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Hypnea musciformis (Wulfen) J. V. Lamour. (Gigartinales, Rhodophyta) responses to gasoline short-term exposure: biochemical and cellular alterations

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ABSTRACT

Presence of toxic compounds in marine coastal waters has increased exponentially since Industrial Revolution. In this way, we aimed to evaluate biochemical and physiological changes occurring within *Hypnea musciformis* after short-term exposure to gasoline. *Hypnea musciformis* was cultivated without gasoline and then exposed to various concentrations of it (0.001 % - 1.0 %, v/v) for periods of 30 min, 1 h, 12 h and 24 h. A Pricncipal Compound Analysis of UV-vis spectral window (200-700 nm) was able to discriminate gasoline-exposed samples according to both exposure time and gasoline concentration. Changes in carotenoid profile composition were observed. Decreased carotenoid content was associated to gasoline exposure time, being lutein and *trans*-β-carotene the major compounds found. Higher gasoline concentrations negatively interfered with phenolic compounds accumulation. In addition, increased gasoline concentrations corresponded to decreased intracellular starch grains content as well as increased its deposition on cell wall external surface. Data obtained allow us to conclude that gasoline can damage *Hypnea musciformis* physiology and cell morphology. This is important, considering *Hypnea musciformis* carotenoids and phenolics are potential biomarkers of environmental stress investigated, as well as its increased cell wall thickness to avoid gasoline diffusion.

Keywords: biochemical stress, cellular organization, gasoline pollution, *Hypnea musciformis*, physiology

Introduction

Increase in human population and industrial development have led to an increase in contaminants in aquatic systems, which is causing a large impact in marine environments and benthic organisms such as seaweeds (Ballesteros *et al*. 2007; Orfanidis *et al*. 2007; Juanes *et al*. 2008; Bahartan *et al*. 2010; Littler *et al*. 2010; Martins *et al*. 2012). Some authors have been using seaweeds as both biomarkers and/or bioindicators for pollution, considering its tolerance or sensitivity to different pollutants (Castilla 1996; Vasquez & Guerra 1996; Owen *et al*. 2012; Anusha *et al*. 2017; Farias *et al*. 2018). Additionally, they have been used as bioindicators of organic micropollutants, as polycyclic

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aromatic hydrocarbons (PAHs), polychlorinated biphenyl (PBCs) and pesticides in contaminated areas (Montone *et al*. 2001; Schweikert & Burritt 2012). According to a review by Lewis & Pryor (2013), 85 microalgae and seaweeds species were studied concerning oil toxicity. Notably, previous studies supported importance of gasoline environmental impact, a petroleum derivative (Paixao *et al*. 2007; Torres *et al*. 2008).

Hydrocarbons in aquatic environments have major anthropogenic origins, such as accidental spills during supply vessels loading or unloading, and coastal zones environmental accidents. Differences in introductions levels are related to impacts intensity, volume, and duration over affected areas. These contaminants can cause adverse effects in marine environments, even when released in small amounts (Haynes & Johnson 2000; Pinto *et al*. 2003). Consequently, presence of petroleum products in marine systems and its ecotoxicological effects are relevant global issues.

Although effects of petroleum hydrocarbons and heavy metals have been extensively studied (O'Brien *et al*. 1976; Ansari *et al*. 2004; Stepanyana & Voskoboinikov 2006; Jarvis & Bielmyer-Fraser 2015; El-Shoubaky & Mohammad 2016; Pilatti *et al*. 2016; Costa *et al*. 2017, Sinaei *et al*. 2018), available data regarding gasoline effects in benthic macroalgae is surprisingly scarce. Gasoline is a complex hydrocarbons mixture containing from 4 to 12 carbon atoms (Oliveira *et al*. 2004; Wiedemann *et al*. 2005). Its toxicity levels are mostly related to low molecular weight of aromatic compounds, such as BTEX (Benzene, Toluene, Ethyl-benzene and Xylene) (Durako *et al*. 1993). Besides, there are other elements responsible for gasoline toxic effects. Among them, heavy metals are present in variable amounts or composition (Pulles *et al*. 2012) being responsible for organisms changes in metabolism and as well as its lower physiological resilience of (Hernandez-Almaraz *et al*. 2014).

In recent years, new approaches have been proposed for coastal areas biomonitoring. In this context, biochemical tools use has shown an interesting alternative for evaluation and proposal of new biomarkers. Seaweeds can synthesize a wide range of primary and secondary metabolites that promote resistance allowing them to survive even in drastically modified ecosystems. Measurement of parameters related to metabolic rates maintenance, such as photosynthesis and antioxidant defenses, are more accurate for diagnosing organism's damage, and can be used to predict damage extent for populations or communities. In recent years, characterization and measurement of chlorophylls, carotenoids (both total or profile content), phenolic compounds and even polysaccharides have been extremely relevant to understanding acclimation capacity and tolerance that allows benthic macroalgae to survive environmental changes resulting from chemical pollution, as observed in an oil spill context (Ramlov *et al*. 2014; Pilatti *et al*. 2016).

Gasoline exposure (Pilatti *et al*. 2016), as well as ultraviolet radiation (Polo *et al*. 2014), heavy metals (Santos *et al*. 2015; Schiavon *et al*. 2017) and diesel oil (Ramlov *et al*. 2014) may promote significant changes in photosynthetic parameters, in low molecular-weight compounds such as carotenoids an accessory pigments, and in cell wall constituents like phenolic compounds. In addition, analysis of morphological changes in response to abiotic stress provides another way to confirm results from biochemical studies as well as those provided by microscopy techniques. Studies evaluating seaweed xenobiotic effects observed development of several strategies in order to tolerate diesel oil and gasoline presence. According to Ramlov *et al*. (2014), longer exposure time (24 h) and concentration of diesel oil (1 %) decreased pigments concentration (chlorophyll and carotenoids), phenolic compounds, and starch grains content in *Hypnea musciformis* as a defense strategy against diesel oil penetration. However, *Ulva lactuca* increased soluble sugars and starch contents suggesting that *U. lactuca* is able to metabolize gasoline hydrocarbons and use them as an energy source. Another investigative tool experiencing a growth on its application is Fourier Transform Infrared Spectroscopy (FTIR). This technique is advantageous once only micro- to nanograms are required for a complete biochemical determination from a range of biological tissues, being a useful tool for algal prospection (Talari *et al*. 2017). Combination of FTIR and multivariate analysis presents an important tool in metabolic profiles discrimination from *Ulva lactuca* exposed to diesel oil and gasoline. Pilatti *et al* (2017) observed that spectral window related to protein absorbance (1700–1500 cm−1) enabled the best discrimination between gasoline-exposed samples regarding exposure time, and between diesel oil-exposed samples.

