

UNIVERSIDADE FEDERAL DO PARANÁ

ISABEL DO PRADO LEITE

ACÚMULO E EFEITOS DE TOXINAS DE DINOFLAGELADOS BÊNTICOS EM
ORGANISMOS MARINHOS, E POSSÍVEIS INTERAÇÕES COM PLÁSTICO MARINHO

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Orientador: Prof. Dr. Luiz Laureno Mafra Junior

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RESUMO

Dinoflagelados bênticos são um grupo de microalgas capazes de produzir potentes biotoxinas que podem gerar efeitos adversos aos ecossistemas e à saúde humana. Os efeitos deletérios aos seres humanos se dão por intoxicação seguindo a ingestão de pescados contaminados, ou por via aérea durante a exposição ao spray marinho contendo células tóxicas ou seus compostos dissolvidos. As toxinas de microalgas podem gerar danos subletais a organismos marinhos como moluscos bivalves e peixes. Essas interações podem ser potencializadas em ambientes marinhos altamente contaminados com lixo plástico, que pode servir de substrato artificial às células bênticas e concentrar toxinas. Assim, o presente estudo teve como um dos objetivos o de investigar experimentalmente a transferência trófica e os efeitos da toxicidade dos dinoflagelados dos gêneros *Prorocentrum* e *Gambierdiscus* ao bivalve *Anomalocardia flexuosa* e ao peixe carnívoro *Pterois volitans*, respectivamente. As dietas dos bivalves foram compostas de densidade altas e moderadas de uma mistura contendo células de *Prorocentrum lima* ou seus compostos dissolvidos, incluindo as toxinas ácido ocadáico e dinophystoxina-1, e da microalga não-tóxica *Tetraselmis* sp. Por sua vez, os peixes-leão (*P. volitans*) foram alimentados com filés do peixe papagaio (*Chlorurus microrhinos*) naturalmente contaminadas com ciguatoxinas. Tanto os bivalves quanto os peixes carnívoros acumularam baixas concentrações de toxinas em seus tecidos durante o período de exposição, sendo os níveis maiores nas glândulas digestivas (bivalves) ou nos fígados (peixes), e menores nos músculos. Enquanto os bivalves apresentaram altas taxas de detoxificação após sete dias de depuração, os peixes ainda continham concentrações similares de toxinas mesmo após seis semanas de depuração. O segundo objetivo principal deste estudo foi o de elaborar um mapa de distribuição da área superficial de micro- e macroplásticos disponível em diferentes regiões geográficas, e avaliar o papel potencial de resíduos plásticos flutuantes como vetores de microalgas bênticas tóxicas e seus compostos. Estimamos que os oceanos abrigam uma vasta área superficial de detritos plásticos flutuantes, representando um substrato potencial para a colonização e um vetor de dispersão de células de microalgas bênticas potencialmente tóxicas e suas toxinas entre as regiões costeiras e oceânicas. Estimamos ainda, um potencial de colonização médio acima de 10^4 células km^{-2} para *Gambierdiscus* spp., *Ostreopsis* cf. *ovata* e *Prorocentrum* spp. em detritos plásticos flutuando nos oceanos. A presença frequente de espécies tóxicas em ambientes marinhos poluídos por plásticos representa, em última análise, um risco aos organismos marinhos e à saúde humana por meio do favorecimento da transferência trófica de toxinas produzidas por microalgas bênticas.

Palavras-chave: Dinoflagelados bênticos. Toxinas algais. Biotoxinas marinhas. *Prorocentrum*. *Gambierdiscus*. Organismos marinhos. Poluição marinha.

ABSTRACT

Benthic dinoflagellates are a group of microalgae capable of producing potent biotoxins that can generate adverse effects on ecosystems and human health. The deleterious effects to humans include intoxication following ingestion of contaminated seafood or airborne exposure to sea spray containing toxic cells or their dissolved compounds. Microalgal toxins can generate sublethal damage to marine organisms such as bivalve mollusks and fish. These interactions can be enhanced in marine environments highly contaminated with plastic litter, which can serve as an artificial substrate for benthic cells and thus concentrate toxins. Therefore, the present study first aimed at experimentally investigating the trophic transfer and toxicity effects of dinoflagellates of the genera *Prorocentrum* and *Gambierdiscus* to the bivalve *Anomalocardia flexuosa* and the carnivorous fish *Pterois volitans*, respectively. The diets of the bivalves were composed of high and moderate densities of a mixture containing *Prorocentrum lima* cells or their dissolved compounds, including the toxins okadaic acid and dinophystoxin-1, and the non-toxic microalgae *Tetraselmis* sp. meanwhile, the lionfish (*P. volitans*) were fed with parrotfish fillets (*Chlorurus microrhinos*) naturally contaminated with ciguatoxins. Both bivalves and carnivorous fish accumulated low concentrations of the toxins in their tissues during the exposure period, the levels were highest in the digestive glands (bivalves) or livers (fish) and lowest in the muscles. While the bivalves showed high detoxification rates after seven days of depuration, the fish still contained similar concentrations of toxins even after six weeks of depuration. The second major goal of this study was to develop a distribution map of the surface area of micro- and macroplastics available in different geographical areas, and to assess the potential role of floating plastics as vectors of toxic benthic microalgae and their compounds. We estimated that ocean waters harbor a vast surface area of floating plastic debris, which represents a potential substrate for colonization and a vector for dispersal of potentially toxic benthic microalgal cells and their toxins between coastal and oceanic regions. Also, we estimated an average potential colonization of up to 10^4 cells km^{-2} for *Gambierdiscus* spp., *Ostreopsis* cf. *ovata* and *Prorocentrum* spp. on plastic debris floating in the oceans. The frequent presence of toxic species in plastic-polluted marine environments ultimately poses a risk to marine organisms and human health by facilitating the trophic transfer of toxins produced by benthic microalgae.

Keywords: Benthic dinoflagellates. Algal toxins. Marine biotoxins. *Prorocentrum*. *Gambierdiscus*. Marine organisms. Marine pollution.

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1. CAPÍTULO 1: INTRODUÇÃO, OBJETIVOS E ESTRUTURA DA TESE

1.1 Introdução geral

Os dinoflagelados bênticos constituem um grupo de microalgas que ocorrem associadas às macroalgas e gramas marinhas (i.e., espécies epífitas), sedimentos de granulometria fina a média, em praias, poças de maré, recifes de corais ou sobre rochas de ambientes transicionais e costeiros, ou ainda em substratos artificiais (FAIMALI et al., 2012; HOPPENRATH et al., 2014; TESTER et al., 2014). As espécies bênticas começaram a ser estudadas com mais frequência na década de 1980, devido a um aumento na incidência da intoxicação conhecida por Ciguatera (CP, da sigla em inglês *Ciguatera Poisoning*). A CP é uma intoxicação alimentar que tem afetado até 500.000 pessoas por ano ao redor do mundo, e está relacionada à produção de ciguatoxinas (CTXs) pelos dinoflagelados do gênero *Gambierdiscus* seguida por seu acúmulo em peixes (YASUMOTO et al., 1987; PARSONS et al., 2012). Desde então, diferentes compostos tóxicos têm sido descritos e associados a diversas espécies produtoras, principalmente às pertencentes aos gêneros *Amphidinium*, *Coolia*, *Gambierdiscus*, *Ostreopsis* e *Prorocentrum*. Estes dinoflagelados têm sido os responsáveis por impactos negativos aos ecossistemas bênticos e/ou intoxicações em humanos por meio de contato primário, pela exposição ao aerossol marinho, ou pelo consumo de pescados contaminados (PARSONS et al., 2012; HOPPENRATH et al., 2014). Como visto para a CP, o consumo de pescados contaminados pode resultar em episódios de intoxicação que podem ser classificadas de acordo com os sintomas e natureza química da toxina envolvida.

As biotoxinas produzidas pelos dinoflagelados são estruturalmente diversas e complexas e, dependendo dos organismos afetados, exibem variados modos de ação, levando a diferentes expressões dos efeitos letais e sub-letais. Os efeitos negativos desencadeados pela exposição de invertebrados marinhos a células tóxicas de *Prorocentrum*, por exemplo, têm sido investigados através de experimentos de laboratório avaliando a cinética das toxinas, mudanças na taxa de filtração e ingestão, assim como alterações histológicas e genéticas (HÉGARET et al., 2007; GALIMANY et al., 2008; ROMERO-GERALDO et al., 2016). Embora, os efeitos das CTXs na fauna marinha sejam menos documentados, foram observados efeitos adversos da exposição a CTX em espécies de peixes juvenis de *Oreochromis* sp. (KELLY et al., 1992) em adultos de *Mugil cephalus* (LEDREUX et al., 2014) e em larvas e embriões do gênero *Oryzias* (MAK et al., 2017; YAN et al., 2017; 2020). As CTXs encontradas no cérebro, fígado e músculos de focas *Monachus schauinslandi* encalhadas (BOTTEIN et al., 2011) sugerem que os mamíferos marinhos também podem sofrer exposição a estas toxinas, que podem persistir dentro das teias alimentares marinhas. Os efeitos tóxicos à exposição a células de *Ostreopsis* spp., durante eventos de florações podem

se manifestar em vertebrados e invertebrados marinhos, como copépodes (GIUSSANI et al., 2016), ouriços-do-mar (SHEARS & ROSS, 2009) e poliquetas (CIMINIELLO et al., 2006; TOTTI et al., 2010). Além disso, a exposição experimental dos mais diversos organismos a células de *Ostreopsis* spp. ou a seus compostos dissolvidos, as palitoxinas (PLTX) e seus análogos, as ovatoxinas (OvTX), quase sempre culmina em algum nível de mortalidade, como verificado em poliquetas (*Dinophilus gyrociliatus*), copépodes (*Tigriopus fulvus*), larvas de craca (*Amphibalanus amphitrite*), euférias de água-viva (*Aurelia* sp.), microcrustáceos (*Artemia salina*) e juvenis de robalo (*D. labrax*) (SIMONINI et al., 2011; FAIMALI et al., 2012; PAGLIARA & CAROPPO, 2012; GIUSSANI et al., 2016).

No Brasil, a produção de diferentes biotoxinas, bem como sua toxicidade para organismos marinhos, já foi comprovada a partir de células de *Prorocentrum*, *Gambierdiscus* e *Ostreopsis* spp. cultivadas em laboratório (NASCIMENTO et al., 2016; GÓMEZ et al., 2017; MENDES et al., 2017; NEVES et al., 2017; PISAPIA et al., 2017; MOREIRA-GONZÁLEZ et al., 2019a; TIBIRIÇÁ et al., 2019), bem como durante florações associadas à contaminação e mortalidade da fauna (FERREIRA, 2006; TIBIRIÇÁ et al., 2019). Outros dinoflagelados tipicamente bênticos que podem produzir toxinas, como *Amphidinium* spp. e *Coolia* spp., também estão presentes no litoral brasileiro, entretanto, apresentam toxicidade relativamente baixa para organismos marinhos (MOREIRA-GONZÁLEZ, 2019b).

Variações interespecíficas na toxicidade vão depender do balanço dos processos de acúmulo, transformação e detoxificação das toxinas nos organismos vetores (MAFRA et al., 2015, 2019). Após a ingestão, os compostos tóxicos podem sofrer transformações durante o processo de digestão. Em seguida, as toxinas podem ser transferidas entre os diferentes tecidos e concentradas em locais específicos, o que pode ser de particular importância sanitária quando tais tecidos se destinam ao consumo humano, como acontece com moluscos bivalves, por exemplo (MAFRA et al., 2015). Apesar de sofrerem transformações, as toxinas produzidas por microalgas são resilientes e termoestáveis, ou seja, sua toxicidade não é reduzida mesmo com o cozimento dos pescados contaminados (VAN DOLAH, 2000). Da mesma forma, a eficiência dos processos de metabolização e detoxificação das toxinas pelos consumidores primários pode influenciar em seu potencial para biomagnificação na cadeia alimentar pelágica e bêntica (BLANCO, 2018). De fato, zooplâncton, peixes herbívoros e organismos suspensívoros como moluscos bivalves podem atuar como vetores de toxinas aos humanos diretamente, ou por meio da transferência dos compostos a organismos de níveis tróficos superiores incluindo cefalópodes e peixes carnívoros. Apesar dos potenciais efeitos negativos das toxinas produzidas pelos dinoflagelados aos organismos marinhos,

ainda são poucos os estudos que avaliaram os processos de acúmulo e eliminação de toxinas e seu potencial de transferência ao longo da cadeia trófica, sobretudo em peixes carnívoros.

A expansão da distribuição geográfica dos dinoflagelados bênticos potencialmente tóxicos tem sido registrada ao longo dos anos (LASSUS et al., 2016). Estudos recentes indicam que a dispersão dos dinoflagelados pelas regiões costeiras e oceânicas pode ser facilitada pela colonização de células em substratos bióticos (e.g. macrófitas) ou em materiais plásticos flutuando nos ambientes marinhos (MASÓ et al., 2003; LARSSON et al., 2018; CASABIANCA et al., 2019; TIBIRIÇÁ et al., 2019). A poluição por plástico representa uma crescente ameaça à qualidade dos ecossistemas marinhos, que já podem conter atualmente >150 milhões de toneladas de lixo plástico acumulado (GALLO et al., 2018). Os detritos plásticos menores variam entre 0,1 μm e 5 mm (microplásticos, MPs), ou ainda menores que 0,1 μm (nanoplásticos). Tais partículas são muito persistentes no ambiente marinho, com tempo de degradação estimado de até 400 anos. Estudos recentes têm demonstrado que os MPs podem ser ingeridos por organismos marinhos de diferentes níveis tróficos, gerando efeitos letais e subletais variados (WANG et al., 2019). A exposição de organismos marinhos às partículas plásticas pode alterar seus padrões de natação, alimentação e/ou respiração, devido à obstrução mecânica ou por intoxicação pós-ingestão.

As partículas plásticas colonizadas por biofilme microbiano podem ser um vetor para dispersão de microalgas potencialmente tóxicas, como os dinoflagelados dos gêneros *Alexandrium*, *Coolia* e *Ostreopsis*, detectados em fragmentos plásticos flutuando na superfície marinha em regiões como o Mar Mediterrâneo (MASÓ et al., 2003, 2016; ZETTLER et al., 2003) e sul do Brasil (LACERDA et al., 2021). Estudos recentes demonstraram que os dinoflagelados bênticos potencialmente tóxicos *Ostreopsis* spp. possuem capacidade de colonizar rapidamente diferentes superfícies plásticas, atingindo densidades celulares moderadas a elevadas (~260 a 4900 células cm^{-2}) (CASABIANCA et al., 2019, TIBIRIÇÁ et al., 2019). Ultimamente, esses substratos artificiais flutuantes podem agir como um vetor de biotoxinas no meio marinho, disponibilizando tais compostos tóxicos para organismos pelágicos. Neste contexto, células aderidas de dinoflagelados tóxicos bênticos e suas toxinas adsorvidas a detritos plásticos marinhos podem representar uma ameaça adicional à biota e à saúde humana.

Além dos efeitos diretos das toxinas e dos detritos plásticos sobre diferentes sistemas dos organismos marinhos, o fato do animal estar intoxicado/estressado pode estimular a produção de espécies reativas de oxigênio (ROS, da sigla em inglês) em diferentes células (DORANTES-ARANDA et al., 2015). Como proteção, as células expostas a ROS são capazes de ativar as substâncias antioxidantes enzimáticas, incluindo a superóxido dismutase (SOD), catalase (CAT), glutathiona peroxidase (GPx), glutathiona S-transferase (GST) e glutathiona redutase (GR), e não-

enzimáticas, como a glutationa (GSH) (DOYOTTE et al., 1997; ABELE; VÁZQUEZ-MEDINA; ZENTENO-SAVÍN, 2012; QIU et al., 2013; SANDRINI-NETO et al., 2016). A determinação do excesso de ROS em um organismo ocorre indiretamente por meio da medição de atividade antioxidante (ex.: CAD, SOD, etc.) ou de produtos da LPO, da desnaturação de proteínas, e de danos ao DNA causados pelo estresse oxidativo (HERMES-LIMA, 2005; ABELE; VÁZQUEZ-MEDINA; ZENTENO-SAVÍN, 2012) (Figura 1.1).

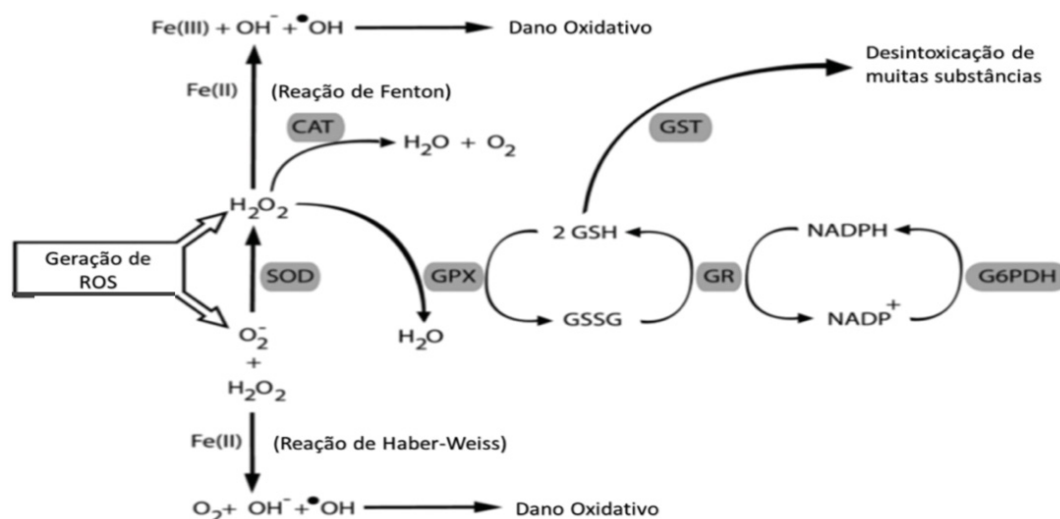


Figura 1.1. Sistema antioxidante e principais enzimas envolvidas. Siglas: CAT - Catalase; SOD – Superóxido dismutase; GPX – Glutathiona peroxidase; GST – Glutathiona S-transferase e GR – Glutathiona redutase. Adaptado de: Hermes-Lima (2005).

Sob condições normais, a produção e a eliminação de ROS se encontram em equilíbrio. O estresse oxidativo ocorre a partir do momento em que o sistema antioxidante não consegue neutralizar o aumento na produção de ROS. Isso resulta em danos reversíveis ou até irreversíveis para as células, podendo levar à morte celular (FERREIRA; MATSUBARA, 1997; ORBEA et al. 2002; LUSHCHAK, 2011; SANDRINI-NETO et al., 2016). Os efeitos deletérios sofridos pelas células dos organismos vão depender, por exemplo, do tipo de organismo, da sua fisiologia, do sistema antioxidante, da alimentação, de fatores ambientais (como salinidade e temperatura) e de sua exposição – paralela ou prévia – a contaminantes e toxinas (ABELE; VÁZQUEZ-MEDINA; ZENTENO-SAVÍN, 2012; SANDRINI-NETO et al., 2016; MEJDOUB et al., 2017). Sob condições ambientais favoráveis, as microalgas tóxicas podem se proliferar rapidamente formando as florações de algas nocivas. Durante esses eventos, a produção das ROS pelos organismos podem atuar como um agente potencializador da toxicidade de microalgas nocivas sobre organismos como peixes e bivalves, por exemplo (DORANTES-ARANDA et al., 2015).

A maioria dos estudos que investigaram o estresse oxidativo em moluscos bivalves tem focado nos efeitos de fatores ambientais adversos como as temperaturas extremas, sobretudo recentemente em face às preocupações com efeitos adversos das mudanças climáticas (ABELE; PUNTARULO, 2004; NOGUEIRA et al., 2017). Efeitos da hipóxia, radiação ultravioleta, xenobióticos, e as respostas de bivalves à exposição a poluentes e toxinas de microalgas também têm sido investigados (Tabela 1.1), sendo poucos os trabalhos que estudaram as respostas de danos oxidativos das toxinas produzidas por microalgas (GORBI et al., 2012, 2013; NÚÑEZ-ACUÑA et al., 2013; QIU et al., 2013). Os biomarcadores de estresse oxidativo de uso mais recorrente na literatura são CAT, SOD, GST e proteínas, analisadas na maioria dos casos em glândulas digestivas e nas brânquias dos bivalves (Tabela 1.1).

Tabela 1.1. Estudos que avaliaram biomarcadores em bivalves, com indicação dos tecidos analisados, dos contaminantes testados, dos biomarcadores avaliados e a referência. A notação “tecido” significa que não houve distinção entre os tecidos analisados.

Gênero	Tecido ¹	Contaminante ²	Biomarcador ³	Referência
<i>Unio</i> (água doce)	BR, GD	Cobre (Cu) e fungicida	CAT, GR, GPx, SOD, GSH, MDA	Doyotte et al., 1997
<i>Mytilus</i> , <i>Crassostrea</i>	GD	HPA e PCB	CAT, SOD, GPX	Orbea et al., 2002
<i>Cerastoderma</i>	BR, GD	Cádmio (Cd)	MT em BR e GD, MDA em GD	Machreki-Ajmi et al., 2008
<i>Cerastoderma</i>	GD, BR	Cd	MT, MDA	Ladhar-Chaadouni et al., 2009
<i>Ruditapes</i>	GD, BR	Cádmio (Cd)	MT	Smaoui-Damak et al., 2009
<i>Ruditapes</i>	BR, massa visceral	Cd, parasita (<i>Himasthla elongata</i>)	MT	Paul-Pont et al., 2010
<i>Mytilus</i>	GD	<i>Ostreopsis</i> (PTX)	CAT, GR, GPX, GST, GSH, MDA	Gorbi et al., 2012
<i>Mytilus</i>	GD	<i>Ostreopsis cf. ovata</i>	CAT, GR, GPX, GST, GSH	Gorbi et al., 2013
<i>Mytilus</i>	hemolinfa (MU adutor)	<i>Alexandrium catenella</i> (STX)	SOD, CAT	Nunez-Acuna et al., 2013
<i>Mytilus</i> , <i>Patinopecten</i>	MU, GD	<i>Alexandrium tamarense</i> (STX)	SOD, CAT, GPX	Qiu et al., 2013
<i>Mytilus</i>	GD	Poluentes, variáveis biológicas	CAT, GST, GRX, LPO	González-Fernández et al., 2015a
<i>Mytilus</i>	GD	Condições nutricionais	CAT, GST, GRX, LPO	González-Fernández et al., 2015b
<i>Cerastoderma</i>	Tecidos	Metais (Cd, Cu, zinco e níquel)	SOD, CAT, MT	Karray et al., 2015
<i>Anadara</i>	GD, BR, GN	Cu, chumbo e Cd	CAT, SOD, GPX	Peng et al., 2015
<i>Mimachlamys</i>	GD	Metais e contaminantes orgânicos	MDA, SOD, GST	Breitwieser et al., 2016
<i>Mytilus</i>	GD	HPA fluoranteno	SOD, CAT, GR, GST, LPO, MDA	González-Fernández et al., 2016
<i>Anomalocardia</i>	GD	Oléo diesel	SOD, CAT, GST, GPx, GSH, LPO	Sandrini-Neto et al., 2016
<i>Anomalocardia</i>	GD	Oléo diesel	SOD, CAT, GST, GPx, LPO	Sardi et al., 2016
<i>Mytilus</i>	BR, GD, MU	Metais pesados	CAT, GST, MDA	Mejdoub et al., 2017
<i>Perna</i>	Tecidos, GD, BR para GSH	Hipoxia	SOD, CAT, GR, GPX, GST, GSH	Nogueira et al., 2017

¹Tecidos: BR = Brânquia; GD = Glândula digestiva; GN = Gônada; MU = Músculo. ²Contaminantes: HPA = Hicarbonetos policíclicos aromáticos; PCB = Bifenilos policlorados; STX = Saxitoxinas; PTX = Palitoxinas. ³Biomarcadores: CAT = catalase; GPx = glutatona peroxidase; GST = glutatona S-transferase; GR = glutatona redutas; GSH = glutatona; SOD = superóxido dismutase; LPO = nível de peroxidação lipídica e MDA = Malondialdeído; MT = Metalotonina.

1.2. Objetivos e hipóteses

Considerando que as toxinas produzidas por dinoflagelados bênticos podem ser acumuladas e biomagnificadas na cadeia trófica, e que a dispersão desses microrganismos pode ser facilitada através da sua colonização em substratos plásticos, gerando um potencial efeito negativo aditivo sobre os organismos marinhos, o presente estudo teve como objetivos principais: (i) aprofundar o conhecimento acerca do acúmulo de toxinas e da toxicidade de dinoflagelados bênticos sobre organismos marinhos de diferentes níveis tróficos (e.g. suspensívoros e predadores) e (ii) avaliar os potenciais de interação entre dinoflagelados bênticos e detritos plásticos como potenciais vetores para a transferência de toxinas algais ao longo das cadeias tróficas marinhas.

Assim, a potencial susceptibilidade dos organismos marinhos aos efeitos das toxinas, associadas ou não à poluição plástica, bem como o possível papel dos organismos marinhos e dos detritos plásticos como vetores das mesmas toxinas para níveis tróficos superiores serão avaliados por meio dos seguintes objetivos específicos:

- Determinar os efeitos letais e sub-letais (respostas fisiológicas e estresse oxidativo), o acúmulo e a detoxificação de toxinas diarreicas – ácido ocadáico (AO) e dinofisistoxinas (DTX) – em moluscos bivalves adultos (*Anomalocardia flexuosa*) expostos experimentalmente ao dinoflagelado bêntico *Prorocentrum lima* ou aos seus compostos tóxicos;
- Determinar as taxas de bioacumulação, biotransformação e detoxificação de ciguatoxinas (CTXs), e seus efeitos deletérios (citotoxicidade e mudanças comportamentais e/ou fisiológicas) em peixes carnívoros alimentados com peixes herbívoros contaminados com CTXs, por meio de uma abordagem experimental;
- Caracterizar e quantificar a distribuição de detritos plásticos na superfície dos oceanos e estimar o potencial de colonização de dinoflagelados bênticos em substrato plástico e sua ocorrência nas diferentes regiões oceânicas associado com detritos plásticos.

As principais hipóteses testadas no presente estudo foram:

- (i) Se alguns organismos marinhos como bivalves e peixes atuam como vetores de intoxicações a consumidores, então eles apresentarão certa resistência aos mecanismos tóxicos quando expostos a dieta contendo toxinas produzidas pelos dinoflagelados bênticos, e acumularão em seus tecidos valores significativamente elevados de toxinas sem sofrer mortalidade;

- (ii) Se tais organismos sofrem efeitos sub-letais decorrentes da exposição aos dinoflagelados bênticos toxigênicos (ou a seus compostos tóxicos), então serão observadas diferentes respostas fisiológicas, comportamentais e de biomarcadores de estresse oxidativo observadas na presença ou ausência de toxinas;
- (iii) Se houver regiões com elevada abundância de partículas plásticas flutuando na superfície dos oceanos, então os plásticos que servem como um substrato para dinoflagelados bênticos potencialmente tóxicos e suas toxinas apresentarão um elevado potencial para atuarem como vetores adicionais para a transferência de toxinas algais ao longo das cadeias tróficas marinhas nestes locais.

1.3. Estrutura da tese

Os resultados da tese estão concentrados na segunda parte da tese, redigida em inglês, que inclui três capítulos que atendem aos seus objetivos específicos. Eles foram redigidos sob a forma de artigos científicos independentes, estruturados conforme as normas de cada periódico onde foram publicados, ou submetidos à publicação. Os periódicos internacionais escolhidos para divulgação dos artigos abordam temas ligados às áreas de toxicologia e poluição marinha, têm fator de impacto (JCR 2020) entre 3.53 e 7.96, e são classificados como Qualis (2013-2016) A1-B2 na área de Biodiversidade da CAPES.

O **segundo capítulo**, intitulado “*Toxin accumulation, detoxification and oxidative stress in bivalve (Anomalocardia flexuosa) exposed to the dinoflagellate Prorocentrum lima*” foi publicado na revista *Aquatic Toxicology*. O artigo aborda os efeitos letais e sub-letais em berbigões infaunais expostos ao dinoflagelado bêntico produtor de toxinas diarreicas – ácido ocadaico (AO) e dinofisistoxinas (DTX) – *Prorocentrum lima*.

O **terceiro capítulo**, “*Experimental evidence of ciguatoxin accumulation and depuration in carnivorous lionfish*”, foi desenvolvido majoritariamente no *Environmental Laboratories* da Agência Internacional de Energia Atômica (IAEA, Mônaco) durante um período de estágio sanduíche, sob a supervisão da Dra. Marie-Yamine Bottein. O artigo avaliou experimentalmente o acúmulo de ciguatoxinas (CTXs) e seus potenciais efeitos deletérios associados em peixes carnívoros, *Pterois volitans*, alimentados com peixes herbívoros naturalmente contaminados com as toxinas, utilizando diferentes metodologias analíticas. O manuscrito foi publicado no periódico *Toxins*, o qual aborda pesquisas relacionadas.

