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Fluoxetine induces photochemistry-derived oxidative stress on *Ulva lactuca*

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Emerging pollutants impose a high degree of stress on marine ecosystems, compromising valuable resources, the planet and human health. Pharmaceutical residues often reach marine ecosystems, and their input is directly related to human activities. Fluoxetine is an antidepressant, and one of the most prescribed selective serotonin reuptake inhibitors globally and has been detected in aquatic ecosystems in concentrations up to 40 μg L^{-1}. The present study aims to evaluate the impact of fluoxetine ecotoxicity on the photochemistry, energy metabolism and enzyme activity of *Ulva lactuca* exposed to environmentally relevant concentrations (0.3, 0.6, 20, 40, and 80 μg L^{-1}). Exogenous fluoxetine exposure induced negative impacts on *U. lactuca* photochemistry, namely on photosystem II antennae grouping and energy fluxes. These impacts included increased oxidative stress and elevated enzymatic activity of ascorbate peroxidase and glutathione reductase. Lipid content increased and the altered levels of key fatty acids such as hexadecadienoic (C16:2) and linoleic (C18:2) acids revealed strong correlations with fluoxetine concentrations tested. Multivariate analyses reinforced the oxidative stress and chlorophyll *a* fluorescence-derived traits as efficient biomarkers for future toxicology studies.

KEYWORDS

antidepressants, macroalgae, oxidative stress, pharmaceuticals, photobiology

Introduction

The ongoing increase in human population with increasing access to therapeutic substances, allied to the inefficient or incomplete removal of anthropogenic-related by-products in wastewater treatment plants (WWTPs), have led to the detection of emerging pollutants (EP) in marine ecosystems (Fatta-Kassinos, Meric and Nikolaou, 2011; Gavrilescu et al., 2015; Kurwadkar et al., 2015; Tang et al., 2019). As reported by EUROSTAT (2017), between 2002 and 2011, over 50% of the manufactured chemicals were considered harmful to ecosystems, with 70% of them having environmental impacts. The toxicology assessment of Pharmaceuticals and Personal Care Products (PPCPs) is of utmost importance due to their environmental persistence and capability of inducing results at lower concentrations, not only in humans but also in an array of other species, from diatoms (Duarte et al., 2020; Feijão et al., 2020; Silva et al., 2020) to molluscs (Fong and Ford, 2014; Moreno-González et al., 2016) and fish (Brooks et al., 2003; Corcoran et al., 2010; Bell et al., 2011; Duarte et al., 2019). PPCPs not only easily dissolve in water but also tend to show characteristics such as high lipophilicity, low biodegradability rate, high bioaccumulation potential, and do not evaporate at normal aquatic temperature and pressure (Kurwadkar et al., 2015). Over the last decade and following the EU's Task Group for the Marine Strategy Framework Directive implementation, recommendations for monitoring emerging contaminants and their effects on marine organisms increased. However, research on PPCPs exposure effects on many marine groups, namely macroalgae is still lacking (Law et al., 2010; Leston et al., 2011, 2014; Wiklund et al., 2011).

Due to the COVID-19 pandemic, an increase in the incidence of mental disorders, such as anxiety and depression, has been reported [e.g., in Canada (Uthayakumar, Kitchen and Gomes, 2021) and in France (Levaillant, 2021)], leading to increased consumption of anti-depressants. Fluoxetine is an antidepressant that selectively inhibits serotonin reabsorption (SSRI) and increases its concentration in the synaptic cleft (Brooks et al., 2003). It is often prescribed to treat various conditions and disorders, such as the abovementioned depression, but also anorexia, nervous bulimia, obsessive-compulsive or panic disorders (El-Bassat et al., 2011). The half-life of fluoxetine in humans and aquatic ecosystems is about 1–4 days (Lancaster and Gonzalez, 1989; Johnson et al., 2005; Sawyer and Howell, 2011) and, given its widespread use, it is frequently detected in aquatic environments (Vasskog et al., 2008; Mezzelani, Gorbi and Regoli, 2018; Reis-Santos et al., 2018). Concentrations can be as high as 40 μg L^{-1} (Ortiz, 2010; aus der Beek et al., 2016) and toxicity to various organisms has been reported (Fent, Weston and Caminada, 2006; Corcoran, Winter and Tyler, 2010). Since pharmaceuticals are designed to achieve results at the lowest possible concentrations in humans (Ebele, Abou-Elwafa Abdallah and Harrad, 2017) it is of the utmost importance to evaluate the consequences of their environmental presence on non-targeted species.

Being at the base of marine ecosystems, macroalgae are responsible for up to 50% of the global oxygen production and are a food and habitat source for numerous marine organisms (Areco, Salomone and Afonso, 2021). Found in the most various regions, from tropical to polar climates, the green macroalga *Ulva lactuca* can grow up to 30 cm wide, attached, sessile or free-floating and is known to form floating communities called green tides (Dominguez and Loret, 2019; Raj, Anandan and Mathew, 2020; Areco, Salomone and Afonso, 2021). Additionally, these macroalgae are used as compost or as an enriching agent in agriculture, as a food source in aquaculture, whereas its by-products (e.g. pigments, lipids and highly charged sulphated polysaccharides, known as Ulvan) find their application in cosmetic and pharmaceutical industries (Alam et al., 2016). From an environmental perspective, *U. lactuca* has been harnessed for biomonitoring Duarte et al., 2021a) and bioremediation of heavy metals (Trinelli, Areco and Dos Santos Afonso, 2013; Trinelli et al., 2019). Its cosmopolitan distribution, fast growth rates and tolerance to abiotic stresses, such as salinity (Bews et al., 2021) and heavy metals (Bonanno, Veneziano and Piccione, 2020; Areco, Salomone and Afonso, 2021), make this species an exceptional model in ecotoxicological studies.

In this context, the objective of this study was to evaluate fluoxetine exposure effects on the photochemistry, energy metabolism, and antioxidant biomarkers of the green macroalga *Ulva lactuca,* as well as to identify valuable and efficient early stress biomarkers of environmental contamination. To evaluate fluoxetine exposure-induced stress on *U. lactuca* photosynthesis, non-invasive high-throughput screening tools, such as pulse amplitude modulated (PAM) fluorometry and laser-induced fluorescence (LIF), were performed, which have been widely used in ecotoxicological studies (Cabrita et al., 2016; Gameiro et al., 2016; Duarte et al., 2021b). The extensive data produced by PAM fluorometry gives insight on light absorption, energy utilization and dissipation, and the state of the photosynthetic machinery, whilst LIF evaluates changes in the fluorescence peak in the red (685 nm) and far-red (735 nm) regions of the spectrum (Lavrov et al., 2012). The fluorescence peak ratio $F_{red/far\text{-}red}$ depends mainly on the chlorophyll content of the sample but is also associated with fatty acid composition variations and other metabolic adaptations (Gameiro, Utkin and Cartaxana, 2015). To further investigate the fluoxetine-induced stress effects on *U. lactuca*, fatty acid composition and membrane saturation parameters were also assessed. Finally, stress resistance-related machinery such as carotenoid content (β-carotene and xanthophylls), total energy reserves quantification, mitochondrial electron transport system activity, and antioxidant enzyme activity were also evaluated following fluoxetine exposure.

Methods

Ulva lactuca growth conditions and ecotoxicological exposure

Ulva lactuca (Ulvaceae, Chlorophyta) was supplied by ALGAplus aquaculture (Ílhavo, Portugal) and maintained in filtered seawater enriched with Provasoli's Enriched Seawater (PES) medium, under constant aeration (Leston et al., 2014). Cultures were maintained in a FytoScope FS 130 growth chamber (Photon Systems Instruments, Drásov, Czech Republic) using a sinusoidal function to simulate sunrise and sunset conditions. Light intensity was set at 40 µmol photons m^{-2} s^{-1} and temperature at 18 ± 1°C (Sand-Jensen, 1988; Feijão et al., 2017; Bonanno, Veneziano and Piccione, 2020).

