



In vitro exposure of *Ulva lactuca* Linnaeus (Chlorophyta) to gasoline – Biochemical and morphological alterations



Fernanda Kokowicz Pilatti ^{a,*}, Fernanda Ramlov ^b, Eder Carlos Schmidt ^c,
Marianne Kreuzsch ^c, Débora Tomazi Pereira ^c, Christopher Costa ^d,
Eva Regina de Oliveira ^a, Cláudia M. Bauer ^a, Miguel Rocha ^d, Zenilda Laurita Bouzon ^c,
Marcelo Maraschin ^a

^a Plant Morphogenesis and Biochemistry Laboratory, Federal University of Santa Catarina, 88049-900 Florianópolis, Brazil

^b Phycology Laboratory, Department of Botany, Federal University of Santa Catarina, 88040-900 Florianópolis, Brazil

^c Department of Cell Biology, Embryology and Genetics, Federal University of Santa Catarina, 88049-900 Florianópolis, Brazil

^d Centre Biological Engineering, School of Engineering, University of Minho, Braga, Portugal

H I G H L I G H T S

- Effect of gasoline on the ultrastructure and metabolism of *Ulva lactuca* was studied.
- Changes in thalli mucilage and cell cytoplasm were noted after exposure to gasoline.
- Carotenoid and polyphenol contents decreased in gasoline-exposed thalli.
- Increase in soluble sugars and starch were positively correlated to time of exposure.

A R T I C L E I N F O

Article history:

Received 11 January 2016

Received in revised form

8 April 2016

Accepted 30 April 2016

Handling Editor: Caroline Gaus

Keywords:

Ulva lactuca

Gasoline

Cytochemistry

Scanning electron microscopy

Carbohydrates

A B S T R A C T

Refined fuels have considerable share of pollution of marine ecosystems. Gasoline is one of the most consumed fuel worldwide, but its effects on marine benthic primary producers are poorly investigated. In this study, *Ulva lactuca* was chosen as a biological model due to its cosmopolitan nature and tolerance to high levels and wide range of xenobiotics and our goal was to evaluate the effects of gasoline on ultrastructure and metabolism of that seaweed. The experimental design consisted of *in vitro* exposure of *U. lactuca* to four concentrations of gasoline (0.001%, 0.01%, 0.1%, and 1.0%, v/v) over 30 min, 1 h, 12 h, and 24 h, followed by cytochemical, SEM, and biochemical analysis. Increase in the number of cytoplasmic granules, loss of cell turgor, cytoplasmic shrinkage, and alterations in the mucilage were some of the ultrastructural alterations observed in thalli exposed to gasoline. Decrease in carotenoid and polyphenol contents, as well as increase of soluble sugars and starch contents were associated with the time of exposure to the xenobiotic. In combination, the results revealed important morphological and biochemical alterations in the phenotype of *U. lactuca* upon acute exposure to gasoline. This seaweed contain certain metabolites assigned as candidates to biomarkers of the environmental stress investigated and it is thought to be a promise species for usage in coastal ecosystems perturbation monitoring system. In addition, the findings suggest that *U. lactuca* is able to metabolize gasoline hydrocarbons and use them as energy source, acting as bioremediator of marine waters contaminated by petroleum derivatives.

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1. Introduction

Marine oil pollution has been receiving attention since the middle of the 19th century with the intensification of tanker operations and oil use, marine tanker collisions, pollutant release from coastal refineries, and continuous operative discharges from

* Corresponding author.

E-mail address: fernanda.kop@gmail.com (F.K. Pilatti).

ships (Torres et al., 2008). The ecological effects of these spills have been subject of considerable laboratory and field research, but determination of toxic concentrations of crude and dispersed oils for seaweeds have received relatively minor efforts and apparently less interest when compared to determining toxicities for faunal species, presumably due to the assumption that animals are more sensitive than plants. However, some studies and reviews have been showing that the sensitivity of organisms is species-specific and depends on the chemical substances used, as algae have found to be more sensitive than animal species in many cases (Eklund and Kautsky, 2003; Lewis and Pryor, 2013).

In marine hard bottom coastal regions, seaweeds form the base of the ecosystem, both as primary producers and as common structural components of the biotope, providing substrate for roe, shelter, and nursery areas for juvenile fishes (Eklund and Kautsky, 2003). Despite of its ecological and economical importance, the knowledge of the toxic effects of xenobiotics on this group of organisms is remarkably low. In their review, Eklund and Kautsky (2003) found that out of 120 compounds tested for toxicity in seaweeds, only 14% consisted on oil. Similarly, Lewis and Pryor (2013) counted less than 10% of entries for toxicity of oils and dispersants for 135 species of aquatic plants, of which 32 were seaweed species. According to these authors, there are no oil-specific standardized phytotoxicity tests, which results in diverse experimental conditions and parameters analyzed, making difficult to compare the results. Nevertheless, common findings in the reviewed papers (Coffey et al., 1977; Batterton et al., 1978; Vandermeulen et al., 1979; Gaur and Singh, 1989; Ansari et al., 1997; Tukaj et al., 1998) indicate that refined oils exhibit increased toxicity on algae than crude oil, especially on their early developmental stages, and toxicity is directly related to growth inhibition and photosynthetic rate decrease.

Although the environmental perturbations of large oil spills to marine waters has received the most attention by the public, regulatory and scientific communities, damages may occur from low level continuous discharges to fresh- and salt-water environments (Lewis and Pryor, 2013). In fact, annually, 48% of the oil pollution in the oceans is due to fuels and 29% to crude oil. Tanker accidents contribute only by 5% of all pollution entering into the sea (Torres et al., 2008). In Brazil, the distribution infrastructure for petroleum, petroleum derivatives, and ethyl alcohol includes inland terminals, waterway terminals, collector centers for ethanol, refineries, and several pipelines. In the inlands, fuel distribution terminals and countless tank trucks are filled daily with gasoline and diesel fuel for nation-wide distribution. Leaks and accidental spills are a regular occurrence during the exploration, production, refining, transport, and storage of petroleum and petroleum products (Vieira, 2007).

In this sense, the lack of knowledge resulting from the scarce information on the effects of fuel oils on seaweed species motivated this investigation. *Ulva lactuca* Linnaeus (Chlorophyta) was chosen as study model for its cosmopolitan occurrence, its tolerance to a wide variety of environmental stress factors, and its ability to survive in highly contaminated places, being a candidate to be a species useful for biomonitoring purposes (Rainbow, 1995). Gasoline was the fuel oil chosen for this study for its worldwide importance, being a complex blend of hydrocarbon compounds, including those known as BTEX (benzene, toluene, ethylbenzene, and xylene) and naphthalene, which make up from 10% to 59% of the fuel, and aliphatic hydrocarbons that make up from 41 to 62% (Tiburtius et al., 2004; Rodrigues et al., 2010). Thus, this study aimed to investigate the biochemical responses and morphological changes of *U. lactuca* exposed *in vitro* to gasoline. Our working hypothesis assumes that *U. lactuca* responds with specific biochemical and morphological changes in its phenotype even upon short exposure

times to the xenobiotic in study.

