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Section: Water Engineering

Lemna minor as a tool for wastewater toxicity assessment and pollutants removal agent

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Abstract

Municipal effluents are of major concern for the quality of the receiving water bodies. In this study the toxicity of municipal effluents was evaluated using the bioassays with aquatic higher plant common duckweed (*Lemna minor* L.). The aim of the study was to evaluate the applicability of *L. minor* for wastewater toxicity testing and it's efficiency in toxicants removal. Toxicity tests were performed on samples of untreated and biologically treated wastewater of small city Šilalė. Chemical analysis recorded high concentrations of nutrients and heavy metals in untreated wastewater. Wastewater treatment significantly reduced (up to 60%) the content of nutrients (N, P), however only slight reduction (up to 10%) in the concentrations of heavy metals was observed. Both, untreated and biologically treated wastewater inhibited the relative growth rate of *L. minor*, affected the biomass of *L. minor* fronds, the content of photosynthetic pigments (chlorophyll a, b) and induced oxidative stress. Undiluted wastewater was extremely phytotoxic and led to the death of test organisms. However due to high concentrations of nutrients (N, P) treated wastewater may even stimulate the growth of *L. minor* and may mask the toxicity of other toxicants (such as heavy metals). It was observed that *Lemna minor* can be used as wastewater phytoremediation agent, as they have the capacity to remove relative high amounts of PO_4^{3-} and NH_4^+ from the wastewater, they may significantly reduce the concentrations of NO_3^- in wastewater and slightly reduce the content of SO_4^{2-}. *L. minor* removed the heavy metals from the wastewater and it was detected that the concentrations of Zn, Mn, Cu and Cd in wastewater were reduced after the growth of *L. minor*, however, the removal of Pb was negligible.

Keywords: bioassays; *Lemna minor*; toxicants removal; toxicity testing; wastewater.

1. Introduction

Municipal effluents are of major concern for the quality of the receiving aquatic ecosystems as effluents from wastewater treatment plants are one of the main sources of anthropogenic chemicals. Municipal wastewater contains a complex variety of organic and inorganic compounds. Traditionally, the control of the quality of effluents discharged in waters was based on physico-chemical analysis of the effluents (e.g. biological and chemical oxygen demand, pH, total dissolved solids, heavy metals, organic compounds, etc.). However, in several countries effluent bioassays are used to assess regulatory compliance with effluent criteria (US, Canada, Germany) [1]. Up to date, more and more countries for routine effluent control use bioassays along with physico-chemical analysis. Potential hazard assessment of effluents in Lithuania is still based on the physic-chemical parameters of the effluents. However, chemical data alone do not allow evaluation of possible toxic effects to biota living in receiving waters. The major problem in controlling wastewater discharges is related to its environmental toxicity. Chemical analysis of wastewater is usually insufficient to provide the information on water quality as a high number of chemical compounds are present and some in concentrations lower than detection limits. It is also impossible to predict the toxicity of complex wastewater using the physico-chemical approach, due to interaction effects of chemicals in mixtures.

Implementation of the Water Framework directive (2000/60/EC) requires ensuring good chemical and biological status of receiving waters. Due this requirement, the whole effluent toxicity assessment is more and more used in routine effluent control and monitoring. Whole effluent toxicity assessment is based on the bioassays for acute and chronic toxicity and is generally accepted as a useful tool for assessing and managing the effluents toxicity. One of the principal advantages of whole effluent toxicity assessment is that bioassays measure the overall toxicity of mixtures, i.e., various interactions (e.g., antagonistic, synergistic) between all effluents components [2]. It is accepted, that toxicity bioassay, using species

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representing the different trophic levels, is the best approach to evaluate the whole toxicity of wastewater. Therefore, the selection of test organisms, representing different trophic levels is crucial. The comparison of wastewater toxicity evaluation using test batteries and the chemical indices revealed good coincidence between the toxicity and chemical-based assessments [3].

