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Effects of Magnesium, Chromium, Iron and Zinc from Food Supplements on Selected Aquatic Organisms

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A B S T R A C T

The aim of this study was to determine the effect of uncontrolled environmental disposal of food supplements containing magnesium (Mg), chromium (Cr), iron (Fe) and zinc (Zn) on selected aquatic organisms including freshwater algae Scenedesmus subspicatus and Raphidocelis subcapitata, water flea Daphnia magna and duckweed Lemna minor. Thirty different food supplements containing Mg, Cr, Fe and Zn were analyzed. Results were expressed as effective concentration 50 (EC_{50}), i.e. growth inhibiting Mg, Cr, Fe and Zn (mg/L) concentration immobilizing 50% of treated organisms. Particular metal EC_{50} differed significantly ($p<0.001$) among study organisms, as follows (in ascending order): Scenedesmus subspicatus EC_{50} Fe (median 46.9 mg/L)<Zn (59.8 mg/L)<Mg (73.0 mg/L)<Cr (88.1 mg/L) (KW-H(3;120) =36.856; $p<0.001$); Raphidocelis subcapitata EC_{50} Fe (median 44.9 mg/L)<Zn (52.6 mg/L)<Mg (62.2 mg/L)<Cr (76.8 mg/L) (KW-H(3;120)=44.0936; $p<0.001$); Daphnia magna EC_{50} Zn (median 59.4 mg/L)<Cr (79.2 mg/L)<Fe (80.8 mg/L) Mg (82.0 mg/L) (KW-H(3;120)=39.2637; $p<0.001$); and Lemna minor EC_{50} Zn (median 131.0 mg/L)<Fe (186.8 mg/L)<Mg (192.5 mg/L)<Cr (240.4 mg/L) (KW-H(3;120)=58.6567; $p<0.001$). Uncontrolled environmental disposal of food supplements containing Mg, Cr, Fe and Zn exerts adverse effects on aquatic organisms. Therefore, legal provisions should regulate both the utilization and disposal of food supplements into the environment.

***Key words**: food supplements, aquatic organisms, ecosystem, toxicity, Croatia*

Introduction

Food supplements provide a concentrated source of nutritive ingredients of specific composition and/or preparation. Food supplements differ from usual foodstuffs by their nutritional characteristics and purpose, and are also known as food additives. By definition, they are intended for food enrichment and health status improvement. Besides vitamins, various elements, mostly those from the group of essential (oligo) elements are most commonly used as food supplements[1–3]. Thus, these elements are necessary for normal function of the human body. Along with other elements of the group, those based on zinc (Zn), iron (Fe), magnesium (Mg) and chromium (Cr) are most commonly used as food supplements and marketed in the form of effervescent tablets, tablets, capsules or pastilles. Because of their beneficial properties, they have been widely used in convalescence and other conditions where they are considered potentially useful or even necessary, especially when their deficiency has been demonstrated or presumed. These products are taken in concentrated form, by food supplements, in recommended dosage[1–5].

In the human body, chromium is involved in the metabolism of fatty acids and insulin. Trivalent chromium is needed for insulin action, as a factor of glucose toler-

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ance and as a cofactor of insulin action by forming a complex with insulin receptors. Additional chromium intake is recommended in the elderly and diabetic patients, at increased physical activity, in pregnancy, lactation and stress. Trivalent chromium is not toxic for plants unless present in a concentration greater than 500 mg chromium *per* kilogram dry matter, whereas the water soluble hexavalent chromium has adverse effects on plants. The toxicity of hexavalent chromium is limited by chromium reduction in the soil, rendering it less soluble and thus less toxic. The recommended daily intake of chromium for adults is 65 μg and maximal recommended intake 150 μg[3,6–8].

Zinc is one of the first microelements in the human body identified as essential (oligo) element, and is found in more than 200 enzymes in the human body. Zinc induces synthesis of low molecular weight proteins in the gastrointestinal mucosa cells and influences protein synthesis in the gastrointestinal tract. The highest zinc concentrations are found in the liver and prostate. The recommended daily intake of zinc is 15 mg in adults and 10 mg in children[3,8–10].

