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MUNICIPAL EFFLUENTS TOXICITY EVALUATION USING HIGHER TERRESTRIAL, AQUATIC PLANTS, AND INVERTEBRATES

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Abstract. The toxicity of Vilnius municipal effluents was evaluated using the bioassays with aquatic invertebrates and terrestrial and aquatic higher plants. Toxicity tests were performed on samples of untreated and biologically treated wastewater. Wastewater toxicity was assessed using seed germination and short-term early seedling growth test of lettuce (*Lactuca sativa* L.), growth inhibition test of *Lemna minor* and the acute microcrustaceans *Daphnia magna* (24-48 h) assay. Undiluted untreated wastewater was severely toxic to *D. magna* and led to death of all exposed organisms (48 h test). The estimated median lethal concentration was 5% WW (20 TU) for untreated wastewater and biologically treated effluents had only slight acute toxicity to *D. magna* (0.80 TU). The inhibition of growth of *L.minor* was detected only in untreated wastewater: EC_{50} for growth rate was 63.4% WW, for biomass – 52.3% WW. The measured endpoints in terrestrial plants test were seed germination, root length, shoot height and total biomass, but only root length was sensitive one. Untreated and biologically treated effluents inhibited the growth of *L.sativa* roots by 70 % and EC_{50} was equalled to be 47.2% WW and 61.1 % WW, respectively. The growth of shoots and total biomass were stimulated due to exposure to effluents; stimulation effect was determined by sufficiently high concentrations of nutrients. The applied bioassays were ranked according to their sensitivity to the effluents as follows: *D. magna* > L. *minor* > *L. sativa.* The treatment of wastewater reduced average toxicity index by 11 times.

Keywords: acute toxicity, bioassays, *Daphnia magna*, effluents, *Lactuca sativa*, *Lemna minor,* toxicity testing.

1. Introduction

Municipal effluents are of major concern for the quality of the receiving aquatic ecosystems. Municipal wastewater contains a complex variety of organic and inorganic compounds. Generally, potential hazard assessment of effluents in Lithuania is based on the physicochemical parameters of eflluents (e.g. biological and chemical oxygen demand, pH, total dissolved solids, heavy metals, organic compounds, etc.) (Jurjonienė *et al.* 2007; Vasarevičius *et al.* 2010). However, chemical data alone do not allow evaluation of possible toxic effects to biota living in receiving waters. Toxicity bioassays, in contrast to physico-chemical analysis, integrate the biological effects of all compounds present and others factors such as bioavailability, toxicants interaction and others. It is accepted, that toxicity bioassay, using species representing the different trophic levels, is the best approach to evaluate the whole toxicity of wastewater.

The whole effluent toxicity (WET) assessment is based on the bioassays for acute and chronic toxicity. The toxicity of effluents mainly is evaluated with luminescent bacteria, algae or higher plants, aquatic invertebrates and vertebrates (fishes). The most popular acute and chronic toxicity bioassays are with aquatic invertebrates (especially crustaceans, e.g.; *Daphnia magna*) (Latif and Licek 2004; Rodriguez *et al.* 2006; Ra *et al.* 2008). As the organism responses are very diverse, the use of test batteries including a set of organisms from several different trophic levels, is highly recommended. Wastewater and treated effluents can effectively be screened for toxicity by a selected battery of tests, and extensive costly chemical analysis may be run only with those samples that reveal suspicious or remarkably high toxicity data.

Animal tests alone are not sufficient to assess environmental hazards. The advantages of plant assays are large array of assessment endpoints (seed germination, biomass, growth, biochemical parameters), low cost and year round testing. Duckweeds (*Lemnaceae*) due to their small size, high multiplication rates and susceptibility to pollutants are one of the most used aquatic plants in toxicity testing procedures of various chemicals and their mixtures. Studies show that duckweeds are very sensitive in various mixtures (wastewater, leachates, etc.) toxicity evaluation (Horvat *et al.* 2007; Radić *et al.* 2011).

