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Effects of selected abiotic factors on Baltic microphytobenthic communities.

Oddziaływanie wybranych czynników abiotycznych na bałtyckie zbiorowiska mikrofitobentosu

The doctoral dissertation

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Summary

The rapid development of the economy, the intensive exploitation of environmental resources and the increasing anthropopressure associated with these phenomena force us to think about future generations. Direct threatening effects of human activity on the marine environment include pollution by undesirable chemicals, increased water temperature associated with climate change, eutrophication, invasions by alien species or overfishing due to overexploitation of populations relative to their self-reproducing capacity (HELCOM 2018; Jakubowska et al. 2002). Close observation of the surrounding environment as well as careful analysis of the impact of anthropogenic factors are important elements in protecting the world around us. The impact of human activity on the environment can be monitored using international guidelines, such as those contained in the Water Framework Directive (2000/60/EC), through the analysis of biological, hydrological or physico-chemical elements. However, for this purpose, it is the best to use indicators based on whole-community studies, as only a sufficiently rich grouping of species with diverse ecological requirements can provide complete information on the effects of factors related to anthropopressure (Pennesi and Danovaro 2017). Hence, the present study undertook to investigate and describe changes in microphytobenthic communities caused by selected factors associated with human activities.

Microphytobenthos in this study is broadly defined as a formation that includes plant microorganisms associated with the seabed or various types of substrate found in the water (Cahoon 2019). It is part of a microecosystem in which, as in any ecosystem, there are interactions between organisms and their environment (Bosserman 1983). It is functionally particularly important, especially for ecosystems of coastal zones, estuaries or shallow seas, as it contains organisms that are important producers and an important part of the trophic chain. Organisms forming microphytobenthic communities are considered reliable indicators of environmental change due to their rapid response to both abiotic and biotic factors (Potapova and Charles 2007). Their use in both monitoring and ecotoxicological studies has a rich and long history dating back to the 19th century (e.g., Dickman 1969; Blanck 1985; Schmitt-Jansen and Altenburger 2005; Schmitt-Jansen and Altenburger 2005; Roubeix et al. 2011; Zhu et al. 2021). Factors of natural or artificial origin not only limit the growth and development of plant microorganisms, but also form the structure and functioning of entire communities. A major advantage of studies using microphytobenthic communities is the simplicity of obtaining data from the environment (Dahl and Blanck 1996; Blanck et al. 2009;

Sylwestrzak et al. 2014b, **PAPERs 1, 2**, **3**). In addition, the literature on research conducted on microphytobenthos constitutes a valuable source of information for practical applications of this formation (e.g., Dahl and Blanck 1996; Underwood et al. 1999, Cohn and McGuire 2000; Underwood et al. 2004; De La Iglesia et al. 2013; Vannoni et al. 2022). In recent years, it has been a significant increase in interest on the use of microphytobenthic communities in ecotoxicological studies testing, among other things, of potentially toxic substances of anthropogenic origin (e.g., Blanck et al. 2009; Araújo et al. 2010; Åsa Arrhenius et al. 2014; Bácsi et al. 2016; Pennesi and Danovaro 2017; Du et al. 2021; Vannoni et al. 2022). For example, experiments have been conducted on marine microphytobenthic communities using antifouling substances and paints (Blanck et al. 2009; Arrhenius et al. 2014), medicines (Pérez et al. 2009), cosmetics (Mason et al. 1996), or herbicide products (Downing et al. 2004). Among other things, these scientific studies contributed to the planning of the dissertation research, in which the bioindication potential of entire communities was used. A completely novelty was the use of communities obtained directly from the environment, interesting results concerning the response of the communities to substances of anthropogenic origin.

The research underlying this dissertation is based on the on an analysis of the impact of factors associated with two types of anthropogenic pressure - pollution caused by the introduction of chemical substances into the environment and temperature increases caused by climate change. To analyse the response of microphytobenthic communities to chemical pollution, three substances from various chemical groups with different modes of action and varying degrees of recognition were selected. One of the substances used was copper (II) chloride (**PAPER 1**). Copper is functionally important for aquatic plant microorganisms and its action mechanism is relatively well recognised (Stauber and Florence 1987; Manimaran et al. 2012; Serwatka et al. 2015; Li et al. 2021). It is a component of many proteins and enzymes involved in metabolic reactions, hence it plays an important role in the metabolism of photosynthetic organisms (Morelli and Scarano 2004). However, in excess, it can interfere with physiological processes. At high concentrations and prolonged exposure, copper ions slow down photosynthesis (Fernandes and Henriques 1991; Guasch et al. 2002) generating oxidative stress by inducing the production of reactive oxygen forms (Morelli and Scarano 2004) and affecting metabolic processes also related to growth (Maksymiec and Krupa 2006) and biochemical composition, including carotenoids, proteins, lipids and carbohydrates (Neethu et al. 2021). Copper ions are used as one of the ingredients in antifouling paints because they negatively affect the condition of microalgae (Traon et al. 2021).

Another substance used was glyphosate in the form of Roundup® (**PAPER 2**). Glyphosate is an organic compound from the phosphonate group, which is a broad-spectrum biological active substance used in many herbicides. Herbicides include many excipients in addition to glyphosate as the active substance. Roundup®, due to its complex composition and rapid degradation rate, can be a source of carbon and nitrogen, and its low concentrations can stimulate microalgal cell growth (Malik et al. 1989; Wong 2000; Berman et al. 2020). A long-term study of pesticide content in surface waters has shown that the most common pesticides are bentazone and glyphosate (Stenström et al. 2021). Glyphosate as a pesticide is used on an increasing scale due to the massive development of agricultural production, the high yield of herbicide formulations containing this substance, their low production cost and the still liberal laws in many countries with highly developed agricultural economies (Brovini et al. 2021). Once in plant cells, this compound inhibits, for example, the production of the enzyme EPSP (5-enolpyruvylshikimate-3-phosphate) synthase, which slows down the organism's formation of aromatic amino acids important for growth and included in the composition of many plant pigments (Franz et al. 1997). Reduced or absent of photosynthetic pigments lead to damage to chloroplast structure and cell degradation (e.g., Sylwestrzak et al. 2015; Kim and Ponomarev 2021).

The last substance was 1-Butyl-3-methyl-imidazolium-chloride [BMIM]Cl, which chemically belongs to the group of ionic liquids (**PAPER 3**). Ionic liquids are substances that are gaining popularity due to their potentially desirable properties such as non-flammability, high thermal and electrochemical stability, low vapour pressure, good conductivity and excellent catalytic properties (Mai et al. 2014; Chen et al. 2020). The properties listed above correspond to the requirements for the products of so-called 'green chemistry'. However, after years of research, the 'green' nature of ionic liquids has been questioned, although the mechanism of the toxic effects of these compounds is still not well understood (Nikitenko et al. 2007; Freire et al. 2010; Kumar et al. 2011; de Jesus and Filho 2022; Maculewicz et al. 2022).

Among the factors related to climate change, the study investigated the impact of a short-term increase in water temperature on the organisms that form microphytobenthic communities (**PAPER 4**). As has been shown, it is not only the steadily increasing temperature that has a huge impact on the environment, but also the frequency and intensity of extreme climatic events such as heat waves (Vieira et al. 2013). Based on studies conducted during the last few years, it has been shown that in summer on the southern Baltic

Sea, average coastal water temperatures are around 19° C, but for short intervals these values rise above 23° C (Siegel et al. 2006; Bradtke et al. 2010; Rak and Wieczorek 2012; Stramska and Białogrodzka 2015). Results from earlier studies suggest that microphytobenthic communities can undergo major changes due to temperature increase. Results from work conducted on diatom-dominated microphytobenthic communities indicate that short-term increases in temperature stimulate photosynthesis, while long-term exposure to higher temperatures leads to a reduction in photosynthetic productivity and to changes in species composition as well as intense cyanobacterial growth (Hicks et al. 2011; Vieira et al. 2013; Cartaxana et al. 2015; Kazanjian et al. 2018).

All factors described above have a profound impact on the functioning of plant micro-organisms, hence their use in testing the response of microphytobenthic communities has adequate justification. It is worth noting that marine organisms associated with brackish environments have so far rarely been used in ecotoxicological tests. The vast majority of such tests are conducted on algal strains, e.g. tests recommended by the OECD (OECD 1984; OECD 2006) or the International Organization for Standardization (ISO 2012). Tests conducted according to standardised methods on selected monocultures are extremely valuable as they allow comparison of the degree of influence of different substances, but give information on the reaction of a few, selected organisms only. Furthermore, many of the species recommended for toxicological testing are maintained for a long time under artificial laboratory conditions to which they adapt, e.g.: *Chlorella vulgaris Beyerinck* (Beijerinck) is maintained in monoculture since 1889 (Beijerinck 1890), *Navicula pelliculosa* (Kützing) Hilse since 1955 (Lewin 1955), and *Selenastrum capricornutum* Printz since 1959 (Guiry 2013). Analysis of the impact of anthropogenic factors on natural microphytobenthic communities is a relatively new and innovative approach used in ecotoxicological testing. Although such studies have been conducted for more than 20 years and the literature is rich, a standard method for conducting these tests has still not been developed and the measurement techniques used during the studies vary (Cibic et al. 2008; Duong et al. 2008; Morin et al. 2017). Within the scope of the doctoral dissertation, a number of tools and methods for conducting ecotoxicological tests on microphytobenthic communities were checked, which undoubtedly represents an interesting contribution to the development of this field of science (Dahl and Blanck 1996; Perkins et al. 2001; Serôdio 2004; Araújo et al. 2010; Arrhenius et al. 2014).

The main objective of the dissertation was to characterise the response of the Baltic Sea microphytobenthic communities to factors described as of anthropogenic origin - substances from different chemical groups, i.e., copper (II) chloride, glyphosate (in the form of Roundup®) and the ionic liquid [BMIM]Cl, as well as for a short-term rapid increase in water temperature.

The study assumes the following research hypothesis:

The introduction of contaminants into the environment in the form of copper (II) chloride, glyphosate (as Roundup®), the ionic liquid [BMIM]Cl and a short-term rapid temperature increase affect the composition and structure of the microphytobenthic communities of the Gulf of Gdańsk, and these changes can be estimated by observing the cells of microorganisms included in microphytobenthos communities.

Conducting the experiments forming the basis of the doctoral dissertation required a number of preliminary studies that allowed the development of the most appropriate methodology for assessing changes in the microphytobenthic communities under the influence of selected factors. The identification of the research material, i.e. the composition and structure of the Baltic microphytobenthos, took place within the scope of Z. Sylwestrzak's master's thesis entitled 'Succession of microphytobenthic communities in the Gulf of Gdańsk (experimental studies)', as well as on the basis of extensive literature of the subject. The experiments conducted in the initial stages of the doctoral dissertation were aimed at testing, among other things:

- the optimal exposure time in the environment of culture slides from which organisms forming microphytobenthic communities were obtained (Sylwestrzak, Zgrundo and Pniewski 2014);
- the influence of the culture medium (seawater plus standard f/2 medium) on the results of ecotoxicological tests conducted on microphytobenthic communities (Sylwestrzak and Pniewski 2014; Sylwestrzak and Zgrundo 2014).

Due to the fact that commonly used methods for assessing the condition of microorganisms, e.g. photosynthetic pigment concentration or photosynthetic activity (PAM fluorescence) (Brotas et al. 2007; Morelle et al. 2018) often did not provide unambiguous results, other less common indicators were tested during the initial study, such as:

- survival rate of representatives of individual microphytobenthos taxa, determined as the ratio of live to dead cells (Sylwestrzak and Zgrundo 2014),
- cell condition of microphytobenthos representatives in relation to the state of chloroplasts. The condition was determined on the basis of changes in the shape and structure of chloroplasts according to previously developed research methodology (Sylwestrzak 2014; Sylwestrzak et al. 2015; Sylwestrzak and Pniewski 2014). Examples and microscopic photographs of cells with correctly formed chloroplasts and abnormally shaped chloroplasts are shown on Fig. 1.

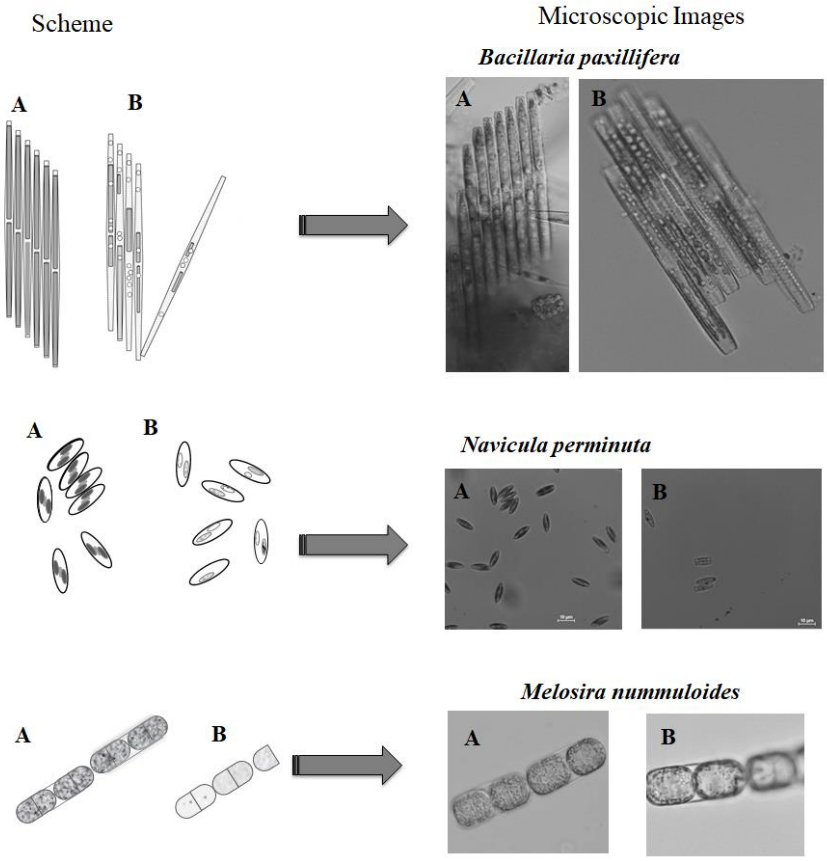

Fig. 1. Examples of cells with correctly formed chloroplasts (A) and abnormally shaped chloroplasts (B) for *Bacillaria paxillifera*, *Navicula perminuta*, *Melosira nummuloides* illustrated by schemes and photographs of the cells made using an optic microscope.

The material used in the targeted tests consisted of microphytobenthic communities obtained from culture slides exposed in the coastal zone of the Gulf of Gdańsk (southern Baltic Sea) for a period of 14 days in July and August 2015. During the exposure of the

culture slides, the water temperature oscillated between 17 and 19 °C and the salinity varied between 7.9 and 8.4 PSU. The methodology of the fieldwork is described in detail in the studies comprising this dissertation (**PAPERs 1, 2, 3 and 4**). After transporting the culture panels to the laboratory, the microphytobenthic communities were scraped from the culture slides using a scalpel, saturated with nitrogen gas to induce hypoxia and sonified. By using the above procedures, animal organisms were eliminated from the microbenthic solution and the test material was standardised (Rosenberg et al. 1991). Microphytobenthos were then placed in 250 ml flasks in 100 ml of filtered seawater collected *in situ*. Irrespective of the experiment, the average community abundance at the start of the tests was 41319 (± 1133), and 95% of the total community were diatoms. The initial concentrations of biogenic compounds in the seawater were: 9.4 $mg \cdot m^{-3}$ $N\text{-}NH_4$, 102 $mg \cdot m^{-3}$ $N\text{-}NO_3$, 36 $mg \cdot m^{-3}$ $P\text{-}PO_4$, 600 $mg \cdot m^{-3}$ $Si\text{-}SiO_4$. During the preliminary studies a biogenic nutrient analysis was carried out before and after the tests. Based on the data obtained, it was shown that during experiments conducted for 7 days, biogenic nutrients are not depleted and do not limit the growth of microalgae (Sylwestrzak 2016). To identify the response of the microphytobenthic communities to the selected chemicals (**PAPERs 1, 2, 3**) concentrations of compounds were used, established based on concentration values that are defined as threatening to the environment (based on standards, e.g. Dz.U.2011.257.1545 or the literature on the subject, i.e. Kulacki and Lamberti 2008; Liu et al. 2015; Skeff et al. 2015) and values whose real effects on plant microorganisms were observed during previous preliminary studies (Sylwestrzak 2012; Sylwestrzak et al. 2014; Sylwestrzak and Zgrundo 2014; Serwatka et al. 2015; Sylwestrzak et al. 2015). The following concentrations were used during the tests: for copper (II) chloride – $2 \cdot 10^{-5}$ $g \cdot dm^{-3}$ and $2 \cdot 10^{-3}$ $g \cdot dm^{-3}$, for glyphosate – 0.042 $g \cdot dm^{-3}$, 0.85 $g \cdot dm^{-3}$ and 8.5 $g \cdot dm^{-3}$, and for the ionic liquid [BMIM]Cl – $1.13 \cdot 10^{-3} g \cdot dm^{-3}$ and $1.75 \cdot 10^{-2}$ $g \cdot dm^{-3}$. In the study presented in **PAPER 4**, the range of temperatures used was selected following measurements carried out in the southern Baltic in recent years (http://www.satbaltyk.pl) and based on publications describing the phenomenon of short-term temperature increases, which can have a strong influence on community composition and structure (Siegel et al. 2006; Bradtke et al. 2010; Rak and Wieczorek 2012; Stramska and Białogrodzka 2015).

Each variant of the experiments described in **PAPERs 1, 2, 3 and 4** was performed three times, and methods of observing the response of microphytobenthic communities to a factor of anthropogenic origin used in **PAPERs 1, 2 and 3** were different from those used in **PAPER 4**. The material spent to analyse the effect of factors considered to be chemical

pollutants was microphytobenthic communities preserved with Lugol's fluid, and observations were made for samples collected after three and seven days (**PAPERs 1 and 2, 3**). Qualitative and quantitative community analysis and chloroplast condition analysis was performed in 50 fields of view in Utermöhl Sedimentation Chambers (2 ml) under a Nikon Eclipse TS100 Inverted Microscope at magnifications of x200, x400. The condition of the cells was determined based on the state of the chloroplasts, grouping them into two categories: cells with normal-shaped chloroplasts (i.e., matching the shapes presented in the publications as typical and normal for the taxa in question) and cells with abnormal-shaped chloroplasts (showing deviations from those presented in the literature). The results of the experiments were presented as the number of all organisms and representatives of each taxon in relation to the number observed in the control solution, according to the principles adopted in the OECD guidelines used to assess the toxicity effects of chemical compounds on plant microorganisms (OECD 2006).

An analysis of the effect of a short-term temperature increase (**PAPER 4**) was carried out on material collected as in **PAPERs 1, 2 and 3**, but using a special laboratory treatment to obtain pure diatom material, which involves the removal of the remaining organisms forming the microphytobenthos. An analysis of the effect of a short-term temperature increase (**PAPER 4**) was carried out on material collected as in **PAPERs 1, 2 and 3**, but using a special laboratory treatment to obtain pure diatom material, which involves the removal of the remaining organisms forming the microphytobenthos. The observation was performed on permanent slides made with Naphrax resin of refractive index ~1.7, using a Nikon 80i Fluorescence Microscope equipped with a DS-U2 camera at x1000 magnification. In this case, up to 300 diatom clades were identified and counted. Other parameters such as photosynthetic activity (determined by changes in chlorophyll fluorescence measured using a Fluorescence Monitoring System (FMS1; Hansatech, Norfolk, UK)), and photosynthetic pigment concentration (qualitative and quantitative analysis of pigments was measured using high-performance liquid chromatography (HPLC) using the Waters system equipped with two Waters 515 pumps and the Waters 2998 Photodiode Array Detector). were also analysed during the test connected with short-term temperature increase. The detailed methodology of the analyses performed is presented in the publication. For the necessary thematic consistency of this dissertation, the results related to the response of diatoms to temperature increase are included hereafter only in relation to community composition and structure.

One of the main elements analysed during the experiments was the total abundance of microphytobenthic communities. In the case of copper (II) chloride (**PAPER 1**) and glyphosate in the form of the Roundup® preparation (**PAPER 2**), the total size of communities in the tested solutions remained at a similar level throughout the study period. Only the community structure, i.e., the number of representatives of individual microalgal taxa, changed. It was observed that taxa sensitive to the test substance were replaced by tolerant or resistant taxa, leading to a change in community structure. A different response was observed for communities treated with [BMIM]Cl ionic liquid, where most of the taxa composing the community under study responded with a reduction in cell number (**PAPER 3**). In the case of a short-term temperature increase, the total community size was practically constant, with only the percentages of individual taxa changing (**PAPER 4**).

Among the dominant species, present in the initial communities and also present in all experiments, mainly diatoms were distinguished, such as: *Bacillaria paxillifera (*O.F.Müller) T.Marsson, *Diatoma moniliformis (*Kützing) D.M.Williams, *Diatoma vulgaris* Bory, *Melosira nummuloides* C.Agardh, *Navicula perminuta* Grunow, *Tabularia fasciculata* (C.Agardh) D.M.Williams and Round. It was observed that the most numerous cyanobacterium was the taxon *Merismopedia* sp. Meyen. Irrespective of the degree of dominance in the initial community, individual taxa showed a different type of response to the presence of an anthropogenic factor. Thus, among other things, a group of organisms was distinguished that were stimulated to grow in the presence of the tested factors. An example of a species that was stimulated by all the factors tested was *N. perminuta*. Indeed, when exposed to ionic liquid, it showed a double increase in cell number under both copper chloride and elevated temperature (**PAPERs 1 and 4**) and eight times increase in number in the presence of glyphosate (**PAPER 2**) as well as a ten times increase in in the presence of [BMIM]Cl ionic liquid number compared to the control solution (**PAPER 3**). Some taxa were stimulated by the presence of chemicals only during the initial phase of testing. For example, the cell number of *Achnanthes brevipes* C.Agardh under a concentration of copper (II) chloride of $2 \cdot 10^{-5}$ g·dm^{-3} increased by 35% on the third day, and on the seventh day already 40% less cells were observed than in the control solution. An unusually large, up to four times, increase in number under copper (II) chloride was observed in *Grammatophora marina* (Lyngbye) Kützing at a concentration of $2 \cdot 10^{-3}$ g·dm^{3} $CuCl_2$ on the third day (**PAPER 1**). Some taxa reacted to the presence of chemicals in the last phase of the tests. For example, in *Navicula ramosissima* (C.Agardh) Cleve, seven times more cells were observed on the seventh day of

testing than in the control solution (**PAPER 3**). Glyphosate at a concentration of 8.5 $g \cdot dm^3$ increased the cell number of *T. fasciculata* by 40% compared to the control solution, on the seventh day. The number of cyanobacteria representatives, i.e., *Merismopedia* sp. and *Spirulina* sp. Turpin ex Gomont, increased several times during the tests (416% and 1750%, respectively). Due to the presence of glyphosate, it is likely that the culture medium was enriched in phosphorus nutrients (Delpy et al. 2022), leading to a complete remodelling and domination of the microphytobenthic community by cyanobacteria (**PAPER 2**).

Among the taxa identified as neutral to agents of anthropogenic origin, in case of copper (II) chloride, were, for example: *Brebissonia lanceolata* (C.Agardh) R.K.Mahoney and Reimer, *Cocconeis pediculus* Ehrenberg, *Fallacia* sp. Stickle and D.G.Mann and *Rhoicosphenia abbreviata* (C.Agardh) Lange-Bertalot (**PAPER 1**). In contrast, glyphosate at the concentrations tested did not affect changes in number in, among others: *Halamphora coffeaeformis* (C.Agardh) Levkov, *T. fasciculata* (**PAPER 2**). Species described as neutral to the applied factors almost weren't observed, in the case of the ionic liquid and short-term temperature increase (**PAPER 3 and 4**).

Several anthropogenically sensitive taxa were identified in the communities tested. Organisms that responded with a reduction in number to the presence of both copper (II) chloride and ionic liquid included *B. paxillifera* and *T. fasciculata* (**PAPERs 1 and 3**). The diatom *M. nummuloides* was a species showing particular sensitivity to the chemicals used, e.g., its count under glyphosate was reduced to only 15% of that in the control sample (**PAPER 2**). Similar reactions were observed for *A. brevipes* and *Cylindrotheca closterium* (Ehrenberg) Reimann, whose cells were not observed in glyphosate and in ionic liquid, on the seventh day of testing. Negative effects of the presence of copper (II) chloride in the culture medium were also observed for representatives of cyanobacteria, e.g., in *Spirulina* sp. a reduction in counts by approximately 40% compared to the control solution was observed (**PAPER 1**). Among the species sensitive to the short-term temperature increase was *G. marina*, whose counts decreased by 43% on the last day of testing compared to the control sample (**PAPER 4**).

The state of chloroplasts, as determined by changes in shape and structure, is an additional indicator that complements information about the cellular condition of the organisms forming the communities used in the experiments. The results obtained indicate that the cell number of individual taxa can remain at a similar level or increase over short

periods of time despite significant impairment of chloroplast function caused by the action of, for example, copper (II) chloride. Changes in chloroplast shape and structure in the presence of copper (II) chloride and ionic liquid were observed in the dominant diatom species *T. fasciculata* (**PAPERs 1 and 3**), while under the influence of glyphosate, changes in chloroplast shape and structure were observed in *B. paxillifera* (**PAPER 2**). By observing chloroplasts, it is possible to gain a better understanding of the response of the taxa forming the communities to the chemical agents used, but it cannot be applied as a stand-alone indicator of community changes.

The research carried out as part of the doctoral dissertation allowed to observe changes in the composition and structure of marine microphytobenthic communities occurring under the influence of factors related to human activity, such as the introduction of chemicals into the environment or short-term increases in temperature, and thus the research hypothesis was confirmed. Microphytobenthos communities are more resistant than single strains of microalgae, a decrease in the number of anthropogenic factors used in single strains was observed at the early stages of research (Sylwestrzak et al. 2015). The decline of sensitive taxa and their replacement by resistant ones indicates the fact that communities as a functional whole are highly resistant to disturbances of anthropogenic origin. Studies based on a variety of microphytobenthos-forming organisms allow us to learn about the richness of responses both at the cellular level (e.g. by assessing the condition of chloroplasts) and at the population level (analysis of community composition and structure). The diatom *N. perminuta* has shown, on the one hand, a particular resistance to a relatively broad spectrum of chemical pollutants and, on the other hand, has flexibly adapted to short-term temperature increases. However, the vast majority of organisms forming the microphytobenthic communities were sensitive to the tested substances of anthropogenic origin. It should also be noted that increased concentrations of the chemicals tested, or increased temperatures favoured the mass growth of only individual taxa (e.g., *G. marina*, *N. perminuta*) or groups of taxa (e.g. cyanobacteria in the case of glyphosate).

The photosynthetic organisms that make up the microphytobenthos are extremely important elements of aquatic ecosystems due, among other things, to their role as primary producers. Understanding and quantifying the response of entire communities is extremely valuable, as it allows us to reliably estimate the changes that may occur under the influence of factors of anthropogenic origin, for each taxon included in their composition. A valuable element of the doctoral dissertation was the testing of different chemical agents based on a

single methodology, which enabled different types of responses to be clearly traced in relation to microorganisms' communities and populations. The Baltic microphytobenthos includes many cosmopolitan taxa and species, which makes it possible to assume that similar responses to the tested agents, presented in the papers forming the basis of the dissertation, will be shown by communities existing in brackish waters of other regions of the world. The use of microphytobenthic communities provides more reliable information on the likely changes that will occur in the environment as a result of human activities than tests conducted on single strains of microalgae. The results obtained in the course of the doctoral dissertation provide new insights into the functioning of microphytobenthic communities under conditions of severe stress caused by factors of anthropogenic origin, as well as enriching knowledge on the possibility of using microorganism communities in ecotoxicology.

Streszczenie

Szybki rozwój gospodarki i intensywna eksploatacja zasobów środowiska oraz związana z tymi zjawiskami nasilająca się antropopresja, zmusza nas do myślenia o przyszłych pokoleniach. Bezpośrednimi groźnymi skutkami wpływu działalności człowieka na środowisko morskie są m.in.: zanieczyszczenie niepożądanymi substancjami chemicznymi, wzrost temperatury wód związany ze zmianami klimatu, eutrofizacja, inwazje gatunków obcych czy przełowienie spowodowane nadmierną eksploatacją populacji w stosunku do zdolności ich samoodtwarzania (HELCOM 2018; Jakubowska i in. 2002). Uważna obserwacja otaczającego nas środowiska i wnikliwa analiza wpływu czynników pochodzenia antropogenicznego są ważnymi elementami ochrony otaczającego nas świata. Wpływ działalności ludzkiej na środowisko można monitorować stosując międzynarodowe wytyczne, jak np. zalecenia zawarte w Ramowej Dyrektywie Wodnej (2000/60/WE), poprzez analizę elementów biologicznych, hydrologicznych czy fizyko-chemicznych. Jednak do tego celu najlepiej zastosować wskaźniki oparte na badaniach całych zbiorowisk, gdyż tylko odpowiednio bogate zgrupowanie gatunków o różnorodnych wymaganiach ekologicznych jest w stanie dostarczyć pełnej informacji o skutkach czynników związanych z antropopresją (Pennesi i Danovaro 2017). Stąd w niniejszej pracy podjęto się zbadania i opisania zmian zachodzących w zbiorowiskach mikrofitobentosu wywołanych wybranymi czynnikami towarzyszącymi działalności człowieka.

