A Comparative Test on the Sensitivity of Freshwater and Marine Microalgae to Benzo-Sulfonamides, -Thiazoles and -Triazoles

Luca Canova [1], Michela Sturini [1], Federica Maraschi [1], Stefano Sangiorgi [2] and Elida Nora Ferri [2,*]

1 Department of Chemistry, University of Pavia, Via Taramelli 12, 27100 Pavia, Italy; luca.canova@unipv.it (L.C.); michela.sturini@unipv.it (M.S.); federica.maraschi@unipv.it (F.M.)
2 Department of Pharmacy and Biotechnology, University of Bologna, Via S. Donato 15, 40127 Bologna, Italy; stefano.sangiorgi9@unibo.it
* Correspondence: elidanora.ferri@unibo.it

Featured Application: These preliminary data on the differences among aquatic microoorganisms in their response to benzo-fused nitrogen heterocyclic pollutants can help in selecting the most suitable biotest for environmental toxicity monitoring activities.

Abstract: The evaluation of the ecotoxicological effects of water pollutants is performed by using different aquatic organisms. The effects of seven compounds belonging to a class of widespread contaminants, the benzo-fused nitrogen heterocycles, on a group of simple organisms employed in reference ISO tests on water quality (unicellular algae and luminescent bacteria) have been assessed to ascertain their suitability in revealing different contamination levels in the water, wastewater, and sediments samples. Representative compounds of benzotriazoles, benzothiazoles, and benzenesulfonamides, were tested at a concentration ranging from 0.01 to 100 mg L^{-1}. In particular, our work was focused on the long-term effects, for which little information is up to now available. Species-specific sensitivity for any whole family of pollutants was not observed. On average, the strongest growth rate inhibition values were expressed by the freshwater *Raphidocelis subcapitata* and the marine *Phaeodactylum tricornutum* algae. *R. subcapitata* was the only organism for which growth was affected by most of the compounds at the lowest concentrations. The tests on the bioluminescent bacterium *Vibrio fisheri* gave completely different results, further underlining the need for an appropriate selection of the best biosensors to be employed in biotoxicological studies.

Keywords: microalgae; *Vibrio fisheri*; benzenesulfonamide; benzothiazole; benzotriazole; biotoxicity tests

1. Introduction

The presence of any xenobiotic compounds in fresh, ground, or marine water represents a threat to organisms living in these ecosystems and, therefore, to human health. Among organic pollutants, a group of high production volume chemicals, benzotriazole (BTRs), benzothiazoles (BTs), and benzenesulfonamides (BSAs), benzo-fused nitrogen heterocyclic compounds containing a benzene ring, has been used for several decades in a large number of industrial activities and consuming products [1].

BTRs are corrosion inhibitors, antifreeze fluids, and aircraft de-icers, and they are employed in photography, plastic production, and dishwasher detergents [2–5]. BTs include vulcanization accelerators, corrosion inhibitors, biocides, UV light stabilizers in textiles and plastics, and precursors in the production of several kinds of pharmaceuticals [6]. BSAs are used for synthesizing dyes, photo-chemicals, disinfectants, and intermediates in the production of pharmaceuticals [7,8]. These polar compounds, some of them resistant to biodegradation, can constitute an environmental threat due to their widespread application and consequent ubiquitous occurrence in various environmental compartments [1,7].

Benzothiazoles, for example, are pervasive and spread into the atmosphere through tire chemical leaching [9]; they have been detected in human matrices, including exhaled breath, adipose tissue, urine, and amniotic samples [10–13]. They can be carcinogenic to humans [14], act as dermal sensitizers [15], and produce allergenic effects and respiratory tract irritation at sufficient exposure [16–18]. Several studies suggested that they show acute toxicity to fish, aquatic plants, and invertebrates at relatively high concentrations [19,20].

Benzotriazoles are spread through the progressive corrosion of metals, act as potent air pollutants, and may give rise to water pollution because of run-off from urban areas and roads [9,21]. Together with benzothiazoles, they were found in the watershed, surface freshwater, drinking water, and at high concentrations in primary and secondary wastewaters [22–26]. Their concentrations range from a few ng L^{-1} in water bodies to hundreds of µg L^{-1} in sewage and indoor dust [6,17,22,26]. Existing evidence suggests that benzotriazoles harm aquatic plants and animals and have estrogenic effects in fish and *Daphnia magna* [6,19,27–29].

Benzenesulfonamides were found in rivers and sewage plants in concentrations up to µg L^{-1} [7,30]. Concerning BSAs, studies exist only for *p*-toluene-sulfonamide (*p*-TSA), which was defined as moderately toxic [8,30], but the large amounts currently used recommend additional tests.

The toxicological and ecotoxicological information is estimated to be scarce, especially concerning chronic effects, although acute aquatic toxicity has been repeatedly reported [19,28,30–34]. Information about toxicity on wildlife is scarce, particularly concerning reptiles, birds, and marine mammals. Recently, benzotriazole UV filters have been found in the blood plasma of fishes, snapping turtles, double-crested cormorants, and bottlenose dolphins from various locations in North America and Europe, thus confirming the widespread diffusion of such compounds in the aquatic environments [35,36].

The increasing amount of potentially harmful pollutants in freshwater and marine environments requires fast and low-cost methods of analysis to carry out effective monitoring activities on the contaminants level. As recommended by the Organization for Economic Co-operation and Development (OECD), additional tests on the bioactivity of new pollutants are mandatory [37]. Advanced chemical technologies are usually the first choice because of their high sensitivity and selectivity, but they are time-consuming, require expensive instruments, and can reveal just the searched compounds [1,7,35]. On the contrary, biomonitoring methods are cheaper, faster, and extremely useful in evaluating the xenobiotics' overall biological effects on the ecosystems [34,38]. Nevertheless, the results obtained from the various employed organisms, even belonging to the same trophic level, must be carefully evaluated and interpreted always keeping in mind that each one will represent just a single aspect of the possible scenario, and then multiple organisms must be investigated.

This paper aimed to carry out a comparative evaluation of the response of four organisms, three microalgae and a marine bioluminescent bacterium, for the presence of seven compounds, separately tested. The compounds belong to the above-mentioned three classes of chemicals: benzothiazole (BT), 2-methylthiobenzothiazole (MeSBT), 2-hydroxybenzothiazole (HOBT), benzotriazole (BTR), 5-methylbenzotriazole (5TTR)), benzenesulfonamide (BSA), and *p*-toluene-sulfonamide (*p*-TSA) (Figure 1).

In detail, we tested the freshwater alga *Raphidocelis subcapitata* (previously *Pseudokirchneriella subcapitata*) and two marine organisms, the diatomea *Phaeodactylum tricornutum*, and the green alga *Dunaliella tertiolecta*. These algal species, are widespread in rivers, deltas, lagoons, and coastal habitats and showed a high degree of habitat tolerance. They are relatively easy to maintain in laboratory cultures and are used as standard toxicity test organisms for organic chemicals [39–42].