O **quarto capítulo**, “*A new look at the potential role of plastic debris as vectors of harmful benthic algae in the ocean*”, trata de uma análise integrada de dados secundários

gerando informações sobre o mapeamento de plásticos nos oceanos e discute as implicações da interação entre a poluição plástica, algas nocivas e toxinas algais para a saúde de organismos marinhos e seres humanos. Os resultados principais incluem um mapeamento da área plástica disponível na superfície de diferentes regiões oceânicas e uma estimativa do potencial de colonização de dinoflagelados bênticos tóxicos selecionados em detritos plásticos flutuando nos oceanos. Este capítulo será submetido ao periódico *Science of the Total Environment*, especializado na publicação de trabalhos focados na interação entre ações antrópicas, o meio ambiente e os organismos que nele vivem.

Por fim, o **quinto capítulo** é uma seção de Considerações Finais que integra os capítulos anteriores, ressaltando a relevância científica dos principais resultados alcançados. E o **sexto capítulo** apresenta recomendações para estudos futuros, seguido de uma lista integrada de **Referências**.

2. CAPÍTULO 2: TOXIN ACCUMULATION, DETOXIFICATION AND OXIDATIVE STRESS IN BIVALVE (*ANOMALOCARDIA FLEXUOSA*) EXPOSED TO THE DINOFLAGELLATE *PROROCENTRUM LIMA*

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Abstract

Prorocentrum lima is a cosmopolitan benthic dinoflagellate capable of producing the diarrhetic shellfish toxins (DSTs) okadaic acid (OA) and dinophysistoxin (DTX). These compounds may cause oxidative stress and accumulate in bivalve tissues, which become vectors of intoxication to human consumers. We investigated DST accumulation, detoxification and oxidative stress biomarkers in clams (*Anomalocardia flexuosa*) experimentally exposed to *P. lima* cells or their compounds. Experimental diets consisted of 6000 cells mL⁻¹ of the non-toxic chlorophyte *Tetraselmis* sp. (C; control condition), and combinations of C with 10 *P. lima* cells mL⁻¹ (T10), 100 *P. lima* cells mL⁻¹ (T100), or to a toxin concentration of ~4 µg OA L⁻¹ and ~0.65 µg DTX-1 L⁻¹ (T100d). Clams were exposed to these diets for 7 days (uptake phase), followed by a 7-day depuration period. No DSTs were detected in clams exposed to treatments C (control) nor to T100d (dissolved compounds) during either uptake or detoxification phase. Conversely, clams exposed to T10 or T100 accumulated, on average, up to 2.5 and 35 µg DST kg⁻¹ in their whole bodies at the end of the uptake phase. These concentrations are ~64 and ~4.5 times lower than the regulatory level of 160 µg OA kg⁻¹, respectively. Accumulated OA quotas were 12–22 times higher in the digestive gland (DG) than in remaining tissues over the uptake phase. Quick toxin transformation was indicated by the early detection of conjugated compounds – DTX-1 and OA esters – in the DG after 6 h of exposure, with OA-ester representing the main compound (30 – 100 %) in that tissue over the experiment. During the depuration period, detoxification rates represented 0.024 h⁻¹, 0.04 h⁻¹ and 0.052 h⁻¹ for OA, DTX-1 and OA-ester, respectively. The activities of catalase, glutathione S-transferase, glutathione peroxidase and the levels of oxidative stress by lipoperoxidation varied similarly in the DG of *A. flexuosa* individuals subjected to T100, T100d and the control condition. However, contrasting antioxidant responses were measured in those exposed to T10. These findings indicate that no oxidative stress was primarily induced by DST-producing dinoflagellates in this clam species under laboratory conditions representative of toxic bloom situations. Even though, possible interactions should be considered under multistressor scenarios.

2.1. Introduction

Diarrhetic Shellfish Poisoning (DSP) is a relatively frequent human intoxication caused by the consumption of seafood, mainly bivalve mollusks, containing okadaic acid (OA) and/or its analogues, the dinophysistoxins (DTXs) (YASUMOTO et al., 1978). Despite being considered non-lethal, these compounds can cause inhibition of serine and threonine protein phosphatases affecting multiple cellular functions (COHEN et al., 1990; VALDIGLESIAS et al., 2013). The most common symptoms of DSP in humans are gastrointestinal disorders, such as diarrhea, vomiting, nausea and abdominal pain, which may persist for up to three days after exposure, depending on the amount of toxin ingested (GERSSEN et al., 2010; VALE and SAMPAYO, 2002; YASUMOTO et al., 1978). In human adults weighing 60 Kg, such symptoms can be induced at doses as low as 48 µg of OA or 32 µg of DTX-1 (MAFRA et al., 2019; VALDIGLESIAS et al., 2013; YASUMOTO et al., 1985). In order to ensure the protection of seafood consumers, a regulatory limit of 160 µg OA kg⁻¹ has been issued for shellfish commercialization in Europe (Regulation EC No. 853/2004, 29) and other countries, including Brazil (FAO/WHO, 2016). In addition to acute intoxication, chronic exposure to diarrhetic shellfish toxins (DSTs) can lead to carcinogenesis and may negatively affect the immune and neurological systems of both humans and marine organisms (TOYOFUKU, 2006; VALDIGLESIAS et al., 2013).

Bivalve mollusks can be highly exposed to DSTs upon active suspension feeding on toxic dinoflagellate cells, mainly of planktonic *Dinophysis* spp., but also benthic *Prorocentrum* spp., during harmful algal blooms (HABs) (REGUERA et al., 2012, 2014; YASUMOTO et al., 1985). Although cases of DSP intoxication are more related to *Dinophysis* blooms, some shellfish poisoning outbreaks may be associated with *Prorocentrum lima* (GAYOSO et al., 2002; KOIKE et al., 1998; LAWRENCE et al., 1998). Particulate matter is usually the main vector of DST uptake in suspension feeders (ALVES and MAFRA, 2018; REGUERA et al., 2014). Accumulation of DSTs in bivalve mollusks occurs mostly in the cells of the digestive gland (DG) following extracellular digestion of particles containing them (reviewed in Blanco, 2018). Although much less efficiently, uptake of dissolved DSTs from seawater is also possible. In this case, toxins may follow distinct absorption routes and tissue distribution, being mainly accumulated in the bivalve DG, followed by the gills, as shown for *Mytilus galloprovincialis* mussels (LI et al., 2018).

Negative effects triggered by the exposure of bivalves to toxic *Prorocentrum* cells have been investigated through laboratory experiments assessing toxin kinetics, changes in bivalve

filtration and ingestion rates, as well as histological and genetic alterations (GALIMANY et al., 2008; HÉGARET et al., 2007; ROMERO-GERALDO et al., 2016). Some of those studies have indicated the digestive gland (DG) as the first – and most – affected bivalve tissue following the ingestion of *Prorocentrum* cells containing DSTs. Following ingestion, toxic compounds can be structurally modified in the DG via enzymatic transformations during the digestion process. In this sense, OA, DTX-1 and DTX-2 are usually esterified with fatty acids forming 7-*O*-acyl ester derivatives (generically known as DTX-3) (KONOKI et al., 2013; ROSSIGNOLI et al., 2011), which are usually less toxic and more easily eliminated than the primary compounds (BLANCO et al., 2018; REGUERA et al., 2014; SVENSSON, 2003). However, these derivatives can be hydrolysed under alkaline conditions such as those found in human gastric system (JØRGENSEN et al., 2005), and thus represent an important source of intoxication for consumers of contaminated bivalves (MAFRA et al., 2019; MANITA et al., 2017; VALE and SAMPAYO et al., 2002; VILLAR-GONZÁLEZ et al., 2008).

The amount of DSTs – in their original or transformed forms – accumulated in bivalve tissues will thus depend on a balance between toxin uptake and transformation/detoxification processes. Following a toxic bloom, bivalves can usually eliminate most or the entire DST burden within a few days (mussels and clams) or weeks (oysters and scallops) (BAUDER et al., 2001; BLANCO et al., 2018; MAFRA et al., 2019). Besides marked inter-specific variations among bivalves (MAFRA et al., 2015, 2019; REGUERA et al., 2014), detoxification rates can also vary according to environmental conditions, such as temperature, salinity, phytoplankton and seston concentration (BRICELJ and SHUMWAY, 1998). Therefore, DST uptake and elimination rates as estimated from laboratory experiments can differ from those estimated for bivalve populations exposed to natural blooms (MAFRA et al., 2015). However, controlled laboratory experiments are still very useful in establishing key relationships between toxin kinetics and selected environmental variables, as well as for determining inter-species variations in toxin accumulation and toxicity among bivalves.

Toxicity of HAB-forming species to bivalves can be enhanced by high levels of algal reactive oxygen species (ROS) (DORANTES-ARANDA et al., 2015), produced by the algal cells and/or by the exposed organisms as part of their antioxidant defense system (LUSHCHAK, 2011; SANDRINI-NETO et al., 2016; VIDAL-LINAN and BELLAS, 2013). Oxidative stress may occur when the antioxidant system fails to neutralize increasing ROS levels, resulting in reversible or irreversible cell damage, and possibly leading to cell death (LUSHCHAK, 2011; SARDI et al., 2016a). In this case, antioxidant enzymes can be used as biomarkers of oxidative stress at cellular level in organisms exposed to xenobiotics, including

algal toxins (MONSERRAT et al., 2007). For this purpose, the activity of catalase (CAT), superoxide dismutase (SOD) and glutathione S-transferase (GST) are the most used biomarkers in studies with bivalves, being commonly quantified in their DGs and gills (GORBI et al., 2012; QIU et al., 2013; SARDI et al., 2016b). Studies on the oxidative stress of algal toxins to bivalves, however, are still scarce (GORBI et al., 2012, 2013; NUÑEZ-ACUÑA et al., 2013; QIU et al., 2013).

The occurrence of HABs in shellfish farming areas may thus threaten the economic stability of this important activity by impairing the health – and growth – of farmed organisms, and by posing an intoxication risk to seafood consumers. In order to prevent human DSP outbreaks, shellfish harvesting bans have been regularly issued in areas where toxic blooms of *Dinophysis* spp. are recurrent, such as southern Brazil (ALVES and MAFRA, 2018; MAFRA et al., 2019). In this region, oysters (*Crassostrea gigas*) and mussels (*Perna perna*) are extensively cultivated, and natural populations of clams (*Anomalocardia flexuosa*) have been increasingly exploited from natural estuarine banks (SILVA-CAVALCANTI and COSTA, 2009). This includes areas where benthic populations of dinoflagellates belonging to the *P. lima* complex are frequent and often abundant (BRUSTOLIN et al., 2014; MOREIRA-GONZÁLEZ et al., 2020). In addition to their commercial value, *A. flexuosa* clams are considered good indicators of marine pollution because their antioxidant enzymes can respond to varying levels of environmental contaminants (BRAGA et al., 2018; SARDI et al., 2016b). In this context, the present investigation aims at evaluating the oxidative stress-related effects, as well as the accumulation, biotransformation and elimination of DSTs in clams (*A. flexuosa*) exposed to *P. lima* cells – or to their intracellular compounds – under laboratory conditions.

2.2. Materials and Methods

2.2.1. Algal culture

The monoclonal culture of *P. lima* (strain LM-046) used in this experiment belongs to the genetic clade “C” (MOREIRA-GONZÁLEZ et al., 2018). It was established from a seaweed sample collected near Recife, Pernambuco State, northeastern Brazil (08° 05' S; 34° 44' W), following procedures described in Tester et al. (2014). Established cultures, in autoclaved filtered seawater (FSW) at 30–32 salinity enriched with 50 % diluted f/2 culture medium (“f/4”) (GUILLARD AND MORTON, 2004), were maintained in a BOD incubator at 26 °C, under $70 \pm 20 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ irradiance and 12 h light: 12 h dark photoperiod. A monoclonal

culture of the non-toxic chlorophycean *Tetraselmis* sp. was maintained at the same conditions described above.

Prior to the exposure experiment, the biomass of both toxic and non-toxic algae was increased using two different systems under constant aeration: (a) 2 L sterile glass bottles for *P. lima*; or (b) 30 L suspended plastic bags for *Tetraselmis* sp., both under 50–85 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ irradiance and 12:12 h photoperiod. *Prorocentrum lima* and *Tetraselmis* sp. cells were always harvested at late exponential phase. At that stage, cultures reached up to 5.0×10^4 and $1.5 \times 10^6 \text{ cells mL}^{-1}$, respectively.

2.2.2. Bivalve sampling and maintenance

Adult individuals of *A. flexuosa* ($n = 400$; shell height (SH) = $20.5 \pm 1.4 \text{ mm}$) were hand-collected from shallow estuarine banks around Mel Island ($25^\circ 32.605'S$, $48^\circ 18.365'W$), in Paraná State, southern Brazil. Sampling was performed in May 2018, when water temperature was 26°C and salinity 30.9. Clams were transported in thermal boxes to the laboratory, and gently washed with 200 ppm sodium hypochlorite (12% free chlorine) for sanitization and removal of sediments and incrustated organisms. Cleaned individuals were placed in aerated tanks, containing 30 L of FSW (cartridge filters, $5 \mu\text{m}$ pore size), at $23 \pm 1^\circ\text{C}$ and 35 ± 2 salinity. The FSW was previously sterilized with 0.05% sodium hypochlorite for 1 h, followed by neutralization with sodium thiosulfate at equivalent molar concentration for 1 h. After 24 h, the specimens were fed *Tetraselmis* sp. at $6000 \text{ cells mL}^{-1}$ twice a day for seven days. During the entire acclimatization period (8 d), the suspension was partially replaced (60% of the total volume) and the tanks cleaned daily. There was a 3.5% mortality rate ($n=14$ out of 400) during this period, especially within the first three days (2.75%).

2.2.3. Experimental setup and design

The experimental design consisted of two factors: diet, with four levels of microalgal exposure (combinations of algal composition and cell density), and time (initial + four levels during either uptake or depuration phase). Animals exposed to different diets were randomly allocated into 12 tanks, with three tanks per diet level (Figure 2.1), and 33 similar-sized individuals (SH $\sim 20.5 \text{ mm}$; mean wet weight = 1.36 g) per tank. Diets consisted of suspensions containing $6000 \text{ cells mL}^{-1}$ of non-toxic *Tetraselmis* sp. cells (C; control condition), and a mixture of C with $10 \text{ P. lima cells mL}^{-1}$ (T10), $100 \text{ P. lima cells mL}^{-1}$ (T100), or to a toxin concentration of $\sim 4 \mu\text{g OA L}^{-1}$ and $0.65 \mu\text{g DTX-1 L}^{-1}$ (T100d), obtained after rupture of 100

P. lima cells mL^{-1} with ultrasonic probe at 130 Hz for 5 min, followed by filtration (PVDE syringe filters, 13 mm, $0.22 \mu\text{m}$) to remove cell debris.

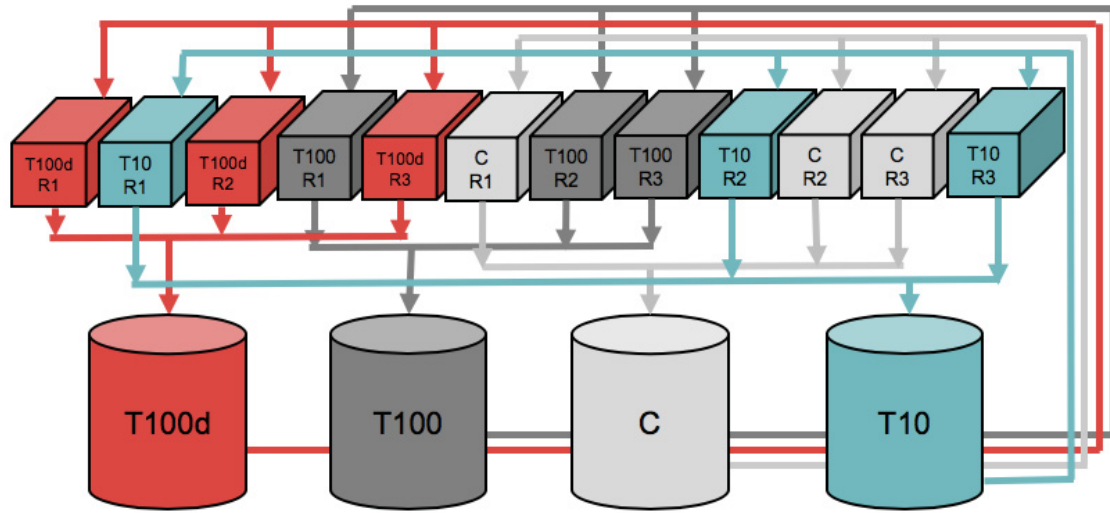


Figure 2.1. Design of the experimental system. Three rectangular tanks (30 L), containing initially 33 clams each, were assigned to each treatment/diet (T) and randomly distributed on an illuminated shelf. The outflow from each tank within the same treatment (replicate, R) was directed to a single cylindrical reservoir (60 L), connected to a peristaltic pump for a constant diet supply. Total biomass offered in each diet was equivalent, but the composition was different. Clams exposed to C (control diet) received exclusively non-toxic *Tetraselmis* sp. cells; those in T10 received a mixed of C with 10 cells mL^{-1} *P. lima*; T100 received mixed of C with $100 \text{ cells mL}^{-1}$ *P. lima*; and T100d received C added to the dissolved contents extracted from *P. lima*, equivalent to $100 \text{ cells mL}^{-1}$.

The experimental system consisted of a set of 30 L plastic tanks. They were randomly allocated to different treatment and replicates, placed on an illuminated shelf ($\sim 40 \mu\text{mol m}^{-2} \text{ s}^{-1}$). The outflow from each tank within the same treatment (diet) was directed to a single reservoir (60 L), in order to maintain uniform food supply among replicates (Figure 2.1). Submerged pumps (Aleas® HM-8101) ensured constant water circulation inside the reservoirs and provided extra aeration to the system. Tanks were cleaned and the suspension partially ($\sim 60\%$) replaced every day, by adding sterile FSW and a volume of culture containing the designated amount of microalgal cells for each diet (or the dissolved contents from *P. lima* in T100d). In addition, diets were constantly supplied using a peristaltic pump (Gilson® Minipuls Evolution), connected to the reservoirs, in order to account for cell losses due to bivalve feeding. Cell density was periodically checked (twice or three times a day) in each reservoir, using microscopic counting for *P. lima* (Olympus® BX-41 light microscope at $100\times$ magnification) or spectrophotometry for *Tetraselmis* sp. (DRAWELL® A007245 at 526 nm wave length). A

calibration curve ($R^2 > 0.99$) was used to convert absorbance values into *Tetraselmis* sp. cell density. If necessary, cell abundance in the reservoirs was corrected by adjusting the peristaltic pump flow rate and/or by adding the required volumes of algal stocks. Room temperature was controlled at $23^\circ\text{C} \pm 1.5^\circ\text{C}$, and the tanks were maintained under a 12h: 12h photoperiod. Water temperature, dissolved oxygen concentration, salinity and pH were monitored daily, using a multi-probe (AT-160, ALFAKIT) and a pHmeter (pH250 - PoliControl). Their values remained constant throughout the experiment and did not vary significantly among treatments (Table S2.1).

2.2.3.1 Toxin uptake phase

After 6, 24, 72 and 192 h of exposure to the experimental diets, three individuals from each tank were sacrificed and dissected, keeping good practices of animal welfare and euthanasia. In addition, initial samples (0 h) were taken prior to the exposure. Bivalves were dissected into three tissue fractions: gills, digestive glands (DGs) and remaining tissues. For each clam, shell height (SH) was measured, and the tissue fractions were individually weighed. Each DG was split into two pieces of similar size through a longitudinal section. One DG fraction from each individual ($n = 3$ per treatment) was combined into a pooled sample and placed into 1.5 mL microcentrifuge tubes for biomarker quantification. The same procedure was adopted with the other DG fraction and the remaining tissues, which were reserved for toxin determination. All tissue samples were immediately frozen and maintained at -80°C until analysis. In addition, 600 mL water samples were taken every day from tanks exposed to diet T100d. Toxins dissolved in the water samples were retained in pre-conditioned C18 cartridges (WAT 020515 Sep-Pak® Plus C18, 55–105 μm , 360 mg), eluted in 1.5 mL of methanol 100%, and stored at -18°C until toxin analysis.

2.2.3.2. Depuration phase

After 192 h of exposure, remaining clams ($n \sim 18$ per tank) were temporarily allocated (for approximately 30 min) in tanks containing fresh FSW, while the experimental tanks were rinsed with high-pressure washers. After cleaning the system, all bivalves were offered a monospecific non-toxic diet (~ 6000 *Tetraselmis* sp. cells mL^{-1}) to evaluate the process of toxin elimination. Similar to the toxin exposure phase, selected tissues were collected from three individuals per tank at this stage and combined into a single sample to measure toxin

concentration and biomarker responses after 12, 24, 72 and 168 h of depuration in a non-toxic diet.

2.2.3.3. *Bivalve biometry, feeding behavior and survival*

Bivalve shell height and soft tissue weight were measured throughout the experiment (from sampled individuals) and did not vary significantly among treatments and over time (Table S2.2). The digestive glands (DG) corresponded, on average, to ~8% of the total soft tissue weight considering all organisms, and 5.8 % for those individuals sampled during detoxification phase (Table S2.2). Clam survival and behavior/feeding patterns (shell opening and cell clearance) were monitored daily. No abnormal, prolonged shell closure was observed in response to the experimental diets. Filtration rates of clams were apparently constant throughout the experiment, based on the daily reduction in algal cell density relative to the number of animals kept in the tanks (data not shown). Only four (out of 324) individuals died during the 13 days of experiment; one from each treatment.

2.2.4. *Toxin analysis*

Initially, intracellular DST concentrations were determined in triplicate from 150 mL aliquots of the *P. lima* cultures after cell retention in microfiber Whatman GF/F filters (47 mm diameter, 0.8 μ m nominal pore size). Filters were stored at -18°C until toxin extraction, when 8 mL of 100 % methanol (HPLC grade) was added to each filter, and cells were disrupted using a Sonic Dismembrator (Cole Parmer® CPX130, 130 Hz) for 5 min at 80 % duty cycle. The extract was centrifuged at $2000 \times g$ for 5 min. Finally, a 2 mL aliquot of the supernatant was passed through a 0.22 μ m PVDE syringe filter to remove cell debris, collected into glass vial and stored at -18°C .

To each sample of DG or remaining soft tissues from both experimental phases ($n = 222$ in total), 99.9 % methanol (HPLC grade) was added in the ratio of 1:9 (w:v). Tissues were triturated by sonication for 3 min, applying pulses of 3 s with 1 s intervals, at 70 % duty cycle. Extracts were then centrifuged for 3 min at $2000 \times g$, and 2.0 mL aliquots of the recovered supernatant were filtered (0.22 μ m PVDE syringe filters) and collected into glass vials. Filtered aliquots were stored frozen until toxin analysis by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). Additional aliquots (1 mL) of each extract were subjected to alkaline hydrolysis by adding 0.125 mL of 2.5 M sodium hydroxide at 76°C for 40 min, followed by the addition of 2.5 M hydrochloric acid (0.125 mL) for pH neutralization. Hydrolysis reactions converted the esterified (conjugated) toxins into their free forms. Both the

original and the hydrolyzed extracts were analyzed by LC–MS/MS as described below, and the concentrations of conjugated DST forms were calculated by subtracting the initial value measured in the original extract from the amount detected in the hydrolyzed extract.

Toxins were determined in a micro-LC system (MicroLC 200 Plus, Eksigent Technologies®) composed of a binary gradient pumping system equipped with 50 μm i.d. tubing for flow rates between 20 and 200 $\mu\text{L min}^{-1}$, a cooled 3-drawer autosampler and a column oven, coupled to a triple quadrupole mass spectrometer (AB Sciex® Q-Trap 4500). High-purity air heated at 350 °C was used as the nebulizing gas. Chromatographic separations were performed using a Halo C-18 column (1.0 $\times$ 50 mm, 2.7 μm ; Eksigent Technologies) at 35 °C. Mobile phases consisted of (A) 100 % ultra-pure water and (B) 95 % aqueous acetonitrile, both containing 2 mM ammonium and 50 mM formic acid. Injection volume was 1 μL and a gradient elution (0.07 mL min^{-1}) was applied as follows: 5% of B for 0.5 min, increasing to 90 % of B, and maintained for 3.5 min before returning to the initial condition until the end of the analysis (7 min in total). The following multiple reaction monitoring (MRM) transitions were monitored with the ion source in negative turbo spray mode: m/z 817.2 $\rightarrow$ 255.0 and 817.2 $\rightarrow$ 113.0 for DTX-1; m/z 803.5 $\rightarrow$ 255.0, and m/z 803.5 $\rightarrow$ 113.0 for OA and DTX-2, and m/z 1041.6 $\rightarrow$ 255.0 for 7-*O*-palmitoyl OA, which is often the most common DTX-3 form (KAMENEVA et al., 2017) and was only screened in the non-hydrolyzed extracts. Concentrations of OA and DTX-1 were calculated from calibration curves made with certified reference material (IMB-NRC, Canada) dissolved in methanol at the following concentrations: 2.62, 5.35, 10.7, 21.4, 42.8 and 68.5 $\mu\text{g L}^{-1}$ for OA, and 5.8, 11.6 and 23.1 $\mu\text{g L}^{-1}$ for DTX-1, using Analyst® 1.6.3 software.

2.2.5. Biochemical biomarkers

Oxidative stress biomarkers were measured in DGs of three individuals randomly collected from each tank at each sampling interval. Pooled DGs samples ($n = 111$) were homogenized (1 g: 10 mL) in 0.1 M phosphate buffer (pH 7.0), using an ultrasound cell disrupting probe (IKA T10 Basic- Ultra-Turrax). The homogenates were centrifuged at 15,000 $\times g$ for 30 min in a refrigerated microcentrifuge (NT 805 - NovaTecnica) at 4 °C. The supernatant was collected and stored at -80 °C until analysis of biomarkers by spectrophotometry at specific absorbance wavelengths. Proteins were quantified at 595 nm following the method described by Bradford (1976). The antioxidant activity of CAT was

measured at 240 nm (AEBI, 1984), SOD at 440 nm (GAO et al., 1998), GST at 340 nm (KEEN et al., 1976) and glutathione peroxidase (GPx) at 340 nm (PAGLIA and VALENTINE, 1967). Finally, the levels of oxidative stress by lipoperoxidation (LPO) were estimated from measurements at 570 nm according to Jiang et al. (1992). A Biotek Synergy HT multimode microplate reader was used for measuring CAT activity, whereas a TECAN Sunrise microplate spectrophotometer was used for quantification of protein, SOD, GST, GPx and LPO.

2.2.6. Data analysis

2.2.6.1. Processing of toxin data

The concentrations ($\mu\text{g kg}^{-1}$) of free and total (conjugated + free form) OA and DTX-1 were calculated for whole individuals at each exposure time. For that, toxin levels measured in DGs and in remaining tissues were multiplied by their respective weights (fresh weight, g), then summed and divided by the total weight (soft tissues) of each individual. When toxin concentrations were below the limit of detection (LOD) in a given tissue fraction, zero values were attributed. Limit of detection and limit of quantification (LOQ) for each toxin were measured statistically as described in Mafra et al. (2009) following the formulae:

$$\text{LOD} = [(3 \cdot \text{SD}/b)] \text{ and } \text{LOQ} = [(10 \cdot \text{SD}/b)]w$$

here “b” is the slope of the calibration curve made of successive dilutions of methanolic reference material solutions (concentration vs. peak area), and “SD” is the standard deviation of peak area values corresponding to repeated runs of the calibration solution with minimum measurable concentration of either OA ($n = 4$) or DTX-1 ($n = 3$). Values of LOD and LOQ corresponded, respectively, to 0.17 and 0.57 pg OA mL^{-1} , and to 1.28 and 4.27 pg DTX-1 mL^{-1} . Detoxification rates in DGs of *A. flexuosa* were calculated following Blanco et al. (2002), using the toxin concentrations at the end of the exposure period (192 h) as the initial levels in each treatment.

