PRELIMINARY RESULTS ON THE ECOTOXIC EFFECTS OF CARBOXYMETHYL CHITOSAN TO COMMON DUCKWEED (*LEMNA MINOR* L.)

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Abstract: *Carboxymethyl chitosan is a hydrophilic modification of chitosan by carboxymethylation, which is a polysaccharide obtained from chitin through deacetylation. Chitin, the second most abundant polysaccharide, is a natural polymer made from chains of N-acetyl D-glucosamine and it is found in the exoskeletons of insects, the cell walls of fungi, and cephalopod beaks. As chitin is insoluble in all regular solvents such as water, organic solvents, mild acidic or basic solution, etc., it is deacetylated to obtain chitosan, which is soluble in dilute aqueous acidic solution (pH<6.5). Chitosan has wide biological applications due to its properties such as biocompatibility and biodegradability, thus the use of acids for the solubilization might induce a toxic effect of the chitosan. By carboxymethylation, chitosan becomes soluble in water at neutral pH, thus no acids are required for solubilization. Due to the potential pollution of the environment caused by the broad medical and pharmaceutical application of carboxymethyl chitosan, the assessment of its ecotoxicity is essential. The ecotoxicological effects of carboxymethyl chitosan (CMCS) were assessed using a growth inhibition assay on Lemna minor (duckweed) conducted according the OECD 221 guideline, followed by fresh and dry weight determination. Ten concentrations of CMCS (1%, 0.5%, 0.1%, 0.05%, 0.001%, 0.005%, 0.0001%, 0.0005%, 0.00001% and 0.00005%) were tested, with 35 duckweed fronds per replicate. After 7 days, the number of fronds (green and chlorosed) and colonies were assessed, the fronds were weighted, after blotting on paper for fresh weight determination, and were kept at 60˚C until no weight change was recorded, for dry weight determination. The results showed both green frond number and total frond number increased with decrease in CMCS concentration. The colony number and the number of fronds with chlorosis decreased with the concentration. Only the highest two concentration (1% and 0.5%) inhibited the growth of fronds, while the other concentrations showed no inhibition, on the contrary, promoting the growth. Regarding the dose-response curve, the calculated E50 value for CMC was 3000.6 mg/L, a high concentration that is not considered toxic.*

Keywords*: ecotoxicity, common duckweed, carboxymethyl chitosan*

INTRODUCTION

The ecotoxicological research, due to the rapid industrial development and being speeded up by severe industrial accidents, is under continuous development. In ecotoxicology, standard guides such as those created by US EPA (United States Environmental Protection Agency), OECD (Organization for Economic Cooperation and Development), ISO (International Organization for Standardization), Government of Canada or ASTM (American Society for Testing and Materials), are used to perform ecotoxicity tests on aquatic and terrestrial organisms. The purpose of these assays is to assess the effects at population level and ecosystem, in practice, the majority of assays being conducted at individual level (WALKER *et al.* 1991, KAHRU *et al.* 2010).

Due to the widespread desire particularly in Europe, of minimizing the vertebrate testing, invertebrates and plants are preferred for ecotoxicity assessments (CRANE *et al.* 2008). The common duckweed, a small free-floating macrophyte, is such an organism. It is preferred

due to its small size, vegetative replication, rapid growth and its adaptability to diverse aquatic conditions (YASEEN *et al.* 2016, HU *et al.* 2018). The growth inhibition assay on common duckweed involves maintaining the duckweed colonies under standard conditions in different concentrations of the tested substance for 7 days, after which the green fronds and fronds with chlorosis are counted. Other gravimetric parameters can be assessed such as colony number, fresh and dry weight, etc., which can be determined quickly and cheaply for other plant species (DATCU and SALA, 2018; DATCU *et al.*, 2019).