Duckweeds (*Lemnaceae*) due to their small size, high multiplication rates, susceptibility to pollutants and duckweeds importance in the aquatic food web, are one of the most used aquatic plants in toxicity testing procedures of various inorganic and organic chemicals and their mixtures. Studies showed that duckweeds are very sensitive in various mixtures (such as, wastewater, leachates, etc.) toxicity evaluation [4, 5]. *Lemna* spp. are the most often used as representatives of the primary producers in the test batteries for wastewater toxicity assessment. The standardised growth inhibition tests with duckweeds have been developed by OECD, ISO and ASTM [6–8]. Moreover, duckweeds are used for aquatic bodies' phytoremediation, wastewater treatment and toxicants removal [9–12].

The main aim of this paper was to evaluate the applicability of common duckweed (*Lemna minor* L.) for wastewater toxicity testing and to assess it's efficiency in toxicants removal from the wastewater.

2. Materials and methods

Samples of untreated (raw) and biologically treated Šilalės municipal effluents were collected in February of 2012. The Šilalės wastewater treatment plant received municipal and industrial wastewater with a population equivalent between 2000 and 10 000. The average yearly discharge is approximately 314 thousands m^3 of effluents.

The wastewater samples were taken to Vytautas Magnus University laboratory and stored in darkness in refrigerator prior to performance of the bioassays.

According to US Environmental Protection Agency (EPA) recommendations for toxicity test a dilution series of five wastewater concentrations (i.e., 100, 50, 25, 12.5 and 6.25% wastewater) and a control was made. Phytotoxicity test was performed with unfiltered wastewater. Phytotoxicity test was performed with aquatic plant common duckweed (*Lemna minor* L.). *L. minor* has been taken from the Vytautas Magnus University laboratory stock culture.

The stock culture of *L. minor* was grown in modified Steinberg medium (ISO/DIS 20079) [7] in growth chambers at 24 °C ± 2°C with a light/dark cycle of 16/8 h. Twenty (20) double-fronded healthy common duckweed (*L. minor*) colonies were transferred to Erlenmeyer flasks containing different concentrations of wastewater. The dilutions of wastewater were performed with Steinberg growth medium of *L. minor*. Experiment has lasted 7 days and has been conducted in 3 replicates and was executed according to OECD protocol [8]. The fronds number has been monitored every day of experiment. Toxicity was recorded as percent inhibition of *L. minor* growth (fronds number and fronds biomass) (relative to control), content of photosynthetic pigments and lipid peroxidation as a result of 7 days exposure to the toxicant (effluents) in its growth medium.

Relative growth rate was calculated from the following Eqn. (1) with measured fronds number (N) at the end (t1) and the start of the test (t0):

$$r = (\ln N_{t_1} - \ln N_{t_0}) / (t_1 - t_0) \tag{1}$$

For dry weight determination, the plants were dried at 60 °C for 48 h up to constant weight.

Content of chlorophylls (a, *b*) was measured spectrofotometrically in 100% acetone extract [13]. Concentration of malondialdehide (MDA), the by-product of lipid peroxidation, was used as biomarker of membrane oxidative damage. The sample of fresh *L.minor* fronds tissue was homogenized with Tris-HCl buffer solution containing 1.5% of PVPP (pH 7.4) and centrifuged at 10 000 g for 30 min at 4 ºC. Equal amounts of tissue extract and 0.5% thiobarbituric acid in 20% trichloroacetic acid (w/v) was mixed and heated at 95 ° C for 30 min. After centrifugation of reaction mixture at 10 000 g for 15 min. absorbance of the colored supernatant was measured at 532 nm and corrected for unspecific turbidity by subtracting the value of absorbance at 600 nm [14].

Results for the toxicity tests were expressed as the concentration of the sample that produced a 50% effect (e.g., growth inhibition) (EC_{50}). EC_{50} values were expressed as a percentage of effluents tested. Toxicity values (EC_{50}) were converted in Toxic Units (TU) (Eq. 2), i.e. inverse of EC_{50} expressed in %:

$$TU = [1/EC_{50}] \times 100 \tag{2}$$

Analysis of anions (Cl^-, NO_2^-, NO_3^-, SO_4^{2-}, PO_4^{3-}) and cations (K^+, Ca^{2+}, NH_4^+, Mg^{2+}) was performed by ion chromatography with conductivity detection (Dionex ISC-1100). pH was measured potentiometrically. Metals (Cu, Ni, Zn, Cd, Mn, Pb) analysis in digested wastewater was performed with Shimadzu AA-6800 atomic absorption spectrometer. Wastewater was digested using the Milestone Ethos One closed vessel microwave system with 6 mL of HNO_3 (65%) and 2 mL of H_2O_2 (30%) in a microwave digestion system at 180 °C for 25 min.

