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Seagrass *Posidonia oceanica* (L.) Delile as a marine biomarker: a metabolomic and toxicological analysis

Chiara Toniolo,[1] Antonella Di Sotto,[2,]† Silvia Di Giacomo,[2] Daniele Ventura,[1] Edoardo Casoli,[1] Andrea Belluscio,[1] Marcello Nicoletti,[1] and Giandomenico Ardizzone[1]

[1]*Department of Environmental Biology, Sapienza University, P.le Aldo Moro 5, 00185 Rome, Italy*
[2]*Department of Physiology and Pharmacology "V. Erspamer", Sapienza University, P.le Aldo Moro 5, 00185 Rome, Italy*

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Abstract. A human-made environmental disaster due to the shipwrecked of Costa Concordia cruise vessel on the Tuscan Island of Giglio (Italy) coast and the possible pollutants release has been feared, so requiring the activation of removal operations and the monitoring of the marine environment. In the present study, the seagrass *Posidonia oceanica* (L.) Delile was used as a bioindicator for the impact of the Costa Concordia accident on the marine and coastal habitat. Different *P. oceanica* samples were collected in the shipwrecked site under different light conditions. Using high-performance thin-layer chromatography, metabolic analysis of the samples was carried out in order to highlight possible changes in the secondary metabolism due to the permanent shading and the presence of pollutant traces. Moreover, sample mutagenicity, as a consequence of the possible absorption of environmental toxicants leaked by the wreck, was assessed by the Ames test. The results highlighted the permanence of the Concordia-induced alteration in the plant secondary metabolites. However, absorption of chemical pollutants and carcinogens was not reported; this point was confirmed by the lack of mutagenic effects found for the samples tested. Our results clearly evidence that the environmental impact of Costa Concordia wreck and removal operations on *P. oceanica* was mainly due to the lack of light in the marine habitat. Present methodological approach, which combines metabolomic and genetic ecotoxicological analysis, could represent a suitable strategy to evaluate the impact of human disasters on the ecosystem and to monitor the environmental changes.

Key words: Ames test; bioindicator; high-performance thin-layer chromatography; mutagenicity; *Posidonia oceanica*.

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† **E-mail:** antonella.disotto@uniroma1.it

Introduction

The night of the 13 January 2012 marks an additional date in the history of marine accidents, due to the shipwreck of the Costa Concordia cruise vessel on the coast of the Tuscan Island of Giglio in Italy. The wreck removal operations started in May 2012: On 17 September 2013, the ship was winched upright to a vertical position through the park-buckling procedure, and less than one year later, at the end of July 2014, the wreck has been refloated and then towed to Genoa Harbor.

Several strategies have been used to improve the removal operations, including drilling to install the submarine supports (anchor blocks and underwater platforms), placing grout bags to stabilize the wreck after the parbuckling, installing caissons on both sides of the wreck for the refloating phase. All these activities have been carried out by several support vessels, used as accommodation, diving and remotely operated vehicle (ROV,

Prometeo-Elettronica Enne Savona) supply. The hull of cruise ship and the other vessels were stopped in the sea for 2 yr, 6 months, and 10 d. As a consequence, the sunlight flow was reduced and the homeostasis of the marine environment was altered. The environmental monitoring works started few months after the accident, in order to preserve the wreck integrity and to prevent the release of sewage and fuel into sea, and to monitor the wreck impact on marine environmental health.

In this context, our research group approached the study of *Posidonia oceanica* (L.) Delile (Fam. Posidoniaceae), an endemic seagrass in the Mediterranean Sea, which has been reported to be a suitable bioindicator for marine ecosystem health, according to previous evidence (Richir et al. 2013). *Posidonia oceanica* develops in shallow waters (from 0 up to 40 m depth) and plays a significant role in coastal habitat. In fact, macrophytes are recognized to be ecosystem engineers as they can provide essential edaphic modifications to the environment, including control of sediment deposition and stabilization of soft bottoms (Jones et al. 1997, Gacia and Duarte 2001, Fonseca and Koehl 2006). Moreover, seagrasses have a pivotal role to enhance benthic structural complexity, improving biodiversity and providing nursery areas for many juvenile fishes (Francour 2000, Beck et al. 2003, Boudouresque 2004). *Posidonia oceanica* effectively accumulates high levels of pollutants and is resistant to pollution, thus persisting in the vicinity of important contamination sources. Being photosynthetic species with a coastal distribution, seagrasses are mainly susceptible to a reduction in water quality and light availability linked to both natural and anthropogenic factors (Ruiz and Romero 2001, Pérez et al. 2008, Lassauque et al. 2010). Given the aforementioned reasons, *P. oceanica* has been identified under Community Legislation (European Council Directive 92/43/CEE, habitat code 1120; *P. oceanica* meadows) as a protected species, and it has been recognized as a good descriptor of environmental quality for coastal waters (European Water Framework Directive 2000/60/EC).

To perform the present study, several descriptors have been used in order to assess the effect of the Costa Concordia shipwreck and the following removal operations on metabolism and growth of *P. oceanica*. The choice of this plant is also advantageous due to the easiest collection and the changes in its secondary metabolite pool under stress conditions (Kuo and McComb 1989, Montefalcone et al. 2008). The metabolomic profile of different samples of *P. oceanica* was studied by high-performance thin-layer chromatography (HPTLC), which represents an efficient method for obtaining multiple phytochemical information of plant extracts (Reich and Schibli 2007, Nicoletti 2011, Nicoletti et al. 2013, Toniolo et al. 2013). Furthermore, taking into account that the exposure of aquatic organisms to environmental pollutants and carcinogenic substances can induce DNA damage by direct genotoxicity or cellular stress, mutagenicity studies were performed on the *P. oceanica* samples, in order to highlight the pollution impact of Costa Concordia wreck and/or other vessels on seagrass.

Materials and Methods

Study area and Posidonia oceanica *collection*

The study was carried out at Island of Giglio (Central Tyrrhenian Sea), few hundred meters north of Giglio Harbor (10°55′16,053″ E, 42°21′40,986″ N; Fig. 1). The study site was located inside the shipyard area delimited during removal operations and was characterized by *P. oceanica* beds extending from 3 m up to 36 m depth; *P. oceanica* distribution has been mapped in detail in August 2012 and in August 2013 (Table 1). The meadow has been exposed to high stress conditions and to a drastic change in ecological factors (i.e., alterations of flow regimes, turbidity, and shading) during the Costa Concordia shipwrecking and its removal operations. Especially, in correspondence with both Costa Concordia wreck and the ASV Pioneer, a large accommodation vessel (100 × 60 m side) was moored for more than one year (from September 2012 to October 2013) close to Costa Concordia's Bow.

Posidonia oceanica samples have been collected by scuba diving during winter 2013 and spring 2014; for each station, three replicates 1 m distant each other have been sampled, constituted each by five shoots and rhizomes, in order to evaluate the metabolic response of the different plant structures.

In order to obtain detailed information about not impacted meadow, specimens have been collected every five meters depth in the control site at Cala Cupa. Upon collection, samples have been

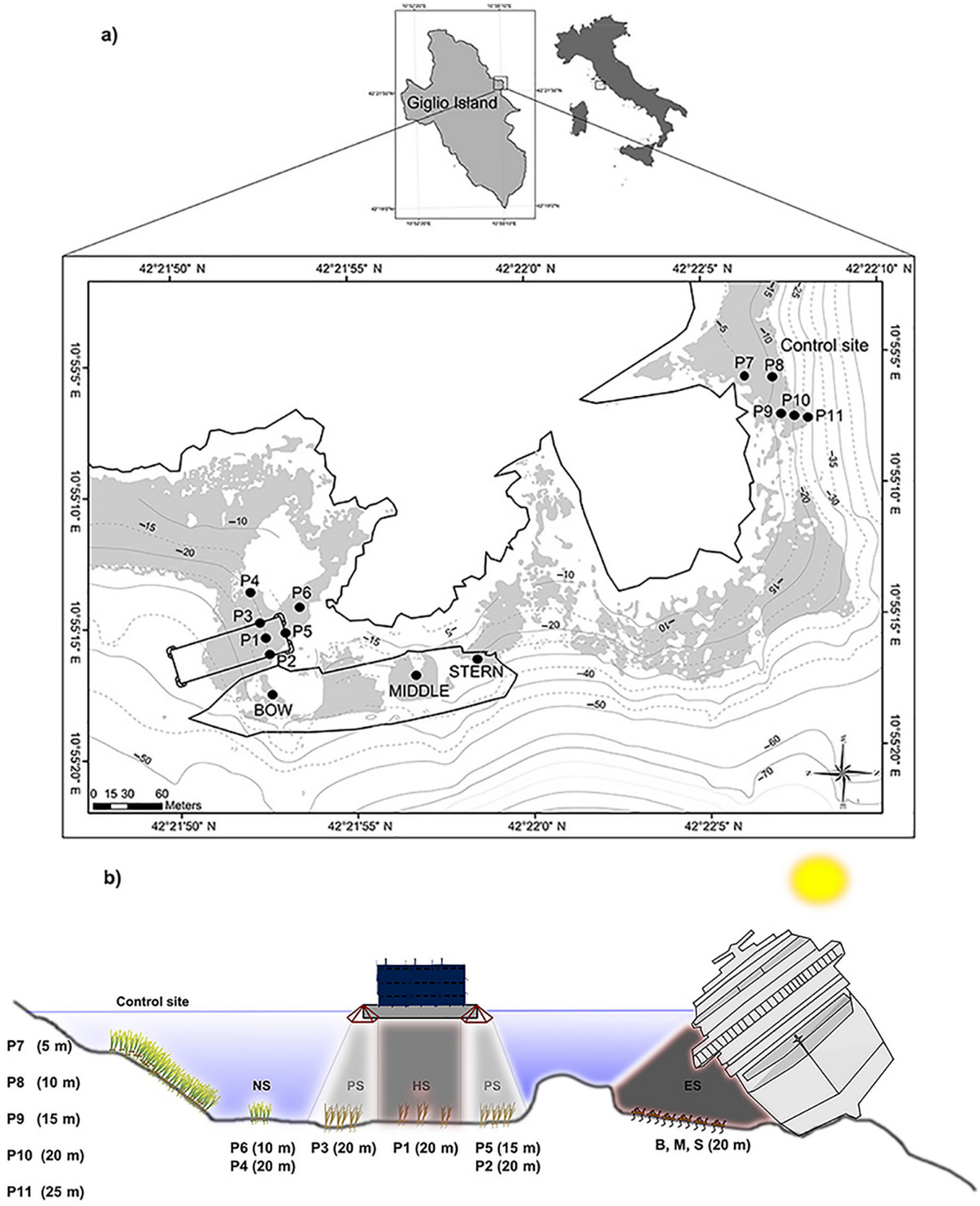

Fig. 1. (a) The study site map. Light gray indicates *Posidonia oceanica* presence on August 2012, whereas Concordia and Pioneer positions are indicated by the two dark shapes. Sampling stations are shown with their identification numbers, and stations under the Costa Concordia wreck are identified as Bow, Middle, and Stern, respectively. (b) A schematic representation of the study area: Different shadow conditions are shown with a different gray gradation. ES, extreme shadow; HS, high shadow; PS, partial shadow; NS, normal shadow. Control site is a distant area not influenced by shadow or any other factor. In the ES zone, samples are at 20 m depth (Bow, Middle, Stern); P1, P2, P3, and P4 are at 20 m depth; P5 at 15 m depth; and P6 at 10 m depth.

