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Effects of Sodium Chloride on Algae and Crustaceans—The Neighbouring Links of the Water Trophic Chain

Łukasz Sikorski

Faculty of Agriculture and Forestry, University of Warmia and Mazury in Olsztyn, Pl. Łódzki 4, 10-727 Olsztyn, Poland; lukasz.sikorski@uwm.edu.pl

Abstract: Salinity limits the habitable living environment for aquatic organisms. Algae and crustaceans are widely used as bioindicators in freshwater environmental risk assessments. This study aimed to use biotests (Algaltoxkit and Daphtoxkit) to determine the effect of sodium chloride (NaCl) on algae *Pseudokirchneriella subcapitata* and crustaceans *Daphnia magna*. Standard biotests were extended to include NaCl effects on algal chlorophyll fluorescence and crustaceans swimming and heart rate. It was found that after 7 days, a 0.24 M of NaCl reduced the growth rate of the algae by 50% (EC_{50}). A NaCl of 0.27 M inhibited the minimum (Fo), maximum (Fm) and variable (Fv) fluorescence by 50%, on average. The crustaceans also responded to NaCl. Those exposed to 0.19 M NaCl during 15 min swam slower by 50% and a 0.27 M immobilised three organisms (EC_{50}). The crustacean immobilisation was less modified by NaCl than swimming. To determine the lethal effect in non-swimming organisms, the heart rate was examined. At 0.35 M of NaCl, all organisms were dead after 30 min, as their hearts did not beat. These studies suggest that physiological and behavioural features are sensitive indicators of the toxic effects of NaCl in algae and crustaceans, before morphological changes are observed.

Keywords: sodium chloride; algae; *Daphnia*; chlorophyll fluorescence; swimming; heart rate

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1. Introduction

Water is one of the most important environmental resources and the availability of good quality water determines the life of organisms. However, salinity limits the habitable living environment for all aquatic organisms [1]. In lakes, chloride is a relatively benign ion at low concentrations but begins to have an ecological impact as concentrations increase to 100 and 1000 mg × L^{-1} [2]. Chloride enters waters with discharges of contaminated post-mining water to the aquatic environment, and with surface run-off from urbanised areas, where it is used for the de-icing of roads. As sodium chloride is a cheap and readily available raw material, it is widely used on roads [3]. After such application, 40–77% of salt is retained in the environment throughout the growing season [4–6]. Salt consumption in Canada is over 6 t × km^{-1} of road per year, while Norway and the United States of America report approximately 5 t × km^{-1}, Sweden reports about 2 t × km^{-1} and Denmark has even lower salt consumption of <1 t × km^{-1} [7]. The food industry, including the dairy, fish and meat sectors, generate high-strength wastewater with an admittedly variable, but short-term, high salt concentration (3.3 g × L^{-1} of sodium chloride), as a result of use of this salt for the disinfection of equipment and food processing in factories [8]. Sodium chloride is also used in commercial aquaculture as a drug to control pathogenic microorganisms by immersing fish in NaCl solutions at concentrations ranging from 0.015 to 35.0 g × L^{-1} [9]. Sodium chloride has been recognised as a compound safer and less toxic to humans and the natural environment than formalin, copper sulphate or hydrogen peroxide [10]. Although sodium chloride after such a treatment does not enter waters directly, it allows numerous exposure changes, i.e., gasometric, haematological and biochemical indicators, including fish behaviour and survivability, to be observed.

However, in the development of plants, chlorine acts as an essential macronutrient although its high content can cause adverse effects, including reduced water uptake capacity in plants (physiological drought, osmotic shock) [11–13] and the inhibition of growth rate [11]. Excessive salt concentration in plant organism tissues also results in a series of morphological changes, inter alia their premature ageing or the inhibition of photosynthesis [14]. Consequently, a high sodium chloride content leads to the dying out of freshwater flora and fauna, particularly in the lowest-situated water bodies in the catchment area [15]. The condition of aqueous ecosystems is a reflection of the processes occurring in their catchment area, which can contribute to the accumulation of contaminants from external inflows in lakes [2]. The salinity of water bodies occurs due to the weathering of naturally saline rocks as well as the aeolian or rainfall deposition (primary salinity). However, secondary salinity occurs as a result of human activities, due to the direct introduction of saline water to the environment, or is a consequence of the change in the water balance of catchment causing the mobilisation of stored salts [16]. As a result, the salt present in the living environment of organisms disturbs their osmotic balance. In response, plants reduce the resources allocated for growth in favour of the elimination of the undesirable ions that penetrate into cells and tissues and increased transport of ions which maintain cell turgor pressure [17]. Although the proper chloride uptake is essential for plants for, inter alia, the regulation of the leaf cell size and protecting chloroplast components from being destroyed by light, in non-halophytic plants, the critical deficiency concentration in the stem is only <0.2 mg $\times$ g^{-1} dry matter [18]. Animals also balance the intake of ion-rich food or liquids with the consumption of ion-poor water and the excretion of excess ions. In vertebrates, if sodium and chlorine ions in the plasma reach a concentration exceeding 100 mM, then aversive channels are activated at a NaCl concentration >150 mM [1]. Invertebrates such as *Daphnia magna* survive and reproduce well only in water with salinity <4 g $\times$ L^{-1} [19]. Hence, a change in sodium chloride concentration in water is one of the early warning indicators of the condition of lakes with significant changes at the local, regional and global levels. It is anticipated that if the presence of chlorides in waters remains at the current level, over the next 50 years, many lakes will exceed the aquatic life threshold criterion for chronic chloride exposure [2], amounting to 230 mg $\times$ L^{-1}, which was established by the US Environmental Protection Agency as early as 1997 [20].

Therefore, a task was undertaken to assess the toxicity of sodium chloride contained in water in relation to the algae *Pseudokirchneriella subcapitata* and crustaceans *D. magna.* These bioindicators colonise freshwater bodies and are widely used in analyses of environmental and toxicological risks in water ecosystems [21–24]. The algae play an important role in the trophic chain where they function mostly as autotrophic organisms that produce organic matter, which is a source of food for animals, including the invertebrates *D. magna*. Then, the energy absorbed from sunlight by the algae flows through the subsequent trophic chain links, thus ensuring the durability of life in the freshwater environment.