Magnesium is an essential element necessary in ion processes in cells and enzyme systems. Magnesium plays a major role in energy metabolism, muscle contraction, transfer of nervous impulses, and bone mineralization, and is a necessary catalyst in a number of cell enzymatic reactions, in fatty acid and protein synthesis, carbohydrate metabolism and calcium regulation in particular. The recommended daily intake of magnesium for adults is 350 mg and maximal recommended intake 600 mg[3,11].

Iron is a constituent of hemoglobin in human blood and is necessary for the action of oxidation system. Body tissues contain 10–12% of iron, which is found in myoglobin and enzymes. In the liver, spleen and bone marrow, iron is found in the form of ferritin and hemosiderin. The level of iron in human body is regulated and maintained by the neurohumoral mechanism, while its absorption is enhanced in the presence of ascorbic acid. The recommended daily intake of iron is 14 mg and maximal recommended intake 30 mg[3,8].

All these elements have a beneficial role in human body functioning and may even be considered necessary to maintain homeostasis. However, if taken in excessive amounts, they may also exert adverse effects on human health, therefore uncontrolled and unnecessary use should not be encouraged[12,13].

There is another, major issue that has not yet been adequately addressed or investigated; it is the question of food additive disposal. These products are found on the market as over-the-counter agents and their utilization is constantly increasing. As food supplements do not belong to drugs (yet being supplied in the form of drugs), they are not subject to toxicological testing, although they contain a broad spectrum of organic and inorganic substances that may, individually or in combination, exert toxic effects on various organisms including human body. Huge amounts of pharmaceutical products and food supplements that are not safe for use in humans are inappropriately disposed of. The problem is even greater taking in consideration the lack of regulations on their controlled disposal in the environment, as these products are simply disposed of as communal waste.

The aim of the present study was to assess the potential adverse effects on some simple aquatic organisms of food supplements enriched with Mg, Cr, Fe and Zn micronutrients having reached water medium by uncontrolled waste disposal. Specific testing included freshwater plankton algae *Scenedesmus subspicatus* and *Raphidocelis subcapitata*, water flea *Daphnia magna* and duckweed *Lemna minor*. Study results were expected to point to the possible ecosystem consequences that may (and do) occur due to the lack of legal provisions on handling and safe disposal of food supplements.

Materials and Methods

Materials

Thirty different food supplements containing Mg, Cr, Fe and Zn were analyzed. The level of Mg in study samples ranged from 133 mg to 250 mg, Fe from 6 mg to 30 mg, Zn from 5 mg to 30 mg, and Cr from 25 μg to 200 μg. Toxicity of each sample was determined at the respective concentration level.

Study organisms

Freshwater green alga *Scenedesmus subspicatus* Chodat is mostly found in the form of colonies, their number being always number two multiple (mostly with 4 or 8 cells). *Raphidocelis subcapitata* is a monocellular green alga. *Raphidocelis subcapitata* is found in colonies of 4 to 16 cells, and occasionally individually. Both are an integral part of freshwater phytoplankton. The freshwater algae *Scenedesmus subspicatus* and *Raphidocelis subcapitata* were cultivated in nutrient medium according to ISO 8692[14]. Plantation culture of test organisms was obtained from culture with exponential growth, so-called preculture that was cultivated 3 days before testing in the same conditions. The preculture cell density was measured immediately before use to calculate the plantation culture of test organisms volume needed to achieve the initial cell density in study solutions of approximately 10^4 cells *per* mL^{-1}. Freshwater samples were neutralized to pH 7.0±0.2, as necessary.

Testing was performed in 250-mL Erlenmeyer flasks containing 50 mL of test medium with initial cell density of 10^4 cells/mL. Each test consisted of six identical control cultures and at least 5 dilutions prepared in triplicate. The flasks were incubated for 72 h at 23±1 °C, at continuous light and shaking (110 min^{-1}) on a rotating shaker (Innova 4340, New Brunswick Scientific, New Jersey, USA). Algal growth was monitored every 24 h in all flasks (including control ones) by cell counting by use of a light microscope and counting chamber (according to Neubauer); pH value was monitored in each study solution at the beginning and at the end of test by use of a pH-meter (pH 526WTW, Weilheim, Germany). The values of inhibition percentage were graphically presented and effective concentration ($EC_{50;}$ the concentration causing 50% inhibition of algal growth) were read off from the lines obtained[14].