The impact of the effluents on living organisms is high and governed by several factors, such as high load of organic matter, heavy metals, high content of nutrients (nitrogen, phosphorous). Toxicity assessment studies have revealed the mutagenic and genotoxic properties of wastewater (Helma *et al.* 1996). Wastewater also can inhibit the growth, feeding rate and reproduction of aquatic organisms and disturb macroinvertebrate community structure and functioning (Lewis *et al.* 1998; Maltby *et al.* 2000). In the water bodies, contaminated by effluents, various biochemical, physiological, reproductive adverse effects are observed and the fish species richness and composition are observed to be lower than in clean ones (Sandstrőm and Neuman 2003; Mower *et al.* 2011).

In Lithuania previously the studies on effluents toxicity were focused on bioassays with algae (Manusadžianas *et al.* 1995; Bartusevičienė and Manusadžianas 2003a), aquatic and terrestrial higher plants (Lakačiauskienė *et al.* 1997; Marčiulionienė and Montvydienė 2002), aquatic invertebrates and test batteries, including several biotests representing different trophic levels (Manusadžianas *et al.* 2000; Bartusevičienė and Manusadžianas 2003b; Manusadžianas et al. 2003; Žaltauskaitė and Vaisiūnaitė 2010).

The main aim of this paper was to determine the toxicity of untreated and biologically treated municipal effluents using seed germination and short-term early seedling growth test of lettuce (*Lactuca sativa* L.), growth inhibition test of *Lemna minor* and the acute microcrustaceans *Daphnia magna* (24-48 h) immobilisation test.

2. Materials and methods

Samples of untreated (raw) and biologically treated Vilnius municipal effluents were collected in February of 2010. The samples were taken to laboratory and stored in darkness in refrigerator prior to performance of the biosassays.

The chemical characteristics of wastewater are presented in Table 1.

According to 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 and part of each sample was filtered (0.45 μm) for acute toxicity test with *Daphnia magna.*

Phytotoxicity test was performed on terrestrial higher plant lettuce (*Lactuca sativa* L.) and aquatic plant duckweed (*Lemna minor* L.). Tests of seed germination and early seedling growth of lettuce were conducted. 50-80 ml of distilled water (as a control) or a testing sample (different concentrations of wastewater) solution was pippeted onto the filter paper fitted on the cotton wool into a 9 cm glass Petri dish. The assays were performed in three replicates.

Thirty healthy looking seeds of lettuce of similar size were placed in each Petri dish on filter paper. The Petri dishes were placed in the dark at 25 °C ± 2°C for 24 h. Afterward the seed germination and the Petri dishes were kept in regime of 25 °C ± 2°C temperature under 16:8 h light:dark for 120h.

Table 1. Chemical characteristics of wastewaters (WW)

	NO_2-N, mg l^{-1}	NO_3-N, mg l^{-1}	NH_4-N mg l^{-1}	PO_4^{3-}, mg l^{-1}
Untreated WW	0.03	1.00	100	18
Biologically treated WW	0.5	20	10	0.6

The following endpoints were measured to evaluate phytotoxicity: seed germination (%), shoot height (mm), root length (mm), and the total plant mass (the total mass of surviving plants in one Petri dish (g)). Plants that did not germinate were not considered in the subsequent analysis of growth.

The standardised growth inhibition tests with duckweed have been conducted according to OECD Guideline 221 (2004). The stock culture of *L. minor* was grown in modified Steinberg medium in growth chambers at 24 °C ± 2°C with a light/dark cycle of 16/8 h. Ten (10) 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 growth medium of *L. minor*. Experiment has lasted 7 days and has been conducted in 3 replicates. The fronds number has been monitored every day of experiment.

Toxicity was recorded as percent inhibition of growth (fronds number and fronds biomass) (relative to control) of *L. minor* as a result of 7 days exposure to the toxicant (effluents) in its growth medium.

Relative growth rate was calculated from the following equation (Eq. 1) with measured fronds number (N) at the end (t_1) and the start of the test (t_0):

$$r = \frac{\ln(N_{t_1}) - \ln(N_{t_0})}{t_1 - t_0} \qquad (1)$$

Acute 24-48 h immobilisation test was performed with *Daphnia magna* according to OECD Guideline 202 (1984). The control was synthetic freshwater (growth medium of *D. magna*). Five (5) organisms (less than 24 h old) were exposed to each dilution sample in 3 replicates. After 24 h and 48 h incubation in light/dark cycle 16:8 h at 20±1 °C, the number of dead individuals was recorded. The test was considered valid if the mortality in control with synthetic freshwater did not exceeded 10 %.

