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Bioaccumulation of tetracycline and degradation products in *Lemna gibba* L. exposed to secondary effluents

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ABSTRACT

The aim of this study was determination of the bioaccumulation capacity of tetracycline (TC) and the degradation products by *Lemna gibba* L. in the pilot-scale reactor that takes the effluent of a municipal wastewater treatment plant (Elazığ city, Turkey). For this aim, *L. gibba* L. which was exposed to secondary clarifier effluent of the treatment plant was harvested from the pilot-scale reactor. Then, the harvested *L. gibba* L. was extracted and passed from solid-phase extraction cartridge and TC, 4-epitetracycline (ETC), 4-epianhydrotetracycline (EATC), and anhydrotetracycline (ATC) concentrations were determined. Maximum TC, ETC, EATC, and ATC concentrations bioaccumulated by *L. gibba* L. were 123 ± 2.0, 129 ± 3.2, 42.7 ± 0.5, and 31.9 ± 0.3 ppb, respectively while minimum TC, ETC, EATC, and ATC concentrations those bioaccumulated by *L. gibba* L. were determined as 99.7 ± 1.2, 111 ± 2.2, 12 ± 0.6, and 8.3 ± 0.1 ppb, respectively. The order of the uptake rate of TC and the degradation products by *L. gibba* L. was determined as follows: ETC > TC > EATC > ATC.

Keywords: Lemna gibba L.; Bioaccumulation; Tetracycline; Treatment; LC–MS–MS

1. Introduction

Tetracyclines (TC), both natural and semisynthetic, form a large group of products produced mainly by *Streptomyces* spp. They have a broad-spectrum of activities including inhibition of many common Gram-positive and Gram-negative bacteria, chlamydia, rickettsie, etc.; they are distinguished mainly for bacteriostatic action caused by inhibition of protheosynthesis [1–3]. TC is one of the most commonly used prophylactic and therapeutic medication for treating human and animal diseases and benefiting agricultural productivity. It is reported that only a small fraction of TC is absorbed or metabolized in the human body [4,5]. Due to their poor absorption, most of them are excreted through feces and urine as unmetabolized parent compound [6].

Municipal wastewater treatment plants (WWTPs) nowadays receive wastewaters that contain a different

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traces of polluting compounds [7]. Mostly, antibiotic traces disposed can not be efficiently treated in conventional WWTPs and enter directly to the receiving environment [8]. The use of antibiotics and growth hormones in human and veterinary medicine has a significant effect on the quality of surface and groundwater [9,10]. The presence of TC and other antibiotics in natural environments can cause bacteria to acquire and transmit antibiotic-resistant genes, which potentially threatens ecosystem functions and human health [10,11].

Alternative advanced technologies for tertiary treatment of WWTP effluents are necessary [12]. In order to remove emerging contaminants which are a broad category of contaminants that includes pharmaceuticals and personal care products, endocrine-disrupting contaminants, perfluorinated compounds, and engineered nanomaterials [13], advanced water reclamation systems (e.g. ozonization, photo–fenton, and reverse osmosis) capable of efficiently eliminating these pollutants have been developed [14]. However, advanced treatment processes require a high level of energy consumption and are expensive to build and maintain. These issues can be overcome by the introduction of biological cleaning systems such as constructed wetlands (CWs), which are typically located after secondary wastewater units [15]. Advanced treatment, downstream of conventional biological process, can significantly improve antibiotics removal before effluent disposal. The installation of treatment techniques to remove antibiotics in wastewaters should also be flexible and allow their implementation not only in urban wastewater treatment plants, but also at important source points such as hospitals and the pharmaceutical industry [16]. The main advantages of CWs are their low operational costs, the fact that they do not require an external energy source and their integration with the landscape. The use of surface flow CWs as tertiary treatment systems have given a similar removal efficiency for emerging pollutants to advanced treatment systems [15,17,18].