2.2.6.2. Integrated biomarker response (IBR) index

The integrated biomarker response (IBR) method (SANCHEZ et al., 2013), was applied to integrate the responses from all biomarkers, simplifying data analysis and interpretation. This index is based on the deviation between an undisturbed (reference) and multiple disturbed states (measured values) (SANCHEZ et al., 2013). In the present study, the IBR method was used to estimate the deviation of biomarker values measured in each treatment – at either uptake or depuration phase – in relation to the values measured in C immediately prior to the experimental

exposure (reference value, X_0). The ratio between the average (X_i) and the baseline values (X_0) for each biomarker was log-transformed (Y_i) in order to reduce variance. Subsequently, Y_i was standardized following the equation

$$Z_i = (Y_i - \mu) / s$$

here “ μ ” is the general mean and “ s ” is the standard deviation for each biomarker response. The deviation biomarker index (A) was then calculated as the difference between Z_i and X_0 . The sum of the absolute A values was finally used to calculate the IBR index for each experimental phase, and A values were marked in star plots, in which an area above 0 reflects an enhancement and an area below 0 indicates an inhibition of a given biomarker response.

2.2.6.3. Statistics

The responses of oxidative stress biomarkers in relation to different diets and exposure times were tested by a repeated measure analysis of variance using treatment (fixed, 4 levels) and time (fixed, 5 levels) as orthogonal factors in each experimental phase. Since the clams were randomly collected, tanks were considered the experimental unit for repeated measures. Mauchly’s test was used to check the sphericity assumption, and the Greenhouse-Geisser correction was used in case of its violation. *A posteriori* comparisons of means for significant terms in the model ($\alpha = 0.05$) were performed using the Scott-Knott test (SCOTT and KNOTT, 1974). All statistical analyses were generated using R 3.6.1 software (R DEVELOPMENT CORE TEAM, 2019) combined with “ez” (LAWRENCE, 2013), “ScottKnott” (JELIHOVSCHI et al., 2014) and “ggplot2” (WICKHAM, 2016) packages.

2.3. Results

2.3.1. Toxin uptake and detoxification

No DSTs were detected in *A. flexuosa* clams sampled from the control treatment (C) during the entire experiment. Likewise, DST levels were always undetectable in clams exposed to T100d treatment, in which toxins were dissolved in seawater. Weight-averaged DST levels in the whole body of *A. flexuosa* attained 2.4 and 35 $\mu\text{g kg}^{-1}$ after 72 h of exposure to toxic *P. lima* cells in T10 and T100, respectively (Figure 2.2). At that time, the average toxin profile in whole clams was composed of 82 % OA and 18 % DTX-1 in T10, and 79 % OA and 21 % DTX-1 in T100 (Figure 2.2). This represents a slight change in relation to what was originally present in *P. lima* cells (84 % OA and 16 % DTX-1), possibly reflecting the faster overall elimination rates for OA relative to DTX-1 (as discussed later), although differential matrix

effect was not investigated for each toxin. Okadaic acid, in its free form, reached $4.1 \mu\text{g kg}^{-1}$ in the DGs after 6 h of exposure to T10 and $66.5 \mu\text{g kg}^{-1}$ after 6 h of exposure to T100. Concentrations were substantially lower during the rest of the uptake phase, and gradually decreased to zero over the depuration phase of the experiment (Figure 2.3A). In the remaining tissues, free OA concentrations were much lower, attaining a maximum of $6 \mu\text{g kg}^{-1}$ at the beginning of the depuration phase in T100 (Figure 2.3B). Free DTX-1 concentrations remained at undetected levels in both DG and remaining tissues of clams throughout the experiment.

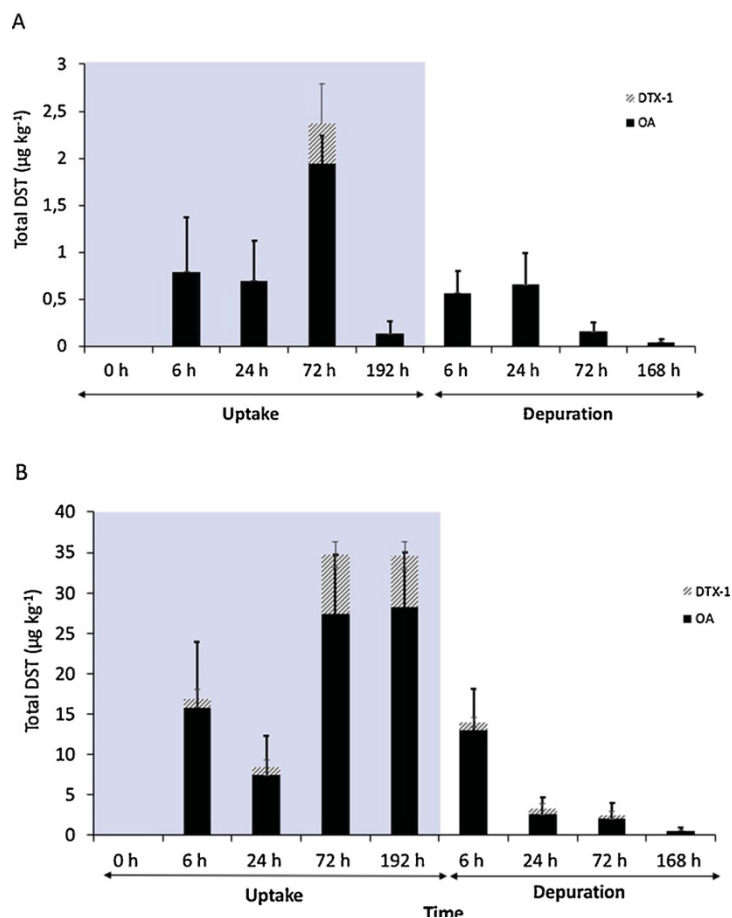


Figure 2.2. Total concentration (mean $\pm$ standard error; $\mu\text{g kg}^{-1}$) of diarrhetic shellfish toxins (DST) in whole body of clams, *Anomalocardia flexuosa*, during 192 h of toxin uptake followed by 168 h of depuration. During uptake phase, clams were exposed to *P. lima* cells at either (A) 10 cells mL^{-1} – T10 or (B) $100 \text{ cells mL}^{-1}$ – T100.

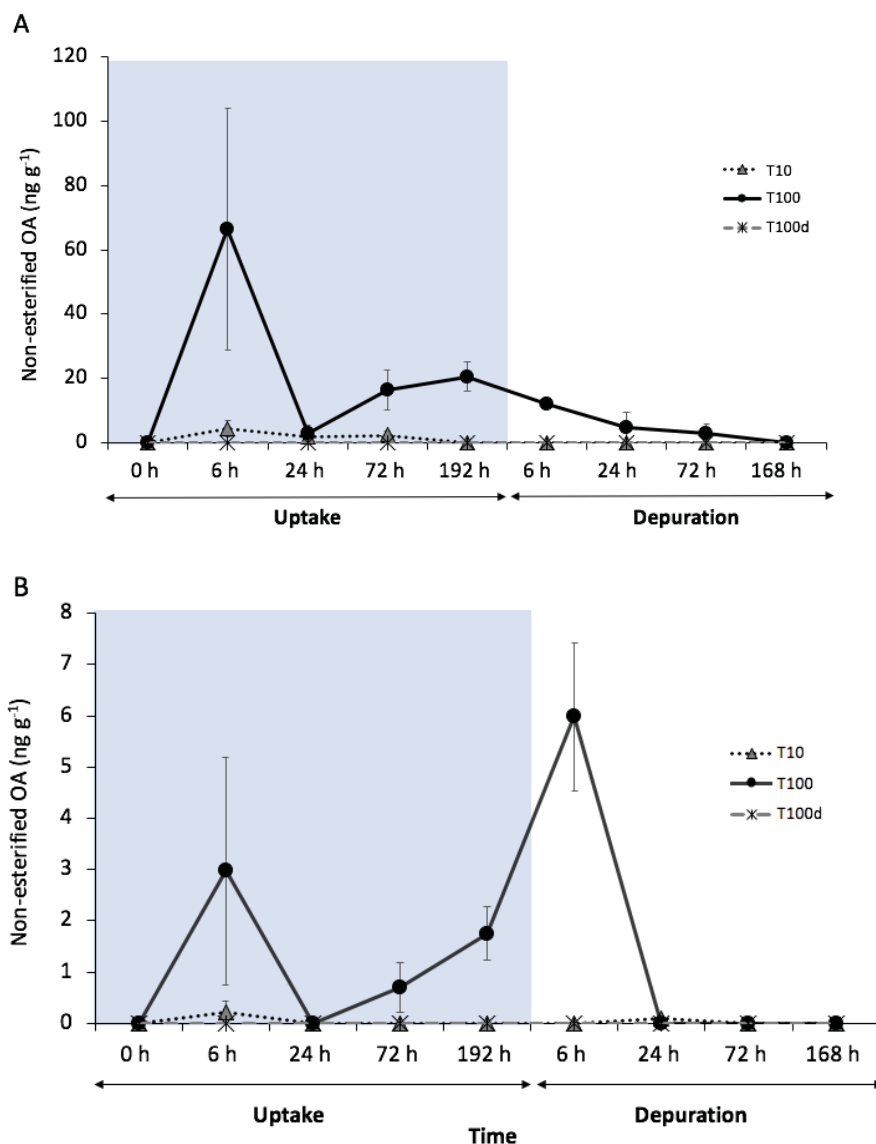


Figure 2.3. Concentrations (mean $\pm$ standard error; $\mu\text{g kg}^{-1}$) of free okadaic acid (OA) in (A) digestive glands and (B) remaining tissues of clams, *Anomalocardia flexuosa*, during 192 h of toxin uptake followed by 168 h of depuration. During uptake phase, clams were exposed to *P. lima* cells at either 10 (T10) or 100 (T100) cells mL^{-1} , or to their cellular contents dissolved in seawater at concentrations equivalent to 100 cell mL^{-1} (T100d).

Clams quickly converted the assimilated toxins into their esterified (conjugated) forms in their DGs. OA-esters became the dominant toxic compounds in DGs after only 24 h of exposure to 100 *P. lima* cells mL^{-1} in T100, and 72 h of exposure to 10 *P. lima* cells mL^{-1} in T10. On average, OA-esters represented up to 97 % of the total OA amount during uptake and early depuration phases, and ~60 % at the end of the depuration period in both treatments (Figure 2.4). Total OA levels (OA + OA-esters) measured in DG of *A. flexuosa* attained 28 $\mu\text{g kg}^{-1}$ after 72 h of exposure to *P. lima* cells in T10 and 567 $\mu\text{g kg}^{-1}$ after 192 h in T100

(Figure 2.4). Similarly, DTX-1 was quickly and fully converted into DTX-1 esters in the DG, reaching detectable levels after 6 h of exposure to toxin-containing cells in T100 and 72 h in T10 (Figure 2.5). Total DTX-1 levels increased until the end of the toxin uptake phase, attaining a maximum average value of $135 \mu\text{g kg}^{-1}$ at the end of that period in T100.

Total OA levels decreased rapidly in both treatments during the depuration phase. Only 3 % of the maximum concentration were detected in clams after 168 h of depuration in T10 and 1.6 % in T100. Detoxification rates – as calculated during the depuration period – reached 0.024 h^{-1} for OA-ester and 0.052 h^{-1} for free OA. Total DTX-1 levels (composed of 100 % DTX-1 esters) also decreased exponentially and became nearly undetectable after 168 h of depuration (Figure 2.5), representing a detoxification rate of 0.04 h^{-1} during that period. The detoxification rate for combined DSTs (OA + DTX-1) in the whole body of *A. flexuosa* was 0.029 h^{-1} in T100, and the DST half-life ($t_{1/2}$) was estimated as 24 h.

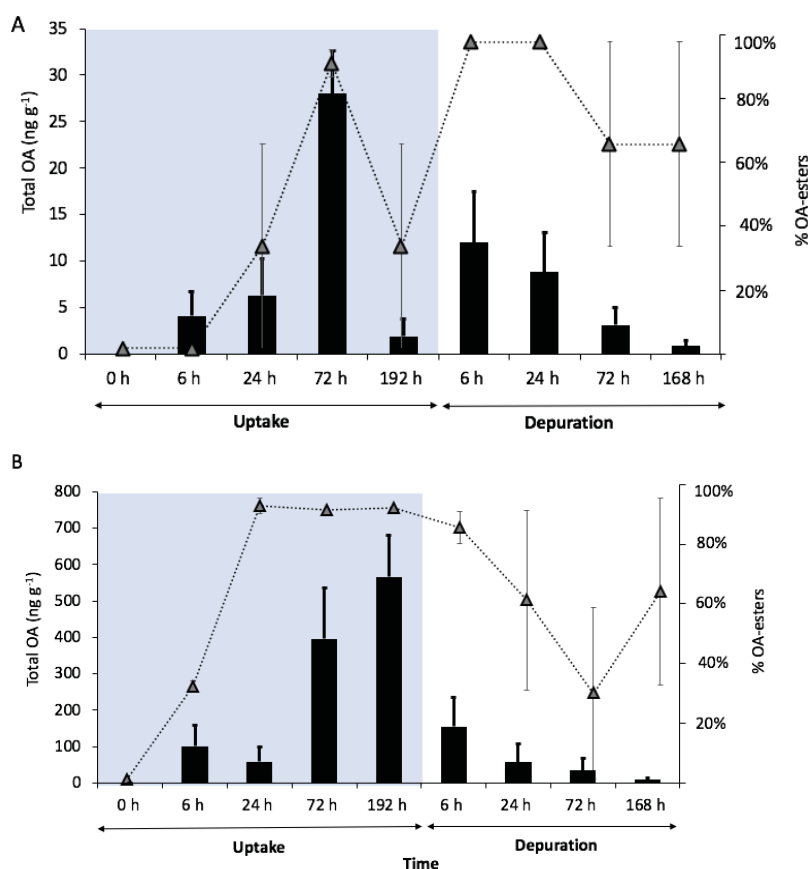


Figure 2.4. Total concentration (black bars; mean $\pm$ standard error, in $\mu\text{g kg}^{-1}$) of okadaic acid (OA + OA-esters), and proportion of OA-esters (grey triangles) in digestive glands of clams, *Anomalocardia flexuosa*, during 192 h of toxin uptake followed by 168 h of depuration. During uptake phase, clams were exposed to *P. lima* cells at either (A) 10 cells mL^{-1} – T10 or (B) 100 cells mL^{-1} – T100.

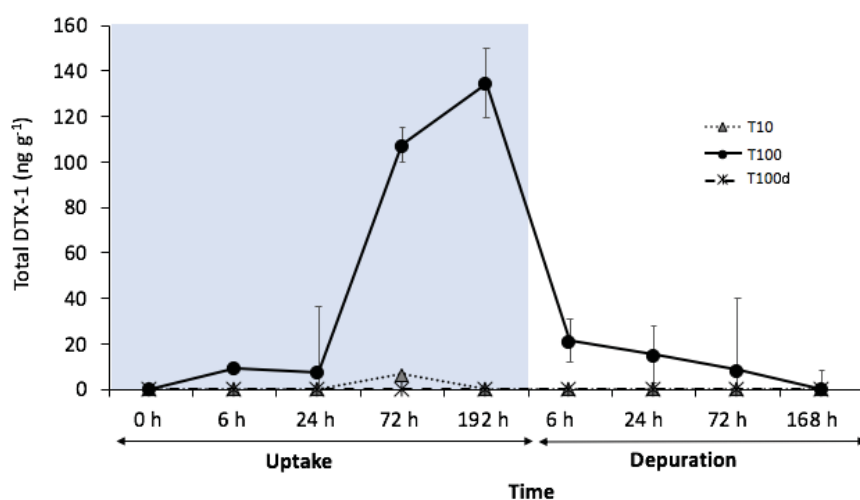


Figure 2.5. Total concentration (mean $\pm$ standard error; $\mu\text{g kg}^{-1}$) of dinophysistoxin-1 (DTX-1 + DTX-1 esters) in digestive glands of clams, *Anomalocardia flexuosa*, during 192 h of toxin uptake followed by 168 h of depuration. During uptake phase, clams were exposed to *P. lima* cells at either 10 (T10) or 100 (T100) cells mL^{-1} , or to their cellular contents dissolved in seawater at concentrations equivalent to 100 cell mL^{-1} (T100d). Toxin profiles were composed of 100 % DTX-1 esters over the entire experiment (i.e., free DTX-1 levels were undetectable).

2.3.3. Biochemical biomarkers

The activities of SOD, CAT, GST and the levels of LPO were similar in *A. flexuosa* individuals exposed to different diet treatments, both during the toxin uptake and depuration phases (Table 2.1 and Figure S2.2). Only the GPx activity varied among diets ($P = 0.0096$), being significantly inhibited in all treatments compared to the control condition during toxin exposure (Table 2.1 and Figure S2.2). Later, during depuration, differences in GPx activity were caused by the interaction between diet treatment and time ($P = 0.0026$). Post-hoc tests revealed an induction of GPx activity in T10, T100 and T100d treatments at early depuration period (i.e., 6–24 h). GPx activity was then inhibited at 72 h of depuration in T100d, and no difference was detected among treatments after 168 h of depuration (Table 2.1 and Figure S2.2).

In contrast, activities and levels of all biomarkers varied significantly over time, except those of SOD during exposure and CAT during depuration (Table 2.1 and Figure S2.2). Most enzymatic antioxidant defenses were induced at later exposure periods: CAT and GST activities were significantly higher at 72 h and 192 h, while GPx activity increased from 24 h until the end of the toxin exposure phase. Levels of LPO did not vary significantly among the sampling times during toxin exposure. In the depuration period, the activity of SOD significantly decreased over time (Table 2.1 and Figure S2.2). Conversely, GST activity and LPO levels significantly increased at early (6 h) and late (168 h) depuration phase compared to intermediate periods (24–72 h) (Table 2.1 and Figure S2.2).

The integrated responses of oxidative stress biomarkers (IBR), as expressed by the deviation index A (Table 2.2), varied among treatments (diets) and over time during both experimental phases. In general, deviation values were greater during the depuration period compared to the uptake phase. In addition, for all treatments, the highest values were recorded at the end (168 h) of the depuration period (Figure 2.6). The integrated responses were similar between treatment T100d and the control diet (C), whereas T10 elicited the most contrasting responses (Figure 2.6).

Table 2.1. Repeated measures analysis of variance for activities of CAT, SOD, GPx, GST and levels of LPO during toxin uptake and depuration phases (n = 3). For Scott-Knott *a posteriori* comparisons: “<” and “>” indicate $P < 0.05$ and “=” indicates $P > 0.05$. Significant terms of interest ($\alpha = 0.05$) are highlighted in bold. † P calculated using the Greenhouse-Geisser correction due to sphericity violation.

	Toxin Uptake					Depuration			
	df	MS	F	P		df	MS	F	P
CAT									
<i>Between subjects</i>									
Treatment	3	152.25	1.707	0.2424	3	130.83	1.048	0.4229	
Residuals	8	89.21			8	124.88			
<i>Within subjects</i>									
Time	4	181.75	3.999	0.0096	3	97.37	0.454	0.7169	
Treatment × Time	12	64.02	1.409	0.2127	9	61.49	0.287	0.9719	
Residuals	32	45.45			24	214.48			
Scott-Knott tests	Ti: 0h = 6h = 24h < 72h = 192h								
SOD									
<i>Between subjects</i>									
Treatment	3	5575.94	0.928	0.4705	3	9003.16	0.302	0.8236	
Residuals	8	6010.60			8	29858.43			
<i>Within subjects</i>									
Time	4	7309.72	0.995	0.4244	3	167757.0	12.288	0.0026 [†]	
Treatment × Time	12	10530.65	1.434	0.2018	9	19495.47	1.428	0.2869 [†]	
Residuals	32	7345.25			24	13651.84			
Scott-Knott tests					Ti: 6h > 24h > 72h = 168h				
GPx									
<i>Between subjects</i>									
Treatment	3	613.69	7.695	0.0096	3	91.03	2.119	0.1761	
Residuals	8	79.76			8	42.96			
<i>Within subjects</i>									
Time	4	1586.05	7.147	0.0081 [†]	3	2283.68	48.544	< 0.001 [†]	
Treatment × Time	12	508.90	2.293	0.0965 [†]	9	344.28	7.318	0.0026 [†]	
Residuals	32	221.92			24	47.044			
Scott-Knott tests	Tr: C > T10 = T100 = T100d Ti: 0h = 6h > 24h = 72h = 192h				Tr (6h): C = T100 < T10 = T100d Tr (24h): C = T10 < T100 = T100d Tr (72h): T10 > T100 > C > T100d Tr (168h): C = T10 = T100 = T100d				
GST									
<i>Between subjects</i>									
Treatment	3	2704.99	1.872	0.2127	3	1548.94	0.339	0.7981	
Residuals	8	1445.21			8	4571.50			
<i>Within subjects</i>									
Time	4	10732.19	5.990	0.0010	3	14202.29	4.319	0.0143	
Treatment × Time	12	908.0	0.507	0.8947	9	797.73	0.243	0.9839	
Residuals	32	1791.57			24	3288.17			
Scott-Knott tests	Ti: 6h = 24 h < 0h = 72h = 192h				Ti: 6h = 168h > 24h = 72h				
LPO									
<i>Between subjects</i>									
Treatment	3	163.72	0.640	0.6101	3	83.21	0.103	0.9562	
Residuals	8	255.71			8	810.24			
<i>Within subjects</i>									
Time	4	882.08	3.137	0.0277	3	1421.93	3.372	0.0350	
Treatment × Time	12	413.55	1.471	0.1867	9	315.12	0.747	0.6634	
Residuals	32	281.21			24	421.66			
Scott-Knott tests	Ti: 0h = 6h = 24h = 72h = 192h				Ti: 6h = 168h > 24h = 72h				

Table 2.2. Sum of absolute values of the estimate deviation biomarker index (A) for *Anomalocardia flexuosa* clams exposed to different treatments/diets (T) over 192 h, followed by 168 h of depuration (D). C was the non-toxic, control diet.

Treatment	6h	24h	72h	192h	6h D	24h D	72h D	168h D
C	3.0	1.8	5.3	6.9	9.8	4.7	6.4	9.5
T10	5.6	3.9	2.3	4.5	1.6	2.0	7.1	7.8
T100	4.6	7.1	8.3	7.7	7.3	5.6	6.8	12.7
T100d	6.1	10.0	5.7	6.9	8.4	7.4	8.4	10.5

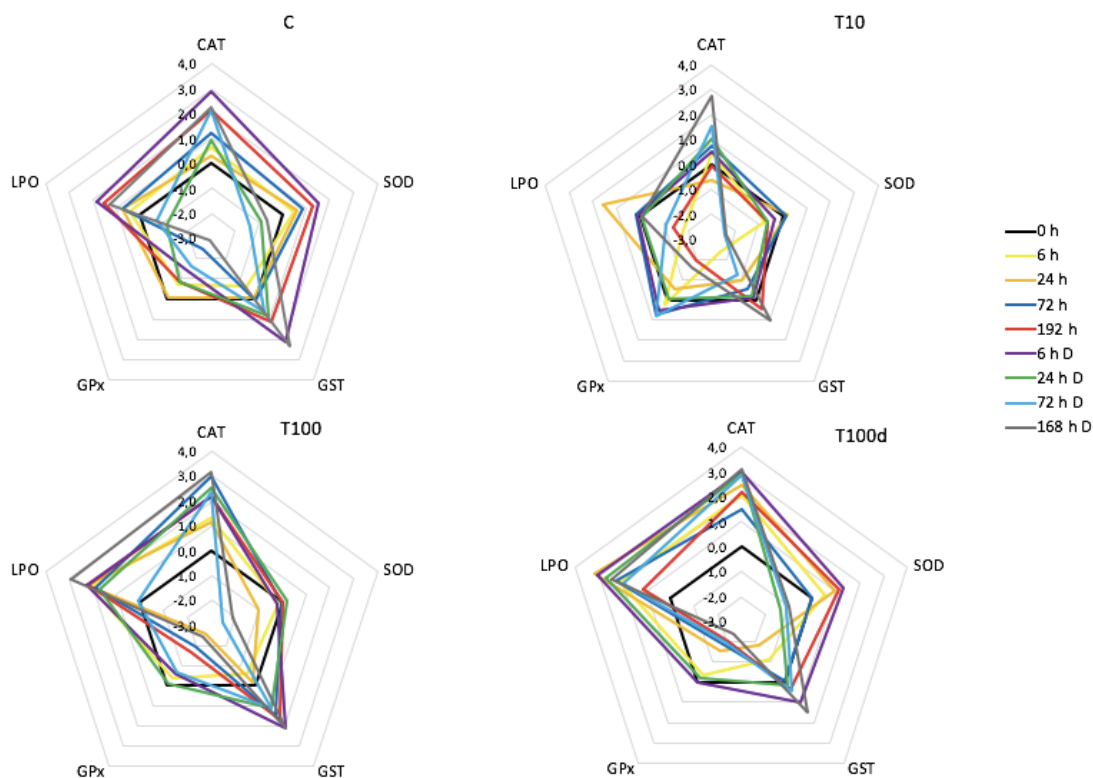


Figure 2.6. Integrated Biomarker Response index (IBR) of oxidative stress biomarkers in digestive glands of *Anomalocardia flexuosa* clams exposed to different treatments/diets (T) over 192 h, followed by 168 h of depuration (D), in relation to the initial condition (time 0). C was the non-toxic, control diet. CAT: catalase; SOD: Superoxide dismutase; GST: Glutathione S-transferase; GPx: Glutathione peroxidase; LPO: Lipoperoxidation.

2.4. Discussion

2.4.1. Toxin kinetics

Average biomass of clams (soft tissues) remained nearly constant throughout the experiment, so that differences in toxin accumulation and elimination, as well as in the sub-lethal negative effects observed throughout the experiment cannot be attributed to any eventual gain or loss of weight (REGUERA et al., 2014; SVENSSON, 2003).

In one of the diets tested in the present study (T100d), seawater in the tanks contained relatively high levels of dissolved DST ($2.7\text{--}7.9\ \mu\text{g OA L}^{-1}$ and $0.45\text{--}1.54\ \mu\text{g DTX-1 L}^{-1}$). In those tanks, exposed *A. flexuosa* individuals actively fed on non-toxic *Tetraselmis* sp. cells over the course of the experiment, however, no DST was detected in their DGs or in remaining soft tissues including the gills. This result indicates that, contrary to what has been suggested for mussels (LI et al., 2018), uptake of dissolved DSTs from seawater by *A. flexuosa* clams should be negligible under ecologically relevant toxin concentrations.

During toxic blooms in the field, adsorption of dissolved toxins on particulate matter has been suggested as an important alternative route for toxin uptake by exposed suspension feeders (MAFRA et al., 2019), including natural populations of *A. flexuosa* (ALVES et al., 2020). In the present study, however, adsorption of dissolved DSTs on *Tetraselmis* cells must have been minimal – if any – during the uptake phase of the experiment. Likewise, the presence of non-toxic microalgal cells (*Isochrysis galbana*) as food had no significant effect on the uptake of dissolved toxins by *M. galloprovincialis* mussels (LI et al., 2018). Taken altogether, these findings suggest that living cells or poorly organic, detrital particles must possess a limited role in DST adsorption and transfer to suspension feeders in natural environments. Effectiveness of DST transfer following adsorption on other particles, including faecal pellets and other highly organic detritus, remains to be demonstrated.

Clams exposed to *P. lima* cells quickly attained greater OA levels in the DG than in the remaining soft tissues. These small organs (5–11 % of the total soft tissue weight) concentrated up to 93 % of the total OA burden in *A. flexuosa*. This is compatible with previous reports of bivalves accumulating large proportions (>70 %) of OA in the DG during toxic dinoflagellate blooms, including mussels, oysters, scallops and other clam species (BAUDER et al., 2001; BLANCO et al., 2007; MAFRA et al., 2015; MOROÑO et al., 2003; MURATA et al., 1982; PILLET et al., 1995; YASUMOTO et al., 1978). In bivalves, the DG is one of the first organs/tissues in contact with toxins after their assimilation from ingested toxic cells (BLANCO, 2018), and its high lipid content increases the affinity for exogenous lipophilic

compounds like DSTs (GOSLING, 2003; SVENSSON and FORLIN, 2004). In our experiment, DGs of *A. flexuosa* accumulated moderate levels ($60\text{--}70\ \mu\text{g kg}^{-1}$) of total OA (OA + OA-esters) only six hours after exposure to the diet containing 100 cells *P. lima* mL^{-1} .

Okadaic acid levels in non-digestive tissues were approximately 10 to 20-fold lower than those accumulated in the DG of *A. flexuosa* during toxin exposure. Even though, OA levels in non-digestive tissues increased substantially and consistently after six hours of depuration in T100 and after 24 h in T10. This suggests active DST transfer among tissues at early depuration stages, as previously documented for oysters, mussels and scallops exposed to DSTs (MAFRA et al., 2015), saxitoxins (LI et al., 2005) and domoic acid (ÁLVAREZ et al., 2020; MAFRA et al., 2010). It is possible that the association of OA with high-density lipoproteins (HDLs) in the DG cells (ROSSIGNOLI and BLANCO, 2010) may have facilitated its transport from the DG to other tissues/organs. These proteins are associated with the transport of other lipophilic substances, like cholesterol to liver and other tissues in humans, for instance, and with their subsequent elimination (JONAS, 2002).