D. tertiolecta (Chlorophyceae, Chlamydomonadales) is a biflagellate green marine microalga, able to grow in severe environments. It lacks a cell wall that may be a potential barrier to the passage of pollutants into the cell [43,44]. It is easy to cultivate, has rapid

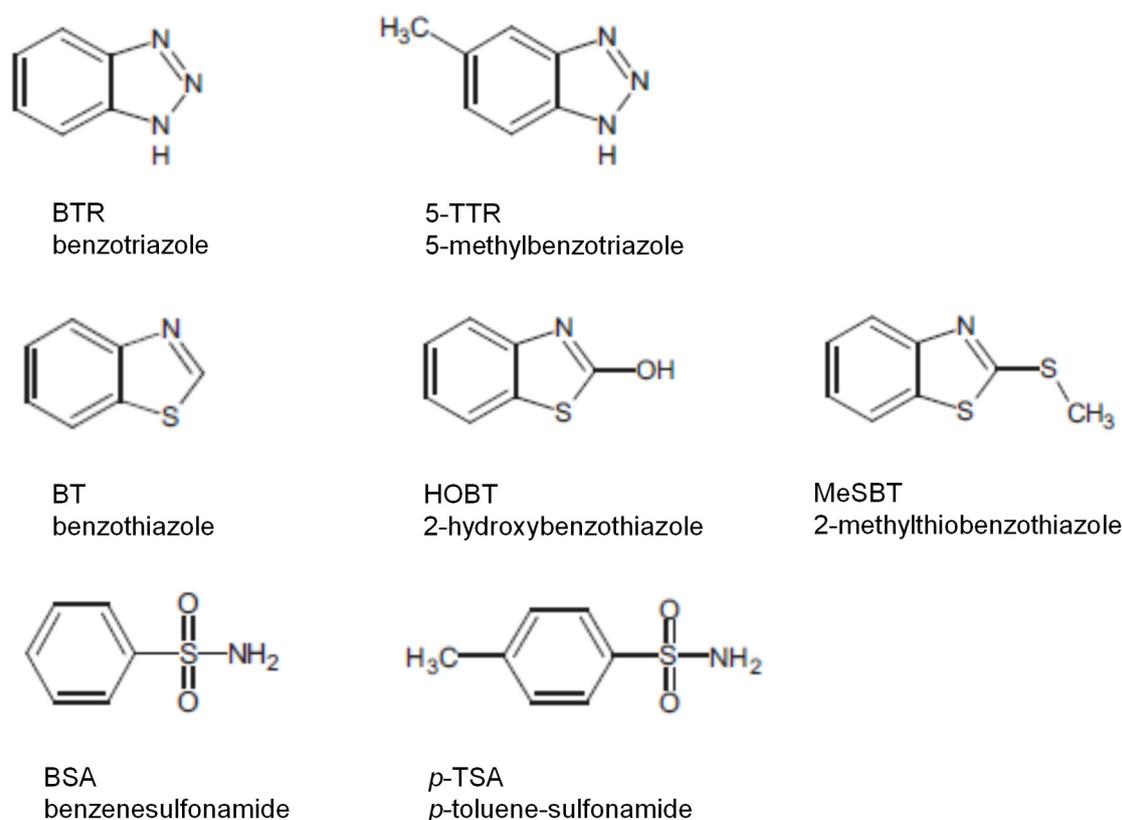growth, and is considered a good indicator to evaluate the toxicity of contaminants present in marine water.

BTR
benzotriazole

5-TTR
5-methylbenzotriazole

BT
benzothiazole

HOBT
2-hydroxybenzothiazole

MeSBT
2-methylthiobenzothiazole

BSA
benzenesulfonamide

p-TSA
p-toluene-sulfonamide

Figure 1. Chemical structures of the tested benzo-fused nitrogen heterocyclic compounds.

R. subcapitata (Chlorophyceae, Sphaeropleales) is a freshwater sessile unicellular green alga. It is easy to cultivate and shows a high growth rate and high sensitivity to several substances. It is one of the green algae representative of oligotrophic and eutrophic environments, a frequently used organism in toxicity studies [45].

P. tricornutum (Bacillariophyta, Bacillariophyceae) is a benthic pennate diatom with a rigid siliceous cell wall. It is the only standardized marine species for wastewater toxicity tests. It is characterized by easy cultivation and sensitivity to pollutants [46].

The well-established dependence of light emission intensity of the bioluminescent bacterium *Vibrio fisheri* from its wellness, i.e., from the presence of harmful or beneficial components, has been exploited to develop one of the most-used toxicity reference assays. *V. fisheri* is a bioluminescent marine bacterium for which light emission intensity is highly influenced by the conditions of its environment. Based on the ascertained inversely proportional reduction in light intensity with the increase in toxicants in solution, the test based on this bacterium has long represented the quicker and easier standardized assay for drinking water quality assessment [47,48].

2. Materials and Methods

2.1. Chemicals

BSA, *p*-TSA, BT, BTR, HOBT, MeSBT, and 5TTR pure compounds were supplied by Sigma-Aldrich (MI, Italy), as well as all chemicals requested to prepare the algae culture media and the bioluminescent bacteria nutrient broth. Thermo Scientific (Vantaa, Finland) supplied the 96-well "Black Cliniplate" microplates for luminescence detection, carried out on the "Victor light 1420" luminescence counter (Perkin-Elmer). Lyophilized aliquots of the luminescent bacteria *V. fischeri* were prepared from fresh cultures at our laboratory, starting from an original batch supplied by the Pasteur Institute (Paris, France). The Istituto

Zooprofilattico Sperimentale of Abruzzo and Molise "G. Caporale" (Teramo, Italy) supplied the three microalgae starting cultures.

2.2. Algal Growth Inhibition Assay

Culture media differed between the three species: for the *D. tertiolecta* the f/2 medium was prepared by adding to sterilized synthetic seawater (Instant Ocean®) a mixture of vitamin (cyanocobalamin + biotin + thiamine) and trace elements [49]. The *P. tricornutum* medium was prepared by adding sodium silicate (30 g L^{-1}) to the *D. tertiolecta* medium. The *R. subcapitata* was cultivated in Jaworski's culture medium [50].

The stock cultures were prepared by inoculating 0.5–1 mL of microalgae suspension on an Erlenmeyer flask containing 250 mL of the respective medium. The flasks were covered with a porous cotton plug and maintained under illumination (white lamp/red lamp Osram Daylight 2 × 36 W plus Osram Gro-Lux lamp 36 W) (8 h light/16 h dark) at 20 °C. The stock cultures were allowed to grow until the required cell density was reached to prepare a fresh stock culture. To perform the assay, a specimen of the stock culture in log phase growth was diluted, obtaining a cell suspension that contained no more than 1.10^3 cells mL^{-1}. Since physiological or metabolic defects can be better evaluated following a prolonged period of growth in spiked culture, a diluted inoculum was employed to let the algae grow for quite a long time before reaching the log phase again. The assays were performed not within the usual interval of 72–96 h, but the growth rate of algae was checked at different intervals after starting the test, about 20 ± 2 (interval A), 27 ± 2 (interval B), and 34 ± 2 days (interval C). The 10 mL test tubes were filled with the appropriate medium containing the desired pollutant's final concentration (100, 50, 25, 10, 5, 1, 0.1, and 0.01 mg L^{-1}), and then the algal culture inoculum (0.1 mL of the diluted suspension) was added. To prepare the controls, the algal inoculum was simply added into the appropriate medium. The tubes were covered with sterilized porous cotton and gauze and kept under the same conditions (light and temperature) as the stock cultures.

We evaluated the algal density, after gentle shaking to homogenize the suspension, by measuring the absorbance of the cultures at 684 nm, an indirect method for cell counting also mentioned in an ISO procedure [51]. The overall experiments were replicated on three independent samples.

The toxic effects were evaluated by calculating the percent of growth inhibition in the samples with respect to the controls according to the absorbance values (Equation (1)).

$$I\% = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \tag{1}$$

where *A* is the absorbance at 684 nm.

The EC$_{50}$ values for each compound were calculated according to Finney, 1971 [52], by using the average value of the % samples inhibition, since the toxic effects are always relative to the respective controls in each experiment.

