Journal of Hazardous Materials 185 (2011) 1187–1197

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Relationships between acute toxicities of para nitrophenol (p-NP) and nitrobenzene (NB) to *Daphnia magna* and *Photobacterium phosphoreum*: Physicochemical properties and metabolites under anaerobic/aerobic sequentials

Delia Teresa Sponza[a,*], Özlem Selcuk Kuscu[b]

[a] *Dokuz Eylül University, Engineering Faculty, Environmental Engineering Department, Buca Kaynaklar Campus, Buca, Izmir, Turkey*
[b] *Department of Environmental Engineering, Engineering and Architecture Faculty, Süleyman Demirel University, Çünür Campus, 32260 Isparta, Turkey*

ARTICLE INFO

Article history:
Received 30 November 2009
Received in revised form
15 September 2010
Accepted 10 October 2010
Available online 15 October 2010

Keywords:
p-Nitrophenol (p-NP)
Nitrobenzene (NB)
Anaerobic migrating blanket reactor (AMBR)
Daphnia magna
Photobacterium phosphoreum

ABSTRACT

In this study, the acute toxicities of nitrobenzene (NB) and para nitrophenol (p-NP) were investigated in a high rate sequential anaerobic migrating blanket (AMBR)/aerobic completely stirred tank reactor (CSTR) using Microtox and *Daphnia magna* tests. After sequential anaerobic and aerobic treatments, the inhibitions in the Microtox bacteria decreased from an initial 78.10–48.20% and 4.00%, respectively, in wastewater containing 40.00 mg/L p-NP. The inhibitions of the influent wastewater containing 60.00 mg/L NB decreased from 72.10% to 45.30% and to 4.00% after anaerobic and aerobic treatment, respectively. The acute toxicity removals were 94% and 93% in the effluent of the whole sequential system, for p-NP and NB, respectively. The acute toxicity in the influent was dependent on the parent NB and p-NP concentrations and ons their physicochemical properties such as hydrophobicity, octanol/water partition coefficient and vapour density for both Microtox bacteria and *Daphnia magna* while the toxicity in the effluent of the anaerobic reactor was strongly dependent on the metabolites of p-NP (p-amino phenol, phenol, NH_4-N) and NB (aniline) for Microtox test. This effluent was not toxic to *Daphnia magna*.

1. Introduction

Nitroaromatic compounds, such as nitrophenol, nitrobenzene and 2,4-Dinitrotoluene (2,4-DNT) are widely used as raw materials or intermediates in the manufacture of explosives, pharmaceuticals, pesticides, pigments, plastics, dyes, wood preservatives, leather and rubber chemicals [1]. Nitrophenol and nitrobenzene were listed by the U.S. Environmental Protection Agency (EPA) as "Priority Pollutants" since they are highly toxic, mutagenic and carcinogenic for the environment and human health [2,3].

Different toxicity tests have been used to determine the effective concentrations of NB and p-NP: Weihua et al., found that the effective concentration affecting 50% (EC_{50}) of the activated sludge was 5.00, 9.50 and 10.70 mg/L for 2-NP, 3-NP and 4-NP, respectively [4]. Razo-Flores et al. reported that nitroaromatics like p-NP and NB were clearly very toxic compounds to methanogens, with IC_{50} values generally ranging from 4.91 to 9.96 mg/L [5]. Donlon et al., found that IC_{50} values (50% inhibition of the acetoclastic methanogens) for p-NP and NB were 8.47 and 9.96 mg/L, respectively [6]. Gemini et al. investigated the biodegradation and detoxification of 40.00 mg/L p-NP by *Rhodococcus wratislaviensis* [7]. 50.00 and 100.00 mg/L p-NP showed high levels of toxicity expressed as EC_{50} for 48 h (2.53 and 1.42 (v/v)), respectively, after aerobic treatment. Ricco et al., evaluated the toxicity of four xenobiotic compounds (3,5-dichlorophenol, formaldehyde, p-nitrophenol and dichloromethane) [8]. The EC_{50} values measured for 4-nitrophenol were 149.90 and 8.76 mg/L, respectively, in activated sludge and *Photobacterium phosphoreum* toxicity tests.