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

$$TU = [1/EC_{50}] \times 100 \qquad (2)$$

In case of low toxicity, i.e. below the 50 % effect level, toxicity units were calculated as proportion of 50 % effect.

Average effluents toxicity index (AvTx) was calculated as the sum of the endpoints values in toxic units divided by the number of tests (Manusadžianas *et al.* 2003).

The toxicity classification of effluents is reported as follows (Persoone *et al.* 2003):

no acute toxicity	TU < 0.4
slight acute toxicity	0.4<TU< 1
acute toxicity	1 ≤ TU< 10
high acute toxicity	10 ≤ TU< 100
very high acute toxicity	≥ TU100

Each species endpoint per effluents solution sample was compared to the corresponding reference sample mean using a Students' *t* test. The difference was significant than $p < 0.05$. A wastewater was considered toxic if a statistically significant reduction occurred in one of the species endpoints relative to the reference.

3. Results and discussion

Clear relationship between lettuce (*Lactuca sativa* L.) seed germination and effluents concentration in solution was not detected. So, this endpoint was excluded from the further toxicity analysis. Generally, seed germination is not as sensitive to various toxicants as others endpoints (Gunderson *et al.* 1997; Meier *et al.* 1997).

Effluents statistically significantly inhibited the growth of lettuce root; the root length in undiluted untreated and biologically treated effluents was by 66-68 % lower than in control (Fig 1). Regression analysis revealed that the root length of *L. sativa* significantly decreases with the concentration of untreated and treated effluents in tested solution ($r > -0.81$, $p < 0.05$).

Calculated EC_{50} for lettuce root growth inhibition was 47.2 % (2.12 TU) and 61.1 % (1.64 TU) in case of raw and treated wastewater, respectively. Results indicate that effluents can be classified as acute toxic for the growth of roots of terrestrial plants.

The shoot height showed no clear dependence on the wastewater concentration in solution (Fig 2). Untreated wastewater slightly inhibited the shoot growth, though the differences from the control were insignificant. Biologically treated wastewater had the stimulatory effect to shoot growth. Many studies have indicated that the shoot system is less sensitive to environmental toxicity than the root system (Wang and Williams 1988; Gunderson *et al.* 1997; Meier *et al.* 1997; Marčiulionienė and Montvydienė 2002).

Exposure to undiluted raw and biologically treated effluents led to increase in shoot and root biomass in comparison with the control (Fig 3).

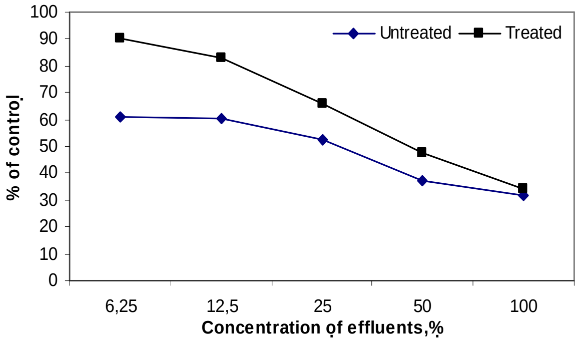

Fig 1. Root length of lettuce (*Lactuca sativa* L.) exposed to different concentration of untreated and biologically treated effluents

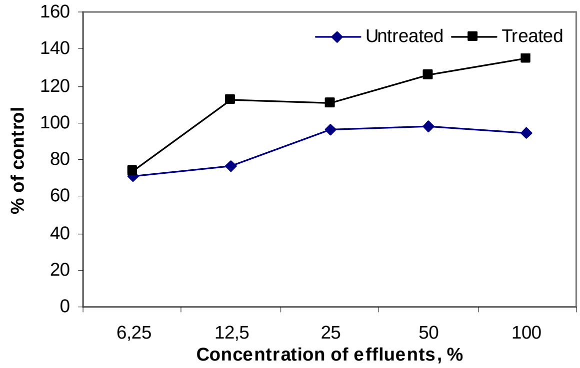

Fig 2. Shoot height of lettuce (*Lactuca sativa* L.) exposed to different concentration of untreated and biologically treated effluents

The weak concentrations of wastewater (6.25-25 %) inhibited the growth of biomass. It indicates that undiluted wastewater contains sufficiently high concentrations of nutrients which stimulate the growth of plants and may mask the adverse effect of other substances in wastewater. Dilution of wastewater decreases the content of nutrients and it results in slower growth of plants. The same pattern when undiluted effluents stimulate the growth and diluted solutions inhibit the growth is characteristic to mixtures containing high amounts of nutrients (Blinova 2000).