2.3. Bioluminescence Inhibition Assay

Lyophilized aliquots of *V. fischeri* containing 3% NaCl were reconstituted with 1 mL of distilled water and re-suspended in 10–30 mL of the nutrient broth (NaCl 15 g, peptone 2.5 g, yeast extract 1.5 g, glycerol 1.5 mL, HEPES 0.01 M in 500 mL, pH 7). An amount of 200 μL of the bacteria suspension and 100 μL of each sample in distilled water plus 3% NaCl or blank (i.e., 3% NaCl in distilled water) were added to the microplate wells. The emitted light was recorded at various intervals between 0–48 h after preparation of the microplate. Each sample was replicated on five independent wells. The concentrations tested on this organism were in the μg L^{-1} range, precisely 100, 50, and 0.1 μg L^{-1}, because of the high sensitivity usually displayed by this assay.

The bioluminescence inhibition percentage (*I*%) was used to express the toxicity of the tested samples and calculated according to the same equation employed for the algae, by using, in this case, the intensity of the emitted light (*L*):

$$I\% = \frac{L_{control} - L_{sample}}{L_{control}} \times 100$$

2.4. Statistical Analyses

Significant differences at 5% and 1% probability levels ($p \leq 0.05$ and 0.01, respectively) were determined via one-way/two-way non parametrical analysis of variance (Kruskal–Wallis test), with data reported as the mean ± standard error. The relationship between growth rate and light absorbance was tested by the Pearson's r correlation test; the predictive value of the growth-absorbance equation and explained variance were tested by the linear regression method. A non-parametrical U–Mann test for unmatched samples was adopted to evaluate pairs comparison. SPSS statistical software version 13.0 was used for all the tests [53].

3. Results

3.1. Preliminary Assessments

We adopted optical density measurements as the index of cells' density inside vessels, and the reliability of the relationship between the growth rate of target species and light absorbance was evaluated by regression analysis. In all cases, we found a highly significant correlation and a very high explained variance (R > 99% s), i.e., a highly predictive correlation between the two variables (Figure 2), as previously described [54]. Consequently, the differences in growth rate expressed as light absorbance between controls and samples have been considered a suitable parameter for describing the toxicant effect on target species. These effects were evaluated after a long time of contact, until one month, to highlight the possible chronic effects produced by these long-lasting pollutants. The environmental concentrations in water usually range from ng to µg L^{-1}, but we tested concentrations in the range 10 µg L^{-1} to 100 mg L^{-1} since, according to the literature, negligible toxic effects are generally reported at the environmental concentrations, so higher ones are employed to analyze the in vitro effects [19,31,34,55].
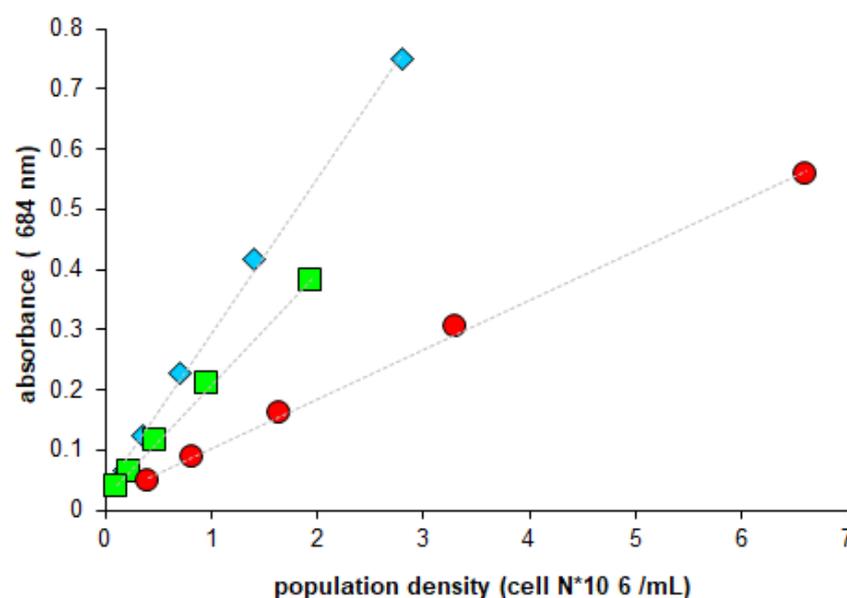

Figure 2. Relationship between target species cell density and light absorbance (♦ *P. tricornutum*, ■ *D. tertiolecta*, ● *R. subcapitata*). All the regression functions are statistically significant at $p < 0.001$ with a high explained variance (R^2$_{Pt}$ = 0.998; R^2$_{Dt}$ = 0.998; R^2$_{Rs}$ = 0.998).

3.2. Growth Response of Dunaliella tertiolecta

The response of the algae to the presence of the different compounds was expressed as the % growth inhibition with respect to the control. The values reported in Figures 3–5 were calculated by comparing the absorbance values recorded during the last measurement (34 ± 2 days).

- Benzenesulfonamides. The algae growth rate was affected by *p*-TSA at the tested concentrations down to 0.1 mg L^{-1} (Figure 3). This concentration reduced the growth rate by about 5%, a quite negligible value. BSA also showed a harmful effect on algae, but the growth rate was decreased with respect to the control only by the concentrations in the range 100–10 mg L^{-1} (K–Wallis $\chi^2_{BSA} = 5.21$, df = 2, $p < 0.05$ $\chi^2_{pTSA} = 5.34$, $p = 0.06$).

- Benzothiazoles. All the benzothiazoles affected *D. tertiolecta* growth rate at the higher concentrations (Mann–U$_{100} = 7$, $p < 0.05$; Mann–U$_{50} = 8$, $p < 0.05$), but the lowest effective concentration was different for the three compounds. Concerning BT, it corresponded to 50 mg L^{-1}, whereas 5 mg L^{-1} was the lowest concentration of HOBT, which reduced the population growth in a significant way. The lowest concentration of MeSBT producing a reduction in the growth rate was the 10 mg L^{-1} one (Figure 4).

- Benzotriazoles. The concentrations in the range 5–100 mg L^{-1} of BTR reduced the growth rate of the algal population, and 5TTR showed to be a little more toxic than BTR since all concentrations were able to produce a minimal but measurable reduction (K–Wallis test; $\chi^2_{BTR} = 5.99$, df = 2, $p = 0.05$ and $\chi^2_{5TTR} = 7.65$, $p < 0.05$) (Figure 5). The effect of some compounds, such as BT and BSA, became significant only at the B or C measuring time, confirming that the toxicity, as well as the possible eutrophic effects, is more frequently evident in these organisms after chronic exposure.
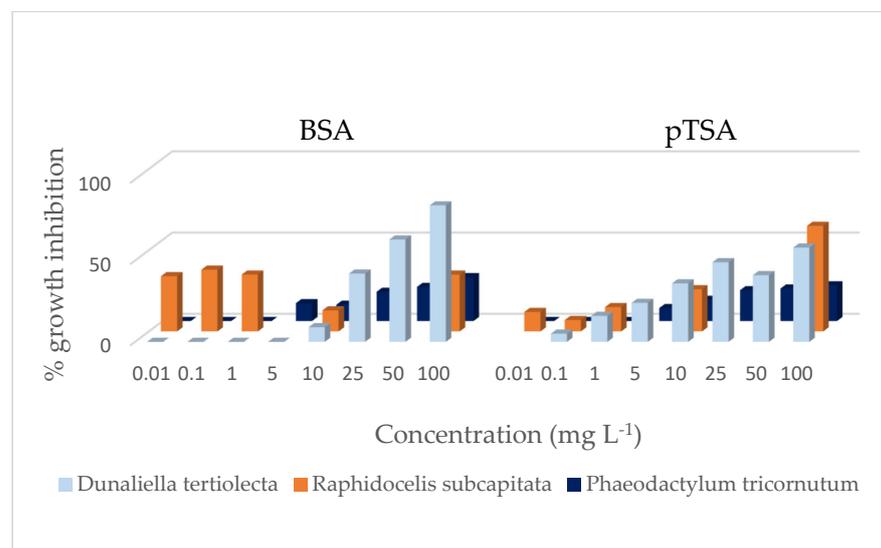
Figure 3. Values of the percentage of growth inhibition produced by the benzenesulfonamides on the three algal species.