This study aimed to determine the effects of the exposure of algae *P. subcapitata* and crustaceans *D. magna*—neighbouring freshwater links of the food chain to sodium chloride at different concentrations, to determine the adverse effects when water is contaminated with this salt. According to OECD and ISO recommendations, evaluations of toxicity should rely on the morphological and behavioural features of tested bioindicators, whereas changes in biochemical features, which are the first indicators of xenobiotics' toxic effects, are not specifically mentioned in these recommendations [21–24]. Therefore, the standard set of biotests was expanded to include an assessment of additional physiological and behavioural features, such as algal chlorophyll fluorescence, crustaceans swimming and heart rate. A thorough knowledge of sodium chloride's effects on tested organisms will contribute to ecotoxicological research and will support evaluations of the relevant environmental risks, by studying and identifying early and sensitive biomarkers of the toxic effects in algae and crustaceans.

2. Materials and Methods

2.1. Chemicals

Sodium chloride (NaCl) was applied in a powder form (assay ≥ 99.0%) (Merck, Warsaw, Poland) at a Molecular Weight of 58.44 g × mol^{-1} [25].

Concentrations 0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30 and 0.35 M of NaCl (0, 2.12, 4.33, 8.52, 12.73, 17.33, 21.26 and 29.29 mS × cm^{-1}, respectively) were prepared in a medium (respectively, for Algaltoxkit and Daphtoxkit), then their electrical conductivity was measured by 20 °C using conductivity/TDS meter model HI 9835 (Hanna Instruments, Woonsocket, RI, USA). The electrical conductivity was determined before biotests and during the experiment.

In the experiment, such a range of sodium chloride concentrations was used to obtain (simultaneously for algae and crustaceans) the dose–response relationship, which first allowed to observe changes in biochemical features, then changes in behavioural and morphological features, to lethal effects.

2.2. Algaltoxkit

Pseudokirchneriella subcapitata plants for the experiment were obtained commercially from the Algaltoxkit F (MicroBioTests Inc., Ghent, Belgium) by the de-immobilisation of small microalgae beads immobilised in an inert matrix.

The toxicity of sodium chloride (Sigma, Merck, Poznań, Poland) to *P. subcapitata* was tested according to the OECD algal growth inhibition test [22] and the ISO Water Quality—Freshwater Algal Growth Inhibition Tests with Unicellular Green Algae [24]. *P. subcapitata* (an algal density of 1.106 cells × mL^{-1}) was grown in 10 cm path-length disposable long cells in polystyrene, containing 25 mL algae-toxicant dilutions in a plant growth chamber (ALL-Round-Al 185-4) illuminated with fluorescent lights (140 µmol photon m^{-2} × s^{-1} PAR) in a light-to-dark cycle of 16 h/8 h (mean maximum temperature of 20 °C during daytime and 16 °C during nighttime) for 7 days. The responses of *P. subcapitata* to NaCl concentrations of 0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30 and 0.35 M (0, 2.12, 4.33, 8.52, 12.73, 17.33, 21.26 and 29.29 mS × cm^{-1}, respectively) were determined based on the biomass growth rate of algae (3, 5 and 7 days) using the optical density at a wavelength of 670 nm (Hitachi U-1800 spectrophotometer, Tokyo, Japan) according to the following formula:

$$Y = (A_{3,5,7} - A_1) \times 100\%/A_1 \quad (1)$$

where: Y—biomass growth rate of algae, %. A_1—optical density at a wavelength of 670 nm for 1 day of the biotest. $A_{3,5,7}$—optical density at a wavelength of 670 nm for days 3, 5 and 7 of the biotest.

Chlorophyll Fluorescence

The chlorophyll fluorescence was measured with a HandyPEA chlorophyll fluorescence system equipped with a Liquid-Phase Chlorophyll Fluorescence Adapter for Handy PEA (Hansatech Instruments Ltd., Pentney, UK). The algae-toxicant dilutions were transferred to 2 mL vials and stored in the dark for 30 min to quench chlorophyll fluorescence. After dark adaptation, chlorophyll was excited at a light intensity of 2500 µmol × m^{-2} × s^{-1}, and minimum chlorophyll fluorescence (Fo), maximum chlorophyll fluorescence (F_m) and variable chlorophyll fluorescence (Fv = Fm − Fo) were determined. The maximum quantum efficiency (Fv/Fm) of PSII (Photosystem II) was determined based on the chlorophyll fluorescence kinetics of *P. subcapitata*.

2.3. Daphtoxkit

Daphnia magna organisms for the experiment were obtained commercially from Daphtoxkit F (MicroBioTests Inc., Ghent, Belgium) by hatching of the ephippia in a growth chamber (ALL-Round-Al 185-4) under continuous illumination (6000 LUX) with a temperature of 20–22 °C for 72 h.

The toxicity of sodium chloride (Sigma, Merck, Poznan, Poland) to *D. magna* was tested according to the OECD Guideline 202 "*Daphnia* sp. Acute Immobilisation Test" [21] and ISO 6341 "Water quality—Determination of the inhibition of the mobility of *D. magna* Straus (Cladocera, Crustacea)—Acute toxicity test" [23]. *Daphnia magna* organisms (6 actively swimming neonates, not older than 24 h) were fed and transferred to plastic plate wells containing 25 mL of standard freshwater with increasing sodium chloride concentration. The responses of *D. magna* to NaCl concentrations of 0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30 and 0.35 M (0, 2.12, 4.33, 8.52, 12.73, 17.33, 21.26 and 29.29 mS $\times$ cm^{-1}, respectively) were determined based on the immobilisation (IM) (which was defined as organisms which were not able to swim after gentle agitation of the liquid for 15 s, even if they could still move their antennae). The IM was determined after 15, 30, 45, 60, 75, 90, 105 and 120 min of the experiment.

2.3.1. Swimming

Daphnia magna swimming (SW) (hopping frequency) was examined by placing the organisms in transparent test tubes with the test solution at a temperature of 20 $\pm$ 2 °C, recording 20 s films using a digital camera (Canon Power Shot A810 HD) and counting (after five seconds from the transfer) the hopping motions. The number of the hops $\times$ min^{-1} was determined after 15, 30, 45, 60, 75, 90, 105 and 120 min of exposure to NaCl concentrations of 0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30 and 0.35 M (0, 2.12, 4.33, 8.52, 12.73, 17.33, 21.26 and 29.29 mS $\times$ cm^{-1}, respectively).

