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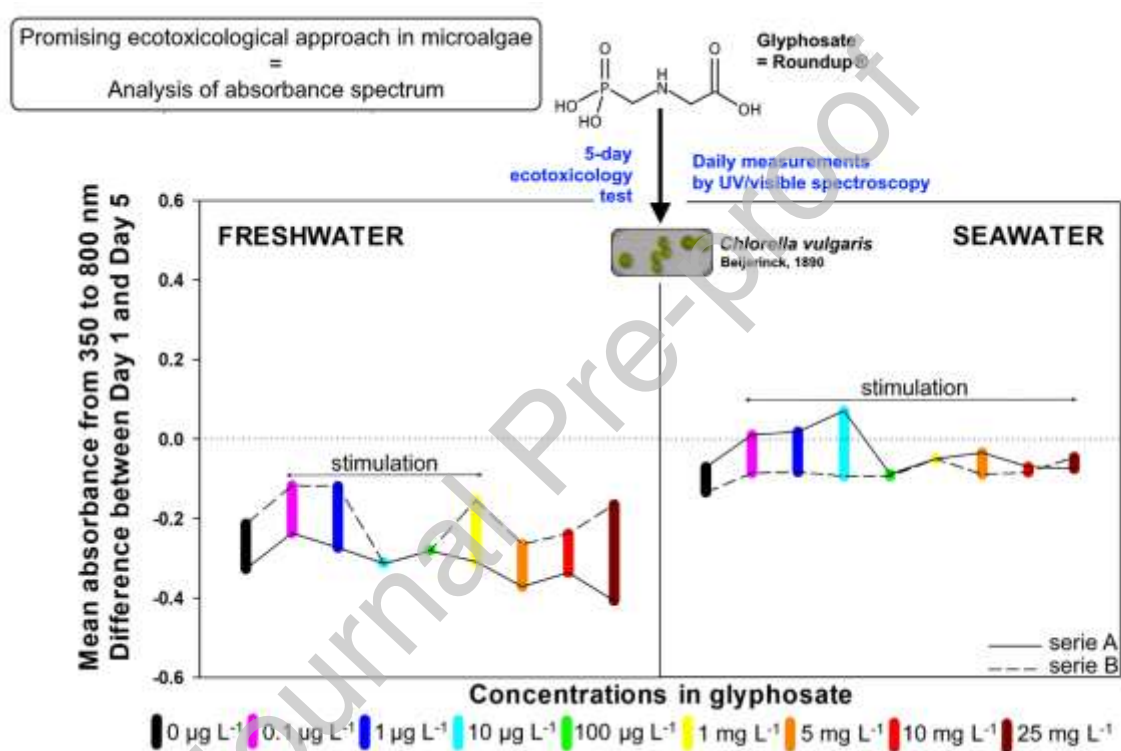
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# Evaluation of Roundup® effects on *Chlorella vulgaris* through spectral changes in photosynthetic pigments in fresh and marine water

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Graphical abstract



## HIGHLIGHTS

- Roundup® caused spectral changes in photosynthetic pigments of *Chlorella vulgaris*.
- Absorption spectra and peak areas were used to monitor toxic effects.
- Cells were stimulated from 10 to 5,000 µg L<sup>-1</sup> in freshwater and inhibited otherwise.

- *C. vulgaris* adapted to seawater was more tolerant above 1 mg L<sup>-1</sup> of glyphosate.
- The dose-response relationship depended on salinity.

## ABSTRACT

Glyphosate is a broad-spectrum herbicide whose use is now restricted because of its potential toxicity to human and environmental health. Its presence is observed even in aquatic ecosystems where this emerging contaminant affects primary producers such as phytoplankton. This study focuses on the cosmopolitan green microalga, *Chlorella vulgaris* Beijerinck, 1890. The ecotoxicological effects of the commercial herbicide, Roundup®, were evaluated in freshwater (FW) and seawater (SW) using ultraviolet/visible spectroscopy over a five-day experiment. A dose-response relationship was investigated on a wide range of concentrations (0.1 to 25,000 µg L<sup>-1</sup>). The population of FW-*Chlorella* was stimulated when exposed to concentrations from 10 to 5,000 µg L<sup>-1</sup> and inhibited otherwise. Chlorophylls and carotenoids exhibited their highest peak area values after four days, before their signal was depleted by day 5, regardless of Roundup® concentration. Conversely, cells of SW-*Chlorella* were stimulated at the highest concentrations (> 1 mg L<sup>-1</sup>), as if glyphosate was acting as a source of phosphorous nutrient. The peak areas of chlorophylls and carotenoids increased in the presence of the contaminant, especially on day 3. The defense mechanisms activated during its adaptation to marine salinity (*e.g.*, decrease in the chlorophyll/carotenoid peak area ratio) could have favored its resilience. Exposure to Roundup® caused a shift in the wavelength at maximum absorbance, which could introduce a bias when measuring peak heights at a single wavelength. The analysis of absorption spectra and the use of peak areas appear to be a promising approach for monitoring changes in photosynthetic pigments.

## KEYWORDS

*Chlorella vulgaris*, Glyphosate, Salinity, Absorption spectrum, Chlorophylls, Carotenoids

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# 1. INTRODUCTION

Glyphosate (N-(phosphonomethyl)glycine) is a broad-spectrum herbicide discovered in 1950 by Swiss chemist Henri Martin. It has been marketed since 1974 in different formulations such as Roundup®, first produced by the Monsanto company and then by the Bayer group, which bought the company in 2018. In 2014, 825,804 tons of glyphosate-based herbicides were sold worldwide including 113,000 tons in the United States alone (Matozzo et al., 2020). Glyphosate is particularly used in agricultural areas where crops are genetically modified and herbicide tolerant. This organic molecule is detected in a wide variety of aquatic environments, from surface water to groundwater (Battaglin et al., 2014; Matozzo et al., 2020). Spread by runoff, soil leaching and aerosols, it is even observed in estuarine and marine environments (Matozzo et al., 2020). Glyphosate is also stored in soils and sediments due to its high affinity for clay and oxide particles, which induces low mobility (Matozzo et al., 2020). Its use is now widespread in urban areas, hence its presence in the effluents of wastewater treatment plants (WWTPs) (Grandcoin et al., 2017; Poiger et al., 2020). Koplin et al. (2006) reported overall removal efficiencies at WWTPs ranging from 71 to 96%. Despite seasonal variations, environmental concentrations remain in the order of a few  $\mu\text{g L}^{-1}$ , suggesting chronic contamination rather than one-time acute events (Matozzo et al., 2020).

Studies on the fate of glyphosate in natural environments have shown its resistance to photolysis and chemical breakdown (Grandcoin et al., 2017). The first degradation pathway results from microbial activities in biofilms and leads to the formation of aminomethylphosphonic acid (AMPA) and glyoxylate after oxidation under aerobic conditions by glyphosate oxidoreductase (Carles et al., 2019; Grandcoin et al., 2017; Matozzo et al., 2020). The second is *via* biodegradation by C-P lyase to sarcosine and inorganic phosphate (Matozzo et al., 2020). The persistence of glyphosate was determined in various environments by evaluating the dissipation half-life (DT50). A range of 7 to

130 days was estimated for soils, indicating rapid degradation (Grandcoin et al., 2017). This duration was greater in seawater at 47-267 days, as well as in rivers at 13-301 days (Matozzo et al., 2020; Mercurio et al., 2014). The metabolite AMPA appears to be more mobile and persistent than its parent molecule (Koplin et al., 2006). Its presence can be detectable for several years instead of a few months for glyphosate (Maggi et al., 2020). These processes are largely influenced by local environmental factors. Maggi et al. (2020) showed that precipitation, pH, and soil organic carbon are the main factors controlling the distribution of contamination hotspots worldwide. Phosphorus availability is also influential as it hinders the biodegradation of glyphosate and promotes the accumulation of AMPA in water (Carles et al., 2019).

