicamente. Contudo, esta maior disponibilidade de hidrocarbonetos na água não se refletiu em maior toxicidade, como indicado pela concentração relativa de metabólitos biliares de HPAs, atividades da EROD e AChE e crescimento dos peixes ao longo de cinco semanas após a exposição.

Assim como observado para o bivalve *A. flexuosa* no Capítulo III, atividades da CAT e GPx no peixe-lobo *A. denticulatus* expostos a óleo não diferiu dos controles. Resultados semelhantes em organismos e abordagens experimentais completamente distintos, sugerem cautela na interpretação da resposta destes biomarcadores no monitoramento da poluição por hidrocarbonetos, como observado por Vidal-Liñán et al. (2010).

O sistema experimental utilizado no Capítulo IV foi concebido e amplamente usado pelo instituto CEDRE (Centre de Documentation de Recherche et

d'Expérimentations sur les Pollutions Accidentelles des Eaux), na França, nos últimos 30 anos para o desenvolvimento de planos de contingência. Períodos de exposição de 48 h e concentrações nominais de óleo de 67 mg L^{-1} foram previamente identificadas como relevantes para respostas de biomarcadores em organismos de regiões temperadas. Os resultados gerados no Capítulo IV validam a aplicação do sistema experimental do CEDRE para espécies de peixes do Ártico.

Finalmente, a detecção de respostas no nível suborganísmico (biomarcadores) juntamente com reduções no crescimento de juvenis do peixe-lobo reportados nesta tese alertam para potenciais efeitos tardios em populações afetadas por derrames de óleo. A demonstração destas respostas subletais em animais expostos destacam a toxicidade muitas vezes oculta de um derrame, que pode afetar populações a médio e longo prazo.

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Anexo I

Leite, D.S., Sandrini-Neto, L., Camargo, M.Z., Thomas, M.C., Lana, P.C., 2014. Are changes in the structure of nematode assemblages reliable indicators of moderate petroleum contamination? Mar. Pollut. Bull. 83, 38–47.

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Are changes in the structure of nematode assemblages reliable indicators of moderate petroleum contamination?

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ABSTRACT

This study assesses through a multiple before-after-control-impact (MBACI) design the effects of diesel oil on the structure of nematode assemblages in unvegetated tidal flats of a subtropical estuary. Oil-exposed treatments were contrasted with controls for a duration of four successive days before and after an experimental spill in three distinct areas of the Paranaguá Estuarine Complex (Southern Brazil). No significant differences were observed in nematode total density, number of taxa and the overall assemblage structure between the control and impact treatments from before to after the experimental spill. This reinforces the idea that, despite being good indicators of environmental stress, free-living marine nematodes are able to tolerate low concentrations of hydrocarbons and to survive in moderately contaminated areas. We also show that robust experimental designs are useful to avoid confounding expected natural variability with the effects of a mild impact.

1. Introduction

Accidents involving oil spills such as the Torrey Canyon in England, Tampico Maru in the United States, Amoco Cadiz in France (Botello and Macko, 1982) and, more recently, the largest spill in history in the Gulf of Mexico, between April and July of 2010 (Mariano et al., 2011), have attracted the interest of the general public and scientists towards oil contamination of the oceans. Previous oil-spilling accidents in Brazil, such as those caused by the ships Norma and Vicuña, which released naphtha, methanol, diesel, and bunker in the Paranaguá Bay in 2001 and 2004, emphasize the need to assess the intensity and extent of damage caused by oil spills, as a first basis for monitoring and control measures.

Estuaries act as sinks for sediment and the associated pollutants from numerous human activities (Yang et al., 2006; Wang et al., 2012). Estuarine habitats are also considered more vulnerable to the impacts of oil spills because the confinement can favor the accumulation of hydrocarbons, mainly in intertidal vegetated areas (Sanz-Lázaro and Marín, 2009).

Oil effects on the benthic macrofauna have been extensively investigated through descriptive (Gómez Gesteira and Dauvin, 2000; Edgar et al., 2003; Zenetos et al., 2004; Andersen et al., 2008; Morales-Caselles et al., 2008; Ocon et al., 2008) and experimental approaches, both in the field (Faraco and Lana, 2003; Schratzberger et al., 2003; Lu and Wu, 2006; Egres et al., 2012) and laboratory (Bhattacharyya et al., 2003). However, few studies have experimentally investigated the effects of exposure to hydrocarbons on meiofaunal organisms (Fleeger and Chandler, 1983; Ansari and Ingole, 2002; Mahmoudi et al., 2005; Ansari et al., 2010; Beyrem et al., 2010; Boufahja et al., 2011).

Meiofaunal organisms in general and nematodes in particular are considered good indicators of contamination for their high abundance and diversity, short generation time, and direct benthic development (Fleeger and Chandler, 1983; Kennedy and Jacoby, 1999; Ansari et al., 2010). In addition, they are present in different sediment types, hydrodynamic conditions, and environments (Bongers and Ferris, 1999). Furthermore, their predominantly benthic life allows for direct contact with components dissolved in the interstitial water through their permeable cuticle (Warwick, 1981; Heip et al., 1985; Vranken and Heip, 1986; Bongers et al., 1991; Bongers and Ferris, 1999). Another advantage of using nematodes in environmental impact studies is the small sample volume necessary for routine studies, thereby allowing a large number of samples to be collected and, thus, ensuring statistical significance

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(Bongers and Ferris, 1999). In this context, the responses from nematode assemblages to environmental changes might provide stronger evidence of oil contamination than those obtained from other animals.

Assessments of the effects of oil spills on marine meiofauna are often contradictory and inconsistent. Overall responses seem to be dependent on the amount of oil spilled, environmental characteristics and target taxonomic groups (Fleeger and Chandler, 1983). Decreases in meiofaunal density and taxonomic richness have been repeatedly reported after experimental oil spills (Boucher, 1980; Danovaro et al., 1995; Mahmoudi et al., 2005) and exposure to sediments contaminated by mineral and synthetic lubricating oils (Beyrem et al., 2010). However, some meiofaunal taxa can be highly tolerant to contamination by hydrocarbons and positively respond to the experimental exposure (Fleeger and Chandler, 1983; Mahmoudi et al., 2005).

