Eva Regina de Oliveira Rodrigues

RESPOSTAS BIOQUÍMICAS E NA ORGANIZAÇÃO CELULAR DA ALGA PARDA *Sargassum cymosum* var. *stenohyllum* (Martius) Grunow (HETEROKONTOPHYTA, FUCALES) À EXPOSIÇÃO À GASOLINA E AO ÓLEO DIESEL

Dissertação submetida ao Programa de Pós-Graduação em Biologia Vegetal da Universidade Federal de Santa Catarina para a obtenção do Grau de Mestre em Biologia Vegetal.
Orientador: Dr. Marcelo Maraschin
Co-orientador: Dr. Paulo Antunes Horta Júnior

Florianópolis
2014

Ficha de identificação da obra elaborada pelo autor,
através do Programa de Geração Automática da Biblioteca Universitária da UFSC.

Rodrigues, Eva Regina de Oliveira
RESPOSTAS BIOQUÍMICAS E NA ORGANIZAÇÃO CELULAR DA ALGA PARDA Sargassum cymosum var. stenohyllum (Martius) Grunow (HETEROKONTOPHYTA, FUCALES) À EXPOSIÇÃO À GASOLINA E AO ÓLEO DIESEL / Eva Regina de Oliveira Rodrigues ; orientador, Marcelo Maraschin ; coorientador, Paulo Antunes Horta Junior. - Florianópolis, SC, 2014.
134 p.

Dissertação (mestrado) - Universidade Federal de Santa Catarina, Centro de Ciências Biológicas. Programa de Pós-Graduação em Biologia Vegetal.

Inclui referências

1. Biologia Vegetal. 2. Alga parda. 3. Sargassum cymosum var. stenophyllum. 4. Perfil metabólico. 5. Respostas ao estresse. I. Maraschin, Marcelo . II. Junior, Paulo Antunes Horta. III. Universidade Federal de Santa Catarina. Programa de Pós-Graduação em Biologia Vegetal. IV. Título.

Este trabalho é dedicado à minha filha Évelin Rodrigues,
pelo apoio incondicional.

AGRADECIMENTOS

Agradeço a minha mãe Cloé Oliveira
Agradeço a meu Orientador Marcelo Maraschin, um mestre nato com quem tenho o privilégio de trabalhar por sua serenidade na resolução de questões. Pela presença e apoio sempre que seus orientados e não orientados precisam. Pelo cultivo da parceria e do auxílio entre os membros de sua equipe e por acreditar em mim.
A Fernanda Ramlov
A professora Zenilda Bouzon por abrir seu laboratório para execução das análises microscópicas e a sua equipe Débora Tomazi, pelo auxílio na microscopia de luz, a Luz Karime Polo e Marthielen Felix, pela amizade e carinho
Agradeço ao. Éder Carlos Schmit, responsável pelo êxito e qualidade das análises microscópicas executadas neste trabalho. obrigada também pelo carinho e pela acolhida em seu grupo de parceiros.
A equipe do Laboratório de Biologia Celular Vegetal
A equipe do Laboratório de Morfogênese e Bioquímica Vegetal pela camaradagem e pronta disposição sempre que precisei de auxílio. Aline Pereira, Bianca Coelho, Virgílio Uarrota, Beatriz Veleirinho, Eduardo Nunes, Manuel Duprá, Claudia Bauer, Luisa, Bruno Bachiega Simone Kobe, Amélia Somensi, Maíra Tomazoli
Agradecimento especial ao meu AMIGO Rodolfo Moresco cuja presença na minha vida é um presente
Ao professor Paulo Horta por ter colocado seu laboratório a disposição para implantação do experimento que resultou neste trabalho e a suas orientadas Cintia Martins , Cintia Lülier, Lidiane Gouveia e Pamela Munoz, da equipe do professor Paulo Horta, pelo apoio e camaradagem todas as vezes que precisei utilizar as dependências do LAFIC.
A Juliana Mazùrkiev, amiga de todas as horas pelo tempo que dispensou na implantação do meu experimento. Ao Anderson Moura, esposo da Fernanda pela coleta do material que deu início ao experimento
A equipe técnica do Laboratório Central de Microscopia Eletrônica da UFSC, em especial a Eliana Oliveira e Thaís Costa..
À professora Maria Alice Neves, coordenadora do Programa de Pós Graduação em Biologia Vegetal, pelo pronto atendimento aos mestrandos e a chefe de expediente Priscila Machado.
Agradecimento especial ao Paulo Ricardo de Medeiros Rodrigues.

"Nascemos para manifestar a glória do Universo que está dentro de nós. Não está apenas em um de nós: está em todos nós. E conforme deixamos nossa própria luz brilhar, inconscientemente damos às outras pessoas permissão para fazer o mesmo."
(Nelson Mandela)

OLIVEIRA, EVA REGINA. **Respostas bioquímicas e na organização celular da alga parda *Sargassum cymosum* var. *stenophyllum* (Martius) Grunow (Heterokontophyta, Fucales) à exposição à gasolina e ao óleo diesel**
Dissertação (Mestrado em Biologia Vegetal) – Programa de Pós-Graduação em Biologia Vegetal, Universidade Federal de Santa Catarina, Florianópolis, 2014

RESUMO

Ocupações e atividades antropogênicas têm contribuído ao aumento do impacto ambiental e da degradação dos ecossistemas marinhos por poluentes químicos (*e.g.*, xenobióticos orgânicos e derivados de petróleo) lançados diretamente ou transportados pelo escoamento de águas pluviais de áreas urbanas. Neste trabalho avaliou-se *in vitro* o impacto na bioquímica e ultraestrutura da alga parda *Sargassum cymosum* var. *stenophyllum* (Martius) Grunow da exposição aguda (30 min, 1 h, 12 h, e 24h) ao óleo diesel e gasolina nas concentrações de 0,001, 0,01, 0,1 e 1% (v/v). Comparativamente ao controle, a exposição aos derivados de petróleo alterou o metabolismo de *S. cymosum* var. *stenophyllum*, considerando os teores de clorofilas *a* e *c*, carotenoides e compostos fenólicos. Os conteúdos de clorofilas mostraram-se elevados em resposta aos tratamentos com óleo diesel, similar à gasolina nos tempos de 12h e 24h de exposição. Os teores de carotenoides totais foram modificados pelos tratamentos em estudo, porém um padrão de expressão fenotípica não foi detectado. De outra forma, uma clara redução nos valores de concentração total de compostos fenólicos resultou da exposição aos agentes poluentes. As análises bioquímicas foram corroboradas pelos resultados das análises por microscopia de luz (ML), eletrônica de varredura (MEV) e eletrônica de transmissão (MET). A análise de imagens revelou o espessamento de parede celular, o aumento no tamanho de cloroplastos, a migração de compostos fenólicos à parede celular, bem como a redução de fisoides e a dilatação das membranas dos tilacoides. Complementarmente, a determinação do perfil metabólico de amostras expostas ao óleo diesel por 24 h, via espectroscopia de ressonância magnética nuclear de ^{1}H(^{1}H-RMN), corroborou os

resultados das análises bioquímicas, onde uma clara alteração metabólica foi detectada à exposição ao óleo diesel, comparativamente à amostra controle. Em seu conjunto, os resultados desta investigação sugerem que a espécie *Sargassum cymosum* var. *stenophyllum* responde de variadas formas à exposição ao óleo diesel e gasolina, constituindo um potencial biomarcador de áreas marinhas afetadas pela contaminação por esses derivados de petróleo.

Palavras-chave: *Sargassum cymosum* var. *stenophyllum.* Derivados de petróleo. Perfil metabólico.

OLIVEIRA, EVA REGINA. Biochemical responses and cellular organization of the brown seaweed *Sargassum cymosum* var. *stenophyllum* when exposed to gasoline and diesel. Dissertation (Master in Plant Biology) – Pos-graduation Program in Plant Biology, Federal University of Santa Catarina, Florianópolis, 2014.

ABSTRACT

Occupations and anthropogenic activities have led to increased environmental impact and degradation of marine ecosystems by chemical pollutants (xenobiotics and petroleum derivatives, *e.g.*) directly disposed into those environments or transported by storm water runoffs from urban areas. In this work, we evaluated the impact on the biochemical and ultrastructural traits of the brown seaweed *Sargassum cymosum* var. *stenophyllum* (Martius) Grunow acutely (30 min, 1h, 12h, and 24h) exposure to diesel and gasoline (0.001, 0.01, 0.1, and 1% - v/v). Comparatively to control treatments, the exposure to petroleum derivatives changed the *S. cymosum* var. *stenophyllum* metabolism regarding the chlorophyll *a* and *c*, carotenoid, and phenolic compounds contents. The chlorophyll amounts showed to be increased following the diesel treatments, similarly to 12h- and 24h-gasoline exposure. The carotenoids also varied in their contents in the treated biomass samples, despite a typical phenotype have not been detected. On the other hand, a clear reduction in the phenolic compounds resulted from the brown alga exposure to those pollutants. Light microscopy, scanning electron microscopy, and transmission electron microscopy analyses corroborate in any extension the biochemical findings. By cell imaging analysis the thickness of the cell wall was detected, as well as the increase in chloroplast size, migration of phenolic compounds toward the cell wall, reduction in the number of physoides, and dilation of thylakoid membranes. Complementally, the metabolic profile of 24h-diesel treated samples of *S. cymosum* var. *stenophyllum* was investigated by ^{1}H nuclear magnetic resonance spectroscopy (^{1}H-NMR), corroborating the biochemical findings, i.e., also revealing a prominent metabolic change in diesel treated samples comparatively to control ones. Taken together, the results of this investigation suggest that Sargassum species cymosum var. stenophyllum responds in many ways to exposure to diesel and gasoline, constituting a potential biomarker of marine areas affected by contamination by these petroleum products.

Keywords: *Sargassum cymosum* var. *stenophyllum*. Petroleum derivative. Metabolic profile

LISTA DE FIGURAS

1 SUMÁRIO

1 INTRODUÇÃO

Da grande variedade de organismos existente no planeta, é importante destacar os de origem marinha, sobretudo as comunidades de algas e sua diversidade. As algas são organismos fotossintetizantes, base da cadeia alimentar e responsáveis pelo equilíbrio dos ecossistemas naturais. Com representantes que vão desde organismos planctônicos, que compõem cerca de 50% da base alimentar dos ecossistemas marinhos (QUARTTERS-GOLLOP *et al*. 2011), até indivíduos de grandes proporções como as pardas da ordem *Laminariales*. Compõem expressivo número de espécies funcionais, com diferentes habilidades para tolerar inúmeros fatores ambientais e resiliência a alterações no meio aquático, inclusive àquelas impostas por atividades humanas (VIDOTTI E ROLLEMBERG, 2004).

As macroalgas ou algas bentônicas constituem uma parte fundamental dos ecossistemas marinhos, como por exemplo na formação de comunidades de costões, no forrageamento e desova ou mesmo como terreno vital para muitas espécies de peixes juvenis .

De grande aplicabilidade, muitas macroalgas marinhas são utilizadas principalmente como fonte de alimento humano. São, ainda, fornecedoras de substâncias que podem conferir alto valor a produtos, entre as quais compostos bioativos e polissacarídeos, os ficocoloides ágar-ágar, ácido algínico e carragenana, principais constituintes das paredes celulares de algas verdes, pardas e vermelhas, utilizados pela indústria (EL GAMAL, 2010).

Habitam locais com fortes interações biológicas e condições abióticas extremas. Para garantir sobrevivência nesses ambientes altamente competitivos as algas são dotadas de diversas estratégias de defesa que são expressas na produção de variado número de metabólitos, à partir de diversas rotas biossintéticas (BARROS *et al.* 2005; RAMLOV, 2010). Muitos desses compostos têm potencial impacto econômico, por exemplo, em ciências de alimentos, na indústria farmacêutica e de produtos. Entre estes compostos estão os ácidos graxos, esteroides, carotenoides, polissacarídeos, lectinas, aminoácidos tipo micosporinas, compostos halogenados e toxinas. (CARDOZO *et al.* 2007).

Os mecanismos de defesa de muitas algas podem atenuar ou neutralizar impactos naturais ou antropogênicos originados de fontes diversas, *e.g.*, metais pesados, xenobióticos orgânicos de diferentes classes e derivados de petróleo, com efeitos estressores significativos

capazes de degradar a integridade ecológica dos ecossistemas marinhos (TORRES *et al.* 2008).

O estudo das alterações fisiológicas e bioquímicas, além da identificação e quantificação de poluentes em organismos base na cadeia trófica, como as algas, pode configurar importante ferramenta diagnóstica do impacto ambiental (HANDY & DEPLEDGE, 1999; TÔRRES *et al.* 2008; FAROOQ *et al.* 2010). Entre os poluentes mais referidos estão os íons de metais pesados (*e.g.*, chumbo, cádmio, mercúrio e cromo). A maior toxicidez desta classe de poluentes é devido a suas propriedades bioacumulativas, biomagnificantes e não biodegradáveis (VOLESKY, 1994; SCHMIDT, 2009; SCHERNER *et al.* 2012). Outros poluentes de grande ocorrência em sistemas aquáticos são os pesticidas, derivados de petróleo e compostos orgânicos, com destaque para os bifenis policlorados e dioxinas (TÔRRES, *et al.* 2008; FAROOQ *et al*. 2010).

Embora a biodegradação de hidrocarbonetos de petróleo tenha sido foco de investigações (MEGHARAJ *et al.* 2000; TÔRRES, 2008), dados relativos à toxicidade destes são limitados considerando o espectro de espécies marinhas (STEPANYAN *et al.* 2006). Além disso, é escassa a literatura quanto à influência dos derivados de petróleo em macroalgas marinhas (RAMLOV, 2010). Segundo Stepanyan e Voskoboinikov (2006), os efeitos da poluição ou contaminação por agentes como derivados de petróleo podem, por exemplo, afetar a biossíntese de clorofila, a atividade fotossintética e o crescimento desses organismos. De fato, ao submeter a Chlorophyta *Ulva pertusa* Kjellman a diferentes concentrações de hidrocarbonetos de petróleo, Wang et al. (2011) observaram alterações na taxa fotossintética e respiratória. Ramlov et al. (2013), examinando as respostas bioquímicas e celulares da Rhodophyta *Hypnea musciformis* (Wulfen) J. V. Lamour exposta *in vitro* a quatro concentrações de óleo diesel, constataram redução na taxa fotossintética, alterações na produção de metabólitos secundários e morfologia dos espécimes analisados. Além desses, estudos com algas pardas relatam resultados positivos na análise de elementos traço e seus acúmulos em tecidos de duas espécies de algas (*Fucus vesiculosus* Linnaeus e *Fucus ceranoides* Linnaeus) expostas ao derramamento de petróleo em seu ambiente natural (VILLARES *et al.* 2007). De forma similar, Pietroletti *et al.* (2010) detectaram mudanças estruturais e metabólicas, como a redução nos teores de clorofila em *Caulerpa racemosa* (Forsskal) J.Agardh , utilizando a espectrofotometria UV-visível e a espectroscopia vibracional de

infravermelho médio, após exposição *in vitro* a hidrocarbonetos e óleo diesel.

Neste contexto, estudos têm demonstrado a importância da utilização de macroalgas como bioindicadoras de poluição (EKLUND; KAUTSKY, 2003). Além de importantes produtores primários, as macroalgas são, por vezes, mais sensíveis a poluentes químicos em relação a outros organismos marinhos (BENENATI, 1990), constituindo um bom modelo de estudo para impactos causados por derivados de petróleo.

Algas da classe Phaeophyceae, as pardas, já têm sido usadas com frequência no monitoramento ambiental e como adsorventes, devido à alta capacidade de acumular metais pesados (ANDRADE *et al.* 2010; STENGEL *et al.* 2004; VIJAYARAGHAVAN *et al.* 2009), propriedades atribuídas a alguns tipos de hidratos de carbono e compostos fenólicos com sítios de ligação a cátions polivalentes (STENGEL *et al.* 2004). Estudo com a alga parda *Padina gymnospora* (Kützing) Sonder exposta a metais pesados evidenciou incrementos de síntese de polissacarídeos de parede celular, comparativamente aos indivíduos em ambientes não poluídos (ANDRADE *et. al.* 2010). Esta resposta bioquímica é considerada uma possível estratégia de proteção para evitar a absorção de metais pesados.

A classe das algas pardas é constituída por organismos pluricelulares predominantemente marinhos, mais comuns em mares frios. Compreendem as algas de maior relevância em águas temperadas e polares, ocorrendo fixadas a substratos ou flutuantes, formando imensas florestas submersas.

Dominam os costões rochosos nas regiões mais frias do globo terrestre. A esse grupo pertencem as algas da ordem *Laminariales*, entre as quais estão as maiores existentes, podendo atingir mais de 25 metros e que formam extensas coberturas a pouca distância da costa, chamadas de *kelps*. Mesmo em regiões tropicais, onde não são predominantes, algas pardas podem formar imensas massas flutuantes. Nessas áreas algumas espécies, como *Sargassum muticum* (Yendo) Fensholt, podem apresentar níveis de crescimento indesejáveis. Em costões com baixa declividade podem estender-se por até 10 quilômetros da costa e em águas claras podem ocorrer desde o nível de maré baixa até 30 metros de profundidade.

Embora constituam grupo monofilético, as algas pardas podem variar de tamanho, desde formas microscópicas até as maiores de todas as macroalgas. Nesses organismos são encontrados além das clorofilas *a* e *c*, compostos carotenoídicos, em maior quantidade a fucoxantina,

xantofila que confere aos membros desse grupo a cor característica, entre marrom e verde-oliva. O material de reserva dessas algas é o carboidrato laminarina, presente em vacúolos (VIDOTTI E ROLLEMBERG, 2004). Importante produto derivado das algas pardas, em especial as de clima temperado, e constituinte das paredes celulares é o polissacarídeo sulfatado alginato, de aplicações bastante importantes entre as quais o uso como estabilizante e emulsificante de alimentos e na formulação de tintas (VIDOTTI E ROLLEMBERG, 2004) . Quanto aos compostos fenólicos, vários estudos, principalmente com espécimes de regiões de clima temperado, têm demostrado que os polifenóis de algas pardas estão envolvidos na defesa química contra herbivoria. Dentre os compostos fenólicos, os florotaninos, em especial o floroglucinol, são os predominantes e encontrados apenas neste grupo de algas. Aos florotaninos são atribuídas, juntamente com as fucanas - polissacarídeos sulfatados complexos encontrados na paredes celulares de algas pardas - propriedades antioxidantes (BALBOA *et al.* 2013). Nessas algas os compostos fenólicos, polímeros de floroglucinol, localizam-se no interior dos fisoides, vesículas com importante papel funcional na constituição de paredes e em mecanismo de reparo de danos (RIVERS, 2007).

Gênero Sargassum

As algas pardas, que predominantemente ocorrem em regiões de clima temperado, têm como um dos representantes tropicais aquelas do gênero *Sargassum*. Ocorrendo tanto em costões rochosos protegidos como em costões expostos à ação das ondas (YONESHIGUE-VALENTIN, 2009), algas do gênero *Sargassum* exercem importante papel ecológico, na composição e distribuição de comunidades de costões rochosos (JACOBUCCI E LEITE, 2002), onde desempenham um papel fundamental na cadeia alimentar marinha, inclusive influenciando a ocorrência de uma diversificada flora e fauna associadas (SZÉCHY *et al.* 2006). Encontrados ao longo da costa brasileira, espécimes desse gênero são característicos na produção de metabólitos secundários que reduzem a palatabilidade das algas para os herbívoros, influenciando, assim, a estrutura das populações desses costões rochosos (COIMBRA, 2006). Nesse gênero, destaque para as algas da espécie *Sargassum cymosum* C. Agardh e variedades, de ampla ocorrência no território nacional e de relevante importância ecológica nos ecossistemas costeiros (YONESHIGUE-VALENTIN *et al*, 2009; MAFRA *et al.* 2010).

Algas do gênero *Sargassum* têm sido aplicadas na formulação de rações (Holdt; Kraan, 2011) e sua biomassa encerra capacidade de biossorção de compostos (Széchy *et al*. 2006; Andrade *et al.* 2010). Além disso, compostos constituintes ou produzidos por espécies de *Sargassum*, têm potencial importância nutracêutica (Matanjun *et al*. 2010).

Metabólitos secundários

Os organismos fotossintetizantes produzem grande variedade de metabólitos secundários, que diferem dos compostos primários por apresentarem ocorrência e distribuição restritas, ou seja, metabólitos secundários específicos são restritos a determinadas espécies de organismos e, muitas vezes, são produzidos em situações especiais.

De um modo geral exercem funções ecológicas importantes contra herbívoros e patógenos ou como atrativos, nas competições ou simbioses. Podem ser divididos em três grupos quimicamente distintos: terpenos, compostos fenólicos e compostos nitrogenados.