Due to strong societal demand, the ecotoxicological effects of glyphosate-based herbicides are now widely studied in terrestrial and aquatic habitats (De Castilhos Ghisi et al., 2020). This emerging contaminant is classified as a chronic aquatic hazard (category 2) and considered toxic to aquatic organisms with long-lasting effects (hazard statement H411) (INERIS, 2014). Since 2015, the World Health Organization has reclassified it as probably carcinogenic to humans (Myers et al., 2016; Van Bruggen et al., 2018). Glyphosate acts as a chelating agent that binds to micro- and macronutrients, altering many plant processes, pathogen resistance and bioavailability of elements in soils (Mertens et al., 2018). In addition, the degradation of Roundup® significantly increases total phosphorus, which promotes the process of eutrophication (Vera et al., 2010). Although toxic to some phytoplankton species, this phosphorus (P) input can be used by marine diatoms (*Emiliania huxleyi*, *Skeletonema costatum*, *Phaeodactylum tricornutum*) to support their growth, beyond that of any dissolved inorganic phosphorus (DIP) availability (Wang et al., 2016). The development of these diatoms, but also of some cyanobacteria and prasinophytes, could be stimulated, potentially affecting the structure of the phytoplankton community (Cruz de Carvalho et al., 2020; Stachowski-Haberkorn et al., 2008; Vera et al., 2010; Wang et al., 2016).

Acute toxicity tests with pure glyphosate or in commercial formulations show lethality at concentrations well above those observed in nature (Matozzo et al., 2020). Focusing upon the foodweb base is important as this is where photoautotrophic microorganisms, essential for healthy aquatic ecosystems, are found (Van Bruggen et al., 2018). In freshwater, green microalgae had a half maximum effective concentration (EC50) of 3.5 mg L<sup>-1</sup> (*Chlorella pyrenoidosa*) and 12.5 mg L<sup>-1</sup> (*Pseudokirchneriella costatum*) after a 96-hour exposure to glyphosate (INERIS, 2014). The no-observed-effect concentration (NOEC) for the latter species was 10 mg L<sup>-1</sup> after 120 hours. In seawater, the data obtained often highlight much lower values. For example, the diatom *Skeletonema costatum* had an EC50 of 0.85 mg L<sup>-1</sup> after a 96-hour exposure to glyphosate and a NOEC of 1.8 mg L<sup>-1</sup> after 120 hours (INERIS, 2014). Moreover, chronic exposure may tend to alter the function of some fundamental metabolic processes without affecting the survival of photoautotrophic microorganisms. The mode of action of glyphosate on these microorganisms is the same as that observed on terrestrial plants. Aromatic amino acid synthesis, respiration, photosynthesis, and chlorophyll production can be disrupted (Van Bruggen et al., 2018). Impaired electron transport in thylakoids and decreased antioxidant capacity inhibit photosynthetic activity and increase lipid peroxidation (Cruz de Carvalho et al., 2020). DeLorenzo et al. (2001) also showed negative impacts on carbon uptake in the two diatoms *Nitzschia* sp. and *Cyclotella meneghiniana*.

The green microalga *Chlorella* sp. is commonly used in ecotoxicological studies due to its ubiquity in marine and continental habitats, from polar to tropical environments (Hodač et al., 2016). Belonging to the first trophic level, this unicellular microorganism participates in the production of organic matter by photosynthesis and is a prey for herbivorous zooplankton. Contaminants such as glyphosate could cause changes in this population, including biomass and photosynthetic efficiency,



which could have cascading effects on the entire ecosystem. Known for being rich in chlorophylls and carotenoids (Safi et al., 2018), the species *Chlorella vulgaris* Beijerinck, 1890 was selected in order to determine a dose-response relationship with Roundup® through the spectral characteristics of photosynthetic pigments. Ultraviolet/visible spectroscopy was used to follow the evolution of the cells for five days without compromising their survival. The characteristics of chlorophyll and carotenoid peaks were analyzed to seek a more informative approach than the usual determination of a single-wavelength absorbance. In addition, the resilience of this cosmopolitan species was assessed in relation to the salinity of the environment in which it occurred (freshwater / seawater).

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## 2. MATERIAL AND METHODS

### 2.1 Contaminant characteristics

The herbicide glyphosate ( $C_3H_8NO_5P$ , CAS Registry Number 1071-83-6) is the active ingredient in the commercial formulation of Roundup®. The solution used in this study had a glyphosate concentration of  $450\text{ g L}^{-1}$ . The solubility of glyphosate is  $10.5\text{ g L}^{-1}$  in water at  $20^\circ\text{C}$  and pH 2, and remains insoluble in most organic solvents (INERIS, 2014). A surfactant, such as polyoxyethylene amine (POEA), is included in glyphosate-based herbicides to facilitate penetration through the plant surface and increase effectiveness. Its main degradation product is aminomethylphosphonic acid, also known as AMPA, which is a weak organic acid with a phosphonate group ( $CH_6NO_3P$ , CAS Registry Number 1066-51-9).

### 2.2 *Chlorella vulgaris* culture

The phytoplankton model organism, *Chlorella vulgaris* Beijerinck, 1890 (5-10  $\mu\text{m}$ ), was used to test the potential ecotoxicological effects of Roundup® in two types of aquatic environment: inland waters and coastal marine areas. Monospecific cultures were performed in the laboratory using the « Teramer®-Start » kit. A first strain was maintained in freshwater (hereafter referred to as FW-*Chlorella*), while the second was adapted to a salty environment equivalent to seawater (hereafter referred to as SW-*Chlorella*). The organisms were kept in an air-conditioned room ( $\sim 20^\circ\text{C}$ ), exposed to a light-dark regime (12h neon light – 12h darkness), oxygenated by bubbling at a controlled air flow, and transplanted regularly. Dust deposition was limited by applying an aluminium foil to the top of the bottles but leaving an opening on the side for air bubbling.

### 2.3 Ecotoxicological test

Two dilution ranges were prepared from the commercial solution of Roundup®, one in ultrapure water and the other in synthetic seawater (salinity 35). The final concentrations of glyphosate to which *Chlorella* cells were exposed were 0, 0.1, 1, 10, 100, 1,000, 5,000, 10,000 and 25,000  $\mu\text{g L}^{-1}$ . A total volume of 6 mL was placed in 15 mL clear tubes at  $\sim 20^\circ\text{C}$  and subjected to the same light-dark regime as for the culture. The caps of the tubes were not tightened to allow gas exchange and the water was oxygenated every day by turning them slowly. No nutrients were added during the five-day test. The concentration of 0  $\mu\text{g L}^{-1}$  represented a control to monitor population changes under experimental conditions without exposure to the contaminant. Two experimental series, A and B, were carried out as duplicates for FW-*Chlorella* and for SW-*Chlorella*.

### 2.4 Spectroscopic measurements

The absorption spectrum of *C. vulgaris* was measured for five days by ultraviolet/visible spectroscopy (SHIMADZU®, UV-1800). Measurements were first made one hour after contact with the contaminant, and then every day at the same time. The acquisition of absorbance values was achieved over a wide range of wavelengths (200 to 900 nm) and at high resolution (every 1 nm) in order to include the peaks corresponding to the main photosynthetic pigments of this green microalga: total carotenoids ( $\sim 390\text{-}540$  nm) and chlorophylls ( $\sim 640\text{-}730$  nm). A blank containing the culture medium was measured for each strain and subtracted from the sample spectra.

### 2.5 Data analysis

UV Probe 2.42 software was used to process the resulting absorption spectra. Peak characteristics

for chlorophylls and total carotenoids (*i.e.*, peak area, peak width at base, peak height, and wavelength at maximum absorbance) were recorded for each strain, series, and concentration.