The analyses of impacts involving oil spills are often carried out after accidents and include descriptions of biological responses from plant and animal communities. Rarely, the pre-disturbance context is adequately known and inferences on the disturbance are made using a simple comparison between previously impacted locations and undisturbed control sites. Consequently, differences in the composition and structure of assemblages might simply reflect background variability preceding the spill (Underwood, 2000). Micro- and meso-scale experimental approaches are, therefore, more appropriate to establish a causal relationship between oil exposure and the biological responses (Glasby and Underwood, 1996). In this study, we investigated the effects of an experimental marine diesel spill on nematode assemblages using a multiple before–after control–impact (MBACI) design (Keough and Mapstone, 1997; Downes et al., 2002). We hypothesized that total density, number of taxa and overall structure of nematode assemblages living in oil-exposed areas would be significantly different from those in control areas, from before and after the experimental spill.

2. Materials and methods

2.1. Study area

The Paranaguá Estuarine Complex on the coast of Paraná State (48°25′W, 25°30′S) is formed by two main axes, the Paranaguá and Antonina Bays (east–west oriented) and the Laranjeiras and Pinheiros Bays (north–south oriented). This system comprises a diversity of estuarine and coastal ecosystems including coastal dunes, mangroves, salt marshes, rocky shores, and extensive tidal flats (Lana et al., 2001).

The Cotinga Channel (Fig. 1) is about 15 km long and receives freshwater input from the Maciel, Guaraguaçú, Correias, Almeidas, and Itiberê Rivers. Noernberg et al. (2006) classified this region as a sub-estuary based on its hydrographic and morphological features. This sub-estuary is composed of many meandering rivers with extensive floodplains, which favors the formation of unvegetated flats mainly through sediment delivery from tidal flows from east to west. Domestic effluents from the city of Paranaguá, where the municipal sewage is still discharged *in natura* in the estuary, reach the Cotinga Channel through the Itiberê River.

The tidal flats used in the experiment are located along the Cotinga Channel; the most internal area is near the mouth of the Guaraguaçú River, the intermediate area is near Rasa Island, and the most external area is near the mouth of the Maciel River (Fig. 1).

2.2. Experimental design and field procedures

We carried out an acute non-cumulative field experiment with the simulation of a single oil spill. Impacted treatments were contrasted with controls in three distinct areas over four successive sampling times, two before and two after the spill (Fig. 2). The MBACI design was used because it is logically capable of separating the effects of the experimental spill from the background environmental variation by using multiple controls and impacted areas (Keough and Mapstone, 1997). The temporal samplings, equally replicated at the times before and after the experimental spill, ensured the correct interpretation of interactions between time and space. The appropriate spatial and temporal replication ensures that the resulting estimates are reliable (Glasby and Underwood, 1996).

Experimental blocks were established in three areas along the Cotinga Channel. Each area included one experimental block corresponding to the impact treatment with the diesel spill and an undisturbed control. The control and impact blocks were established 40 m apart in each area and were positioned at similar tidal levels. Each block consisted of 12 1-m^2 plots with centralized experimental units of 0.35 × 0.35 m (Fig. 1). Plots were arranged in rows with a delimited pathway to avoid trampling and additional disturbances during sampling. Four of these 12 1-m^2 plots in each block were randomly assigned and actually used for sampling (Fig. 1).

The experiment was conducted during low tide, with the simulation of a single spill in early 2010, followed by the monitoring of biological responses between control and impact treatments in pre-established temporal scales two days before and two days after the oil exposure. In each centralized experimental unit of the impact treatment, 2500 ml of marine fuel oil, commercially named Marine Diesel Oil (MDO), was uniformly poured using a garden watering can. Maritime diesel oil is largely used as a fuel by small and medium vessels and in the auxiliary engines of large vessels. Marine fuel oil is produced by mixing of heavy oil fractions obtained by atmospheric distillation with fractions from secondary crude oil processing. The spilled oil was contained by wooden square artifacts properly allocated to prevent its dispersion and cross-contamination of the control treatments.

2.3. Biological sampling and processing

Four replicated cores were sampled for meiofaunal analyses from each randomly assigned treatment plot (control and impact), in the three unvegetated tidal flats during each of the four sampling times (1 and 2 days before and 1 and 2 days after the experimental oil spill) (Fig. 2). Meiofauna samples were taken using a corer 2.5 cm in diameter and 5 cm in height. Samples were processed according to the procedure proposed by Somerfield and Warwick (1996). Samples were first fixed in 4% formaldehyde and then were sieved through a 63-μm mesh. The retained material was separated using colloidal silica (Ludox TM 50) diluted to a specific gravity of 1.15 g cm^{-3}; this procedure was repeated three times. The final supernatant sample was transferred to a Dollfus plate, and 100 individuals (or all individuals if the total number was less than 100) were removed and diaphanized according to De Grisse (1969). Subsequently, permanent slides with approximately 10 individuals were assembled, and nematodes were counted and identified at the genus level under a stereomicroscope. The identification keys by Platt and Warwick (1983, 1988) and Warwick et al. (1998) were used. Finally, the total abundance of each species was calculated from the ratio between the frequency of each species among the 100 individuals and the total number in each sample.

Three sediment-replicated cores were collected from each treatment at each sampling time for chlorophyll-*a* and phaeopigment analyses; these samples were kept frozen until the analysis. Pigments were extracted from sediment samples with 10 ml of 100% acetone (Strickland and Parsons, 1972). The chlorophyll-*a* and phaeopigment concentrations were estimated using the equation described by Lorenzen (1967).