2.3.2. Heart Rate

The heart rate (BPM) of the organisms was examined using a microscope (Nikon Eclipse E200, Tokyo, Japan) with a photo camera (Nikon E8400) attached to the eyepiece. The *D. magna* individuals were placed on a glass slide in a drop of the test solution at a temperature of 20 $\pm$ 2 °C, and 20 s films were recorded to be later used to count the number of *D. magna* heartbeats. The number of heart beats $\times$ min^{-1} was determined within 20 s after 15, 30, 45, 60, 75, 90, 105 and 120 min of exposure to NaCl concentrations of 0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30 and 0.35 M (0, 2.12, 4.33, 8.52, 12.73, 17.33, 21.26 and 29.29 mS $\times$ cm^{-1}, respectively.

2.4. Statistical Analysis

The experiment was conducted in 6 replicates. Results were expressed as mean $\pm$ standard deviation (SD). The results were statistically evaluated using analysis of variance ANOVA (F test) for one factor (to Fo, Fm, Fv and Fv/Fm) and two factors (to Y, SW, IM, BPM). The factors of the experiment were: time and concentration used. Significant differences were determined by the Tukey test at the level $p < 0.01$. Research results: Y, Fo, Fm, Fv and Fv/Fm of algae *P. subcapitata* and the SW, IM and BPM of crustaceans *D. magna* exposed to NaCl were performed using the STATISTICA 13.3 statistical package (TIBCO Software Inc., Palo Alto, CA, USA). Effective concentration (EC_x) and lethal concentration (LC_x) data were analysed with a selected regression model (obtained from a dose–response plot) to calculate the concentrations at 20%, 50% and 90% response levels.

3. Results

3.1. Pseudokirchneriella Subcapitata

The electrical conductivity of the tested NaCl concentrations did not change during the experiment and was invariably: 0; 2.12; 4.33; 8.52; 12.73; 17.33; 21.26 and 29.29 mS $\times$ cm^{-1}, respectively.

After three, five and seven days of the experiment, it was demonstrated that increasing sodium chloride concentrations and time modified the Y of algae *P. subcapitata* (Table 1). On day 3 of the experiment, the algal Y in the control sample was 182%. The algae grew increasingly slower under exposure to NaCl. Even at the lowest concentrations of 0.05 and 0.10 M, the algal Y amounted to 153.05% and 141.84%, respectively (Figure 1).

Table 1. Analysis of variance (ANOVA) for morphological and biochemical features of algae *Pseudokirchneriella subcapitata* exposed to sodium chloride (NaCl).

Source of Variation	*Pseudokirchneriella subcapitata*				
	Growth Rate (Y)	Minimum Chlorophyll Fluorescence (Fo)	Maximum Chlorophyll Fluorescence (Fm)	Variable Chlorophyll Fluorescence (Fv)	Maximum Quantum Efficiency (Fv/Fm)
	F-Value				
Intercept	16,973.32 *	8082.986 *	5207.053 *	4248.882 *	136,826.6 *
Concentration NaCl (C)	644.88 *	277.819 *	192.971 *	162.972 *	71.3 *
Time (T)	2496.63 *	-	-	-	-
C × T	125.88 *	-	-	-	-

C—concentration, T—time, C × T—interactions between the factors, * significant at $p < 0.01$.

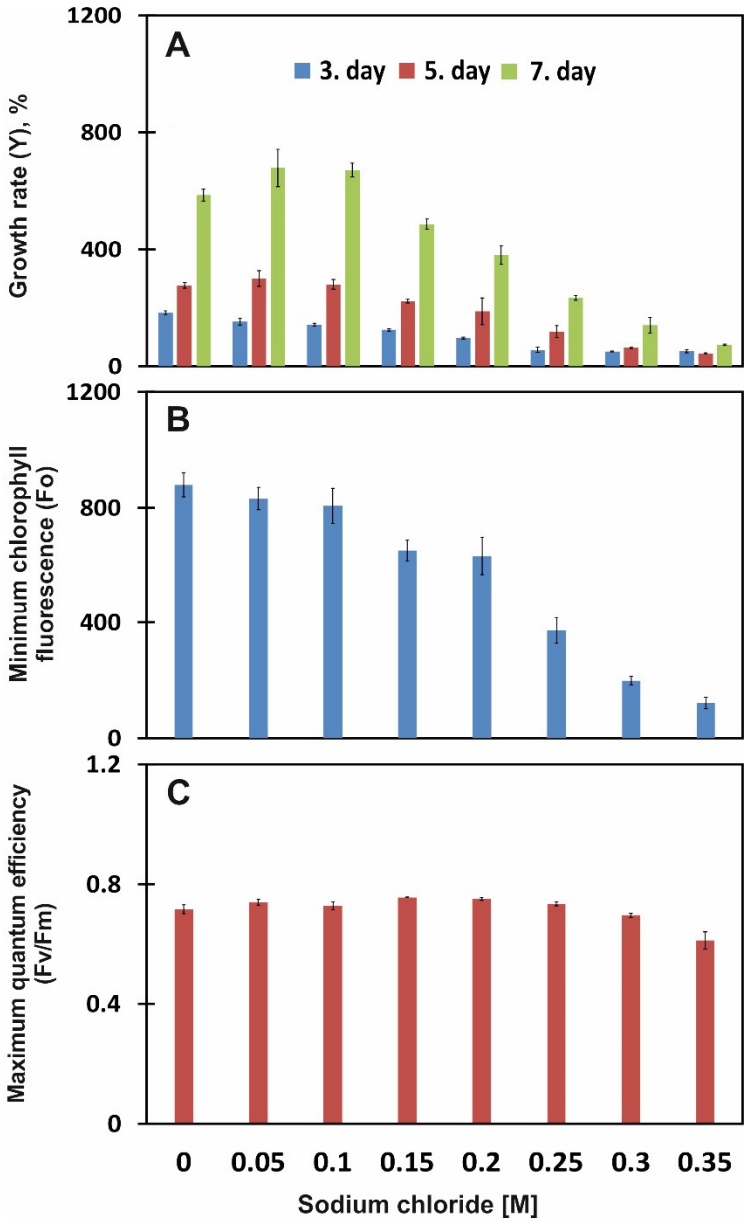

Figure 1. (**A**)—Growth rate (Y), (**B**)—minimum chlorophyll fluorescence (Fo) and (**C**)—maximum quantum efficiency (Fv/Fm) of PSII of algae (*Pseudokirchneriella subcapitata*) exposed to sodium chloride (NaCl) concentrations (0–0.35 M). Data points represent the mean ± SD, $n = 6$.