Over the bivalve digestion process, OA, DTX-1 and DTX-2 may undergo a series of transformations, including the esterification with fatty acids forming 7-O-acyl derivatives (often called DTX-3) and hybrid diol-fatty acid esters (TORGERSEN et al., 2008). Conjugation of DSTs with endogenous molecules allow further release of more polar compounds through the route of xenobiotic elimination, a common process in several bivalves (BLANCO, 2018; MOROÑO et al., 2003). In our study, the high proportions of OA-esters (>97 %) and DTX-1 esters (100 %) measured in DG samples after 24 h and 6 h, respectively, of exposure to *P. lima* cells are indicative of quick transformation of DSTs by *A. flexuosa*. For DTX-1, whose concentrations were ~5 times lower than those of OA in *P. lima* cells, the free form was already undetectable after only 6 h of exposure to the highest *P. lima* cell density ($100\ \text{cells mL}^{-1}$). At that time, free OA concentrations in the DG were relatively high (up to $104\ \mu\text{g kg}^{-1}$), dropping to $\leq 29\ \mu\text{g kg}^{-1}$ thereafter. Likewise, field studies have shown that wild *A. flexuosa* clams, as well as farmed mussels, scallops and oyster can accumulate moderate amounts of free OA at initial stages of *Dinophysis* spp. blooms, and then build up increasing concentrations of OA-esters over extended exposure periods (ALVES and MAFRA, 2018; LINDEGARTH et al., 2009; MAFRA et al., 2019; SUZUKI and MITSUYA, 2001). Accumulation of DST esters would be even more pronounced in bivalves exposed to *P. lima* cells already containing great proportions of toxins in their esterified forms, as reported in selected *P. lima* strains (WU et al., 2020), but not in the strain used in the present study (LM-046).

Concentrations of OA-esters dropped quickly and their relative contribution to the total OA burden gradually decreased in the DG of *A. flexuosa* during the depuration period. In contrast, relatively low concentrations ($<10 \mu\text{g kg}^{-1}$) of OA in its free form persisted for a longer period in that same tissue. Such a pattern has been previously described as a two-compartment detoxification process in oysters, mussels and scallops (BAUDER et al., 2001; MAFRA et al., 2015; MARCAILLOU-LE BAUT et al., 1993), and may involve the existence of high-affinity toxin binding sites. Estimated depuration rates for DST-esters are, in general, higher than those of their free forms in bivalves (reviewed in VALE et al., 2008), but the opposite has been also reported (LINDEGARTH et al., 2009). Here, we estimate two-fold faster detoxification rates for OA-esters relative to its free form, and 30 % faster detoxification of OA-esters relative to DTX-1 esters.

Whole *A. flexuosa* individuals eliminated DSTs (OA + DTX-1) at slightly faster rates (0.029 h^{-1}) than bivalves like mussels, *P. perna* (0.023 h^{-1}), and oysters, *C. gigas* (0.010 h^{-1}) and *Crassostrea gasar* (0.004 h^{-1}) under equivalent laboratory conditions (MAFRA et al., 2015). Detoxification rates under natural, field conditions, however, are usually lower (MAFRA et al., 2015), partially due to re-contamination during and after the toxic bloom. In our experimental study, quick DST elimination can be associated with the intense digestive activity and metabolic fecal losses elicited by the constant replacement of non-toxic *Tetraselmis* sp. cells during depuration (BLANCO et al., 1997; HAWKINS et al., 1990), as described in scallops, *Pecten maximus* (Bougrier et al., 2003). In addition, feeding the clams with a background level of non-toxic microalgae may have prevented them from acquiring higher toxin levels due to a toxin diluting effect (BAUDER et al., 2001).

During the present investigation, *A. flexuosa* clams contained much lower whole-body DST levels (up to $35 \mu\text{g kg}^{-1}$) than the regulatory limit for human consumption ($160 \mu\text{g kg}^{-1}$). Nevertheless, under exceptional circumstances, such as those recorded during a massive *Dinophysis* spp. bloom (up to $5200 \text{ cells mL}^{-1}$) in southern Brazil, wild *A. flexuosa* clams may accumulate much higher OA levels (up to $1300 \mu\text{g kg}^{-1}$), although still below those accumulated by co-occurring mussels and oysters (MAFRA et al., 2019). Such lower capacity for DST accumulation by *A. flexuosa* clams must be associated with their fast toxin transformation and elimination rates, as reported in the present study.

2.4.2. Sublethal effects to exposed clams - oxidative stress

The presence of DSTs – in either particulate or dissolved forms – caused no apparent feeding inhibition to exposed clams. Neither abnormal shell closure nor decreasing clearance of non-toxic *Tetraselmis* cells from suspension was noticed throughout the 7-day toxin exposure period. Slightly greater levels of dissolved DSTs ($9.2 \mu\text{g OA L}^{-1} + 13.2 \mu\text{g DTX-1 L}^{-1}$), in contrast, caused marked clearance rate inhibition in the mussel *M. galloprovincialis* (LI et al., 2018), suggesting that *A. flexuosa* may be relatively resistant to these compounds.

Variations in the activity of antioxidant enzymes may indicate high-efficiency antioxidant defense mechanisms in bivalves exposed to algal toxins, including bay scallops (*Argopecten irradians*) exposed to domoic acid-producing *Pseudo-nitzschia* cells (SONG et al., 2020), freshwater clams (*Diplodon chilensis*) exposed to microcystin-LR (SABATINI et al., 2011) and mussels (*Mytilus galloprovincialis*) exposed to palytoxin-like compounds in *Ostreopsis* cf. *ovata* cells (GORBI et al., 2012, 2013). In the present study, significant temporal variations were reported in the activity of CAT during depuration and SOD during exposure to DSTs, and the activity of GST, GPx and LPO levels during both toxin exposure and depuration periods in the DG of *A. flexuosa*. This may be related to increasing ROS production induced to protect the cells from oxidative damage. However, there was no clear distinction in the antioxidant responses of *A. flexuosa* clams exposed to non-toxic phytoplankton cells alone (control), or in the presence of high *P. lima* cell abundances ($100 \text{ cells mL}^{-1}$), contrary to what has been reported for algal cells containing palytoxin-like compounds (GORBI et al., 2013). Moreover, clams exposed to dissolved compounds from *P. lima* exhibited levels of oxidative stress biomarkers comparable to those maintained under control condition throughout the experiment, except for GPx activity at early depuration phase (6–24 h). This indicates no obvious negative effects from dissolved DSTs at ecologically relevant concentrations in this bivalve species. Thus, temporal variation in oxidative stress biomarkers, as described herein for clams, might be due to undetermined factors during their long-term maintenance under laboratory conditions, or to complex environmental stressors present at the site where they were collected.

In this study, clams exposed to either 10 or 100 *P. lima* cells mL^{-1} —or to their dissolved compounds – increased their GPx activity at early depuration and their GST and LPO levels at late depuration phase. This would be mainly related to the recovery of clam's antioxidant system over the depuration period. Activity of GPx, SOD and CAT were induced in the DG

and muscles of *M. galloprovincialis* to eliminate the high production of ROS upon exposure to *A. tamarense* cells containing paralytic shellfish toxins (PSTs) (QIU et al., 2013). In contrast, when exposed to DST-producing *P. lima* cells, *Crassostrea ariakensis* oysters maintained their GST activity unchanged in the DG (ZOU et al., 2015). The high GST activity and LPO levels observed in animals exposed to all diets tested in the present study (including the control condition), may suggest a chronic exposure to ROS and cellular damage in DG, induced by an intensified metabolism or detoxification process of exogenous compounds other than DSTs (SARDI et al., 2020).

Our results suggest a limited capacity of DSTs to induce oxidative stress in exposed *A. flexuosa* under laboratory conditions. Thus, *A. flexuosa* clams may possess some mechanism to avoid oxidative stress generated by DSTs. The co-occurrence of *A. flexuosa* and the dinoflagellates *Dinophysis* spp. and *Prorocentrum* spp. in southern Brazil suggest repeated exposure of this clam to DSTs in the field (MAFRA et al., 2019; MOREIRA-GONZÁLEZ et al., 2018). This may have led this species to develop an efficient mechanism to deal with toxic compounds, in evolutionary time, as indicated for other bivalves exposed to PSTs (BRICELJ et al., 2005).

2.5. Conclusion

The present results highlight the limited capacity for DST accumulation in *A. flexuosa* from toxin-containing *P. lima* cells, and negligible uptake when the toxins were dissolved in seawater. Low toxin accumulation was mostly related to quick toxin transformation and elimination. Such efficient detoxification processes might be strategic for the management of DST-contaminated bivalve stocks in aquaculture and harvesting sites. In addition to fast DST detoxification, *A. flexuosa* exhibited no apparent negative responses upon exposure to these compounds via either particulate or dissolved routes. *Anomalocardia flexuosa* may thus become an interesting alternative resource in areas frequently affected by DST-producing dinoflagellates blooms, such as southern Brazil.

3. CAPÍTULO 3: EXPERIMENTAL EVIDENCE OF CIGUATOXIN ACCUMULATION AND DEPURATION IN CARNIVOROUS LIONFISH

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Abstract

Ciguatera poisoning is a food intoxication associated with the consumption of fish or shellfish contaminated, through trophic transfer, with ciguatoxins (CTXs). In this study, we developed an experimental model to assess the trophic transfer of CTXs from herbivorous parrotfish, *Chlorurus microrhinos*, to carnivorous lionfish, *Pterois volitans*. During a six-week period, juvenile lionfish were fed naturally contaminated parrotfish fillets at a daily dose of 0.11 or 0.035 ng CTX3C equiv. g⁻¹, as measured by the radioactive receptor binding assay (r-RBA) or neuroblastoma cell-based assay (CBA-N2a), respectively. During an additional six-week depuration period, the remaining fish were fed a CTX-free diet. Using r-RBA, no CTXs were detectable in muscular tissues, whereas CTXs were measured in the livers of two out of nine fish sampled during exposure, and in four out of eight fish sampled during depuration. Time-point pooled liver samples, as analyzed by CBA-N2a, confirmed the accumulation of CTXs in liver tissues, reaching 0.89 ng CTX3C equiv. g⁻¹ after 41 days of exposure, followed by slow toxin elimination, with 0.37 ng CTX3C equiv. g⁻¹ measured after the six-week depuration. These preliminary results, which need to be pursued in adult lionfish, strengthen our knowledge on CTX transfer and kinetics along the food web.

3.1. Introduction

Gambierdiscus spp. are benthic dinoflagellates that may produce lipophilic ciguatoxins (CTXs), as well as other bioactive compounds, including maitotoxins (MTXs), gambierone, gambieroxides, gambierol, and gambieric acid (CUYPERS et al., 2008; HOPPENRATH et al., 2014; MOROHASHI et al., 2000; MURRAY et al., 2019; SOLIÑO & COSTA 2018; WATANABE et al., 2013). Ciguatoxins are considered the primary cause of Ciguatera poisoning (CP) in humans, affecting up to 500,000 fish consumers every year, including rare lethal cases (EFSA, 2010; FRIEDMAN et al., 2017; PARSONS et al., 2012; YASUMOTO et al., 1987). Affected people may experience temporary and/or persistent/recurrent neurological effects resulting from CTXs binding to voltage-gated sodium channels in excitable tissues

(DECHRAOUI et al., 1999; KUMAR-ROINÉ et al., 2011; LOMBET et al., 1987; POLI et al., 1986; AU et al., 2016). Ciguatera symptoms include the tingling of body extremities and/or cold allodynia and, in more severe cases, cardiovascular and respiratory insufficiency leading to coma and death (KUMAR-ROINÉ et al., 2011). Chronic neurological manifestations may persist for weeks, months, and in severe cases, years after the first exposure to CTXs (FRIEDMAN et al., 2017; CHINAIN et al., 2019; DICKEY et al., 2010). Symptoms may vary geographically, according to the dominant toxin profile in each region. Gastrointestinal disorders such as nausea, vomiting, abdominal pain, and diarrhea are predominantly manifested in the Caribbean region, where fish are contaminated by the so-called Caribbean ciguatoxins (C-CTXs). In contrast, in the Pacific and Indian Oceans, neurotoxic symptoms may predominate following the consumption of fish containing P-CTXs and I-CTXs, respectively. Neurotoxic symptoms may include hallucinations mostly among victims from the Indian Ocean (FRIEDMAN et al., 2017).

The effects of CTXs on marine fauna are less documented. In fish, adverse effects from exposure to CTX have been observed in fresh/brackish water species such as blueheads, *Thalassoma bifasciatum* (DAVIN et al., 1986), juveniles of *Oreochromis* sp. (KELLY et al., 1992), larvae and embryos of the genus *Oryzias* (COLMAN et al., 2004; EDMUNDS et al., 1999; MAK et al., 2017; YAN et al., 2017; 2020), and adult *Mugil cephalus* (LEDREUX et al., 2014). Ciguatoxins found in the brain, liver, and muscles of stranded *Monachus schauinslandi* monk seals (BOTTEIN et al., 2011) suggest that marine mammals may also suffer from CTX exposure, and that these compounds persist within the complex marine food webs. However, marine species experientially fed with CTX (*Naso brevirostris* and *Epinephelus coioides*) did not seem sensitive to the effects of CTX (CLAUSING et al., 2018; LI et al., 2020). The fish resistance mechanism to CTX is still unknown.

Following the ingestion of *Gambierdiscus* cells by herbivorous and omnivorous organisms, CTX-like toxins are biotransformed and transmitted along the trophic food webs to top-chain carnivores, including fish, such as groupers and snappers, and marine mammals (BOTTEIN et al., 2011; DECHRAOUI-BOTTEIN et al., 2005; SANCHEZ-HENAO et al., 2020; LI et al., 2020; YOGI et al., 2011). More than 400 species of fish are suspected as potential vectors of CP to humans (HALSTEAD, 1978). Coral reef fish known to accumulate CTXs include barracuda, grouper, snapper, moray eel, parrotfish, trevally, and wrasse (CLAUSING et al., 2016; FAO/WHO, 2020). In general, higher-level carnivorous fish exhibit greater toxin concentrations than smaller fish and herbivores (CHAN et al., 2011; DÍAZ-ASENCIO et al., 2019; LEWIS & HOLMES, 1993), but this is not always the case (CHINAIN et al., 2010; 2020;

DARIUS et al., 2007; GABORIAU et al., 2014; MAK et al., 2013). The complex processes and kinetics involving CTX accumulation, elimination, and trophic transfer to carnivores are still poorly understood and limited to field observations (MAK et al., 2013) and scarce laboratory studies (LI et al., 2020).

Lionfish, *Pterois volitans* (Scorpaenidae), are carnivorous fish with venom-containing spines (SAUNDERS & TAYLOR, 1959). They are native to the Indo-Pacific region and were mostly likely introduced through aquarium releases into the Atlantic Ocean, becoming invasive to the Caribbean Sea and the Gulf of Mexico (SNYDER & BURGESS et al., 2007; WHITFIELD et al., 2002). Due to the absence of natural predators for lionfish in reef ecosystems where they have been recently introduced, their use as a fishery resource has been stimulated to reduce the adverse ecological impacts on reef communities (HARDISON et al., 2018; MORRIS & WHITFIELD, 2009). However, as with many other species, lionfish may accumulate CTXs and reach toxin concentrations above safety levels for human consumption, as recommended by the U.S. Food and Drug Administration (0.1 ng C-CTX1 equiv. g⁻¹, or 0.01 ng CTX1B equiv. g⁻¹) (FDA, 2011). Concentrations of CTXs in lionfish vary geographically, primarily depending on the local abundance and composition/dominance of *Gambierdiscus* species. In the Caribbean Ciguatera-endemic region, for instance CTX accumulation in lionfish muscles can be highly frequent ($\geq 40\text{--}50\%$), and reach levels up to 0.3 ng C-CTX1 equiv. g⁻¹ (HARDISON et al., 2018; ROBERTSON et al., 2014). This suggests that *P. volitans* could become a common vector for CP in that region, where other top predators (e.g. snapper, barracuda, and grouper) can accumulate even higher CTX levels, posing a risk to human health (HARDISON et al., 2018; ROBERTSON et al., 2014). Those toxin-equivalent concentrations must be considered carefully, however, because the venoms produced by lionfish itself can potentially interfere with the toxicity bioassays commonly used to quantify CTX. To avoid false positive results on CTX tests, cooking the fillets of lionfish is recommended before testing for the presence of ciguatoxin. This procedure denatures the scorpaenitoxins, leaving only CTX-related toxicity if present (WILCOX & HIXON, 2014). Regardless, at least one case of CP following the consumption of lionfish has been already confirmed (BOUCAUD-MAITRE et al., 2018).

Considering the ecological relevance of *P. volitans* associated with its potential threats as an invasive species and emerging CP vector, this species was selected in the present study to study the transfer of CTX from a prey to a carnivorous reef fish predator. The potential effects of CTXs, as well as their transfer, accumulation, and elimination were investigated during a long-term (twelve weeks) laboratory feeding experiment using naturally CTX-contaminated

steephead parrotfish, *Chlorurus microrhinos* (formerly *Scarus gibbus*) fillets as source of toxins. The presence and concentration of CTXs were evaluated in liver and muscle tissues of juvenile *P. volitans* during both toxin uptake and depuration phases, using distinct analytical methods, such as radioligand-receptor binding assay (r-RBA), neuroblastoma cell-based assay (CBA-N2a), and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

3.2. Materials and methods

3.2.1. Fish acclimation and maintenance

Juvenile lionfish, *Pterois volitans* (Scorpaenidae), originating from Bali, Indonesia, were acquired from a supplier (Tropic Nguyen, France). The juvenile lionfish were selected based on maintenance and individual replication needs considering the laboratory setting and aquarium size. Upon reception, the lionfish were acclimated for approximately two weeks in a 2000 L open-circuit tank. The tank was filled with flowing 1 μm filtered seawater at 400 L h⁻¹, 25 $\pm$ 0.5 °C, pH 8.1 $\pm$ 0.1 and a salinity of 39, and maintained under a 12: 12 h (light: dark) cycle and permanent aeration. Fish were fed once a day during acclimation in amounts higher than needed for maintenance (equivalent to 5% of their body weight). The food consisted initially of living prey (guppy or seabream). Then, inert frozen food (*Antherina boyeri* and krill) was gradually incorporated into the diet before the final transition to the frozen, CTX-containing parrotfish (*Chlorurus microrhinos*) fillet.

3.2.2. Preparation of the experimental diets

The toxic material used for the feeding experiments consisted of naturally CTX-contaminated fillets of the parrotfish *Chlorurus microrhinos* collected from Moruroa Atoll (Tuamotu Archipelago, French Polynesia). Parrotfish were selected based on the CTX levels in their fillets. Then, 7.8 kg (wet weight) of selected fillet were homogenized using an industrial mixer to obtain a toxic, homogenous fish fillet matrix. Toxicity of the prepared food was evaluated by means of cytotoxicity using CBA-N2a and binding affinity using r-RBA composed of 3 $\times$ 10 g and 8 $\times$ 5 g aliquots of the dried homogenate, respectively.

Different food preparations using Gelly Belly™ (Gel Food, Florida Aqua Farms, Inc., USA) and frozen food were tested. Gelly Belly™ consists of a gelatin-based food mixed with microalgae, seaweeds, fish, and krill meal, with added vitamins and minerals. The most appropriate food presentation for lionfish proved to be homogenized cube-shaped frozen fillets.

Frozen food dices were individually offered to lionfish using forceps, which were instantaneously caught and ingested without significant particle loss.

The food provided to the individuals in the control group and to all remaining lionfish during the depuration phase was composed of homogenized fillet of farmed seabream (*Sparus auratus*; ~120-140 g total body weight), originated from Turkey and supplied by Relais d'Or (France). After homogenization, seabream fillets were fashioned into cubes, placed in plastic bags, and kept at -18 °C, until being offered to the carnivorous fish as frozen cubes. A sample of seabream fillets was collected and further tested for CTX presence.

3.2.3. Experimental design

After acclimation, *P. volitans* individuals ($n = 48$; 5.05 – 34.0 g initial wet weight) were distributed among seven aquaria, allocating six fish per tank, except in two tanks where ten and eight fish were placed due to the smaller size of some individuals (~5 g) (Figure 3.1). This step aimed at obtaining similar fish sizes and total living biomass ($15.2 \text{ g} \pm 9.52$, mean $\pm$ SE) across the experimental tanks. Tanks were randomly assigned to either toxin-containing ($n = 5$ tanks) or control diet ($n = 2$ tanks), each containing 100 L of filtered seawater maintained under constant aeration and flow (100 L h^{-1}), at the same conditions of temperature, salinity, and pH used during the acclimation phase. To reduce animal stress, three tubes of $10 \text{ cm} \times 20 \text{ cm}$ (D $\times$ L) assembled as a pyramidal structure were placed inside the aquaria to provide shelter for the fish (Figure 3.1). Finally, the aquarium walls were covered with opaque plastic coating to limit potential stress caused by the human presence in the laboratory. Standards of animal welfare were rigorously maintained throughout the experiment. All procedures were carefully conducted to minimize handling and reduce physiological stress.

After six weeks of exposure to either the toxin-containing or control diet, fish entered the depuration stage, during which they received non-contaminated frozen food (homogenized seabream fillets) for an additional 6-week period. Fish feedings were carefully conducted throughout the experiment, assuring quick capture (usually $< 5 \text{ min}$) and no significant particle rejection. Some individuals in the control group were maintained over the entire duration of the experiment (12 weeks) and served as reference for possible visual behavior alterations and/or intoxication symptoms relative to dosed fish. The behavioral pattern was observed daily in all tanks during the feeding period throughout the 12-week experiment. During the exposure and depuration phases, the number of deaths was computed in relation to the total number of fishes at the beginning of the experiment, while the percentage of fish rejecting the toxic food considered the average number of individual remaining in each experimental condition.

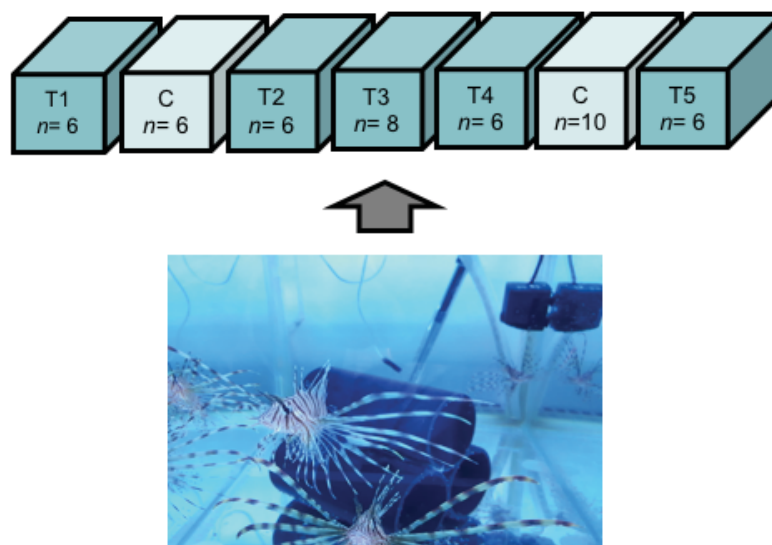


Figure 3.1. Design of the experiment, with five tanks initially containing six or eight fish (n) assigned to the CTX-exposed treatment (T) and two tanks for control (C) containing six fish and ten fish (n). The image represents a picture of the juvenile lionfish (*Pterois volitans*) individuals and their shelters made of dark tubes in one of the experimental tanks.

3.2.3.1. Feeding experiment

During the experiment, all lionfish individuals were fed in a constant proportion to 4–5% of their body weight per day, as recommended for juvenile fish. The amount of food supplied was adjusted weekly based on the average fish weight for each tank. The lionfish were individually fed, always in the afternoon (2:00 p.m.), for five days a week. Individuals were observed over 30 min to assure complete food ingestion after every feeding procedure. At the end of this time interval, any rejected food was removed from the tank during the daily cleaning procedure. Lionfish usually consumed the entire portion of toxic food offered in less than 15 min. In addition, the tanks were connected to an open system under a constant seawater flow. Thus, any residual toxin eventually released from the food or excreted by the fish would be quickly eliminated from the tanks. Therefore, the source of CTX to the exposed fish was considered limited to the food pathway.

After 30 days of exposure, four CTX-exposed and three control lionfish were randomly collected from the control tanks. Likewise, between two and five individuals from the exposed group were sampled each time, at the end of the exposure period (41 days) and after 8, 29, and 43 days of the following depuration stage. Finally, three additional control individuals were collected after 8, 29, and 43 days of the depuration stage (Table 3.1). Fish euthanasia was

achieved using an overdose of eugenol, and the death was confirmed by the absence of respiratory movements (GREEN et al., 2012). Lionfish were dissected immediately following death to ensure the integrity of collected tissues. For toxin analysis, muscle and liver samples were individually weighed (wet weight) and stored in plastic tubes at -18 °C.

Table 3.1. Number of CTX-exposed juvenile *Pterois volitens* individuals (*n*) sampled after a given exposure or depuration period. In addition, fish fed non-toxic food (control) were sacrificed after 30 days of exposure and after 8, 29, and 43 days of depuration period (3 individuals at each time).

Periods of experiment	Cumulative number of experimental weeks	Days of exposure/depuration	Cumulative number of feeding episodes Toxic/control food	<i>n</i>
Exposure	5	30 days of exposure	22 / 0	4
	6	41 days of exposure	29 / 0	5
	7	8 days of depuration	29 / 6	3
Depuration	10	29 days of depuration	29 / 20	3
	12	43 days of depuration	29 / 30	2

3.2.4. Toxin determination in food and exposed fish

3.2.4.1. Sample extraction

The extraction of CTXs from the muscle and liver samples followed the procedures described in previous studies (CLAUSING et al., 2018; IAEA-TECDOC-1729, 2013; DÍAZ-ASENCIO et al., 2018). Briefly, each tissue sample from individual lionfish was extracted as a whole when the tissues weighed < 4 g, or partially, in 4 g aliquots after homogenization with T-25 digital Ultra Turrax (IKA Works, Germany). Tissue samples were then cooked in Falcon tubes in a water bath at 70 °C for 15 min, homogenized in acetone (3 mL g⁻¹ tissue) using a sonication probe (Branson digital probe cell breaker) for 2 min at 30% duty, and centrifuged for 3 min at 1400 × g. The supernatant was recovered from the tubes and the tissue pellet was homogenized twice again in acetone, as previously described. After three successive extraction steps, the supernatant fractions were combined and evaporated under nitrogen gas flow (Turbovap) in a water bath at 60°C. Dried extracts were resuspended in 5 mL of aqueous methanol (MeOH/H₂O 90:10), and the lipids were removed by solvent-solvent separation (three times) after the addition of an equal volume of *n*-hexane. The 1:1 solvent-solvent fraction was separated (3×) into an aqueous phase (MeOH/H₂O 60:40) and dichloromethane (DCM). Finally, the organic phase (DCM), containing the CTXs, was evaporated with nitrogen, resuspended in pure MeOH to 10 g tissue equivalent (TE) mL⁻¹ and stored at -18 °C until further analysis. Frozen homogenized fillet cubes of parrotfish (toxic food) and seabream (control

food) were extracted following the same procedure described for lionfish tissues, adjusting the sonication time to 20 min to ensure complete fish cell lysis.

3.2.4.2. Toxin analysis

3.2.4.2.1. radioligand-Receptor Binding Assay (r-RBA)

The presence and quantification of CTXs in parrotfish (*C. microrhinos*) fillet homogenates (toxic food) and in selected extracts of fish muscle and liver were first determined using the r-RBA (DECHRAOUI-BOTTEIN & CLAUSING, 2017). This detection method is based on the binding competition between CTXs from the sample and a radiolabeled brevetoxin (tritiated PbTx-3) for their common receptor on the voltage gated sodium channel (Na_v) using a porcine brain homogenate (Sigma Aldrich, St. Louis, USA) membrane preparation (DECHRAOUI et al., 1999, DECHRAOUI BOTTEIN et al., 2017).

