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Ecotoxicological effects of new generation pollutants (nanoparticles, amoxicillin and white musk) on freshwater and marine phytoplankton species

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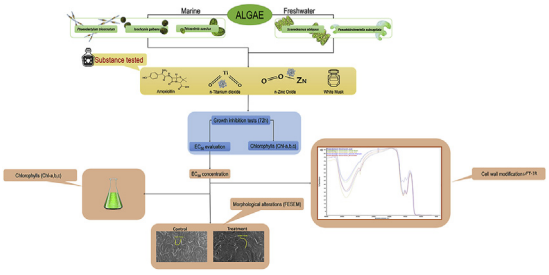
HIGHLIGHTS

- Effects of new generation pollutants on five phytoplankton species were determined.
- EC_{50} values for amoxicillin and white musk were reported for the first time.
- μFT-IR showed a marked spectral alteration of treated cultures compared to controls.
- A significant decrease in chlorophylls in all species exposed to EC_{50} was observed.
- Changes in cell size, when significant, were an increase in maximum cell length.

GRAPHICAL ABSTRACT



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ABSTRACT

Phytoplankton occupies a key trophic level in aquatic ecosystems. Chemical impacts on these primary producers can disrupt the integrity of an entire ecosystem. Two freshwater (*Pseudokirchneriella subcapitata*-Ps and *Scenedesmus obliquus*-S) and three marine (*Phaeodactylum tricornutum*-P, *Isochrysis galbana*-I, *Tetraselmis suecica*-T) microalgae species were exposed to dilutions of four chemicals: nanoparticles (n-TiO_2, n-ZnO), amoxicillin (antibiotic), and white musk (personal care fragrance) to determine the half maximal effective concentration (EC_{50}) after 72 h of exposure under standardized and controlled environmental conditions. Cell cultures were exposed to EC_{50} to determine sublethal effects (72 h) based on biochemical (chlorophylls *a, b, c*), molecular (changes in outer cell wall structure), and morphological alterations. We report for the first time EC_{50} values for nanoparticles in not standardized species (S, I and T) and for amoxicillin and white musk in all tested species. Standardized species (Ps and P) were less sensitive than non-standardized in some cases. Fourier-transformed infrared spectroscopy showed a marked spectral alteration (from 10.44% to 90.93%) of treated cultures compared to negative controls; however, principal component analysis disclosed no differences in molecular alteration between the five microalgae species or the two aquatic habitats considered. There was a significant decrease in

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chlorophylls content in all species exposed to EC_{50} compared to controls (Kruskal Wallis test; $p < 0.05$). There was a significant increase in cell-size (Mann–Whitney U test; $p < 0.05$) in I, P and T exposed to white musk and S exposed to amoxicillin. Findings highlight ecotoxicological risks from new generation pollutants for primary producers in aquatic ecosystems.

1. Introduction

Primary producers occupy a key trophic level in aquatic ecosystems. Environmental impacts on phytoplankton can disrupt energy transfer toward the upper levels of the trophic web (Cadotte et al., 2011; Li et al., 2020). Although single-cell primary producers in aquatic ecosystems account for approximately 0.2% of the biomass produced by primary producers on Earth, their turnover rate is much higher than in terrestrial environments (Field et al., 1998; Shurin et al., 2006). Also, phytoplankton is an indicator of environmental quality in ecological assessment (Cadotte et al., 2011; Stevenson, 2014).

The ecological fitness of phytoplankton communities is lower after exposure to chemicals and new generation pollutants (Miller et al., 2012; Echeveste et al., 2016; Romero et al., 2020). Plastic nanoparticles, antibiotics, and personal care fragrances are among the new generation compounds impacting aquatic ecosystems worldwide. Ubiquitous in municipal wastewater treatment plant discharge, they are an emerging threat to ecosystem conservation (Li et al., 2004; Abbassi et al., 2016; Elizalde-Velàzquez et al., 2016).

Single-celled algal species are widely used bioindicators in ecotoxicology as they demonstrate stress responses at different levels of biological organization (ANPA, 2000; Moreira-Santos et al., 2004). Ecotoxicological tests on such species are standardized by the National and International Organization for Standardization (i.e., UNI EN ISO 10253) and the Organization for Economic Cooperation and Development. Despite the generally good reproducibility, variability among replicates has been reported in some cases (Knauer et al., 2005; Kraufvelin et al., 2006). Tests employing unicellular phytoplanktonic species are used to assess water and effluent quality, and waste and their associated effects (Stauber et al., 2002; Eisentraeger et al., 2003; Moreira-Santos et al., 2004). Furthermore, ecotoxicological tests are recognized as a key step in evaluating sediment quality (i.e, Gonçalves et al., 2013). Inhibition of the growth rate of phytoplanktonic primary producers is considered an effect of chemical stress (Bonin et al., 1986; Knauer and Hommen, 2012). Small phytoplanktonic species are more sensitive to chemical stress than larger algal cells (Othman et al., 2012).

Renzi et al. (2014) reported that conventional and standardized endpoints such as the inhibition of growth after 72 h of chemical exposure to a marine algal species (*Phaeodactylum tricornutum*; Phaeodactylaceae) are less informative about the potential effects of chemical substances than early and sublethal effects based on the quantification of photosynthetic complex and biovolume, which allows for better detection of sublethal effects on aquatic ecosystems (Knauer and Hommen, 2012). Recent studies have reported that standardized endpoints are not useful to detect sublethal effects such as morphological and physiological changes (Mecozzi et al., 2008; Knauer and Hommen, 2012) since toxicants affect mainly biochemical and growth processes in exposed cells (Falasco et al., 2009).

Renzi et al. (2014) found that physiological and morphological changes are reliable quantitative ecotoxicological endpoints in diatoms (*P. tricornutum*) exposed to metals and surfactants. The efficiency of the photosynthetic complex (Geider et al., 1993) proved a useful tool to determine early stress in algal species exposed to chemicals (Streiber et al., 2002; Katsumata et al., 2006; Renzi et al., 2014). Reduction of cellular biovolume is another ecotoxicological endpoint with an ecological meaning: a stress-induced decrease in body size results in less energy transfer towards higher trophic levels, with knock-on effects upwards from the lower to the higher trophic levels (Cloern and Dufford, 2005).

With this study we examined four new generation chemicals of high ecological impact (nanoparticles, antibiotics, personal care fragrances) that municipal wastewater treatment plants emit into aquatic environments (Yang and Metcalfe, 2006; Liu et al., 2013; Abbassi et al., 2016; Elizalde-Velázquez et al., 2016). Data for the effects these chemicals have on microalgae species at diverse levels of biological organization are unknown or considered unsatisfactory for determining their environmental risk.

Nanoparticles (1–100 nm in size) is an umbrella term for a heterogeneous group of chemicals (Moore, 2006; Pettitt and Lead, 2013). Artificial titanium dioxide (TiO_2) and zinc oxide (ZnO) nanoparticles are ubiquitous in the environment (Gottschalk et al., 2009). They find widespread industrial use (Nowack and Bucheli, 2007; Kumar et al., 2014) in medicines and personal care products, plastic, rubber, paints, and glass (Hossain et al., 2014). Recent studies have highlighted their impact on aquatic ecosystems (Mukherjee and Acharya, 2018) and the inverse relation with particle dimension (Sun et al., 2009). A more standardized approach (Renzi and Guerranti, 2015) is needed to determine their effect on ecosystems (Wang et al., 2014).