2. Material and methods

2.1. Algal material

Thalli of *Ulva lactuca* were collected in April 2013 at Armação Beach (27°49'96" S, 48°50'01" W), Florianópolis, SC, southern Brazil, packed in plastic flasks containing seawater and immediately transferred to the Plant Cell Biology Laboratory (LaBCEV-UFSC). Seaweed samples were rinsed with sterile seawater and manually cleaned up for epibionts removal. Culture conditions during acclimation and experimental periods consisted in sterile seawater enriched with 4 mL L⁻¹ (v/v) of von Stosch medium (VSES, Edwards, 1970), water continuous aeration, 24 ± 2 °C, illumination from above with fluorescent lights (Philips C-5 Super 84 16W/840), PAR at 80 μmol photons m⁻² s⁻¹ (Li-cor light meter 250, USA), and 12 h photocycle (starting at 8: 00 a.m.). For acclimation, algal biomass was cultivated in PET flasks (25 g biomass L⁻¹ of seawater) during 10 days.

2.2. Experimental design

A certified quality control common gasoline sample was purchased from Petrobras[®] at a commercial gas station in Florianópolis (Santa Catarina state, southern Brazil) and its chemical profile was determined by gas chromatography (GC, 2010-2, Shimadzu), using a flame ionization detector. The gasoline sample presented a typical hydrocarbon composition (Tables 1 and 2), with paraffinic hydrocarbons that include the alkane series, the naphthene hydrocarbons comprising the cycloalkanes and the aromatic ones covering all compounds that contain one or more ring structures similar to benzene, aside from ethanol (35.1%, v/v) whose addition to gasoline is regulated by Brazilian National Agency of Petroleum.

The experimental design was as previously described by Ramlov et al. (2013) and consisted in the combination of four concentrations of gasoline (0.001%, 0.01%, 0.1%, and 1.0% – v/v, nominal concentrations) and four times of exposure to the pollutant (30 min, 1 h, 12 h, and 24 h), resulting in a contingency table with 16 groups. Each group consisted in 5 replicates, i.e., 2 g of *U. lactuca* thalli cultivated in Erlenmeyer flasks containing the previously described nominal concentrations of gasoline and enriched seawater (described in section 2.1) at final volume of 400 mL. Flasks apertures were closed with plastic film, but not sealed, and water aeration system was not closed, aiming at to mimic the real conditions found in case of environmental pollution cause by gasoline in aquatic ecosystems. Therefore, gasoline compounds volatilization was not impeded and dissolved hydrocarbons were not measured over the experimental period. At the end of the experiment, algal biomasses were removed from the flasks and thalli surfaces were gently patted dry with paper towel. From each group, two portions of thalli (1 cm × 1 cm) were collected for microscopy procedures and the remaining biomass was aliquoted (1 g, FW) in

Table 1
Hydrocarbon and ethanol contents (% v/v) in Petrobras[®] common gasoline.

Compounds	Concentration (% v/v)
Ethanol (C ₂ H ₆ O)	35.1
Paraffinic hydrocarbons (C _n H _{2n+2})	9.7
Isoparaffinic hydrocarbons	18.6
Aromatic hydrocarbons (C _n H _{2n-6})	15.6
Naphtenic hydrocarbons	10.9
Olefinic hydrocarbons (C _n H _{2n})	6.4
Unidentified hydrocarbons	3.7

Table 2
Types of hydrocarbon categories (%) in Petrobras® common gasoline according to the number of carbon atoms in the molecule.

Number of carbons	Hydrocarbon categories				
	Paraffinic (%)	Isoparaffinic (%)	Aromatic (%)	Naphtenic (%)	Olefinic (%)
C4	0.14	0.04	–	–	0.05
C5	1.73	3.16	–	0.26	3.00
C6	2.26	3.94	0.56	3.63	1.52
C7	2.39	3.69	3.04	3.77	1.35
C8	1.52	4.84	3.52	2.15	0.34
C9	0.78	1.84	3.06	0.97	0.10
C10	0.32	1.08	4.80	0.12	–
C11	0.16	–	0.46	–	–
C12	0.21	–	0.16	–	–
C13	0.18	–	–	–	–
Total	9.70	18.60	15.60	10.90	6.40

packs, immediately frozen in liquid nitrogen (LN), and kept at -80°C for further analysis. For the control group, 10 g fresh thalli were separated in aliquots directly from the acclimation flasks, surface dried with paper towels, and frozen (-80°C).

2.3. Light microscopy and cytochemistry

Samples were processed and stained according to Schmidt et al. (2009). For that, thallus samples were fixed using a 2.5% paraformaldehyde (w/v) solution in 0.1 M phosphate buffer (pH 7.2), in a vacuum chamber, overnight, at 4°C . Subsequently, samples were washed in phosphate buffer, dehydrated in increasing series of ethanol aqueous solutions, pre-infiltrated with ethanol/Histoiresin (Leica Histoiresin, Heidelberg, Germany) solution (1: 1, v/v) for 4 h, and then infiltrated with 100% Histoiresin for 24 h. Samples were included in Histoiresin by adding the polymerizer at room temperature, for 2–3 h. Thallus sections (5 μm thickness) were stained with Toluidine Blue (TB-O) 0.5% (w/v) (Merck Darmstadt, Germany), pH 3.0, for acidic polysaccharides detection and Coomassie Brilliant Blue (CBB) 0.4% (w/v) in Clarke's solution (Serva, Heidelberg, Germany) for proteins. Slides were cemented with Canada balsam and investigated with an Epifluorescent microscope (Olympus BX 41) equipped with the Image Q Capture Pro 5.1 Software (Qimaging Corporation, Austin, TX, USA).

2.4. Scanning electronic microscopy (SEM)

Samples underwent fixation and dehydration steps as described in section 2.3. Subsequently, they were dried with hexamethyldisilazane, transferred to aluminum stubs, coated with gold, and analyzed under SEM JSM 6390 LV (JEOL Ltd., Tokyo, Japan) at 10 kV.

2.5. Biochemical analyses

2.5.1. Desalination and grinding

Prior to analysis, the whole biomass was desalinated with 0.5 M ammonium formate for 30 s, profusely rinsed with distilled water, and gently dried with paper towels. For extractions, algal biomass was ground using mortar, pestle, and liquid nitrogen.