Mikrofitobentos w pracy zdefiniowano w szeroki sposób jako formację, w której skład wchodzą mikroorganizmy roślinne związane z dnem lub różnego rodzaju podłożem znajdującym się w wodzie (Cahoon 2019). Jest on częścią mikroekosystemu, w którym zachodzą interakcje pomiędzy organizmami, a ich środowiskiem jak w każdym ekosystemie (Bosserman 1983). Ma on ogromne znaczenie pod względem funkcjonalnym, zwłaszcza dla ekosystemów stref przybrzeżnych, estuariów czy płytkich mórz, ponieważ w jego skład wchodzą organizmy, które są istotnymi producentami i ważnym elementem łańcucha troficznego. Organizmy tworzące zbiorowiska mikrofitobentosu uważa się za wiarygodne wskaźniki zmian środowiskowych ze względu na ich szybką reakcję na czynniki zarówno abiotyczne jak i biotyczne (Potapova i Charles 2007). Ich wykorzystanie zarówno w badaniach monitoringowych, jak i ekotoksykologicznych, ma bogatą i długą historię sięgającą XIX wieku (np. Dickman 1969; Blanck 1985; Schmitt-Jansen i Altenburger 2005; Roubeix i in. 2011; Zhu i in. 2021). Czynniki pochodzenia naturalnego czy sztucznego nie tylko ograniczają wzrost i rozwój mikroorganizmów roślinnych, ale również kształtują strukturę i

funkcjonowanie całych zbiorowisk. Dużą zaletą badań z wykorzystaniem zbiorowisk mikrofitobentosu jest łatwość ich pozyskiwania ze środowiska (Dahl i Blanck 1996; Blanck i in. 2009; Sylwestrzak i in. 2014a, **ARTYKUŁ 1, 2**, **3**). Ponadto literatura przedmiotu dotycząca badań prowadzonych na mikrofitobentosie to cenne źródło informacji w praktycznych zastosowaniach tej formacji (np. Dahl i Blanck 1996; Underwood i in. 1999, Cohn i McGuire 2000; Underwood i in. 2004; De La Iglesia i in. 2013; Vannoni i in. 2022). W ostatnich latach znacząco wzrosło zainteresowanie zastosowaniem zbiorowisk mikrofitobentosu w badaniach ekotoksykologicznych testujących między innymi potencjalnie toksyczne substancje pochodzenia antropogenicznego (np. Blanck i in. 2009; Araújo i in. 2010; Åsa Arrhenius i in. 2014; Bácsi i in. 2016; Pennesi i Danovaro 2017; Du i in. 2021; Vannoni i in. 2022). Na przykład, na zbiorowiskach morskiego mikrofitobentosu przeprowadzano eksperymenty z wykorzystaniem substancji i farb antyporostowych (Blanck i in. 2009; Arrhenius i in. 2014), leków (Pérez i in. 2009), kosmetyków (Mason i in. 1996), czy preparatów chwastobójczych (Downing i in. 2004). Między innymi te prace naukowe stanowiły przyczynek do zaplanowania badań w ramach dysertacji, w których wykorzystano potencjał bioindykacyjny całych zbiorowisk. Dużą nowością było zastosowanie zbiorowisk pozyskanych bezpośrednio ze środowiska, co pozwoliło uzyskać nowe, interesujące wyniki dotyczące reakcji zbiorowisk na substancje pochodzenia antropogenicznego.

Badania stanowiące podstawę pracy doktorskiej zdecydowano oprzeć się o analizę wypływu czynników związanych z dwoma typami presji antropogenicznej – zanieczyszczeniem wywołanym wprowadzaniem substancji chemicznych do środowiska i wzrostami temperatury wywołanymi zmianami klimatycznymi. Do analizy reakcji zbiorowisk mikrofitobentosu na zanieczyszczenia chemiczne wybrano trzy substancje z różnych grup chemicznych o odmiennym sposobie oddziaływania i zróżnicowanym stopniu rozpoznania. Jedną z zastosowanych substancji był chlorek miedzi (II) (**ARTYKUŁ 1**). Miedź ma istotne znaczenie funkcjonalne dla wodnych mikroorganizmów roślinnych, a mechanizm jej działania jest stosunkowo dobrze rozpoznany (Stauber i Florence 1987; Manimaran i in. 2012; Serwatka i in. 2015; Li i in. 2021). Jest składnikiem wielu białek i enzymów, biorących udział w szlakach metabolicznych, stąd odgrywa on istotną rolę w metabolizmie organizmów fotosyntetyzujących (Morelli i Scarano 2004). Jednak w nadmiarze, może zaburzać procesy fizjologiczne. Przy wysokich stężeniach oraz długotrwałej ekspozycji jony miedzi hamują fotosyntezę (Fernandes i Henriques 1991; Guasch i in. 2002) generując stres oksydacyjny poprzez indukcję produkcji reaktywnych form tlenu (Morelli i Scarano 2004) i wpływają na

procesy metaboliczne związane również ze wzrostem (Maksymiec i Krupa 2006) oraz na skład biochemiczny, m.in. zawartość karotenoidów, białek, lipidów i węglowodanów (Neethu i in. 2021). Jony miedzi wykorzystuje się jako jeden z składników farb antyporostowych, ponieważ negatywnie oddziałują na kondycję mikroglonów (Traon i in. 2021).

Kolejną zastosowaną substancją był glifosat w postaci preparatu Roundup® (**ARTYKUŁ 2).** Glifosat to związek organiczny z grupy fosfonianów, który jest substancją o szerokim spektrum aktywności biologicznej stosowaną w wielu herbicydach. W skład środków chwastobójczych obok glifosatu, jako substancji aktywnej, wchodzi wiele substancji pomocniczych. Roundup® ze względu na swój złożony skład oraz szybkie tempo rozpadu może być źródłem węgla i azotu, a jego niskie stężenia mogą stymulować wzrost komórek mikroglonów (Malik i in. 1989; Wong 2000; Berman i in. 2020). W trakcie wieloletnich badań nad zawartością pestycydów w wodach powierzchniowych wykazano iż najczęściej występujące pestycydy to bentazon oraz glifosat (Stenström i in. 2021). Glifosat jako pestycyd jest stosowany na coraz większą skalę ze względu na masowy rozwój produkcji rolnej, wysoką wydajność preparatów chwastobójczych z tą substancją, ich niski koszt produkcji oraz wciąż liberalne prawo w wielu krajach z wysoko rozwiniętą gospodarką rolną (Brovini i in. 2021). Związek ten po przedostaniu się do komórek rośliny hamuje np. produkcję enzymu syntetazy EPSP (5-enolopirogroniano-szikimo-3-fosforanu), która spowalnia tworzenie przez organizm aminokwasów aromatycznych ważnych dla wzrostu oraz wchodzących w skład wielu barwników roślinnych (Franz i in. 1997). Zmniejszona ilość lub brak barwników fotosyntetycznych prowadzi do uszkodzenia struktury chloroplastów i degradacji komórek (np. Sylwestrzak i in. 2015b; Kim i Ponomarev 2021).

Ostatnią substancją był chlorek 1-butylo-3-metyloimidazoliowy [BMIM]Cl, który pod względem budowy chemicznej przynależy do grupy cieczy jonowych (**ARTYKUŁ 3)**. Ciecze jonowe to substancje, które zyskują coraz większą popularność ze względu na ich potencjalnie pożądane właściwości takie jak: niepalność, wysoka stabilność termiczna i elektrochemiczna, niska prężność par, dobre przewodnictwo i doskonałe właściwości katalityczne (Mai i in. 2014; Chen i in. 2020). Wymienione powyżej własności odpowiadają wymaganiom stawianym produktom tzw. zielonej chemii. Jednak po latach badań „zielony" charakter cieczy jonowych został zakwestionowany, chociaż mechanizm toksycznego oddziaływania tych związków wciąż nie jest dobrze poznany (Nikitenko i in., 2007; Freire i in., 2010; Kumar i in., 2011; de Jesus i Filho, 2022; Maculewicz i in., 2022).

Spośród czynników związanych ze zmianami klimatycznymi, w ramach pracy badano wpływ krótkookresowego nagłego wzrostu temperatury wody na organizmy tworzące zbiorowiska mikrofitobentosu (**ARTYKUŁ 4)**. Jak wykazano ogromne znaczenie dla środowiska ma nie tylko stale wzrastająca temperatura, ale także częstotliwość i intensywność ekstremalnych zjawisk klimatycznych takich jak fale upałów (Vieira i in. 2013). Na podstawie badań prowadzonych w ostatnich latach wykazano, że latem w południowym Bałtyku średnie temperatury wody przybrzeżnej wynoszą ok. 19° C, ale w krótkich przedziałach czasu wartości temperatury wzrastają powyżej 23° C (Siegel i in. 2006; Bradtke i in. 2010; Rak i Wieczorek 2012; Stramska i Białogrodzka 2015). Wyniki badań prowadzonych we wcześniejszych latach sugerują, iż zbiorowiska mikrofitobentosu mogą podlegać dużym zmianom spowodowanym wzrostem temperatury. Wyniki prac prowadzonych na zbiorowiskach mikrofitobentosu zdominowanych przez okrzemki wskazują, że krótkookresowe wzrosty temperatury stymulują fotosyntezę, natomiast długotrwała ekspozycja na wyższą temperaturę prowadzi do zmniejszenia wydajności fotosyntezy oraz do zmian w składzie gatunkowym i intensywnego rozwoju sinic (Hicks i in. 2011; Vieira i in. 2013; Cartaxana i in. 2015; Kazanjian i in. 2018).

Opisane powyżej czynniki mają ogromny wpływ na funkcjonowanie mikroorganizmów roślinnych, stąd wykorzystanie ich do testowania reakcji zbiorowisk mikrofitobentosu ma odpowiednie uzasadnienie. Warto podkreślić, że organizmy morskie związane ze środowiskiem brakicznym dotychczas rzadko były stosowane w testach ekotoksykologicznych. Przeważającą część testów ekotoksykologicznych prowadzi się na szczepach glonów, np. testy rekomendowane przez OECD (OECD, 1984; OECD, 2006) lub Międzynarodową Organizację Normalizacyjną (ISO 2012). Testy prowadzone według ustandaryzowanych metodyk na wybranych monokulturach są niezwykle cenne ponieważ pozwalają na porównanie stopnia wpływu różnych substancji, jednak dają informację tylko na temat reakcji nielicznych, wybranych organizmów. Ponadto wiele gatunków rekomendowanych w testach toksykologicznych jest utrzymywanych przez długi czas w sztucznych warunkach laboratoryjnych, do których się adaptują np.: *Chlorella vulgaris Beyerinck* (Beijerinck) jest utrzymywana w monokulturze od 1889 roku (Beijerinck 1890), *Navicula pelliculosa* (Kützing) Hilse od 1955 roku (Lewin 1955), a *Selenastrum capricornutum* Printz od 1959 roku (Guiry 2013). Analiza wpływu czynników pochodzenia antropogenicznego na naturalne zbiorowiska mikrofitobentosu to stosunkowo nowe, innowacyjne podejście stosowane w testach ekotoksykologicznych. Pomimo, iż takie badania

prowadzi się od ponad 20 lat, to wciąż nie opracowano standardowej metodyki prowadzenia testów, a stosowane w trakcie badań techniki pomiarowe są różnorodne (Cibic i in. 2008; Duong i in. 2008; Morin i in. 2017). W ramach pracy doktorskiej przetestowano szereg narzędzi i metodyk prowadzenia testów ekotoksykologicznych na zbiorowiskach mikrofitobentosu, co niewątpliwie stanowi interesujący wkład w rozwój tej dziedziny nauki (Dahl i Blanck 1996; Perkins i in. 2001; Serôdio 2004; Araújo i in. 2010; Åsa Arrhenius i in. 2014;).

Głównym celem dysertacji było scharakteryzowanie reakcji zbiorowisk mikrofitobentosu bałtyckiego wywołanej oddziaływaniem czynników określanych jako czynniki pochodzenia antropogenicznego - substancji z różnych grup chemicznych, tj. chlorku miedzi (II), glifosatu (w postaci Roundup®) i cieczy jonowej [BMIM]Cl oraz krótkookresowego nagłego wzrostu temperatury wody.

W pracy założono następującą hipotezę badawczą:

Wprowadzenie do środowiska zanieczyszczeń w postaci: chlorku miedzi (II), glifosatu (jako Roundup®), cieczy jonowej [BMIM]Cl oraz krótkookresowy nagły wzrost temperatury wpływają na skład i strukturę zbiorowisk mikrofitobentosu Zatoki Gdańskiej, a zmiany te można oszacować poprzez obserwację komórek mikroorganizmów wchodzących w skład zbiorowisk mikrofitobentosu.

Przeprowadzenie eksperymentów stanowiących podstawę rozprawy doktorskiej wymagało wykonania szeregu wstępnych badań, które pozwoliły na opracowanie najodpowiedniejszej metodyki do oceny zmian zachodzących w zbiorowiskach mikrofitobentosu pod wpływem wybranych czynników. Rozpoznanie materiału badawczego tj. składu i struktury mikrofitobentosu bałtyckiego miało miejsce w ramach realizacji pracy magisterskiej Z. Sylwestrzak zatytułowanej „Sukcesja zbiorowisk mikrofitobentosu w Zatoce Gdańskiej (badania eksperymentalne)", a także na podstawie bogatej literatury przedmiotu. Eksperymenty przeprowadzone w początkowych etapach pracy doktorskiej miały na celu przetestowanie m. in.:

- optymalnego czasu ekspozycji w środowisku szkiełek hodowlanych, z których pozyskiwano organizmy tworzące zbiorowiska mikrofitobentosu (Sylwestrzak, Zgrundo i Pniewski 2014);

- wpływu medium hodowlanego (woda morska i standardowa pożywka f/2) na wyniki testów ekotoksykologicznych prowadzonych na zbiorowiskach mikrofotbentosu (Sylwestrzak i Pniewski, 2014; Sylwestrzak i Zgrundo 2014).

Ze względu na fakt, iż powszechnie stosowane metody oceny kondycji mikroorganizmów, np. koncentracja barwników fotosyntetycznych lub aktywność fotosyntezy (fluorescencja typu PAM) (Brotas i in. 2007; Morelle i in. 2018), często nie dostarczały jednoznacznych wyników, dlatego w trakcie wstępnych badań przetestowano inne mniej popularne wskaźniki, takie jak:

- przeżywalność przedstawicieli poszczególnych taksonów mikrofitobentosu, wyznaczaną jako stosunek komórek żywych do martwych (Sylwestrzak i Zgrundo 2014).
- kondycja komórek przedstawicieli mikrofitobentosu w odniesieniu do stanu chloroplastów. Kondycję określano na podstawie zmian w kształcie i strukturze chloroplastów zgodnie z wcześniej opracowaną metodyką badań (Sylwestrzak 2014; Sylwestrzak i Pniewski 2014; Sylwestrzak i in. 2015). Przykłady i fotografie mikroskopowe komórek z prawidłowo wykształconymi chloroplastami i chloroplastami o nieprawidłowym kształcie przedstawiono na Fig. 1.

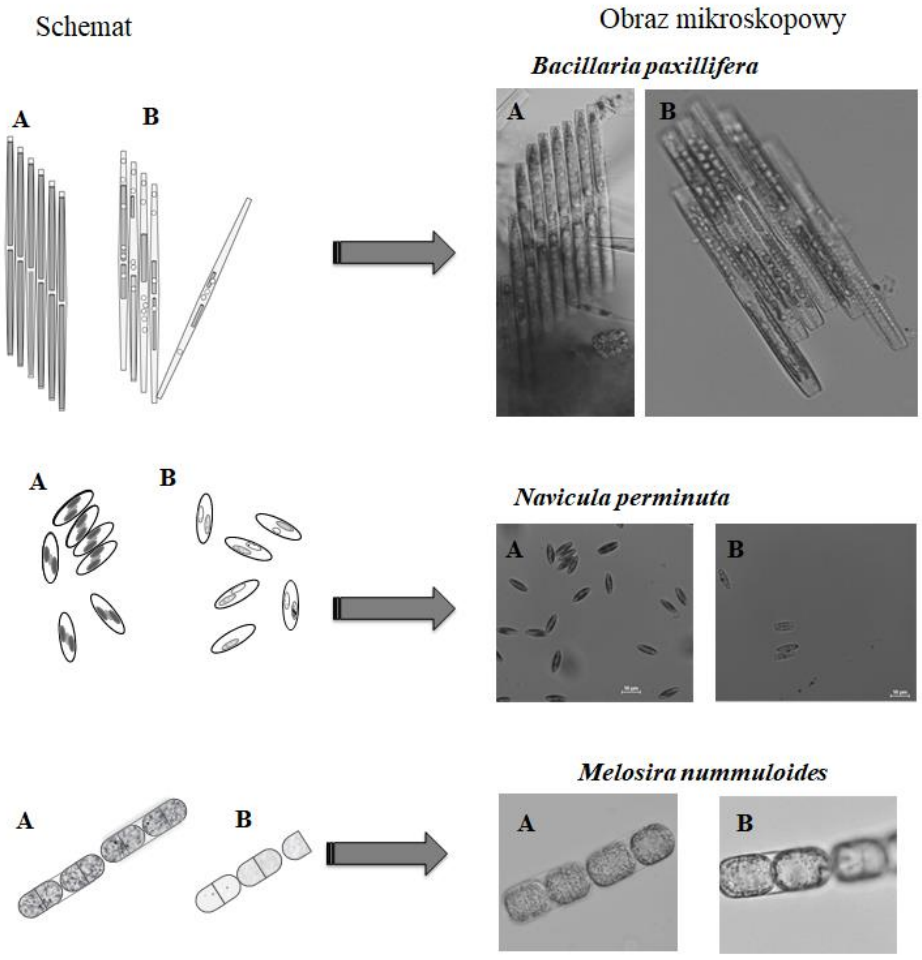

Fig. 1. Przykłady komórek z prawidłowo wykształconymi chloroplastami (A) i chloroplastami o nieprawidłowym kształcie (B) dla *Bacillaria paxillifera, Navicula perminuta, Melosira nummuloides* zobrazowane w postaci schematu i zdjęć komórek spod mikroskopu świetlnego.

Materiał wykorzystany w docelowych testach stanowiły zbiorowiska mikrofitobentosu pozyskane ze szkiełek eksponowanych w przybrzeżnej strefie Zatoki Gdańskiej (południowy Bałtyk) przez okres 14 dni w lipcu i sierpniu 2015 r. W trakcie ekspozycji szkiełek temperatura wody oscylowała pomiędzy wartościami 17°C i 19 °C, a zasolenie zmieniało się w zakresie od 7,9 do 8,4 PSU. Metodykę prac terenowych szczegółowo opisano w artykułach wchodzących w skład niniejszej dysertacji (**ARTYKUŁ 1, 2, 3 i 4)**. Po przetransportowaniu paneli hodowlanych do laboratorium zbiorowiska mikrofitobentosu zeskrobywano ze szkiełek hodowlanych skalpelem, wysycano gazowym azotem w celu wywołania hipoksji oraz poddawano sonifikacji. Dzięki zastosowaniu powyższych procedur z roztworu mikrofitobentosu wyeliminowano organizmy zwierzęce, a materiał badawczy został

ujednolicony (Rosenberg i in. 1991). Następnie mikrofitobentos umieszczano w 250 ml kolbach w 100 ml przefiltrowanej wody morskiej zebranej *in situ*. Niezależnie od eksperymentu średnia liczebność zbiorowisk w momencie rozpoczęcia testów wynosiła 41319 (± 1133), a 95% całego zbiorowiska stanowiły okrzemki. Wyjściowe stężenia związków biogenicznych w wodzie morskiej wynosiły: 9,4 $mg \cdot m^{-3}$ $N\text{-}NH_4$, 102 $mg \cdot m^{-3}$ $N\text{-}NO_3$, 36 $mg \cdot m^{-3}$ $P\text{-}PO_4$, 600 $mg \cdot m^{-3}$ $Si\text{-}SiO_4$. W trakcie badań wstępnych przeprowadzono analizę składników biogennych przed rozpoczęciem i po zakończeniu testów. Na podstawie uzyskanych danych wykazano, iż podczas eksperymentów prowadzonych przez 7 dni składniki biogenne nie są wyczerpywane i nie limitują wzrostu mikroglonów (Sylwestrzak 2016). W celu rozpoznania reakcji zbiorowisk mikrofitobentosu na wybrane substancje chemiczne (**ARTYKUŁ 1, 2, 3**) zastosowano stężenia związków ustalone na podstawie wartości koncentracji, które są określane jako zagrażające środowisku (na podstawie norm np. Dz.U.2011.257.1545 lub literatury przedmiotu, tj. Kulacki i Lamberti, 2008; Liu i in., 2015; Skeff i in., 2015) oraz wartości, których realny wpływ na mikroorganizmy roślinne obserwowano w trakcie wcześniejszych badań wstępnych (Sylwestrzak 2012; Sylwestrzak i Pniewski 2014; Sylwestrzak i Zgrundo 2014; Serwatka i in. 2015; Sylwestrzak i in. 2015a, 2015b). W testach zastosowano następujące stężenia: dla chlorku miedzi (II) - $2 \cdot 10^{-5}$ $g \cdot dm^{-3}$ i $2 \cdot 10^{-3}$ $g \cdot dm^{-3}$, dla glifosatu - 0,042 $g \cdot dm^{-3}$, 0,85 $g \cdot dm^{-3}$ i 8,5 $g \cdot dm^{-3}$ oraz dla cieczy jonowej [BMIM]Cl - $1{,}13 \cdot 10^{-3} g \cdot dm^{-3}$ i $1{,}75 \cdot 10^{-2}$ $g \cdot dm^{-3}$. W badaniach zaprezentowanych w **4 ARTYKULE** zakres zastosowanych temperatur wybrano na podstawie pomiarów prowadzonych na południowym Bałtyku w ostatnich latach (http://www.satbaltyk.pl) oraz na podstawie publikacji opisujących zjawisko krótkookresowych wzrostów temperatury, które mogą mieć duży wpływ na skład i strukturę zbiorowisk (Siegel i in. 2006; Bradtke i in. 2010; Rak i Wieczorek 2012; Stramska i Białogrodzka 2015).

Każdy wariant doświadczeń opisywanych w **ARTYKUŁACH 1, 2, 3 i 4** wykonywano w 3 powtórzeniach, jednak inne metody obserwacji reakcji zbiorowisk mikrofitobentosu na działanie czynnika pochodzenia antropogenicznego zastosowano w **ARTYKUŁACH 1, 2 i 3,** a inne w **ARTYKULE 4.** Do analizy wpływu czynników uznawanych jako zanieczyszczenia chemiczne wykorzystywano materiał, który stanowiły zbiorowiska mikrofitobentosu konserwowane płynem Lugola, a obserwacje przeprowadzano dla próbek zebranych po trzech i siedmiu dniach (**ARTYKUŁ 1 i 2, 3)**. Analizę jakościową i ilościową zbiorowisk oraz analizę kondycji chloroplastów wykonano w 50 polach widzenia w komorach sedymentacyjnych Utermöhl (2 ml) pod mikroskopem ze światłem odwróconym

Nikon Eclipse TS100 przy powiększeniach x200, x400**.** Kondycję komórek wyznaczano na podstawie stanu chloroplastów grupując je w dwóch kategoriach: komórki z chloroplastami o prawidłowym kształcie (zgodne z kształtami przedstawianymi w publikacjach jako typowe i prawidłowe dla danych taksonów) oraz komórki z chloroplastami o nieprawidłowym kształcie (wykazujące odstępstwa od prezentowanych w literaturze przedmiotu). Wyniki eksperymentów przedstawiano jako liczebność wszystkich organizmów i przedstawicieli poszczególnych taksonów w odniesieniu do liczebności obserwowanej w roztworze kontrolnym zgodnie z zasadami przyjętymi w wytycznych OECD stosowanych do oceny wpływu toksyczności związków chemicznych na mikroorganizmy roślinne (OECD 2006).

Analizę wpływu krótkookresowego wzrostu temperatury (**ARTYKUŁ 4**) przeprowadzono na materiale zebranym jak w przypadku **ARTYKUŁÓW 1, 2 i 3,** jednak z zastosowaniem specjalnej preparatyki laboratoryjnej mającej na celu uzyskanie czystego materiału okrzemkowego, co wiąże się z usunięciem pozostałych organizmów wchodzących w skład mikrofitobentosu. Obserwację w preparatach stałych wykonanych z zastosowaniem żywicy Naphrax o współczynniku załamania światła ~1,7 przeprowadzono pod mikroskopem świetlnym Nikon 80i wyposażonym w kamerę DS-U2 przy powiększeniu x1000. W tym przypadku identyfikowano i zliczano do 300 okryw okrzemek. W trakcie testu związanego z krótkookresowym wzrostem temperatury analizowano także inne parametry, takie jak: aktywność fotosyntetyczna (określana za pomocą zmian fluorescencji chlorofilu *a* mierzonych z zastosowaniem fluorymetru Fluorescence Monitoring System (FMS1; Hansatech, Norfolk, UK)) oraz koncentracja barwników fotosyntetycznych (analiza jakościowa i ilościowa barwników została wykonana metodą wysokosprawnej chromatografii cieczowej (HPLC) z wykorzystaniem systemu Waters wyposażonego w dwie pompy Waters 515 oraz detektor fotodiodowy Waters 2998 Photodiode Array Detector). Szczegółową metodykę wykonanych analiz przedstawiono w publikacji. Ze względu na niezbędną spójność tematyczną niniejszej dysertacji w dalszej części tekstu uwzględniono wyniki związane z reakcją okrzemek na podwyższenie temperatury tylko w odniesieniu do składu i struktury zbiorowiska.

Jednym z podstawowych elementów analizowanych w doświadczeniach była całkowita liczebność zbiorowisk mikrofitobentosu. W przypadku chlorku miedzi (II) (**ARTYKUŁ 1**) i glifosatu w postaci preparatu Roundup® (**ARTYKUŁ 2**) w testowanych roztworach całkowita liczebność zbiorowisk pozostawała na podobnym poziomie podczas całego okresu badań. Zmiany dotyczyły jedynie struktury zbiorowisk czyli liczebności

przedstawicieli poszczególnych taksonów mikroglonów. Zaobserwowano, że taksony wrażliwe na działanie zastosowanej substancji zastąpione zostały taksonami tolerancyjnymi lub odpornymi, co doprowadziło do zmiany struktury zbiorowiska. Odmienną reakcję obserwowano w przypadku zbiorowisk poddanych działaniu cieczy jonowej [BMIM]Cl, gdzie większość taksonów wchodzących w skład badanego zbiorowiska reagowała zmniejszeniem liczebności komórek (**ARTYKUŁ 3**). W przypadku krótkookresowego wzrostu temperatury całkowita liczebność zbiorowisk była praktycznie stała, a jedynie zmieniały się udziały procentowe poszczególnych taksonów w całkowitej liczebności zbiorowisk (**ARTYKUŁ 4**).

Wśród gatunków dominujących, obecnych w wyjściowych zbiorowiskach, a także występujących we wszystkich eksperymentach wyróżniono głównie okrzemki, m. in.: *Bacillaria paxillifera (*O.F.Müller) T.Marsson*, Diatoma moniliformis (*Kützing) D.M.Williams, *Diatoma vulgaris* Bory, *Melosira nummuloides* C.Agardh, *Navicula perminuta* Grunow, *Tabularia fasciculata* (C.Agardh) D.M.Williams i Round. Najliczniej obserwowaną sinicą był takson *Merismopedia* sp. Meyen. Niezależnie od stopnia dominacji w zbiorowisku wyjściowym poszczególne taksony wykazały rożny typ odpowiedzi na obecność czynnika pochodzenia antropogenicznego. Stąd wyróżniono między innymi grupę organizmów stymulowanych do wzrostu w obecności testowanych czynników. Przykładem gatunku, na który stymulujący wpływ miały wszystkie testowane czynniki była *N. perminuta*. Na przykład pod wpływem cieczy jonowej wykazała dwukrotne zwiększenie liczebności komórek zarówno pod wpływem chlorku miedzi jak i podwyższonej temperatury (**ARTYKUŁ 1 i 4**) oraz ośmiokrotne zwiększenie liczebności w obecności glifosatu (**ARTYKUŁ 2)** a także dziesięciokrotne zwiększenie liczebności w obecności cieczy jonowej [BMIM]Cl w stosunku do roztworu kontrolnego (**ARTYKUŁ 3**). Niektóre taksony były stymulowane obecnością substancji chemicznych jedynie w początkowej fazie testów. Na przykład liczba komórek *Achnanthes brevipes* C.Agardh w stężeniu $2 \cdot 10^{-5}$ g·dm^{-3} chlorku miedzi (II) zwiększyła się o 35% w trzeciej dobie, a w siódmej obserwowano już o 40% mniej komórek niż w roztworze kontrolnym. Niezwykle duży, aż czterokrotny, wzrost liczebności pod wpływem chlorku miedzi obserwowano u *Grammatophora marina* (Lyngbye) Kützing w stężeniu $2 \cdot 10^{-3}$ g·dm^3 $CuCl_2$ w trzeciej dobie (**ARTYKUŁ 1**). Część taksonów reagowała na obecność substancji chemicznych w ostatniej fazie testów. Na przykład u *Navicula ramosissima* (C.Agardh) Cleve w siódmej dobie testów obserwowano siedmiokrotnie więcej komórek niż w roztworze kontrolnym. (**ARTYKUŁ 3**). Glifosat wpływał na zwiększenie

liczebności komórek *T. fasciculata* o 40% w stosunku do roztworu kontrolnego w stężeniu 8,5 $g \cdot dm^3$ w siódmej dobie. Liczebność przedstawicieli sinic, tj. *Merismopedia* sp. i *Spirulina* sp. Turpin ex Gomont, uległa wielokrotnemu zwiększeniu podczas trwania testów (odpowiednio o 416% i 1750%). Dzięki obecności glifosatu prawdopodobnie doszło do wzbogacenia medium hodowlanego w fosforowe związki biogenne (Delpy i in. 2022), co prowadziło do całkowitej przebudowy i zdominowania zbiorowiska mikrofitobentosu przez sinice (**ARTYKUŁ 2**).

Wśród taksonów zidentyfikowanych jako obojętne na działanie czynników pochodzenia antropogenicznego, w przypadku chlorku miedzi (II), wyróżniono np.: *Brebissonia lanceolata* (C.Agardh) R.K.Mahoney i Reimer, *Cocconeis pediculus* Ehrenberg, *Fallacia* sp. Stickle i D.G.Mann oraz *Rhoicosphenia abbreviata* (C.Agardh) Lange-Bertalot (**ARTYKUŁ 1**). Z kolei glifosat w testowanych stężeniach nie wpływał na zmiany w liczebności m.in. u: *Halamphora coffeaeformis* (C.Agardh) Levkov, *T. fasciculata* (**ARTYKUŁ 2)**. W przypadku cieczy jonowej i krótkookresowego nagłego wzrostu temperatury niemal nie obserwowano gatunków określanych jako obojętne w stosunku do zastosowanych czynników (**ARTYKUŁ 3, 4**).