Growing attention has been directed to antibiotics in human and veterinary medicine and their biologically active forms as environmental micropollutants thanks to a better understanding of their impact on waterbodies (Sicuro et al., 2020). Drugs enter the aquatic environment mainly through direct disposal by domestic and hospital sewage. Another major point of entry, similar in meaning but more delocalized throughout a geographical area, is via the discharge of sewage from intensive farming and effluents from intensive aquaculture plants, where the use of veterinary medicines is common practice (Wie et al., 2011; Santi et al., 2019). Amoxicillin is an antibiotic in the β-lactam class, a group of semi-synthetic penicillin that act on bacterial wall synthesis. Current scientific knowledge is insufficient to fully assess the risks that amoxicillin poses to the environment (Elizalde-Velázquez et al., 2016).

Synthetic musk, known primarily as white musk, is a class of synthetic fragrances the perfume industry uses to produce the scent of musk deer or other natural musk. These compounds form the basic notes in the formulas of many fragrances on the perfumery market. White musk xylene is a synthetic substance that simulates the scent produced by musk deer but is harmful to both the environment and humans alike. It is used in the mineral oil and the fuel industry and in cosmetics as a perfume agent and for dry cleaning. Xylene musk is not only highly persistent and bioaccumulates in tissues, but it also contains bis(2-ethylhexyl) phthalate (DEHP), a toxic substance that can impair male fertility. It is harmful to the environment, especially when incorrectly

processed and disposed. In animals it can impair thyroid and reproductive function and reduce offspring survival and alter development (Wu et al., 2006).

The aim of this study was to determine the lethal and sublethal effects of new generation pollutants (nanoparticles, antibiotic, white musk) on freshwater (*Pseudokirchneriella subcapitata* and *Scenedesmus obliquus*) and marine (*Phaeodactylum tricornutum*, *Isochrysis galbana* and *Tetraselmis suecica*) phytoplankton species. Response to standardized cellular growth was evaluated to measure physiological, biochemical, molecular and morphological sublethal effects in the experimentally exposed microalgae species.

2. Material and methods

2.1. Experimental design

Multiple effects following exposure of unicellular algal species to four chemicals of ecological interest were measured. Five algal species were exposed: two freshwater species (*Pseudokirchneriella subcapitata, Scenedesmus obliquus)* and three (*Phaeodactylum tricornutum*, *Isochrysis galbana, Tetraselmis suecica*) marine species. Two species, *P. subcapitata* and *P. tricornutum*, are used for standardized ecotoxicological tests according to UNI EN ISO 10253:2017 (UNI, 2017). The five species in their phase of exponential growth were exposed to dilutions of four chemicals: nanoparticles (n-TiO_2, n-ZnO), antibiotic (amoxicillin), and personal care fragrance (white musk) to determine the half maximal effective concentration (EC_{50}) compared to controls (unexposed algal cultures) using as endpoint the growth inhibition after 72 h of exposure under standardized and controlled environmental conditions. Cell cultures exposed to EC_{50} were used to explore sublethal effects (72 h) of biochemical (photosynthetic complex efficiency; spectrophotometry), molecular changes in the outer cell wall structure (μFT-IR microscopy), and morphological changes, such as changes in structure and mean body-size (FESEM, field emission scanning electron microscopy).

2.2. Features of algal species

Phaeodactylum tricornutum Bohlin. This marine species is found in three different morphotypes: oval, fusiform, and triradiate (Francius et al., 2008; Bartual et al., 2011). It contains chlorophyll-*a* and chlorophyll-*c* as photosynthetic pigments and β-carotene and three xanthophylls (fucoxanthin, diatoxanthin, diadinoxanthin) as accessory pigments that give this species its golden-brown colour.

Tetraselmis suecica (Kylin) Butch. This marine green microalgae species is widely used in aquaculture for feeding molluscs and crustacean larvae (Chini Zittelli et al., 2006). It contains chlorophyll-*a* and chlorophyll-*b* as photosynthetic pigments and carotenoids and xanthophylls as accessory pigments.

Isochrysis galbana Parke. This freshwater species is ellipsoidal in shape, with a very thin fragile cell wall, two apical flagella generally of the same length, and a smaller flagellum, the aptonema which has a variety of functions, including attachment to a substrate (Qiang and Richmond, 1994; Bendif et al., 2013; Nomura et al., 2019). It contains chlorophyll-*a* and chlorophyll-*c* as photosynthetic pigments and fucoxanthin as carotenoid.

Pseudokirchneriella subcapitata (Korshikov) F.Hindák. This freshwater green microalga species has a half-moon shape and is normally found in unicellular form. It is the best known and most frequently used species in ecotoxicological tests because of its rapid growth rate and sensitivity to toxicants. It proliferates by forming four daughter cells (autospores) through multiple fission after two nuclear divisions (Yamagishi et al., 2017). It contains chlorophyll-*a* and chlorophyll-*b* as photosynthetic pigments.

Scenedesmus obliquus Kützing. This freshwater species has elongated, rounded or cylindrical cells; sexual reproduction is by longitudinal binary division, although sexual reproduction is reported in some families of the genus *Scenedesmus*. It contains chlorophyll-*a* as the principal pigment, chlorophyll-*b*, carotene, and xanthophyll.

2.3. Chemicals and concentrations

For this study we used four new generation chemicals of high ecological impact: n-TiO_2 (CAS n. 13463-67-7, Caelo, powder, particle size <21 nm, surface area 35–65 m^2/g); n-ZnO (CAS n. 1314-13-2, Caelo, powder, particle size <100 nm, surface area 15–25 m^2/g); amoxicillin (EG S.p.A., pharmaceutical powder), and white musk (Farmalabor, n. 01574A_00001_0100FLBCBIT, pure liquid. Nanoparticles were the same commercial raw products that Renzi and Blašković (2019) tested on *Daphnia magna*. Exposure concentrations were determined after a preliminary range-finding test to define scalar dilution of exposure; EC_{50} was calculated using the following concentration: 1.56, 3.13, 6.25, 12.50, 25, 50 mg/L (TiO_2); 0.63, 1.25, 2.50, 5, 10, 20 mg/L (ZnO); 10, 20, 200, 2000, 4000 mg/L (amoxicillin); 4, 8, 80, 800, 8000, 16000 mg/L (white musk).

2.4. Growth inhibition tests

Standardized algal species. Standardized ecotoxicological tests on algal species (*Phaeodactylum capricornutum,* marine; *Pseudokirchneriella subcapitata,* freshwater) were performed following UNI EN ISO 10253:2017 (UNI, 2017) slightly modified (using ASTM algal medium) and UNI EN ISO 8692:2012 (UNI, 2012), respectively; controlled and standardized variables were fixed and monitored (Table 1).

Non-standardized algal species. Ecotoxicological tests were performed following standardized UNI EN ISO 10253:2017 (UNI, 2017) methods also on non-standardized species. Two marine (*Isochrysis galbana* and *Tetraselmis suecica*) and one freshwater (*Scenedesmus obliquus*) algal species were tested. Variables were standardized and controlled as reported for standardized species by setting the parameters according to the reference method (UNI EN ISO 10253:2017, marine, and UNI EN ISO 8692:2012, freshwater).

