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A study of the effects of sodium alginate and sodium carboxymethyl cellulose on the growth of common duckweed (*Lemna minor* L.)

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Abstract: Sodium alginate (ALG) and sodium carboxymethyl cellulose (CMC) are two polysaccharides that have a wide range of applications, which could lead to accidental pollution of the environment, making the assessment of their potential ecotoxicity imperative. The present study assesses the effects of ALG and CMC on the growth response of common duckweed (*Lemna minor* L.). The results emphasize that both polysaccharides can be classified as practically nontoxic based on their EC_{50} values, with ALG having a relatively higher toxicity compared to CMC. It was also observed that high doses of 1, 5 and 10 mg mL^{-1} of the two polysaccharides produced growth inhibitory effects against common duckweed. The toxicity of biopolymers against common duckweed, measured as EC_{50} values, seems to be correlated to the hydrophobicity of the monomers building the polymer. The EC_{50} values increase linearly with increasing water solubility (log *S*) values and decrease linearly with the lipophilicity (log *P*) values.

Keywords: ecotoxicity; growth inhibition; biopolymer, EC_{50}.

INTRODUCTION

Sodium alginate (ALG) and sodium carboxymethyl cellulose (CMC) are two polysaccharides. Alginates are natural polysaccharides that mainly occur in the cell walls of brown algae. The main biological source of sodium alginate is represented by brown algae species, such as *Macrocystis pyrifera*, *Ascophyllum nodosum* and *Laminaria* sp., but bacterial species, such as *Pseudomonas* sp. and

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Azotobacter sp., can also produce alginates.[1,2] Alginates are salt equivalents of alginic acid, an anionic polysaccharide composed of two repeating units. These units, D-mannuronate and L-guluronate, form homopolymeric blocks of either D-mannuronate, or L-guluronate, interspersed with alternating monomers. This structure makes alginic acid a linear binary copolymer.[3,4] Alginates have a wide range of applications, especially in the pharmaceutical and biomedical industries. Alginates can be used as drug and protein delivery systems, wound dressings, in cell culture and tissue regeneration, as 3D bioprinting bioink, adsorbents for the removal of different compounds from aqueous solutions, *etc*.[4–6]

Carboxymethyl cellulose is an anionic water-soluble cellulose ether, one of the most important cellulose derivatives. CMC is produced by the reaction of chloroacetic acid, or its sodium salt, with alkali cellulose swollen in aqueous NaOH.[7–9] Sodium carboxymethyl cellulose is a copolymer. *β*-D-glucose and *β*-D-glucopyranose 2-*O*-(carboxymethyl)-monosodium salt are the two monomers of CMC, these being linked *via* *β*-1,4-glycosidic bonds. The two monomers are not randomly distributed along the macromolecule, the substitution of the –OH groups with −COOH groups taking place slightly predominantly at the C-2 of glucose.[4,5] CMC has applications in industries such as pharmaceutical (as a suspending agent or tablet excipient), medical (in infection control or wound healing), cosmetic (as suspending agent or for the increase of viscosity), food (acting as water-binder and thickener), textile industry (as coatings), detergents (acting as a suspending agent), paper (in coating colours that improve the surface properties of paper), *etc*.[7,8,10,11]

All the applications of ALG and CMC could lead to accidental pollution of the environment with these two polysaccharides, the assessment of their potential ecotoxicity therefore being imperative.

There are a multitude of standardized ecotoxicity tests for both terrestrial and aquatic environments. The duckweed growth inhibition assay is one of these tests, in which duckweed colonies are exposed for 7 days to the test substance and several parameters can be analysed, such as number of green fronds, percent inhibition of growth rate and half maximal effective concentration.[12]

A simple search regarding the words "alginate", "carboxymethyl cellulose", "ecotoxicity" and "duckweed" in the Web of Science Core Collection (Web of Science, Clarivate Analytics) for the period 2000–2021 revealed that the number of published scientific articles regarding each of these terms is constantly increasing (Fig. 1).

Due to the wide range of applications of ALG and CMC, as well as the increased importance of protecting the environment and analysing the ecotoxicity of potential pollutants, investigating the potential ecotoxicological effects of sodium alginate and carboxymethyl cellulose is essential. The available data in the Web of Science Core Collection on these subjects are scarce, only a few articles being published about ALG or CMC and duckweed, but they do not describe the potential ecotoxicological effects of these polysaccharides towards duckweed. To the best of our knowledge, the current study is the only one that addresses the effects of sodium alginate and sodium carboxymethyl cellulose on the growth of *Lemna minor*.