The significantly reduction of algal Y by 50% (EC_{50}) occurred at a NaCl concentration of 0.21 M (Tables 2 and 3). On the other hand, the lowest Y_3 was noted from the NaCl concentration of 0.25 M to the highest concentration of 0.35 M. At these concentrations, the algal Y amounted to an average of 53% (Figure 1).

Table 2. The growth rate (Y), minimum chlorophyll fluorescence (Fo), maximum chlorophyll fluorescence (Fm), variable chlorophyll fluorescence (Fv) and maximum quantum efficiency (Fv/Fm) of algae *Pseudokirchneriella subcapitata* exposed to sodium chloride (NaCl). The table contains the mean of the examined features and in superscript the level of significance (a–o).

Feature		Sodium Chloride, M							
		0	**0.05**	**0.1**	**0.15**	**0.20**	**0.25**	**0.3**	**0.35**
Growth rate (Y), %	3 day	183.17 ^{fgh}	153.05 ^{efg}	141.84 ^{def}	124.59 ^{de}	96.61 ^{bcd}	55.93 ^{ab}	50.16 ^{a}	51.59 ^{ab}
	5 day	276.93 ^{jk}	300.16 ^{k}	280.13 ^{jk}	223.54 ^{hi}	188.71 ^{ghi}	119.04 ^{cde}	62.97 ^{ab}	44.5 ^{a}
	7 day	586.23 ^{n}	679.11 ^{o}	672.01 ^{o}	487.01 ^{m}	381.01 ^{l}	233.95 ^{ij}	141.25 ^{def}	74.15 ^{abc}
Min. chlorophyll fluorescence (Fo)	7 day	880.00 ^{d}	832.17 ^{d}	806.83 ^{d}	652.33 ^{c}	633.33 ^{c}	373.50 ^{b}	199.00 ^{a}	121.50 ^{a}
Max. chlorophyll fluorescence (Fm)	7 day	3120.00 ^{e}	3203.67 ^{e}	2982.00 ^{d}	2689.00 ^{cd}	2551.83 ^{c}	1413.83 ^{b}	659.00 ^{a}	319.17 ^{a}
Var. chlorophyll fluorescence (Fv)	7 day	2239.50 ^{de}	2371.17 ^{e}	2175.17 ^{cde}	2036.33 ^{cd}	1918.17 ^{c}	1040.00 ^{b}	459.50 ^{a}	197.33 ^{a}
Max. quantum efficiency (Fv/Fm)	7 day	0.72 ^{bc}	0.74 ^{cde}	0.73 ^{cd}	0.76 ^{e}	0.75 ^{de}	0.74 ^{cde}	0.70 ^{b}	0.61 ^{a}

Values that do not share the same superscript letter (a–o) are significantly different ($p < 0.05$).

Table 3. Effect of sodium chloride (NaCl) on growth rate (Y), minimum chlorophyll fluorescence (Fo), maximum chlorophyll fluorescence (Fm), variable chlorophyll fluorescence (Fv) and maximum quantum efficiency (Fv/Fm) of algae *Pseudokirchneriella subcapitata*.

Feature		Sodium Chloride, M		
		EC_{20}	**EC_{50}**	**EC_{90}**
Growth rate (Y)	3 day	0.08	0.21	0.39
	5 day	0.13	0.24	0.38
	7 day	0.14	0.24	0.37
Min. chlorophyll fluorescence (Fo)	7 day	0.22	0.29	0.39
Max. chlorophyll fluorescence (Fm)	7 day	0.17	0.26	0.34
Var. chlorophyll fluorescence (Fv)	7 day	0.18	0.26	0.34
Max. quantum efficiency (Fv/Fm)	7 day	0.39	0.51	0.63

On day 5 of the experiment, the algal Y in the control sample was higher than on day 3 and amounted to 276.93%. On day 5 of the exposure, the lowest tested NaCl concentrations (0.05 M and 0.10 M) slightly insignificantly stimulated the algal growth. At these concentrations, the Y were 300.16% and 280.13%, respectively (Figure 1, Table 2). The stimulation of growth resulted in a slightly higher effective concentration of 0.24 M which significantly inhibited the algal growth by 50% (Table 3). At the highest tested NaCl concentration (0.35 M), the algal Y rate reached a value of 44% and was over six times lower than that for the control algae (Figure 1).

On the last day (day 7) of the exposure of algae *P. subcapitata* to NaCl, the algal Y in the control sample was 586.23%. Plants exposed to NaCl at 0.05 and 0.10 M were significantly stimulated (Table 2). Their Y was the highest and amounted to an average of 675.56%. At concentrations of 0.15 M and higher, NaCl was already phytotoxic (Figure 1). A concentration of 0.24 M inhibited the Y by 50% (EC_{50}) (Table 3). On the other hand, at

the highest concentration (0.35 M), the algae only grew to 74.15%, i.e., almost seven times less than in the control sample (Figure 1).

On day 7 of the experiment, a measurement of the chlorophyll fluorescence of algae exposed to sodium chloride in the medium was taken. It was demonstrated that Fo, Fm, Fv and Fv/Fm were modified by NaCl concentration (Table 1). The Fo value decreased with an increase in the concentrations of this xenobiotic. The highest Fo value (880) was noted in the control sample (Figure 1) and the NaCl concentrations of 0.22 M and 0.29 M significantly inhibited the Fo by 20% (EC_{20}) and 50% (EC_{50}), respectively (Tables 2 and 3). The highest NaCl concentration (0.35 M) in the medium resulted in a reduction in Fo value to 121.5 (Figure 1).

The lowest NaCl concentration (0.05 M) did not reduce the algal Fm, which was the highest in the entire experiment (3203.67) (Table 4). The Fm value subsequently significantly declined (Table 2). The NaCl concentration of 0.17 M reduced Fm by 20% (EC_{20}) and a NaCl concentration of 0.26 reduced Fm by 50% (EC_{50}) compared with the control sample (Table 3). In most contaminated samples (0.30 and 0.35 M of NaCl), Fm only amounted to 659 and 319.17, respectively (Table 4).

Table 4. The algae *Pseudokirchneriella subcapitata* exposed to 0–0.35 M of sodium chloride (NaCl) and the effect of sodium chloride (NaCl) on maximum chlorophyll fluorescence (Fm) and variable chlorophyll fluorescence (Fv) of plants. Data points represent the mean ± SD, $n = 6$.