Em organismos marinhos, compostos que particularmente despertam o interesse comercial, além dos primários (polissacarídeos, lipídeos e ácidos graxos e proteínas), incluem compostos do metabolismo secundário como pigmentos e compostos fenólicos. Tendências recentes na pesquisa de medicamentos de fontes naturais têm apontado as algas como promissores organismos para fornecimento de novos compostos bioquimicamente ativos (CARDOZO *et al.* 2007). A composição e concentrações químicas das populações de algas naturais são influenciadas por interações abióticas espaciais e temporais nos parâmetros ambientais, incluindo luz, temperatura , nutrientes e salinidade e também intervenções antropogênicas, bem como interações bióticas (Stengel *et al*. 2011). Neste sentido, a análise das possíveis alterações em níveis de compostos secundário como os fenólicos e os carotenoídicos, bem como de clorofilas (*a* e *c* no caso das algas pardas) podem servir como parâmetros para avaliar possíveis alterações nos organismos produtores.

A determinação do perfil metabólico parcial, associada à análise do processo de estresse de macroalgas marinhas expostas a derivados de petróleo é considerada uma estratégia adequada à identificação de compostos candidatos a marcadores bioquímicos associados ao estresse derivado da exposição aqueles poluentes. Assim, este trabalho utilizou como modelo de estudo o cultivo *in vitro* da macroalga *Sargassum cymosum* var. *stenophyllum* (Martius) Grunow e suas interações com

derivados de petróleo, gerando informações relevantes ao entendimento dos efeitos daqueles poluentes em bases bioquímicas e morfológicas.

1.1 CARACTERIZAÇÃO SUMÁRIA DOS DERIVADOS DE PETRÓLEO EM ESTUDO

1.1.1 Óleo Diesel

O óleo diesel é um combustível derivado do petróleo, inflamável e usado em motores de combustão interna e ignição por compressão. Consiste em uma das frações obtidas por destilação no refino do petróleo. É constituído basicamente por hidrocarbonetos, em proporções que variam conforme as características de ignição requeridas (Petrobrás Distribuidora).

1.1.2 Gasolina

Combustível automotivo composto basicamente por uma mistura de hidrocarbonetos e, em menores quantidades, por produtos oxigenados, nitrogenados, com enxofre e compostos metálicos. A gasolina é produzida à partir do refino do petróleo através de complexos processos constituídos de várias etapas que definem os tipos de gasolina. Na composição deste combustível os hidrocarbonetos utilizados são mais leves comparativamente aqueles do óleo diesel. As dimensões das cadeias carbônicas estendem-se de C6 a C12, sendo constituída majoritariamente por octano C_8H_{18} (Petrobrás Distribuidora).

2 HIPÓTESE

A exposição de espécimes da macroalga *Sargassum cymosum* var. *stenophyllum* aos agentes poluentes óleo diesel e gasolina causa alterações significativas em seu metabolismo e organização celular.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar as respostas bioquímicas e morfológicas da alga parda *Sargassum cymosum* var. *stenophyllum* quando exposta a derivados de petróleo, *e.g.*, óleo diesel e gasolina.

3.2 OBJETIVOS ESPECÍFICOS

- Determinar a produção de metabólitos secundários, *e.g*. carotenoides e compostos fenólicos em talos de *S. cymosum* expostos *in vitro* a gasolina e óleo diesel, via espectrofotometria de UV-visível..
- Quantificar clorofilas *a* e *c*, em talos de *S. cymosum* var. *stenophylum* expostos *in vitro* a gasolina e óleo diesel, via espectrofotometria de UV-visível.
- Identificar alterações citológicas e na organização celular em amostras de *S. cymosum* var. *stenophylum* expostas aos derivados de petróleo em estudo, via microscopia de luz, eletrônica de varredura e eletrônica de transmissão.
- Prospectar alterações de perfis metabólicos de talos de *S. cymosum* var. *stenophylum* consoante aos tratamentos em estudo, via espectroscopia de ressonância magnética nuclear de ^{1}H.

4 MATERIAL E MÉTODOS

4.1 OBTENÇÃO DO MATERIAL BIOLÓGICO

Espécimes de *S. cymosum* var. *stenophyllum* foram coletados na praia de Ponta das Canas, Florianópolis, estado de Santa Catarina, região sul do Brasil (27º23'34" S, 48º26'11" W) (Figuras 1 e 2), em 12 de setembro de 2012, acondicionados em recipientes com água do mar e transferidos ao Laboratório de Ficologia (LAFIC/CCB) da Universidade Federal de Santa Catarina, UFSC.

4.2 PREPARO DA BIOMASSA

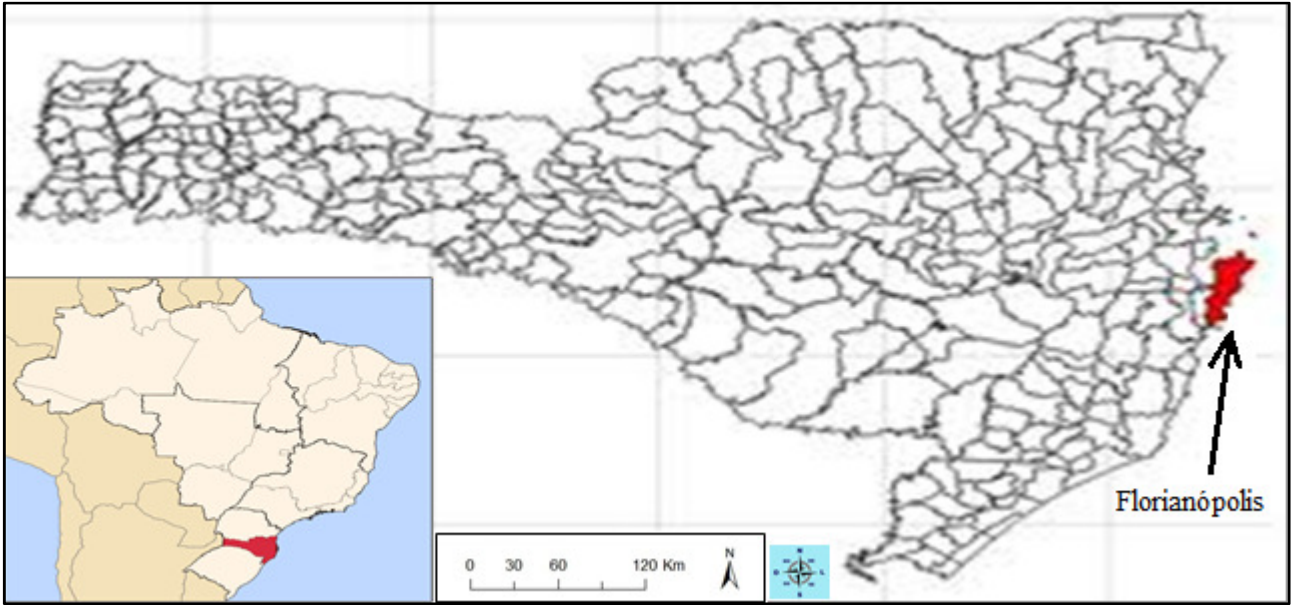
Em laboratório, as amostras foram limpas manualmente para remoção de epibiontes e cultivadas, *in vitro,* em água do mar enriquecida com solução de von Stosch (VSES), preparada segundo Edwards (1970). O enriquecimento consistiu da adição de 4 mL de VSES (50%) a 1000 mL de água do mar esterilizada. Este meio foi utilizado à aclimatação das amostras em estudo, sob condições de 24°C ± 2, fotoperíodo de 12 h, irradiância, ao dia, de 80 µmol de fótons.m^2s^{-1} e salinidade de 34 ups (± 1 ups) (unidade padrão de salinidade), sob agitação constante, durante três semanas. As trocas de meio de cultura foram feitas a cada 5 dias.

4.3 IMPLANTAÇÃO DO EXPERIMENTO

A escolha das concentrações, bem como o detalhamento do presente experimento, seguiu as etapas desenvolvidas em projeto piloto prévio conduzido por Ramlov *et al.* (2013).

Após o período de aclimatação, talos (2g, massa fresca) dos espécimes foram acondicionados em Erlenmeyers contendo 400 mL de água do mar, nas mesmas condições do período de aclimatação, com adição de gasolina ou óleo diesel nas concentrações de 0.001; 0.01; 0.1 e 1% (v/v), ao longo de uma série temporal, i.e., t_{zero} (controle, ausência de gasolina ou óleo diesel), 30 min., 1h, 12h e 24h (Figura 2). Cada tratamento foi constituído de cinco repetições simultâneas. Ao término dos períodos de incubação, alíquotas de 1 g (peso fresco) das amostras foram coletadas, imediatamente congeladas em nitrogênio líquido e transferidas a freezer -80ºC até posterior análise. As amostras controle (n = 5) foram coletadas diretamente do meio de cultura, sem adição de gasolina ou óleo diesel, após 24 horas do início do experimento.

Figura 1. Mapa do estado de Santa Catarina destacando Florianópolis (em vermelho no mapa)



Fonte: www.rpsc.ufsc.br

Figura 2. Mapa de Florianópolis com a localização da praia de Ponta das Canas (A); Praia de Ponta das Canas (B); Banco de *Sargassum cymosum* (C).

www.cbers.inpe; cifonauta.cebimar.usp

Figura 3. Etapas sequenciais à implantação dos experimentos: A) Detalhe da biomassa fonte de explantes amostrais de *Sargassum cymosum* var. *stenophyllum*; B) Amostras de cultivo *in vitro* de talos de *S. cymosum* em meio de cultura contendo 0.001, 0.01,0.1 e 1 % de gasolina, sob aeração; C) Amostras de segmentos de talos de *S. cymosum* em cultivo *in vitro* na presença de óleo diesel (0.001, 0.01, 0.1 e 1% - v/v, frascos dispostos sequencialmente [→] em função das concentrações apresentadas).

4.4 ANÁLISES BIOQUÍMICAS

4.4.1 Extração e quantificação de clorofilas a e c

Para extração das clorofilas foi utilizada acetona (grau p.a. - padrão analítico, 4ºC, 3mL/225mg, peso fresco, n = 5). A biomassa amostral foi triturada na presença de N_2 líquido, incubada em banho de gelo (10 min, ausência de luz) e centrifugada (12000*g*, 5 min). O sobrenadante foi recolhido e medidas as absorbâncias nos comprimentos de onda de 630 ηm, 647 ηm e 664 ηm. Os teores de clorofilas foram calculados de acordo com a equação de Jeffrey and Humphrey (1975).

4.4.2 Extração e quantificação de carotenoides

A extração de carotenoides seguiu, com modificações, protocolo anteriormente descrito por Kuhnen et al. (2009).

A biomassa amostral (1g, peso fresco, n = 5) foi imersa em N_2 líquido, macerada na presença de 10 mL de álcool metílico (p.a.) e incubada (1h, ausência de luz). O extrato organossolvente foi filtrado em suporte de celulose (∅ poro 14µm), sob vácuo. O filtrado foi transferido a espectrofotômetro UV-visível para leitura das absorbâncias nos comprimentos de onda de 200-700 ηm. Os valores de absorbância a 450 ηm foram selecionados para posterior quantificação do teor total de carotenoides, utilizando curva padrão de β-caroteno (Sigma-Aldrich, St. Louis, MO, EUA - 0,5 a 10 $\mu g.mL^{-1}$, $y = 0.167x$, $r^2 = 0,99$). As análises foram realizadas em quintuplicatas.

4.4.3 Extração e quantificação de fenólicos totais

Amostras (1g, peso fresco, n = 5) foram adicionadas de 10 mL de álcool metílico 80% (v/v), maceradas em cadinho com N_2 líquido e incubadas (1h, ao abrigo da luz) para extração dos compostos fenólicos. A mistura amostral foi centrifugada (12000*g*, 5 min) e o sobrenadante recolhido. Os conteúdos de fenólicos totais foram determinados pelo método colorimétrico de Folin-Ciocalteau (λ = 725ηm), conforme descrito por Rhandir *et al.* (2002). O cálculo dos teores dos analitos utilizou curva-padrão de floroglucinol (Sigma-Aldrich, St. Louis, MO, EUA – 100 - 1250 $\mu g.mL^{-1}$, $y = 0.0004x$, $r^2 = 0,997$).

4.5 ANÁLISES DE RESSONÂNCIA MAGNÉTICA NUCLEAR DE 1H (1H-RMN)

Para caracterização do perfil metabólico foram selecionadas amostras do maior tempo de exposição da alga ao óleo diesel, considerando-se os motivos acima descritos (item 4.3.4). As análises de ressonância magnética nuclear (RMN) de ^{1}H foram realizadas no Laboratório de RMN, no Departamento de Química da Universidade Federal de São Carlos (SP) e a metodologia experimental utilizou os procedimentos descritos por Kuhnen *et al.* (2010). Os espectros de ressonância magnética nuclear foram obtidos em equipamento Bruker DRX-400.

4.6 ANÁLISES CITOQUÍMICAS E MORFOLÓGICAS

4.6.1 Microscopia de luz (ML)

Amostras de filoides (~ 5mm de espessura) controle e tratadas com gasolina e óleo diesel foram fixadas *overnight* em solução de 2,5% de paraformaldeído em tampão fosfato (0,1 M - pH 7,2), conforme descrito por Schmidt *et al.* (2009). Posteriormente, as amostras foram desidratadas em série crescente de soluções aquosas de etanol (30% - 100%) e infiltradas com historresina (Leica Historresina, Heidelberg, Alemanha) e etanol (1:1) por 4 horas e historresina por mais 24 horas. As amostras dispostas em blocos de historresina foram seccionadas em micrótomo manual modelo Leica RM 2135, com navalhas de tungstênio. As secções com espessura de 4µm foram distendidas em lâminas de vidro com gotas de água à temperatura ambiente e secas a 37ºC. Em seguida, as secções (4µm de espessura) foram coradas com Azul de Toluidina [AT-O, 0,5% (m/v), pH 3,0 - Merck Darmstadt, Alemanha - Schmidt *et al.* 2010] e visualizadas em microscópio de epifluorescência (Olympus BX 41) equipado com sistema de captura de imagem (Image Capture Q Pro 5.1).

4.6.2 Microscopia eletrônica de varredura (MEV)

O procedimento de fixação do material amostral à análise por MEV foi idêntico ao utilizado à ML (OURIQUES & BOUZON, 2000). As amostras foram desidratadas com uma série etanólica, secas em equipamento de ponto crítico (EM-DPC-030, Leica, Heidelberg, Alemanha) e analisadas em microscópio de varredura JSM 6390 LV

(JEOL Ltd., Tóquio, Japão, 10 kV). A eventual adsorção/ligação da gasolina e do óleo diesel à parede celular das amostras foi avaliada em espectrômetro de energia dispersiva de raios-X acoplado ao microscópio de varredura (MEV-EDX, NORAN System 7 EDS analyzer, Thermo Scientific), porém sem pós-fixação das amostras em tetróxido de ósmio ou metalização, i.e., revestimento com ouro.

4.6.3 Microscopia eletrônica de transmissão (MET)

As amostras (~ 5mm de espessura) foram fixadas em solução composta de glutaraldeído (2,5% -v/v) paraformaldeído (2,0%, v/v) $CaCl_2$ 5 mM em tampão de cacodilato de sódio 0,075 M (pH 7,2), suplementada com 0,2 M de sacarose e 1% (m/v) de cafeína, *overnight* (OURIQUES & BOUZON, 2000). O material foi pós-fixado com solução de tetróxido de ósmio a 1% (m/v), durante 4h, desidratado numa série graduada de acetona e embebida em resina de Spurr. Seções ultrafinas (70ηm) foram coradas com acetato de uranila aquoso 2% (m/v), seguido por citrato de chumbo 2% (m/v). Quatro repetições foram feitas para cada grupo experimental e duas amostras de cada repetição foram examinados em microscópio de transmissão JEM 1011 (JEOL Ltd., Tóquio, Japão, 80 kV). As semelhanças elevadas com base na comparação das repetições dos tratamentos sugeriram que a análise ultraestrutural é confiável.

5 ANÁLISES ESTATÍSTICAS

Os dados foram analisados por análise de variância bifatorial (ANOVA) e teste de Tukey. Todas as análises estatísticas foram realizadas utilizando o pacote de software Statistica (versão 6.0), considerando-se $p \leq 0,05$. A homogeneidade da variância foi testada pelo teste de Levene. Os dados derivados das análises bioquímicas dos tratamentos de 24 horas com óleo diesel foram submetidos a técnicas de análise multivariada (análise de componentes principais), utilizando-se *scripts* implementados em linguagem estatística R. Optou-se por submeter apenas os dados do experimento com diesel à análise multivariada, devido a similaridade dos resultados estatísticos deste com o experimento com gasolina.

CAPÍTULO I

Respostas bioquímicas e na organização celular da alga parda *Sargassum cymosum* var. *stenophyllum* quando exposta à gasolina

Artigo a ser submetido à publicação em revista científica

Biochemical and morphological responses of the brown seaweed *Sargassum cymosum* var. stenophyllum when exposed to gasoline

Eva Regina Oliveira[a], Fernanda Ramlov[b], Éder Carlos Schmidt[c], Débora Tomazi[c], Claudia Marlene Bauer[a], Rodolfo Moresco[a], Fernanda Pilatti Kokowicz[a], Zenilda Laurita Bouzon[c], Paulo Antunes Horta[b], Marcelo Maraschin[a]

[a]Plant Morphogenesis and Biochemistry Laboratory, Federal University of Santa Catarina, 88049-900, P.O. Box 476, Florianopolis, SC, Brazil.

[b]Phycology Laboratory, Department of Botany, Federal University of Santa Catarina, 88049-900, P.O. Box 476, Florianopolis, SC, Brazil.

[c]Plant Cell Biology Laboratory, Department of Cell Biology, Embriology and Genetics, Federal University of Santa Catarina, 88049-900, P.O. Box 476, Florianopolis, SC, Brazil.

Corresponding author: +55 48 37214812
E-mail address: ginagro@gmail.com

ABSTRACT

Coastal ecosystems and marine communities are the first environments affected by chemical pollutants dumped directly these environments by humans or transported by storm water runoff and from urban areas and activities. In this context, , biochemical and morphological effects on the brown alga *Sargassum cymosum* var. stenophyllum when exposed to gasoline doses of 0.001, 0.01, 0.1 and 1% (v / v) over 30 min, 1 h, 12 h and 24 h were determined in in vitro culture. An increase in chlorophyll content in all treatments was noted, more pronounced in 1% gasoline 1pm. Concentrations of total carotenoids varied during the treatment and does not following a pattern according to the gas concentrations tested. In turn, the total concentration of phenolic compounds been found to be a slight increase for the treatment gasoline/1h / 0.1%.All other treatments had lower levels compared with control plants.The biochemical analyzes were corroborated by light (ML) microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The image analysis revealed a cell wall thickness, the increase in chloroplast size, the migration of phenolic compounds toward the cell wall, as well as the reduction of physodes and the dilation of thylakoid membranes. The X-ray microanalysis identified the elements C, N, O, Na, and K both on the cell surface and in the inner parts of the cell wall, but a pattern of ultrastructural distribution was not detected for the studied treatments. Taken together, the findings herein described point to *S. cymosum* var. *stenophyllum* as useful bioindicator in marine areas affected by pollution from gasoline.

Keywords: *Sargassum cymosum*, petroleum derivate, metabolic changes, environmental damage

1 INTRODUCTION

Several studies have been performed in recent years on the environmental impacts in coastal areas and the sources thereof. Great attention has been given to acute stress events, *e.g.*, oil spills or toxic algae bloom, pesticides originating from agricultural areas, heavy metals contamination, and anti-fouling paints used on ships (Crowe *et al.* 2000; Islam; Tanaka, 2004). Frequent sources of pollution of anthropogenic origin are the petroleum-derived fossil fuels like gasoline that is often spilled in coastal areas, dragged by rains or floods.

On rocky shores, the proximity to urban or industrial areas and harbors with high human intervention is a potential source of damage to that coastal environment, beyond the naturally hostile environment for the fauna and flora communities that live there. Among others, macroalgae are important communities in coastal ecosystems, since they have a strategic relevance in recovering of environments stressed by pollutants derived from anthropogenic activities.

The strategic role of seaweeds to recover polluted environments comes in several forms, since these organisms are potential monitors or impacted environments transformers, either as adsorbents or absorbents recyclers of contaminants (Lee *et al.*, 2004; Torres *et al.* 2008). In this context, the brown macroalgae have this potential and Fucales order is mentioned because of its abundance along the Brazilian coastal line, such as noticed for the species belonging to the *Sargassum* genus (*e.g.*), known for providing polysaccharides and secondary metabolites of biotechnological importance (Andrade *et al.* 2010).

