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Ecotoxicological effects evoked in hydrophytes by leachates of invasive *Acer negundo* and autochthonous *Alnus glutinosa* fallen off leaves during their microbial decomposition

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ABSTRACT

Throughout 90-day biodegradation under microaerobic conditions, invasive to Lithuania species boxelder maple (*Acer negundo*) leaves lost 1.5-fold more biomass than that of autochthonous black alder (*Alnus glutinosa*), releasing higher contents of N_{tot}, ammonium and generating higher BOD_7. Boxelder maple leaf leachates were characterized by higher total bacterial numbers and colony numbers of heterotrophic and cellulose-decomposing bacteria than those of black alder. The higher toxicity of *A. negundo* aqueous extracts and leachates to charophyte cell (*Nitellopsis obtusa*), the inhabitant of clean lakes, were manifested at mortality and membrane depolarization levels, while the effect on H^+-ATPase activity in membrane preparations from the same algae was stronger in case of *A. glutinosa*. Duckweed (*Lemna minor*), a bioindicator of eutrophic waters, was more sensitive to leaf leachates of *A. glutinosa*. Fallen leaves and leaf litter leachates from invasive and native species of trees, which enter water body, affect differently microbial biodestruction and aquatic vegetation in freshwater systems.

1. Introduction

Lake ecosystems experience various influences from terrestrial surroundings. One of these includes the input of natural organic material, dissolved organic carbon (DOC) and nutrients originated, for example, from falling into water leaves or leachates from leaf litter affecting aquatic organisms (Pflugmacher et al., 2001; Earl et al., 2012). When leaves enter water and degrade, biotic and abiotic processes are involved in the formation and removal of dissolved organic matter. Particulate detritus interacts with the biotic, i.e., bacteria, fungi, invertebrates (Gessner et al., 1999) and abiotic, i.e., temperature, water flow, soil/water chemistry (Allard and Moreau, 1986) components of ecosystems and thus regulates the rate of breakdown.

During leaf decomposition, water-soluble chemical compounds are released into the water, which may evoke diverse responses of aquatic organisms including microorganisms that participate in biodegradation processes. Some of these compounds are humic substances, capable of generating reactive oxygen species and, for instance, affect physiology and photosynthesis of aquatic plants (Pflugmacher et al., 1999). Recently, it was shown that fallen leaf degradation extracts from common oak *Quercus robur* and European beech *Fagus sylvatica* have inhibiting effects on the photosynthetic activity of *Vesicularia dubyana* (Nimptsch and Pflugmacher, 2008). Extracts from *Phragmites australis* and *Q. robur* leaves were found to affect the antioxidative system and photosynthetic rate of *Ceratophyllum demersum* (Kamara and Pflugmacher, 2007). Grigutytė et al. (2009) explored the effect of the *Fagus sylvatica* leaf extracts on oxidative stress enzyme activities in charophyte *Nitellopsis obtusa.* Some hydrophytes such as *V. dubyana*, *C. demersum* or *N. obtusa* seem to be intolerant to leaf litter leachates. These findings suggest that decomposing leaves may be an important environmental factor influencing certain plant species and thus community structure within freshwater ecosystems.

Climate warming evokes beneficial conditions for invasive species to dominate over native species by replacing them in ecological relationships. Other factors, such as human trade and travel, may promote their dissemination in the environment (Burrows et al., 2012). The invasive to Lithuania boxelder maple

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Acer negundo that became commonly distributed after its escape from cultivation in 1963 (Gudžinskas, 1998), eagerly colonises ecotones such as coastal zone of lakes and rivers. In Lithuania, these zones are dominated by autochthonous black alder *Alnus glutinosa* (Prieditis, 1997). It has been mentioned that *A. negundo* litter decomposes well and may accelerate litter mineralisation in contrast to the species with hardly decomposable leaves (Mędrzycki, 2007). Therefore, the impacts of native and invasive species from terrestrial surroundings might be different to biota of aquatic ecosystem.

