


## Article

# Are Toxic Substances Always Toxic? Case Studies of Different Organismal Responses Based on Brackish-Water Microphytobenthic Communities from the Baltic Sea

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**Abstract:** In this manuscript, we present the results of ecotoxicological tests conducted on the Baltic microphytobenthos community consisting largely of cosmopolitan species showing the responses at the community (taxonomic composition and structure), population (abundance), and cellular (chloroplast shape) levels. The tests were carried out for three chemicals with different modes of action on photosynthetic organisms, i.e., copper (II) chloride (at concentrations of  $2 \cdot 10^{-5} \text{ g} \cdot \text{dm}^{-3}$  and  $2 \cdot 10^{-3} \text{ g} \cdot \text{dm}^{-3}$ ), glyphosate in the form of Roundup® (concentrations ranging from  $4.2 \cdot 10^{-2}$  to  $8.5 \text{ g} \cdot \text{dm}^{-3}$ ), and the ionic liquid [BMIM]Cl (at concentrations of  $1.13 \cdot 10^{-3} \text{ g} \cdot \text{dm}^{-3}$  and  $1.75 \cdot 10^{-2} \text{ g} \cdot \text{dm}^{-3}$ ). The results of the study indicate that the responses of microphytobenthos at each level analysed are highly variable and difficult to predict a priori based on experiments performed on single strains. In addition, it was shown that microphytobenthic communities are relatively resistant to the substances tested, which is due to taxonomic richness and the resulting potential for substitution of sensitive taxa by more resistant taxa. The results obtained in the tests also indicate that the number of cells of individual taxa can remain at a similar level or increase over short periods of time despite the deformation of chloroplasts in a numerous group of cells.

**Keywords:** ecotoxicity; toxicity tests; microphytobenthic communities; diatoms; cyanobacteria; copper chloride; glyphosate; ionic liquid



**Citation:** Sylwestrzak, Z.; Zgrundo, A. Are Toxic Substances Always Toxic? Case Studies of Different Organismal Responses Based on Brackish-Water Microphytobenthic Communities from the Baltic Sea. *Appl. Sci.* **2023**, *13*, 12238. <https://doi.org/10.3390/app132212238>

Academic Editor: Leonel Pereira

Received: 22 September 2023

Revised: 3 November 2023

Accepted: 4 November 2023

Published: 11 November 2023



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## 1. Introduction

More than 300 million tons of industrial chemicals were used in the EU in 2018, of which more than two-thirds were classified by Eurostat as hazardous to health [1]. Many of these substances end up in surface waters and affect aquatic organisms through, for example, morphological changes, reduced reproductive capacity of aquatic mollusks, changes in animal behavior, or changes in the composition and structure of microphytobenthic communities [2]. Unfortunately, the effects of such changes are often difficult to predict.

Toxicological tests are currently carried out worldwide on a variety of organisms belonging to different levels of the food web, ranging from plants, herbivores, and carnivores to bacteria [3,4]. Photosynthetic organisms are particularly important for ecosystems due to the fact that they provide oxygen, participate in biogeochemical cycles, and provide food for other organisms. Hence, changes in plant organisms under the influence of various factors will not only limit their growth but may also directly affect the structure and functioning of the entire ecosystem [5]. Until now, ecotoxicological tests have mainly been conducted on monocultures of algae isolated from the environment. These are extremely valuable but provide information on the response of organisms only in terms of the fundamental niche, e.g., [6]. The use of whole communities in ecotoxicological testing is rare, despite the fact that they are easily obtained from the environment and do not require the labour-intensive and costly steps of isolating and maintaining individual strains in the laboratory [7–9]. Conducting toxicological tests that take into account

whole communities makes it possible to understand the response of organisms in the context of actually existing interpopulation interactions, e.g., competition (antagonistic interaction) and symbiosis (non-antagonistic interaction). However, whole-community tests are still rare due to various challenges, including the lack of a recommended consistent methodology, the acquisition of a sizeable data set for a high number of taxa representing a variety of taxonomic groups with different life strategies, and the associated problems in interpreting the results. The microphytobenthos, which is the main element studied in this work, is ecologically very important for aquatic ecosystems, including those of coastal zones, estuaries, or shallow seas, as it is an important primary producer associated with the bottom. Organisms forming microphytobenthic communities, due to their short life cycles, are considered good indicators of environmental change [10], and their analysis provides important information on the state of the marine environment [11]. It is worth emphasizing that the tested Baltic microphytobenthic community includes many cosmopolitan species, which makes it possible to assume that similar reactions to selected chemical substances will be observed in communities occurring in brackish waters in other regions of the world.

The study aimed to test the effects of three compounds from different chemical groups with different levels of toxicity on natural microphytobenthic communities. The first substance used in the tests was copper in the form of copper (II) chloride. European refined copper consumption reached around 3.78 million metric tonnes in 2021, but consumption in the region has been gradually declining over the past decade [12]. Copper (II) chloride is produced on an industrial scale by the chlorination of copper. The use of copper chloride is very wide.  $\text{CuCl}_2$  is used as a wood preservative, fungicide, insecticide, and herbicide. Due to its oxidising properties, it is used as a purifying agent, e.g., in water treatment, and as a deodorising agent to prevent odour in the petroleum industry. Copper (II) chloride is an important element in the production of vinyl chloride and dichloroethane and has applications in the synthesis of organic and inorganic compounds as a catalyst. It is also used in pyrotechnics as a blue-green colouring agent [13]. Copper is a compound of very high functional importance to aquatic plant microorganisms and is a substance with a relatively well-recognised effect and mechanism of action, e.g., [14–17]. Copper has an essential role in the metabolism of photosynthetic organisms and is a component of many proteins and enzymes involved in a number of metabolic pathways [18]. However, high concentrations and prolonged exposure to copper ions inhibits photosynthesis and disrupts physiological processes [19,20]. Copper compounds are used as algicides, as they are toxic in large quantities to algae [21]. Copper (II) chloride may be a genotoxic compound, as it causes chromosomal aberrations and mitotic cycle disruption in cells of *A. cepa* model organisms [22]). Copper chloride also negatively affects aquatic and soil organisms [23]. In particular, denitrifying bacteria have been found to be very sensitive to its presence. Copper (II) chloride at a concentration of  $0.95 \text{ mg} \cdot \text{dm}^{-3}$  causes 50% inhibition (IC<sub>50</sub>) of the metabolic activity of denitrifying microorganisms [24]. The US Environmental Protection Agency allows concentrations of no more than 1.3 ppm of aqueous copper ions in drinking water. Ingestion of copper ions in excessive quantities in humans can cause headache, diarrhoea, decreased blood pressure and fever, and, in extreme cases, haemolysis [25].