The endpoint of this analysis was the inhibition of growth of the algal species after exposure to a chemical substance. Species in their exponential growth phase were exposed to the test substance and put under continuous light for 72 h in controlled temperature conditions. Every 24 h the samples were shaken, and cell density was measured by spectrophotometry (Onda UV-30 scan spectrophotometer, optical length 10 cm, Bormac s.r.l., Carpi, Italy) and calculated by light absorbance at a wavelength of 670 nm. Tests were performed in triplicate for each concentration ($n = 3$). Spectrophotometric response was calibrated using a cell density versus absorbance curve developed on tested algal stock by Bruker's chamber counts at each of the 10 points in scalar dilution of 10^6 cell/mL stock. The initial concentration of the specimens was 10^4 cells/mL, and the sample concentration was 100%. Details on the calculation to determine the inhibition percentage of growth (I %) are reported in Renzi et al. (2014). A specific concentration of nutrients was added to the sample; the nutrients were not added to the negative controls because they were included in the medium. At the end of the 72-h exposure period, the growth rate of the control and the exposed cultures was calculated. Growth and growth inhibition were quantified by measuring algae biomass as a function of time estimated by cell count or optical density. The endpoint of the test was the growth inhibition percentage (I%), expressed as the logarithmic increase in biomass (average specific

Table 1
Variable of interest defined by the method. Endpoint: inhibition of cell growth; Test duration: 72 h. PSU denotes practical salinity unit.

Species	Method	Salinity (PSU)	Temperature (°C)	pH (unit)	Illumination
P. tricornutum	UNI EN ISO 10253:2017	30 ± 1	20 ± 2	8.0 ± 0.2	24/24 h light 10000 lux from both sides
P. subcapitata	UNI EN ISO 8692:2012	2 ± 1	23 ± 2	8.1 ± 0.2	24/24 h light 6000–10000 lux from both sides

growth rate) during the exposure period. From the average growth rates recorded in a series of test solutions, the concentration (expressed as a percentage or mg/L) that yields a given percentage of growth rate inhibition (i.e., 50%) compared to negative controls can be determined and expressed as EC_{50}.

Quality assurance and quality control (QA/QC). A QA/QC protocol was applied during the experiments to ensure methodological quality of the experimental results. The Bioscience Research Centre is a certified laboratory (ISO 9001:2015, TÜV) and applies strict control procedures under UNI EN ISO 17025:2018 guidelines to ensure data quality. The QA/QC tests were performed as described in the reference methods. Tests were performed following the general quality criteria applied by the laboratory. During the test, negative controls (algal culture medium) and positive controls (potassium dichromate) were set up. The test is considered valid when the algal concentration in the negative control after 72 h of growth is > 16 times the starting concentration for all of the species tested. For the positive controls, the standardized reference values for comparison of performance were available only for *P. capricornutum* and *P. subcapitata;* the tests are considered validated if the EC_{50} of the positive control is 20.1 ± 5.3 mg/L (*P. tricornutum)* and 1.19 ± 0.27 mg/L (*P. subcapitata*). Table 2 presents the results of the tests of the negative and the positive controls.

2.5. Biochemical analyses

Chlorophylls (Chl-*a, b, c*) were used as stress biomarkers. Levels in cell cultures were determined following the APAT CNR IRSA 9020 (APAT, 2003) method at the end of the inhibition of growth test (72 h). About 50 mL of exposed algal species were filtered on glass fibre filters (pore diameter, 0.45 μm) by vacuum pump, extracted with acetone (Sigma Aldrich, St. Louis, MO. USA, analytic grade) kept on ice and in the dark for 2 h at +4 °C to prevent pigment alterations. The supernatant was collected, and the spectrophotometric absorbance measured at 630, 647, 664, 750 nm (Onda UV-20 UV/Vis spectrophotometer, Bormac). Chlorophyll content was calculated according to the APAT CNR IRSA 9020 (APAT, 2003) method as follows:

Chl-*a* = {[11.85 (Abs 664–750) −1.54 (Abs 647–750) - 0.08 (Abs 630–750)] v}/V·L
Chl-*b* = {[21.03 (Abs 647–750) - 5.43 (Abs 664–750) - 2.66 (Abs 630–750)] v}/V·L
Chl-*c* = {[24.52 (Abs 630–750) - 7.60 (Abs 647–750) - 1.67 (Abs 664–750)] v}/V·L

Where: Abs is the absorbance measured at the set wavelength, v is the volume (mL) of acetone extract, V is the sample volume (L), L is the optical path (cm). Data are expressed as mg/m^3 per compound. The limit of quantification (LOQ) was 0.001 mg/m^3.

The percentage of Chl-*a, b, c* expressed by the cells was normalized to normal expression in negative controls. The percentages were used to determine the EC_{50} for the chlorophylls. Moreover, the cell cultures were exposed to EC_{50} to determine sublethal effects of biochemical alterations (photosynthetic complex efficiency).

2.6. Molecular changes

Fourier-transformed infrared spectroscopy (μFT-IR) has proved useful to determine molecular changes in algal species exposed to chemical stress (Mecozzi et al., 2008). The percentage of spectral match between exposed algae and controls was taken as a proxy of molecular changes in the outer wall cell structure after exposure. About 25 mL of algal cell cultures were collected on Anodisc™ (AlO_2, 0.2 μm, Whatman™, Cytiva, Little Chalfont, Bucks., UK) membrane filters, air-dried (35 °C), and quantified by FT-IR microscopy (Nicolet iN10 MX FT-IR microscope, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a liquid nitrogen-cooled MCT-A detector. With this sensitive technique a small volume of cell cultures can be filtered on the disk and tests performed directly on cells without preliminary extraction or pre-treatment. Changes in characteristic peaks were determined by comparing the untreated algae culture with the algae exposed to EC_{50} of each chemical. The spectra readings of the cell cultures were collected by the transmission mode (acquisition range, 4000-1200 cm^{-1}) from about 30 replicates to determine the mean spectra of the controls and exposed cultures. Integration was performed using Omnic™ Picta™ software (Thermo Fisher Scientific). Mean matches (%) were calculated by averaging the coupled matches of the spectral replicates (single spectra collected per type, $n = 30$). The peak height in absorbance at each wavelength was calculated to obtain a mean spectrum for each condition and for comparison to the negative controls (Mecozzi et al., 2008).

2.7. Morphological changes

Scanning electron microscope measurements are useful to

Table 2
Quality assurance/quality control data for negative and positive controls. SD denotes standard deviation.

Species	Negative Control (Inhibition %, 72 h)			Positive Control ($K_2Cr_2O_7$) EC_{50} mg/L	
	Substance	Mean	SD	*p*-value	95% confidence limit
Phaeodactylum tricornutum	ASTM cell culture medium	0.0	0.87	18.80	16.76–20.84
Pseudokirchneriella subcapitata	ISO freshwater cell culture medium	0.0	3.43	3.07	1.96–4.19
Isochrysis galbana	f/2 cell culture medium	0.0	1.41	30.61	27.85–33.36
Tetraselmis suecica	ASTM cell culture medium	0.0	1.11	23.86	21.01–26.71
Scenedesmus obliquus	ISO freshwater cell culture medium	0.0	0.30	1.34	1.13–1.74

determine changes in structure and mean body size (Hillebrand et al., 1999). Untreated and exposed cultures were analysed by field emission scanning electron microscopy (Merlin FE-SEM, Carl Zeiss, Oberkochen, Germany) to detect changes in structure and/or body size at ultra-high resolution (better than 1 nm). About 25 mL of algal cell cultures were collected on sterile nitrocellulose fibre filter disks (pore diameter, 0.45 μm, Sartorius, Göttingen, Germany) and fixed with two drops of Lugol solution (100 g/L KI + 50 g/L I_2) to protect cellular structure from deterioration. The samples were kept at 4 °C until analysis. FE-SEM was coupled with a wavelength dispersive and energy dispersive spectrometer (EDS) to have ultrastructural and microanalytical data (X-Max 50 EDS system, Oxford Instruments, Abingdon, UK). With the combination of charge compensation in-situ, Zeiss Microscopy has engineered a solution for imaging non-conductive specimens that allows for both microscopic investigations and microanalysis (mapping of the elemental distribution) on samples without the need for covering with a conductive layer. This technique provides topographical and elemental information at magnifications within ~10–300,000 Å with virtually unlimited depth of field. High-resolution micrographs collected with an in-lens secondary electron detector (InLens, Zeiss) were used to measure maximum cellular length (Nikon NIS-Element D software, Tokyo, Japan) on a representative number of algal cells ($n = 10$) with a 3% error in body size.