Worldwide, *Hypnea* species are important phycolloids sources, especially *kappa* carrageenan, holding significant economic value for Brazilian marine exploitation (Marinho-Soriano 2017). *Hypnea musciformis* is the best-known species from *Hypnea* genus, being reported from many tropical and subtropical shores. This species is widely distributed in southern Brazil near petroleum terminals, and therefore exposed to possible petroleum spills and its derivatives. Previous study with diesel oil, another petroleum derivative, showed a toxic effect and metabolic changes in *H. musciformis* seaweed in *in vitro* essays (Ramlov *et al*. 2014). Diesel oil composition is variable, according to source of crude oil and process of refining. Its main composition is a hydrocarbons mixture, as gasoline, but with prevailing 15 to 25 carbon atoms chains (Yasin *et al*. 2012). Both fuels (diesel and gasoline) have potentially toxic PAHs fractions for organisms. However, solubility and fractioning of those PAHs differs according to chemical properties and interaction with water. In addition, Yang *et al*. (1997) demonstrated that in water system, there are variations on temperature and pressure, making of PAHs dissociation

ability different among fuels, with higher solubility found for gasoline fractions. Considering that small molecules has an elevated mobility in aqueous systems, is expected that gasoline dissociated fractions, containing shorter carbon chains, even in low concentrations and in short-term exposure, present more toxic effects for organisms, when compared to diesel effects.

Considering its wide distribution, including both impacted and pristine areas, *H. musciformis* works as a pollution biomarker in Brazilian marine coastal areas, mainly for heavy metal biomonitoring (Bouzon *et al*. 2012; Santos *et al*. 2015). Even though, reports regarding gasoline biochemical damages on physiology of this specie still absent. For these reasons, necessity of characterization of metabolic deviations for a purpose of environmental biomarkers application emerges. Thus, this study aimed to evaluate impact of gasoline on biochemical responses as well as morphological changes of *H. musciformis*, improving metabolic deviations baseline from potential fuel contaminations on coastal zone. Our hypothesis is that *H. musciformis* responds with specific biochemical and morphological changes in its phenotype, even under short-term exposure to gasoline, once species is related to presents physiological harmful responses under other petroleum derivatives.

Materials and methods

Algal material

Hypnea musciformis (Wulfen) J.V. Lamouroux samples were collected at Ponta das Canas Beach (27°23'34"S 48°26'11"W), Florianópolis, Santa Catarina state, Southern Brazil, in October 2011 from subtidal rocks. Samples were transported at room temperature, in dark containers, to the laboratory where epiphytes cleaning and distilled water rinsing was performed. Sterilized seawater enriched with von Stosch's seawater enrichment medium (Edwards 1970) was used as culture medium. Cultures were incubated at 25 ± 2 °C under a photon flux density (PFD) of 80 ± 5 μmol photons m^{-2} s^{-1}, provided by cool-white fluorescent lamps with a 12 h photocycle (starting at 7 a.m.), with continuous aeration. PFD was measured with a quantum photometer (LI-185, Li-Cor Inc., Nebraska, USA) equipped with an underwater quantum sensor (LI-193 SA, Li-Cor Inc., Nebraska, USA). Culture medium renewal was performed weekly. Thalli segments were cultivated under the same conditions for 14 days (experimental acclimation period) before their use in gasoline oil experiments.

Experimental design

A certified quality control common gasoline sample was purchased from Petrobras® at a commercial gas station in Florianópolis and its chemical profile was determined by gas chromatography (GC, 2010-2, Shimadzu, Quioto, Japan), using a flame ionization detector. Gasoline sample presented a typical hydrocarbon composition, described by and Pilatti *et al*. (2016).

Ramlov *et al*. (2014) previously described experimental design. *H. musciformis* apical thalli portions were selected (2.0 g) and cultivated in 400 mL seawater for 30 min, 1 h, 12 h and 24 h with gasoline oil at concentrations (v/v) of 0.001 %, 0.01 %, 0.1 %, and 1 %. Experiments were carried out in growth chambers (347 CDG; Fanem, Sao Paulo, Brazil) at 25 ± 2 °C under PFD of 80 ± 5 μmol photons m^{-2} s^{-1}, provided by cool-white fluorescent lamps with a 12 h photocycle, and continuous aeration. Flask apertures were closed with plastic film, but not sealed, and the water aeration system was not closed. Experimental design not included an emulsifier, once we consider that this compound is not present in environment immediately after oil spill, and we focus on short-term responses to gasoline toxicity. All experimental procedures aimed to mimic real conditions found in a gasoline spill context in aquatic ecosystems. Control plants (no gasoline oil added) were cultivated under same conditions described above. Three replicates were made for each experimental group. At the end of experiment, algal biomasses were removed from flasks and immediately frozen in liquid nitrogen (LN), and kept at -80 °C for further analysis.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra from samples were collected on a Bruker IFS-55 (Model Opus. v 5.0, Bruker Biospin, Massachusetts, EUA) equipped with DGTS detector and single reflection system (45° angle of incidence), with total ATR reflectance attenuation accessory (golden gate). Spectra from five replicates *per* sample were collected in a 4000-400 cm^{-1} spectral window, with a 4 cm^{-1} resolution.

Spectra processing used Essential FTIR (v.1.50.282) software and considered the definition of spectral window of interest (3000-600 cm^{-1}), baseline correction, normalization and optimization of signal/noise ratio (smoothing).

Carotenoids and phenolic analysis

Carotenoids were extracted from samples (1.0 g fresh mass, n=3) using a hexane: acetone (1:1, v/v) solution containing 100 mg L^{-1} *tert*-butyl hydroxytoluene (BHT). Organosolvent extracts were filtered through a cellulose membrane in order to remove particles, followed by solvent removal under an N^2 flux. Dry residue was then solubilized in hexane (3 mL) and, prior to chromatographic analysis, methanol 10 % KOH (100 μL mL^{-1}) was added to 1 mL of organosolvent extract in order to obtain complete carotenoid saponification (3 h, dark, room temperature), which allowed better analyte identification by HPLC. Further,

this solution was triple washed with distilled-deionized water. Non-esterified extract was collected, concentrated (N^2 flux), and re-solubilized in hexane (100 μL) for further chromatographic analysis as previously described by Kuhnen *et al*. (2009). A concentrated sample (10 μL, n=3) was injected into liquid chromatograph (Shimadzu LC-10A, Quioto, Japan) equipped with a C^{18} reverse-phase column (Vydac 218TP54; 250 mm x 4.6 mm, O 5 μm, 30 oC), protected by a 5 μm C^{18} reverse-phase guard column (Vydac 218GK54) and a UV-visible detector (450 nm). Elution was performed with MeOH:CH3CN (90:10, v/v) at a flow rate of 1 $mL.min^{-1}$. Carotenoid identification (β-carotene, α-carotene, lutein, zeaxanthin, and β-cryptoxanthin) was performed using retention times and co-chromatography of standard compounds (Sigma-Aldrich, St. Louis, MO, USA), as well as by analogy with other reports of carotenoid analysis by RP-HPLC-UV-visible light under similar conditions (Scott & Eldridge 2005; Hulshof *et al*. 2007). Carotenoid quantification was based on standard curves, e.g., lutein standard curve (0.5 - 45 μg mL^{-1}; y= 7044x; r^2 = 0.999) for lutein, zeaxanthin and β-cryptoxanthin quantification and β-carotene standard curve (0.01-12 μg mL^{-1}; y = 1019x; r^2 = 0.998) for β- and α-carotene quantification.