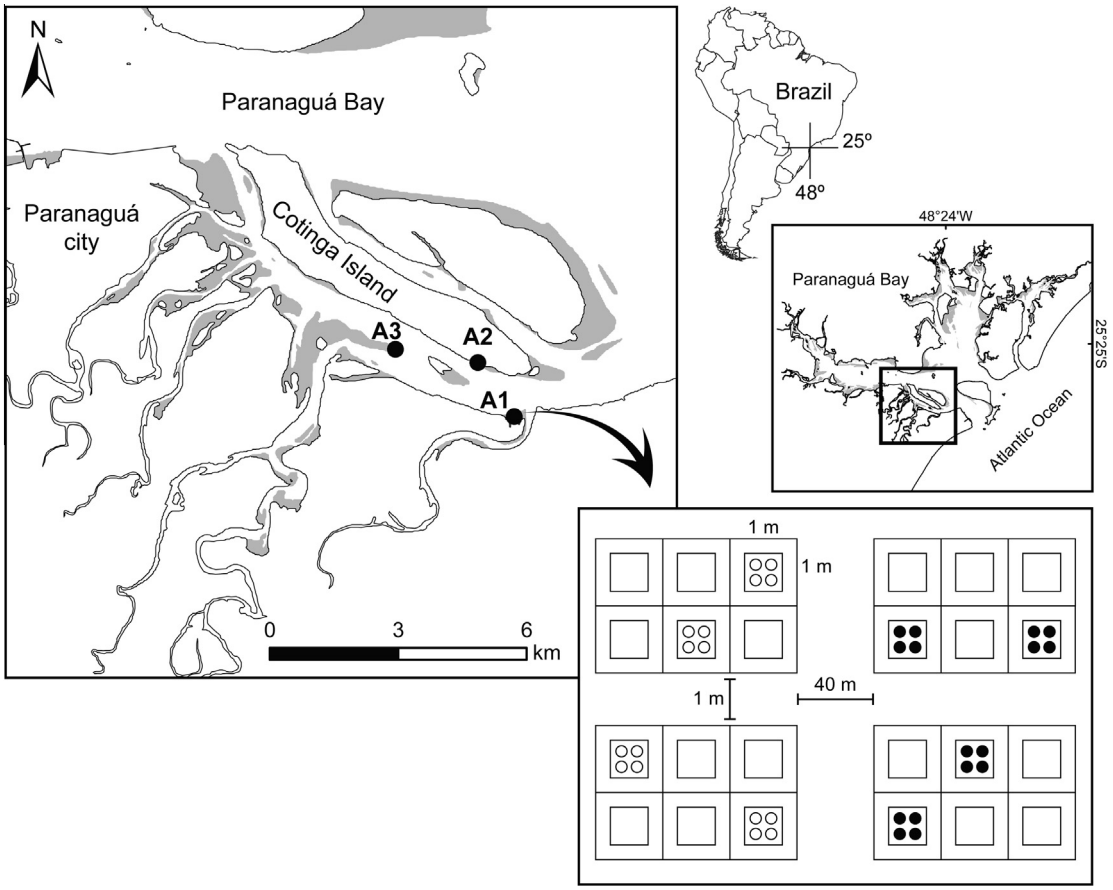

Fig. 1. Map of the Paranaguá Estuarine Complex with the location of intertidal flats and schematic representation of plots with control (○) and impact (●) experimental units.

2.4. Sampling and processing of physicochemical variables

Sediment samples were collected before and after oil exposure from both control and impact treatments for grain size analysis and determination of organic matter content. Grain size analysis was conducted by pipetting and sieving (Suguio, 1973) and granulometric parameters (i.e., sediment grain size in phi, sorting and clay percentage) obtained using SysGran software, version 3.0 (Camargo, 2006). The organic matter content was determined by differences between the initial and final weights after burning 5 g of sediment at 550 °C for 1 h.

Additional sediment samples were collected before and after the experimental oil spill from both treatments to determine the aliphatic hydrocarbon concentrations. The analytical procedures for sample preparation and determination of aliphatic hydrocarbons were performed according to the methods described by UNEP (1991) and Martins et al. (2004). The levels of hydrocarbon contamination in the sediments were estimated using the concentration of total aliphatics, concentration of unresolved complex mixture (UCM), and association between even and odd chain alkanes (CPI).

Water salinity and temperature (both in sediment and water) were measured *in situ* on all sampling days using a precision digital thermometer and a portable refractometer.

2.5. Data analysis

The total density of nematodes, number of taxa, and density of dominant (three taxa comprising 57% of total density) and constant taxa (three taxa comprising only 15% of total density but

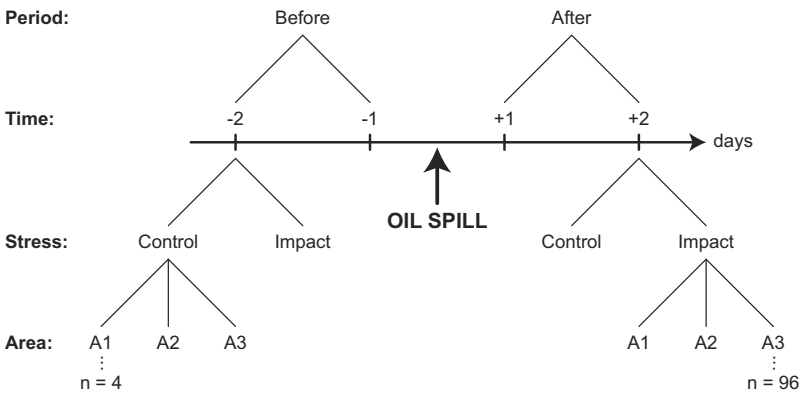

Fig. 2. Multiple before–after control–impact (MBACI) experimental design used in this study.

present in nearly all samples) were analyzed separately using analysis of variance. ANOVA was also applied to test for differences in the concentration of chlorophyll-*a* and phaeopigments. The linear model consisted of four factors: Stress (two levels, fixed and crossed – control and impact), Period (two levels, fixed and crossed – before and after), Areas (three levels, random and nested within Stress) and Times (two levels, fixed and nested within Period). In such a design, the impact is identified as the interaction Stress × Period indicating an overall difference between the impacted areas compared to controls from before to after the experimental oil spill.

Degrees of freedom, mean square estimates and *F*-ratios for the MBACI model were calculated according to Keough and Mapstone (1997) and Downes et al. (2002) in the R environment (R Core Team, 2012). The homogeneity of variances was verified with Cochran's test, and data were transformed when necessary. To meet the homoscedasticity assumption, *Parodontophora* densities were $\ln(x+1)$ transformed; densities of *Pseudolella* and the concentration of phaeopigments were transformed to square-root.

Differences among nematode assemblages were tested by permutational multivariate analysis of variance (Anderson, 2001) using the same linear model from the univariate analyses through the PERMANOVA software, version 1.6 (Anderson, 2005). A non-metric, multidimensional scaling analysis (nMDS) was performed to visualize the main variation trends of nematode assemblages between treatments and periods. All multivariate analyses used the dissimilarity coefficient of Bray–Curtis with $\ln(x+1)$ transformed data.