2.8. Statistical analyses

Data were entered into Excel® program (Microsoft, Redmond, WA, USA) for descriptive statistics (i.e., mean, standard deviation, percentage). Calculation sheets were validated and blocked before the use to minimize random mistakes. The Kolmogorov–Smirnov test was performed to determine whether our dataset was well-modelled by normal distribution. When calculated, the EC_{50} for the positive controls and the exposed cultures was determined from the concentration-effect curves by application of the lognormal model using TRAP (Analysis type = Tolerance Distribution; Model shape = Gaussian distribution) software. Principal component analysis (PCA) was performed to check for trends in average transmittance (obtained by μFT-IR) of controls (untreated cultures) and exposed cultures (n-TiO_2, n-ZnO, amoxicillin, white musk). PCA plots were created to summarize the molecular effects of the chemicals (treatments) on the five phytoplanktonic species considering the two groups (treatments and controls), the five species and the two habitats (marine and freshwater). Since the null hypothesis for normal distribution could not be rejected, a non-parametric Mann–Whitney *U* test was used to reveal morphological changes (difference in maximum cellular length) between exposed and control cultures. The Kruskal–Wallis test was used to show differences in chlorophyll levels between the exposed and the control cultures, followed by the Conover-Iman post hoc test. Statistical analyses were performed using R software (version 1.1.463, RStudio, Inc., Boston, MA, USA).

3. Results

3.1. EC_{50} and growth inhibition tests

The EC_{50} of the antibiotic (amoxicillin) was 20–200 mg/L in *P. tricornutum* (marine) and *S. obliquus* (almost 20 mg/L, freshwater); 10–20 mg/L affected 50% of exposed cells in *T. suecica, I. galbana* (about 20 mg/L, marine), and *P. subcapitata* (about 10 mg/L, freshwater). The EC_{50} was about 25 mg/L of TiO_2 for *P. subcapitata*, while lower concentrations, about 12.5 mg/L, affected *T. suecica* and *I. galbana* and 12.5–25.0 mg/L affected *P. tricornutum*. Differently, *S. obliquus* was more sensitive to TiO_2 (about 6.25 mg/L). The species most sensitive to ZnO was *P. subcapitata* (about 5.0 mg/L) in a freshwater environment (*S. obliquus,* range 5–10 mg/L), and *I. galbana* (about 2.5 mg/L) in a marine environment (*P. tricornutum* and *T. suecica,* range 2.5–5 mg/L). Differently, white musk was highly toxic for *T. suecica* and *I. galbana* marine species (about, 5.0 and 2.5 mg/L, respectively) followed by about 800 mg/L for the other species. Table 3 presents the data on inhibition of growth.

3.2. Biochemical analyses

Table 4 presents the EC_{50} calculated from the inhibition of growth (%) compared to the EC_{50} calculated from the biochemical biomarkers of stress (Chl-*a, b, c*). Photosynthetic pigments are reported for algal species in which their production is documented by the literature (paragraph 2.2.). The biomarker concentration in the exposed cultures was normalized to the levels in the negative controls of the same species by calculating the percentage of change in the specific biochemical biomarker. The changes, expressed as percentage (%), were used to calculate the EC_{50} of each biomarker (toxicant concentration that produces a change of 50% in the biomarker concentration in the exposed cells compared to the concentration in the negative controls). The EC_{50} for Chl-*a* under the tested concentrations were not calculable for *I. galbana*, *S. obliquus,* and *P. subcapitata*, Plausible explanations are that either the effects were lower than the EC_{50} at the maximum concentration of chemical (grey, Chl-*b* in *T. suecica, S. obliquus, P. subcapitata*), or the stress biomarker is higher than the EC_{50} at the lowest concentration of chemical (red, Chl-*a*, *I. galbana, S. obliquus, P. subcapitata*; Chl-*c*, *P. tricornutum*). The results were similar to the values recorded using inhibition of growth, except at levels of Chl-*a* in species *I. galbana*, *S. obliquus, P. subcapitata*.

Generally, in cultures exposed at the EC_{50} there was a significant decrease in Chl-*a* in all microalgae for all chemicals compared to controls (Table 5) (Kruskal–Wallis test, p-value<0.05), except for *P. subcapitata* which showed no significant change (Kruskal–Wallis test, p-value>0.05). There was a significant decrease in Chl-*b* in *T. suecica* after exposure to all chemicals (Conover-Iman test, p-value<0.05), in *S. obliquus* control after exposure to amoxicillin (Conover-Iman test, p-value<0.05), and in *P. subcapitata* control after exposure to amoxicillin, TiO_2 and ZnO (Conover-Iman test, p-value<0.05). Chl-*c* was detected only in the control group of *I. galbana* and *P. tricornutum*, whereas Chl-*c* concentrations were lower than the LOQ for all chemicals.

3.3. Molecular changes

In Fig. 1 are presented two examples of the spectra overlay between the negative control and the exposed species; spectra refer to the outer cell wall structure. Table 6 presents the results of the mean spectral match (%) between the spectra of cultures exposed to toxicants compared to negative controls. The data are given for each species at the EC_{50} for all chemicals. FT-IR spectroscopy revealed a wide range in spectral alteration (from 10.44% to 90.93%).

Principal component analysis used to check for trends in transmittance (μFT-IR spectra) disclosed changes in the photosynthetic complex by the chemicals. The first PCA (Fig. 2a) showed that the first (Dim1) and the second (Dim2) components accounted for meaningful amounts of the total variance (93.4%): Dim1 explained 57.5% of the total variance and Dim2 35.9%. The exposed and the control groups are arranged by overall spectral response. There was a clear separation between the two groups: the controls are located in the left half of the plot and the exposed groups in the right half. Also, the overlap of confidence ellipses (95%) of the four treatments

Table 3
Inhibition of growth (%) after exposure to different chemical concentrations. Effects closest to the EC_{50} (mg/L) are given in bold.