W testowanych zbiorowiskach zidentyfikowano wiele taksonów wrażliwych na działanie czynników pochodzenia antropogenicznego. Organizmami które reagowały zmniejszeniem liczebności na obecność zarówno chlorku miedzi jak i cieczy jonowej były m.in.: *B. paxillifera* i *T. fasciculata* (**ARTYKUŁ 1 i 3**). Okrzemka *M. nummuloides* była gatunkiem wykazującym szczególną wrażliwość na zastosowane substancje chemiczne, np. jej liczebność pod wpływem glifosatu zmniejszyła się do zaledwie 15% w stosunku do liczebności w próbie kontrolnej (**ARTYKUŁ 2**). Podobne reakcje obserwowano u *A. brevipes* i *Cylindrotheca closterium* (Ehrenberg) Reimann, których komórek nie obserwowano w glifosacie i w cieczy jonowej w siódmej dobie testów. Negatywne skutki obecności chlorku miedzi (II) w medium hodowlanym obserwowano także w przypadku przedstawicieli sinic, np. u *Spirulina* sp. obserwowano zmniejszenie liczebności w stosunku do roztworu kontrolnego o około 40% (**ARTYKUŁ 1).** Wśród gatunków wrażliwych na krótkookresowy wzrost temperatury wyróżniono *G. marina,* której liczebność zmniejszyła się o 43% w ostatnim dniu testów w stosunku do próby kontrolnej (**ARTYKUŁ 4**).

Stan chloroplastów określany poprzez zmianę w kształcie i strukturze to dodatkowy wskaźnik uzupełniający informację o kondycji komórek organizmów wchodzących w skład

zbiorowisk wykorzystanych w eksperymentach. Uzyskane wyniki wskazują, że liczba komórek poszczególnych taksonów może pozostawać na podobnym poziomie lub wzrastać w krótkich okresach czasu pomimo znacznego upośledzenia funkcji chloroplastów wywołanego działaniem np. chlorku miedzi (II). Zmiany w kształcie i strukturze chloroplastów w obecności chlorku miedzi (II) i cieczy jonowej obserwowano u dominującego gatunku, tj. okrzemki *T. fasciculata* (**ARTYKUŁ 1 i 3**), podczas gdy pod wpływem glifosatu zmiany w kształcie i strukturze chloroplastów obserwowano u *B. paxillifera* (**ARTYKUŁ 2**). Dzięki obserwacji chloroplastów można lepiej poznać reakcję taksonów wchodzących w skład zbiorowisk na zastosowane czynniki chemiczne, jednak nie może być on stosowany jako samodzielny wskaźnik zmian zachodzących w zbiorowiskach.

Przeprowadzone w ramach realizacji pracy doktorskiej badania pozwoliły na potwierdzenie hipotezy badawczej poprzez zaobserwowanie zmian w składzie i strukturze zbiorowisk morskiego mikrofitobentosu zachodzących pod wpływem czynników związanych z działalnością człowieka, takich jak: wprowadzenie substancji chemicznych do środowiska czy krótkookresowe wzrosty temperatury. Zbiorowiska mikrofitobentosu charakteryzują się większą odpornością niż pojedyncze szczepy mikroglonów, u których obserwowano zmniejszenie liczebności pod wpływem zastosowanych czynników pochodzenia antropogenicznego już na wczesnych etapach testów (Sylwestrzak i in. 2014b). Ustępowanie taksonów wrażliwych i zastępowanie ich odpornymi wskazuje na fakt dużej wytrzymałości zbiorowisk jako funkcjonalnej całości na zaburzenia pochodzenia antropogenicznego. Badania oparte na rozmaitych organizmach tworzących mikrofitobentos pozwalają poznać bogactwo reakcji zarówno na poziomie komórkowym (np. poprzez ocenę stanu chloroplastów) jak i populacyjnym (analiza składu i struktury zbiorowisk). Okrzemka *N. perminuta* wykazała szczególną odporność na stosunkowo szerokie spektrum zanieczyszczeń chemicznych z jednej strony, a z drugiej elastycznie przystosowała się do krótkookresowego wzrostu temperatury. Jednak przeważająca część organizmów tworzących zbiorowiska mikrofitobentosu była wrażliwa na testowane substancje pochodzenia antropogenicznego. Uwagę zwraca fakt, że zwiększona koncentracja testowanych substancji chemicznych czy wzrost temperatury sprzyjał masowemu rozwojowi jedynie pojedynczych taksonów (np. *G. marina, N. perminuta*) lub grup taksonów (np. sinic w przypadku glifosatu).

Organizmy fotosyntetyzujące tworzące mikrofitobentos są niezwykle istotnymi elementami ekosystemów wodnych ze względu m.in. na ich rolę jako pierwotnych producentów. Poznanie i opisanie reakcji całych zbiorowisk jest niezwykle cenne, ponieważ

umożliwia wiarygodnie oszacować zmiany, które mogą wystąpić pod wpływem czynników pochodzenia antropogenicznego w odniesieniu do każdego taksonu wchodzącego w ich skład. Wartościowym elementem pracy doktorskiej jest testowanie różnych czynników chemicznych w oparciu o jedną metodykę co umożliwiło wyraźne prześledzenie różnych typów reakcji w odniesieniu do zbiorowisk i populacji mikroorganizmów. W skład mikrofitobentosu bałtyckiego wchodzi wiele taksonów i gatunków kosmopolitycznych co pozwala przypuszczać, że podobne reakcje na wpływ testowanych czynników, przedstawione w artykułach stanowiących podstawę dysertacji, wykażą zbiorowiska występujące w wodach brakicznych w innych rejonach świata. Wykorzystanie zbiorowisk mikrofitobentosu pozwala uzyskać bardziej wiarygodne informacje na temat prawdopodobnych zmian, które pojawią się w środowisku w wyniku działalności człowieka, niż testy prowadzone na pojedynczych szczepach mikroglonów. Uzyskane podczas realizacji pracy doktorskiej wyniki dają nowe spojrzenie na funkcjonowanie zbiorowisk mikrofitobentosu w warunkach silnego stresu wywołanego czynnikami pochodzenia antropogenicznego jak i wzbogacają wiedzę na temat możliwości wykorzystania zbiorowisk mikroorganizmów w ekotoksykologii.

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Copper chloride (II) effect on the composition and structure of marine microphytobenthic communities

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Abstract To assess the temporary effects of the increased copper ion inflow on estuarine microphytobenthic communities, ecotoxicological tests were conducted using natural microphytobenthic assemblages obtained from an artificial substratum exposed to the waters of the southern Baltic Sea (Gulf of Gdańsk). The applied copper ion concentrations reflected permitted copper values established for waters of a good ecological status ($2{\cdot}10^{-5}$ g Cu·dm^{-3}), and the maximum copper concentrations which, according to the current environmental regulations, are allowed to be discharged into the environment ($2{\cdot}10^{-3}$ g Cu·dm^{-3}). In the studied communities, diverse responses of single species to $CuCl_2$ exposure were recorded, including both growth inhibition and stimulatory effects as well. Despite the shift in the community composition and structure, total cell number remained at a similar level. The results of our investigations suggest that microphytobenthic assemblages are resistant to $CuCl_2$ which is facilitated by the shift in the community composition resulting from the increasing cell number of copper tolerant species.

Keywords Copper chloride · Toxic effect · Microphytobenthos · Microalgal communities · Baltic Sea · Algal growth inhibition test

Highlights
- Algae assemblages in the high CuCl2 concentration change their structure.
- High concentration CuCl2 does not decrease growth assemblages.
- Tested taxa have higher resistance in assemblages than tested particular species.

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Introduction

The intense development of new technologies and industries results in many substances being released into the atmosphere, soil, and surface waters that cause disadvantageous, often difficult to evaluate environmental changes. A variety of research techniques are used to assess the impact anthropogenic substances have on the environment such as investigating the condition of assemblages in situ based on specially constructed indicators (e.g., Charles et al., 2020; Serra et al., 2010) or toxicological tests performed in laboratories. Microalgal toxicological tests are performed in accordance with, for example, the guidelines of the Organisation for Economic Co-operation and Development (OECD) (OECD, 2011) or regional recommendations such as standard water quality ISO CEN (EN 8692:2012). OECD recommends conducting tests on monocultures of

specific strains of freshwater microalgae and using parameters such as growth, cell biomass, fluorescence, and optical density to determine the impact of toxic substances. This is why ecotoxicological studies have been conducted to date most frequently using single monocultures and freshwater strains (e.g., Stauber & Florence, 1987; Yu et al., 2007). However, because of the increasingly rapid progression of the degradation of the world's oceans, studies that examine marine strains are also being introduced (e.g. Manimaran et al., 2012; Cid et al., 1995; Latała & Nędzi, 2009). Studies of this type are extremely valuable, but they only provide information on the reactions of single strains within the range of the so-called basic niche, which means that they do not take into consideration relationships with other organisms or the limiting influence of a variety of environmental factors. Only studies that consider entire assemblages permit organisms to respond more reliably. Tests on whole microphytobenthic assemblages permit assessing the reactions of numerous species from many taxonomical groups simultaneously, which is why the results of such studies are of a considerably wider scope in the context of environmental contamination than are those of single species (Arrhenius et al., 2014; Clements & Newman, 2002).

Experiments on Baltic microalgae assemblages were conducted with reference to copper, which has a very high functional significance for aquatic plant microorganisms, and its impact and mechanism of action is relatively well recognized (e.g., Stauber & Florence, 1987; Manimaran et al., 2012; Masmoudi et al., 2013; Serwatka et al., 2015). Copper plays an important role in the metabolism of photosynthetic organisms, and it is a component of many proteins and enzymes that participate in various metabolic pathways (Morelli & Scarano, 2004). However, depending on concentrations, it is either an essential micronutrient or a toxic substance. As a micronutrient, it is present in algal enzymes (e.g., oxidases) and is responsible for the transport of electrons occurring in mitochondria and thylakoid membranes (e.g., part of plastocyanin) (Pinto et al., 2003). However, excess amounts of this element can disrupt many physiological processes. At high concentrations and long-term exposures, copper ions inhibit photosynthesis (Fernandes & Henriques, 1991; Guasch et al., 2002) generating oxidative stress through the induction of reactive oxygen species (Morelli & Scarano, 2004) and affecting metabolic processes associated with growth (Maksymiec & Krupa, 2006). In large quantities, the salts of this element act as algaecides by inhibiting the growth of algae and as a consequence causing unfavorable, long-lasting changes in the aquatic environment at different levels of trophic networks (Kierzkowski et al., 2000).

The novelty of the present research is that it assesses the condition of an entire assemblage of marine microphytobenthos through the comprehensive analysis of the changes in the composition and structure of assemblages combined with an assessment of the condition of individual cells. Copper chloride was used for the study because of its well-documented specificity of interactions with freshwater and marine algae. Thanks to the indicators used, the results of our research are unique and worthy of special attention in light of progressing water pollution associated, inter alia, with the large number of new substances that are being introduced into the environment and the increasing importance of marine resources to the global economy.

Materials and methods

Study area and sampling

Microphytobenthos assemblages were sampled in July and August 2015 in the Gulf of Gdańsk, in Sopot. The study material was collected as described in Sylwestrzak et al. (2021). In brief, a specially designed culture panel (Fig. 1B) was exposed in the waters of the Gulf of Gdańsk 300 m from the shore (54°26′49″N, 8°34′24″E) (Fig. 1A) for 2 weeks. During the exposure period, the temperature varied within the range of 17–19 °C, and salinity changed between 7.9 and 8.4. After the exposition period ended, the panel was carefully collected and immediately transported to the laboratory for further processing.

Preparation of microalgal suspensions and experiment design

Microphytobenthic communities growing on the panels were scraped off with a scalpel and suspended in seawater collected in situ, previously filtered through the Whatman GF/C filters, and subsequently autoclaved. The pH of seawater was 7.8, and concentrations of the nutrient compounds in seawater

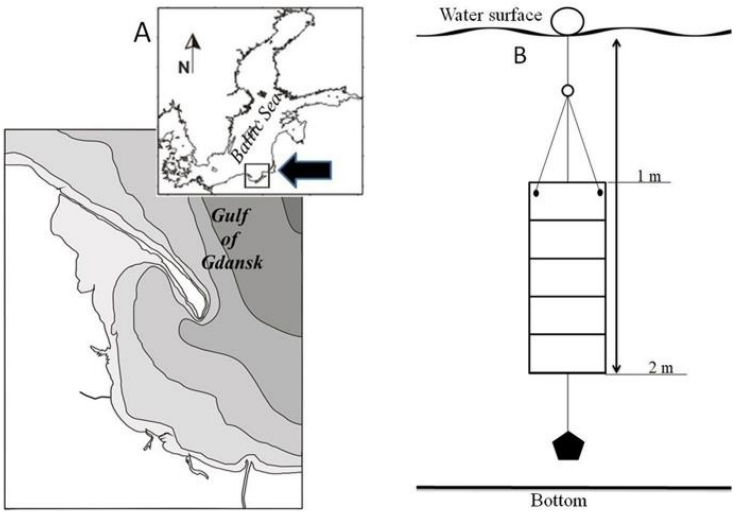


Fig. 1 **A** Location of the station where the culture panel was exposed to the waters of the Gulf of Gdańsk. Inset shows the location of the Gulf of Gdańsk in the Baltic Sea. **B** Diagram of the culture panel exposed to the waters of the Gulf of Gdańsk

were N-NH4 9.4 mg·m-3, N-NO3 102 mg·m-3, P-PO4 36 mg·m-3, and Si-SiO4 600 mg·m-3; because of such high nutrient values, no culture medium was added to the seawater. Currently, due to the very low concentrations of copper (1.3·10–7–1.1·10–6 g·dm-3) in the environment, this compound is not monitored and thus not regularly reported (Bakierowska et al., 2020). The microalgal suspension of 100 ml volume was then placed in 250-ml flasks. The suspension was sonicated with an impulse force that permitted mixing the cells thoroughly but avoided weakening or destroying them (Pniewski, 2015). Next, flasks with algal suspensions were saturated with nitrogen for 30 s to eliminate heterotrophic microorganisms (Connolly et al., 2004; Rosenberg et al., 1991) and then kept in a thermostatic chamber for a period of 72 h to acclimate to light (PAR – 60 µmol photons m^{-2} s^{-1}, photoperiod L:D 16:8) and temperature conditions (18 °C ± 1 °C, 8 PSU). The initial mean abundance of microalgal cells in suspension was as high as 38,800 ± 700 cells/ml. After the acclimation period, the microphytobenthic species were subjected to CuCl2 toxicity tests as follows: control – microphytobenthic species in 100 ml filtered seawater; test solutions – microphytobenthic species in 100 ml filtered seawater at two copper chloride (II) concentrations, i.e., 2·10–5 and 2·10–3 g·dm-3. The lower concentration of copper (2·10–5 g·dm-3) was selected based on previously published values shown to cause inhibitory effects in microalgal strains typically used for ecotoxicological tests; this concentration is also relevant from the environmental point of view as it coincides with the limit value for copper ions in surface waters of the highest water quality classes in Poland (Ustaw & Polskiej, 2019). The higher Cu concentration (2·10–3 g·dm-3), on the other hand, was chosen based on the results of preliminary experiments carried out on the Baltic microphytobenthic communities and indicated as having a substantial influence on the species composition (Serwatka et al., 2015; Sylwestrzak et al., 2015). Furthermore, it is important to note that this value complied with the legal regulations specifying the amount of copper that could be discharged into the environment (from 1·10–3 to 5·10–3 g·dm-3 depending on the source of pollution) (Ustaw & Polskiej, 2019). All experimental treatments were performed in three replicates.

Microscopic analysis

Qualitative and quantitative changes in assemblage composition and structure, i.e., changes in taxonomic composition and taxa abundance, were the primary parameters used to assess the changes in the microphytobenthic species. All microscopic

examinations were conducted on material preserved in Lugol solution after 1, 3, and 7 days for 50 fields of vision in sedimentation Utermöhl chambers (2 ml) under a Nikon Eclipse TS100 inverted light microscope at magnifications of 200× and 400× following Organisation for Economic Co-operation and Development (OECD) guidelines for assessing the effects of chemical toxicity on plant microorganisms and the Utermöhl method (Utermöhl, 1958). Microalgae were identified using various taxonomic keys and floras (Pliński & Hindák, 2010; Pliński & Komárek, 2007; Snoeijs & Balashova, 1998; Snoeijs & Kasperovičiene, 1996; Snoeijs & Potapova, 1993, 1995; Snoeijs & Vilbaste, 1994; Witkowskiet al., 2000).

Additionally, the condition of microalgal cells was assessed based on their chloroplast state (Sylwestrzak et al., 2021); three replicates after 1st, 3rd, and 7th day were examined to classify the state of the chloroplasts in all cells present in 50 fields of vision under a Nikon Eclipse 80i microscope fitted with a Nikon DSU2 camera at a magnification of 400×. Cells were separated into three groups, i.e., (1) live cells with normal chloroplasts, (2) live cells with abnormal chloroplasts, and (3) dead cells. Here, only the results obtained for the cell groups with normal and abnormal chloroplasts are presented (Fig. 2).

Statistical analysis

The obtained data were processed with MS Excel. Student's *t* test was performed to verify significant differences between means using STATISTICA version 10 (StatSoft, Inc.), analysis of principal component analysis (PCA) was performed with the Canoco5 statistical program (Lepš & Šmilauer, 2003), and similarity percentage (SIMPER) was performed with the PRIMER-e (Ter Braak & Šmilauer, 2003).

Results

Quantitative and qualitative analysis of assemblages

During the experiment, a total of 39 microalgae species was identified, including 33 diatom, 4 cyanobacterium, and 2 green alga taxa. The full list of taxa identified is in Appendix 1.

The highest mean abundance of 41,200 ± 1800 microalgal cells (per 1 ml suspension) was observed at the beginning of the tests (Fig. 3A). On the 3rd and 7th day of the experiment, statistically significant differences in the cell numbers were observed between control and treatment solutions ($p < 0{,}05$). It is noteworthy that the observed differences were less than 20% of the total cell count.

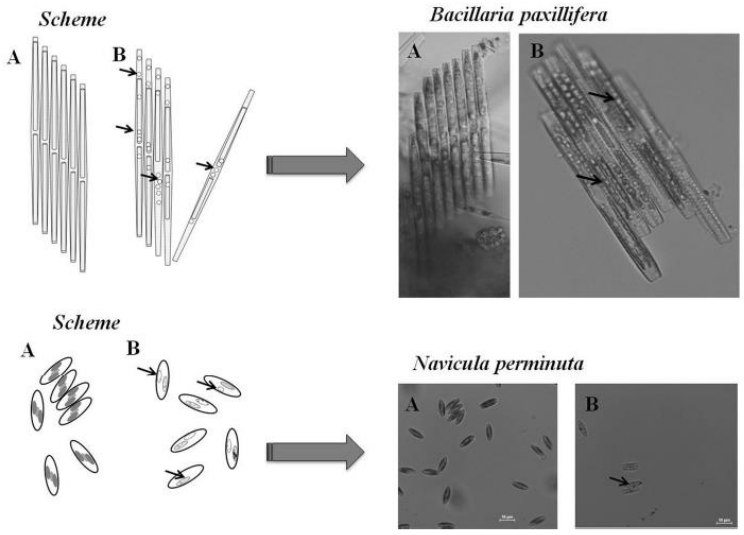


Fig. 2 Scheme and cells of selected taxa with normal chloroplasts (**A**) and with abnormal chloroplasts (**B**)

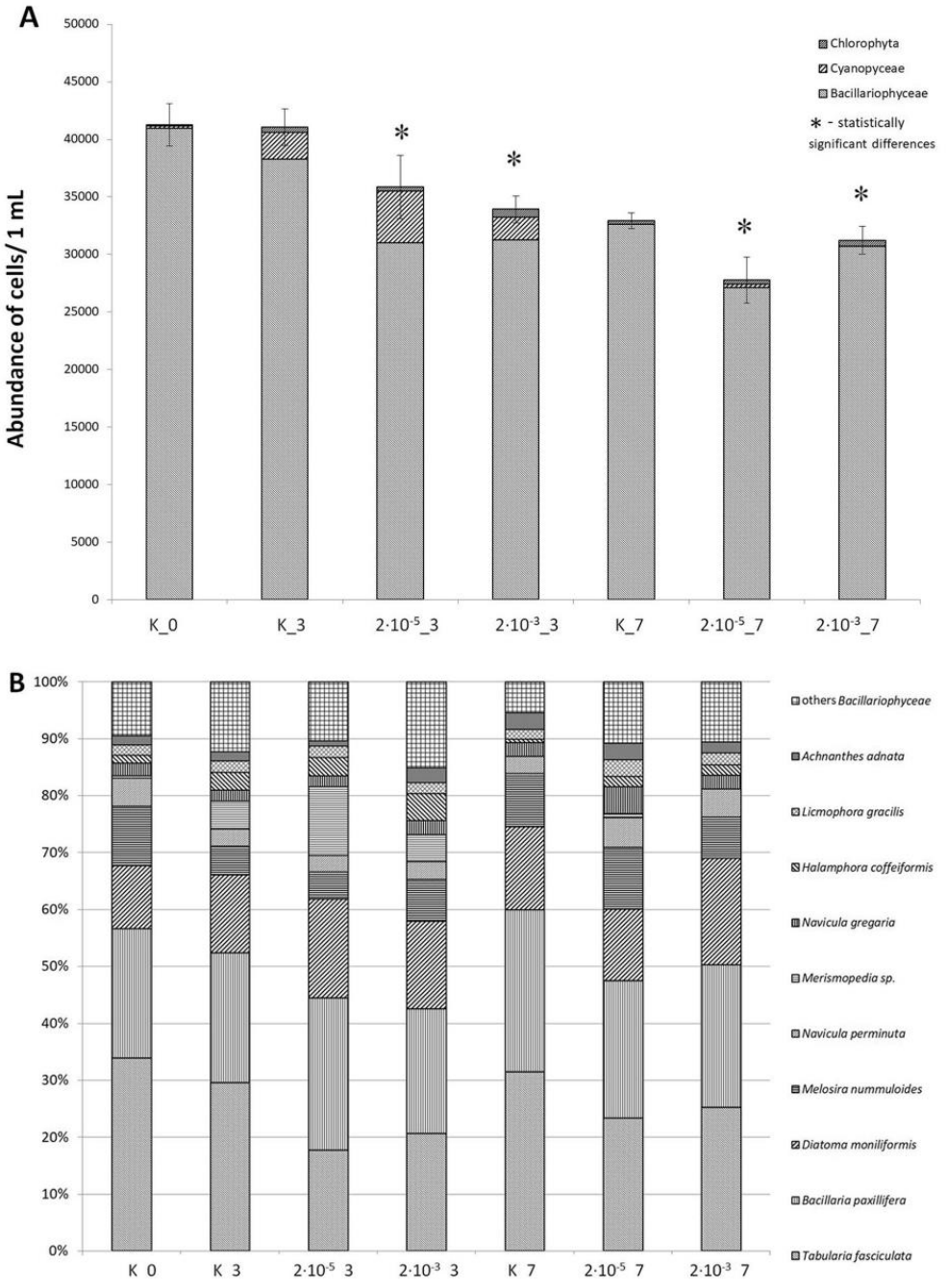
The assemblages analyzed were dominated by diatoms, which constituted from 86% up to 99% of all the cells counted. However, on the 3rd day of tests, the abundance of cyanobacteria was significantly high and their number increased to up 13% of the total cell counts ($CuCl_2$ solution of $2{\cdot}10^{-5}$ g·dm^{-3}). Green algae were present throughout the experiment, but their abundance only once exceeded 2% of the total cell counts (differences between days were not statistically significant). SIMPER analysis showed that the composition and structure of the analyzed communities were similar.

Two species, namely *Bacillaria paxillifera* (O.F. Müller) T. Marsson and *Tabularia fasciculata* (C.Agardh) D.M. Williams & Round, were characterized by the highest abundance (ca. 25% of the cell count) during the course of the experiments (Fig. 3B). For both species, the highest number of cells was recorded in the control solution; the highest abundance of *B. paxillifera* (30%) was observed on the 7th day, while *T. fasciculata* had the highest cell number (34%) at the beginning of the experiments. The cell number of *Diatoma moniliformis* (Kützing) D.M. Williams increased during the experiment and reached its peak on the 7th day at the $CuCl_2$ concentration of $2{\cdot}10^{-3}$ g·dm^{-3} (19% of total abundance). *Melosira nummuloides* (Kützing) D.M. Williams contributed substantially to the community structure with cell count between 5 and 11%. Remaining species were characterized by the number of cells lower than 5% (e.g., *Navicula perminuta* Grunow (3–5%), *Achnanthes adnata* Bory (1–3%), and *Halamphora coffeiformis* (C. Agardh) Kützing (1–5%). Among species belonging to other taxonomic groups, only *Merismopedia* sp. had a noticeable input into the community structure; its cell number increased above the value of 5% on the 3rd day of the experiment (both control and treatments); subsequently, the abundance of the species decreased.

PCA showed those species for which growth was limited in samples treated with $CuCl_2$ e.g., *B. paxillifera*, *Cylindrotheca closterium* (Ehrenberg) Reimann and J.C. Lewin, *Entomoneis paludosa* (W. Smith) Reimer, *T. fasciculata*, *Nitzschia dissipata* (Kützing) Rabenhorst, and *Navicula meniscus* Schumann (Fig. 4, left part of diagram). The second group constituted species which were stimulated by the presence of $CuCl_2$, such as *Brebissonia lanceolata* (C. Agardh) R.K. Mahoney and Reimer, *Cocconeis pediculus* Ehrenberg, *Fallacia* sp., *Grammatophora marina* (Lyngbye) Kützing, and *Rhoicosphenia abbreviata* (C. Agardh) Lange-Bertalot, and their abundance increased in comparison with control solution even at the highest concentration of copper chloride (central and upper right part of diagram with left upper part). There were also taxa which showed various responses depending on the day and concentration used (lower right part of diagram). This group was composed of species such as *A. adnata*, *Melosira nummuloides* C. Agardh, and *Navicula perminuta* Grunow.

Abundance of selected taxa

In the further part of the work, selected species of microalgae are presented. Based on previous research, selected species of microalgae are a permanent element of the microphytobenthos of the Gulf of Gdańsk, and their size allows the observation of chloroplasts. Among the dominant species in the assemblages, tested *T. fasciculata* was the most abundant at the beginning of the experiment (14,000 cells/1 ml). This was followed 2 days later by a reduction in the abundance of about 40% ($p < 0.05$) in all treatments, but on the subsequent days of the experiment, the abundance of this species remained at a relatively similar level (Fig. 4A). This taxon exhibited a reduction of growth in Cu treatments in comparison to the control solution. A similar number of *B. paxillifera* cells was observed in the control solution and at the concentration of $2{\cdot}10^{-5}$ g·dm^{-3} after 3 days of the experiment. However, on the 7th day, the cell number was significantly lower compared to the control solution. The lowest number of cells was observed after 7 days at a copper chloride concentration of $2{\cdot}10^{-5}$ g·dm^{-3} at approximately 80% of control. At the $CuCl_2$ concentration of $2{\cdot}10^{-5}$ g·dm^{-3}, the number of *E. paludosa* cells decreased by 5% on the 3rd day and by 86% on the 7th day of the experiment, while at the concentration of $2{\cdot}10^{-3}$ g·dm^{-3}, decreases were by 12% and 79% on the 3rd and 7th days, respectively. Furthermore, under control conditions, the number of *N. meniscus* cells increased throughout the experiment. In all treatments, after the 3rd day, the abundance of the species did not differ from the control, whereas on the 7th day, the species disappeared completely. Other taxa of smaller abundance which showed limited growth in solution with $CuCl_2$ were *C. closterium* and *N. dissipata*.


A
Abundance of cells/ 1 mL
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Chlorophyta
Cyanopyceae
Bacillariophyceae
* - statistically significant differences
*
K_0
K_3
2·10-5_3
2·10-3_3
K_7
2·10-5_7
2·10-3_7
B
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
K 0
K 3
2·10-5 3
2·10-3 3
K 7
2·10-5 7
2·10-3 7
others Bacillariophyceae
Achnanthes adnata
Licmophora gracilis
Halamphora coffeiformis
Navicula gregaria
Merismopedia sp.
Navicula perminuta
Melosira nummuloides
Diatoma moniliformis
Bacillaria paxillifera
Tabularia fasciculata

◂**Fig. 3** **A** Abundance of microalgae: K_0, the moment the experiment began; K_3, control solution after three days of the test; K_7, control solution after 7 days of the test and the concentration of copper chloride of $2{\cdot}10^{-5}$ g·dm^{-3} tested after 3 and 7 days, $2{\cdot}10^{-5}$_3 and $2{\cdot}10^{-5}$ _7, and of $2{\cdot}10^{-3}$ g·dm^{-3} tested after 3 and 7 days, $2{\cdot}10^{-3}$_3 and $2{\cdot}10^{-3}$_7. Statistically significant differences were denoted with the asterisk. **B** Contribution of the 9 most abundant species to the total community cell count

The next group consisted of species the abundance of which increased in the presence of copper chloride compared to control conditions (Fig. 5A). A decrease in *N. perminuta* Grunow abundance was observed in all treatments after three days (13% relative to the control at the concentration of $2{\cdot}10^{-5}$ g·dm^{-3} and 14% at the concentration of $2{\cdot}10^{-3}$ g·dm^{-3}), but after 7 days, a strong increase in the abundance of cells of this taxon was noted in both $CuCl_2$ concentrations (50% relative to the control at the concentration of $2{\cdot}10^{-5}$ g·dm^{-3} and 58% at the concentration of $2{\cdot}10^{-3}$ g·dm^{-3}). This group was also represented by *G. marina*, the abundance of which decreased in the control solution as the experiment progressed, while in the solutions of the tested substance, a fourfold increase in the number of cells of this taxon was noted in comparison to that in the control from the same day. This group also included the following diatom species: *Brebissonia lanceolata*, *Cocconeis pediculus*, *Halamphora coffeiformis*, *R. abbreviata*, and the green alga *Scenedesmus* sp.

The last group identified comprised species that were characterized by various responses depending on the day and concentration (Fig. 5B). For instance, *A. adnata* and *M. nummuloides* increased their abundance in the control solution and at the $CuCl_2$ concentration of $2{\cdot}10^{-5}$ g·dm^{-3}, whereas at the higher $CuCl_2$ concentration, their cell number declined. The opposite response was found for *Rhopalodia brebissonii* (Ehrenberg) O. Müller; in the control solution and the higher concentration of copper chloride, its cell number dropped, while it increased at the $CuCl_2$. A table with the abundance of all species is provided in Appendix 2.