Daphnia magna is an integral part of freshwater zooplankton and is fed on phytoplankton. During most of the year the community consists of females, which can produce eggs by parthenogenesis at short time intervals. The culture of *Daphnia magna* Straus was used in the study[15]. The water used for dilution had the following characteristics: pH 7.8±0.2; hardness 250 mg/L±25 mg/L (expressed as $CaCO_3$); Ca to Mg ratio approximately 4:1; and level of dissolved oxygen 7.9 mg L^{-1}. Water for dilution was aerated up to saturation of oxygen concentration or pH stabilization; pH was adjusted to 7.8±0.2 with NaOH solution and/or HCl concentration of 1 mol dm^{-3}, as needed; pH was determined by use of a WTW256-pH meter. Study water samples were neutralized. The test was performed in glass tubes (160x15 mm) containing 10 mL of test medium. Each test consisted of four controls and at least five dilutions prepared in quadruplicate. Five daphnia were put in each tube, thus providing 2 mL of test solution *per* animal. During the test, daphnia were kept in the dark, at a temperature of 20±2 °C and deprived of food for 48 h. The number of motile daphnia was monitored in each tube every 24 h. Immobile daphnia were recorded and transferred to clean water. Some daphnia started moving after some time and were considered survivors. Other immobile animals were examined under magnifying glass to check for possible limb movements and heart beats. If heart beats were recorded, the animal was considered as survivor, and if there were no movements it was considered dead. Oxygen concentration in test tubes was measured immediately upon counting immobile animals. Dissolved oxygen was measured by use of oxygen electrode (WTW Model OXI 3000). At the end of 48-h testing, the percentage of immobility was calculated for each concentration relative to the total number of daphnia used. The 48-h EC_{50} was determined by an appropriate statistical method (logit analysis)[15].

Common duckweed or aquatic lentil (*Lemna minor* L.) is a small, simple structure and free-floating monocot. The plant body resembles small leaves of up to 1 cm in length. The root consisting of fine threads is 1–5 cm long. The main role of the root is to maintain the plant in horizontal position. Another role is photosynthesis, since many root cells contain chloroplasts. *Lemna minor* L. grows in freshwater, mostly stagnant water. Aquatic lentil was cultivated in nutrient medium according to ISO/CD 20079[16]. Study substances were added to nutrient medium before autoclaving. Each concentration of study chemicals and controls (nutrient medium without the addition of study substances) was prepared in eight replicates. One healthy plant colony was inoculated on sterilized and cool medium. The gain in the number of plants was determined by counting all, even very tiny macroscopically visible plants during 7-day experiment. The data obtained were entered in the following equation:

$$\text{Plant count gain} = \frac{\text{Plant count on day n} - \text{plant count on day 1}}{\text{Plant count on day 1}}$$

(n = 3, 5, 8, 10, 12, 14)

Results were expressed as the mean plant count gain in eight Erlenmeyer flasks ± standard deviation. The study endpoints were EC_{50} values for all tests at 7 days, 72 h and 48 h[16].

Statistical methods

Data were processed by use of Kolmogorov-Smirnov test for normality of distribution, followed by nonparametric Kruskal-Wallis test. The level of significance was set at $P<0.05$. The Statistica software, version 8.0 (StatSoft Inc., Tulsa, OK, USA) was employed on data analysis.

Results

Study results are presented as EC_{50}, i.e. Mg, Cr, Fe and Zn concentration (mg/L) that inhibited growth and immobilized 50% of treated organisms (Table 1 and Figures 1–4). Study metals yielded statistically significantly different ($p<0.001$) EC_{50} in all study organisms, as follows (in ascending order): *Scenedesmus subspicatus* EC_{50} Fe (median 46.9 mg/L; mean 59.7 mg/L)<Zn (median 59.8 mg/L; mean 60.7 mg/L)<Mg (median 73.0 mg/L; mean 76.7 mg/L)<Cr (median 88.1 mg/L; mean 85.9 mg/L) (KW-H(3;120)=36.856; $p<0.001$) (Figure 1); *Raphidocelis subcapitata* EC_{50} Fe (median 44.9 mg/L; mean 59.4 mg/L)<Zn (median 52.6 mg/L; mean 50.5 mg/L) <Mg (median 62.2 mg/L; mean 67.5 mg/L)<Cr (median 76.8 mg/L; mean 78.6 mg/L) (KW-H(3;120)=44.0936; $p<0.001$) (Figure 2); *Daphnia magna* EC_{50} Zn (median 59.4