A one-way analysis of variance (ANOVA) was used to assess the concentration effect on estimated endpoints. Significant differences between controls and samples, treated with wastewater, were determined by the Dunett's test and were considered to be significant at $p < 0.05$. Regression analysis was used to detect the relationship between wastewater concentration and estimated parameters. All the statistical analysis was carried out using Statistica software.

3. Results and discussion

3.1. Chemical characteristic of wastewater

Concentrations of principal anions and cations in untreated and biologically treated wastewater are presented in Table 1. Generally, analysing wastewater chemistry the focus is on the concentrations of nutrients (nitrogen and phosphorous). Nitrites were detected only in treated wastewater and their concentration did not exceed the maximum allowable concentrations [15]. Biological treatment decreased the concentrations of principal contaminants, however several of them maximum allowable concentration are exceeded (NH_4^+ and PO_4^{3-}). The levels of phosphates and ammonium in the Šilalė effluents were substantially higher than those detected in other Lithuania wastewater treatment plants (Vilnius, Kaunas, Šilutė, Kretinga) effluents [16-18].

Table 1. Chemical characteristics of untreated and biologically treated wastewater (in mg l^{-1})

	NO_2^-	NO_3^-	NH_4^+	PO_4^{3-}	SO_4^{2-}	Cl^-	Mg^{2+}	Ca^{2+}	K^+	pH
Untreated wastewater	n.d.[a]	0.05	88.34	19.38	50.16	251.30	19.05	88.91	29.66	7.3
Treated wastewater	0.42	0.02	78.75	11.79	70.02	212.88	17.47	78.57	32.58	7.4

[a] – not detected.

Biological treatment efficiently decreased the concentrations of nutrients in the effluents. The ammonium concentration decreased by 11%, nitrates – by 60%, phosphates – by 40%. The concentrations of other compounds were also reduced, however the changes were less pronounced. The concentration of Cl- was reduced by 15%, Mg2+ – by 8%, Ca2+ – by 12%, respectively.

Analysis of the heavy metals concentrations in the wastewater revealed that Ni (1.62 mg l-1) was the most abundant metal in the untreated wastewater and the other metals were ranked in decreasing order Pb>Mn>Cu>Cd>Zn (Fig. 1). Biological treatment reduced the concentrations of the several heavy metals, however Cd, Zn and especially Pb content in the treated wastewater were higher.

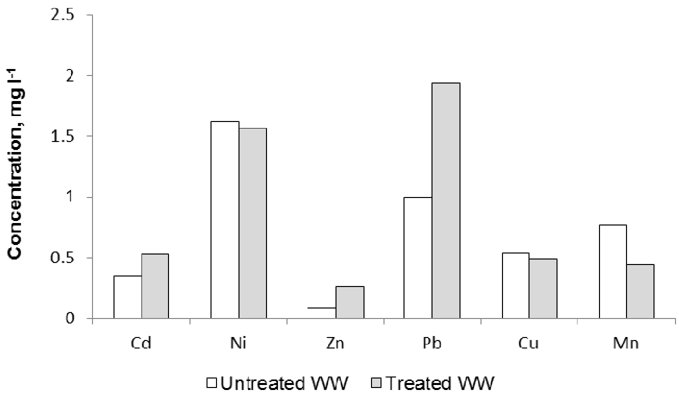

Fig. 1. Metals concentrations (mg l^{-1}) in untreated and biologically treated wastewater

Mn was the most efficiently removed heavy metal from wastewater as it concentration was reduced by 43%. Cu and Ni were removed with very low efficiency, i.e., by 9% and 7%, respectively. However the concentration of Ni in treated wastewater 7.5 times exceeded the threshold value (0.2 mg l^{-1}) [15]. The threshold values were also exceeded by Cd (13 times) and Pb (1.9 times). The heavy metals concentrations in Šilalė wastewater were considerably higher than that detected in the wastewater of Šiauliai [19].