Table 1. Mean shoot density and percentage of regression measured for extreme shading (ES), high shading (HS), partial shading (PS), and normal shading (NS) conditions and for control site (CS).

Sites (stations), depth	2012	2013	Regression percentage
ES (B, M, S), 20 m	0	0	100.0
HS (1), 20 m	200.5 ± 43.1	3.1 ± 1.6	98.5
PS (2), 20 m	202.2 ± 40.2	40.7 ± 7.4	79.9
PS (3), 20 m	198.4 ± 37.3	5.0 ± 2.3	97.5
PS (5), 20 m	228.0 ± 32.6	42.6 ± 8.5	82.3
NS (4), 20 m	200.2 ± 40.0	164.1 ± 27.2	18.0
NS (6), 10 m	237.8 ± 33.3	202.5 ± 45.6	14.8
CS (7–11), 5–25 m	From 400.2 to 171.7		0.0

stored in plastic beakers with marine water in order to maintain the functionality of vital structures until laboratory analyses. Analyses have been performed no more than 12 h after the samples were received in the laboratory. After careful washing and cleaning, each sample has been separated into the following parts (separately analyzed): leaves (aerial part) and rhizomes (ground parts). Voucher samples of the collected materials have been deposited at the Herbarium of Sapienza, under the identification number MN21/2014.

Sampling stations are shown in Fig. 1a, b; sampling design was based on the following shading conditions:

1. Extreme shading (ES) condition occurs under the Costa Concordia starboard side. Here, rhizomes (no living plants were present) were collected close to the Bow, Middle, and Stern of the wreck (stations indicated as B, M, and S).
2. High shading (HS) condition occurs under ASV Pioneer Vessel. Both leaves and rhizomes were collected (station P1).
3. Partial shading (PS) condition develops at the edge of ASV Pioneer hull shadow. Both leaves and rhizomes were collected at the margin of the Pioneer shadow (stations P2, P3, and P5).
4. Natural shading (NS) condition develops few tens meters away to the ASV Pioneer hull shadow. Both leaves and rhizomes are non-directly influenced by the ships shadow (stations P4 and P6).
5. Control site was free from any shading influence. Both leaves and rhizomes were collected along a depth gradient from 5 to 25 m (stations P7, P8, P9, P10, and P11).

These conditions were <5% (ES), 30% (HS), 40% (PS), and 100% (NS), respectively, of photosynthetic photon flux density measured at the same depth in the control site, completely not affected by shadow. Light intensity measures were carried out by a portable underwater data logger (Hobo Onset).

Sample preparation

Before HPTLC analyses, the samples were carefully washed, dried with a gentle air flux, and immediately extracted; for each sample, 2.5 g of fresh leaves and rhizomes was extracted with 10 mL of aqueous EtOH (70%) at room temperature for 48 h, and the obtained extracts were filtered and evaporated under vacuum until total dryness. Then, each dry extract was dissolved in methanol till obtaining a concentration of 30 mg/mL. The solutions were stored at −5°C until analyzed.

In order to perform mutagenicity analyses, plant material (5 g) was extracted with 10 mL of methanol (100% v/v) for 24 h; then, the extracts were concentrated to dryness using a rotary vacuum evaporator. In order to perform the analysis, the residues were dissolved in dimethylsulfoxide (DMSO; 100% v/v), chosen as the most suitable solvent.

Chemicals and reagents

All the substances including the amino acids histidine (≥99% purity), tryptophan (≥98% purity), and biotin (≥99% purity), the mutagens 2-nitrofluorene (2NF; 98% purity), 2-aminoanthracene (2AA; 96% purity), sodium azide (SA; > 99.5% purity), and methyl methanesulfonate (MMS; 99% purity), the media bacteriological agar, nutrient broth, and nutrient agar were supplied by Sigma-Aldrich (St. Louis, Missouri, USA). The S9 fraction (the liver postmitochondrial supernatant of rats treated with the mixture phenobarbital/β-naphthoflavone to induce the hepatic microsomal enzymes) was supplied and certified by Moltox (Molecular Toxicology, Boone, North Carolina, USA).

To perform the bacterial reverse mutation assay, 2NF and 2AA were dissolved in DMSO, while SA and MMS were prepared in deionized

water. The S9 metabolic activator was prepared just before use by adding phosphate buffer (0.2 mol/L) 500 μL, deionized water 130 μL, KCl (0.33 mol/L) 100 μL, $MgCl_2$ (0.1 mol/L) 80 μL, S9 fraction 100 μL, G6P (0.1 mol/L) 50 μL, and NADP (0.1 mol/L) 40 μL. The mixture was kept on ice during testing.

Chemical study

High-performance thin-layer chromatography analysis.—High-performance thin-layer chromatography analysis was performed according to Di Sotto et al. (2016). Filtered solutions of extracts and standards were applied with Linomat 5 (CAMAG, Muttenz, Switzerland) connected to a nitrogen tank. The HPTLC plates (glass plates silica gel 60 F_{254}; Merck, Darmstadt, Germany) were developed in ethyl acetate/dichloromethane/acetic acid/formic acid/water (100:25:10:10:11 *v/v*) using the automatic and reproducibly developing chamber ADC 2, saturated with the same mobile phase for 20 min at 25°C. The developing solvents (i.e., type of solvents and ratios) were carefully optimized before the analyses. The length of the chromatogram run was 70 mm from the point of application.

The developed layers were derivatized with a selected solution, including natural product reagent (NPR; 1 g diphenylborinic acid aminoethylester in 200 mL of ethyl acetate) and anisaldehyde/sulfuric acid (1 mL *p*-anisaldehyde, 10 mL H_2SO_4, 20 mL AcOH in 170 mL MeOH). Finally, the plates were dried for 5 min at 120°C before inspection. All the treated plates were inspected under a UV light at 254 or 366 nm or under reflectance and transmission white light (WRT), respectively, at a Camag TLC visualizer, before and after derivatization. WinCATS software 1.4.4 was used for the documentation of derivatized plates. Therefore, for each plate, at least six different visualizations were obtained. Owing to the available space, only some images of the plates were reported but others can be obtained under request.

Densitometric analysis.—For the densitometric analysis, the scanner was set at 300 nm, after a multi-wavelength scanning between 190 and 800 nm in the absorption mode had been preliminarily tried. Minimum background compensation was performed on the *x*-axis during the scanning. The sources of radiation were deuterium and tungsten lamps. The slit dimension was kept at 6.00 × 0.45 mm, and the scanning speed used was 100 mm/s (Del Serrone et al. 2007).

Phytochemical study.—The extracts were separated by column chromatography in silica gel using CH_2Cl_2/CH_3OH 95:5 (v/v) as the mobile phase. Separations were monitored by TLC in the same solvent. Pure fractions were identified on the basis of mass spectrometry (MS) and nuclear magnetic resonance (NMR) (^1H and ^{13}C) data (Maggi et al. 2017). Spectra and data used for the identification of marker constituents are available upon request.

Mutagenicity study

The mutagenic power of different *P. oceanica* samples, in terms of ability to induce point mutations at the gene level, was evaluated in vitro by the bacterial reverse mutation assay (Ames test). Experiments were carried out on different *Salmonella Typhimurium* and *Escherichia coli* strains. These bacteria can detect mutagens acting with different mutation mechanisms, because they carry genotypes that make them sensitive to different mutational events (Di Sotto et al. 2009, 2014). Taking into account that bacteria are unable to metabolize inactive chemicals via cytochrome P450, an exogenous metabolic activation system S9 was also included in the Ames test. This allows evaluating whether a CYP450-mediated biotransformation is responsible for the generation of mutagenic compounds (Di Sotto et al. 2009).

Bacterial strains.—A set of five strains, *S. Typhimurium* TA1535 (hisG46 chl1005 rfa1001), TA1537 (hisC3076 chl1007 rfa1003), TA98 (hisD3052 chl1008 rfa1004 pKM101), and TA100 (hisG46 chl1005 rfa1001 pKM101), and *E. coli* WP2*uvr*A (trpE65 uvrA155), was used. The strains TA1535 and TA1537 were supplied by the Department of Pharmacology, University of Bologna (Italy), while TA98, TA100, and WP2*uvr*A were provided by the Research Toxicological Centre (Pomezia, Rome, Italy). After confirmation of the genotypes by the strain check assay (Di Sotto et al. 2013), the permanent cultures of each strain were prepared and then frozen. The working cultures, prepared from the permanent ones, were incubated overnight (16 h) at 37°C, to reach a concentration of approximately 1×10^9 bacteria/mL. In each experiment, the number of viable cells for each strain was determined according to Di Sotto et al. (2014). Viability of

the tested strains TA1535, TA1537, TA98, TA100, and WP2*uvr*A, expressed as viable cells/plate, was of 275.1 ± 8.7, 343.7 ± 14.2, 240.0 ± 13.1, 209.0 ± 9.5, and 371.5 ± 12.9, respectively.

Preliminary assays.—Preliminarily, the solubility of all the extracts in the final mixture was assessed. Insolubility was defined as the formation of a precipitate of the substance in the final mixture under the test conditions and evident to the unaided eye (Di Sotto et al. 2009).