2.5.2. Chlorophylls a and b

Chlorophylls were extracted according to Hiscox and Israelstam (1979), with modifications. Fresh thalli (100 mg) were added to 10 mL dimethyl sulfoxide (DMSO) and incubated for 30 min, at 40°C . Absorbances of extracts were measured at 645 nm and 663 nm ($n = 3$) in a UV–visible spectrophotometer (Spectrumlab D180) and concentration of chlorophylls was calculated according to Arnon (1949).

2.5.3. Carotenoids

Carotenoids were extracted from fresh thalli (1 g) using 10 mL of hexane: acetone (1: 1, v/v) solution containing *tert*-butyl-hydroxytoluene (BHT) 0.001% (w/v), for 1 h (Ramlov et al., 2013). Extracts were filtered and solvent was evaporated under N_2 flux. To improve identification of the analytes by HPLC, saponification of carotenoids was performed prior to chromatographic analysis. The dry residue was solubilized in 3 mL hexane prior to chromatographic analysis and 10% KOH in methanol was added ($100\ \mu\text{L}\ \text{mL}^{-1}$) to the organosolvent extract, followed by incubation for 3 h, in the dark, at room temperature. The solution was washed (3x) with distilled-deionized water, the non-esterified extract collected, concentrated under N_2 flux, and re-solubilized in hexane ($100\ \mu\text{L}$) for chromatographic analysis as previously described (Ramlov et al., 2013). Samples ($10\ \mu\text{L}$, $n = 3$) were injected into liquid chromatograph (Shimadzu LC-10A) equipped with a C18 reverse-phase column (Vydac 218TP54; $250\ \text{mm} \times 4.6\ \text{mm}\ \varnothing$, $5\ \mu\text{m}$ particle, 30°C), protected by a $5\ \mu\text{m}$ C18 reverse-phase guard column (Vydac 218GK54), and a UV–visible detector (450 nm). Elution was performed with $\text{MeOH}:\text{CH}_3\text{CN}$ (90: 10, v/v) at a flow rate of $1\ \text{mL}\ \text{min}^{-1}$. Carotenoid identification (α -carotene, β -carotene, lutein, zeaxanthin, and β -cryptoxanthin) was performed using retention times and co-chromatography of standard compounds (Sigma-Aldrich, USA), as well as by analogy with other reports of carotenoid analysis under similar conditions (Scott and Eldridge, 2005; Hulshof et al., 2007). Carotenoid contents were calculated based on external standard curves, e.g., lutein standard curve ($0.5\text{--}45\ \mu\text{g}\ \text{mL}^{-1}$; $y = 7044x$; $r^2 = 0.999$) for lutein, zeaxanthin, and β -cryptoxanthin and β -carotene standard curve ($0.01\text{--}12\ \mu\text{g}\ \text{L}^{-1}$; $y = 1019x$; $r^2 = 0.998$) for α - and β -carotene quantification.

2.5.4. Polyphenols

Polyphenols were extracted from 1 g powdered fresh thalli by incubating samples for 1 h in 10 mL methanol (80%, v/v). The extract was recovered by filtration on cellulose paper under reduced pressure and polyphenols were determined according to Schiavon et al. (2012), with modifications. $100\ \mu\text{L}$ of methanolic extracts were added to $75\ \mu\text{L}$ Folin-Ciocalteu reagent, $825\ \mu\text{L}$ 2% sodium carbonate (w/v), and incubated for 1 h, in the dark. Absorbance was measured at 750 nm ($n = 3$) in a microplate reader (ThermoPlate TP-Reader) and concentration of polyphenols was calculated using an external gallic acid standard curve ($50\text{--}500\ \mu\text{g}\ \text{mL}^{-1}$; $y = 0.0044x$; $r^2 = 0.999$).

2.5.5. Soluble sugars and starch

Soluble sugars were extracted from fresh thalli (50 mg) with methanol: chloroform: water solution (12: 5: 3, v/v/v) and starch was extracted with perchloric acid 30% (v/v) from the pellet resultant of the soluble sugars extraction, as described by Trevelyan

and Harrison (1952). Determination of soluble sugars and starch concentrations in extracts was performed through the colorimetric anthrone reaction (0.2% anthrone in sulfuric acid, w/v) and absorbance was recorded at 630 nm ($n = 6$) in a UV–visible spectrophotometer (Spectrumlab D180). Concentrations of the analytes were calculated using external standard curves for glucose (10–200 $\mu\text{g mL}^{-1}$; $y = 0.0088x$; $r^2 = 0.998$) and starch (0.5–5 mg mL^{-1} ; $y = 0.34x$; $r^2 = 0.998$).

2.6. Statistical analysis

One-way and multifactorial analysis of variance (ANOVA) followed by the *post hoc* Tukey test and Pearson's correlation test were performed on the biochemical data, considering the confidence level of 95% ($p \leq 0.05$). Further, data was normalized and tested for principal component analysis (PCA) and hierarchical clustering analysis (HCA). All statistical analysis and graphics design were carried out using scripts written in R language (v. 3.1.1) employing tools designed by the authors at the University of Minho (Portugal) and UFSC in Brazil (the scripts used are freely available as a CRAN package named *specmine*), as well as the functions from the packages “devtools” (Wickham and Chang, 2015), “agricolae” (Mendiburu, 2014), and “ggplot2” (Wickham, 2009). The data and the HTML reports generated from the analysis (using R Markdown features) are provided in [supplementary material](#), making the analysis fully reproducible.

3. Results and discussion

The hydrocarbons commonly present in petroleum and their derivatives are paraffins (15%–60%), naphthenes (30%–60%), and aromatics (3%–30%), with asphaltics making up the remainder. However, one could note that the percentages of hydrocarbon types in petroleum derivatives (gasoline, diesel oil, kerosene, e.g.) can vary greatly, giving them a quite distinct compound profile depending upon geographic origin of the petroleum and the refinery processes used, for instance. In this sense, in view of the eventual chemical heterogeneity of gasoline marketed all over the world, caution should be taken when analyzing comparatively the pollutant impact of this xenobiotic in aquatic systems. Moreover, in this regard it is worth mentioning the peculiar trait of adding ethanol up to 35% (v/v) to gasoline in Brazil, a practice regulated by governmental agencies.

3.1. Morphological alterations

In the pursuit for cellular injuries caused by acute exposure of *U. lactuca* thalli to gasoline, we started examining cell morphology using cytochemistry techniques and scanning electronic microscopy (SEM). Toluidine Blue (TB-O) staining showed metachromatic reaction to the cell wall resulting in purple color in control and treated cells (Fig. 1a–m). This reaction indicates the presence of acidic polysaccharides such as ulvan. Metachromatic reaction was also observed in granules dispersed throughout the cytoplasm. This cytochemical test made also possible to detect cytoplasmic retraction, specially where the two layers of cells join together (Fig. 1b–m). When stained with Coomassie Brilliant Blue (CBB), protein structures of cytoplasm were noted, as well as a thin outer layer on the cell wall (Fig. 2a–m). In control samples (Fig. 2a), the cytoplasm was well distributed around the vacuole, while cells exposed to gasoline (Fig. 2b–m) exhibited their cytoplasm agglutinated. SEM analysis of the thalli's surface in the control sample (Fig. 3a) exhibited well-marked contours of the cell wall and turgid cytoplasm. In their turn, the samples exposed to gasoline (Fig. 3b–m) presented drastic changes in the mucilage deposited

on the cell surface. Besides, cell wall contours were lost and the surface was found to be wrinkly in some samples (Fig. 3b, d, f–k) and smooth in others (Fig. 3c, e, l, m).