3.2. Toxicity testing with Lemna minor

Low concentrations of untreated wastewater (6.25–12.5%) slightly stimulated the growth of *L. minor* and the total fronds number was by 5–12.7% higher than in control (Fig. 2A). The higher concentrations of untreated wastewater inhibited the growth of the fronds and at the end of the experiment the fronds number was significantly lower than in control ($p<0.05$). At the highest concentrations (50 and 100%) new fronds developed only in the first 2–3 days of the treatment and after that they died.

Exposure to biologically treated wastewater reduced *L. minor* fronds number in comparison with the control group (Fig. 2B). The plants exposure to the highest concentrations of treated wastewater (50–100%) has led to their death. There was no significant differences between *L. minor* fronds number in the control and in the treatments with 6.25–12.5% (Dunnett's test, $p>0.05$). In the treatment with 25%, the total fronds number was significantly lower than in the control treatment ($p<0.05$).

Relative growth rate of *L. minor* during the whole 7 days exposure period was significantly affected by untreated and biologically treated wastewater (ANOVA, untreated WW: $F = 222.99$, $p < 0.001$; treated WW: $F = 69.82$, $p < 0.001$) (Fig. 3A).

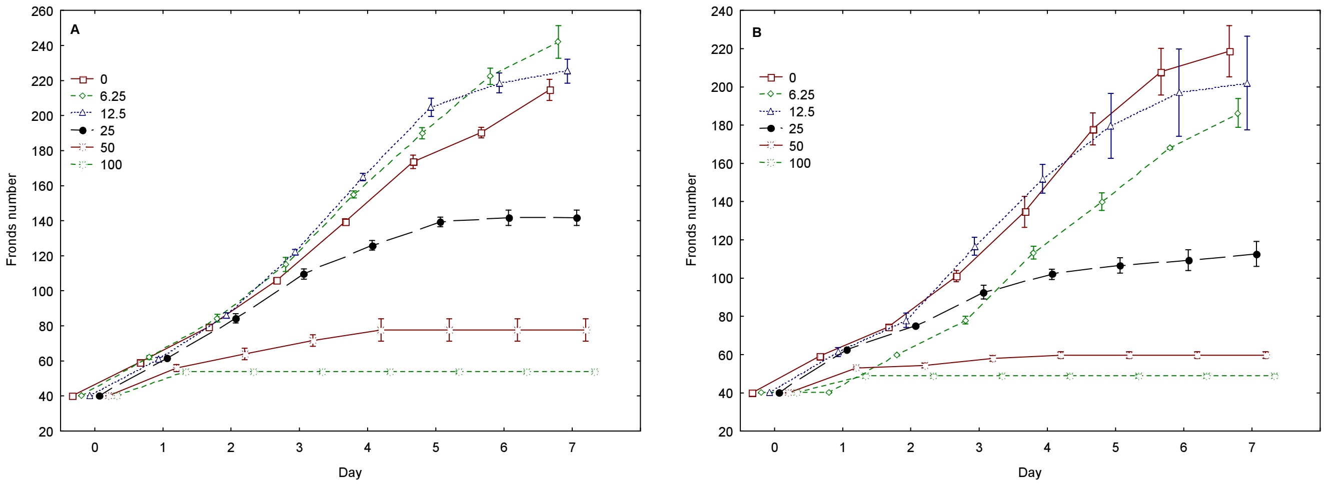

Fig. 2. Fronds number of *Lemna minor*, exposed to A) untreated and B) biologically treated wastewater for 7 days

Exposure to 25% of untreated wastewater in medium led to a decrease of growth rate by 25% in comparison with control plants ($p < 0.05$). The same concentration of treated wastewater reduced the growth rate by almost 40%. The regression analysis showed that 7-day *L. minor* relative growth rate significantly decreased with wastewater concentration in medium (untreated WW: $R^2 = 0.90$, $p < 0.001$; treated WW: $R^2 = 0.84$, $p < 0.001$). Calculated EC_{50}, effective concentration resulting in relative growth rate reduction by 50%, was 57.13% for untreated wastewater and 47.20% for treated wastewater, i.e. TU 1.75 and TU 2.12, respectively. Results indicate that both untreated and treated wastewater can be classified as acute toxic for the growth of aquatic plants [20].