Secondary metabolites such as carotenoids and phenolic compounds are involved in plant defense mechanisms in situations of stress and herbivory (Pereira *et al.* 2000). According to Reddy *et al.* (2009), brown algae usually synthesize a wide range of metabolites in response to abiotic stresses. Thus, the brown alga *Sargassum cymosum* var. *stenophyllum*, frequently found in marine ecosystems in Santa Catarina State (southern Brazil), was chosen as a biological model in this study to evaluate the effects of acute exposure to gasoline in their biochemical, cytological and ultrastructural features.

2 MATERIAL AND METHODS

2.1 COLLECTION, GROWING ALGAE AND IMPLEMENTATION OF THE EXPERIMENT

S. cymosum var. *stenophyllum* samples were collected in September 2012 at Ponta das Canas beach (Florianópolis, Santa Catarina State, southern Brazil - 27º23'34" S, 48º26'11" W), immediately stored at 4ºC and transferred to the Laboratory of Phycology (Federal University of Santa Catarina - UFSC). After cleaning the thallus segments, samples were transferred to culture medium supplemented with von Stosch solution (50%) (Edwards, 1970) and acclimated for three weeks under continuous aeration at 24ºC±2°C, daily photosynthetically active irradiation (PAR) at 80 µmol photons.$m^{-2}.s^{-1}$ (Li-cor light meter 250, USA), and 12h-photoperiod. The salinity was 34 ups (± 1 ups) (standard salinity unit). The exchange of culture medium were made every 5 days. After acclimatization, thallus segments (2g, fresh weight) were grown in flasks containing 400 mL of culture medium and gasoline at 0.001%, 0.01%, 0.1%, and 1% (v/v) for 30min, 1h, 12h, and 24h under the same experimental conditions mentioned in the acclimatization step. Each treatment consisted of five simultaneous replications and the control plants were cultured on culture medium and collected after 24 hours of initiation of the experiment. At the end of the experiment, thallus samples were collected, immediately frozen in liquid N_2 and stored at -80°C until analysis. Control samples (n = 5) were collected directly from the culture medium without addition of petrol, 24 hours after the beginning of the experiment.

2.2 PIGMENTS ANALYSES

The chlorophylls *a* and *c* were extracted from 225mg-fresh biomass crushed and macerated in cold acetone for 10 minutes in the dark. The resulting extract was centrifuged (12.000*g*, 5 min), the supernatant collected for spectrophotometric reading of absorbances at 630ηm, 647ηm, and 664ηm and further calculation of the contents according to the equation of Jeffrey and Humphrey (1975).

For the determination of carotenoids, 1g of biomass (dry weight) was ground in liquid N_2 and soaked in 10mL of methyl alcohol for 1h, protected from light, at room temperature. The extract was filtered on paper filter paper (14 µm pore ∅) under vacuum. The filtrate was read in its absorbance at 450ηm and the quantification of the total carotenoids

was taken from the external standard curve of β-carotene (Sigma, 0.5 to 10 μg.mL^{-} - r^2 = 0.99, y = 0.167x). The results were expressed as mg β-carotene/g biomass (dry weight). The analyses were performed with five replicates per treatment.

The extraction for the detection and quantification of phenolic compounds was performed with biomass samples (1g, fresh weight, n = 5) soaked in 10 mL of 80% methyl alcohol (v/v) for 1h. The methanolic extract was centrifuged (12000g, 5min) and the supernatant collected. The colorimetric method for determination of total phenolic contents used the Folin Ciocalteau reagent and the method previously described by Rhandir et. al (2002). The absorbances were read at 750ηm, followed by calculating the concentrations of the analytes using a phloroglucinol external standard curve (Sigma-Aldrich, St. Louis, MO, USA - 100–1250 μg.mL^{-1}, y = 0.0004x; r^2 = 0.997).

Light microscopy (lm) and cytochemistry analysis

Light microscopy analysis (LM) used 5 mm length-samples on average fixed overnight with 2.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) as previously described (Schmidt *et al.*, 2009). The samples were further subjected to dehydration in increasing ethanol solutions, infiltrated with historesin (Leica Historesin, Heidelberg, Germany), sectioned (5μm length), stained with Toluidine Blue (TB-O) 0.5%, pH 3.0 (Merck Darmstadt, Germany), and examined with an Epifluorescent microscope (Olympus BX 41) equipped with Image Q Capture Pro 5.1 Software (Qimaging Corporation, Austin, TX, USA - Schmidt et al, 2010). The reliability in the LM analysis is suggested by the similarity observed among the replicates (5) of each treatment.

Scanning electron microscope (sem)

For scanning electron microscopy (SEM) the procedures for sample preparation were the same described for TEM. After dehydration with ethanol series, samples were critical point dried in CPD-EM-030 apparatus (Leica, Heidelberg, Germany), followed by the visualization of the samples under SEM JSM 6390 LV (JEOL Ltd., Tokyo, Japan, at 10 kV) microscope. The evidence of gasoline adsorption/binding in the cell wall was evaluated by SEM (Noran Instruments Analiser System) coupled to an energy dispersive spectrometer X-ray (SEM-EDX), without post-fixation in osmium tetroxide samples or coated with gold.

Transmission electron microscope (TEM)

The material for transmission electron microscopy (TEM) analysis consisted of 5mm length-samples on average, fixed in 2.5% glutaraldehyde, 2.0% paraformaldehyde, and 5 mM $CaCl_2$ in 0.075 M sodium cacodylate buffer (pH 7.2) plus 0.2 M sucrose and caffeine 1% overnight (Ouriques & Bouzon, 2000). Next, the material was post-fixed in 1% osmium tetroxide for 4h, dehydrated in a graded acetone series and embedded in Spurr resin. Thin sections were stained with aqueous uranyl acetate followed by lead citrate. Four replicates were made for each experimental group and two samples per replication were examined under TEM JEM 1011 (JEOL Ltd., Tokyo, Japan, at 80 kV) microscope. Similarities observed in the comparison between repetitions of each individual treatment suggest that the ultrastructural analyzes were reliable.

3 STATISTICAL ANALYSES

Data were analyzed by bifactorial Analysis of Variance (ANOVA) and Tukey test. All statistical analyses were performed using the Statistica software package (Release 6.0), considering $p \leq 0.05$. Homogeneity of the variance was tested using Levene's test.

4 RESULTS

4.1 PIGMENTS ANALYSIS

Effects of interaction between time of exposure and concentration of gasoline in *S. cymosum* var. *stenophyllum* were significant (Table 1). By comparing the data of the treated plants to control ones, an increase in the concentration of the chlorophylls *a* and *c* was found (Table 2), except for the exposure by 30 minutes, at 1% gasoline. In relation to control, regarding the carotenoid compounds a clear tendency was not detected in the data set as shown in Table 2. The quantification of total phenolic compounds showed a reduction in the amounts of these metabolites in treated plants comparatively to control ones. Such an effect was more prominent in longer exposure times (Table 2).

Table 1. Two-away ANOVA of pigment concentrations in *Sargassum cymosum* var. *stenophyllum* exposed to gasoline (0.001, 0.01, 0.1 and 1%) in times of 30min, 1h, 12h and 24h.

		Chlorophyll *a*		Chlorophyll *c*		Carotenoids		Phenolic	
Variable	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Concentration	1	13546.4	0.00	12450.9	0.00	14904.4	0.00	29.0	0.00
Time	1	1625.5	0.00	4279.2	0.00	14541.2	0.00	22.9	0.00
Concentr. x Time	1	7824.4	0.00	3605.8	0.00	9235.7	0.00	8.1	0.00

Table 2. Contents of chlorophylls *a* and *c* (µg.g^{-1}, fresh weight biomass), carotenoids (µg.g^{-}, dry weight), and total phenolics (µg.g^{-}, dry weight) of *S. cymosum* var. *stenophyllum* exposed to gasoline (0.001% - 0.01% - 0.1% - 1%, v/v) for 30min, 1h, 12h, and 24h. Values are the mean ± standard deviation (n = 5). The letters indicate significant differences (Tukey test, p ≤ 0.05, comparing control and treated plants).

Time	Gasoline (%)	Chlorophyll *a* (µg.g^{-1})	Chlorophyll *c* (µg.g^{-1})	Total carotenoids (µg.g^{-1})	Total phenolics (µg.g^{-1})
	Control	87.65±1.11o	40.71±2.29l	32.63±0.05g	23.77±3.32a
	0.001	194.69±3.39d	130.75±2.62d	33.77±0.05f	14.75±2.19c
30 min	0.01	166.08±0.21g	160.81±0.25b	33.91±0.04f	13.80±3.88c
	0.1	337.80±3.00a	409.78±2.79a	25.49±0.08l	20.02±3.08ab
	1	58.16±0.17p	25.65±0.42m	26.93±0.06j	16.90±0.96c
	0.001	113.54±2.80m	53.65±1.23i	34.83±0.06e	14.55±3.71a
1h	0.01	80.75±0.98e	82.06±0,.2g	36.87±0.04d	17.97±0.34bc
	0.1	151.65±1.08i	151.30±1.14c	43.14±0.08c	26.42±1.96ef
	1	247.08±0.39b	160.63±0.65b	30.16±0.20h	11.75±0.77e
	0.001	144.11±2.54j	61.58±3.18h	20.00±0.02n	5.85±1.64e
12h	0.01	109.84±0.45n	42.37±0.20j	9.79±0.09a	7.62±0.69e
	0.1	161.15±1.70h	128.49±3.60d	6.50±0.07b	8.07±0.55e
	1	179.23±1.62e	102.04±3.03e	21.43±0.05m	3.77±0.51e
	0.001	125.13±0.74l	61.13±0.66h	14.41±0.05o	10.00±1.11d
24h	0.01	237.46±0.50c	128.17±0.67d	33.84±0.11f	9.45±1.30de
	0.1	170.21±2.46f	157.31±4.14b	29.90±0.03h	9.20±062cd
	1	129.34±0.87k	93.62±2.17f	29.04±0.07i	1.30±0.22f

Light microscopy (LM) and cytochemistry analysis

LM of control and treated samples of *S. cymosum* stained with Toluidine Blue showed a metachromatic reaction in the cell wall, suggesting the presence of acidic polysaccharides such as alginic acid and sulfated fucan (Figure 1 a, 2 b-m). In their turn, the gasoline-treated plants exposed during 1h and 24h revealed an increase in the lenticular cell wall thickness (Figure 2 b-e, j-m). In the cytoplasm of cortical cells of the control samples a large quantity of dark blue and yellow physodes was observed (Fig.1 a, arrows). In the cytoplasm of cortical cells of treated samples it was also possible to observe the migration of physodes toward cell surface (Figure 2 b-m, arrows). However, plants exposed to 1% gasoline/1h showed a reduction in the physodes number (Figure 2 e).

Scanning electron microscope (SEM)

When observed under scanning electron microscopy (SEM), the surface of cortical cells of *S. cymosum* control samples appeared smooth (Figure 3 a). In contrast, gasoline-exposed specimens (Figure 3 b-m) showed an irregular surface and disrupted cell walls, apparently the result of gasoline absorption. These results indicate that exposure to gasoline may cause changes in mucilage of *S. cymosum*.

The results of X-ray microanalysis identified the elements C, N, O, Na, and K both on the cell surface and in the inner parts of the cell wall, but a pattern of ultrastructural distribution was not found for the studied treatments. However, microanalysis revealed proportionally increased levels of carbon in gasoline-treated plants comparatively to control ones, especially at the highest concentrations of gasoline, suggesting the eventual adsorption of that pollutant by ultrastructural components on the cell surface.

Transmission electron microscope (TEM)

Observed under transmission electron microscopy (Figure 4 a-b 5 c,d), control samples of *S. cymosum* revealed the abundant presence of chloroplasts, mitochondria, and physodes preserved, as well a thick cell wall (Figure 4 a, b). The sulfated polysaccharides such as alginic acid and fucans (Figure 4 b) were found to occur as an amorphous matrix with concentric microfibrils forming the cell wall. Importantly, cells with abundant physodes (Figure 4 c) and thylakoids with three bands

organization typically expected to occur in brown algae (Figure 4 d) were detected.

On the other hand, the samples exposed to gasoline for 24h at the concentrations assayed displayed ultrastuctural changes in respect to control (Figure 5 a-f). For example, an increase in the size of chloroplasts and few and dispersed physodes were noticed (Figure 5), as well as the presence of apparently crystalline bodies in the cytoplasm (Figure 5 b, arrows) and phenolic compounds in the cell wall (Figure 5 c, arrows). It was also observed the increase in lipid bodies (plastoglobuli) in thylakoids (Figure 5 d, arrows). Morphologically, it can be noted the swollen of thylakoid membranes and the increased size of plastoglobuli (Figure 5 e, arrows). Another alteration detected referred to the presence of large vacuoles in areas with reduced number of physodes.

Figure 1. Light micrografies of the transversal sections of thallus stained with TB-O of control; The cell walls (CW) of cortical cells (CC) show metachromatic reaction and in the cytoplasm the presence of physodes is highlighted by arrows.

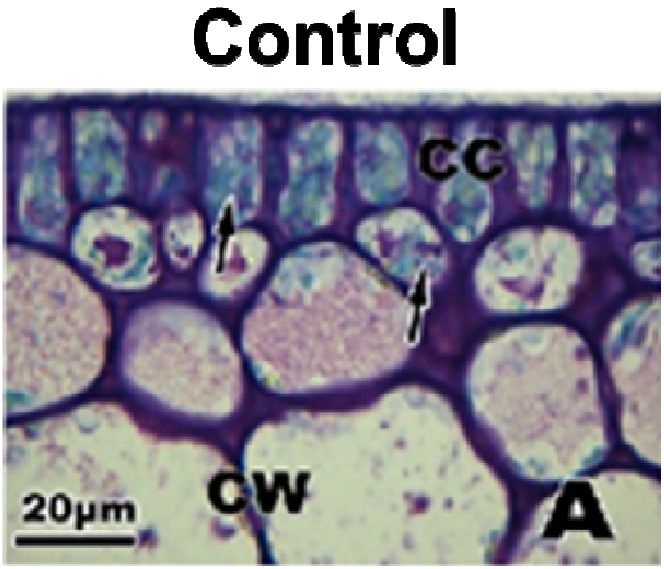


Figure 2. Light micrografies of the transversal sections of phylloid stained with TB-O of treated plants (B-M) of *S. cymosum* var. *stenophyllyum* exposed to gasoline. Detail of gasoline-treated plant cells in respect to the metachromatic reaction in the cell wall and the physodes migration. One also can observe the thickening of the walls in the treated segments.

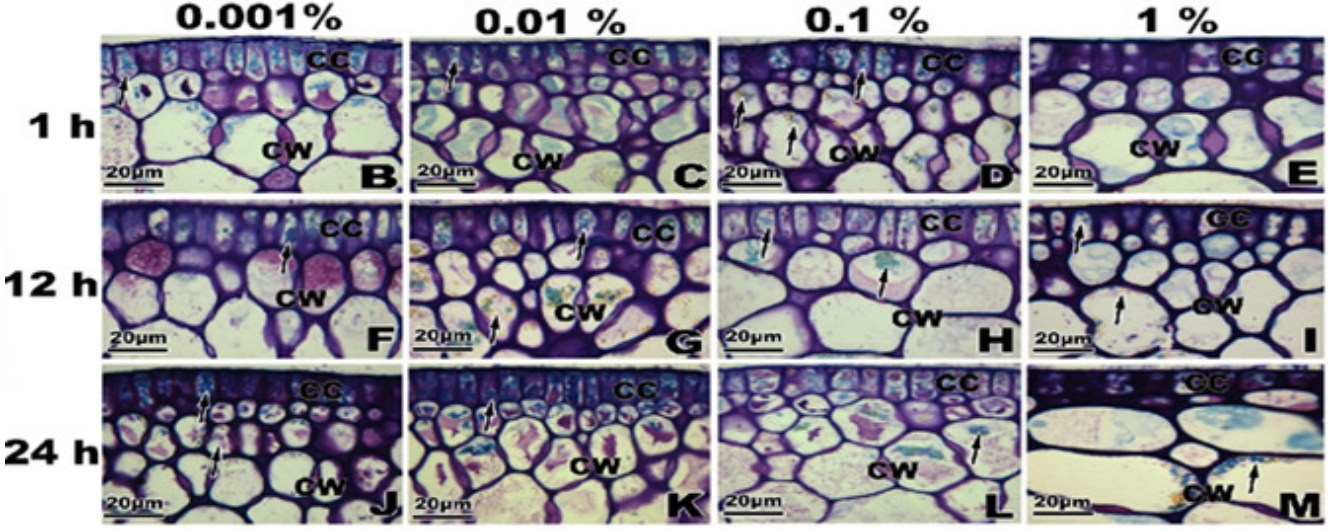


Figure 3. Scanning electron microscopy (SEM) images of thallus segments of control (a) and exposed to gasoline plants (b-m) of *S. cymosum* var. *stenophyllum*. Detail of the surface topography of cortical cell walls showing a smooth aspect in control plants (a). The cell surface appears to be irregular in plants treated with gasoline comparatively to untreated ones (b-m).

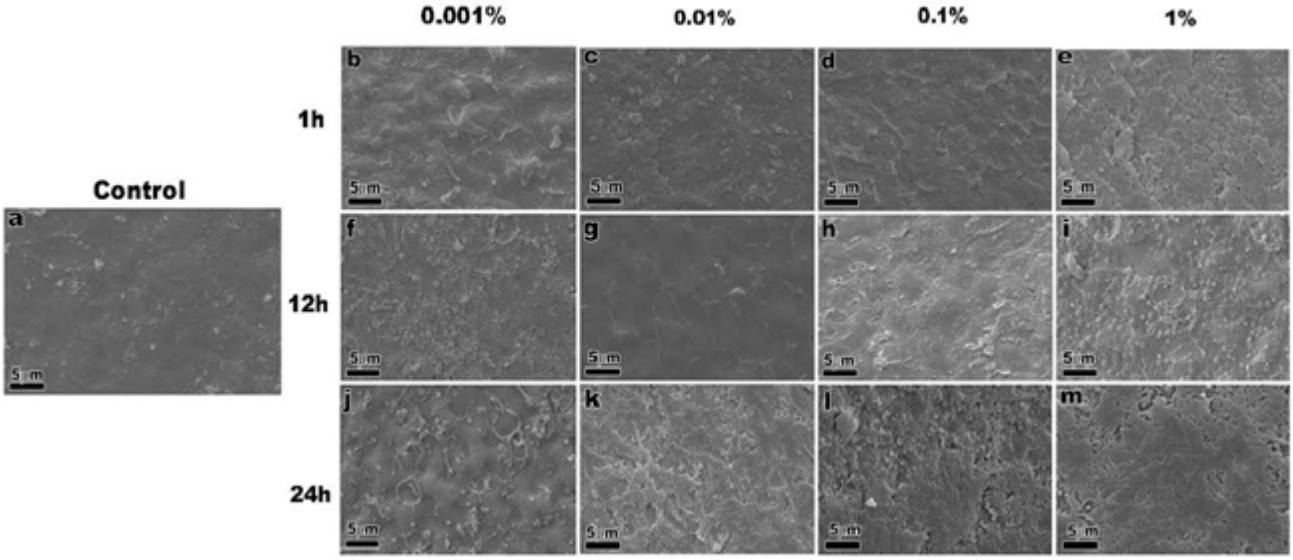


Table 3. X-ray microanalysis of the cell wall surface and internal cell wall revealing the presence of elements carbon, nitrogen, oxygen, sodium, and potassium in thallus samples of *S. cymosum* var. *stenophyllum* cultured *in vitro*.