As the first and main physical barrier against mechanical and chemical threats, seaweed mucilage is expected to readily exhibit evidences of disturbances in the surroundings. Even a short period of exposure (1 h) to the smallest concentration of gasoline tested (0.001%) was able to produce dramatic changes in *U. lactuca*'s surface when compared to control sample. Gasoline might comprise high levels of BTEX, which are small apolar molecules that can easily diffuse through cell wall and cell membrane, displacing fatty acid molecules and causing membrane disruption. One of the consequences of membrane disruption is the leakage of cell-sap into intercellular space, resulting in the loss of cell turgor (Baker, 1970). Taking into account the amount of BTEX found in the studied gasoline (15.6%, v/v), this finding is thought to be one of the causes for the alteration observed in *U. lactuca*'s mucilage by SEM images in samples exposed to gasoline, as cytoplasm retraction was detected in cytochemical analysis as well. Despite the fact that benzene is by far the most harmful compound in gasoline, it has been claimed that due to low background concentrations of BTEX in water, their rapid volatilization and degradation processes and low to moderate toxicity, the overall risk to the aquatic environment is considered low.

On the other hand, one cannot rule out the possibility that changes observed by SEM in the mucilage after exposure to gasoline result from the adsorption of hydrocarbons to the polysaccharides of the cell wall, or to the augment of accumulation of those macromolecules as a physical barrier against the penetration of gasoline into the cells. Both effects were observed *in situ* in many seaweed species after oil spills (O'Brien and Dixon, 1976). As shown herein, the hypothesis of increased accumulation of cell wall's polysaccharides as a protection mechanism is reinforced by the observation of metachromatic granules in TB-O cytochemical analysis. The metachromatic reaction observed in the granules is possibly due to the synthesis and exportation of polysaccharides to the cell wall, as suggested by Schmidt et al. (2012). Although the algae might be enhancing the mucilage production and accumulation in the cell wall as a physical protection, it is still plausible to assume that gasoline is able to penetrate cells and disrupt their structures. First evidences for this are found in CBB cytochemical analysis, which stains proteins present in organelles membranes. In control samples, CBB staining marked protein structures in the cytoplasm and their shapes surrounding the vacuole are visible. Interestingly, cytoplasm of cells exposed to gasoline became shapeless and cells agglutinated after increasing time of exposure and concentration of the pollutant. Importantly, one should keep in mind the high content of ethanol (~35% v/v) of the studied gasoline, which might also contribute to disrupt the cell/organelle membranes due to its well known destructive effect of the hydrophobic core of proteins and of the hydrogen bonding pattern of those macromolecules, leading to their denaturation.

3.2. Biochemical analysis

The contents of total chlorophylls (chlorophyll *a* + chlorophyll *b*), total carotenoids, polyphenols, soluble sugars, and starch are plotted in Fig. 4a–e. Moreover, variables means, standard deviation and Tukey test results by group are displayed in Table 1 of [supplementary material](#). Tables 3 and 4 contain the results of one-way ANOVA by factor (time of exposure and concentration) and multifactorial ANOVA, respectively. Table 5 shows the results of Pearson's correlation between biochemical variables and factors. Finally, carotenoid profiles obtained by RP-HPLC are presented in Table 2 of [supplementary material](#) with Tukey test results.

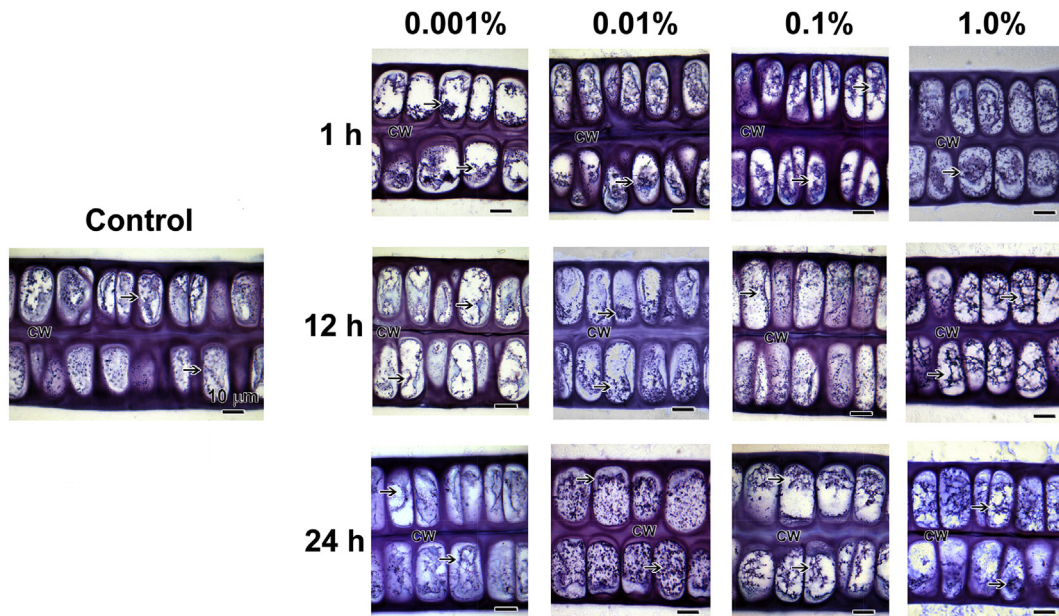


Fig. 1. Light microscopy of cross sections of both cell layers of control (a) and gasoline-treated (b–m) *U. lactuca* thallus stained with TB-O. CW = cell wall. Arrows indicate small metachromatic granules evenly spread in cytoplasm (a) and metachromatic clusters in shrunk cytoplasm (b–m). Scale bars = 10 μ m.