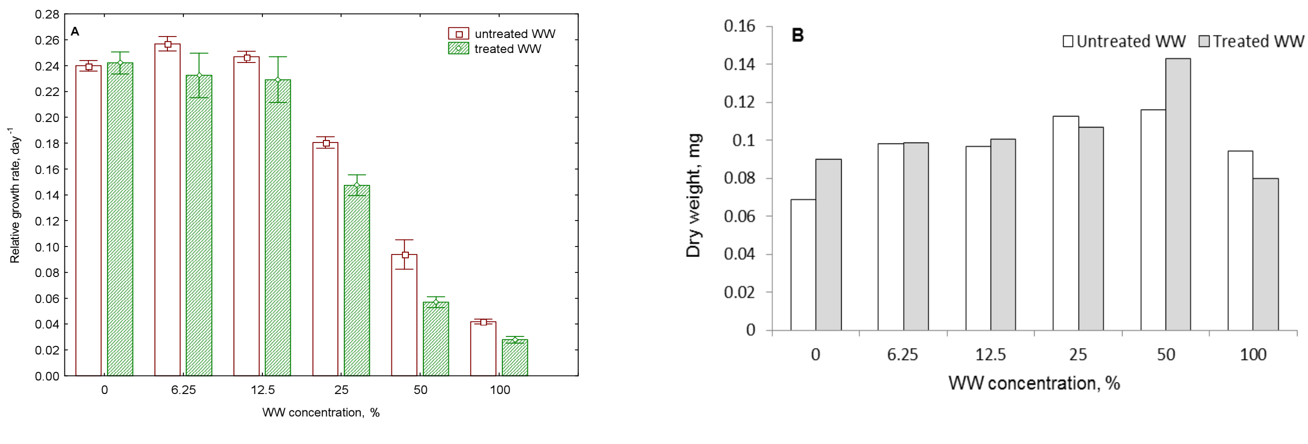

Fig. 3. Relative growth rate (day^{-1}) (A) and frond dry weight (mg) (B) of *Lemna minor* exposed to untreated and biologically treated wastewater for 7 days

Both untreated and treated wastewater stimulated the duckweeds biomass production and no adverse effect was observed (Fig. 3B). Stimulatory effect was more pronounced in the treatment with treated wastewater, the biomass was increased in the range of 21% and 2.66 times. Solutions containing different concentrations of untreated wastewater caused the increase of biomass growth by 16–42%.

No significant adverse impact on the content of photosynthetic pigments was observed (Fig. 4AB). The analysis of the photosynthetic pigments was performed only in the treatments with 6.25–2% of wastewater, as in other treatment with higher wastewater concentration in the medium there was no enough biomass for chemical analysis. The lowest concentrations (6.25%) of raw untreated wastewater have caused very negligible and insignificant changes in photosynthetic pigment content compared with the control plants (by 5.7–5.9%). Whereas, exposure to the same concentration of treated wastewater resulted in higher content of chlorophylls *a* and *b* (by 31-46%) than in control plants. In the leaves of *L. minor* exposed to 12.5–25% of untreated wastewater content of chlorophyll *a* increased by 20.75–29.62%, chlorophyll b – by 23.53–58.82% compared with the control ($p < 0.05$). Exposure to the solution containing 12.5–25% of biologically treated wastewater had more pronounced stimulatory effect on the content of chlorophylls: content of chlorophyll *a* increased by 53.85–71.79%, chlorophyll *b* by – 46.15–84.62%.