For phenolic compounds analysis, 1.0 g samples (fresh weight, n=3) were extracted using 80 % methanol acidified with 1 % HCl, for 1 h, under agitation. Total phenolic contents were determined using Folin–Ciocalteau reagent (Sigma-Aldrich, St. Louis, USA) as previously described by Randhir *et al*. (2002). Absorbance was read at 725 nm and an external epigallocatechin gallate standard curve (Sigma-Aldrich, St. Louis, USA) (50 –1000 μg mL^{-1}; y = 0.0002x; r^2 = 0.999) was used for concentration determination purposes.

Microscopic analysis

For light microscopy (LM), control samples and treated individuals of *Hypnea musciformis* were fixed using a solution (v/v) of 2.5 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) overnight. Subsequently, we carried out an increasing series of ethanol aqueous solutions to dehydrate the samples. After dehydration, samples were infiltrated with Historesin (Leica Historesin, Heidelberg, Germany). Sections (5 μm of thickness) were stained with Periodic Acid-Schiff (PAS) used to identify neutral polysaccharides (Gahan 1984) and examined under an Epifluorescent (Olympus BX 41, Tokyo, Japan) microscope equipped with Image Q Capture Pro 5.1 Software (Qimaging Corporation, Austin, USA).

For scanning electron microscope (SEM) visualization, samples were fixed with a solution of 2.5 % glutaraldehyde (v/v) in 0.1 M sodium cacodylate buffer (pH 7.2) plus 0.2 M sucrose overnight (Santos *et al*. 2015). We used an ethanolic solution series to dehydrate samples in order to dry at Critical EM-CPD-030 point (Leica, Heidelberg, Germany). Samples (n=4, surface of thallus) were examined under SEM JSM 6390 LV (JEOL Ltd., Tokyo, Japan, at 10 kV).

Statistical analysis

Obtained data were summarized, and dataset homogeneity of variance was assessed using Levene's test. Factorial analysis of variance (ANOVA) was performed for carotenoids and total phenolic compounds, followed by Tukey *post-hoc* multiple comparison test ($p<0.05$) when appropriate (Zar 1999), using STATISTICA 7.0 software (StatSoft). We built descriptive models through unsupervised methods, i.e., principal component analysis. For UV-visible scanning spectrophotometry dataset (200 – 700 nm), were used MVSP (Kovcomp, UK) statistical packages and FTIR processed spectra using scripts written in R language (v. 3.1.1) and employing tools designed by authors at University of Minho (Portugal) and UFSC in Brazil (scripts used are freely available as a CRAN package named *specmine*).

Results

Unsupervised multivariate analysis

FTIR spectral profile analysis (Fig. 1) for studied treatments revealed the presence of several chemical constituents in 3000 cm^{-1} – 600 cm^{-1} regions. At this range, signals commonly associated to protein occurrence (1650 cm^{-1} and 1550cm^{-1}, related to primary amines), lipids (2800 cm^{-1}- 2900 cm^{-1}) and carbohydrates (1200 cm^{-1} - 950 cm^{-1}) were identified. FTIR spectra from control and gasoline treatments look very similar and this fact turns any discrimination analysis based on chemical traits into a hard task. Principal component analysis (PCA, Figs. 2, 3) was applied to FTIR dataset as a strategy to reduce data dimensionality without losing relevant information (Figs. 2A; 3A). Only a feeble discrimination of samples could be observed by plotting PCA as a function of gasoline concentration or exposure time for lipids, proteins, and the carbohydrates region (Figs. 2B-D, 3B-D). However, considering only proteins and carbohydrates region, control samples were separate from those exposed to gasoline (Figs. 2C-D; 3C-D).

As another data analysis possibility, carotenoid organosolvent extracts UV-visible scanning dataset was used for PCA analysis. Preliminary descriptive models were built by calculating principal components for whole dataset (200–700 nm) in order to build a descriptive model where relationships between *Hypnea musciformis* exposure time to gasoline treatments and its effects on metabolic profile could be detected. Scores plot (PC1 vs. PC2) containing 71.83 % of dataset variability revealed differences in chemical composition, mainly for *H. musciformis* samples cultivated for 12 h and 24 h in 1 % gasoline oil, which were separated in (PC1-, 2-)

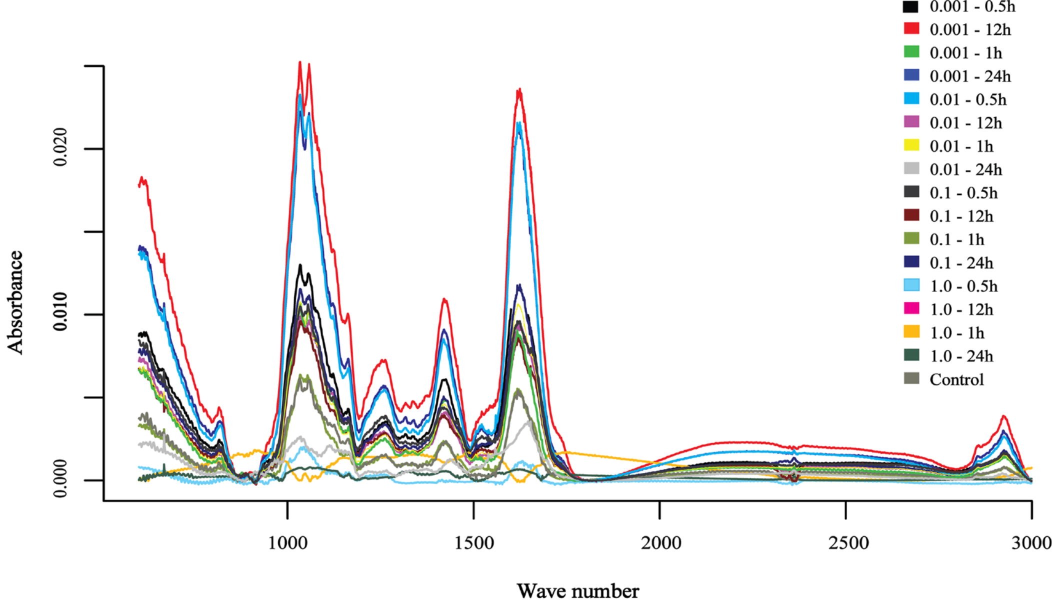

Figure 1. ATR-FTIR spectra in 3000–600cm^{-1} wavenumber region from *Hypnea musciformis* exposed to gasoline (0.001 % - 1 %, v/v) for 30 min, 1 h, 12 h, and 24 h.