The second substance used in the tests was glyphosate in the form of Roundup®. Glyphosate in the form of Roundup is the most popular herbicide worldwide, with 825.8 million kilograms of glyphosate used worldwide in 2014 [26]. In Poland, 92 glyphosate-based herbicides are currently registered, with more still being registered. The amount of herbicides used worldwide is increasing despite the fact that the use of glyphosate has been shown not to lead to a positive effect on yields compared to alternative weed control methods such as mechanical and thermal weed control [27]. Glyphosate is the active substance used in non-selective herbicides. It is an extremely effective compound that has a broad spectrum of biological activity. The popularity of Roundup® herbicide has increased with the spread of genetically modified crops in recent years [28]. Therefore, it is very important to study the combined effect of the active substance glyphosate and the other components of the preparation, as this is the form to which the natural environment is

most often exposed. Once in the plant, glyphosate inhibits, for example, the production of the enzyme EPSP (5-enolpyruvate-shikimo-3-phosphate) synthetase, which is responsible for the formation by plants of aromatic amino acids that are important for their growth and are included in the composition of many plant pigments [29] or the activity of microsomal ATPases [28]. Glyphosate at concentrations above  $400 \mu\text{g}\cdot\text{dm}^{-3}$  is potentially toxic to some aquatic species, including amphibians and fish [30–32]. Glyphosate and its breakdown product, so-called AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), are observed in crops in non-target plant species even after the recommended withdrawal period. It is of concern that both glyphosate and AMPA have also been detected in crop plants and in the leaves of trees of native forests after glyphosate application to neighbouring crops [33]. The presence of glyphosate in marine ecosystems, as reported in some studies, e.g., [34], indicates its widespread use and high potential for spreading. However, glyphosate and AMPA pose a low risk to mammals, mainly due to low absorption through the skin and gastrointestinal tract [33,35]. Both substances are excreted in the urine and have a half-life of 3 to 15 h without any structural changes [36]. For these reasons, coupled with the acute toxicity data set, glyphosate and AMPA have been classified by the EPA in the category of least toxic substances (Category IV; virtually non-toxic and non-irritant) [33].

The third substance used was the ionic liquid 1-butyl-3-methylimidazolium chloride ([BMIM]Cl). Substances in this group are the new generation of polar organic solvents and catalysts referred to as ‘green chemistry’. Ionic liquids are ‘designer solvents’ in which the right choice of anions and cations allows the creation of chemical compounds with appropriate properties depending on the future application [37–39]. Due to this unique feature, it is difficult to determine the range of applications for ionic liquids worldwide. One very good example of the use of ionic liquids in cellulose processing is provided, for example, by [40]. However, after years of research, their ‘green’ nature has been questioned due to their negative environmental impact [41–43]. The properties of ionic liquids (e.g., good solubility, stability) result in the potential for these substances to accumulate in aquatic ecosystems, where they may enter, e.g., with wastewater [44,45]. Ionic liquids can pose a potential hazard by contaminating water and soil through accidental spills or ineffective wastewater treatment [46]. In studies conducted by different teams, imidazolium and pyridinium cations have been shown to adsorb on various types of soils and marine sediments, e.g., [47–50]. Furthermore, soil microorganisms also present in wastewater have been shown to have the capacity to degrade imidazole ionic liquids, e.g., [37,51]. However, as shown by some authors [52], the way ionic liquids are degraded in the environment is determined by numerous biotic and abiotic factors. Certain classes of imidazole cations of ionic liquids inhibit, in humans, the activity of acetylcholinesterase AchE, an enzyme that breaks down one of the primary neurotransmitters, acetylcholine, into choline and acetic acid residues. It is produced by the liver, among others, and is used in diagnostics as a marker of the liver’s efficiency in protein production [53]. In addition, 1-octyl-MIM bromide has been shown to cause liver damage in mice [54].

The research we conducted was aimed at updating the knowledge of the response of microphytobenthic communities to the presence in the environment of substances of anthropogenic origin with different toxic potential. The results of the study indicate that at the level of communities, populations, and cells, the responses of microphytobenthos are very diverse and difficult to predict a priori on the basis of experiments performed on single strains. An important aspect of the work is its versatility, because the taxa comprising the tested community are cosmopolitan (identified in various coastal waters in the world ocean). In addition, the laboratory tests performed made it possible to systematise knowledge of the methodological issues involved in this type of experimentation and indicate that the newly applied indicator based on the deformation state of chloroplasts does not correspond to the criteria of low labour and time consumption met by indicators commonly used in ecotoxicological tests.

## 2. Materials and Methods

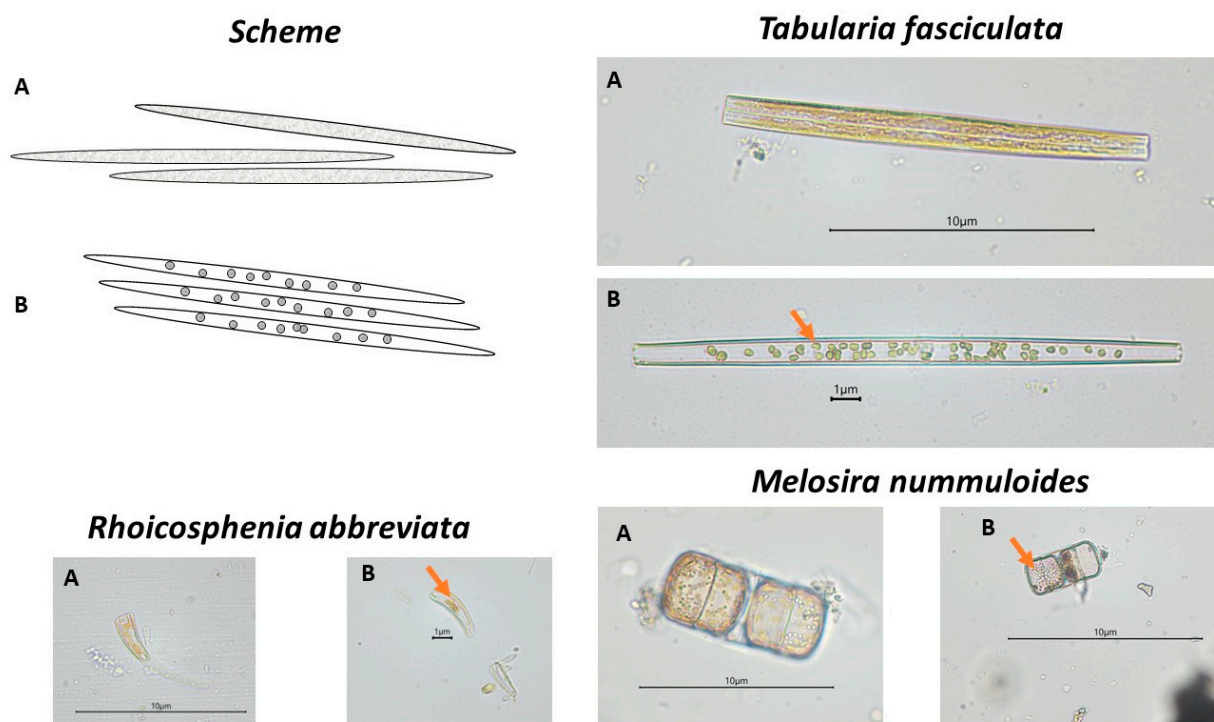
In the first phase of the work, a number of preliminary tests were carried out during which the most appropriate exposure time of the culture panels in the environment [55], the duration of the ecotoxicological test, and the culture medium [9] were selected and the analytical methods used to assess the changes occurring in the communities were optimised [56].

