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Evaluation of Municipal Effluent Toxicity Using Higher Plants and Invertebrates

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Toxicity of Kaunas municipal effluent was evaluated using bioassays with aquatic invertebrates and terrestrial higher plants. Toxicity tests were performed on samples of both untreated and mechanically and biologically treated wastewater. Wastewater toxicity was assessed using seed germination and short-term early seedling growth tests of lettuce (*Lactuca sativa* L.) and acute microcrustaceans *Thamnocephalus platyurus* (24 h) assays. Undiluted untreated wastewater was severely toxic to *T.platyurus* and led to death of all exposed organisms. Twofold decrease in wastewater concentration in the solution (from 100 % to 50 %) led to sharp transition between extremely toxic to medium toxic to tested organisms. In four and more times diluted wastewater only 10-20 % of the total exposed *T. platyurus* died. Measured endpoints in higher plants tests were: seed germination, root length, shoot height and total biomass. Raw sewage was slightly toxic (0.6 TU) to the root growth of lettuce, but mechanically and biologically treated effluents exhibited no adverse effects or even started to stimulate the growth. The growth of shoots and total biomass were stimulated due to exposure to effluents. Stimulation effect was determined by sufficiently high concentrations of nutrients.

Key words: *bioassays, effluent, micro-biotesting, acute toxicity, toxicity testing.*

1. Introduction

Municipal wastewater contains a complex variety of organic and inorganic compounds. The composition of municipal wastewater varies significantly both in terms of place and time. In Lithuania hazard assessment of wastewater is based on physicochemical parameters e.g. biological oxygen demand (BOD), chemical oxygen demand (COD), pH, total dissolved solids. However, during recent years it has become generally accepted that chemical data alone do not allow evaluation of toxic effects. Toxicity bioassays, in contrast to physico-chemical analysis, integrate biological effects of all compounds present and other factors such as bioavailability, toxicants interaction and the others. A toxicity bioassay, using the species representing different trophic levels, is a best approach to evaluate the whole toxicity of wastewater.

The whole wastewater (effluent) toxicity assessment is based on bioassays for acute and chronic toxicity. Toxicity of wastewater is mainly evaluated with algae or higher plants (Wang&Williams 1988), luminescent bacteria *Photobacterium phosphoreum* (Ince&Erdoğdu 1998), aquatic vertebrates (fish) (Dwyer et al. 2005), but the most popular acute and chronic toxicity bioassays are with aquatic invertebrates (especially crustaceans) (Latif&Licek 2004, Rodriguez et al. 2006, Ra et al. 2008) and test batteries (Sherry et al. 1997, Castillo et al. 2000). Previously in Lithuania the studies on wastewater toxicity were focused on bioassays with algae (Manusadžianas et al. 1995, Bartusevičienė&Manusadžianas 2003a), aquatic and terrestrial higher plants (Lakačiauskienė ir kt. 1997, Marčiulionienė&Montvydienė 2002), aquatic

invertebrates and test batteries, including several biotests representing different trophic levels (Manusadžianas et al. 2000, Bartusevičienė&Manusadžianas 2003b, Manusadžianas et al. 2003).

The impact of effluents on living organisms is high and governed by several factors, such as a high load of organic matter, heavy metals, high content of nutrients (nitrogen, phosphorous). Comparative toxicity assessment studies have revealed genotoxic properties of wastewater (Helma et al. 1996). Wastewater can also 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 rivers contaminated by effluents, the fish species richness and composition are observed to be lower than in clean rivers. These effects in fish populations are caused by reproductive dysfunction and recruitment failure (Adams 1992).

This paper aims at determining toxicity of both untreated and mechanically and biologically treated municipal effluents using lettuce (*Lactuca sativa* L.) seed germination and early seedling growth tests and acute *Thamnocephalus platyurus* mortality tests.

2. Materials and methods

Samples of untreated (raw) and mechanically and biologically treated Kaunas municipal effluents were collected in January of 2009. The samples were taken to the laboratory and stored in darkness in refrigerator prior to performance of biosassays.

Chemical characteristics of wastewater are presented in Table 1.