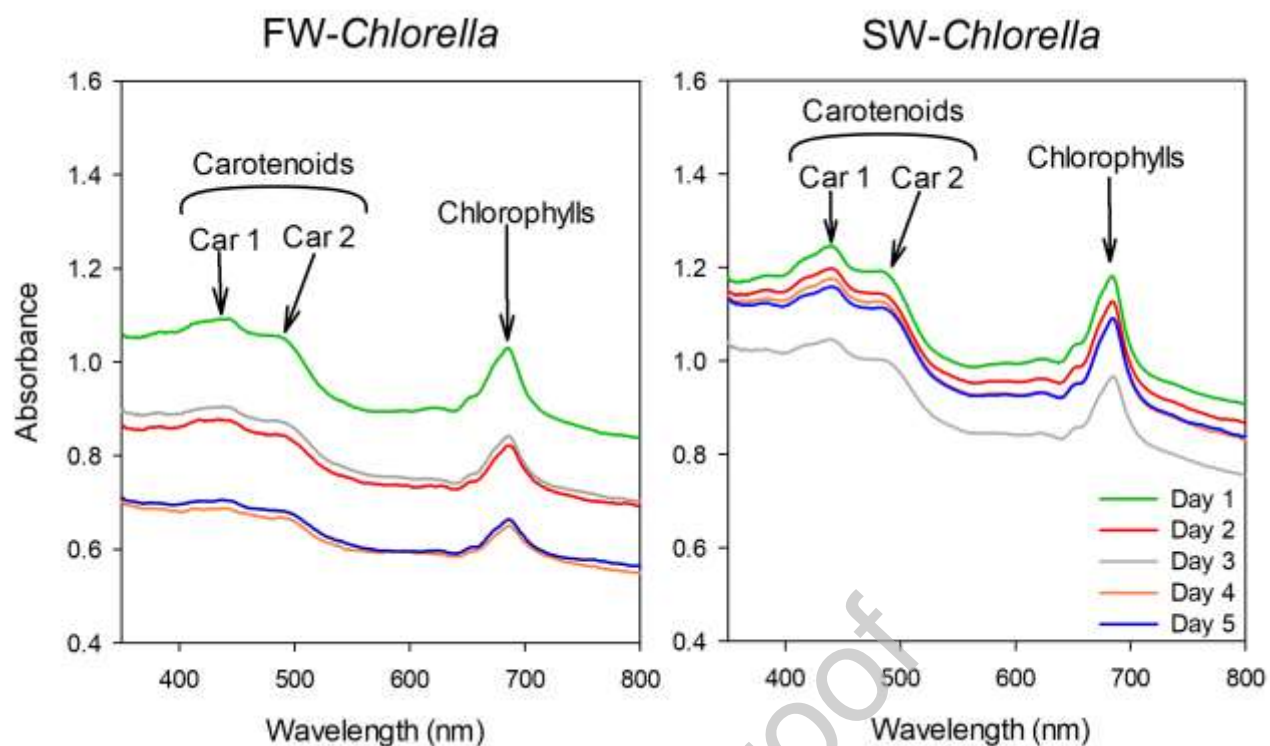
Principal component analysis (PCA) with Pearson's correlation coefficient as the similarity index was performed with XLSTAT 2019.1.1.62917 (Microsoft Excel®, Addinsoft). The objective was to assess which spectral measurements (*i.e.*, peak area, peak height, peak width at base, wavelength at maximum absorbance for chlorophylls and carotenoids, as well as the ratio of chlorophyll to carotenoid peak areas) were most indicative of a potential effect of glyphosate on *C. vulgaris*. FW-*Chlorella* and SW-*Chlorella* data were analyzed together to better understand the difference in response with salinity. Variables were standardized using the skewed standard deviation to overcome the difference in units. A distance biplot was constructed to represent approximate Euclidean distances between the variables.

### 3. RESULTS

#### 3.1 Influence of salinity on *Chlorella vulgaris*

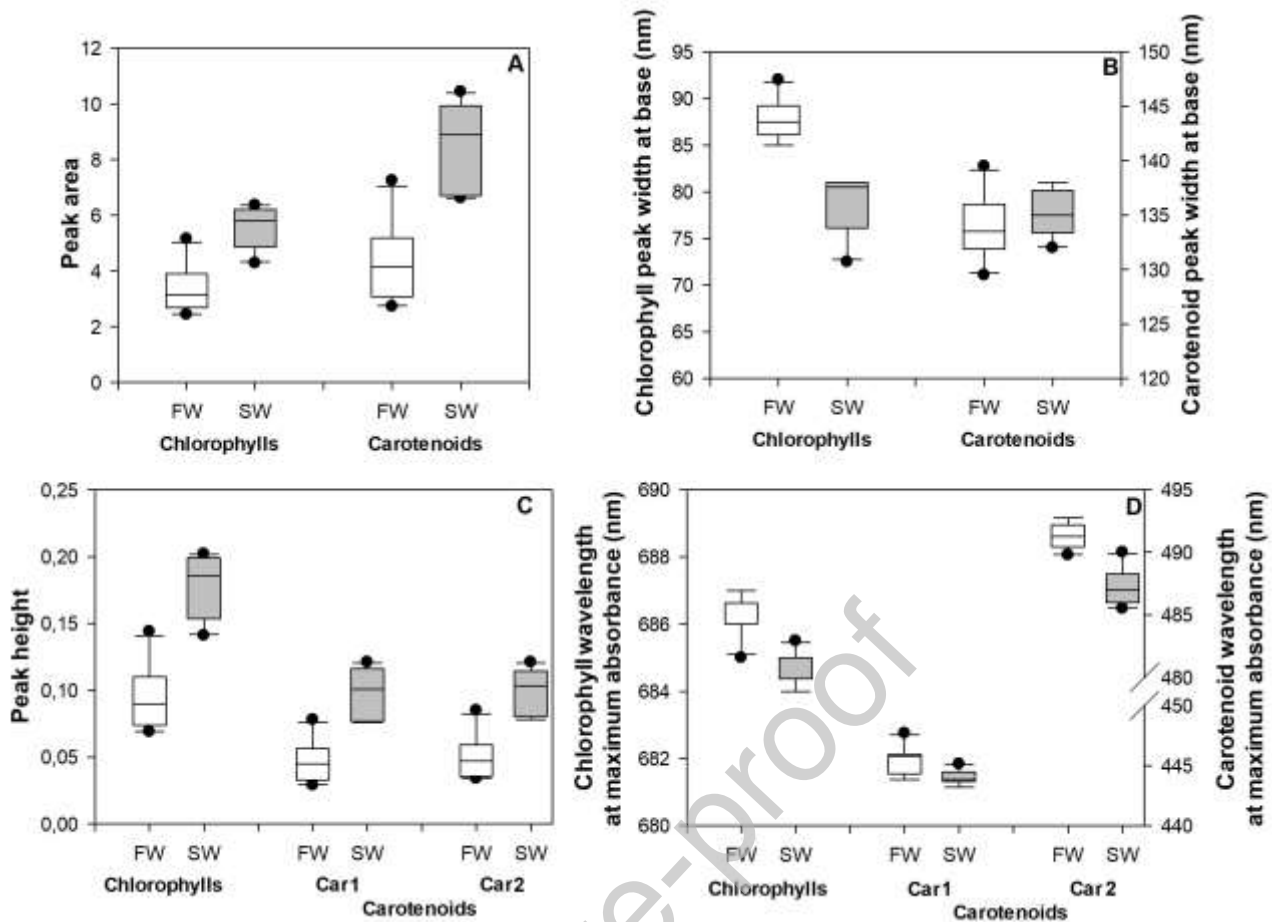
Before performing the ecotoxicological tests, we evaluated the effect of salinity on the population dynamics of *Chlorella vulgaris* for five days without any exposure to Roundup®. Then, the results obtained in the presence of glyphosate were compared to this control experiment. The absorption spectra changed as a function of time (from day 1 to day 5), but also as a function of the culture medium. **Figures 1 and 2** represent the different spectral characteristics measured with the freshwater and seawater controls: absorption spectra between 350 and 800 nm (**Figure 1**), as well as peak areas (**Figure 2A**), peak widths at base (**Figure 2B**), peak heights (**Figure 2C**), and wavelengths at maximum absorbance (**Figure 2D**) for each photosynthetic pigment identified.

Absorption spectra had a similar appearance in freshwater and seawater with two overlapping peaks - Car 1 and Car 2 - representing total carotenoids (391-538 nm for FW-*Chlorella*, 391-534 nm for SW-*Chlorella*) and a better-defined peak representing chlorophylls (638-733 nm for FW-*Chlorella*, 638-720 nm for SW-*Chlorella*) (**Figure 1**). In both cases, duplicates A and B demonstrated the same spectral profile, as well as the same trend. For carotenoids, the height and wavelength at maximum absorbance were determined for each Car 1 and Car 2 peak, while the area and width at the base were evaluated for these two peaks together. Photosynthetic pigment peaks were slightly less spread out at the base for SW-*Chlorella*. The mean absorbance value between 350 and 800 nm was lower in freshwater, which is the natural environment of *Chlorella*, than in seawater ( $0.747 \pm 0.115$  and  $0.992 \pm 0.114$ , respectively).