### 2.1. Field and Laboratory Works

The microphytobenthos used in the laboratory study was collected from slides exposed in the coastal zone of the southern Baltic Sea (Gulf of Gdańsk) at a station located at 54°26'49" N, 8°34'24" E for a period of 14 days in summer (August) 2015. The methodology of the field work is presented in detail in Sylwestrzak et al. [57]. After transporting the panels to the laboratory, the material was prepared for further testing. After sonification and nitrogen desaturation to remove animal organisms, microphytobenthos cells of 44,273 ( $\pm 2151$ ) in 1 mL were placed in 250 mL flasks in 100 mL of seawater collected in situ. The measured concentrations of biogenic compounds in seawater were: N-NH<sub>4</sub> 9.4 mg·m<sup>-3</sup>, N-NO<sub>3</sub> 102 mg·m<sup>-3</sup>, P-PO<sub>4</sub> 36 mg·m<sup>-3</sup>, Si-SiO<sub>4</sub> 600 mg·m<sup>-3</sup>. Based on preliminary tests, it was shown that such concentrations of biogenic salts do not limit the growth of microalgae [57]. Light conditions with a PAR irradiance of 60  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  and an L:D photoperiod of 16:8 were obtained using an artificial light source: Phillips halogen lamps (OSRAM L 36W/640-1).

In the tests, copper (II) chloride, glyphosate, and the ionic liquid BMIM[Cl] were used to compare effects of substances from different chemical groups with distinct mechanisms of action on living organisms and varying levels of toxicity on natural microphytobenthic communities. The concentration values of the test substances were selected on the basis of maximum acceptable concentrations of selected compounds in the environment introduced by legislation [58] and the literature [37,59] as well as on the basis of previous studies of our own [55]. The following concentrations were used in the ecotoxicological tests: for copper (II) chloride,  $2 \cdot 10^{-5} \text{ g} \cdot \text{dm}^{-3}$  and  $2 \cdot 10^{-3} \text{ g} \cdot \text{dm}^{-3}$ ; for glyphosate,  $4.2 \cdot 10^{-2} \text{ g} \cdot \text{dm}^{-3}$ ,  $8.5 \cdot 10^{-1} \text{ g} \cdot \text{dm}^{-3}$ , and  $8.5 \text{ g} \cdot \text{dm}^{-3}$ ; and for ionic liquid [BMIMCl],  $1.13 \cdot 10^{-3} \text{ g} \cdot \text{dm}^{-3}$  and  $1.75 \cdot 10^{-2} \text{ g} \cdot \text{dm}^{-3}$ . All variants of the experiment were carried out in 3 replicates. The values used were high but were intended to reflect a sudden one-off influx of high concentrations of test substances into the environment. Undertaking the study, it was assumed that the microphytobenthic communities extracted from the environment had not adapted to the elevated values of the compounds tested. For example, the content of copper ions in the area of their cultivation is at the level of  $1.3 \cdot 10^{-7}$ – $1.1 \cdot 10^{-6} \text{ g} \cdot \text{dm}^{-3}$  [60]. In turn, Skeff et al. [59] showed that in river mouths in the Baltic Sea, concentrations of glyphosate range from  $2.8 \cdot 10^{-8} \text{ g} \cdot \text{dm}^{-3}$  to  $1.68 \cdot 10^{-6} \text{ g} \cdot \text{dm}^{-3}$ , while APMA is detected in all of them. In studies conducted in rivers in Germany, 1310 different ionic liquid pollutants were identified, but only ca. 20 compounds were detected in concentrations of up to  $\mu\text{g} \cdot \text{dm}^{-3}$  [61].

Observations of changes in the composition and structure of microphytobenthic communities were carried out using the principles adopted in the OECD Guidelines for the Assessment of the Effects of Chemical Toxicity on Plant Microorganisms [62]. The microalgae were identified using various taxonomic keys and floras [63–70]. Qualitative and quantitative analysis of communities was conducted using a Nikon (Japan) 80i microscope equipped with a DS-U2 camera at  $\times 400$  magnification. In addition, changes in the shape of chloroplasts of microphytobenthic cells were analysed according to a previously developed research methodology [9,55]. The state of the chloroplasts, determined by a change in shape and structure, was introduced as an additional indicator to supplement information on the cellular condition of the organisms composing the communities. Examples in the form of diagrams and microscopic photographs of cells with normally formed chloroplasts and chloroplasts with abnormal shape are shown in Figure 1.



**Figure 1.** Diagrams and photographs of plant microorganisms with normally formed chloroplasts (A) and with abnormal chloroplasts (marked with an arrow) (B). *Tabularia fasciculata* (Bacillariophyceae) in control solution at the start of the experiment (A) and after 7 days of testing at a concentration of  $8.5 \text{ g}\cdot\text{dm}^{-3}$  glyphosate (B); *Rhoicosphenia abbreviata* (Bacillariophyceae) in control solution at the start of the experiment (A) and after 7 days at a concentration of  $1.75\cdot 10^{-2} \text{ g}\cdot\text{dm}^{-3}$  ionic liquid [BMIM]Cl (B); *Melosira nummuloides* (Coscinodiscophyceae) in control solution at the start of the experiment (A) and after 7 days at a concentration of  $2\cdot 10^{-3} \text{ g}\cdot\text{dm}^{-3}$   $\text{CuCl}_2$  (B).

## 2.2. Statistical Analysis

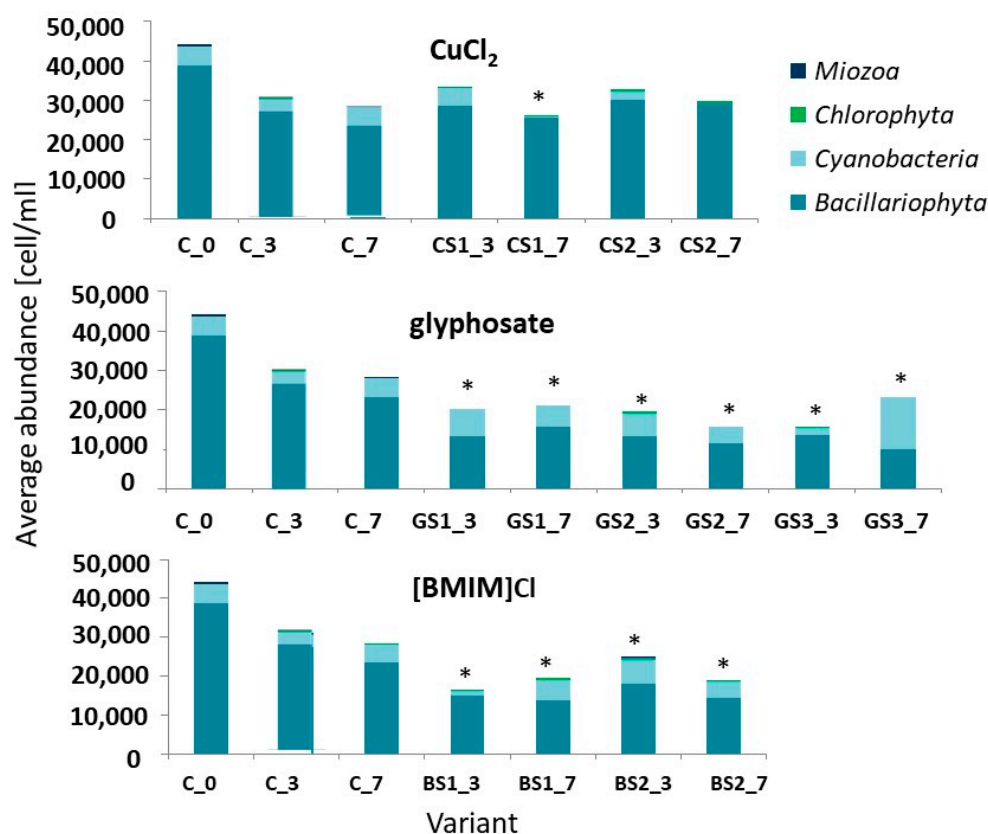
The data obtained were processed with MS Excel. Student's *t*-test was performed to compare the significance of differences in cell numbers between tested substances' concentrations and the control solution and to designate differences among successive test days with STATISTICA version 10 (StatSoft, Kraków, Poland).