The assay was performed on a microplate according to the method reported in (IAEA-TECDOC-1729, 2013), modified according to previous studies (CLAUSING et al., 2018; DÍAZ-ASENCIO, L. et al., 2018; DECHRAOUI BOTTEIN & CLAUSING, 2017). First, 35 μL of phosphate buffered saline (PBS-Tween®) with bovine serum albumin (BSA) (1 g L^{-1}) was added to each well of a 96-well filtration microplate (MultiScreen HTS FB Filter Plate MSFBN6B50, Millipore) to moisturize the membrane filter. Subsequently, 35 μL of either the standard CTX3C provided in dried form by Wako-Pure Chemicals, Osaka, Japan (2.85×10^{-9} to 1.92×10^{-12} M), a solution of PbTx-3 (Latoxan, Rosam, France) (1.8×10^{-8} M) used as an internal assay Quality Control (QC) (3×10^{-9} M in assay) or the diluted samples were added to each corresponding well, after vortex-mixing and sonication. Likewise, only one concentration of fish sample was tested, i.e., 0.6 g tissue equiv. mL^{-1} for the parrotfish toxic food and 0.6 tissue equiv. mL^{-1} and 0.12 g tissue equiv. mL^{-1} for lionfish muscle and liver, respectively. Each sample was tested in duplicate wells in one experiment. Then, 35 μL of the working solution of [^3H] PbTx-3 (1 nM assay concentration) and 195 μL of the diluted brain membrane homogenate (0.8 mg protein mL^{-1}) were sequentially added to all wells. After incubation for 1 h at 4 °C, each well was washed (3 $\times$) with 200 μL of ice-cold phosphate buffered saline solution (PBST) and filtered using a MultiScreen HTS vacuum collector system (Millipore, Massachusetts, USA) to remove the excess radiotracer [^3H] PbTx-3. Only the membrane receptor-bound toxin molecules were retained in the filter at this stage. After filtration, the microplate was placed on a counting cassette (Perkin-Elmer rigid 96 plate 14105), with 50 μL of liquid scintillant (Optiphase, Perkin-Elmer) per well. Finally, the plate was incubated in the

dark at room temperature for 2 h prior to quantification of the radioactivity in a beta-microplate counter (MicroBeta², Perkin-Elmer, Massachusetts, USA).

3.2.4.2.2. Liquid Chromatography Coupled with Tandem Mass Spectrometry (LC-MS/MS)

Analysis by LC-MS/MS was performed on liver samples of lionfish and in CTX-contaminated parrotfish homogenates to confirm the presence of CTXs in the extracts. Aliquots of the extracts remaining from the r-RBA analysis (10–100 μL) were dried off with nitrogen gas and re-suspended with the following volume of 90% aqueous MeOH: 200 μL for lionfish liver samples and 500 μL for parrotfish homogenate, yielding a final concentration of 0.5–2.6 g liver equiv. mL^{-1} and 0.4–1.1 g flesh equiv. mL^{-1} , respectively. Different dilutions reflected the amount of matrix available in each case. Toxin determination was performed on a UHPLC system (UFLC Nexera, SHIMADZU, Japan) coupled to a hybrid triple quadrupole-linear ion-trap API4000 Qtrap mass spectrometer (ABSciex[®], Framingham, MA, USA), equipped with a TurboV[®] electrospray ionization source.

Eluents consisted of deionized water (A) and 95% acetonitrile (B), both containing 2 mM ammonium formate and 50 mM formic acid. The following linear elution gradient was run at 0.4 mL min^{-1} through a Zorbax Eclipse Plus C18 column ($50 \times 2.1 \text{ mm}$, $1.8 \mu\text{m}$, $95\text{\AA}$; Agilent Technologies, Santa Clara, CA, USA), maintained at 40°C : 78 to 88% B in 10 min, held at 88% B for 4 min, decreased back to 78% in 1 min and held during 5 min to equilibrate. Samples were kept at 4°C during the analysis, and 5 μL aliquots were injected into the system. Scheduled MRM scanning (90 s detection window; 2 s target scan time) was applied in positive electron spray ionization (ESI+) mode, using the following optimized parameters: curtain gas at 25 psi, ion spray at 5500 V, turbo gas temperature at 300°C , gas 1 and 2 at 40 and 60 psi, respectively, declustering potential at 105 V and entrance potential at 10 V. A list of MRM transitions (m/z) scanned in ESI+ for the detection of CTX-like compounds is given in Table 3.2, along with the respective collision energy (CE) and targeted retention time values. Instrument control, data processing, and analysis were conducted using Analyst software 1.6.2 (SCIEX, CA, USA). Due to the lack of standards, calibration curves of CTX3C (Wako, Japan) were applied to calculate the concentration of every compound detected, assuming an equivalent molar response for the other analogs.

Table 3.2. List of MRM transitions (m/z) used in ESI+ to detect CTXs by LC-MS/MS.

Compound	MRM transitions (m/z)		Precursor ion species	Targeted time (min)	CE (eV)
	Precursor ion (Q1)	Product ion (Q3)			
CTX3C or CTX3B	1040.6	1005.6	$[M+NH_4]^+$	11.2	30
	1023.6	1005.6	$[M+H]^+$		20
	1023.6	125.1			50
M- <i>seco</i> -CTX3C	1041.6	1023.6	$[M+H]^+$	4.7	20
	1041.6	1005.6			30
	1041.6	125.1			50
2-hydroxyCTX3C	1058.6	1005.6	$[M+NH_4]^+$	5.0	30
	1058.6	1023.6			20
	1058.6	125.1			50
51-hydroxyCTX3C	1056.6	1021.6	$[M+NH_4]^+$	6.3	30
	1039.6	1021.6	$[M+H]^+$		20
	1039.6	1003.6			20
CTX4A or CTX4B	1078.6	1043.6	$[M+NH_4]^+$	12.8	30
	1061.6	1043.6	$[M+H]^+$		20
	1061.6	125.1			50
54-deoxyCTX1B or 52- <i>epi</i> -54-deoxyCTX1B	1112.6	1077.6	$[M+NH_4]^+$	6.8	20
	1112.6	1059.6			30
	1112.6	95.1			90
CTX1B	1128.6	1093.6	$[M+NH_4]^+$	3.2	20
	1128.6	1075.6			30
	1128.6	95.1			90
C-CTX1 or C-CTX2	1141.4	1123.4	$[M+H]^+$	4.4	30
	1123.4	1105.6	$[M+H-H_2O]^+$		30
	1123.4	1087.6			30
2,3-dihydro-2,3-dihydroxyCTX3C	1074.6	1039.6	$[M+NH_4]^+$	6.0	30
	1057.6	1039.6	$[M+H]^+$		20
	1057.6	125.1			50
CTX3C analog 1	1040.6	1005.6	$[M+NH_4]^+$	10.0	30
	1023.6	1005.6	$[M+H]^+$		20
	1023.5	125.1			50
CTX3C analog 2	1040.6	1005.6	$[M+NH_4]^+$	8.5	30
	1023.6	1005.6	$[M+H]^+$		20
	1023.5	125.1			50
CTX3C analog 3	1040.6	1005.6	$[M+NH_4]^+$	7.5	30
	1023.6	1005.6	$[M+H]^+$		20
	1023.5	125.1			50

3.2.4.2.3. Cell based assay on neuroblastoma (CBA-N2a)

The neuroblastoma (N2a) CCL 131 cell line (ATCC) was used in this study, and the CBA-N2a was performed following the protocol described by the authors of (VIALLOON et al., 2020). Briefly, the 60 inner wells of several 96-well microplates were seeded with 200 μ L of a 5% FBS culture medium at an initial cell density of $50,000 \pm 10,000$ cells well⁻¹ (exponential growth phase) and left to grow for 26 h in an incubator at 37°C and 5% CO₂. Among the several microplates run in parallel, one microplate served as the Reference Cell Viability (RCV) control

to establish the initial cell viability of N2a cells using an MTT assay (VIALLOON et al., 2020). For each microplate, the mean absorbance of DMSO control (12 outer wells filled with DMSO only) was subtracted from each raw absorbance value (60 inner wells containing N2a cells), and all viability data were expressed in net absorbance data.

The remaining microplates were treated as follows. The growth medium was renewed by the addition of 200 μL of 2% FBS culture medium with 90 μM of ouabain (O) and 9 μM of veratridine (V) for non-destructive treatment in the OV^+ condition (bottom half of the microplate). In the OV^- condition, the growth medium was renewed by the addition of 200 μL of 2% FBS culture medium (upper half of the microplate). Appropriate controls in both OV conditions, namely COV^- and COV^+ , were established by adding 10 μL of 2% FBS culture medium to verify the final cell viability and the non-cytotoxicity of the OV treatment, respectively, in the absence of CTXs. The implementation of additional quality check controls (QC) to both OV^- and OV^+ conditions, namely QCOV^- and QCOV^+ , was also undertaken to check for the specific effect of voltage gated sodium channel (VGSC) activators. Basically, 10 μL of PbTx3 (Latoxan, France) at 0.1 $\mu\text{g mL}^{-1}$ were added in triplicate wells in both OV conditions to reach a final concentration of 4760 pg PbTx3 mL^{-1} in the wells. As CTX3C was tested in parallel with the fish samples, eight-point 1:2 serial dilutions of the CTX3C standard (Institut Louis Malardé) and sample stock solutions were prepared (100 μL per concentration) using a U-bottom 96-well microtiter. Then, 10 μL of CTX3C concentrations were directly added in triplicate wells under the non-destructive OV^+ condition (85.7/8.57 μM final concentrations) and not under the OV^- condition, since no cytotoxicity occurs in the absence of the O/V treatment (VIALLOON et al., 2020). The final concentrations of CTX3C tested ranged from 0.15 pg mL^{-1} to 19.05 pg mL^{-1} . The three parrotfish (toxic food) aliquots were tested with final concentrations ranging from 74 ng mL^{-1} to 9524 ng mL^{-1} of dry extract (corresponding to 0.23 to 29.76 mg liver equiv. mL^{-1}). Each concentration was tested in triplicate wells in both OV conditions, in three independent microplates run the same day.

Liver extracts were further analyzed by CBA-N2a. To this end, all liver extracts prepared from individual fish collected from a single tank at the same sampling interval (days 30, 41 of the exposure phase and days 8, 29 and 43 of the depuration phase). Pooled samples ranged from 0.11– 0.51 g fresh weight equivalent of fish liver. These samples were re-suspended in a solution of 5 μL MeOH, 15 μL dimethyl sulfoxide (DMSO), and 2% fetal bovine serum (FBS) RPMI medium, providing concentrations of sample stock solutions ranging from 0.442 g fresh liver equiv. mL^{-1} to 1.567 g fresh liver equiv. mL^{-1} , with no more than 10% of solvent used during the serially two-fold dilution procedure. As dry extract weights were not

available for lionfish livers, fresh fish liver equivalents could only be considered to estimate the concentration ranges tested. Pools of lionfish liver from the exposure experiment were tested together in one CBA-N2a experiment. In the same way, pooled liver from the depuration phase were also tested together in another CBA-N2a experiment. For each CBA-N2a experiment, CTX3C was tested in parallel with the lionfish liver samples, each concentration tested in triplicate wells in both OV conditions. Full dose-response curves were used for accurate CTX quantification. These two CBA-N2a experiments could not be repeated due to the very small amount of extract available. In all microplates, peripheral wells received 200 μ l of sterile distilled water, and microplates were left to incubate overnight for about 19 h prior to the determination of the final cell viability using the MTT assay with an incubation time of 45 minutes.

3.2.4.3. Data Analysis

3.2.4.3.1. *r*-RBA

The *r*-RBA result of each extract was determined after the assay performance was confirmed by the Quality Control (QC) together with the additional parameters of the CTX3C standard curve (Supplementary Materials, Figure S2), such as the effective concentration inducing a 50% values effect (EC_{50}), and the slope of the linear portion of the standard curve. Sample CTX quantification was only completed when the CPM measurement of a sample dilution fell on the linear part of the CTX3C standard curve and the relative standard deviation (*RSD*) of the triplicate CPM values was verified to be less than 30%. The Hill equation was used to convert CPM values into CTX concentrations.

Data analysis was performed in GraphPad Prism version 6.0 (San Diego, USA), allowing the determination of the parameters of each CTX3C competition sigmoidal curve (using 4 parameters). The detection limit (LOD) and the limit of quantification (LOQ) of the CTX-like composite in fish muscle and liver samples were determined based on the relative standard deviation (*rSD*) of the top plateau (B_{max}) of the dose-response curve according the method described by the authors of Díaz-asencio et al. (2018), following the equations: $LOD = B_{max} - 3 * rSD$; and $LOQ = B_{max} - 10 * rSD$. The values of LOD and LOQ were not estimated in this study. However, previous publications have reported LOQ values corresponding to 0.32 ng CTX3C equiv. g^{-1} (CLAUSING et al., 2018) and 1.5 ng CTX3C equiv. g^{-1} (DÍAZ-ASENCIO et al., 2019) and LOD values of 0.75 ng CTX3C equiv. g^{-1} (DÍAZ-ASENCIO et al., 2018).

3.2.4.3.2. LC-MS/MS

A calibration curve was calculated from the successive dilutions of a CTX3C standard

solution (Wako, Tokyo, Japan) in MeOH at concentrations ranging from 12.5 to 200 ng mL⁻¹. The limit of detection (LOD) and limit of quantification (LOQ) were calculated statistically based on the following formulae: $LOD = 3.3 * std / S$; and $LOQ = 10 * std / S$, where “std” is the standard deviation “S” and the slope of the calibration curve composed of successive dilutions of the CTX3C reference material. The calculated values for LOD and LOQ corresponded to 0.57–1.14 ng CTX3C mL⁻¹ and 1.74 ng CTX3C mL⁻¹, respectively. This was equivalent, respectively, to 0.22–1.14 ng CTX3C mL⁻¹ and 0.67–3.48 ng CTX3C g⁻¹ of lionfish liver, and to 0.54–1.54 and 1.64–4.71 ng CTX3C g⁻¹ of parrotfish flesh.

3.2.4.3.3. CBA-N2a

Net absorbance data were used to establish the full sigmoidal dose-response curves for CTX3C, lionfish liver extracts, and parrotfish toxic food. The CTX3C and fish samples were tested in parallel in the same assay. The maximum concentration of the liver tissue (MCE) that did not induce unspecific mortality on N2a cells was determined in OV⁻ and OV⁺ conditions using the fish control sample from the exposure phase of the experiment. This concentration was further used to establish the concentration range of the fish liver samples to use for the assay in the depuration experiment. The maximum concentration of dry extract (MCE) was defined as 20 mg mL⁻¹ from the fish liver control at 42 days. The limit of detection (LOD) and the limit of quantification (LOQ) of the CTX-like toxicity in the fish liver samples, expressed in ng CTX3C equiv. g⁻¹ of fish liver were determined according to the following equations: $LOD = (EC_{80}/MCE)$ and $LOQ = (EC_{50}/MCE)$, where EC₈₀ and EC₅₀ are the values obtained for CTX3C toxin standard, and were determined at 0.06 ± 0.01 and 0.12 ± 0.02 ng CTX3C equiv. g⁻¹, respectively. The composite cytotoxicity in the fish liver samples, expressed in ng CTX3C equiv. g⁻¹ of fish liver, was then estimated based on the $(EC_{50} \text{ of CTX3C} / EC_{50} \text{ of fish liver})$ equation.

3.3 Results and Discussion

3.3.1. Experimental setting and fish behavior

The experimental feeding encompassed a 12-week period, including 6 weeks of exposure to CTX and 6 weeks of depuration. A gradual decrease in feeding activity was observed over the course of the experiment, in both CTX exposed and control fish, although the propensity was greater among exposed fish (Figure 3.2). Increased food rejection episodes were indeed registered, mainly after the fifth week of experiment, during the late exposure

period. The proportion of non-eating fish was 62% in the group exposed to CTX and 32% in the control group at late exposure phase (Figure 3.2). Such changes in feeding behavior were not observed in previous experiments using herbivorous fish (*Naso brevirostris*) or carnivorous groupers (*Epinephelus coioides*) exposed to diets containing 0.4 ng CTX3C equiv. g⁻¹ over 16 weeks and ~1.0 ng CTXs equiv. g⁻¹ day⁻¹ (mean of CTX1B, CTX-2, CTX3 quantifications) over 30 days of exposure, respectively (LI et al., 2020; CLAUSING et al., 2018). However, the food rejection episodes observed during the present experiment did not seem to affect the CTX uptake and accumulation, since toxin levels were in fact detected in fish liver at the final exposure stage due to prolonged feeding (4-5 weeks) on CTX-containing parrotfish flesh (Table 3.3).

Although laboratory conditions appeared to be optimal and constant throughout the experiment, unexpected deaths were registered among both control and exposed lionfish (15 out of 48 individuals in total). Mortality also affected individuals belonging to a separate batch, received after the beginning of the experiment, maintained under similar conditions in the facility for future experiments. The specimens used in this study may have become more sensitive and prone to diseases such as bacterial infections when transferred to a laboratory setting. Consequently, these unexpected fish mortalities led to the loss of experimental replicates. Despite the reduction of replicates and considering that (i) fish showed no signs of suffering during the experiment, (ii) CTX-contaminated matrices were difficult to obtain, and (iii) published information of CTX trophic transfer under controlled conditions are still very limited, we decided to maintain the experimental protocol. Diet comparisons (i.e. exposed vs. control group) were thus achieved using randomly sampled individual fish as replicates.

Increased food rejection episodes may have contributed to the fish deaths observed during our experiment, although this could not be confirmed. Indiscriminate, sporadic mortality episodes registered during the experiment seemed unrelated to the exposure to CTX. Out of the 32 CTX-dosed fish, 11 fish died, mostly on week 7 (after 1 week of depuration). Similarly, 4 out of 16 control fish died, but in this case most deaths were registered on week 12, at the end of the experiment. The cumulative proportion of fish mortalities in the exposed group (36%) was similar to that of the control (28%).

During the entire experiment, exposed lionfish exhibited no clear signs of acute intoxication, such as erratic swimming, rapid gill movement, loss of equilibrium, and fin paralysis, as described in CTX-exposed mullet *Mugil cephalus* (LEDREUX et al., 2014). Likewise, in natural environments, CTX-contaminated fishes, including lionfish in the Caribbean region (ROBERTSON et al., 2014), as well as snapper and grouper on Pacific coral

reefs (MAK et al., 2013), have been found exhibit no signs of intoxication. Thus, coral reef fish species facing repeated exposure to CTXs in the natural environment may have developed resistance mechanisms to these compounds, perhaps binding them to particular soluble proteins in the skeletal muscle (BANNER et al., 1963; HAHN et al., 1992), or storing them in compartments where they would be biologically unavailable.

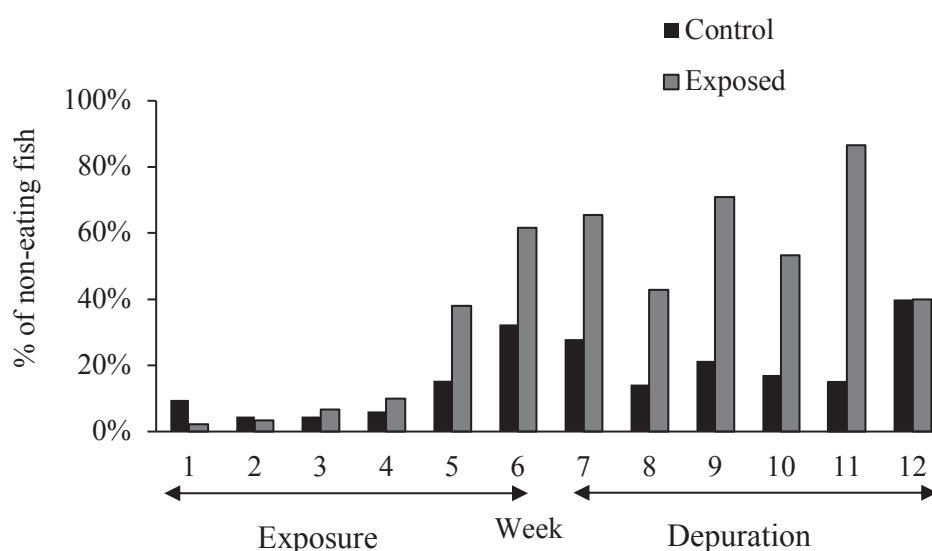


Figure 3.2. Proportion of control and CTX-exposed lionfish rejecting the food in relation to the number of remaining individuals at a given time during both the exposure and depuration periods.

3.3.2. Toxin levels in the food and fish dosing

The average CTX content in the naturally contaminated parrotfish (*Chlorurus microrhinos*) fillet homogenate (toxic food) was estimated as 2.27 ± 0.4 ng CTX3C equiv. g⁻¹ (coefficient of variation, CV = 17.6%) by r-RBA, with no significant difference ($p < 0.05$) among the eight subsamples tested. Analysis by CBA-N2a confirmed the presence of CTXs in the CTX-contaminated food, estimated at a concentration of 0.70 ± 0.02 ng CTX3C equiv. g⁻¹ ($n = 3$) with CV of 2.8% (Supplementary Materials, Figure S1). Unfortunately, the toxin levels in parrotfish flesh were too close to the LOD and LOQ values by LC-MS/MS (up to 1.5 ng CTX3C equiv. g⁻¹ and 4.7 ng CTX3C equiv. g⁻¹ of flesh, respectively), to allow unambiguous determination of CTXs using this technique. As expected, the CTX-free food (control) exhibited no activity by r-RBA analysis in our experimental conditions.

Given the toxin content in the CTX-contaminated food and the daily ration of 0.05 g food per g of fish, individuals from the CTX-exposed group received a dose equivalent to 0.11

ng CTX3C equiv. g⁻¹ d⁻¹ according to r-RBA estimations, or 0.035 ng CTX3C equiv. g⁻¹ d⁻¹ by CBA-N2a quantification. The dose administrated herein can be representative of an exposure that can eventually occur in the reef environment (i.e. environmentally relevant dose), as high levels of CTXs (up to 10.7 ng CTX3C equiv. g⁻¹) have been reported in parrotfish (*C. microrhinos*) sampled from French Polynesia islands (CHINAIN et al., 2020).

Considering the average amount of food supplied and the toxin content in the experimental diet, the total toxin ingested per lionfish (initial body weight: 15.8 ± 9.6 g; n = 32) was estimated by r-RBA as 1.74 ng CTX3C equiv. day⁻¹, totalizing 52.2 ng CTX3C equiv. and 71.3 ng CTX3C equiv. over 30 and 41 days, respectively, assuming that the fish accept all food portions offered. Based on the CBA-N2a analysis each exposed lionfish may have ingested, on average, a 3.2-fold lower dose.

3.3.3. Toxin levels in fish tissues

CTX were detectable by r-RBA in the liver of fish, yet not consistently. When quantified by this assay, toxin concentrations in the livers of exposed lionfish reached 9.4 ng CTX3C equiv. g⁻¹ at day 41 of exposure to CTX-contaminated food. The highest toxin concentration (9.77 ng CTX3C equiv. g⁻¹) was measured at late depuration phase (Table 3.3). As expected, CTX was not detected in the livers of control fish using either r-RBA (LOD: 0.75 ng CTX3C equiv. g⁻¹) or LC-MS/MS (LOD: up to 1.14 CTX3C equiv. g⁻¹ of liver). In fact, this latter analytical method proved to be the least sensitive one. Unfortunately, the limited sample volumes remaining from other technique, as well as the relatively low concentrations of individual CTX compounds present in our samples, together with the matrix effect, did not allow us to make any further clarification on the toxin profile of CTX-exposed fish. In French Polynesia, CTX composition of steephead parrotfish *C. microrhinos* typically includes CTX3C type compounds (71.1% as CTX3C and its M-*seco* form) and, to a lesser extent, CTX4A type (28.8% as CTX4A and its M-*seco* form) (FAO/WHO, 2020; CHINAIN et al., 2021). However, CTX profiles in fish are species-specific and can differ from regional variations (IKEHARA et al., 2017; YOGI et al., 2014). The typical toxin profile reported in *Gambierdiscus* species found in Pacific coral reef is also mostly composed of the less polar CTXs (48% P-CTX3C, 34% 49-epiCTX3C and 13% CTX4A) (FAO/WHO, 2020).

Following CTX absorption, toxin metabolization in fish may affect the depuration and elimination process (LEWIS & HOLMES, 1993; IKEHARA et al., 2017). Of note, as suggested in the present study for lionfish, low CTX depuration rates were also reported in other carnivorous fishes including experimentally exposed orange-spotted grouper (*Epinephelus*

coioides) (LI et al., 2020), as well as wild-caught moray eels *Gymnothorax javanicus* (*Lycodontis javanicus*) (LEWIS et al., 1991) and red snapper (*Lutjanus bohar*) (BANNER et al., 1966). For the latter species, the fish would require up to 30 months to completely eliminate the acquired toxin load. Moreover, the livers of contaminated orange-spotted groupers showed faster elimination rates for CTX1B, 52-*epi*-54-deoxyCTX1B, and 54-deoxyCTX1B, which are already highly metabolized toxins, compared to other tissues such as skin, gills and muscles, indicating different CTX elimination rates among tissues of exposed fish over 30 days (LI et al., 2020).

Table 3.3. Detection and quantification of ciguatoxins in individual lionfish livers during the experimental exposure and depuration periods by r-RBA analysis.

Periods of experiment	Fish #	Days of exposure/depuration	Fish total weight (g)	Liver wet weight (g) (% contribution)	Toxin concentration (ng CTX3C equiv. g ⁻¹)
Exposure	1	30	20.3	0.53 (3%)	<LOD
	2	30	16.9	0.45 (3%)	7.08
	3	30	10.0	0.30 (3%)	<LOD
	4	30	5.56	0.08 (1.5%)	<LOD
	5	41	21.0	0.74 (3.5%)	<LOD
	6	41	14.4	0.33 (2%)	<LOD
	7	41	17.6	0.39 (2%)	9.43
	8	41	14.4	0.40 (3%)	<LOD
	9	41	9.08	0.22 (2%)	<LOD
Depuration	10	8	19.4	0.65 (3%)	9.30
	11	8	15.0	0.61 (4%)	9.42
	12	29	16.7	0.55 (3%)	8.93
	13	29	8.72	0.02 (0.2%)	<LOD
	14	29	9.81	0.11(1.1%)	<LOD
	15	43	21.2	0.34 (2%)	9.77
	16	43	10.3	0.18 (2%)	<LOD

**LOD = limit of detection of the r-RBA was 0.75 ng CTX3C equiv. g⁻¹

Pooled liver extracts from control fish caused no cytotoxic effects on N2a cells in either OV⁻ or OV⁺ conditions throughout the experiment. Conversely, sigmoidal dose-response curves with a negative slope were obtained under the OV⁺ condition for liver extracts from fish sampled after 30 and 41 days of exposure to toxic food (Figure 3.3), and from those sampled after a depuration period of 8, 29, and 43 days (Figure 3.4). Based on the CTX-like composite toxicity using the CBA-N2a results, toxin concentrations in pooled lionfish livers were estimated as 1.36 ng CTX3C equiv. g⁻¹ and 0.89 ng CTX3C equiv. g⁻¹ after 30 and 41 days of exposure to toxin-containing food (herbivore *C. microrhinos* parrotfish fillets). Toxin concentration values decreased gradually over the depuration period, reaching 0.81 ng CTX3C

equiv. g⁻¹, 0.52 ng CTX3C equiv. g⁻¹ and 0.37 ng CTX3C equiv. g⁻¹ in pooled extracts from 8, 29 and 43 days of depuration on non-toxic food, respectively (Table 3.4). Considering the decrease in CTX-like cytotoxicity corresponding to a 0.44 ng CTX3C equiv. g⁻¹ difference between days 8 and 43 of depuration (see Table 3.4), it would take an additional 35-day depuration period for lionfish livers to reach the CBA-N2a LOD of 0.06 ng CTX3C equiv. g⁻¹. The kinetics of CTX elimination in lionfish liver, however, should be examined with caution considering: i) the limited number of time points for sampling during depuration stage (8, 23 and 43 days after last exposure), ii) the use of pooled samples in CBA-N2a due to an insufficient amount of remaining individual tissues, and iii) the limited amount of pooled tissues available, which did not allow for replication of the quantification of CTX in livers by CBA-N2a.