Starting from the highest soluble concentration, a cytotoxicity assay was performed in order to find the highest concentration to study in the mutagenicity test. A cytotoxic effect was evaluated as a reduction (>30%) in the number of revertant colonies and as a change in the auxotrophic background growth (background lawn), in comparison with the control plates (Di Sotto et al. 2009). To perform the test, solutions of test substances (50 μL) were added to an overnight culture (100 μL) and S9 mixture or phosphate buffer (0.1 mol/L; 500 μL). The mixture was pre-incubated under shaking at 37°C for 30 min, and then, it was added with top agar (2 mL) containing 10% of histidine/biotin (0.5 mmol/L) for TA 98 and TA 100, and 10% of tryptophan (0.5 mmol/L) for WP2*uvr*A; finally, it was poured onto a minimal agar plate. After incubation at 37°C for 48 h, the plates were examined, the background lawn was observed, and the bacterial colonies were scored.

Mutagenicity assay.—The mutagenicity was assayed by the pre-incubation method (Di Sotto et al. 2009, 2014), starting from the highest non-toxic concentration (dilution factor from 1:2 to 1:5). The vehicle DMSO (2% v/v) was used as negative control. The mutagens SA (1 μg/plate for TA1535 and TA100), 9AA (50 μg/plate for TA1537), 2NF (2 μg/plate for TA98), MMS (500 μg/plate for WP2*uvr*A), 2AA (1 μg/plate for TA98 and TA100 and 10 μg/plate for TA1535, TA1537, and WP2*uvr*A) were used as positive controls, in order to verify the bacteria susceptibility to a known genotoxic damage. The experiments were repeated at least twice, and each concentration was tested in triplicate.

To perform the test, an overnight culture (100 μL) was added to the solutions of the samples tested (50 μL) plus S9 mixture or phosphate buffer (0.1 mol/L; 500 μL). The mixture was gently vortexed in a sterile tube and then incubated under shaking at 37°C for 30 min. After the pre-incubation, the tubes were added with top agar (2 mL) containing 10% of histidine/biotin (0.5 mmol/L) for TA98 and TA100, and 10% of tryptophan (0.5 mmol/L) for WP2*uvr*A, then gently vortexed, and poured onto a minimal agar plate. The plates were incubated at 37°C for 72 h and then examined. The histidine- or tryptophan-independent revertant colonies and the viable cells were scored, and the bacterial background lawn was observed. A positive response in the mutagenicity assay was defined as an increase (at least twofold above the vehicle) in the histidine- or tryptophan-independent revertant colonies (Di Sotto et al. 2009).

Statistical analysis

All values are expressed as mean ± standard error. One-way analysis of variance (one-way ANOVA), followed by Dunnett's multiple comparison post-test, was used to verify the significance of a positive response. A P value <0.05 was considered statistically significant. Statistical analysis was performed with GraphPad Prism (version 4.00) software (GraphPad Software, San Diego, California, USA).

Results and Discussion

Human-mediated impacts lead to different responses considering the observation scale. At macroscale, a dramatic coverage and limited reduction in *Posidonia oceanica* meadows have been reported along Latium coast (Ardizzone et al. 2006). At the finer scale, physiological responses are mainly photoacclimatation to low light conditions, mechanisms to reduce the respiratory demand of lower carbon-fixing leaves, concentration and relative proportion of *chlorophyll a* and *b*, reduction in carbohydrates concentration, and imbalance in the carbon economy and nitrogen accumulation (Dalla Via et al. 1998, Ruiz and Romero 2001).

In the HPTLC analytic approach, the comparison of fingerprints was the core of the analysis. Quantitative differences can be better evidenced and considered after densitometric conversion.

The analysis of leaves (Fig. 2) shows an evident depression of the intensity of several spots, corresponding to a minor production of the secondary metabolites in shadowed stations. No leaves were

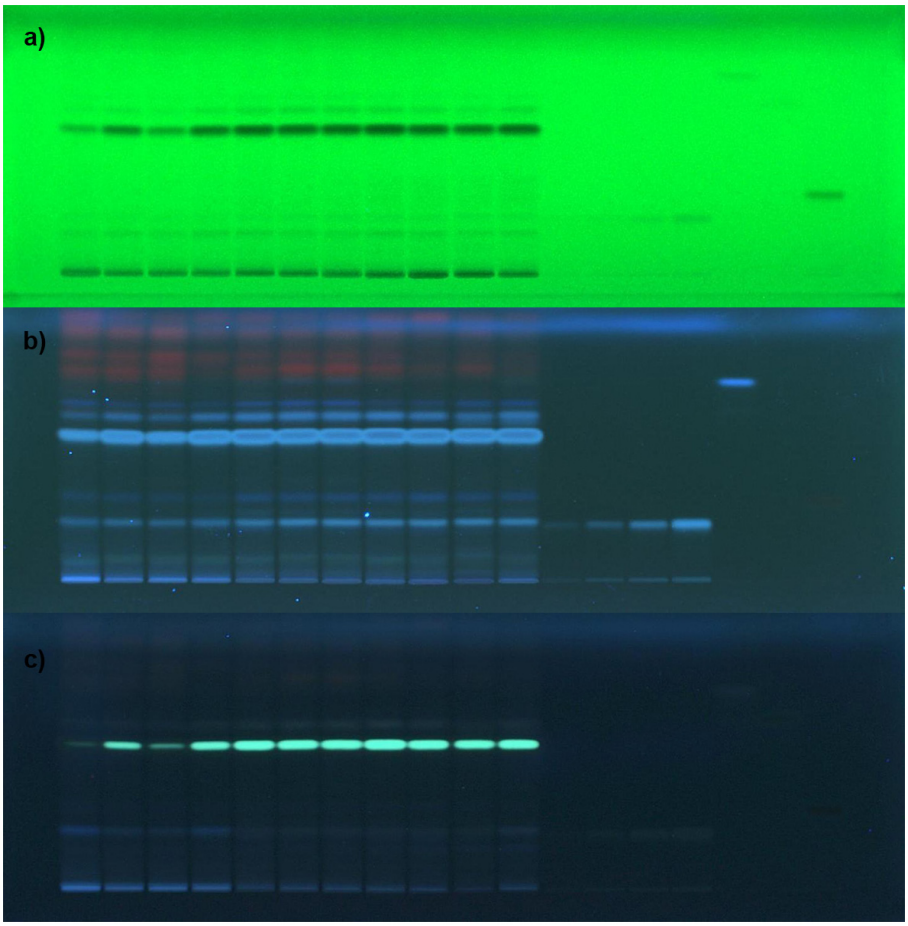

Fig. 2. High-performance thin-layer chromatography plates of *Posidonia oceanica* leaf (L) extracts. Tracks: 1. P1L; 2. P2L; 3. P3L; 4. P4L; 5. P5L; 6. P6L; 7. P11L; 8. P10L; 9. P9L; 10. P8L; 11. P7L; 12. chlorogenic acid (CGA) 0.25 mmol/L; 13. CGA 0.5 mmol/L; 14. CGA 1 mmol/L; 15. CGA 2 mmol/L; 16. caffeic acid; 17. epicatechin; 18. isoquercitrin. (a) Visualization, UV 254 nm, without derivatization. (b) Visualization, UV 366 nm, without derivatization. (c) Visualization, UV 366 nm, derivatization with natural product reagent.

collected below Costa Concordia wreck because no living plants were present (ES). Samples P1 and P3 (HS and PS, respectively) were evidently affected, and they show a minor concentration of secondary metabolites, in particular the fluorescent spot at Rf 0.50 (Fig. 2c), just in the middle of the tracks, evidences a detriment of intensity. These spots are usually assigned to polymeric polyphenols of high MW, and these substances are considered very important in the plant metabolism as a natural defense against external oxidant agents present in the environment or internal reactive oxygen species (ROS) produced as a consequence of stress condition (Grace and Logan 2000). Instead, samples P2, P4, P5, and P6 (PS and NS) were more similar to the references of *Posidonia* leaves at stations P7–P11 (control site). As expected, the production of this kind of substances was mostly affected in samples HS and PS, in particular P1 and P3, than in sample NS, which could mean a deterioration of the health status of the meadow due to the decrease in irradiance. Concerning the stations P7–P11, corresponding to the samples not affected and therefore used as standards, the presence of a higher concentration of polyphenols is due to the higher intensity of sunlight. Attention was also focused on chlorogenic acid (CGA) production. This compound is

ubiquitous in plant metabolism and therefore usually neglected in the phytochemical studies. However, recently, the importance of this polyphenol was evidenced, being CGA used as a marker of the metabolic changes (Grace and Logan 2000) and studied for the biological activity (Farah et al. 2008, Mollica et al. 2016). Several authors clearly underlined its key role in plant reactions to environmental O_3 changes (Del Moral 1972) and, together with foliar phenols, its implication as a biochemical indicator has been suggested (Sager et al. 2005), although quantitative changes can be different for each polyphenol (Moglia et al. 2008).

The analysis of rhizomes (Figs. 3, 4) confirms previous considerations about samples of leaves concerning the production of metabolites. In this case, each underground corm was divided, separating the external part, due to death tissues and to the residues of the old leaves, and the internal part; in fact, the analysis of the external portions showed no accumulation of any important metabolite, and therefore, attention was focused on the internal tissues of the rhizomes. Samples P3 to P11 (PS, NS, and control; Fig. 3) revealed a strong concentration of polymeric polyphenols, in particular catechin and epigallocatechin (black spots, respectively, at Rf 0.63 and 0.32); instead, samples P1 and P2 (HS and PS) showed a minor production of them. In contrast, these metabolites were practically absent in corms of samples collected under the wreck (PB, PM, and PS-ES), remembering that no living plants were present under the wreck because these plants had only rhizomes without leaves. This is in total agreement with previous consideration on the effect of sunlight exposition, and confirmed by the presence of (+)-catechin, considered a low MW polyphenol and a precursor of polymeric polyphenols, such as condensed tannins.

