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RESEARCH ARTICLE

Toxicological effects of active and inert ingredients of imazethapyr formulation Verosil® against *Scenedesmus vacuolatus* (Chlorophyta)

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Abstract

Imazethapyr, a selective systemic herbicide, is widely used in agriculture and it is frequently detected in water bodies close to application areas. Like other agrochemicals, imazethapyr is commercialized in formulations containing a mixture of additives that increase the effectiveness of the active ingredient. These complex mixtures may cause adverse effects on non-target primary producers, such as microalgae, when they reach freshwater bodies. The aim of this study was to assess the effects, separately, of the formulation Verosil®, the formulation additives, and technical-grade imazethapyr, in the acidic form or as ammonium salt, on the microalga *Scenedesmus vacuolatus* (Chlorophyta). Verosil®, formulation additives, and acid imazethapyr significantly inhibited the growth of *S. vacuolatus* (Verosil® > formulation additives > acid imazethapyr) and caused morphological alterations from 2 mg L^{-1}, 4 mg L^{-1}, and 60 mg L^{-1} onwards, respectively. Verosil® and formulation additives caused the most adverse effect including membrane disorganization, cytoplasm contraction, cell wall thickening, thylakoidal membrane disaggregation, and starch granule accumulation. In addition, Verosil® and formulation additives increased the chl *a*/chl *b* ratio, indicating possible alterations in photosystems as a stress response. The carotene/chl *a* ratio was also increased in microalgae exposed to both Verosil® and formulation additives, suggesting an antioxidant response to these toxic compounds. All these results support the hypothesis that the formulation additives contribute significantly to the toxicity and alterations caused by the commercial formulation Verosil® on *S. vacuolatus*.

Keywords Green algae · Herbicide · Imidazolinone · Non-ionic Surfactant · *Scenedesmus* · Formulation additives

Introduction

Imazethapyr is a selective systemic herbicide that belongs to the imidazolinone group. This herbicide inhibits the enzyme acetohydroxyacid synthase (EC 2.2.1.6) in plants, thus interfering with the biosynthesis of the branched-chain amino acids valine, leucine, and isoleucine (Ortiz et al. 2017; Xie et al. 2018). The effect of these types of herbicides causes cell death by blocking amino acid biosynthesis, which is a target for the disruption of plant metabolism (Shaner et al. 1984). Imazethapyr is the most widely used imidazolinone due to its high activity at low application rates and its broad-spectrum of weed control in soybean and other legume crops (Gupta et al. 2014; Hess et al. 2010), as well as its persistence in the soil (Souza et al. 2016). However, it has scarcely been researched by the scientific community and little information on its environmental fate is available. A study conducted in the USA by Battaglin et al. (2000) to assess herbicide occurrence in water bodies near agricultural

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Highlights
- The effects of the imazethapyr formulation Verosil® and its components were tested separately.
- *Scenedesmus vacuolatus* growth was inhibited by imazethapyr and formulation adjuvants.
- Imazethapyr acid and its ammonium salt had little toxic effect on *S. vacuolatus*.
- Verosil® or its adjuvants alone alter *S. vacuolatus* morphology and ultrastruture.
- Verosil® or its adjuvants altered S. vacuolatus pigments.

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areas showed that imazethapyr filters into the groundwater and it is one of the most frequently detected herbicides (present in 71% of all analyzed samples) in the two months following applications. The concentrations detected reached 0.158 μg L^{-1} in ground water and were up to 0.690 μg L^{-1} in streams. In a similar study in the south of Brazil, Almeida et al. (2019) detected up to 6.54 μg L^{-1} of imazethapyr in surface water bodies sampled in different months of the year. These are part of the very few studies addressing the presence of imazethapyr in the environment. It has been demonstrated that agricultural practices are one of the main diffuse non-point sources of pollution (Larsen et al. 2013). Consequently, agrochemicals, such as herbicides, may also reach water bodies indirectly through drift or surface runoff or directly by washing the tanks of the sprayers (Vera et al. 2010).

In freshwater bodies, imazethapyr may cause adverse effects on non-target primary producers, such as microalgae, due to the similarity of the metabolic pathways in these organisms with those of plants (Rutherford and Krieger-Liszkay, 2001). Phytoplanktonic microalgae are important models for studying the toxicity of aquatic pollutants due to their role in the maintenance of aquatic food chains (DeLorenzo et al. 2001; Cetın et al. 2013).

Microalgae are very useful organisms in ecotoxicological studies due to their sensitivity to different contaminants, their relatively short life cycle, and the ease of handling them in laboratory cultures (Lewis 1995). Different species of *Scenedesmus* are among the most widely distributed planktonic green microalgae in aquatic environments in the world and are also used in ecotoxicological assays (Staveley and Smrchek 2005). In particular, *Scenedesmus vacuolatus* (formerly *Chlorella fusca* var. vacuolata) corresponds to one of the most frequently detected species in the phytoplankton of water bodies near agricultural fields and this makes it a suitable model to evaluate the effect that herbicides may have on phytoplanktonic microalgae. Additionally, the effects of several contaminants (including metals and herbicides) on this microalga have been studied in our laboratory and alterations in the growth rate, chlorophyll content, and morphology have been recorded (Iummato et al. 2019; Sabatini et al. 2009). Therefore, the analysis of the effect of imazethapyr-based herbicides may expand the understanding of the microalga responses to herbicides in current use.

The most common toxic effects observed in microalgae exposed to herbicides may include alteration in the growth rate, changes in cell morphology, destruction of chlorophyll and inhibition of its synthesis, and a reduction in photosynthesis (Chen et al. 2013; Iummato et al. 2019; Prado et al. 2011; Romero et al. 2011; Sabatini et al. 2009). Imazethapyr, as well as other imidazolinone herbicides, has been proven to have toxic effects on plants (Qian et al. 2011) and non-target organisms, such as rotifers (Reimche et al. 2015), tadpoles (Carvalho et al. 2019; Pérez-Iglesias et al. 2015), and even mammalian cells (Soloneski et al. 2017). Although, information about its toxicity on microalgae is scarce, growth inhibition effects have been reported in some blue-green algae, green algae, and diatoms (PMRA 2010; Stone et al. 2019; Xie et al. 2018). Furthermore, Magdaleno et al. (2015) have previously demonstrated that the imazethapyr formulation Verosil® inhibits *Pseudokirchneriella subcapitata* growth in a concentration dependent manner.

Most of the studies performed to assess herbicide toxicity focus on the active ingredient. However, herbicides are commercialized in formulations, which are complex mixtures that increase the effectiveness of the active ingredient. Although several studies showed the contribution of formulation additives to the toxicity of some herbicides (Lipok et al. 2010; Pereira et al. 2009; Tsui and Chu, 2003), toxicological information comparing the effects of technical-grade imazethapyr versus its commercial formulations is scarce. There is even less available data when it comes to formulation additives since manufacturers are not required to disclose their composition. There is much speculation about the toxicity of the formulation additives, but specific data cannot be obtained.

Alkylphenol or ethoxylated alkylphenol ethers are among the most commonly used additives that act as surfactants (Krogh et al. 2003). These molecules are highly versatile and can interact with all cellular components resulting in adverse biological effects (Priac et al. 2017). For example, Krogh et al. (2003) studied the action of several non-ionic surfactants on photosynthetic microorganisms and they demonstrated that most of these compounds have toxic effects on microalgae.

The objective of this study was to explore the differential effects of formulation Verosil®, the formulation additives, and technical-grade imazethapyr, in the acid form or as ammonium salt, on the microalga *S. vacuolatus* (Chlorophyta). We studied the growth, pigment content, morphology, and ultrastructure of this alga, under exposure to the mentioned agrochemicals, with the hypothesis that the formulation additives contribute significantly to the toxicity as well as alterations that could be caused by the commercial formulation Verosil® to a phytoplanktonic microalga.