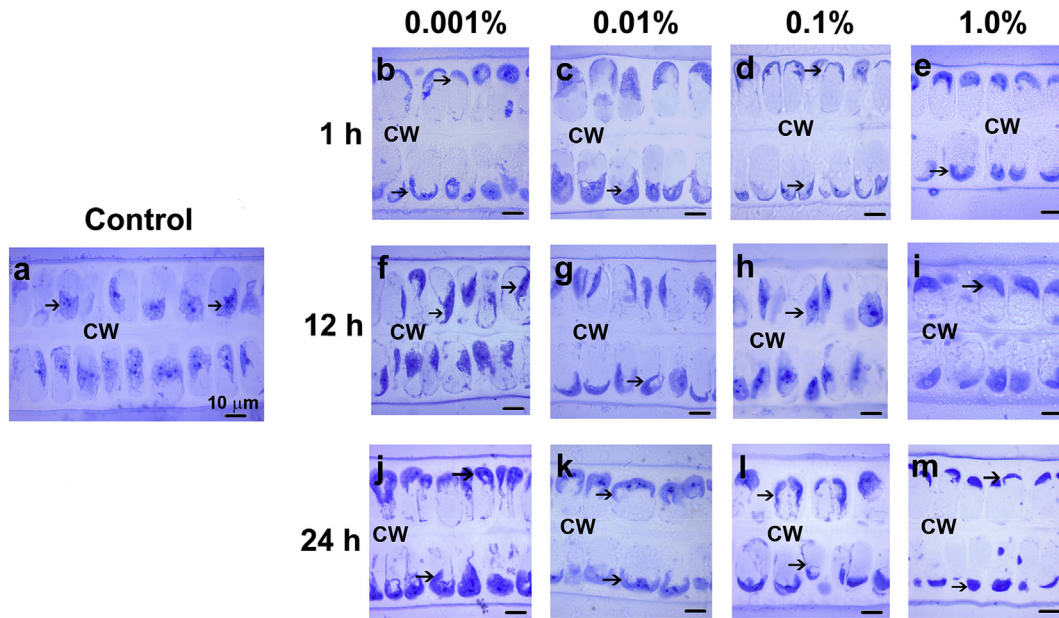


Fig. 2. Light microscopy of cross sections of both cell layers of control (a) and gasoline-treated (b–m) *U. lactuca* thallus stained with CBB. CW = cell wall. Arrows indicate cytoplasm well distributed around the vacuole (a) and warped, agglutinated cytoplasm (b–m). Scale bars = 10 μ m.

3.2.1. Secondary metabolites responses

Along with cell ultrastructural changes, biochemical responses have also been the target of investigations regarding the effect of xenobiotics on aquatic organisms. In plants and algae, the amounts of photosynthetic pigments are the main biochemical variables analyzed due to their vital importance for these organisms, together with secondary metabolites responsible for cell defense; polyphenols being usually the most important group in this role. In our study, chlorophylls *a* and *b* and phenolic contents have been determined, as well the carotenoid profile by liquid chromatography.

One-way ANOVA with *post hoc* Tukey test (Table 1, supplementary material) indicated differences ($p \leq 0.05$) between the control and gasoline-treated groups as to their amounts of chlorophyll *a* and *b*, as well as their summation. Chlorophyll *a* and *b* were then tested for Pearson's correlation and the result indicated that these pigments are highly correlated to each other ($r = 0.99$, p -value ≤ 0.05). Thus, only their summation (total chlorophylls) was used in further statistical analysis. The amounts found are shown in Fig. 4(a). Results of one-way ANOVA with *post hoc* Tukey test of samples grouped by time of exposure and by concentration (Table 3) were not significant ($p \leq 0.05$) and negative and low

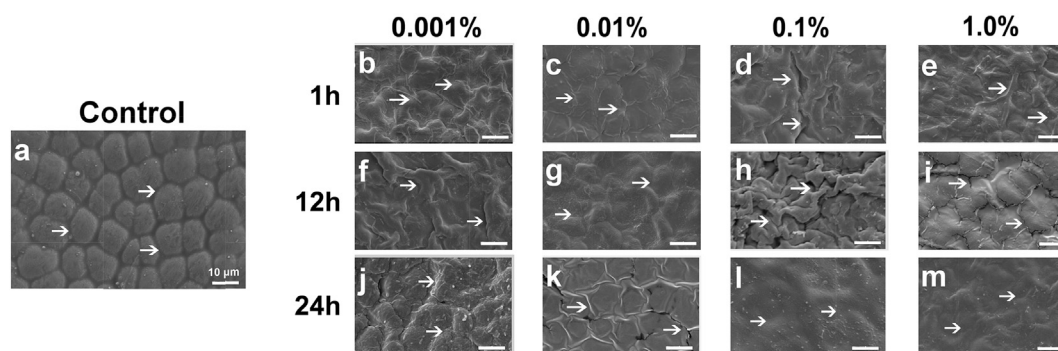


Fig. 3. Scanning electron microscopy (SEM) images of control (a) and gasoline-treated (b–m) *U. lactuca* thallus surface. Arrows indicate contours of the cell wall and turgid cytoplasm in control (a) and wrinkles, cracks, and smoothing in gasoline-treated thallus surface (b–m).