Duckweed plants are common in the aquatic environment, especially in quiescent water bodies and are divided into four genera: *Spirodela, Wolffiella, Lemna*, and *Wolffia*. There are approximately 40 species worldwide. Duckweed plants are widely distributed in the world from the tropical to the temperate zones, from fresh water to brackish estuaries, and throughout a wide range of trophic conditions [19,20].

Lemna gibba L. is an important, fast growing tested organism. It is an aquatic plant and is relevant to many aquatic environments, including lakes, streams, and effluent. Applications of *L. gibba* L. in wastewater treatment was found to be very effective in the removal of soluble salts, organic matter, heavy metals and in eliminating suspended solids, algal abundance, and total and fecal coliform densities [21,22].

Only recently has extensive research been carried out on plant uptake and assimilation of pharmaceuticals in CWs. To date, there has been little quantitative evaluation of the ability of plants to assimilate and translocate pharmaceuticals, and available data on plant uptake is limited to only a few pharmaceutical compounds and plant species. Additionally, most studies on plant uptake of pharmaceuticals have been done in hydroponic solutions [23,24].

To our knowledge, this is the first study for the determination of the bioaccumulation capacity of TC and the degradation products by *L. gibba* L. in the pilot-scale reactor which takes the secondary effluents of a WWTP. The aim of the study is to assess the bioaccumulation capacity of *L. gibba* L. for TC and the degradation products in the effluents of a municipal WWTP as an operational alternative.

2. Materials and methods

2.1. Reagents

The pharmaceutical formulations of TC contain small amounts of impurities namely 4-epitetracycline (ETC), anhydrotetracycline (ATC), and 4-epianhydrotetracycline (EATC) [25]. Degradation productions of pharmaceuticals can be considered as contaminants contributing to these complex mixtures that are present [26–28].

In this study, TC, 98%, ETC, 97%, ATC, 97%, and EATC, 97% were analyzed. TC was purchased from Sigma-Aldrich (USA). The degradation products were purchased from Acros Organics (NJ, USA). Methanol from Carlo Ebra, methylene chloride from Fisher Chemical, acetonitrile and formic acid from J.T. Baker (USA) were all of HPLC grade. Hydrochloric acid (HCl) (J.T. Baker), sodium hydroxide (NaOH) (Acros Organics), ammonia solution ($NH_3.H_2O$) (Carlo Ebra), and ethylenediamine tetraacetic acid disodium (Na_2EDTA) were all of analytical reagent grade and purchased from Sigma-Aldrich (USA). Solid-phase extraction (SPE) cartridges, Oasis HLB (500 mg, 6 cm^3), and Oasis MAX (60 mg, 3 cm^3) cartridges were used in the study. They were purchased from Waters Corporation (Milford, MA, USA). The ultrapure water used through the study was supplied by Zeneer power water purification system.

2.2. Municipal wastewater treatment plant

The chosen WWTP in the study is located in Elazığ city, Turkey. Schematic diagram of the studied WWTP

was shown in Fig. 1. Elazığ municipal wastewater treatment plant (EMWWTP) was projected according to the conventional activated-sludge system. In the EMWWTP, wastewater flows of about 36,000 and 54,000 m^3/d (varies seosonally) are treated in a series of pre-treatment (screens, a grit chamber), primary clarifier and cyclic activated sludge system, followed by a secondary clarifier, before being discharged into a surface water (Kehli stream) and eventually into Keban Dam Lake, which is an important source for city to supply water (drinking, usage and irrigation water) from it [29]. Recently, the presence of antibiotics in the influent of EMWWTP [30] and the presence of TC in EMWWTP [31] was determined.

2.3. Pilot-scale reactor

The pilot-scale reactor used in the study was established after the secondary clarifier in EMWWTP to take the effluent of the treatment plant. EMWWTP secondary effluent was given to the pilot-scale reactor by a pump and the wastewater flow rate was regulated by a timer. The dimensions of the pilot-scale reactor were as follows; diameter of 41 cm and water depth of 3 cm (Fig. 2). *L. gibba* L. was weighed by wet weight (50 g) and added to the pilot-scale reactor. Hydraulic retention time of the pilot-scale reactor was regulated to 3 h.