Cell condition in selected taxa

In species in which abundance decreased in the presence of copper chloride, their physiological condition also decreased. The analysis of the condition carried out for *T. fasciculata* indicated the progressive degradation of chloroplasts as the experiment continued for each treatment, and only 30% of the cells observed in the copper chloride concentration of $2{\cdot}10^{-5}$ g·dm^{-3} had unchanged chloroplasts after 7 days (Fig. 6A). No high chloroplast degradation was noted in *B. paxillifera* in the control solution since after 7 days only 10% of the cells had damaged chloroplasts, and in the copper chloride solutions, only a maximum of 20% of the cells had damaged chloroplasts. In *E. paludosa* at the $CuCl_2$ concentration of $2{\cdot}10^{-5}$ g·dm^{-3} on the 3rd day of the experiment, 40% of cells had degenerated chloroplasts, and on the 7th day, it was 75%. At the concentration of $2{\cdot}10^{-3}$ g·dm^{-3}, the cells were more affected, and on the last day of the experiment, only 13% of cells had normally developed chloroplasts. On the 7th day of the experiment at the lower $CuCl_2$ concentration, 48% of *N. meniscus* cells had degenerated chloroplasts, while at the concentration of $2{\cdot}10^{-3}$ g·dm^{-3}, 70% had them.

In the group of diatoms stimulated with $CuCl_2$, the structure of the chloroplasts was unchanged, or some alterations occurred only in a small fraction of the cells (Fig. 6B). The majority of *N. perminuta* cells with degraded chloroplasts were observed after 7 days of the tests (less than 30%, $CuCl_2$ solution of $2{\cdot}10^{-5}$ g·dm^{-3}). Whereas in the species *G. marina*, changes in cell condition were observed only at the beginning of the experiment.

Discussion

There are many reports in the world literature on toxicological tests conducted on microalgal marine plankton, mainly diatoms and green algae (Halling-Sørensen, 2000; Peterson et al., 1994), and on freshwater microalgal monocultures (e.g., Stauber & Florence, 1987; Yu et al., 2007; Manimaran et al., 2012;). Although common, tests on monocultures provide information on the reactions of organisms only within the range of the so-called basic niche, while only studies that consider whole assemblages facilitate understanding the response of organisms more reliably. The current experiments that tested the effect of copper chloride (II) were performed on Baltic microalgal assemblages harvested directly from the environment and transferred to the laboratory where, after mixing and incubation, they were tested in flasks. The results can be applied to all oceanic regions with similar salinity or

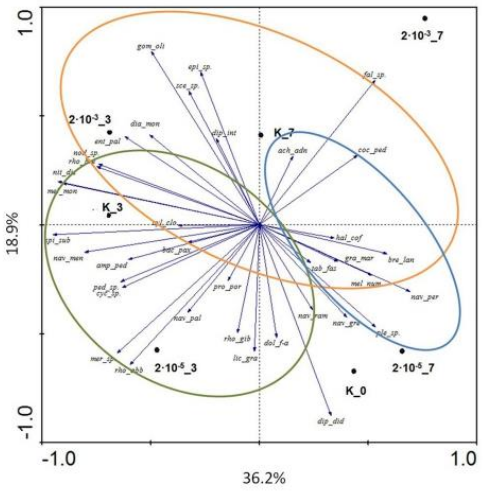


Fig. 4 PCA based on the microphytobentos abundance data. Percentage explained variation λ1 = 36,20%, λ2 = 0.18,9%, and λ3 = 16,8%. K_0, the moment the experiment began; K_3, control solution after 3 days of the test; K_7, control solution after 7 days of the test and the concentration of copper chloride of $2{\cdot}10^{-5}$ g·dm^{-3} tested after 3 and 7 days, $2{\cdot}10^{-5}$_3 and $2{\cdot}10^{-5}$ _7, and of $2{\cdot}10^{-3}$ g·dm^{-3} tested after 3 and 7 days, $2{\cdot}10^{-3}$_3 and $2{\cdot}10^{-3}$_7

trophic conditions because all the species identified in the assemblages are cosmopolitan and observed not only in the waters of the Gulf of Gdańsk and the Baltic Sea (e.g., Arrhenius et al., 2014; Lange-Bertalot et al., 2003; Majewskaet al., 2012; Zgrundo et al., 2008) but also in the world oceans (e.g., Andersson & Kautsky, 1996; Witkowskiet al., 2000).

This research was conducted on copper, which is a highly functionally important element that plays an important role in aquatic plant microorganism metabolism. However, its impact depends on its concentration and accessibility in the environment. On the one hand, it is an essential micronutrient, the lack of which limits growth and development, while on the other, it is a dangerous substance in doses that exceed the demand and the detoxification capabilities of organisms. Hence, copper is used as an effective algicide and is an active ingredient in many antifouling products used for ship maintenance. It is estimated that these products are the main source of copper pollution in the Baltic Sea (Andersson & Kautsky, 1996). The concentration of copper in the Gulf of Gdańsk sediments in the 1990s was between 21 and 57 ppm (average 41 ppm, 0.041 g·dm^{-3}). In later studies, on heavy metal concentrations in the Gulf of Gdańsk sediments, the copper content detected was 19–45 µg·g^{-1} sediment dry weight (Szefer et al., 2009). Heavy metals occurring in surface sediments play important roles because they can interfere with ecological balance by inhibiting many processes in organisms at different trophic levels. However, there are few reports in the world literature on copper ion concentrations in natural waters. To the best of our knowledge, there are currently no reports on copper ion concentrations in the Baltic Sea since the values observed do not drastically exceed generally accepted norms. Sources made available as part of monitoring rivers in Poland cite average Cu values in 2007–2009 of $1.0{\cdot}10^{-5}$ g·dm^{-3} (Wisłoka River in Mielec, Poland) (Kaniuczak & Augustyn, 2011). Currently, no measurements of copper chloride (II) concentrations are performed in the Gulf of Gdańsk region on regular basis due to the very low concentrations ($1{,}3{\cdot}10^{-7}$–$1{,}1{\cdot}10^{-6}$ g·dm^{-3}) (Bakierowska et al., 2020), but they were done regularly in the 1990s since there was a high risk of elevated concentrations of this substance. The lowest concentrations tested in the present study were only twice as high as maximum values reported in waters in Poland; hence, the tests performed currently reflect the reactions of microorganisms to the maximum concentrations observed in the environment.

Copper in high doses limits the development of the cells of individual taxa, and as demonstrated in the current experiment, the concentration of $2{\cdot}10^{-5}$ g·dm^{-3} $CuCl_2$ after 7 days caused a decrease in the size of the community by 13% compared to the control solution. Whereas in the tests Sabater et al. (2002) conducted on freshwater communities in a solution of copper ions with a concentration of $1.5{\cdot}10^{-5}$ g·dm^{-3}, a significant change in the structure of the community was noted with the number of the diatoms *Ulnaria ulna* and *Achnanthes minutissima* decreasing significantly after 7 days of the experiment. In the case of the assemblages collected from the waters of the Gulf of Gdańsk, the changes were of a different nature; i.e., the reconstruction of the entire assemblage was observed, and the number of cells of resistant taxa increased significantly, e.g., *G. marina* and *N. perminuta*, while the number of *B. paxillifera* cells decreased slightly. After 7 days of

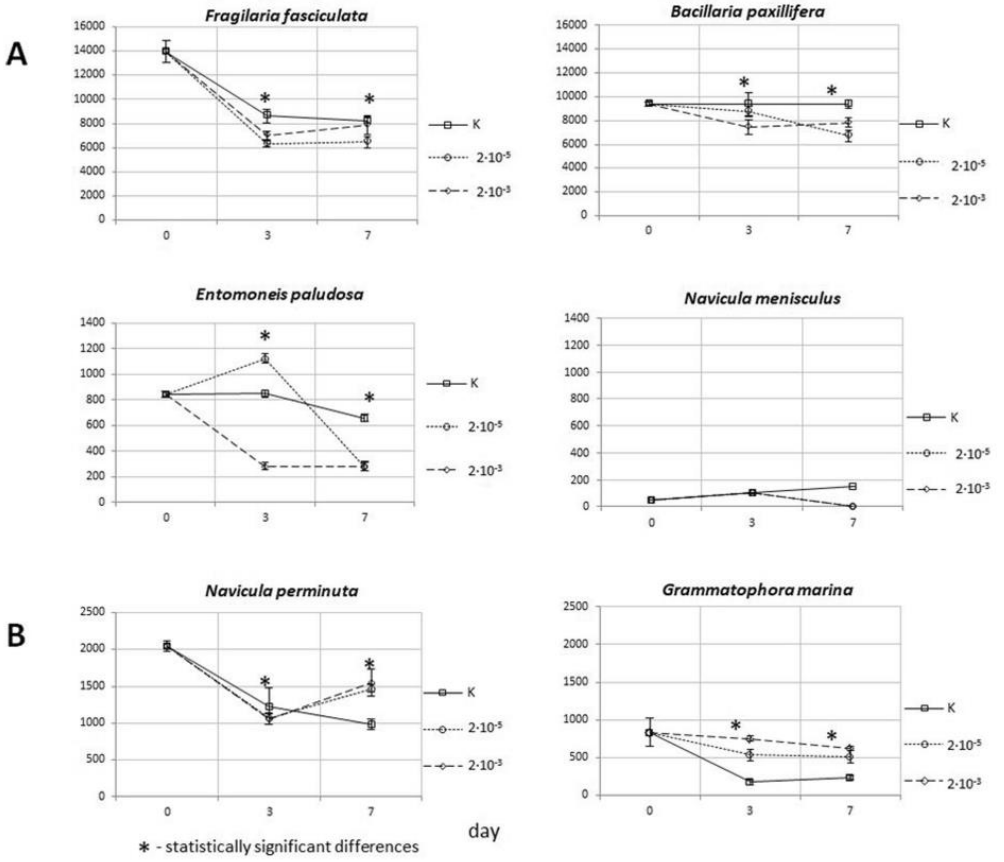


Fig. 5 Abundance of cells of selected taxa in the control solution: K_0, the moment the experiment began; K_3, after 3 days of the test in the control solution; K_7, after 7 days of the test, and the concentrations of copper chloride tested of $2{\cdot}10^{-5}$ g·dm^{-3} $CuCl_2$ (marked on the diagram 2.10^5_3) and $2{\cdot}10^{-3}$ g·dm^{-3} $CuCl_2$ (2.10^3_3) after 3 days of tests and the concentrations of copper chloride tested of $2{\cdot}10^{-5}$ g·dm^{-3} $CuCl_2$ (2.10^5_7) and $2{\cdot}10^{-3}$ g·dm^{-3} $CuCl_2$ (2.10^3_7) after 7 days of tests. Statistically significant differences observed each day between the control solution and $CuCl_2$ treatments were denoted with the asterisk. **A** Species in which copper chloride (II) inhibited growth, **B** species in which copper chloride (II) stimulated growth

the tests, no decreases in the total cell number of the organisms tested were observed in the $2{\cdot}10^{-3}$ g·dm^{-3} solution; however, the changes noted in assemblage structure were much more pronounced. The species that responded to $CuCl_2$ treatment by limiting their growth (e.g., *C. closterium*, *R. abbreviata*, *U. ulna*) on the 3rd and 7th day of the experiment were replaced by more resistant species (e.g., *C. placentula*, *G. marina*, *N. perminuta*).

An interesting aspect of the research was the analysis of cell condition in relation to abundance. It was demonstrated that species characterized by a slight decrease in abundance, e.g., *B. paxillifera* exhibited statistically significant chloroplast degradation (23% relative to the control in a solution of copper ions with a concentration of $1.5{\cdot}10^{-5}$ g·dm^{-3} after 3 days and 7% at the concentration of $1.5{\cdot}10^{-5}$ g·dm^{-3} and 25% at the concentration of $1.5{\cdot}10^{-3}$ g·dm^{-3} after 7 days).

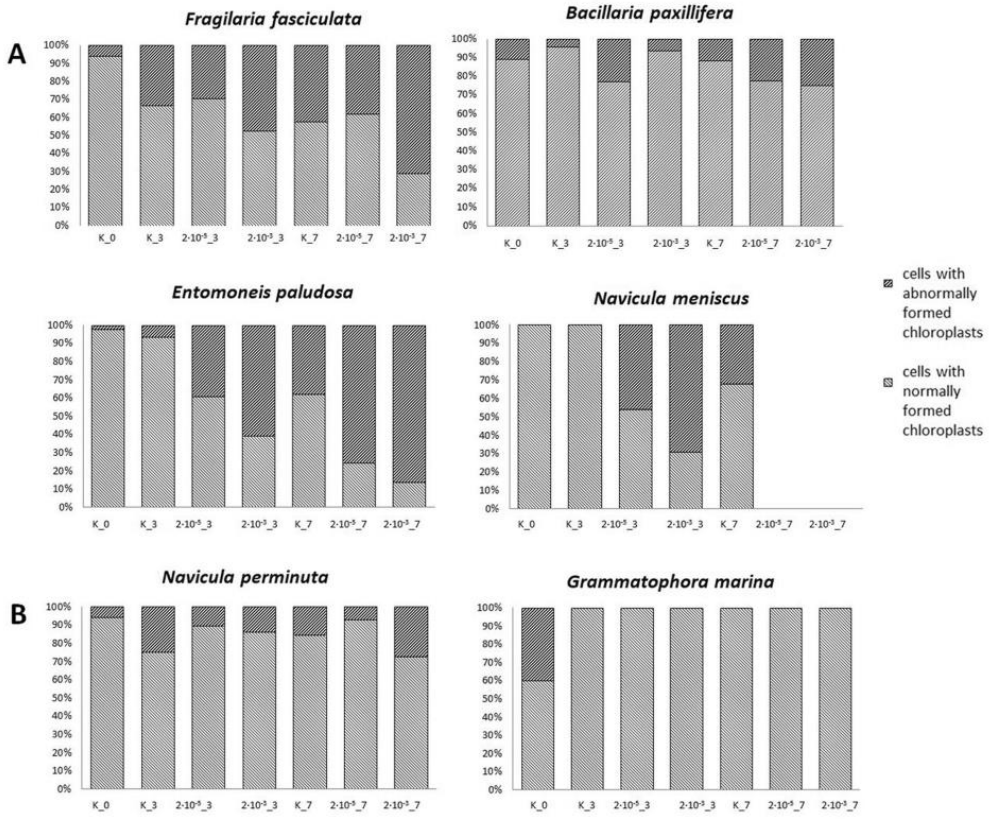


Fig. 6 Changes in condition of selected species expressed as the percentage share of cells with deformed chloroplasts in control solution: K_0, the moment the experiment began; K_3, after 3 days of the test in the control solution; K_7, after 7 days of the test: and the concentrations of copper chloride tested of $2{\cdot}10^{-5}$ g·dm^{-3} $CuCl_2$ (marked on the diagram 2.10^5_3) and $2{\cdot}10^{-3}$ g·dm^{-3} $CuCl_2$ (2.10^3_3) after 3 days of tests and the concentrations of copper chloride tested of $2{\cdot}10^{-5}$ g·dm^{-3} $CuCl_2$ (2.10^5_7) and $2{\cdot}10^{-3}$ g·dm^{-3} $CuCl_2$ (2.10^3_7) after 7 days of tests. **A** Species in which copper chloride (II) inhibited growth, **B** species in which copper chloride (II) stimulated growth

The assessment of the physiological condition based on the changes in the chloroplast shape was also performed in the experiment describing the influence of Roundup on the microphytobenthos (Śliwińska et al., 2016). As in this study, among species in which chloroplast degradation correlated with their abundance, *B. paxillifera*, *F. fasciculata*, and *N. perminuta* were also found.

The relatively high resistance of microphytobenthos assemblages to the substance tested probably stemmed from the relationships among the species and the extensive structure of the community tested, understood as a multitude of species and a diversity of genera. As was shown in previous studies, monocultures of *B. paxillifera* were characterized by much lower resistance to the effects of copper chloride (II), i.e., after 7 days of testing at a concentration of $1{\cdot}10^{-3}$ g·dm^{-3} $CuCl_2$, the number of cells decreased by 40% (Śliwińska et al., 2016). In laboratory tests, *N. perminuta* also exhibited much lower resistance to copper ions at a concentration of $1{\cdot}10^{-3}$ g·dm-$^{-3}$ $CuCl_2$; the lowest number of cells was

observed after 7 days (15% compared to the control solution) (Sylwestrzak et al., 2021; Zgrundo et al., 2017). Yu et al. (2007) report a similar relationship from their studies of the role of algal interactions, including cyanobacteria, in multi-species microalgal assemblages and determinations of algal sensitivity to the presence of copper ions based on measurements of the inhibited production of certain cellular enzymes. Analyses of *Microcystis aeruginosa* were conducted at the same initial cell density and on the same surfaces with the presence or absence of two species of green algae, *Chlorella pyrenoidosa* and *Scenedesmus obliquus*, as the differentiating factor in the test treatments. These authors demonstrated that the EC_{50} for *M. aeruginosa* in a multispecies assemblage was significantly higher compared to that in monocultures of this species and that reactions to copper ion metabolism were mediated by interspecific interaction. Microphytobenthic assemblages are characterized by a large number of species, even in the early stages of their development, which means that the reaction to toxic substances differs from that in monocultures. This fact was also confirmed in an experiment on Baltic microphytobenthos (Arrhenius et al., 2014). The presence of many different diatom and cyanobacteria species in the assemblages meant that the microorganisms tolerated considerably high concentrations of copper ions than did the algae in tests commonly performed on monocultures.

Interestingly, the differences in the sensitivity to the substance tested among species did not depend on cell size, cell wall type, or taxonomic group. The rate of absorption of selected ions by cell membranes and internal detoxification mechanisms were possibly the factors that influenced copper toxicity significantly as is suggested by Levy et al. (2007). The results presented in the paper also do not indicate a clear relationship between cell size and its resistance to the presence of high concentrations of copper chloride. In both cases, species that were smaller, e.g., *N. perminuta*, or larger, e.g., *B. paxillifera*, were characterized by high survival rates.

Conclusions

The microphytobenthos assemblages studied at two $CuCl_2$ concentrations that were approximately 10–1,000 times higher than those currently observed in the environment did not respond to the presence of the toxicant as expected. There was only a small decrease in overall abundance; however, the numbers of individual species showed decreases and increases which ultimately led to a change in community structure. The tests performed allowed recording of various responses in different species caused by the presence of $CuCl_2$. Based on these responses, two main groups of organisms were distinguished, namely taxa that growth was inhibited to varying degrees (e.g., slightly *Bacillaria paxillifera*, *Tabularia fasciculata*, and *Licmophora gracilis* and significantly *E. paludosa* and *N. meniscus*) and stimulated (e.g., *Grammatophora marina*, *Navicula perminuta*) by the presence of $CuCl_2$. The results suggested that the cell number of taxa can remain at similar levels or increase over short periods of time despite the significant impairment of chloroplast function.

Furthermore, some of the taxa showed much higher resistance to $CuCl_2$ compared to their responses in ecotoxicological tests performed using only single species. The change observed in the microphytobenthos structure suggested that assemblages can survive in the environment even at quite high toxic substance concentrations by substituting sensitive taxa with indifferent ones.

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Author contributions Conceptualization, ZS, AZ, and FP; methodology, ZS, AZ, and FP; software, ZS; validation, ZS, AZ, and FP; formal analysis, ZS, AZ, and FP; investigation, ZS; resources, ZS and AZ; writing — original draft preparation, ZS, AZ, and FP; writing — review and editing, ZS, AZ, and FP; visualization, ZS; supervision, AZ; project administration, ZS; funding acquisition, ZS and AZ. All authors have read and agreed to the published version of the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Declarations

Conflict of interest The authors declare no competing interests.

Appendix 1. List of taxa

	Taxa	Author
Bacillariophyta	*Achnanthes adnata*	Bory
	Achnanthes lemmermannii	Hustedt
	Amphora pediculus	(Kützing) Grunow
	Amphora sp.	Kützing
	Bacillaria paxillifera	(O.F. Müller) T. Marsson
	Brebissonia lanceolata	(C. Agardh) R.K. Mahoney and Reimer
	Cocconeis pediculus	Ehrenberg
	Cyclotella sp.	(F.T. Kützing) A. de Brébisson
	Cylindrotheca closterium	(Ehrenberg) Reimann and J.C. Lewin
	Diatoma moniliformis	(Kützing) D.M. Williams
	Diploneis didyma	(Ehrenberg) Ehrenberg
	Diploneis interrupta	(Kützing) Cleve
	Entomoneis paludosa	(W. Smith) Reimer
	Epithema sp.	Kützing
	Fallacia sp.	Kütz
	Gomphonema olivacea	(Hornemann) Rabenhorst
	Grammatophora marina	(Lyngbye) Kützing
	Halamphora coffeiformis	(C. Agardh) Mereschkowsky
	Licmophora gracilis	(Ehrenberg) Grunow
	Melosira moniliformis	C. Agardh
	Melosira nummuloides	C. Agardh
	Navicula gregaria	Donkin
	Navicula meniscus	Schumann
	Navicula palpebralis	Brébisson ex W. Smith
	Navicula perminuta	Grunow
	Navicula ramosissima	(C. Agardh) Cleve
	Nitzschia dissipata	(Kützing) Rabenhorst
	Pleurosigma sp.	W. Smith
	Proschkinia poretzkajae	(Koretkevich) D.G. Mann
	Rhoicosphenia abbreviata	(C. Agardh) Lange-Bertalot
	Rhopalodia gibba	(Ehrenberg) O. Müller
	Surirella brebissonii	Krammer and Lange-Bertalot
	Tabularia fasciculata	(C. Agardh) D.M. Williams and Round
Cyanobacteria	*Dolichospermum flosaquae*	(Brébisson ex Bornet and Flahault) P. Wacklin, L. Hoffmann and J. Komárek
	Merismopedia sp.	(Turpin) Meneghini
	Nodularia sp.	Mertens ex Bornet and Flahault
	Spirulina subsalsa	Oersted ex Gomont
Chlorophyta	*Pseudopediastrum boryanum*	(Turpin) E. Hegewald
	Scenedesmus sp.	Meyen

Appendix 2. Table with the abundance of all species

Taxa	K_0	K_3	K_7	$2{\cdot}10^{-5}$ _3	$2{\cdot}10^{-5}$ _7	$2{\cdot}10^{-3}$ _3	$2{\cdot}10^{-3}$ _7
Achnanthes adnata	700	667	933	323	818	900	600
Achnanthes lemmermanni	0	1500	0	0	0	0	0
Halamphora coffeiformis	592	1250	217	1158	492	1575	567
Amphora pediculus	15	0	0	200	0	50	0
Amphora sp.	150	0	0	0	0	0	200
Dolichospermum flosaquae	31	29	0	27	78	21	15
Bacillaria paxillifera	9383	9350	9367	9583	6713	7450	7817
Brebissonia lanceolata	25	50			125		50
Cocconeis pediculus	0	0	0	0	190	50	100
Cyclotella sp.	0	50	0	50	0	0	0
Diatoma moniliformis	4533	5650	4833	6250	3500	5208	5867
Diploneis didyma	25			50	123		
Diploneis interrupta	0	0	0	50	0	50	50
Entomoneis paludosa	342	383	133	325	48	267	75
Epithema sp.		100				50	100
Fallacia sp.							200
Tabularia fasciculata	13,992	12,158	10,350	6350	6472	6975	7867
Gomphonema olivaceum	50	50	75	50	0	200	175
Grammatophora marina	833	175	233	533	508	750	617
Licmophora gracilis	733	833	567	700	820	658	650
Melosira moniliformis	165	400	200	100	0	283	0
Melosira nummuloides	4325	2050	3083	1683	3006	2492	2250
Merismopedia sp.	150	2025	0	4325	200	1608	0
Navicula gregaria	913	825	783	700	1309	875	750
Navicula meniscus	50	100	150	100	0	100	0
Navicula palpebralis	0	0	0	50	0	0	0
Navicula perminuta	2050	1225	983	1067	1471	1050	1550
Navicula ramosissima	400	150	50	467	145	125	350
Nitzschia closterium	450	133	100	0	0	100	0
Nitzschia dissipata	67	50	75	50	0	92	0
Nodularia sp.	0	15	0	0	0	38	0
Pediastrum sp.	0	100	0	50	0	0	0
Pleurosigma sp.	175	125	75	50	298	0	50
Proshkinia porotzkaje	67	175	0	100	174	238	75
Rhoicosphenia abbreviata	192	183	150	475	300	667	338
Rhopalodia gibba	0	150	0	0	0	300	0
Rhopalodia brebissonii	738	483	250	517	609	750	400
Scenedesmus sp.	100	400	300	325	380	692	500
Spirulina subsalsa	11	242	10	155	7	290	6

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Instytut Oceanografii Uniwersytetu Gdańskiego

Zakład Funkcjonowania Ekosystemów Morskich

OŚWIADCZENIE

Oświadczam, że wkład w powstanie niżej wymienionej publikacji naukowej: Sylwestrzak Z., Zgrundo A. **Pniewski, F.** 2022. *Copper chloride (II) effect on the composition and structure of marine microphytobenthic communities*. Environmental Monitoring and Assessment, 194(6), pp.1-15

wchodzącej w skład rozprawy naukowej Pani Zuzanny Sylwestrzak pt. stanowił około 10% całości i obejmował:

-pomoc w opracowaniu koncepcji badań laboratoryjnych,

-współpraca przy interpretacji wyników i przygotowaniu manuskryptu.

……

dr Aleksandra Zgrundo Gdynia, 18.10.2022

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Zakład Funkcjonowania Ekosystemów Morskich

OŚWIADCZENIE

Oświadczam, że wkład w powstanie niżej wymienionej publikacji naukowej: Sylwestrzak Z., **Zgrundo A**. Pniewski, F. 2022. *Copper chloride (II) effect on the composition and structure of marine microphytobenthic communities*. Environmental Monitoring and Assessment, 194(6), pp.1-15

wchodzącej w skład rozprawy naukowej Pani Zuzanny Sylwestrzak pt. stanowił około 35% całości i obejmował:

-pomoc zaplanowaniu badań, pomoc w realizacji prac terenowych.

-współpraca w interpretacji wyników i przygotowaniu manuskryptu.

Publication 2

Sylwestrzak Z., Zgrundo A. Pniewski, F. 2021. Ecotoxicological studies on the effect of Roundup®(glyphosate formulation) on marine benthic microalgae. International Journal of Environmental Research and Public Health, 18(3), p.884.

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Article

Ecotoxicological Studies on the Effect of Roundup® (Glyphosate Formulation) on Marine Benthic Microalgae

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Abstract: Glyphosate is a very effective herbicide and the main active ingredient in Roundup®—the most extensively used herbicide in the world. Since glyphosate is highly water soluble it reaches water bodies easily in surface water runoff. This prompted us to undertake an experiment to evaluate the effects of glyphosate in Roundup® on natural communities of marine microphytobenthos. Microphytobenthos communities were obtained from the environment, and after transporting them to the laboratory and acclimatizing them, they were tested under controlled conditions. Changes in microphytobenthos composition and structure and the deteriorating condition of the cells of community-forming organisms (assessed by analyzing changes in chloroplast shape) were used to assess the impact of Roundup® on endpoints. The tests indicated that microphytobenthic communities were relatively resistant to herbicide. The species richness of the communities probably enabled them to rebuild effectively. Sensitive species were replaced by those more tolerant of glyphosate. Only at the highest glyphosate concentration (8.5 $g \cdot dm^{-3}$) tested was a strong negative effect noted that limited community abundance and eliminated some of the organisms. The dominant diatoms in the communities were replaced by intensively developing cyanobacteria, which ultimately comprised nearly 60% of all the cells observed in the communities.

Keywords: toxic effect; marine microphytobenthos; microalgal communities; Baltic Sea; algal growth inhibition test; ecotoxicological tests; glyphosate; Roundup®

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1. Introduction

Glyphosate is the most extensively used herbicide in the world. It easily reaches water bodies through surface runoff waters and this affects photosynthetic microorganism communities that are often the foundation of the functioning of aquatic ecosystems. Hence, monitoring the reactions of microorganisms to glyphosate is an important element of environmental management [1]. Since relatively little is known about the impact glyphosate has on communities of aquatic organisms, and especially those of marine organisms, the aim of this study was to assess the impact of glyphosate in the popular herbicide Roundup® on the condition of the natural microphytobenthos in the Baltic Sea.

Glyphosate is the active ingredient in non-selective herbicides that also contain many excipients, and it is an extremely effective compound that has a broad spectrum of biological activity. The popularity of the herbicide Roundup®, the main active ingredient of which is glyphosate, has grown with the proliferation of genetically modified crops [2]. Since it is believed that the combined effects of glyphosate and the expedients in products containing it are greater than those of glyphosate alone [3], we decided to assess the impact the product Roundup® itself has on the environment. The increasing potential exposure of ever larger segments of society to glyphosate and the development of molecular test methods have both contributed to a growing interest in this substance in the context of its biological activity and glyphosate metabolites [4]. The concentration of glyphosate in European surface waters varies within the range of $0.67 \cdot 10^{-7}$ $g \cdot dm^{-3}$ and $9 \cdot 10^{-6}$ $g \cdot dm^{-3}$ depending on the sampling approach and measuring methods [5].

Chemically, glyphosate is N-phosphonomethyl glycine, which is highly hydrophilic. Its solubility is 10–15.7 $g \cdot L^{-1}$ at 25°, and its half-life in aquatic environments is less than seven days [6]. Herbicides containing the active ingredient glyphosate are very effective. After entering the plant, glyphosate inhibits the production of the enzyme EPSP synthase (5-enolpyruvylshikimate-3-phosphate). Inhibiting the activity of this enzyme prevents plants from forming the aromatic amino acids that are important for their growth and that are components of many plant pigments [7]. The reduced amount or lack of photosynthetic pigments affects the structure and functioning of chloroplasts [8]. Glyphosate also causes plant desiccation [9].