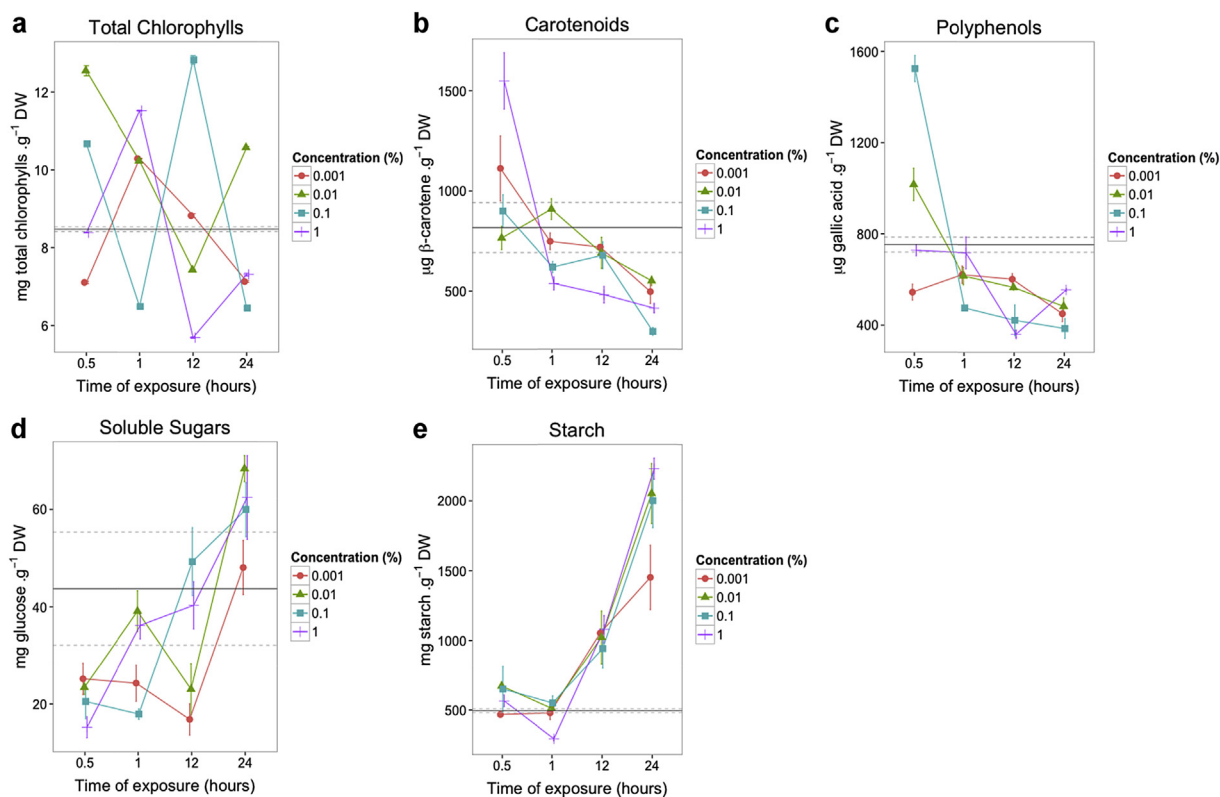


Fig. 4. (a) Total chlorophylls, (b) carotenoids, (c) polyphenols, (d) soluble sugars, and (e) starch contents of *U. lactuca* thallus. In each plot, control group content is represented by the horizontal continuous dark line and its upper and lower standard deviation (SD) are represented by dashed lines. Gasoline-treated groups are represented by dots according to the time of exposure (X axis) and concentration of gasoline (dot shapes). Vertical line in each dot represents upper and lower SD for the respective group.

correlations between the time of exposure (-0.31) and concentration (-0.18) of gasoline were observed (Table 5). Nonetheless, multifactorial ANOVA revealed that the interaction of the factors time of exposure and pigment concentration was able to explain around 75% of the variance observed in the data set of the chlorophyll contents (Table 4).

As carotenoids play important roles in plant cell physiology, not only in the photosynthesis but also in cell structure and antioxidant defense, we performed HPLC analysis to identify and quantify those secondary metabolites. Carotenoid profiles are discriminated in Table 2 of supplementary material, along with the results of one-way ANOVA and Tukey test, as total carotenoids contents are plotted in Fig. 4b. Lutein, β -cryptoxanthin, α -carotene, *trans*- β -carotene, and *cis*- β -carotene were identified in all groups.

Furthermore, an unidentified compound with retention time 3.3–3.5 min was not detected in thalli exposed to 0.01% and 0.1% of gasoline for 30 min, and zeaxanthin was not detected in 0.001%, 0.01%, and 0.1% gasoline-treated samples for 1 h. It was possible to detect a decrease in lutein content only after 12 h and 24 h of exposure, as zeaxanthin seemed to be more sensitive with reduced contents in all groups exposed to gasoline. β -cryptoxanthin concentration decreased just after 1 h exposure and continued doing so until the end of the experiment. α -carotene, *trans*- β -carotene, and *cis*- β -carotene showed to be augmented after 30 min of exposure to gasoline, but longer exposure times (12 h) led to reduced amounts of *cis*- and *trans*- β -carotene. Although an increment in total carotenoid content has been noted after 30 min and 1 h of exposure to gasoline, concentrations decreased after 12 h and 24 h (Table 1,

Table 3
One-way ANOVA and Tukey test performed on total chlorophylls, total carotenoids, polyphenols, soluble sugars, and starch contents in *U. lactuca* grouped by time of exposure (30 min, 1 h, 12 h, 24 h) and by concentration of gasoline (0.001%–1.0%, v/v). C = control (non exposed group). Confidence level of 95% was considered. p-values presented refer to ANOVA. Groups with significant differences in Tukey test ($p \leq 0.05$) are shown in the last column.

		p-values	Groups with significant difference (Tukey test)
By time of exposure	Total chlorophylls	2.142E-01	–
	Carotenoids	4.281E-04	24 h – C; 1 h – 30min; 12 h – 30min; 24 h – 30min; 24 h – 1 h
	Polyphenols	3.511E-06	1 h – 30min; 12 h – 30min; 24 h – 30min
	Soluble sugars	3.446E-11	30min – C; 12 h – 30min; 24 h – 30min; 24 h – 1 h; 24 h – 12 h
	Starch	1.621E-22	12 h – C; 24 h – C; 12 h – 30min; 24 h – 30min; 12 h – 1 h; 24 h – 1 h; 24 h – 12 h
By concentration	Total chlorophylls	0.168	–
	Carotenoids	0.866	–
	Polyphenols	0.611	–
	Soluble sugars	0.477	–
	Starch	0.601	–

Table 4
Multifactorial ANOVA performed on total chlorophylls, total carotenoids, polyphenols, soluble sugars, and starch contents in *U. lactuca*, considering the effect of time of exposure and concentration of gasoline separately and combined. Confidence level of 95% was considered ($p \leq 0.05$).

	Time of exposure	Concentration	Time of exposure: concentration	Residuals
Variance explained				
Total chlorophylls	0.116	0.125	0.758	0.000
Carotenoids	0.606	0.034	0.316	0.044
Polyphenols	0.488	0.047	0.451	0.015
Soluble sugars	0.714	0.041	0.185	0.060
Starch	0.906	0.019	0.049	0.027
p-values				
Total chlorophylls	1.228E-41	8.850E-43	2.754E-53	
Carotenoids	2.383E-19	1.887E-04	5.773E-13	
Polyphenols	1.256E-24	3.278E-10	5.670E-22	
Soluble sugars	9.464E-17	9.390E-04	2.697E-07	
Starch	5.360E-25	4.844E-04	2.061E-05	

Table 5
Pearson's correlation performed on total chlorophylls, total carotenoids, polyphenols, soluble sugars, and starch contents in *U. lactuca* not exposed (control) and exposed to gasoline (0.001–1%, v/v) for 30 min, 1 h, 12 h, and 24 h. Confidence level of 95% was considered. (*) indicates p-value ≤ 0.05 ; (***) indicates p-value ≤ 0.01 .

	Time of exposure	Concentration
Total chlorophylls	–0.31	–0.18
Carotenoids	–0.65 ***	–0.03
Polyphenols	–0.51 *	–0.08
Soluble sugars	0.75 ***	0.09
Starch	0.95 ***	0.08

supplementary material). One-way ANOVA of total carotenoid contents of samples grouped by time of exposure (Table 3) indicated significant differences ($p \leq 0.05$, Tukey test) for the 30 min-exposure treatment with respect to other exposure times, as well as between the 24 h-exposed samples, control group, and that exposed for 30 min. Multifactorial ANOVA indicated that the time of exposure alone explained about 60% of the variance associated with the total concentration of carotenoids and its combination with the variable concentration of gasoline was able to increase that value by 31% (Table 4). In addition, a negative linear correlation coefficient ($r = -0.65$) between total carotenoids and time of exposure to gasoline was observed (Table 5, $p \leq 0.05$).