2.4. Plant collection

The aquatic plant *L. gibba* L. that identified according to the procedure in Flora of Turkey [32] was collected from local fresh water bodies (referred as natural water in Table 1) in Elazığ region (Turkey). *L. gibba* L. was settled to the jerrycan with a volume of 2 L and then brought to the laboratory of Environmental Engineering Department, Fırat University. In the laboratory, the plants collected from the natural environment were washed with distilled water to remove the probable pollutants on the plant surface before they were placed into the pilot-scale reactor.

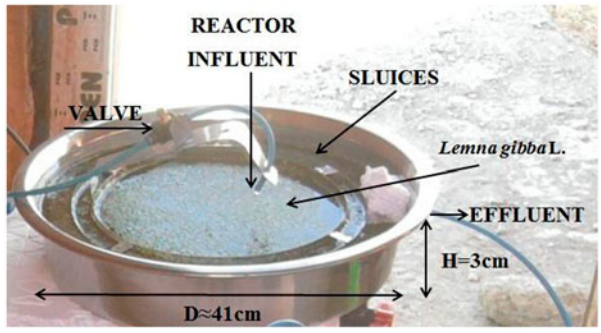

Fig. 2. Pilot-scale reactor.

2.5. Plant extraction

The extraction of the plant samples taken from the pilot-scale reactor everyday during the study period was performed by the method used by Lillenberg et al. [33]. 250 mg of dried *L. gibba* L. was extracted with 10 mL of 1:1 (v/v) mixture of acetonitrile and 1% acetic acid, then homogenized with laboratory homogenizer DIAX 900 (Heidolph Instruments, Germany) 25,000 rpm, sonicated (5′), vortexed (1′) and centrifuged at 8,000 rpm. The supernatant was then separated and dried by nitrogen stream. Approximately 15 mL of 1% acetic acid was added to the 1 mL of evaporation residue.

2.6. Solid-phase extraction

Analyses were performed by ultrafast liquid chromatography–tandem mass spectrometry (UFLC-MS/MS) (Shimadzu Prominence UFLC coupled to 3,200 QTRAP, Applied Biosystems) and SPE using the method reported by Jia et al. [34]. The samples were filtered with a glass microfiber filter (0, 7 μm, Whatman, Maidstone,

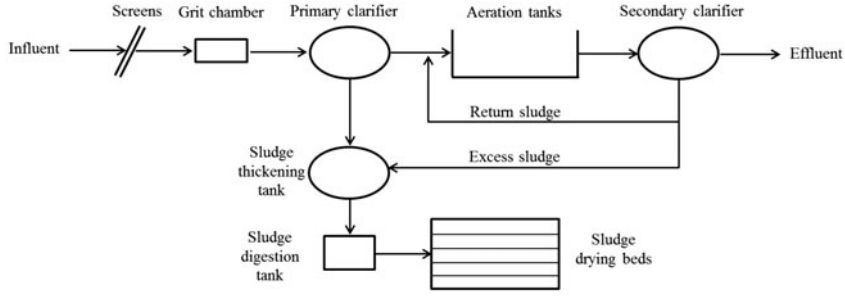

Fig. 1. Schematic diagram of the studied WWTP.

Table 1
Physicochemical properties of the effluent of EMWWTP and the natural water

Parameter	Unit	Natural water	Wastewater (average ± SD) (n = 13)
pH	–	7.54 ± 0.08	7.66 ± 0.12
Temperature	(°C)	19 ± 0.7	20.3 ± 0.20
BOD_5	mg/L	3.5 ± 0.1	42.7 ± 1.30
COD	mg/L	7.6 ± 0.3	81.5 ± 1.10
TOC	mg/L	3.0 ± 0.2	33.2 ± 0.8
$O\text{-}PO_4^{-3}$	mg/L	0.2 ± 0.05	7.1 ± 0.13
NH_4^+-N	mg/L	<0.05	0.055 ± 0.001
NO_2^--N	mg/L	<0.05	0.70 ± 0.02
NO_3^--N	mg/L	0.84 ± 0.01	2.52 ± 0.08
TC	ppb	ND	10.7 ± 0.4
ETC	ppb	ND	51.6 ± 0.2
EATC	ppb	ND	13.3 ± 0.3
ATC	ppb	ND	10.2 ± 0.3

Note: ND: Not dedected, SD: Standard deviation.