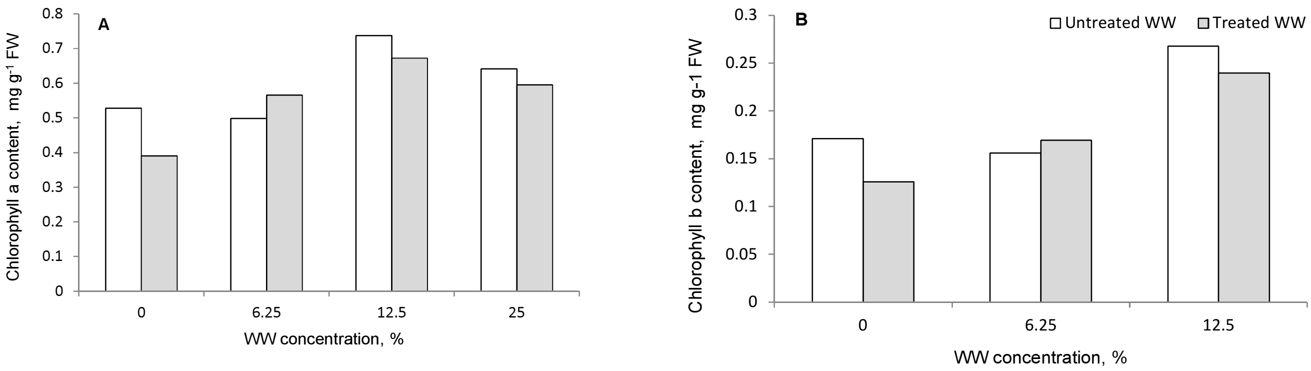

Fig. 4. Content of photosynthetic pigments chlorophyll *a* (A) and chlorophyll *b* (B) (mg g^{-1} FW) in L. minor exposed to untreated and biologically treated wastewater for 7 days

Exposure to all wastewater samples significantly increased the content of malondialdehyde (MDA) in the tissue of *L. minor* fronds (Fig. 5). There was no significant difference between the levels of MDA in *L. minor* exposed to untreated or biologically treated wastewater. The exposure to undiluted raw and biologically treated wastewater increased the content of MDA by 1.7-fold.

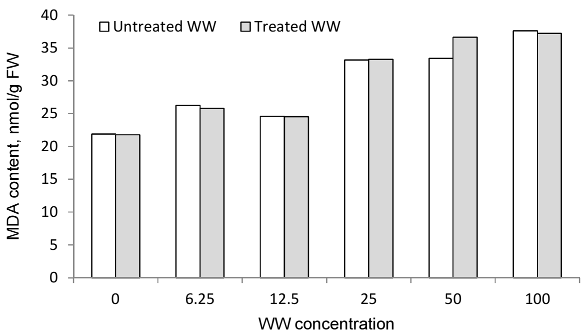

Fig. 5. Content of MDA in the fronds of *L. minor* exposed to untreated and biologically treated wastewater for 7 days

After *L. minor* exposure to industrial wastewater, the inhibition of growth, based on frond number count and biomass, content of photosynthetic pigments and induction of lipid peroxidation, was recorded [21].

The frond count may give misleading results, because any new protruded frond is count and this count does not reflect whether the plant is alive or dead. This was the case of our study, as in the highest concentrations the duckweeds developed new fronds only at the beginning of the test and after 2-3 days the fronds were dead. Moreover, under stress conditions the fronds may be smaller but the total number quite high. Due to this biomass calculation sometimes may be more appropriate endpoint to measure toxicity. Though during this study stimulation of biomass was recorded and this may be due to high content of nutrients. Low concentrations of untreated wastewater stimulated the growth of *L. minor* due to high content of nutrients (N, P) (Table 1). Whereas, biological treatment reduced the nutrient content and this resulted in slower growth of plants.

3.3. Toxicants removal by Lemna minor

Phosphorous was the most efficiently removed nutrient from wastewater, as it concentration was reduced by almost 100%. Nitrates and ammonium were removed with lower efficiency, i.e., by 58.3% and 50.2–75.3%, respectively (Fig. 6A). One of the aims of the wastewater treatment is to reduce nutrients such as nitrogen and phosphorus levels in effluents to a protective level of the receiving water body. As we can see, this goal was not achieved during the biological wastewater treatment (Table 1). *L. minor* application in wastewater treatment reduced the level of phosphorous to this level, however the concentrations of ammonium the effluents after the treatment with *L. minor* exceeded the maximum allowable level by 3.89 times. *L. minor* nutrients removal potential from wastewater are reported to be approximately 50% of total N, 80–90%

of ammonia and 50–60% of total phosphorous [9], [22]. Öbek and Hasar (2002) [23] examined the capacity of *L. minor* in phosphorous removal from treated wastewater and the initial concentration of phosphate decreased form 15 mg l^{-1} to 0.05 mg l^{-1} at the end of 8 days of treatment. The capacity of other *Lemna* spp. species *Lemna gibba* to remove nitrogen from wastewater was detected to be between 42%–62% of total nitrogen, depending on initial nitrogen concentrations [11]. The data of our study is at the same level or even higher.