Materials and methods

Preparation of test solutions

All assayed chemicals were provided by the company Agrofina S. A. (CABA, Argentina). The herbicide formulation used was the commercially available Verosil®, containing 11.57% (w/v) imazethapyr (2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-ethyl-3-pyridine

carboxylic acid ammonium salt), and 25% (v/v) non-ionic surfactants. Bioassays were performed to analyze the effect of Verosil® and the effect of the active ingredients and the additives applied, independently. Imazethapyr ammonium salt (IMAS, CAS number 101917–66-2), acid imazethapyr (AIM, CAS number: 81335–77-5), and the non-ionic surfactant additive mixture present in the Verosil® formulation (AF), provided by Agrofina S. A., were also tested separately. The company informed that the AF is a mixture of ethoxylated alkylphenol ethers 25% (p/v), although the full composition of the mixture remained confidential. To facilitate the comparison between formulations and tested compounds, all concentrations are expressed as acid imazethapyr equivalents (IME, i.e., the concentration of acid imazethapyr that is contained—or would be contained—in the tested solution). In the case of AF, the amount added in each Erlenmeyer flask corresponds to the amount of additive present in the Verosil® formulation when adding the indicated IME equivalents.

Bioassays were carried out by preparing dilutions in Bold´s basal medium (BBM), pH 6.5 (Bischoff and Bold 1963), from stock solutions of 5 g L^{-1} IME for Verosil®, 4 g L^{-1} IME for IMAS, and 1 g L^{-1} IME for AIM. Tests were also carried out with AF from a stock solution considering the amount of additive that would be present in the 5 g L^{-1} IME Verosil® test, but without imazethapyr (to test the effect of the additives alone).

Chemical analysis

Experimental imazethapyr concentrations were verified for Verosil®, IMAS, and AIM on samples from the bioassayed flasks taken before carrying out the test and again after an incubation period of 96 h. Quantification was performed by high-performance liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS) at INTI (Instituto Nacional de Tecnología Industrial), Buenos Aires, Argentina. The analysis was performed on a Waters Quattro Premier XE spectrometer (Waters, Milford, MA, USA) equipped with a Waters 2695 binary pump plus auto sampler. The HPLC separation was carried out using an XTerra MS C18 column (Waters, Milford, MA, USA, 100 mm × 2.1 mm, 3.5 µm). The mobile phase consisted of (A) water/acetonitrile (90:10 with 0.1% formic acid) and (B) acetonitrile/0.1% formic acid. An increasing linear gradient (v/v) of solvent B was used ($t_{(min)}$, %B): (0, 20), (4.5, 40) with a flow rate of 0.3 mL/min and a column temperature of 40 °C. The injection volume was 10 µL. The mass instrument was operated in electrospray positive ion mode, with a source of 3 kV, a cone of 30 V, and 150 °C of the source temperature. Nitrogen was used for desolvation as well as for the cone gas. The detection and quantification of imazethapyr was performed using the multiple reaction monitoring (MRM) mode and ion precursor-product ion of 290–177 m/z. The collision gas was argon with a flow rate of 0.2 mL/min and the collision energy was 25 eV. Calibration samples were prepared dissolving Imazethapyr PESTANAL® (analytical standard) in the culture medium to compensate for matrix effects. The correlation coefficients (r^2) of the calibration curves were higher than 0.995. Nominal and measured concentrations are shown in Table 1.

Microalga cultivation

The BAFC CA 4 strain of *S. vacuolatus* (Chlorophyceae, Chlorophyta) was obtained from the Culture Collection of the Laboratorio de Biología de Protistas, Departamento de Biodiversidad y Biología Experimental, belonging to the Centro de Recursos Genéticos of Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. The microalga was maintained in BBM agar medium at 24 ± 1 °C with a 12:12 light/dark photoperiod. To perform the different tests, cultures were initiated in 150 mL of sterilized liquid BBM medium (pH 6), inoculating with the previously mentioned stock. Liquid cultures were incubated under continuous cool-white fluorescent light (80 µmol photons m^{-2} s^{-1}) and constant agitation in an orbital shaker (145 rpm) at 24 ± 1 °C. Once the exponential phase of growth was reached (10 to 15 days, average cell density of 6 10^6 cells mL^{-1}), they were used to initiate the different tests, after determination of their cellular density by cell counting.

Algal bioassays

Bioassays were performed axenically in 500-mL Erlenmeyer flasks containing 300 mL of sterilized BBM culture medium, adding a volume of exponential culture (an average

Table 1 Experimental concentrations of Verosil® formulation acid imazethapyr and imazethapyr ammonium salt at the beginning of the assay and after 96 h

Nominal concentration	Experimental concentration at T_{0h} (mg IME L^{-1} ± SD)	Experimental concentration at T_{96h} (mg IME L^{-1} ± SD)
Verosil® (2 mg IME L^{-1})	1.7 (±0.24)	1.8 (±0.19)
Verosil® (6 mg IME L^{-1})	4.9 (±0.98)	5.7 (±0.64)
IMAS (100 mg IME L^{-1})	96.0 (±6.08)	106.5 (±7.1)
IMAS (500 mg IME L^{-1})	468 (±62.4)	437.1 (±58.3)
AIM (20 mg IME L^{-1})	18.0 (±1.15)	17.0 (±1.40)
AIM (60 mg IME L^{-1})	58.0 (±5.00)	50.5 (±7.54)

T_{0h} initial time of assay, T_{96h} 96 h after the beginning of the assay, *IME* acid imazethapyr equivalents. *AIM* acid imazethapyr, *IMAS* imazethapyr ammonium salt, *SD* standard deviation

of 1.5 mL) to reach an initial cell density of 30,000 *S. vacuolatus* cells per mL.

The microalga was exposed to Verosil® (0, 2, 4, 6, and 8 mg IME L^{-1}), IMAS (0, 100, 300, and 500 mg IME L^{-1}), AIM (0, 20, 40, 60, and 100 mg IME L^{-1}), or AF (0, 4, 8, and 12 mg IME L^{-1}). The concentrations used for the analysis were established with data from preliminary bioassays that tested a greater range of concentrations, in order to use concentrations lower and higher than the IC_{50} (estimated toxicant concentration that cause 50% reduction in growth compared to a control). In the assay with Verosil®, an additional control was included that only contained the maximum additive mixture used (equivalent to 8 mg IME L^{-1}).

There were no changes in the pH after addition of the agrochemicals. The cultures (3 replicates per treatment) were maintained at 24 ± 1 °C with constant illumination (80 µmol photons m^{-2} s^{-1}) and agitation in an orbital shaker (145 rpm). After 96 h of exposure, aliquots from each culture were separated in order to evaluate the different parameters (1 mL for cell growth analysis, 10 mL for light microscopy analysis, 40 mL for pigment content analysis, and 249 mL for ultrastructure analysis). The bioassays were repeated two to four times to corroborate the data obtained.

Algal growth assessment

The cell number was evaluated by direct counting in the Neubauer chamber, using a Leica DM 500 light microscope at 400 × (Geis et al. 2000). Counting at least 25 squares ensured an error of less than 10% (Venrick 1978). Growth results are expressed as percentages of algal growth relative to the control value.

The IC_{50} was estimated by the linear interpolation method (EPA-821-R-02–013 2002). In addition, inhibitory concentrations 10 and 20 (IC_{10} and IC_{20}) were calculated using the GraphPad Prism 5.01 program.

Morphology and ultrastructure analysis

S. vacuolatus morphology and ultrastructure were analyzed from cultures grown in BBM liquid medium containing the previously mentioned agrochemicals. The morphological observation and photomicrography were performed with a Leica DM 500 light microscope equipped with a Leica ICC50 digital camera.

The cell surface area and volume were estimated by measuring the cell diameter of 100 randomly chosen cells per replicate (3 replicates) of two separate bioassays in images obtained by light microscopy and analyzed using the Leica Application Suite software version 1.8.0. Calculation of the cited parameters was carried out according to Hillebrand et al. (1999), considering the *S. vacuolatus* cells as spherical bodies.