	C	*N*	*O*	*Na*	*K*	*C*	*N*	*O*	*Na*	*K*
	Cell wall surface					***Internal cell wall***				
Control	28.1 ± 2.6	33.1 ± 2.6	31.8 ± 2.8	2.6 ± 0.5	4.4 ± 1.3	34.0 ± 3.6	29.4 ± 4.0	30.7 ± 1.5	2.5 ± 0.3	3.4 ± 3.0
0.001%/1h	21.1 ± 4.7	39.0 ± 6.8	29.1 ± 2.8	4.5 ± 0.4	6.3 ± 1.2	32.9 ± 1.3	25.1 ± 9.2	35.0 ± 0.7	3.3 ± 0.4	3.7 ± 0.6
0.01%/1h	30.7 ± 6.6	30.4 ± 7.1	31.9 ± 0.9	3.0 ± 0.2	4.0 ± 1.0	45.3 ± 1.0	13.0 ± 1.9	35.5 ± 2.0	3.4 ± 0.3	2.8 ± 0.5
0.1%/1h	26.9 ± 0.4	38.9 ± 2.8	28.9 ± 2.0	1.8 ± 0.5	3.5 ± 0.9	28.0 ± 3.6	36.7 ± 4.7	28.8 ± 2.6	2.5 ± 0.2	4.0 ± 0.6
1%/1h	31.4 ± 3.3	26.1 ± 4.1	32.3 ± 2.1	3.7 ± 0.4	6.5 ± 1.4	40.9 ± 1.3	21.1 ± 2.1	29.8 ± 1.8	3.4 ± 0.4	4.8 ± 0.9
0.001%/12h	23.8 ± 2.9	32.0 ± 3.4	31.0 ± 3.0	9.0 ± 0.5	4.2 ± 1.6	26.3 ± 3.3	33.2 ± 4.1	29.5 ± 2.1	4.1 ± 0.4	6.9 ± 1.4
0.01%/12h	38.7 ± 5.9	19.2 ± 3.8	32.4 ± 2.4	3.5 ± 0.6	6.2 ± 1.7	48.8 ± 2.7	10.4 ± 1.1	33.3 ± 1.9	2.9 ± 0.5	4.6 ± 0.8
0.1%/12h	24.5 ± 2.0	37.3 ± 2.2	29.2 ± 2.5	2.6 ± 0.4	6.4 ± 1.0	35.3 ± 1.7	24.7 ± 2.0	32.3 ± 1.2	2.9 ± 0.5	4.8 ± 0.7
1%/12h	33.2 ± 6.3	23.6 ± 7.0	31.9 ± 1.2	3.7 ± 0.4	7.6 ± 1.6	42.9 ± 0.9	13.1 ± 1.5	36.4 ± 1.7	3.0 ± 0.1	4.6 ± 0.2
0.001%/24h	33.2 ± 2.8	19.5 ± 3.3	33.4 ± 1.4	4.0 ± 0.4	9.9 ± 1.3	46.8 ± 3.0	14.6 ± 1.1	32.1 ± 1.5	3.3 ± 0.4	3.2 ± 0.6
0.01%/24h	29.0 ± 5.2	29.2 ± 3.2	30.4 ± 7.9	5.8 ± 0.5	5.6 ± 1.2	40.7 ± 2.0	21.4 ± 3.3	31.0 ± 2.1	2.3 ± 1.1	4.6 ± 0.7
0.1%/24h	31.3 ± 2.5	27.1 ± 3.8	31.9 ± 2.1	3.5 ± 0.3	6.2 ± 1.1	36.6 ± 0.3	22.0 ± 1.4	31.4 ± 0.3	3.3 ± 0.4	6.7 ± 1.5
1%/24h	34.2 ± 2.3	25.3 ± 1.4	32.6 ± 1.2	3.1 ± 1.0	4.8 ± 0.5	31.5 ± 0.8	24.5 ± 2.8	34.9 ± 2.9	2.6 ± 0.5	6.5 ± 1.0

Figure 4. Micrografies of transmission electron microscopy (TEM) of *S. cymosum* var. *stenophyllum* of control plants. a. Note the cells showing a large quantity of chloroplasts (C), mitochondria (M, and arrows), physodes (Ph), and thick cell wall (CW). b.

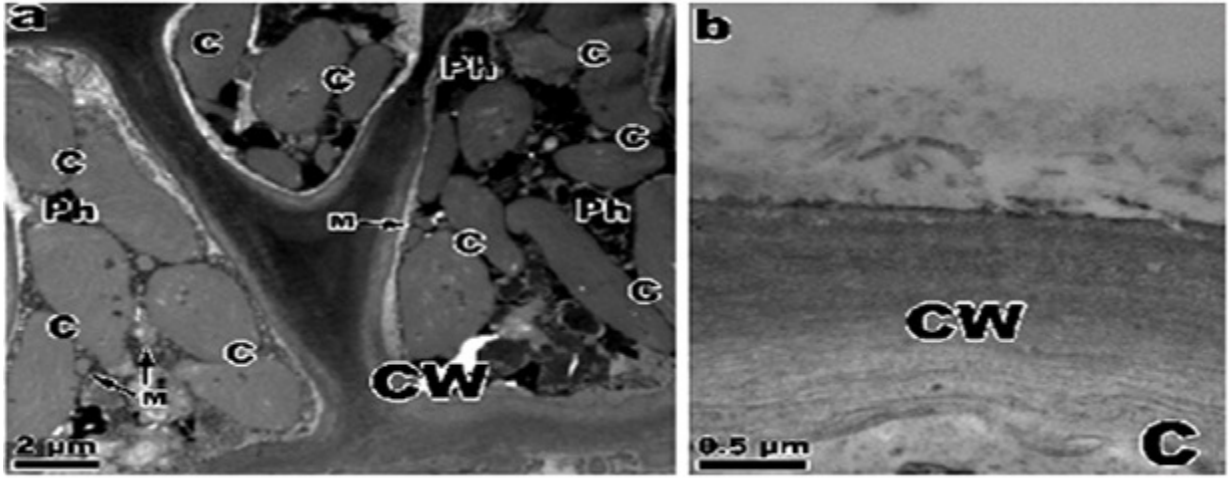


Figure 5. Micrografies of transmission electron microscopy (TEM) of *S. cymosum* var. *stenophyllum* of control plants.. Detail of thick cell wall (CW) and well preserved mitochondria. c. Note the presence of phenolic compounds in physodes. d. Note the chloroplast internal organization of thylakoids in three bands into the chloroplasts (arrows).

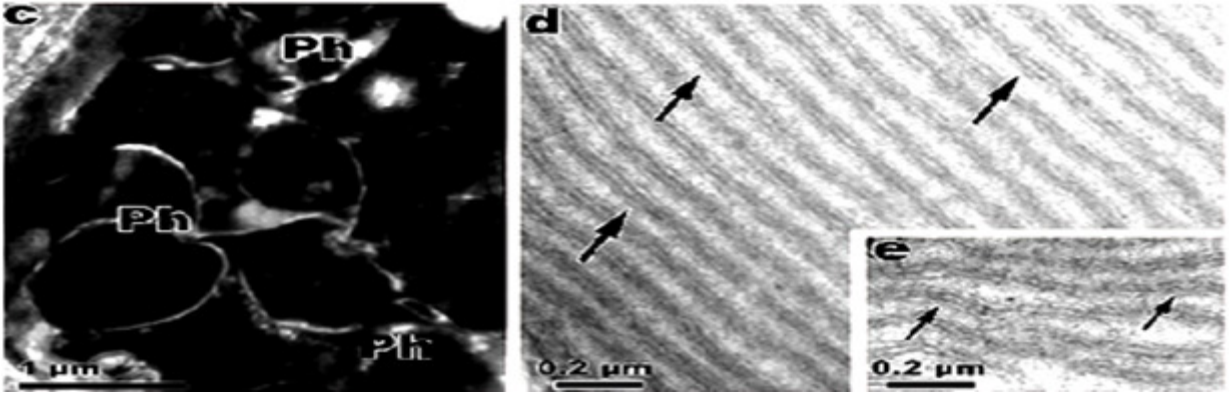


Figure 6. Transmission electron microscopy (TEM) images of *S. cymosum* var. *stenophyllum* plants treated with 24h of exposure to gasoline (v/v - 0.001 - a , 0.01 - b and 0.1 - c. Observe the cells showing a large quantity of chloroplasts (C), physodes (Ph), and thick cell wall (CW). b-c

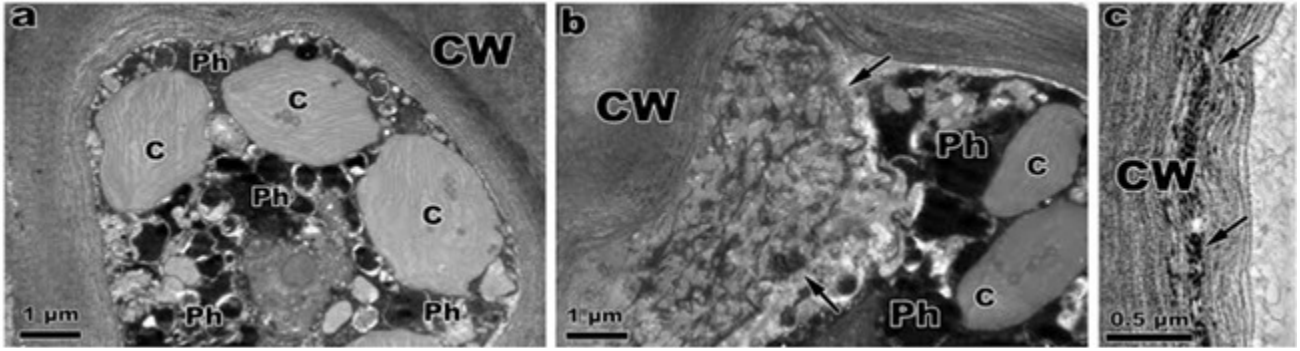
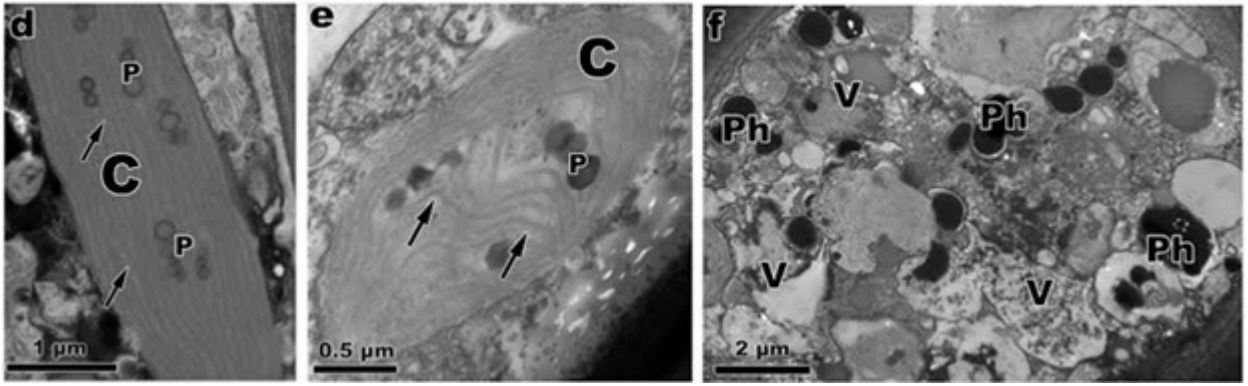


Figure 7. Transmission electron microscopy (TEM) images of *S. cymosum* var. *stenophyllum* plants treated with 24h of exposure to gasoline. (v/v - 0.01 - d, 1% - e and 0.001 - f,). Note the presence of phenolic compounds in the cell wall (arrows). d. Observe the increase of plastoglobuli (P) and intact thylakoids (arrows). e. Detail of chloroplasts showing thylakoids dilation (arrows). f. Note the presence of vacuole (V) with electron dense material near the physodes.



5 DISCUSSION

Brown algae like *Sargassum* spp have the ability to adsorb toxic substances such as heavy metals and oils (Andrade et al, 2010). This study aimed to evaluate the biochemical and morphological changes of *S. cymosum* var. *stenophyllum* sharply when exposed to gasoline. Important metabolic and morphological changes were detected after exposure to the pollutant, for example, increased chlorophyll content (except for the 1% concentration at time 30 minutes) and the reduction in the amount in all treatments phenolic compounds. A reduction of carotenoids in long exposure times compared with the control. These events are consistent with the image analysis through transmission electron microscopy, which revealed an increase in the size of chloroplasts, a phenotype eventually related to the increase of chlorophyll amounts.

A possible increase in the chlorophylls *a* and *c* up to 24h of exposure and the reduction in levels of carotenoids may suggest the use of carbon skeletons of these compounds in other metabolic route in the early hours of stress (Hamilton, 2001).

In respect to the marked reduction in the contents of phenolic compounds for all the exposure times, previous studies report that algae of the genus *Sargassum* tend to release those metabolites into the medium as a defense mechanism upon the action of stressor agents. Besides, it is claimed that eventually damage repair routes might be triggered by the exposure to that petroleum derivative in the biological model in study, taking into account the results from SEM which revealing irregularities and cracks in the cell surfaces structures of treated samples. Further, this evidence was confirmed by LM and TEM analyses showing the migration of phenolic compounds toward the cell wall, also including the leakage of these compounds to that ultrastructural cell component. Other evidence from TEM images refers to the increase of plastoglobuli and lipid bodies, assumed as an adaptive biochemical mechanism expressed by *S. cymosum* var. *stenophyllum* under the adverse conditions as herein shown. Indeed, according to Qiang Hu *et al.* (2008), the lipid bodies are a form of carbon storage in plants under stress.

Another ultrastructural modification detected through LM and TEM refer to the cell wall thickening, a phenomenon previously reported by Andrade *et al.*, (2010). The authors describe the overproduction and accumulation of polysaccharides in the cell walls of *Padina gymnospora* as a protection mechanism against heavy metal toxicity. The X-ray microanalysis detected increased concentrations of carbon and coincident

reduction in nitrogen levels when exposed to either fuel compared to the control samples. These alterations may confirm the hypothesis of the nitrogen balance and carbon, that say the availability these nutrients determine the concentrations of secundary metabolites in plant tissues (Hamilton *et al.* 2001). Taking together, these findings suggest being the brown alga *Sargassum cymosum* var. *stenophyllum* a biological model candidate to assist in monitoring and evaluation of damage in areas impacted by petroleum derivatives pollution such as gasoline.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the staff of the Central Laboratory of Electron Microscopy (LCME - Federal University of Santa Catarina, Florianopolis, Santa Catarina, Brazil) for the use of their facilities. The first author holds a Master's scholarship from CAPES, Fernanda Ramlov holds a postdoctoral fellowship from CAPES. Marcelo Maraschin is a CNPq fellowship. This study is part of the MSc dissertation of the first author.

REFERENCES

Andrade LR; Leal RN, Noseda, DM (2010) Brown algae overproduce cell wall olysaccharides as a protection mechanism against the heavy metal toxicity. Marine Pollution Bulletin 60:1482–148.

Barros MP, Pinto E, Sigaud-Kutner TCS, Cardozo KHM & Colepicolo P (2005) Rhythmicity and oxidative/nitrosative stress in algae. Biological Rhythm Research 36: 67-82.

Crowe TP, Thompson RC, Bray S and Hawkins SJ (2000) Impacts of anthropogenic stress on rocky intertidal communities. Journal of Ethnopharmacology **7:**273–297.

Edwards, P (1970) Illustrated guide to the seaweeds and seagress in the vicinity of Porto Aransas, Texas. Contribution of Marine Sciences Austin 15: 1-228.

Eklund, BT & Kautsky, L(2003) Review on toxicity testing with marine macroalgae and the need for method standardization—exemplified with copper and phenol. Marine Pollution Bulletin 46:171-181.

Farooq, U, Kozinski, JA, Khan, MA, Athar, M (2010) Biosorption of heavy metal ions using wheat based biosorbents – A review of the recent literature. Bioresource Technology 101:5043–5053.

Hamilton JG, Zangerl AR, DeLucia EH (2001) The carbon–nutrient balance hypothesis: its rise and fall. Ecology Letters, 4:86-95.

Handy RD, Depledge, MH (1999) Physiological responses: their measurement and use as environmental biomarkers in ecotoxicology. Ecotoxicology 8:329-349.

Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. The Plant Journal 54: 621–639.

Islam MD S, Tanaka M (2004) Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: a review and synthesis. Marine Pollution Bulletin 48:624–649.

Jeffrey SW, Humphrey GF (1975) New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. Biochemie und physiologie der pflanzen, 167:191-194.

Mafra Jr, LL & Cunha, SR (2002)Bancos de *Sargassum cymosum* (Phaeophyceae) na enseada de Armação do Itapocoroy, Penha, SC: biomassa e rendimento em alginato. - Brazilian Journal of Aquatic Science and Technology 6:111-119.

Ouriques LC; Bouzon ZL (2000) Stellate chloroplast organization in Asteronema breviarticulatum (ectocarpales, phaeophyta). Phycologia 39:267-271.

Pereira RC, Bianco EM, Bueno LB, Pereira RC, Oliveira MAL, Pamplona OS, Gama BAP(2010) Associational defense against herbivory between brown seaweeds.Phycologia 49:424–428.

Raize O, Argaman Y, Yannai S (2004) Mechanisms of biosorption of different heavy metals by brown marine macroalgae. Biotechnology and Bioengineering 87:4-7.

Randhir R, Preethi S, Kalidas S (2002) L-DOPA and total phenolic stimulation in dark germinated fava bean in response to peptide and phytochemical elicitors. Process Biochemistry 37:1247-1256.

Reddy P, Urban SB, MP, Pinto, E, Sigaud-Kutner, TCS, Cardozo, KHM & Colepicolo, P (2005) Rhythmicity and oxidative/nitrosative stress in algae. Biological Rhythm Research 36:67-82.

Schmidt EC, Scariot LA, Rover T, Bouzon, ZL (2009) Changes in ultrastructure and histochemistry of two red macroalgae strains of *Kappaphycus alvarezii* (Rhodophyta, Gigartinales), as a consequence of ultraviolet B radiation exposure. Micron 40:860-869.

CAPÍTULO II

Efeitos do óleo diesel sobre a bioquímica e organização celular da alga parda *Sargassum cymosum* var. *stenophyllum*.

Artigo a ser submetido ao Journal of Applied Phycology

Effects of diesel oil on the biochemistry and cellular organization of the brown alga *Sargassum cymosum* var. *stenophyllum.*

Eva Regina Oliveira[a], Fernanda Ramlov[b], Éder Carlos Schmidt[c], Débora Tomazi[c], Claudia Marlene Bauer[a], Rodolfo Moresco[a], Fernanda Pilatti Kokowicz[a], Zenilda Laurita Bouzon[c], Paulo Antunes Horta[b], Marcelo Maraschin[a]

[a]Plant Morphogenesis and Biochemistry Laboratory, Federal University of Santa Catarina, 88049-900, P.O. Box 476, Florianopolis, SC, Brazil.

[b]Phycology Laboratory, Department of Botany, Federal University of Santa Catarina, 88049-900, P.O. Box 476, Florianopolis, SC, Brazil.

[c]Plant Cell Biology Laboratory, Department of Cell Biology, Embriology and Genetics, University of Santa Catarina, 88049-900, P.O. Box 476, Florianopolis, SC, Brazil.

Corresponding author: +55 48 37214812
E-mail address: ginagro@gmail.com

ABSTRACT

The impact of acute diesel (0001, 0:01, 0.1 and 1%) exposure in biochemistry and cellular organization of the brown seaweed *Sargassum cymosum* var. *stenophylum* were evaluated in vitro on times of 30min, 1h, 12h and 24h. Were evaluate chlorophyll *a* and *c*, as well as carotenoid and phenolic contents, in this species. Chlorophyll content increased following diesel treatments. No typical phenotype was detected for carotenoid compounds, but a clear reduction in phenolic compounds was observed by electron microscopy and cytochemical analysis ultrastructural changes, such as thickening and accumulation of phenolic compounds in the cell wall, irregularities on the cell surface, and an increased number of vacuoles, were detected. In a second and complementary approach, the metabolic profile of *S. cymosum* samples treated for 24 hours was determined by nuclear magnetic resonance spectroscopy (1H-NMR) which showed a significant reduction in qualitative profile of metabolites in the samples treated compared with control, corroborating the biochemical findings. *S. cymosum* var. *stenophyllum* would be a potential candidate biomarker in marine areas affected by diesel pollution.

Keywords: *Sargassum cymosum*, metabolic profile, oil pollution, abiotic stress

1 INTRODUCTION

Increased population density along coastal areas is a main cause of anthropogenic impact in such regions. By their high toxicity and bioaccumulation, the ions of heavy metals are the most common marine contaminants found (Ritter et al., 2008), along with pesticides, xenobiotics, and oil spills (Stepanyan & Voskoboinikov, 2006). In particular, diesel oil, a petroleum derivative, is the focus of this work, and its environmental impact and importance is supported by previous studies (Megharaj et al. 2000; Tôrres, 2008; Rodrigues et al. 2010).

Among benthic marine organisms, algae form the base of the food chain, and these species are very sensitive to environmental impacts (McCormick & Cairns 1994; Cheng & Yang 2006). In particular, marine macroalgae are essential in establishing the balance and resilience of coastal ecosystems. Accordingly, they are able to develop strategies against stressors as expressed by the production of various metabolites, making these organisms the most promising bioindicators of organic and inorganic pollutants (Cheng, Yang 2006; Torres et al. 2008). Therefore, some species of algae can be considered as biological markers to monitor the effect of stressor agents on habitats and communities.

Various approaches to detect algal responses to pollutants, including heavy metals, xenobiotics, and hydrocarbons, have been adapted. One important approach detects changes in the production of metabolites potentially involved in the biochemical mechanisms of algae defense (Ryzhik, 2011; Le Lann et al. 2012). For instance, carotenoids, pigments of the photosynthetic apparatus, and accessory phenolic compounds have been cited (Steinberg; Altena 1992). On the other hand, the analysis of morphological alteration or damage in algae exposed to abiotic stressors provides another means of corroborate the results of biochemical studies.