3. Results

3.1. Environmental variables and photosynthetic pigments

The sediment of the experimental areas was mainly composed of fine and very fine sand with a low organic matter content (1.2–4.7%). The sediment composition varied slightly among the areas; Area 1 showed a higher percentage of fine sand than the other areas (Table 1).

The water and sediment temperatures remained relatively uniform throughout the study period, ranging from 28 to 29 °C in the water and from 19 to 27 °C in the sediment. A slight salinity gradient was observed from the most external Area 1 (salinity ranging from 26 to 30) to the most internal Area 3 (salinity ranging from 22.5 to 25).

No significant differences were observed in the concentrations of chlorophyll-*a* and phaeopigments in any term of the MBACI model ($P > 0.05$ in all cases). The concentrations of phaeopigments were higher than the chlorophyll-*a* concentration in all cases, ranging from an undetectable amount (hereafter referred as 0) to 157.34 $\mu g\, g^{-1}$ in the control, and from 0 to 301.81 $\mu g\, g^{-1}$ in the impact treatment. Chlorophyll-*a* concentrations were often below the detection limit and varied from 0 to 65.91 $\mu g\, g^{-1}$ in the control, and from 0 to 65.59 $\mu g\, g^{-1}$ in oil-exposed sediments.

3.2. Aliphatic hydrocarbons

The sum of total aliphatics ranged from 1.42 to 2.77 $\mu g\, g^{-1}$ of dry sediment in the control treatment, and from 2.31 to 30.8 $\mu g\, g^{-1}$ in the impact treatment (Table 2). The presence of UCM (unresolved complex mixture), with values exceeding 60% of total aliphatics, were only recorded in the oil-exposed areas one day after the experimental spill (Table 2).

The CPI (Carbon Preferential Index) varied from 5.81 to 6.31 in the control treatment, and from 5.64 to 6.40 in the impact treatment before the spill (Table 2), indicating the predominance of *n*-alkanes in higher plants. However, one day after the experimental spill the CPI value approached 1.0 in the impact treatment in Areas 2 and 3, which indicates an oil-genic influence (Table 2).

3.3. Nematodes

A total of 166,980 individuals were counted, belonging to 33 different genera numerically dominated by *Terschellingia*, *Spirinia* and *Sabatieria*. Overall, no significant differences were detected in total density of nematodes, number of taxa, and density of dominant and constant genera between the impacted and control treatments from before to after the experimental spill. No patterns of decreasing total density and the total number of taxa were observed (Fig. 3). However, a sharp decrease in the total density of nematodes was evident after the impact, only in Area 2, followed by a fast recovery two days after the spill (Fig. 3).

The total density and number of taxa showed no significant differences for the main effects. The main source of variability was associated with the spatial heterogeneity, with significant differences observed among areas (Table 3).

No significant variation was recorded in the density of the dominant taxa (i.e., *Terschellingia*, *Spirinia* and *Sabatieria*) that could be unequivocally attributed to the experimental spill (Fig. 4). *Terschellingia* density varied only between periods, regardless of the effects of treatments or areas (Table 3). Significant differences were detected in the density of *Sabatieria* among areas and sampling times, whereas densities of *Spirinia* differed only among areas (Table 3).

The three more constant genera (i.e., *Parodontophora*, *Metachromadora* and *Pseudolella*) showed responses similar to the patterns from the numerically dominant taxa, without significant reductions or variations that could be attributed to the experimental spill (Fig. 5; Table 3). Densities varied mainly among areas, especially for *Pseudolella*, which presented a relatively high and constant density in Areas 1 and 2, and extremely low in Area 3.

Similar to the patterns observed in the univariate analyses, the nMDS ordination diagram and PERMANOVA results showed no

Table 1
Percentages of granulometric fractions and organic matter content in sediment samples of the studied areas (A1, A2, A3) between control and oil-exposed treatments, both before and after the experimental spill.

	Before						After					
	Control			Impact			Control			Impact		
	A1	A2	A3	A1	A2	A3	A1	A2	A3	A1	A2	A3
Coarse sand	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Medium sand	0.2	0.0	0.1	0.1	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0
Fine sand	28.9	6.2	1.1	26.2	6.2	14.2	35.7	6.3	0.8	8.3	14.2	20.3
Very fine sand	57.6	75.7	78.7	54.8	84.8	77.3	55.0	82.1	87.6	82.2	73.4	74.9
Silt	9.5	12.9	7.6	6.9	5.8	3.8	7.1	7.6	8.8	3.1	4.3	2.9
Clay	3.6	4.5	12.2	11.8	2.6	4.5	1.8	3.5	2.6	6.2	7.9	1.8
Organic matter	3.6	4.7	2.9	2.9	3.4	4.6	2.6	2.1	1.2	4.2	2.7	2.1

Table 2
Concentrations and evaluation parameters applied to aliphatic hydrocarbons in sediment samples of the studied areas (A1, A2, A3) between control and oil-exposed treatments, both before and after the experimental spill. AHs, total aliphatic hydrocarbons ($\mu g\, g^{-1}$ dry weight); UCM, unresolved complex mixture ($\mu g\, g^{-1}$ dry weight); CPI, Carbon Preferential Index.

	Before						After					
	Control			Impact			Control			Impact		
	A1	A2	A3	A1	A2	A3	A1	A2	A3	A1	A2	A3
AHs	1.88	2.77	1.42	3.98	2.31	2.67	1.93	2.55	1.84	10.69	30.88	16.42
UCM	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.34	20.9	10.7
CPI	5.81	6.31	5.91	5.64	6.40	5.87	5.84	6.12	5.79	4.90	2.38	2.62

n.d. = not detected.