For the ultrastructure analysis, material was prepared as described by Juárez et al. (2011). Cells were harvested by centrifugation at 3000 × *g* for 15 min, and fixed in a 2% (w/v) glutaraldehyde-paraformaldehyde solution in BBM (filtered through a 0.22-µm pore millipore membrane), with a gentle vacuum at room temperature for 2 h and then it was left at 5 °C for 24 h. The fixed material was washed with BBM, post-fixed in 1% OsO_4 (osmium tetroxide) at room temperature

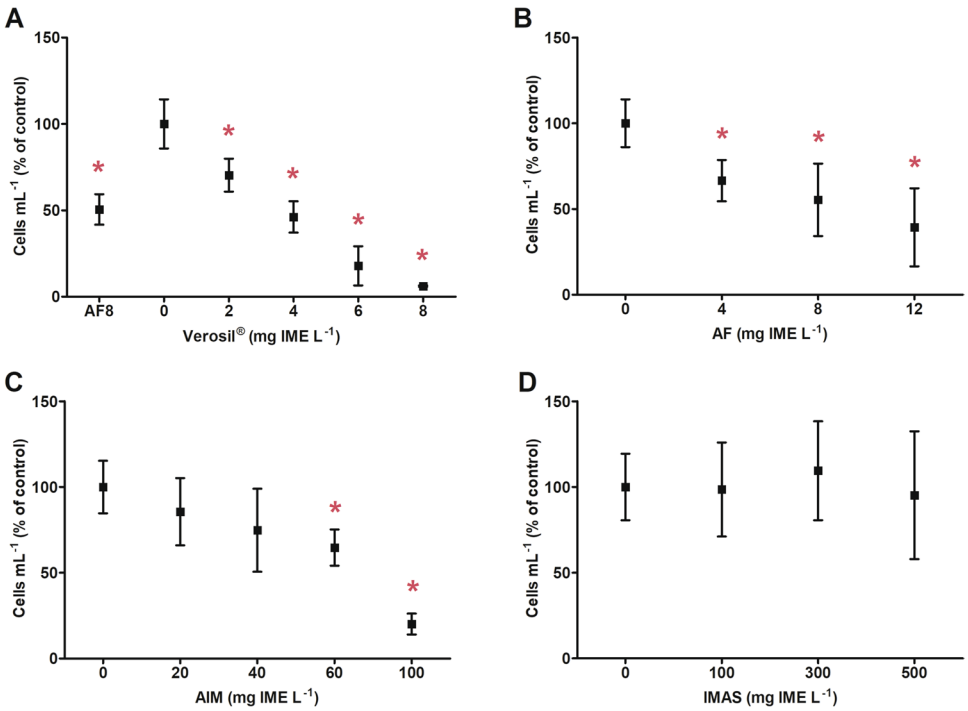

Fig. 1 Culture growth (cells mL^{-1}) of *S. vacuolatus* after 96-h exposure to different treatments. **A** Imazethapyr formulation Verosil® and its additives in a concentration of 8 mg IME L^{-1} (AF8), **B** additive mix of the formulation (AF), **C** acid imazethapyr (AIM), and **D** imazethapyr ammonium salt (IMAS). Data is expressed as means ± SD as percentage of the control value, $n = 12$ (4 bioassays with 3 replicates each). Significant differences between control and treatments are indicated by asterisks ($p < 0.05$). IME, acid imazethapyr equivalents

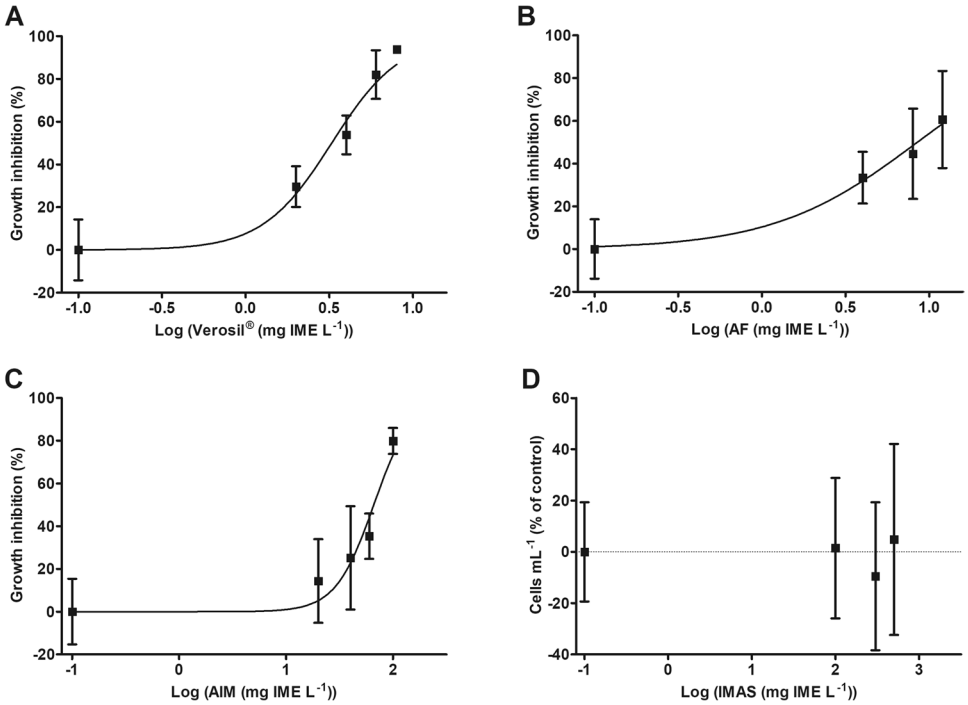

Fig. 2 Concentration–response curve and best-fit line as determined by nonlinear regression analysis of each treatment for *Scenedesmus vacuolatus*. Each data point represents the mean ± SD, $n = 12$ (4 bioassays with 3 replicates each). Solid lines represent the best-fit line as determined by nonlinear regression analysis. IME: acid imazethapyr equivalents

for 1 h and dehydrated gradually with acetone 10% to 100% solutions. This material was embedded in low viscosity Spurr resin. Ultrathin sections were cut with a glass knife on an ultramicrotome and stained with uranyl acetate and lead citrate (Reynolds 1963). The observations were carried out in the transmission electron microscope Zeiss—EM109T at the Laboratorio Nacional de Investigación y Servicios de Microscopía Electrónica (LANAIS—MIE), Facultad de Medicina, Universidad de Buenos Aires.

Pigment content

Cells were harvested and then resuspended and ground in 80% acetone. After 1 h at 4 °C in the dark, the extracts were clarified by centrifugation at 3,000 × *g* for 10 min, and their absorbance was read at 663.2, 646.8, and 470 nm in a UV/vis Shimadzu spectrophotometer. The chlorophyll *a*, chlorophyll *b*, and carotene concentrations were calculated according to Lichtenthaler (1987). The results were expressed as percentages relative to the control value.

Statistical analysis

For each quantitative test, the means and standard deviations were determined. Normality and homoscedasticity were tested using the probes of Shapiro–Wilk and Bartlett, respectively. In some cases, data was transformed using log 10 to stabilize the variances between groups prior to statistical analyses. GraphPad Prism version 5.00 software was used for statistical analysis. Data was analyzed by one-way ANOVA, followed by Dunnett's post hoc test analysis to determine the significant differences between treatments (Sokal and Rohlf 1995). The differences were considered significant when $p < 0.05$.

Results

Growth

Verosil®, AF, and AIM had an inhibitory effect on *S. vacuolatus* growth in a concentration dependent manner. Algal cell concentration was significantly diminished from 2 mg IME L^{-1}, 4 mg IME L^{-1} and 60 IME mg L^{-1} onwards, by

Table 2 Inhibitory concentrations of Verosil® formulation and its components on *Scenedesmus vacuolatus*

Agrochemical	IC_{50} (CI_{95}) (mg IME L^{-1})	IC_{20} (CI_{95}) (mg IME L^{-1})	IC_{10} (CI_{95}) (mg IME L^{-1})
Verosil®	3.25 (2.94–3.63)	1.58 (0.73–2.53)	1.20 (0.62–2.29)
AF	6.94 (5.56–8.66)	2.09 (1.32–3.57)	1.12 (0.40–2.21)
AIM	66.1 (56.2–77.8)	38.0 (26.9–49.0)	26.3 (19.1–37.2)
IMAS	–	–	–

IC_{50}, IC_{20}, and IC_{10}: inhibitory concentration 50, 20, and 10 respectively. CI_{95} 95% confidence interval, *IME* acid imazethapyr equivalents, *AF* additive mix of the formulation, *AIM* acid imazethapyr, *IMAS* imazethapyr ammonium salt

exposure to Verosil®, AF, and AIM respectively. IMAS had no effect on growth at the assayed concentrations (Fig. 1).