England). After filtration, 16 mL sample was added with 0.5 g/L Na_2EDTA, and acidified to pH 3.0 with hydrochloric acid. Oasis HLB cartridges were preconditioned with 6 mL of methylene chloride, 6 mL of methanol and 6 mL of ultrapure water containing 0.5 g/L Na_2EDTA (adjusted to pH 3.0 with HCl). The samples were passed through these HLB cartridges. The flow rate was approximately 3 mL/min. The HLB cartridges were rinsed with 10 mL of ultrapure water. They were dried under a flow of nitrogen and then eluted with 6 mL of methanol. The eluates were collected in an amber vial and dried under a gentle flow of nitrogen. They were reconstituted to 0.3 mL with methanol. The extracts were diluted to 8 mL by ultrapure water (adjusted to pH 7.0 with 5% $NH_3{\cdot}H_2O$). The solutions were then applied to the Oasis MAX cartridges (preconditioned with 1 mL of methanol, 1 mL of 5 N NaOH, and 1 mL of ultrapure water). All cartridges were rinsed with 1 mL of 5% $NH_3{\cdot}H_2O$, followed by 1 mL of methanol. Elution was performed with 3 mL of acetonitrile/water containing 1% formic acid (50/50, v/v) mixed reagents. The extracts were concentrated to 1.5 mL under a stream of nitrogen and measured with UFLC-MS/MS soon after they were prepared.

2.7. UFLC–MS/MS

Concentrations of TC and the degradation products (ETC, ATC, EATC) in duckweed (*L. gibba* L.) samples were analyzed using UFLC-MS/MS. Separation of TC and the degradation products was achieved with a Waters ACQUITY UPLC BEH C18 column (1.7 μm; 2.1 mm × 100 mm). The injection volume was 10 μL (full loop). The mobile phases were Acetonitrile (A) and ultrapure water containing 0.1% formic acid (v/v) (B). The gradient was as follows. The initial 10% A was increased linearly to 20% in 5 min, a further 20% A was increased to 90% over 4 min and kept for 0.5 min, followed by an increase to 100% A and held for 1 min. Finally the gradient was returned to the initial conditions of 10% A and held for 2 min to allow for equilibration. The flow rate was 0.2 mL/min. The column was maintained at 30˚C and the sample room temperature was 20˚C. Mass spectrometry was performed using a AB Applied Biosystems (triple-quadrupole) detector equipped with an electrospray ionization.

The concentration range of the calibration standarts were 0.1, 0.5, 1, 2, 3, 4, 5, 10, 30, 50, 100, 300, and 500 μg/L. Mean coefficients of determination (R^2) were 0.9761, 0.9850, 0.9996, and 0.9998 for ATC, EATC, ETC, and TC, respectively.

2.8. Statistical analyses

Experimental results were analyzed using the IBM SPSS Statistics 21 programme (USA) and values shown are the means of three replicates. Each point is the mean of three replicates. Error bars indicate the standard deviation.

3. Results and discussion

Physicochemical properties of the effluent of EMWWTP and the natural water are given in Table 1.

TC and degradation products were obtained in the effluents of EMWWTP. TC, ETC, EATC, and ATC concentrations in the secondary effluents were 10.7 ± 0.4, 51.6 ± 0.2, 13.3 ± 0.3, and 10.2 ± 0.3 ppb, respectively.

In the present study, TC, ETC, EATC, and ATC bioaccumulated in plants (*L. gibba* L.) were in unit of ppb (dry weight).