Humans release many chemicals into the seas, and these can cause varying degrees of environmental deterioration. Currently, much research is being conducted to expand knowledge about the state of and threats to the marine environment, and modern techniques used in water monitoring allow for the early detection of sources that threaten water bodies. Ecotoxicological studies are one of the tools used to assess the impact of chemical substances on aquatic environments. To date, most ecotoxicological studies have been performed on monocultures and single strains [10–14]. While these studies are extremely valuable, they provide information about the reaction of organisms only within the range of the so-called potential niche. Only studies that take into account whole communities make it possible to identify more reliably organism responses that reflect processes occurring in the environment, as they also include interactions among organisms. Furthermore, it has been shown that [15,16], microorganisms maintained as monocultures undergo microevolution changing their genetic makeup and thus phenotypic features. The evidence was also provided that "in-culture" evolution can finally led to establishing a strain optimally adapted to culturing conditions loosing adaptations typical of natural populations. Consequently, laboratory studies describe responses of altered organisms non-existing in the natural environment. Therefore, in our research we conducted toxicological tests on entire microphytobenthos communities obtained from the environment. Providing information on the response of the entire ecological assemblage to a toxic substance, such glyphosate and expedients from Roundup® in high concentrations, will contribute to a better understanding of environmental responses to herbicides introduced by humans that have significant negative impacts on terrestrial plants and human itself.

2. Materials and Methods

2.1. Field Work

Experiments on the impact glyphosate has on Baltic microalgae were conducted on microphytobenthic assemblages obtained from the environment. The microphytobenthos used in laboratory studies was obtained from glass slides exposed to the waters of the Gulf of Gdańsk (southern Baltic Sea) for a period of 14 days in the summer (Figure 1A). The water temperature during incubation was approximately 17–19 °C, and salinity ranged from 7.9 to 8.4 PSU. The culture panel (100 × 40 × 10 cm) with microscope slides (76 × 26 × 1 mm) was deployed at a depth of approximately 1–2 m (Figure 1B) at a distance of about 300 m from the shore at a station located at 54°26′49″N, 8°34′24″E (Figure 1A). The panel with the microscope slides on which microphytobenthos had grown were immediately placed in large containers filled with sea water collected in situ so that the slides remained immersed in the water. These were transported carefully so they reached the laboratory undisturbed. Transport time was about 15 min, and laboratory work began immediately upon their delivery.

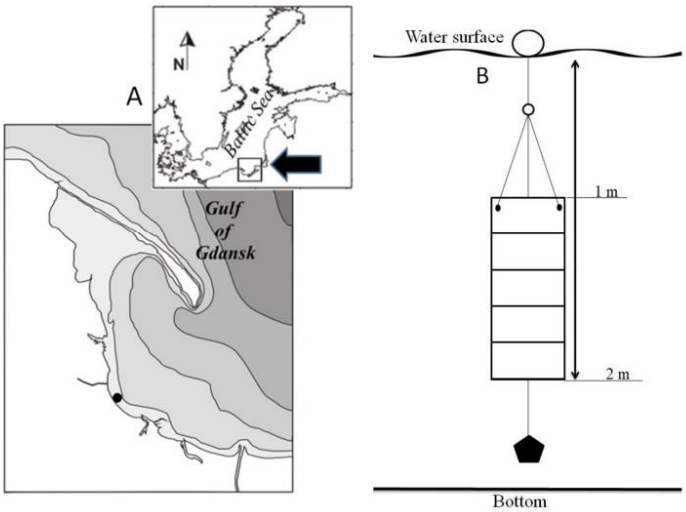

Figure 1. (**A**) Location of the station where the culture panel was exposed to the waters of the Gulf of Gdańsk. Inset shows the location of the Gulf of Gdańsk in the Baltic Sea. (**B**) Diagram of the culture panel exposed to the waters of the Gulf of Gdańsk.

2.2. Preparation of Microalgal Suspensions and Experiment Design

After transporting the panels to the laboratory, the microphytobenthic communities growing on the microscope slides were scraped off with a scalpel. The microphytobenthos collected were sonicated with an impulse force that permitted mixing the cells thoroughly but avoided weakening or destroying them [Pniewski, unpublished research]. After sonication for a period of two minutes, the microphytobenthos was placed in 250 mL flasks in 100 mL of sea water collected in situ and filtered with Whatman GF/C filters and subsequently autoclaved. The natural concentrations of the nutrient compounds in sea water were: N-NH_4 9.4 mg·m^{-3}, N-NO_3 102 mg·m^{-3}, P-PO_4 36 mg·m^{-3}, Si-SiO_4 600 mg·m^{-3}. With such high nutrient values, the decision was made not to add the culture medium to the solution because the additional nutrients would not have been used by the organisms within seven days. Additionally, excess nutrients could have caused additional stress for the organisms that comprised the microphytobenthos communities, which was something we wanted to avoid. Flasks with algal suspensions were saturated with nitrogen for 30 s to eliminate animal microorganisms following the standard method [17]. Then the communities were acclimated in a thermostatic chamber for a period of 72 h. The initial mean abundance of microalgal cells in flasks calculated was as high as 38,800 cells/mL ± 700.

After the acclimation period, the microphytobenthos was subjected to glyphosate toxicity tests as follows: control—microphytobenthos assemblage in 100 mL filtered seawater; test solutions—microphytobenthos assemblage in 100 mL filtered seawater at three solution of glyphosate (Roundup®) of 0.042 g·dm^{-3}, 0.85 g·dm^{-3}, and 8.5 g·dm^{-3}. All experimental variants were performed in three replicates. The glyphosate concentrations were selected based on current literature on the subject and results of previously carried out experiments [8,13,18–21]. The lowest concentration of glyphosate (0.042 g·dm^{-3}) was selected based on previously published values shown to cause inhibitory effects in microalgal strains typically used for ecotoxicological tests [8,17]. The concentration of 0.85 g·dm^{-3} was selected as a dose having weak but still noticeable effect on microphytobenthic communities [19,21]. The highest glyphosate concentration (8.5 g·dm^{-3}), was chosen based on the results of preliminary experiments carried out on the Baltic microphytobenthic communities and indicated as having a substantial influence on the species composition [18,20].

2.3. Microscopic Analysis

Qualitative and quantitative changes in assemblage structure, i.e., changes in taxonomic composition and taxon abundance, were the primary parameters used to assess the changes in the microphytobenthos. Observations of the assemblages preserved in Lugol solution were performed in triplicate after one, three, and seven days for 50 fields of vision in sedimentation chambers (2 mL) under an Eclipse TS100 inverted light microscope (Nikon, Tokyo, Japan at magnifications of ×200 and ×400 according to principles in Organisation for Economic Co-operation and Development (OECD) guidelines for assessing the effects of chemical toxicity on plant microorganisms [22]. In each field of vision all cells were counted and identified. Cell numbers were counted according to the Utermöhl method [23] and Helcom [24] guidelines in which units are considered to be cells or threads at 100 µm in length. The microalgae were identified using [25–32]. Additionally, analysis of the condition of microalgal cells occurring in the microphytobenthos were conducted in three replicates after one, three, and seven days by observing the state of the chloroplasts in all cells present in 50 fields of vision under a Nikon Eclipse 80i microscope fitted with a Nikon DSU2 camera at a magnification of ×400. Observations were conducted based on previously developed research methodology [8,18]. During the observations, three groups of cells were identified: live cells with normal chloroplasts, live cells with abnormal chloroplasts, and dead cells. In this article, only the results for the cell groups with normal and abnormal chloroplasts are presented (Figure 2).

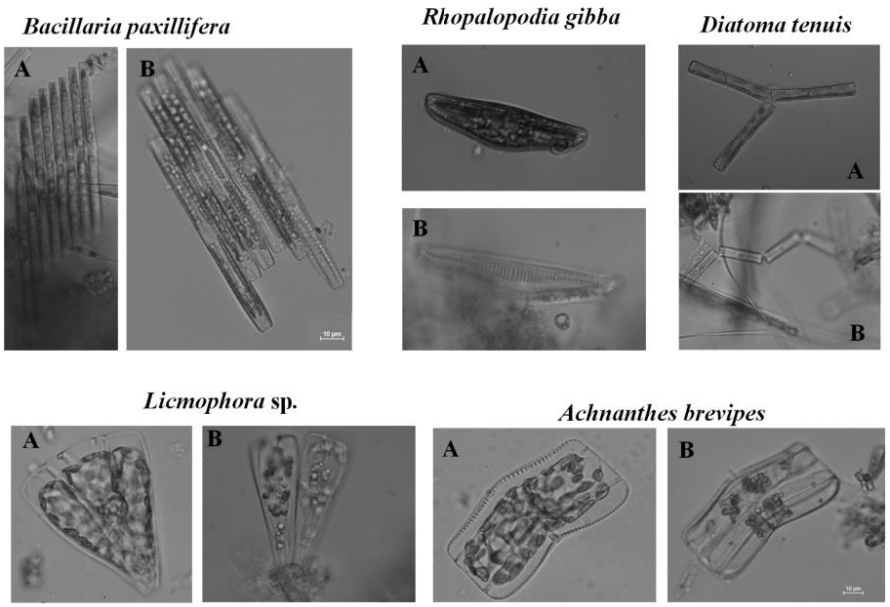

Figure 2. Cells of selected taxa with normal chloroplasts (A) and with abnormal chloroplasts (B).

2.4. 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 glyphosate concentrations and the control solution and to designate differences among successive test days with STATISTICA version 10 (StatSoft, Inc., Tulsa, OK, USA).

3. Results

3.1. Quantitative and Qualitative Analysis of Assemblages

During the experiment, a total of 58 microalgae species was identified, including 45 diatom, nine cyanobacterium, and two green alga taxa and representatives of Myzozoa (*Peridinium* sp.) and Haptophyta (*Prymnesium* sp.). The full list of taxa identified is in Appendix A (Table A1). Appendix A (Table 2) presents the mean abundance and standard deviation of selected taxa.

The highest mean abundance of 44,000 cells ± 2000 was observed at the beginning of the tests (Figure 3). Surprisingly, differences in cell abundance on days three and seven in the 0.042 g·dm^{-3} glyphosate solution were statistically insignificant and almost identical at 46% and 47% of the control samples, respectively. The smallest abundance was observed after day three in the concentration of 8.5 g·dm^{-3} glyphosate at 50% of the control cell abundance (statistically significant difference, $p > 0.05$), but at this concentration the number of cells increased to 82% of the control value on day seven of the tests.

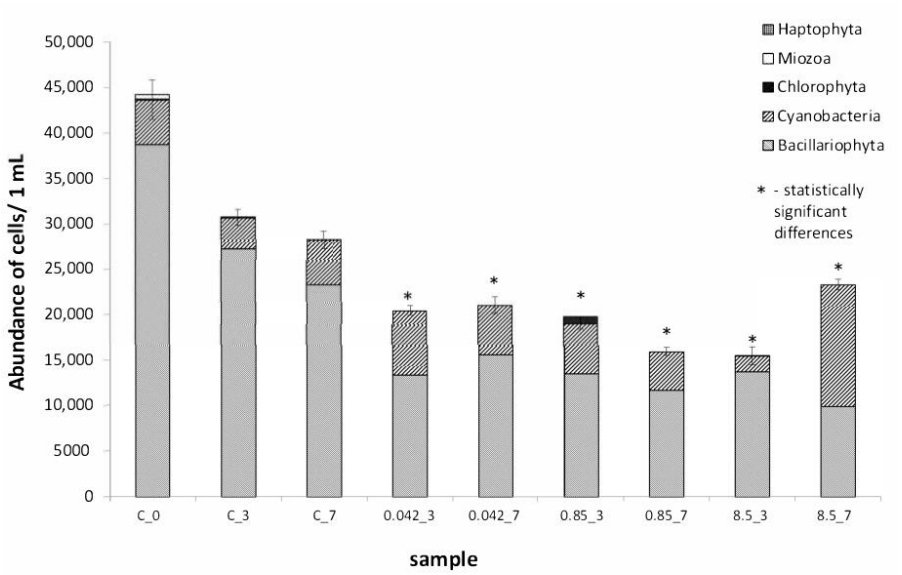

Figure 3. Abundance of microalgae in 1 mL suspension in control solutions: C_0—the start of the experiment; C_3—after three days of the test; C_7—after seven days of the test; and in the concentrations of glyphosate tested: of 0.042 g·dm^{-3} glyphosate (0.042_3) after three and (0.042_7) after seven days of tests; 0.85 g·dm^{-3} glyphosate (0.85_3) after three and (0.85_7) after seven days of tests; 8.5 g·dm^{-3} glyphosate (8.5_3) after three and (8.5_7) after seven days of tests. Statistically significant differences were denoted with the asterisk.

The microphytobenthos assemblages analyzed were dominated by diatoms, which constituted from 65 to 88% of all the cells counted (in the control solution on day three). Only on day seven at a concentration of 8.5 g·dm^{-3} did they account for 43% of total abundance. The abundance of cyanobacteria in the control solution did not exceed 18% (the most were observed on day seven of the experiment), while at concentrations of 0.042 g·dm^{-3} and 0.85 g·dm^{-3} they constituted from 26 to 35% of total abundance. The most cyanobacteria were observed on day seven of the experiment in the solution at a concentration of 8.5 g·dm^{-3} at 57%.

3.2. Abundance of Selected Taxa

The microphytobenthic communities tested were dominated by diatoms such as *Tabularia fasciculata* and *Bacillaria paxillifera* that were 18 and 17%, respectively, of the entire community at the start of the experiment. The abundance of *T. fasciculata* on day 0 was 8000 ± 93 cells/1 mL) (Figure 4A). On subsequent days of the experiment in the concentration of 0.85 g·dm^{-3} glyphosate, no significant differences were noted in changes in the number of cells of this taxon (1% more on day three and 4% less on day seven in comparison to the control solution). In the 0.042 g·dm^{-3} solution 30% fewer cells than in the control solution were observed on day three, but by day seven there were 30% more. A different reaction was observed in the highest concentration tested, and on day three abundance was observed to increase by 40%, and on day seven there were 29% more cells in the control solutions. Increases in *B. paxillifera* abundance of 69% were observed in the control solution on day three, but on day seven abundance was once again similar to that at the start of the experiment. The abundance of this species decreased in comparison to the control from 65 to 74% in all of the concentrations tested on day three and on day seven from 67 to 73% (Figure 4A). Representatives of cyanobacteria *Merismopedia* sp. contributed a large share to the communities tested; at the beginning of the experiment *Merismopedia* sp. cells were 7% of all the those observed. An increase in the numbers of these cyanobacterium cells of 27% was observed in the control solution on day seven of the experiment (Figure 4 B). The presence of glyphosate had a stimulatory effect on the growth of the numbers of *Merismopedia* sp. cells, for example, the number of cells increased by approximately four times on day three at concentrations of 0.042 g·dm^{-3} and 0.85 g·dm^{-3}. In the highest concentration tested of 8.5 g·dm^{-3} on day seven three times the number of *Merismopedia* sp. cells were observed than in the control. On the other hand, the number of units of other cyanobacterium of the genera *Spirulina* was small and did not differ in either the control or in the glyphosate solutions throughout the experiment (except for the cultures exposed to the glyphosate concentration of 0.85 g·dm^{-3} at which on day three the cell number was twice as low compared to the control solution). However, on day seven of the experiment at the concentration of 0.85 g·dm^{-3} an increased number of cells of this species was observed at 1750% of the control values (Figure 4B). *Halamphora cofeiformis* was identified as a tolerant species since small changes in numbers were observed at low glyphosate concentrations, for example, on day three numbers of it were comparable to those observed in the control solution. Only at a concentration of 8.5 g·dm^{-3} on day seven was a decrease observed in the number of cells to 56% fewer than in the control solution (Figure 4C). Some species, such as *Navicula perminuta*, turned out to be resistant to the applied Roundup® concentrations and after an initial decrease, on day seven the cell number significantly increased. At concentrations of 0.042 g·dm^{-3} and 0.85 g·dm^{-3} growth was seven and eight times, respectively, higher in comparison to the control (Figure 4C). During the experiment, decreases in the numbers of cells of especially sensitive species, such as *Diatoma tenuis*, were noted in all concentrations by approximately 40% in comparison to the control solution; however, no large differences in numbers were observed among concentrations or on subsequent days of the tests (Figure 4D). Results were similar for *Melosira nummuloides* in which the highest numbers (24% fewer cells than in the control solution) were observed on day three at the concentration of 0.042 g·dm^{-3}, while on day seven the number of cells decreased by 76%. At the concentration of 0.85 g·dm^{-3} from 65 to 85% fewer cells were noted than in the control solution, and there were 82% fewer cells of this taxon at the highest concentration tested.

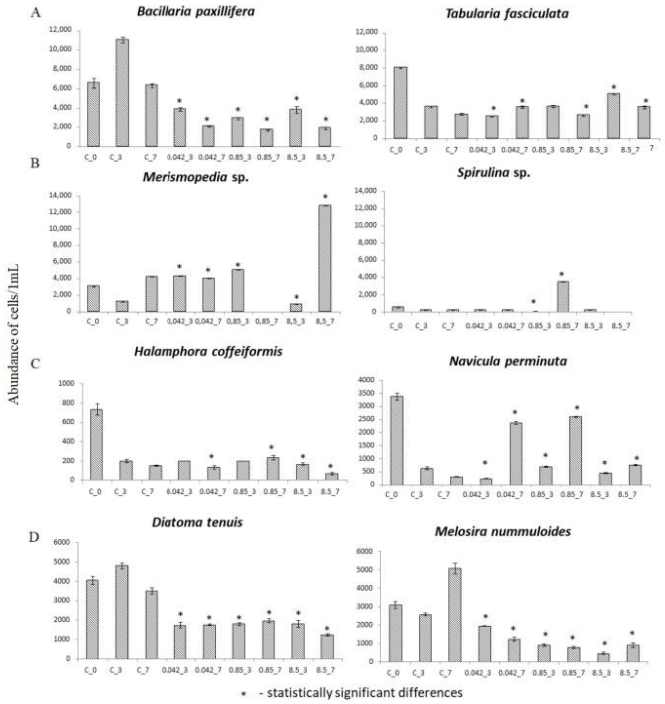

Figure 4. Abundance of cells of selected taxa in control solution: C_0—the start of the experiment; C_3—after three days of the test; C_7—after seven days of the test; and in glyphosate concentrations of glyphosate tested of: 0.042 g·dm^{-3} glyphosate (0.042_3) after three and (0.042_7) after seven days of tests; 0.85 g·dm^{-3} glyphosate (0.85_3) after three and (0.85_7) after seven days of tests; 8.5 g·dm^{-3} glyphosate (8.5_3) after three and (8.5_7) after seven days of tests. Statistically significant differences were denoted with the asterisk. (**A**)—dominant species on which glyphosate had a negative effect on growth, (**B**)—cyanobacteria in which glyphosate stimulated growth selectively, (**C**)—resistant species on which glyphosate had a positive effect on abundance, (**D**)—sensitive species on which glyphosate had a negative effect on abundance.

3.3. Cell Condition in Selected Taxa

The analysis of chloroplast condition in selected taxa indicated differences among the main dominants. *Bacillaria paxillifera* cells were in good condition, and the number of cells with damaged chloroplasts was small and did not exceed 15% of live cells at most concentrations. Only at the concentration of 8.5 g·dm^{-3} was chloroplast degradation noted in 30% of live cells on day three and in 94% on day seven. In *Tabularia fasciculata* degraded chloroplasts were observed in 30 to 40% of live cells in the control on days three and seven and at concentrations of 0.042 g·dm^{-3} and 0.85 g·dm^{-3}. On the other hand, at a concentration of 0.85 g·dm^{-3} on day seven, 45% of cells had abnormal chloroplasts, while as many as 85% did in the concentration of 8.5 g·dm^{-3} (Figure 5A). Among the cyanobacteria *Merismopedia* sp. and *Spirulina* sp. all cells examined appeared to be normal. Among the diatom species identified as resistant to the effects of glyphosate, such as *Halamphora coffeiformis* and *Navicula perminuta*, cells with deformed chloroplasts were less than 60% with the exception of cells in the concentration of 8.5 g·dm^{-3} (up to 100%). Interestingly, *N. perminuta* cells were in worse condition than, for example, those of *T. fasciculata* on day three of the experiment (Figure 5C). Evident effects of glyphosate on

chloroplasts were observed in both sensitive (*Diatoma tenuis*, *Melosira nummuloides*) and resistant diatoms mainly at the concentration of 8.5 g·dm^{-3}.

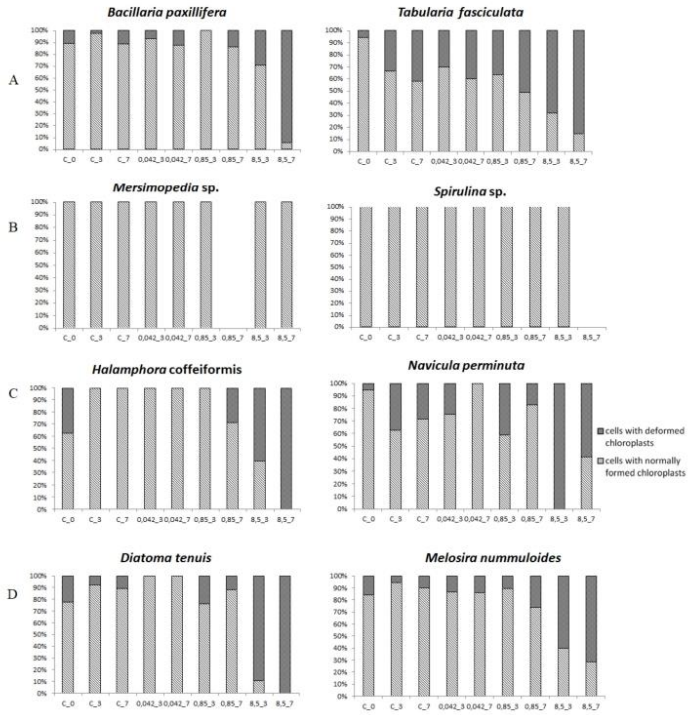

Figure 5. Changes in the condition of selected species expressed as the percentage share of cells with normal chloroplasts and abnormal chloroplasts in the control solution (**C**) and in the tested glyphosate solutions (0.042 g·dm^{-3}, 0.85 g·dm^{-3}, and 8.52 g·dm^{-3}) at the start of the experiment and after three and seven days of the tests. (**A**)—dominant species on which glyphosate had a negative effect on growth, (**B**)—cyanobacteria in which glyphosate stimulated growth selectively, C—resistant species on which glyphosate had a positive effect on abundance, (**D**)—sensitive species on which glyphosate had a negative effect on abundance.

4. Discussion

Toxicological tests on microalgae (most commonly planktonic marine diatoms) are conducted widely throughout the world [1,10,33,34]. Many of these tests are conducted on single strains, which only provide information regarding so-called potential niches. Only research that takes into consideration entire assemblages make it possible to obtain a more reliable picture of the reactions that occur among the taxa tested. To date, most tests conducted on glyphosate toxicity have focused on organisms inhabiting fresh waters [1,14], but our study concentrated on marine and brackish water organisms forming microphytobenthic communities in the southern Baltic (Gulf of Gdańsk). In our opinion, this is particularly interesting because marine coastal zones are the main areas that receive most of the pollution from surface water runoff. For example, the Gulf of Gdańsk, which is the area from which the communities used in the study were collected, is the basin that receives the waters of Poland's largest rivers, including the Vistula that collects pollutants from a surface area of 193,960 km^2, which is more than half of the country [35]. Large quantities of glyphosate potentially reach the Baltic, but studies of glyphosate in water bodies are

not conducted widely. A few researchers have performed such studies, and their results confirm that there is glyphosate in open waters [5,36]. Researchers have studied the content of glyphosate and aminomethylphosphonic acid (AMPA) (the main glyphosate metabolite) in river mouths in the Baltic Sea and revealed that there was glyphosate in most of them at concentrations ranging from $2.8 \cdot \times 10^{-8}$ g·dm^{-3} to $9 \cdot \times 10^{-5}$ g·dm^{-3}, while APMA was detected in all of them. While the half-life of glyphosate in aquatic environments under aerobic conditions is less than seven days [6], in soils its half-life ranges from two to 174 days [37]. The recommended single dose for resistant weeds is 21.6 g 100 m^2, Coupe and coworkers [38] suggested that with the water runoff form agriculture areas even (up to) 0.86% of a Roundup® dose can be directly transported into the surface waters. Thus, the concentrations used in our study can correspond to concentrations of glyphosate after applying the herbicide Roundup®. Intense precipitation, river runoff, and the fact that this compound can accumulate in soils for as many as 174 days can lead to very high concentrations of glyphosate in water bodies.

Glyphosate affects plant growth by inhibiting the production of aromatic amino acids that halts the production of protein [39]. It also inhibits the production and activity of the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthetase [40] that halts the synthesis of compounds that are important for plant growth, such as phenylalanine, tyrosine, and tryptophan, which are found in many plant pigments, flavonoids, and anthocyanins [7]. The reduced amounts or lack of photosynthetic pigments has a negative impact on the structure and functioning of chloroplasts [18]. Additionally, Roundup® contains surfactants such as isopropylamine salt (IPA) and polyoxyethylene amines (POEA) that are added to the product to increase its effectiveness [1,41]. Surfactants are often considered manufacturer trade secrets, and the precise chemical compositions and actions of these compounds are currently unknown. Therefore, only studying the effects of Roundup® as a product can answer questions about how it affects organisms and the impact it has on them.

Based on the qualitative and quantitative analyses of natural microphytobenthos communities, several groups of microalgae were identified that were the most important. Diatoms were the most abundant in communities in the control solution, they constituted, on average, about 80% of the entire community. Cyanobacteria were the second most frequently observed group of microorganisms in each sample. The remaining groups of microorganisms contributed small shares to the communities and were not permanent elements of the communities studied. Their abundance did not exceed 1.5%. An interesting relationship between diatoms and cyanobacteria was observed in the glyphosate solution at a concentration of 8.5 g·dm^{-3}; the number of cyanobacterial cells increased substantially between days three and seven. Our observations are reflected in the research of other authors. For example, Forlani [42] showed that some cyanobacteria could break down glyphosate into simpler compounds and then use them as a source of phosphorus, which might contribute to rapid increases in their numbers. These glyphosate properties and its long half-life in soils (up to 174 days) could contribute to increased concentrations of it in water bodies. Following weed suppression in spring, heavy precipitation and surface water runoff into rivers transports this substance to water bodies where it can be used as a source of phosphorus and carbon thus contributing to cyanobacterial blooms whose harmful effects are widely known. This assumptions is reflected in studies on the toxicity of various products in which the active ingredient is glyphosate. Gonzalez [1] observed increased cyanobacterial numbers and decreased numbers of *Chlorophyta* and *Bacillariophyta* in all tests performed using glyphosate at a concentration of just 0.003 g·dm^{-3}. In turn, Berman [43] observed large quantities of picoplanktonic cyanobacteria in post-agricultural areas, which were linked to the glyphosate in their soils and waters. In our tests on marine microphytobenthic communities, the intensive development of cyanobacteria (e.g., *Merismopedia* sp., *Spirulina subsalsa*) in higher glyphosate concentrations could have been caused by increased amounts of phosphorus, nitrogen, or carbon that these organisms could have obtained from glyphosate. It is interesting that during our research we observed

a significant increase in the number of *Spirulna* sp. cells on day seven in the concentration of 0.85 g·dm^{-3} that replaced the cells of another cyanobacterium—*Merismopedia* sp.

The analysis of the structure of communities exposed to glyphosate indicated that some groups of organisms were particularly resistant to this substance (e.g., *Merismopedia* sp., *Navicula perminuta*, *Entomoneis paludosa*), but there were also many other organisms in which rapid chloroplast degradation and cell growth inhibition were noted (e.g., *Navicula ramosissima*, *Diatoma tenuis*) (Table 2). The Roundup® safety data sheet states that the EC_{50} value for *Selenastrum capricornutum* for 72-h exposure is 0.014 g·dm^{-3} [37]. Glyphosate toxicity tests on *Scenedesmus quadricauda* indicated that small glyphosate concentrations (0.002 g·dm^{-3}) stimulated photosynthesis and chlorophyll *a* synthesis [11]. In our tests in a concentration trpile as high of 0.042 g·dm^{-3} on, for example, we observed increased numbers of cells in the cyanobacterium *Merismopedia* sp. implying that conditions were favorable for this organism, which meant more intense photosynthesis and chlorophyll *a* synthesis. As previously mentioned, reports in the literature indicate that Roundup® can be a source of carbon or nitrogen, and low concentrations of it can stimulate microalgal cell growth [44,45]. On the other hand, Sáenz et al. [46] revealed that at a concentration of 0.1 g·dm^{-3} it caused the total cessation of the growth of the green alga *S. quadricauda* after 96 h of exposure. In turn, Hernando [47] reported that tests conducted over seven days on *Chlorella pyrenoidosa*, another green alga species, indicated that this alga is more resistant to the effects of Roundup® since the EC_{50} for this species is 0.189 g·dm^{-3}. In our study we showed that the cells of green algae of the genus *Scenedesmus* were present on day seven of the tests at all tested concentrations (data not presented), which suggested that even very high concentrations of glyphosate did not eliminate these taxa from microorganism communities. In laboratory ecotoxicological studies conducted on the diatom species *B. paxilifera* isolated from the Baltic Sea, it was shown that a glyphosate concentration of 0.05 g·dm^3 caused a decrease in cell numbers of 51% after seven days in comparison to control conditions [13]. However, in our experiment on communities we observed growth inhibition of *B. paxilifera* on day seven of the tests of 22% at a concentration of 0.042 g·dm^{-3} and of 23% of the control value at a concentration of 0.85 g·dm^{-3}, while at a concentration of 8.5 g·dm^{-3} we observed 51% fewer cells than in the control solution. It is an interesting fact that at lower concentrations despite substantial growth inhibition few damaged chloroplast cells were noted (12 and 14%, respectively). Differences were noted with the concentration of 8.5 g·dm^{-3} in which abnormal chloroplasts were observed in as many as 94% of *B. paxilifera* cells. Because the life cycles of aquatic microorganisms are short (from one to several days), ecotoxicological studies often represent chronic toxicity even with relatively short periods of exposure of three to five days [48]. While the manufacturers of Roundup® indicate that the effects of the product are evident in target plants within seven to 10 days (yellowing and desiccation are noted that indicate, i.e., chloroplast degradation), plant death does not occur for up to three weeks [37].

Studies conducted on communities of freshwater periphyton indicated that natural communities adapted to stress factors, such as toxic substances, by altering community structure through the robust development of cyanobacteria and the inhibition of diatom growth [49]. Our observations in the current experiment were identical. Additionally, during the study we also examined the condition of chloroplasts, which indicated that usually advanced degradation only occurred at the highest concentration of 8.5 g·dm^{-3} (i.e., in *Halamphora coffeaeformis*, *Bacillaria paxilifera*, *Diatoma tenuis*, and *Melosira nummuloides*).

