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Cultivating duckweed *Lemna minor* in urine and treated domestic wastewater for simultaneous biomass production and removal of nutrients and antimicrobials

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ABSTRACT

Duckweed *Lemna minor* was cultivated in human urine (HU) and the effect of urine type, dilution factor, temperature, existence of macro- and microelements on growth rate was investigated. The simultaneous removal of nutrients and selected antimicrobials was also studied in experiments with HU and treated domestic wastewater, while the starch and protein content of biomass was determined. Higher growth rates were observed at 24 °C, using HU stored for 1 d and with dilution factor equal to 1:200. In experiments with HU and wastewater, the removal of COD, total phosphorus and total nitrogen exceeded 80%, 90% and 50%, respectively, while ciprofloxacin and sulfamethoxazole were eliminated by more than 80%. The main removal mechanism for the former antimicrobial was photodegradation, while plant uptake and biodegradation seem to be of significant importance for the latter. Crude protein content reached 31.6% in experiments with HU and biomass harvesting, while starch content was enhanced when duckweed was transferred to water for 21 d, reaching 47.1%.

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1. Introduction

Constructed wetland technology is a promising alternative treatment process for removing conventional and non-conventional pollutants from wastewater (Stefanakis et al., 2011; Avila et al., 2014). Among different plant-based systems, duckweed ponds are of special interest as they achieve significant removal of major pollutants and heavy metals (Sekomo et al., 2012; Zhang et al., 2014). Recent studies have also reported the removal of emerging contaminants such as pharmaceuticals and personal care products in these systems due to several biotic and abiotic mechanisms (Reinhold et al., 2010; Zhang et al., 2014). Additionally, duckweeds can produce biomass with high crude protein content due to their ability to metabolize ammonia directly from water body (Mohedano et al., 2012), while they can accumulate high percentages of starch, a fact that allow their use for bioethanol production (Xu et al., 2011; Ge et al., 2012).

In domestic wastewater, 85% of total N and 50% of total P originate from human urine (HU), indicating that separately collected HU could be used for nutrients recovery and crop production (Liu et al., 2013; Zhang et al., 2013). When urine leaves the human body it contains urea, inorganic ions, natural organic metabolites as well as traces of antimicrobials and other synthetic organic chemicals that are related to health protection and human habits. Nonetheless, literature data for urine composition vary widely; the main characteristics of HU are: pH 5–8, urea 5000–9000 mg L^{-1}, NH_4^+-N 250–8100 mg L^{-1}, COD 8000–10,000 mg L^{-1}, K^+ 1300–3100 mg L^{-1} and TP 350–2000 mg L^{-1} (Chang et al., 2013; Tuantet et al., 2014a; Zhang et al., 2013). It is worth mentioning that urine composition changes during transportation and storage, leading to an increase of pH and NH_4^+-N due to hydrolysis and a decrease of Mg due to precipitation of crystals. Regarding antimicrobials, parent compounds as well as their metabolites have been detected in HU at concentrations ranging up to some hundreds mg L^{-1}, depending on medical treatment (Gika et al., 2010; Cazola-Reyes et al., 2014).

In some recent studies, HU has been used for cultivating aquatic microorganisms in order to produce biomass that can be valorized as biofertilizer, biochemicals and biofuels. Tuantet et al. (2014a) studied the growth of *Chlorella sorokiniana* using different types of urine and in the presence of additional trace elements. Moreover, they achieved continuous cultivation of these microalgae, producing biomass that contained up to 53% w/w and 25% w/w proteins and total fatty acids, respectively (Tuantet et al., 2014b). In another study, Zhang et al. (2014) used fresh urine to cultivate *Chlorella sorokiniana*, recovering in biomass 80.4% and 96.6% of N and P, respectively; while Chang et al. (2013) reported cultivation of

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Spiroulina platensis in HU under autotrophic and mixotrophic conditions, achieving significant NH_4^+-N, P and urea removal as well as high protein content. On the other hand, there is no information for the cultivation of duckweed using HU, as well as for the characteristics of produced biomass and the removal of nutrients and antimicrobials in such systems.

Based on the above, the main objective of this study was to investigate duckweed's *Lemna minor* growth using HU. Experiments were conducted using different types of urine (fresh, hydrolyzed, stored, and synthetic) and the effect of several parameters such as urine dilution, temperature, existence of macro- and microelements on growth rate was investigated. The efficiency of *Lemna minor* to remove nutrients (COD, total N, NH_4^+-N, total P) and selected antimicrobials (sulfamethoxazole, SMX and ciprofloxacin, CIP) from HU and treated domestic wastewater was also studied; while the content of produced biomass on protein and starch was determined.