For example, the Phaephyceae class, brown algae. Have often been used in studies related to the biosorption of pollutant agents in preparation for the removal of oil in contaminated waters, otherwise known as bioremediation (Raize et al. 2004; Vijayaraghavan et al. 2009; Mishra et al. 2012). Indeed, the most promising tools for the recovery of contaminated marine waters involve technologies such as bioremediation (Wrabel; Peckol 2000) and biotransformation of aquatic systems (Pinto et al. 2003; Vidotti; Rollemberg 2004).

The brown algae predominate in temperate regions. In the tropical regions have *Sargassum* as one of the representatives. Occurs both on rocky shores protected as headlands exposed to wave action (Yoneshigue-Valentin, 2009).

Found along the Brazilian coast, specimens of the genus *Sargassum* play a key role in the marine food chain, including influencing the occurrence of a diverse flora and fauna associated (Szechy *et al*. 2006). They also produce secondary metabolites that reduce the palatability of algae to herbivores, thus influencing the structure of the populations of these rocky shores (Coimbra, 2006)

In this context, the present study aimed to evaluate the impacts of acute exposure of the brown alga *Sargassum cymosum* var. *stenophyllum* (Martius) to diesel oil, focusing on ultrastructural and metabolic traits.

2 MATERIAL AND METHODS

2.1 BIOLOGICAL SAMPLES - COLLECTION AND CULTURE CONDITIONS

Specimens of *Sargassum cymosum* var. *stenophyllum* were collected in September 2012 at Ponta das Canas Beach (Florianópolis city, Santa Catarina State, southern Brazil - 27º23'34" S, 48º26'11" W) and taken to the Laboratory of Phycology (LAFIC - Federal University of Santa Catarina). Samples were cleaned and transferred to culture medium supplemented with von Stosch solution 50%, according to Edwards (1970), acclimated for 21 days under continuous aeration at 25ºC ± 2°C, with daily photosynthetically active irradiation (PAR) at 80 mol photons m-2 s-1 (Li-color light meter 250, USA), and photoperiod of 12 h. The salinity was 34 ups (± 1 ups) (standard salinity unit). The exchange of culture medium were made every 5 days.

2.2 TREATMENTS

After acclimation, thallus segments of *Sargassum cymosum* var. *stenophyllum* (2g, fresh weight) were grown in flasks containing 400 mL of sterile seawater plus diesel oil at concentrations (v/v) of 0.001%, 0.01%, 0.1%, and 1%. The algae were exposed to that pollutant for 30 min, 1h, 12h, and 24h under the same experimental conditions noted above throughout the acclimation period. Each treatment consisted of five simultaneous replications and control plants were grown in culture medium for 24 hours. At the end of the experiment, samples were collected, immediately frozen in liquid nitrogen and stored at -80 ° C until analysis.

2.3 BIOCHEMICAL ANALYSIS

2.3.1 Extraction and quantification of chlorophyll a and c

Chlorophylls a and *c* were extracted with cold acetone *(4ºC, 3mL/*225mg fresh weight, n = 5). The mixture was incubated on ice for 10 minutes, followed by centrifugation (12000g/5min). The supernatant was collected, and its absorbance values were measured at 630nm, 647nm, and 664nm in order to calculate the total content of chlorophylls, according to the equation of Jeffrey and Humphrey (1975) FÓRMULA.

2.3.2 Extraction and quantification of carotenoids

To 1g of biomass (fresh weight) in nitrogen, 10 mL of methyl alcohol were added, followed by incubation for 1h at room temperature. Afterwards, the organosolvent extract was recovered through filtration on cellulose support (14µm pore ∅) in vacuo. The filtrate (3 ml aliquots) was scanned (wavelengths of 200-700 nM UV - visible) and the absorbance values at 450 nm was selected for further quantification of the total content of carotenoids. For calculation purposes, a standard curve of β - carotene (Sigma - Aldrich, St. Louis, MO, USA - 0.5 to 10 µg.mL - 1, y = 0.167x, r2 = 0.99) was built and previously described by Kuhnen (2009). The analyses were carried out in quintuplicate and the results expressed as mg β-carotene/g samples (dry weight).

2.3.3 Extraction and quantification of phenolic compounds

Phenolic compounds were extracted by incubating fresh alga samples (1.0 g, n = 5) in 80% methanol solution (v/v) for 1h. After centrifugation (12000g, 5min), the supernatant was collected and Folin-Ciocalteau reagent added to determine the total content of phenolic compounds, as previously described by Rhandir et al. (2002). The absorbance was read at 725 ηm, and a phloroglucinol standard curve (Sigma-Aldrich, St. Louis, MO, USA - 100–1250 µg.mL–1, y = 0.0004x, r2 = 0.997) was built for further calculation of the concentrations.

2.3.4 Detection and quantification of carotenoids by high performance liquid chromatography – HPLC

This analysis was performed on samples treated with diesel oil for 24 h and control. To accomplish this, aliquots (10µL) of the methanolic extract (see item 1.3.2) were injected into a liquid chromatograph (LC Shimzadu - 10A) equipped with a C18 reverse phase column (Vydac 201TP54, 250mm x 4.6mm ∅) fitted to a guard column (Vydac 218GK54 5mm) and a UV-Visible detector operating at 450 nm. Elution was performed with methanol: acetonitrile (90: 10, v/v) as the mobile phase at a flow rate of 1 ml.min-. The identification of carotenoids was performed by comparison with the retention time of standard compounds, e.g., lutein (Sigma-Aldrich, St. Louis, MO, USA) and fucoxanthin, in this case only for comparison purposes retention

time. The quantification of carotenoids was performed using the external standard curve of lutein (2.5 to 50 µg.mL-1 - r2 = 0.99, y = 7044x) considering the area under the peaks of interest for the calculation of concentrations of analytes for further chromatographic analysis as previously described (Kuhnen et al. 2009). The values of the content of carotenoids (mg per g, dry weight) were determined from the average of three consecutive injections for each sample.

2.4 SPECTROSCOPIC ANALYSIS BY 1H NUCLEAR MAGNETIC RESONANCE (1H-NMR)

Extracted following the same protocol used for carotenoids (see item 1.3.2). The extracts (3mL) were centrifuged (5000 rpm/5min), and the supernatant recovered and lyophilized, followed by the addition of 750 uL of deuterated methanol and centrifugation (5000 rpm/10min). The supernatant (700 uL) was transferred to NMR tubes (5 mm internal ∅), followed by high resolution ^{1}H-NMR analysis. The ^{1}H-NMR spectra were recorded on a 400 MHz Bruker Advanced spectrometer, as previously described (Kuhnen et al., 2010).

2.5 MICROSCOPIC ANALYZES

Light microscopy (LM) and cytochemistry

Samples (~ 5 mm length) were fixed in 2.5% paraformaldehyde in 0.1 M (pH 7.2) phosphate buffer overnight, following the description by Schmidt *et al.* (2009). Subsequently, the samples were dehydrated in increasing series of aqueous ethanol solutions and infiltrated with Historesin (Leica Historesin, Heidelberg, Germany). Then, 5µm length-sections were stained with 0.5% Toluidine Blue (TB-O, w/v), pH 3.0 (Merck Darmstadt, Germany), as previously described (Schmidt *et al.* 2010), and investigated with an epifluorescent microscope (Olympus BX 41) equipped with an Image Q Capture Pro 5.1 Software (Qimaging Corporation, Austin, TX, USA). Similarities based on the comparison of individual treatments with replicates suggested that the LM analyses were reliable.

2.5.1 Transmission electron microscope (TEM)

Samples (5mm in length) were fixed in a solution composed by 2.5% glutaraldehyde, 2.0% paraformaldehyde, and 5 mM CaCl2 in 0.075M sodium cacodylate buffer (pH 7.2) plus 0.2M sucrose and 1% caffeine and left to stand overnight (Ouriques and Bouzon, 2000). The material was post-fixed with 1% osmium tetroxide for 4h, dehydrated in a graded acetone series and embedded in Spurr's resin. Thin sections were stained with aqueous uranyl acetate 2% (m⁄v), followed by lead citrate 2% (m⁄v), according to Reynolds (1963). Four replicates were made for each experimental group, and two samples per replication were examined under TEM JEM 1011 (JEOL Ltd., eTokyo, Japan, at 80 kV). Similarities based on the comparison of individual treatments with replicates suggest that the ultrastructural analyses were reliable.

2.5.2 Scanning electron microscope (SEM)

The fixation procedure for SEM observations was identical to that used for TEM. The samples were dehydrated with ethanolic series, dried in a critical point apparatus (EM-CPD-030, Leica, Heidelberg, Germany), and examined under SEM JSM 6390 LV (JEOL Ltd., Tokyo, Japan, at 10 kV). The eventual adsorption/binding of diesel to the cell wall was determined by using SEM (NORAN System 7 EDS analyzer, Thermo Scientific) coupled to an energy dispersive X-ray spectrometer (SEM-EDX), without post-fixing the samples in osmium tetroxide 1% (m⁄v) or coating with gold.

3 STATISTICAL ANALYSIS

Data were analyzed by bifactorial Analysis of Variance (ANOVA) and Tukey test. All statistical analyses were performed using the Statistica software package (Release 6.0), considering $p \leq 0.05$. Homogeneity of the variance was tested using Levene's test. The biochemical dataset was further subjected to multivariate statistical analysis following an unsupervised method, i.e., the principal components analysis (PCA), by implementing the required script using the R language (v.2.15.2).

4 RESULTS

Table 1. Two-away ANOVA of pigment concentrations in *Sargassum cymosum* var. *stenophyllum* exposed to diesel (0.001, 0.01, 0.1 and 1%) in times of 30min, 1h, 12h and 24h.

		Chlorophyll *a*		Chlorophyll *c*		Carotenoids		Phenolic	
Variable	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Concentration	1	35665.0	0.00	34568.6	0.00	2249.9	0.00	68.9	0.00
Time	1	8326.0	0.00	15184..9	0.00	402.6	0.00	21.1	0.00
Concentr. x Time	1	3018.0	0.00	4527.4	0.00	219.2	0.00	3.5	0.00

4.1 QUANTIFICATION OF CHLOROPHYLL A AND C

The chlorophyll contents strongly increased in all treatments in comparison to control, showing the sensitivity of this biochemical target to diesel exposure (Table 2). Indeed, both short (30min) and long (24h) exposure times caused changes in both concentration of contents chlorophyll a and c compared to control. As shown in Table 2, chlorophyll a/chlorophyll c ratio is about 2 units in control plants, while this value meaningfully oscillates (e.g., 0.9 – 30min/0.001%, 1.1 - 24h/0.001%) in the treated samples. The exposure of *S. cymosum* to 0.001% diesel oil for 24h led to an excess of chlorophyll a (5.3 orders of magnitude) and c (10.5 orders of magnitude) contents relative to control. A slight decrease in the other concentrations at this exposure time was observed, but all significantly differed from the control ($p<0.05$).

4.2 QUANTIFICATION OF CAROTENOIDS

Shorter exposure times (30min and 1h) at concentrations from 0.001 to 0.1% stimulated carotenoid biosynthesis in *S. cymosum*, while for longer treatment times (12 h and 24h), a uniform response was not detected in the studied samples. Interestingly, the plants treated with a 1% concentration of diesel oil seemed to be more sensitive to the stress

imposed by diesel oil with respect to the amounts of the pigments of interest, allowing the speculation that higher concentrations might lead to more pronounced biochemical changes and toxic effects to the cells (Table 1).

4.3 QUANTIFICATION OF PHENOLIC COMPOUNDS

The phenolic compounds significantly decreased in all treatments compared to control (Table 2), typically indicating that the biosynthesis and accumulation of such secondary metabolites were impaired. Furthermore, for the 30min and 1h exposure times, no differences were detected in the total amounts of target compounds, irrespective of diesel oil concentrations, a result not observed for longer exposure times of 12 and 24h.

Table 2. Contents of chlorophylls *a* and *c* (µg.g^{-}, fresh weight biomass), carotenoids (µg.g^{-}, dry weight) and total phenolics (µg.g^{-}, dry weight) of *S. cymosum* var. *stenophyllum* exposed to diesel oil (0.001%, 0.01%, 0.1%, 1% - v/v) for 30min, 1h, 12h, and 24h. Values are the mean ± standard deviation (n = 5). Same letters indicate no significant difference according to the Tukey test (p < 0.05).

Time	Treatments (%)	Chlorophyll *a* (µg.g^{-1})	Chlorophyll *c* (µg.g^{-1})	Carotenoids (µg.g^{-1})	Total phenolics (µg.g^{-1})
	Control	87.65 ± 1.11o	40.71 ± 0.23o	32.63 ± 0.24f	20.90 ± 3.32a
	0.001	275.26 ± 1.10i	293.76 ± 0.57c	34.69 ± 0.23de	12.53 ± 0.26cd
30 min	0.01	282.67 ± 0.96h	245.45 ± 0.57f	40.85 ± 0.69c	13.96 ± 1.16bc
	0.1	208.68 ± 1.14l	198.26 ± 0.26m	40.67 ± 0.45c	13.88 ± 0.57b
	1	338.50 ± 0.77e	197.09 ± 0.92h	30.19 ± 0.22g	13.03 ± 1.54b
	0.001	415.04 ± 2.35b	277.55 ± 4.40d	40.50 ± 0.26c	5.38 ± 0.54de
1h	0.01	296.90 ± 0.89g	148.71 ± 1.71j	34.54 ± 0.22e	5.15 ± 0.41e
	0.1	217.18 ± 1.69k	146.69 ± 4.50k	42.67 ± 0.61b	5.42 ± 0.19e
	1	194.62 ± 0.26m	193.29 ± 0.65n	33.92 ± 0.45f	4.33 ± 0.51e
	0.001	278.93 ± 1.00hi	157.92 ± 0.53i	31.14 ± 0.42g	9.16 ± 0.66e
12h	0.01	189.27 ± 0.36n	139.41 ± 0.27l	30.38 ± 0.39g	3.65 ± 0.27e
	0.1	269.51 ± 0.01j	255.33 ± 0.95e	44.84 ± 0.70a	10.66 ± 0.45e
	1	309.03 ± 1.46f	210.83 ± 0.41g	26.37 ± 0.24h	4.33 ± 0.16e
	0.001	463.06 ± 5.66a	426.26 ± 1.76a	35.70 ± 0.34d	8.10 ± 0.36e
24h	0.01	356.85 ± 2.10c	290.72 ± 0.75c	30.21 ± 0.52g	12.80 ± 0.16cd
	0.1	354.08 ± 2.10c	242.53 ± 0.82f	41.37 ± 1.07c	10.47 ± 0.83e
	1	349.09 ± 2.85d	310.38 ± 1.57b	25.53 ± 0.55h	15.13 ± 0.64b

4.4 DETECTION AND QUANTIFICATION OF CAROTENOIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY - HPLC

The chromatogram shown in Figure 1 represents a typical carotenoid profile of the studied *S. cymosum* samples. Having regard to the retention time observed fucoxanthin (*ie*, 3.7 minutes), the identity of the chromatogram peak was assigned to this compound. Indeed, brown seaweed species are well known sources for this pigment. However, the xanthophylls lutein also has shown a quite near peak at 3.8 min, so that

the unambiguous determination of the identity of the compound should be confirmed by mass spectrometry and NMR analysis, in greater detail. The carotenoid contents calculated for *S. cymosum* samples treated for 24 h revealed that diesel oil concentrations higher than 0.001% (v/v) meaningfully change the biosynthesis and accumulation of those metabolites, even though a phenotypic pattern can not be determined.

Figure 1. HPLC chromatogram of S. cymosum var. stenophyllum organosolvent extract after 24 hours of exposure to diesel fuel, which shows a peak at 3.7 min attributed fucoxanthin pigment, taking into account the retention time of the standard compound.

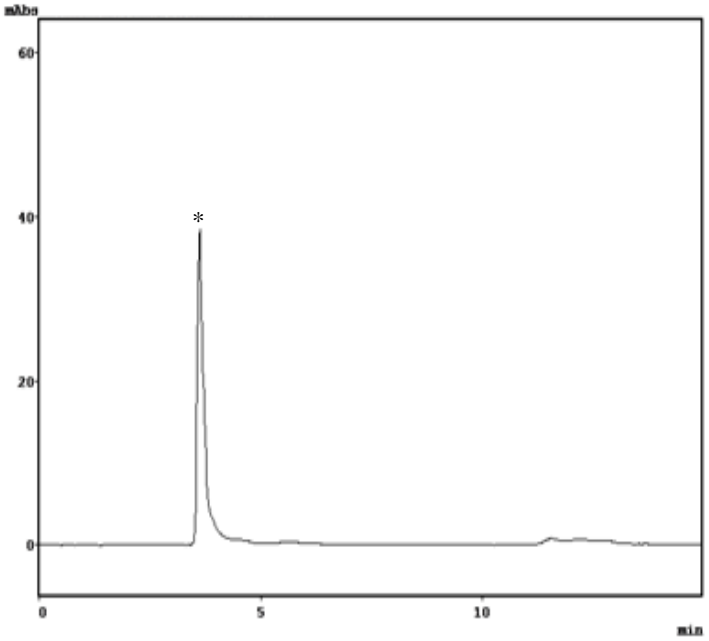


Table 3. Concentration of fucoxanthin (µg.g-, dry weight biomass) determined by HPLC in the organosolvent extract (methyl alcohol) of diesel oil (0.001, 0.01, 0.1, 1%)-treated samples of *S. cymosum* after 24h exposure and control (no diesel). The values represent the mean ± standard deviation of three consecutive injections (10 µL). Same letters indicate no significant difference according to the Tukey test ($p < 0.05$)

Compound	Diesel concentration (%)	Peak average area	Concentration ($\mu g.g^{-}$)
Fucoxanthin (Rt = 3.7 min)	Control	2211642.10	31.39±2.94ab
	0.001	2018212.67	28.65±0.32b
	0.01	1404125.67	19.93±2.92c
	0.1	2641992.33	37.51±3.75a
	1	2156240.00	30.61±2.98b

4.5 SPECTROSCOPIC ANALYSIS BY 1H NUCLEAR MAGNETIC RESONANCE (NMR)

The NMR spectra revealed a larger number of chemical shifts in control samples compared to the diesel-treated plants (Table 4), suggesting a toxic effect on the metabolic processes of the brown alga derived from the suppression of some biosynthetic pathways. For the resonances of the aliphatic region (0 to 3.00 ppm) where, for example, signals from amino acids, alcohols, and organic acids e.g., are found a reduction by ~28% (0.001% diesel) was detected in the number of 1H peaks. Similarly, higher diesel concentrations from 0.01 to 1% suppressed the number of 1H resonances by 26%-36%, showing a clear negative effect on alga metabolism. The number of anomeric resonances (3.00 to 5.50 ppm), usually attributed to sugar compounds, such as monosaccharides, also showed a decrease (about 37%) by exposure of the algal biomass to diesel oil. On the other hand, the biosynthetic pathways associated with compounds having aromatic rings in their structure (5.50 to 8.50 ppm – e.g., phenolic acids, tryptophan, and tyrosine) seemed to be less prone to the toxic effects of this pollutant (Tables 6).

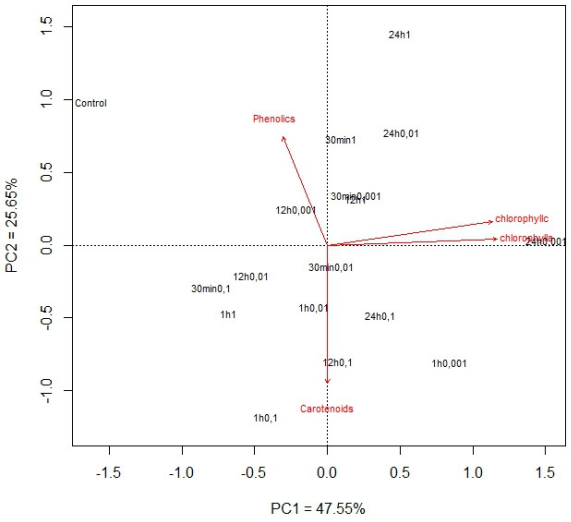
Table 4. Number of resonances detected in the ^{1}H-NMR spectra of the organosolvent extract of *S. cymosum* var. *stenophyllum* 24h-exposed to diesel oil, according to the aliphatic, anomeric, and aromatic spectral regions. Total number of chemical shifts (ppm) are shown in (Table 6).