Based on the tests we performed, the microphytobenthos communities were relatively resistant to glyphosate. Their high species variability meant that they were able to rebuild communities. Sensitive species were replaced by ones that were more resistant to glyphosate or were able to break down glyphosate and use this compound as a source of essential nutrient salts [42]. Tsui and Chu [41] showed that the toxicity of Roundup® might not only stem from the glyphosate content, but also from isopropylamine (IPA) salts and polyoxyethylene amines (POEA). In studies of microphotoautotrophs from marine or freshwater communities, Lipok et al. [50] demonstrated that IPA salts were more

toxic than glyphosate. Although algae are more susceptible to the herbicidal effect of IPA and glyphosate salts than non-photosynthetic organisms, they are able to activate defense mechanisms so they can survive in environments in which these compounds occur. Algae have similar metabolic pathways to higher plants (e.g., they synthesize aromatic amino acids), which also makes them susceptible to glyphosate [41]. However, as already mentioned, some species, such as some cyanobacteria, can use the substances into which glyphosate decomposes. The second strategy is the robust development of resistant or tolerant organisms that can occupy the vacant niches of organisms that glyphosate eliminates. In studies conducted on monocultures in fresh and brackish waters, Tsui and Chu [41] demonstrated that not until the concentration of glyphosate (from Roundup®) was as high as 7.2 $g \cdot dm^3$ was microbiological life totally destroyed. Despite the changes in structure we observed in the present experiment, the communities were able to function in the concentration of 8.5 $g \cdot dm^3$ glyphosate, and their numbers were smaller than the control by approximately 50% on day three and 20% on day seven. Concentrations observed in the environment to date [36] are many times lower, but even they have an impact on microalgae functioning in communities. The breakdown of glyphosate produces nutrients that enrich the environment by stimulating growth in many microalgal species. Therefore, even small concentrations of glyphosate that reach the environment can disrupt species equilibrium by stimulating the growth of selected taxa, and not, as might be expected, by reducing the number of many species. However, even high concentrations (8.5 $g \cdot dm^3$) did not cause substantial degradation in the relatively rich community we studied (58 taxa), but only caused it to rebuild with the robust development of cyanobacteria. Harmful algal blooms form an increasing problem in many aquatic environments, both freshwater and marine. The massive development of cyanobacteria in the Baltic Sea is associated with increasing eutrophication. While several bloom-forming algae are toxic, non-toxic algal blooms can also have a negative impact on the environment, as depletion of O_2 and formation of toxic sulfide during bloom decaying, leading to degradation of many elements of ecosystem [51,52].

The results obtained here can be applied to all marine regions with similar salinity or trophic conditions as all the species identified in the studied assemblages are cosmopolitan and observed not only in the waters of the Gulf of Gdansk and the Baltic Sea [53–56], but also in the world oceans [30,57].

5. Conclusions

Tests performed on microphytobenthic communities revealed that they were relatively resistant to the effects of the glyphosate in Roundup®. The species richness of the communities permitted them to rebuild quickly and effectively by replacing sensitive species with tolerant and resistant ones. Organisms were divided into three groups depending on their reactions: those that were neutral to the effects of glyphosate (e.g., *Tabularia fasciculata*, *Halamphora coffeaeformis*), those that were stimulated by glyphosate (cyanobacteria such as *Merismopedia* sp. and *Spirulina* sp. and diatoms such as *Navicula perminuta*), and those that were sensitive to the presence of glyphosate (*Bacillaria paxilifera*, *Diatoma tenuis*, *Melosira nummuloides*). High glyphosate concentrations of 8.5 $g \cdot dm^{-3}$ had a negative impact on some organisms and strongly limited diatom growth. However, even this high concentration was preferred by some organisms and facilitated the mass development of cyanobacteria, which dominated the communities by day seven. The analysis of chloroplast condition indicated that their advanced degradation was usually noted at the highest concentration of 8.5 $g \cdot dm^{-3}$ (e.g., *Halamphora coffeaeformis*, *Bacillaria paxilifera*, *Diatoma tenuis*, *Melosira nummuloides*).

Author Contributions: Conceptualization, Z.S., A.Z. and F.P.; methodology, Z.S., A.Z., F.P.; software, Z.S.; validation, Z.S., A.Z. and F.P.; formal analysis, Z.S.; investigation, Z.S.; resources, Z.S. and A.Z.; writing—original draft preparation, Z.S., A.Z.; writing—review and editing, A.Z.; visualization, Z.S.; supervision, A.Z.; project administration, Z.S.; funding acquisition, Z.S., A.Z. All authors have read and agreed to the published version of the manuscript.

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

Appendix A

Table A1. List of taxa identified in the material studied.

Group of Algae	Taxon	Author
Bacillariophyta	*Achnanthes brevipes*	Bory
	Achnanthes lemmermannii	Hustedt
	Amphora ovalis	(Kützing) Kützing
	Amphora pediculus	(Kützing) Grunow
	Amphora sp.	Kützing
	Bacillaria paxillifera	(O.F.Müller) T.Marsson
	Berkeleya rutilans	(Trentepohl ex Roth) Grunow
	Brebissonia lanceolata	(C.Agardh) R.K.Mahoney & Reimer
	Chaetoceros wighamii	Brightwell
	Cocconeis pediculus	Ehrenberg
	Cocconeis placentula	Ehrenberg
	Cocconeis sp.	Ehrenberg
	Cyclotella sp.	(Kützing) Brébisson
	Cylindrotheca closterium	(Ehrenberg) Reimann & J.C.Lewin
	Diatoma moniliformis	Kützing
	Diatoma tenuis	C.Agardh
	Diatoma vulgaris	Bory
	Diploneis didyma	(Ehrenberg) Ehrenberg
	Diploneis interrupta	(Kützing) Cleve
	Encyonema prostratum	(Berkeley) Kützing
	Entomoneis paludosa	(W.Smith) Reimer
	Epithemia gibba	(Ehrenberg) Kützing 1844
	Epithemia sp.	Kützing
	Fallacia sp.	Kütz
	Gomphonella olivacea	(Hornemann) Rabenhorst
	Grammatophora marina	(Lyngbye) Kützing
	Gyrosigma sp.	Kützing

Table A1. *Cont.*

Group of Algae	Taxon	Author
	Halamphora cofeiformis	(C.Agardh) Mereschkowsky
	Licmophora sp.	C.Agardh
	Melosira moniliformis	C.Agardh
	Melosira nummuloides	C.Agardh
	Navicula gregaria	Donkin
	Navicula palpebralis	Brébisson ex W.Smith
	Navicula perminuta	Grunow
	Navicula ramosissima	(C.Agardh) Cleve
	Navicula sp.	Bory de Saint-Vincent
	Nitzschia sigma	(Kützing) W.Smith
	Opephora sp.	Petit
	Planothidium delicatulum	(Kützing) Round & Bukhtiyarova
	Pleurosigma aestuarii	(Brébisson ex Kützing) W.Smith
	Pleurosigma sp.	W. Smith
	Proschkinia poretzkajae	(Koretkevich) D.G.Mann
	Rhoicosphenia abbreviata	(C.Agardh) Lange-Bertalot
	Tabularia fasciculata	(C.Agardh) D.M.Williams & Round
	Tryblionella sp.	(Grunow)
Cyanobacteria	*Anabaena* sp.	Bory ex Bornet & Flahault
	Merismopedia sp.	Meyen
	Microcystis sp.	Lemmermann
	Nodularia sp.	Mertens ex Bornet & Flahault
	Oscillatoria sp.	Vaucher ex Gomont
	Spirulina major	Kützing ex Gomont
	Spirulina subsalsa	Oersted ex Gomont
	Woronichinia sp.	A.A.Elenkin
Myzozoa	*Peridinium* sp.	Ehrenberg
Chlorophyta	*Pseudopediastrum boryanum*	(Turpin) E.Hegewald
	Scenedesmus sp.	Meyen
Haptophyta	*Prymnesium* sp.	N.Carter

Table 2. Average abundance and standard deviations of selected taxa.

Average Abundance	C_0	C_3	C_7	0.042_3	0.042_7	0.85_3	0.85_7	8.5_3	8.5_7
Bacillaria paxillifera	6550	11,033	6333	3867	2100	2900	1700	3800	1900
Diatoma tenuis	4050	4800	3500	1733	1750	1800	1967	1800	1250
Melosira nummuloides	3080	2567	5067	1950	1233	900	767	450	900
Navicula perminuta	3367	633	300	250	2367	700	2600	450	767
Tabularia fasciculata	8033	3600	2733	2533	3567	3633	2633	5033	3533
Cylindrotheca closterium	5233	200	100	100	400	150	100	667	700

Table 2. *Cont.*

Average Abundance	**C_0**	**C_3**	**C_7**	**0.042_3**	**0.042_7**	**0.85_3**	**0.85_7**	**8.5_3**	**8.5_7**
Entomoneis paludosa	300	267	100	150	100	133	0	300	0
Merismopedia sp.	3060	1200	4200	4300	4000	5000	0	900	12,800
Halamphora cofeiformis	733	200	150	200	133	200	233	167	67
Scenedesmus sp.	1100	450	400	0	600	0	600	600	600
Spirulina major	83	100	100	100	100	100	100	100	0
Spirulina subsalsa	483	100	100	100	100	0	3400	100	0
Standard Deviations	**C_0**	**C_3**	**C_7**	**0.042_3**	**0.042_7**	**0.85_3**	**0.85_7**	**8.5_3**	**8.5_7**
Bacillaria paxillifera	476	311	200	178	66	132	70	332	98
Diatoma tenuis	208	147	156	146	49	72	116	170	64
Melosira nummuloides	201	80	297	21	110	70	64	64	121
Navicula perminuta	126	49	14	7	57	20	14	21	21
Tabularia fasciculata	93	111	85	76	133	140	85	87	188
Cylindrotheca closterium	283	0	0	0	14	21	0	46	0
Entomoneis paludosa	10	12	0	7	0	6	0	20	0
Merismopedia sp.	111	57	0	0	26	0	0	14	0
Halamphora cofeiformis	59	17	7	0	15	0	25	15	12
Scenedesmus sp.	99	7	0	0	28	28	0	0	0
Spirulina major	4	0	0	0	0	0	0	0	0
Spirulina subsalsa	99	0	0	0	0	0	0	0	0

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Instytut Oceanografii Uniwersytetu Gdańskiego

Zakład Funkcjonowania Ekosystemów Morskich

OŚWIADCZENIE

Oświadczam, że wkład w powstanie niżej wymienionej publikacji naukowej: Sylwestrzak Z., Zgrundo A. **Pniewski, F.** 2021. *Ecotoxicological studies on the effect of Roundup® (glyphosate formulation) on marine benthic microalgae.* International Journal of Environmental Research and Public Health, 18(3), p.884.072.wchodzącej w skład rozprawy naukowej Pani Zuzanny Sylwestrzak pt. stanowił około 10% całości i obejmował:

-pomoc w opracowaniu koncepcji badań laboratoryjnych,

-współpraca przy interpretacji wyników i przygotowaniu manuskryptu.

……

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Instytut Oceanografii Uniwersytetu Gdańskiego

Zakład Funkcjonowania Ekosystemów Morskich

OŚWIADCZENIE

Oświadczam, że wkład w powstanie niżej wymienionej publikacji naukowej: Sylwestrzak Z., **Zgrundo A**. Pniewski, F. 2021. *Ecotoxicological studies on the effect of Roundup® (glyphosate formulation) on marine benthic microalgae.* International Journal of Environmental Research and Public Health, 18(3), p.884.072.wchodzącej w skład rozprawy naukowej Pani Zuzanny Sylwestrzak pt. stanowił około 30% całości i obejmował:

-pomoc zaplanowaniu badań, pomoc w realizacji prac terenowych,

-pomoc w interpretacji wyników i redagowaniu manuskryptu.

Publication 3

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Article

Effects of the Ionic Liquid [BMIM]Cl on the Baltic Microphytobenthic Communities

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Abstract: Ionic liquids (IL) are regarded as the solution to the modern world's need to create and use compounds that exhibit a range of desirable properties while having a low environmental impact. However, recent reports are shattering the image of ionic liquids as environmentally friendly substances, especially in relation to the aquatic environment, revealing their potentially toxic effects. To assess the potential environmental impact of ILs, we conducted an experiment involving 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), a substance considered to be the least hazardous among the imidazolium chloride ILs, on Baltic microphytobenthic communities. Microphytobenthos collected from the environment was tested under controlled laboratory conditions, and both the cell counts and the chloroplast condition were used as endpoints. It was shown that [BMIM]Cl at concentrations of 10^{-3} and 10^{-2}, considered safe based on a cumulative impact assessment, has a negative effect on the condition of the microalgal cells and causes a reduction in population size. Although, under the influence of [BMIM]Cl, only a small proportion of the species was eliminated from the communities, only two species among those important to the communities showed resistance to this compound and eventually began to dominate the communities.

Keywords: ionic liquid; IL; [BMIM]Cl; microphytobenthos; microalgal communities; microphytobenthic communities; toxic effect; ecotoxicological test; environmental pollution

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1. Introduction

The concept of sustainable development was introduced at the end of the 20th century, and it is based on the idea of social and economic development, which assumes that while meeting the needs of contemporary societies it will not limit the development opportunities for future generations. Sustainable development assumes a parallel development of the economy, society, and the environment. In line with these assumptions, more and more environmentally friendly substances, such as ionic liquids (ILs), have started to be used in industry. These are substances that are gaining increasing recognition and researchers in many scientific fields are interested in them as they are characterized by a set of specific properties, such as low vapor pressure, non-flammability, thermal and electrochemical stability, good conductivity, and catalytic properties [1,2]. They are used in various chemical processes, where they represent a new alternative to traditional organic solvents [3]. ILs are often called 'designer solvents'; the appropriate selection of anions and cations allows for the creation of a suitable chemical compound, depending on the future application [4,5]. However, with regard to ILs' specific features (e.g., high solubility, thermal stability, and/or poor biodegradability in water), they may potentially pollute the aquatic environment [6]. Science has known of ILs as solvents since 1914 [7]. However, the first stable ILs were described in 1995 [5]. Since then, there has been a rapid increase in interest in these substances, especially in terms of their effects on human health and the environment, as in, e.g., [6–15]. A major threat from ionic liquids is their low degradation rate. For example, a 28-day experiment showed a complete lack of biodegradation of [BMIM]Cl [7]. As a result, after years of research, their "green" status has been questioned [8–10]. Inadequate

wastewater treatment, accidental spillage, or improper storage of waste contaminated with ILs can lead to the release of these substances into the environment where they subsequently cause negative effects in the ecosystem [1]. Numerous studies have proven the deleterious effects of ILs towards microalgae [16–21]. The toxicity of ILs depends on temperature and pH. Under conditions described as moderate, i.e., room temperature and pH close to neutral, these substances are stable. During industrial processes, the physicochemical conditions can change, and it has been shown that an acidic condition (pH about 3) and high temperature (of the range between 60 °C and 100 °C) accelerate the hydrolysis of the ILs, causing an increase in their toxicity. The IL used in this study was 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), which is characterized by a relatively short alkyl chain [9]. The toxicity of ILs increases with the elongation of the alkyl chain [2]; hence, the IL used here is not considered to be a highly toxic substance [22]. However, its toxicity is comparable to that of chlorinated organic substances, such as dichloromethane and chloroform; thus, it is more dangerous to the environment than common solvents of organic origin (e.g., acetone, methanol, and ethanol). Due to its properties, [BMIM]Cl is used in cellulose processing, among other areas. Although the ionic liquid itself can be recovered to a very high degree during industrial applications (such as the one mentioned above), the very process of creating the imidazole cation involves the use of large amounts of natural organic materials, energy, and solvents, causing harmful emissions to both air and water as well [7].

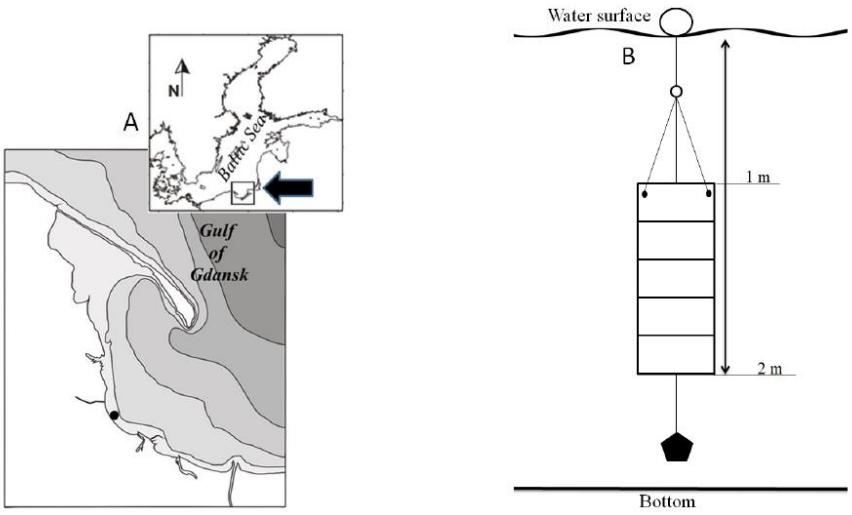
The photosynthetic organisms that form the microphytobenthos are extremely valuable elements of aquatic ecosystems due to their role as primary producers, combined with oxygen production and CO_2 reduction. Understanding the joint response of photosynthetic organisms forming a microphytobenthic community is extremely important in order to reliably estimate the changes that may occur following the introduction of this increasingly common substance into the environment and to assess the associated risks.

Monitoring the response of organisms to potentially toxic substances introduced into the ecosystem is an important part of environmental quality control. To date, investigations into the toxicity of ionic liquids have provided information on the response of single algal strains under laboratory conditions, e.g., [21–23]. Previous ecotoxicological studies conducted on marine microphytobenthic communities in the Baltic Sea have tested substances such as irgarol 1051, Sea-Nine™211 (DCOIT), and TBT (trin-butyltin) [24,25]. However, most of the studies used only communities developed at salinities typical of marine waters, i.e., 32–36 PSU. Only studies conducted to determine the impact of glyphosate and copper ions on microphytobenthic communities were performed on organisms collected from environments with salinities around 8 [26,27]. Further ecotoxicological tests on other potentially toxic substances carried out on microphytobenthic communities typical of brackish waters, which are considered species-minimum waters for macrozoobenthos and macroalgae and aquatic higher plants, but which support an abundance and diversity of planktonic microorganisms [28], are an interesting contribution to the existing knowledge. In the case of ILs, the salinity aspect is extremely important because the toxicity of ILs increases in inverse proportion to the salinity [16–21]. At high salinity values, the toxicity of ILs decreases, probably due to the reduced permeability of the microalgal cell membranes which limits the migration of harmful cations [29]. Our study was designed to provide a general picture of the response of multispecies microalgal communities to an IL considered to be of relatively low toxicity, i.e., 1-butyl-3-methylimidazolium chloride—[BMIM]Cl, under brackish water conditions, and to complement the existing knowledge on the potential risks arising from the widespread use of this substance. For that purpose, observations were made at the population level, i.e., the change in species composition and the community dominance structure were determined, and at the cell level, i.e., the condition of the chloroplasts was analyzed.

2. Materials and Methods

2.1. Study Area and Field Works

The experiment investigating effects of the IL [BMIM]Cl on microphytobenthic communities is one of a series of tests based on the identical methodology described in detail in [26]. The experiments were conducted in parallel on communities with identical species composition to allow for the comparison of the results. In brief, the study material was collected from glass slides mounted on a dedicated culture panel (Figure 1b) exposed in the Gulf of Gdańsk waters at a distance of 300 m from the shore (54°26′49″ N, 8°34′24″ E) (Figure 1a) for two weeks. During this time, the temperature and salinity changed within limited ranges, i.e., 17–19 °C and 7.9–8.4 PSU, respectively. The 2-week incubation period allowed for the acquisition of a relatively rich and diverse microphytobenthic community but was still devoid of organisms such as fouling macroalgae and fauna that will eventually dominate the surface of any substrate in marine waters in the long term.



(**a**) (**b**)

Figure 1. (**a**) Sampling site. The black dot indicates the location of the culture panel during exposure in the Gulf of Gdańsk (54°26′51″ N 18°34′33″ E). (**b**) The design of the culture panel used in this study.

2.2. Microalgal Material Preparation Procedure and Experimental Design

In the laboratory, the microphytobenthic communities were removed from the microscope slides by scraping them off with a scalpel. Subsequently, the microalgal cells were re-suspended in the seawater collected at the sampling site, which was first filtered through a glass filter (Whatman GF/C) and then autoclaved. The obtained microphytobenthos suspension was then sonicated, which allowed for the disruption and removal of cell aggregates. The sonication power was carefully chosen in order not to weaken or damage the cells [26].

The experiment was carried out in 250 mL flasks filled with 100 mL of microalgal suspension. Each microphytobenthos culture was insufflated with nitrogen for 30 s to remove heterotrophic microorganisms [30,31]. At the beginning of the experiment, the mean microalgal cell abundance was 38,800 cells/mL ± 700. Before the experiment, flasks with microalgal suspension were maintained in a thermostatic chamber for 72 h at constant light, temperature, and salinity conditions (i.e., 60 µmol photons·m^{-2}·s^{-1} with a photoperiod L:D 16:8 h, 18 °C ± 1 °C, and 8 PSU, respectively) to let the communities acclimate to the

experimental conditions. The natural concentrations of the nutrient compounds in the sea water were: N-NH_4 9.4 mg·m^{-3}, N-NO_3 102 mg·m^{-3}, P-PO_4 36 mg·m^{-3}, and Si-SiO_4 600 mg·m^{-3}. As the nutrient concentrations in the natural Baltic water were sufficiently high to maintain the microphytobenthos community during the experiment, any kind of culture medium was not added. This was also dictated by the fact that the high nutrient content could facilitate the growth of random species rapidly responding to the increase in nutrient concentrations.

After the acclimation phase, the [BMIM]Cl toxicity tests were performed according to the following design: control—microphytobenthic assemblages kept in filtered sea water without the addition of the tested IL and test solutions—microphytobenthic assemblages treated with two [BMIM]Cl concentrations, i.e., 1.13×10^{-3} g·dm^{-3} and 1.75×10^{-2} g·dm^{-3}. The lower [BMIM]Cl concentration selected for the experiment was previously proven to have significant effects on the species composition of the Baltic microphytobenthic communities [32]. The higher concentration of [BMIM]Cl was inferred from previously published values indicated as having inhibitory effects on algae [18,33]. All experimental treatments were carried out in triplicates.

2.3. Microscopic Analysis

Qualitative and quantitative changes in assemblage composition and structure, i.e., changes in taxonomic composition and taxa abundance, were the primary parameters used to assess the changes in microphytobenthos. Microscopic analysis was conducted on microalgal material preserved in Lugol solution. The observations were conducted in all cultures on the third and seventh experiment day. Fifty fields of view were checked in Utermöhl chambers (2 mL) using a Nikon Eclipse TS100 inverted light microscope (magnifications of ×200 and ×400). All cells were counted and identified as laid out in the Utermöhl method [34] and Helcom [35] guidelines (cells or threads of 100 µm length are treated as units). Species were identified using appropriate keys and floras [36–43].

The analysis of the microalgal cell condition was also performed. For this purpose, the state of chloroplasts was observed and classified in one of three classes of cells: (1) live cells with normal chloroplasts, (2) live cells with abnormal chloroplasts, and (3) dead cells. Here, the results obtained for the two first cell classes are reported (Figure 2). The microalgal cell condition was evaluated in all cells counted in 50 fields of vision under a Nikon Eclipse 80i microscope fitted with a Nikon DSU2 camera at a magnification of ×400.

2.4. Statistical Analysis

Differences between means were verified with the Student's *t*-test using STATISTICA version 10 (StatSoft Polska Sp. z o.o., Kraków, Poland). Principal component analysis (PCA) was carried out with the Canoco 5 (Microcomputer Power, Ithaca, NY, USA) [44,45] and similarity percentage (SIMPER) with the PRIMER-e (PRIMER-e, Auckland, New Zealand).

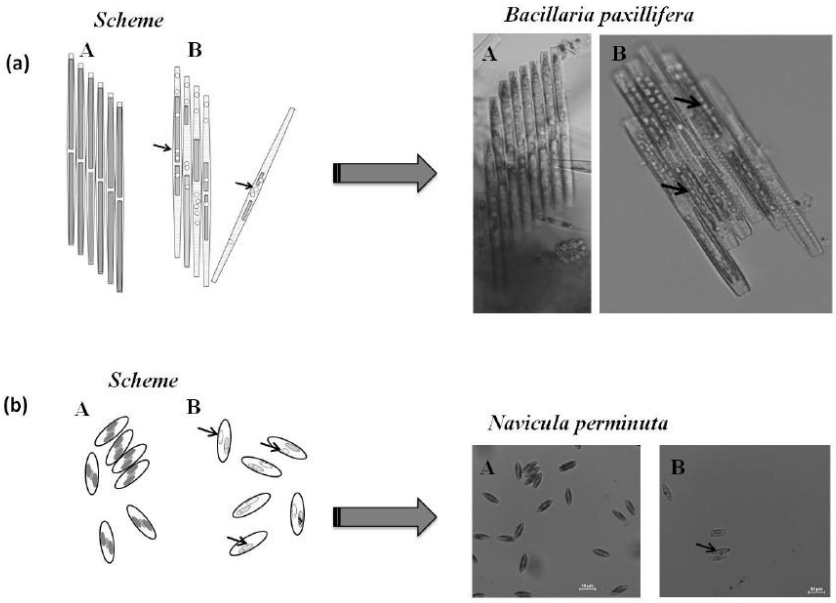


Figure 2. Examples of cells of *Bacillaria paxilifera* (**a**) and *Navicula perminuta* (**b**) with normal (A) and abnormal chloroplasts (B). Chloroplasts treated as abnormal have a deformed shape compared to the ones correctly formed. The shape of the chloroplast itself and thus its alternations are species- and/or genus-specific.

3. Results

3.1. Analysis of Taxonomic Composition and Structure

A total of 46 microalgae species were identified, including 35 diatoms, 6 cyanobacteria, and 2 green algae taxa, as well as representatives of dinoflagellates (*Peridinium* sp.) and haptophytes (*Prymnesium* sp.) (the list of all identified taxa is in the Appendix A (Table A1).

At the beginning of the experiment, the highest number of cells, 38,800 ± 700 cells/mL, was found (Figure 3). On the third day, a 47% decrease in the number of microalgae cells was observed in the concentration of 1.13×10^{-3} g·dm^{-3}, while in the concentration of 1.75×10^{-2} g·dm^{-3} [BMIM]Cl, a drop by only 21% was observed. On the seventh day of testing, a similar abundance of microalgae was observed in both concentrations, 23–26% less than in the control solution. All differences in abundance between the concentrations tested and the control solution were statistically significant ($p < 0.05$).

The microphytobenthic communities were heavily dominated by diatoms, constituting from 70% to 92% of all the observed photosynthetic microorganisms. In the control cultures, the abundance of cyanobacteria did not exceed 10% (the highest number was observed on the third day of the experiment). On the seventh day, at both [BMIM]Cl concentrations, i.e., 1.13×10^{-3} g·dm^{-3} and 1.75×10^{-2} g·dm^{-3}, they represented 23% and 28% of the total abundance, respectively. The abundance of *Prymnesium* sp. (Haptophyta) did not exceed 0.3% during the whole experiment, while the abundance of *Peridinium* sp. (Dinophyceae) in the control solution at the start of the tests was only 0.6%.

SIMPER similarity analysis showed a high similarity in community composition and structure at the control and both ionic liquid concentrations during the experiment. The average similarity was calculated as high as 71.92%. Based on the PCA analysis performed on the quantitative data, it was found that in addition to the concentration of the ionic liquid, the duration of the experiment also influenced the transformation of communities

(Figure 4). At the start of the experiment (point K_0, right part of the graph), the community was the richest (i.e., it was characterized by the highest number of species). On the third day of testing (upper left part of the graph), an increased proportion of cyanobacteria was observed (e.g., marked in graph as *cya_sp.*, *mic_sp.*, *spi_mai*, *wor_sp.*). The group of organisms dominating on the seventh day of testing (lower left part of the graph) included, among others, a diatom characterized by a relatively high resistance to ionic liquid—*Navicula perminuta* (marked as *nav_per*). However, based on the PCA analysis, it was not possible to delineate groups of organisms that were unequivocally sensitive or tolerant to the IL tested.

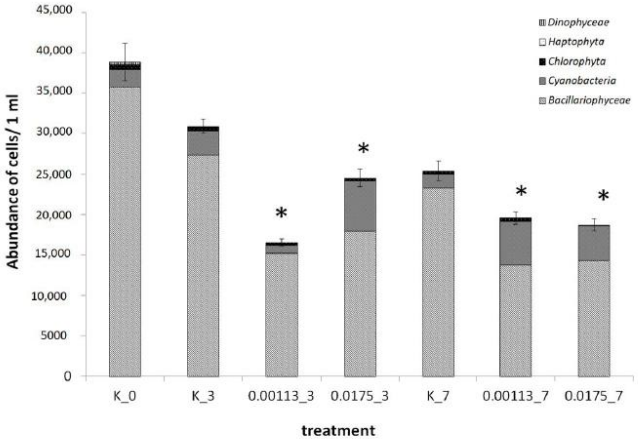


Figure 3. Abundance of microalgae. K indicates control cultures; the numbers 0.00113 and 0.0175 indicate cultures of lower (1.13×10^{-3} g·dm^{-3}) and higher (1.75×10^{-2} g·dm^{-3}) tested [BMIM]Cl concentrations, respectively. The number after the underline denotes the day of the experiment. Statistically significant differences were marked with the asterisk. In each case, the value from the experimental variant was compared with the value for the control sample on the same day ($p = 0.000002$ for 0.00113_3 vs. K_3, $p = 0.005617$ for 0.0175_3 vs. K_3, $p = 0.005178$ for 0.00113_7 vs. K_7, $p = 0.001357$ for 0.0175_7 vs. K_7).