TABLE 1
RESULTS OF ALL EFFECTIVE TOXICITY TESTS (EC_{50}) oF Mg, Cr, Fe AND Zn AS MICRONUTRIENTS IN FOOD SUPPLEMENTS ON ALL TEST ORGANISMS (mg/L)

Micronutrient	*Scenedesmus subspicatus*		*Raphidocelis subcapitata*		*Daphnia magna*		*Lemna minor*	
	X±SD	Median	X±SD	Median	X±SD	Median	X±SD	Median
Mg	76.7±14.0	73.0	67.5±12.9	62.2	80.9±12.5	82.0	198.6±34.4	192.5
Cr	85.9±4.9	88.1	78.6±13.1	76.8	86.7±17.1	79.2	235.7±26.8	240.4
Fe	59.7±25.8	46.9	59.4±26.8	44.9	73.2±17.4	80.8	174.5±46.6	186.8
Zn	60.7±9.2	59.8	50.5±7.1	52.6	61.0±9.4	59.4	138.2±31.8	131.0

mg/L; mean 61.0 mg/L)<Cr (median 79.2 mg/L; mean 86.7 mg/L)<Fe (median 80.8 mg/L; mean 73.2 mg/L)< Mg (median 82.0 mg/L; mean 80.8 mg/L) (KW-H(3;120)= 39.2637; $p<0.001$) (Figure 3); and *Lemna minor* EC_{50} Zn (median 131.0 mg/L; mean 138.2 mg/L)<Fe (median 186.8 mg/L; mean 174.5 mg/L)<Mg (median 192.5 mg/L; mean 198.6 mg/L)<Cr (median 240.4 mg/L; mean 235.7 mg/L) (KW-H(3;120)=58.6567; $p<0.001$) (Figure 4).

Comparison of study organisms revealed freshwater algae (*Scenedesmus subspicatus* and *Raphidocelis subcapitata*) to be more sensitive to the action of study metals than the more complex large water flea and duckweed (*Daphnia magna* and *Lemna minor*), as expected. Comparison of study metals (Mg, Zn, Fe and Cr) indicated Fe in aquatic medium to be most aggressive for algae (*Scenedesmus subspicatus* and *Raphidocelis subcapitata*), followed by Zn, Mg and Cr. However, Zn showed highest toxicity for more complex organisms (*Daphnia magna* and *Lemna minor*), followed by Fe, Mg and Cr.

Quite unexpectedly, the alga Scenedesmus subspicatus and water flea *Daphnia magna* showed comparable sensitivity to Zn (median 59.8 *vs*. 59.4 mg/L; mean 60.7 *vs*. 61.0 mg/L).

Discussion

The present study revealed that uncontrolled use and disposal of dietary supplements may entail manifold ad-

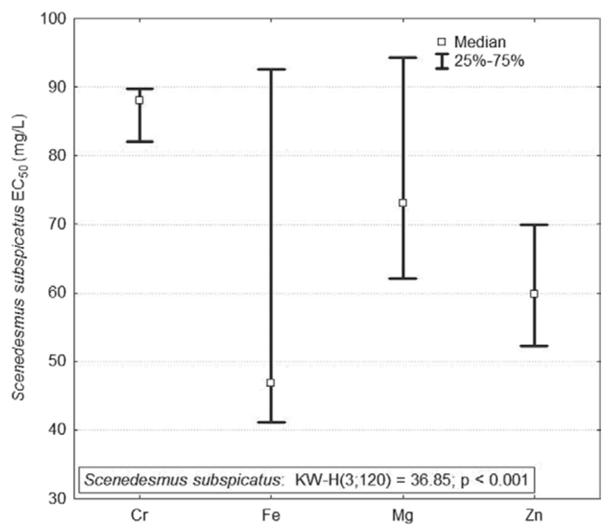

Fig. 1. Graphic presentation of effective toxicity tests (EC50) of Cr, Fe, Mg and Zn as micronutrients in food supplements on the test organism Scenedesmus subspicatus (mg/L).