3.3. Growth Response of Raphidocelis subcapitata

Benzenesulfonamides, benzothiazoles, and benzotriazoles. All the pollutants showed detrimental effects on the alga and inhibited, even if not always with a linear trend, the growth rate. In the whole tested range, 0.01–100 mg L^{-1}, a variable and apparent reduction in population viability was observed after exposure to benzenesulfonamides (K–Wallis test; $\chi^2_{BSA} = 5.1$, $p = 0.072$; $\chi^2_{pTSA} = 5.31$, $p = 0.06$; Figure 3) and benzothiazoles (K–Wallis test; $\chi^2_{BT} = 6.41$, $p < 0.05$; $\chi^2_{HOBT} = 6.77$, $p < 0.05$; $\chi^2_{MeSBT} = 5.99$, $p = 0.05$; Figure 4). Differently from the other two organisms, this alga showed an unexpected higher sensitivity towards

most parts of the compounds at lower concentrations. Only in the case of 5TTR, which significantly affected its growth (K–Wallis test; $\chi^2_{BTR} = 10.1$, $p < 0.01$; $\chi^2_{5TTR} = 8.05$, $p < 0.05$; Figure 5), the percent of inhibition values were in a relatively perfect linear relation with the respective concentrations.
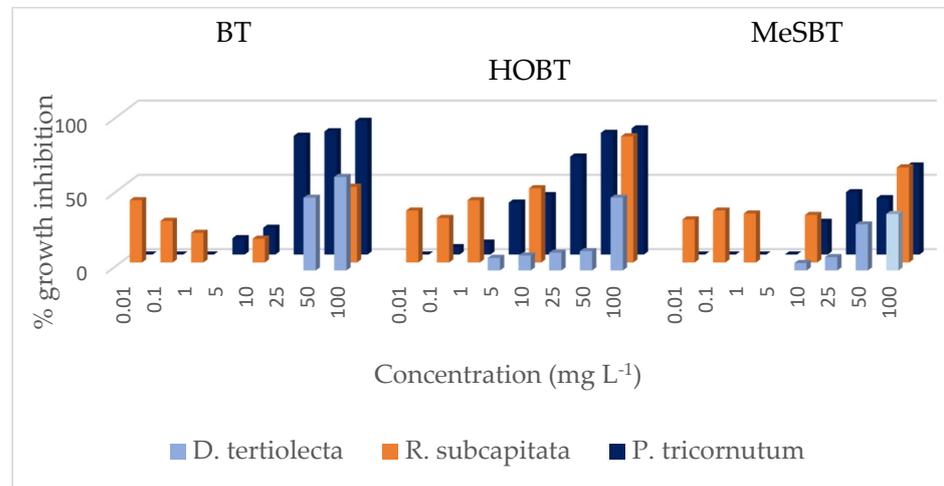

Figure 4. Values of the percentage of growth inhibition produced by the three benzothiazoles on the three algal species.
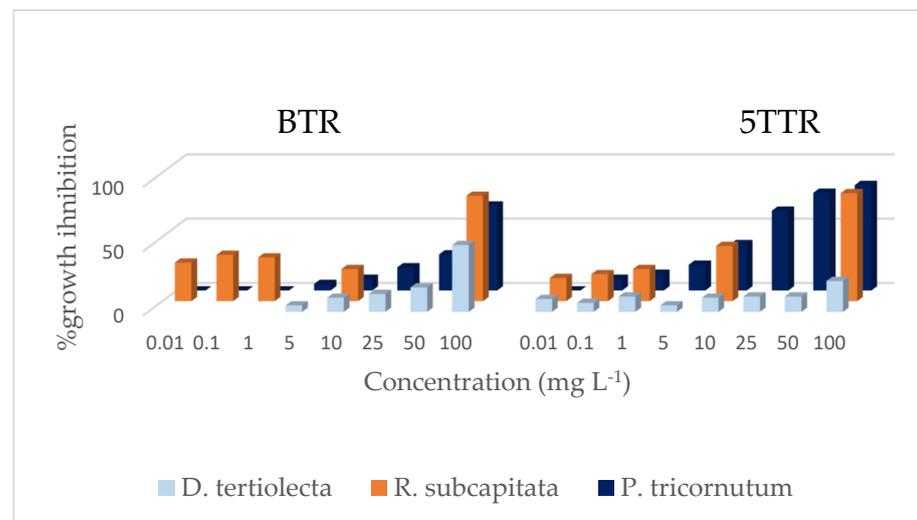

Figure 5. Values of the percentage of growth inhibition produced by the two benzotriazoles on the three algal species.

3.4. Growth Response of Phaeodactylum tricornutum

- Benzenesulfonamides. The toxicity of the two tested compounds on the marine diatom can be considered very similar in both the absolute values of growth inhibition and the lowest effective concentration, which was 5 mg L^{-1} in both cases (Figure 3).
- Benzothiazoles. These compounds showed quite different effects on algal growth. The BT and HOBT higher concentrations produced inhibition values above 80% (K–Wallis test; $\chi^2_{BT} = 6.20$, $p < 0.05$; $\chi^2_{HOBT} = 6.31$ $p < 0.05$; Figure 4). A measurable effect was produced until the 5 mg L^{-1} concentration, and marginal inhibition can be ascribed to the 1 mg L^{-1} solution in the case of HOBT. MeSBT resulted in being the least toxic, and the lowest effective concentration was the 10 mg L^{-1} one (K–Wallis test; $\chi^2_{MeSBT} = 5.84$ p = ns; Figure 4).

- Benzotriazoles. To this alga, the 5TTR solutions resulted in being toxic at all concentrations between 0.1 and 100 mg L^{-1}, producing % inhibition values quite regularly, dependent on the benzothiazole concentration (K–Wallis test; $\chi^2_{5TTR} = 7.15$, $p < 0.05$; Figure 5).

BTR resulted in being slightly less toxic, and the 5 mg L^{-1} solution was the lowest one affecting the population growth (K–Wallis test; $\chi^2_{BTR} = 6.24$, $p < 0.05$; Figure 5).

In Table 1, we reported the EC_{50} values of the various compounds for the three algae. It is easier to understand that the less sensitive strain was the marine green algae *D. tertiolecta*. Better sensitivity was expressed by the freshwater alga and by the diatomea, the latter already known to be a sensitive microorganism among the marine ones.

Table 1. EC_{50} values of the various compounds calculated for the three algae, in mg L^{-1}. The value "> 100" means that the maximum concentration tested (100 mg L^{-1}) produced an inhibition lower than 50%.