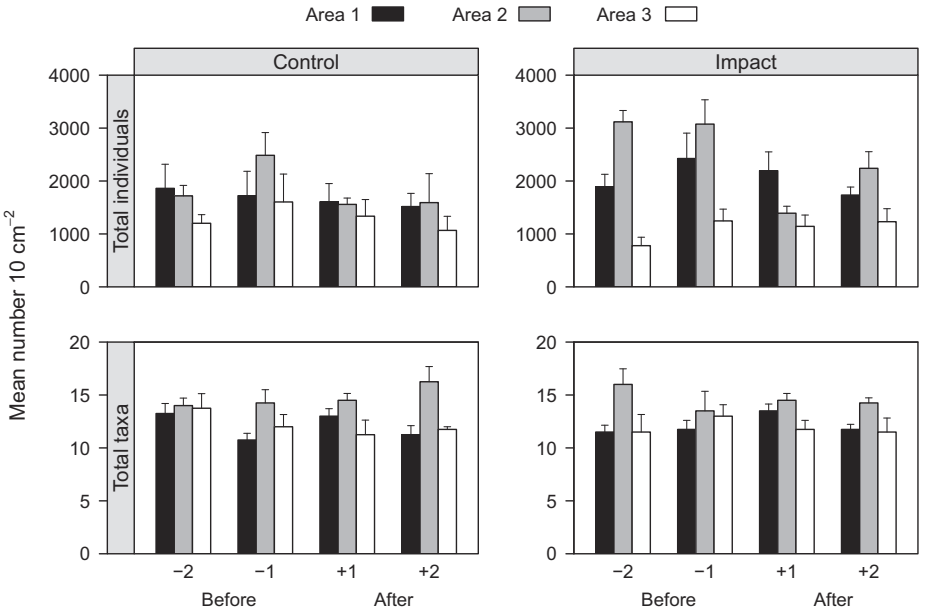

Fig. 3. Mean (SE, $n = 4$) density of nematodes, and number of taxa in the three areas of control and impact treatments, from before (−2, −1 days) to after (+1, +2 days) the experimental oil spill.

evidence of differences related to the experimental spill, but revealed a remarkable difference in the structure of nematode assemblages among the studied areas (Fig. 6; Table 4).

4. Discussion

We rejected the hypothesis that total density, number of taxa and overall structure of nematode assemblages in oil-exposed areas would be significantly different from those in control areas, from before to after an experimental spill. No alterations in the structure of nematode assemblages were identified that could be unequivocally attributed to the experimental contamination by diesel fuel, at least in the spatial and temporal scales adopted.

The manipulative MBACI model (Keough and Mapstone, 1997; Downes et al., 2002), applied both in the univariate and multivariate analyses, showed that differences were due more to the spatial variability and heterogeneity among areas than to the experimental spill itself. No significant reductions or increases in the densities of the dominant and constant taxa were recorded after the experimental spill, except in Area 2. In this area, a decrease in the overall density of nematodes was observed reflected by the responses from the *Parodontophora*, *Terschellingia*, and *Spirinia* genera after the impact, yet detected on day 1 and followed by a fast recovery on day 2. This type of response is defined by Underwood (2000) as a "pulse disturbance", i.e., a short-term effect with a sudden drop in the density of organisms followed by a fast recovery.

The rapid recovery of benthic communities after small-scale disturbances is well known in the local literature and has been reported in previous experiments (Faraco and Lana, 2003; Egres et al., 2012; Gern and Lana, 2013; Sandrini-Neto and Lana, 2014). This rapid recovery is usually associated with the active migration of juvenile and adult animals from adjacent areas to the experimental plots (Negrello Filho et al., 2006; Sandrini-Neto and Lana, 2014), larval recruitment (Carman et al., 2000), or tolerance to toxic compounds by recolonizing species (Schratzberger et al., 2003; Beyrem et al., 2010). In the case of small animals devoid of larval development such as nematodes, the possibility of active/passive transport of resuspended adults and juveniles by currents from nearby areas should also be considered as a relevant recolonization mechanism (Thomas and Lana, 2011).

The time scales used in our experiment were short and, therefore, there was not enough time for the development and subsequent direct meiofaunal recruitment. In this context, the persistence or rapid recolonization of affected assemblages certainly occurred because the dominant and constant species were tolerant to small concentrations of oil, rapidly migrating, or being passively transported from adjacent areas.

Many factors can explain the broad variability of species-specific responses to contaminants. For instance, sediment texture and seawater properties can affect contaminant bioavailability (Langston and Spence, 1994), which in turn depends on partitioning between the sediment, pore water and overlying water (Austen

Table 3
Summary of analysis of variance ($n = 4$ replicate cores) of the MBACI model for total density of nematodes (a), total number of taxa (b) and densities of dominant (c–e) and constant taxa (f–h).

Source		(a) Total individuals		(b) Total taxa		(c) *Spirinia*	
	df	MS	*F*	MS	*F*	MS	*F*
Stress = S	1	1708800.67	0.380	0.38	0.011	64325.26	0.223
Period = P	1	3400548.17	2.809	0.00	0.000	204333.76	1.605
Areas(Stress) = A(S)	4	4500681.21	10.379***	35.56	8.002***	287923.08	3.498*
Times(Period) = T(P)	2	662541.33	1.740	4.27	0.845	115620.05	2.523
S × P	1	77520.67	0.064	0.00	0.000	6256.51	0.049
S × T(P)	2	107506.83	0.282	3.02	0.598	5682.89	0.124
A(S) × P	4	1210545.04	3.179	5.00	0.990	127317.17	2.779
A(S) × T(P)	8	380769.96	0.878	5.05	1.137	45820.38	0.557
Residual	72	433621.83		4.44		82302.06	
		(d) *Terschellingia*		(e) *Sabatieria*		(f) *Metachromadora*	
	df	MS	*F*	MS	*F*	MS	*F*
Stress = S	1	34352.67	0.353	4959.37	0.048	6936.00	0.048
Period = P	1	348968.17	11.256*	39366.00	2.501	2301.04	0.100
Areas(Stress) = A(S)	4	97387.42	2.399	103005.34	3.093*	144438.74	23.049***
Times(Period) = T(P)	2	89347.08	3.923	128509.69	5.243*	5271.04	0.370
S × P	1	6337.50	0.204	39528.17	2.511	9841.50	0.426
S × T(P)	2	3408.33	0.150	8195.85	0.334	22095.08	1.553
A(S) × P	4	31003.96	1.361	15739.30	0.642	23122.86	1.625
A(S) × T(P)	8	22777.83	0.561	24512.24	0.736	14228.66	2.271*
Residual	72	40594.78		33298.53		6266.62	

		(g) *Parodontophora*		(h) *Pseudollela*	
	df	MS	*F*	MS	*F*
Stress = S	1	2.65	0.231	7.22	0.015
Period = P	1	3.76	4.464	5.99	1.153
Areas(Stress) = A(S)	4	11.48	5.558***	468.16	40.520***
Times(Period) = T(P)	2	0.63	0.250	6.80	0.475
S × P	1	2.67	3.178	7.62	1.467
S × T(P)	2	1.46	0.584	5.35	0.374
A(S) × P	4	0.84	0.337	5.20	0.363
A(S) × T(P)	8	2.50	1.211	14.31	1.239
Residual	72	2.06		11.55	

Significance codes.
* $P < 0.05$.
*** $P < 0.001$.

and McEvoy, 1997) and also of the sediment organic carbon content (Di Toro et al., 1991).