Diesel concentration (%)	Aliphatic (0.00 – 3.00 ppm) region	Anomeric (3.00 – 5.50 ppm) region	Aromatic (5.50 – 8.50 ppm) region
Control	126	106	27
0.001	91	72	23
0.01	81	67	26
0.1	82	67	31
1	93	62	26

4.6 PRINCIPAL COMPONENT ANALYSIS

Biochemical data were subjected to multivariate analysis using an unsupervised method, i.e., principal components analysis (PCA). The principal components 1 and 2 (PC1 and PC2) accounted for 73% of sample variance of the dataset (Figure 2).

Figure 2 Factorial distribution of PC1 and PC2 for biochemical variables in *S. cymosum* in vitro, exposed to concentrations of diesel oil for 24 hours.



4.7 MICROSCOPIC ANALYZES

Light microscopy (LM) and cytochemistry

Control samples of *S. cymosum* stained with Toluidine Blue (TB-O) showed a metachromatic reaction in the cell wall, indicating the presence of acidic polysaccharides, such as alginic acid and sulfated fucan (Figure 3a). When stained with TB-O, the samples treated with diesel oil presented a reaction in the cell wall similar to that of control plants (Figure 3 b-m). Moreover, the diesel oil-treated plants cultivated during 24h showed an increase in lenticular cell wall thickness (Figure 3 j-m). In the cytoplasm of cortical cells of control samples, a large quantity of dark blue and yellow physodes was observed (Figure 6a, arrows), as cortical cells of treated samples showed a migration of the physodes from the cytoplasm toward the cell surface (Figure 3 b-m, arrows). Thallus samples cultivated at 1% diesel for 1h presented a reduction in the number of physodes (Figure 3e).

4.7.1 Scanning electron microscope (SEM) and X-ray microanalysis

When observed by scanning (SEM) electron microscopy, the walls of cortical cells showed a smooth surface in the samples of *S. cymosum* of control (Figure 4a). In contrast, samples exposed to diesel oil (bm Figure 4) revealed a rough surface of cells and disrupted cell walls, apparently resulting from the absorption of oil. This finding indicates that plants exposed to diesel undergo changes in mucilage that coats the rod. The results of X-ray microanalysis of *S. cymosum* have to be considered as qualitative. The elements C, N, O, Na, K occurred on both cell surface and internal to the cell wall, but a clear pattern of distribution according to diesel treatments was not detected (Table 5).

4.7.2 Transmission electron microscope (TEM)

When observed by transmission electron microscopy (Figure 5a-d), the cytoplasm of control *S. cymosum* cells was filled with large chloroplasts, small mitochondria, and a large quantity of physode (Fig. 5a). These cells were surrounded by a thick cell wall (Figure 5a-b) formed by concentric microfibrils embedded in an amorphous matrix which consisted of sulfated polysaccharides, such as alginic acid and

sulfated fucan (Figure 5b), confirming the previous findings by LM of samples stained with TB-O. The mitochondria showed well-developed cristae membranes (Figure 5b), and large physodes were observed in the cytoplasm (Figure 5c). As shown in Figure 5a, the cells that presented an increase in the number of chloroplasts were larger and exhibited the typical structure of brown algae with aggregated thylakoids in bands three to three (Figure 5c).

After exposure to diesel during 24h in the four concentrations, changes in the ultrastructural organization of *S. cymosum* were detected (Figure 6a-i). A large quantity of vacuoles was observed in treated cells (Figure 6a), as well as the deposition of phenolic compounds in the cell wall (Figure 6 b-c). These cells presented well-preserved mitochondria (Figure 6d), and the thylakoid membranes showed no dramatic ultrastructural damage, except for a certain dilation (Figure 6 d-e, arrows), as the number of plastoglobuli was observed to increase in the chloroplasts (Figure 6e). The cytoplasm of treated samples (Figure 6 f-h) was denser compared to control, and a large quantity of vacuoles with electron dense material was observed. Finally, in the cytoplasm, crystallized structures were observed in treated cells (Figure 6i).

5 DISCUSSION

Exposure of brown alga *Sargassum cymosum* var. stenophylum to diesel oil resulted in observable changes at different levels and in all analyzed parameters. The chlorophyll content increased in all treatments compared to control. Were expected reduction in the concentrations of this pigment, since the stress conditions increase the production and accumulation of reactive oxygen species (ROS), triggering oxidative damage to biomolecules, as well as to the photosynthetic apparatus, Solovchenko et al. (2007). This reduction was observed in Polo et al (2013), who exposed *S. cymosum* in stress by UV rays and different salinities.

For carotenoids, the changes differed from those observed in chlorophyll in content, not showing a pattern in responses over treatments. These pigments may play an important protective activity of the photosynthetic apparatus during stress conditions. For longer exposure times, it was not possible to detect a homogeneous response of *S. cymosum*, eventually revealing an impairment of metabolic control associated with that biosynthetic pathway. Moreover, the total contents of those pigments and the amounts of fucoxanthin detected in the samples did not follow a pattern resulting from the diesel treatments.

The phenolic compounds declined significantly with exposure to diesel oil in all treatments. The phenolic compounds declined significantly with exposure to diesel oil in all treatments. These results were also consistent because the diesel oil-treated samples appeared to concentrate such metabolites along the cell walls outside the physodes, as shown by imaging analysis. These metabolites are also related to the mechanism of protection of the photosynthetic apparatus, with potent antioxidant activity (Le Lann et al. 2012) and antiherbivory (Pavia; Toth, 2000; Lüder; Clayyon, 2004) in *H. musciformis*. The observed decrease in the phenolic contents of *S. cymosum* may be an indication of loss of these compounds in the process of repairing damage caused by the action of diesel on the surface of the tissue (figure 4). Already in 1977 Fagenberg and Dawies observed accumulation of vesicles, including the physodes on the surface of cells in the wound of a stalk of brown seaweed. In studying fucus vesiculosus, Ryzhik et al. (2012) reported the loss of phenolic compounds into the environment, possibly as a defense mechanism. It is possible that contact with diesel oil caused an intensification of the process of peeling in the alga's cell surface (Grande et al. 2012), as herein observed.

In this study, we observed a thickening of the cell walls in the treated stalk segments shown in the LM images (figure 3) that may be evidence of structural defense mechanisms. Interestingly these results was described by Schmidt et al. (2010) in study with *Kappaphycus alvarezii* exposed to ultraviolet-B. Similar results were found by Ramlov et al. (2011, 2013) who studied the exposure of the red algae *Graciliaria dominguensis* to light irradiation and *Hypnea musciformis* to diesel oil.this phenomenon was also observed by Andrade et al. (2010) with *Padina gymnospora*, reporting changes in cell wall constituents, likely indicative of plant response to diesel oil deposition in their ultrastructural component.

Previous study (Grande et al., 2012) demonstrated that brown algae like *Sargassum cymosum* have the ability to mitigate structural and physiological effects of short-term exposure to toxic chemicals, including diesel oil. This particular properties of adsorption (Liu et. al. 2011), can make them suitable for use as adsorption substrates to remove oil spillage along coastal areas.

Furthermore, the cytoplasmic shrinkage and the migration of physodes (storage place of phenolics) toward the cell wall herein described for the samples exposed to higher diesel concentrations is also thought to be a survival response of *S. cymosum* var. s*tenophyllum* to that pollutant (figures 5 and 6).

Analysis of TEM images revealed a slight dilation of thylakoid in diesel-treated plants compared to control, while maintaining the overall integrity of the structure of chloroplasts. In any extension, such findings might be correlated to the increased chlorophyll contents found in samples after exposure to that pollutant. Indeed, exposing the brown alga *S. cymosum* to diesel oil resulted in observable changes at different levels and in all analyzed parameters.

Although principal components 1 and 2 (PC1 and PC2) accounted for 73% of sample variance of the dataset, the description model built was not able to discriminate among the samples according to diesel treatments. Such findings prompted us to speculate that a certain biochemical similarity might derive from common mechanisms of responses to the stressor agent (Figure 2). However, the clear separation between control and treated plants indicates a prominent change in metabolic processes caused by diesel oil exposure.

The results obtained from NMR analysis confirmed that distinct metabolic profiles result from exposure of *S. cymosum* var. stehophyllum to the studied xenobiotic. Based on the number of chemical shifts detected in the ^{1}H-NMR spectra, a prominent reduction of metabolites seems to occur in all diesel-treated plants, allowing the inference of toxic effect on cell metabolism compared to control (Table 4).

X-ray microanalysis detected the elements C, N, O, Na, and K both on the cell surface and internal to the cell wall, but not affording a pattern of distribution according to the diesel treatments investigated. The increase in the concentrations of carbon and coincident reduction in nitrogen levels, as detected by this microanalysis when exposed to the diesel compared to the control

samples, can confirming the hypothesis may be carbon-nitrogen balance, whereby the availability controlling the concentrations of these nutrients in plant tissues secondary (Hamilton et al. 2001) metabolites. The increase in oxygen levels from the same analyzes, may mean increased respiratory rate, ode to corroborate the displayed morphological changes in the chloroplasts of samples submitted for fuels (Table 5).

Taken together, the results of this study showed that the brown alga *S. cymosum* var. *stenophyllum* makes use of defense mechanisms, both biochemical and morphological, in order to resist stress conditions imposed by diesel oil contamination. According to some authors (Voskoboinikov & Stepanyan, 2006), the adaptation mechanisms of this brown alga to oil-polluted marine environments are still not fully elucidated. However, our findings reinforce the suggestion that this species is a promising candidate as a bioindicator of coastal areas affected by diesel pollution.

Figure 3. Light microscopy of the transversal sections stained with TB-O of control (a).

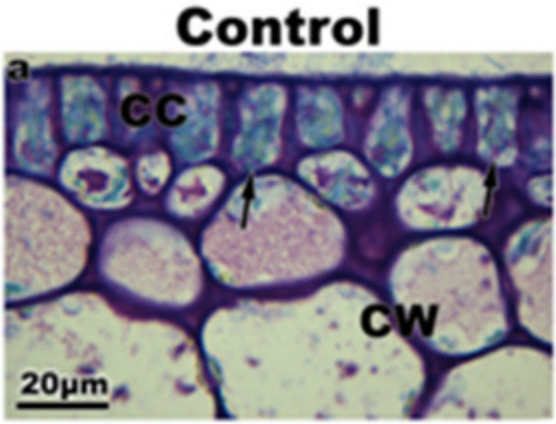


Figure 4. Light microscopy of the transversal thallus sections stained with TB-O and exposed to diesel concentrations (b-m) of *S. cymosum* var. *stenophyllum*. The cell walls (CW) of cortical cells (CC) show metachromatic reaction (a). In the cytoplasm the presence of physodes is highlighted (arrows, b-m). Details of the plants treated with diesel in respect to the metachromatic reaction in the cell wall and the physodes migration.

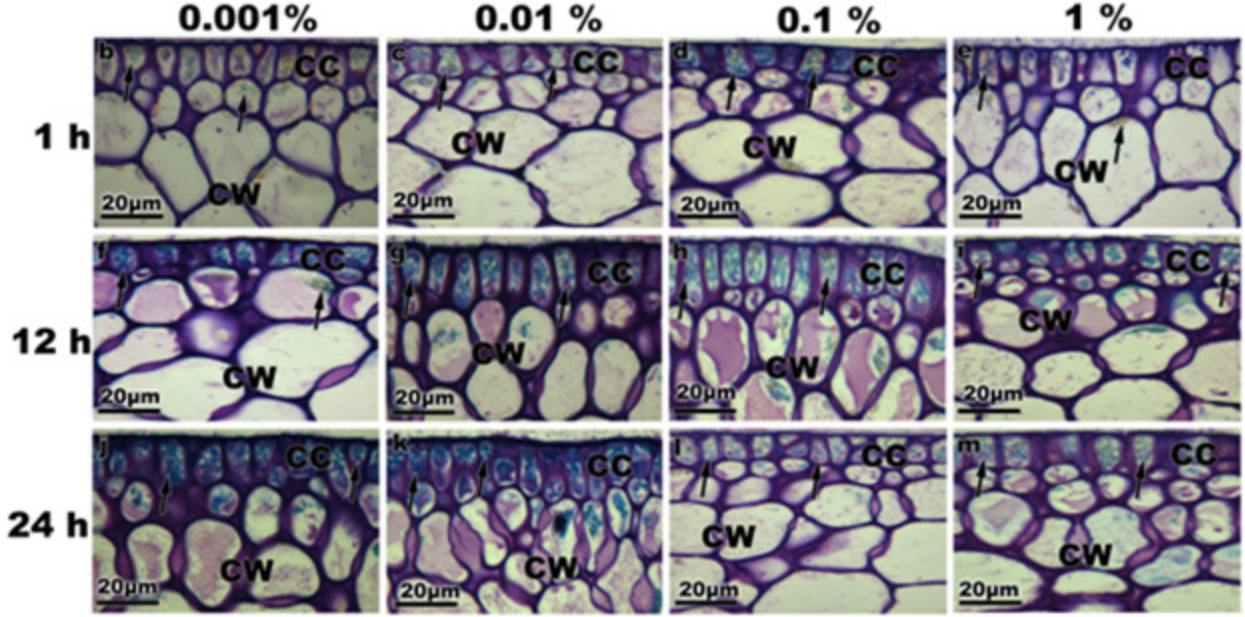


Figure 5. Scanning electron micrographs of control (A) and stalk exposed to diesel concentrations for 1 hour (a- e) *S. cymosum* var. *stenophyllum*. Detail of control surface topography of cortical cell walls showing a smooth aspect (a) and topography of diesel-treated plants showing an irregular surface after diesel treatments (b - e).

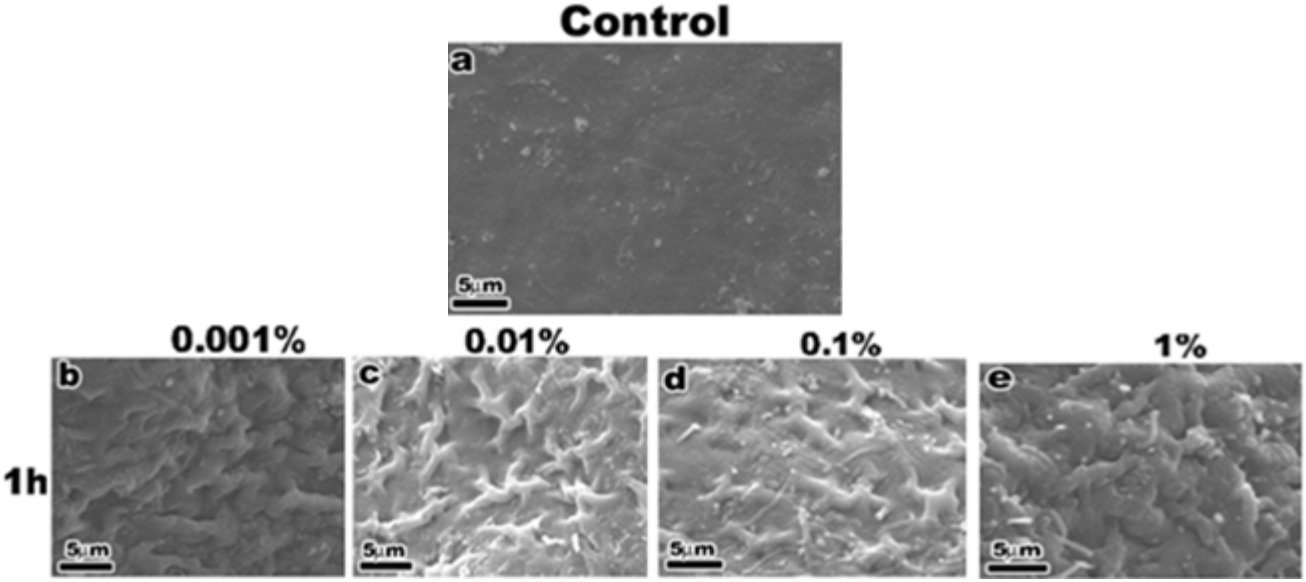


Figure 6. Scanning electron micrographies of thallus exposed to diesel concentrations of 12h and 24h (f-m) of *S. cymosum* var. *stenophyllum*. Detail topography of diesel-treated plants showing an irregular surface after diesel treatments (f-m).

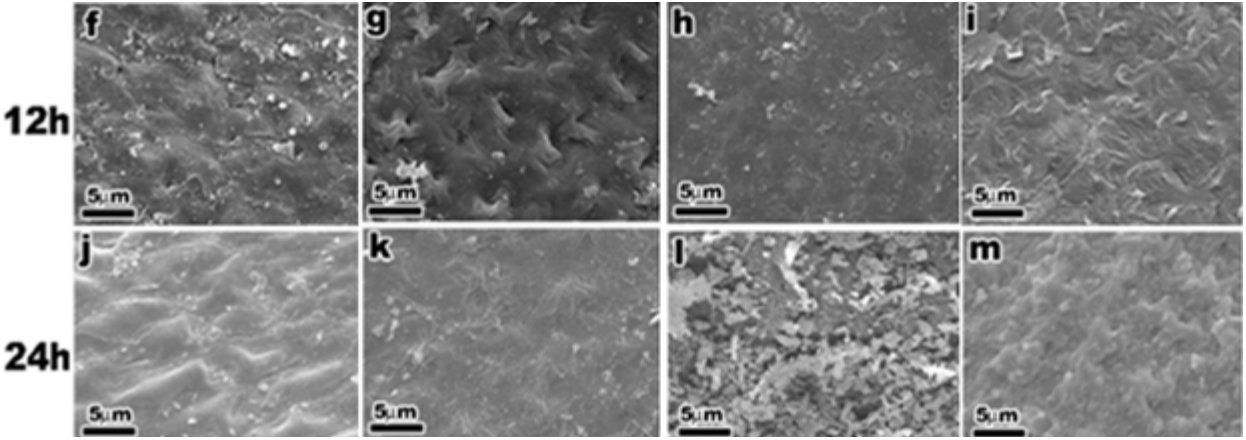


Table 5. X-ray microanalysis of the cell wall surface and internal cell wall revealing the presence of elements carbon, nitrogen, oxygen, sodium, and potassium in thallus samples of *S. cymosum* var. *stenophyllum* cultured *in vitro*.

	C	*N*	*O*	*Na*	*K*	*C*	*N*	*O*	*Na*	*K*
	Cell wall surface					*Internal cell wall*				
Control	28.1±2.6	33.1±2.6	31.8±2.8	2.6±0.5	4.4±1.3	34.0±3.6	29.4±4.0	30.7±1.5	2.5±0.3	3.4±3.0
1h/0.001%	44.8±1.6	6.3±0.5	38.2±1.8	3.4±0.2	7.1±0.3	47.5±0.7	3.7±0.7	38.9±0.5	3.5±0.2	6.3±0.5
1h/0.01%	41.0±2.0	13.9±6.5	36.0±3.3	3.1±0.6	5.9±1.0	48.4±1.0	8.2±0.5	35.4±0.3	3,0±0.4	5.3±0.7
1h/0.1%	42.9±5.4	12.3±1.3	34.2±4.0	3.0±0.3	7.5±1.3	43.5±0.9	9.1±0.8	36.4±0.4	2.9±0.2	8.1±0.6
1h/1%	45.4±0.8	6.3±1.1	40.3±1.1	3.2±0.3	5.7±0.7	49.9±2.5	6.2±2.4	35.9±3.6	2.9±0.5	5.0±0.6
12h/0.001%	36.9±2.6	27.1±3.3	34.0±1.3	3.3±0.5	5.5±0.4	38.6±1.4	17.0±1.8	35.3±0.8	3.3±0.2	5.9±0.3
12h/0.01%	44.0±0.8	9.9±4.3	39.4±2.4	4.4±0.9	2.3±0.1	49.1±0.7	8.8±1.6	36.1±1.4	4.0±0.4	1.9±0.3
12h/0.1%	23.5±1.0	36.5±1.5	29.7±1.2	2.5±1.3	7.8±0.7	36.1±1.6	23.4±3.3	32.9±1.8	3.0±0.5	4.5±0.3
12h/1%	33.2±2.5	22.4±1.4	33.2±0.6	5.0±0.5	8.0±1.1	38.4±1.2	15.8±0.8	35.1±2.0	4.1±0.3	6.6±0.8
24h/0.001%	25.2±2.8	36.3±3.2	29.3±2.3	3.0±0.3	6.4±0.8	36.2±1.9	27.6±0.5	28.0±0.5	2.5±0.7	4.9±0.3
24h/0.01%	29.1±2.2	32.9±3.5	30.0±2.1	3.9±0.4	4.1±0.4	40.5±0.6	20.0±2.0	32.5±0.9	2.4±0.3	4.5±0.4
24h/0.1%	34.0±4.6	17.4±2.3	38.7±4.2	3.2±0.6	6.7±0.8	47.7±2.1	9.6±2.1	35.4±1.4	2.9±0.4	4.3±0.7
24h/1%	24.6±2.6	33.5±2.3	32.7±1.3	3.3±0.4.	5.9±1.0	33.8±2.0	25.1±1.4	34.7±1.4	2.9±0.6	3.5±0.5

Figure 7. Transmission electron microscopy (TEM) images of *S. cymosum* var. *stenophyllum* control plants (a-c). Note the cells showing a large quantity of chloroplasts (C), mitochondria (M, and arrows), physodes (Ph), and thick cell wall (CW) (a). Details of the thick cell wall (CW) and well preserved mitochondria (b). Note the presence of phenolic compounds in physodes (c). Note the chloroplast internal organization of thylakoids in three bands into the chloroplasts (arrows, d).