Together with the secondary metabolites, attention was focused on lipids, considering their importance as metabolic resources reservoir. Using

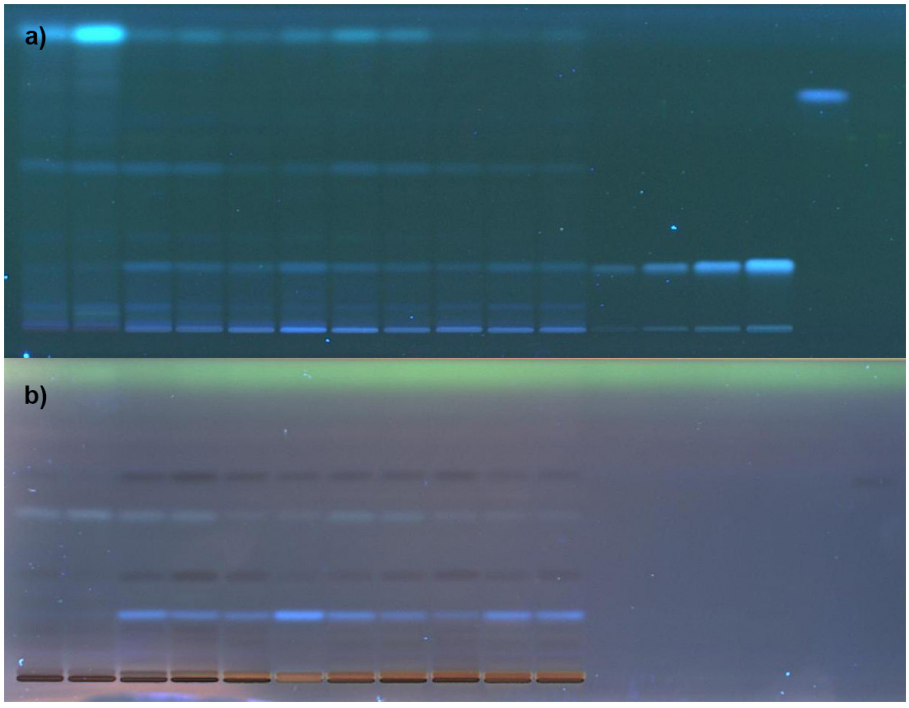

Fig. 3. High-performance thin-layer chromatography plates of *Posidonia oceanica* rhizome (R) extracts. Tracks: 1. P1R; 2. P2R; 3. P3R; 4. P4R; 5. P5R; 6. P6R; 7. P11R; 8. P10R; 9. P9R; 10. P8R; 11. P7R; 12. chlorogenic acid (CGA) 0.25 mmol/L; 13. CGA 0.5 mmol/L; 14. CGA 1 mmol/L; 15. CGA 2 mmol/L; 16. caffeic acid; 17. epicatechin. (a) Visualization, UV 366 nm, without derivatization. (b) Visualization, UV 366 nm, derivatization with natural product reagent and anisaldehyde/sulfuric.

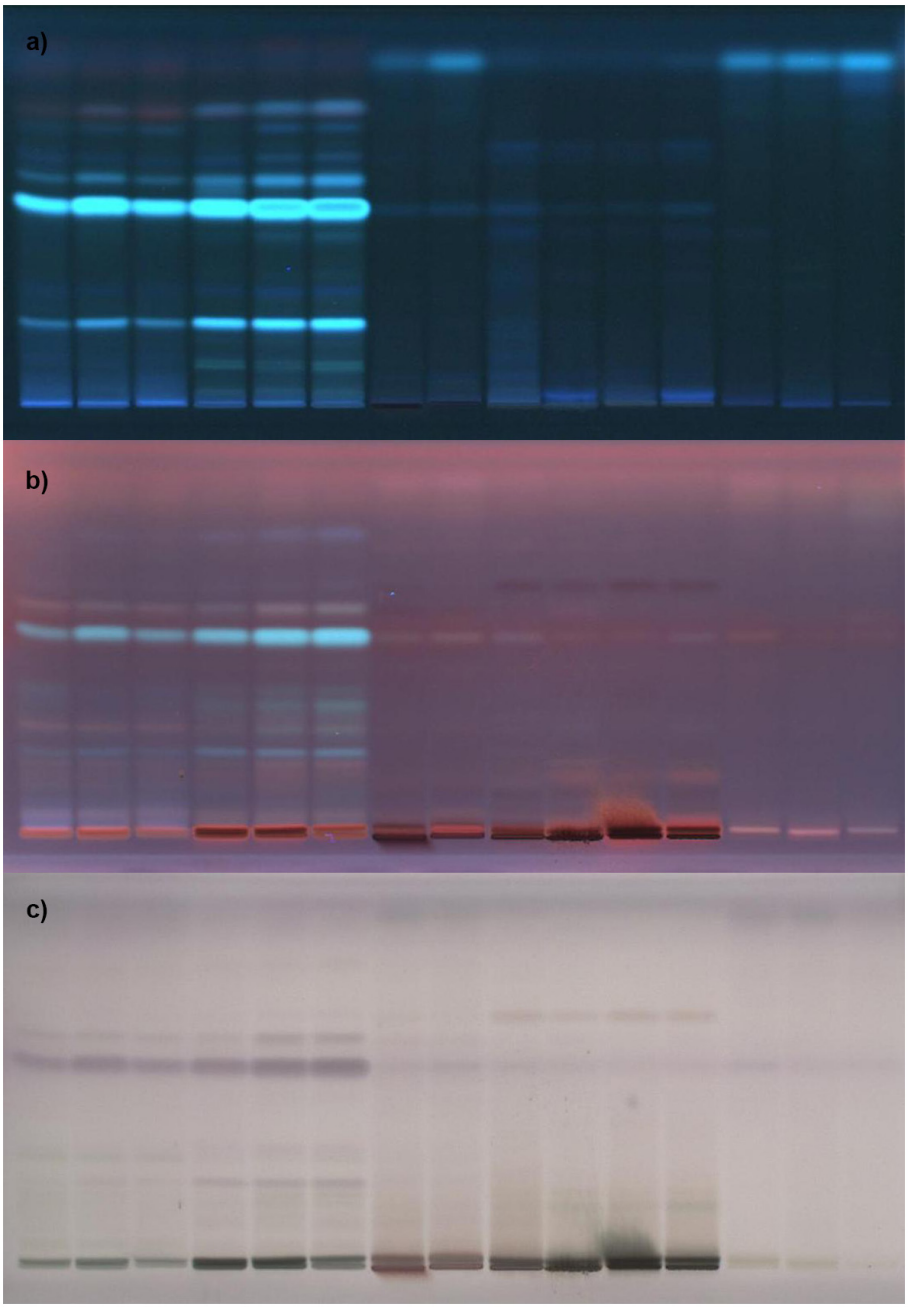

Fig. 4. High-performance thin-layer chromatography plates of *Posidonia oceanica* extracts of leaf (L) and rhizome (R) tracks: 1. P1L; 2. P2L; 3. P3L; 4. P9L; 5. P10L; 6. P11L; 7. P1R; 8. P2R; 9. P3R; 10. P9R; 11. P10R; 12. P11R; 13. PB; 14. PM; 15. PS; (a) visualization, UV 366 nm, without derivatization. (b) Visualization, UV 366 nm, derivatization with natural product reagent. (c) Visualization, WRT light, derivatization with natural product reagent.

a targeted analysis, besides the aforementioned minor production of several metabolites (in particular in the leaves), an accumulation of lipids can be evidenced in corms of plants mainly deprived of light. The accumulation of these lipids was present particularly in samples under the Pioneer platform blue spots (Rf 0.88) in samples P1-P2 and PB, PM, and PS (Fig. 4a); these compounds

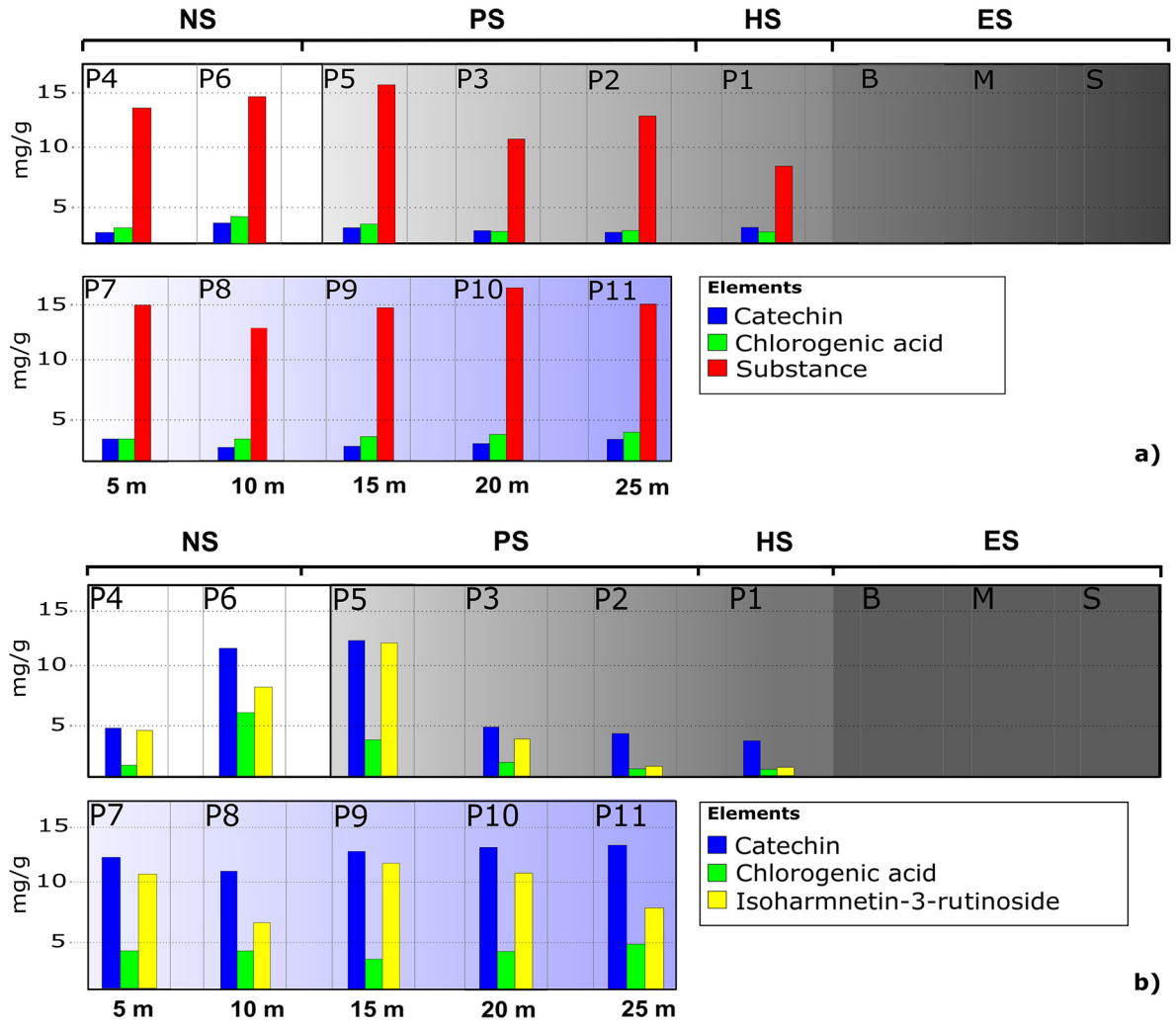

Fig. 5. Densitometric conversion after high-performance thin-layer chromatography analyses on (a) leaves and (b) rhizomes; the shades of gray below plots' background indicate different shading conditions. ES, extreme shadow; HS, high shadow; PS, partial shadow; NS, normal shadow.

correspond to fatty alcohols and acids. This situation can have two explanations: (1) the very evident schlerotization of the corms, as an extreme tentative of preservation, with the accumulation of substances such as suberin in the root endodermis, and (2) the necessity to limit the metabolic production to basic constituents, due to the stress conditions (Díaz et al. 1997).