The degradation of wastewaters contaminated with these pollutants is very difficult since they are resistant to conventional treatment processes [9]. In spite of their recalcitrant nature, microorganisms have developed enzymatic mechanisms to degrade nitro compounds under anaerobic conditions. Recent studies showed that NB and p-NP could be efficiently biodegraded with anaerobic technology [10–15]. In this study, it was found that p-NP transformed to p-amino phenol (p-AP) in the AMBR reactor under anaerobic conditions. A large part of the p-AP produced in the AMBR reactor was converted to phenol and ammonia in the same anaerobic reactor. The remaining part of the p-AP was degraded in the aerobic reactor. A complete mineralization of p-AP (E = 100%) was found in the aerobic CSTR reactor. The contribution of the aerobic reactor to the treatment in the sequential reactor system was

* Corresponding author. Tel.: +90 232 4127119; fax: +90 232 4531143.
E-mail address: delya.sponza@deu.edu.tr (D.T. Sponza).

0304-3894/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2010.10.030

not only the removal of p-NP remaining from AMBR, but probably also the degradation of the remaining p-AP to phenol and ammonia. Moreover, this reactor oxidized the ammonia produced in the anaerobic AMBR reactor to nitrite (NO_2-N), and nitrate (NO_3-N) via nitrification. The untreated p-NP remaining from the anaerobic reactor was completely removed in the aerobic reactor [16]. NB was reduced completely to aniline in the anaerobic AMBR reactor. Then aniline was rapidly mineralized in the aerobic process [17]. The behavior of the mixed population indicated that NB was reduced to aniline via hydroxiaminobenzene in the first step, and, in the second, oxidative step, aniline was mineralized to catechol with meta cleavage.

The AMBR was developed as a high rate anaerobic reactor with compartmentalisation, continuous flow and simple design [18]. The AMBR is a flow-through reactor consisting of three to five compartments. It is operated without a hydraulic upflow pattern for mixing and granular sludge development, which eliminates gas–solid separation and the feed distribution systems. The flow is reversed periodically to prevent the biomass accumulating in the final compartment. Because of this approach the biomass is able to migrate with the flow over the horizontal plane of the system. This promotes granulation and eventually gave the AMBR its name. The flow over the horizontal plane of the reactor is reversed once a week. A weekly change in flow direction was chosen to prevent the phase separation, to prevent a pH drop due to total volatile fatty acid (TVFA) accumulation in the initial compartment and to prevent the biomass levels increasing due to anticipated biomass migration between compartments [18]. Hence effluent recycling is not required to control pH in the initial compartments. This is advantageous because effluent recycling changes the compartmentalized reactor from a system that approaches plug flow conditions into a system that approaches completely mixed conditions. The properties aforementioned for the AMBR cause an increase in removal efficiencies [18,19]. High COD concentrations and toxic substances could be treated at short HRTs using AMBRs [10,11,20].

Although there are several studies concerning the toxicities of NB and p-NP [7,8,21], the relationships between removal efficiencies of NB, p-NP, their intermetabolite products and acute toxicities in a high rate sequential anaerobic AMBR/aerobic CSTR reactor system have not been thoroughly investigated. Furthermore, comparative toxicity tests between different trophic levels have not been researched for NB and p-NP in an AMBR reactor system. The effects of metabolites produced from the p-NP and NB on the acute toxicity removals in the sequential AMBR/CSTR reactors have also not been investigated before. Therefore, this study was undertaken to gain insight into these matters.

The aim of this study was to determine (i) the interactions between the removal efficiencies of AMBR and CSTR reactors treating 40.00 mg/L p-NP and 60.00 mg/L NB and their metabolic products and acute toxicities and (ii) the reasons for actual acute toxicities of p-NP and NB to *Daphnia magna* and *Photobacterium phosphoreum*.