The inhibitory concentrations for the different agrochemicals, estimated from the concentration–response curves (Fig. 2), are shown in Table 2. Verosil® showed the lowest IC_{50}, followed closely by that of AF, whereas AIM was one order of magnitude higher than Verosil® and AF. The IC_{20} showed the same ranking as IC_{50}, but there was an inversion in the order between Verosil® and AF for IC_{10}, even though the confidence intervals overlapped almost completely. In cultures exposed to IMAS, none of the tested concentrations affected cell growth significantly.

Morphology and ultrastructure

Light microscopy showed several morphological alterations in the algal cells exposed to the assayed agrochemicals (Fig. 3). Cells in the control cultures (Fig. 3A, B) showed the typical characteristics for the species (Shihira and Krauss, 1965): a spherical shape of 5 to 12 µm diameter, a laminar chloroplast in the form of a surrounding net containing one pyrenoid covered by a starch sheath.

In cultures treated with Verosil®, an increase in cell volume was observed at the higher concentrations (4, 6, and 8 mg IME L^{-1}). Cells with disorganized plastids, some

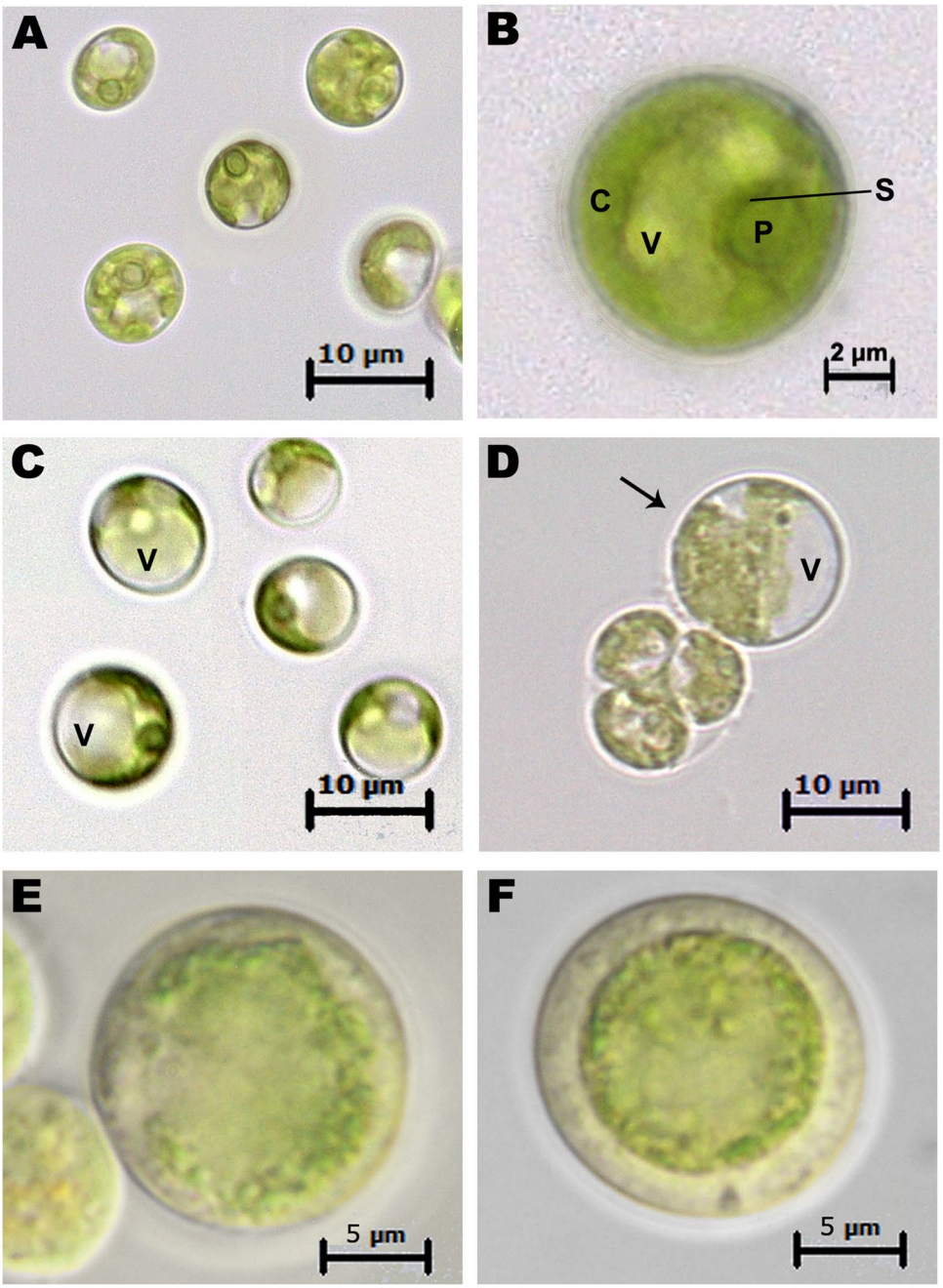

Fig. 3 Light microscopy of *Scenedesmus vacuolatus* under different treatments. **A**, **B** Overview and detail of cells under control conditions, showing chloroplast (C), pyrenoid (P) covered by a starch sheath (S), and vacuole (V). **C**, **D** Cells exposed to high concentrations of Verosil® formulation, formulation additives (AF) or acid imazethapyr AIM). Note the cells with larger volume (arrow) and enlarged vacuoles (V). **E**, **F** Detail of large volume cells exposed to high concentrations of Verosil® formulation, formulation additives (AF), and acid imazethapyr (AIM), showing a disorganized plastid (**E**) and thickened cell wall (**F**)

decolored cells, some cells with thickening of the cell wall and some cases of cytoplasmic contraction were also observed (Fig. 3D-F).

In cells exposed from 4 to 8 mg IME L^{-1} of AF, an increase in cell vacuolization and cell volume was observed (Fig. 3C, D). There were also cells with disorganized plastids and cell wall thickening (Fig. 3E, F). Cells exposed to low concentrations of IMA had normal morphology, whereas a slight increase in the degree of vacuolization was observed at 60 mg IME L^{-1} and 100 mg IME L^{-1} (Fig. 3C). On the other hand, the cultures exposed to 100 and 500 mg L^{-1} IMAS had normal morphology.

Additionally, the cell volume was determined for microalgae exposed to Verosil® (Fig. 4). The volume increased significantly compared with the control ($p < 0.05$, control value: 286.7 ± 62.8 µm^3) from 6 mg IME L^{-1} onwards, whereas the surface/volume ratio decreased significantly ($p < 0.05$) at the same concentration (0.84 ± 0.07 in the control and 0.69 ± 0.03 in the 6 mg IME L^{-1}). Treatment with AF did not produce any significant variation in the volume or surface/volume ratio of control cells.

The *S. vacuolatus* ultrastructure was analyzed by transmission electron microscopy and the control culture cells showed the typical, distinctive characteristics of this species (Fig. 5): the presence of a unique excentric nucleus (N) with an electrodense nucleolus (Fig. 5B); a laminar parietal chloroplast with lamellae formed of stacked thylakoid groups (Fig. 5C); a pyrenoid embedded in the chloroplast matrix surrounded by two to three starch granules and the cell wall 100–160 nm thick with a trilaminar structure (Fig. 5D).