Chemical	Concentration (mg/L)	Marine species			Freshwater species	
		P. tricornutum	T. suecica	I. galbana	S. obliquus	P. subcapitata
Amoxicillin	4000	100.00	100.00	100.00	100.00	100.00
	2000	100.00	100.00	100.00	100.00	100.00
	200	100.00	100.00	100.00	100.00	100.00
	20	**38.61**	**83.88**	**59.88**	**43.97**	100.00
	10	−0.86	10.64	10.22	31.24	**43.38**
TiO_2	50.00	100.00	100.00	100.00	100.00	100.00
	25.00	100.00	100.00	100.00	80.78	**49.38**
	12.50	**20.17**	**57.38**	**53.64**	66.24	39.72
	6.25	3.48	9.37	15.07	**40.60**	35.62
	3.13	−16.70	−21.09	1.48	25.81	29.15
	1.56	−26.36	−36.24	−18.58	14.46	22.86
White Musk	16,000	100.00	100.00	100.00	100.00	100.00
	8000	100.00	100.00	100.00	87.71	100.00
	800	**50.27**	100.00	73.61	**54.51**	**48.97**
	80	27.27	**43.09**	**39.06**	32.74	43.98
	8	−11.70	14.63	12.21	17.97	31.37
	4	−20.50	−56.41	−1.34	−8.43	25.42
ZnO	20.00	100.00	100.00	100.00	100.00	100.00
	10.00	100.00	100.00	100.00	**71.59**	100.00
	5.00	**63.72**	**51.15**	83.41	38.12	**48.92**
	2.50	12.53	10.93	**52.93**	24.93	43.04
	1.25	−17.43	−20.88	23.93	18.14	37.62
	0.63	−20.97	−34.62	−11.32	−15.15	30.13

Table 4
EC_{50} calculated from inhibition of growth and biochemical analyses. The symbol "-" means that the pigment is absent in the algal species. NC denotes not calculable results. Red highlight: effects probably lower than EC_{50} at maximum concentration tested; grey highlight: stress biomarker higher than EC_{50} at the lowest concentration tested.

Test		Chemical	EC_{50}	Marine species			Freshwater species	
				P. tricornutum	T. suecica	I. galbana	S. obliquus	P. subcapitata
Inhibition algal growth (%)		Amoxicillin	mg/mL	0.03	0.02	0.03	0.03	0.01
		TiO_2	mg/L	15.23	11.69	11.78	11.76	29.14
		ZnO	mg/L	4.41	4.87	1.82	6.42	3.59
		White Musk	mg/mL	1.01	0.12	0.76	2.87	0.77
Biochemical analyses (Chlorophylls)	Chl-*a*	Amoxicillin	mg/mL	7.16	7.46	NC	NC	NC
		TiO_2	mg/L	13.86	18.54	NC	NC	NC
		ZnO	mg/L	20.36	3.75	NC	NC	NC
		White Musk	mg/mL	0.04	11.11	NC	NC	NC
	Chl-*b*	Amoxicillin	mg/mL	–	NC	–	NC	NC
		TiO_2	mg/L	–	4.69	–	NC	NC
		ZnO	mg/L	–	15.13	–	NC	NC
		White Musk	mg/mL	–	NC	–	NC	NC
	Chl-*c*	Amoxicillin	mg/mL	6.00	–	NC	–	–
		TiO_2	mg/L	NC	–	NC	–	–
		ZnO	mg/L	NC	–	NC	–	–
		White Musk	mg/mL	NC	–	NC	–	–

suggests similar transmittance and/or effects of the chemicals on the outer cell wall structure. The other two PCA plots (Fig. 2b and c) show the changes in spectral response by the five algal species and the two habitats (marine and freshwater). There was a similar trend in spectral response due to the overlap of confidence ellipses, indicating a similar effect at the molecular level.

3.4. Morphological changes

Table 7 presents the morphological changes (maximum cell size). The data are expressed as the mean maximum cellular length ±standard deviation (SD). An example of the measurements in the negative controls and the exposed cultures are shown in Fig. 3. Measurements were taken at concentrations similar to the EC_{50} and changes in length (increased/reduced) were considered significant. Significant increase in cell size were observed in *I. galbana*, *P. tricornutum* and *T. suecica* exposed to white musk and in *S. obliquus* exposed to amoxicillin compared to the negative controls (Mann–Whitney *U* test; $p < 0.05$). A non-significant change in cell size was noted in *P. subcapitata* (Table 7).

4. Discussion

Ecotoxicological tests rely on organisms that are widespread in the environment, ecologically relevant, sensitive to chemicals, and easy to test under laboratory conditions (Walsh and Merrill, 1984; EPA, 1991). In the present study we performed ecotoxicological tests to determine the EC_{50} after exposure to four new generation pollutants in five algae species in marine and freshwater environments. The response of phytoplankton communities to a chemical stressor holds ecological interest (Cadotte et al., 2011; Stevenson, 2014) since they are key species in regulating energy flows in aquatic ecosystems. The new generation chemicals we tested in this study are widely used in industry and ubiquitous in municipal wastewater effluents. Few studies (mainly on nanoparticles) (Miller et al., 2012; Sendra et al., 2017) or other data about their effects on

Table 5
Chlorophyll (Chl-*a*, Chl-*b*, Chl-*c*) concentration (mean ± standard deviation; mg/m^3) in freshwater (*Pseudokirchneriella subcapitata*-Ps, *Scenedesmus obliquus*-S) and marine (*Phaeodactylum tricornutum*-P, *Isochrysis galbana*-I, *Tetraselmis suecica*-T) microalgae exposed to EC_{50} of the four chemicals for 72 h (C denotes control; A amoxicillin; M white musk; T titanium dioxide; Z zinc oxide). LOQ (limit of quantification) = 0.001 mg/m^3. Lowercase letters denote differences revealed by the Conover-Iman post hoc test.

Species	Treatment	Chl-*a* (mg/m^3)	Chl-*b* (mg/m^3)	Chl-*c* (mg/m^3)
I	C	0.13 ± 0.01^a	–	0.02 ± 0.03
I	A	0.04 ± 0.02^b	–	<LOQ
I	M	0.08 ± 0.01^b	–	<LOQ
I	T	0.06 ± 0.02^b	–	<LOQ
I	Z	0.05 ± 0.01^b	–	<LOQ
P	C	0.20 ± 0.02^a	–	0.02 ± 0.02
P	A	0.10 ± 0.01^b	–	<LOQ
P	M	0.06 ± 0.01^b	–	<LOQ
P	T	0.09 ± 0.03^b	–	<LOQ
P	Z	0.10 ± 0.02^b	–	<LOQ
S	C	0.23 ± 0.02^a	0.54 ± 0.02^a	–
S	A	0.08 ± 0.01^b	0.20 ± 0.02^b	–
S	M	0.23 ± 0.02^a	0.58 ± 0.01^a	–
S	T	0.23 ± 0.01^a	0.54 ± 0.02^a	–
S	Z	0.20 ± 0.02^a	0.47 ± 0.03^a	–
Ps	C	0.10 ± 0.02^a	0.45 ± 0.02^a	–
Ps	A	0.06 ± 0.01^a	0.11 ± 0.02^b	–
Ps	M	0.11 ± 0.02^a	0.45 ± 0.03^a	–
Ps	T	0.10 ± 0.03^a	0.20 ± 0.02^b	–
Ps	Z	0.11 ± 0.02^a	0.36 ± 0.04^b	–
T	C	0.13 ± 0.02^a	0.41 ± 0.03^a	–
T	A	0.05 ± 0.01^b	0.09 ± 0.01^b	–
T	M	0.05 ± 0.01^b	0.08 ± 0.02^b	–
T	T	0.05 ± 0.02^b	0.11 ± 0.04^b	–
T	Z	0.04 ± 0.03^b	0.08 ± 0.02^b	–

phytoplankton have been reported to date. Data are available for standardized species (*P. tricornutum* and *P. subcapitata*) while non-standardized species have been largely unexplored.