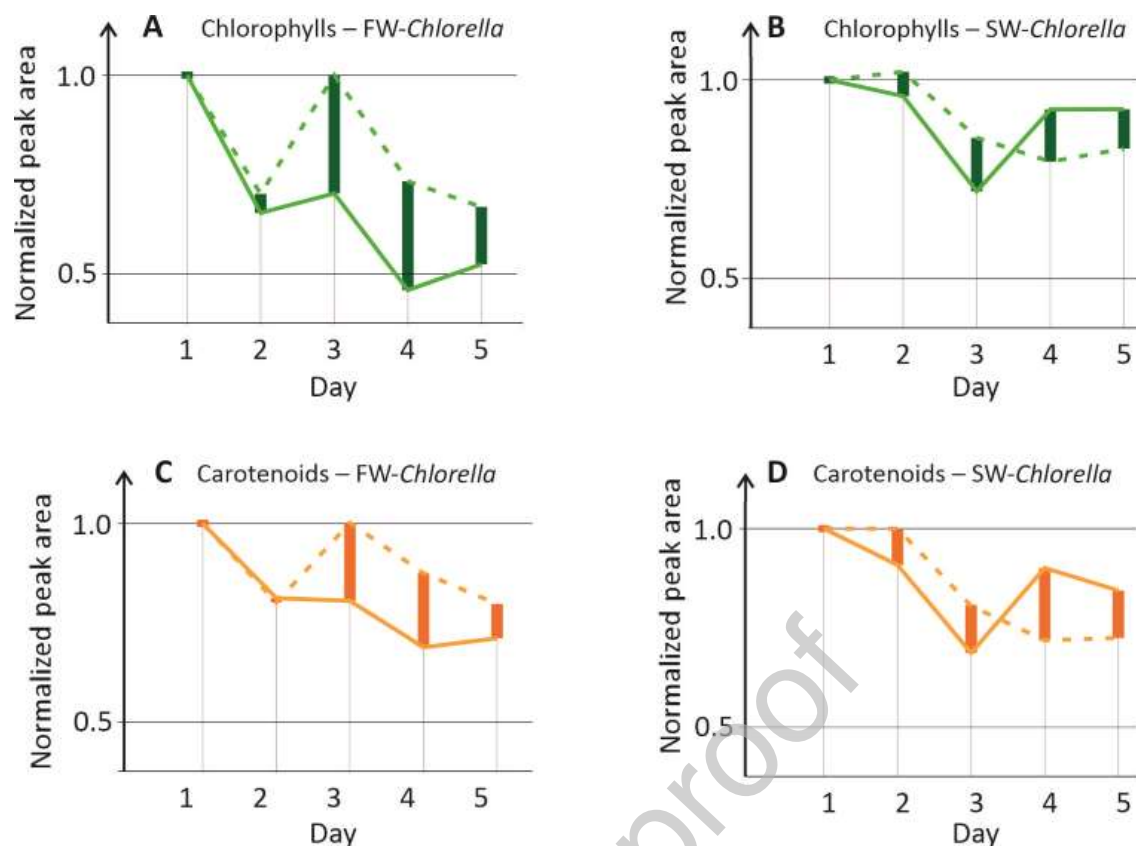
**Figure 1.** Absorption spectra of the freshwater (FW-*Chlorella*) and seawater (SW-*Chlorella*) controls (series A) from day 1 to day 5.

Statistics on the entire data set (series A and B) (**Figure 2**) showed that, as with the absorbance values, the peak areas of photosynthetic pigments were lower in FW-*Chlorella* ( $3.34 \pm 0.84$  for chlorophylls,  $4.25 \pm 1.39$  for carotenoids) than in SW-*Chlorella* ( $5.56 \pm 0.75$  for chlorophylls,  $8.52 \pm 1.49$  for carotenoids) (**Figure 2A**). The same pattern was observed for the peak height of chlorophylls ( $0.094 \pm 0.024$  in freshwater,  $0.177 \pm 0.023$  in seawater) and carotenoids ( $0.048 \pm 0.015$  in freshwater,  $0.099 \pm 0.017$  in seawater) (**Figure 2C**). However, with respect to the peak width at the base, chlorophylls exhibited higher values in freshwater (around 9 nm higher), whereas carotenoids had similar values in both environments. There was also a slight shift in the wavelength at maximum absorbance for chlorophylls and carotenoids (**Figure 2B**). Thus, values were higher in FW-*Chlorella* than in SW-*Chlorella* with an average deviation of 1.4 nm for chlorophylls, 1.1 nm for Car 1 and 2.9 nm for Car 2 (**Figure 2D**).



**Figure 2.** Characteristics of chlorophyll and carotenoid peaks for the FW-*Chlorella* (white) and SW-*Chlorella* (grey) controls: peak area (A), peak width at base (B), peak height (C), and wavelength at maximum absorbance (D). The center boxes show data between the 25<sup>th</sup> and 75<sup>th</sup> percentiles with the median represented by a line. The whiskers extend to the 10<sup>th</sup> and 90<sup>th</sup> percentiles. The dots represent the 5<sup>th</sup> and 95<sup>th</sup> percentiles. FW – freshwater, SW – seawater.

The time course of the spectra during the five-day experiment was also different (**Figure 3**). Despite the significant differences observed between the two series, FW-*Chlorella* showed a decrease in absorbance values on the second day, followed by a stabilization on day 3, then a further decrease on the last two days. SW-*Chlorella* was maintained for the first two days at high absorbance values and decreased on the third day.



**Figure 3.** Change in peak area as a function of time. The peak area is normalized to the peak area on the first day. Plain lines – series A, dashed lines – series B. The vertical bars indicate the difference between series A and B.

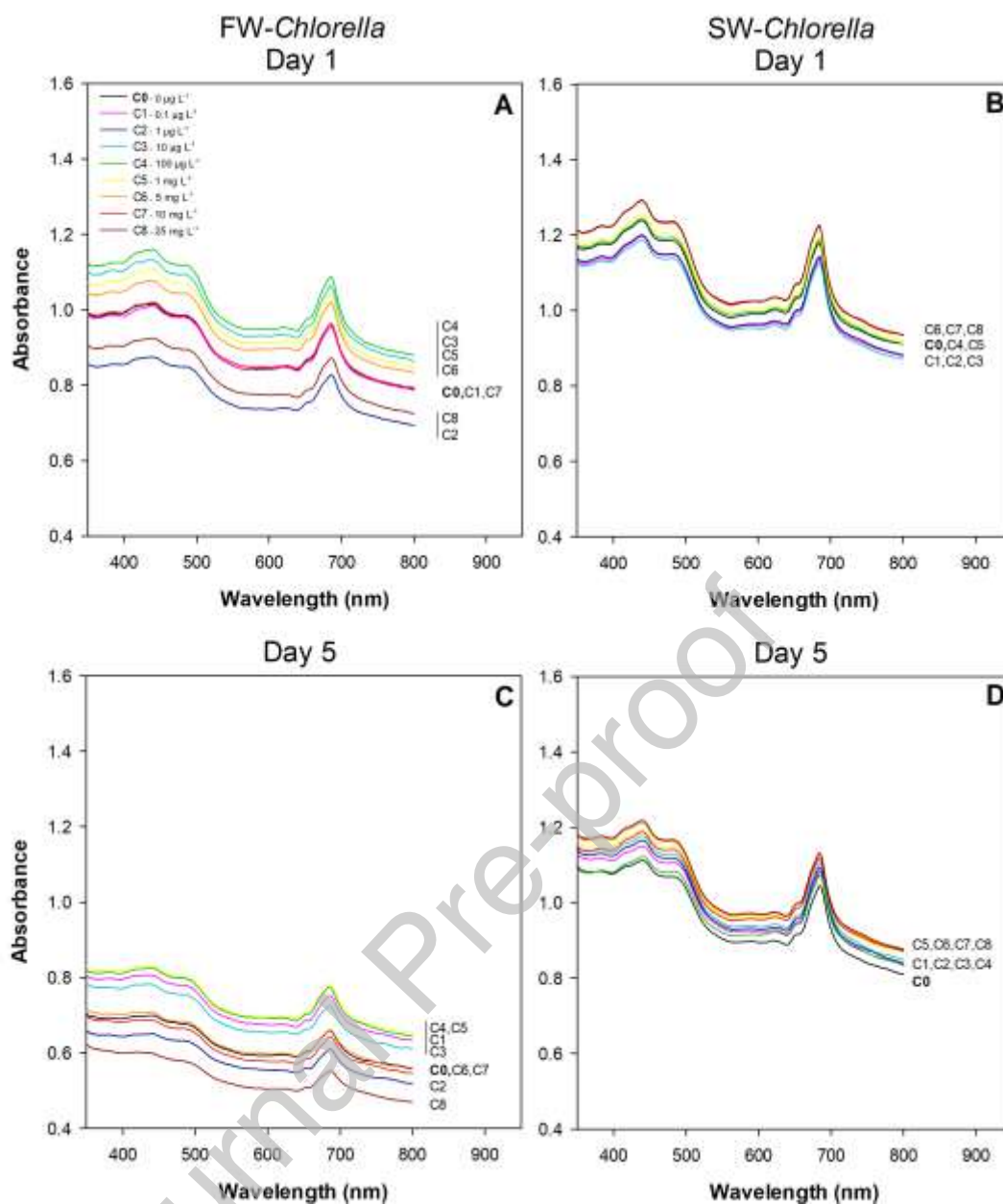
### 3.2 Evolution of *C. vulgaris* exposed to Roundup® contamination

The temporal evolution of the population can be determined from the average absorbance calculated from the A and B series (Figure 4). The absorption spectra exhibit a baseline drift over the measurement time. These shifts are often due to variable light scattering between samples, especially in the case of heterogeneous particle sizes. However, this does not affect the processing of the spectra.