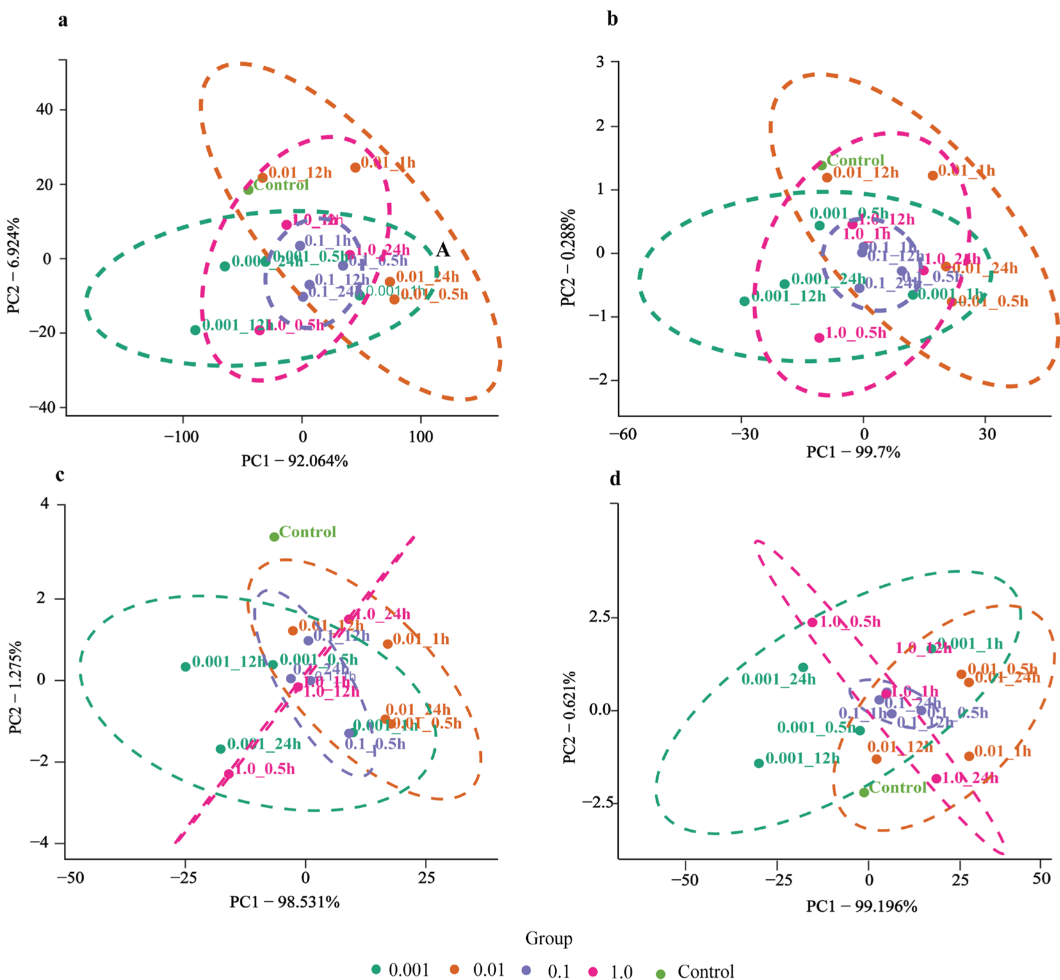

Figure 2. PCA scores scatter plot of ATR-FTIR dataset (3000–600 cm^{-1}) as gasoline concentration function. **A.** full spectrum; **B.** lipid region; **C.** protein region; **D.** carbohydrates region.

and (PC1+, PC2-) axes, respectively (Fig. 4). PC1 loadings reveal the major important wavenumbers, which explain the distinction of the previously found samples. Loadings indicated that 326 to 350 nm and 419 to 440 nm are possibly associated to observed discrimination. Although the whole spectra provide a good starting point for an exploratory data analysis, it is often necessary to analyze specific regions of the spectra to understand discrimination obtained by PCA. Groups formed in this exploratory data analysis led us to investigate details of carotenoids profile and phenolic compounds.

Biochemical analyses

Interaction effects between exposure time and gasoline concentrations in *Hypnea musciformis* were significant for total carotenoids content (Tab. 1). Shorter exposure times (30 min and 1 h) and treatments with 0.001 % to 0.1 % of gasoline for 12 h stimulated carotenoid accumulation in *H. musciformis* compared to control (Tab. 2). Interestingly, treated-samples with 1 % gasoline concentration for 12 h and for 24 h were more sensitive to stress imposed by gasoline (Tab. 2).

Carotenoid profile formed by xanthophylls lutein, zeaxanthin, and β-cryptoxanthin and carotenes, α-carotene, *trans*-β-carotene, and *cis*-β-carotene was detected in *Hypnea musciformis* biomass for both gasoline-treated samples and control (Tab. 2). Lutein and *trans*-β-carotene were the major

Table 1. Two-way ANOVA of total carotenoids and phenolic compounds of *Hypnea musciformis* exposed to gasoline (control, 0.001 % - 1 %, v/v) for 30 min, 1h, 12h and 24h.

Variable	Carotenoids*			Phenolic compunds		
	df	F	*p*-Value	df	F	*p*-Value
Concentration (C)	4	93.82	0.00	4	19.704	0.00
Time (T)	3	896.31	0.00	3	10.731	0.00
C x T	12	1603.40	0.00	12	2.52	0.02

*Value is the sum of carotenoids identified by HPLC.