## 3. Results

One of the main elements analysed in the experiments was the qualitative and quantitative composition of the microphytobenthic communities. A total of 58 microalgal taxa were identified during the study (Appendix A). In the initial community, 87% of the taxa were represented by diatoms, 11% by cyanobacteria, and green algae accounted for less than 1%. Single Haptophyta cells were also identified, but their maximum percentage contribution to the community was 0.3%; hence, they were not included in the graph.

### 3.1. Response at Community Level

In the case of copper (II) chloride in the solutions tested, the total abundance of communities remained at a similar level throughout the study period, irrespective of the variant tested (Figure 2). Only the community structure, i.e., the abundance of representatives of individual microalgal taxa, changed. Glyphosate in the form of the Roundup® preparation influenced the reduction in the community abundance and its remodelling. At the highest applied concentration of  $8.5 \text{ g}\cdot\text{dm}^{-3}$  of glyphosate, an increase in the proportion of blue-green algae to 51% of the total community was observed. In the case of communities treated with the ionic liquid [BMIM]Cl, most of the taxa composing the community under study responded with a reduction in cell abundance.



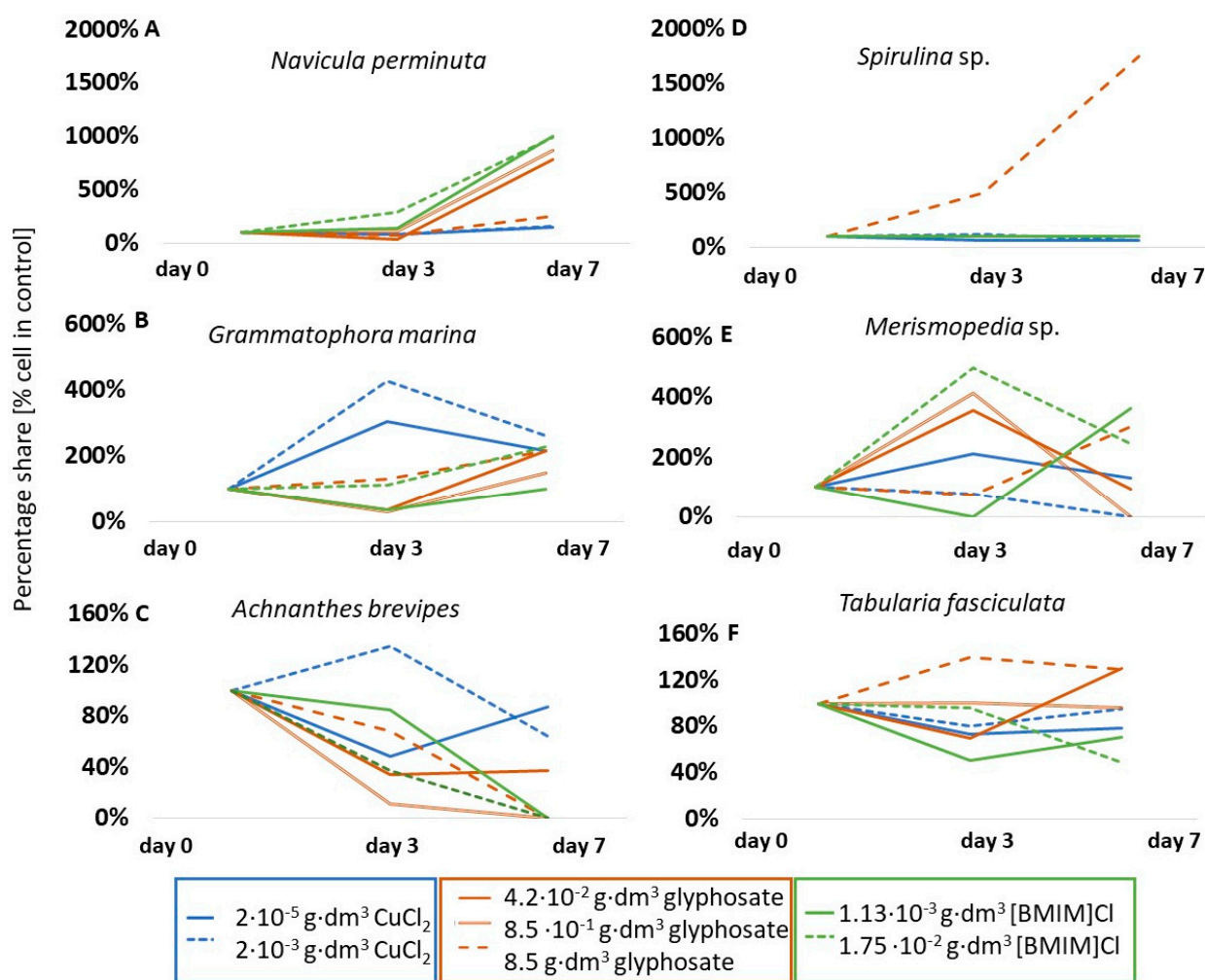
**Figure 2.** Average abundance of taxonomic groups in communities during tests. Designations used: the first part of the caption encodes the concentration, e.g., C—control solution,  $2 \cdot 10^{-3}$ —CuCl<sub>2</sub> concentration; the second part encodes the day of the test—e.g., \_3 is the third day, \_7 is the seventh day. Statistically significant differences were marked with \* symbol.

### 3.2. Response at Population Level

Among the dominant species present in the initial communities and present in all variants of the experiments, mainly diatoms were observed. The most abundant species were *Bacillaria paxillifera*, *Diatoma moniliformis*, *Diatoma vulgare*, *Navicula perminuta*, *Tabularia fasciculata* (Bacillariophyceae), and *Melosira nummuloides* (Coccosphyceae), among others. Among Cyanobacteriota (formerly Cyanobacteria), the most abundant taxa were *Spirulina sp.* and *Merismopedia sp.* Regardless of the degree of dominance in the initial community, individual taxa showed different types of response to the presence of the tested substance. Three types of response were identified: an increase in abundance, which was interpreted as growth stimulation; no change in abundance, interpreted as no response ('indifferent' taxa); and a decrease in abundance, interpreted as growth inhibition.