Compound	*Raphidocelis subcapitata*	*Dunaliella tertiolecta*	*Phaeodactylum tricornutum*
BSA	>100	57	>100
PTSA	92	>100	>100
BTR	67	>100	81
5TTR	38	>100	30
BT	>100	>100	41
MESBT	86	>100	75
HOBT	16	>100	32

3.5. Effects of the Compounds on Vibrio fisheri Light Emission Intensity

In Figure 6, the light emission of the various compounds at 0.1 µg L^{-1} was compared to that of the control at the typical acute (1 h) and chronic toxicity (24 h) intervals, respectively. Moreover, the last record of emission intensity was taken at 48 h after the bacteria–compound contact. It is possible to observe that the majority of the compounds at this concentration have a eutrophic effect on these organisms, or at least stimulate the light emission intensity. Indeed, their vitality was not affected by this concentration, even at the longest possible contact time for this assay. In detail, any significant differences in light emission between the control bacterial cultures and any of the cultures spiked with each compound ($\chi^2_{1h} = 7.53$; df = 7; $p = 0.38$) were observed after 1 h of contact, while strong differences were observed after 24 h ($\chi^2_{24h} = 21.41$; df = 7; $p = 0.003$) and 48 h ($\chi^2_{48h} = 21.32$; df = 7; $p = 0.003$).
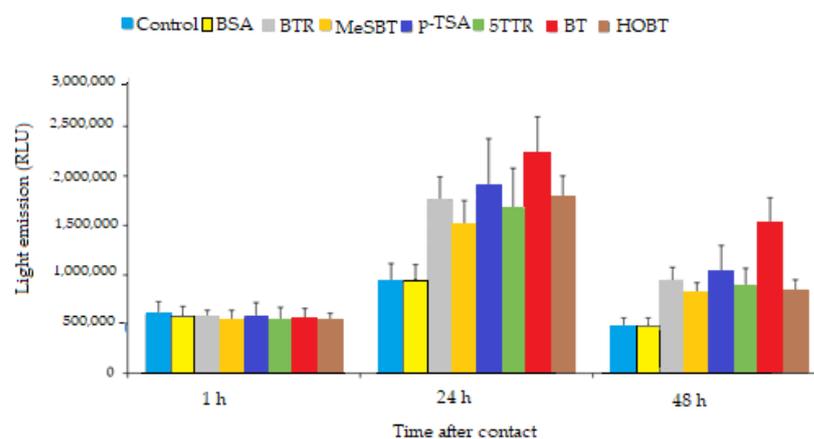

Figure 6. The light emission intensity of the *V. fisheri* suspensions in contact with the seven benzofused nitrogen heterocycles, all at the 0.1 µg L^{-1} concentration.

The observed results in the presence of the 50 and 100 µg L^{-1} solutions showed a more complicated behavior. At the usual acute toxicity interval, no clear inhibition effect was observed (Figure 7A); after 48 h of contact, the majority of the compounds produced a more or less pronounced reduction in light intensity (inhibition) in the range of 10–72%, but HOBT, at both concentrations, greatly stimulated it. BT produced a similar eutrophic effect only at the 50 µg L^{-1} concentration (Figure 7B).
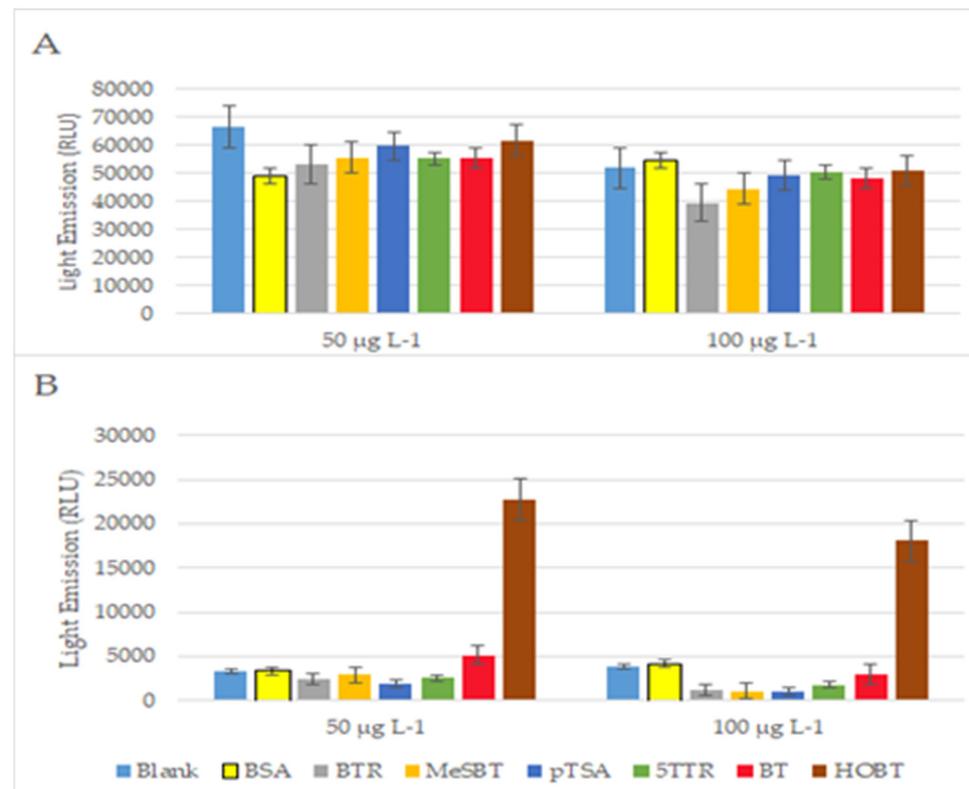

Figure 7. Bioluminescent bacteria light emission intensities recorded at short toxicity intervals (3 h (**A**) and 48 h (**B**)) after contact with the various compounds at the 50 and 100 µg L^{-1} concentration. With the exception of BSA, showing no effect, HOBT and BT at 50 µg L^{-1}, shown eutrophic effects, and other compounds resulted in a clearer toxic effect.

4. Discussion

As stated recently, toxicological data for microalgae are scanty when compared with other taxa [56]. Most of the information on the toxicity of several pollutants in the aquatic environments, the compounds under study included, comes from tests carried out on crustaceans and fish, although algae can be more sensitive than animals to aquatic toxicity [57–60]. Another problem is the prevalence in lab repositories of freshwater species with high tolerance to the pollution that have been frequently used as a model in ecotoxicological studies [56]. These species can be unsuitable surrogates for estuarine or marine microalgae [61].

A way to directly compare our data with those of the literature is not easy to find, since these three algae have never employed together, just two on three sporadically. Moreover, we focused our work on the long-term effects. Previous studies included different species of microalgae [19,34], or sometimes *Raphidocelis subcapitata* [62–64]. Very often, one of the tests in the panel employed to evaluate the biotoxicity is the Microtox® one [62,65,66] based on *Vibrio fisheri*, but it is an acute test (15 min), for which results are very far from our chronic toxicity data.

Nevertheless, we tested a wide range of concentrations of the investigated pollutants similarly to the most part of previous studies and, consistently with them, the first conclu-

sion was that all the compounds had some effects at the higher concentrations, but at the levels detected in freshwaters, they did not pose a risk for aquatic ecosystems.

The tested algae should be applied, for example, to reveal the high concentrations found in wastewaters from sewage treatment plants. Sensitivity for specific families of pollutants was not observed, and each species showed different behaviors with respect to the three groups of compounds. It must be underlined that the differences in the response of microorganisms to the same or to similar pollutants may depend on various factors, ranging from lipid peroxidation to metabolic energy deficiency, decreased photosynthetic energy, or endocrine disrupting effects, and are therefore species-specific and related to the ecology and physiology of the organism [67]. Except for benzenesulphonamides, the freshwater alga, in particular, and the diatomea seemed to be the most sensitive to these compounds, not only concerning the degree of growth inhibition produced by the high concentration, but mainly with respect to the response to the lowest ones. In this family of pollutants, benzothiazoles and benzotriazoles showed the most marked effect on our algal species. According to our expectations, the freshwater *Raphidocelis subcapitata* was more sensitive to benzo-fused nitrogen heterocyclic pollutants than the marine ones, which are well adapted to fast changes in the chemical balance of their habitat.