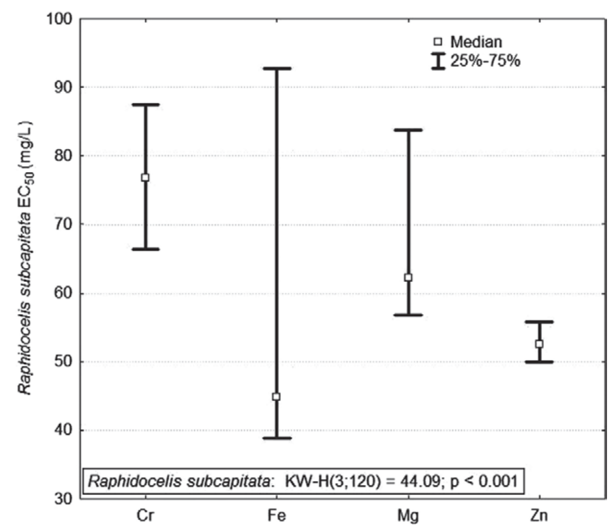

Fig. 2. Graphic presentation of effective toxicity tests (EC50) of Cr, Fe, Mg and Zn as micronutrients in food supplements on the test organism Raphidocelis subcapitata (mg/L).

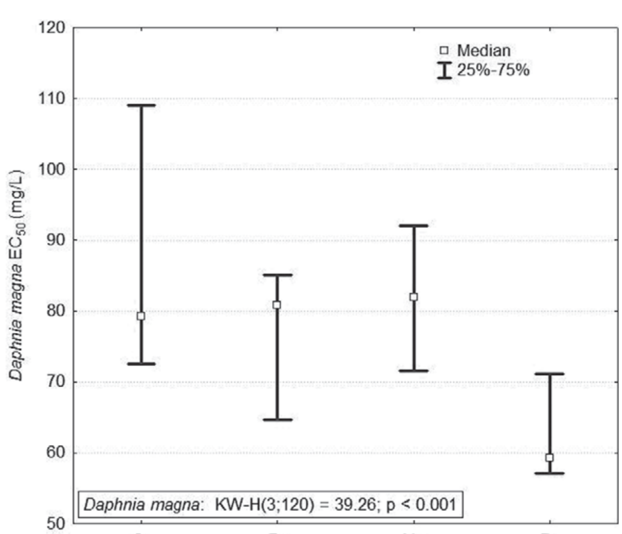

Fig. 3. Graphic presentation of effective toxicity tests (EC50) of Cr, Fe, Mg and Zn as micronutrients in food supplements on the test organism Daphnia magna (mg/L).

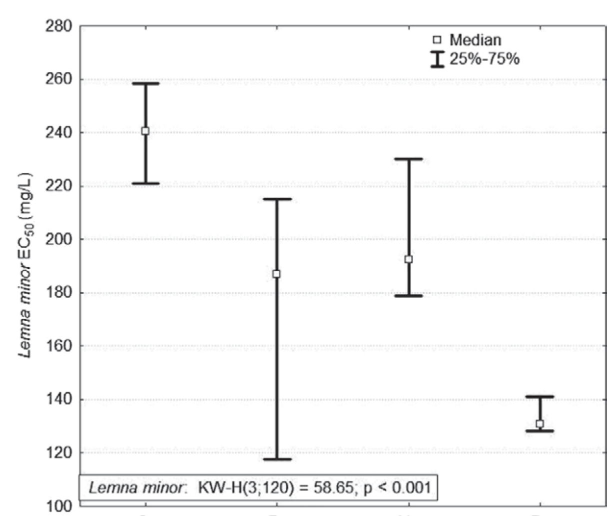

Fig. 4. Graphic presentation of effective toxicity tests (EC50) of Cr, Fe, Mg and Zn as micronutrients in food supplements on the test organism Lemna minor (mg/L).

verse consequences for both the environment and directly or indirectly for human health. Direct adverse effects on human health result from their large-scale use without proper indications and knowledge about the interactions among various dietary supplements, and with other food ingredients or medication. There are literature reports on cases of food supplement poisoning, some of them with lethal outcome[12,13]. These data point to the need of legal regulation in the field and better knowledge of the issue among users and all those pretended experts expected to provide due opinion and advice. The financial aspect is by no means negligible, as people spend rather high amounts of money buying products that not only fail to lead to favorable health effects as expected, but may even threaten it[12]. On the other hand, these products definitely elicit adverse effects on the ecosystem, thus indirectly threatening human health and welfare sooner or later.