The bioaccumulated TC concentrations by *L. gibba* L. are given in Fig. 3.

The maximum TC concentration bioaccumulated in *L. gibba* L. exposed to secondary effluent was determined at day 3 (Fig. 3). TC concentration at day 3 was determined as 123 ± 2.0 ppb. The lowest TC concentration was 99.7 ± 1.2 ppb at day 13. TC bioaccumulation by *L. gibba* L. differed because of the changes in TC concentrations of secondary effluent that flows to the pilot-scale reactor. This study is the first study in the scientific literature which deals with the bioaccumulation of TC and the degradation products by *L. gibba* L. Therefore, the results obtained could not be discussed directly with any study and the discussion was done by other studies about the elimination of some antibiotics in various plants. In general, the results given for various antibiotics in the literature are significantly lower than the results those we found probably because of the different characteristics of the antibiotics, wastewater, or solutions investigated and plants used. Park et al. [35] were reported the concentrations of sulfamethoxazole antibiotics between 0.08 and <2.5 ng/plant g for *Acorus* and <2.5 ng/plant g for *Typha*. They were also reported the concentrations of triclosan as < 10 ng/plant g for both *Acorus* and *Typha*. In the study of Liu et al. [36] which investigated the elimination of veterinary antibiotics from swine wastewater in the vertical flow CWs planted with *hybrid pennisetum*, sulfamethazine content in vegetation was 10 ng/g. Liu et al. [37] were studied the accumulation capacity of *Phragmites australis* which exposed to ciprofloxacin HCl, oxytetracycline HCl, and sulfamethazine at various concentrations (0.1, 1, 10, 100, and 1,000 μg/L) in nutrient solutions. The accumulated amounts of antibiotics in plants those exposed to concentration of 1,000 μg/L were 13,834, 6,901, and 2,047 ng/g dry weight, respectively. Lower amounts were reported for concentration of 0.1 μg/L (345, 165, and 24 ng/g dry weight, respectively). Higher results than aforementioned ones were reported by Migliore et al. [38] who obtained similar results to ours. Migliore et al. [38] were investigated the effect of 100 mg/L flumequine on *L. salicaria* growth. In their study, flumequine contents in *L. salicaria* were 64.9, 31.6, and 15.7 μg/g dry weight (ppm), respectively after exposure times of 10, 20, and 30 days. In the same study, after a 35 day period, flumequine contents in plants were 13.3, 8.7, 0.7, 0.3, and 0.2 μg/g dry weight (ppm), respectively at 5,000, 1,000, 500, 100, and 50 μg/L concentrations of flumequine. Boonsaner and Hawker [39] investigated the mechanism of uptake and accumulation of zwitterionic TCs by *Oryza sativa* L. Their results showed that the TCs were present only in rice roots, but not in the shoots after 15 day (with a test concentration of 50 mg/L TC). Kim et al. [40] reported a whole-body concentration of TC in algae (*P. Subcapitata*) as 7.02 ± 0.95 ng/mg ww when exposed to 1 mg/L TC.

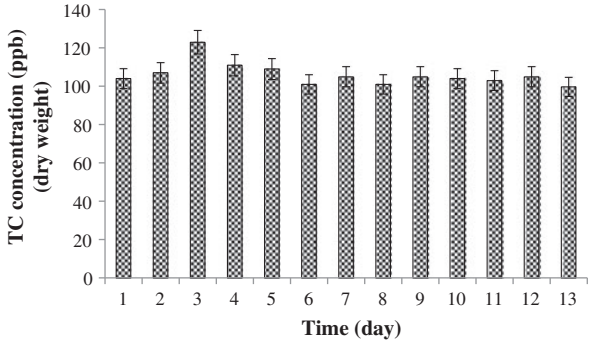

Fig. 3. TC bioaccumulation by *L. gibba* L.