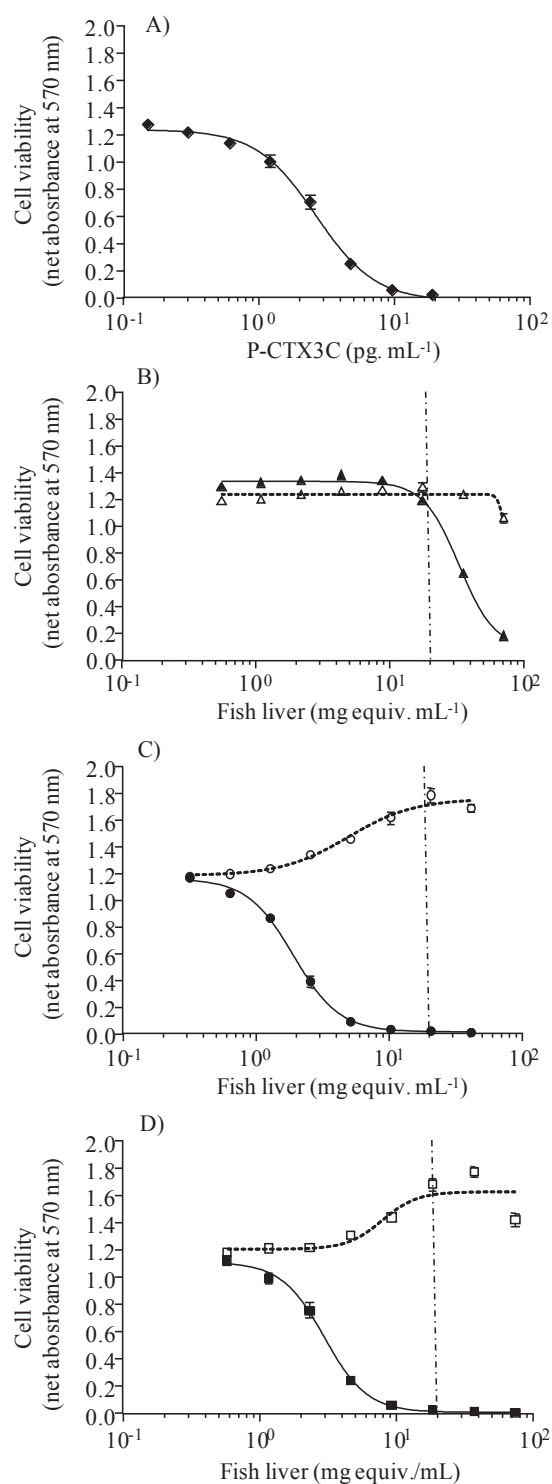


Figure 3.3. Quantification of CTX in fish liver during the exposure phase of the experiment. Dose-response curves of N2a cells when exposed to increasing concentrations of fish liver extracts, obtained from the exposure phase of the experiment, in OV^- (open symbols) and OV^+ (solid symbols) conditions; (A) CTX3C ($\blacklozenge$), (B) non-exposed control fish at day 42 (pool of three specimens) ($\Delta/\blacktriangle$), (C) exposed fish at day 30 (pool of four specimens) ($\circ/\bullet$), and (D) exposed fish at day 41 (pool of five specimens) ($\square/\blacksquare$). Data represent the mean $\pm$ SD of one assay, with each point run in triplicate. The dotted vertical line corresponds to the maximum concentration of liver tissue (MCE) that does not induce a matrix effect on the assay, which was estimated at 20 mg mL^{-1} of fish liver extracts.

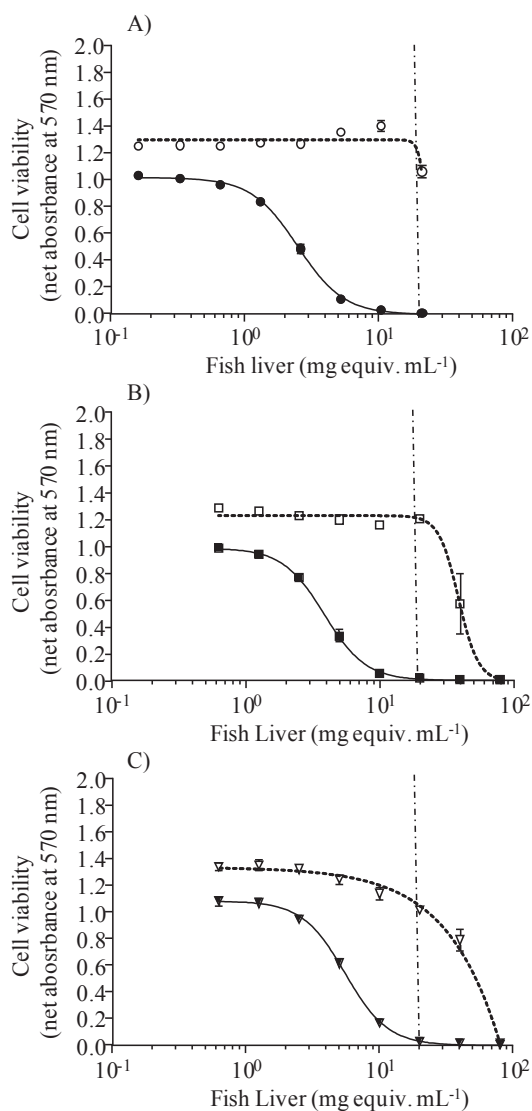


Figure 3.4. Quantification of CTX in fish liver during the depuration phase of the experiment. Dose-response curves of N2a cells when exposed to increasing concentrations of pooled fish liver extracts, obtained from the depuration stage of the experiment, in OV⁻ (open symbols) and OV⁺ (solid symbols); (A) 8 days of depuration (pool of three specimens) ($\circ/\bullet$), (B) 29 days of depuration (pool of three specimens) ($\square/\blacksquare$), and (C) 43 days of depuration (pool of two specimens) ($\Delta/\blacktriangle$). Data represent the mean $\pm$ SD of one assay, with each point run in triplicate. The dotted vertical line corresponds to the maximum concentration of liver tissue (MCE) that does not induce the matrix effect, which was established at 20 mg mL⁻¹ of fish liver extracts.

Table 3.4. Estimation of the EC₅₀ values and the CTX-like concentration of the pooled lionfish liver samples, as determined by the CBA-N2a analysis of the control (CTX-free food) and during exposure (CTX-contaminated food) and depuration (CTX-free food) periods of the experiment.

Periods of experiment	Days of exposure/depuration	Pooled samples (n)	Fish liver weight per pool (g)	EC ₅₀ (mg equiv. mL ⁻¹)	Toxin concentration (ng CTX3C equiv. g ⁻¹)
Exposure	30	4	0.47	1.93	1.36
	41	5	0.19	2.97	0.89
Control	42	2	0.50	ND*	<LOD**
	8	1	0.51	ND*	<LOD**
Depuration	8	2	0.23	2.49	0.81
	29	3	0.13	3.88	0.52
	43	2	0.11	5.46	0.37

*ND= not determined as control samples gave no cytotoxicity at the MCE in both OV conditions. **LOD = limit of detection of the CBA-N2a (0.06 ± 0.01 ng CTX3C equiv. g⁻¹).

Livers of lionfish belonging to the CTX-exposed group weighed, on average, 0.64 g (3.4% of the total fish body weight), resulting in a toxin burden of up to 4.53 ng CTX3C equiv. and 6.04 ng CTX3C equiv. in this organ after 30 and 41 days of exposure, respectively (based on the r-RBA results). When referring to the CBA-N2a results, the average toxin burden in pooled livers ranged from 0.87 ng CTX3C equiv. to 1.90 ng CTX3C equiv. after 30 and 41 days of exposure, respectively. This represents a retention of 8.6% (r-RBA) or 6.8% (CBA-N2a) of the ingested toxin burden at the end of the exposure period. Recent studies have reported that carnivorous grouper (*Epinephelus coioides*) fish, fed over 30 days, demonstrated a toxin burden of ~ 1 ng CTX1B g⁻¹ d⁻¹ a greater toxin dose than that used in our experiment (0.11 ng CTX3C equiv. g⁻¹ d⁻¹ or 0.035 ng CTX3C equiv. g⁻¹ d⁻¹ from r-RBA or CBA-N2a analysis) and accumulated up to 25% of the ingested toxin load in the livers and 10% in muscles (LI et al., 2020). In contrast, after 16 weeks of dietary exposure to 0.4 ng CTX3C equiv. g⁻¹ d⁻¹, juvenile *Naso brevirostris* accumulated $\sim 2\%$ of the exposed CTX3C amounts in muscular tissue (CLAUSING et al., 2018).

In the present study, toxin concentrations in the muscles of lionfish belonging to the dosed group were always below the limit of detection, as determined by r-RBA. The sample amounts remaining from this first analysis were deemed insufficient for a supplementary evaluation by CBA-N2a. In natural environments, lionfish muscles were found to contain relatively high CTX levels (≥ 0.24 ng C-CTX1 equiv. g⁻¹), indicating the potential of this species to cause human poisoning events (HARDISON et al., 2018; BOUCAUD-MAITRE et al., 2018). When experimentally exposed to 4-fold higher weight-specific daily doses of CTXs than those administered in our experiment, the herbivorous coral reef fish, *Naso brevirostris*,

accumulated up to 3.24 ± 0.59 ng CTX3C equiv. g^{-1} in muscular tissues (CLAUSING et al., 2018). This value is within the range usually found among naturally contaminated fish in French Polynesia (GABORIAU et al., 2014). Moreover, in CTX-exposed *N. brevirostris*, the total amount of toxins in muscles increased linearly over 16 weeks of toxin exposure (CLAUSING et al., 2018). The muscles of *N. brevirostris* quickly eliminated the incorporated ciguatoxins, contrary to what has been observed for mullets (*Mugil cephalus*) after nine toxic feedings with gel food containing *Gambierdiscus* cells (LEDREUX et al., 2014). Finally, muscular tissues of groupers (*Epinephelus coioides*) incorporated the equivalent of 0.34 ng CTX1B $\text{g}^{-1} \text{d}^{-1}$ after 30 days of exposure to 1 ng CTX1B $\text{g}^{-1} \text{d}^{-1}$ (LI et al., 2020). In the Caribbean region, the highest C-CTX1 levels detected in the muscles of wild-caught fish were 0.24 ng g^{-1} in grey snapper (*Lutjanus griseus*), 0.3 ng g^{-1} C-CTX1 equivalents in lionfish (*P. volitans*), 0.9 ng g^{-1} in grouper (Serranidae), 13.8 ng g^{-1} in black jack (*Caranx lugubris*) and 49 ng g^{-1} in barracuda (*Sphyraena barracuda*) (ROBERTSON et al., 2014; POTTIER et al., 2002; 2003; SOLIÑO et al., 2015).

Despite the loss of replication and the need for a longer period of experimentation, our results indicate CTX transfer from contaminated prey to carnivorous *P. volitans*. Toxins concentrated into lionfish livers, while their transport to the muscles could not be reliably assessed. In other fish species, CTXs appear to be primarily accumulated in the liver, being secondarily distributed to the muscles and other tissues as the concentrations increase (LI et al., 2020).

3.4. Conclusions

The accumulation of CTX in the livers of juvenile lionfish *Pterois volitans* became evident after five weeks of feeding toxin-contained parrotfish *Chlorurus microrhinos* fillets. The toxin levels in the muscles of lionfish were always below the limits of detection, as determined by r-RBA, suggesting potential differential tissue distribution over the experiment. However, this could not be confirmed by a more sensitive method, such as CBA-N2a due to the insufficient amount of flesh. Lionfish retained detectable toxin levels in their livers at 43 days after the last exposure, indicating a slow elimination process. No acute intoxication signs were observed throughout the experiment, suggesting the resistance of *P. volitans* to CTXs.

4. CAPÍTULO 4: A NEW LOOK AT THE POTENTIAL ROLE OF PLASTIC DEBRIS AS VECTORS OF HARMFUL BENTHIC ALGAE IN THE OCEAN

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Abstract

The increasing amounts of plastic debris floating in the oceans represent a significant surface area for the potential colonization of a variety of marine organisms and the adsorption of chemical compounds. Plastic debris can act as a potential substrate for harmful microorganisms, such as potentially toxic benthic microalgae and their compounds. In this study, we (i) develop a distribution map of the surface area of micro- and macroplastics available in different geographical areas; and (ii) assess the potential role of floating plastics as potential vectors for transfer of toxic benthic microalgae and their compounds along marine trophic chains. In total, a plastic surface area of 972 km² is potentially available for colonization of microorganisms in surface waters of the global ocean. We estimated an average potential colonization of 5.4×10^4 , 2.4×10^5 , 4.3×10^5 cells km⁻² for *Gambierdiscus* spp., *Ostreopsis* cf. *ovata* and *Prorocentrum* spp. on floating plastic debris in the oceans, respectively. Thus, plastic debris floating in superficial waters, especially in places such as Mediterranean Sea, Caribbean, Australia, French Polynesia and Korea, can act as vectors for the dispersal of potentially toxic dinoflagellates of the genera *Ostreopsis*, *Gambierdiscus* and *Prorocentrum*. We suggest that the potential negative implications of such association can become a major threat to marine ecosystems and human health as plastic pollution and benthic harmful algal blooms have both increased substantially over the past decade.

4.1. Introduction

The global production of plastic materials has been growing exponentially over the last 60 years, reaching >300 million tons in 2014, and this amount is expected to increase 6-fold by 2050 (UNEP, 2016; PLASTICS EUROPE, 2018). The input of plastics in the oceans comes mainly from marine activities such as aquaculture, fishing and shipping (HINOJOSA & THIEL, 2009). Per year, approximately 5000 fishing nets and plastic traps are lost in the NW Atlantic, up to 31,000 in the NE Pacific, and 20,000 nets in the Caribbean (UNEP 2009). On the coast of Galicia (NW Spain), on average, 25% of the plastic litter on the beaches comes from fisheries and aquaculture (GAGO et al., 2014). Another substantial fraction of the marine plastics can be

of terrestrial origin, reaching the oceans mainly through runoff from rivers, such as the Rhine River, which can carry an average of 893,000 plastic particles per km² into the North Sea (MANI et al., 2015). Rivers are responsible for an increment of ~1.8 million tons of plastic waste per year in the oceans (LEBRETON et al., 2017).

The high durability of these synthetic polymers favors their extended permanence in the oceans, generating a series of adverse effects on marine ecosystems. Plastic debris poses a threat to marine organisms through the ingestion and entanglement, leading to lethality in most severe cases (WANG et al., 2019). Macroplastics (> 5 mm) mainly affect larger pelagic and benthic marine organisms including fish, sea turtles, seabirds, and marine mammals (WRIGHT et al., 2013; ANDERSON et al., 2016). In Greece, 60% of the stranded sperm whales contained macroplastic debris in their stomachs, consisting mainly of plastic bags (46%) (ALEXIADOU et al., 2019). Smaller plastic particles, composed of nanoplastics (< 0.1 µm) and microplastics (MPs; 0.1 µm - 5 mm), together represent the largest portion of plastic debris amounts in the oceans (> 90%). They can be found in both sediments and the water column of any marine region (VAN CAUWENBERGHE et al., 2015; ERIKSEN et al., 2014; ANDERSON et al., 2016), with most millimeter-sized plastic particle concentrated within the first few meters in the upper water layer (REISSER et al., 2014a). Recent studies have shown that microplastics can be ingested or adhered to marine organisms of different trophic levels, causing lethal and sublethal effects (TAYLOR et al., 2016; WANG et al., 2019). The harmful effects of plastic particle exposure on marine animals include reduction of feeding, growth and reproduction rates (WANG et al., 2019). In addition, microplastics, due to their strong hydrophobic properties, can adsorb highly toxic organic pollutants and facilitate their transfer along the trophic chain (ENGLER et al., 2012; GALLO et al., 2018). These chemicals can induce reproductive and growth disorders and eventually cause lethality of distinct marine organisms (GALLOWAY & HANDY, 2003; GALLO et al., 2018).

Plastic particles can be transferred along the trophic chain and reach human consumers of contaminated seafood. Trophic transfer of microplastics to seafood has been demonstrated in the laboratory (FARRELL & NELSON, 2013; SETALA et al., 2014; TOSETTO et al., 2017), and up to 360 - 470 microplastic particles kg⁻¹ have been reported in commercially farmed bivalve mollusks in Europe (VAN CAUWENBERGHE & JANSSEN, 2014). In addition, smaller MPs and nanoplastics can aggregate on or enter phytoplankton cells, inhibiting their growth, either through physical blockage, oxidative stress, genetic changes or by reducing their photosynthetic efficiency (LAGARDE et al., 2016; ZHAO et al., 2019). Conversely, macroplastics can act as artificial substrates for the growth of microorganisms and may harbour

high cell abundances of potentially toxic microalgae, mainly benthic dinoflagellates (YOKOTA et al., 2017; CASABIANCA et al., 2019; TIBIRIÇÁ et al., 2019).

Approximately 170 microalgal taxa are capable of forming harmful algal blooms, impairing ocean and human health. Among them, around 45 species of toxigenic benthic dinoflagellates may colonize distinct natural and artificial substrata including marine plastic litter (HOPPENRATH et al., 2014). Benthic dinoflagellates are widely distributed in tropical and temperate environments and produce a vast array of potent biotoxins associated with seafood-borne human poisoning outbreaks worldwide (LASSUS et al., 2016). Benthic harmful algal blooms (BHABs) have experienced a global increase in frequency and geographical distribution in recent years, mainly due to anthropogenic activities favoring the introduction of exotic species and climate changes, especially global warming (LASSUS et al., 2016). Most BHAB-forming species reach their optimum growth at high temperature (i.e., 25-30 °C) and would benefit from increasing periods of warming (e.g. TESTER et al., 2010). In this context, plastic debris floating in the surface of the oceans may become an important vector of toxic microalgal cells and their compounds, posing an additional threat to marine ecosystem quality and human health. This is especially relevant for benthic dinoflagellates, which are naturally limited by the availability of suitable substratum and light on the ocean floor.

In this study we revisited the literature and related datasets on marine plastic debris and plastic algae colonization aiming at (i) quantifying the surface area of micro- and macroplastic substrate available for colonization by microorganisms and toxin adsorption in different geographical areas; and (ii) estimating the cell density of key toxin-producing benthic dinoflagellates potentially colonizing plastic debris in different coastal regions of the world. We focused our estimates on three taxa of wide distribution for which records on plastic colonization are available, namely *Prorocentrum lima*, *Ostreopsis* cf. *ovata* and the genus *Gambierdiscus*. To reach more reliable estimates, we developed, for each species, environmental suitability models to account for natural limitations on their spatial distribution.

4.2. Methods

4.2.1. Estimating the surface area of plastic debris in the oceans

The surface area provided by marine plastic debris (SP), in cm² Km⁻², was calculated based on reported counts of plastic particles floating in the world's oceans (ERIKSEN et al., 2014; LEBRETON 2021). To be more precise, we used the spatial outputs of an oceanographic model calibrated with empirical data from coastal and oceanic waters that estimated the global

distribution of plastic debris belonging to four different size classes (see Eriksen et al., 2014 for details). Since our focus was on plastic particles passive of ingestion by marine biota, we restricted our analyses to smaller macroplastics (4.76-200 mm; originally denominated “mesoplastics” in Eriksen et al., 2014) and two size categories of microplastics (small MPs: 0.33-1 mm and large MPs: 1.01-4.75 mm). Plastic counts were obtained from sampling in the upper 2 m of water, where the vast majority of millimeter-sized plastics are concentrated (REISSER et al., 2014a).

To calculate SP, we also needed information on the shape of plastic items within each size class. Plastic items were then classified according to the most common shapes found in the oceans into beads, fibers, fragments, or films. The amount of items of each shape at the ocean surface was estimated by taking into account the percentage of these items reported in the superficial waters of subtropical gyres for both microplastics and macroplastics (ERIKSEN et al., 2013). Plastic shapes were approximated to the following geometrical forms for calculation of average surface areas: spheroids (beads), cylinders (fibers) or cuboids (fragments and films). The arithmetic average size within each size category (i.e., 0.665 mm for small MPs, 2.88 mm for large MPs, and 102.4 mm for macroplastics) was attributed to the length of each geometrical shape, and the other dimensions were calculated by applying the average Length:Width:Height ratios described in Kooi & Koelmans (2019) for beads (1:0.80:0.68), films (1:0.55:0.0505) and fragments (1:0.55:0.505). For fibers, we used the interquartile range diameter from fibers extracted from seawater, reported in Suaria et al. (2020), to determine the radius for small MPs (0.0075 mm) and large MPs or macroplastics (0.0102 mm).

The average surface area was calculated for each shape and size category and multiplied by the corresponding amounts of plastic items estimated for each $1^\circ \times 1^\circ$ oceanic grid cell. Finally, we used polygons from oceanic basins and the Large Marine Ecosystems (LMEs) to obtain regional SP values. At all spatial units (i.e. grid cell, LMEs or ocean basin), SP values were divided by the corresponding area and expressed in $\text{cm}^2 \text{ km}^{-2}$.

4.2.2. Microalgal colonization

To estimate the cell density of our selected benthic species potentially colonizing plastic debris in surface waters, we associated the plastic surface area available with their reported colonization on plastic substrates. The maximum colonization values (i.e., cell density on plastic substrates) reported in the literature and used in our analyses varied from 16 cells cm^{-2} for *Prorocentrum* spp., 9 cells cm^{-2} for *Ostreopsis* cf. *ovata* and 2 cells cm^{-2} for *Gambierdiscus* spp. (CASABIANCA et al., 2019; TESTER et al., 2014). For *Ostreopsis* cf. *ovata*, specifically,

higher cell densities have been reported on plastic substrates (up to 8400 cells cm⁻²; TIBIRIÇÁ et al. 2019), but they were obtained from passive plastic samplers placed next to the bottom during massive blooms and were thus not considered here.

Although the association of the field observations of cell densities with the estimated plastic surface area, as described above, allowed us to estimate the colonization potential of toxic microalgal cells on plastic debris floating in surface waters worldwide, the simple substrate availability does not guarantee the successful colonization and survival of the target organism since plastic debris may be located in - or travel across - waters outside the environmental requirements of the species. Furthermore, in the case of benthic microalgae, colonization would be most likely restricted to shallow coastal areas. To account for natural limitations in species distribution, we employed here an adapted version of the Relative Environmental Suitability model (KASCHNER et al., 2006; READY et al., 2010). This model relies on environmental envelopes computed for each species and returns an index of environmental suitability for each individual grid cell varying between 0 and 1, which can be used to penalize the colonization potential of each target species. To calculate the environmental envelopes, we extracted the annual average values of temperature, salinity, nitrate, phosphate and the photosynthetically active radiation (PAR) at the seabed from the exact locations where the target species have already been recorded. We used only records from locations with average depth between 0 and 100 m. For records out of this range, we searched for the closest point within this range inside a 1° buffer zone to account for inaccurate coordinate information. Then, we used the estimated environmental envelopes of our benthic species to project their environmental suitability at surface waters. Spatial data for all variables at the bottom and surface were obtained from Bio-ORACLE (ASSIS et al., 2017) using the *sdm* predictors R package (BOSCH, 2020). Values of PAR at the surface were recalculated to 1 m depth, following Assis et al. (2017), to represent the radiation incident at the upper 2 m water layer, where the amounts of plastic items were estimated from. Spatial distribution of the target species was retrieved from the Ocean Biodiversity Information System (OBIS) and complemented with data from published literature listed in AlgaeBase (<https://www.algaebase.org/>). For *Ostreopsis* cf. *ovata*, which had the least number of occurrences in OBIS among the target species, we also recovered information available in additional review papers: Rhodes (2011) and Pavaux et al. (2020).

4.3. Results and Discussion

4.3.1. Mapping plastic debris floating in the ocean

Since 1950, only a small portion (~9%) of the total plastic produced worldwide (~8300 million metric tons) has been recycled, and most of it (70%) ends up in the environment, with around 5% (~8 million tons per year) reaching the oceans (JAMBECK et al., 2015; GEYER et al. 2017; GALLO et al., 2018). Eriksen et al. (2014) estimated that approximately 5 trillion plastic particles (>250,000 tons) were floating in the oceans in 2007-2013, what may be seen as a conservative estimate. Van Sebille et al. (2015), for instance, estimated a value up to ten times higher (15-51 trillion plastic particles). In any case, studies have indicated that the amount of plastic debris in the ocean surface is at concentrations below what would expect from the reported rates of plastic production and entry in marine environments, suggesting that there are processes facilitating its loss (CÓZAR et al, 2014; ERIKSEN et al., 2014). Factors may be facilitating the removal of plastic material from the ocean surface include fragmentation, biodegradation by microorganisms (ZETTLER et al., 2013; HARSHVARDHAN & JHA 2013; BALASUBRAMANIAN et al., 2010), ingestion by marine organisms (COLE, 2013), and sedimentation, enhanced or not by biofouling, as 50% of plastics discarded in landfills are already denser than seawater (BARNES et al., 2009). Therefore, understanding the ultimate fate of plastic debris in the ocean and knowing the total surface area they represent are imperative for determining the extent of their environmental impacts (CÓZAR et al, 2014; ERIKSEN et al., 2014), especially those aggravated by adhered microorganisms and/or adsorbed toxic substances.

Based on counts of plastic items floating in the oceans, as reported by Eriksen et al. (2014), and on the proportion of plastic items of different shapes (BURNS & COXALL, 2018; ERIKSEN et al., 2013), we estimated that plastic debris provides a plastic surface area of $972 \times 10^{10} \text{ cm}^2$ (972 km²) in the global ocean (Table 4.1). This value represents only ~4% of the total plastic surface area estimated by Zhao et al. (2020) ($2.5 \times 10^{14} \text{ cm}^2$). These authors estimated the plastic surface area based on the amount of plastics reported by Eriksen et al. (2014), like this study. However, they assumed that all particles are spherical, including those >200 mm, while in our study we took into account various particle shapes (see Methods for details), and we ignored items greater than 200 mm, which cannot be easily ingested by marine organisms and are generally denser than seawater, settling quickly and reaching shorter distances (BARNES et al., 2009). Thus, our estimations result in a more conservative, but, in our view, more realistic estimation of the plastic substrate area available in the global ocean. In

any case, the massive amount of plastic debris floating in the oceans indicates an extensive and potentially long-lasting surface area for colonization by microorganisms and for adsorption of chemical compounds.

Table 4.1. Estimate of the plastic surface area ($\text{cm}^2 \text{ km}^{-2}$) and the total area of plastics ($\times 10^{10} \text{ cm}^2$) available in the surface of different oceanic regions, arranged in decreasing order of total plastic area: NP = North Pacific, NA = North Atlantic, IO = Indian Ocean, SP = South Pacific, SA = South Atlantic, AO = Arctic Ocean, and SO = Southern Ocean.

	NP	NA	IO	SP	SA	AO	SO	Global Ocean
Size (mm)	Total plastic surface area ($\times 10^{10} \text{ cm}^2$)							
0.33 - 1.00	0.650	0.440	0.470	0.250	0.110	0.010	<0.010	1.93
1.01 - 4.75	18.2	12.2	12.8	6.61	2.94	0.140	<0.010	53.1
4.76 - 200	287	269	190	114	51.8	5.34	0.180	917
Total	306	282	203	121	54.8	5.48	0.180	972
	Plastic surface area ($\text{cm}^2 \text{ km}^{-2}$)							
0.33 - 1.00	79.8	94.6	67.1	28.7	28.0	4.54	0.020	53.6
1.01 - 4.75	2232	2615	1839	754	733	106	0.350	1469
4.76 - 200	35104	57302	27075	12981	12917	4100	80.7	25365
Total	37416	60011	28981	13764	13678	4210	81.0	26887
Type	Total plastic surface area ($\times 10^{10} \text{ cm}^2$)							
Fiber	0.310	0.290	0.210	0.120	0.060	0.010	<0.010	1.00
Bead	3.24	2.91	2.16	1.27	0.580	0.050	<0.010	10.2
Film	51.2	47.8	33.8	20.3	9.23	0.950	0.030	163
Fragment	251	231	167	99.0	45.0	4.47	0.140	797
	Plastic surface area ($\text{cm}^2 \text{ km}^{-2}$)							
Fiber	38.0	61.1	29.4	14.0	13.9	4.29	0.080	27.3
Bead	396	620	309	145	144	42.2	0.780	283
Film	6264	10189	4835	2314	2302	726	14.2	4521
Fragment	30718	49142	23808	11291	11218	3437	65.9	22056

Microplastics and macroplastic items are complex and diverse in terms of composition (polymer type), shape, and size. Fibers are frequently reported in surface waters, accounting for 14%, on average, of the total number of floating items in the South Pacific subtropical gyre (ERIKSEN et al, 2013). Conversely, in the Arctic Sea, synthetic microfibers, usually composed of polyester, may account for as much as 92% of the total number of plastic items (ROSS et al., 2021). However, the relative contribution of microfibers may be overestimated in some studies, since plastic particles are commonly characterized by visual assessment, the simplest and cheapest method available (HIDALGO-RUZ et al., 2012). Suaria et al. (2020) reported that only ~8% of the fibers present in the oceans are in fact synthetic, while most are composed of natural polymers including cellulose and those of animal origin. Moreover, despite their high occurrence in aquatic environments (~52%) (BURNS & BOXALL, 2018), fibers are normally very thin and thus represent less than 1% of the plastic surface available in the oceans for

colonization by microorganisms and/or adsorption of chemical compounds (Table 4.1). Fragments appear to be much more representative in the ocean in terms of surface area. In fact, these items which account for approximately 79% of all plastic items floating in the Canary Islands, Spain (GONZÁLEZ-HERNÁNDEZ et al., 2020), and in the South Pacific subtropical gyre (ERIKSEN et al., 2013), represent 82% of the total surface area of floating plastic available in the ocean (Table 4.1). Plastic films account for 5% of the number of plastic debris in the South Pacific subtropical gyre (ERIKSEN et al., 2013), although representing over 16 % of the total plastic surface area in the ocean (Table 4.1). Finally, the relative contributions of spheres/spheroids (1%) to the total plastic surface area available in the ocean (Table 4.1) is very close to their abundance (2%) in the South Pacific subtropical gyre (ERIKSEN et al., 2013). In terms of particle size, although microplastics (0.33-4.75 mm) are the main constituent (> 90%) among the plastic debris in the oceans (ERIKSEN et al., 2014), macroplastics (4.76-200 mm) correspond to approximately 917 of the 972 km² (94%) of the total plastic surface area floating in the ocean (Table 4.1). This represents a significant area that can be made available for colonization by harmful benthic algae, and act as vectors of toxins for marine organisms, including those living in more superficial and open waters, where cell abundance is frequently low (TESTER et al., 2014).

