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Metabolomics of *Ulva lactuca* Linnaeus (Chlorophyta) exposed to oil fuels: Fourier transform infrared spectroscopy and multivariate analysis as tools for metabolic fingerprint

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ABSTRACT

Fossil fuels, e.g. gasoline and diesel oil, account for substantial share of the pollution that affects marine ecosystems. Environmental metabolomics is an emerging field that may help unravel the effect of these xenobiotics on seaweeds and provide methodologies for biomonitoring coastal ecosystems. In the present study, FTIR and multivariate analysis were used to discriminate metabolic profiles of *Ulva lactuca* after in vitro exposure to diesel oil and gasoline, in combinations of concentrations (0.001%, 0.01%, 0.1%, and 1.0% - v/v) and times of exposure (30 min, 1 h, 12 h, and 24 h). PCA and HCA performed on entire mid-infrared spectral window were able to discriminate diesel oil-exposed thalli from the gasoline-exposed ones. HCA performed on spectral window related to the protein absorbance (1700–1500 cm^{-1}) enabled the best discrimination between gasoline-exposed samples regarding the time of exposure, and between diesel oil-exposed samples according to the concentration. The results indicate that the combination of FTIR with multivariate analysis is a simple and efficient methodology for metabolic profiling with potential use for biomonitoring strategies.

1. Introduction

Metabolome comprises the full suite of low molecular weight organic metabolites synthesized by a biological system, as metabolomics is the well-established science field concerned on its study (Fiehn, 2001). Within this field, four main strategies for analysis are considered: metabolomics itself, which is the non-biased identification and quantification of all the metabolites of a living cell or organism at a given moment; metabolite profiling is the identification and quantification of the metabolites from a given class of compounds or a metabolic pathway; target metabolite analysis aims to identify and quantify one or few specific metabolites related to a metabolic reaction; metabolic fingerprinting is a rapid, high-throughput global analysis which aims at obtaining enough information to unravel metabolic alterations, without pursuing to get metabolites identification or quantitative data for all biochemical pathways (Fiehn, 2001; Dunn and Ellis, 2005). Nuclear magnetic resonance (NMR), mass spectrometry (MS), and Fourier transform infrared spectroscopy (FTIR) are analytical devices with enough high resolution to handle critical information for metabolic fingerprinting (Fiehn, 2001). According to Bundy et al. (2009), environmental metabolomics is the application of metabolomics to characterize the interaction of living organisms with their environment. It enables a rapid, unbiased, and simultaneous measurement of many metabolites, and therefore differs substantially from traditional biochemical methods that typically detect only one or a few metabolites. As a result, metabolomics is a particularly powerful approach for discovering biomarker profiles of toxicant exposure and disease, and for identifying the metabolic pathways involved in such processes (Viant, 2007).

Although considerable information about the metabolome of terrestrial plants, specially crop species and *Arabidopsis thaliana*, has been available in latest years, few marine macrophytes, e.g., seaweeds and seagrasses, have been examined using metabolomics technologies. Seaweeds play important ecological roles in nutrient cycling, carbon sequestration, sediment stabilization, and habitat provision to marine fauna. These organisms also experience a diverse range of environmental fluctuations, threats from invasive species and pathogens, and anthropogenic stress. The currently available studies have been focused mostly on metabolomics of seaweeds' economically valuable compounds, as some information on their responses to environmental stress starts to emerge in literature (Kumar et al., 2016). Marine pollution due to oil and oil derivatives is a global concerning since the 19th century.

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Besides eventual big oil spills that gain people, governments, and media attention, small leaks occur frequently during oil extraction, processing, transport, and storage, constituting silent sources of contamination. The toxic effects of these xenobiotics are widely studied in marine fauna, but information regarding to the macroalgae is scarce (Eklund and Kautsky, 2003; Torres et al., 2008; Lewis and Pryor, 2013). Thus, investigations on the effects of oil derivatives on seaweeds and developing simple methodologies to unravel the metabolic damages thereof in these organisms is central for biomonitoring marine ecosystems and interceding in case of contamination. The present work aimed at to determine the metabolic profiles of *Ulva lactuca* thalli exposed to diesel oil and gasoline through FTIR, followed by the construction of discriminant models using multivariate statistical techniques.

2. Material and methods

Thalli of *Ulva lactuca* were collected in April 2013 at Armação Beach (27°74′96″ S, 48°50′01″ W), in Florianópolis (Santa Catarina State, southern Brazil), packed in plastic flasks containing seawater and immediately transferred to the Plant Cell Biology Laboratory, where they were 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, 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.

Both certified quality control common gasoline and diesel oil samples were purchased from Petrobras at a commercial gas station in Florianópolis (Santa Catarina state, southern Brazil) and their chemical profiles were determined by gas chromatography (GC 2010-2, Shimadzu) using a flame ionization detector and described elsewhere. The gasoline sample presented a typical hydrocarbon composition, 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 (Pilatti et al., 2016). The diesel sample composition presented hydrocarbons compounds ranging from C6 to C30, with predominance of C8 to C18-chain long hydrocarbons (Ramlov et al., 2013).