At the beginning of the experiment, Ø1.5 cm algae discs were cut and placed in 24-well transparent plates with 3 ml PES medium for 48 h to allow algal recovery after cut-induced stress. After the first 24 h, the algal medium was replaced.

At the end of the 48-h acclimation period, the discs were separated into one control group and five treatment groups (N = 30 samples per group). The samples were exposed to several fluoxetine concentrations (0, 0.3, 0.6, 20, 40, and 80 µg L^{-1}) that not only mimic environmental conditions, but also allow the assessment of future effects throughout a relevant gradient (Johnson et al., 2007; El-Bassat et al., 2011; Minguez et al., 2014; aus der Beek et al., 2016; Grzesiuk, Wacker and Spijkerman, 2016; Grzesiuk et al., 2018; Feijão et al., 2020). The exposure lasted 48 h to avoid nutrient deficiency and salinity increase according to Han *et al.* (2007). At the end of the exposure period, samples were monitored non-invasively using bio-optical methods (*see* next section) and immediately flash-frozen in liquid nitrogen for further analysis.

Photochemical assessment

At the end of the 48-h trial, all samples were dark-adapted for 15 min prior to PAM fluorometry measurements. To evaluate fluoxetine-exposure related effects on photochemistry, namely light absorption, chemical (potential) energy utilization and energy dissipation, the pre-programmed OJIP protocol available in the FluorPen FO100 (Photon System Instruments, Drásov, Czech Republic) was used. This standard protocol involves exposing the sample to short-duration modulated saturating light at 3500 µmol photons m^{-2} s^{-1} to induce a fluorescence curve known as the Kautsky Effect (Zhu et al., 2005; Feijão et al., 2020). From this analysis, a set of variables is obtained through the FluorPen1.1 software (Table 1).

Laser-Induced Fluorescence (LIF) spectra of *U. lactuca* discs were obtained through a frequency-doubled pulsed Nd:YAG laser Quantel Ultra 532-30-20-H-N and an optical receiver based on a commercial palm-size Ocean Optics USB4000 spectrometer as described by Gameiro *et al.* (2016). Reflected fluorescence was collected by a light-gathering optical train based on optomechanical elements from Thorlabs Inc. (Lavrov et al., 2012; Utkin et al., 2013; Gameiro et al., 2016). The spectrometer was tuned and calibrated using a mercury argon calibration source Mikropack CAL-2000 to demonstrate high sensitivity (approximately 55 photons per count) and resolution (0.19 nm per channel).

The analysis consists of exposing the sample to 30 mJ, 7 ns monochromatic (532 nm) radiation pulses, generated at approximately 20 Hz rate. To avoid disturbing effects due to reaction centre (RC) closure and excitation annihilation, the pulsed radiation was kept at a sufficiently low single-pulse fluence (Rosema et al., 1998) regulated by the dimension of the laser spot on the targeted surface. LIF-derived parameters such as the $F_{red}/F_{Far\text{-}red}$ (F_{685}/F_{735}) ratio and maximum fluorescence (F_{max}) were obtained.

Pigment profiling

To assess fluoxetine-induced changes in pigment abundance, samples were freeze-dried for 48 h and then extracted with 3 ml of pure acetone for 24 h in the dark at −20°C to prevent pigment degradation (Cabrita et al., 2016, 2017; Feijão et al., 2020). Following centrifugation (4000 × *g* for 15 min at 4°C), supernatants were analysed using a UV-1603 spectrophotometer (Shimadzu, Kyoto, Japan) and read from 350 to 750 nm (0.5 nm steps). The obtained absorbance spectrums were introduced in the Gauss-Peak Spectra (GPS) fitting library and analysed through the algorithm developed by Küpper et al. (2007), enabling the detection of the most abundant pigments such as chlorophyll *a* (chl *a*) and *b*, pheophytin *a* (phaeo *a*), β-Carotene, zeaxanthin, antheraxanthin, violaxanthin, and auroxanthin.

Fatty acid analysis

Direct trans-esterification of *U. lactuca* discs was performed by adding freshly prepared methanol sulfuric acid (97.5:2.5, v/v) and subjecting the samples to 70°C for 60 min. Petroleum ether and Milli-Q water were added to recover the fatty acids methyl esters (FAMEs). Followed by a brief homogenization using the vortex and 5 min centrifugation (5000 × *g*), the upper phase was dried under a nitrogen flow in an SBH200D/2 sample concentrator (Stuart, Staffordshire, United Kingdom) at 30°C (Duarte et al., 2021a). FAMEs were resuspended in an appropriate amount of hexane and analysed in a Varian 430-GC gas chromatograph (Varian, Inc., CA, United States) equipped with a hydrogen flame ionization detector (FID) set at 300°C. The temperature of the injector was set to 270°C, with a split ratio of 50. The fused-silica capillary column (50 m ×

TABLE 1 Fluorometric analysis parameters and their description.

OJIP-test	
Area	Corresponds to the oxidized quinone pool size available for reduction and is a function of the area above the Kautsky plot
N	Reaction centre turnover rate
S_M	Corresponds to the energy needed to close all reaction centres
δR_0	Probability of an electron moving from quinone B (Q_B) to the Photosystem I (PSI) end-electron acceptor
P_G	Grouping probability between the two PSII units
ABS/CS	Absorbed energy flux per cross-section
TR/CS	Trapped energy flux per cross-section
ET/CS	Electron transport energy flux per cross-section
DI/CS	Dissipated energy flux per cross-section
RC/CS	Number of available reaction centres per cross-section

0.25 mm; WCOT Fused Silica, CP-Sil 88 for FAME; Varian, Inc.) was maintained at a constant nitrogen flow of 2.0 ml min^{-1} and the oven set at 190°C. Fatty acid identification was performed by comparison of retention times with standards (Sigma-Aldrich), and chromatograms were analysed by the peak surface method, using the Galaxy software. The internal standard used was pentadecanoic acid (C15:0) (Feijão et al., 2017). The saturation-derived variables (saturated fatty acids, SFA; unsaturated fatty acids, UFA; monounsaturated fatty acids, MUFA; Polyunsaturated fatty acids, PUFA; SFA/UFA; PUFA/SFA) were determined and the double bond index (DBI) was calculated as follows:

$$DBI=\frac{2\times(\%\,\text{monoenes}+2\times\%\text{dienes}+3\times\%\text{trienes}+4\times\%\text{tetraenes}+5\times\%\text{pentaenes})}{100}$$