Table 1. Chemical characteristics of wastewaters (WW)

	BOD_7, mg l^{-1}	COD, mg l^{-1}	P_{total}, mg l^{-1}	NH_4-N, mg l^{-1}
Untreated WW	129	na	5.5	na
Mechanically treated WW	139	389	4.9	27
Biologically treated WW	7.1	34	0.37	9.8

na – not analyzed

According to the EPA recommendations for a toxicity test a dilution series (six) of five wastewater concentrations (i.e., 100, 50, 25, 12.5 and 6.25 % wastewater) and a control sample were prepared. Phytotoxicity tests were performed with unfiltered wastewater and a part of each sample was filtered (0.45 μm) for acute toxicity tests with *Thamnocephalus platyurus.*

Phytotoxicity tests were performed on higher plant lettuce (*Lactuca sativa* L.). Tests on seed germination and early seedling growth were conducted with chosen plants. 50-80 ml of distilled water (as a control) or a testing sample (different concentrations of wastewater) solution was pipetted onto the filter paper fitted on the cotton wool into a 9 cm glass Petri dish. The assays were performed in 3 replicates per treatment (N dilutions) and 1 control. Thirty healthy looking seeds of lettuce of a similar size were placed in each Petri dish on filter paper. Petri dishes were placed in the dark at 25 °C ± 2°C for 24 h. Afterwards the seed germination and Petri dishes were kept in under the regime of 25 °C ± 2°C temperature and 16:8 h light:dark exposure for 120 h.

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

An acute toxicity test was performed with Thamnotoxkit F^{TM}, a 24 h mortality test with the anostracan crustacean *Thamnocephalus platyurus.* The test was performed according to the Toxkit Standard Operational Procedures (Thamnotoxkit F^{TM}). The crustacean toxicity test to assess the mortality of fairy shrimps *Thamnocephalus platyurus* was conducted by hatching them from cysts after 20-22 h of incubation at 25 °C in synthetic freshwater (growth medium of fairy shrimps) under continuous illumination (3000-4000 lux). The assays were performed in 3 replicates per treatment (N dilutions) and 1 control. The control was synthetic freshwater (growth medium of fairy shrimps). 10 organisms were exposed to each dilution sample in 3 replicates. After 24 h incubation under dark conditions at 25 °C, the number of dead larvae was recorded. The test was considered valid if mortality in a control with synthetic freshwater did not exceed 10 %.

Results of toxicity tests were expressed as the concentration of the sample that produced a 50% effect (growth inhibition, mortality) (EC_{50} or LC_{50}). EC_{50} (LC_{50}) values were expressed as the percentage of wastewater tested. Toxicity values (LC_{50}) were converted into Toxic Units (TU) (Eq. 1), i.e. inverse of LC_{50} expressed in %:

$$TU = [1/EC50] \times 100 \quad (1)$$

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

Toxicity classification (Persoone et al. 2003) is reported as follows:

no acute toxicity	$TU < 0.4$
slight acute toxicity	$0.4 < TU < 1$
acute toxicity	$1 \leq TU < 10$
high acute toxicity	$10 \leq TU < 100$
very high acute toxicity	$\geq TU100$

Each species endpoint per wastewater solution sample was compared to the corresponding reference sample mean using Students' *t* test. The difference was significant when p was < 0.05. Wastewater was considered phytotoxic if a statistically significant

reduction occurred in one of the species endpoints relative to the reference.

3. Results and discussion

Lettuce *Lactuca sativa* L. seed germination in the control sample was 90%. Exposition to different wastewater solutions inhibited the seed germination by 4 to 40 % in comparison with the germination rate in the control solution. However, no clear relationship between seed germination and effluents concentration in the solution was detected. Generally, seed germination is not so much sensitive to various toxicants as to the other endpoints (Kapustka et al. 1995, Gunderson et al. 1997, Meier et al. 1997, Marčiulionienė&Montvydienė 2002).

Undiluted wastewater statistically inhibited the growth of lettuce root significantly; the root length in undiluted wastewater was by 30 % lower than in the control sample (Fig. 1), i.e. slight toxicity (0.6 TU). Weak concentrations of wastewater (6.25-12.5 %) had no significant adverse effect on the growth of roots. Regression analysis revealed that the root length decreases with the concentration of wastewater in the tested solution ($r = -0.32$, $p < 0.05$).

The adverse impact of treated effluents on the growth of lettuce roots was less pronounced. The root length in undiluted mechanically and biologically treated effluents was by 14 and 11% lower, respectively, than in the control sample. Diluted treated effluents had a stimulatory effect on the root growth.