Total density and the number of nematode taxa only differed among experimental areas. These differences highlight the importance of physical, chemical, and biological gradients that create great spatial and temporal heterogeneity for the resident fauna (Rodil et al., 2006; Gingold et al., 2010). Nematode assemblage structure varies greatly at distinct spatial and temporal scales as a response to the scale-dependent nature of most ecological processes (Blome et al., 1999). Overall, density and diversity of nematodes are regulated by sediment-related variables, particularly the grain size (Steyaert et al., 1999). Coarser sediments promote more diverse nematode assemblages, whereas finer sediments are characterized by low diversity but generally high densities (Coull and Chandler, 1992; Steyaert et al., 1999). There were, however, no clear relationships between nematode patterns and grain-size characteristics of the sediments in our study, despite a slight difference in granulometry among experimental areas.

Similar to the patterns described for density and number of nematode taxa, the densities of the dominant taxa (i.e., *Terschellingia*, *Spirinia* and *Sabatieria*) did not suffer significant changes that could be attributed to the experimental oil spill. *Terschellingia* densities varied significantly between sampling times regardless of treatment or area suggesting some environmental change affecting all treatments and areas in a similar way. Similarly, no significant variations were detected in the densities of the constant genera *Pseudolella*, *Parodontophora* and *Metachromadora* because of the experimental oil spill. Again, other sources of environmental variability proved to be more important because heterogeneity was observed among the experimental areas.

The non-metric multidimensional scaling (nMDS) showed a clear separation of the areas, indicating that the spatial variability in the structure of assemblages was more important than variation putatively introduced by the oil spill. The multivariate analysis reinforces the pattern found in the univariate analyses, which in almost all cases showed significant differences among the studied areas.

Hydrocarbon analyses indicated that oil-exposed plots were effectively contaminated, although the persistence of contaminants also varied significantly between areas. According to Volkman et al. (1992), concentrations of total aliphatic hydrocarbons below 10 $\mu g\ g^{-1}$ indicate that sediments are not impacted by hydrocarbons, whereas values greater than 100 $\mu g\ g^{-1}$, along with the presence of UCM, indicate oil contamination. All control treatments presented values below 10 $\mu g\ g^{-1}$ indicating no hydrocarbon contamination. However, the impact treatments in all areas presented intermediate values between 10 and 100 $\mu g\ g^{-1}$, which indicate small alterations in the environment.

The presence of UCM is usually associated with degraded or weathered petroleum residues and provides strong evidence for petroleum contamination in sediment samples (Readman et al., 2002; Azimi et al., 2005; Maioli et al., 2011). In this study, the

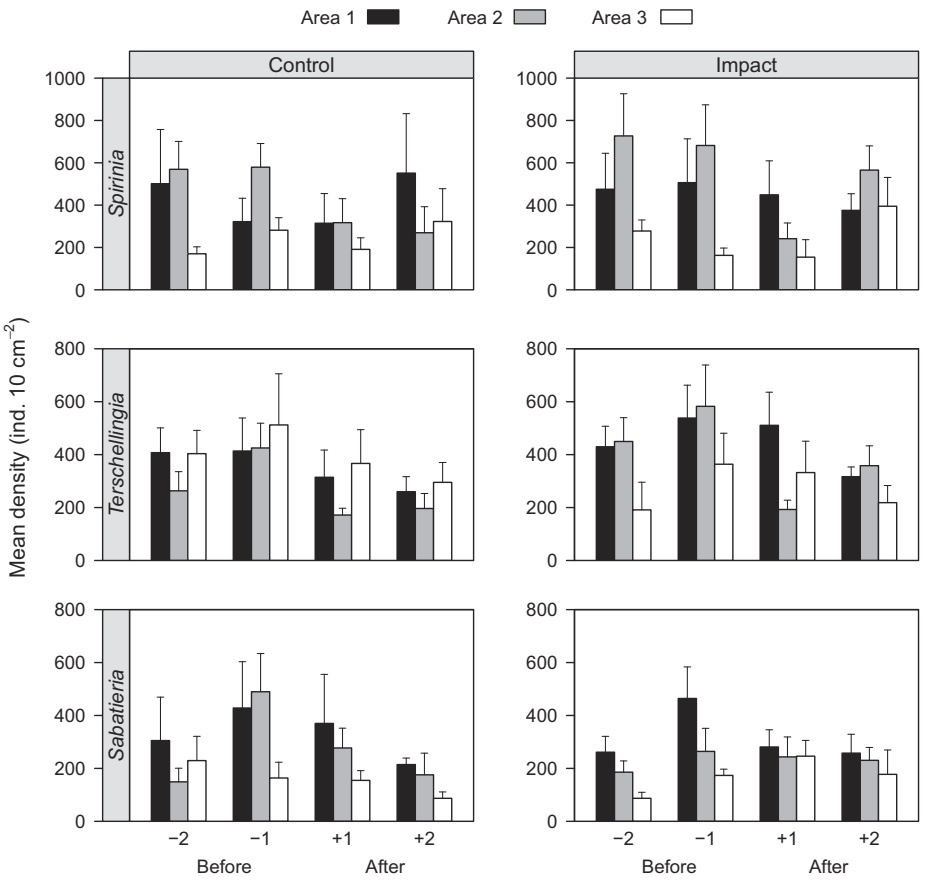

Fig. 4. Mean (SE, $n = 4$) density of dominant nematode taxa in the three areas of control and impact treatments, from before (−2, −1 days) to after (+1, +2 days) the experimental oil spill.

presence of UCM with values exceeding 60% of total aliphatics were only recorded in the impact treatments one day after the experimental spill, indicating the contamination caused by petroleum hydrocarbons.