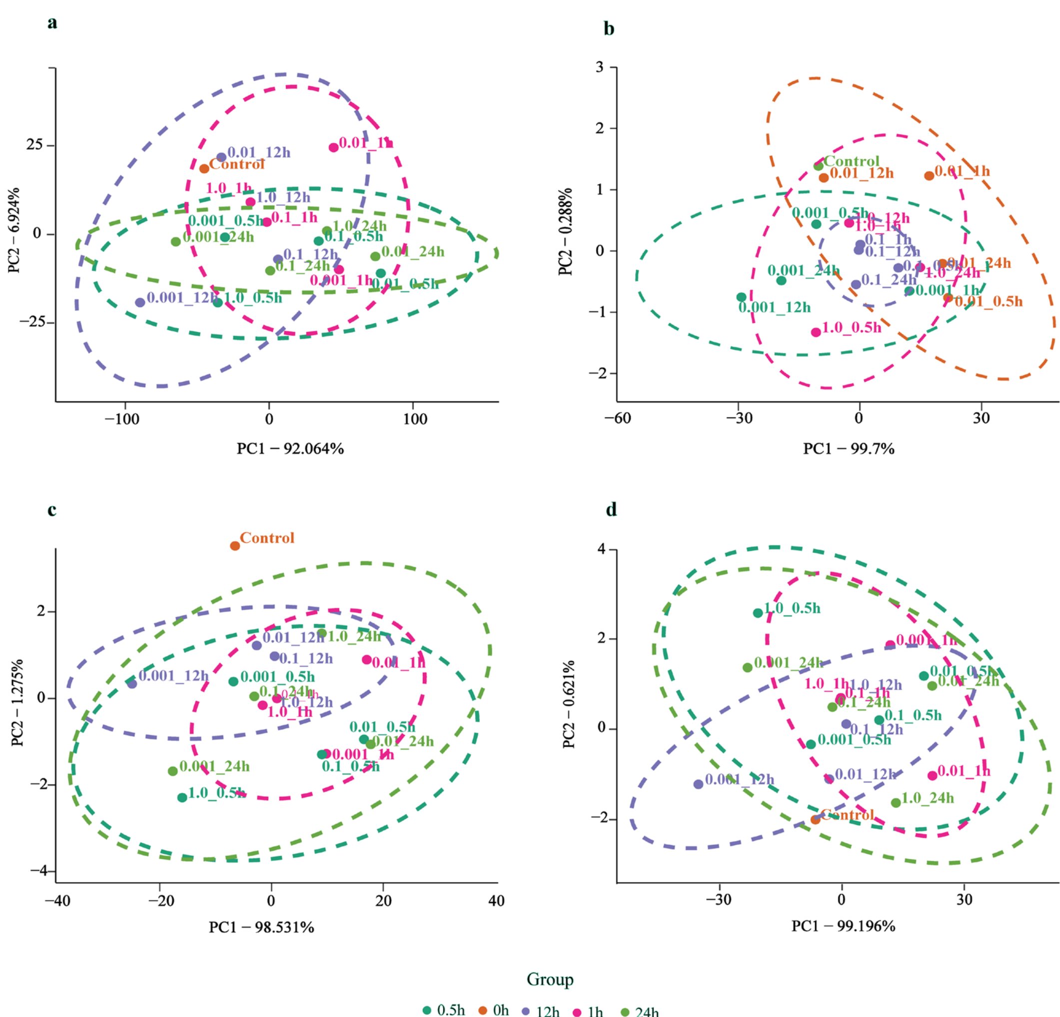

Figure 3. PCA scores scatter plot of ATR-FTIR dataset (3000 – 600 cm^{-1}) as gasoline time exposure. **A.** full spectrum; **B.** lipid region; **C.** protein region; **D.** carbohydrates region.

compounds found. Zeaxanthin, β-cryptoxanthin, α- and *cis*-β-carotene were accumulated only in lesser amounts (Tab. 2). Shorter exposure times (i.e., 30 min and 1 h) combined to higher gasoline concentration (i.e, 0.1 and 1 %) stimulated lutein and *trans*-β-carotene production (Tab. 2). On the other hand, *H. musciformis* cultivated at 24 h along the gasoline concentrations gradient showed a strong lutein content decrease, with a reduction, or no detection, of other carotenoid compounds (Tab. 2).

Bifactorial ANOVA analysis of phenolic compounds showed significant interaction between exposure time and gasoline concentration (Tab. 1). These compounds decreased in gasoline-treated samples, with larger phenolic compounds concentration decreases observed for both higher gasoline concentrations treatments and exposure time (12 and 24 h – 1 %) (Tab. 2).

Morphological analyses

Hypnea musciformis control samples stained with Periodic Acid-Schiff (PAS) exhibited a positive neutral polysaccharide reaction. These neutral polysaccharides, for its distribution and structural conformation, are an indicative of cellulose and other mucilaginous substances. Interestingly, PAS

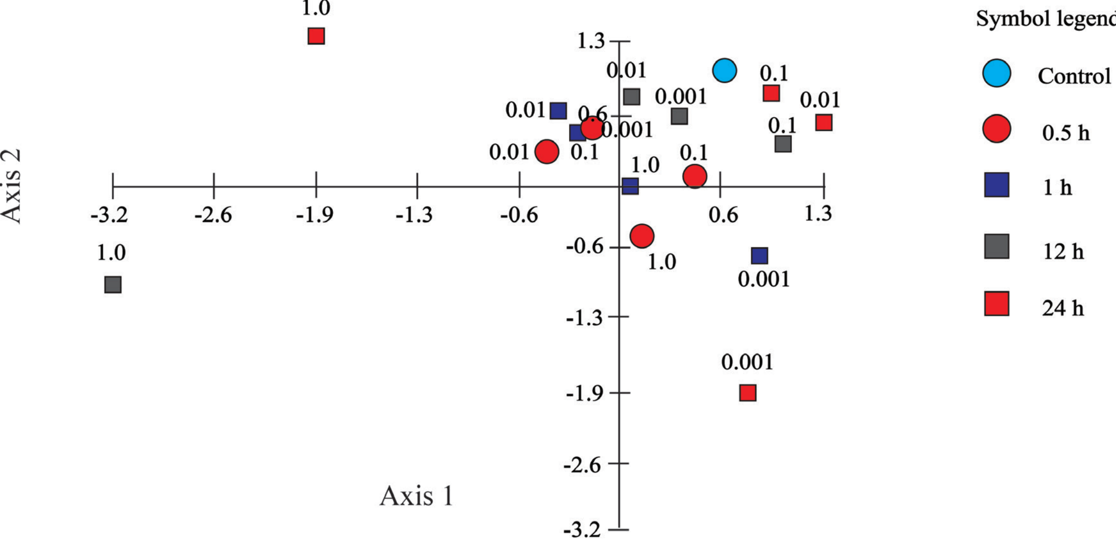

Figure 4. PCA scores scatter plot of UV-visible scanning spectrophotometry dataset (200 – 700 nm) from *Hypnea musciformis* exposed to gasoline (0.001 % - 1 %, v/v) for 30 min, 1 h, 12 h, and 24 h.

Table 2. Total carotenoids and total phenolic compounds (μg.g^{-1}, dry weight) contents of *Hypnea musciformis* exposed to gasoline (control, 0.001 % - 1 %, v/v) for 30 min, 1h, 12h and 24h. Values are means ± SD (n=3). Lowercase letters indicate significant differences among treatments (Tukey's *a posteriori* test, $p \leq 0.05$).