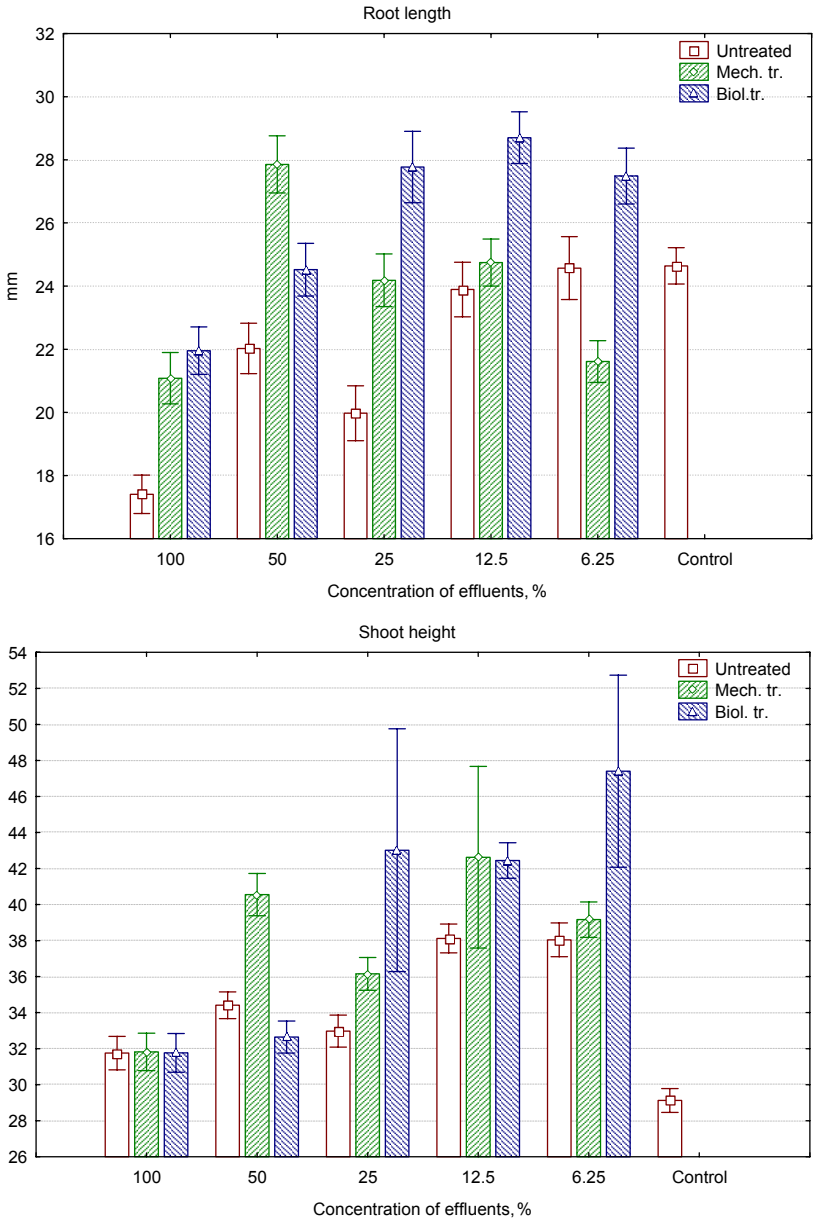

Fig. 1. Root length and shoot height of lettuce (Lactuca sativa L.) exposed to different concentrations of both untreated and mechanically and biologically treated effluents

A significant increase in mean lettuce shoot height was observed in all tested untreated and treated effluents solutions (Fig. 1). The height of shoot of lettuce exposed to 100 % concentration of wastewater was by 9 % longer than that in the control group. The best shoot growth was observed in a case of 6.25-12.5 % concentration of wastewater when the height of shoot was by 30 – 60 % longer than that in the control group ($p<0.05$). That stimulation effect could be explained by sufficiently high concentrations of nutrients and low concentrations of toxic substances. Many studies have indicated that the root system is more sensitive to environmental toxicity than the shoot system (Wang&Williams 1988, Kapustka et al. 1995, Gunderson et al. 1997, Meier et al. 1997, Marčiulionienė&Montvydienė 2002).

Replicates of lettuce seedlings were pooled because of their limited mass to obtain a single mass of the species for each replicate series. A statistically significant increase in mean total biomass of lettuce was observed after the exposure to all tested concentrations of effluents (Fig. 2).