Sodium Chloride, M		Maximum Chlorophyll Fluorescence (Fm)	Variable Chlorophyll Fluorescence (Fv)	Visible Symptoms
0	Mean ±SD	3120.00 158.84	2239.50 145.41	
0.05	Mean ±SD	3203.67 244.44	2371.17 209.02	
0.1	Mean ±SD	2982.00 326.87	2175.17 269.73	
0.15	Mean ±SD	2689.00 148.60	2036.33 111.88	
0.2	Mean ±SD	2551.83 288.50	1918.17 224.57	
0.25	Mean ±SD	1413.83 155.44	1040.00 112.61	
0.3	Mean ±SD	659.00 62.77	459.50 47.83	
0.35	Mean ±SD	319.17 73.25	197.33 54.07	

The Fv index was modified by NaCl analogously to the Fm. The highest Fv values were noted in the control sample and at the lowest concentration (0.05 M) and they amounted to 2239.50 and 2371.17, respectively (Table 4). Fv then in NaCl concentration $\geq$ 0.15 M significantly declined to reach, at a concentration of 0.34 M, a value 90% lower than that of the algae growing without xenobiotic (Tables 2 and 3).

The toxicity of NaCl was not reflected in measurements of the Fv/Fm, i.e., the most sensitive index of the photosynthetic apparatus photochemical activity. This index in the control sample (and in concentrations ranging from 0.05 to 0.25 M) amounted to an average

of 0.73, while that measured for the algae most exposed to NaCl (0.35 M) Fv/Fm was significantly decreased to 0.61 (Figure 1, Table 2).

3.2. *Daphnia Magna*

The electrical conductivity of the tested NaCl concentrations did not change during the experiment and was invariably: 0; 2,12; 4.33; 8.52; 12.73; 17.33, 21.26 and 29.29 mS × cm^{-1}, respectively. The ANOVA demonstrated that SW, IM and BPM was changed by the NaCl concentration and duration of the test (Table 5).

Table 5. Analysis of variance (ANOVA) for morphological and biochemical features of crustaceans *Daphnia magna* exposed to sodium chloride (NaCl).

Source of Variation	*Daphnia magna*		
	Swimming (SW)	**Immobilisation (IM)**	**Heart Rate (BPM)**
	***F*-Value**		
Intercept	132,001.1 *	983,526.6 *	216,317.8 *
Concentration NaCl (C)	17,409.4 *	130,207.4 *	14,005.1 *
Time (T)	298.6 *	3837.0 *	3813.4 *
C × T	126.4 *	1101.7 *	170.7 *

C—concentration, T—time, C × T—interactions between the factors, * significant at $p < 0.01$.

Daphnia magna SW (hopping) in the control sample was, on average, 308 hops × min^{-1}, with the lowest value of 260.33 hops × min^{-1} noted at 30 min and the highest level of about 330 hops × min^{-1} at 60 and 105 min (Figure 2). In contaminated samples, the *D. magna* swam significantly, with increasingly less intensely (Table 6).

Table 6. The swimming (SW), immobilisation (IM) and heart rate (BPM) of crustaceans *Daphnia magna* exposed to sodium chloride (NaCl). The table contains the mean of the examined features and in superscript the level of significance (a–z).

Feature		Sodium Chloride, M							
		0	**0.05**	**0.1**	**0.15**	**0.20**	**0.25**	**0.3**	**0.35**
Swimming (SW) Hops × min^{-1}	15 min	275.00 ^{m}	230.00 ^{nij}	185.00 ^{g}	185.00 ^{g}	147.00 ^{ef}	82.00 ^{c}	77.67 ^{c}	0.00 ^{a}
	30 min	260.33 ^{lm}	241.67 ^{jk}	223.00 ^{hi}	223.00 ^{hi}	11.00 ^{ab}	19.00 ^{b}	0.00 ^{a}	0.00 ^{a}
	45 min	328.00 ^{op}	273.50 ^{m}	219.00 ^{hi}	219.00 ^{hi}	11.33 ^{ab}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}
	60 min	329.67 ^{p}	273.84 ^{m}	218.00 ^{h}	218.00 ^{h}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}
	75 min	302.83 ^{n}	245.58 ^{kl}	188.33 ^{g}	188.33 ^{g}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}
	90 min	313.00 ^{no}	233.34 ^{ijk}	153.67 ^{f}	153.67 ^{f}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}
	105 min	330.00 ^{p}	232.67 ^{hijk}	135.33 ^{de}	135.33 ^{de}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}
	120 min	320.00 ^{op}	224.34 ^{hi}	128.67 ^{d}	128.67 ^{d}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}
Immobilisation (IM) organism	15 min	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	2.67 ^{e}	4.33 ^{f}	6.00 ^{i}
	30 min	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	5.33 ^{g}	5.67 ^{h}	6.00 ^{i}	6.00 ^{i}
	45 min	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	5.67 ^{h}	6.00 ^{i}	6.00 ^{i}	6.00 ^{i}
	60 min	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	6.00 ^{i}	6.00 ^{i}	6.00 ^{i}	6.00 ^{i}
	75 min	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	6.00 ^{i}	6.00 ^{i}	6.00 ^{i}	6.00 ^{i}
	90 min	0.00 ^{a}	0.20 ^{b}	0.40 ^{c}	0.80 ^{d}	6.00 ^{i}	6.00 ^{i}	6.00 ^{i}	6.00 ^{i}
	105 min	0.00 ^{a}	0.17 ^{b}	0.34 ^{c}	0.67 ^{d}	6.00 ^{i}	6.00 ^{i}	6.00 ^{i}	6.00 ^{i}
	120 min	0.00 ^{a}	0.17 ^{b}	0.34 ^{c}	0.67 ^{d}	6.00 ^{i}	6.00 ^{i}	6.00 ^{i}	6.00 ^{i}
Heart rate (BPM) Beats × min^{-1}	15 min	366.00 ^{uw}	373.50 ^{w}	381.00 ^{wz}	396.00 ^{z}	354.00 ^{tu}	211.00 ^{fg}	252.00 ^{klm}	170.00 ^{e}
	30 min	331.00 ^{rs}	341.25 st	351.50 ^{tu}	372.00 ^{w}	218.00 ^{fgh}	90.00 ^{d}	45.00 ^{c}	0.00 ^{a}
	45 min	252.00 ^{klm}	275.25 ^{no}	298.50 ^{p}	345.00 st	286.00 ^{op}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}
	60 min	229.00 ^{hij}	241.75 ^{jkl}	254.50 ^{lm}	280.00 ^{no}	186.00 ^{e}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}
	75 min	235.00 ^{hijk}	242.75 ^{jkl}	250.50 ^{klm}	266.00 ^{mn}	105.00 ^{d}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}
	90 min	227.00 ^{fghij}	239.75 ^{ijkl}	252.50 ^{lm}	278.00 ^{no}	95.00 ^{d}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}
	105 min	229.00 ^{hij}	224.25 ^{fghi}	219.50 ^{fgh}	210.00 ^{f}	29.00 ^{bc}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}
	120 min	228.00 ^{ghij}	251.50 ^{klm}	275.00 ^{no}	322.00 ^{r}	20.00 ^{b}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}

Values that do not share the same superscript letter (a–z) are significantly different ($p < 0.05$).