Oxidative stress biomarkers

Fluoxetine-exposed and control *U. lactuca* discs were homogenized in a mortar and pestle using liquid nitrogen. The soluble protein fraction was extracted at 4°C in 500 µl of a previously mixed solution consisting of 50 mM sodium phosphate buffer (pH 7.6) with 0.1 mM Na-EDTA. Following sample centrifugation at 15,000 *g* for 10 min at 4°C (Sigma 2–16K, Sigma Laborzentrifugen GmbH, Germany), the supernatant was collected. To assess the enzymatic activity of catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APx, EC 1.11.1.11), superoxide dismutase (SOD, EC 1.15.1.1) and glutathione reductase (GR, EC 1.8.1.7), measurements were performed at 25°C in an Epoch 2 Microplate Reader (BioTek Instruments, VA, United States). Measurement of CAT activity was performed according to Teranishi *et al.* (1974) by monitoring H_2O_2 consumption and consequent decrease in absorbance at 240 nm (ε = 39.4 M^{-1} cm^{-1}). Determination of APx activity was performed according to Tiryakioglu *et al.* (2006) through measurement of ascorbate oxidation observed as a decrease in absorbance at 290 nm (ε = 2.8 mM^{-1} cm^{-1}). Following the protocol of Marklund and Marklund (1974), SOD activity was assayed by monitoring pyrogallol reduction shown as an absorbance increase at 325 nm. Pyrogallol autoxidation was read without enzymatic extract throughout the reading interval to allow the comparison of results. To determine GR activity, glutathione-dependent oxidation of NADPH and its decrease in absorbance at 340 nm (ε = 6.22 mM^{-1} cm^{-1}) were monitored according to Edwards, Rawsthorne and Mullineaux (1990). The oxidative ratio was determined as:

$$Oxidative\ ratio\ =\ \frac{SOD}{CAT\ +\ APx}$$

To evaluate changes in lipid peroxidation, *U. lactuca* discs were homogenized in 1 ml of fresh solution containing 0.5% of Thiobarbituric acid (TBA) and 20% Trichloroacetic acid (TCA). Samples were placed at 90°C for 30 min in an Accublock Digital Dry Bath (Labnet Inc., CA, United States). After stopping the reaction on ice and the following centrifugation at 15,000 *g* for 10 min at 4°C, samples were analysed in a UV-1603 spectrophotometer (Shimadzu, Kyoto, Japan). Absorbances were measured at 532 and 600 nm and the concentration of malondialdehyde (MDA) was determined according to Heath and Packer (1968) using the molar extinction coefficient, 155 mM^{-1} cm^{-1}.

Energy balance

Energy available

Using 1 ml of Mili-Q water (18.2 MΩ cm), discs were homogenized by mortar and pestle. Aliquots from each sample were used to assess lipid, carbohydrate and protein contents and electron transport system (ETS) activity. Mili-Q water was used as a reaction blank. The spectrophotometric measurements were performed at 25°C in a synergy H1 Hybrid

Multi-Mode microplate reader (Biotek® Instrument, Vermont, United States).

To assess energy available (E_a), the results obtained regarding lipid, carbohydrate, and protein content (following the methodologies described in De Coen and Janssen (1997), were transformed into energetic equivalents. The conversion was performed using the respective combustion energies as reported by Gnaiger (1983): 17,500 mJ mg carbohydrates^{-1}, 24,000 mJ mg protein^{-1}, and 39,500 mJ mg lipid^{-1}.

$$Ea = carbohydrates + lipids + proteins \ (\mathrm{mJ\ mg^{-1}FW})$$

Energy consumption

The consumption and metabolism of cellular oxygen are directly linked to mitochondrial ETS activity. Therefore, energy consumption (Ec) was determined using ETS data (for each 2 µmol of INT-formazan formed, 1 µmol of O_2 was consumed), converting the result into energetic equivalents using the specific oxyenthalpic equivalents for an average lipid, protein and carbohydrate mixture (480 kJ mol O_2^{-1}) reported by Gnaiger (1983). ETS assessment protocol was performed as described by King and Packard (1975) with major modifications (Aderemi et al., 2018).

$$Ec = ETS\ activity \ (\mathrm{mJ\ mg^{-1}FW\ h^{-1}})$$

Cellular energy allocation

The cellular energy allocation (CEA), standardized to mg^{-1} FW, was determined based on the previous lipid, carbohydrate and protein content measurements and respective ETS activity (Verslycke, Ghekiere and Janssen, 2004).

$$CEA = \frac{Ea}{Ec}$$

Statistical analysis

Spearman correlation coefficients and statistical significance among biophysical and biochemical parameters were obtained. Non-parametric Kruskal–Wallis tests with Bonferroni *post hoc* comparisons were also performed. These analyses were performed in R studio 1.4.1717 *via* packages "corrplot" (Wei and Simko, 2021) and "agricolae" (De Mendiburu and Simon, 2015). Canonical Analysis of Principal Coordinates (CAP) was performed to evaluate the ability of biochemical and biophysical traits to successfully classify discs according to fluoxetine concentration exposure. The benefit of this multivariate approach is its robustness in analysing heterogeneous data, which is why it is frequently used to compare different sample groups based on their intrinsic characteristics (Duarte et al., 2021c; Pires et al., 2021). Multivariate statistical analysis procedures were conducted using Primer 6 software (Clarke and Gorley, 2006).

Results

Fluoxetine exposure impact on *U. lactuca* photochemistry

Regarding the Kautsky curve fluorescence data points, it is possible to observe that all samples exposed to fluoxetine reveal higher fluorescence emission and curve shape changes compared to the control samples (Figure 1A). The oxidized quinone pool (area) was lower in samples exposed to the two highest fluoxetine concentrations (40 and 80 µg L^{-1}), with no statistical difference between them (Figure 1B). The energy needed to close all reaction centres (Figure 1C, S_M) decreased in samples exposed up to 0.6 µg L^{-1} of fluoxetine, then increased, registering the highest value at 80 µg L^{-1}. The turnover rate of dark-adapted reaction centres (N) and the probability of an electron moving from quinone B (Q_B) to the Photosystem I (PSI) end-electron acceptor (δR_0) followed the same pattern (Figures 1D,E). The dysconnectivity between two PSII antennae increased, as evidenced by an increase in grouping probability (P_G) in samples exposed to the two highest fluoxetine concentrations tested (Figure 1F).

When exposed to increasing concentrations of fluoxetine, *U. lactuca* suffered a decrease in energy absorption (ABS/CS, Figure 2A) starting at 0.3 µg L^{-1}, with a maximum decrease observed at the two highest fluoxetine concentrations tested (40 and 80 µg L^{-1}). Samples exposed to these highest concentrations also revealed a strong decrease in trapped (TR/CS) and transported (ET/CS) energy (Figures 2B,C). Dissipated energy flux (DI/CS, Figure 2D) decreased when *U. lactuca* was exposed to low fluoxetine concentrations (0.3 and 0.6 µg L^{-1}), returning to control levels at higher concentrations (Figure 2D). Oxidized reaction centre density (RC/CS) was significantly depleted in samples exposed to the two highest fluoxetine concentrations (40 and 80 µg L^{-1}), registering the lowest value at 80 µg L^{-1} (Figure 2E).

The obtained fluorescence spectra of fluoxetine-exposed *U. lactuca* samples revealed that exposure to the two lowest fluoxetine concentrations (0.3 and 0.6 µg L^{-1}) induced a fluorescence increase at 685 nm when compared to unexposed samples, registering the highest value in samples exposed to 0.3 µg L^{-1} of fluoxetine (Figure 3A). Fluoxetine concentrations above 20 µg L^{-1} induced an abrupt decrease at the same wavelength (Figure 3A). Maximum absorbance at the red region (Figure 3B) revealed a slight increase in samples exposed to the lowest concentrations, followed by a statistically significant decrease in those exposed to the highest concentrations (40 and 80 µg L^{-1}) while within the far-red region (Figure 3C) fluoxetine-exposed samples only showed a statistically significant decrease at 20 µg L^{-1}. Wavelength deviation where maximum red and far-red fluorescence was attained (Figures 3D,E respectively) presented an overall sinusoidal shape, with positive values in samples exposed to