For an easier comprehension, the analyses carried out on leaf and rhizome samples are summarized in Fig. 5a, b, after densitometric conversion. Changes in flavonoid and phenolic compounds of *P. oceanica* were principally due to nutrient enrichment (Cannac et al. 2006) and to competition with algal species, such as *Caulerpa taxifolia* (Vahl) C. Agardh and *Caulerpa racemosa* (Forsskål) J. Agardh (Dumay et al. 2004). Although other studies need to be carried out on the chemistry of *P. oceanica*, it is likely that ecological factors have a potential influence on secondary metabolites; therefore, they may be used as indicators of seagrass health status (Agostini et al. 1998, Heglmeier and Zidorn 2010, Bitam et al. 2012).

The results of HPTLC analysis on the metabolism of *Posidonia* plants highlighted that the

environmental impact of Costa Concordia wreck and removal operations on *P. oceanica* was mainly due to the absence of light. The results were confirmed by the mutagenicity studies. For this purpose, no signs of insolubility were highlighted for all the samples tested up to the concentration of 5000 μg/plate (corresponding to 1887 μg/mL). All the samples were cytotoxic at the concentration of 5000 μg/plate in all the strains tested, both in the absence and in the presence of the S9 metabolic activator (data not shown). In spite of the other samples, both leaves and rhizomes of samples 1 and 2, at concentrations of 500 and 1000 μg/plate, significantly reduced the number of spontaneous revertant colonies in TA1537 and TA100 strains in the absence of S9, so exhibiting signs of cytotoxicity (Table 2). The presence of the metabolic activator reduced the cytotoxicity, being the samples not cytotoxic at 500 μg/plate in both strains (Table 3). The cytotoxicity profile can be ascribed to a different composition of leaves and rhizomes of P1 and P2 with respect to the other samples tested: This allows us to hypothesize that some changes in P1 and P2 samples of *P. oceanica* occurred, due to the absorption of some cytotoxic compounds released into environment or to the production of toxic plant metabolites by altered metabolic pathways. These compounds may be detoxified by the CYP450 enzymes, added to the system by the S9 mixture.

When tested at non-toxic concentrations (ranging from 10 to 1000 μg/plate) in the mutagenicity assay, the samples did not increase the number of revertant colonies in all strains tested both in the absence and in the presence of the metabolic activator S9 (Appendix S1: Tables S1 and S2). This allows us to hypothesize that the samples are lacking for components able to induce point mutations, through frameshift, base substitution, cross-linking, and oxidative mechanisms. Conversely, the mutagens 2NF, SA, 9AA, MMS, and 2AA significantly increased the number of revertant colonies with respect to vehicle (Appendix S1: Tables S1 and S2). Although just few studies have been carried out on *P. oceanica* DNA variation due to pollution induction, this plant seems to have a quick response especially to cadmium and mercury exposition (Maestrini et al. 2002, Greco et al.

Table 2. Effect of leaves and rhizomes from *Posidonia oceanica* on the spontaneous revertant colonies of *Salmonella Typhimurium* TA1535, TA1537, TA98, and TA100 and *Escherichia coli* WP2*uvr*A, in the absence of the metabolic activator S9.

		Number of revertant colonies				
Sample	μg/plate	TA1535	TA1537	TA98	TA100	WP2*uvr*A
Leaves						
P1	10	57.0 ± 2.1	39.7 ± 2.3	40.0 ± 2.3	106.7 ± 4.4	45.3 ± 2.9
	50	58.0 ± 3.6	38.3 ± 1.8	39.7 ± 3.8	106.0 ± 4.3	46.7 ± 2.9
	100	56.3 ± 1.8	38.3 ± 2.6	38.7 ± 1.8	103.7 ± 3.8	44.3 ± 3.5
	500	47.7 ± 5.2	24.3 ± 2.0†	38.7 ± 3.7	72.0 ± 5.0†	42.7 ± 2.7
	1000	51.0 ± 2.1	21.0 ± 2.3†	36.7 ± 2.0	66.4 ± 2.6†	35.3 ± 3.2
P2	10	57.3 ± 1.2	38.3 ± 2.0	39.0 ± 2.9	105.3 ± 4.2	48.7 ± 2.6
	50	58.3 ± 3.4	38.3 ± 1.7	39.7 ± 3.2	106.3 ± 3.8	48.3 ± 3.2
	100	61.3 ± 2.3	38.0 ± 3.2	38.3 ± 2.9	105.7 ± 4.3	47.7 ± 4.0
	500	64.0 ± 2.3	27.3 ± 2.0†	39.3 ± 3.8	75.3 ± 4.2†	49.3 ± 3.2
	1000	52.3 ± 1.4	22.5 ± 1.3†	35.0 ± 2.3	74.3 ± 3.8†	47.0 ± 2.3
P3	10	64.0 ± 4.6	41.3 ± 2.4	38.7 ± 2.9	121.3 ± 5.2	48.0 ± 3.0
	50	69.3 ± 1.3	37.7 ± 1.9	40.0 ± 2.3	121.0 ± 5.7	53.3 ± 2.4
	100	59.7 ± 4.6	37.3 ± 2.6	38.7 ± 1.3	119.0 ± 2.5	52.0 ± 1.1
	500	58.7 ± 2.4	35.7 ± 2.3	38.3 ± 2.7	117.3 ± 1.3	49.3 ± 1.8
	1000	58.0 ± 4.2	37.3 ± 2.0	35.3 ± 2.3	101.3 ± 4.5	41.3 ± 3.0
P4	10	58.0 ± 5.0	38.3 ± 2.6	37.0 ± 3.2	119.3 ± 5.2	45.0 ± 2.1
	50	60.0 ± 4.2	36.7 ± 2.6	38.3 ± 2.9	121.7 ± 5.2	52.7 ± 1.8
	100	60.0 ± 2.3	38.7 ± 2.7	39.7 ± 2.3	119.7 ± 2.6	53.3 ± 4.4
	500	60.7 ± 1.8	37.7 ± 2.9	38.7 ± 1.3	116.7 ± 2.7	44.7 ± 3.7
	1000	59.3 ± 2.7	37.3 ± 1.8	37.7 ± 2.6	101.0 ± 5.1	44.0 ± 3.5

(Table 2. *Continued*)