2. Material and methods

2.1. Experimental lab-scale reactor and seed

The AMBR reactor consisted of a rectangular tank (interior dimensions: length = 45 cm, height = 20 cm, width = 15 cm) with an active volume of 13.50 L. It was constructed from a stainless steel container, which was divided into three equal compartments by two vertical stainless steel sheets. Round openings with a diameter of 2.50 cm located in the rear of the stainless steel sheets were placed at a height of 0.50 cm from the bottom to create sufficient contact between the biomass and the substrate. The AMBR was provided with six equidistant sampling ports along its length at heights of 5 and 20 cm from the base. Three compartments were mixed equally every 15 min at 60 rpm with a mixer (Heidolph) to ensure gentle mixing. The paddles in the mixer further enhanced the mixing. A minimum of three compartments was required in the AMBR before the flow was reversed in order to maintain enough contact time between microorganisms and influent feed. The flow over the horizontal plane of the reactor was reversed once a week. A weekly change in flow direction was chosen to prevent a pH drop due to TVFA build up in the initial compartment and to prevent the biomass migration between compartments. The influent feed was pumped to the AMBR using a peristaltic pump. The mechanical mixers were operated for 15 min and then they were stopped for 15 min throughout continuous operation of the AMBR reactor to reduce the electrical energy used. The samples were withdrawn from the sample points of the AMBR reactor after stopping the mixing process in the AMBR reactor to prevent biomass losses. Four automatic ball valves, with an internal diameter of 2.54 cm, were used to open and to close the influent and effluent ports. The gas produced was collected via a porthole on the top of the reactor. The operating temperature of the AMBR reactor was maintained at a constant temperatue ($37.00 \pm 1\,^{\circ}C$) by placing the AMBR reactor on a heater. The outlet of the AMBR was connected to a glass U-tube to maintain the hydraulic conditions of the effluent wastewater used as feed in the CSTR reactor. The effluent of the anaerobic AMBR reactor was used as the influent of the aerobic CSTR reactor. The aerobic CSTR reactor was made of stainless steel and consisted of an aerobic (effective volume = 9.00 L) and a settling compartment (effective volume = 1.32 L). The CSTR reactor was continuously fed with the wastewater from the base by a feeding pump. The aerobic reactor was aerated by an air pump and porous diffusers to maintain the DO concentrations between 4.00 and 6.00 mg/L. The effluent wastewater from the aeration tank to the sedimentation tank passed through holes in a plate inclined at 45° to the horizontal axis. Effluent leaving the sedimentation tank was collected in an effluent tank.

Granular anaerobic sludge used as seed in the AMBR reactor was obtained from an up-flow anaerobic sludge blanket reactor at the Pakmaya Yeast Baker Factory in Izmir, Turkey. The total suspended solid (TSS) and volatile suspended solid (VSS) concentrations of the feed sludge were 45.00 and 35.00 g/L, respectively, in the AMBR. The specific methanogenic activity of the feed sludge was measured as 0.34 g COD/g VSS day.

2.2. Composition of synthetic wastewater

Two different nitroorganic compounds (p-NP and NB) were used in this study. Although the influent COD concentration was kept constant at 3000.00 mg/L with glucose, the influent COD concentrations varied between 3000.00 ± 101 mg/L (mean ± SD) and 3447.00 ± 61 mg/L (mean ± SD) with 40.00 mg/L p-NP and 60.00 mg/L NB concentration since p-NP and NB gives additional COD to synthetic wastewater. Glucose was used as carbon substrate to provide the electrons for the reduction of p-NP and NB during continuous operation. In addition, a Vanderbilt mineral salts medium was added to the feed wastewater to improve the growth of methanogens in the AMBR reactor. The Vanderbilt mineral medium was prepared in distilled water by dissolving 0.40 g $MgSO_4$, 0.40 g NH_4Cl, 0.40 g KCl, 0.30 g Na_2S, 0.08 g $(NH_4)_2HPO_4$, 0.05 g $CaCl_2$, 0.04 $FeCl_2$, 0.01 g $CoCl_2$, 0.01 g KI, 0.01 g $Na(PO_3)_6$, 0.50 mg $AlCl_3$, 0.50 mg $MnCl_2$, 0.50 mg $CuCl_2$,0.50 mg $ZnCl_2$, 0.50 mg NH_4VO_3, 0.50 mg $NaMoO_4$, 0.50 mg H_3BO_3, 0.50 mg $NiCl_2$, 0.50 mg $NaWO_4$, 0.50 mg Na_2SeO and 0.01 g cystein per liter [22]. Sodium thioglycollate (0.50 mg/L) was added to the feed in order to maintain the anaerobic conditions necessary for operating the anaerobic AMBR reactor under the desired reducing environ-

Table 1
Operating conditions of the AMBR and AMBR/CSTR reactor system treating p-NP (a) and NB (b).