Environmental effects of various types of contaminants have for years been investigated on simple test organisms by use of standardized tests. These test organisms include those used in the present study (green algae *Scenedesmus subspicatus* and *Raphidocelis subcapitata*; large water flea *Daphnia magna*; and duckweed *Lemna minor*)[14,16–37] and some other organisms such as algae, worms, fish, etc.[17,22,25,26,31,37,38]. These organisms are tested for the effects of phthalates[22], vertebrate hormones[23], presence of chelating compounds[27], conditions and methods of test organism culture[28], waste[29], industry[32], radioactive isotopes[39], herbicides[21,40], etc.

However, tests for metal toxicity are most commonly employed[17–20,24–27,29–31,33,34,36–38,40]. These include testing for the effects of 'toxic' metals such as Cd, Pb, Ni and Hg[20,24,25,29,30,33,34] and some heavy metals such as Zn, Cr, Al, Cu and Fe, which play a role in human body functioning, but are associated with cases of human poisoning[17,18,20,24–27,30,31,33,36–38,40].

Our results confirmed those reported elsewhere, in particular those stating that standardized tests on the test organisms used in the present study are very useful, relatively easy to perform and rather comparable with other similar studies[17–37,39,40].

However, our study tackled some specific aspects that have not been addressed elsewhere. First, there are relatively few studies using more than one test organism[17,21,22,25,26,31], while those using more than two test organisms are extremely rare[26]. In contrast to the only such a study available, where all test organisms were algae, we used two green algae (*Scenedesmus subspicatus* and *Raphidocelis subcapitata*), large water flea (*Daphnia magna*), and a plant, aquatic lentil (*Lemna minor*) as a more complex organism. As expected and reported from other studies, *Lemna minor* proved most resistant to the effect of study metals[21,41,42], whereas *Daphnia magna* showed higher sensitivity to the study contaminants as compared with freshwater test fish[25]. It was quite surprising that large water flea *Daphnia magna* was as sensitive to Zn effect as freshwater alga *Scenedesmus subspicatus*, which is of a much simpler structure.

Our choice of study metals (Cr, Zn, Fe and Mg) was unique because, to our knowledge, the toxicity of these four metals has not been tested elsewhere; the more so, our search of the literature yielded no report on Mg testing. It may seem quite conceivable because there is no regulation on the maximum allowable concentration (MAC) of Mg or it is set very high (in food, water and soil), since this element is considered safe and its daily dietary intake necessary (essential element), including drinking water[11,43–46]. However, considering our study results we are not inclined to share this opinion on complete safety of Mg. Not only Mg proved more toxic for the test organisms than Cr, but the Mg EC_{50} (median 62.2–192.5 mg/L; mean 76.7–198.6 mg/L) was lower than the Mg concentration present in most food supplements enriched with this element (particular products tested contained 133–250 mg Mg).

Despite the 'known' toxic effect on humans, including carcinogenic risk[6–8], Cr showed lowest toxicity for the test organisms. It is consistent with some studies where Cr showed low or moderate toxicity to test organisms[24,33]; however, other studies point to its high toxicity[26]. The toxicity of Cr is influenced by its valence, medium pH, and presence (or absence) and concentration of chelating compounds such as ethylenediaminetetraacetic acid (EDTA) used as antidotes in metal poisoning[19].

As an essential element, Fe is involved in oxygen transport and cellular oxidation systems[8,47]. Major reasons for iron supplementation include Fe loss in menstruation cycle, pregnancy and lactation, and intensive growth and development[8,47]. Morse *et al*. report on 20,000 children aged 6 years or younger to have taken Fe supplements, unfortunately, with many cases of poisoning and even lethal outcome[48]. Prescribing and using Fe supplements is therefore considered highly questionable, emphasizing the need of additional education on Fe biology and mechanisms of Fe supplementation.

Iron also exerts toxic effects in the environment[49], with pronounced effect on reduced biodiversity in aquatic medium[49]. Our study yielded similar findings, as Fe showed highest toxicity of all study metals on the freshwater algae *Scenedesmus subspicatus* and *Raphidocelis subcapitata*, whereas only Zn showed higher toxicity on more complex test organisms. It appears strange that there is no legal restriction for farmland Fe concentration[44,45], while Fe MAC in drinking water is only 200 μg/L (0.2 mg/L)[46], which is several times below the Fe concentration found in Fe supplements (6–30 mg) that are disposed in the environment without any control measure.