One-way ANOVA of polyphenol contents showed significant increases compared to control for 30 min-samples exposure to 0.01% and 0.1% of gasoline ($p \leq 0.05$, Tukey test), followed by a significant decrease after 12 h and 24 h of exposure in all concentrations assayed (Fig. 4c). Samples grouped by time of exposure (Table 3) differed ($p \leq 0.05$, Tukey test) in their polyphenol concentrations in *U. lactuca* exposed to gasoline for 30 min

comparatively to those with longer exposure times (1 h, 12 h, and 24 h). According to the multifactorial ANOVA (Table 4), the factor time of exposure alone (48%) and its interaction with the concentration of gasoline (45%) showed to be important to explain the variance of polyphenol contents observed. Corroborating with these results, a negative linear correlation coefficient ($r = -0.51$) for the polyphenol contents and time of exposure was found (Table 5, $p \leq 0.05$).

The displacement of fatty acids followed by the disruption of cell and organelle membranes increases the distance between enzymes and photosystem components, i.e. chlorophylls and carotenoids, interrupting important metabolic functions, such as photosynthesis and respiration, as these processes depend on the enzyme activity and integrity of cytoplasmic and sub-cellular membranes (O'Brien and Dixon, 1976). Additionally, one could speculate that the damage caused by the Brazilian gasoline herein investigated to the cell and organelle membranes, mostly to the protein fraction thereof, might be also increased due to its relevant content of ethanol. Decrease in chlorophylls content has been associated to deleterious effects of many xenobiotics in water as reported for *U. lactuca* exposed to cadmium and copper, for *H. musciformis* exposed to diesel, and *Hydrilla verticillata* exposed to toluene, xylene, and ethylbenzene (Han et al., 2008; Kumar et al., 2010; Yan and Zhou, 2011; Ramlov et al., 2013). Nevertheless, our measurements of photosynthetic pigments of *U. lactuca* exposed to gasoline revealed fluctuations in chlorophyll *a* and *b* contents along the time of exposure and concentrations studied, without a pattern of response.

Regarding the carotenoid profile, the metabolites investigated fluctuated in their contents without a linear association with any of the factors investigated. However, total carotenoid contents started decreasing after 12 h and 24 h of exposure to gasoline, suggesting a

biochemical damage to their biosynthesis and accumulation pathways. For these observations, it seems that photosynthetic pigments in *U. lactuca* resist to acute gasoline injuries. Given the importance of these pigments in plant metabolism, it is expected that they are able to cope with some level of injuries and carotenoids might be involved in mechanisms of protection against these threats, once they stabilize and protect the lipid phase of thylakoid membranes, which contributes to preserve the functionality of photosynthetic machinery (Frank and Cogdell, 1996; Havaux, 1998).

Carotenoids also play an important role in non-enzymatic defense against oxidative damage, together with phenolic compounds, which are synthesized in plants as a response to ecological and physiological pressures (Khoddami et al., 2013; Petrusa et al., 2013). Increase in carotenoid and polyphenol contents have been associated to oxidative stress due to pollutants such as metals and hydrocarbons. Increase in these metabolites were also attributed to protection against oxidative stress in *U. lactuca* and *H. verticillata* exposed to selenium, cadmium, and toluene (Kumar et al., 2010; Yan and Zhou, 2011; Schiavon et al., 2012). However, the exposure to gasoline led to a decrease in carotenoid and polyphenol contents in *U. lactuca* as shown herein. These results suggest that *U. lactuca*'s carotenoids and polyphenols are sensitive to gasoline treatment even for a short time of exposure and could be used as biomarkers to this type of pollution in biomonitoring programs with this species.

3.2.2. Carbohydrates metabolism – the main detoxification pathway in *U. lactuca*?

In order to gain more insight on *U. lactuca* responses after exposure to gasoline, we decided to investigate the cell carbohydrate pool (i.e., soluble sugars and starch), since soluble sugars are crosslinked to energetic, storing, and detoxifying pathways.

Content of soluble sugars in 30 min- and 1 h-treated samples significantly decreased compared to control group ($p \leq 0.05$, one-way ANOVA, *post-hoc* Tukey test), increasing afterward and achieving significantly higher values after 24 h (Fig. 4d). One-way ANOVA of samples grouped by time of exposure indicated differences between samples exposed for 30 min to gasoline in respect to control and groups exposed for 12 h and 24 h. The latter was also significantly different from groups exposed for 1 h and 12 h (Table 3). The factor time of exposure explained about 71% of the variance obtained for soluble sugars (Table 4), while a relevant correlation coefficient between soluble sugars and time of exposure, i.e. 0.75, has been detected (Table 5).

Starch content (Fig. 4e) in *U. lactuca* exposed to gasoline for 12 h and 24 h significantly increased compared to control (Table 1, supplement material, $p \leq 0.05$, Tukey test). Results of one-way ANOVA applied to samples grouped by time of exposure to gasoline showed that both times of exposure (12 h and 24 h) differed from control and 30 min- and 1 h-treated samples still differing from each other ($p \leq 0.05$ - Table 3). Multifactorial ANOVA revealed that around 90% of the starch data set variance is explained by the time of exposure (Table 4), and a high linear correlation coefficient ($r = 0.95$) has been found between the starch content and time of exposure to the xenobiotic (Table 5).

Desmodium armatum cultures exposed to diesel and gasoline exhibited decrease in chlorophylls and carotenoids contents, but continued growing for 9 days, which was assigned to the assimilation of the hydrocarbons in seaweed metabolism as carbon source (Kureyshevich and Guseynova, 2008). It is possible that *U. lactuca* has shown similar ability to detoxify gasoline hydrocarbons and use them as a carbon source. The ability to metabolize organic compounds has already been reported for the green algae *Chlorella vulgaris*, *Scenedesmus platydiscus*, *Scenedesmus quadricauda*, and

Selenastrum capricornutum (Haritash and Kaushik, 2009). The initial steps in the metabolism of aromatic hydrocarbons in plant cells include hydroxylation and glycosylation, followed by the cleavage of the aromatic ring, with the formation of aliphatic products that go through further modifications ending up in the tricarboxylic acid cycle (Korte et al., 2000).