Carboxymethyl chitosan (CMCS) is a hydrophilic derivative of chitosan, obtained through carboxymethylation (SHARIATINIA 2018). Chitosan, a polysaccharide consisting of two monomer units, D-glucosamine and N-acetyl-D-glucosamine, is obtained by the deacetylation of chitin (ZHAO et al. 2011). Thus the source of CMCS is chitin, the next most abundant polysaccharide after cellulose, obtained mainly from the shells of crustaceans (MUXIKA *et al.* 2017). The many applications of chitosan are due to its amine and hydroxyl groups and its cationic nature (ZHAO et al. 2011).

By improving chitosan's solubility by carboxymethylation, the obtained CMCS having numerous biomedical applications such as wound healing, drug/gene delivery, tissue engineering and bioimaging (SHARIATINIA 2018). Due to its wide range of applications, CMCS represents a possible pollution source for the environment. Also, the study of ecotoxicity and of CMCS has increased over the years (Figure 1), but the scientific literature reveals only a small number of articles that approached the ecotoxicity of CMCS, directly or indirectly. Thus, the study of its ecotoxicity is essential.

Figure 1. Number of articles published about ecotoxicity and carboxymethyl chitosan (CMCS) in ISI Web of Science (Clarivate Analytics)

MATERIAL AND METHODS

Carboxymethyl chitosan was supplied by Santa Cruz Biotechnology (cat. no. sc-358091) and tested in ten concentrations: 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005%, 0.0001% and 0.00005%.

Common duckweed (*Lemna minor* L.) was used for the assessment of ecotoxicity of CMCS. Culture of duckweed was maintained under standard conditions, as described in OECD and EPA guidelines (OECD-221 2006, EPA-850.4400 2012).

The ecotoxicity of CMCS was assessed through a growth inhibition assay conducted in accordance with the OECD guideline (OECD-221 2006), the chosen positive control being 0.5% zinc chloride (Carl Roth, cat. no. 3533).

For each concentration, 35 fronds were used / vessel to monitor the effects of CMCS over the period of 7 days, expressed as number of green fronds, number of fronds with chlorosis, total number of frond and number of colonies, followed by the determination of fresh and dry weights.

The obtained data was analyzed and the percent inhibition of growth rate was calculated as described in the OECD guideline (OECD-221 2006). Using the total number of fronds, a dose-response curve was plotted using Microsoft Office Excel 365 with Solver Add-in, and the median effective concentration (EC_{50}) was calculated.

The fresh weight was measured by blotting the fronds on a paper, followed by measuring the weight using an analytical balance. The dry weight was obtained by drying the fronds in an oven at 60˚C for several days, until no change in weight over time was observed.

RESULTS AND DISCUSSIONS

The aim of this study was to assess the ecotoxicity of carboxymethyl chitosan on common duckweed. The number of chlorosed fronds increased with the increase in concentration of CMCS, while the number of green fronds decreased (Figure 2).

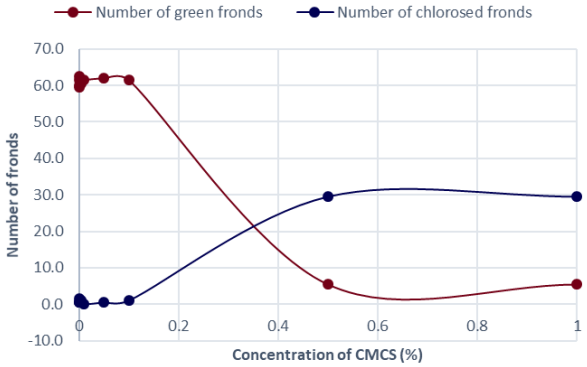

Figure 2. The effect of CMCS on the number of green fronds and fronds with chlorosis

While the number of colonies increased as the concentration of CMCS increased, the total number o fronds decreased (Figure 3). A high number of colonies can signify an increase in common duckweed population, but only if the number of fronds is also increasing. If the number of fronds is not increasing with the number of colonies, then the higher number of colonies signify the break-up into single fronds.