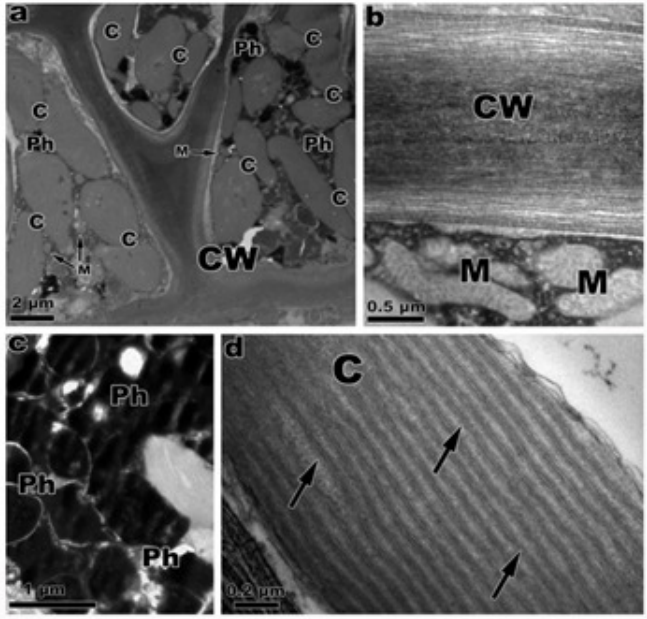


Figure 8. Transmission electron micrographic images of *S. cymosum* var. *stenophyllum* plants after 24h of exposure to diesel. Observe the cells showing a large quantity of chloroplasts (C), physodes (Ph), vacuoles (V), and thick cell wall (CW) (a). Notice the presence of phenolic compounds in the cell wall (arrows, b-c). Treated with 0.1 % of diesel (b, e, f). Treated with 0.01 % of diesel; (c and d). Note the mitochondria (M) association with chloroplast (arrows, d). Observe the increase of plastoglobuli (P) and thylakoids dilation (arrows, e).

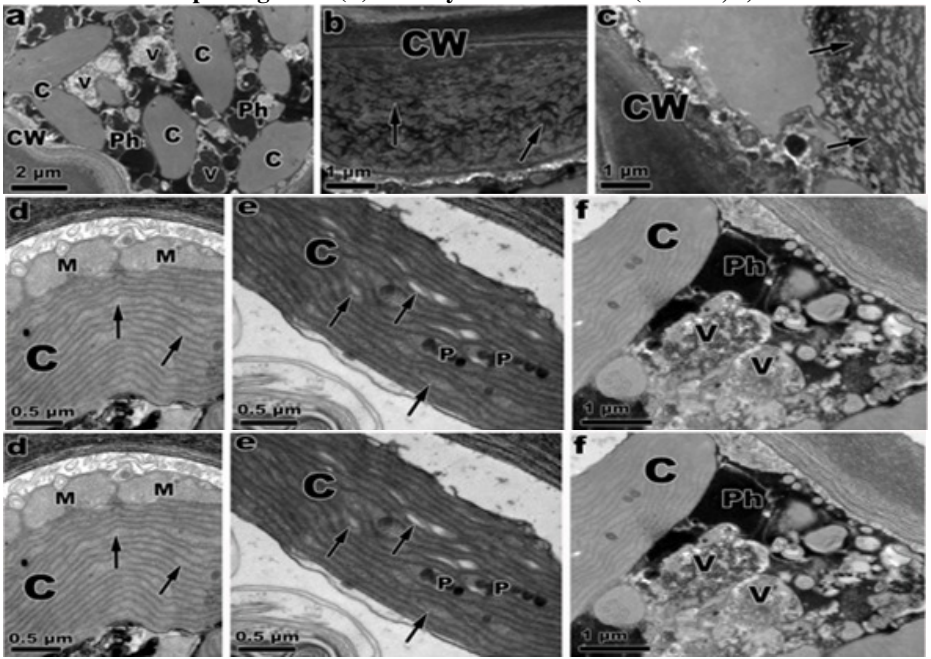


Figure 9. Transmission electron micrographic images of *S. cymosum* var. *stenophyllum* plants after 24h of exposure to diesel treated with 1% of diesel (g and i) treated with 0.001 % of diesel oil.. Note the presence of vacuole with electron dense material near to the physodes (Ph) (g-h). Note the crystallized structures in the cytoplasm (i).

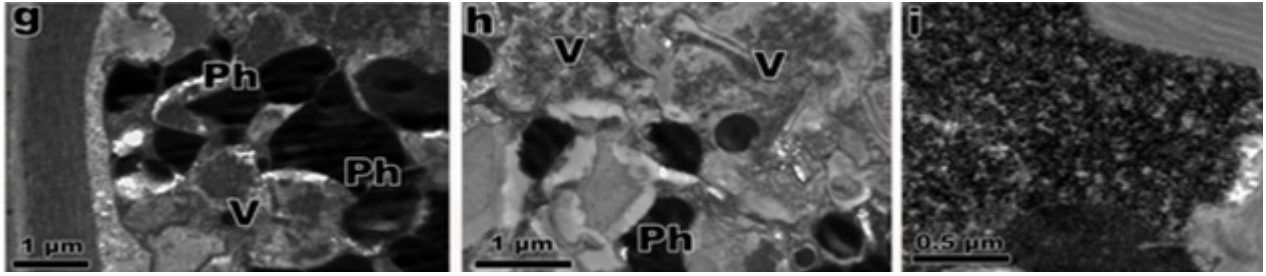


Table 6. ^{1}H-NMR spectra of control and 24h-diesel *in vitro* treated samples of *S. cymosum* var. *stenophyllum*

	Chemical shifts		
Diesel concentration (%)	Aliphatic region 0 - 3.00 ppm	Anomeric region 3.00 - 5.50 ppm	Aromatic region 5.50 - 8.50 ppm
Control	0.07 0.13 0.15 0.16 0.62 0.66 0.67 0.69 0.71 0.72 0.73 0.76 0.77 0.78 0.79 0.81 0.83 0.84 085 0.86 0.87 0.88 0.89 0.90 0.92 0.94 0.96 0.97 0.98 0.99 1.00 1.01 1.02 1.05 1.07 1.09 1.10 1.12 1.13 1.14 1.16 1.18 1.19 1.20 1.21 1.22 1.23 1.24 1.25 1.28 1.29 1.30 1.33 1.37 1.41 1.42 1.44 1.45 1.48 1.50 1.52 1.53 1.55 1.56 1.58 1.62 1.64 1.66 1.68 1.69 1.71 1.72 1.79 1.81 1.82 1.84 1.86 1.87 1.89 1.95 2.00 2.01 2.02 2.04 2.05 2.06 2.07 2.10 2.12 2.13 2.15 2.16 2.17 2.20 2.21 2.22 2.23 2.24 2.26 2.27 2.29 2.30 2.31 2.32 2.33 2.35 2.37 2.39 2.42 2.44 2.46 2.47 2.53 2.54 2.56 2.58 2.60 2.62 2.75 2.77 2.79 2.81 2.84 2.85 2.90 2.99	3.02 3.03 3.18 3.24 3.25 3.27 3.28 3.31 3.39 3.41 3.44 3.46 3.47 3.49 3.53 3.54 3.55 3.61 3.62 3.64 3.65 3.66 3.67 3.69 3.70 3.72 3.73 3.75 3.78 3.79 3.81 3.82 3.83 3.84 3.86 3.88 3.89 3.90 3.91 3.92 3.93 3.96 3.97 3.98 3.99 4.00 4.01 4.02 4.04 4.05 4.07 4.08 4.10 4.11 4.12 4.13 4.14 4.15 4.17 4.19 4.20 4.22 4.23 4.27 4.28 4.30 4.31 4.32 4.37 4.39 4.40 4.44 4.47 4.49 4.53 4.57 4.59 4.64 4.72 4.82 4.86 5.07 5.08 5.11 5.12 5.16 5.18 5.19 5.21 5.22 5.24 5.25 5.26 5.27 5.30 5.32 5.33 5.34 5.35 5.36 5.37 5.38 5.39 5.40 5.42 5.43	5.52 5.58 6.26 6.51 6.59 6.61 6.88 6.92 6.98 7.00 7.07 7.08 7.09 7.11 7.12 7.13 7.14 7.16 7.18 7.26 7.28 7.30 7.32 7.35 7.36 7.52 7.53 7.54 7.55 7.72 7.74

Chemical shifts			
Diesel concentration (%)	Aliphatic region 0 - 3.00 ppm	Anomeric region 3.00 - 5.50 ppm	Aromatic region 5.50 - 8.50 ppm
0.001	0.07 0.15 0.16 0.69 0.71 0.73 0.75 0.76 0.77 0.81 0.83 0.84 0.85 0.86 0.87 0.88 0.89 0.90 0.92 0.94 0.96 0.97 0.98 0.99 1.00 1.01 1.02 1.03 1.04 1.06 1.07 1.09 1.11 1.14 1.16 1.18 1.23 1.24 1.25 1.28 1.29 1.30 1.33 1.37 1.40 1.41 1.42 1.44 1.45 1.48 1.50 1.51 1.52 1.53 1.55 1.56 1.58 1.60 1.62 1.63 1.64 1.66 1.67 1.68 1.71 1.72 1.74 1.76 1.77 1.84 1.87 1.95 2.04 2.05 2.06 2.10 2.15 2.17 2.19 2.20 2.21 2.23 2.26 2.27 2.29 2.31 2.32 2.33 2.35 2.84 2.85	3.28 3.35 3.39 3.49 3.51 3.53 3.54 3.55 3.57 3.61 3.62 3.63 3.64 3.65 3.66 3.67 3.69 3.70 3.72 3.73 3.75 3.76 3.78 3.79 3.81 3.82 3.83 3.84 3.86 3.88 3.89 3.90 3.92 3.93 3.95 3.97 3.98 3.99 4.00 4.02 4.05 4.08 4.10 4.11 4.13 4.14 4.15 4.16 4.19 4.22 4.23 4.28 4.29 4.30 4.31 4.32 4.33 4.36 4.37 4.38 4.40 4.67 4.82 4.86 5.17 5.19 5.30 5.35 5.36 5.37 5.39 5.44	5.58 6.51 6.61 6.98 7.00 7.07 7.09 7.11 7.13 7.14 7.26 7.28 7.29 7.30 7.32 7.35 7.36 7.52 5.53 7.54 7.72 7.72 7.73
0.01	0.07 0.13 0.15 0.16 0.69 0.71 0.73 0.76 0.78 0.83 0.84 0.85 0.86 0.88 0.90 0.92 0.94 0.96 0.97 0.98 0.99 1.00 1.01 1.03 1.05 1.08 1.11 1.14 1.16 1.23 1.25 1.28 1.30 1.33 1.37 1.41 1.42 1.48 1.49 1.52 1.53 1.55 1.56 1.58 1.60 1.62 1.64 1.66 1.69 1.71 1.72 1.76 1.84 1.97 2.05 2.10 2.17 2.20 2.21 2.23 2.26 2.27 2.2 9 2.31 2.32 2.33 2.35 2.27 2.42 2.44 2.47 2.51 2.54 2.56 2.58 2.60 2.62 2.77 2.81 2.84 2.85	3.03 3.28 3.35 3.39 3.43 3.49 3.51 3.53 3.54 3.55 3.61 3.63 3.65 3.67 3.70 3.72 3.73 3.75 3.78 3.79 3.81 3.82 3.83 3.84 3.86 3.88 3.89 3.90 3.93 3.97 3.98 4.00 4.02 4.05 4.08 4.10 4.11 4.13 4.15 4.16 4.19 4.20 4.22 4.23 4.28 4.29 4.31 4.32 4.36 4.37 4.40 4.65 4.72 4.82 4.86 4.97 5.07 5.11 5.16 5.17 5.19 5.26 5.30 5.35 5.36 5.37 5.44	5.58 6.29 6.51 6.59 6.61 6.98 7.00 7.70 7.09 7.11 7.12 7.13 7.14 7.19 7.26 7.32 7.35 7.52 7.53 7.54 7.65 7.7. 7.71 7.72 7.73 7.74

Chemical shifts			
Diesel concentration (%)	Aliphatic region 0 - 3.00 ppm	**Diesel concentration (%)**	Aliphatic region 0 - 3.00 ppm
0.1	0.07 0.13 0.15 0.16 0.69 0.72 0.73 0.76 0.78 0.84 0.85 0.86 0.88 0.89 0.90 0.92 0.94 0.96 0.97 0.98 0.99 1.00 1.01 1.05 1.06 1.08 1.11 1.14 1.16 1.23 1.26 1.29 1.30 1.33 1.37 1.42 1.43 1.48 1.49 1.51 1.53 1.55 1.56 1.58 1.60 1.62 1.64 1.68 1.71 1.72 1.76 1.77 1.93 2.05 2.06 2.10 2.17 2.26 2.27 2.29 2.31 2.33 2.35 2.37 2.42 244 2.47 2.49 2.51 2.53 2.54 2.56 2.58 2.60 2.62 2.75 2.77 2.79 2.81 2.82 2.84 2.99	3.02 3.20 3.28 3.33 3.36 3.40 3.49 3.52 3.53 3.54 3.55 3.61 3.62 3.67 3.70 3.72 3.73 3.75 3.78 3.79 3.81 3.82 3.83 3.84 3.89 3.94 3.96 3.98 4.00 4.02 4.05 4.08 4.10 4.11 4.12 4.13 4.14 4.15 4.17 4.20 4.22 4.23 4.27 4.28 4.30 4.31 4.37 4.40 4.63 4.65 4.69 4.72 4.82 4.86 4.94 5.11 5.16 5.17 5.19 5,26 5.27 5.30 5.34 5.36 5.37 5.39 5.44	5.58 6.30 6.46 6.51 6.59 6.61 6.90 6.92 6.98 7.00 7.07 7.09 7.11 7.12 7.13 7.14 7.19 7.26 7.32 7.35 7.52 7.53 7.55 7.60 7.62 7.65 7.68 7.70 7.72 7.73 7.74
1	0.07 0.13 0.15 0.44 0.69 0.71 0.76 078 0.84 0.85 0.86 0.88 0.90 0.94 0.96 0.97 0.98 1.00 1.01 1.05 1.08 1.11 1.12 1.14 1.16 1.23 1.26 1.28 1.30 1.33 1.37 1.40 1.42 1.45 1.48 1.49 1.51 1.53 1.55 1.56 1.58 1.60 1.62 1.64 1.66 1.69 1.71 1.72 1.84 2.01 2.02 2.5 2.06 2.0 10 2.17 2.21 2.26 2.27 2.28 2.30 2.32 2.33 2.35 2.37 2.42 2.44 2.47 2.51 2.54 2.56 2.58 2.60 2.62 2.66 2.76 2.77 2.79 2.82 2.84 2.85 2.99	3.02 3.18 3.28 3.32 3.38 3.40 3.41 3.46 3.49 3.53 3.54 3.55 3.62 3.63 3.67 3.70 3.72 3.73 3.75 3.78 3.79 3.81 3.82 3.83 3.84 3.90 3.93 3.98 4.00 4.02 4.05 4.08 4.10 4.11 4.13 4.15 4.16 4.19 4.20 4.22 4.23 4.28 4.29 4.31 4.37 4.40 4.65 4.69 4.72 4.82 4.94 5.07 5.11 5.16 5.17 5.19 5.26 5.30 5.34 5.36 5.37 5.44	5.58 6.51 6.59 6.61 6.90 6.98 7.00 7.07 7.09 7.11 7.12 7.13 7.14 7.19 7.26 7.32 7.35 7.49 7.52 7.53 7.54 7.60 7.62 7.65 7.72 7.73

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the staff of the Central Laboratory of Electron Microscopy (LCME) of the Federal University of Santa Catarina, Florianopolis, Santa Catarina, Brazil, for the use of their facilities and the Nuclear Magnetic Resonance Laboratory of Federal University of São Carlos. The first author holds a Master's scholarship from CAPES. Fernanda Ramlov holds a postdoctoral fellowship from CAPES. Marcelo Maraschin is a CNPq fellowship. This study is part of the MSc dissertation of the first author.

6 REFERENCES

Andrade LR, Leal RN, Noseda M , Duarte MER, Pereira MS, Mourão PAS, Farina M, Amado Filho GM (2010) Brown algae overproduce cell wall polysaccharides as a protection mechanism against the heavy metal toxicity. Marine Pollution Bulletin 60:1482–1488.

Chen JP & Yang L (2006) Study of a heavy metal biosorption onto raw and chemically modified *Sargassum* sp. via spectroscopic and modeling analysis. Langmuir 22:8906-8914.

Crowe TP, Thompson RC, Bray S and Hawkins SJ (2000) Impacts of anthropogenic stress on rocky intertidal communities. Journal of Ethnopharmacology **7:**273–297.

Edwards, P (1970) Illustrated guide to the seaweeds and seagress in the vicinity of Porto Aransas, Texas. Contribution of Marine Sciences Austin 15: 1-228.

Fagerberg, WR, Dawes, CJ (1977) Studies on *Sargassum* II. Quantitative ultrastructural changes in differentiated stipe cells during wound regeneration and regrowth Protoplasma, 92: 211-227.

Grande H, Reis M, Jacobucci GB (2012) Small-scale experimental contamination with diesel oil does not affect the recolonization of *Sargassum* (Fucales) fronds by vagile macrofauna. Zoologia 29:135–143.

Hamilton JG, Zangerl AR, DeLucia EH (2001) The carbon–nutrient balance hypothesis: its rise and fall. Ecology letters, 4:86-95.

Jeffrey SW, Humphrey GF (1975) New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. Biochemie und physiologie der pflanzen, 167:191-194.

Kuhnen S, Lemos PMM, Campestrini LH, Ogliari JB, Dias PF, Maraschin M, (2009) Antiangiogenic properties of carotenoids: a potential role of maize as functional food. Journal of functional foods 1(3): 284–290.

Le Lann K, Ferret C, VanMee E, Spagnol C, Lhuillery M, Payri C, Stiger-Pouvreau V (2012) Total phenolic, size-fractionated phenolics and fucoxanthin content of tropical Sargassaceae (Fucales, Phaeophyceae) from the South Pacific Ocean: Spatial and specific variability. Phycological Research 60**:** 37–50.

Liu H, Yang F, Zheng Y, Kang J, Qu J, Chen JP (2011) Improvement of metal adsorption onto chitosan/*Sargassum* sp. composite sorbent by an innovative ion-imprint technology. Water Research 45:145-154.

Lüder UH and Clayton MN (2004) Induction of phlorotannins in the brown macroalga *Ecklonia radiata* (Laminariales, Phaeophyta) in response to simulated herbivory—the first microscopic study. Planta 218:28–937.

Matanjun P, Mohamed S, Muhammad k, Mustapha NM (2010) Comparison of Cardiovascular Protective Effects of Tropical Seaweeds, *Kappaphycus alvarezii*, *Caulerpa lentillifera*, and *Sargassum polycystum*, on High-Cholesterol/High-Fat Diet in Rats. Journal of Medicinal Food 13(4): 792-800.

McCormick PV and Cairns Jr (1994) Algae as indicators of environmental change. Journal of Applied Phycology 6:509-526.

Megharaj M, Singleton I, McClure NC, Naidu R (2000) - Influence of Petroleum Hydrocarbon Contamination on Microalgae and Microbial Activities in a Long-Term Contaminated Soil. Arch. Environ. Contam. Toxicol. 38:439–445.

Mishra PK and Mukherji S (2012) Biosorption of diesel and lubricating oil on algal biomass. Biotech 2:301–310

Ouriques LC, Bouzon ZL (2000) Stellate chloroplast organization in asteronema breviarticulatum comb. Nov. (ectocarpales, phaeophyta). Phycologia, v. 39:267-271.

Pavia H and Toth G B (2000) Inducible Chemical Resistance to Herbivory in the Brown Seaweed *Ascophyllum nodosum.* Ecology, 81:3212–3225.

Pinto E, Sigaud-Kutner TCS, Leitão MAS, Okamoto, O.K., Morse, D, Colepicolo, P (2003). Heavy metal-induced oxidative stress in algae. Journal Phycology, 39:1008–1018.

Poppe F, Schmidt RAM, Hanelt D (2003) Effects of UV radiation on the ultrastruture of several red algae. Phycological Research, 51:11-19.