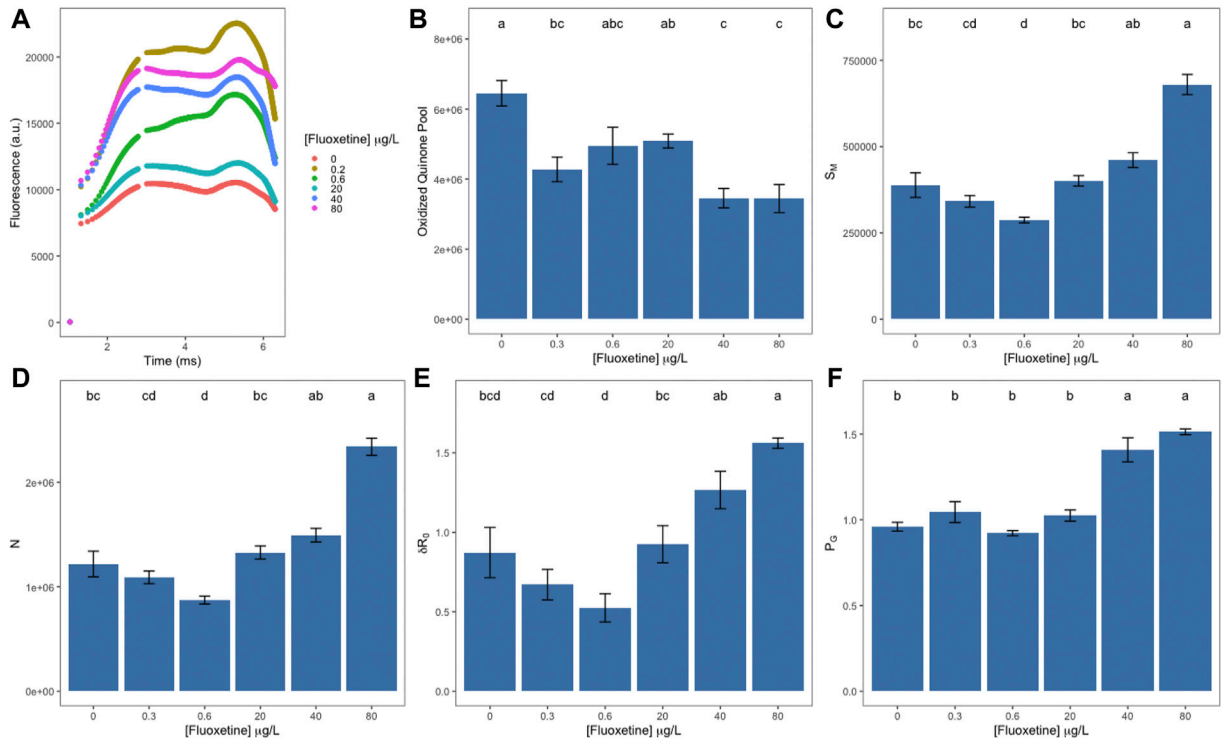

FIGURE 1
OJIP-derived parameters of Ulva lactuca following a 48-h exposure to different fluoxetine concentrations. **(A)** Kautsky chlorophyll a fluorescence induction curves. **(B)** Oxidized quinone pool. **(C)** Energy needed to close all reaction centres (SM). **(D)** Reaction centre turnover rate (N). **(E)** Probability of moving an electron from quinone B (QB) to the PSI end-electron acceptor (δR0). **(F)** Grouping probability (PG). Average ± standard error, $N = 5$; different letters indicate significant differences at $p < 0.05$.

lower concentrations and negative in samples exposed to the highest concentrations of fluoxetine. The F_{685}/F_{735} ratio slightly decreased with increasing concentrations, registering the lowest value in samples exposed to the highest fluoxetine concentration (Figure 3F). Maximum fluorescence presented a statistically significant decrease in samples exposed to fluoxetine concentrations above 20 µg L^{-1} (Figure 3G). Maximum wavelength and its deviation did not show any statistically significant differences between control and fluoxetine-exposed samples (Figures 3H,I).

Photosynthetic pigments and carotenoid concentration

Photosynthetic pigments such as chlorophyll *a* and b (chl *a* and chl *b*) as well as phaeophytin *a* (phaeo *a*), a degradation product of chl *a*, did not show any statistically different variation induced by fluoxetine exposure (Supplementary Figures S1A–C). Moreover, the xanthophyll cycle-related pigments and the de-epoxidation state did not show any statistically significant variation (Supplementary Figures S1D–I).

Fatty acid composition and membrane fluidity

The most abundant fatty acids found in *U. lactuca* are the unsaturated palmitic acid (C16:0), the polyunsaturated linolenic acid (C18:3), followed by the hexadecatrienoic (C16:3) and monounsaturated oleic (C18:1) acids (Supplementary Figure S2A). With the exception of increased oleic acid concentration, fluoxetine exposure did not significantly affect the fatty acid relative abundance, double bond index (DBI) and unsaturation ratios (SFA/UFA and PUFA/SFA) of exposed macroalgae (Supplementary Figures S2B–D).

Oxidative stress biomarkers

Samples exposed to the highest fluoxetine concentrations revealed reduced catalase and increased ascorbate peroxidase activities (Figures 4A,B, respectively). When exposed to 40 and 80 µg L^{-1} of fluoxetine, samples registered a steep decrease in superoxide dismutase activity, with a

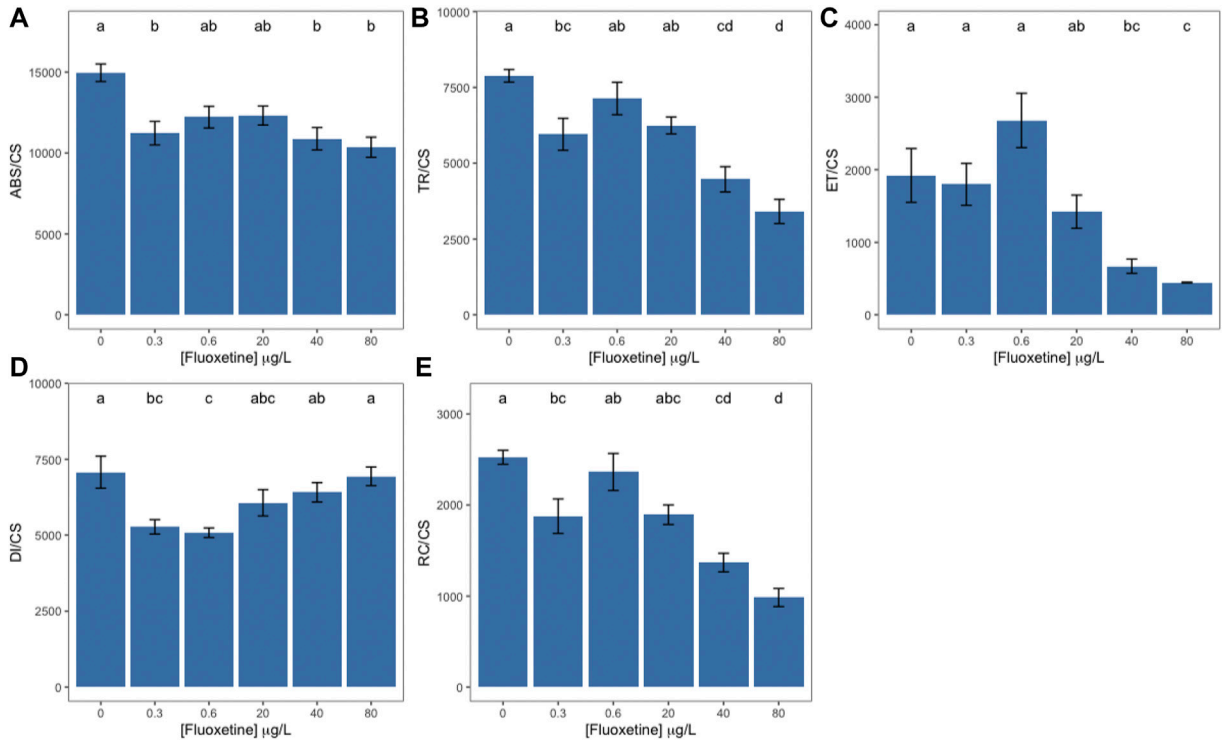

FIGURE 2
Ulva lactuca phenomological energy fluxes following a 48-h exposure to different fluoxetine concentrations. **(A)** Energy absorption per cross section (ABS/CS). **(B)** Trapped energy per cross section (TR/CS). **(C)** Electron transport per cross section (ET/CS). **(D)** Energy dissipation per cross section (DI/CS). **(E)** Number of reaction centres available per cross-section (RC/CS). Average ± standard error, $N = 5$; different letters indicate significant differences at $p < 0.05$.

simultaneous decrease of the oxidative ratio to zero (Figures 4C,D). Glutathione reductase activity tended to increase with increasing exogenous fluoxetine concentrations (>0.3–80 µg L^{-1}) (Figure 4E). Lipid peroxidation products also increased significantly registering the highest value in samples exposed to 40 µg L^{-1} (Figure 4F).