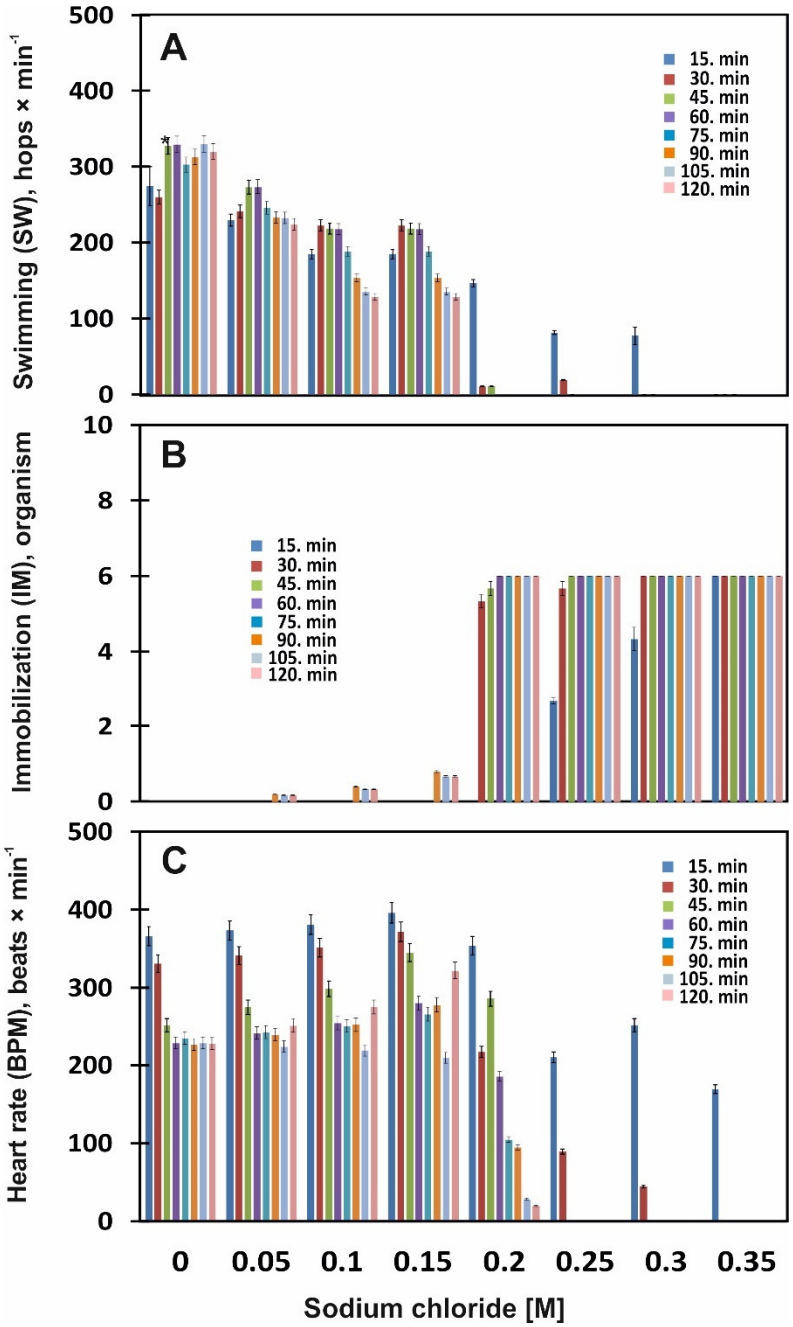

Figure 2. (**A**)—Swimming (SW), (**B**)—immobilisation (IM) and (**C**)—heart rate (BPM) of crustaceans (*Daphnia magna*) exposed to sodium chloride (NaCl) concentrations (0–0.35 M). Data points represent the mean ± SD, $n = 6$.

Only a NaCl concentration of 0.07 M reduced SW by 20% (15 min EC_{20}) after 15 min, while a slightly higher concentration of 0.10 M reduced SW by 50% (120 min EC_{50}) after 120 min (Table 7). An abrupt inhibition of SW was noted at a concentration of 0.2 M after 30 min. In this sample, only 11 hops × min^{-1} were noted, as compared to 147 hops × min^{-1} after 15 min. At a NaCl concentration of 0.35 M (the highest test concentration), only 15 min were sufficient to prevent the crustaceans from SW (Figure 2).

Table 7. Effect of sodium chloride (NaCl) on swimming (SW), immobilisation (IM) and heart rate (BPM) of crustaceans *Daphnia magna*.

Feature		Sodium Chloride, M		
		EC_{20}	EC_{50}	EC_{90}
Swimming (SW)	15 min	0.07	0.19	0.34
	30 min	0.09	0.17	0.26
	45 min	0.07	0.15	0.22
	60 min	0.09	0.15	0.20
	75 min	0.08	0.14	0.20
	90 min	0.05	0.12	0.19
	105 min	0.04	0.10	0.19
	120 min	0.03	0.10	0.19
Immobilisation (IM)	15 min	0.22	0.27	0.33
	30 min	0.16	0.18	0.22
	45 min	0.16	0.18	0.20
	60 min	0.16	0.18	0.20
	75 min	0.16	0.18	0.20
	90 min	0.13	0.17	0.20
	105 min	0.14	0.17	0.20
	120 min	0.14	0.17	0.20
		LC_{20}	LC_{50}	LC_{90}
Heart rate (BPM)	15 min	0.12	0.21	0.29
	30 min	0.13	0.20	0.27
	45 min	0.20	0.23	0.26
	60 min	0.19	0.22	0.25
	75 min	0.18	0.20	0.24
	90 min	0.16	0.20	0.25
	105 min	0.18	0.21	0.24
	120 min	0.18	0.20	0.23

The loss of SW ability was reflected in the immobilisation of organisms. No immobilised individuals were noted in the control sample. On the other hand, as early as after 15 min at a NaCl concentration of 0.25 M, almost three organisms were significantly immobilised (Figure 2, Table 6). The *D. magna* IM was less modified by NaCl than SW. The effective NaCl concentration responsible for the IM of 50% of individuals ranged from 0.27 M in 15 min to 0.17 M of NaCl after 120 min of exposure (Table 7). A concentration of 0.35 M, at which all the organisms (i.e., six individuals) exhibited no movement from 15 min to the end of the experiment, had the strongest effect on the *D. magna* (Figure 2).