On the contrary, an unexpected result was the absence of acute adverse effects on the *Vibrio fisheri* light emission, at least at the 50 and 100 μg L^{-1} concentrations. A detrimental effect was observed only at the chronic toxicity intervals (24 and 48 h), and was not produced by all compounds. In fact, two compounds showed at that time a stimulation of light emission, and this phenomenon is quite frequent when the tested compounds do not produce an acute toxicity effect toward the *V. fisheri*. Over time, the light induced degradation or the bacterial metabolism transform the organic pollutants into carbon sources for bacteria. This could be the effect produced by all compounds but BSA at the 0.1 μg L^{-1} concentration, or, in this case, it possibly involves the hormesis effect [68]. The *V. fisheri* test is considered highly sensitive, but, in this case, the response of acute toxicity tests was surprising: there was no toxicity, and no detected risks. Actually, benzotriazoles at the concentrations present in some WWTP effluents were estimated to pose a risk to *V. fisheri* [69], but it was not so evident by using solutions of the pure compounds. This result is a more evident example of how different the effects of any chemical on organisms belonging to the various, and even the same, trophic levels can be.

On the other hand, we estimate that it is important to underline that the most significant results from the compounds under study were obtained from all organisms after chronic exposure intervals, confirming the rationale of our experimental design and the need for long-term assays in evaluating the environmental impact. The selection of the most suitable organism and bioassay(s) design must be accurate and, in any case, based on multiple tests.

The results of this investigation should be helpful in determining the suitability of the various available organisms as environmental biomarkers of these and similar chemicals. Based on these preliminary data, we can state that the three microalgae species are unsuitable for rapidly evaluating the effect of the pure benzo-fused nitrogen heterocyclic compounds at the low contamination levels of surface and drinking waters.

However, the continuous and large use of these compounds can lead to their accumulation in particular compartments, such as sludge and sediments, and their hydrophilicity helps in the transfer to the water. Our plan is to investigate the possible onset of physiological and/or somatic effects of these benzo-fused nitrogen heterocycles in algae grown for various cycles in media containing the low concentration detected in freshwater or coastal environments. These effects, when permanent, would be employed as witnesses of long-term pollution. Moreover, the same experiments must be repeated at similar concentrations in real samples, to exactly evaluate the influence of the co-pollutant traces.

References

1. Hidalgo-Serrano, M.; Borrull, F.; Marcé, R.M.; Pocurull, E. Presence of benzotriazoles, benzothiazoles, and benzensulfonamides in surface water samples by liquid chromatography coupled to high-resolution mass spectrometry. *Sep. Sci. Plus* **2019**, *2*, 72–80. [CrossRef]
2. Richardson, S.D.; Kimura, S.Y. Water analysis: Emerging contaminants and current issues. *Anal. Chem.* **2016**, *88*, 546–582. [CrossRef]
3. Bruzzoniti, M.C.; Sarzanini, C.; Rivoira, L.; Tumiatti, V.; Maina, R. Simultaneous determination of five common additives in insulating mineral oils by high-performance liquid chromatography with ultraviolet and coulometric detection. *J. Sep. Sci.* **2016**, *39*, 2955–2962. [CrossRef]
4. Janna, H.; Scrimshaw, M.D.; Williams, R.J.; Churchley, J.; Sumpter, J.P. From dishwasher to tap? Xenobiotic substances benzotriazole and tolyltriazole in the environment. *Environ. Sci. Technol.* **2011**, *45*, 3858–3864. [CrossRef]
5. Careghini, A.; Mastorgio, A.F.; Saponaro, S.; Sezenna, E. Bisphenol A, nonylphenols, benzophenones, and benzotriazoles in soils, groundwater, surface water, sediments, and food: A review. *Environ. Sci. Pollut. Res. Int.* **2015**, *62*, 5711–5741. [CrossRef]
6. Liao, C.; Kim, U.-J.; Kannan, K. A review of environmental occurrence, fate, exposure, and toxicity of benzothiazoles. *Environ. Sci. Technol.* **2018**, *52*, 5007–5026. [CrossRef] [PubMed]
7. Herrero, P.; Borrull, F.; Pocurrull, E.; Marcé, R.M. An overview of analytical methods and occurrence of benzotriazoles, benzothiazoles and benzosulfonamides in the environment. *Trends Anal. Chem.* **2014**, *62*, 46–55. [CrossRef]
8. Richter, D.; Massmann, G.; Taute, T.; Duennbier, U. Investigation of the fate of sulfonamides downgradient of a decommissioned sewage farm near Berlin, Germany. *J. Contam. Hydrol.* **2009**, *106*, 183–194. [CrossRef] [PubMed]
9. Asheim, J.; Vike-Jonas, K.; Gonzalez, S.V.; Lierhagen, S.; Venkatraman, V.; Veivåg, I.L.S.; Snilsberg, B.; Flaten, T.P.; Asimakopoulos, A.G. Benzotriazoles, benzothiazoles and trace elements in an urban road setting in Trondheim, Norway: Re-visiting the chemical markers of traffic pollution. *Sci. Total Environ.* **2019**, *649*, 703–711. [CrossRef] [PubMed]
10. Ferrario, J.B.; DeLeon, I.R.; Tracy, R.E. Evidence for toxic anthropogenic chemicals in human thrombogenic coronary plaques. *Arch. Environ. Contam. Toxicol.* **1985**, *14*, 529–534. [CrossRef]
11. Asimakopoulos, A.G.; Wang, L.; Thomaidis, N.S.; Kannan, K. Benzotriazoles and benzothiazoles in human urine from several countries: A perspective on occurrence, biotransformation, and human exposure. *Environ. Int.* **2013**, *59*, 274–281. [CrossRef]
12. Wang, L.; Asimakopoulos, A.G.; Kannan, K. Accumulation of 19 environmental phenolic and xenobiotic heterocyclic aromatic compounds in human adipose tissue. *Environ. Int.* **2015**, *78*, 45–50. [CrossRef]
13. Li, X.; Wang, L.; Asimakopoulos, A.G.; Sun, H.; Zhao, Z.; Zhang, Z.; Zhang, L.; Wang, Q. Benzotriazoles and benzothiazoles in paired maternal urine and amniotic fluid samples from Tianjin, China. *Chemosphere* **2018**, *199*, 524–530. [CrossRef]
14. Sorahan, T. Cancer risks in chemical production workers exposed to 2-mercaptobenzothiazole. *Occup. Environ. Med.* **2009**, *66*, 269–273. [CrossRef]
15. Wang, X.; Suskind, R.R. Comparative studies of the sensitization potential of morpholine, 2-mercaptobenzothiazole and 2 of their derivatives in guinea pigs. *Contact Dermat.* **1988**, *19*, 11–15. [CrossRef] [PubMed]
16. U.S. Consumer Product Safety Commission (CPSC). Sensory and Pulmonary Irritation Studies of Carpet System Materials and Their Constituent Chemicals. 2006. Available online: http://www.cpsc.gov/LIBRARY/FOIA/FOIA98/os/3519926D.pdf (accessed on 7 July 2021).
17. Wan, Y.; Xue, J.; Kannan, K. Benzothiazoles in indoor air from Albany, New York, USA, and its implications for inhalation exposure. *J. Hazard. Mater.* **2016**, *311*, 37–42. [CrossRef] [PubMed]
18. Wang, L.; Asimakopoulos, A.G.; Moon, H.B.; Nakata, H.; Kannan, K. Benzotriazole, benzothiazole, and benzophenone compounds in indoor dust from the United States and East Asian countries. *Environ. Sci. Technol.* **2013**, *47*, 4752–4759. [CrossRef] [PubMed]
19. Seeland, A.; Oetken, M.; Kiss, A.; Fries, E.; Oehlmann, J. Acute and chronic toxicity of benzotriazoles to aquatic organisms. *Environ. Sci. Pollut. Res.* **2012**, *19*, 1781–1790. [CrossRef]
20. Durjava, M.K.; Kolar, B.; Arnus, L.; Papa, E.; Kovarich, S.; Sahlin, U.; Peijnenburg, W. Experimental assessment of the environmental fate and effects of triazoles and benzotriazole. *Altern. Lab. Anim.* **2013**, *41*, 65–75. [CrossRef]