2. Methods

2.1. Chemicals and culture

Analytical standards of SMX and CIP hydrochloride were purchased from Sigma–Aldrich (Steinheim, Germany). The physicochemical properties of two selected antimicrobials can be found in Table S1. Stock solutions were prepared in methanol (Fisher, USA). Culture of *Lemna minor L.*, clone St. was donated by Federal Environment Agency (Berlin, Germany). Before their use in urine and wastewater experiments, the duckweed cultures were grown for 4 weeks in Swedish standard (SIS) sterile growth medium (Table S2) according to the conditions described by OECD Guideline 221 (OECD, 2006). All salts used for *Lemna minor* growth medium were purchased by Fluka (Heidelberg, Germany). HPLC grade water was prepared in the laboratory using a MilliQ/MilliRO Millipore system (Bedford, USA). Regenerated Cellulose (RC) filters (0.2 μm, 4 mm) for antimicrobials analysis were purchased from Phenomenex (Torrance, CA, USA). HU and secondary treated wastewater used in this study were collected from the University Campus (Lesvos island, Greece).

2.2. Experiments with Lemna minor

2.2.1. Role of different parameters on Lemna minor growth rate

Experiments were initially conducted to investigate the optimal conditions for cultivating *Lemna minor* in urine. Different dilution factors (1:2, 1:5, 1:10, 1:25, 1:50, 1:100, 1:150, 1:200, 1:250) of HU and synthetic urine (SU) were tested and the growth rates of *Lemna minor* were calculated. HU was used in three different forms (fresh, hydrolyzed, stored for 1 day at 4 °C), while not hydrolyzed SU was prepared according to Table S3. Hydrolysis of HU was achieved by continuous mixing on a shaker for 30 min at 30 °C (Tuantet et al., 2014a). Experiments were also performed at different temperatures (12 °C, 18 °C, 24 °C and 30 °C), different initial mass of duckweed (0.5 g, 1.0 g and 1.5 g) and in the presence of different macroelements (Fe, Ca, Mg) and mixture of microelements (B, Mn, Mo, Zn, Cu, Co). The experimental conditions used in each experiment are reported in Table 1.

All experiments were conducted in triplicate in glass Petri dishes (12 cm diameter), containing 100 mL of each tested media. Each Petri dish was inoculated with 12 healthy fronds of *Lemna minor* or appropriate mass of duckweed and incubated in a temperature-controlled incubator under continuous illumination with fluorescent lamps. The pH was adjusted to 7, using HCl or NaOH.

2.2.2. Nutrients and antimicrobials removal in Lemna minor experiments with urine and wastewater

Experiments with SIS medium, HU and secondary treated domestic wastewater were conducted in Petri dishes to investigate the elimination of COD, urea, NH_4^+-N, TN and TP and the removal of two antimicrobials from different classes commonly found in HU (SMX and CIP) in the presence of *Lemna minor* (Table 2). The substances were chosen according to previous studies as two of the most often used antimicrobials in Greece (Iatrou et al., 2014) that are not totally removed during conventional wastewater treatment (Thomaidi et al., 2015). The duration of the experiments was 14 days and the tested concentration for antimicrobials was 50 $\mu g\,L^{-1}$. Before the addition of target antimicrobials, toxicity tests were conducted for a wide range of concentrations (SMX: 2–2000 $\mu g\,L^{-1}$; CIP: 50–450 $\mu g\,L^{-1}$) to investigate possible toxicity of these compounds to *Lemna minor*.

Aqueous samples for the determination of nutrients and antimicrobials were taken at different time intervals, while biomass samples were taken at the beginning and at the end of the experiment to characterize duckweed for crude protein and starch content. To investigate the role of biomass harvesting on removal of nutrients and antimicrobials, experiments were also conducted with HU and harvesting of 0.5 g biomass at Days 5 and 10. To study the role of abiotic factors on the removal of antimicrobials, additional experiments were conducted in the absence of duckweed for all tested media (Table 2).

Table 1
Experimental protocol applied in *Lemna minor* growth rate experiments (number of replicates: 3).

Experiment	Type of urine	Dilution factor	Temperature (°C)	Initial number of leafs/ initial mass of duckweed (g)	pH	Duration (d)	Macro-, micro elements
A	Fresh HU	1:2, 1:5, 1:10, 1:25, 1:50, 1:100, 1:150, 1:200, 1:250	24	12 leafs	7	7	No addition
	Hydrolyzed HU, Stored HU, SU	1:150, 1:200, 1:250					
B	Stored HU, SU	1:200	12, 18, 24, 30	12 leafs	7	7	No addition
C	Stored HU	1:200	24	0.5 g, 1 g, 1.5 g	7	7	No addition
D	Stored HU	1:200	24	0.5 g	7	10	Fe[a] Ca[b] Mg[c] B, Mn, Mo, Zn, Cu, Co[d]

[a] 0.17 $mg\,L^{-1}$.
[b] 9.8 $mg\,L^{-1}$.
[c] 7.4 $mg\,L^{-1}$.
[d] B: 0.17 $mg\,L^{-1}$, Mn: 0.056 $mg\,L^{-1}$, Mo: 0.004 $mg\,L^{-1}$, Zn: 0.011 $mg\,L^{-1}$, Cu: 0.013 $mg\,L^{-1}$, Co: 0.002 $mg\,L^{-1}$.

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