Treatments		Carotenoids*	Lutein	Zeaxanthin	Rt 7.4	Trans-β-carotene	Cis-β-carotene	α-carotene	Phenolic compound
Time	Gasoline concentration								
	Control	5.18±0.57fg	1.54±0.30	0.53±0.02	0.17±0.02	1.65±0.13	0.33±0.03	0.96±0.08	5.85±0.70abc
30'	0.001 %	9.02±0.36ef	2.58±0.16	0.40±0.03	0.33±0.01	2.98±0.10	0.59±0.04	2.13±0.08	6.76±1.78a
	0.01 %	12.20±0.17d	4.34±0.22	0.84±0.07	0.36±0.00	3.33±0.04	0.67±0.02	2.66±0.03	5.99±0.73ab
	0.1 %	8.17±0.56ef	2.48±014	0.34±0.09	0.27±0.02	2.63±0.23	0.52±0.04	1.93±0.17	4.34±0.66bc
	1 %	16.15±0.63ab	6.77±0.22	1.74±0.02	0.48±0.11	3.66±0.27	0.68±0.05	2.82±0.23	4.55±1.06abc
1h	0.001 %	17.82±0.62a	6.00±0.27	1.43±0.16	0.72±0.17	5.01±0.32	0.98±0.06	3.67±0.24	5.51±0.69abc
	0.01 %	9.91±0.69e	2.62±0.07	0.74±0.15	0.32±0.03	3.05±0.23	0.59±0.02	2.42±0.16	5.74±0.98abc
	0.1 %	14.76±0.49b	5.19±0.21	1.09±0.15	0.52±0.15	3.87±0.16	0.76±0.05	3.35±0.07	4.53±0.50bc
	1 %	17.25±1.10a	5.47±0.03	1.72±0.22	0.58±0.15	5.07±0.32	0.96±0.09	3.30±0.22	3.97±0.28c
12h	0.001 %	6.90±0.057f	2.41±0.17	0.72±0.16	0.25±0.01	1.76±0.15	0.15±0.32	1.43±0.12	5.76±0.67c
	0.01 %	10.54±1.04de	2.28±0.25	0.51±0.05	0.39±0.07	3.67±0.43	0.32±0.03	2.91±0.33	4.53±0.35bc
	0.1 %	10.55±0.84de	2.30±0.29	0.73±0.13	0.27±0.01	4.10±0.31	0.78±0.06	2.17±0.16	3.37±0.35bc
	1 %	0.83±0.12h	0.52±0.03	0.08±0.03	-	0.18±0.03	0.97±0.08	0.06±0.01	1.95±0.18d
24h	0.001 %	3.56±0.34g	1.25±0.20	0.42±0.02	0.12±0.01	1.03±0.12	-	0.56±0.07	5.34±0.18abc
	0.01 %	1.76±0.07h	0.58±0.08	0.06±0.03	-	0.56±0.03	0.19±0.03	0.58±0.02	6.09±0.49ab
	0.1 %	0.99±0.47h	0.60±0.24	0.11±0.01	-	0.20±0.00	-	0.13±0.11	4.00±0.21c
	1 %	0.43±0.03h	0.21±0.01	0.21±0.01	-	-	-	-	1.82±0.44d

*Value is the sum of carotenoids identified by HPLC.

showed a useful cytochemical staining for proposed analysis of cellular carbohydrates distribution. At cytoplasmic level, neutral polysaccharides represented by floridean starch grains were observed both in control (Fig. 5A) and gasoline-treated samples (Fig. 5B-M), and was possible to detect a variation in number and disposition of polysaccharides for gasoline treatments (Fig. 5B-M). For 1 % gasoline-treated samples (Fig. 5B, F, J), reduction in starch grains number through exposure time was observed (1 to 24h). Interestingly, in contrast, treatments with lower gasoline dilutions (0.001 %, 0.01 % and 0.1 %) showed a slow response to starch grains synthesizes and accumulation as well as an increase in cortical cells number was observed for these treatments. This reaction was observed especially for 0.01% (Fig. 5C, G, K) and 0.1 % (Fig. 5D, H, L).

SEM data showed *Hypnea musciformis* from control treatment had a regular cell walls surface, with no exudation evidence (Fig. 6A). On the other hand, gasoline-exposed samples presented evidence of intracellular deposition on cell wall extern surface (Fig. 6B-M). This effect showed more pronounced after 1 h exposure for all gasoline dilutions (Fig. 6B-E). As previously described and correlated to

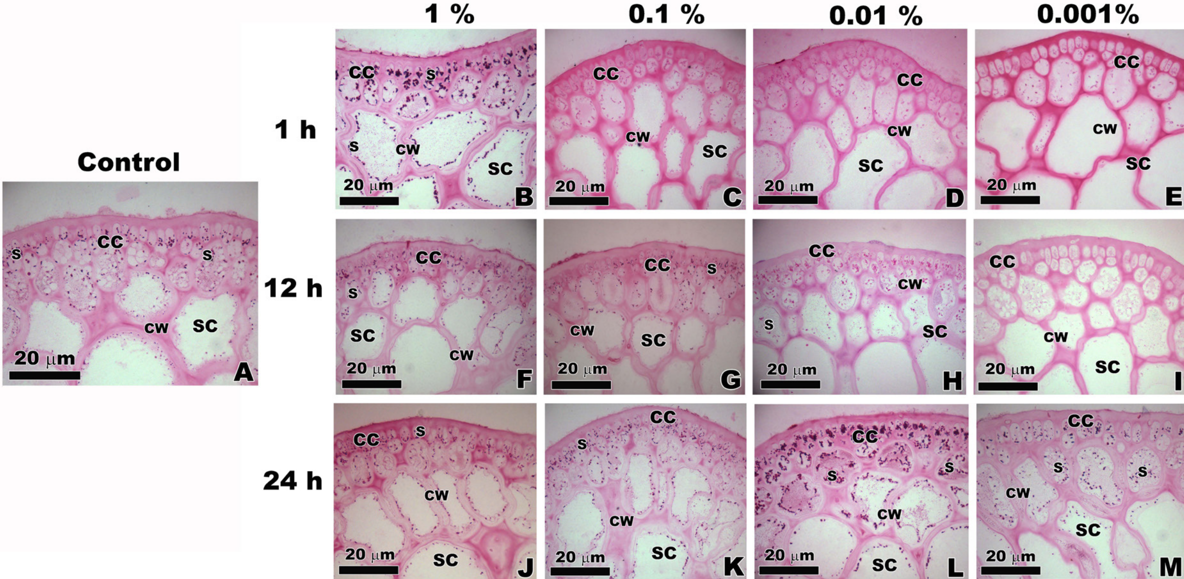

Figure 5. Light microscopy from *Hypnea musciformis* control transverse sections (**A**) and thallus exposed to different gasoline concentrations (0.001% - 1%, v/v) for 30 min, 1 h, 12 h, and 24 h (**B-M**). Sections were Periodic Acid-Schiff (PAS) stained (**A**). Observe PAS-positive reaction for floridean starch grains (S) on cytoplasm and positive reaction to cell walls (CW) polysaccharides. (**B-E**) Cortical and subcortical cells details. Note punctual floridean starch grains and no apparent cell walls thickness. (**F-M**) Note apparent increase on cortical and subcortical cells cell wall thickness from samples cultivated with gasoline during 12 h (F-I) and 24 h (J-M).