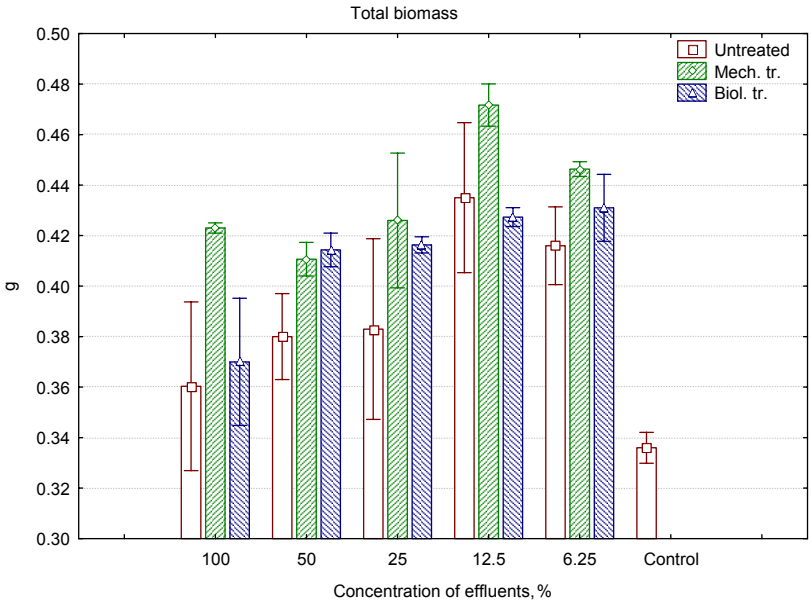

Fig. 2. Total biomass of lettuce (Lactuca sativa L.) exposed to different concentrations of both untreated and mechanically and biologically treated effluents

An increase in total biomass amounted to 8 % in undiluted untreated wastewater and to 24.3 % in the lowest concentration wastewater. The stimulatory effect of mechanically and biologically treated effluents was somewhat higher than that of untreated wastewater. Mass increase in samples of mechanically and biologically treated effluents was from 13 to 40 % in comparison with the control sample.

According to our study results, it may be concluded that root length is one of the most sensitive endpoint in comparison with seed germination, the growth of the shoot system and plant biomass. The results have revealed that undiluted untreated wastewater is slightly phytotoxic. In 50 % concentration of effluent in the solution, the category of its toxicity falls to the non-toxic level or even in some cases stimulation is observed.

Undiluted untreated wastewater showed very high (100 %) lethal toxicity to *Tamnocephalus platyurus* (Fig. 3). Twofold decrease in wastewater concentration in the solution (from 100 % to 50 %) resulted in a sharp decrease in crustacean mortality by a factor of 2.5, and transition between extremely toxic to moderately toxic doses of toxicants to tested organisms was observed. Mortality of fairy shrimps in the solutions containing 12.5-25 % of effluent was relatively low and the difference in mortality in the control group was statistically insignificant ($p>0.05$). The lowest tested concentration of wastewater (6.25 %) as well as in the growth medium of *T. platyurus* (control solution) led to the death of 10 % of *T. platyurus.*

Mechanical and biological treatment of effluents had sharply reduced their toxicity. Mortality of *T. platyurus* was lower than 45 % even at a 100 % concentration. Diluted treated effluents were slightly toxic to fairy shrimps.

LC_{50} was calculated only for untreated wastewater, whereas mechanically and biologically treated effluents had the effect level below 50%. Calculated LC_{50} value for *T. platyurus* was equalled to 51.45 % of untreated wastewater in the solution. Results have indicated that untreated wastewater has acute toxicity to tested aquatic invertebrates (TU = 1.94) (Persoone et al. 2003). Toxic units in mechanically and biologically treated effluents were 0.95 and 0.6, respectively, i.e. the latter were slightly acute toxic. The similar results have been obtained during the other study of Kaunas municipal untreated wastewater toxicity (Bartusevičienė, Manusadžianas 2003b) and they support our findings that untreated wastewater can be classified as toxic to *T.platyurus*.

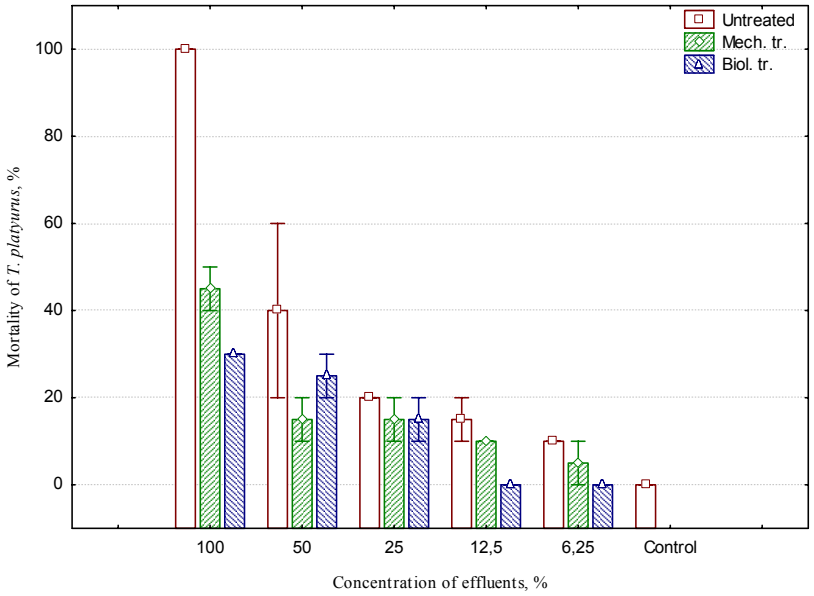

Fig. 3. Mortality of Thamnocephalus platyurus exposed to different concentrations of both untreated and mechanically and biologically treated effluents