For FW-*Chlorella*, a decrease was observed between day 1 and day 5 (Figures 4A and 4C). At the beginning of the experiment, the spectra of the lowest and highest concentrations of glyphosate (0.1, 1, 10,000 and 25,000  $\mu\text{g L}^{-1}$ ) were equal to or lower than the spectrum of the control (Figure



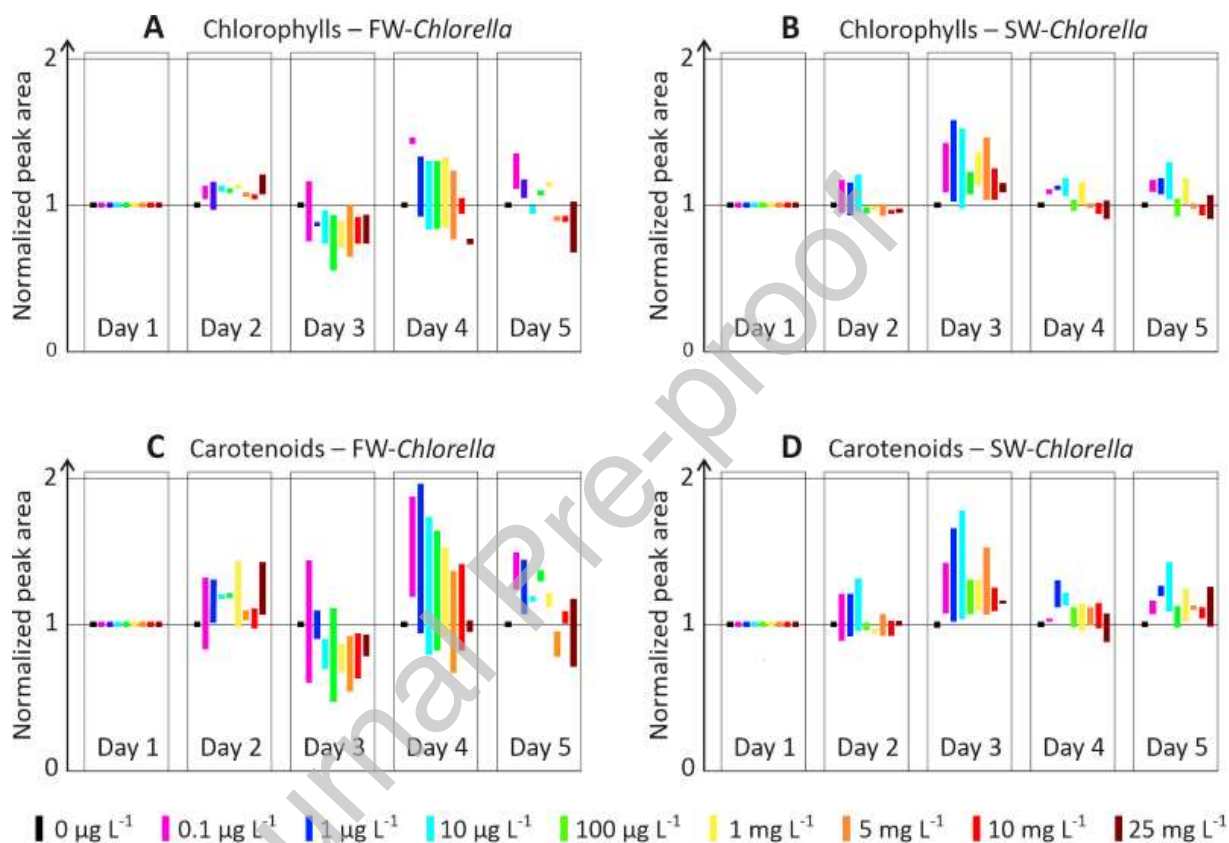
**4A**). Conversely, cell stimulation was observed at intermediate concentrations (10, 100, 1,000 and 5,000  $\mu\text{g L}^{-1}$ ). Five days later, the trend was similar, but with recovery occurring with the 0.1  $\mu\text{g L}^{-1}$  concentration (**Figure 4C**). *SW-Chlorella* appeared to maintain its population throughout the experiment with absorbance values of  $1.04 \pm 0.11$  and close spectra for all concentrations (**Figures 4B and 4D**). On the first day, the spectra obtained for 0.1, 1 and 10  $\mu\text{g L}^{-1}$  were lower than the control spectrum (**Figure 4B**). An increase in absorbance values with increasing glyphosate concentration was then observed, with the spectra of 100 and 1,000  $\mu\text{g L}^{-1}$  being at the same level as the control spectrum and those of 5, 10 and 25  $\text{mg L}^{-1}$  exceeding it. On the fifth day, the control spectrum ended up with the lowest absorbance values (**Figure 4D**). The spectra of 0.1, 1, 10 and 100  $\mu\text{g L}^{-1}$  were located just above the control spectrum, followed by those of 1, 5, 10 and 25  $\text{mg L}^{-1}$ . Furthermore, the difference between series A and B emphasized some instability for *FW-Chlorella*. This corresponded to 10-20% in the average absorbance values (350-800 nm) for five of the nine concentrations tested on day 1 and only one concentration on day 5. This difference reached more than 20% on four occasions on days 2, 3 and 4. In 65% of cases, it remained below 10%. For *SW-Chlorella*, the percentages were systematically lower than 10%, and even 5% in 68% of cases.



**Figure 4.** Average absorption spectra for all concentrations on days 1 (A-B) and 5 (C-D) in FW-Chlorella (A-C) and SW-Chlorella (B-D).

In terms of peak area changes as a function of time, the normalized values oscillated around the control for FW-Chlorella (Figures 5A and 5C), with a larger amplitude for carotenoids than for chlorophylls. The impact of glyphosate appeared to emerge at the end of the experiment with the lowest concentrations above the control (0.1, 100 and 1,000  $\mu\text{g L}^{-1}$ ) and the highest below (5, 10 and 25  $\text{mg L}^{-1}$ ). An exception was noted at 10  $\mu\text{g L}^{-1}$  with a normalized peak area lower than the

control for chlorophylls and higher for carotenoids. For *SW-Chlorella*, the peak areas of the contaminated solutions presented higher values than the control, especially on day 3 (**Figures 5B and 5D**). An inverse dose-effect relationship to that observed with the absorption spectra was demonstrated, as the normalized peak areas slowly decreased with increasing glyphosate concentration, which was particularly marked on days 4 and 5.