Sample	µg/plate	Number of revertant colonies				
		TA1535	TA1537	TA98	TA100	WP2*uvr*A
P5	10	57.3 ± 4.0	37.3 ± 2.6	37.3 ± 2.6	120.3 ± 5.2	49.3 ± 3.3
	50	59.7 ± 5.0	37.3 ± 2.4	38.3 ± 3.3	121.3 ± 6.3	53.3 ± 1.3
	100	64.7 ± 2.7	39.0 ± 2.1	40.0 ± 2.1	120.0 ± 2.5	41.3 ± 3.5
	500	65.3 ± 3.2	36.3 ± 3.5	38.0 ± 1.1	116.0 ± 3.0	52.0 ± 3.0
	1000	57.3 ± 4.0	37.3 ± 2.4	35.7 ± 1.9	100.3 ± 3.3	46.0 ± 3.0
P6	10	64.0 ± 3.5	38.7 ± 2.6	37.0 ± 2.9	118.7 ± 5.2	50.7 ± 2.2
	50	68.0 ± 2.3	39.3 ± 2.4	38.7 ± 3.2	118.0 ± 4.7	50.7 ± 2.4
	100	64.3 ± 3.5	38.7 ± 2.0	39.0 ± 1.1	115.0 ± 4.0	49.3 ± 2.9
	500	56.7 ± 2.4	37.3 ± 1.8	41.7 ± 2.0	117.0 ± 4.0	52.0 ± 3.0
	1000	61.3 ± 3.5	36.0 ± 3.1	37.7 ± 3.0	102.7 ± 6.7	55.3 ± 2.9
P10m	10	51.7 ± 2.0	39.3 ± 2.6	38.3 ± 2.9	115.0 ± 2.6	46.7 ± 1.3
	50	53.3 ± 0.7	40.7 ± 1.3	38.7 ± 3.5	117.3 ± 3.5	52.0 ± 3.5
	100	52.7 ± 1.3	39.3 ± 2.0	38.3 ± 1.8	116.7 ± 2.7	48.0 ± 2.3
	500	56.0 ± 2.0	37.7 ± 1.4	41.0 ± 2.3	116.3 ± 4.1	49.3 ± 3.2
	1000	56.0 ± 1.1	39.3 ± 1.2	37.0 ± 2.9	106.0 ± 3.0	46.7 ± 2.6
P15m	10	59.3 ± 1.9	42.0 ± 3.0	37.3 ± 3.5	120.3 ± 5.2	46.7 ± 2.6
	50	57.0 ± 3.0	37.3 ± 2.1	39.0 ± 3.5	120.0 ± 4.0	46.3 ± 2.7
	100	57.7 ± 1.4	39.0 ± 2.1	38.7 ± 1.4	120.0 ± 2.5	46.7 ± 1.3
	500	57.0 ± 4.2	39.3 ± 3.2	41.0 ± 2.3	119.3 ± 2.3	49.3 ± 2.7
	1000	57.3 ± 2.3	36.3 ± 5.3	37.3 ± 2.9	101.0 ± 3.5	46.7 ± 3.5
P20m	10	61.3 ± 1.9	40.3 ± 2.7	37.3 ± 2.9	120.0 ± 4.6	48.0 ± 1.1
	50	59.0 ± 3.0	38.7 ± 1.9	39.0 ± 2.9	120.7 ± 5.8	44.7 ± 3.5
	100	57.7 ± 3.2	38.0 ± 2.3	38.7 ± 1.4	119.3 ± 5.2	42.7 ± 2.4
	500	60.3 ± 4.5	38.0 ± 2.9	40.7 ± 1.8	118.7 ± 2.7	47.3 ± 3.5
	1000	56.3 ± 2.3	36.8 ± 5.6	36.7 ± 3.2	117.3 ± 5.3	48.7 ± 2.4
P25m	10	59.3 ± 2.6	38.0 ± 2.6	37.7 ± 2.4	114.7 ± 4.8	45.7 ± 3.2
	50	58.3 ± 2.4	37.7 ± 1.9	37.7 ± 3.2	119.7 ± 2.6	48.0 ± 3.0
	100	59.3 ± 3.5	38.3 ± 2.0	39.7 ± 1.2	120.0 ± 2.3	47.7 ± 3.0
	500	58.3 ± 3.8	38.0 ± 2.3	39.7 ± 2.0	121.0 ± 3.2	47.0 ± 1.7
	1000	57.7 ± 2.0	36.7 ± 4.2	38.3 ± 2.7	107.3 ± 4.0	49.3 ± 1.8
P30m	10	60.0 ± 4.2	38.3 ± 2.6	38.3 ± 2.3	115.3 ± 2.9	48.7 ± 2.4
	50	60.7 ± 4.0	39.3 ± 2.6	38.0 ± 3.5	110.0 ± 4.6	48.0 ± 3.0
	100	58.0 ± 1.1	40.0 ± 1.1	39.0 ± 3.5	113.0 ± 7.0	47.0 ± 3.8
	500	61.3 ± 4.4	38.7 ± 2.3	41.0 ± 2.3	114.7 ± 5.8	48.7 ± 3.2
	1000	53.3 ± 1.3	36.7 ± 5.2	38.0 ± 2.6	100.0 ± 4.2	47.3 ± 3.2
Rhizomes						
P1	10	59.0 ± 2.6	37.0 ± 6.0	37.5 ± 4.5	108.9 ± 7.1	51.0 ± 1.0
	50	61.3 ± 2.6	39.5 ± 3.5	40.5 ± 2.5	104.3 ± 6.3	50.0 ± 4.0
	100	61.0 ± 2.1	41.0 ± 6.0	36.5 ± 4.5	104.5 ± 7.4	50.0 ± 4.0
	500	60.7 ± 2.0	23.0 ± 1.0[t]	40.0 ± 2.0	69.0 ± 2.0[t]	44.7 ± 5.8
	1000	58.0 ± 1.5	12.5 ± 3.5[t]	37.5 ± 3.5	61.0 ± 8.0[t]	50.7 ± 4.8
P2	10	60.0 ± 2.1	41.0 ± 2.0	36.0 ± 3.0	107.9 ± 4.8	51.0 ± 6.1
	50	60.0 ± 2.9	38.5 ± 2.5	36.5 ± 1.5	102.8 ± 7.9	53.0 ± 9.0
	100	61.3 ± 2.6	36.0 ± 2.0	35.5 ± 6.5	99.0 ± 3.0	49.3 ± 6.6
	500	55.0 ± 2.0	24.0 ± 2.0[t]	39.5 ± 1.5	70.0 ± 6.0[t]	46.7 ± 7.0
	1000	57.3 ± 1.4	19.0 ± 1.0[t]	36.0 ± 2.0	52.0 ± 4.0[t]	46.7 ± 6.4
P3	10	62.0 ± 8.0	39.0 ± 2.0	37.0 ± 2.0	115.1 ± 9.4	46.0 ± 2.0
	50	57.0 ± 5.0	37.0 ± 2.0	35.0 ± 2.9	101.0 ± 8.4	46.0 ± 4.0
	100	60.0 ± 0.0	38.5 ± 2.5	37.0 ± 6.0	101.1 ± 4.4	50.0 ± 4.0
	500	56.7 ± 5.3	39.0 ± 3.0	35.5 ± 3.5	104.0 ± 4.0	49.0 ± 5.0
	1000	62.0 ± 8.0	42.0 ± 4.3	37.0 ± 4.2	96.0 ± 4.0	48.0 ± 6.1

(Table 2. *Continued*)

Sample	μg/plate	Number of revertant colonies				
		TA1535	TA1537	TA98	TA100	WP2*uvr*A
P4	10	61.5 ± 6.5	41.0 ± 2.0	41.0 ± 3.0	101.3 ± 8.7	53.3 ± 1.3
	50	62.0 ± 9.3	38.0 ± 4.0	36.5 ± 5.5	100.0 ± 9.5	53.0 ± 1.0
	100	58.0 ± 8.0	35.0 ± 2.0	37.5 ± 1.5	109.0 ± 1.0	44.0 ± 5.3
	500	61.0 ± 3.0	34.0 ± 2.0	36.5 ± 4.5	99.3 ± 5.3	47.3 ± 2.9
	1000	61.0 ± 3.0	39.0 ± 4.0	38.0 ± 4.0	103.3 ± 6.8	48.0 ± 3.0
P5	10	59.5 ± 3.5	40.0 ± 2.8	41.0 ± 2.0	105.9 ± 7.6	47.0 ± 5.0
	50	59.0 ± 2.0	42.5 ± 1.5	39.0 ± 6.0	107.7 ± 9.1	45.0 ± 3.0
	100	56.0 ± 2.0	34.5 ± 5.5	40.0 ± 3.0	101.6 ± 1.1	46.9 ± 4.0
	500	63.5 ± 5.5	38.0 ± 4.0	40.0 ± 2.0	98.0 ± 4.0	46.0 ± 6.0
	1000	56.0 ± 2.0	40.0 ± 2.0	42.0 ± 6.0	101.0 ± 5.0	42.0 ± 2.0
P6	10	60.0 ± 6.0	44.5 ± 2.5	41.5 ± 6.5	109.7 ± 5.8	44.0 ± 2.0
	50	63.5 ± 2.5	42.5 ± 1.5	43.0 ± 4.0	108.7 ± 7.3	45.0 ± 3.0
	100	64.0 ± 1.0	42.5 ± 4.5	43.0 ± 6.0	103.8 ± 5.0	43.0 ± 5.0
	500	56.5 ± 2.5	42.0 ± 3.0	41.5 ± 6.5	104.4 ± 6.3	44.0 ± 3.0
	1000	56.5 ± 1.5	44.5 ± 3.5	37.0 ± 1.0	119.2 ± 0.8	48.5 ± 3.5
P10m	10	63.0 ± 2.0	40.0 ± 5.0	44.0 ± 2.0	106.6 ± 3.2	50.0 ± 4.0
	50	62.0 ± 7.0	42.0 ± 3.0	40.5 ± 5.5	110.6 ± 2.3	48.0 ± 6.0
	100	62.5 ± 3.5	38.5 ± 2.5	38.0 ± 5.0	109.3 ± 3.6	45.0 ± 6.0
	500	60.0 ± 6.0	42.0 ± 0.0	34.0 ± 2.0	112.0 ± 4.0	44.0 ± 2.0
	1000	57.0 ± 0.0	38.0 ± 0.0	39.5 ± 1.0	114.0 ± 4.0	42.0 ± 8.0
P15m	10	55.0 ± 8.0	41.0 ± 2.0	38.5 ± 6.5	107.5 ± 6.8	44.0 ± 4.0
	50	63.0 ± 2.0	42.0 ± 5.0	41.0 ± 8.0	118.1 ± 4.8	48.0 ± 5.0
	100	62.5 ± 3.5	42.0 ± 5.0	40.5 ± 4.5	112.1 ± 3.7	45.0 ± 7.0
	500	61.0 ± 4.0	39.5 ± 3.5	40.5 ± 6.5	116.7 ± 3.4	43.0 ± 6.0
	1000	65.5 ± 3.5	38.5 ± 2.5	38.0 ± 1.0	110.0 ± 4.3	40.7 ± 4.7
P20m	10	61.5 ± 5.5	39.0 ± 1.9	40.5 ± 1.5	112.4 ± 1.0	44.8 ± 6.0
	50	60.5 ± 2.5	38.0 ± 6.2	41.0 ± 4.0	113.3 ± 8.6	46.0 ± 4.0
	100	58.5 ± 2.5	36.5 ± 5.5	39.5 ± 4.5	104.6 ± 9.9	43.0 ± 6.0
	500	60.3 ± 2.3	39.0 ± 3.3	36.0 ± 4.0	102.0 ± 6.0	42.0 ± 8.6
	1000	59.0 ± 4.0	40.5 ± 1.7	33.5 ± 5.5	102.0 ± 4.0	48.0 ± 7.2
P25m	10	60.5 ± 8.5	37.3 ± 4.0	41.0 ± 3.0	101.5 ± 4.2	42.0 ± 2.0
	50	58.5 ± 2.5	38.2 ± 3.0	42.5 ± 3.5	111.0 ± 4.9	43.0 ± 3.0
	100	58.5 ± 8.5	41.4 ± 3.0	40.5 ± 2.5	111.1 ± 9.5	45.0 ± 7.0
	500	59.0 ± 1.0	39.0 ± 3.0	34.0 ± 5.0	103.0 ± 9.9	48.0 ± 2.0
	1000	57.5 ± 5.5	34.0 ± 2.5	36.5 ± 1.5	100.7 ± 4.9	44.0 ± 4.0
P30m	10	58.5 ± 9.5	38.0 ± 2.0	42.5 ± 1.5	101.0 ± 7.0	45.6 ± 7.8
	50	58.0 ± 8.0	31.5 ± 4.5	42.5 ± 2.5	109.4 ± 3.5	43.5 ± 7.1
	100	59.5 ± 6.5	29.7 ± 3.0	41.0 ± 3.0	104.6 ± 3.9	44.0 ± 0.0
	500	56.0 ± 4.0	30.0 ± 2.0	41.0 ± 9.0	102.0 ± 2.0	43.0 ± 8.0
	1000	54.0 ± 2.0	30.5 ± 3.0	40.0 ± 4.0	103.1 ± 2.5	46.0 ± 10.0
2-NF	2.0	–	–	162.7 ± 4.8	–	–
Sodium azide	1.0	2057 ± 43.3	–	–	1547 ± 48.1	–
9-AA	50.0	–	102 ± 2.3	–	–	–
Methyl methanesulfonate	500.0	–	–	–	–	507.0 ± 4.7
Vehicle‡		60.0 ± 2.3	39.3 ± 1.3	38.7 ± 2.3	106.7 ± 5.8	45.3 ± 3.5

Notes: Values are expressed as mean ± SEM (n = 6 plates). Cells with no value were not tested.