Plastic debris, mainly composed of low-density polymers (<1 g cm⁻³) of high durability, can persist in the oceans for decades (HAMMER et al., 2012; ANDRADY, 2011; CÓZAR et al., 2014). Among the different polymers, the most abundant and/or frequent in the oceans are polyethylene (PE: 42%), polypropylene (PP: 25%), glass (PS: 5%), as well as polystyrene (OS), polyester (PEST) and polyamide (PA), together corresponding to 28% (ERNI-CASSOLA et al., 2019). Thus, the nature of these plastic materials facilitates their accumulation and permanence in ocean surface waters for long periods, reaching great distances (LAGANÀ et al. 2019; ONDA et al. 2020). The areas of greatest plastic accumulation are ocean gyres (LAW et al., 2010; CARPENTER et al., 1972; CÓZAR et al., 2014; ERIKSEN et al., 2014). The North Pacific gyre, situated between Hawaii and California and known as the "Great Pacific Garbage Patch", has an average density of 678,000 plastic items per km² and an area of 1.6 million km² (approximately the size of Iran, the 16th largest country in the world). This gyre concentrates ~1.7 trillion plastic items in total, representing 34% of the total amount of floating plastics in the oceans (MOORE et al., 2001; LEBRETON et al., 2018). According to our estimates, the Northern Hemisphere alone contains 60% of the total area of available plastic substrate in the oceans. From that, 31% are concentrated in the Pacific region (Table 4.1), the most populated coastline in the world, with one-third of the global coastal population (CIESIN, 2012).

Likewise, the Indian Ocean, the third largest of the world, contains 21% of the total plastic surface area, mostly because of the plastic debris accumulated in its gyre and along its highly populated coastline (Figure 4.1).

Plastic accumulation in surface waters also occurs in enclosed bays, gulfs, seas, and watersheds. Although the Mediterranean Sea represents less than 1% of the total marine area (EAKINS & SHARMAN, 2010), it harbors ~ 5% of the total plastic area available worldwide resulting from concentrations of plastic surface area ($10^6 \text{ cm}^2 \text{ km}^{-2}$) as high as those found in large ocean gyres (Figure 4.1). Notably, our estimates of plastic surface area for the Mediterranean Sea (Table S1), as based on the world's counts of plastic debris by Eriksen et al. (2014), may be taken as conservative. Suaria and Aliani (2014) estimated that > 60 million macroplastic items (25 items per km^2) would be floating in the Mediterranean Sea, which is five times higher than the value reported by Eriksen et al. (2014). Other marine water bodies harboring high concentrations of plastic surface area - in the order of $10^6 \text{ cm}^2 \text{ km}^{-2}$ - include the Red Sea, the Southeast U.S. continental shelf, Gulf of Thailand, Bay of Bengal, and the South China Sea (Figure 4.1 and Table S1). In contrast, polar and subpolar regions including Antarctica, Hudson Bay, Beaufort Sea and the Bering Sea exhibit much lower values, in the order of $10^4 \text{ cm}^2 \text{ km}^{-2}$ or lower (Figure 4.1 and Table S1).

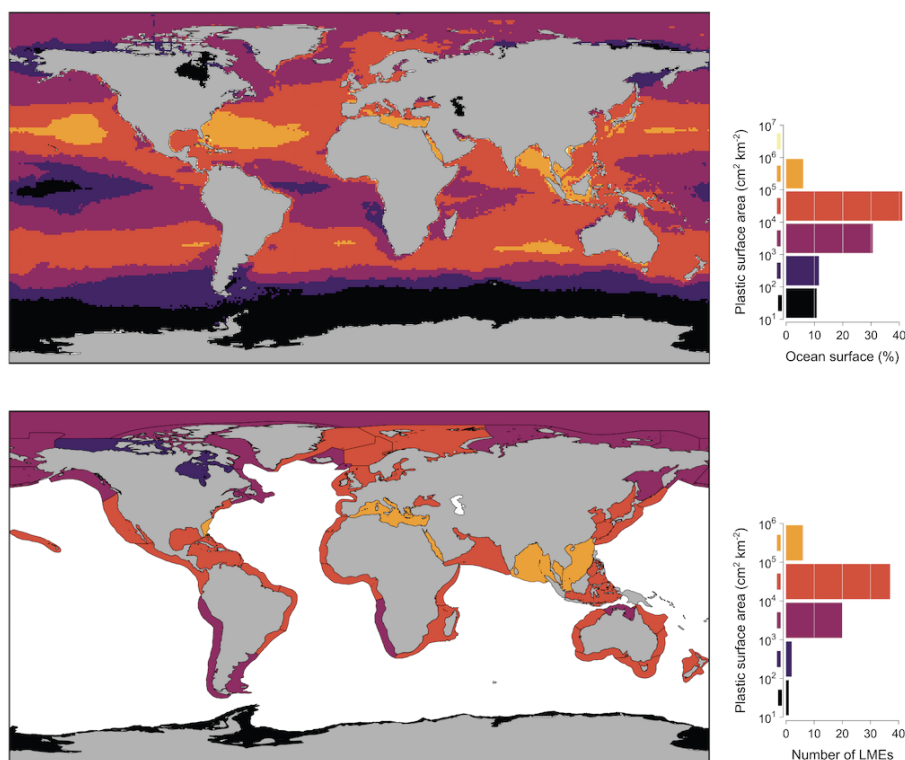


Figure 4.1. Plastic surface area estimation for the global ocean at $1^\circ \times 1^\circ$ resolution (top) and for each Large Marine Ecosystems (LME; bottom). Maps legend show the oceanic area covered by, or the frequency of LMEs within, each level of plastic surface area available in surface waters.

Lebreton et al. (2018) showed that plastics in coastal surface waters are more recent materials compared to those found in ocean gyres, and predominantly composed of PE (> 50%) (RIPKEN, et al., 2021; HANNINEN, et al., 2021). The plastic objects (i.e., macroplastics) most commonly found on the beaches are food wrappers, cigarette butts, plastic bottle caps, plastic cups and plates (Ocean Conservancy and International Coastal Cleanup, 2020). Most (> 77%) plastic debris that are recently discarded on the beaches remain floating over in nearshore (10 km) waters (ONINK et al., 2021) and travel as much as 160 km along the coast (VAN SEBILE et al., 2015). Usually, these debris are dragged from one beach to another, being subject to continuous friction and fragmentation into smaller particles. In this process, they are also heavily colonized by microorganisms (ZETTLER et al., 2013) including benthic toxigenic dinoflagellates (MASÓ et al, 2003; 2016, REISSER et al., 2014b; CASABIANCA et al., 2019; ONDA et al., 2020).

4.3.2. Plastics as alternative substrates for colonization by microorganisms

Plastics floating on the surface of the oceans can be colonized by diverse marine microorganisms, including potentially toxic microalgae (GREGORY 2009; OBERBECKMANN et al. 2015). The hydrophobic nature, low density, and high surface area of plastics facilitate biofilm formation and adsorption of organic and inorganic compounds in the ocean (EICH et al., 2015; GALGANI et al. 2019; THIEL & GUTOW 2005; KAISER et al. 2017; FAZEY & RYAN 2016; OGONOWSKI et al, 2018; OBERBECKMAN et al, 2018; KIRSTEN et al., 2018). During colonization, microorganisms form an organic coating layer and quickly change the plastic's physical-chemical properties, mainly increasing its hydrophilicity and decreasing its buoyancy from positive to neutral (LOBELLE & CUNLIFFE, 2011). Over time, successional processes lead to the loss and replacement of new species, transforming the structure and the interactions within the complex community living on the plastic surface, referred to as the "plastisphere" (ZETTLER et al. 2013; ONDA & SHARIEF, 2020).

The colonization of plastics in marine environments starts after the first contact of microorganisms with the plastic surface, usually happening between a few hours and weeks (DANG & LOVELL 2000; LOBELLE & CUNLIFFE, 2011). The composition of organisms adhering to plastic surfaces can be quite different from the community present in the water surrounding the plastic (ZETTLER et al. 2013; BRYANT et al. 2016; MCCORMICK et al. 2014; MUTHUKRISHNAN et al. 2019). Diatoms are the most frequent group of microalgae

(present in up to 94% of the samples) among the colonizers of different types of plastic debris (MASÓ et al., 2006; AMARAL-ZETTLER et al., 2020; ZHAO et al., 2020; SCHLUNDT, et al., 2020; EICH et al., 2015; CARPENTER & SMITH 1972; CARSON et al. 2013; ZETTLER et al. 2013; ONDA et al., 2020), followed by cyanobacteria (BRYANT et al., 2016) and dinoflagellates (58%) (REISSER et al., 2014b). However, a sampling method for benthic dinoflagellates using a piece of nylon mesh placed close to the bottom, as described in Tester et al. (2014), demonstrated that dinoflagellate cells are replaced by epiphytic diatoms and detritus after 48 h following initial colonization, suggesting a quick succession process.

Plastics provide a long-lived substrate and can maintain community adherence for long periods even in oligotrophic waters, such as open oceans. The current amount and increasing inputs of plastics into the oceans every year may facilitate the survival of non-native colonizing species by providing persistent and motile substrates that disperse slowly throughout the oceans, allowing for acclimatization of attached organisms (BARNES 2002; GALL & THOMPSON 2015). Physical processes such as currents, winds, and storms facilitate the dispersal of plastics, increasing the dispersal potential of these nonnative species. Barnes (2002) estimated that plastic debris in the oceans can double the dispersal of adhering invasive organisms in the subtropics and triple it at higher latitudes. Thus, plastic particles can be a vector for the dispersal of potentially toxic microalgae cells and cysts, such as dinoflagellates of the genera *Alexandrium*, *Coolia*, *Ostreopsis*, *Ceratium*, *Gymnodinium*, and *Protoperidinium*, which have been detected adhering to plastic fragments floating in the waters of the Mediterranean Sea (MASÓ et al, 2003; 2016), Atlantic Ocean (ZETTLER et al., 2003), Australia (REISSER et al., 2014b) and the Philippines (ONDA et al., 2020).

Quantification of potentially toxic microalgae cells in plastic debris, particularly related to transport capacity, is still poorly understood. However, this becomes critical towards assessing the implications of plastics on oceanic and coastal ecosystems. By integrating estimates of plastic distribution in the oceans and data from colonization studies of microalgal cells on plastic substrates (Table 4.2), we estimate the following average colonization potentials for *Gambierdiscus*, *Ostreopsis* cf. *ovata*, *Prorocentrum*, *Alexandrium* spp. and *Pseudo-nitzschia* spp. on plastic debris in the global ocean: 5.4×10^4 , 2.4×10^5 , 4.3×10^5 , 1.3×10^6 , and 1.8×10^8 cells km⁻², respectively. We focused our activities on the species of *Prorocentrum lima*, *Ostreopsis* cf. *ovata* and the genus *Gambierdiscus*, due their wide distribution worldwide for which records on floating plastic colonization are available in the ocean. These three taxa produced toxic compounds associated with a broad toxic spectrum, causing frequent and/or severe human intoxication.

Table 4.2. Review of the available literature on the maximum amounts of marine microalgae cells adhered to plastic/artificial substrates (cm²) present in oceanic regions. PE = polyethylene, OS = polystyrene, PP = polypropylene.

Genus/Species	Colonization (cells cm ⁻²)	Time of colonization	Toxin adsorption	Type of plastic material	Collection site/experiment	Reference*
<i>Gambierdiscus</i>	< 2	24 h		artificial substrate fiberglass screen	Indian Ocean and North Atlantic	1
<i>Prorocentrum</i>	< 16	24 h		artificial substrate fiberglass screen	Indian Ocean and North Atlantic	1
<i>Ostreopsis</i> cf. <i>ovata</i>	1-9 < 260		1-62 ng PLTX cm ⁻²	PE: 55%, OS: 24%, PP: 9%	Mediterranean Sea	2
<i>Ostreopsis</i> cf. <i>ovata</i>	mean 8400	24 h		flexible PP plastic	Atlantic Ocean	3
<i>Ostreopsis</i> cf. <i>ovata</i>	mean 80,000		0.012 ngPLTX cell ⁻¹	artificial substrate fiberglass screen	Atlantic Ocean	3
<i>Ostreopsis</i>	< 2	24 h		artificial substrate fiberglass screen	Indian Ocean and North Atlantic	1
Other taxa						
<i>Alexandrium pacificum</i>	1-30		0.001-0.072 ng cm ⁻²	PE: 55%, OS: 24%, PP: 9%	Mediterranean Sea	2
<i>Alexandrium minutum</i>	7-73		0.001-0.072 ng cm ⁻²	PE: 55%, OS: 24%, PP: 9%	Mediterranean Sea	2
<i>Pseudo-nitzschia multistriata</i>	1-6606		5 - 443 ng cm ⁻²	PE: 55%, OS: 24%, PP: 9%	Mediterranean Sea	2
<i>Lingulodinium polyedrum</i>	< 1			PE: 55%, OS: 24%, PP: 9%	Mediterranean Sea	2
<i>Gonyaulax spinifera</i>	< 1			PE: 55%, OS: 24%, PP: 9%	Mediterranean Sea	2
Diatoms	mean 990			rigid PP	Atlantic Ocean	3
	mean 280			flexible PP plastic	Atlantic Ocean	3

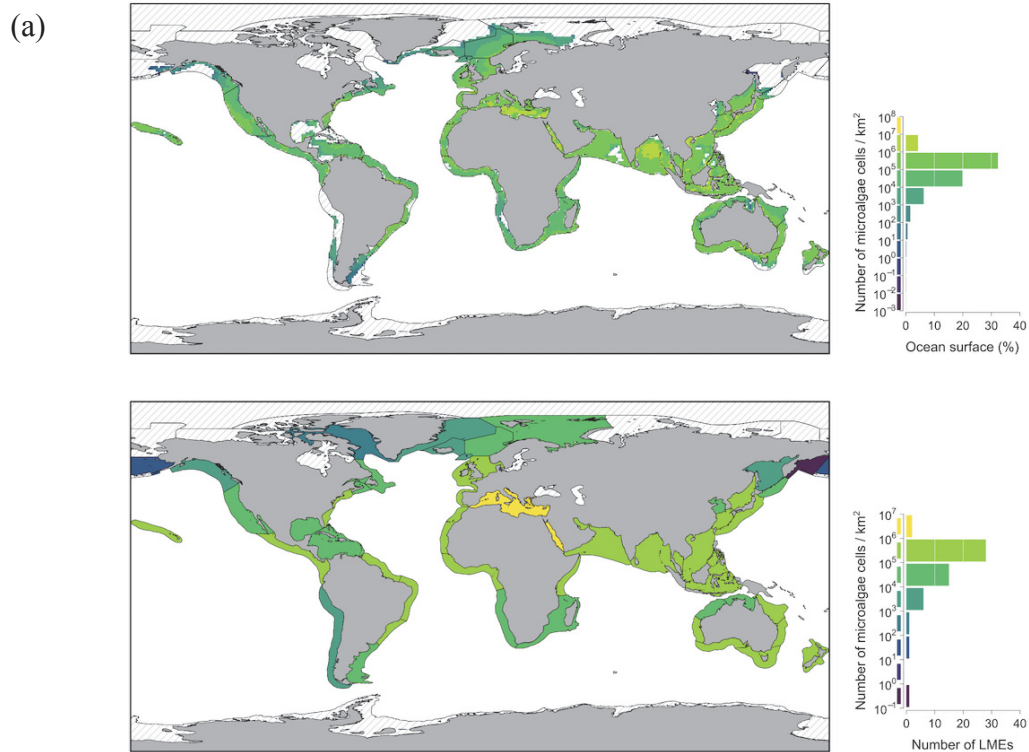
*Fonte: 1 - Tester et al., 2014; 2 - Casabianca et al., 2019; 3 - Tibiriçá et al., 2019

Recent studies have shown that the potentially toxic epibenthic dinoflagellate *Ostreopsis cf. ovata* has the ability to rapidly colonize (i.e., within a few hours) different plastic surfaces, preferably those of more flexible composition (PP and LDPE) reaching moderate to high cell densities (~ 260 to 4900 cells cm^{-2}) (CASABIANCA et al., 2019, TIBIRIÇÁ et al., 2019). Thus, the increasing occurrence and recent geographic expansion of *Ostreopsis* spp. may be facilitated by their rapid cell adhesion at high densities to plastic materials present in the marine environment (MASÓ et al., 2003; GRANÉLI et al., 2011; CASABIANCA et al., 2019; TESTER et al., 2014). During a massive bloom in southern Brazil, *Ostreopsis cf. ovata* was dominant on the surface of plastics left in situ for 24 h (TIBIRIÇÁ et al., 2019), suggesting that dinoflagellates can colonize plastic during BHAB.

Casabianca et al. (2019) reported a direct relationship between the abundance of harmful algae colonizing plastic substrates and their intracellular toxin content in the Mediterranean Sea. Moreover, dissolved okadaic acid (OA) produced by dinoflagellates of the genera *Prorocentrum* and *Dinophysis* can sorb onto plastic fragments, mainly composed of polystyrene, after 48 h of exposure (COSTA et al., 2020). Thus, the rapid colonization of microalgae and/or the adsorption of their toxic compounds on plastics freshly released into the water column may be of great concern, especially at sites subject to recurrent blooms of potentially toxic microalgae. Tester et al. (2014) defined potential risk levels for human poisoning events (BHABs) as abundance values of *Gambierdiscus*, *Ostreopsis* and *Prorocentrum* on plastic substrates reaching 50-530, 200 and 30 cells cm^{-2} , respectively.

In general, colonization of floating plastic debris by harmful benthic dinoflagellates is expected to be maximum in regions with concurrently high environmental suitability and plastic availability (Figure 4.2). Considering the colonization and toxicity potential of *Ostreopsis cf. ovata* (Table 4.2), and the plastic surface area estimated for the Mediterranean Sea (Table S1), where massive blooms (e.g. 530 cells cm^{-2}) of this species are recurrent, we estimate a colonization potential of 4.2×10^7 cells km^{-2} (or 8.6×10^6 cells km^{-2} during blooms) for this species on plastic debris floating in the Mediterranean Sea. Furthermore, the toxin adsorption capacity associated with this species was estimated as 1×10^7 ng PLTX km^{-2} in the same region. Other places with moderate to high colonization potential (10^5 to 10^6 cells km^{-2}) for *O. cf. ovata* include the Red Sea, South Asia, Australia and Brazil (Figure 4.2.a) Our estimates also indicate a high colonization potential (10^7 cell km^{-2}) for *Prorocentrum lima* in the Mediterranean Sea, where high cell densities of the toxin-producing species *P. lima* have been reported (BEN-GHARBIA et al., 2016; BARBIER et al., 1999). *Prorocentrum* spp. are also common and abundant along the coasts of the USA, Southeast Asia and Australia, where the potential for

plastic colonization was also high in our models (Figure 4.2.b). In Australia, cells of potentially harmful benthic *Prorocentrum* species can remain viable and be dispersed across the ocean attached to floating macrophytes (Larsson et al., 2018), a process that may be enhanced by plastic pollution. Finally, CTX-producing *Gambierdiscus* spp. exhibited higher plastic colonization potential values (10^6 cell km^{-2}) in geographical areas where they are frequently reported (Figure 4.2.c), such as the Caribbean (LITAKER et al., 2009), French Polynesia (CHINAIN et al., 1999), Australia (SEGER et al., 2019), and Hong Kong (CHAN, 2014). Ultimately, floating plastic debris colonized by benthic toxic microalgae may act as vectors of biotoxins in the marine environment, making such toxic compounds available to pelagic organisms and human consumers of seafood.



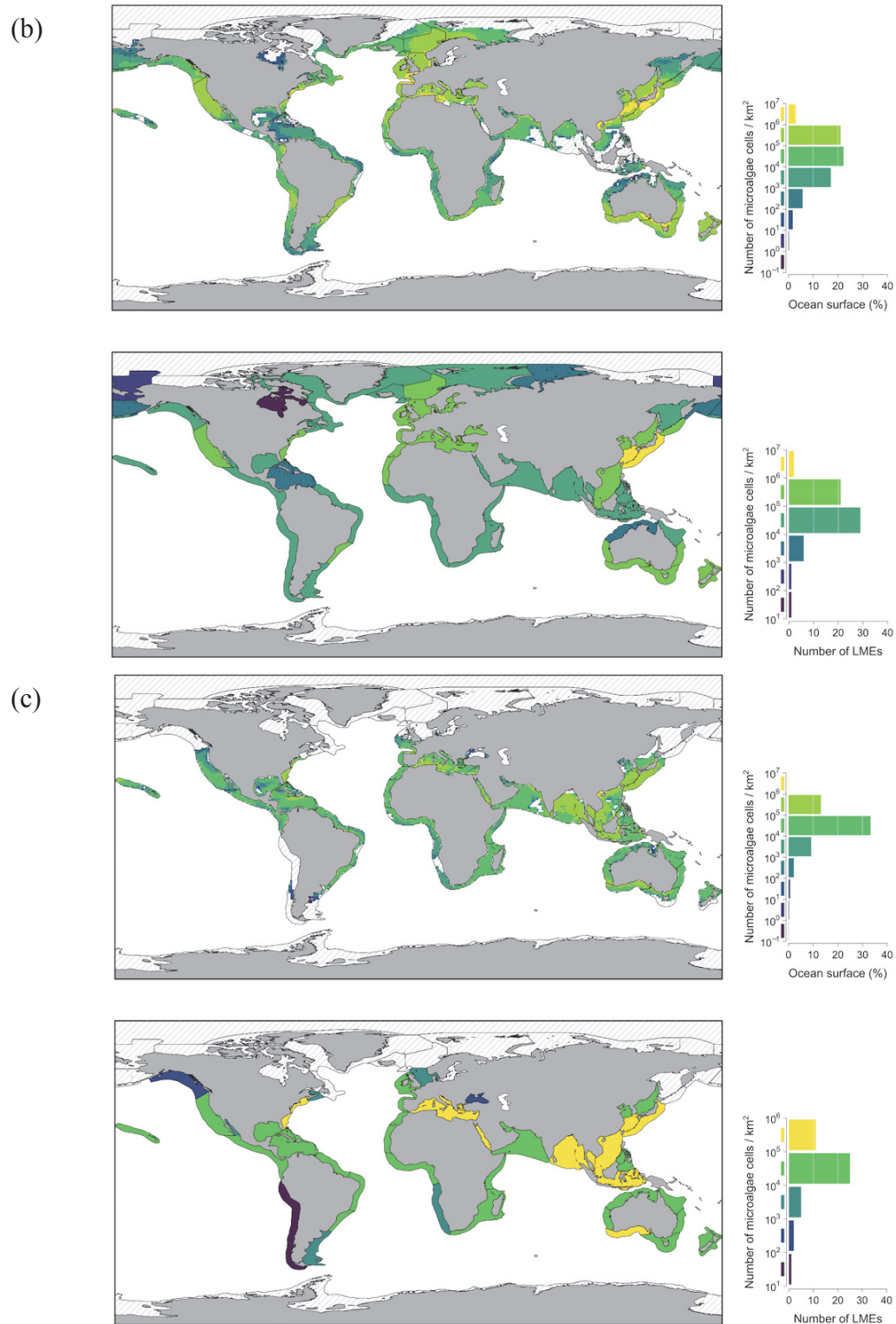


Figure 4.2: Spatial distribution and cell density estimation for *Ostreopsis* cf. *ovata* (a), *Prorocentrum lima* (b) and *Gambierdiscus* (c) on plastic debris in coastal surface waters. For each species, the estimations are represented at 1° x 1° resolution (upper) and for each Large Marine Ecosystems (LME) (bottom panels). Map legend show the coastal area covered by, or the frequency of LMEs within, each level of cell density estimation.

4.3.3. Implications for marine biota and human health

The persistence of plastic debris in marine ecosystems may lead to lethal and sub-lethal effects on organisms of different trophic levels, from plankton to top predators (NELMS, et al. 2018; DERRAIK, 2002; WANG et al., 2019). Damage can be physiological through ingestion of microplastics (see review in LUSHER et al., 2015) or toxic effects, including changes in swimming patterns, feeding, growth, reproduction, oxidative stress, and lethality (PAWAR et al., 2016; ELTEMSAH & BOHN, 2019; DERRAIK, 2002; GALLO et al., 2018; GALLOWAY, 2003; CHOI et al., 2019). Besides the negative effects elicited by the plastic itself, the ingestion of biotoxin-coated plastic items by marine organisms and their consumers may cause additional health damages.

In the ocean, as discussed in Section 4.3.2, plastic debris can be readily covered by toxin-producing benthic dinoflagellates, indicating an alternate route for toxin transfer to marine fauna via ingestion (EICH et al., 2015; MICHELS et al., 2018; OBERBECKMANN; OSBORN; DUHAIME, 2016). Among them, *Prorocentrum* spp. can produce polycyclic ethers, namely okadaic acid (OA) and dynophysotoxins, which are potent inhibitors of serine and threonine protein phosphatases, causing acute gastrointestinal poisoning in humans and inducing cytotoxicity (LEE et al., 2016; VALDIGLESIAS et al., 2013). These dinoflagellates exhibit a wide geographical distribution in tropical and temperate areas (HOPPENRATH, et al., 2014), thus their cells or compounds onto plastic debris can have their dispersal facilitate along the coast and ingestion by marine animals.

Species of the genus *Gambierdiscus* can produce potent neurotoxic compounds, including maitotoxins, considered the most toxic non-proteic compounds (HOLLAND et al., 2013), and ciguatoxins, responsible for the Ciguatera poisoning (CP) in humans following the consumption of contaminated fish (HOPPENRATH et al., 2014; LASSUS et al., 2016). On average, around 50,000 cases of CP are recorded annually, mainly in endemic areas of the Pacific Ocean, Indian Ocean and the Caribbean Sea, from 35 °N to 35 °S (MARCAILLOU et al., 2001). In fact, recurrent CP regions correspond to sites with high occurrences of CTX-producing dinoflagellate species, as reported here. Annually, Australia has reported ~ 140 CP cases (SEGER et al., 2019), French Polynesia ~ 200 cases per 10,000 people (CHATEAU-DEGAT et al., 2007), and Hong Kong 10.2 cases per 1,000,000 people, on average (CHAN, 2014).

Ostreopsis cf. *ovata* produces one of the most potent biotoxins known, the palytoxin (PLTX) and its analogues, the ovatoxins (OvTXs) (HOPPENRATH et al., 2014). High cell densities of *O. cf. ovata* have been found associated with plastic litter in the Mediterranean Sea,

suggesting potential risks to marine fauna and human health related to this association in the region. Indeed, these toxic compounds can cause mortality of marine organisms and human intoxication, related to contact with contaminated seawater, inhalation of sea spray, or ingestion of contaminated seafood (CIMINIELLO et al., 2012).

As reported here, marine plastic litters may be covered by moderate to large amounts of toxic algal cells. Thus, the ingestion of toxic microalgae-containing plastic litter can cause chronic and acute intoxication in animals and humans and should therefore be considered as a potential threat to marine ecosystems and human health. Nor et al. (2021) estimated that global dietary and inhalation rates of microplastic (up to 5 mm) in children and adults can reach 553 and 883 particles inhabitant⁻¹ day⁻¹, respectively. Seafood ingestion should be considered the main route of toxin-coated plastic intoxication to humans. Oysters and mussels, for instance, may contain up to ~0.5 microplastic particles g⁻¹ of soft tissues (VAN CAUWENBERGHE & JANSSEN, 2014). Another risk associated with plastics debris are the *Vibrio* bacteria that preferentially colonize biotic surfaces covered with phytoplankton. Thus, mucous aggregates of *Ostreopsis* cells covering plastic surfaces, for example, may secondarily contribute to their colonization by *Vibrio* spp., creating a multi-contaminant particle. This would ultimately increase the potential risk for intoxication of marine organisms or humans by consumption of contaminated seafood, and for infection by direct contact with water highly polluted with plastics, especially at places with recurrent harmful algal blooms.