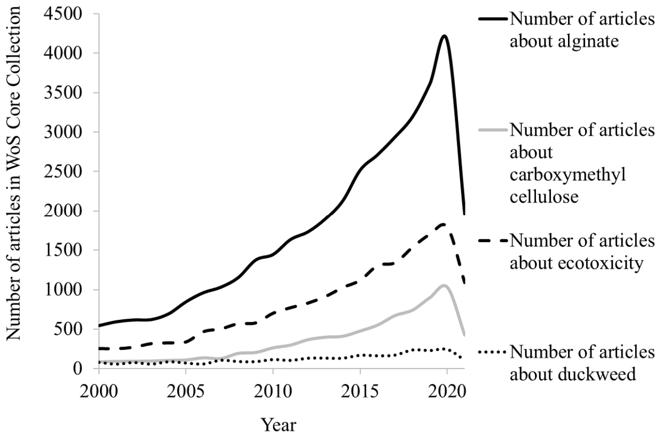

Fig. 1. Bibliometric analysis of research in 2000–2021 from the Web of Science Core Collection (Web of Science, Clarivate Analytics) on alginate, carboxymethyl cellulose, ecotoxicity and duckweed.

The aim of this research was to study the effects of sodium alginate and carboxymethyl cellulose on the growth response of common duckweed (*Lemna minor* L.), highlighting the potential ecotoxic effects of these two biopolymers.

EXPERIMENTAL

Materials

Sodium alginate from brown algae (Cat. No. W201502) and medium viscosity sodium carboxymethyl cellulose (Cat. No. C4888) were purchased from Sigma–Aldrich and used as supplied. Zinc chloride ($ZnCl_2$, Cat. No. 3533) was purchased from Carl Roth.

Tested polysaccharides

Ten concentrations of ALG and CMC were tested: 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001 and 0.0005 mg mL^{-1}.

Duckweed culture

Common duckweed, *Lemna minor* L., was used as the test organism for the assessment of growth response to ALG and CMC. The duckweed was collected from a natural habitat and was surface sterilised and cultured in the laboratory for several months before being used in the ecotoxicity tests. The duckweed culture was maintained under standard conditions, as described in the OECD[13] and EPA guidelines,[14] under continuous cool white fluorescent lighting at a temperature of 24±2 °C.

Duckweed growth inhibition test

The growth response of duckweed to ALG and CMC was assessed through a growth inhibition assay conducted in accordance with an OECD guideline,[13] the chosen positive control being 0.5 % $ZnCl_2$. The exposure conditions were the same as the culture conditions, in accordance with the OECD[13] and EPA guidelines.[14] All experiments were performed in triplicate and the data are presented as mean values.

A number of 35 fronds per test vessel for each tested concentration, negative and positive control, were used to assess the effects of ALG and CMC. After an exposure period of 7 days, the effects of ALG and CMC on the total number of fronds and on the number of green fronds were analysed. The total number of fronds and the number of green fronds were determined by manual counting.

Ecotoxicity data analysis

The data regarding the total number of fronds was used for the calculation of the average specific growth rate (μ) as described in the OECD[13] guideline. μ was calculated based on the measurement variable in the test or control vessel at time i (N_i, the beginning of the test) and at time j (N_j, the end of the test), and on the entire test period (t) from i to j, using Eq. (1):

$$\mu_{i-j} = \frac{\ln N_j - \ln N_i}{t} \tag{1}$$

Dose-response curves were plotted, and the half maximal effective concentration was calculated based on the total number of fronds (EC_{50}) and on the average specific growth rate (E_rC_{50}) using Microsoft Office Excel 365 with Solver Add-in. Based on the calculated EC_{50} values, the tested polysaccharides were classified into one of the aquatic ecotoxicity categories (Table I) according to U.S. EPA.[15]

TABLE I. Toxicity categories for aquatic ecotoxicity according to U.S. EPA[15]

Categories according to U.S. EPA	EC_{50} / mg L^{-1}
Very highly toxic	<0.1
Highly toxic	0.1–1
Moderately toxic	>1–10
Slightly toxic	>10–100
Practically nontoxic	>100