Based on SIMPER analysis, the most important species in the communities were distinguished in terms of abundance (Appendix A, Table A2). The most abundant species was *Bacillaria paxilifera* (up to 36% on the third day in the control solution) (Figure 5). The second most abundant species was *Tabularia fasciculata* (up to 23% of all cells on the initial day of the experiment). The abundance of *Diatoma vulgaris* ranged from 9% to 17% throughout the experiment. For *Melosira nummuloides*, the highest number of cells was observed on the seventh day of testing in the control solution (20% of all cells). Interesting changes were observed in the case of *N. perminuta*; the proportion of this species in the initial community did not exceed 8%, but on the seventh day at both [BMIM]Cl concentrations, i.e., 1.13×10^{-3} g·dm^{-3} and 1.75×10^{-2} g·dm^{-3}, the share of this taxon increased to 15% and 16%, respectively. The highest share of *Cylindrotheca closterium* in the community was observed at the start of the experiment (13%), but on subsequent days the share did not exceed 8%. In the case of *Navicula gregaria*, a maximum share of 6% was observed at a concentration of 1.13×10^{-3} g·dm^{-3} on the seventh day of testing. While the share of the only representative of cyanobacteria, *Spirulina major*, did not exceed 1% during the whole experiment.

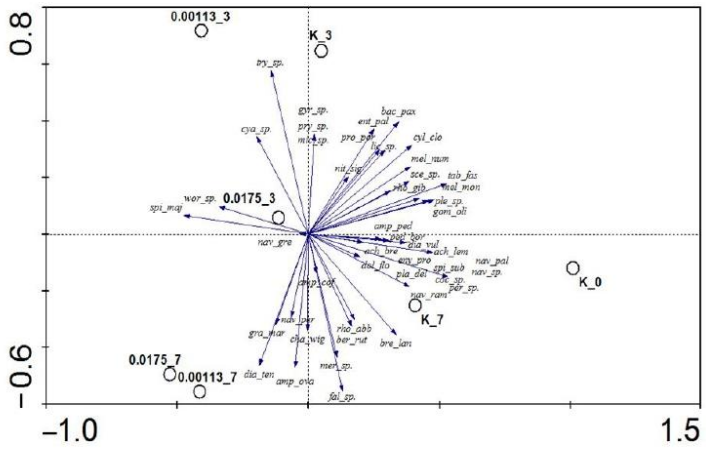


Figure 4. PCA (principal component analysis) of microphytobenthos species composition (based on the abundance data) from control and [BMIM]Cl treatments cultures. Percentage explained variation λ1 = 26%, λ2 = 22.4% λ3 = 17.9%. K indicates control cultures, 0.00113 and 0.0175 indicate cultures of lower (1.13×10^{-3} g·dm^{-3}) and higher (1.75×10^{-2} g·dm^{-3}) tested [BMIM]Cl concentrations, respectively. The number after the underline denotes the day of the experiment. Taxon codes are included in Table A1 in Appendix A.

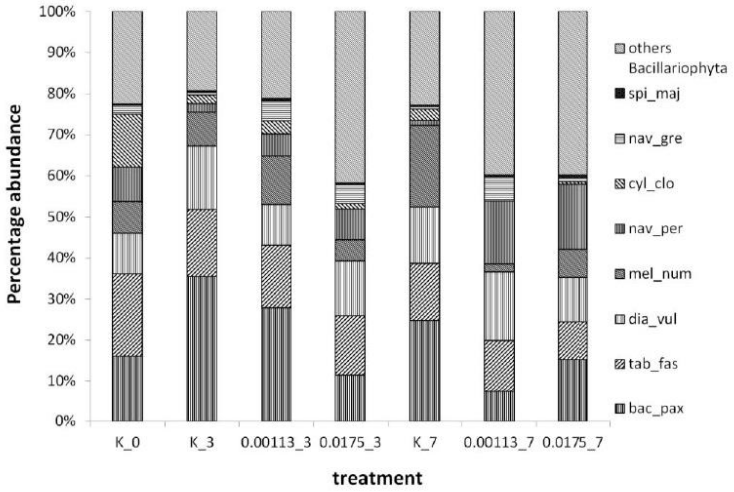


Figure 5. Percentage abundance of the 8 most important species distinguished in the tested communities based on SIMPER analysis. Labels used: spi_maj—*Spirulina major*, nav_gre—*Navicula gregaria*, cyl_clo—*Cylindrotheca closterium*, nav_per—*Navicula perminuta*, mel_num—*Melosira nummuloides*, dia_vul—*Diatoma vulgaris*, tab_fas—*Tabularia fasciculata*, bac_pax—*Bacillaria paxilifera*.

3.2. Abundance of Selected Taxa

In the presence of the IL, most of the taxa showed a statistically significant reduction in their abundance. Only for two taxa, i.e., *N. perminuta* and *Navicula ramosissima*, did the abundance in the [BMIM]Cl solutions increase compared to the control solution (Figure 6a,b). Hence, they were identified as tolerant species to this IL. The abundance

of *N. perminuta* in the control solution on the seventh day decreased by 91% relative to the start of the experiment, while in both concentrations of the ionic liquid abundances of about 89% relative to the initial abundance were recorded. On the third day, in the lower [BMIM]Cl concentration cultures (1.13×10^{-3} g·dm^{-3}), the cell abundance was 142% of the number in the control solution, while at the concentration of 1.75×10^{-3} g·dm^{-3} it was as high as 289%. On day seven, as much as a tenfold rise in the cell abundance was observed for this taxon as compared to the control solution. A similar growth stimulation was noted on day three for *N. ramosissima*, but on day seven, the cell number was only 45% and 64% of that in the control depending on the [BMIM]Cl concentration.

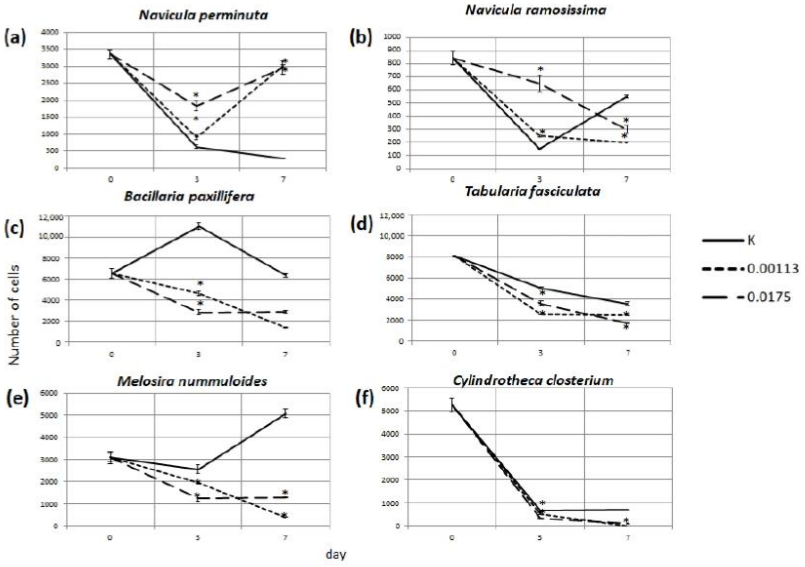


Figure 6. Number of cells of selected microalgae during experiment: K—control solution; 0.00113—the concentration of 1.13×10^{-3} g·dm^{-3} [BMIM]Cl; 0.0175—the concentration of 1.75×100^{-2} g·dm^{-3} [BMIM]Cl. Statistically significant differences between the control solution and [BMIM]Cl treatments are marked with the asterisk. (**a**,**b**)—tolerant species positively affected by the ionic liquid; (**c**–**f**)—sensitive species negatively affected by the ionic liquid.

Figure 6c–f presents changes in the abundance of selected taxa considered most important in the communities in relation to the abundance based on the SIMPER analysis. The reduction in cell numbers in the [BMIM]Cl solutions tested indicated statistically significant ($p < 0.05$) growth inhibition. Such a response was characteristic of almost all the organisms observed in the microphytobenthic communities. However, depending on the taxon and concentration used, either a gradual reduction in abundance over time (e.g., *B. paxillifera* and *M. nummuloides* in the solution of 1.13×10^{-3} g·dm^{-3} [BMIM]Cl) or a reduction in abundance followed by an initial increase (e.g., *B. paxillifera* and *M. nummuloides* in the solution of 1.75×10^{-2} g·dm^{-3} [BMIM]Cl or *T. fasciculata* in the solution of 1.13×10^{-3} g·dm^{-3} [BMIM]Cl) was observed. The most dramatic changes in the abundance of *C. closterium* were noted. In the solution of IL of 1.13×10^{-3} g·dm^{-3}, no living representatives of this species were observed on day seven, and in the 1.75×10^{-2} g·dm^{-3}, only 14% of the abundance in the control solution was noted.

3.3. Cell Condition in Selected Taxa

Analysis of the chloroplast state in the cells provided complementary information on the differences in the response of the various taxa to [BMIM]Cl. In species considered tolerant, i.e., *N. perminuta* and *N. ramosissima*, a small number of cells with abnormally shaped chloroplasts were observed (Figure 7a,b). Interestingly, in the case of *N. perminuta*, cells with abnormally shaped chloroplasts were mainly observed in the control solution (e.g., up to 38% of all cells on the third day) and were not present or were only in small proportions in the IL solutions (up to 20% in the solution of 1.13×100^{-3} g·dm^{-3} [BMIM]Cl). Similarly, in *N. ramosissima*, abnormally shaped chloroplasts were not observed in the cells at the beginning of the experiment. Deformed chloroplasts were present in the cells on the third day in the solution of 1.75×100^{-2} g·dm^{-3} [BMIM]Cl (15% of cells) and on the seventh day in the control solution (18% of cells) and in the solution of 1.13×100^{-3} g·dm^{-3} (50% of cells).

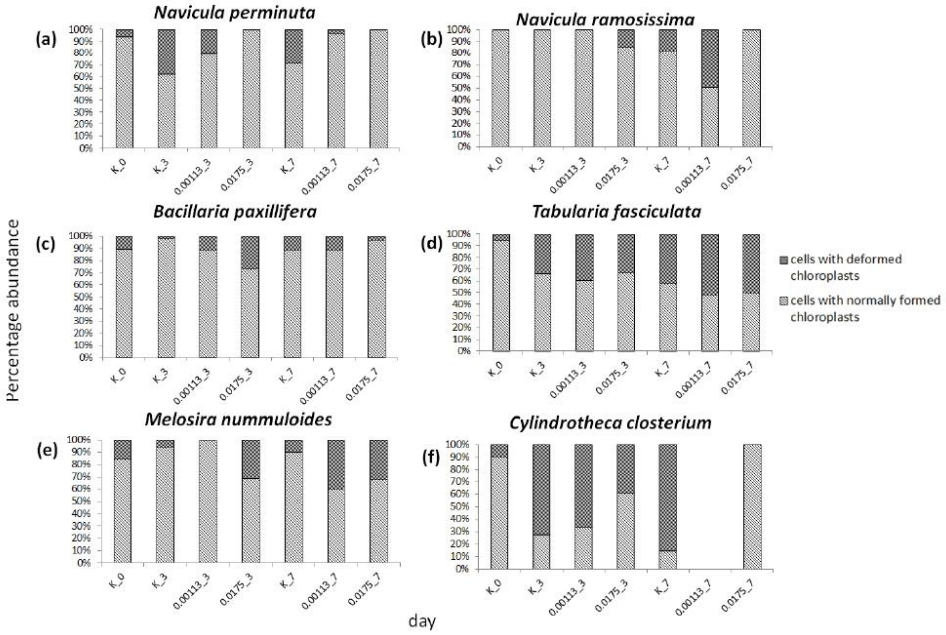


Figure 7. Condition of selected species shown as the percentage of cells with normal and abnormal chloroplasts. K indicates control cultures; 0.00113 and 0.0175 indicate cultures of lower (1.13×100^{-3} g·dm^{-3}) and higher (1.75×100^{-2} g·dm^{-3}) tested [BMIM]Cl concentrations, respectively. (**a**,**b**)—tolerant species positively affected by the ionic liquid; (**c**–**f**)—sensitive species negatively affected by the ionic liquid.

For the taxa considered sensitive, cells with deformed chloroplasts were observed irrespective of the solution and day of the experiment (Figure 7c–f). For example, in *B. paxillifera* less than 15% of cells were characterized by deformed chloroplasts. Only in the solution of 1.75×100^{-2} g·dm^{-3} [BMIM]Cl on the third day was chloroplast degradation observed in 27% of the living cells. In *T. fasciculata*, except for at the beginning of the experiment, cells with degraded chloroplasts accounted for about 30–40% of all the cells. However, the highest number of cells with degraded chloroplasts was observed on the last day of testing in both IL solutions (about 50% of all cells). Similarly, for *M. nummuloides* in the control solution and at the beginning of the experiment, cells with abnormally

formed chloroplasts made up a small proportion of the population (0–16%). In contrast, in the solution of 1.13×100^{-3} g·dm^{-3} [BMIM]Cl, up to 40% of the cells with damaged chloroplasts were observed on the seventh day and in the solution of 1.75×100^{-2} g·dm^{-3}, 31% and 32% of cells on the third and seventh day, respectively. A significantly worse cell condition was observed in *C. closterium* as compared to the previously described species. In most of the solutions tested, cells with abnormally shaped chloroplasts accounted for about 40–85% of all the cells. Only at the solution of 1.75×100^{-2} g·dm^{-3} [BMIM]Cl did all of the cells have chloroplasts of normal shape, but the population size was low.

4. Discussion

ILs as solvents with salt structures have been known since 1914 [7]. However, the first stable ILs were described in 1995 [5]. The beginning of the 21st century brought the possibility of designing chemical compounds combining required biological properties with preferred physicochemical characteristics. Currently, these substances are being studied on a massive scale, as evidenced by the number of peer-reviewed publications e.g., [6,9,10,12–14]. Furthermore, the list of their potential applications as reaction media in many industrial fields is growing [9,12]. ILs have also found applications in medicine due to their antibacterial, antifungal, anticholinergic, and local anesthetic activities and agrochemistry as bactericides, fungicides, herbicides, plant growth stimulants, or wood preservatives [9,46]. Although ILs are popular in research and economics, this does not necessarily correspond to the amount of research related to the monitoring of these compounds in the environment and the subsequent risk assessment; the number of papers focusing on the presence of ILs or their compounds in the environment remains small [10,47–50]. In one of such studies, 1310 pollutants were identified in riverine waters in Germany, among which ca. 20 different compounds belonging to ILs were detected in concentrations of up to μg·dm^{-3} [48]. In addition, in the United States, based on analyses of sediments from lakes located within the state of Minnesota, it was shown that the concentration of the IL C4-PYR was 0.053 μg·dm^{-3} [51]. In this context, one of the ILs, i.e., 1-octyl-3-methyl imidazolium, is of particular significance as it was identified not only in environmental samples [52] but also in human blood [53].

The picture is completed by the fact that imadazolium-based ILs, such as [BMIM]Cl tested here, have a low rate of biodegradation and are resistant to photodegradation [54–56]. Previous studies have shown that ILs, after eventual emission into the environment, may behave similarly to some persistent organic pollutants [57]. An extremely important aspect is also the fact that the technology to effectively remove ILs from wastewater is still being developed [4]. Hence, it is to be expected that, due to the increasing popularity of ILs, they will be used widely and, consequently, will be uncontrollably introduced into the aquatic environment, remaining there for a long time due to the difficulties associated with water treatment and their poor biodegradability. Therefore, it is of paramount importance to investigate the ILs' toxicity under environmental conditions and not only under controlled laboratory conditions [58]. Our tests on the effects of the IL [BMIM]Cl, considered to be relatively harmless [32,33,57], on the whole communities of microphytobenthos collected from the environment, allowed us to determine the response of a wide spectrum of microorganisms and not just single strains as in standard ecotoxicological tests. Changes in the tested communities at the population and cellular level show in a more reliable way the direction of the changes to which the microphytobenthos, an important component of the marine ecosystem, is subjected.

In a study using cumulative impact assessment, it was found that the [BMIM]Cl turned out to be the least hazardous among the imidazolium chloride ionic liquids with the Safe Environmental Concentration (SEC) as high as 750×100^{-3} mmol/L, which corresponds to the concentration of 1.31×100^{-3} g·dm^{-3} [59]. In this study, however, it was shown that the concentration of 1.13×100^{-3} g·dm^{-3} already reduced the abundance of dominant species by 20% to 60% within 3 days and up to 75% within 7 days. Only two diatom species of higher abundances showed resistance and gained a quantitative advantage in the studied

communities. The number of cells of *N. perminuta*, for example, increased, reaching almost 290% of the abundance in the control solution on the third and 1000% on the seventh day of the experiment. As a result, the total abundance of cells comprising the communities decreased by only 25%. Although the composition of the communities remained similar, the abundance structure changed due to the strong dominance of tolerant taxa—apart from diatoms, cyanobacteria also showed some resistance. Similar observations regarding the substitution of sensitive taxa by tolerant or indifferent taxa were found during experiments on the same Baltic microalgal communities, testing the effects of copper chloride [27] and glyphosate [26]. However, effects induced by the aforementioned substances were also manifested by a drastic shift in species composition, i.e., the increased contribution of cyanobacteria to the total community abundance.

Interesting changes were observed for the second taxon selected as resistant to the presence of the IL [BMIM]Cl—*N. ramosissima*. On the third day of the experiment, its cell numbers increased significantly, but on the seventh day, the cell abundance again decreased in the IL concentrations tested. Such a reaction may indicate depletion of the IL molecules, the presence of which has a stimulating effect on the test organisms. For example, in toxicological tests conducted by [20] on *Scenedesmus obliquus*, low concentrations of ILs have been shown to stimulate cells for biological activity (e.g., by changing the activity of catalase and superoxide dismutase). However, this may also be a response related to the competitive activity of other taxa, such as the predominant *N. perminuta*. Similarly, [21] selected from their study several species for which the IL [BMIM]Cl was practically harmless, i.e., the cyanobacterium *Anabaeana cylindrica* and the green alga *Chlorella pyrenoidosa*, and in the case of the green alga *Dunaliella salina*, they concluded that it was relatively harmless.

Typically, the reactions of taxa to toxicants tested in communities are milder than in laboratory tests conducted on monocultures [26,27,60]. In our studies, conducted on communities grown in nature, *B. paxillifera* cell counts decreased by 55% on the seventh day of the experiment in the solution of 1.75×100^{-2} g·dm^{-3} [BMIM]Cl. A very similar response was observed in toxicological tests conducted on a monoculture of *B. paxillifera* isolated from the Baltic Sea—the concentration of 1.75×100^{-2} g·dm^{-3} decreased the growth of the strain by 58% on the seventh day of testing [61]. A similarly large reduction was shown for the same concentration in *T. fasciculata* (50% reduction on the seventh day). Moreover, [19] observed an inhibition of 50% cell growth (EC50) in the planktonic diatom *Skeletonema marinoi* at the concentration of 0.1 mM [BMIM]Cl (1.745×100^{-2} g·dm^{-3}). In turn, for the green alga *Chlorella pyrenoidosa*, 50% inhibition of growth (IC50) was shown for the concentration of 21.4×100^{-2} g·dm^{-3} [21]. The phenomenon of the same species reacting the same way to a substance regardless of how it is cultured and tested (individually or in communities) may indicate that the communities as a whole, but also the individual components of the community, do not necessarily have mechanisms to protect them from the toxic effects of the IL under testing.

Exposure time was also shown to be a variable having an effect on the action of the IL because, as reported by [62], excessive accumulation of the IL in microorganisms increases its effect. In the case of *M. nummuloides*, which was part of the tested community in our study, a significant increase in cell number was observed in the control solution during the experiment, while on the third day at the [BMIM]Cl solution of 1.75×100^{-2} g·dm^{-3} 50% fewer cells were observed, while on the seventh day the abundance decreased to 8% of the abundance in the control solution. A similarly rapid abundance reduction response to [BMIM]Cl was observed for the green alga *Dunaliella salina* [21]. This implies that there is a group of sensitive species in natural marine phytobenthic communities that may be rapidly eliminated from the environment while exposed to ILs, e.g., the aforementioned *M. nummuloides* or *C. closterium*.

The cell condition index used in our study, which consists of an assessment of the chloroplast state, confirmed the observations based on cell abundance. In the species considered sensitive, the percentage of cells with deformed chloroplasts was much higher than in the resistant species, e.g., up to a half of the observed cells of *T. fasciculata* on the

seventh day of testing had degraded chloroplasts at both solutions of [BMIM]Cl. The negative effect on the chloroplast condition was confirmed by toxicity studies on five ILs ([Cnmim]Cl, n = 6, 8, 10, 12, 16). Ultrastructural morphology performed during the study revealed IL negative effects on various cellular structures, e.g., chloroplast grana became loose and mitochondria and their intermembranes swelled [22]. Other studies also confirmed that chloroplast damage can be considered as an indicator of microalgal degradation [27,63].

Tests carried out on the Baltic Sea microphytobenthic communities from the Gulf of Gdańsk made it possible to assess the effect of the IL [BMIM]Cl on the microorganisms comprising this formation. The study showed that 1-butyl-3-methylimidazolium chloride is a relatively harmful substance for the entire community, which contradicts the assessment carried out by reports based on cumulative impact assessment [47,59,64]. Thus, we have confirmed that even ILs considered to be of relatively low hazard have a significant impact on the aquatic environment. Hence, we are convinced that this group of compounds requires special attention in the context of testing its effects on different ecosystem components, its bioaccumulation, and its fate in the environment, as suggested recently by a growing group of authors [9,11,64].

5. Conclusions

During this study, the toxic influence of [BMIM]Cl on the marine microphytobenthic communities was demonstrated. The majority of species comprising the tested community reacted negatively to the presence of [BMIM]Cl at concentrations between 10^{-3} and 10^{-2} g·dm^{-3}, with a reduction in cell abundance and a deterioration in cell condition. Only in the case of two dominant diatom species, *N. perminuta* and *N. ramosissima*, was a stimulation of growth observed. In conclusion, the IL [BMIM]Cl on a short time scale contributes to a reduction in the abundance of species representing diverse taxonomic groups, which translates into the decrease in the total abundance and biomass of the microphytobenthic communities. However, despite the elimination of individual taxa, it does not lead to the degradation of entire communities but to their transformation into communities strongly dominated by a few resistant taxa.

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Appendix A

Table A1. List of taxa identified in the studied microphytobenthic communities with codes used in statistical analysis.

Group of Organisms	Taxon Code	Taxon Name	Author
Bacillariophyta	*ach_bre*	*Achnanthes adnata*	Bory
	ach_lem	*Achnanthes lemmermannii*	Hustedt
	amp_ova	*Amphora ovalis*	(Kützing) Kützing
	amp_ped	*Amphora pediculus*	(Kützing) Grunow
	bac_pax	*Bacillaria paxillifera*	(O.F. Müller) T. Marsson
	ber_rut	*Berkeleya rutilans*	(Trentepohl ex Roth) Grunow
	bre_lan	*Brebissonia lanceolata*	(C. Agardh) R.K. Mahoney & Reimer
	cha_wig	*Chaetoceros wighamii*	Brightwell
	coc_sp.	*Cocconeis* sp.	Ehrenberg
	cyl_clo	*Cylindrotheca closterium*	(Ehrenberg) Reimann & J.C. Lewin
	dia_ten	*Diatoma tenuis*	C. Agardh
	dia_vul	*Diatoma vulgaris*	Bory
	eny_pro	*Encyonema leibleinii*	(C. Agardh) W.J. Silva, R. Jahn, T.A.V. Ludwig, & M. Menezes
	ent_pal	*Entomoneis paludosa*	(W. Smith) Reimer
	fal_sp.	*Fallacia* sp.	Kützing
	gom_oli	*Gomphonella olivacea*	(Hornemann) Rabenhorst
	gram_mar	*Grammatophora marina*	(Lyngbye) Kützing
	gyr_sp.	*Gyrosigma acuminatum*	(Kützing) Rabenhorst
	amp_cof	*Halamphora coffeiformis*	(C. Agardh) Mereschkowsky
	lic_sp.	*Licmophora gracilis*	(Ehrenberg) Grunow
	mel_mon	*Melosira moniliformis*	C. Agardh
	mel_num	*Melosira nummuloides*	C. Agardh
	nav_gre	*Navicula gregaria*	Donkin
	nav_pal	*Navicula palpebralis*	Brébisson ex W. Smith
	nav_per	*Navicula perminuta*	Grunow
	nav_ram	*Navicula ramossisima*	(C. Agardh) Cleve
	nav_sp.	*Navicula* sp.	Bory
	nit_sig	*Nitzschia sigma*	(Kützing) W. Smith
	pla_del	*Planothidium delicatulum*	(Kützing) Round & Bukhtiyarova
	ple_sp.	*Pleurosigma* sp.	W. Smith
	pro_por	*Proschkinia poretzkajae*	(Koretkevich) D.G. Mann
	rho_abb	*Rhoicosphenia abbreviata*	(C. Agardh) Lange-Bertalot
	rho_gib	*Rhopalodia gibba*	(Ehrenberg) O. Müller
	tab_fas	*Tabularia fasciculata*	(C. Agardh) D.M. Williams & Round
	try_sp.	*Tryblionella*	W. Smith
Cyanobacteria	*dol_flo*	*Dolichospermum flosaquae*	(Brébisson ex Bornet & Flahault) P. Wacklin, L. Hoffmann & J. Komárek
	cya_sp.	*Cyanobacteria*	
	mer_sp.	*Merismopedia* sp.	(Turpin) Meneghini
	mic_sp.	*Microcystis* sp.	Lemmermann
	spi_maj	*Spirulina major*	Meyen
	spi_sub	*Spirulina subsalsa*	Oersted ex Gomont
	wor_sp.	*Woronichinia* sp.	A.A. Elenkin
Chlorophyta	*ped_bor*	*Pseudopediastrum boryanum*	(Turpin) E. Hegewald
	sce_sp.	*Scenedesmus* sp.	Meyen
Dinophyceae	*per_sp.*	*Peridinium* sp.	Ehrenberg
Haptophyta	*pry_sp.*	*Prymnesium* sp.	N. Carter

Table A2. Results of the similarity analysis calculated with SIMPER. Average similarity: 71.92%.

Species	Av. Abundance	Av. Similarity	Sim/SD	Contribution%	Cumulative%
Bacillaria paxillifera	6.06	6.13	8.84	8.52	8.52
Tabularia fasciculata	5.93	6.06	9.60	8.43	16.94
Diatoma vulgaris	5.73	5.89	9.08	8.19	25.13
Melosira nummuloides	5.09	5.02	7.19	6.98	32.12
Navicula perminuta	4.94	4.84	5.10	6.73	38.85
Merismopedia sp.	5.11	4.50	1.85	6.26	45.10
Halamphora coffeiformis	3.58	3.56	6.10	4.94	50.05
Navicula gregaria	3.73	3.52	4.99	4.90	54.94
Navicula ramossisima	3.54	3.40	6.27	4.72	59.67
Cylindrotheca closterium	3.99	3.17	1.78	4.40	64.07
Grammatophora marina	3.23	3.16	7.58	4.40	68.47
Spirulina major	2.38	2.55	7.16	3.55	72.01

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Zakład Funkcjonowania Ekosystemów Morskich

OŚWIADCZENIE

Oświadczam, że wkład w powstanie niżej wymienionej publikacji naukowej: Sylwestrzak, Z., Zgrundo, A., **Pniewski, F.**, 2022. *Effects of the Ionic Liquid [BMIM] Cl on the Baltic Microphytobenthic* Communities. Journal of Marine Science and Engineering, 10(9), p.1223. wchodzącej w skład rozprawy naukowej Pani Zuzanny Sylwestrzak pt. stanowił około 5% całości i obejmował:

-pomoc w interpretacji wyników i redagowaniu manuskryptu.

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dr Aleksandra Zgrundo Gdynia, 18.10.2022

Instytut Oceanografii Uniwersytetu Gdańskiego

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OŚWIADCZENIE

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-pomoc zaplanowaniu badań, pomoc w realizacji prac terenowych.

-współpraca w interpretacji wyników i przygotowaniu manuskryptu.

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ORIGINAL ARTICLE

Influence of short periods of increased water temperature on species composition and photosynthetic activity in the Baltic periphyton communities

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Abstract
Periphyton plays a vital ecological role in shallow, well-lit ecosystems which are vulnerable to rapidly changing environmental conditions, including raising temperature due to global warming. Nevertheless, little is known on the effect of increased temperatures on the taxonomic structure and functioning of periphytic communities. In this study, the influence of short-term temperature increase on the species composition and photosynthetic activity of the Baltic periphytic communities was investigated. The collected communities were exposed to increased temperature of 23 °C (ca. 4 °C above the summer average) for 72 h. After this time, species composition of the communities was studied under light microscope and their photosynthetic performance was evaluated using PAM fluorometry. Results showed that the biomass of cyanobacteria slightly increased. There were significant changes in the abundance of diatom species, among which *Fragilaria fasciculata* and *Navicula ramosissima*, were negatively affected by the elevated temperature and their cell number significantly decreased, whereas, *Diatoma moniliformis* and *N. perminuta* were stimulated by the increased temperature. Additionally, a shift towards higher abundance of smaller taxa was also observed. The higher quantum yield of photosystem II (PSII) (higher ΦPSII) accompanied by the lower value of non-photochemical quenching (NPQ) observed in communities kept at 23 °C showed more efficient photosynthesis. This was further confirmed by the changes in rapid light curves (higher photosynthetic capacity, $rETR_{max}$, and photoacclimation index, E_k). The obtained data constitute evidence that short periods of increased temperature significantly affect the structure and functioning of the Baltic periphyton.

Keywords Periphyton · Chlorophyll fluorescence · Diatoms · Temperature · Baltic Sea

Introduction

Periphyton assemblages (algal biofilms) can be found on a variety of substrata submerged in water. They can thrive on a solid surface-water interface by excreting extracellular polymeric substances. Mature periphytic assemblages have three-dimensional structure, including taxa of different growth forms (Tuji 2000; Gulzar et al. 2017). Periphyton can be responsible for majority of primary production especially in shallow well-lit habitats, in such aquatic environments as lakes, rivers, coastal waters etc. (Dodds et al. 1999). It is also an important source of food for invertebrates (Gulzar et al. 2017). Interacting with the surrounding environment periphyton affects a concentration of nutrients and provides oxygen (Gaiser 2009). Furthermore, periphyton, due to its short life cycle, quickly responds to pollution and changes in environmental conditions, and thus it is often used as an indicator of water quality (Gulzar et al. 2017).