The CPI is used to determine the origin of compounds taking into account the concentrations of hydrocarbons with odd carbon chains over the even carbon chains in *n*-alkanes of greater molecular mass (C_{25}–C_{34}) (Wang et al., 1999). Values around 1.0 and high concentrations of total aliphatics indicate an anthropogenic origin of *n*-alkanes from petrogenic contamination (Wang et al., 1999), whereas values greater than 4.0 indicate a biogenic origin of *n*-alkanes associated with terrigenous input (Hostettler et al., 1999). This index ranged from 5.81 to 6.31 before the spill in the control treatments and from 5.64 to 6.40 in the impact treatments, indicating that in all cases the hydrocarbons were essentially of terrigenous biogenic origin before the spill. The value approached 1.0 after the spill in the impact treatments, particularly in Areas 2 and 3, indicating the influence of the oil spill in these areas.

Analyses of the effects of oil spills on meiofauna have generated contradictory or inconsistent results due to different approaches applied to different environments. According to Coull and Chandler (1992), hydrocarbon effects on meiofauna depend on the oil type, crude oils being less toxic than refined oil. Results are also affected whether or not meiofauna are exposed in the field or laboratory conditions. Toxicant dosage should be higher in field exposure than *in vitro* in order to detect toxic effects (Coull and Chandler, 1992). Finally, pollutant effects on meiofauna depend on taxon sensitivity. Generally, the response of nematodes to pollution is not uniform and relatively weak when compared to other meiofauna groups, especially copepods (Coull and Chandler, 1992).

Some studies have reported decreases in the density and diversity of taxa while others reported no significant effects or even increases in the densities of organisms. Fleeger and Chandler (1983) carried out an experiment of crude oil spillage over a bank of *Spartina alterniflora* in Louisiana and recorded increased densities in the dominant meiofaunal groups. In a microcosm experiment, Mahmoudi et al. (2005) demonstrated that responses of nematode species to diesel exposure were varied. They observed that some taxa (e.g., *Chaetonema* sp.) are extremely sensitive to the effects of diesel oil, whereas others (e.g., *Daptonema* spp.) showed increased densities, which could suggest opportunistic behavior. Danovaro et al. (1995) recorded a decrease in the densities of the majority of taxa and defined the meiofauna as being extremely sensitive to oil impact.

The use of different contaminants in manipulative experiments can also lead to contradictory results. Nematode assemblages seemed to be more affected by synthetic lubricant oils that contain highly toxic additives (Thompson et al., 2007), which are often more recalcitrant to biodegradation than the base oil (Powell et al., 2005). A series of laboratory microcosm experiments showed that *Daptonema trabeculosum* and *Spirinia gerlachi* were eliminated with synthetic lubricant oils. However, densities of *Spirinia gerlachi* only increased in synthetic lubricant oils "used" and "clean" and *Terschellingia longicaudata* increased in "pure" synthetic lubricant. Therefore, these species were categorized as "intolerant" or "resistant", depending on the type of contaminant.

The experimental areas are subjected to constant tidal influence and are located near the mouth of rivers, which can accelerate the dispersion and dilution of oil. These conditions can also favor rapid recolonization of organisms moving from nearby areas. Thomas and Lana (2011) showed that nematodes from the same region

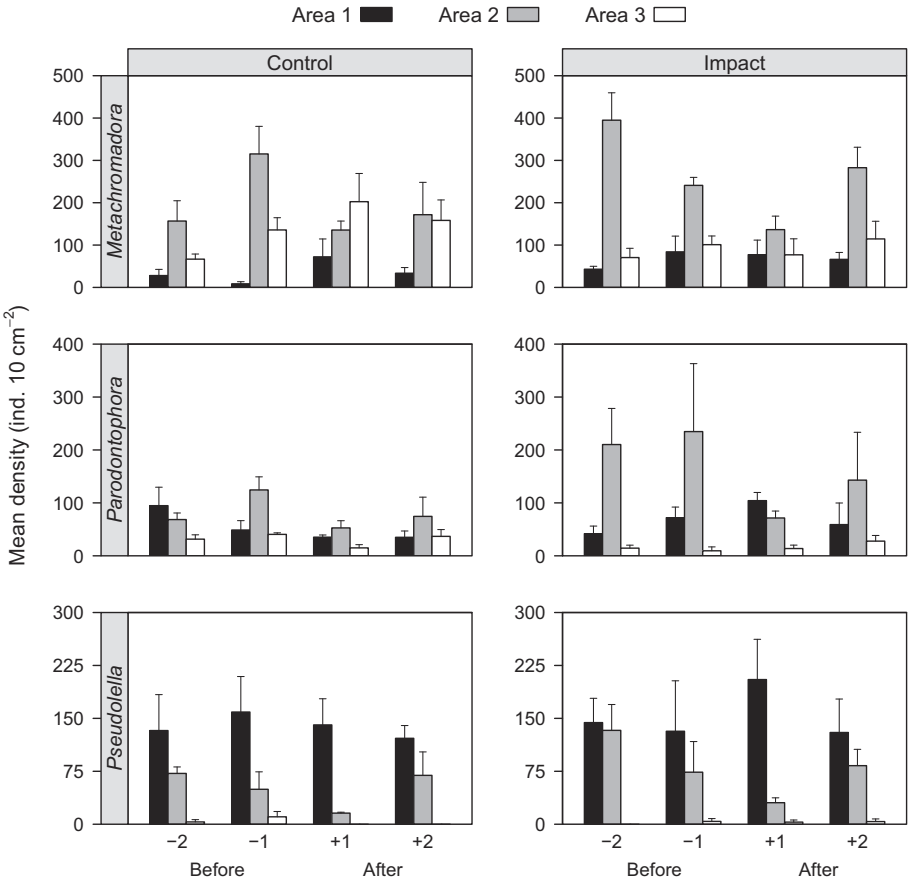

Fig. 5. Mean (SE, $n = 4$) density of the constant nematode taxa in the three areas of control and impact treatments, from before (−2, −1 days) to after (+1, +2 days) the experimental oil spill.