21. Han, X.; Xie, Z.Y.; Tian, Y.H.; Yan, W.; Miao, L.; Zhang, L.L.; Zhu, X.W.; Xu, W. Spatial and seasonal variations of organic corrosion inhibitors in the Pearl River, South China: Contributions of sewage discharge and urban rainfall runoff. *Environ. Pollut.* **2020**, *262*, 114321. [CrossRef] [PubMed]

22. Matamoros, V.; Jover, E.; Bayona, J.M. Occurrence and fate of benzothiazoles and benzotriazoles in constructed wetlands. *Water Sci. Technol.* **2010**, *61*, 191–198. [CrossRef]

23. Wang, L.; Zhang, J.; Sun, H.; Zhou, Q. Widespread occurrence of benzotriazoles and benzothiazoles in tap water: Influencing factors and contribution to human exposure. *Environ. Sci. Technol.* **2016**, *50*, 2709–2717. [CrossRef]

24. Zhao, X.; Zhang, Z.F.; Xu, L.; Liu, L.Y.; Song, W.W.; Zhu, F.J.; Li, Y.F.; Ma, W.L. Occurrence and fate of benzotriazoles UV filters in a typical residential wastewater treatment plant in Harbin, China. *Environ. Pollut* **2017**, *227*, 215–222. [CrossRef]

25. Felis, E.; Sochacki, A.; Magiera, S. Degradation of benzotriazole and benzothiazole in treatment wetlands and by artificial sunlight. *Water Res.* **2016**, *104*, 441–448. [CrossRef]

26. Karthikraj, R.; Kannan, K. Mass loading and removal of benzotriazoles, benzothiazoles, benzophenones, and bisphenols in Indian sewage treatment plants. *Chemosphere* **2017**, *181*, 216–223. [CrossRef]

27. Gatidou, G.; Oursousdou, M.; Stefanatou, A.; Stasinakis, A.S. Removal mechanisms of benzotriazoles in duckweed Lemna minor wastewater treatment systems. *Sci. Total Environ.* **2017**, *596–597*, 12–17. [CrossRef]

28. Giraudo, M.; Douville, M.; Cottin, G.; Houde, M. Transcriptomics, cellular and life-history responses of *Daphnia magna* chronically exposed to benzotriazoles: Endocrine distrupting potential and molting effects. *PLoS ONE* **2017**, *12*, e0171763. [CrossRef]

29. Duan, Z.; Xing, Y.; Feng, Z.; Zhang, H.; Li, C.; Gong, Z.; Wang, L.; Sun, H. Hepatotoxicity of benzotriazole and its effect on the cadmium induced toxicity in zebrafish *Danio rerio*. *Environ. Pollut.* **2017**, *224*, 706–713. [CrossRef] [PubMed]

30. Meffe, R.; Kohfahl, C.; Hamann, E.; Greskowiak, J.; Massmann, G.; Dünnbier, U.; Pekdeger, A. Fate of para-toluenesulfonamide (*p*-TSA) in groundwater under anoxic conditions: Modelling results from a field site in Berlin (Germany). *Environ. Sci. Pollut. Res.* **2014**, *21*, 568–583. [CrossRef]

31. Zeng, F.; Sherry, J.P.; Bols, N.C. Evaluating the toxic potential of benzothiazoles with the rainbow trout cell lines, RTgill-W1 and RTL-W1. *Chemosphere* **2016**, *155*, 308–318. [CrossRef]

32. Molins-Delgado, D.; Díaz-Cruz, M.S.; Barceló, D. Removal of polar UV stabilizers in biological wastewater treatments and ecotoxicological implications. *Chemosphere* **2015**, *119S*, S51–S57. [CrossRef]

33. Yang, T.; Mai, J.; Wu, S.; Liu, C.; Tang, L.; Mo, Z.; Zhang, M.; Guo, L.; Liu, M.; Ma, J. UV/chlorine process for degradation of benzothiazole and benzotriazole in water: Efficiency, mechanism, and toxicity evaluation. *Sci. Total Environ.* **2021**, *760*, 144304. [CrossRef] [PubMed]

34. Gatidou, G.; Anastopoulou, P.; Aloupi, M.; Stasinakis, A.S. Growth inhibition and fate of benzotriazoles in *Chlorella sorokiniana* cultures. *Sci. Total Environ.* **2019**, *663*, 580–586. [CrossRef] [PubMed]

35. Loos, R.; Tavazzi, S.; Mariani, G.; Suurkuusk, G.; Paracchini, B.; Umlauf, G. Analysis of emerging organic contaminants in water, fish, and suspended particulate matter (SPM) in the Joint Danube Survey using solid-phase extraction followed by UHPLC-MS-MS and GC-MS analysis. *Sci. Total Environ.* **2017**, *607–608*, 1201–1212. [CrossRef]

36. Lu, Z.; De Silva, A.O.; Zhou, W.; Tetreault, G.R.; de Solla, S.R.; Fair, P.A.; Houde, M.; Bossart, G.; Derek, C.; Muir, G. Substituted diphenylamine antioxidants and benzotriazole UV stabilizers in blood plasma of fish, turtles, birds and dolphins from North America. *Sci. Total Environ.* **2019**, *647*, 182–190. [CrossRef]

37. OEDC. Advisory Document of the Working Group on Good Laboratory Practice on the Management, Characterisation and Use of Test Items. Environment Directorate Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology. 2018. Available online: https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2018)6&doclanguage=en (accessed on 7 July 2021).

38. Camuel, A.; Guieysse, B.; Alcántara, C.; Béchet, Q. Fast algal eco-toxicity assessment: Influence of light intensity and exposure time on *Chlorella vulgaris* inhibition by atrazine and DCMU. *Ecotoxicol. Environ. Saf.* **2017**, *140*, 141–147. [CrossRef]

39. Chhetri, R.K.; Baun, A.; Andersen, H.R. Algal toxicity of the alternative disinfectants performic acid (PFA), peracetic acid (PPA) chlorine dioxide (ClO_2) and their by-products hydrogen peroxide (H_2O_2) and chlorite (ClO_2^-). *Int. J. Hyg. Environ. Health* **2017**, *220*, 570–574. [CrossRef]

40. DeLorenzo, M.E.; Keller, J.M.; Arthur, C.D.; Finnegan, M.C.; Harper, H.E.; Winder, V.L.; Zdankiewicz, D.L. Toxicity of the antimicrobial compound triclosan and formation of the metabolite methyl-triclosan in estuarine systems. *Environ. Toxicol.* **2008**, *23*, 224–232. [CrossRef]

41. ISO 14442:2006. Water Quality—Guidelines for Algal Growth Inhibition Tests with Poorly Soluble Materials, Volatile Compounds, Metals, and Wastewater. Available online: https://www.iso.org/standard/34814.html (accessed on 7 July 2021).