Metals cause membrane depolarization and cytoplasmic acidification, leading to disruption of cellular homeostasis (Pinto et al., 2003). Metals bind to proteins to form a metal–protein complex that can affect enzymatic systems, growth, photosynthesis, respiration, reproduction, nutrient assimilation, and molecular synthesis (Morin, 2003). Excesses Zn can totally or partially inhibit growth (Stauber and Florence, 1990) and photosynthesis (Nguyen-Deroche et al., 2009). Metals affect growth and photosynthesis differently (Cid et al., 1995); the mechanism of action of metals is increased permeability which causes loss of organic matter (Steeman-Nielsen and Wium-Andersen, 1971), loss of potassium (Rai et al., 1981), and reduced uptake of essential elements and compounds such as manganese (Sunda and Huntsman, 1983) and silicic acid (Rueter and Morel, 1981). Differently, organic chemicals, such as surfactants, alter lipid and biological membranes and induce cell lysis (Pavlić et al., 2005).

Titanium dioxide (TiO_2) and zinc oxide (ZnO) nanoparticles are largely added as an anti-UV filter (TiO_2) in cosmetics and as an antimicrobial agent (ZnO). Statistically, the annual production of ZnO nanoparticles is between 550 and 5550 tons and the yield is approximately 10–100 times higher than that of any other nanomaterial (Piccinno et al., 2012). Their use was always considered safe; since at the end of the last century, in vitro tests showed that they induced DNA damage under particular conditions (Dunford et al., 1997). Furthermore, exposure to ZnO nanoparticles has been found to induces effects also on humans; for example, they

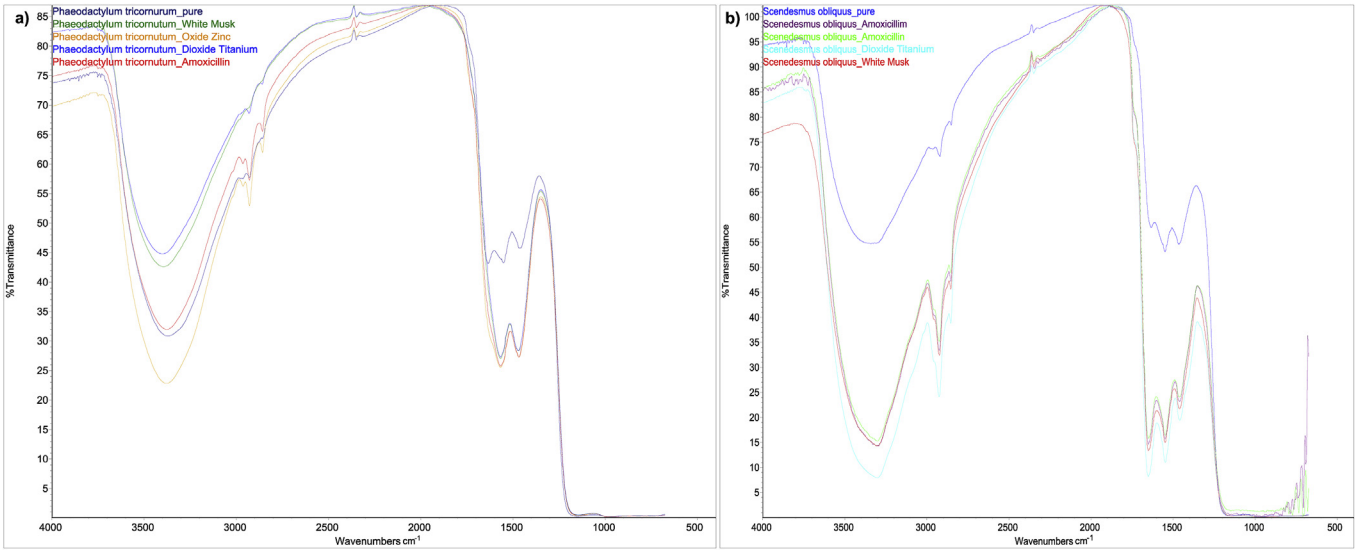

Fig. 1. Molecular changes observed by μFT-IR. Example of spectra overlay on *P. tricornutum* (a) and *S. obliquus* (b) exposed to the four chemicals here tested at EC_{50}.

Table 6
Mean percentage of spectral match between spectra collected on exposed cell cultures and negative controls. Data were recorded at exposure to the EC_{50} dose and are reported as the mean percentage of match between spectra (n = 30); 100% means identity. Data are referred to the global spectral response.

Treatment	Percentage match	Marine species			Freshwater species	
		P. tricornutum	T. suecica	I. galbana	S. obliquus	P. subcapitata
Amoxicillin	%	90.03 (0.71)	90.32 (1.10)	87.52 (0.35)	10.44 (4.29)	–
TiO_2	%	88.28 (0.55)	90.06 (2.27)	–	42.33 (2.31)	87.48 (2.33)
ZnO	%	89.63 (0.13)	89.36 (0.64)	89.39 (0.18)	73.93 (5.91)	88.54 (0.14)
White Musk	%	88.84 (0.04)	90.93 (0.09)	89.71 (0.45)	77.87 (3.44)	89.38 (0.04)