An example of a species that was stimulated by all the substances tested was *Navicula perminuta* (Figure 3A). This species showed an increase in cell number, by approximately 50% relative to the control, under the influence of copper (II) chloride, an eightfold increase in abundance in the presence of glyphosate, as well as a tenfold increase in abundance in the presence of ionic liquid [BMIM]Cl on the seventh day of testing. An unusually large, up to fourfold, increase in abundance under the influence of copper chloride was also observed in *Grammatophora marina* (Bacillariophyceae) (Figure 3B) on the third day, and, in the case of glyphosate and ionic liquid ( $1.75 \cdot 10^{-2} \text{ g} \cdot \text{dm}^{-3}$ ), a twofold increase on the seventh day of testing. Some taxa were stimulated by the presence of chemicals only during the initial phase of testing. For example, the number of *Achnanthes adnata* (formerly *Achnanthes brevipes*) (Bacillariophyceae) cells in the presence of copper (II) chloride was 135% of the abundance in the control sample on the third day, but only 64% on the seventh day (Figure 3C). Other taxa responded differently depending on the substance

and/or concentration. For example, glyphosate stimulated the cells of representatives of cyanobacteria to increase in abundance very intensively. Cell abundance of *Spirulina* sp. increased by 17.5 times that of the control on the last day (Figure 3D), and abundance of *Merismopedia* sp. increased by about four times for lower concentrations on the third day (Figure 3E). However, the other substances did not affect the change in abundance (neutral response for *Spirulina* sp. in [BMIM]Cl) or had an inhibitory effect (*Spirulina* sp. and *Merismopedia* sp. in CuCl<sub>2</sub>). Similarly different responses were observed for the diatom *T. fasciculata* (Figure 3F). In the case of glyphosate, a stimulation of up to 40% increase in cell numbers relative to the control solution was observed at the highest concentration, no response was observed at intermediate concentrations, and growth inhibition was observed at the lowest concentration. In contrast, copper chloride had no significant effect on changes in abundance for this taxon ( $p < 0.05$ ).



**Figure 3.** Percentage share of cells of selected microalgal taxa compared to control solutions. Note that the changes in scale in each graph are due to different degrees of change in population size.

The group of taxa that showed a lack of response to copper (II) chloride also included, for example, *Diatoma moniliformis* ( $p < 0.05$ ) (Figure 4A) and *Bacillaria paxillifera* ( $p < 0.05$ ) (Figure 5B). However, in the presence of glyphosate and ionic liquid, few taxa showed a lack of response, such as the aforementioned *Spirulina* sp. which, at  $0.85 \text{ g} \cdot \text{dm}^{-3}$  glyphosate and at both ionic liquid concentrations used, did not change its abundance over the course of the tests. A table showing the results of the significance tests for the species in question is included in Appendix B.

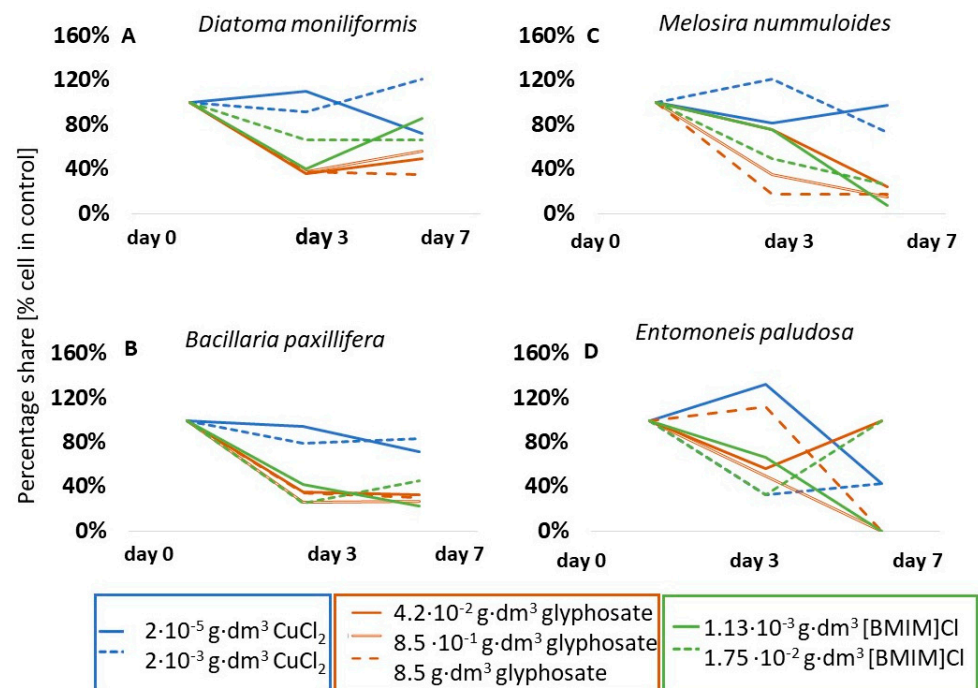


Figure 4. Percentage share of cells of selected microalgal taxa compared to control solutions.

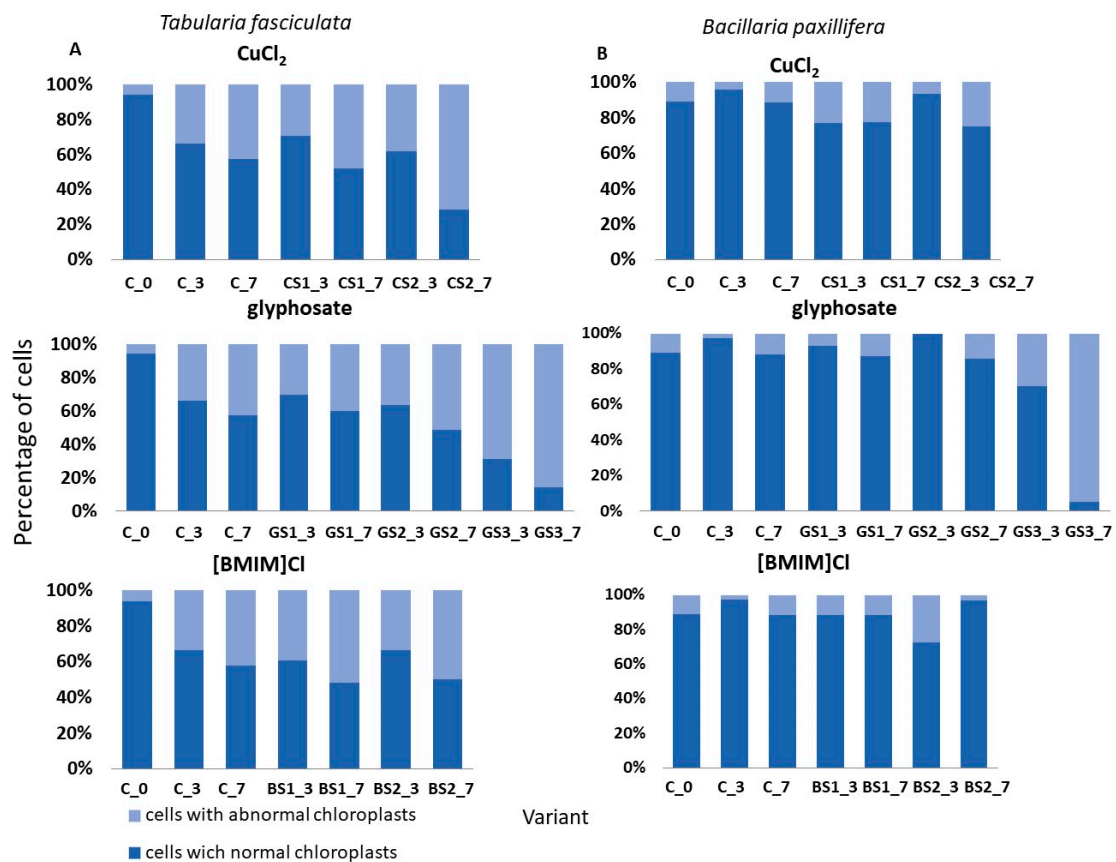


Figure 5. Changes in the condition of selected species expressed as the percentage share of cells with normal chloroplasts and abnormal chloroplasts in the control solution (C) and in the tested substances. Designations used: C—control solution,  $2 \cdot 10^{-3}$ — $\text{CuCl}_2$  concentration; the second part encodes the day of test—e.g.,  $_3$  is the third day of test,  $_7$  is the seventh day of test. (A) *Tabularia fasciculata*. (B) *Bacillaria paxillifera*.