According to our study results, it may be concluded that root length is the most sensitive endpoint in comparison with seed germination, the growth of shoot system and plant biomass. The results revealed that undiluted untreated wastewater is phytotoxic. In case of 50 % concentration of effluents in solution, the effluent's toxicity category falls to non-toxic level or even stimulation is observed. Biological treatment significantly reduced the phytotoxicity of effluents to terrestrial plants.

The adverse impact of effluents to the growth of *Lemna minor* was more pronounced than in the case of terrestrial plants. The duckweeds exposed to undiluted raw wastewater showed 100 % mortality. Some studies indicate that *L. minor* is very sensitive to effluents and mortality sometimes reaches 100 % (Wang and Williams 1988).

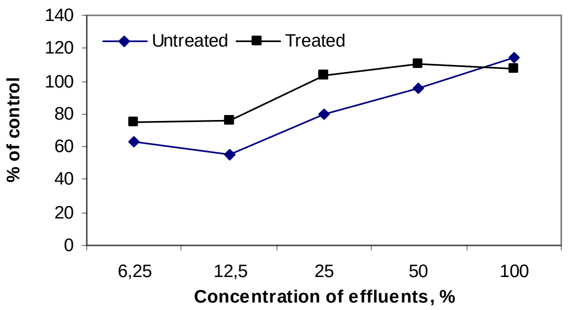

Fig 3. Total biomass of lettuce (*Lactuca sativa* L.) exposed to different concentration of untreated and biologically treated effluents

The regression analysis revealed that number of fronds during the test period was significantly affected by time (duration of exposure) and concentration of wastewater in solution (untreated wastewater: fronds number = 22.4 + 7.2 × *day* – 0.13 × concentration (R^2 = 0.95, p = 0,00); treated wastewater: fronds number = 17.6 + 9.1 × *day* + 0.11 × concentration (R^2 = 0.88, p = 0,00)).

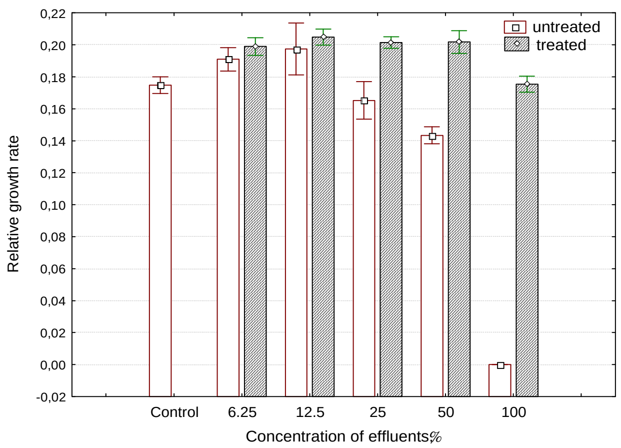

Fig 4. The relative growth rate and biomass of *L. minor* exposed to different concentration of untreated and biologically treated effluents

Relative growth rate of *L. minor* decreased with increasing concentration of untreated wastewater in solution (Fig 4). The growth rate of *L. minor* exposed to the solution containing 50 and 25 % of untreated wastewater was by 18 % and 5.6 %, respectively, lower than in control. Low concentrations of untreated wastewater in solution induced more intense growth than in control. Calculated EC_{50} for *L. minor* growth inhibition was 55.3 %, i.e. TU 1.81. Results indicate that raw wastewater can be classified as acute toxic for the growth of aquatic plants.

Exposure to biologically treated effluents increased the growth of *L. minor* by 12-17 % and no adverse impact on growth rate was detected.

In *L. minor* growth inhibition test any new visible daughter frond is count. As under stress conditions very small fronds may be protruded, so frond number may underestimate the toxic effect to duckweed. Due to this disadvantage, the biomass evaluation is more suitable for toxicity assessment.