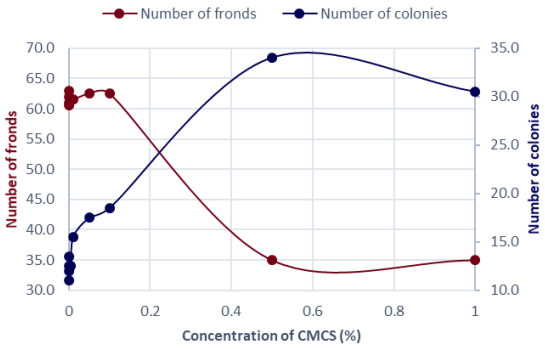

Figure 3. The effect of CMCS on the total number of fronds and colonies

The two highest CMCS concentrations caused similar effect to the positive control, while the rest of the concentrations not only allowed the survival of the fronds but even caused an increase in their number (Figure 4).

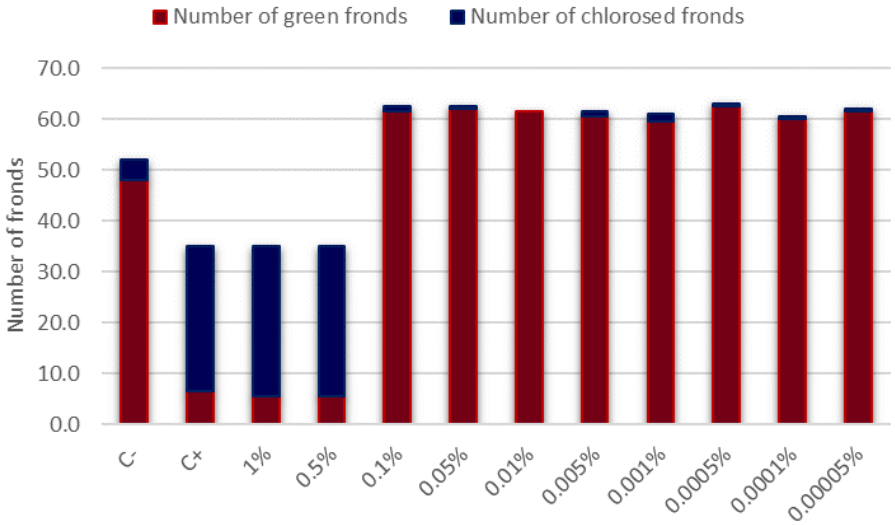

Figure 4. The number of green fronds and fronds with chlorosis for the ten tested concentrations of CMCS in comparison with the negative and positive controls

The highest two concentration had a percent inhibition of growth rate slightly higher than the positive control. The rest of the tested concentrations did not inhibit the growth rate at all, even promoting the frond growth (Figure 5).

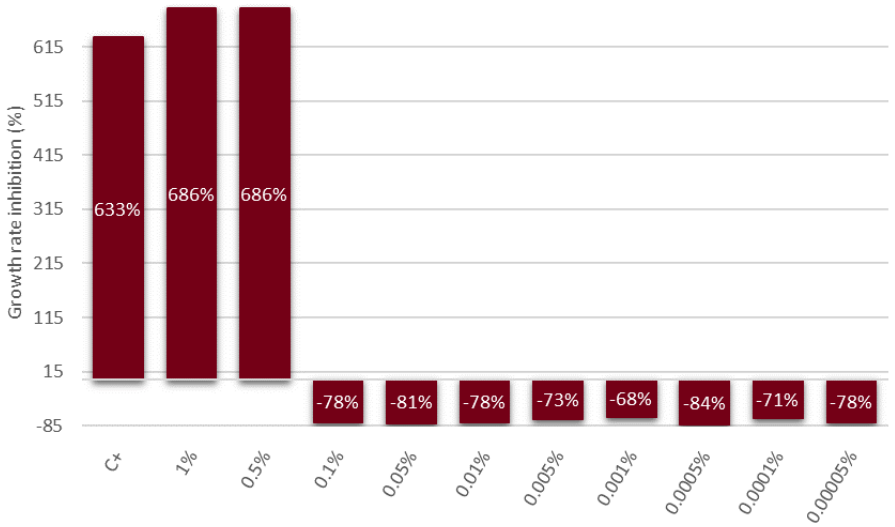

Figure 5. Percent inhibition of growth rate of the ten tested concentrations of CMCS in comparison with the positive control

The fresh and dry weight of fronds treated with CMCS followed a similar pattern, the dry weight values being about 15 times smaller than the fresh weight values (Figure 6).