† Toxicity, evaluated as a reduction (>30%) in the number of revertant colonies and as a change in the auxotrophic background lawn), in comparison with the control plates.

‡ DMSO, 50 μL/plate.

Table 3. Effect of leaves and rhizomes from *Posidonia oceanica* on the spontaneous revertant colonies of *Salmonella Typhimurium* TA1535, TA1537, TA98, and TA100 and *Escherichia coli* WP2*uvr*A, in the presence of the metabolic activator S9.

Sample	μg/plate	Number of revertant colonies				
		TA1535	TA1537	TA98	TA100	WP2*uvr*A
Leaves						
P1	10	37.3 ± 1.8	34.0 ± 2.3	68.0 ± 2.6	69.7 ± 2.6	51.7 ± 2.9
	50	39.0 ± 2.3	35.3 ± 2.4	69.3 ± 2.9	72.0 ± 3.5	54.0 ± 3.5
	100	38.0 ± 3.2	34.3 ± 1.8	69.0 ± 2.3	71.0 ± 3.5	53.7 ± 2.3
	500	35.7 ± 3.8	33.0 ± 2.1	68.7 ± 3.8	68.0 ± 2.3	55.0 ± 3.2
	1000	35.3 ± 2.4	21.3 ± 1.8	67.0 ± 3.8	47.0 ± 2.6	44.3 ± 2.7
P2	10	38.3 ± 1.8	34.0 ± 1.7	68.0 ± 4.0	71.0 ± 2.9	53.0 ± 3.2
	50	38.0 ± 2.3	36.3 ± 2.6	71.0 ± 3.2	70.7 ± 3.5	53.3 ± 3.8
	100	38.3 ± 2.3	34.3 ± 2.6	68.7 ± 3.2	72.0 ± 4.4	54.0 ± 1.7
	500	35.3 ± 2.0	32.7 ± 2.6	67.7 ± 3.5	66.3 ± 2.0	51.0 ± 2.1
	1000	33.7 ± 2.6	26.0 ± 2.1	66.0 ± 2.9	44.0 ± 2.6	38.0 ± 3.2
P3	10	38.3 ± 3.0	34.7 ± 1.7	68.3 ± 2.3	70.3 ± 2.0	50.7 ± 3.0
	50	40.0 ± 4.2	35.0 ± 2.1	68.0 ± 3.2	71.7 ± 3.5	53.3 ± 2.7
	100	39.3 ± 2.7	35.7 ± 2.6	69.7 ± 2.6	72.3 ± 5.0	57.3 ± 2.9
	500	38.7 ± 4.0	34.7 ± 4.4	69.0 ± 2.9	67.0 ± 2.6	56.3 ± 4.0
	1000	36.7 ± 2.7	36.7 ± 1.0	64.3 ± 2.9	65.0 ± 2.3	61.3 ± 1.3
P4	10	36.7 ± 2.7	34.3 ± 2.3	69.3 ± 1.8	70.0 ± 2.5	57.3 ± 3.5
	50	35.3 ± 2.4	37.7 ± 2.3	70.3 ± 2.7	70.7 ± 2.3	59.3 ± 4.8
	100	36.7 ± 1.3	35.7 ± 2.3	69.3 ± 3.2	72.0 ± 3.8	55.0 ± 3.6
	500	36.3 ± 2.6	34.3 ± 2.6	68.7 ± 3.0	67.0 ± 3.0	56.0 ± 3.5
	1000	36.0 ± 2.0	35.0 ± 2.3	65.0 ± 2.9	65.3 ± 2.9	57.3 ± 3.5
P5	10	36.0 ± 3.1	33.3 ± 2.0	69.0 ± 3.5	71.0 ± 1.7	53.3 ± 1.8
	50	35.3 ± 3.5	34.0 ± 1.2	71.0 ± 2.5	70.7 ± 4.0	53.3 ± 3.7
	100	36.0 ± 3.5	33.3 ± 2.6	68.0 ± 3.2	72.3 ± 3.8	51.7 ± 3.5
	500	36.7 ± 1.3	34.3 ± 2.3	69.7 ± 2.0	67.0 ± 2.6	57.0 ± 3.2
	1000	36.7 ± 1.8	34.0 ± 1.0	64.7 ± 3.2	66.3 ± 2.0	57.3 ± 2.6
P6	10	38.0 ± 4.0	34.0 ± 2.6	68.0 ± 2.3	70.7 ± 2.3	57.0 ± 2.6
	50	37.3 ± 2.4	35.3 ± 2.4	70.7 ± 1.3	69.7 ± 4.0	56.7 ± 2.9
	100	42.0 ± 3.5	33.7 ± 2.6	68.7 ± 2.0	73.3 ± 3.8	60.3 ± 2.6
	500	36.8 ± 1.3	32.7 ± 3.8	69.0 ± 2.6	68.0 ± 2.6	57.3 ± 2.4
	1000	39.3 ± 1.3	32.7 ± 3.6	64.7 ± 3.0	67.0 ± 2.9	56.3 ± 2.3
P10m	10	36.7 ± 2.9	33.7 ± 1.7	73.0 ± 4.4	71.3 ± 2.0	55.3 ± 4.4
	50	38.7 ± 2.9	34.7 ± 2.0	75.3 ± 2.9	71.7 ± 4.1	54.3 ± 3.4
	100	37.7 ± 2.0	36.0 ± 1.7	71.0 ± 2.6	73.0 ± 3.2	57.3 ± 4.1
	500	37.7 ± 3.5	34.0 ± 1.7	68.7 ± 1.4	66.3 ± 2.8	57.3 ± 4.0
	1000	39.7 ± 3.3	31.7 ± 2.2	65.3 ± 2.6	65.3 ± 2.9	54.7 ± 3.2
P15m	10	36.7 ± 3.5	33.0 ± 2.3	68.7 ± 1.4	71.7 ± 2.4	54.7 ± 3.5
	50	38.0 ± 3.5	34.3 ± 2.6	69.7 ± 0.9	72.7 ± 2.6	56.0 ± 4.3
	100	37.0 ± 2.5	35.7 ± 2.0	73.3 ± 1.8	68.0 ± 4.1	57.3 ± 3.8
	500	38.7 ± 2.9	37.0 ± 2.1	69.3 ± 3.8	65.3 ± 2.6	54.7 ± 3.3
	1000	34.7 ± 3.7	33.7 ± 2.6	66.7 ± 3.2	72.3 ± 2.0	49.3 ± 6.7
P20m	10	37.3 ± 3.5	36.3 ± 1.4	70.3 ± 1.4	70.7 ± 3.0	56.0 ± 4.3
	50	34.3 ± 1.4	36.3 ± 1.7	68.7 ± 1.4	71.3 ± 4.0	55.7 ± 4.1
	100	38.0 ± 4.0	35.3 ± 2.3	72.3 ± 2.3	73.3 ± 4.2	57.3 ± 4.0
	500	36.7 ± 2.4	34.3 ± 2.0	68.3 ± 3.2	68.0 ± 2.6	50.7 ± 3.5
	1000	41.3 ± 1.3	33.7 ± 3.8	65.3 ± 2.6	66.3 ± 2.0	52.3 ± 4.4
P25m	10	35.3 ± 3.2	35.0 ± 2.1	69.3 ± 2.3	72.3 ± 4.0	54.0 ± 2.3
	50	35.7 ± 2.0	33.7 ± 2.0	69.7 ± 3.8	74.3 ± 3.2	53.7 ± 3.3
	100	35.7 ± 3.5	34.7 ± 1.7	68.7 ± 2.4	73.0 ± 3.2	56.3 ± 4.3
	500	37.7 ± 1.4	34.7 ± 1.8	68.7 ± 3.5	69.3 ± 1.4	54.7 ± 2.7
	1000	33.7 ± 1.4	35.0 ± 2.6	64.0 ± 3.0	68.0 ± 1.5	49.7 ± 5.9

(Table 3. *Continued*)