Computation of physicochemical parameters

The SwissADME[16] web tool was used for the computation of some physicochemical parameters, such molecular weight, lipophilicity as log P values and water solubility as log S values, based on the simplified molecular-input line-entry system (SMILES) formulas of the analysed compounds. These parameters were determined for the monomers of the two tested polysaccharides, as well as monomers of two other polymers, carboxymethyl chitosan and polyacrylamide, in order to assess the influence of the physicochemical properties on the ecotoxicity of these compounds. The SMILES of the monomers and all chemical structures were built using ACD/ChemSketch freeware.[17]

Statistical analysis

For the statistical analysis of the data, the PAST[18] software was used. The normality of the data was tested using the Shapiro–Wilk W test. The distribution analysis was followed by an ANOVA analysis which included Levene's, Tukey's posthoc, Kruskall–Wallis and Mann–

–Whitney U tests. If the data were normally distributed, the homogeneity of variance among treatments was determined through Levene's test and an analysis of variances was determined through Tukey's posthoc test. Otherwise, non-parametric tests were performed (Kruskall–
–Wallis and Mann–Whitney U tests). The difference was considered significant only if the *p* value was smaller than 0.05 ($p < 0.05$). All correlation analysis were done through Pearson Linear *r* correlation test.

RESULTS AND DISCUSSION

The percentage of green fronds in the samples treated with the two polysaccharides, compared to the negative control, was approximately 90 %, except for the three highest tested concentrations: 1, 5 and 10 mg mL^{-1}. The percentages of the three highest concentrations of ALG were 83, 56 and 33 %, respectively, and for CMC they were 88, 57 and 2 %, respectively (Fig. 2).

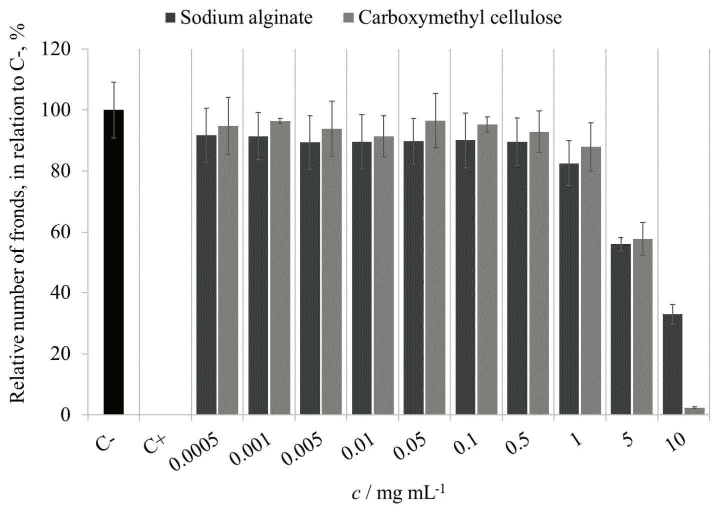

Fig. 2. Percentage of green fronds compared to the negative control for ALG and CMC, as well as the negative and positive controls.

The statistical analysis of normality of the data regarding the number of green fronds showed that the data for ALG were normally distributed, while the data for CMC were not normally distributed. Thus, the ALG data was analysed by parametric tests, while the CMC data was analysed by non-parametric tests.

The Levene's test of homogeneity of variance among treatments had a *p* value of 0.03 (<0.05) for ALG, and the Kruskall–Wallis test had a *p* value of 0.01 (<0.05) for CMC, the variances being significantly different. Thus, Tukey's posthoc test was performed for ALG, and the Mann-Whitney U tests for CMC (Table II).

The total number of fronds decreases in a relatively linear manner with the concentrations of both ALG (R^2 = 0.89) and CMC (R^2 = 0.92), Fig. 3. ALG produces a stronger decrease in the number of fronds compared to CMC, which highlights the higher toxic effect of ALG on common duckweed.

TABLE II. Results of Tukey's posthoc test for ALG (above diagonal) and of Mann–Whitney U tests for CMC (below diagonal). Statistically significant data ($p < 0.05$) are written in bold

c / mg mL^{-1}	10	5	1	0.5	0.1	0.05	0.01	0.005	0.001	0.0005
	p values									
10		0.2333	**0.0001**	**0.0022**	**0.0000**	**0.0007**	**0.0022**	**0.0008**	**0.0004**	**0.0055**
5	**0.0324**		0.2032	**0.0243**	**0.0216**	**0.0155**	**0.0243**	**0.0171**	**0.0093**	**0.0253**
1	**0.0111**	**0.0369**		0.9984	0.9975	0.9976	0.9984	0.9983	0.9883	0.9930
0.5	**0.0111**	**0.0369**	0.6742		1	1	1	1	1	1
0.1	**0.0174**	**0.0498**	0.3169	0.4529		1	1	1	1	1
0.05	**0.0179**	0.0518	0.2187	0.7133	0.8845		1	1	1	1
0.01	**0.0174**	**0.0498**	0.6213	1	0.6552	0.3807		1	1	1
0.005	**0.0109**	**0.0358**	0.6752	1	0.7110	1	0.8041		1	1
0.001	**0.0314**	0.0765	0.2909	0.2909	0.5784	0.8584	0.4673	0.7628		1
0.0005	**0.0179**	0.0518	0.5403	1	0.8845	0.7715	1	0.8049	0.8584	