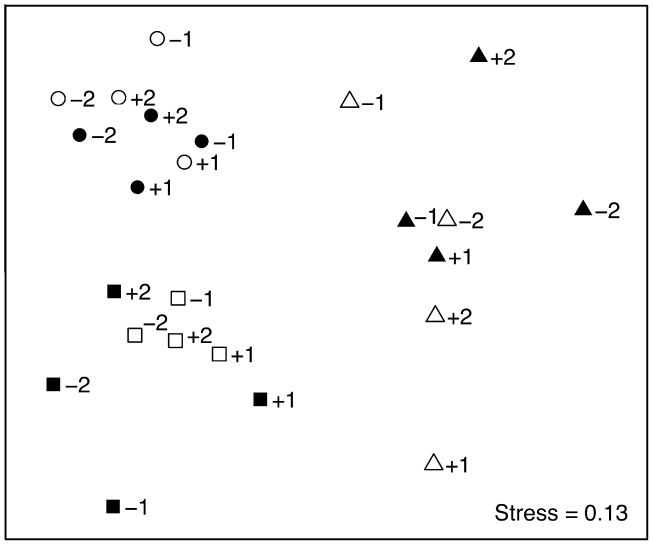

Fig. 6. Non-metric multidimensional scaling (nMDS) of nematode assemblages based on a Bray–Curtis similarity matrix of ln($x + 1$) transformed data comparing the control (Area 1 = ○; Area 2 = □; Area 3 = △) and impact (Area 1 = ●; Area 2 = ■; Area 3 = ▲) treatments from before (−2, −1 days) to after (+1, +2 days) the experimental oil spill.

can disperse up to 2 m when carried over by currents in the water column during a single tidal event. This distance would be enough for organisms from other areas to quickly move towards the impact treatment areas and recolonize them in a few hours.

Table 4
Summary of PERMANOVA (9999 permutations, $n = 4$ replicate cores) of the MBACI model based on Bray–Curtis dissimilarities of ln($x + 1$) transformed nematode densities.

Source	*df*	MS	*Pseudo-F*
Stress = S	1	698.17	0.141
Period = P	1	1203.20	3.624*
Areas(Stress) = A(S)	4	4948.90	10.706***
Times(Period) = T(P)	2	1149.80	1.990
S × P	1	558.71	1.683
S × T(P)	2	567.36	0.982
A(S) × P	4	331.98	0.718
A(S) × T(P)	8	577.96	1.250
Residual	72	462.26	

Significance codes.
* $P < 0.05$.
*** $P < 0.001$.

In this sense, the experimental analysis showed that the local nematode assemblages displayed a resilient behavior, being able to withstand small concentrations of hydrocarbons or rapidly recolonize impacted areas. The rapid recovery of the impacted areas is probably associated with the dynamics of the studied areas, which favors the dispersion of pollutants through intense tidal currents and enables a rapid meiofaunal recolonization.

5. Conclusions

By comparing oil-exposed treatments and controls through an MBACI design, we showed that marine free-living nematodes in unvegetated tidal flats of a subtropical estuary are resilient to oil

disturbance. Despite being considered good indicators of environmental impacts, these organisms were able to tolerate low concentrations of hydrocarbons in sediments and to survive in moderately petroleum-contaminated areas. There were no significant changes in overall abundance and number of taxa between control and impacted treatments from before to after the oil spill. Significant differences in the structure of nematode assemblages were more related to the spatial and temporal variability than to the presence of oil contaminants in the sediment. However, it should be stressed that observed patterns are potentially scale-dependent, both in space and over time.

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Anexo II

Licença ambiental para a realização dos derrames experimentais

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 32152-1	Data da Emissão: 01/12/2011 16:16

Dados do titular

Nome: Leonardo Sandrini Neto	CPF: 046.509.149-06
Título do Projeto: Avaliação da confiabilidade de indicadores bênticos na detecção de contaminação por hidrocarbonetos	
Nome da Instituição : UNIVERSIDADE FEDERAL DO PARANÁ	CNPJ: 75.095.679/0001-49

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Reconhecimentos das áreas e amostragens piloto	12/2011	01/2012
2	Instalação e manutenção dos experimentos, amostragens	02/2012	12/2013
3	Experimentos e amostragens complementares	01/2014	07/2014

De acordo com o art. 33 da IN 154/2009, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Observações e ressalvas

1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
2	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
3	Este documento somente poderá ser utilizado para os fins previstos na Instrução Normativa IBAMA n° 154/2007 ou na Instrução Normativa ICMBio n° 10/2010, no que especifica esta Autorização, não podendo ser utilizado para fins comerciais, industriais ou esportivos. O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior.
4	A autorização para envio ao exterior de material biológico não consignado deverá ser requerida por meio do endereço eletrônico www.ibama.gov.br (Serviços on-line - Licença para importação ou exportação de flora e fauna - CITES e não CITES). Em caso de material consignado, consulte www.icmbio.gov.br/sisbio - menu Exportação.
5	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.
6	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio e o material biológico coletado apreendido nos termos da legislação brasileira em vigor.
7	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico. Veja maiores informações em www.mma.gov.br/cgen.
8	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.

Locais onde as atividades de campo serão executadas

#	Município	UF	Descrição do local	Tipo
1		PR	Baia de Paranagua	Fora de UC

Atividades X Táxons

#	Atividade	Táxons
1	Coleta/transporte de espécimes da fauna silvestre in situ	Malacostraca (*Qtde: 150), Cnidaria (*Qtde: 20), Sipuncula (*Qtde: 20), Ostracoda (*Qtde: 200), Bivalvia (*Qtde: 150), Polychaeta (*Qtde: 2000), Echiura (*Qtde: 20), Nemertea (*Qtde: 50)

* Qtde. de indivíduos por espécie/localidade/unidade de conservação, a serem coletados durante um ano.

Material e métodos

1	Método de captura/coleta (Invertebrados Aquáticos)	Draga, pegador (Van veen, Box corer, Holme, Petersen, etc.)

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Destino do material biológico coletado

#	Nome local destino	Tipo Destino
1	UNIVERSIDADE FEDERAL DO PARANÁ	

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Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº154/2007, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	Qtde.	Tipo de amostra	Qtde.	Data

* Identificar o espécime no nível taxonômico possível.

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