A significant inhibition of growth was presented by one of the dominant diatom species, *B. paxillifera*, in both copper chloride and ionic liquid tests (Figure 4B). In contrast, growth inhibition of 15% in the presence of glyphosate and as much as 92% in the presence of ionic liquid was observed for the diatom *M. nummuloides* (Figure 4C). For *A. brevipes*, no cells of this taxon were observed in glyphosate and in ionic liquid on the seventh day of tests (Figure 3C). The species *Entomoneis paludosa* (Bacillariophyceae) also reacted negatively to the presence of ionic liquid and showed a 57% reduction in abundance on the seventh day of tests, while glyphosate induced a complete growth inhibition of this taxon (Figure 4D). It is noteworthy that, in the case of ionic liquid, more than 75% of the identified taxa were sensitive to its effects, as manifested by growth inhibition.

### 3.3. Response at the Cellular Level

The effects of the tested chemicals on changes in the shape of chloroplasts in cells of the selected two dominant diatom taxa are presented in Figure 5. Although the abundances of *T. fasciculata* and *B. paxillifera* were similar and constituted about 17% of the total community at the start of the tests, their responses at the cellular level differed depending on the substance used and between concentrations. Chloroplast deformations were observed in the presence of all tested substances in both species (Figure 5A). However, on the seventh day at a copper chloride concentration of  $2 \cdot 10^{-3} \text{ g} \cdot \text{dm}^{-3}$ , 71% of the cells had abnormally formed chloroplasts, while at the highest concentration of glyphosate, up to 86% of the cells had abnormally formed chloroplasts. In contrast, in *B. paxillifera*, only 6% to 24% of cells with abnormally shaped chloroplasts were observed at the copper chloride and ionic liquid concentrations tested, while glyphosate at  $8.5 \text{ g} \cdot \text{dm}^{-3}$  caused changes in chloroplasts in 95% of cells (Figure 5B).

## 4. Discussion

As shown in previous studies, the presence of substances of anthropogenic origin in river basins, estuaries, or coastal zones can alter community structure and primary microphytobenthic production, even if the pollution does not exceed acute toxicity levels [71]. To test the response of microphytobenthic communities developing in brackish waters of the Baltic Sea, compounds were selected whose observed concentrations are not currently very high, but whose popularity continues to grow. Previously, copper has been used in antifouling products used for ship maintenance, which have been a source of copper compounds released into marine waters [72]. Despite its use being banned [73], the use of copper ions in many other industries means that the risk of introducing them in quantities exceeding the detoxification capacity of organisms into surface waters is increasing. For example, in recent years, there has been an increased interest in copper-based nanoparticles for applications in cosmetics (cream additives), industry (e.g., metal coatings, inks, and food packaging plastics), and medicine (e.g., disinfection and antimicrobial coatings) [74–76]. Coastal ecosystems and estuaries are also increasingly exposed to herbicide contamination [71]. A multiyear study of pesticide content in surface waters has shown that the most common pesticides are bentazone and glyphosate [77]. Glyphosate as a pesticide is used on an increasing scale due to the massive expansion of agricultural production, the high yield of herbicide formulations with this substance, their low cost of production, and the still liberal laws in many countries with highly developed agricultural economies [78]. In the meantime, ionic liquids are gaining popularity due to their reputation as ‘green chemistry’ and are finding use in a variety of chemical processes, where they represent a new alternative to traditional organic solvents [79]. However, inadequate treatment of wastewater or accidental spillage of ionic liquids can cause negative effects on the ecosystem [43].

### 4.1. Response at Community Level

Microphytobenthic communities are specific microecosystems with a complex taxonomic structure. Each taxon has a unique tolerance to a specific environmental stressor, so it can be expected that the response of communities will vary according to the stressor and its

intensity. Tests showed that copper (II) chloride mainly altered the taxonomic composition of the community, with no significant change in total cell abundance. Similar observations on compositional and structural changes in freshwater microalgal communities treated with copper ions were carried out by Sabater et al. [80]. In our tests, we have shown that in the presence of Roundup® the proportion of cyanobacteria increased significantly. On the one hand, glyphosate may have eliminated diatoms from the community as cyanobacteria took advantage of the niches that formed; on the other hand, glyphosate decomposes relatively quickly, and cyanobacteria show a competitive advantage to the introduction of an additional carbon source, which is in line with the results of studies conducted on the toxicity of glyphosate on six species of Cyanobacteriota [81]. In our tests, we also showed that the ionic liquid [BMIM]Cl causes a reduction in the abundance of the entire community, which is dominated by diatoms. Latała et al. [82] also suggested that diatoms, in particular, are extremely sensitive to ionic liquids.

#### 4.2. Response at Population Level

Among the dominant species present in the initial community and present during the experiments, mainly diatoms and single cyanobacterial species were distinguished, including numerous cosmopolitan taxa, e.g., *B. paxillifera*, *N. perminuta*, *N. ramossissima*, *T. fasciculata*, *M. nummuloides*, and *S. subsalsa* [83]. Irrespective of the degree of dominance in the initial community, cell size, or taxonomic group, individual taxa showed a variety of responses to the presence of the chemicals tested. The rate of uptake of selected ions across cell membranes and the internal detoxification mechanism were likely to be factors that significantly influenced the toxicity of the tested substances [84]. We distinguished three main types of response to the presence of potentially toxic chemicals. Some organisms responded with an increase in cell numbers relative to control samples. This was found to be a response characteristic of resistant species in which the chemical stimulates growth. Some taxa remained indifferent to the presence of the toxicant and showed no significant change in abundance. The last group was made up of sensitive taxa, which reacted by decreasing cell numbers under the influence of the test substances. Unfortunately, in the case of our tests, we are unable to determine whether the selected substances stimulated growth or reduced competition, which ultimately manifested itself in an increase in population size.

An example of a particularly resistant species, which was stimulated by all the factors tested, was *N. perminuta*. Other resistant taxa that increased their abundance, contributing to the remodelling of the community structure were, e.g., *G. marina* (copper chloride II), *Cylindrotheca closterium*, *Spirulina* sp. (glyphosate), and *Merismopedia* sp. ([BMIM]Cl). Plants, including aquatic plants, have many defence mechanisms that enable them to survive under unfavourable conditions. As a result of oxidative stress caused by pollution, the activation of detoxification mechanisms and defensive antioxidant processes are observed, which enable microalgae cells to defend themselves, but these are taxon-specific responses [85]. Important regulatory factors in the adaptation strategies of algae to chemical stress are auxins and cytokinins, whose production rates vary between species [86]. Taxa identified as indifferent to the tested chemicals in which these mechanisms were likely to have acted included: *C. pediculus*, *M. nummuloides*, and *N. gregaria* in the case of copper (II) chloride. In contrast, glyphosate did not affect changes in abundance in, e.g., *Licmophora* sp. and *T. fasciculata*. In the case of ionic liquid, no species described as indifferent were observed.