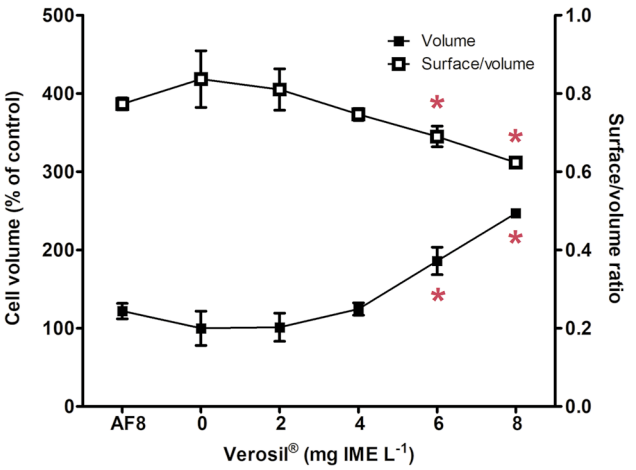

Fig. 4 Cell volume and surface/volume ratio of *Scenedesmus vacuolatus* exposed to different concentrations of the formulation Verosil® and its formulation additives (AF). Control values: 286.7 ± 62.8 µm^3 and 0.84 ± 0.07 µm^3 respectively. Data is presented as the mean ± SD, $n = 6$ (2 bioassays with 3 replicates each). Asterisks indicate significant differences from the control ($p < 0.05$) determined on the basis of crude data for volume and based on the transformed data for surface/volume ratio. AF8: formulation additives in a concentration of 8 mg IME L^{-1}. IME, acid imazethapyr equivalents

Observations performed by light microscopy of the cells treated with 4 to 8 mg IME L^{-1} of Verosil® and 4 mg to 8 mg IME L^{-1} of AF were corroborated by ultrastructure analysis (Fig. 6). A general disorganization in the internal membranes (Fig. 6A), thickening of the cell wall (cw) reaching up to 250 nm, loss of the stacking pattern of thylakoid membranes (Fig. 6B), contraction of the cytoplasm, and starch granule accumulation (Fig. 6C) were observed in cells treated with Verosil®. There was also a slight alteration in the nuclear organization and loss of nuclear membrane integrity (Fig. 6D). Cells treated with AF showed starch granule accumulation and cytoplasmic contraction was observed (Fig. 6E), as well as disorganized thylakoids, thickening of the cell wall (up to 250 nm), and deposition of lipidic droplets (Fig. 6F).

Pigments

Cells treated with 6 mg IME L^{-1} Verosil® or with 4 mg IME L^{-1} AF showed a significant increase with respect to the control ($p < 0.05$, control values: 2.45 ± 0.10 and 2.05 ± 0.11 respectively; Fig. 7A, C) in the chl *a*/chl *b* ratio. The carotenes/chl *a* ratio increased compared with the control at all Verosil® and AF concentrations ($p < 0.05$, control values: 0.29 ± 0.01 and 0.25 ± 0.02, respectively; Fig. 7B, D). In cultures exposed to AIM, a significant decrease was observed compared with the control ($p < 0.05$; control value: 2.16 ± 0.08, Fig. 7E) for the chl *a*/chl *b* ratio starting from 60 mg IME L^{-1}, whereas the carotene/chl *a* ratio was not modified (control value: 0.24 ± 0.03, Fig. 7F). On the other hand, IMAS did not cause any significant alteration in the chlorophyll ratio, nor in the carotene/chl *a* ratio (control value: 2.29 ± 0.24 and 0.23 ± 0.02 respectively; Fig. 7G, H).

Discussion

Previous literature on the effects of imazethapyr and other imidazolinones in microalgae is scarce and mostly focused on growth inhibition. Besides, there is no data available on alterations in other parameters, such as pigment balance, cell morphology, and ultrastructure.

Technical-grade imazethapyr or imazethapyr formulation showed very low toxicity in *Mycrocsytis aeruginosa* (IC_{20}: 14–20 mg L^{-1}, Xie et al. 2018), *Selenastrum capricornutim* (EC_{50}: 22.4 mg L^{-1}), and *Navicula pelicullosa* (EC_{50}: 22.9 mg L^{-1}, PMRA 2010). In addition, Stone et al. (2019) studied the imidazolinone imazapic in low concentrations (up to 1.1 mg L^{-1}) and found no growth inhibition in the chlorophytes *Chlorella* sp., *Pediastrum duplex*, and *Monoraphidium arcuatum*. Our study showed that both the

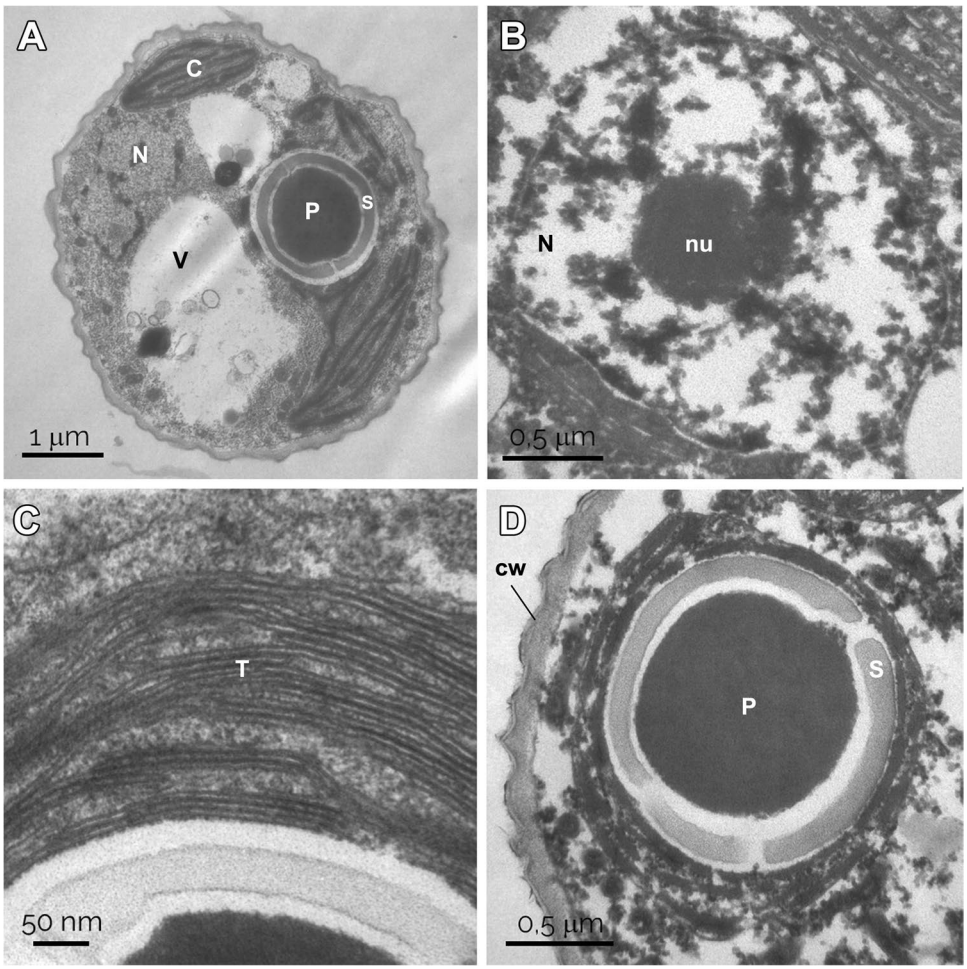

Fig. 5 Transmission electron microscopy of *Scenedesmus vacuolatus* under control conditions. **A** Overview. **B** Detail of the nucleus structure. **C** Detail of chloroplast and lamellae formed by stacked thylakoids. **D** Detail of the pyrenoid surrounded by the starch granules (V) vacuole, (P) pyrenoid, (C) chloroplast, (N) nucleus, (nu) nucleolus, (T) thylakoids, (s) starch granules, and (cw) cell wall