Nowadays, the environment is undergoing a dramatic change due to global warming, including not only continually increasing temperatures but also the frequency and intensity of climate extreme phenomena such as heat waves (Vieira et al. 2013). Recent studies proved temperature increase in the Baltic and have shown that in summer the average temperatures of ca. 19 °C in the Southern Baltic are recorded. However, occasionally short periods (several days) of temperatures above 23 °C can be also observed (http://www.satbaltyk.pl) (Siegel et al. 2006; Bradtke et al. 2010; Woźniak et al. 2011; Rak and Wieczorek 2012; Stramska

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and Białogrodzka 2015). Despite the ecological importance of periphyton, little is known on the influence of increased temperature on its structure and functioning. The analyses of diatom dominated benthic communities from intertidal mudflats have shown that transient higher temperatures stimulated its photosynthesis, whereas, prolonged high temperature exposer led to the biomass decrease, photosynthesis impairment and changes in species composition promoting growth of cyanobacteria (Hicks et al. 2011; Vieira et al. 2013; Cartaxana et al. 2015). In subtidal systems a shift towards heterotrophic benthic communities was also recorded (Hancke and Guld 2004). Such strong effects observed for benthic microalgae suggest that periphyton may also undergo significant temperature-driven changes.

Thus, the aim of this study was to investigate the influence of short-term temperature increase on the structure and photosynthetic activity of the Baltic periphytic communities. In this study, species composition of periphyton kept at control (18 °C) and elevated (23 °C) temperatures was investigated and their photosynthetic activity was assessed by means of variable chlorophyll fluorescence.

Materials and methods

Sampling site, microalgal suspension preparation and experimental setup

Periphytic communities were collected from outdoors cultivation panels (Sylwestrzak et al. 2018) submerged at the depth of ca. 2 m in the littoral zone of the Gulf of Gdańsk, ca. 300 m from the shore near Sopot (the Baltic, 54°26′N, 18°33′E). Periphyton was grown on glass slides (2 cm × 8 cm) for a week in July 2015. After the exposition period, panels were collected and transported to the laboratory where microalgae were gently scrapped from the glasses and resuspended in the Baltic water of salinity ca. 8 collected at the sampling site and filtered through the sterile membrane filters with pore size of 0.45 μm (Millipore Sterile HA Filters). To prepare periphyton cultures 100 ml Erlenmeyer flasks were filled with 50 ml of microalgal suspension. Subsequently, they were kept under constant irradiance (provided by fluorescent lamps Phillips 40 W) and temperature conditions, i.e. 60 μmol photons m^{-2} s^{-1} (as measured by a quantum meter LiCor LI-198 with cosine collector) in 16 h light:8 h dark cycles and 18 °C (a water temperature measured during the sampling), for three days. After acclimatization period, on the starting day of the experiment species composition, photosynthetic pigment content and photosynthetic activity was analyzed in three randomly chosen out of six periphyton cultures. Subsequently, the cultures were divided into two groups; one of them was moved to higher temperature, i.e. 23 °C, whereas the second one was further kept at 18 °C. Both groups of cultures were incubated under experimental conditions for another 3-day period. During this time light conditions remained the same. All measurements were carried out in triplicates.

The study of periphyton species composition

Species composition of each replicate was studied under light microscope Nikon 80i equipped with DS-U2 camera using 40× objective. In order to establish the relative abundance of cyanobacteria and microalgae at least 300 cells were counted. The biovolume of counted species was calculated according to Olenina et al. (2006). Subsequently, the biomass (wet weight) of each taxonomic group was derived based on an assumption of a plasma density of 1 g cm^{-3} across all taxa (HELCOM 2013). To study diatoms permanent slides were prepared; periphyton samples were treated with hydrogen peroxide at 30–90 °C for 3 – 6 h, then rinsed with water, mounted in Naphrax (Battarbee 1986) and analyzed with the same microscope under 100× oil immersion objective, counting at least 300 frustules.

Photosynthetic pigment analyses

Ten ml aliquots of periphyton samples were filtered through GF/C Whatman glass filters (25 mm diameter) under low vacuum then frozen and stored at −20 °C until further processing. Pigments were extracted using 90% acetone as described in Pniewski et al. (2015). Subsequently, pigments were analyzed using Waters HPLC system equipped with Water 2998 Photodiode Array Detector. Pigments were separated using reverse phase chromatography (RP-HPLC) (250 mm × 4.6 mm LiChrospher®100 RP-100 endcapped column) following optimized protocol by Stoń and Kosakowska (2002). The HPLC system was calibrated using pigment standards purchased from The International Agency for 14C Determination DHI Institute for Water and Environment in Denmark. Pigments were identified from their retention times and absorbance spectra, and quantified according to the procedure provided by Mantoura and Repeta (1997).

Measurements of chlorophyll a fluorescence

Measurements of chlorophyll *a* fluorescence were carried out using a computer-operated Fluorescence Monitoring System (FMS1; Hansatech, Norfolk, UK). The device provides amber light with emission maximum at 594 nm to excite fluorescence and a PIN-photodiode at wavelengths beyond 700 nm to detect it. An integral halogen lamp (8 V/20 W) provides actinic as well as saturating irradiance. Light was measured with a Li-Cor LI-189 quantum-meter with a cosine collector. All measurements were made with 5.5-mm-diameter Fiberoptic kept perpendicularly to the biofilm at the constant distance of 4 mm.

Similarly as with pigment analysis, five ml aliquots of periphyton cultures were filtered through GF/C Whatman glass filters (6 mm diameter) under low vacuum and placed in the thermoregulated DW2 chamber. Periphyton samples were dark-adapted for 15 min to measure the maximum quantum yield (Fv/Fm) of photosystem II (PSII) (Genty et al. 1989). Subsequently, samples were illuminated with the actinic light of 60 μmol photons m^{-2} s^{-1} for 8 min in order to reach a steady-state and the effective quantum yield of PSII in the light-adapted state (ΦPSII) and non-photochemical quenching (NPQ) were measured. After the light period, samples were exposed to 9 increasing light intensities from 10 to 1280 μmol photons m^{-2} s^{-1} in order to construct rapid light curve (RLCs) with light duration step of 10 s and at each light step the relative electron transport rate (rETR) was calculated (Ralph and Gademann 2005; Lefebvre et al. 2011). To quantitatively compare RLC curves empirical data were mathematically fitted to the model of Jassby and Platt (1976) and photosynthetic parameters, i.e. the maximum relative electron transport rate ($rETR_{max}$), the initial slope of the rETR vs. E response curve (α), the light saturation index (E_k) were estimated (Sakshaug et al. 1997).

Statistics

To compare the mean values of the analyzed parameters Student t-test was used. First, the means calculated on the starting and the last day of the experiment for cultures kept at 18 °C were compared to track statistically significant changes occurring due to the incubation period itself. Subsequently, the mean values of all parameters calculated for periphytn cultures maintained at different temperatures, i.e. 18 and 23 °C, on the third day of the incubation period were compared to assess the effect of temperature on the studied assemblages. All statistical analyses were performed using Statistica 10 (StatSoft Inc., USA).

Results

After the 3-day acclimatization period the biomass of the communities, as measured by the chlorophyll *a* (Chl*a*) concentration, stabilized (ca. 0.36 μg Chl*a* ml^{-1}). There was no change in community biomass (0.36 ± 0.02 μg Chl*a* ml^{-1}) at the lower temperature (18 °C) on the third day of cultivation period compared to the starting day. In communities exposed to the higher temperature (23 °C) Chl*a* concentration only slightly decreased (0.30 ± 0.06 μg Chl*a* ml^{-1}) compared to the communities maintained at the lower temperature (Student t-test; $p > 0.05$). Furthermore, there were slight, but statistically not significant, changes regarding species and photosynthetic pigment composition as well as parameters estimated to describe periphyton photosynthetic activity in communities kept at the lower temperature compering the starting (K 18 °C) and the last day of the experiment (E 18 °C). Clear differences emerged only when the communities from different temperature treatments were compared at the end of the experiment (Student t-test; $p < 0.05$).

Diatoms dominated in the studied assemblages contributing more than 91% to their biomass. The remaining biomass was due to the cyanobacterial input and with the increase in temperature the ca. 2.5-fold increase in cyanobacterial biomass form 3.8% at 18 °C to 8.4% at 23 °C was observed. Different temperature conditions altered the composition and structure of the assemblages. The total number of 29 species was identified in the samples. Under each experimental treatment species richness was 20–28. Among the identified species cyanobacteria were represented by three genera, i.e. *Anabaena*, *Merismopedia* and *Spirulina*. The remaining species were diatoms of which twelve species constituted more than 90% of the cell count (Fig. 1). The diatom composition showed that at both temperatures assemblages were dominated by species of medium size (1000–5000 $μm^3$), 84 and 77.5% at 18 °C and 23 °C, respectively, and at the elevated temperature the proportion of smaller diatoms (with volume below 1000 $μm^3$) significantly increased (by ca. 7%) up to 17.7% (Student t-test; $p > 0.05$). Diatoms of bigger size (>5000 $μm^3$) occurred less frequently and their abundance remained relatively constant. The most abundant species were: *Bacillaria paxillifera* (O.F.Müller) T.Marsson 1901, *Diatoma moniliformis* (Kützing) D.M.Williams 2012, *Frgailaria fasciculata* (C.Agardh) Lange-Bertalot 1980, *Navicula perminuta* Grunow in Van Heurck 1880, *N. ramosissima* (C.Agardh) Cleve 1895. Among them, the diatom *B. paxillifer* was not

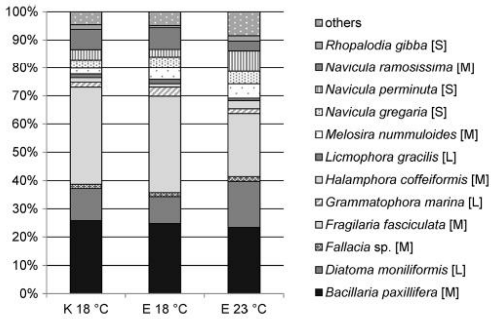

Fig. 1 Diatoms species composition of the periphyton communities; K 18 °C – the initial community observed on the starting day of the experiment, E 18 °C – the community developed after 3-day incubation period at the temperature of 18 °C, E 23 °C – the community developed after 3-day incubation period at the temperature of 23 °C. Letters denote the size class of species; S – small (<1000 $μm^3$), M – medium (1000–5000 $μm^3$), B – large (>5000 $μm^3$)

affected by temperature and its abundance remained stable (ca. 24%). Two species, i.e. *F. fasciculata* and *N. ramosissima*, were negatively affected by the elevated temperature and their cell number significantly decreased (Student t-test, $p < 0.05$), whereas *D. moniliformis* and *N. perminuta* were stimulated by temperature and their amount increased (Student t-test, $p < 0.05$).

Regarding the fact that the changes in the species composition of communities kept at the lower temperature (18 °C) throughout the experiment were strongly limited, there were no statistically significant changes in their photosynthetic performance also (Fig. 2, Table 1). Comparing lower and higher temperature treatments it was shown that Fv/Fm changes were not statistically significant. Samples kept at the higher temperature showed higher values of the quantum yield of PSII (ΦPSII), whereas in case of the non-photochemical quenching the reverse pattern was observed and its 2-fold decline was recorded. The analysis of RLCs showed that higher temperature increased periphyton photosynthetic activity (Fig. 2, Table 1). The $rETR_{max}$ values increased by ca. 34%, whereas α declined by ca. 5%. The behavior of both aforementioned parameters determined the change in the E_k value causing its increase at higher temperature conditions reaching 272 μmol photons m^{-2} s^{-1}. Changes in variable fluorescence, and by extension in fluorescence parameters and RLCs, are dependent on the activity of xanthophyll cycles. The xanthophyll pool ((Dt + Dd)/Chl*a*) varied slightly during the experiment; at the higher temperature (E 23 °C) (Dt + Dd)/Chl*a* decreased by ca. 10% (Student t-test, $p < 0.05$) compared to the lower one (E 18 °C), whereas the de-epoxidation state of xanthophylls (Dt/Dt + Dd) decreased by ca. 20% (Student t-test, $p < 0.05$) (Fig. 3).

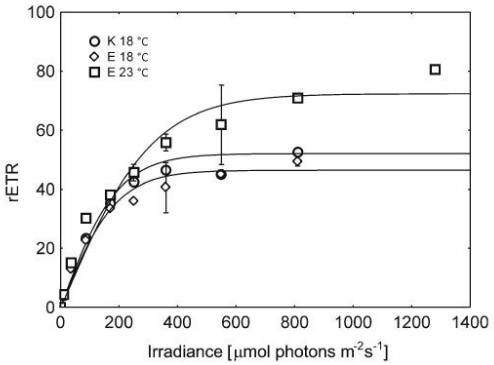

Fig. 2 Temperature-driven changes in the shape of RLCs (rapid light curves) measured for the periphyton communities; K 18 °C – the initial community observed on the starting day of the experiment, E 18 °C – the community developed after 3-day incubation period at the temperature of 18 °C, E 23 °C – the community developed after 3-day incubation period at the temperature of 23 °C

Table 1 Fluorescence parameters measured for the periphyton communities; K 18 °C – the initial community observed on the starting day of the experiment, E 18 °C – the community developed after 3-day incubation period at the temperature of 18 °C, E 23 °C – the community developed after 3-day incubation period at the temperature of 23 °C. Fv/Fm – the maximum quantum yield of PSII, ΦPSII – the quantum yield of PSII, NPQ – non-photochemical quenching, α – the initial part of RLCs (rapid light curves), $rETR_{max}$ – the maximum relative electron transport rate and E_k – the index of photoacclimation

	E 18 °C Mean ± SE	E 23 °C Mean ± SE	*P**
Fv/Fm	0.445 ± 0.007	0.476 ± 0.014	0.121
ΦPSII	0.293 ± 0.010	0.372 ± 0.009	**0.004**
NPQ	0.45 ± 0.02	0.21 ± 0.05	**0.012**
α	0.276 ± 0.017	0.262 ± 0.019	0.388
$rETR_{max}$	46.8 ± 5.7	70.7 ± 7.4	**0.011**
E_k	170 ± 22	272 ± 48	**0.028**

* Statistically significant differences were shown for $P<0.05$ in bold font

Discussion

Elevated temperature affected the composition of the studied communities, with diatoms remaining the dominant taxonomic group. The relative abundance of cyanobacteria increased. Similar observations were reported by Cartaxana et al. (2015) who investigated the microphytobenthos community of the Tages estuary. Previous studies have proved that cyanobacteria can be favored under higher temperature conditions (e.g. Latała and Misiewicz 2000; Van der Grinten et al. 2005). On the other hand, Piggott et al. (2015) reported that the raised temperature had no positive effect on cyanobacteria in periphyton communities. Furthermore, diatom communities were also affected by increased temperature. In this study, there was no dramatic shift in the community structure; nevertheless, the relative abundance

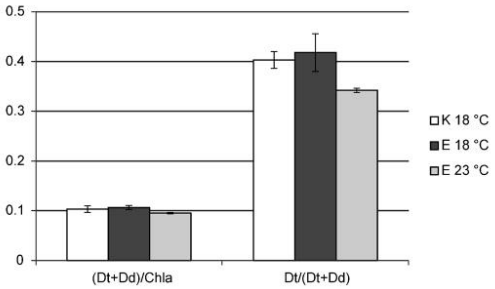

Fig. 3 Temperature-driven changes of the xanthophyll cycle pigments in the periphyton communities; K 18 °C – the initial community observed on the starting day of the experiment, E 18 °C – the community developed after 3-day incubation period at the temperature of 18 °C, E 23 °C – the community developed after 3-day incubation period at the temperature of 23 °C; Dd – diadinoxanthin, Dt - diatoxanthin

of eight species was significantly affected (Fig. 1.), including two *Navicula* species, i.e. *N. perminuta* and *N. ramosissima*. Laboratory experiments showed that the former species grew well within a wide temperature range reaching high cell number at 15–25 °C. Furthermore, the higher the light intensity (up to 150 μmol photons m^{-2} s^{-1}) was the higher the number of cells was observed (Biskup, unpublished). The latter one, on the other hand, was found in the Baltic proper to be forming large colonies under ice cover (Snoeijs and Kautsky 1989), which may suggest its affinity for lower temperatures. Such divers responses of individual species to elevated water temperatures were further reflected in the proportions of the diatom size classes. The amount of large and medium taxa declined in favor of small ones. Overall, there were no negative effects at the community level, while the responses to elevated temperature were species-specific, in agreement with Piggott et al. (2015).

The elevated temperature clearly increased photosynthetic activity of algal communities. Fluorescence measurements showed that when acclimated to culturing light conditions (constant irradiance of 60 μmol photons m^{-2} s^{-1}) the steady-state quantum yield of PSII (*Φ*PSII) reached higher values at higher temperature (Table 1). *Φ*PSII provides information on the proportion of the light absorbed by chlorophyll associated with PSII and used for photochemistry (Maxwell and Johnson 2000). Temperature changes may alter concentration and activity of the electron transport chain as well as Calvin-Benson cycle enzymes (most notably RUBISCO) directly affecting *Φ*PSII (Davison 1991). Therefore, its increase under higher temperature conditions indicated an efficient use of the absorbed photons, and thus higher overall photosynthetic capacity of periphyton.

Furthermore, the higher *Φ*PSII values corresponded with the decline of NPQ (Table 1). Non-photochemical quenching is a photoprotective mechanism which enables safe dissipation of the excessive light energy through the operation of the xanthophyll cycle; in diatoms it involves a reversible conversion of diadinoxanthin into photoprotecive diatoxanthin under high light conditions (Serôdio et al. 2005). In this study, an effective use of absorbed light, as shown by higher *Φ*PSII, lowered the necessity for the effective energy dissipation. This is further supported by the changes in the photosynthetic pigments composition. The de-epoxidation state of xanthophylls (Dt/Dt + Dd) indicated a lower relative amount of diatoxanthin (Fig. 3), explaining lower capacity of periphyton for NPQ build-up under higher temperature (van Leeuwe et al. 2011).

The effective adjustment of the photosynthetic activity of periphyton was also confirmed by RLCs variations. Under limiting light intensities, photosynthetic efficiency of studied communities did not differ significantly as seen from the similar α values (Table 1, Fig. 2), similarly to Vieira et al. (2013). Changes in the α values are mainly controlled by light conditions which affect photosynthetic pigments' composition, concentration and packaging, while temperature plays less important role (Henley 1993). With further increase in light intensity, ETR values also increased reaching the maximum value (ETR_{max}). The saturated part of the light curves is mainly controlled by the carbon metabolism which is an enzyme mediated process (Davison 1991), thus under higher temperature it may operate more rapidly, subsequently leading to the higher ETR_{max} values (Kirk 1996). The light saturation index E_k reflects the optimum light intensity at which microalgae balance the light absorption and its usage and thus E_k is considered to be an indicator of their photoacclimation status (Henley 1993; Serôdio et al. 2006). Since α values remained constant at both applied temperatures the observed E_k changes were the result of the ETR_{max} values increase, in congruence with Salleh and McMinn (2011).

Previous studies showed that temperature may differently affect benthic microalgal communities and its influence may be modified by other factors. Longer exposure of microphtobenthos to elevated temperatures (4–6 °C above seasonal average) often had detrimental effects leading to the decrease in assemblage biomass and causing a shift in the community structure (e.g. Defew et al. 2004; Hicks et al. 2011; Cartaxana et al. 2015; Piggott et al. 2015). The results of this study showed that even short-term increase in water temperature may induce small but still significant changes in the structure of the community (by promoting species preferring higher temperatures and reducing those less resistant to heat stress). Such changes may be facilitated by species-specific photosynthetic responses of dominant species (e.g. Salleh and McMinn 2011). Furthermore, it may be also speculated that several consecutive short-term periods of higher temperature may have even more profound effects, overall leading to much more pronounced changes in community species composition. Gradually accumulated over longer period of time, small changes in the primary producers communities could finally elicit effects observed at higher-trophic levels (Armitage and Fogg 2004; Shurin et al. 2012).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Zakład Funkcjonowania Ekosystemów Morskich

OŚWIADCZENIE

Oświadczam, że wkład w powstanie niżej wymienionej publikacji naukowej: Pniewski, F. and Sylwestrzak, Z., 2018. Influence of short periods of increased water temperature on species composition and photosynthetic activity in the Baltic periphyton communities. Biologia, 73(11), pp.1067-1072.wchodzącej w skład rozprawy naukowej Pani Zuzanny Sylwestrzak pt. stanowił około 50% całości i obejmował:

- realizację prac laboratoryjnych, tj.

-pomoc w interpretacji wyników i redagowaniu manuskryptu.

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- Sylwestrzak Z., Zgrundo A., Pniewski F. 2014. Rozdział 9. *Zastosowanie mikrofitobentosu w testach ekotoksykologicznych – metodologia,* [W:] Bochentyn B.,Walkowicz M. (red.), Metodologia badań wykorzystywana przez młodych naukowców, Creativetime, Kraków, ISBN: 978-83-63058-43-2, str.74-81 (monografia).

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• Sylwestrzak Z., Zgrundo A., Pniewski F., Latała A. 2015. *Wpływ glifosatu na wodne mikroorganizmy roślinne – przegląd dotychczasowych badań.* [W:] Kuczera M., Piech K. (red.) Zagadnienia aktualnie poruszane przez młodych naukowców 3, Creativetime, Kraków, ISBN: 978-83-63058-50-0, str. 227-230.

• Sylwestrzak Z., Zgrundo A., Jurowska J., Śliwińska S., Pniewski F., Latała A. 2015. *Ocena kondycji zbiorowisk mikrofitobentosu jako metoda monitoringu zanieczyszczeń w Morzu Bałtyckim*. [W:] Dera J., Ostrowska M., Krajowa konferencja Bałtyk 2015, Wydawnictwo Instytutu Oceanologii PAN, CISBN 978-83-941037-1-2.

• Śliwińska S., Bubak I., Sylwestrzak Z., Pniewski F., Latała A. 2015. *Allelopathic effects and anthropogenic substances on cyanobacteria and microalgae in aquatic ecosystems.* European Journal of Phycology, vol 50, Suppl. 1, pp. 187.

• Serwatka M., Zgrundo A., Sylwestrzak Z., Śliwińska S. 2015. *Effect of $CuCl_2$ on growth and motility of the marine diatom Cylindrotheca closterium (Ehrenberg) Lewin and Reimann.* European Journal of Phycology, vol 50, Suppl. 1, pp. 170.

• Sylwestrzak Z., Zgrundo A., Pniewski F., Lejk K., Latała A. 2016. *Wpływ glifosatu w postaci preparatu Roundup na zbiorowiska mikrofitobentosu Zatoki Gdańskiej – nowe*

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• Śliwińska S., Sylwestrzak Z., Zgrundo A., Pniewski F., Latała A. 2016. *The effects of allelochemicals and selected anthropogenic substances on the diatom Bacillaria paxillifera.* Edukacja Biologiczna i Środowiskowa 1/2016, ISSN 1643-8779, str. 23-30.

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• Zgrundo A., Sylwestrzak Z., Pniewski F., 2017. *Effects of copper chloride (II), glyphosate and ionic liquid on mixed algal cultures*. Phycologia, vol. 56 No. 4 supplement, str. 207.

• Pniewski F., Sylwestrzak Z. 2018. *Influence of short periods of increased water temperature on species composition and photosynthetic activity in the Baltic periphyton communities*. Biologia, 73(11), 1067-1

• Sylwestrzak Z., Zgrundo A. Pniewski, F. 2021. *Ecotoxicological studies on the effect of Roundup®(glyphosate formulation) on marine benthic microalgae.* International Journal of Environmental Research and Public Health, 18(3), p.884.072.

• Zgrundo A., Sylwestrzak Z., Kulasiewicz A. 2022. *Mikroflora porośłowa [w:] Zatoka Pucka, Aspekty świata ożywionego, tom III* [red. Bolałek J., Burska D.], Wydawnictwo Uniwersytetu Gdańskiego, Gdańsk, str.89-108.

• Sylwestrzak Z., Zgrundo A. Pniewski, F. 2022. *Copper chloride (II) effect on the composition and structure of marine microphytobenthic communities.* Environmental Monitoring and Assessment, 194(6), pp.1-15.

• Sylwestrzak, Z., Zgrundo, A. and Pniewski, F., 2022. *Effects of the Ionic Liquid [BMIM] Cl on the Baltic microphytobenthic communities.* Journal of Marine Science and Engineering, 10(9), p.1223.

CONFERENCES:

Author and co-authorship of 12 oral presentation and 13 posters presented at 24 scientific conferences, including 11 international and 13 national.

National

- II Interdyscyplinarna Akademicka Konferencja Ochrony Środowiska (IAKOŚ), 17-20.03.2017, Gdańsk
- Krajowa konferencja Bałtyk 2015, 14-16.10.2015, Sopot
- Ogólnopolska Konferencja Hydrologiczna z okazji Światowego Dnia Wody, 22.03.2017, Poznań
- Nowe wyzwania dla polskiej nauki – spojrzenie młodych naukowców, 3.04.2016, Gdańsk
- Wpływ młodych naukowców na osiągnięcia polskiej nauki, Nowe trendy w naukach przyrodniczych, VIII edycja, 11.04.2015, Gdańsk
- Innowacyjność w naukach biologicznych, inżynieryjnych, humanistycznych i społeczno-ekonomicznych oraz w rolnictwie i naukach o ziemi, 25.03.2015, Olsztyn
- I Toruńskie Sympozjum Doktorantów Nauk Przyrodniczych, 20-22.03.2015, Toruń,
- XIII Sympozjum Młodych Oceanografów, 28.11.2014, Gdynia
- Wpływ młodych naukowców na osiągnięcia polskiej nauki, VI Edycja, 26.04.2014, Gdańsk
- Wpływ młodych naukowców na osiągnięcia polskiej nauki, V Edycja, 1.12.2013, Poznań
- XII Sympozjum Młodych Oceanografów, 29.11.2013 Gdynia
- XI Sympozjum Młodych Oceanografów, 30.12.2012, Gdynia

International

- 37th International Conference of Polish Phycologists, 22-25.05.2018, Kraków-Dobczyce, Poland

- 11th International Phycological Congress, 13-19.08.2017 Szczecin, Poland
- 36th International Conference of Polish Phycologists, 24-27.05.2017, Lublin – Kazimierz Dolny, Poland
- 35th International Conference of Polish Phycologists, 1-4.06.2016,Łódź – Stryków, Poland
- International Sopot Youth Conference, 20.05.2016, Sopot, Poland
- 6th European Phycological Congress (EPC6), Aquatic Biodiversity and Ecosystems: Evolution, Interactions & Global Change, 30.08- 4.09.2015, London, UK
- 34th International Conference of Polish Phycologists, 18–21 05.2015, Rzeszów–Polańczyk, Poland
- 33rt International Conference of Polish Phycologists, 19 –22.06.2014, Gdynia – Cetniewo, Poland
- 32nd International Conference of Polish Phycologists, 20-23.05.2012, Konin – Mikorzyn, Poland
- Mares Conference – Marine Ecosystem Health and Conservation, 17-21.10.2012, Olhão, Portugal
- 31st International Conference of Polish Phycological Society, 2012, Olsztyn, Poland

SCHOLARSHIPS

- Doctoral scholarship awarded by the University of Gdańsk (2013-2018)
- Doctoral scholarship from pro-quality grant awarded by University of Gdańsk (2013-2017)
- Scholarship for the best PhD students awarded by the by University of Gdańsk (20123-2018)

MEMBERSHIP IN SCIENTIFIC ORGANIZATION

2018 - present	Scientific Society “Semper aqua” (president)
2017 - present	Federation of European Phycological Societies Member
2017- present	Polish Phycological Society
2016 - present	Polish Hydrobiological Society
2013-2015	Studenckie Koło Naukowe Oceanografów, UG (supervisor)
2013-2016	Wydziałowa Rada Doktorantów

TEACHING EXPERIENCE

- Classes with students of Oceanography and Aquaculture –business and technology (University of Gdańsk):
  - Ecology (2013-2022)
  - Hydroecology (2021-2022)
- Jean Monnet educational and research project "Children's University for Europe" (2012-2013)
- Tutor of student internships, Faculty of Oceanography and Geography (2015)

POPULAR-SCIENCE EVENTS:

- Workshops "Nature of Puck Bay" and "Sea animals under pressure - sea turtles" on Święto Morza, 25.06.2016, Instytut Oceanografii UG, Gdynia (2016)
- Workshops "Nature of Puck Bay" and "Sea animals under pressure - sea turtles" on Dzień Ryby w Helu, 30.07.2016, Hel (2016)
- Workshops “Green inhabitants of the Baltic Sea - what plants can be found on the seashore” and ”In the depth or at the bottom? Baltic algae life strategies" Warsztaty Oceanograficzne dla Młodzieży,12.09.2016, Institute of Oceanography, Gdynia (2016)

- Scientific booth "Baltic - the Mediterranean Sea of Northern Europe" with Studenckie Koło Naukowego Oceanografów (supervisior), XVI Piknik, XIII Bałtycki Festiwal Nauki, Gdynia (2015).

- Scientific booth "Explore the Sea with Pirates" with Studenckie Koło Naukowe Oceanografów (supervisior), XVI Piknik, XII Bałtycki Festiwal Nauki, Gdynia (2014).

- Scientific booth "What is hiding at the bottom of the Baltic Sea?", "Everyone can protect the sea (II)" XVI Piknik, XI Bałtycki Festiwal Nauki, Gdynia (2014).

COURSES

- Workshop "Cyanobacteria, diatoms and green algae" on 32nd International Conference of Polish Phycologists, 20-23.05.2013, Konin - Mikorzyn (2013)

- Academic course "Blue biotechnology of microorganisms" Faculty of Oceanography and Geography, University of Gdańsk, 1.02 - 30.04.2013, Gdynia (2013)

- Seminar „Ocenianie w dydaktyce akademickiej" implemented as part of a series of meetings " Dobre zwyczaje akademickie w naukach przyrodniczych" (2014)

- „Teacher training courses" Faculty of Oceanography and Geography, University of Gdańsk (2014)

- Course "Improve the performance of your research. How test tubes, plates and pipette tips affect the results of your experiments" Eppendorf, Gdańsk, Polska (2015).

- Course „Microscopic Imaging Techniques Workshop", Department of Algology and Mycology University of Łódź, 06.2016. Łódź (2016)

OTHERS

- Scientific director of the Interdisciplinary Student Scientific Expedition I-SEA (Interdisciplinary student's expedition along Adriatic shore) 11-20.07.2014 (2014)