4.4. Conclusions

A vast plastic surface area is available in superficial waters of the global ocean. The Northern Hemisphere contains the large total area of available plastic substrate in the oceans, mainly concentrated in the Pacific region. Also, we estimated concentrations of plastic surface area for the Mediterranean Sea, Red Sea, the Southeast U.S. continental shelf, Gulf of Thailand, Bay of Bengal, and the South China Sea as high (10⁶ cm² km⁻²) as those found in large ocean gyres, indicating an extensive and potentially long-lasting surface area for colonization by microorganisms and for adsorption of chemical compounds. Plastic debris can be colonized by toxigenic benthic algae such as *Gambierdiscus* spp., *Ostreopsis* cf. *ovata* and *Prorocentrum lima*. According to our estimates, these epibenthic dinoflagellates show a colonization potential up to 10⁴ cells km⁻² on plastic debris floating in the oceans. Thus, floating plastics can act as vectors for transfer of toxins produced by benthic microalgae along marine trophic chains. The ingestion of toxic microalgae-containing plastic litter by marine animals and humans may lead

to chronic and acute intoxication and become a major threat to marine ecosystems and human health.

5. CONSIDERAÇÕES FINAIS

O presente trabalho buscou ampliar o conhecimento sobre a cinética e a transferência trófica das toxinas produzidas pelos dinoflagelados bênticos dos gêneros *Prorocentrum* e *Gambierdiscus* sobre organismos marinhos, adicionando duas publicações ao tema (LEITE et al., 2021a; 2021b). Além disso, buscou contribuir para o entendimento de como os detritos plásticos nos ambientes marinhos podem atuar como potenciais vetores para a transferência de dinoflagelados bênticos como *Gambierdiscus* spp., *Prorocentrum lima* e *Ostreopsis cf. ovata* e seus compostos tóxicos ao longo das cadeias tróficas.

Os trabalhos descritos nesta tese revelaram níveis detectáveis de toxinas nos tecidos dos berbigões infaunais (*Anomalocardia flexuosa*) e em peixes-leão carnívoros (*Pterois volitans*) após aproximadamente uma semana de exposição experimental às células de *Prorocentrum lima* produtoras de toxinas diarreicas (DSTs), ou cinco semanas sob uma dieta de filés de peixe-papagaio naturalmente contaminados com ciguatoxinas (CTXs), respectivamente. Assim como os berbigões apresentaram capacidade limitada em acumular DSTs a partir dos dinoflagelados, os peixes carnívoros acumularam baixas concentrações de CTXs nos fígados. Nem os bivalves nem tampouco os peixes exibiram respostas adversas claras quando expostos à dieta tóxica, mostrando resistência às toxinas mesmo sendo expostos a dosagens relativamente altas de toxinas. Desta forma, a primeira hipótese da tese foi parcialmente confirmada, ou seja, os organismos consumidores se mostraram resistentes, embora tenham acumulado em seus tecidos concentrações limitadas de toxinas. Já a segunda hipótese formulada no início da tese, especificamente para berbigões, foi refutada. As respostas de biomarcadores de estresse oxidativo decorrentes da exposição de células de dinoflagelados bênticos toxigênicos ou seus compostos tóxicos dissolvidos na água do mar não foram significativamente diferentes das respostas observadas na ausência de toxinas (condição controle).

Indivíduos de berbigões e peixes-leão apresentaram evidências de distribuição diferenciada das toxinas entre os tecidos, como previamente reportado para outras espécies de bivalves (e.g., MAFRA et al., 2015) e peixes carnívoros (LI et al., 2020). Contudo, ao longo do experimento com peixes-leão, variadas limitações metodológicas impediram uma análise quantitativa da distribuição de CTXs entre diferentes tecidos. A distribuição destas toxinas entre

os tecidos dos indivíduos juvenis não pode ser confirmada por métodos mais sensíveis como o CBA-N2a, devido a quantidade insuficiente de amostras restantes após as análises por r-RBA.

Outro resultado relevante apresentado nessa tese foi que, durante os sete dias de depuração, os berbigões apresentaram uma taxa de detoxificação (cerca de 3% por hora) mais eficiente do que a de outras espécies de bivalves (e.g. *P. perna*, *C. gigas* e *C. gasar*) expostas a condições laboratoriais semelhantes (MAFRA et al., 2015). Pelo contrário, os peixes-leão apresentaram um lento processo de detoxificação das CTXs nos fígados. CTXs foram detectadas nesse tecido mesmo após 45 dias de depuração. O tempo de eliminação de toxinas parece superior ao que fora reportado anteriormente para outras espécies de peixes carnívoros como a garoupa *Epinephelus coioides*, que teve um período de depuração estimado em torno de 30 dias (LI et al., 2020). Apesar dos níveis de CTX relativamente baixos que foram detectados em fígados de peixes-leão neste estudo, essa espécie pode representar um risco para intoxicação em humanos. Em trabalhos anteriores, níveis mais elevados de CTXs foram medidos em indivíduos coletados em ambientes naturais, e pelo menos um caso confirmado de CP foi associado ao consumo de peixes desta espécie na Ilha de Guadalupe (HARDISON et al., 2018; BOUCAUD-MAITRE et al., 2018). Por outro lado, *A. fleuxosa*, por seu baixo potencial em acumular DSTs, pode representar um interessante recurso alimentar alternativo em áreas frequentemente afetadas por florações de dinoflagelados produtores destas toxinas, como a costa sul do Brasil. Isso pode ajudar a reduzir o impacto econômico do fechamento de áreas de cultivo de espécies comerciais de moluscos bivalves.

Até o momento da elaboração da presente tese, embora se tenha vasta documentação da colonização por microorganismos e da adsorção de compostos químicos em partículas plásticas (e.g., GREGORY 2009; ENGLER 2012), poucos estudos haviam evidenciado a colonização de microalgas potencialmente nocivas e a adesão de toxinas em detritos plásticos (e.g. MASÓ et al., 2003; 2016; ZETTLER et al., 2013; REISSER et al. 2014a; ONDA et al., 2020). Existe, portanto, uma lacuna importante a ser preenchida na relação entre os temas de poluição marinha e saúde dos oceanos. Assim, buscando contribuir para resolver essa questão, estimamos que uma expressiva área superficial plástica está disponível para a potencial colonização de microorganismos, incluindo espécies de microalgas bênticas produtoras de toxinas. Os dinoflagelados *Ostreopsis* cf. *ovata*, *Gambierdiscus* spp. e *Prorocentrum lima* apresentam um elevado potencial de colonização ($<10^4$ células por km^2) nos detritos plásticos flutuando nos oceanos, onde existem maiores concentrações de plásticos e maior adequabilidade ambiental dessas espécies. Em última análise, uma diversidade de outras espécies toxigênicas podem apresentar um potencial semelhante, como sugerem estudos com os gêneros *Alexandrium*,

Coolia, *Gymnodinium* e *Protoperidinium* detectados em detritos plásticos flutuando nas águas do Mar Mediterrâneo (MASÓ et al, 2003; 2016), Oceano Atlântico (ZETTLER et al., 2003), Austrália (REISSER et al., 2014b) e Filipinas (ONDA et al., 2020). Assim, a presente tese confirmou a hipótese de que as quantidades de plástico atualmente disponíveis na superfície dos oceanos representam um importante vetor alternativo para a transferência de dinoflagelados bênticos e seus compostos tóxicos ao longo das cadeias tróficas marinha, com grande potencial em causar impactos negativos aos ecossistemas e à saúde humana.

6. RECOMENDAÇÕES PARA TRABALHOS FUTUROS

- Estudos que avaliem *in situ* as respostas de biomarcadores de estresse oxidativo em berbigões *A. flexuosa*, em períodos de alta abundância de dinoflagelados produtores de DSTs – *Prorocentrum* spp. ou *Dinophysis* spp. – poderão contribuir para o entendimento dos efeitos adversos decorrentes da exposição desses dinoflagelados em tais organismos.
- Trabalhos complementares fazem-se necessários para se compreender a cinética e transferência de CTXs ao longo da cadeia trófica marinha, sobretudo em peixes carnívoros adultos.
- Apesar de peixes-leão acumularem baixas concentrações de toxinas mediante exposição experimental, testes de campo com estes organismos devem ser considerados, já que eles podem representar um potencial vetor de intoxicação em humanos pela transferência trófica.
- Estudos que avaliem os potenciais impactos da associação entre plásticos e dinoflagelados tóxicos aos organismos marinhos de diferentes níveis tróficos são fortemente encorajados. Em particular, deve-se incluir a avaliação da presença e – principalmente – a quantificação de células de microalgas potencialmente nocivas e suas toxinas sobre detritos plásticos marinhos.

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ANEXOS

ANEXO I: Material suplementar do Capítulo 2

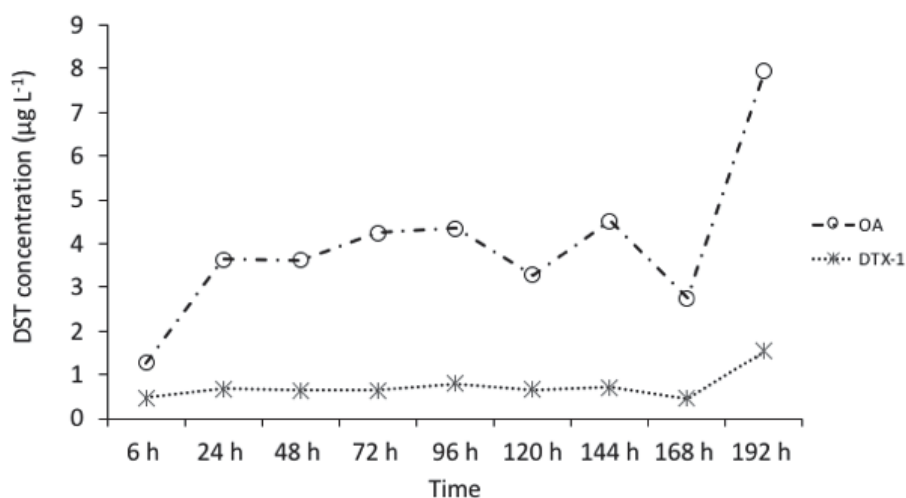
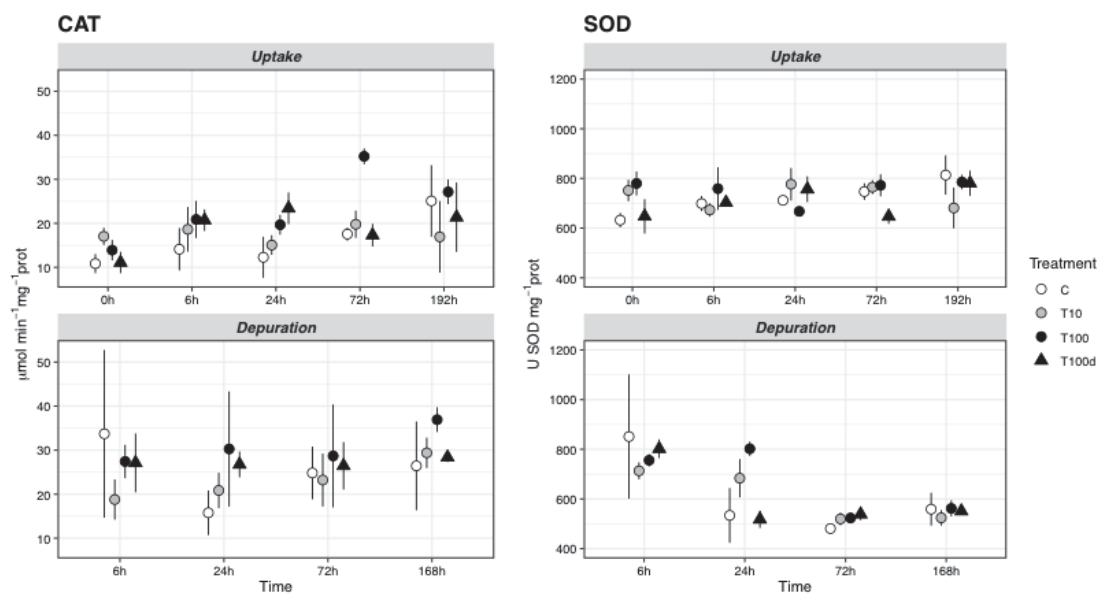


Figure S2.1. Concentration ($\mu\text{g L}^{-1}$) of diarrhetic shellfish toxins (DST) dissolved in seawater of the tanks comprising treatment T100d over the uptake phase of the experiment. Toxin was added to the tanks after disrupting *P. lima* cells at concentrations equivalent to those contained in 100 cells mL^{-1} .



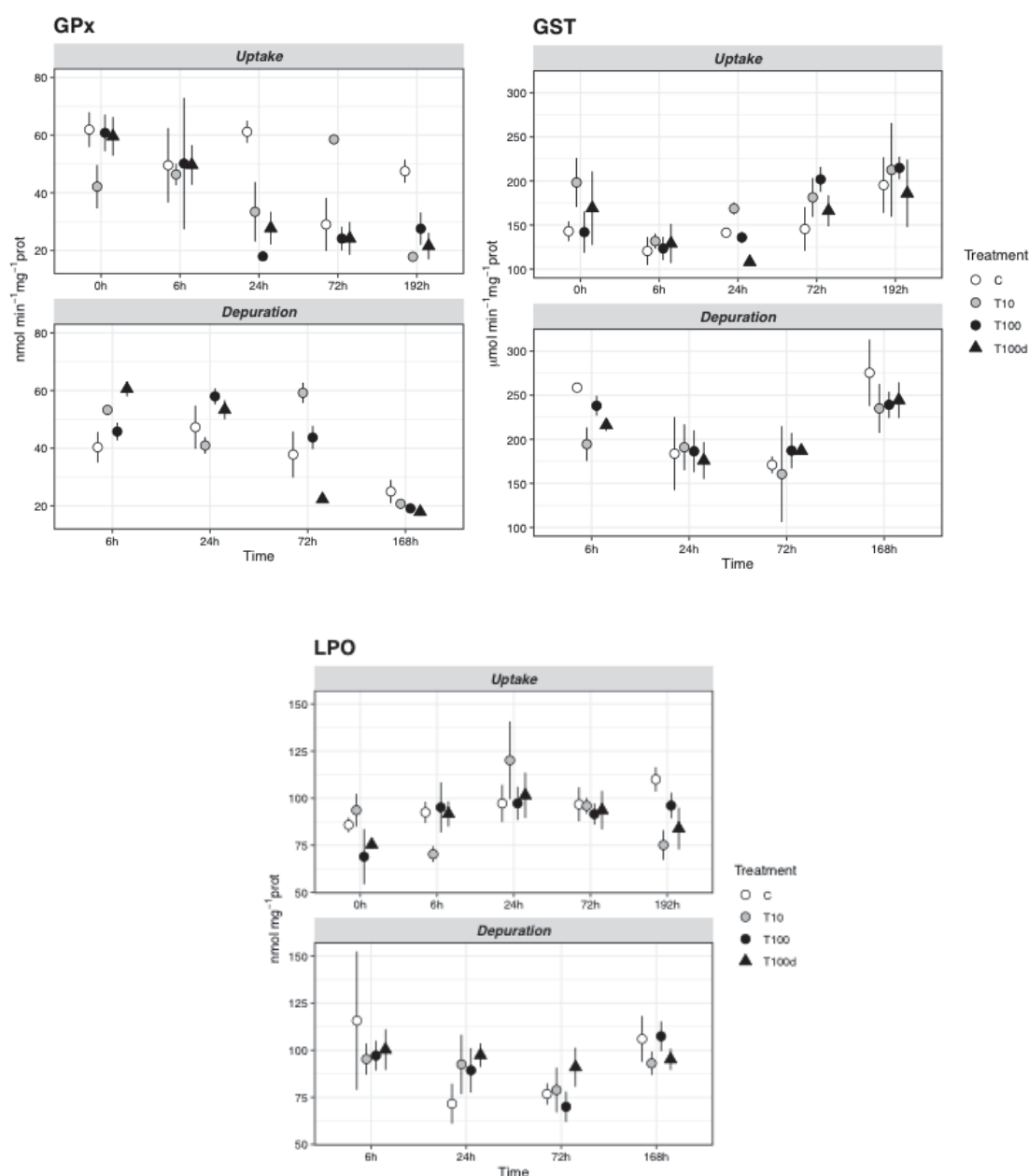


Figure S2.2. Mean $\pm$ SD of **CAT**: Catalase activity ($\mu\text{mol min}^{-1} \text{mg protein}^{-1}$); **SOD**: Superoxide dismutase activity (unity mg protein^{-1}); **GST**: Glutathione S-transferase activity ($\text{nmol min}^{-1} \text{mg protein}^{-1}$); **GPx**: Glutathione peroxidase activity ($\text{nmol min}^{-1} \text{mg protein}^{-1}$); **LPO**: lipoperoxidation ($\text{nmol min}^{-1} \text{mg protein}^{-1}$) among the sampling times (in hours) in all diets tested, including the control condition (C), within each experimental phase – uptake or depuration. During uptake phase, clams were exposed to *P. lima* cells at either 10 (T10) or 100 (T100) cells mL^{-1} , or a toxin concentration of $\sim 4 \mu\text{g OA L}^{-1}$ and $\sim 0.65 \mu\text{g DTX-1 L}^{-1}$ (T100d).

Table S2.1. Physical-chemical parameters monitored over the entire experimental period. Values represent the time-averaged ($\pm$ standard deviation) measurements ($n = 42$) from three tanks per treatment (diet): C (control), T10 (C + 10 cells mL^{-1} *P. lima*), T100 (C + 100 cells mL^{-1} *P. lima*) and T100d (C + toxin concentration of $\sim 4 \mu\text{g OA L}^{-1}$ and $\sim 0.65 \mu\text{g DTX-1 L}^{-1}$).

Treatment	pH	Salinity	OD (mg L^{-1})	Temperature ($^{\circ}\text{C}$)
C	8.0 ± 0.1	35.0 ± 0.3	7.0 ± 0.1	23.3 ± 0.1
T10	8.1 ± 0.1	35.3 ± 0.3	7.0 ± 0.1	23.3 ± 0.1
T100	8.1 ± 0.1	35.3 ± 0.2	6.9 ± 0.1	23.2 ± 0.1
T100d	8.1 ± 0.0	35.3 ± 0.3	6.9 ± 0.1	23.2 ± 0.1

Table S2.2. Mean ($\pm$ SD) wet weight (g) of digestive glands (DG) and remaining tissues of *Anomalocardia flexuosa*, as well as the relative (%) weight of DG in relation to the total soft tissue weight, mean shell height (SH), and number of individuals (n) collected in treatments C, T10, T100 and T100d during the uptake and depuration phases of the experiment.

Treatments	Weight (g)		% DG	SH (mm)	
	DG	Other tissues			n
Uptake					
C	0.16±0.04	1.48±0.30	10.7	20.7±1.75	45
T10	0.16±0.04	1.38±0.26	11.4	20.5±1.20	45
T100	0.15±0.03	1.34±0.23	11.3	20.4±1.32	45
T100d	0.13±0.04	1.42±0.26	9.4	20.2±1.07	45
Mean	0.15±0.04	1.41±0.26	10.7	20.4±1.34	45
Depuration					
C	0.07±0.015	1.33±0.36	5.4	20.5±1.07	36
T10	0.08±0.018	1.29±0.23	5.9	20.5±1.45	36
T100	0.07±0.015	1.35±0.24	5.3	20.9±1.68	36
T100d	0.07±0.014	1.29±0.27	5.7	20.4±1.25	36
Mean	0.07±0.016	1.31±0.27	5.6	20.6±1.34	36

ANEXO II: Material suplementar do Capítulo 3

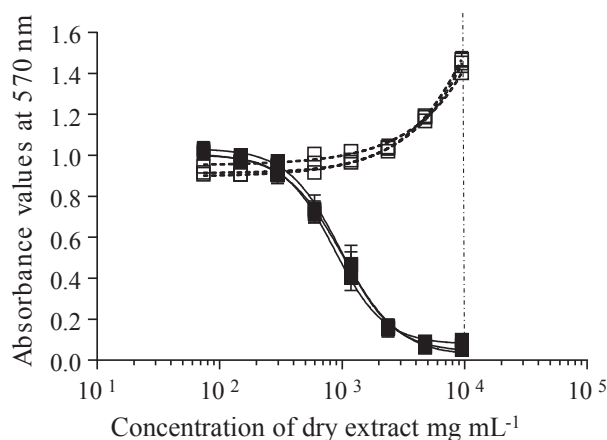


Figure S3.1: Dose-response curves of N2a cells when exposed to increasing concentrations of parrotfish flesh extracts (toxic food) in OV^- (open symbols) and OV^+ (solid symbols) conditions at $85.7/8.75 \mu M$ (final concentrations). Data represent the mean $\pm$ SD of each aliquot tested, with each point run in triplicate. Absorbance values were measured at 570 nm via the MTT assay, after a 45 min MTT incubation time. The initial cell viability was 1.054 ± 0.020 in the RCV control. The mean final cell viability was 0.927 ± 0.023 , in absence of O/V treatment (COV^-), and 1.031 ± 0.029 in the presence of non-destructive O/V treatment (COV^+), respectively. The dotted vertical line corresponds to the MCE established at $10\,000 \text{ mg mL}^{-1}$ of fish flesh extracts avoiding non-specific cytotoxicity in both conditions of OV treatments. The LOD and LOQ values in fish flesh were 0.03 ± 0.01 and $0.06 \pm 0.02 \text{ ng CTX3C equiv. g}^{-1}$ (Viallon et al., 2020).

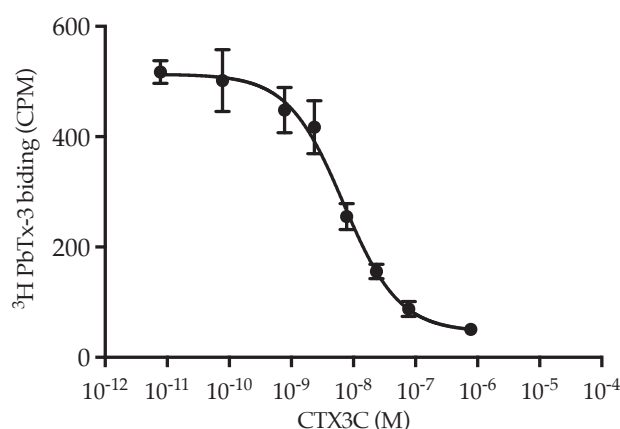


Figure S3.2: Sigmoidal dose-response curve of r-RBA used to quantify the concentration of the CTX in fish liver and muscle during the experiment (GraphPad Software, Inc., La Jolla, California, USA).

ANEXO III: Material suplementar do Capítulo 4

Table S4.1. Estimate of the plastic surface area (cm² km⁻²) available in the surface of different Large Marine Ecosystems (LME), arranged in decreasing order of total plastic area.

LME	Size (mm)			Type				Total
	0.33 - 1.00	1.01 - 4.75	4.76 - 200	Fiber	Bead	Film	Fragment	
Red Sea	241	6911	189982	200	2007	33719	161206	197134
Southeast U.S. Continental Shelf	282	7916	177770	189	1917	31602	152259	185969
Gulf of Thailand	522	15594	155357	174	1906	27916	141476	171473
Mediterranean Sea	340	9764	151981	164	1718	27121	133081	162086
Bay of Bengal	336	9629	111595	124	1328	19999	100111	121561
South China Sea	341	10098	97315	109	1202	17498	88944	107755
Kuroshio Current	207	5769	81898	89.36	939	14632	72213	87875
East China Sea	286	8118	72912	82.54	916	13130	67188	81317
Indonesian Sea	228	6554	69857	77.85	845	12537	63180	76640
Sulu-Celebes Sea	228	6498	64537	72.37	792	11598	58801	71264
Gulf of Mexico	116	3195	66013	70.57	719	11744	56791	69325
South West Australian Shelf	148	4003	58792	64.01	671	10499	51709	62944
Caribbean Sea	117	3352	59182	63.75	657	10545	51384	62650
Northeast U.S. Continental Shelf	115	3161	53871	58.14	601	9603	46885	57148
Iberian Coastal	61	1596	48424	51.02	507	8589	40933	50080
Sea of Japan	128	3580	38145	42.51	461	6846	34504	41854
South Brazil Shelf	121	3497	38077	42.36	459	6831	34364	41696
Celtic-Biscay Shelf	63.3	1668	39621	42.11	425	7040	33846	41353
Insular Pacific-Hawaiian	67.7	1761	38491	41.05	416	6844	33017	40319
Southeast Australian Shelf	81.9	2207	36351	39.31	408	6482	31710	38639
Arabian Sea	91.9	2487	33571	36.75	388	6002	29722	36149
New Zealand Shelf	60.1	1564	29308	31.48	323	5219	25359	30932
North Sea	43.4	1121	27786	29.48	297	4936	23689	28950
Guinea Current	85.8	2599	25587	28.71	315	4599	23329	28272
West Central Australian Shelf	60.9	1594	26205	28.34	294	4673	22865	27860
Baltic Sea	82.2	2246	24841	27.6	298	4455	22388	27169
Pacific Central American Coastal	31.9	912	25369	26.8	267	4502	21517	26314
North Brazil Shelf	51.3	1475	19448	21.32	225	3478	17249	20974
East Central Australian Shelf	35.9	910	19235	20.54	209	3421	16530	20181
Norwegian Sea	25.3	631	18505	19.52	194.5	3283	15665	19162
Canary Current	21.3	525	18239	19.14	189	3232	15345	18786
East Brazil Shelf	27.4	720	15686	16.73	170	2789	13458	16434
Yellow Sea	225	6260	9634	16.11	259	1913	13931	16120
Somali Coastal Current	36.7	994	15010	16.31	171	2679	13175	16041
Oyashio Current	19.9	502	15311	16.13	160	2715	12942	15834
Northeast Australian Shelf	26.7	689.8	15064	16.06	163	2679	12923	15780
Black Sea	426	12345	2999	15.42	357	954	14444	15771
California Current	20.3	507	14571	15.38	153.42	2586	12345	15099

Northwest Australian Shelf	18.4	450	11804	12.5	125.33	2096	10039	12273
Barents Sea	13.5	330	10575	11.12	110	1875	8922	10919
Gulf of California	14.1	357	9971	10.53	105	1769	8457	10342
Agulhas Current	17.7	435	9836	10.48	106.05	1748	8425	10289
Greenland Sea	12.1	292	9874	10.37	102.6	1750	8315	10178
North Australian Shelf	9.5	236	8582	9	88.71	1520	7210	8828
Humboldt Current	17.2	433	5608	6.16	65.38	1003	4984	6058
Faroe Plateau	5.8	133	5501	5.75	56.37	974	4604	5640
Iceland Shelf and Sea	5.56	128	5218	5.45	53.51	924	4369	5351
Labrador Newfoundland	4.64	106	4909	5.12	49.98	869	4097	5021
Scotian Shelf	3.81	88.29	4645	4.83	46.95	822	3864	4737
Central Arctic	4.77	108	4551	4.75	46.59	806	3807	4664
Gulf of Alaska	1.84	42.72	4312	4.44	42.54	761	3548	4357
Canadian High Arctic - North Greenland	4.45	103	4080	4.27	41.93	723	3419	4187
Kara Sea	3.9	88.7	3696	3.86	37.85	654	3092	3788
Patagonian Shelf	14.1	386.04	3254	3.71	41.52	587	3023	3655
Sea of Okhotsk	5.54	137.15	3451	3.66	36.78	613	2940	3593
Canadian Eastern Arctic - West Greenland	2.78	62.54	2783	2.9	28.39	493	2325	2849
Benguela Current	7.81	192.96	2643	2.89	30.51	472	2337	2843
East Siberian Sea	2.3	52.03	2298	2.4	23.45	406	1919	2351
Laptev Sea	2.31	51.9	2258	2.36	23.06	399	1887	2312
Aleutian Islands	0.18	3.47	2298	2.35	22.16	405	1872	2301
West Bering Sea	0.13	2.46	1728	1.77	16.66	304	1407	1730
East Bering Sea	0.1	1.81	1617	1.65	15.58	285	1317	1619
Northern Bering Chukchi Seas	0.89	19.59	1051	1.09	10.62	185	874	1071
Beaufort Sea	0.49	10.23	625	0.65	6.28	110	518	636
Hudson Bay Complex	0.13	2.7	207	0.21	2.07	36.69	171	210
Antarctica	0.02	0.34	79.8	0.08	0.78	14.08	65.23	80.16
Total	90.5	2560	34505	37.78	399	6169	30550	37156