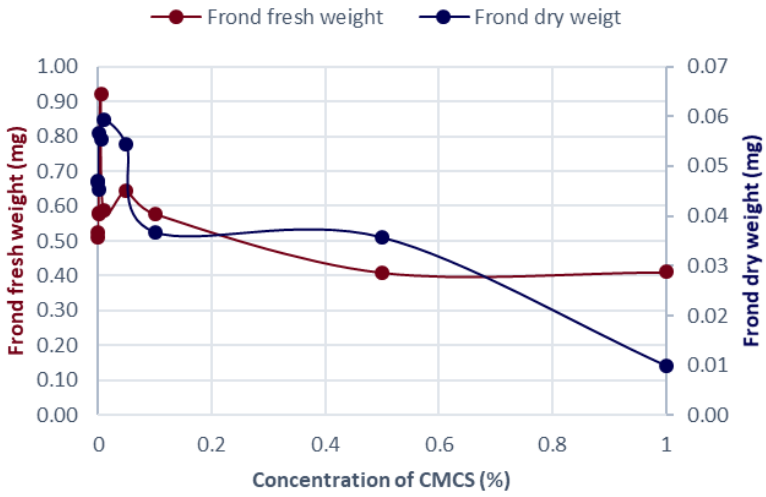

Figure 6. The effect of CMCS on the fresh and dry weight of fronds

The EC50 of CMCS was calculated from the dose-response curve plotted using the total number of fronds and the CMCS concentrations (Figure 7). The calculated EC50 value for CMCS was 3000.67 mg/L.

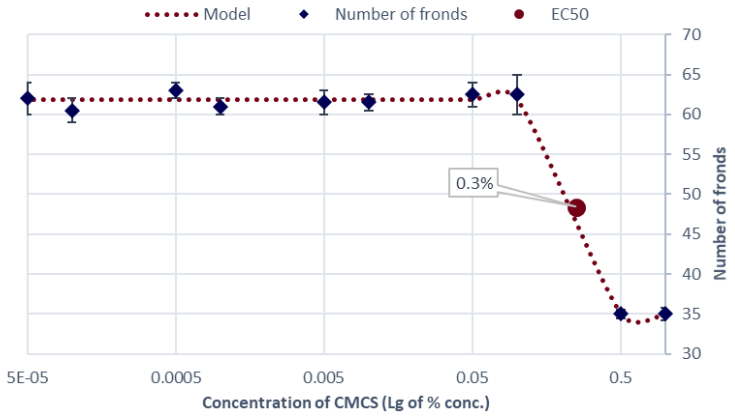

Figure 7. Dose-response curve based on the total frond number and EC_{50} of CMCS. The model represents the fitted data calculated from the total frond number

Carboxymethyl chitosan has a very low toxicity in accordance with its EC_{50} value (3000.67 mg/L), comparable with that of sodium chloride as described by Godoy *et al.* 2017, its EC_{50} being 3981.4 mg/L (GODOY *et al.* 2017). Thus it is unlikely that CMCS could be classified as a toxic substance as its median effective concentration value is only about 1.3 times smaller than that of sodium chloride, and it is about 2000 times larger than potassium dichromate's and about 300 times larger than zinc chloride's (INCE *et al.* 1999, GODOY *et al.* 2017).

CONCLUSIONS

The aim of this study was the assessment of the ecotoxicity of carboxymethyl chitosan, due to its many biomedical applications.

Both green frond number and total frond number increased with decrease in CMCS concentration, while the colony number and the number of fronds with chlorosis decreased.