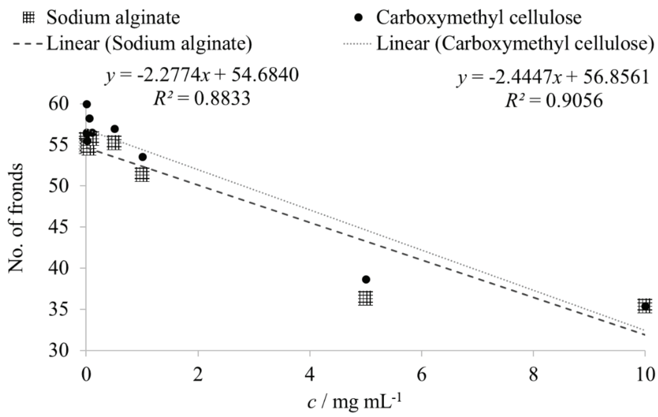

Fig. 3. The effect of the ALG and CMC concentration on the total number of fronds.

The Pearson Linear r correlation test showed a strong negative correlation between the total number of fronds and the concentration of ALG (–0.94) and CMC (–0.95), with a high probability of correlation for both ALG ($p = 5.4\times10^{-5}$) and CMC ($p = 2.3\times10^{-5}$).

Dose-response curves were plotted for both ALG (Fig. 4) and CMC (Fig. 5) based on the total number of fronds and on the average specific growth rate. The half median effective concentrations were calculated based on the total number of fronds (EC_{50}, Figs. 4 and 5) and on the average specific growth rate (E_rC_{50}) (Table III).

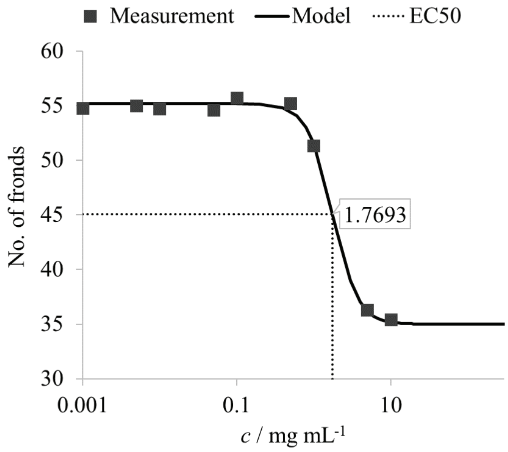

Fig. 4. Dose-response curve of total number of fronds and the concentration of ALG.

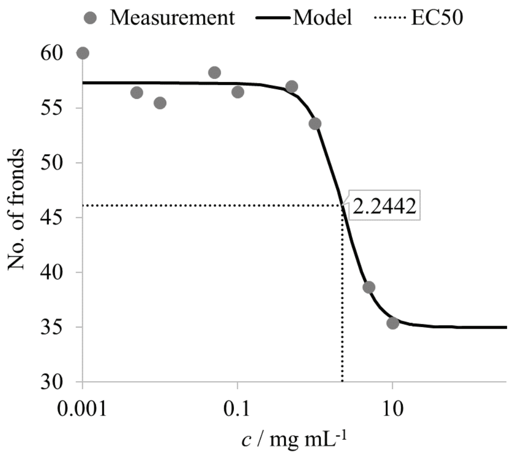

Fig. 5. Dose-response curve of the total number of fronds and the concentration of CMC.

According to the U.S. EPA15 toxicity categories for aquatic ecotoxicity, ALG and CMC can be classified as practically nontoxic based on the obtained EC_{50} values, ALG being slightly more toxic than CMC, having lower EC_{50} and ErC_{50} values.