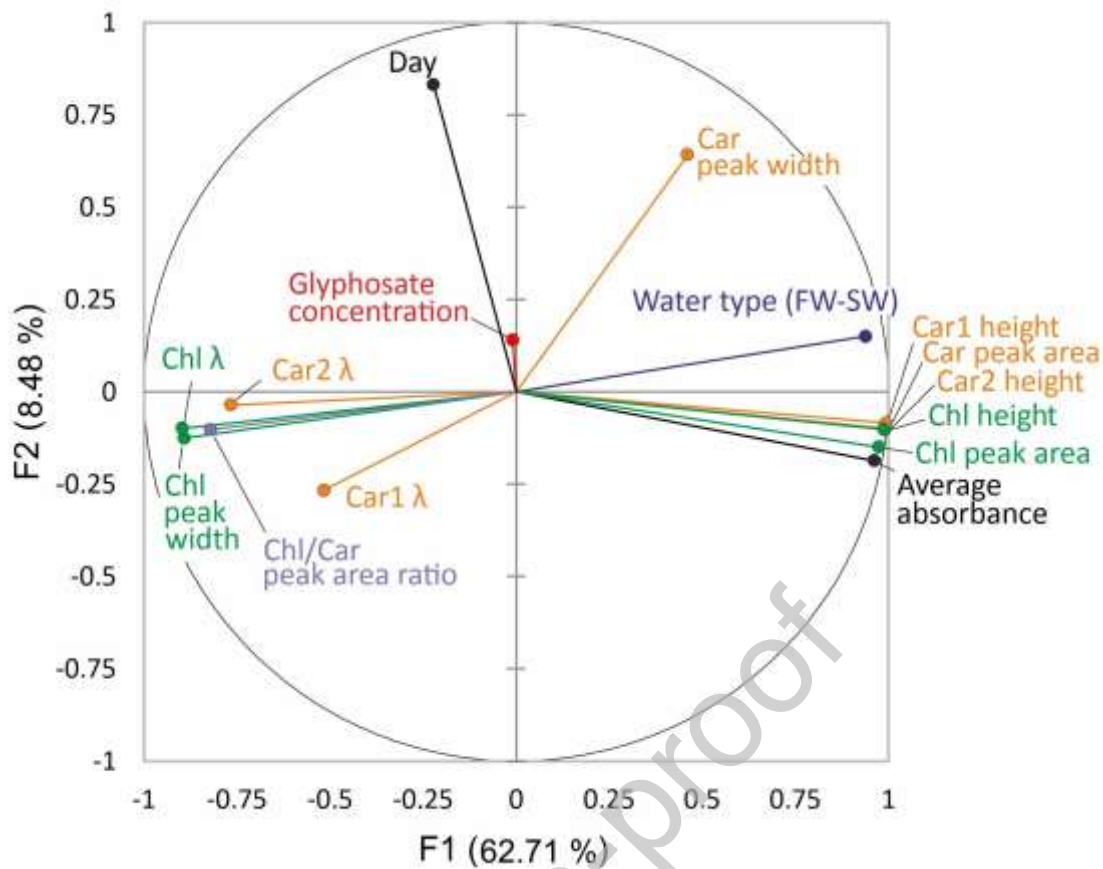
**Figure 5.** Change in normalized peak area as a function of time, for all glyphosate concentrations for chlorophylls (A-B) and carotenoids (C-D) in *FW-Chlorella* (A-C) and *SW-Chlorella* (B-D). Peak area is normalized to the peak area of control (0 µg L<sup>-1</sup>). The vertical bars indicate the difference between series A and B.

The analysis of the other spectral features allowed us to know more precisely which component of the peak area can be modified in case of environmental perturbation. For *FW-Chlorella*, the decrease in peak areas between the lowest concentration tested ( $3.52 \pm 0.79$  at 0.1 µg L<sup>-1</sup>) and the

highest ( $2.63 \pm 0.85$  at  $25 \text{ mg L}^{-1}$ ) corresponded to a peak height crush of 23% for chlorophylls and 27-28% for carotenoids. For SW-*Chlorella*, the extension of peak areas for chlorophylls (from  $5.75 \pm 0.38$  at  $0.1 \mu\text{g L}^{-1}$  to  $6.17 \pm 0.59$  at  $25 \text{ mg L}^{-1}$ ) and for total carotenoids (from  $8.64 \pm 0.97$  at  $0.1 \mu\text{g L}^{-1}$  to  $9.62 \pm 1.17$  at  $25 \text{ mg L}^{-1}$ ) was both in height (6% for chlorophylls, 8 and 12% for Car 1 and Car 2 respectively) and in width (1.1 nm for chlorophylls, 0.9 nm for total carotenoids). Carotenoids also showed a shift in the wavelength value of their two peaks, which decreased by 0.8-0.9 nm between the lowest and highest concentrations tested.

### 3.3 Use of peak areas as a bioindicator in ecotoxicology

We performed a principal component analysis (PCA) on the FW-*Chlorella* and SW-*Chlorella* data to assess which spectral variables were most relevant to consider (**Figure 6**). The first two factorial axis explained 71.19% of the total variance, which was related to the F1 axis (62.71%). The Pearson's correlation coefficient between Car 1 height, Car 2 height, Car peak area, Chl height, Chl peak area, and average absorbance were all higher than 0.97; so, the description of the evolution of one over time applied to the others. For the chlorophyll and carotenoid peaks, there was an anticorrelation between wavelength and height. When the peak was higher, there was a shift in wavelength at maximum absorbance to a lower value. The chlorophyll/carotenoid peak area ratio was positively correlated with the wavelength at maximum absorbance and the width at the base of the chlorophyll peak ( $\rho > 0.77$ ), indicating that the variations in the chlorophyll peak were larger than those in the carotenoid peak. This analysis also confirmed the influence of water salinity on absorbance values and spectral characteristics of photosynthetic pigments, especially for chlorophylls ( $\rho > 0.84$ ).

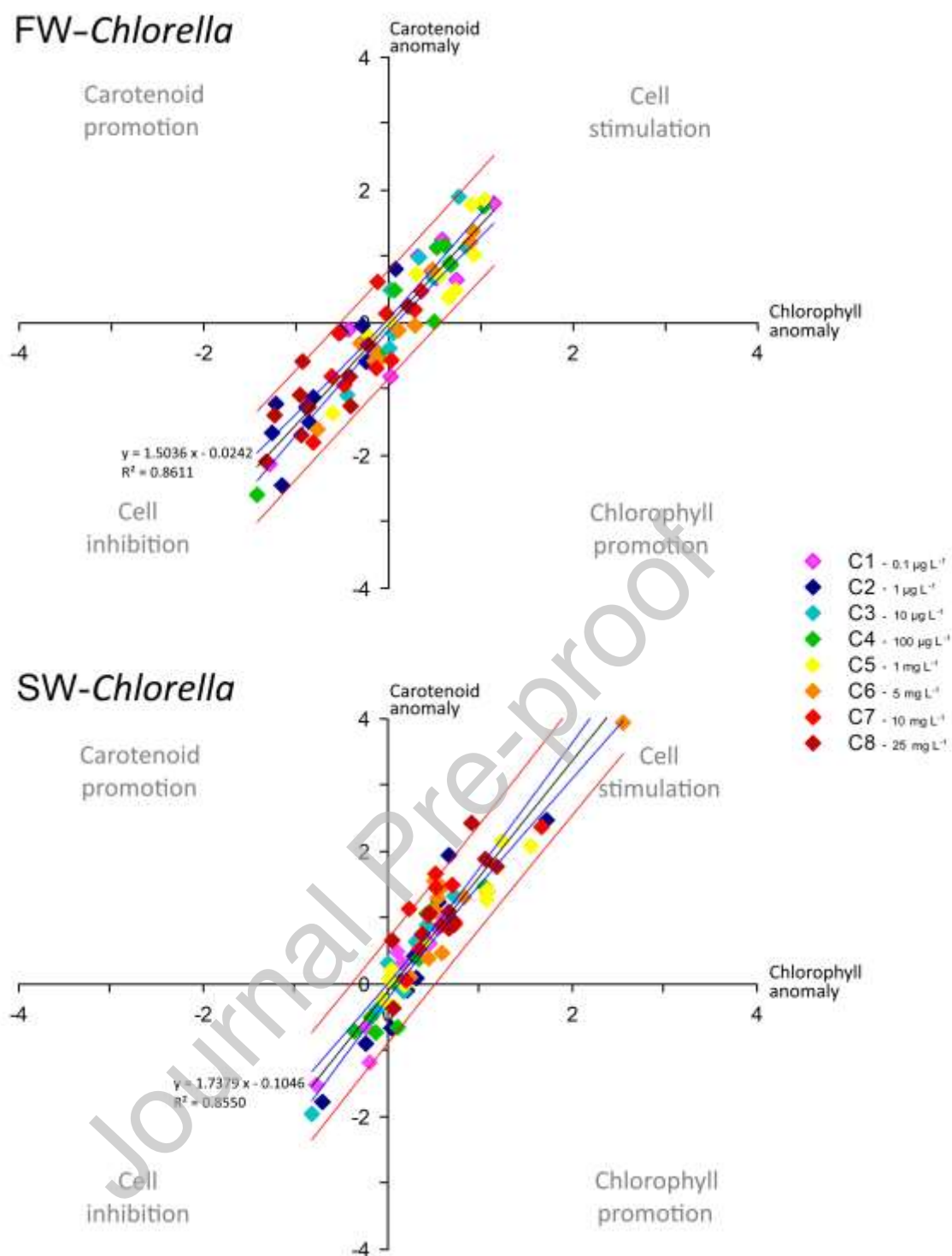


**Figure 6.** Principal component analysis (PCA) performed on the spectral features for the entire data set. Chl – chlorophylls, Car – carotenoids,  $\lambda$  – wavelength at maximum absorbance, height – peak height, peak width – peak width at base.