Sample	µg/plate	Number of revertant colonies				
		TA1535	TA1537	TA98	TA100	WP2*uvr*A
P30m	10	36.7 ± 3.5	36.0 ± 2.1	67.7 ± 4.1	74.0 ± 3.6	61.3 ± 2.7
	50	36.7 ± 3.5	35.3 ± 2.6	71.3 ± 3.5	75.3 ± 2.9	59.3 ± 3.0
	100	36.0 ± 3.6	36.3 ± 1.8	69.0 ± 4.0	76.0 ± 3.2	57.3 ± 3.5
	500	35.0 ± 1.5	33.3 ± 2.4	69.3 ± 2.9	72.3 ± 2.6	56.0 ± 2.3
	1000	34.3 ± 1.3	34.7 ± 2.0	65.7 ± 3.5	68.7 ± 1.8	61.3 ± 2.9
Rhizomes						
P1	10	34.5 ± 2.5	31.0 ± 2.0	62.9 ± 6.5	73.4 ± 7.2	58.0 ± 6.0
	50	35.5 ± 0.5	31.5 ± 6.5	64.2 ± 2.6	73.0 ± 7.6	51.0 ± 5.0
	100	41.5 ± 3.5	39.5 ± 2.5	65.5 ± 3.8	69.3 ± 9.1	52.0 ± 6.0
	500	40.0 ± 1.0	31.5 ± 3.5	60.0 ± 2.0	74.0 ± 6.0	61.0 ± 8.0
	1000	38.5 ± 4.5	16.5 ± 2.5†	58.0 ± 1.0	42.0 ± 5.0†	54.0 ± 4.0
P2	10	42.5 ± 6.5	40.0 ± 3.0	54.8 ± 4.5	68.6 ± 8.5	59.0 ± 3.0
	50	36.0 ± 4.0	35.5 ± 3.5	60.3 ± 7.8	67.9 ± 9.2	52.0 ± 2.0
	100	40.0 ± 4.0	39.0 ± 4.0	59.9 ± 1.3	70.0 ± 6.0	57.0 ± 3.0
	500	39.3 ± 3.5	33.5 ± 2.5	62.5 ± 5.5	71.2 ± 4.2	54.0 ± 10.0
	1000	37.3 ± 2.4	23.0 ± 2.0†	56.2 ± 4.5	47.0 ± 4.0†	58.0 ± 2.0
P3	10	33.0 ± 1.0	34.5 ± 0.5	61.0 ± 2.0	66.3 ± 7.9	55.0 ± 2.0
	50	36.0 ± 0.0	34.5 ± 3.5	59.0 ± 5.0	70.0 ± 8.0	57.0 ± 4.0
	100	34.0 ± 4.0	39.5 ± 1.5	57.0 ± 1.0	74.0 ± 4.0	51.0 ± 7.0
	500	36.0 ± 4.0	38.0 ± 2.0	59.2 ± 4.5	73.0 ± 6.0	52.0 ± 6.0
	1000	38.0 ± 6.0	34.0 ± 0.5	58.2 ± 4.5	64.0 ± 6.3	50.0 ± 8.0
P4	10	38.0 ± 2.0	38.0 ± 5.0	62.9 ± 2.6	67.0 ± 9.0	59.0 ± 8.0
	50	35.5 ± 3.5	33.5 ± 3.5	59.8 ± 1.7	64.3 ± 6.7	59.0 ± 3.0
	100	34.0 ± 4.0	32.0 ± 5.0	61.8 ± 2.4	69.3 ± 9.1	58.0 ± 4.0
	500	34.0 ± 2.0	34.5 ± 2.5	64.0 ± 2.0	72.0 ± 7.0	54.0 ± 4.0
	1000	36.0 ± 4.0	38.0 ± 0.0	60.0 ± 18.0	64.0 ± 4.0	57.0 ± 4.0
P5	10	39.5 ± 1.5	40.0 ± 9.0	57.7 ± 2.2	65.8 ± 7.4	58.0 ± 4.0
	50	35.5 ± 1.5	39.0 ± 4.0	60.5 ± 1.5	69.1 ± 8.9	57.0 ± 1.0
	100	34.0 ± 2.0	39.5 ± 1.5	62.8 ± 1.2	65.7 ± 7.4	58.0 ± 6.0
	500	36.0 ± 2.0	38.0 ± 2.1	64.0 ± 4.0	69.0 ± 7.0	60.0 ± 2.0
	1000	32.0 ± 2.0	32.0 ± 1.5	64.5 ± 2.5	66.0 ± 9.6	58.0 ± 2.0
P6	10	34.0 ± 2.0	37.0 ± 6.0	60.3 ± 2.6	64.3 ± 5.9	59.0 ± 2.6
	50	35.0 ± 2.0	32.5 ± 5.5	62.9 ± 5.2	73.8 ± 5.0	52.2 ± 2.0
	100	33.5 ± 2.5	39.0 ± 4.0	65.5 ± 2.6	71.0 ± 6.4	54.8 ± 3.2
	500	37.0 ± 1.0	31.0 ± 2.0	61.2 ± 2.6	68.4 ± 7.5	53.4 ± 5.2
	1000	35.0 ± 3.0	36.5 ± 1.5	63.0 ± 5.2	68.8 ± 4.2	53.0 ± 2.6
P10m	10	36.0 ± 1.0	36.0 ± 9.0	59.0 ± 2.6	72.4 ± 7.9	54.8 ± 3.2
	50	40.5 ± 2.5	40.0 ± 8.0	62.2 ± 1.9	66.8 ± 8.0	54.2 ± 1.9
	100	35.0 ± 2.0	36.5 ± 2.5	64.8 ± 3.2	64.2 ± 8.4	52.2 ± 5.8
	500	36.5 ± 2.5	39.0 ± 3.0	58.0 ± 2.0	62.0 ± 2.0	58.0 ± 6.0
	1000	38.5 ± 4.5	38.0 ± 3.0	63.0 ± 1.0	72.0 ± 8.0	53.0 ± 1.0
P15m	10	38.0 ± 4.0	38.0 ± 2.5	64.8 ± 3.2	68.5 ± 2.8	59.0 ± 6.5
	50	37.5 ± 1.5	33.0 ± 2.0	62.2 ± 1.9	67.6 ± 1.9	51.6 ± 3.9
	100	35.5 ± 2.5	39.5 ± 2.5	62.2 ± 5.8	71.3 ± 1.3	57.0 ± 3.2
	500	38.0 ± 3.0	37.5 ± 1.5	58.6 ± 3.9	68.3 ± 9.5	58.6 ± 4.0
	1000	37.0 ± 2.0	33.0 ± 2.0	58.8 ± 4.5	74.9 ± 6.0	53.4 ± 3.4
P20m	10	35.5 ± 2.5	40.5 ± 2.5	59.0 ± 6.5	69.1 ± 3.5	50.3 ± 2.6
	50	36.0 ± 1.0	33.5 ± 2.5	61.6 ± 3.9	70.4 ± 9.1	51.6 ± 6.5
	100	37.0 ± 5.0	34.5 ± 4.5	57.0 ± 3.2	67.6 ± 1.9	59.0 ± 1.3
	500	33.0 ± 2.0	40.5 ± 1.5	60.0 ± 4.0	66.0 ± 2.0	52.0 ± 2.6
	1000	35.0 ± 3.0	39.0 ± 1.0	61.0 ± 3.0	66.0 ± 6.0	50.0 ± 8.0

(Table 3. *Continued*)

Sample	μg/plate	Number of revertant colonies				
		TA1535	TA1537	TA98	TA100	WP2*uvr*A
P25m	10	33.0 ± 2.0	38.5 ± 2.5	60.3 ± 2.6	66.3 ± 6.3	59.0 ± 1.3
	50	36.0 ± 3.0	37.5 ± 2.5	61.6 ± 6.5	70.3 ± 6.6	51.6 ± 7.8
	100	37.5 ± 1.5	39.0 ± 2.0	59.0 ± 1.3	69.2 ± 3.4	53.5 ± 7.1
	500	33.0 ± 2.0	38.0 ± 3.5	65.3 ± 4.5	66.8 ± 8.0	54.7 ± 3.9
	1000	39.5 ± 2.5	36.0 ± 1.0	64.0 ± 3.2	74.5 ± 5.5	55.2 ± 2.7
P30m	10	36.5 ± 2.5	37.0 ± 3.0	59.0 ± 1.3	71.5 ± 4.5	50.3 ± 5.2
	50	32.5 ± 3.5	35.5 ± 1.5	61.6 ± 7.8	72.5 ± 9.5	51.6 ± 3.9
	100	32.5 ± 2.5	37.5 ± 2.5	63.5 ± 7.1	69.0 ± 7.0	59.0 ± 3.9
	500	34.0 ± 2.0	32.0 ± 4.0	64.0 ± 0.0	64.0 ± 8.0	54.0 ± 2.0
	1000	38.0 ± 4.0	30.5 ± 0.5	62.0 ± 6.0	64.0 ± 4.0	55.5 ± 1.5
2-aminoanthracene	1.0	–	–	326.7 ± 5.8	222.7 ± 9.3	–
	10.0	315.5 ± 11.4	304. ± 16.7	–	–	277.3 ± 11.8
Vehicle‡		35.3 ± 3.5	34.0 ± 2.3	68.0 ± 2.3	69.3 ± 7.4	53.3 ± 3.0

Notes: Values are expressed as mean ± SEM (n = 6 plates). Cells with no value were not tested.

† Toxicity, evaluated as a reduction (>30%) in the number of revertant colonies and as a change in the auxotrophic background lawn, in comparison with the control plates.

‡ DMSO, 50 μL/plate.

2012). Genome alterations were clearly visible after few days; therefore, our data, collected one year after the accident, suggest no modification in *P. oceanica* DNA. Present data are reassuring about the leakage of genotoxic substances from the shipwreck and suggest that the strategy applied for retaining the pollution in Island of Giglio environment near the wreck has been suitable.

Conclusions

Determination of man-mediated impacts in environmental changes is usually a complex challenge. *Posidonia oceanica* represents a good indicator in order to assess the impact on natural environment due to its sensitivity to pollution and to change in habitat. Furthermore, the secondary metabolism was selected in consideration of its known linkage to response of the organism to environmental pressure. Finally, an adequate analytic method was necessary to measure the metabolic replay.

Actually, HPTLC utilization is focused on the analysis of herbal and pharmaceutical marketed products, but its potentiality in biologic themes is high and deserves to be explored. The fingerprints comparison showed the metabolic effects of light default. To our knowledge, this is one of the first examples of application of the fingerprint HPTLC for measuring an environmental impact. As a matter of fact, the application was successful, albeit some changes were necessary in order to adapt this chemical approach to biologic problems.

All the reported results clearly evidence that long-term shadowing significantly affected the secondary metabolism of *P. oceanica*. Secondary metabolites decreased proportionally to the degree of light reduction, highlighting the pivotal role of irradiance on the seagrass health. Furthermore, considering the lack of mutagenic evidence, likely due to the absorption of pollution agents, it is possible to suppose a natural habitat recovery in HS and PS stations, where canopy is thinner and sparser, as reported for other studies (Fitzpatrick and Kirkman 1995, Gacia et al. 2012). In fact, several rhizomes in these affected areas have been found in good condition and are able to restore the meadow.

However, checking the evolution of the biological system in the next years, by collecting new seagrass samples in the same stations inside study area to evaluate for phytochemical changes and toxicological effects, is crucial to monitor the actual impact of the naval accident on the aquatic ecosystems.

In the present study, we proposed an integrated methodological approach, including both metabolomic and the genetic ecotoxicological analysis of the samples from the affected areas, which can be easily applied to define the impact of human disasters on the ecosystem and to monitor the environmental changes in the years.

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