Energy balance

Although no statistically significant change in the carbohydrate content was observed, macroalgae discs showed an increase in lipid content starting at the exogenous fluoxetine concentration of 0.6 µg L^{-1} and peaking at 40 µg L^{-1} (Figures 5A,B). Protein content registered a significant decrease in samples exposed to intermediate fluoxetine concentrations (0.6–40 µg L^{-1}), whilst samples exposed to 80 µg L^{-1} showed no statistically significant difference (Figure 5C). The mitochondrial electron transport (ETS) revealed a tendency to decrease in fluoxetine-exposed samples (Figure 5D) while the available energy (Ea) and cellular energy allocation did not vary significantly (Figures 5E,F).

Biomarker integrated profiles

To analyse the relationship between the biophysical and biochemical traits, Spearman correlations are presented in Figure 6. Exogenous exposure to fluoxetine induced strong negative correlations among the fluorometric data, namely the LIF-derived parameters and the OJIP-derived energy fluxes, except for the dissipated energy flux. The strong negative trend was also evident for the oxidative enzymatic activities, specifically for catalase and superoxide dismutase activities. Although no statistically significant variations were observed in pigment abundance, the highest positive Spearman correlations were registered in the xanthophyll cycle. Similarly, to the latter, despite the lack of statistical differences in the fatty acid-derived data, a strong positive correlation between the exogenous fluoxetine concentration and membrane fluidity, here evaluated *via* the double bond index (DBI), was observed. Additionally, several correlations were found between other analysed traits namely, strong negative correlations between the xanthophyll cycle pigments and fluorometric variables (the F_{685}/F_{735} ratio and energy fluxes), and strong positive correlations between the F_{685}/F_{735} ratio and

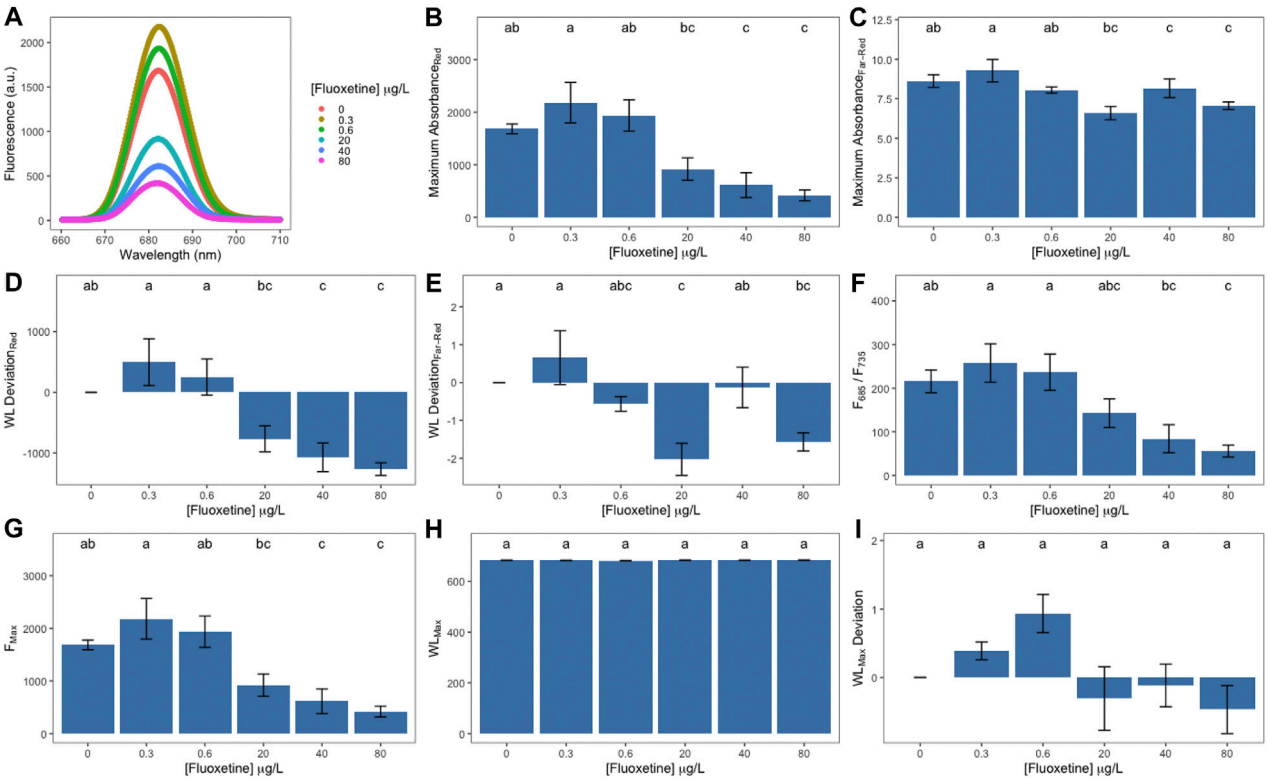

FIGURE 3
LIF-derived parameters of Ulva lactuca following a 48-h exposure to different fluoxetine concentrations. **(A)** Relative fluorescence. **(B)** Maximum absorbance at the red region. **(C)** Maximum absorbance at the far-red region. **(D)** Wavelength deviation at the red region. **(E)** Wavelength deviation at the far-red region. **(F)** Red/Far-red fluorescence ratios (F685/F735). **(G)** Maximum fluorescence (Fmax). **(H)** Maximum wavelength (WLMax). **(I)** Maximum wavelength deviation (WLMax Deviation). Average ± standard error, $N = 5$; different letters indicate significant differences at $p < 0.05$.

fatty acids (C16:2, C18:2, DBI), enzyme activity (CAT and SOD), lipids, proteins, and therefore Ea and CEA.

Multivariate Canonical Analysis of Principal Coordinates

The multivariate Canonical Analysis of Principal Coordinates (CAP) allowed the evaluation of the efficiency of the various photochemical and biochemical datasets to be used as biomarkers to accurately separate the different fluoxetine exposure groups. Biochemical and biophysical traits were analysed in groups, namely PAM and LIF (Figures 7A,B), fatty acids relative abundance (Figure 7C), pigment concentration (Figure 7D) and the oxidative stress data (Figure 7E). Oxidative stress biomarkers had the highest classification accuracy (>80%) in distinguishing the different exposure groups, followed by the Kautsky fluorescence data (>70%) (Figure 7F). Fatty acids relative abundance presented the lowest accuracy when classifying *U. lactuca* samples according to the fluoxetine exposure to which they were subjected (<25%).

Discussion

Early detection of xenobiotic toxicity in marine environments throughout the trophic web is paramount given the constant re-introduction, persistence, unknown interactions with non-targeted species, and synergistic effects of emerging pollutants such as PPCPs (Bi et al., 2018; Duarte et al., 2021b; Pires et al., 2021). Not only is it valuable to have the possibility of efficiently assessing toxicity through non-destructive high-throughput physical techniques, such as PAM fluorometry and LIF, but these methods allow for fast insights on known chemical pathways, due to the intrinsic correlation between energy conversion, oxidative stress, and biomass production.