Regarding the percent inhibition of growth rate, only the highest two concentration (1% and 0.5%) inhibited the growth of fronds, while the other concentrations showed no inhibition, on the contrary, promoting the growth.

In addition, the calculated E_{50} value from the dose-response curve, for CMC was 3000.6 mg/L, a high concentration that is not considered toxic.

ACKNOWLEDGEMENTS

This work was supported by Grant: ID 4/2017, PN3-P3-284, "Biotechnological tools implementation for new wound healing applications of byproducts from the crustacean seafood processing industry" – ChitoWound.

BIBLIOGRAPHY

CRANE, M., HANDY, R. D., GARROD, J., OWEN, R., 2008 - Ecotoxicity test methods and environmental hazard assessment for engineered nanoparticles. Ecotoxicology, 17 (5): 421-437.

DATCU, A.D., IANOVICI N., ALEXA E., SALA F., 2019 - Nitrogen fertilization effects on some gravimetric parameters for wheat. AgroLife Scientific Journal, 8(1): 87-92.

DATCU, A.D., SALA F., 2018 - Studies regarding the influence of nitrogen fertilizing dose on some ecophysiological parameters for Triticum aestivum. Research Journal of Agricultural Science 50(4): 105-110.

EPA-850.4400, 2012 - Aquatic Plant Toxicity Test Using Lemna spp.

GODOY, A.A., CARVALHO, L.B.D., KUMMROW, F., PAMPLIN, P.A.Z., 2017 - Sodium chloride as a reference substance for the three growth endpoints used in the Lemna minor L. (1753) test. Revista Ambiente & Água, 12: 8-16.

HU, C., LIU, L., LI, X., XU, Y., GE, Z., ZHAO, Y., 2018 - Effect of graphene oxide on copper stress in Lemna minor L.: evaluating growth, biochemical responses, and nutrient uptake. Journal of Hazardous Materials, 341: 168-176.

INCE, N.H., DIRILGEN, N., APIKYAN, I.G., TEZCANLI, G., ÜSTÜN, B., 1999 - Assessment of Toxic Interactions of Heavy Metals in Binary Mixtures: A Statistical Approach. Archives of Environmental Contamination and Toxicology, 36 (4): 365-372.

KAHRU, A., DUBOURGUIER, H.C., 2010 - From ecotoxicology to nanoecotoxicology. Toxicology, 269 (2): 105-119.

MUXIKA, A., ETXABIDE, A., URANGA, J., GUERRERO, P., DE LA CABA, K., 2017 - Chitosan as a bioactive polymer: Processing, properties and applications. International Journal of Biological Macromolecules, 105 (Part 2): 1358-1368.

OECD-221, 2006 - Lemna sp. Growth Inhibition Test.

SHARIATINIA, Z., 2018 - Carboxymethyl chitosan: Properties and biomedical applications. International Journal of Biological Macromolecules, 120: 1406-1419.

WALKER, C.H., GREIG-SMITH, P.W., CROSSLAND, N.O., BROWN, R., 1991 - Ecotoxicology. Animals and Alternatives in Toxicology: Present Status and Future Prospects. M. Balls, J. Bridges and J. Southee. London, Macmillan Education UK**:** 223-251.

YASEEN, D.A., SCHOLZ, M., 2016 - Shallow pond systems planted with Lemna minor treating azo dyes. Ecological Engineering, 94: 295-305.

ZHAO, L.M., SHI, L.E., ZHANG, Z.L., CHEN, J.M., SHI, D.D., YANG, J., TANG, Z.X., 2011 - Preparation and application of chitosan nanoparticles and nanofibers. Brazilian Journal of Chemical Engineering, 28: 353-362.

UTAMI, D., KAWAHATA, A., SUGAWARA, M., JOG, R.N., MIWA, K., MORIKAWA, M., 2018 - Effect of Exogenous General Plant Growth Regulators on the Growth of the Duckweed Lemna minor. Frontiers in Chemistry, 6 (251)