Peak area anomalies of each photosynthetic pigment were then calculated (**Figure 7**). They corresponded to the difference between the experimental and control peak areas. In the upper right part of the graph (chlorophyll anomaly  $> 0$  and carotenoid anomaly  $> 0$ ), the increase of both values indicated a stimulation of photosynthetic cells. Conversely, in the lower left (chlorophyll anomaly  $< 0$  and carotenoid anomaly  $< 0$ ), their decrease corresponded to inhibition. In both cases, the contribution of photosynthetic pigments remained balanced. Finally, carotenoids appeared promoted in the upper left (chlorophyll anomaly  $< 0$  and carotenoid anomaly  $> 0$ ), while chlorophylls took the lead in the lower right (chlorophyll anomaly  $> 0$  and carotenoid anomaly  $< 0$ ). Regardless of the environment in which the cells evolved, chlorophyll and carotenoid anomalies were distributed

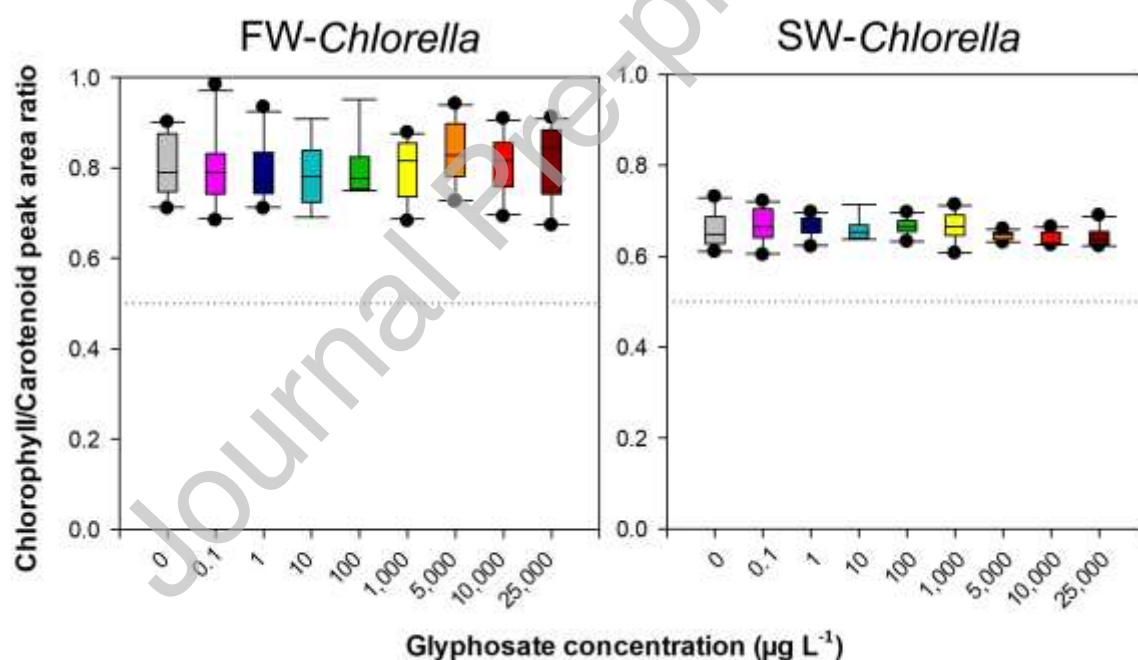
along a line from a zone of inhibition (anomalies < 0) to a zone of stimulation (anomalies > 0). In our ecotoxicological test, no imbalance in the contribution of photosynthetic pigments was observed. The two inverse trends previously highlighted were confirmed by this bioindication tool. The lowest and two highest concentrations (1, 10,000 and 25,000  $\mu\text{g L}^{-1}$ ) negatively affected FW-*Chlorella*, whereas intermediate concentrations stimulated them (**Figure 7A**). In contrast, SW-*Chlorella* showed a clear increase in chlorophyll and carotenoid anomalies above 1  $\text{mg L}^{-1}$  (**Figure 7B**).

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**Figure 7.** Peak area anomalies of chlorophylls and carotenoids for FW-*Chlorella* (top) and SW-*Chlorella* (bottom) exposed to Roundup®. The peak area anomaly was the difference between the experimental and control peak areas. The linear regression lines (black) are surrounded by a 95% confidence band (blue) and a 95% prediction band (red).

The association of chlorophyll and carotenoid peak areas in a ratio showed that the contribution of photosynthetic pigments was mainly affected by the growing environment (**Figure 8**). Over the whole experiment, the contribution of chlorophylls to carotenoids appeared particularly high for FW-*Chlorella* with an average ratio value of  $0.804 \pm 0.070$ , while it reached  $0.656 \pm 0.027$  for SW-*Chlorella*. The temporal evolution of this ratio remained similar with an increase of 8.2% in freshwater and 6.8% in seawater between day 1 and day 5. The lack of imbalance in the contribution of photosynthetic pigments that was demonstrated by the analysis of peak area anomalies was confirmed by the chlorophyll/carotenoid peak area ratio. For FW-*Chlorella*, the ratio was slightly higher above  $1 \text{ mg L}^{-1}$  of glyphosate, with median values ranging from 0.816 to 0.845 (*versus* 0.790 for the control). For SW-*Chlorella*, the median value of the chlorophyll/carotenoid peak area ratio was approximately  $0.653 \pm 0.013$  for all concentrations tested.



**Figure 8.** Chlorophyll/carotenoid peak area ratio for all concentrations tested in FW-*Chlorella* and SW-*Chlorella*. The center boxes show data between the 25<sup>th</sup> and 75<sup>th</sup> percentiles, with the median represented by a line. The whiskers extend to the 10<sup>th</sup> and 90<sup>th</sup> percentiles. The dots represent the 5<sup>th</sup> and 95<sup>th</sup> percentiles.



## 4. DISCUSSION

Standard ecotoxicology experiments on phytoplankton presented by the Organisation for Economic Co-operation and Development (OECD) consist of a growth inhibition test (OECD, 2011). The cell biomass is evaluated by counting under a microscope equipped with a counting chamber. Automated systems, such as flow cytometry, can also be used for pico- and nanophytoplankton (0.2 to 20  $\mu\text{m}$ ). An optical measurement is only carried out as a complement to appreciate the evolution of the biomass, provided that there is a good correlation between the counted biomass and the chlorophyll concentration. This type of result is intended to obtain the classical indices of ecotoxicology. The EC50 and EC10 (effective concentration) represent contaminant concentrations that result in a 50% and 10% reduction in biomass growth compared to the control (ChemSafetyPRO, 2017). The NOEC (no-observed-effect concentration) indicates the highest concentration for which no toxic effects are detected (ChemSafetyPRO, 2017). As part of the REACH regulation in Europe (Registration, Evaluation and Authorisation of Chemicals), these indices are then used to calculate the PNEC (predicted no-effect concentration) which is the highest concentration of contaminant that is safe for aquatic environments (ChemSafetyPRO, 2017). In this research work, we tried to develop an approach to assess the ecotoxicological impact on a metabolic function, such as photosynthesis, rather than on population growth. Altering this fundamental process could have negative consequences at the base of the foodweb, which can cascade to higher trophic levels and affect the functioning of aquatic ecosystems.

Pigment content is usually assayed after extraction by high performance liquid chromatography (HPLC) (Mahmoud et al., 2016; Picazo et al., 2013; Safi et al., 2014). Nevertheless, this technique leads to the destruction of the cells and prevents the monitoring of their physiological changes according to the studied perturbation. Microalgae were best preserved when analyzed by ultraviolet/visible spectroscopy. It should be noted that this method remains less accurate than

HPLC, both in terms of quality and quantity (Dos Santos et al., 2003). Two groups of photosynthetic pigments - chlorophylls and carotenoids - can be identified on the absorption spectrum of *Chlorella vulgaris*. For optimization purposes, the absorbance of photosynthetic pigments is typically measured at a single wavelength, either for biomass assessment or for calculation of spectral indices (Kaeofoon et al., 2021; Wong et al., 2017; Zhou et al., 2019).