González-Pleiter et al. [41] reported that TC is very toxic for the green alga with an exposure concentration as low as 32 ± 8 μg/L. Lu et al. [42] investigated the effect of TCs on growth of water hyacinth (*Eichhornia crassipes*). It was reported that there was not any visible symptoms of phytotoxicity after 20 day experiment and the water surface was completely covered by the plants after 6 days of the experiment. The dry weight of roots and aerial parts were significantly inhibited by 21 and 10%, respectively, in the presence of high-TCs. Boonsaner and Hawker [39] reported that 100 mg/L TC concentration may be toxic or detrimental to the rice plant (*O. sativa* L.) as judged by wilting, decolorization of leaves or defoliation.

The annual load of the target compound in EMWWTP can be calculated from the equation below:

$$L = Q.C \tag{1}$$

where L is the annual load of the target compound in wastewater (kg/year), Q is the wastewater flow (m^3/year), and C is the concentration of the target compound in wastewater (kg/m^3).

The maximum target compound bioaccumulated by *L. gibba* L. can be calculated from the equation below:

$$L_b = Q.C_p \tag{2}$$

where L_b is the maximum target compound bioaccumulated (kg/year), Q is the wastewater flow

(m^3/year), and C_p is the concentration of the target compound in plant (kg/m^3).

When estimation of the annual loading of TC in EMWWTP is done according to the daily wastewater flow of about 36,000 and 54,000 m^3/d, TC load will be about between 140.6 and 210.9 kg/year, respectively. In view of the circumstances, the maximum TC bioaccumulation by *L. gibba* L. will be estimated as 1,616.2 and 2,424.3 kg/year for wastewater flow of 36,000 and 54,000 m^3/d, respectively.

Bioaccumulated ETC concentrations by *L. gibba* L. are given in Fig. 4.

TCs could be converted into their epimers in the plants, animals, and environment. Conversion from antibiotics to their epimers in plants depends on plant biological activity [36]. The maximum ETC concentration bioaccumulated by *L. gibba* L. was 129 ± 3.2 ppb at days 3, 4 and 12. The minimum ETC concentration was determined as 111 ± 2.2 ppb at day 9. When TC and ETC bioaccumulations of *L. gibba* L. were compared, it could be said that *L. gibba* L. bioaccumulated ETC more efficiently than TC. Annual loading of ETC in EMWWTP is estimated and ETC load is obtained about between 678 and 1,017 kg/year. Thus, the maximum ETC bioaccumulation by *L. gibba* L. will be 1,695.1 and 2,542.5 kg/year for wastewater flow of 36,000 and 54,000 m^3/d, respectively.

EATC concentrations bioaccumulated by *L. gibba* L. are given in Fig. 5.

The highest EATC bioaccumulation by *L. gibba* L. was determined as 42.7 ± 0.5 ppb at day 1 while the lowest EATC bioaccumulation was 12 ± 0.6 ppb at day 10 (Fig. 5). When EATC and TC bioaccumulation was compared, it was seen that uptake rate of TC by *L. gibba* L. was higher. Similarly, when ETC and EATC accumulation was compared it was seen that uptake rate of ETC by *L. gibba* L. was higher. Loading of EATC in EMWWTP is estimated annually and EATC load is calculated about between 174.8 and 262.1 kg/year. The

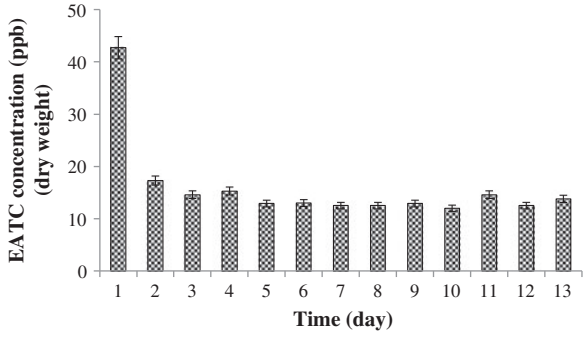

Fig. 5. EATC bioaccumulation by *L. gibba* L.

maximum EATC bioaccumulation by duckweed will be 561.078 and 841.617 kg/year for wastewater flow of 36,000 and 54.000 m^3/d, respectively.