When exposed to gasoline, *U. lactuca* presented a decrease in soluble sugars content after 30 min and 1 h. After 12 h exposure, soluble sugar amount was similar to control groups and after 24 h exposure higher contents in respect to control group were detected. It is possible that during the first hours of exposure, a great amount of soluble sugars has been driven by the cell to the glycosylation of gasoline hydrocarbons. As the penetration of these molecules in the cells implied disrupting the cell membranes and possibly blocked photosynthesis, cellular levels of soluble sugars could not be maintained in this pathway. Nevertheless, increased soluble sugars levels after 12 h exposure to gasoline might be due to the production of such metabolites during the detoxification process. As a consequence of progressive membrane damages and decoupling of enzymatic chains, soluble sugars are unable to go through the respiration pathway and accumulate in the cytoplasm. Based on the starch content measurements, the excess of soluble sugars probably has been driven to starch synthesis, once this pathway works with soluble enzymes and might be relatively unaffected by gasoline treatment. Unfortunately, no similar investigation aiming to investigate the carbohydrates metabolism of algae exposed to hydrocarbons was found in the literature so far to help further discussion.

3.3. Unsupervised multivariate analysis – a picture of biochemical profile

In an attempt to mend the pieces obtained by the biochemical analysis, we performed and compared two unsupervised multivariate analysis methods over the studied biochemical variables. Hierarchical clustering analysis (Fig. 5a) was able to separate in one branch of the dendrogram the thallus samples exposed to 0.01%, 0.1%, and 1.0% of gasoline for 24 h. In the neighbor branch, thallus samples treated with 0.001% of gasoline for 24 h were set together with all groups of thalli exposed to gasoline for 12 h. A third branch encompassed all groups exposed to gasoline for 1 h, those exposed to 0.001% of gasoline for 30 min, and control thalli. Thalli exposed to 0.01%, 0.1%, and 1.0% of gasoline for 30 min were set in a fourth branch, closer to the former one.

Principal components analysis shown lesser discriminating power in respect to hierarchical clustering analysis, but yet returned interesting results available in supplement material. PC1 and PC2 together were able to explain around 77% of the sample variance, while near 96% was achieved by adding the extra contributions of PC3 and PC4, suggesting the relevance of all biochemical parameters involved in the model (Table 3, supplement material). Groups exposed to 0.01%, 0.1%, and 1.0% of gasoline for 24 h grouped in negative PC1, far from the axis (Fig. 5b), while control and groups exposed to 1 h and 12 h were close to PC1 axis. By plotting the variables as vectors, it was found that soluble sugars and starch strongly contributed for the sample grouping in negative PC1, while total chlorophylls, total carotenoids, and polyphenols contributed mostly for a positive PC1. Total chlorophylls and polyphenols contributed for a negative PC2, as the contents of carotenoids contributed for a positive PC2. Unsupervised methods are in agreement with the analysis of variance and the correlation results, reinforcing that the time of exposure was the decisive factor affecting *U. lactuca* metabolism, and that 12 h of exposure to gasoline was a turning point for the metabolic alterations found.

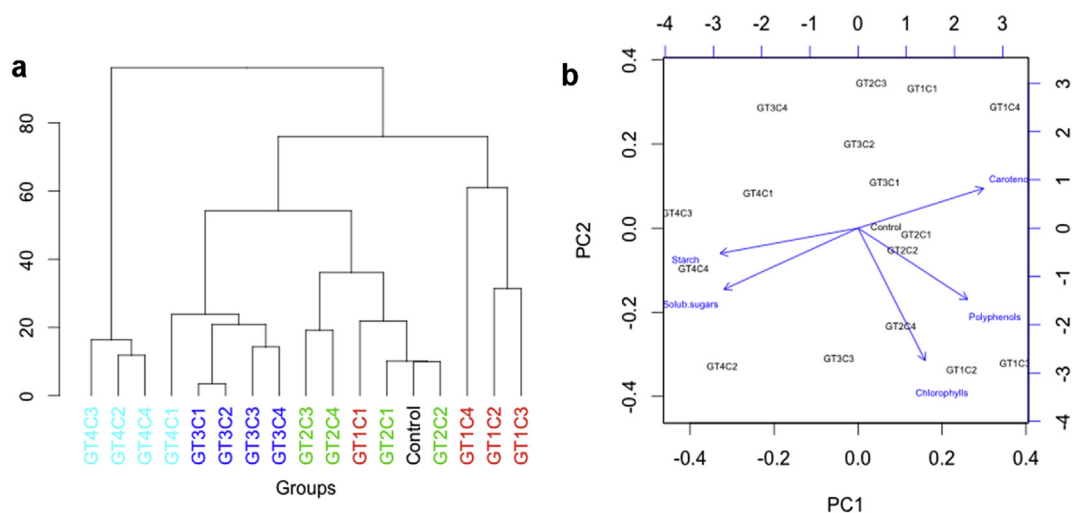


Fig. 5. a) Dendrogram of hierarchical cluster analysis of biochemical variables of control (black leaf) and gasoline-treated *U. lactuca* thallus. Group labels: G = gasoline; Time of exposure: T1 = 30min (red leaves), T2 = 1 h (green leaves), T3 = 12 h (blue leaves), T4 = 24 h (cyan leaves); Concentration of gasoline (C1 = 0.001%; C2 = 0.01%; C3 = 0.1%; C4 = 1.0%); b) PCA of biochemical variables of control and gasoline-treated *U. lactuca* thallus. Group labels defined as above. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Final considerations

Literature concerning the toxicity of oil and its derivatives in seaweeds highlights that tolerance or susceptibility to these hydrocarbons is species-dependent, in addition to the importance of developing standard methods that allow comparing results and including more effectively the macroalgae in the scenario of environmental monitoring programs (O'Brien and Dixon, 1976; Baker, 1970; Eklund and Kautsky, 2003). Although *U. lactuca* is considered to be a tolerant species to a wide range of environmental pollutants, including heavy metals (Han et al., 2008), susceptibility to gasoline was observed in our short-term experiments through morphological alterations and decreasing in carotenoid and polyphenol contents after 12 h and 24 h of exposure. Nonetheless, our findings on soluble sugars and starch contents indicate that this macroalgae is able to detoxify these hydrocarbons and possibly use them as a carbon source in its metabolism. Further chronic exposure and recovery experiments are needed to confirm our findings, however our results shed a light on an important detoxifying pathway in macroalgae that remains poorly comprehended and might help to elucidate the crucial role of this species in bioremediation of aquatic environments.

Acknowledgements

To Coordination for the Improvement of Higher Education Personnel (CAPES) for financial support. Authors are thankful to the Central Laboratory of Electron Microscopy (LCME) and Multi-User Laboratory for Studies in Biology (LAMEB) at Federal University of Santa Catarina. The research fellowships granted to M. Maraschin (Proc. 307099/2015-6) and Fernanda Ramlov (Proc. 473408/2013-9) by National Council of Technological and Scientific Development (CNPq) are also acknowledged. This work was partially funded by CNPq and FCT agreement through the PropMine (Proc. 490383/2013-0) and PVE (Proc. 407323/2013-9, Dr. Miguel Rocha) grants.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.chemosphere.2016.04.126>.

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