This preliminary study proposes a compromise between fine analysis by HPLC and succinct analysis by single-wavelength spectroscopy. The influence of an environmental disturbance could then be determined by ultraviolet/visible spectroscopy on the optical characteristics of the absorption spectrum. The actual state of phytoplankton cells would be adequately represented by the spectroscopic characterization of the photosynthetic pigments. Here we used four parameters to describe a peak: area, width at base, height, and wavelength at maximum absorbance. Peak area and peak height were strongly correlated, while the wavelength at maximum absorbance can be shifted due to environmental factors. For instance, this was the case for the SW-*Chlorella* carotenoid peaks that shifted ~1 nm with increasing glyphosate concentration. Unlike peak areas, absorbance values could then be underestimated *via* their usual determination at a single wavelength and introduce a bias in data interpretation. Although it is not yet possible to link the peak width to a particular process, the shift in wavelength at maximum absorbance indicated a change that could have implications for the energy transfer within photosystems and the quantum yield of CO<sub>2</sub> uptake (Liu and Van Iersel, 2021). These spectroscopic parameters were used to refine the diagnosis of the impact of a contaminant on phytoplankton species. They may then be employed as a marker of an altered photosynthetic apparatus, as environmental disturbances can affect the efficiency of light-to-biomass conversion (Vecchi et al., 2020). In this context, the measurement of the absorption spectrum by spectroscopy appeared as an accessible monitoring tool to perform daily analyses on the numerous samples needed to obtain an ecotoxicological response.

This approach was applied to evaluate the potential impact of Roundup® on the unicellular green microalga, *Chlorella vulgaris* Beijerinck, 1890. This species is commonly used as a model organism in ecotoxicological tests (Kaeboon et al., 2021; Silva et al., 2009; Wu et al., 2016). It contains chlorophylls *a* and *b*, as well as carotenoids such as violaxanthin, neoxanthin, astaxanthin, canthaxanthin, lutein,  $\beta$ -carotene, and xanthophyll (Mahmoud et al., 2016; Mendes et al., 1995). This phytoplanktonic species presents good sensitivity to contaminants and is easily cultured in the laboratory. Mensah et al. (2013) found that among a panel of seven microalgae, *Chlorella* species were the most sensitive to Roundup® exposure. Cellular inhibition in FW-*Chlorella* by the highest concentrations ( $> 5 \text{ mg L}^{-1}$ ) was consistent with the EC50 of  $3.85 \text{ mg L}^{-1}$  reported by Reno et al. (2016) for *C. vulgaris* contaminated with Roundup Ultramax®. Our study also showed an increase in absorbance values at intermediate concentrations (10 to  $5,000 \mu\text{g L}^{-1}$ ) indicating, in this case, a stimulation of the FW-*Chlorella* population. Since no nutrients were added during the five-day experiment, glyphosate could be used as a source of phosphorus (P) by organisms in contact with less toxic concentrations. Indeed, bacteria convert glyphosate to inorganic phosphate (Pi) through the action of C-P lyase and make it bioavailable in water (Matozzo et al., 2020). Phosphorus (P) uptake and assimilation from glyphosate biodegradation were observed in freshwater cyanobacteria, with changes in gene expression and enzyme activity (Drzyzga and Lipok, 2018; Qiu et al., 2013). This input is a significant problem for freshwater ecosystems where it promotes eutrophication (Vera et al., 2010). However, it can be considered as a "welcome contaminant" especially in marine habitats, such as the Mediterranean Sea, where this element is often limiting for the development of primary producers (Djaoudi et al., 2018).

Another objective of this study was to test the potential ecotoxicological effects of Roundup® on the same phytoplankton organism in two types of aquatic environment: inland waters and coastal marine areas. A typical freshwater strain (FW-*Chlorella*) was therefore successfully adapted to a

salinity similar to that of seawater (SW-*Chlorella*). Microalgae of the genus *Chlorella* are known to implement tolerance mechanisms under persistent high salinity conditions (Shetty et al., 2019). Since it cannot change cell volume due to a rigid cell wall, osmoregulation occurs through the production of organic solutes (e.g., trehalose) and the accumulation of inorganic ions. The production of these osmoprotective molecules and the exclusion of Na<sup>+</sup> consume a lot of energy inducing an increase in the storage of lipids and total proteins. High salinity can then lead to stunted growth, loss of photosynthesis (i.e., chlorophyll breakdown and carotenoid accumulation), and downregulation of oxidative phosphorylation (Almutairi et al., 2021; Li et al., 2018). This loss of photosynthesis was confirmed in our study by the decrease of the chlorophyll/carotenoid peak area ratio in SW-*Chlorella*. Nevertheless, Farghl et al. (2015) pointed out that induction of antioxidants helps cells overcome the negative effects of sodium, as shown by growth behavior. Prior to our experiment, SW-*Chlorella* achieved cell recovery after a few weeks of adaptation with greater growth and spectral characteristics than the original FW-*Chlorella* strain. These changes in metabolic functioning may contribute to improve tolerance of SW-*Chlorella* exposed to Roundup®. A dose-response relationship was demonstrated with stimulation of the SW-*Chlorella* population with increasing glyphosate concentration. Conversely, the marine diatom *Phaeodactylum tricornutum* showed a decrease in cell density with only 250 µg L<sup>-1</sup> of Roundup Pronto® (Cruz de Carvalho et al., 2020). Its photosynthetic activity was inhibited due to the decrease of antioxidant capacities, which were instead induced in SW-*Chlorella* to cope with salinity stress.

In addition, SW-*Chlorella* exhibited higher carotenoid content, as well as specific changes in their spectral characteristics with an increase in peak area, a shift in wavelength at maximum absorbance, and a spreading of the peak width at the base as a function of glyphosate concentration. Carotenoid accumulation has previously been reported as a defense mechanism when *C. vulgaris* is exposed to a salty environment (Almutairi et al., 2021). These have been shown to be photoprotective antioxidant pigments used when cells are exposed to environmental stressors (Cruz de Carvalho et

al., 2020). They increase energy dissipation by non-photochemical processes and prevent possible photoinhibitory events. Among carotenoids, lutein appears to be positively affected by glyphosate contamination (Mysore Doddaiah et al., 2013). In the carotenoid biosynthetic pathway, glyphosate inhibits the activity of lycopene  $\beta$ -cyclase which prevents the synthesis of  $\beta$ -carotene from lycopene. The synthesis of  $\alpha$ -carotene, from which lutein is derived, is then enhanced. In addition, glyphosate can impair the chlorophyll biosynthetic pathway through magnesium (Mg) and iron (Fe) deficiency due to the formation of insoluble metal complexes (Kaeofoon et al., 2021; Mertens et al., 2018). Electron transport, photosystem activities (PSI and PSII), and NADH and NADPH pools could also be negatively affected (Kaeofoon et al., 2021). Exposure to Roundup® would then force a transfer of energy from photosynthesis to energy consumption. The adaptation of *C. vulgaris* to seawater may therefore have increased its resilience to this environmental disturbance.

## 5. CONCLUSION

The introduction of glyphosate into aquatic ecosystems constitutes a disruption of the phytoplankton compartment at the base of the foodweb. In this study, an unusual dose-response relationship was demonstrated for *Chlorella vulgaris* grown in freshwater, *i.e.* in its natural environment, with a stimulation at intermediate concentrations of Roundup® (10 to 5,000  $\mu\text{g L}^{-1}$ ) and an inhibition otherwise. Marine-adapted *C. vulgaris* were stimulated in the presence of glyphosate, particularly at high concentrations ( $> 1 \text{ mg L}^{-1}$ ). This emerging contaminant could then be used as a source of phosphorus. The defense mechanisms activated by SW-*Chlorella* to adapt to seawater may have increased its resilience to exposure levels that could be toxic in freshwater. It should be noted that the maximum intensity of these effects was not reached for the concentrations tested.

In addition, our exploration of spectral changes in photosynthetic pigments revealed a shift in the wavelength at maximum absorbance in response to environmental stresses. Thus, unlike the peak area, the determination of peak height could be underestimated with the usual technique of single-wavelength absorbance. Changes in peak shape (*e.g.*, crushing with a decrease in peak height, spreading with an increase in peak width at base) may also be an indicator of altered photosynthetic efficiency. The analysis of absorption spectrum and the use of peak areas appear to be a promising approach to monitoring the evolution of ecotoxicological effects. This preliminary work must be continued and confirmed by applying this method to other emerging contaminants such as pharmaceutical substances.

## AUTHOR CONTRIBUTIONS STATEMENT

Floriane Delpy contributed to the implementation of methodology, the realization of experiments, the data processing, the writing of original draft, and the review of manuscript.

Yves Lucas contributed to the data processing, the writing of original draft, and the review of manuscript.

Patricia Merdy contributed to the funding acquisition, the implementation of methodology, the supervision of experiments, the data processing, the writing of original draft, and the review of manuscript.

#### **Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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