R Core Team: R: A language and environment for statistical computing, *R Foundation for Statistical Computing* 2013, Vienna, Austria, http://www.R-project.org

Ramlov F, Souza JC, Faria AVF, Maraschin M, PA Horta, Yokoya N S (2011) Growth and accumulation of carotenoids and nitrogen compounds in *Gracilaria domingensis*(Kütz.) Sonder ex Dickie (Gracilariales, Rhodophyta) cultured under different irradiance and nutrient levels. Brazilian Journal of Pharmacognosy 21:255-261.

Ramlov F, Carvalho TJG, Schmidt EC, Martins CDL, Kreusch MG, Rodrigues ERO, Bouzon ZL, Horta PA, Maraschin M (2013) Metabolic and Cellular Alterations Induced by Diesel Oil in *Hypnea musciformis* (Wulfen) J. V. Lamour. (Gigartinales, Rhodophyta). Journal of applied Phycology, doi: 10.1007/s10811-013-0209-y, accessed on 22/01/2014.

Raize O, Argaman Y, Yannai S (2004) Mechanisms of biosorption of different heavy metals by brown marine macroalgae. Biotechnology and Bioengineering 87:4-7.

Randhir R, Preethi S, Kalidas S (2002) L-DOPA and total phenolic stimulation in dark germinated fava bean in response to peptide and phytochemical elicitors. Process Biochemistry 37:1247-1256.

Reynolds, E. S. (1963) The use of lead citrate at light pH as an electron opaque stain
in electron microscopy. Journal Cell Biology 17: 208-212.

Ritter A, Goulitquer S, Salaun J-P, Tonon T, Correa JA, Potin P (2008) Copper stress induces biosynthesis of octadecanoid and eicosanoid oxygenated derivatives in the brown algal kelp *Laminaria digitata*. New Phytologist 180:809–821.

Rodrigues RV, Miranda-Filho KC, Gusmão EP, Moreira CB, Romano LA, Sampaio LA (2010) Deleterious effects of water-soluble fraction of petroleum, diesel and gasoline on marine pejerrey *Odontesthes argentinensis* larvae. Science of the total environment, 408:2054-2059.

Ryzhik IV (2012) The metabolic activity of cells of Fucus vesiculosus Linnaeus, 1753 (Phaeophyta: Fucales) from the Barents Sea under conditions of oil pollution. Russian Journal of Marine Biology 38: 96-100.

Schmidt EC, Scariot LA, Rover T, Bouzon, ZL (2009) Changes in ultrastructure and histochemistry of two red macroalgae strains of *Kappaphycus alvarezii* (Rhodophyta, Gigartinales), as a consequence of ultraviolet B radiation exposure. Micron 40: 860-869.

Schmidt ÉC, Nunes BG, Maraschin M and Bouzon ZL (2010) Effect of ultraviolet-B radiation on growth, photosynthetic pigments, and cell biology of *Kappaphycus alvarezii* (Rhodophyta, Gigartinales) macroalgae brown strain. Photosynthetica 48:161-172. .

Steinberg PD & van Altena I (1992) Tolerance of marine invertebrate herbivores to brown algal phlorotannins in temperate Australasia. Ecological Monographs, 62:189–222.

Stepaniyan OV (2008) Effects of crude oil on major functional characteristics of macroalgae of the Barents Sea. Russian Journal of Marine Biology 34:131-134.

Stepaniyan OV & VoskoboinikovGM (2006) Effect of oil and oil products on morphofunctional parameters of marine macrophytes. Russian Journal of Marine Biology, 32: S32–S39.

Torres M A, Barros MP, Campos SCG, Pinto E, Rajamani S, Sayre RT, Colepicolo P (2008) Biochemical biomarkers in algae and marine pollution: A review. Ecotoxicology and Environmental Safety 71: 1-15.

Vijayaraghavan, K, Teo TT, Balasubramanian R, Joshi UM (2009) Application of *Sargassum* biomass to remove heavy metal ions from synthetic multi-metal solutions and urban storm water runoff. Journal of Hazardous Materials, 164:1019–1023.

Vidotti, EC, Rollemberg MCE (2004) Algas: da Economia nos Ambientes Aquáticos à Bioremediação e a Química Analítica. Química Nova, 27:139-145.

Volesky, B (1994) Advances in biosorption of metals: Selection of biomass types. FEMS Microbiology Reviews 14:4291–302.

Voskoboinikov G M, Matishov GG, Bykov OD, Maslova TG, Sherstneva OA and Usov AI (2004) Resistance of marine macrophytes to oil Pollution. Doklady Biological Sciences 397:340–341.

Wrabel ML and Peckol P (2000) Efects of bioremediation on toxicity and chemical composition of Nº 2 fuel oil: growth responses of the brown alga *Fucus vesiculosus*. Marine Pollution Bulletin 40:135-139.

7 DISCUSSÃO E CONCLUSÕES GERAIS

A bem citada capacidade das algas, entre as quais as do gênero *Sargassum*, de adsorção de substâncias como óleos e metais pesados (WRABEL (2000); VIJAYARAGHAVAN (2009); WANG; CHEN (2009) e de complexar compostos como alguns níveis de hidrocarbonetos, está relacionada a sua conformação estrutural e fisiológica, uma vez que por estruturas como organelas e paredes celulares aciona mecanismos de defesa que vão de alterações morfológicas como espessamento de paredes, relatado em trabalhos como o de Andrade *et al.* (2010), com exposição de *Padina gymnospora* a metais pesados e de Ramlov *et al.* (2013), que submeteram a carragenófita *Hypnea musciformis* a diferentes concentrações de óleo diesel. Outro indício do ajuste morfofisiológico em algas são a dilatação de membranas, migração de corpos celulares, aumento e dilatação de organelas e até alternância no aumento da produção de elementos e compostos de importância estratégica na ativação de defesas, quando expostas a situações de estresse e riscos iminente.

Neste estudo em que *Sargassum cymosum* foi exposta a dois derivados de petróleo, óleo diesel e gasolina, foi possível observar algumas alterações comuns às duas condições, por vezes mais intensas em um combustível do que em outro. Exemplo disto ficou demonstrado nas análises bioquímicas, onde o padrão de aumento de compostos, neste caso clorofilas, e redução de outros, como os compostos fenólicos e até mesmo oscilação nos teores de carotenoides, foram eventos que repetiram-se à exposição da alga a ambos os derivados de petróleo. Tais ocorrências sugerem que as alterações bioquímicas seguem um padrão quando esta alga é submetida a risco de dano, como ocorre quando em contato com derivados de petróleo, i.e., óleo diesel e gasolina.

Embora as oscilações nos teores de carotenoides se repetissem aos primeiros tempos de exposição (30min e 1h) a óleo diesel e também a gasolina, acima desses tempos as respostas adotaram diferentes intensidades entre um tratamento e outro. Enquanto na exposição ao diesel as oscilações continuaram nos demais tempos de exposição (12h e 24h), com valores acima e abaixo dos teores verificados no controle, nas amostras expostas a gasolina as oscilações ocorreram com valores inferiores ao do controle, ou seja, sob contato por 12 horas ou mais com gasolina, a alga mostrou decréscimo mais acentuado de carotenoides, sugerindo efeito tóxico importante deste combustível sobre *S. cymosum* em períodos acima de 12 horas, mesmo a baixas concentrações.Os resultados de redução dos compostos detectados em RMN das amostras

com 24 horas de exposição ao óleo diesel, quando comparados ao controle, denotam abalo no metabolismo da alga com prejuízo desses compostos à medida em aumentam as concentrações de óleo diesel e, portanto, de risco.

A redução nos teores de fenólicos totais, sugerida em estudos prévios como indício de perda para o meio, repetiu-se em ambos os tratamentos com aparente redução mais acentuada dos fisoides nos tratamentos com gasolina, bem como maior vacuolação nesses tratamentos. O aumento de vacúolos e no número de plastoglóbulos, foram verificados, também, por Santos et al (2013), ao expor a agarófita *Gracilaria domingensis* a concentrações de cádmio. O aumento de vacúolos pode sugerir maior necessidade de compartimentalização de compostos nocivos a planta, como mais uma estratégia de proteção. O aumento de tamanho e ligeira alteração morfológica dos cloroplastos também ficaram mais evidenciados, através das visualizações em microscopia eletrônica de transmissão, nos tratamentos com gasolina. Quanto ao aumento nas concentrações de carbono e coincidente redução nos níveis de nitrogênio, detectadas por microanálise de raio-X nas amostras expostas aos dois combustíveis, quando comparadas ao controle, podem estar confirmando a hipótese do balanço carbono-nitrogênio, segundo a qual, a disponibilidade desses nutrientes controla as concentrações de metabólitos secundários em tecidos vegetais (HAMILTON *et al.* 2001). Já o aumento nos níveis de oxigênio à partir das mesmas análises, pode significar aumento da taxa respiratória, que ode corroborar com a alteração morfológica visualizada nos cloroplastos das amostras submetidas aos combustíveis.

8 CONSIDERAÇÕES FINAIS

Em resposta a hipótese proposta neste trabalho, diante dos resultados obtidos pode-se inferir que os derivados de petróleo, óleo diesel e gasolina causam alterações fisiológicas e ultraestruturais detectáveis na alga parda *Sargassum cymosum* var. *stenophyllum.* A série de métodos de análise usadas neste trabalho para investigação das alterações ocorridas em *S. cymosum*, reforçam a consistência dos resultados obtidos.

Os achados neste trabalho consistem em primeiros dados das alterações causadas na alga parda *Sargassum cymosum* var. *stenophyllum*, em clima subtropical, quando submetida a exposição aguda, *in vitro*, aos derivados de petróleo aqui estudados. E os indícios são de que esta espécie é uma potencial candidata a monitoramento de áreas contaminadas por óleo diesel ou gasolina.

AGRADECIMENTOS:

A CAPES pelo apoio financeiro através do Programa Nacional de Apoio e Desenvolvimento da Botânica (PNADB/2009) e aos Coordenadores do Projeto "*Avaliação dos impactos da urbanização sobre a biodiversidade marinha: uma análise sob a perspectiva do fitobentos*", Profª Fanly Fungyi Chow e Prof. Paulo Antunes Horta Junior, pela disponibilidade da bolsa de mestrado.

9 REFERÊNCIAS GERAIS

ALE, M.T.; MARUYAMA, A.T.; TAMAUCHI, H.; MIKKELSEN, J.D. ; MEYER, A.S. Fucoidan from *Sargassum* sp. and *Fucus vesiculosus* reduces cell viability of lung carcinoma and melanoma cells in vitro and activates natural killer cells in mice in vivo. **International Journal of Biological Macromolecules,**v.49, p. 331– 336, 2011.

ANDRADE L.R.; LEAL R.N.; NOSEDA, D. M.; E.R. Brown algae overproduce cell wall polysaccharides as a protection mechanism against the heavy metal toxicity. **Marine Pollution Bulletin,**v.60, p. 1482–1488, 2010.

BARROS, M.P.; PINTO, E., SIGAUD-KUTNER; T.C.S., CARDOZO, K.H.M. & COLEPICOLO, P. Rhythmicity and oxidative/nitrosative stress in algae. **Biological Rhythm Research**v. 36: 67-82, 2005.

BENENATI, F. - Keynote address: plants—keystone to risk assessment. **Plants for toxicity assessment. American Society for testing and material**, W.R. ASTM STP 1091, Philadelphia, pp. 5–13, 1990. google books - acessado em 02⁄01⁄2014 .

DUARTE, M.E.R.; CARDOSO, M.A.; NOSEDA, M.D. Structural studies on fucoidans from the brown seaweed *Sargassum stenophyllum*. **Carbohydrate Research** 333: 281–293, 2001.

EDWARDS, P 1970 Illustrated guide to the seaweeds and seagress in the vicinity of Porto Aransas, Texas. **Contribution of Marine Sciences Austin** 15: 1-228.

EKLUND, B.T.; KAUTSKY, L Review on toxicity testing with marine macroalgae and the need for method standardization—exemplified with copper and phenol. **Marine Pollution Bulletin,**46: 171-181, 2003

EL GAMAL A. A., - Biological importance of marine algae, Review article. **Saudi pharmaceutical journal**, 18: 1–25, 2010.

FAGERBERG, W. R.; DAWES, C. J. Studies on *Sargassum* II. Quantitative ultrastructural changes in differentiated stipe cells during wound regeneration and regrowth **Protoplasma**, 92 (3-4): 211-227, 1977.

FAROOQ , U.; KOZINSKI, J. A.; KHAN, M.A.; ATHAR, M. Biosorption of heavy metal ions using wheat based biosorbents – A review of the recent literature. **Bioresource Technology,** 101: 5043–5053, 2010.

HAMILTONJG, ZANGERL A.R.; DELUCIA EH The carbon–nutrient balance hypothesis: its rise

HANDY & DEPLEDGE Physiological responses: their measurement and use as environmental biomarkers and fall. **Ecology letters**, 4: 86-95, 2001.

HANDY & DEPLEDGE, 1999. Physiological Responses: Their Measurement and Use as Environmental Biomarkers in Ecotoxicology. **Ecotoxicology**, 8: 329-349, 1999.

JEFFREY, S.W., HUMPHREY, G.F.new Spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. **Biochemie und physiologie der pflanzen**, 167: 191-194, 1975.

KANG K, PARK Y, HWANG HJ, KIM SH, LEE JG, SHIN H-C Antioxidative properties of brown algae olyphenolics and their perspectives as chemopreventive agents against vascular risk factors. **Archives of Pharmacology Research**, v. 26, p. 286-293, 2003.

KUHNEN S, LEMOS PMM, CAMPESTRINI LH, OGLIARI JB, DIAS PF, MARASCHIN M - Antiangiogenic properties of carotenoids: a potential role of maize as functional food. **Journal of functional foods** 1(3): 284–290, 2009.

KUHNEN S, OGLIARI J.B.; DIAS P.F.; SANTOS M.S.; FERREIRA A.G.; BONHAM C.C.; WOOD, K.V.; MARASCHIN M: Metabolic fingerprint of Brazilian maize landraces silk (stigma/styles) using NMR spectroscopy and Chemometric Methods. **Journal of Agricultural and Food Chemistry 58**: 2194-2200, 2010.

MAFRA JR, L.L & CUNHA, S.R. - Bancos de *Sargassum cymosum* (Phaeophyceae) na enseada de Armação do Itapocoroy, Penha, SC:

biomassa e rendimento em alginato. **Brazilian Journal of Aquatic Science and Technology,** 6: 111-119, 2002.

MATANJUN P, MOHAMED S, MUHAMMAD K, MUSTAPHA NM, - comparison of cardiovascular protective effects of tropical seaweeds, *Kappaphycus alvarezii*, *Caulerpa lentillifera*, and *Sargassum polycystum*, on high-cholesterol/high-fat diet in rats. **Journal of medicinal food** 13(4): 792-800, 2010.

MEGHARAJ, M.; SINGLETON, N.C.; MCCLURE, N.R. Influence of petroleum hydrocarbon contamination on microalgae and microbial activities in a long-term contaminated soil. **Arch. Environment Contamination Toxicology**, 38: 439–445, 2000.

OURIQUES L.C.& BOUZON Z.L. Stellate chloroplast organization in Asteronema breviarticulatum comb. Nov. (Ectocarpales, Phaeophyta). **Phycologia**, v. 39:267-271, 2000.

PETROBRÁS DISTRIBUIDORA DE PRODUTOS AUTOMOTIVOS - disponível em <http://www.br.com.br/wps/portal/portalconteudo/produtos/automotivos/gasolina>. Acesso em 20 jan 2014.

PIETROLETTI, M.; CAPOBIANCHI, A.; RAGOSTA, E.; MECOZZI, M. Preliminary evaluation of hydrocarbon removal power of *Caulerpa racemosa* in seawater by means of infrared and visible spectroscopic measurements. **Spectrochimica Acta Part,**v. 77, p. 673-679, 2010.

RAMLOV, F. (2010) Variação sazonal dos carotenóides e compostos fenólicos e estudos fisiológicos em diferentes estádios reprodutivos de *Gracilaria domingensis* (Kütz.) Sonder ex Dickie (Gracilariales, Rhodophyta). Tese (Doutorado) **-- Instituto de Botânica da Secretaria de Estado do Meio Ambiente - Biodiversidade**, São Paulo-SP.

RAMLOV F, CARVALHO TJG, SCHMIDT EC, MARTINS CDL, KREUSCH MG, RODRIGUES ERO, BOUZON ZL, HORTA PA, MARASCHIN M (2013) Metabolic and cellular alterations induced by diesel oil in *Hypnea musciformis* (Wulfen) J. V. Lamour. (Gigartinales, Rhodophyta)**. Journal of applied Phycology,** doi: 10.1007/s10811-013-0209-y, accessed on 22/01/2014.

RANDHIR R, PREETHI S, KALIDAS S (2002) L-DOPA and total phenolic stimulation in dark germinated fava bean in response to peptide and phytochemical elicitors. **Process Biochemistry** 37: 1247-1256.

REVIERS, B. Biologia e Filogenia das Algas. ARTMED, Porto Alegre, 2006.

SCHERNER, F. ; RIUL, PABLO ; BOUZON, ZENILDA ; BLAKENSTEYN, A. ; PAGLIOSA, PAULO ROBERTO ; OLIVEIRA, EURICO C DE ; HORTA, P. A. Herbivory in a rhodolith bed: a structuring factor?. **Pan-American Journal of Aquatic Sciences**, v. 5, p. 358-366, 2010.

SCHMIDT, E.C.;SCARIOT, L.A.; ROVER, T.; BOUZON, Z.L. Changes in ultrastructure and histochemistry of two red macroalgae strains of *Kappaphycus alvarezii* (Rhodophyta, Gigartinales), as a consequence of ultraviolet B radiation exposure. **Micron,**v.40, v. 860-869, 2009.

STENGEL, D.B.; MACKEN, A.; MORRISON, L.; MORLEY, N. Zinc concentrations in marine macroalgae and a lichen from western Ireland in relation to phylogenetic grouping, habitat and morphology. **Marine Pollution Bulletin,v.** 48, p. 902–909, 2004.

STEPANYAN O.V.; VOSKOBOINIKOV, G.M Effect of oil and oil products on morphofunctional parameters of marine macrophytes. **Russian Journal of Marine Biology,** vol.32, p.32–39, 2006.

TORRES, M.A.; BARROS, M.P.; CAMPOS, S.C.G.; PINTO, E.; RAJAMANI, S.; SAYRE, R.T.;COLEPICOLO P. Biochemical biomarkers in algae and marine pollution: A REVIEW. **Ecotoxicology and Environmental Safety,** v 71, p. 1-308, 2008.

VIDOTTI, E.C.; ROLLEMBERG M., E. Algas: da economia nos ambientes aquáticos à bioremediação e a química analítica. **Química Nova**, v. 27, p. 39-145, 2004.

VIJAYARAGHAVAN, K.; TEO T.T.; BALASUBRAMANIAN R.; JOSHI U.M. Application of *Sargassum* biomass to remove heavy metal ions from synthetic multi-metal solutions and urban storm water runoff. **Journal of Hazardous Materials**,v.164p.1019–1023, 2008.

VILLARES, R.; REAL, C.; FERNÁNDEZ, J.A.; ABOAL, J.; CARBALLEIRA, A. Use of an environmental specimen bank for evaluating the impact of the Prestige oil spill on the levels of trace elements in two species of Fucus on the coast of Galicia (NW Spain). **Science of the Total Environment,** v.374, p.379-387**,** 2007.

VOLESKY, B Advances in biosorption of metals: selection of biomass types. FEMS. **Microbiology Reviews**,14:4291–302, 1994.

VOSKOBOINIKOV G M, MATISHOV GG, BYKOV OD, MASLOVA TG, SHERSTNEVA OA AND USOV AI Resistance of marine macrophytes to oil Pollution. **Doklady Biological Sciences** 397:340–341,2004.

YONESHIGUE-VALENTIN, Y; NASSAR, C.A.G.Aspectos populacionais de *Sargassum vulgare* C. Agardh (Ochrophyta, Fucales) na Ponta do Arpoador - Rio de Janeiro. **Oecologia Australis**, v. 12, p. 291-298, 2009.

WANG, J.; CHEN, C. Biosorbents for heavy metals removal and their future. **Biotechnology Advances,v.** 27, p. 195–226, 2009.

WRABEL M.L.; PECKOL P. Efects of bioremediation on toxicity and chemical Composition of No 2 fuel oil: growth responses of the brown alga *Fucus vesiculosus*. Marine Pollution Bulletin 40(2):135-139, 2000.

ZHAO X., DONG S.., WANG J.., LI F., CHEN A. , LI B. A comparative study of antithrombotic and antiplatelet activities of different fucoidans from *Laminaria japônica*. **Thrombosis Research,** 129: 771–778, 2011.