To determine the lethal effect in the organisms, the effect of increasing NaCl concentrations on the *D. magna* BPM was examined. During the first 90 min of the test, in both control crustaceans and those exposed to NaCl at concentrations ranging from 0.05 to 0.15 M, the BPM increased significantly (Table 6). After 120 min, in the control sample, the average BPM was, in the entire test, 228 beats $\times$ min^{-1}, while at a concentration of 0.15 M NaCl, it was an average of 322 beats $\times$ min^{-1} (Figure 2). At higher concentrations of the tested xenobiotic (i.e., >0.2 M), the organisms exhibited significantly cardiac dysfunction as early as after 15 min of exposure (Table 6). In the entire test, the BPM was reduced by 50% (LC_{50}) and 90% (LC_{90}) due to being exposed to 0.21 and 0.25 M NaCl (Table 7). In turn, at concentrations of 0.3 and the highest of 0.35 M of the xenobiotic, all the organisms were dead after 45 and 30 min, respectively, because their hearts had stopped beating (Figure 2).

4. Discussion

When a stress-inducing toxic substance appears in the environment, organisms begin to defend themselves against its adverse effects, for which they expend their energy. The defense processes take place at the expense of energy which should be spent on the development; therefore, the growth rate decreases and functioning is impaired [17]. Thus,

increasing (≥0.12 M) NaCl concentrations inhibited the Y of unicellular organisms, algae *P. subcapitata* (Table 3). The literature indicates that increasing NaCl concentrations are also inhibitors of the development of other algae such as *Chlorella vulgaris*, *Chlorella salina*, *Chlorella emersonii* and *Scenedesmus opoliensis* [26]. A similar response was exhibited by a lower vascular plant, the common duckweed *Lemna minor* L. The growth rate of this plant is also inhibited by NaCl [27], although the fresh weight of plants at the lowest concentrations (ranging from 3.91 to 31.25 mM) shows an opposite trend and is slightly stimulated. In this study, the algae exposed to NaCl at 0.05 and 0.10 M were also stimulated, which manifested itself in a Y higher than that noted for the control sample (Figure 1). However, after seven days, exceeding the NaCl concentration of 0.14 M resulted in the lowest observed effect concentration (LOEC). The LOEC is defined as the lowest applied concentration of a chemical compound, which reduces the measured response by more than 20% [28]. Sometimes, even such plant response to salinity is not observed, as certain plant species tolerate environmental contamination by NaCl. This tolerance involves the formation of certain defense mechanisms. These include a reduction in the absorption of this chemical, or the accumulation of substances that help maintain the osmotic balance and protect cell structures against excessive water loss [29], including rapidly changing the volume during high salinity stress by adjusting the intracellular ion and glycerol concentration, eventually restoring the suitable cell turgor pressure [26]). To mitigate the effects of salinity, plants also accumulate osmoprotectants, including soluble carbohydrates, in their tissues, especially monosaccharides and sucrose in common duckweed, and cyclitols in yellow lupin [27]. However, this concept of defense against salinity has no effect when the NaCl concentration in water is 0.12 M or more (Figure 1). It was demonstrated that a NaCl concentration of 0.24 M inhibited the algal Y by 50% (EC_{50}) (Table 3). Observation of such plant responses to NaCl and the slowing of cell division, reduced size, ceased motility and triggered palmelloid formation, allowing both the toxicity of the salt and the degree of aquatic environment degradation to be estimated [26,27].

However, morphological features represent the secondary effects of xenobiotics' toxic influence. The increasingly greater inhibition of algal growth was probably due to the disturbance of photosynthetic activity, caused by NaCl. It was demonstrated that this xenobiotic inhibited by an average of 50% the minimum Fo, Fm and Fv at a concentration of 0.27 M and the Fv/Fm of photosynthesis at a concentration (EC_{50}) almost two times higher (Table 3). A chloride concentration of 0.15 M in the leaves of tobacco triggers a parabolic change in chlorophyll fluorescence, wherein after 24 h the Fv/Fm was significantly reduced as compared to the control (by 34.56%). However, after 48 h, the Fv/Fm value was slightly higher but still reduced by 21.33% [18]. The increase in the photosynthetic rate in higher plants may be due to a release of large quantities of photosynthetic products, including large quantities of glycerol. On the other hand, the inhibition of photosynthesis due to salinity causes deficiency in different cations, production of reactive oxygen species which interfere with various biochemical and physiological processes, including osmotic stress [26]. High xenobiotic contents in water in the form of hypertonic and hyperosmotic stress causes the dehydration of common duckweed tissue and an increase in dry matter content. Such an effect on the common duckweed leaves is exhibited by glyphosate in the form of isopropylamine salt, which, at concentrations of 20 and 40 μM, simultaneously inhibited the photochemical activity of photosystem II (PSII) by 62% and 95%, respectively [30].

The adverse effects of water salinity do not only affect plants. Most animal organisms at various levels of complexity are also unable to adapt to high salinity in the environment. The toxicity induced by salinity takes the form of multiple detrimental effects that can be observed in ecosystems. This resulted in decision to carry out a study concerning the effect of increasing NaCl concentrations on the SW, IM and BPM of crustaceans *D. magna*. *Daphnia magna* swim with a hopping motion. In their SW, two motion phases are distinguished: the action of the antennae which moves the body upwards and forwards, and downwards. The tested crustaceans repeat both motion phases in the same order [31]. The SW (hopping) of *D. magna* in the control sample was demonstrated to average 307.36 hops × min^{-1}

(Figure 2), while within 15 min a NaCl concentration of 0.07 M reduced the SW by 20% (15 min EC_{20}). At the same time, the inhibition of SW in half of the organisms (EC_{50}) was observed at a NaCl concentration of 0.19 M, while at the end of the test, the same effect occurred at half of the concentration (120 min EC_{50} = 0.10 M) (Table 7). In a study by Bownik et al. [31], control crustaceans (*D. magna*) at the beginning of the test, after 2 and 24 h exhibit the same frequency of hopping (approximately 210 hops × min^{-1}). The study also reports that behavioural biomarkers (SW track density, SW speed, turning angles and hopping frequency) are a sensitive feature of *D. magna* exposed to procaine penicillin [31]. In this study, of all the *D. magna* testing effects, it was the SW that was the most sensitive of all the tested features (Table 7). Organisms that do not swim due to NaCl become defenseless, as NaCl disrupts the relationship between the predator and the prey. It increases the likelihood and frequency with which the predator and a potential prey meet each other [32].