Zinc is an essential element for human body functioning; however, its excessive intake is associated with toxicity. Excessive Zn absorption suppresses Cu and Fe absorption, while free Zn ion in aquatic medium is very toxic for plants, intervertebrates, and even fish[17,18,24–26,30,31]. Our study also pointed to high Zn toxicity; as compared with Fe, Mg and Cr, Zn exerted highest toxicity for more complex test organisms, water flea (*Daphnia magna*) and duckweed (*Lemna minor*). We point to the unexpectedly high sensitivity of the water flea *Daphnia magna*, which was comparable to that of the alga *Scene-*

desmus subspicatus. On comparison of the two test algae, *Raphidocelis subcapitata* proved more sensitive to Zn action than *Scenedesmus subspicatus*, which is consistent with the results reported by Lukavsky et al.[26]. In our study, Zn showed by far lower EC_{50} as compared with other study metals (the difference ranging from 9 mg/L to nearly 100 mg/L) in more complex test organisms. Therefore, it is quite surprising that Zn MAC is 3 mg/L in drinking water and even 2000 mg/kg (2 g/kg) in mud intended for farmlands. Uncontrolled disposal of food supplements containing 5–30 mg Zn is therefore considered an issue of high concern.

The findings of this study point to the need of legal regulations for the manufacture, sale, utilization, and in particular controlled and appropriate disposal of food supplements containing Mg, Cr, Fe and Zn, not only in the Republic of Croatia, but also in the European Union, where such legal provisions are also lacking. Although belonging to essential elements, the study metals showed toxic potential and effects on certain simple aquatic organisms, which cannot but reflect, sooner or later, on the entire ecosystem and on human health and quality of life in general.

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UČINCI MAGNEZIJA, KROMA, ŽELJEZA I CINKA IZ DODATAKA PREHRANI NA ODABRANE VODENE ORGANIZME

SAŽETAK

Cilj ovog istraživanja bio je utvrditi učinak nekontroliranog okolišnog odlaganja dodataka prehrani koji sadrže magnezij (Mg), krom (Cr), željezo (Fe) i cink (Zn) na odabrane vodene organizme, uključujući slatkovodne alge *Scenedesmus subspicatus* i *Raphidocelis subcapitata*, vodenu buhu *Daphnia magna* i vodenu leću *Lemna minor*. Analizirano je trideset različitih dodataka prehrani koji sadrže Mg, Cr, Fe i Zn. Rezultati su izraženi kao efektivna koncentracija 50 (EC50), tj. koncentracija Mg, Cr, Fe i Zn (mg/L) koja inhibira rast odnosno imobilizira 50% izloženih organizama. EC50 pojedinih metala značajno se razlikovala ($p<0.001$) među ispitivanim organizmima, kako slijedi (uzlazno): *Scenedesmus subspicatus* EC50 Fe (medijan 46,9 mg/L)<Zn (59.8 mg/L)<Mg (73.0 mg/L)<Cr (88.1 mg/L) (KW-H(3;120)= 36,856, $p<0.001$); *Raphidocelis subcapitata* EC50 Fe (medijan 44.9 mg/L)<Zn (52.6 mg/L)<Mg (62.2 mg/L)<Cr (76.8 mg/L) (KW-H(3;120)=44,0936, $p<0.001$); *Daphnia magna* EC50 Zn (medijan 59.4 mg/L)<Cr (79.2 mg/L)<Fe (80.8 mg/L)<Mg (82.0 mg/L) (KW-H(3;120)=39,2637, $p<0.001$) i *Lemna minor* EC50 Zn (medijan 131.0 mg/L)<Fe (186.8 mg/L)<Mg (192.5 mg/L)<Cr (240.4 mg/L) (KW-H(3;120)=58,6567, $p<0.001$). Nekontrolirano odlaganje dodataka prehrani koji sadrže Mg, Cr, Fe i Zn u okoliš ima negativne učinke na vodene organizme. Stoga, zakonskim odredbama treba regulirati kako uporabu tako i odlaganje dodataka prehrani u okoliš.