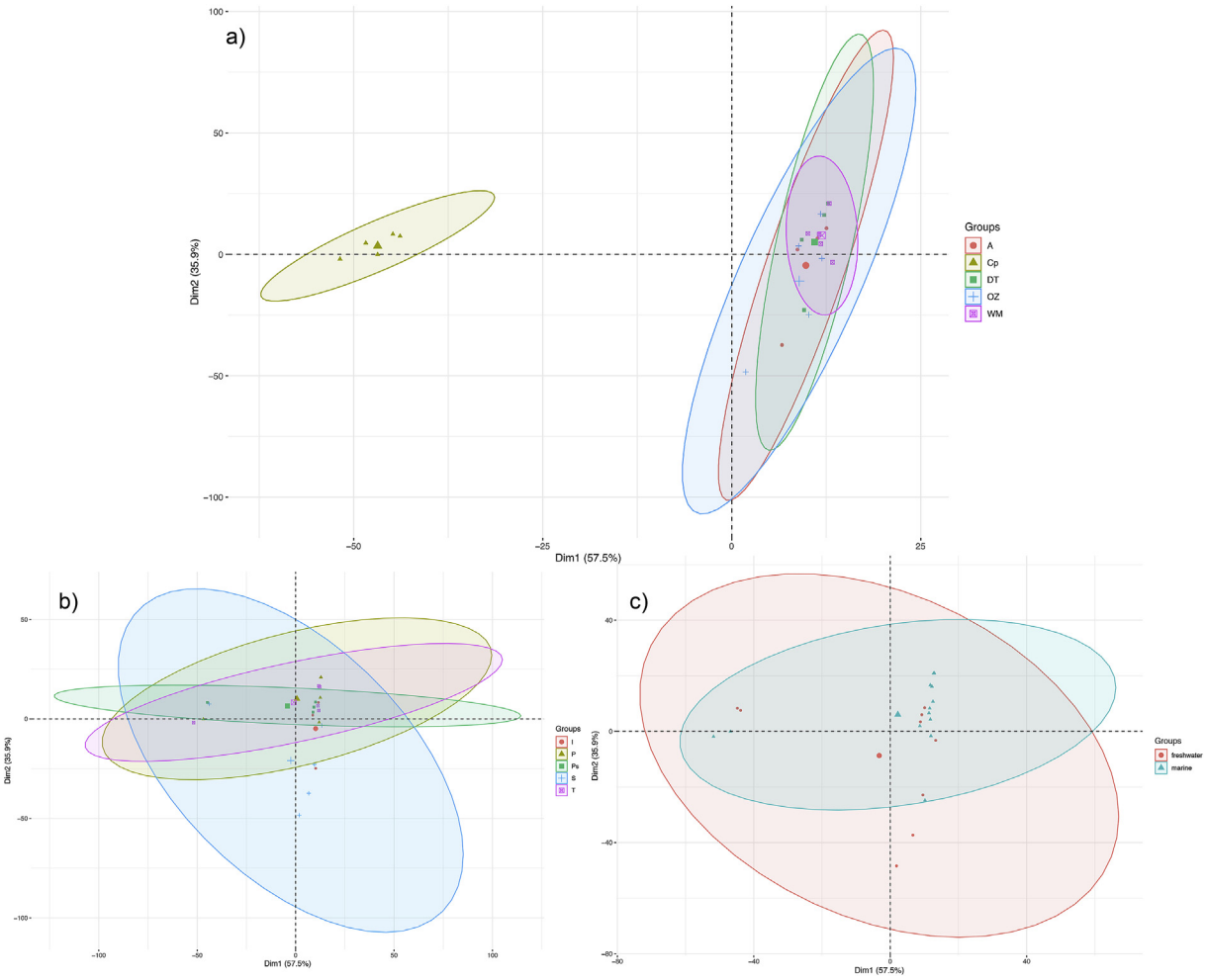

Fig. 2. Principal component analysis of average transmittance (obtained by μFT-IR). a) Clusters are based on controls (untreated cultures = Cp) and exposed cultures (DT = n-TiO_2; OZ = n-ZnO; A = amoxicillin; WM = white musk); b) clusters based on the five plankton species (I=*Isochrysis galbana;* P=*Phaeodactylum tricornutum*; Ps = *Pseudokirchneriella subcapitata*; S=*Scenedesmus obliquus*; T = *Tetraselmis suecica*); c) clusters based on aquatic habitat (freshwater and marine). Confidence ellipses (95%) plot values for each group.

Table 7
Morphological changes observed by field emission scanning electron microscopy. Mean (n = 10) of maximum cellular length expressed in μm ±standard deviation (SD). Comparison of data from exposed and control cultures is reported with associated level of significance (Mann–Whitney U test, p-value). NS denotes not significant; *$p < 0.05$; **$p < 0.01$.

		Mean (μm)	SD	p-value	Significance
Isochrysis galbana	Negative controls	2.00	0.24	–	–
	Amoxicillin	2.85	0.59	0.458	NS
	White musk	3.73	0.12	0.010	**
Phaeodactylum tricornutum	Negative controls	15.95	4.49	–	–
	White musk	22.41	0.89	0.020	*
	TiO_2	21.18	4.76	0.090	NS
	ZnO	15.62	3.85	0.294	NS
Scenedesmus obliquus	Negative controls	6.92	0.45	–	–
	Amoxicillin	8.20	0.50	0.004	**
	White musk	7.10	1.00	0.600	NS
	TiO_2	7.62	1.06	0.129	NS
	ZnO	7.33	0.68	0.085	NS
Tetraselmis suecica	Negative controls	10.27	0.63	–	–
	Amoxicillin	13.11	1.19	0.240	NS
	White musk	11.54	0.12	0.013	**
	TiO_2	11.43	1.79	0.419	NS
	ZnO	11.64	2.13	0.292	NS
Pseudokirchneriella subcapitata	Negative controls	8.52	1.65	–	–
	White musk	8.64	1.48	0.721	NS
	TiO_2	7.58	1.08	0.289	NS
	ZnO	7.92	1.10	0.221	NS

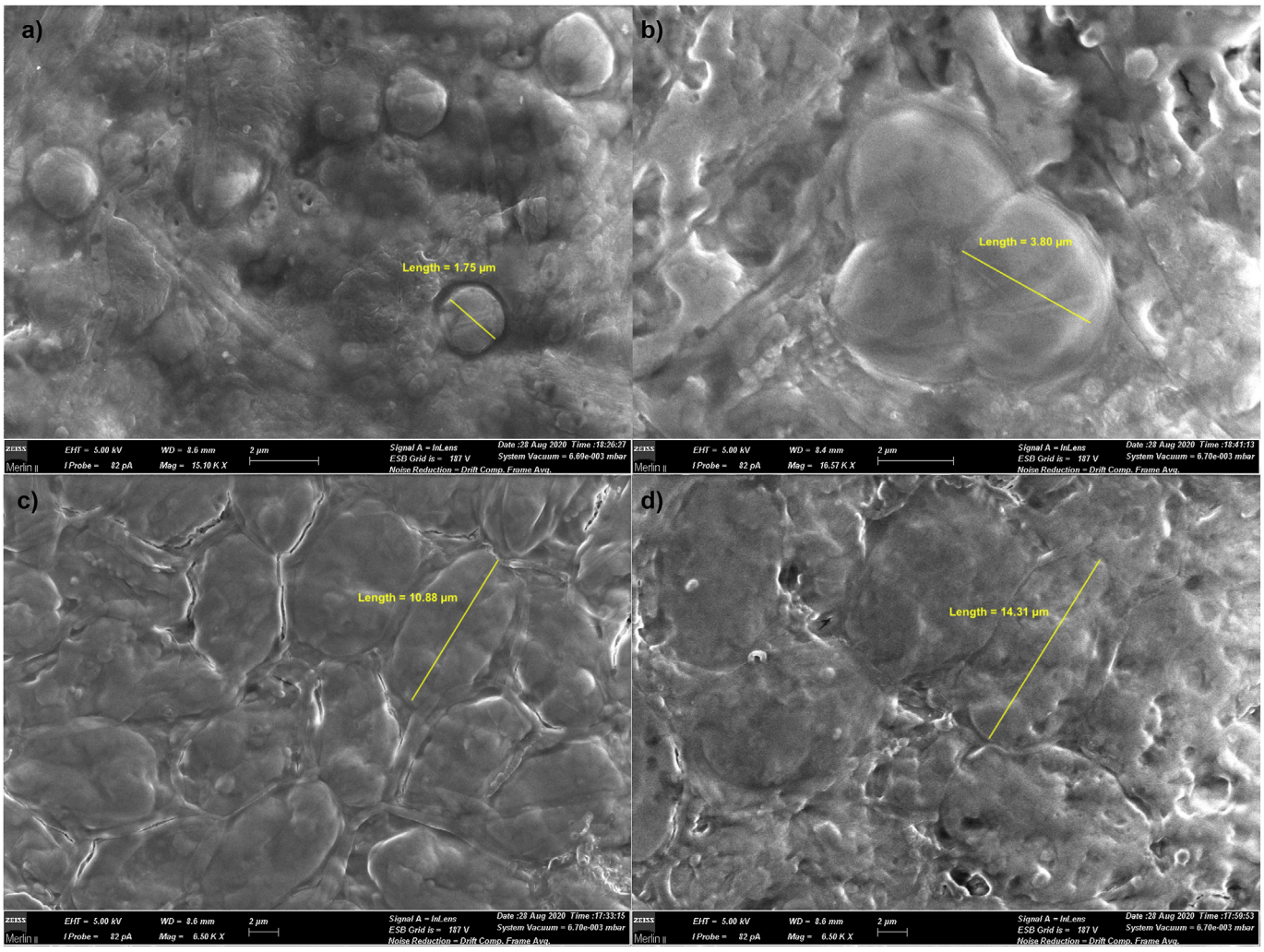

Fig. 3. Morphological changes in algal species. Structural changes are shown for *Isochrysis galbana* (a; negative controls-normal cells) exposed to white musk (b) and for *Tetraselmis suecica* (c; negative controls-normal cells) exposed to white musk (d).

can cause corrosive gastroduodenal lesions (Liu et al., 2006) and pulmonary toxicity (Moos et al., 2010). Recent studies on functionalized commercial nanoparticles considered representative of their environmental behaviour (Nowack and Bucheli, 2007) showed that the nanoparticles can affect aquatic species (i.e., *D. magna*, Renzi and Blaskovic, 2019) and induce mortality.