A study by Yu et al. [87] showed that multispecies algal communities with interspecies interactions respond differently to stress caused, for example, by the presence of chemicals. The authors determined the sensitivity of algae to the presence of copper ions by measuring the inhibition of the production of certain cellular enzymes and showed, among other things, that the EC<sub>50</sub> for *Microcystis aeruginosa* (Cyanobacteriota) in the community was significantly higher compared to monocultures of this species and that cell metabolism is also affected by interspecies interactions. Also, our previous studies indicate that taxa in communities have a higher resistance to the stressors tested than individual microalgal strains [45]. Microphytobenthic communities usually consist of a large number of species,

even at early stages of succession [88]; hence, it is to be expected that the response of taxa to the presence of toxic substances in communities will be different than in monocultures and further enhanced or weakened by competitive relationships. In our tests, we showed that high concentrations of Roundup® contributed to complete community conversion. Cyanobacteriota were particularly resistant to this preparation. The presence of glyphosate probably resulted in enrichment of the culture medium with phosphorus biogenic compounds [89], leading to a dominance of the microphytobenthic community by them (e.g., *Spirulina* sp.). Similar results were obtained in community tests conducted in mesocosms, where a receding of diatom species in favour of cyanobacteria was observed [90], and in tests on pesticide toxicity in South American lakes [91]. It is probable that the receding of diatoms contributes to the release of ecological niches that cyanobacteria exploit, as in the case of our study; e.g., *Spirulina* sp. and *Mersimopedia* sp. showed an increase in abundance.

Copper at high doses reduces cell growth of individual taxa, as it inhibits photosynthesis [19]. In the experiment presented here, at concentrations of  $2 \cdot 10^{-5} \text{ g} \cdot \text{dm}^{-3}$  and  $2 \cdot 10^{-3} \text{ g} \cdot \text{dm}^{-3}$ , the presence of copper resulted in a 57% reduction in the abundance of *E. paludosa* compared to the control solution after seven days, while no representatives of *C. closterium* were observed. In tests conducted on microalgae isolated from the Cochin estuary in India, it was shown that the  $\text{EC}_{50}$  for the diatom *Nitzschia closterium* (synonym of *Cylindrotheca closterium*) was  $2.045 \cdot 10^{-6} \text{ g} \cdot \text{dm}^{-3}$  [92]. In tests on freshwater communities [80] at a concentration of  $1.5 \cdot 10^{-5} \text{ g} \cdot \text{dm}^{-3}$  copper ions, a significant change in community structure was observed with a marked decrease in the abundance of the diatoms *Synedra ulna* and *Achnanthes minutissima* after seven days of testing. In our tests on brackish-water microphytobenthic communities, the presence of copper ions at a concentration of  $2 \cdot 10^{-5} \text{ g} \cdot \text{dm}^{-3}$  induced a reduction in the abundance of diatom species, e.g., *B. paxillifera* and *T. fasciculata*, and at concentrations of  $2 \cdot 10^{-5} \text{ g} \cdot \text{dm}^{-3}$  and  $2 \cdot 10^{-3} \text{ g} \cdot \text{dm}^{-3}$  of the cyanobacterium *Spirulina* sp. In the course of the experiments described here, it was shown that many diatom species, including *A. brevipes*, *B. paxillifera*, *M. nummuloides*, and *Peridinium* sp., are sensitive to glyphosate in the formulation Roundup® at concentrations ranging from  $4.2 \cdot 10^{-2}$  to  $8.5 \text{ g} \cdot \text{dm}^{-3}$ . Glyphosate-based herbicides are chemical stressors for microalgae that can affect the structure of, among others, freshwater plankton [93]. Furthermore, a study by Fan et al. in [94] showed that the ionic liquids [C10IM]Cl and [C10MIM]Cl, at concentrations ranging from  $0.2 \cdot 10^{-4} \text{ g} \cdot \text{dm}^{-3}$  to  $0.1 \cdot 10^{-3} \text{ g} \cdot \text{dm}^{-3}$ , inhibited microalgal growth by 50%. In our tests, a reduction in abundance at concentrations of  $1.13 \cdot 10^{-3} \text{ g} \cdot \text{dm}^{-3}$  and  $1.75 \cdot 10^{-2} \text{ g} \cdot \text{dm}^{-3}$  [BMIM]Cl was observed on the third day in dominant taxa such as *B. paxillifera* and *T. fasciculata*, while some taxa, such as *A. brevipes* and *C. closterium*, were not observed at all on the seventh day of testing.

#### 4.3. Response at the Cellular Level

An innovative aspect of the study was the analysis of the state of chloroplasts, which provides insight into the response of the taxa comprising the communities to the applied chemical agents at the cellular level. Based on the tests carried out, it was observed that species, despite a similar response at the population level, i.e., similar changes in abundance, showed different proportions of cells with deformed chloroplasts compared to cells with normally formed chloroplasts. For example, in *T. fasciculata*, the proportion of cells with abnormally shaped chloroplasts ranged from 30% to 70% for copper (II) chloride, from 30% to 85% for glyphosate, and from 10% to about 50% for ionic liquid. In contrast, in *B. paxillifera*, the number of cells with abnormally shaped chloroplasts was 20% to 30% higher than in *T. fasciculata* at the same concentrations. Information on chloroplast structure damage induced by the presence of chemicals can be found in the literature, e.g., [55,95], but the value of such indicators seems small in relation to the effort and time required for the analysis.

## 5. Conclusions

Based on tests conducted on microphytobenthic communities with chemicals with documented toxic effects on living organisms, changes were observed both at the community level (taxonomic composition and structure) and at the cellular level (chloroplast shape). The relatively high resistance of microphytobenthic communities to the tested substances was due to the taxonomic richness of the studied formation. Sensitive taxa (e.g., *E. paludosa*, *M. nummuloides*) were replaced by species with higher resistance (e.g., *N. perminuta*, *Spirulina* sp.) so that the community abundance did not undergo significant changes. Changes in the taxonomic composition and community structure of Baltic microphytobenthos depended on the substance tested and its concentration. The results obtained in the tests indicate that the number of cells of individual taxa can remain at a similar level or increase over short periods of time despite significant deformation of chloroplasts. The novel method of analysing the degree of chloroplast deformation makes it possible to observe early responses of cells to a stress factor before the population size noticeably changes, but the analysis of chloroplast status is difficult and time-consuming; hence, its potential is limited.

**Author Contributions:** Conceptualization, Z.S. and A.Z.; methodology, Z.S. and A.Z.; software, Z.S. and A.Z.; validation, Z.S. and A.Z.; investigation, Z.S.; resources, Z.S. and A.Z.; data curation, Z.S.; writing—original draft preparation, Z.S. and A.Z.; writing—review and editing, Z.S. and A.Z.; visualization, Z.S.; supervision, A.Z. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by a Research Projects for Young Scientists from the Faculty of Oceanography and Geography, University of Gdańsk (No. 538-G245-B888-15, 538-G245-B209-16, 538-G245-B567-17).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** All authors have read and agreed to the published version of the manuscript.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.

**Acknowledgments:** We would like to express our gratitude to Filip Pniewski for his valuable suggestions during the development of this research work.

**Conflicts of Interest:** The authors declare no conflict of interest.

## Appendix A

**Table A1.** List of taxa identified during experiments with various variants of experiments (+ sign refers to presence).