The experimental design was as previously described by Pilatti et al. (2016) and consisted in combining four concentrations of gasoline or diesel oil (0.001%, 0.01%, 0.1%, and 1.0% - v/v, nominal concentrations) and four times of exposure to that pollutant (30 min, 1 h, 12 h, and 24 h). Each group consisted of 5 replicates, i.e., 2 g of *U. lactuca* thalli cultivated in Erlenmeyer flasks containing the previously described nominal concentrations of the xenobiotic and enriched seawater 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 contamination caused by these pollutants in aquatic ecosystems. Therefore, gasoline and diesel oil 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. Biomass aliquots (1 g, FW) were immediately frozen in liquid nitrogen (LN) and kept at −80 °C for further analysis. An experimental design pilot test and subsequent experiments were performed by our research group sampling control thalli at each time of exposure, but no significant metabolic difference was detected between those samples (data not published). Thus, in order to optimize the experimental protocol and lab resources, control group consisted on 10 g fresh thalli collected directly from the acclimation flasks, surface dried with paper towels, separated in aliquots, frozen in LN, and kept at −80 °C.

Table 1
Labels for control group (C) and groups exposed to 0.001% (*C1*), 0.01% (C2), 0.1% (C3), and 1.0 (*C4*) of diesel oil (D) or gasoline (G) for 30 min (T1), 1 h (T2), 12 h (T3), and 24 h (T4).

		Time of exposure					
		Control	30 min	1 h	12 h	24 h	
Concentration	Control	C	–	–	–	–	Control
	0.001%	–	DT1C1	DT2C1	DT3C1	DT4C1	Diesel
			GT1C1	GT2C1	GT3C1	GT4C1	Gasoline
	0.01%	–	DT1C2	DT2C2	DT3C2	DT4C2	Diesel
			GT1C2	GT2C2	GT3C2	GT4C2	Gasoline
	0.1%	–	DT1C3	DT2C3	DT3C3	DT4C3	Diesel
			GT1C3	GT2C3	GT3C3	GT4C3	Gasoline
	1.0%	–	DT1C4	DT2C4	DT3C4	DT4C4	Diesel
			GT1C4	GT2C4	GT3C4	GT4C4	Gasoline

Fuel-exposed groups were labeled according to the xenobiotic treatments of thalli considering a combination of the variables time of exposure and concentration of the xenobiotic as described in Table 1.

FTIR spectra were obtained from lyophilized biomass (0.5–1.0 g) and KBr tablets, in a Fourier transform spectrometer FTLA2000 ABB Bomem, equipped with attenuated total reflectance (ATR) accessory. A KBr tablet was used for baseline spectrum acquisition. Spectra were acquired in transmittance mode, from 4000 to 400 cm^{-1}, with 2.04 cm^{-1} resolution, resulting in 1765 points. 128 co-added scans before Fourier transformed were collected for each spectrum, and 5 spectra of each sample were obtained.

Data pre-processing, multivariate analysis and plots were performed in R language (v. 3.2.3), using package "specmine" (Costa et al., 2015). Three datasets were used: i) full dataset, which contains spectra from control group and from samples exposed to gasoline and to diesel oil; ii) gasoline dataset, which contains spectra from control group and from samples exposed to gasoline; iii) diesel oil dataset, which contains spectra from control group and from samples exposed to diesel oil. All spectra were initially transformed from transmittance to absorbance. Next step was assessing combinations of normalization, baseline correction, and smoothing algorithms and test them for PCA and HCA, searching for the best samples segregation (data not showed). Full dataset pre-processing consisted in data normalization using median, followed by baseline correction with modified polynomial fitting (MPF) method. Pre-processing of gasoline and diesel oil datasets consisted solely on baseline correction with asymmetric least squares (ALS) method. After pre-processing, principal component analysis (PCA) and hierarchical clustering analysis (HCA) were performed on each dataset. Besides the entire mid-infrared spectral window (4000 to 400 cm^{-1}), spectral windows of interest underwent multivariate analysis separately, i.e., proteins (1700–1500 cm^{-1}), lipids (3000–2800 cm^{-1}), carbohydrates (1200–900 cm^{-1}) and fingerprint region (800–500 cm^{-1}), which is named for its bands, formed by the whole molecules vibration (Harborne, 1998; Pistorius et al., 2009). Reports with all the analysis and results were generated with R Markdown and are available in the supplementary material.

3. Results and discussion

Initially, multivariate analysis performed over full dataset aimed to detect differences between diesel oil- and gasoline-exposed thalli's metabolic profiles. Fig. 1 shows all spectra analyzed after pre-processing, with the indication of spectral windows related to the absorbance of carbohydrates, proteins, lipids, and fingerprint. PCA, a multivariate analysis technique is a mathematical algorithm that reduces the dimensionality of datasets by developing a smaller number of uncorrelated artificial variables, called principal components (PCs), that account for most of the variance in the observed variables. Samples can then be plotted, making possible to visualize similarities and differences between samples and whether they can be grouped (Ringner, 2008; Ami et al., 2012).

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