The micro nutrients were also rather efficiently removed from both untreated and treated wastewater. Whereas, the increase in potassium content in the solution of untreated wastewater after the growth of *L. minor* was observed (by 17.83%). This phenomenon may be explained by the fact that potassium is not very tightly bound in structural tissues or enzyme complexes [24] and it is easily leached from the plants tissues.

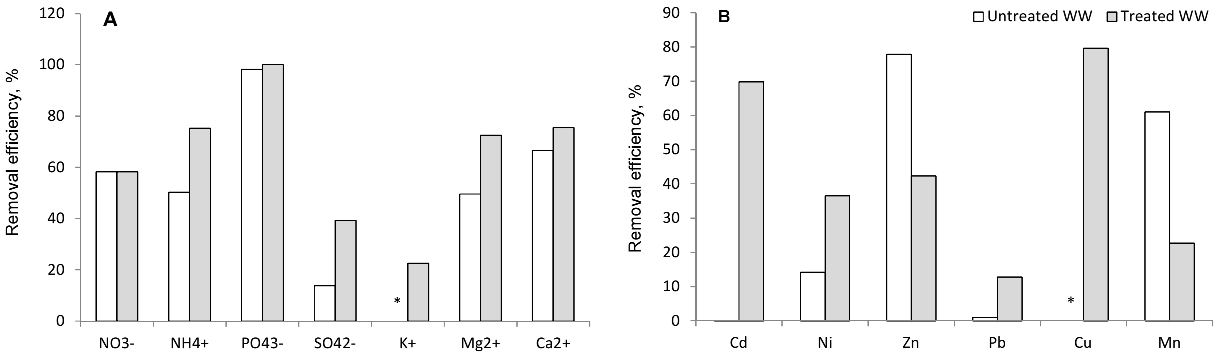

Fig. 6. Removal efficiency of nutrients (A) and heavy metals (B) by *L. minor* exposed to untreated and biologically treated wastewater for 7 days

Cd and Pb were almost not removed from the untreated wastewater, though the removal from biologically treated wastewater consisted of 12.7% and 69.8%, respectively. Zn was the most efficiently removed, depending on the initial Zn concentration *Lemna minor* removed between 42.3–77.8% of Zn. Cu concentration after the experiment has slightly increased by 11.1%.

As after the biological treatment the levels of Ni, Cd, Pb exceeded the maximum allowable concentrations, so this indicate the need of applying new treatment technologies for reducing the levels of chemicals in wastewater. After the 7 days of effluents exposure to *L. minor* treatment, the concentrations of these metals were significantly reduced. However, the maximum allowable concentrations of Ni, Cd and Pb were exceeded by 4.95, 4 and 15 times, respectively. The results suggests that more efficient heavy metals removal may be achieved be increasing the time of *L. minor* treatment. The studies show that *Lemna minor* is is a good bioaccumulator of Zn, Cd and Cu, but a relatively poor accumulator of Ni and Pb [22].

4. Conclusions

Lemna minor has been shown to be a potential scavenger of nutrients and heavy metals from wastewater and may be used in wastewater treatment systems.

Chemical analysis recorded high concentrations of nutrients and heavy metals in untreated and biologically treated wastewater. Wastewater treatment significantly reduced the content of macronutrients (N, P) and micronutrients, however only slight reduction in the concentrations of heavy metals was observed. Wastewater treatment did not reduce nutrients and heavy metals levels in effluents to a protective level of the receiving water body. The application of *L. minor* in wastewater treatment was shown to effectively reduce the content of nutrients and heavy metals. Moreover, the application of *L. minor* in wastewater treatment not only removes the chemicals from the wastewater, but give us the information about the toxicity of the wastewater as well. Both, untreated and biologically treated wastewater inhibited the relative growth rate of *L. minor*, affected the biomass of *L. minor* fronds, the content of photosynthetic pigments (chlorophyll a, b) and induced oxidative stress. It may be conclude that *Lemna minor* can be used as wastewater phytoremediation agent.

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