Verosil® formulation and its formulation additives, AF, had a toxic effect on the microalga *S. vacuolatus* (Chlorophyta) at all the concentrations tested. The IC_{50} for Verosil® was 3.25 IME mg L^{-1} (CI_{95}: 2.94–3.63), which is more than 100 times lower than the application concentration recommended in the field (500 mg IME L^{-1}). Magdaleno et al. (2015) previously demonstrated that Verosil® formulation exerts high toxicity on *Pseudokirchneriella subcapitata*, showing an IC_{50} close to 1 mg L^{-1}, while the IC_{50} determined for *S. vacuolatus* in this study shows that this species is more tolerant to Verosil® than *P. subcapitata*. Considering the scarce published studies addressing the environmental concentration of imazethapyr in water bodies, we think that more knowledge should be produced in this area before ensuring the potential harm or safety of this herbicide. However, the IC_{50} determined in our study indicates that Verosil® is toxic for aquatic organisms according to the EU Directive 93/67/EEC (Technical Guidance Document in Support of Commission Directive 93/67/EEC 1996). Although the few reports on environmental concentrations of imazethapyr are in the order of µg L^{-1}, there is information that makes us suspect possible scenarios with concentrations that are toxic to microalgae, such as *S. vacuolatus*. Ulrich et al. (2013) demonstrated that the quantity of herbicide that reaches water bodies is highly dependent on the amount of rainfall between the application and sampling times and that it can reach up to 7% of the amount applied. Additionally, imazethapyr adsorption by the organic soil particles, together with high rainfall, might allow a considerable percentage of the herbicide to run off into the surface water (Espy et al. 2011; Souza et al. 2016). Although the IC_x estimates in this study were done with a number of toxicant concentrations just below those recommended by USEPA and OECD (EPA-821-R-02–013 2002; OECD 2011), the repeatability and the high fit to the sigmoid function allow us to consider them reliable and these IC_x parameters provide valuable information to understand the toxicity of chemical compounds as well as the sensitivity of different species. Taking into account these results, the environmental fate information mentioned above and that the minimum recommended concentration for Verosil® field application is 500 mg IME L^{-1}, it could be possible to find concentrations close to the IC_{10}, IC_{20} and IC_{50} determined for *S. vacuolatus.*

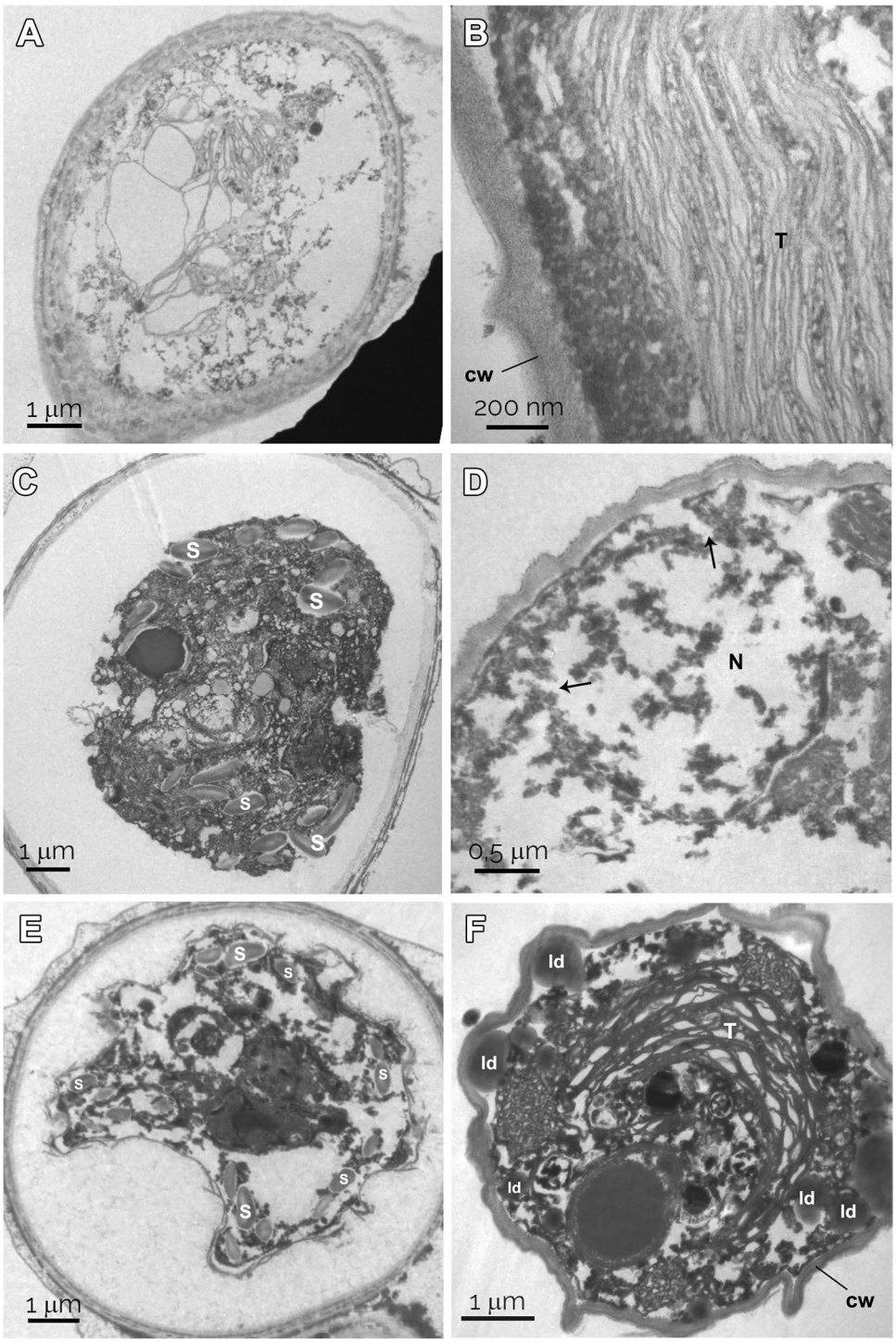

Fig. 6 Transmission electron microscopy of *Scenedesmus vacuolatus* exposed to the Verosil® formulation and exposed to the Verosil® formulation additives. **A**, **B**, **C**, **D** Verosil® (4 mg IME L^{-1}). **E**, **F** Formulation additives (8 mg IME L^{-1}). **A** Cell showing general internal membranes disorganization. **B** Detail of the thickened cell wall (cw) and disaggregated thylakoidal membranes (T). **C** Cell with contracted cytoplasm and starch granules accumulation (s). **D** Detail of nucleus (N) showing an altered morphology and loss of the membrane integrity (black arrow). **E** Cell showing contraction of the cytoplasm and accumulation of starch granules (s). **F** Cell with disorganized thylakoid (T) membranes, thickened cell wall (cw), and numerous lipid droplets (ld). IME, acid imazethapyr equivalents

Formulation additives are often considered by manufacturers to be inert in terms of their contribution to toxicity in non-target organisms. Although there are some studies that support this assertion (Cedergreen and Streibig 2005; Williams et al. 2000), other publications have reported toxic effects of the pesticide adjuvants (including ethoxylate surfactants) on microalgae, such as *S. subcapitata* (EC_{50}: 1.5–3.5 mg L^{-1}), *M. aeruginosa* (EC_{50}: 0.1–0.6 mg L^{-1}), *N. pelliculosa* (EC_{50}: 0.1–0.6 mg L^{-1}), *S. obliquus* (EC_{50}: 5.2–9.7 mg L^{-1}), and *S. subspicatus* (EC_{50}: 0.32–0.74 mg L^{-1}) (Krogh et al. 2003; Lürling et al., 2011; Pavlic et al. 2005). Furthermore, several publications have shown that the toxicity of commercial formulations of different herbicides is greater than that of the active ingredient they contain (Gorzerino et al. 2009; Pereira et al. 2009). The antecedents on herbicide effects mainly focus on glyphosate, one of the