In humans, the mode of action of SSRIs, such as fluoxetine has been extensively detailed and is reported to induce a serotonin concentration increase at the synaptic cleft due to

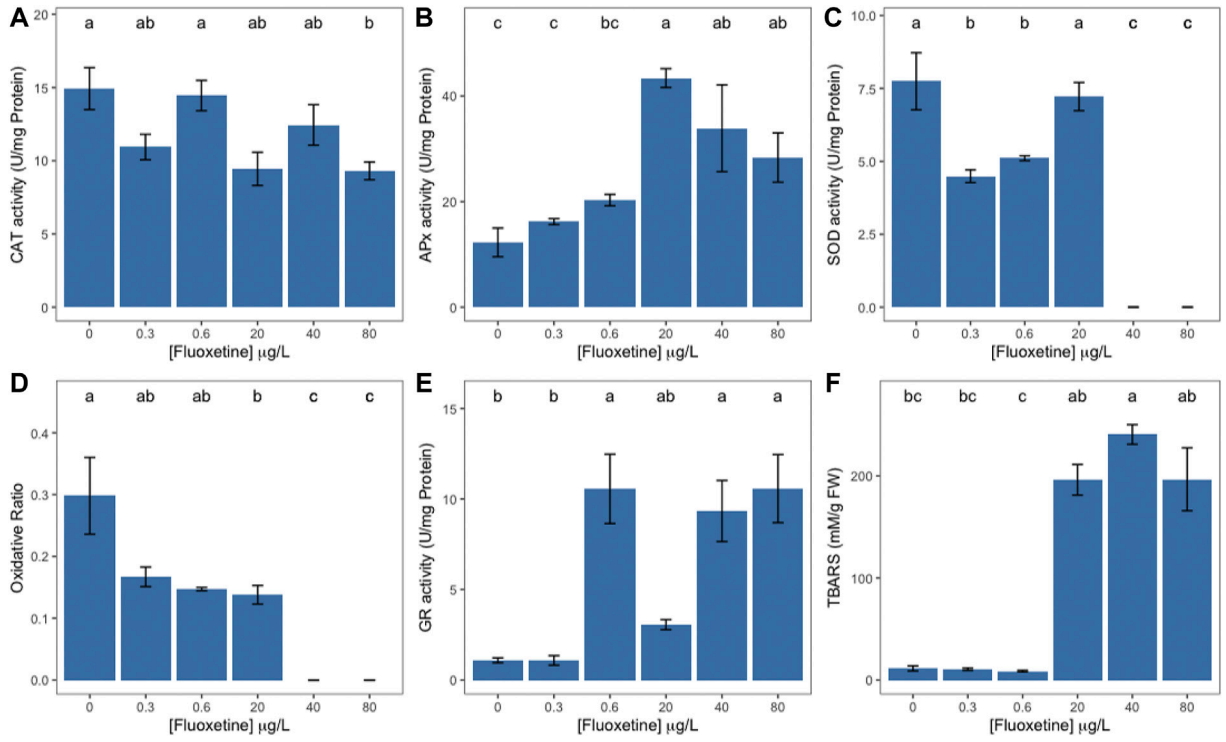

FIGURE 4
Ulva lactuca oxidative stress enzyme activity and lipid peroxidation products following a 48-h exposure to different fluoxetine concentrations. **(A)** Catalase (CAT). **(B)** Ascorbate Peroxidase (APx). **(C)** Superoxide Dismutase (SOD). **(D)** Oxidative ratio. **(E)** Glutathione reductase (Gr). **(F)** Thiobarbituric Acid Reactive Substances (TBARS). Average ± standard error, $N = 3$; different letters indicate significant differences at $p < 0.05$.

its reabsorption inhibition (Brooks et al., 2003). However, non-targeted organisms, such as unicellular microalgae or multicellular macroalgae may respond differently to fluoxetine exposure when compared to other organisms that are structurally, chemically and genetically more complex (Fent, Weston and Caminada, 2006; Minguez et al., 2014; Bi et al., 2018). In the present study, *U. lactuca* exposed to increasing exogenous fluoxetine concentrations showed a significant dose-related decrease in energy absorption and trapped energy flux. A similar response has already been reported in the microalgae *Phaeodactylum tricornutum,* exposed to the same fluoxetine concentrations (Feijão et al., 2020), which is often associated with lower efficiency in light-harvesting and exciton energy generation from captured photons (Duarte et al., 2021d) and commonly reported in microalgal exposure to other PPCPs (Silva et al., 2020) and pesticides (de Carvalho et al., 2020). The photosynthetic response seems to mirror the metabolic changes occurring in *P. tricornutum*, namely a decrease in the oxidized quinone pool and RC density, with a significantly increased quinone turnover and energy needed to close all RCs. The connectivity between two PSII antennae gradually decreased (inversely to P_G) as exogenous fluoxetine concentrations increased (Feijão et al., 2020). The outcome of PSII antenna dysconnectivity lies in the inevitable electron transport impairment (Duarte et al., 2017), and is associated with the abovementioned reduced quinone pool and the turnover rate causing quinone overreduction and increased electron leakage to O_2 and subsequent superoxide radical (O_2·−) formation (Cleland and Grace, 1999; Takagi et al., 2016; Ivanov, Borisova-Mubarakshina and Kozuleva, 2018). However, due to the cessation of SOD activity observed in the macroalgae samples exposed to the highest fluoxetine concentrations, quinone overreduction might have occurred due to the increased content in lipid peroxidation products, despite generally increased antioxidant enzymes activities (APx, GR). This often leads to the activation of photoprotective mechanisms such as β-carotene mobilization to quench singlet oxygen ($^1O_2$) resulting from lipid degradation (Telfer et al., 2002; Telfer, 2014; Pospíšil, 2016). While β-carotene content did not increase, a strong positive correlation was observed with exogenous fluoxetine concentration, indicating that at higher fluoxetine concentrations *U. lactuca* might prioritize carotenoid production to counteract photosynthesis-derived oxidative stress.