Our findings for EC_{50} in standardized algal species exposed to nanoparticles are consistent with the literature. Comparison among nanoparticles is difficult because of differences in particle size and/or whether functionalized/not-functionalized raw materials are tested (Renzi and Guerranti, 2015). Wang et al. (2016) reported that n-TiO_2 inhibits growth at concentrations below 20 mg/L in *P. tricornutum*. Severe effects were observed after the first day (12.65 mg/L); the EC_{50} was about 168 mg/L after 72 h of exposure. Li et al. (2017) reported that exposure to 100 nm n-ZnO could affect *P. tricornutum* diatoms more than bulk ZnO and Zn-ions at an EC_{50} of 1.09 (0.96–1.57) mg/L.

There are sparse data for standardized algal species from freshwater environments and for non-standardized species. Aruoja et al. (2009) reported an EC_{50} of 5.83 mg/L for n-TiO_2 and 0.02 mg/L for n-ZnO, respectively, in *P. subcapitata*. In their study on non-standardized species, Li et al. (2017) reported that exposure to 100 nm of n-ZnO could affect *T. suecica* at a higher EC_{50} of 3.91 (3.66–4.14) mg/L, thus showing less sensitivity of this species compared to *P. tricornutum* diatoms. To the best of our knowledge, data on the effects of exposure to amoxicillin and white musk on algal species are lacking; this precluded comparison of our and previous findings.

We observed differences in tolerance to chemicals by the algae species. The algae reported most tolerant to heavy metal pollution is *P. tricornutum* (Falasco et al., 2009). Our study underlines the need to widen the panel of phytoplanktonic species to correctly assess the impacts by new generation pollutants on phytoplanktonic communities in natural environments. A wider panel would also guard against simplification and underestimation of environmental risks when tolerant species are employed as indicators of the entire trophic level response.

Hou et al. (2019) reported that the mechanism of action of n-TiO_2 NP toxicity to organisms is three-fold: 1) reactive oxygen species (ROS) formation, 2) damage to cell wall and lipid peroxidation of cell membranes, and 3) damage to biological macromolecules. Renzi et al. (2014) found a reduction in chloroplast dimension in marine *P. tricornutum* species exposed to metals (Zn and Cu). This observation was shared by other studies on chloroplasts exposed to heavy metals (Sicko-Goad, 1982; Rachlin et al., 1984; Chan and Wong Ling, 1987; Rai et al., 1990; Visviki and

Rachlin, 1992) and provided evidence for a correlated alteration in chlorophyll content. The population endpoint (growth curve) at 72 h was affected to a greater extent than the photosynthetic endpoint at 24 h (Othman et al., 2012).

Moreover, the biochemical biomarkers we employed in the present study provided a good proxy of early-occurring stress in the species tested here. Physiological endpoints were detected earlier and showed greater sensitivity of response than the traditional endpoint (growth-rate inhibition). Renzi et al. (2014) found a certain consistency between the sensitivity of a photosynthetic complex and biovolume endpoint responses. The early stress biomarkers were found to be sensitive to the chemicals, particularly in the non-standardized species. This is consistent with previous studies that reported that the photosynthetic complex was a sensitive physiological endpoint in the detection of stress responses at sublethal doses (Renzi et al., 2014).

Chlorophyll contents were used as an indicator for physiological stress because photosynthesis is known to be decreased at elevated concentrations of certain pollutants (Monni et al., 2001). The significant decrease in chlorophyll content we noted in the microalgal species indicates a change in photosynthetic activity. All the chemicals at EC_{50} caused a significant decrease in chlorophyll in *I. galbana*. Similarly, Moro et al. (2020) observed a decrease in chlorophyll content in *I. galbana* cultures treated with oxytetracycline. The reduction in chlorophyll content is noteworthy since it is an important marker for evaluating the effects of pollutants on algal photosynthesis and respiration (Moro et al., 2020).

Biometry (morphological features) can demonstrate species health and response to environmental stressors (Lugoli et al., 2012; Dromph et al., 2013; Vadrucci et al., 2013). Recent studies reported that exposure to metals can affect the proliferation and the morphology of algal species; the ensuing changes in cell size often reflect an increase in biovolume in freshwater and marine algae (Machado and Soares, 2014). In contrast, a low sensitivity of size trait was observed (Weiner et al., 2004). Biovolume is an endpoint that can demonstrate early detect chemical stress on phytoplankton communities (Larson and Passy, 2005; Roselli et al., 2013; Renzi et al., 2014). However, there is no link between pollutant-induced toxicity and cell size (Levy et al., 2007, 2008, 2008; Renzi et al., 2014).

The molecular changes we observed are consistent with the literature and highlight a general spectral alteration in a range of 10–91% of the mean match. The PCA suggested, however, that no differences in molecular changes could be detected in the five planktonic species. Our results are consistent with the literature and show that spectral IR acquisition is a cost-effective, rapid technique to detect structural and biochemical effects in species exposed to sublethal concentrations. Alterations in IR spectra in the range of 3000 cm^{-1} are associated with $-CH_3$ and $-CH_2$ stretching and are due to alterations in lipids and proteins. In contrast, alterations in regions of about 1600 cm^{-1} are associated with C=O stretching and protein damage (Mecozzi et al., 2008).

5. Conclusions

With the present study we defined for the first time the EC_{50} for nanoparticles in non-standardized algal species and for amoxicillin and white musk in all the species tested. Species sensitiveness to the same chemicals differed notably: the standardized species were less sensitive than the non-standardized species in some cases. This suggests that attention is warranted in data extrapolation to the whole trophic level. The biochemical biomarkers were sensitive and better able than inhibition of growth to detect stress at sublethal levels. Changes in cell size, when significant, were an increase in maximum cell length. Finally, μFT-IR microscopy was useful in detecting sublethal stress and molecular changes in the exposed algal species.

Credit author statement

Andrea Broccoli: Investigation; Methodology; Writing - Original Draft, Serena Anselmi: Conceptualization; Investigation; Methodology; Writing- Reviewing and Editing, Andrea Cavallo: Investigation; Methodology; Writing- Reviewing and Editing Vittoria Ferrari: Investigation; Methodology; Writing- Reviewing and Editing Daniela Prevedelli: Methodology; Supervision; Writing-Reviewing and Editing, Paolo Pastorino: Conceptualization; Data curation; Writing- Reviewing and Editing, Monia Renzi: Funding acquisition; Investigation; Conceptualization, Supervision; Writing- Reviewing and Editing. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

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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