Effluents adversely affected the biomass of duckweeds (Fig 5). Solutions containing different concentrations of untreated wastewater caused the inhibition of biomass growth by 10-100%. The relationship between the dry weight of *L. minor* (expressed as % of control) and the concentration of untreated wastewater in solution was strong and statistically significant (r = - 0.94, p = 0.00). Calculated EC_{50} for *L. minor* biomass inhibition was 54.42%, i.e. TU 1.84. Results indicate that raw wastewater can be classified as acute toxic for the growth of aquatic plants biomass.

There was no significant decrease in dry weight of duckweeds exposed to biologically treated effluents. The increase in weight of frond consisted in 44 % in undiluted treated effluents, though lower concentrations of treated effluents in solution caused slight statistically insignificant decrease in frond weight (6-16 %).

The study has shown that untreated wastewater might be classified as acute toxic for terrestrial and aquatic plants, though the high concentrations of nutrients in wastewater may mask the effects of other toxicants. Biological treatment of wastewater reduces the phytotoxicity and no significant adverse effect to terrestrial and aquatic plants has been observed after exposure to biologically treated effluents. It shows that after the treatment, the effluent's toxicity to higher plants category falls from acute toxic to non-toxic level or even stimulation is observed.

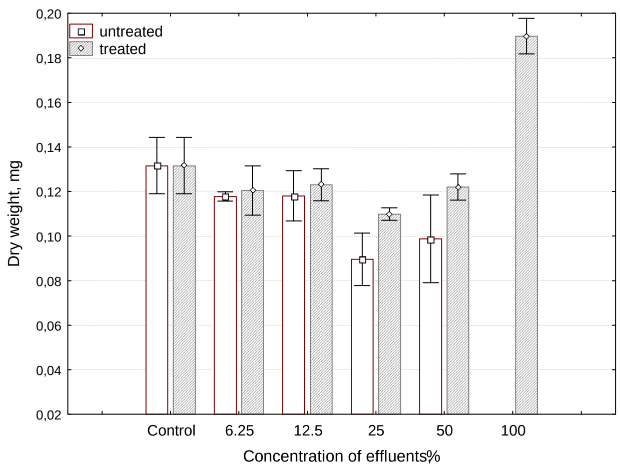

Fig 5. The dry weight of *L. minor* (mass of one frond, mg) exposed to different concentration of untreated and biologically treated effluents

L. minor proved to be more sensitive to aquatic pollutants than terrestrial plants. The possible reason for such phenomenon might be that tested substances are taken up directly through the leafy fronds and terrestrial plants take up the substance mostly by roots (Naumann *et* *al.* 2007). Undiluted untreated wastewater showed very high (80% 24h and 100% 48h) lethal toxicity to *Daphnia magna* (Fig 6).

Calculated 24h LC_{50} value for *D. magna* was equaled to 57% of untreated wastewater in solution. Results indicate that untreated wastewater has acute toxicity to tested aquatic invertebrates (TU = 1.75). Mortality of *D. magna* dramatically increased with time and 48h LC_{50} value for *D. magna* was equaled to 5% of untreated wastewater in solution, i.e. TU = 20, very high acute toxicity.

Biological treatment of effluents had sharply reduced their toxicity. The mortality of *D. magna* after 24 h was 30% and 40% after 48 h in 100% concentration of effluents. LC_{50} was not calculated for treated effluents, as biologically treated effluents had below 50% effect level. Toxic units in case of biologically treated effluents were 0.6 (24h) and 0.8 (48h), i.e. slight acute toxicity. However, when the $EC_{50} > 100\%$, there can be a risk of long-term toxicity and this should be evaluated by means of chronic toxicity tests (e.g.; daphnia reproduction test).

The similar results were obtained during the other Kaunas municipal untreated wastewater toxicity study (Bartusevičienė and Manusadžianas 2003b) and they support our findings that untreated wastewater can be classified as toxic to crustaceans.

The results of logistic regression show that the risk of death of tests organisms (*D. magna*) increases with the concentration of effluents in solution (untreated effluents: $\chi2 = 14.28$, $p = 0.0001$; biol. tr. effluents: $\chi2 = 10.57$, $p = 0.001$).