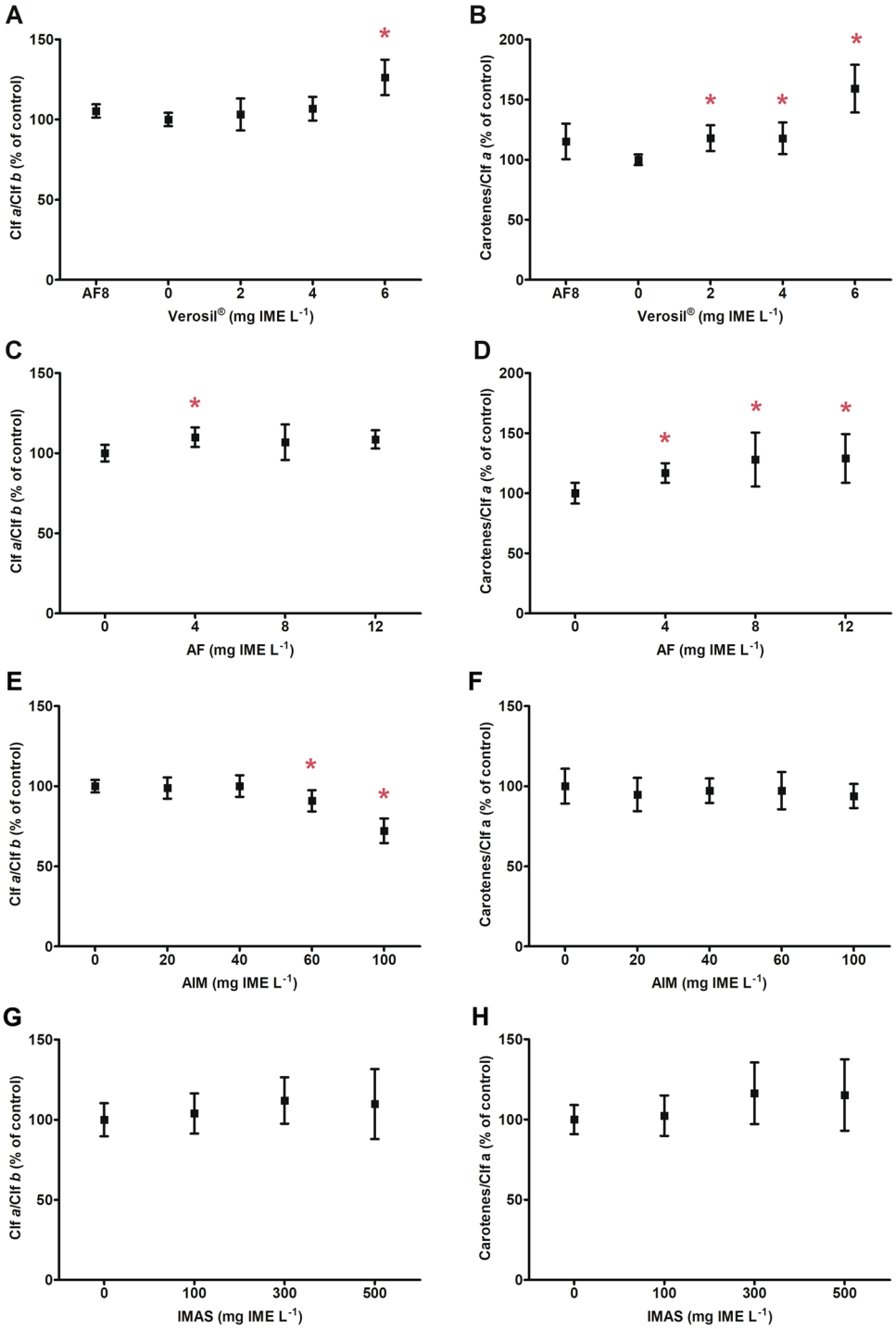

Fig. 7 Pigments in *Scenedesmus vacuolatus* exposed to different concentrations of the Verosil® formulation, its formulation additives (AF), acid imazethapyr (AIM), and imazethapyr ammonium salt (IMAS). Chlorophyll *a*/chlorophyll *b* ratio (Clf *a*/clf *b*) for cells exposed to Verosil® and its additives in a concentration of 8 mg IME L^{-1} (AF8) (**A**), AF (**C**), AIM (**E**), and IMAS (G) and carotenes/chlorophyll *a* ratio (carotenes/clf *a*) for cells exposed to Verosil® and AF8 (**B**), AF (**D**), AIM (**F**), and IMAS (H). Control values: 2.45 ± 0.10 (**A**); 0.29 ± 0.01 (**B**); 2.05 ± 0.11 (**C**); 0.25 ± 0.02 (**D**); 2.16 ± 0.08 (**E**); 0.24 ± 0.03 (**F**); 2.29 ± 0.24 (**G**); 0.23 ± 0.02 (**H**). Data is presented as the mean ± SD, $n = 9$ (3 bioassays with 3 replicates each). Asterisks indicate significant differences from the control ($p < 0.05$) determined based on the transformed data for **A**, **B**, and **D**, and based on the raw data for **C**, **E**, **F**, **G**, and **H**. IME, acid imazethapyr equivalents

most used pesticides (Benbrook 2016; de Castilhos Ghisi et al. 2020; Nunes Rezende et al. 2021). Studies with different formulations of glyphosate and its active ingredients on *Scenedesmus acutus*, *S. quadricauda*, and S. vacuolatus, and also on the algal periphytic community, showed that the toxicity of the formulations was higher than that of the active ingredients (González et al. 2019; Iummato et al. 2019; Sabatini et al. 2009; Sáenz et al. 1997). In addition, Howe et al. (2004) and Tsui and Chu (2003) demonstrated that the additive POEA plays a crucial role in the toxicity of the glyphosate commercial formulation, and Gao and Tam (2011) demonstrated that the nonylphenol surfactant negatively affects the growth of *Selenastrum capricornutum* and *Chlorella vulgaris*. Results from our study are in agreement with the research mentioned above: the IC_{50} values for Verosil® formulation and AF are very close, whereas

IMAS showed no toxicity at any of the concentrations tested. Hence, we could assume that AF plays a crucial role in the toxicity of the Verosil® formulation and that IMAS plays a role as an AF enhancer in *Scenedesmus vacuolatus*. This arises considering that Verosil® is composed by FA + IMAS and IMAS by itself did not show toxicity, then its presence in the formulation could be responsible for enhancing the toxicity of FA, leading Verosil® to present a greater toxicity (observed as decrease in the IC_{50}).

We verified that there is differential toxicity between the different chemical forms of imazethapyr. For example, the acidic form of imazethapyr (AIM) has a more severe effect on the morphology and physiology of microalgae than the imazethapyr ammonium salt (IMAS). Something similar happens with the herbicide glyphosate, in which its acid form is more toxic than the isopropylamine salt form (González et al. 2019). However, this difference has been attributed to the drop in pH promoted by glyphosate in its acidic form. In our study, the pH of the culture medium did not show any significant variation in the bioassay concentrations and so the differential toxicity is due to other factors. Although little information exists on the influence of the chemical form of a pesticide on its toxicity, it has been shown in sulfonylureas that the uncharged forms can cross the membranes freely and exert their toxicity. In contrast, the charged forms would not cross the membranes, which has been linked to its lower toxicity in *Chlorella fusca* var. *vacuolata* (currently *S. vacuolatus*) (Fahl et al. 1995). Given that microalgae have anionic intra and extracellular ligands (Perales-Vela et al. 2006; Pistocchi et al. 2000), it is possible that the ionic forms of imazethapyr (anionic) may either be rejected by the cell or fail to cross the membranes due to their charge. On the other hand, it has been shown that imazethapyr has more possibilities of interaction with other molecules in its neutral form (Bresnahan et al. 2000), favoring its chances to enter the cells. Thomas et al. (2020) have postulated that the low toxicity of the imidazolinone imazapic to microalgae is related with its interaction of the active ingredients with the cations in the medium, leading to lower its bioavailability for uptake. Therefore, the differential effect between the two forms of imazethapyr (IMA and IMAS) observed in this study could be related to their charge and chances to enter *S. vacuolatus* cells, However, further studies should be performed to fully understand the interactions between these active ingredients, the medium, and the cellular components.

The effects produced by the different forms of imazethapyr on *S. vacuolatus* included alterations in its morphology. Observations by light microscopy showed different effects with the toxics tested and the most marked alterations also occurred with Verosil® and AF. These were also confirmed by analysis of the ultrastructure. Wall thickening, up to twice as thick as that of the control cultures, was observed in cells exposed to the agrochemicals mentioned. Iummato et al. (2019) also observed thickening of cell walls in *S. vacuolatus* exposed to a glyphosate formulation. Pesticides have also been reported to cause alteration of cell walls in other microalgae (Asselborn et al. 2015; Debenest et al. 2008). The thickening of the cell wall could be a response that would decrease the entry of toxics into the cell (Pereira et al. 2013; Wang et al. 2004). Contraction of the cytoplasm was also observed in this study, which had already been reported by other authors in microalgae exposed to other xenobiotics (Liu and Xiong 2009; Qian et al. 2009). This alteration has been proposed as an indicator of the loss of plasma membrane integrity against stress situations (Qian et al. 2009). However, further studies on membrane permeability would be needed to corroborate this statement.