To investigate possible ecotoxicological effects of fallen leaves leachates, two species characterized by different bioindicatory value were chosen: duckweed *Lemna minor*, a cosmopolitan species capable to spread in nutrient-rich stagnant marshes, bogs lakes and quiet streams including sewer outlets rich in organic matter (Environment Canada, 1999) and charophyte *N. obtusa*, a bioindicator of clear oligo- or mesotrophic waters (Van den Berg et al., 1998; Pybus et al., 2003). Macrophytic green algae *N. obtusa* form underwater benthic plantations of fresh and brackish waters. The bioelectrical response of charophyte cell is rapid and highly sensitive to chemical compounds and can be used as a relevant endpoint to assess the toxicity of aquatic samples (Manusadžianas et al., 1999). Good correlation between electrophysiological response (90-min 50%-depolarization of resting potential) and cell lethality (96-h LC50) induced by chemical stressors has been previously shown (Manusadžianas et al., 2002, 2007).

By linking lake and lake shore ecosystems, and in accordance with the fact that decomposing leaves are an additional source of allochthonous dissolved organic carbon and presumably toxicity agent, we sought to compare the prolonged impacts of boxelder maple *Acer negundo* and black alder *Alnus glutinosa* leaves on (1) basic physical–chemical parameters and microbial development in the leachates obtained during the 90-day leaf biodegradation; (2) charophyte *N. obtusa* and duckweed *L. minor* by assessing leaf aqueous extract- and biodegraded leaf leachate-induced algae lethality, cell membrane depolarization and H^+-ATPase activity responses, and duckweed growth inhibition.

2. Materials and methods

2.1. Preparation of leaf aqueous extracts and biodegraded leaf leachates

Fallen off leaves of *Alnus glutinosa* and *Acer negundo* were collected in Rudnia (Varėna district, Lithuania) during autumn of 2010. After transportation to the laboratory they were cleaned from tree debris, air-dried and roughly chopped. The leaves (100 g of dry weight/L) were placed into 5-L plastic containers filled with medium containing 0.1 mM KCl, 1.0 mM NaCl, 0.5 mM $CaCl_2$ and 1.0 mM TRIS, pH 7.5 (adjusted with NaOH or HCl). The containers were shaken for 48 h, the obtained extracts were paper filtered and centrifuged (20,000 × *g*, 10 min, 4 °C) to remove sediments, then supernatant was 0.45 μm and 0.2 μm filtered. Leaf aqueous extracts were stored in sterile 2-L bottles at 4–8 °C.

To obtain biodegraded leaf leachates, the leaves (10 g of dry weight/L) were placed into half-filled 40-L plastic containers filled with water from littoral zone of mezotrophic Lake Balsys. Before the experiment, the lake water (LW) was filtered through fourfold mill silk (pore size 20 μm) to remain bacteria and remove phytoplankton, zooplankton and hyphomycetes.

2.2. Design of the experimentation with biodegraded leaf leachates

For leaf biodegradation experiment, three treatments were chosen: LW (control), LW + *A. glutinosa* leaves, LW + *A. negundo* leaves. During the experiment, the media in all treatments were aerated everyday for 2 h. The experiment was run in dark at 11–13 °C for 90 days. Chemical [O_2, NO_2^-, NO_3^-, NH_4^+, inorganic nitrogen (N_{in}), total nitrogen (N_{tot}), PO_4^{3-}, total phosphorus (P_{tot}), DOC, BOD_7] and microbiological (total number of bacteria, heterotrophic and cellulose–degrading bacteria) parameters of media were analysed once every 10 days from days 0–40 and on 60th and 90th day. The temperature and pH of the leachates were measured every day. All chemical and microbial analyses were carried out in duplicate. At the end of experiment, the leaf litter mass loss in the treatments was determined. The mass loss was calculated after drying the leaves at 60 °C to constant weight and was expressed as a percentage of the initial dry mass.

For the detection of the development of allochthhonous bacteria (brought with leaves) the filtered (0.45 μm, Millipore) and sterilized (20 min, 120 °C) LW was used.