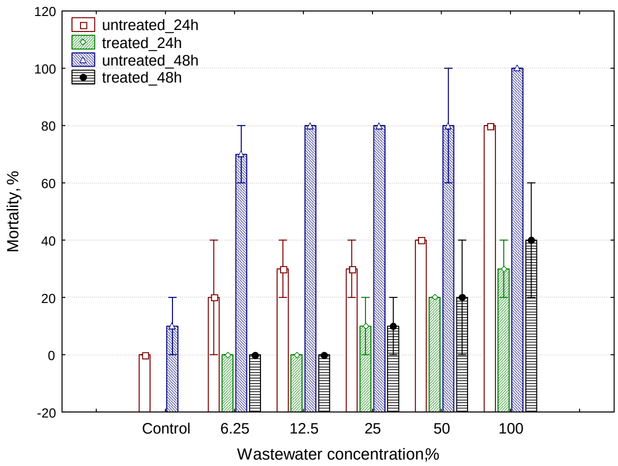

Fig 6. Mortality of *Daphnia magna* 24-48 h exposed to different concentrations of untreated and biologically treated effluents

In calculations of average toxicity index (AvTx) onlythe most sensitive endpoints were included: root growth of *L. sativa*, biomass of *L. minor* and mortality of *D. magna* after 48 h exposure. Calculated average toxicity index (AvTx) for untreated wastewater was 7.98, i.e.; acute toxicity level. The treatment had sharply decreased the effluents toxicity and AvTx was only 0.81 – slight acute toxicity level.

The applied bioassays were ranked according to their sensitivity to the effluents: *D. magna* > *L. minor* > L. *sativa*. The study revealed that aquatic invertebrates are more sensitive for whole effluent toxicity than terrestrial and aquatic plants. The growth of roots of *Lactuca sativa* was sensitive only to undiluted wastewater though the other endpoints showed the stimulation effect. One of the possible reasons could be the high concentrations of plant nutrients (nitrogen, phosphorus) in wastewater and the toxic effect could be masked by the high nutritional potential of complex wastewater and the stimulation of growth. However, as primary contaminants in the effluents were nutrients and if only acute toxicity test with aquatic invertebrates were used in assessment, the phytotoxic and phytostimulatory effects would not have been detected. The ability of effluents to stimulate growth of plants might indicate a potential to perturb receiving ecosystems through enhancement effects. Furthermore, biotests with aquatic and terrestrial plants may give additional important information on the possible effect of effluents on the eutrophication process in receiving waters.

4. Conclusions

The results of comparative bioassays sensitivity study show that applied *L. sativa*, *L.minor* and *D.magna* bioassays were ranked according to their sensitivity to the effluents: *D. magna* > *L. minor* > L. *sativa*. The study revealed that aquatic invertebrates are more sensitive for whole effluent toxicity than terrestrial and aquatic plants.

The results of the early *L. sativa* seedlings growth test show that only root length was sensitive endpoint. Vilnius municipal untreated and treated effluents was phytotoxic to root growth of *L. sativa* (untreated – 2.12 TU, treated – 1.64 TU) and stimulated the growth of shoot and biomass. However, low short-term toxicity may indicate that there can be a risk of long-term toxicity which should be evaluated by a means of chronic toxicity tests.

L. minor growth inhibition test showed more sensitivity to effluents than early *L. sativa* seedlings growth test. Effluents were of moderate toxicity to *L. minor* growth rate (1.81 TU) and biomass (1.84 TU).

The results of the acute crustaceans *D. magna* test show that Vilnius municipal untreated wastewater is of high acute toxicity to aquatic invertebrates and may pose relatively serious hazards to receiving waters. Calculated 48h LC_{50} was equaled 5% of untreated wastewater in solution (20 TU). It was found that biological treatment almost detoxified wastewater, i.e. 0.8 TU.

Average toxicity index (AvTx) for untreated wastewater was 7.98, i.e.; acute toxicity level. The biological treatment had sharply decreased the effluents toxicity and AvTx was only 0.81 – slight acute toxicity level.

As primary contaminants in effluents were nutrients, if only animal species were used in toxicity assessment, the phytotoxic or phytostimulatory effects would not have been detected. So, the potential ecological impact of effluents would have been underestimated. Due to a number of potential toxicants in such complex substances (as wastewater), further investigations based on the toxicity testing using a battery of tests is recommended.

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