TABLE III. EC_{50} and E_rC_{50} values for ALG and CMC

Polysaccharide	EC_{50} / mg L^{-1}	E_rC_{50} / mg L^{-1}
ALG	1769.3	1786.8
CMC	2244.2	2532.7

In order to assess the possible correlation between some physicochemical parameters of the polysaccharides and their toxicity against common duckweed, specific literature for ecotoxicity data obtained for other biopolymers was searched. This proved difficult due to the lack of information on the ecotoxicity of other polysaccharides towards duckweed or even polymers. Thus, data from two identified articles: the effect of carboxymethyl chitosan (CMCS) on *L. minor* L.[19] and the effect of polyacrylamide (PAM) on *Lemna aequinoctialis* Welw[20] were included in this study.

The SMILES formulas necessary for the computation of the physicochemical properties were generated for the monomers of the four polymers: sodium *β*-D-mannuronate (Fig. 6, **a**) and sodium *α*-L-guluronate (Fig. 6, **b**) for ALG;

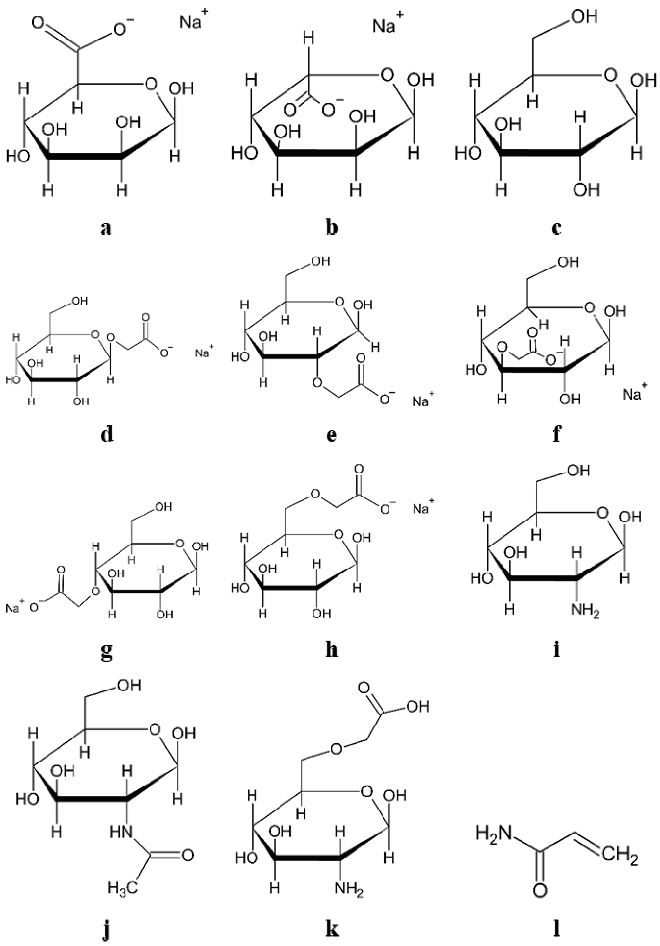

Fig. 6. Chemical structures of the monomers used for the computation of the physicochemical parameters using SwissADME: **a**) sodium *β*-D-mannuronate and **b**) sodium *α*-L-guluronate for ALG; **c**) D-glucose, **d**) sodium 1-*O*-carboxymethyl-D-glucose, **e**) sodium 2-*O*-carboxymethyl-D-glucose, **f**) sodium 3-*O*-carboxymethyl-D-glucose, **g**) sodium 4-*O*-carboxymethyl-D-glucose and **h**) sodium 5-*O*-carboxymethyl-D-glucose for CMC; **i**) D-glucosamine, **j**) *N*-acetyl-D-glucosamine and **k**) *O*-carboxymethyl-D-glucosamine for CMCS; **l**) acrylamide for PAM.

D-glucose (Fig. 6, **c**) and sodium carboxymethyl-D-glucose (Fig. 6, **d**–**h**) for CMC; D-glucosamine (Fig. 6, **i**), *N*-acetyl-D-glucosamine (Fig. 6, **j**), *O*-carboxymethyl-D-glucosamine (Fig. 6, **k**) for CMCS; acrylamide (Fig. 6, **l**) for PAM.

Starting from the SMILES formulas, the values of molecular weight, log *P* and log *S* for the monomers of the investigated biopolymers were computed. Besides these parameters, log *P* and log *S* strongly affect the EC_{50} value. The EC_{50} values increased linearly (R^2 = 0.82) with increasing log *S* value (Fig. 7) and linearly decreased (R^2 = 0.96) with the log *P* values (Fig. 8).