When exposed to the highest concentrations of Verosil®, *S. vacuolatus* also showed an increase in cell volume and vacuolization. Similarly, Iummato et al. (2019) and Romero et al. (2011) observed an increase in the cell size accompanied by an increase in vacuolization in *S. vacuolatus* and *Chlorella kessleri*, respectively, exposed to a glyphosate formulation. Comparable results were obtained for *Chlorella vulgaris* exposed to the herbicide isoproturon (Rioboo et al. 2002), *Pseudokirchneriella subcapitata* exposed to the herbicide metalochlor (Machado and Soares 2020), *S. obliquus* exposed to the FFD-6 surfactant (Lürling et al. 2011), and for *Ankistrodesmus gracilis* exposed to the insecticide chlorpyrifos (Asselborn et al. 2015). It has been suggested that some herbicides produce an uncoupling between cell growth and cell division, inducing an increase in volume per cell due to the accumulation of macromolecules (Asselborn et al. 2015; Rioboo et al. 2002). Nonetheless, further studies should be performed to elucidate whether the analyzed herbicide has an impact on cell division.

The increase in starch granules was very marked in the cultures treated with Verosil® and AF. The same effect was observed in *S. vacuolatus* treated with a glyphosate formulation (Iummato et al. 2019). This has also been reported in *Ankistrodesmus gracilis* (Asselborn et al. 2015) treated with the chlorpyrifos insecticide and in *C. pyrenoidosa* (Liu and Xiong 2009) and *C. vulgaris* (Qian et al. 2008) treated with metolachlor and glufosinate herbicides, respectively. The formation of starch granules in *Arabidopsis thaliana* (model plant) exposed to imazethapyr has been shown to be caused by an accumulation of carbon skeletons, which cannot be properly processed for protein formation by inhibition of the enzyme that participates in the synthesis of branched-chain amino acids. However, the possibility that the formulations tested exert some inhibitory effect on the starch hydrolysis enzymes, leading to their accumulation (Qian et al. 2011), should not be ruled out. In addition to the starch accumulations, the chloroplasts of some cells exposed presented altered morphology.

A disorganization of the thylakoid membranes, which had a torn arrangement, was observed instead of the compactly stacked membrane system. The exposure of *S. vacuolatus* to a glyphosate formulation produced an identical effect on the thylakoid membranes (Iummato et al. 2019). Qian et al. (2008) also observed alterations in the thylakoids of *C. vulgaris* treated with the herbicide glufosinate and in some cases they observed total loss of the chloroplasts.

Since thylakoids contain the pigments that capture light (antenna molecules), an alteration in their structure could have an impact on their balance. Cells treated with AIM showed a decrease in the chl *a*/chl *b* ratio. A similar response has been observed in *C. pyrenoidosa* treated with the herbicide metolachlor (Liu and Xiong 2009) and in *C. kessleri* treated with the herbicide glyphosate (Romero et al. 2011). Sabatini et al. (2009) also observed a decrease in the chl *a*/chl *b* ratio of *S. vacuolatus* exposed to copper and proposed that this was related to damage to photosystems and the antenna complex. Conversely, *S. vacuolatus* showed an increase in the chl *a*/chl *b* ratio when exposed to Verosil® and AF. Comparable results were reported in *C. kessleri* exposed to the pesticide lindane (Mostafa and Helling 2002) and in *C. vulgaris* exposed to cadmium (Cheng et al. 2016). During the process of chlorophyll degradation under stress conditions, chl *b* may be converted into chl *a*, thus resulting in an increase in the chl *a*/chl *b* ratio (Eckardt 2009).

Considering that the balance of the pigments can change in autotrophic organisms facing changes in environmental conditions (Basa et al. 2014) and that the chl *a*/chl *b* ratio may be altered selectively (increased or decreased) in response to chemical stresses (Mostafa and Helling 2002), the results in this study, together with those of others mentioned above, suggest that the alterations in pigment levels depend on the xenobiotic and the species.

A change in the chl *a*/chl *b* ratio can be used as an indicator of alterations in photosystem II in Chlorophyta (Rutherford and Krieger-Liszkay 2001; Vera et al. 2015). It has been reported that pollutants can damage the thylakoid membranes or the synthesis of structural proteins of the antenna centers and this alters the relationship between pigments (Juárez et al. 2008; Romero et al. 2011), which could explain the different alterations in the pigments observed in *S. vacuolatus* when exposed to the studied agrochemicals. Magnusson et al. (2010) observed for six herbicides and their degradation products that the inhibition of photosynthetic activity occurred in a matter of minutes in marine microalgae. The inhibition of photosynthetic activity could be triggered by an alteration in the pigments (Rutherford and Krieger-Liszkay 2001) and therefore, the pigment alterations observed in *S. vacuolatus* in this study could be related to alterations that were also observed in its cell growth and morphology.

Cells treated with Verosil® and AF also showed an increased the carotene/chl *a* ratio. These results have also been reported in microalgae exposed to herbicides, such as *C. vulgaris* exposed to isoproturon (Rioboo et al. 2002) and *S. vacuolatus* exposed to a commercial formulation containing glyphosate (Iummato et al. 2019). Carotenoid pigments play an important role in dissipating excess energy from excited chlorophyll molecules and reactive oxygen species, which could otherwise harm algae (Magnusson et al. 2008). These pigments are involved in the response to light intensity and, for example, it has been proposed that an increase in the carotenoid levels of cucumbers exposed to glyphosate could be an adaptive response to prevent photooxidative damage in the chloroplasts (Sacała 2011). Given the role of carotenes in the protection of photosystems, the increase in the content of these molecules observed in *S. vacuolatus* could indicate a possible participation of carotene in the antioxidant response of this algae against the formation of reactive oxygen species. The findings in the present study, as well as the extensive background on the alternations induced by pesticides on the redox balance in organisms (Gao and Tam 2011; Iummato et al. 2019; Qian et al. 2008, 2009; Romero et al. 2011), have encouraged us to perform new studies to try to understand whether oxidative stress participates in the toxicity mechanism of imazethapyr.

Conclusions

The present study is a contribution to the understanding of the impact of imazethapyr, as an active ingredient or in a formulation, on a phytoplanktonic microalga. We have demonstrated that both the Verosil® formulation and the formulation additives (AF) alone are toxic to *Scenedesmus vacuolatus* at low concentrations and cause alterations in both growth and cell morphology. We also corroborated that the AF contribute greatly to the toxicity of the Verosil® formulation, since the active ingredient has little effect at high concentrations on the physiology and morphology of the microalga. We have also verified that there is differential toxicity between the different chemical forms of imazethapyr. Thus, the acidic form of imazethapyr (AIM) has a more severe effect on the morphology and physiology of the microalga than the imazethapyr ammonium salt (IMAS).

Modern pesticides greatly contribute to agricultural development, and they are less toxic than previous generations of pesticides. However, there are very few antecedents of the effect of imazethapyr and its formulations on non-target organisms. The results obtained here represent not only a contribution to the understanding of the effect of this herbicide on microalgae but a valuable set of data to take informed decision by water quality regulation agencies. Toxicity studies on these agrochemicals should be continued to further understand the toxicity mechanisms and the impact of these findings in aquatic ecosystems and deepen

this new data. In this work we have shown that additives play a fundamental role in harming key organisms that are the basis of food chains. It would be desirable that agrochemical manufacturers should not only invest in the search for new active ingredients, but also target investments in the search, synthesis, and application of more environmentally friendly adjuvants and additives. The continuity of the agro-industrial value chain depends on combining economic and environmental aspects, which is why the concept of sustainable development takes vital importance.

Author contribution Anabella Victoria Fassiano performed the overall experiments and also handled the data analysis, manuscript writing, and revision, as well as approval of the final version for publication. Hugo March helped acquiring the chemicals used in the assays, the results validation and interpretation of data, and revision of the manuscript. Marina Santos participated in the analytical determination of imazethapyr concentrations in the culture medium and helped to draft the manuscript. Ángela Beatriz Juárez and María del Carmen Ríos de Molina conceived the study, participated in its design and co-ordination, funding acquisition, performed critical interpretation of data, helped to draft the manuscript and revision, and approved the final version for publication. All authors read and approved the final manuscript.

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Data availability The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Informed consent, or human or animal rights are not applicable to this study, neither ethical approval.

Competing interests The authors declare no competing interests.

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