ATC concentrations bioaccumulated by *L. gibba* L. are given in Fig. 6.

Maximum and minimum ATC bioaccumulations were 31.9 ± 0.3 ppb at day 1 and 8.3 ± 0.1 ppb at day 10, respectively (Fig. 6). Concentrations of EATC bioaccumulated were higher than the concentrations of ATC bioaccumulated by *L. gibba* L. for a period of 13 days. It was clear that uptake rates of ETC and TC by *L. gibba* L. were also higher than ATC. ATC was the least uptaken compound by *L. gibba* L. This situation is probably because of the structure of ATC. It is accepted that metabolites are generally less toxic than the parent compound. But, they often have significant activity, as reported for the TC degradation product ATC [26]. ATC had an EC_{50} value for sewage sludge bacteria approximately three times lower than the EC_{50}value of the parent compound TC [4].

According to the daily wastewater flow of about 36,000 and 54,000 m^3/d, the estimation of the loading of ATC in EMWWTP is about between 134 and 201 kg/year, respectively. Therefore, the maximum ATC bioaccumulation by *L. gibba* L. can be estimated

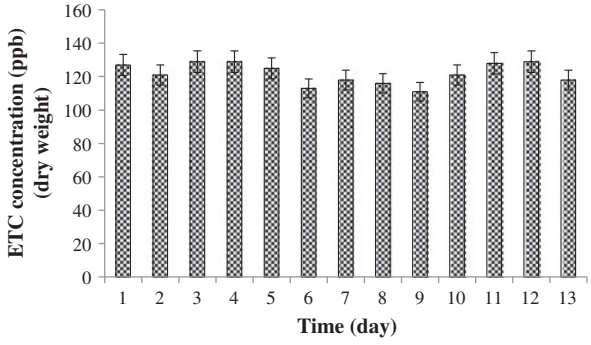

Fig. 4. ETC bioaccumulation by *L. gibba* L.

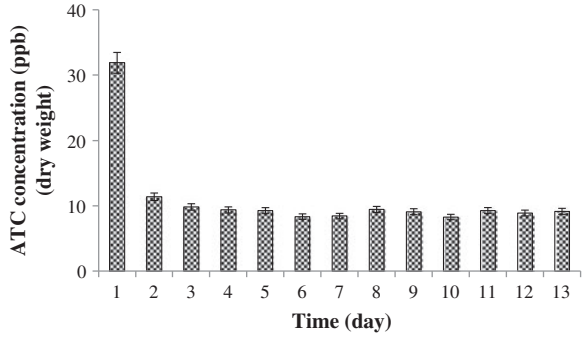

Fig. 6. ATC bioaccumulation by *L. gibba* L.

for wastewater flow of 36,000 and 54,000 m^3/d as 419.2 and 628.8 kg/year, respectively.

As a result, there was significant difference between data obtained. TC and ETC concentrations were siginificantly higher than the concentrations of EATC and ATC in plants. TC and ETC concentrations were about between 100 and 130 ppb while EATC and ATC concentrations were about between 8 and 43 ppb.

4. Conclusions

The amounts of TC and the degradation products bioaccumulated were determined in *L. gibba* L. which was exposed to the secondary effluents of EMWWTP. According to the results of our study, the highest TC, ETC, EATC, and ATC concentrations bioaccumulated by *L. gibba* L. were 123 ± 2.0, 129 ± 3.2, 42.7 ± 0.5, and 31.9 ± 0.3 ppb while the minimum concentrations were 99.7 ± 1.2, 111 ± 2.2, 12 ± 0.6, and 8.3 ± 0.1 ppb, respectively. The order of uptake rate of TC and the degradation products by *L. gibba* L. was determined as ETC > TC > EATC > ATC. As a result, TC and degradation products were efficiently bioaccumulated by *L. gibba* L.

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