2.3. Physical–chemical analysis

Leachate temperature and pH were measured using a portable universal MultiLine f/Set-3 meter (WTW). Chemical analysis was performed in a certified analytical laboratory (JSC Water Investigations, Vilnius, Lithuania) according to standard methods of water investigation (Merkienė and Čeponytė, 1994). Phosphate was assessed via molybdate ascorbic acid method (ISO 6878). The total phosphorus concentration was measured using persulphate-H_2SO_4 digestion and molybdate ascorbic acid methods. Nitrate was determined by Ion Chromatography (ISO 10304-1), ammonium – by manual spectrometric method ISO 7150-1. The total nitrogen was determined using potassium persulfate-$K_2S_2O_8$ digestion following Cd–Cu reduction to NO_2 (ISO 11905-1), dissolved organic carbon – by the dichromate oxidation method according to ISO 15705. Dissolved oxygen concentration was determined by electrometric method (EN 25813). Humic acid content analysis was performed according to Faithfull (2002) in a certified analytical laboratory (JSC Labtarna, Vilnius).

2.4. Microbial analysis

Total number of bacteria were enumerated under epifluorescence microscopy after 4′,6-diamidino-2-phenylindole (DAPI) staining on 0.2 μm black Millipore filters (Porter and Feig, 1980). At least 200 bacterial cells in 20 fields were counted at 1000 × using an epifluorescence microscope (Olympus IX70).

Dominant morphotypes of bacteria were estimated qualitetively by visual inspection under an Olympus CH40 phase-contrast microscope at 1000× magnification, then the size of bacteria of dominant morphotype was measured. Heterotrophic bacteria were incubated on 10× diluted agar nutrient medium at 25 °C for 120 h (Kuznetsov and Dubinina, 1989). After incubation the bacterial colonies were counted and colonies forming units were calculated in 1 mL (CFU/mL). The number of cellulose-degrading bacteria (CDB) was determined by inoculation of 0.1 mL water sample on solid Hetchinson medium (g/L): K_2HPO_4 – 1.0, $CaCl_2$ – 0.1, $MgSO_4$ – 0.3, $FeCl_3$ – 0.01, $NaNO_3$ – 2.5, agar Difco–20 (pH 7.2), C source – 1% Carboxy Metyl Cellulose (CMC). Petri dishes were incubated at 25 °C for 2 weeks. After incubation, the numbers of visible colonies in Petri dishes were recorded. For the recognition of CDB colonies, 5 mL of 0.1% Congo red and 5 M NaCl were used in each plate (Kluepfel, 1988).

2.5. Charophyte algae material

The freshwater charophyte *N. obtusa* (Desv.) was harvested from Lake Obelija, Lithuania (Kostkevičienė and Sinkevičienė, 2008) in autumn 2010. Prior to experiment, internodal cells were separated from neighbouring cells. After separation from thalloma, single cells were kept at room temperature (20 ± 2 °C) in glass containers filled with equal parts of non-chlorinated tap water, lake water and artificial pond water (APW) containing (mM): 0.1 KH_2PO_4, 1.0 $NaHCO_3$, 0.4 $CaCl_2$, 0.1 $Mg(NO_3)_2$, and 0.1 $MgSO_4$ (pH 7–7.4) (Vorobiov and Manusadžianas, 1983).

2.6. N. obtusa cell lethality testing

Lethality response of macrophytic algae cells of *N. obtusa* was investigated up to eight days. The APW medium was used as control. Single internodal cells (each 4–15 cm in length) were placed on Petri dishes (10 cells per dish, 2–4 replicates), preadapted for 1–2 days in APW and then were kept at room temperature (18–22 °C) in the dark. The preadaptation in APW before the test allowed discarding occasionally dead cells, which had been injured during the transfer to Petri dishes. Survival of the cells was checked daily by gently picking up each cell with a spatula. A cell was judged to be dead when picked up if there was disappearance of turgor pressure, a state in which a cell bends on the spatula. The APW in each Petri dish was changed 2 and 4 days after the beginning of the exposures. The 8-days median lethal concentration (8-d LC50) was calculated for each treatment.

2.7. Electrophysiological experiments

Bioelectrical activity of up to 32 living internodal cells was measured simultaneously according to K^+-anaesthesia method (Shimmen et al., 1976), modified for multichannel recording with extra cellular chlorinated silver wire electrodes. The discrete values of cell membrane (plasmalemma) potential from distinct cells were taken every second. After amplification, the output signals were channelled into a PC by means of controller. The data on kinetics of cell transmembrane resting potential (RP) of all 32 cells were plotted on a display in graphic form for visual control and stored for further analysis. The details of computer-assisted experimental setup have been published previously (Manusadžianas et al., 1999, 2002).