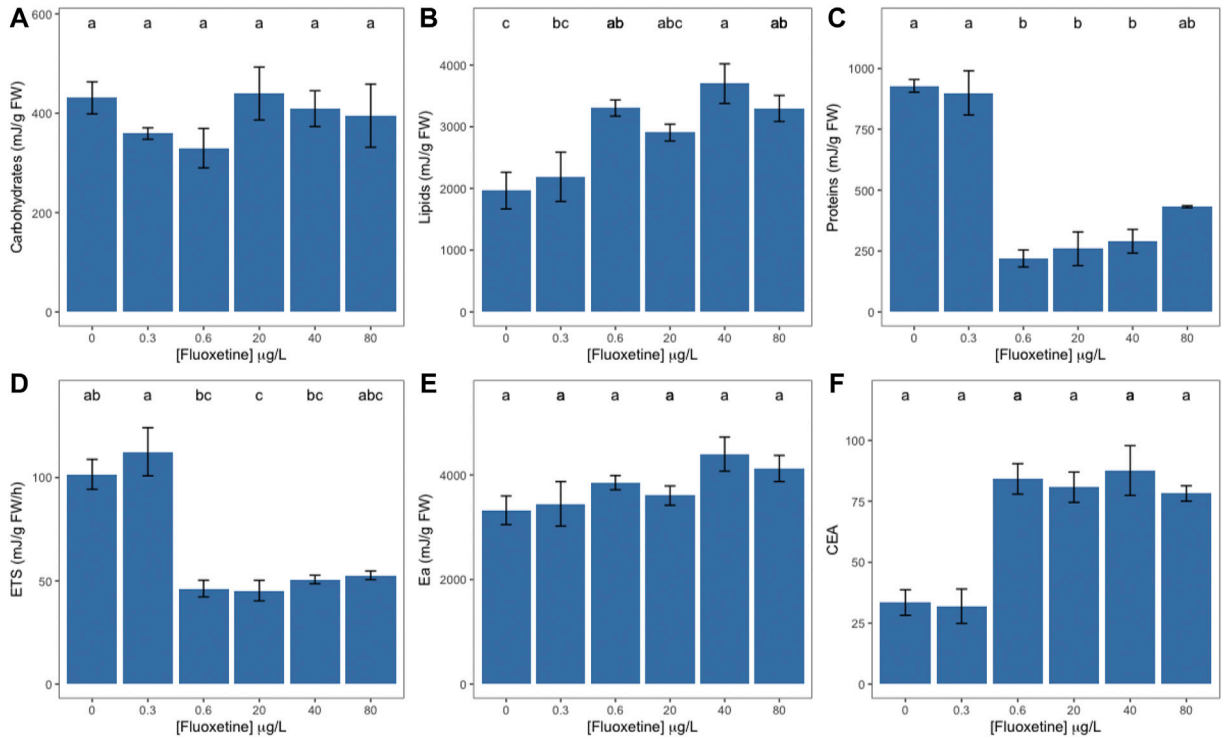

FIGURE 5
Ulva lactuca energetic metabolism at the mitochondria level of Ulva lactuca following a 48-h exposure to different fluoxetine concentrations. **(A)** Carbohydrates. **(B)** Lipids. **(C)** Proteins. **(D)** Electron Transport System (ETS). **(E)** Available energy (Ea). **(F)** Cellular energy allocation (CEA). Average ± standard error, $N = 3$; different letters indicate significant differences at $p < 0.05$.

Regarding the other H_2O_2-scavenging enzyme, namely CAT, fluoxetine exposure appears to have a more prominent effect on its activity at higher organizational levels when comparing the response of *P. tricornutum* to *U. lactuca*. Catalase activity seemed to be less affected in *P. tricornutum,* where it remained stable than in macroalgae like *U. lactuca* which showed a significant decrease in CAT activity at the highest exogenous fluoxetine concentration of 80 µg L^{-1} (Feijão et al., 2020). In *P. tricornutum*, APx, which has a higher affinity for H_2O_2 (Das and Roychoudhury, 2014), revealed a significant increase at the highest exogenous fluoxetine concentration, while in *U. lactuca* it was the only H_2O_2-scavenging enzyme that increased its activity when exposed to fluoxetine concentrations above 20 µg L^{-1} (Feijão et al., 2020). GR is a versatile enzyme with high reductive potential involved in the reduction of glutathionedisulfide to glutathione which can scavenge numerous ROS (H_2O_2, $^1O_2$, $O_2{\cdot}-$) and has been reported to be involved in multiple cellular processes, including xenobiotic detoxification (Mullineaux and Rausch, 2005). In *U. lactuca*, GR activity increased and showed a strong positive correlation with exogenous fluoxetine concentration, highlighting the occurrence of fluoxetine-induced stress.

Variations in chl content have been reported in photosynthetic organisms following exposure to fluoxetine and other SSRIs, such as freshwater plankton colonizing quartz rocks (Yang et al., 2019), algal biofilms (Richmond et al., 2019), and diatoms (Feijão et al., 2020). In this study, while chl *a* content did not vary, the $F_{Red}/F_{Far\text{-}Red}$ which often detects chl *a* content variations (Chappelle et al., 1984; Hák, Lichtenthaler and Rinderle, 1990) and is highly sensitive to stressors such as dehydration in plants and macroalgae, did decrease in samples exposed to higher fluoxetine concentrations revealing possible underlying molecular changes in known metabolic pathways such as anthocyanin content and lipids (Bartošková et al., 1999; Gameiro et al., 2016). This decrease in the $F_{Red}/F_{Far\text{-}Red}$ ratio was mostly due to a more pronounced decrease in fluorescence in the red region which is often related to changes in PSII electronic transitions. However, a decrease in fluorescence was also observed in fluoxetine-exposed samples in the far-red region which implies changes in vibrational energy sublevels (Govindjee, 1995; Franck, Juneau and Popovic, 2002).

The total lipid content was significantly increased in fluoxetine-exposed samples at most concentrations above 0.6 µg L^{-1}, showing a strong negative correlation with the $F_{Red}/F_{Far\text{-}Red}$ ratio. While available energy and CEA did not show any significant changes, both revealed strong positive correlations with exogenous fluoxetine

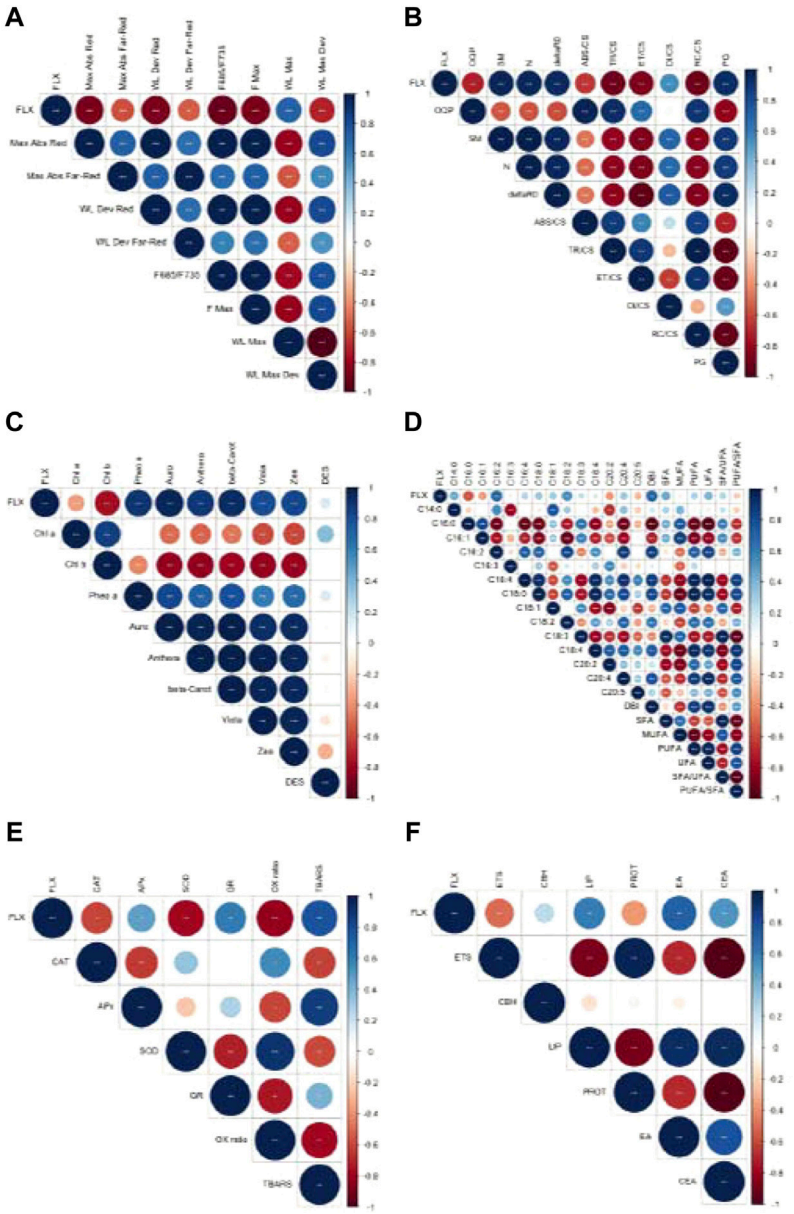

FIGURE 6
Ulva lactuca Spearman correlations with fluoxetine exposure. **(A)** LIF-derived variables. **(B)** PAM-derived variables. **(C)** Pigment concentration variables. **(D)** Fatty acid analysis variables. **(E)** Oxidative stress variables. **(F)** Energy metabolism variables. Shades of blue indicate positive correlations while shades of red indicate negative correlations between parameters. *N* = 30 (fluorometric data); *N* = 3 (pigments, oxidative stress and energetic biomarkers and fatty acids); asterisks indicate statistically significant differences at $p < 0.05$ (*), 0.01 (**) and 0.001 (***).

concentration, implying a bigger net energy budget for plant growth and division (Verslycke, Ghekiere and Janssen, 2004). However, given the low photosynthetic ET/CS and negative trend in the mitochondrial ETS, in energetic terms, it seems that fluoxetine exposure might be reducing both autotrophic energy production and respiratory activity (Duarte et al., 2020).