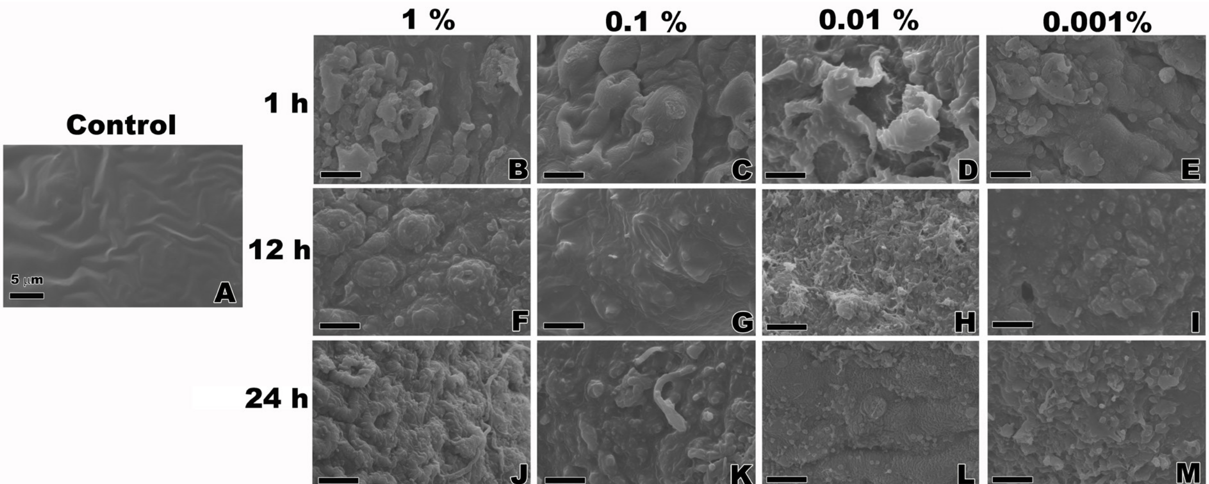

Figure 6. Thallus surface scanning electron microscopy (SEM) from *Hypnea musciformis* control (**A**) and exposed to different gasoline concentrations (0.001 % - 1 %, v/v) for 30 min, 1 h, 12 h, and 24 h (**B-M**). (**A**) Cortical cell walls surface topography detail from control showing a rough pattern. (**B-M**) Gasoline treated samples topography showing irregular surface following gasoline treatments.

PAS stained samples, is possible to suggest that cell wall surface deposition is major composed by polysaccharides like structural cellulose or even for carrageenan.

Discussion

According to our present results, *Hypnea musciformis* exposed to gasoline treatments showed different structural and metabolic responses associated with defensive mechanisms against gasoline toxicity. Even under short-term exposure, we detected and measured antioxidant compounds variation that demonstrates *H. musciformis'* ability to avoid oxidative stress at cellular level, and the efficient mechanism of synthesis for physical defense.

In this study, principal component analysis (PCA) was applied to FTIR and UV-visible light dataset as a strategy to reduce data dimensionality without losing relevant information. Nevertheless, a clear discrimination of samples was found only for UV-visible light dataset, which leads us to believe that primary metabolism was less affected by short-term exposure to gasoline than secondary metabolism. We observed that protein and carbohydrate regions from control samples presented certain differences when compared to treated samples. Although FTIR analyzes do not discriminate treatments, we can observe that there was a separation between treatments and control for proteins and carbohydrates region. Thus, we inferred FTIR as an important analytical tool for metabolic impact prospection in seaweeds exposed to environmental changes. Simonescu (2012) compiled data from growth and wide range of application of FTIR in environmental studies; analyses can be applied for identifying pollutant chemical characteristic (Simonescu 2012). We use FTIR and multivariate analysis to discriminate metabolic profiles of *Ulva lactuca* after *in vitro* exposure to diesel oil and gasoline. As a result, Pilatti *et al*. (2017) observed that both PCA and HCA performed on entire mid-infrared spectral window were able to discriminate diesel oil-exposed thalli from the gasoline-exposed ones. In addition to this, HCA performed in the spectral window related to protein absorbance (1700-1500 cm^{-1}) allowed the best discrimination between samples exposed to gasoline and diesel oil. Furthermore, FTIR has improving its application on algae biochemistry and physiology determination for its characterization as well as to identify a relationship between chemical profile and environmental conditions (Mecozzi *et al*. 2012; Ramsey *et al*. 2012; Shefer *et al*. 2017).

Samples could be clearly discriminated by UV-visible light reflecting mainly differences in secondary metabolism composition. Figure 4 shows the existence of two sample groups according to their similarities, where we observe a cluster of *H. musciformis* samples cultivated for 12 and 24 h with 1 % of gasoline. Loading values correlated with PC1 refer to 326 to 350 nm and 419 to 440 nm, which can be indicative of phenolic compounds and carotenoids, respectively. Due to results from this exploratory analysis, we chose these two classes of compounds to further investigate.

Despite of our biochemical results did not show a clear dose-dependent response for carotenoids, carotenoid content did seems to be related to gasoline exposure time. Total carotenoid content increased during 30 min and 1 h exposure when compared to control treatment, and decreased with increasing time exposure for 12 and 24 h. Moreover, *H. musciformis* carotenoid profile was also altered by the different gasoline treatments. Changes encompassed lutein and trans-β-carotene increase during 30 min and 1 h exposure, as well as a decrease in these compounds after 12 and 24 h-exposure. Carotenoids are recognized as cellular defenses against ROS, both by photooxidative protection (Baranska *et al*. 2013) and degenerative effects of pollutants such as heavy metals (Sytar *et al*. 2013). According to Frank & Cogdell (1996) and Havaux (1998), carotenoids are involved in protection mechanisms against injuries, as they stabilize and protect thylakoid membranes lipid phase, consequently contributing to preserve photosynthetic machinery functionality. We suggest that carotenoids increase or accumulation following 30 min and 1 h exposure acts as a defense mechanism against gasoline exposure. However, after 12 and 24 h exposure, total carotenoid content decreased significantly, particularly at 1 % gasoline concentration, which is a strong indicator of gasoline toxic effects correlated to exposure time. Carotenoid contents decrease related to exposure time was also observed for *U. lactuca* (Pilatti *et al*. 2016), suggesting carotenoids are sensitive to gasoline treatment exposure. We could also hypothesize that a decrease in carotenoid concentration is related to activation of another defense mechanism, via reallocation of resources for different antioxidant compounds synthesis.