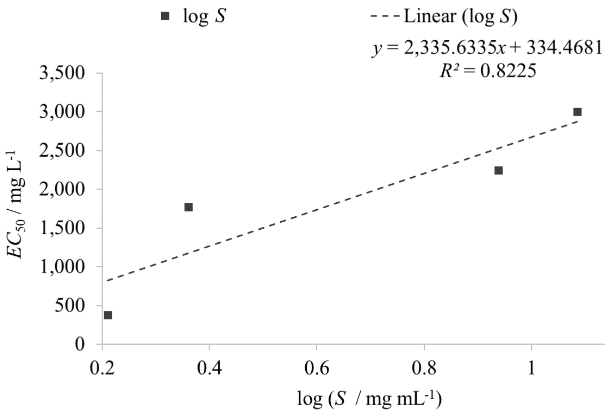

Fig. 7. The effect of log *S* values on the EC_{50} values.

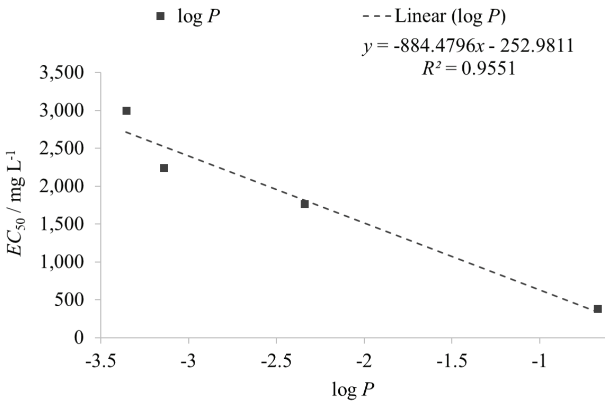

Fig. 8. The effect of log *P* values on the EC_{50} values.

Statistical analysis revealed that only log *P* showed a statistically significant (p = 0.0227) correlation (–0.97) with the EC_{50} values.

CONCLUSIONS

Based on the EPA toxicity categories for aquatic ecotoxicity and taking into account the EC_{50} values, this study shows that both sodium alginate and carboxymethyl cellulose can be classified as practically nontoxic. However, it was observed that the high concentrations (1, 5 and 10 mg mL^{-1}) of the two polysaccharides had some growth inhibitory effects on duckweed. It was also observed that sodium alginate had a relatively higher toxicity compared to carboxymethyl cellulose. The computational data reveals that the physicochemical parameters log *S* and log *P* influence the EC_{50} values. The EC_{50} values increased linearly with increasing log *S* value and decreased linearly increasing log *P* values, only the correlation between EC_{50} and the log *P* values being statistically significant. Although both polysaccharides can be classified as being practically non-toxic, these had concentration-dependent adverse effects on duckweed. These effects could pose a risk to the environment in the event of accidental pollution due to the widespread use of ALG and CMC. These effects must be taken into consideration in the processing of waste containing these polysaccharides in significant concentrations, which highlights the importance of the present results.

ИЗВОД

ПРОУЧАВАЊЕ УТИЦАЈА НАТРИЈУМ-АЛГИНАТА И НАТРИЈУМ-КАРБОКСИМЕТИЛ ЦЕЛУЛОЗЕ НА РАСТ ОБИЧНЕ СОЧИВИЦЕ (*Lemna minor* L.)

BIANCA V. BOROS[1], NATHALIE I. GRAU[2], ADRIANA ISVORAN[1], ADINA D. DATCU[3], NICOLETA IANOVICI[3] и VASILE OSTAFE[1]

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Натријум-алгинат (ALG) и натријум-карбоксиметил целулоза (CMC) су два полисахарида са широким спектром примене, који може довести до случајног загађења животне средине, па је процена њихове потенцијалне екотоксичности императив. Ова студија процењује ефекте ALG и CMC на раст обичне сочивице (*Lemna minor* L.). Резултати указују да се оба полисахарида могу класификовати као практично нетоксична на основу вредности EC_{50}, при чему ALG има релативно већу токсичност у односу на CMC. Такође је примећено да високе дозе ова два полисахарида, од 1,5 и 10 mg mL^{-1}, производе инхибиторне ефекте на раст обичне сочивице. Чини се да је токсичност биополимера против обичне сочивице, мерена као EC_{50} вредност, у корелацији са хидрофобношћу мономера који граде полимер. Вредности EC_{50} линеарно расту са повећањем вредности log *S* и линеарно се смањују са вредностима log *P*.

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