Neutral lipids (NL) which include triacylglycerol (TAG) seem to be a big component (55%) of some Chlorophyta such as *Ulva armoricana* (Kendel et al., 2015) and often increase under stress conditions in macroalgae and higher plants (Klok et al., 2014; Du and Benning, 2016; Ferreira et al., 2021), so it cannot be excluded the possibility that the lipid content increase might be associated with NL content increase reinforcing the prevalence of fluoxetine-induced stress. Although lipid profiling was not assessed, the strong positive correlations between key fatty acids and fluoxetine exposure should be highlighted, namely the C16:2 and C18:2 fatty acids.

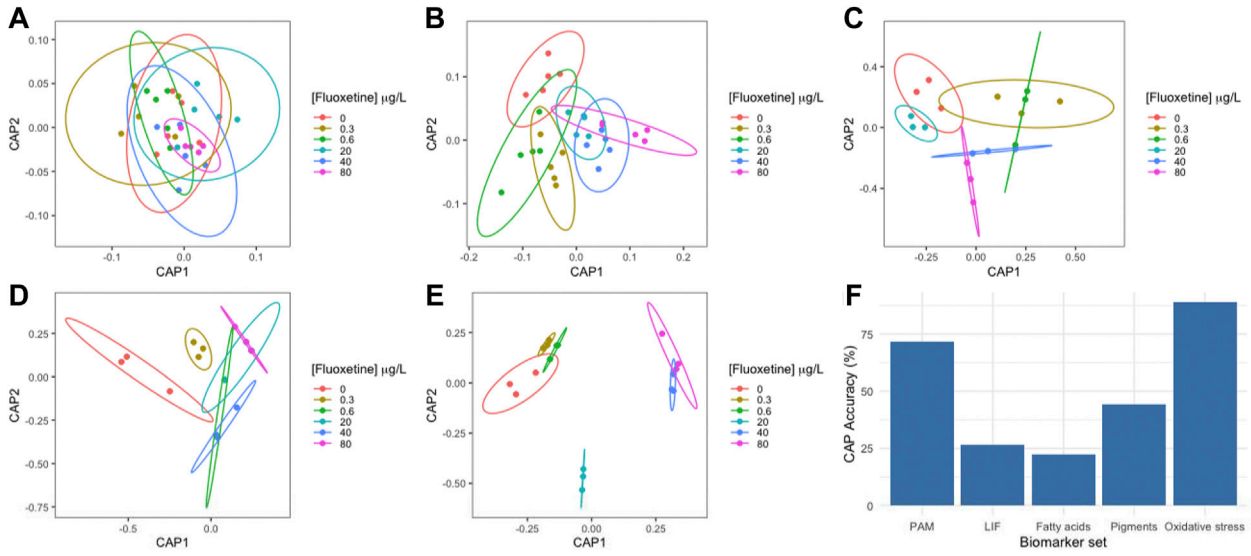

FIGURE 7
Ulva lactuca Canonical Analysis of Principal components (CAP) following a 48-h exposure to different fluoxetine concentrations. **(A)** PAM-derived variables. **(B)** LIF-derived variables. **(C)** Fatty acid profile. **(D)** Pigment profile. **(E)** Oxidative stress biomarkers. **(F)** CAP accuracy. *N* = 5 (PAM and LIF); *N* = 3 (Fatty acids, pigments, and oxidative stress).

Digalactosyldiacylglycerol (DGDG) was found to be the only polar lipid with C16:2 in *U. lactuca* from the Adriatic and Sea of Japan making this fatty acid a possible biomarker for DGDG content variations (Kostetsky et al., 2018). Similarly, although not exclusive from this lipid class, C18:2 was found mostly present in DGDG in *U. lactuca* (Barkina et al., 2020). The galactolipids DGDG and monogalatosyldiacylglycerol (MGDG) are the most abundant lipids in plant thylakoid membranes and they are reported to be responsible for photosystem stabilization and electron modulation at the quinone level (Sakurai et al., 2007; Kern and Guskov, 2011; Mizusawa and Wada, 2012). More importantly, DGDG is also known to protect against Mn cluster dissociation in the O_2-evolving complexes and therefore negative impacts on its content can impair PSII activity (Sakurai et al., 2007). Although membrane fluidity (DBI) did not change, a strong positive correlation was observed with fluoxetine exposure reinforcing the hypothesis that *U. lactuca* might be prioritizing membrane integrity over photosynthesis stabilization.

Aside from all the physiological meaning in terms of a possible mode of action of fluoxetine on non-target organisms, namely macroalgae, it is also important to highlight the possibility of using some of these metabolic features as potential fluoxetine exposure biomarkers. Overall, the biomarker CAP analysis demonstrated that chlorophyll *a* fluorescence (PAM: >70% correct classification accuracy) and oxidative stress-derived biomarkers (Oxidative stress: 100%) are the best non-invasive and invasive techniques, respectively, to assess fluoxetine exposure in *U. lactuca*. Bio-optical techniques such as PAM have the advantage to allow performing a non-invasive evaluation of the organisms, producing a high amount of data that can efficiently act as biomarker variables or be integrated into multivariate biomarker indexes (Duarte, et al., 2021c; Duarte et al., 2021d; Lemos, 2021), and used for exposure assessment. The assessed oxidative stress biomarkers have been demonstrated to be reliable from diatoms to plants exposed to stressors such as heavy metals, pharmaceuticals and pesticides (de Carvalho et al., 2020; Pires et al., 2021). Thus, bio-optical and oxidative stress-derived biomarkers are highlighted as efficient approaches for future fluoxetine exposure toxicity assessments in *U. lactuca*.

Conclusion

It is clear from this study that exogenous exposure to fluoxetine causes significant stress on the energy metabolism of *U. lactuca*. The negative impacts led to increased oxidative stress and activation of photoprotective pathways such as antioxidant enzyme activity. Non-invasive high-throughput techniques like PAM and LIF have the potential to detect signs of fluoxetine-induced stress before its classical tangible markers such as chl *a* content and fatty acid abundance suffered abrupt declines or variations. The present study highlights the effects of fluoxetine exposure on a non-target organism that are concomitant with the effects that SSRI has on other organisms ranging from diatoms to fish. Therefore, the potential impact of fluoxetine on the trophic chain is of concern. Given the negative bearings observed after a short-term exposure analysed in this work, it could be expected that long-term fluoxetine

exposure and possible synergistic effects with other EPs commonly found in aquatic environments, may potentiate fluoxetine toxicity to macroalgae and other marine biota, affecting not only macroalgae communities but also the trophic webs and ecosystems of which they are part.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, upon reasonable request.

Author contributions

BD, VF, and PR-S conceived and designed the experiments. EF, RC, ID, AU, and SN performed the experiments and data processing. EF wrote the manuscript. AM, MC, AU, IC, JM, and ML provided technical and editorial assistance. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.963537/full#supplementary-material

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