Not only was NaCl demonstrated to inhibit SW but also to cause systematic IM of organisms. Those exposed to a NaCl concentration <0.2 M were potentially still able, up to 75 min of the test (Figure 2, Table 7), to swim away to lower-saline areas or migrate in search of food. As reported by Bownik et al. [31], the early inhibition of SW is probably a consequence of neurotoxic changes. This was the effect of NaCl at a concentration of 0.27 M, since after 15 min it immobilised up to three organisms (EC_{50}) (Figure 2, Table 7). Many toxicity tests which use crustaceans assume the IM to be the endpoint and refer to the concentrations responsible for organism IM as a lethal concentration (LC). For example, Schuytema et al. [19] report that the test duration (2–21 days) had a slight effect on the range of salinity LC_{50} value in relation to *D. magna* Straus, and the LC_{50} for all tests was on average 6.6 g × L^{-1}. The EC_{50} values of salinity (as NaCl) for *D. magna* and *Daphnia longispina*, which amount to 5.9 and 2.9 g × L^{-1}, respectively, and are responsible for acute IM, are referred as mortality values [33].

As demonstrated by this study, non-swimming *D. magna* still exhibit a heartbeat. After 45 min of exposing these crustaceans to 0.2 M NaCl, when almost all organisms are immobilised (an average of 5.67 organisms), the BPM is still 286 beats × min^{-1} (Figure 2). This proves that the IM of organisms can be an indicator of SW inhibition but cannot be an indicator of mortality. The *D. magna* whose environment was not contaminated throughout the test exhibited a BPM of 262.13 beats × min^{-1} (Figure 2). The control *D. magna* organisms in a study by Bownik et al. [31] were characterised by a different (almost twice as high) heart rate (approximately 420 beats × min^{-1}). However, those exposed for 24 h to almost 1.2 g × L^{-1} of procaine penicillin exhibited only 164 beats × min^{-1}. Meanwhile, the heart of *D. magna* exposed to a NaCl concentration ≤0.15 beat faster by an average of 20% than that of control organisms and, after exceeding a NaCl concentration of 0.2 M, it gradually decreases over time (Figure 2). Similarly, caffeine at a concentration of 1% as early as after 7 min significantly increases the heart rate, while 5% ethanol produces an inversely proportional response. *Daphnia magna* organisms are sensitive to substances modifying the effects of neurotransmitters, as their heart, similarly to that in humans, is myogenic, and its rhythm is generated by a specialised heart muscle. The increased BPM in *D. magna* may result from the generation of greater activity of the sympathetic nervous system, while a decreased BPM may be due to the suppression of the baroreceptor reflex sensitivity [34]. *Daphnia* organisms are the foundation of the food chain in aquatic ecosystems and if NaCl in water (LOEC—at an average concentration >0.13 M) (Table 7) has an adverse effect on their behaviourism and physiological reactions, not only does it affect these organisms but also the entire ecosystem.

5. Conclusions

The applicated toxicity tests demonstrated that sodium chloride was not neutral towards the algae *P. subcapitata* and crustaceans *D. magna*.

A NaCl concentration of 0.29 M inhibited the Fo by 50%, and a NaCl concentration of 0.26 M inhibited (by 50%) the Fm and Fv. In the second experiment, i.e., the Daphtoxkit

test, organisms exposed to NaCl swam increasingly slower and within 15 min reached EC_{50} = 0.19 M. At the same time, at a NaCl concentration EC_{50} = 0.27 M, three out of six tested organisms were immobilised. The *D. magna* immobilisation was less modified by NaCl than SW, and BPM was a better measure of *D. magna* mortality than IM. As demonstrated by the BPM tests, after 45 min almost all *D. magna* individuals at a NaCl concentration of 0.2 M were immobilised, yet their hearts kept beating until 90 min.

The extension of the standard Daphtoxkit to include BPM tests and of the Algaltoxkit to include the testing of Fv/Fm (which, of all the chlorophyll fluorescence indicators, responded most poorly to sodium chloride) indicates the adaptability of the tested organisms to low water salinity. However, NaCl concentrations >0.19 M and >0.13 M are toxic (LOEC) to algae and crustaceans, respectively.

The comparison of the responses of these neighbouring links of the trophic chain shows the synergistic effect of NaCl on the aquatic ecosystem. The number of algae *P. subcapitata* (characterised by the high bioproductivity of producers), reduced due to the presence of NaCl will result in the simultaneous inhibition of the survivability of the first-order consumers, i.e., the crustaceans *D. magna*, which are the basic component of zooplankton. Ultimately, such an ecological imbalance exacerbates the problems existing in water bodies, e.g., eutrophication. Just as eutrophication initially leads to a disproportion of species in aquatic ecosystems, particularly an increase in phytoplankton, the presence of NaCl leads to the simultaneous dying out of both phyto- and zooplankton.

These studies suggest that physiological and behavioural features, additionally tested in the experiment, such as algal chlorophyll fluorescence, crustaceans swimming and heart rate are early and sensitive indicators of the toxic effects of NaCl in algae and crustaceans, before morphological changes are observed. The results of this study also confirm that the Algaltoxkit and Daphtoxkit biological methods are useful analytical tools for evaluating the toxicity of NaCl and predicting the consequences of the chemical contamination of freshwater bodies based on the calculated toxicity indicator values.

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Abbreviations

NaCl	sodium chloride
Y	biomass growth rate of algae
Fo	minimum chlorophyll fluorescence
Fm	maximum chlorophyll fluorescence
Fv	variable chlorophyll fluorescence
Fv/Fm	maximum quantum efficiency
IM	immobilisation
SW	swimming (hopping frequency)
BPM	heart rate
EC_x	effective concentration associated with x% response (20%, 50%, 90%)
LC_x	lethal concentration associated with x% response (20%, 50%, 90%)

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