Another metabolite evaluated in this study was total phenolic compounds. In contrast to results observed for total carotenoids, accumulation of phenolic compounds was dose-dependent. In general, phenolic compounds concentration decreased in all treatments compared to control with a more pronounced decrease for treatments at higher gasoline concentrations (0.1 and 1 %). This result suggests that treatments did not activate phenolic accumulation, but in opposite way, causes phenolic compound consumption or degradation as a cell defense antioxidant mechanism against reactive oxygen species (ROS). In parallel, Ramlov *et al*. (2014) reported an increase in carotenoids and phenolic compounds content when they exposed *H. musciformis* to diesel oil in the same conditions tested in present work. These previous effects, resultant from another petroleum derivative, reinforce the application the of metabolic deviation analyses methods to evaluate *H. musciformis* stress state in the presence of contaminants. Moreover, other authors have associated carotenoid and polyphenol contents increase to oxidative stress due to pollutants, such

as metals and hydrocarbons (Kumar *et al*. 2010; Yan & Zhou 2011; Schiavon *et al*. 2012). Finally, Pilatti *et al*. (2016) reported gasoline exposure led to a decrease in carotenoid and polyphenol content in *U. lactuca* L. and suggested that carotenoids and polyphenols of this species are sensitive to gasoline treatments even for a short-term exposure. With all this in mind, both carotenoids and polyphenols possibly are useful biomarkers for fuels pollution, as gasoline or diesel, being easily applied as measuring indicators in biomonitoring programs with *H. musciformis* and for *U. lactuca*.

Interestingly, these parameters showed a similar behavior at each time exposure but presented different variation ranges. While total carotenoids had a strong reduction in concentration for each time of exposure, phenolic compounds showed the same tendency, but less pronounced for lower gasoline doses. This fact reinforces our hypothesis that carotenoids were used as an energy source for phenolic synthesis. Another possible fate for carotenoids is chemical chelating on cytoplasm. Recent studies have pointed out that carotenoid fractions could act as chelator for ionic substances, with storage in vacuoles or exudation to the extracellular matrix (Britton 2008). Those considerations support our results and justify reductions at both fraction and total carotenoids contents. Nevertheless, phenolic compounds were not accumulated in high amounts, probably as primary biochemical defenses against gasoline toxicity. As previously described, phenolic compounds have chemical availability for ionic ligations, and its complex chemical-phenol exudation is a detoxification mechanism (Balboa *et al*. 2013). Thereby, our results demonstrated that both parameters are good indicators of metabolic injury and are related to early defensive mechanisms, able to avoid more extensive damage under gasoline short-term exposure.

Microscopy techniques applied here were effective in demonstrating morphological changes caused by short-term gasoline exposure. As observed for PAS-stained samples cytochemistry, an increase in starch grains number could be associated with *Hypnea musciformis* ability to synthesize polysaccharides for chelating and immobilizing toxic compounds present in gasoline. In turn, number variation through time indicates that polysaccharides could be exported to cell wall, in an exudation mechanism. Under SEM, morphological changes in *H. musciformis* cell wall surface were also observed. A change in mucilage or arrangement of cell walls polysaccharides that coats thallus was probably caused by gasoline exposure. This mischaracterization was probably caused by the interaction between gasoline components within negatively charged chemical groups in the cell wall polymers. Furthermore, gasoline might comprise high levels of BTEX hydrocarbons (Benzene, Toluene, Ethyl-benzene and Xylenes), which are small nonpolar molecules. They can diffuse through cell wall and cell membrane, displacing fatty acid molecules and cause membrane disruption. As demonstrated by Pilatti *et al*. (2016), the high ethanol content (~35 %, v/v) present in gasoline might contribute to the cell/organelle membranes disruption, due to its known destructive effect to proteins hydrophobic core, leading to its denaturation. Therefore, PAS stained samples corroborate physical mechanisms role against toxicity, once cell wall polysaccharides increasing is a mechanism to sequestrate and immobilize ions avoiding membrane permeability (Andrade *et al*. 2010; Bouzon *et al*. 2012).

One of the consequences of membrane disruption is cell sap leakage into intercellular space, resulting in cell turgor loss (Baker 1970). Besides, increased cell wall deposition could be related to our previous observations, in which matrix polysaccharides were exudate and accumulated on thallus surface, as a physical defensive mechanism to avoid gasoline internalization and/or of stressful compounds chelation by cell wall. In turn, Santos *et al*. (2015) reported floridean starch grains increase in *H. musciformis* in response to Cu and Pb exposure, as well as changes in cellular roughness patterns. Structural analyses demonstrated that *H. musciformis* morphological alterations could be associated with a class of chemical contamination. However, in all described cases, the species had physical ability to defend against stress factors to which it was exposed.

Considering results of this research together to related works (Pilatti *et al*. 2016; 2017), it is possible to infer that gasoline exposure effects are related to exposure time, while diesel oil effects are related to its concentration.

In general, our findings reinforce *Hypnea musciformis* susceptibility to damage caused by petroleum derivatives exposure. In our short-term experiments, analysis showed alterations in total carotenoids content and phenolic compounds as a response to gasoline stress. These compounds assigned as good candidates for environmental stress biomarkers, since their upper or down production are related to both concentration and gasoline exposure time. In addition, microscopy analysis revealed starch grains number increase and changes in mucilage or cell walls polysaccharides arrangement that coats thallus as a defense strategy against gasoline diffusion.

Conclusion

Gasoline is a stressor able to cause potential damage to *Hypnea musciformis* physiology and cell morphology. During short-term exposure, *H. musciformis* presented a relative increase in cell wall thickness to avoid gasoline diffusion and decreased carotenoid and polyphenol contents after 12 and 24 h exposure. As already observed by our research group, carotenoids and phenolic compounds accumulation monitoring might be an effective strategy to detect diesel oil and gasoline contamination in marine environments (Ramlov *et al*. 2014; Pilatti *et al*. 2016). These compounds showed variations among samples

exposed to diverse conditions of simulated gasoline spills contamination that possibly could occurs in environment. Especially for *H. musciformis*, we emphasize the potential use of biochemical metabolites monitoring as a biomarker of impacted environments by biofuels or gasoline, the need for further investigations, and its potential as a biomonitor of coastal systems, once it is cosmopolitan species with high interest for biotechnology uses and ecological restoration of locally degraded ecosystems.

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