Taxa	Author	CuCl <sub>2</sub>	Roundup®	[BMIM]Cl
<i>Bacillariophyta</i>				
<i>Achnanthes brevipes</i>	Bory	+	+	+
<i>Achnanthes lemmermannii</i>	Hustedt	+	+	+
<i>Amphora ovalis</i>	(Kützing) Kützing		+	+
<i>Amphora pediculus</i>	(Kützing) Grunow	+	+	+
<i>Amphora</i> sp.	Kützing	+	+	
<i>Bacillaria paxillifera</i>	(O.F.Müller) T.Marsson	+	+	+

Table A1. Cont.

Taxa	Author	CuCl <sub>2</sub>	Roundup®	[BMIM]Cl
<i>Berkeleya rutilans</i>	(Trentepohl ex Roth) Grunow		+	+
<i>Brebissonia lanceolata</i>	(C.Agardh) R.K.Mahoney & Reimer	+	+	+
<i>Chaetoceros wighamii</i>	Brightwell		+	+
<i>Cocconeis pediculus</i>	Ehrenberg	+	+	
<i>Cocconeis placentula</i>	Ehrenberg		+	
<i>Cocconeis</i> sp.	Ehrenberg		+	+
<i>Cyclotella</i> sp.	(Kützing) Brébisson	+	+	
<i>Cylindrotheca closterium</i>	(Ehrenberg) Reimann & J.C.Lewin	+	+	+
<i>Diatoma moniliformis</i>	Kützing	+	+	
<i>Diatoma tenuis</i>	C.Agardh		+	+
<i>Diatoma vulgare</i>	Bory		+	+
<i>Diploneis didyma</i>	(Ehrenberg) Ehrenberg	+	+	
<i>Diploneis interrupta</i>	(Kützing) Cleve	+	+	
<i>Encyonema prostratum</i>	(Berkeley) Kützing		+	+
<i>Entomoneis paludosa</i>	(W.Smith) Reimer	+	+	+
<i>Epithemia gibba</i>	(Ehrenberg) Kützing		+	
<i>Epithemia</i> sp.	Kützing	+	+	
<i>Fallacia</i> sp.	Kütz	+	+	+
<i>Gomphonella olivacea</i>	(Hornemann) Rabenhorst	+	+	+
<i>Grammatophora marina</i>	(Lyngbye) Kützing	+	+	+
<i>Gyrosigma</i> sp.	Kützing		+	+
<i>Halamphora coffeiformis</i>	(C.Agardh) Mereschkowsky	+	+	+
<i>Licmophora gracilis</i>	(Ehrenberg) Grunow	+		+
<i>Licmophora</i> sp.	C.Agardh		+	
<i>Melosira moniliformis</i>	C.Agardh	+	+	+
<i>Melosira nummuloides</i>	C.Agardh	+	+	+
<i>Navicula gregaria</i>	Donkin	+	+	+
<i>Navicula meniscus</i>	Schumann	+		
<i>Navicula palpebralis</i>	Brébisson ex W.Smith	+	+	+
<i>Navicula perminuta</i>	Grunow	+	+	+
<i>Navicula ramosissima</i>	(C.Agardh) Cleve	+	+	+
<i>Navicula</i> sp.	Bory de Saint-Vincent		+	+
<i>Nitzschia dissipata</i>	(Kützing) Rabenhorst	+		
<i>Nitzschia sigma</i>	(Kützing) W.Smith		+	+
<i>Opephora</i> sp.	Petit		+	
<i>Planothidium delicatulum</i>	(Kützing) Round & Bukhtiyarova		+	+
<i>Pleurosigma aestuarii</i>	(Brébisson ex Kützing) W.Smith		+	
<i>Pleurosigma</i> sp.	W. Smith	+	+	+

Table A1. Cont.

Taxa	Author	CuCl <sub>2</sub>	Roundup®	[BMIM]Cl
<i>Proschkinia poretzkajae</i>	(Koretkevich) D.G.Mann	+	+	+
<i>Rhoicosphenia abbreviata</i>	(C.Agardh) Lange-Bertalot	+	+	+
<i>Rhopalodia gibba</i>	(Ehrenberg) Otto Müller	+		+
<i>Surirella brebissonii</i>	Krammer & Lange-Bertalot	+		
<i>Tabularia fasciculata</i>	(C.Agardh) D.M.Williams & Round	+	+	+
<i>Tryblionella</i> sp.	(Grunow)		+	+
<b>Cyanobacteria</b>				
<i>Dolichospermum flos-aquae</i>	(Bornet & Flahault) P.Wacklin	+	+	+
<i>Cyanobacteria</i> sp.				+
<i>Merismopedia</i> sp.	Meyen	+	+	+
<i>Microcystis</i> sp.	Lemmermann		+	+
<i>Nodularia</i> sp.	Mertens ex Bornet & Flahault	+	+	
<i>Oscillatoria</i> sp.	Vaucher ex Gomont		+	
<i>Spirulina major</i>	Kützing ex Gomont		+	+
<i>Spirulina subsalsa</i>	Oersted ex Gomont	+	+	+
<i>Woronichinia</i> sp.	A.A.Elenkin		+	+
<b>Dinophyceae</b>				
<i>Peridinium</i> sp.	Ehrenberg		+	+
<b>Chlorophyta</b>				
<i>Pseudopediastrum boryanum</i>	(Turpin) E.Hegewald			+
<i>Scenedesmus</i> sp.	Meyen	+	+	
<b>Haptophyta</b>				
<i>Prymnesium</i> sp.	N.Carter		+	+

## Appendix B

Table A2. Tables of the statistical analysis results for chosen taxa.

Taxon	Day of Experiment	CuCl <sub>0.0002</sub> g/dm <sup>3</sup>	CuCl <sub>0.002</sub> g/dm <sup>3</sup>	Glyphosate <sub>0.042</sub> g/dm <sup>3</sup>	Glyphosate <sub>0.85</sub> g/dm <sup>3</sup>	Glyphosate <sub>8.5</sub> g/dm <sup>3</sup>	[BMIM]Cl <sub>0.00113</sub> g/dm <sup>3</sup>	[BMIM]Cl <sub>0.0175</sub> g/dm <sup>3</sup>
<i>Achnanthes brevipes</i>	3	0	0	0	0	0	0	0
	7	0.033	0	0	0	0	0	0
<i>Bacillaria paxillifera</i>	3	0.011	0	0	0	0	0	0
	7	0	0	0	0	0	0	0
<i>Diatoma moniliformis</i>	3	0.0001	0.012	0	0	0	0	0
	7	0	0	0	0	0	0	0
<i>Entomoneis paludosa</i>	3	0	0	0	0	0	0	0
	7	0	0	1	0	0	0	1
<i>Grammatophora marina</i>	3	0	0	0	0	0	0	0
	7	0	0	0	0	0	1	0
<i>Melosira nummuloides</i>	3	0	0	0	0	0	0	0
	7	0.322	0	0	0	0	0	0
<i>Merismpoedia</i> sp.	3	0	0	0	0	0	0	0
	7	0	0	0.0589	0	0	0	0
<i>Navicula perminuta</i>	3	0	0	0	0.0002	0	0	0
	7	0	0	0	0	0	0	0
<i>Spirulina</i> sp.	3	0	0	1	1	0	1	1
	7	0	0	1	0	0	1	1
<i>Tabularia fasciculata</i>	3	0	0	0	0.682	0	0	0.093
	7	0	0	0	0.238	0	0	0

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