The results of logistic regression show that the risk of death of test organisms increases with the concentration of effluents in the solution (untreated effluents: $\chi 2 = 57.48$, $p = 0.0001$; mech. tr. effluents: $\chi 2 = 11.85$, $p = 0.001$; biol. tr. effluents: $\chi 2 = 8.99$, $p = 0.003$).

The study has revealed that aquatic invertebrates are more sensitive to the whole effluent toxicity assessment than terrestrial plants. *T. platyurus* is indicated to be more sensitive to effluents than the other crustaceans (*Daphnia magna*) or rotifers (*Brachionus calyciflorus*) (Latif et al. 1995, Blinova 2000; Manusadžianas et al. 2000). However, as primary contaminants in the effluents were nutrients and if only acute toxicity tests with aquatic invertebrates were used in the assessment, the phytotoxic and phytostimulatory effects would not have been detected. The growth of roots of *Lactuca sativa* was sensitive only to undiluted wastewater, while the other endpoints showed the stimulation effect. One of the possible reasons could be high concentrations of plant nutrients (nitrogen, phosphorus) in wastewater and a toxic effect could be masked by a high nutritional potential of complex wastewater and stimulation of growth. Biotests with aquatic and terrestrial plants may give additional important information on a possible effect of effluents on the eutrophication process in receiving waters (Blinova 2000). Similar results were obtained by G.C. Castillo et al. (2000), they found that Lactuca sativa was not very sensitive to wastewater, while aquatic invertebrates were more sensitive.

4. Conclusions

Results of the tests on early *Lactuca sativa* seedlings growth show that Kaunas municipal untreated wastewater has relatively low short-term phytotoxicity, i.e. below a 50 % effect, or even stimulation. 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.

Results of the acute crustaceans *T. platyurus* tests show that Kaunas municipal untreated wastewater is toxic to aquatic invertebrates and may pose relatively serious hazards to receiving waters. Calculated LC_{50} equalled 51.45 % of wastewater in the solution (TU = 1.94). Mechanical and biological treatment of effluents had sharply reduced toxicity of effluents (below 50 % of the effect level).

Due to a number of potential toxicants in such complex substances as wastewater further studies based on toxicity testing with a battery of tests are recommended.

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Municipalinių nuotekų toksiškumo testavimas aukštesniaisiais augalais ir bestuburiais

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(gauta 2010 m. rugpjūčio mėn.; atiduota spaudai 2010 m. rugsėjo mėn.)

Kauno municipalinių nuotekų toksiškumas buvo vertinamas remiantis biotestais, naudojant vandens bestuburius ir sausumos aukštesniuosius augalus. Buvo vertinamas nevalytų, mechaniškai ir biologiškai išvalytų nuotekų toksiškumas. Nuotekų toksiškumas vertintas sėklų dygimo ir trumpalaikio ankstyvojo sėjamosios salotos (*Lactuca sativa* L.) augimo ir ūmaus toksiškumo mikrovėžiagyvių *Thamnocephalus platyurus* (24 h) testais. Neskiestos nevalytos nuotekos buvo ypatingai toksiškos *T.platyurus* ir sukėlė visų testuojamų organizmų žūtį. Du kartus sumažinta nuotekų koncentracija mėginyje (nuo 100 % iki 50 %) gerokai sumažino nuotekų toksiškumą ir nuotekos tapo vidutiniškai toksiškos testuojamiems organizmams. Nuotekas praskiedus daugiau kaip 4 kartus, žuvo tik 10–20 % veiktų *T. platyurus.* Aukštesniųjų augalų bioteste buvo vertintas sėklų dygimas, šaknų ilgis, daigelių aukštis ir bendra biomasė. Nevalytos nuotekos buvo nelabai toksiškos (0,6 TU) sėjamosios salotos šaknelės augimui, tačiau mechaniškai ir biologiškai išvalytos nuotekos neigiamo poveikio nedarė arba netgi skatino augimą. Nuotekos skatino daigelių ir biomasės augimą. Skatinamasis efektas gali būti paaiškintas pakankamai didelėmis biogeninių medžiagų koncentracijomis nuotekose.