42. OEDC. Freshwater alga and cyanobacteria, growth Inhibition test (n.201). Guidelines for Testing of Chemicals, Section 2; OECD: Paris, France. Available online: https://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-2-effects-on-biotic-systems_20745761 (accessed on 18 August 2021).

43. Borowitzka, M.A.; Siva, C.J. The taxonomy of the genus *Dunaliella* (Chlorophyta, Dunaliellales) with emphasis on the marine and halophilic species. *J. Appl. Phycol.* **2007**, *19*, 567–590. [CrossRef]

44. Manzo, S.; Miglietta, M.L.; Rametta, G.; Buono, S.; Di Francia, G. Toxic effects of ZnO nanoparticles towards marine algae *Dunaliella tertiolecta*. *Sci. Total Environ.* **2013**, *445–446*, 371–376. [CrossRef] [PubMed]

45. Leliaert, F.; Smith, D.R.; Moreau, H.; Herron, M.D.; Verbruggen, H.; Delwiche, F.; De Clerck, O. Phylogeny and molecular evolution of the green algae. *Crit. Rev. Plant. Sci.* **2012**, *31*, 1–46. [CrossRef]
46. Singh, J.; Saxena, R.C. An Introduction to microalgae: Diversity and significance. In *Handbook of Marine Microalgae*, 1st ed.; Kim, S.K., Ed.; Academic Press: New York, NY, USA, 2015; pp. 11–24. [CrossRef]
47. ISO 11348-3:2007. Water Quality—Determination of the Inhibitory Effect of Water Samples on the Light Emission of Vibrio Fischeri (Luminescent Bacteria Test). Available online: https://www.iso.org/standard/40518.html (accessed on 7 July 2021).
48. Johnson, B.T. Microtox® Acute Toxicity Test. In *Small-Scale Freshwater Toxicity Investigations*; Blaise, C., Férard, J.F., Eds.; Springer: Dordrecht, Germany, 2005. [CrossRef]
49. Algae Research and Supply. F/2 Medium. Available online: https://algaeresearchsupply.com/pages/f-2-media (accessed on 7 July 2021).
50. CCLA. Culture Collection of Autotrophic Organisms. The Jaworskis' Medium. Available online: https://ccala.butbn.cas.cz/en/jaworskis-medium (accessed on 7 July 2021).
51. International Standards Organization. ISO N. 8692/2004 amended by ISO 8692/2012. Water Quality—Freshwater Algal Growth Inhibition Test with Unicellular Green Algae. Available online: https://www.iso.org/standard/54150.html (accessed on 7 July 2021).
52. Finney, D.J. *Probit Analysis*, 3rd ed.; Cambridge University Press: New York, NY, USA, 1971.
53. Steel, R.G.; Torrie, J.H. *Principles and Procedures of Statistics*, 3rd ed.; Mc-Graw Hill: New York, NY, USA, 2000.
54. Riberio Rodrigues, L.H.; Arenzon, A.; Raya-Rodriguez, M.T.; Ferreira Fontoura, N. Algal density assessed by spectrophotometry: A calibration curve for the unicellular algae *Pseudokirchneriella subcapitata*. *J. Environ. Chem. Ecotoxicol.* **2011**, *3*, 225–228. [CrossRef]
55. Montesdeoca-Esponda, S.; Álvarez-Raya, C.; Torres-Padrón, M.E.; Sosa-Ferrera, Z.; Santana-Rodríguez, J.J. Monitoring and environmental risk assessment of benzotriazole UV stabilizers in the sewage and coastal environment of Gran Canaria (Canary Islands, Spain). *J. Environ. Manag.* **2019**, *233*, 567–575. [CrossRef]
56. Flood, S.; Burkholder, J.; Cope, G. Assessment of atrazine toxicity to the estuarine phytoplankter *Dunaliella tertiolecta* (Chlorophyta), under varying nutrient conditions. *Environ. Sci. Pollut. Res.* **2018**, *25*, 11409–11423. [CrossRef] [PubMed]
57. Lewis, M.A. Chronic Toxicities of Surfactants and Detergents Builders to algae—A Review and Risk Assessment. *Ecotoxicol. Environ. Saf.* **1990**, *20*, 123–140. [CrossRef]
58. Lewis, M.A. Use of Freshwater Plants for Phytotoxicity Testing—A Review. *Environ. Pollut.* **1995**, *87*, 319–336. [CrossRef]
59. Blanck, H.; Wangberg, S.A. Pattern of Cotolerance in Marine Periphyton Communities established under Arsenate Stress. *Aquatic Toxicol.* **1991**, *21*, 1–14. [CrossRef]
60. Walsh, G.E. Principles of Toxicity Testing with Marine Unicellular Algae. *Environ. Toxicol. Chem.* **1988**, *7*, 979–987. [CrossRef]
61. Graymore, M.; Stagnitti, F.; Allinson, G. Impacts of atrazine in aquatic ecosystems. *Environ. Int.* **2001**, *26*, 483–495. [CrossRef]
62. Corsi, S.R.; Geis, S.W.; Loyo-Rosales, J.E.; Rice, C.P. Aquatic toxicity of nine aircraft deicer and anti-icer formulations and relative toxicity of additive package ingredients alkylphenol ethoxylates and 4,5-methyl-1H-benzotriazoles. *Environ. Sci Technol.* **2006**, *40*, 7409–7415. [CrossRef]
63. Azizian, M.F.; Nelson, P.O.; Thayumanavan, P.; Williamson, K.J. Environmental impact of highway construction and repair materials on surface and ground waters. Case study: Crumb rubber asphalt concrete. *Waste Manag.* **2003**, *23*, 719–728. [CrossRef]
64. European Chemicals Agency (ECHA). N-cyclohexylbenzothiazol-2-sulphenamide, CAS No: 95-33-0, EINECS No.202-411-2. *Summary Risk Assessment Report, Final Report, May.* 2008. Available online: https://echa.europa.eu/documents/10162/48e99dc2-8d09-47e5-b167-61e8d2b13fd1 (accessed on 7 July 2021).
65. Reemtsma, T.; Fiehn, O.; Kalnowski, G.; Jekel, M. Microbial transformations and biological effects of fungicide-derived benzothiazoles determined in industrial wastewater. *Environ. Sci. Technol.* **1995**, *29*, 478–485. [CrossRef]
66. Pillard, D.A.; Cornell, J.S.; Dufresne, D.L.; Hernandez, M.T. Toxicity of benzotriazole and benzotriazole derivatives to three aquatic species. *Wat Res.* **2001**, *35*, 557–560. [CrossRef]
67. Pérez, P.; Fernández, E.; Beiras, R. Use of Fast Repetition Rate Fluorometry on Detection and Assessment of PAH Toxicity on Microalgae. *Water Air Soil Pollut.* **2010**, *209*, 345–356. [CrossRef]
68. Ray, S.D.; Farris, F.F.; Hartmann, A.C. Hormesis. In *Encyclopedia of Toxicology*, 3rd ed.; Wexler, P., Ed.; Elsevier: Amsterdam, The Netherlands, 2014; pp. 944–948. [CrossRef]
69. Molins-Delgado, D.; Távora, J.; Diaz-Cruz, M.S.; Barceló, D. UV filters and benzotriazoles in urban aquatic ecosystems: The footprint of daily use products. *Sci. Total Environ.* **2017**, *601–602*, 975–986. [CrossRef]