Runs	Period (days)	HRT	OLR	NP con.	NPLR	SRT	HRT	OLR	NP con.	NPLR	SRT	HRT	SRT
		Anaerobic (AMBR)					Aerobic (CSTR)					AMBR/CSTR	
(a)													
Run 1	35	10.38	0.31	40.00	3.85	340	6.92	0.06	2.51	1.86	20	17.30	360
Run 2	33	5.19	0.60	40.00	7.71	280	3.46	0.12	2.67	1.89	20	8.65	300
Run 3	27	3.40	0.93	40.00	11.76	180	2.37	0.68	2.99	1.99	20	5.77	200
Run 4	34	2.40	1.31	40.00	16.67	151	1.33	0.48	3.00	2.00	20	3.73	171
Run 5	32	1.50	2.14	40.00	26.67	125	1.00	0.43	3.18	2.13	20	2.50	145
Run 6	25	1.00	3.25	40.00	40.00	110	0.67	0.25	3.38	2.56	20	1.67	130
(b)													
Run 1	15	10.38	0.31	60.00	5.78	757	6.92	0.06	3.22	0.35	17.3	17.30	777
Run 2	16	5.19	0.60	60.00	11.56	384	3.46	0.12	4.00	0.40	8.65	8.65	404
Run 3	15	3.40	0.93	60.00	17.14	238	2.37	0.68	5.45	0.47	5.77	5.77	258
Run 4	16	2.40	1.31	60.00	30.00	198	1.33	0.48	5.90	0.50	3.73	3.73	218
Run 5	16	1.50	2.14	60.00	40.00	103	1.00	0.43	6.00	0.57	2.50	2.50	123
Run 6	16	1.00	3.25	60.00	60.00	65	0.67	0.25	6.12	0.62	1.67	1.67	85

OLR: Organic loading rate (kg COD/m^3 day), HRT: Hydraulic retention time (day), SRT: Solid retention time (day), NPLR: p-NP loading rate, NBLR: NB loading rate, NP: p-NP concentration (mg/L), NB con.: NB concentration (mg/L).
OLR = 2.14 kg COD/m^3 day, NPLR = 26.67 g p-NP/m^3 day, NBLR = 40 g NB/m^3 day (operational conditons throughout toxicity studies).

ment [22]. The desired alkalinity and neutral pH were obtained by the addition of 5000.00 mg/L $NaHCO_3$ to the feed media [22].

2.3. Operating conditions

Operating conditions for the AMBR and sequential AMBR/CSTR reactor system treating p-NP and NB are summarized in Table 1. Firstly, the AMBR/CSTR reactor system was operated for 186 days at six different HRTs (17.30, 8.65, 5.77, 3.73, 2.50 and 1.67 days) at a constant 40.00 mg/L p-NP concentration, corresponding to COD and p-NP loading rates varying between 0.31–3.25 and 3.85–40.00 g/m^3 day, respectively [10]. Secondly, the AMBR/CSTR reactor system was operated over 94 days at the same HRTs (17.30, 8.65, 5.77, 3.73, 2.50 and 1.67 days) at constant (60.00 mg/L) NB concentration corresponding to COD and NB loading rates varying between 0.31–3.25 kg/m^3 day and 5.78–60.00 g/m^3 day, respectively [11] (see Table 1). In both runs, the removals of p-NP, NB, the metabolites produced through the sequential anaerobic AMBR/aerobic CSTR reactor system, and the acute toxicities were measured.

2.4. Analytical methods

TSS, VSS and soluble COD were measured following Standard Methods [23]. Biogas production was measured by passing the gas through distilled water containing 2.00% (v/v) H_2SO_4 and 10.00% (w/v) NaCl [24]. Methane production was measured by using distilled water containing 3.00% NaOH (w/v) [5]. In the beginning of the studies the methane content in the biogas was determined by a Dräger (Stuttgart, Germany) Pac-Ex methane gas analyzer and with a GC–MS (HP Agilent -1100). The methane gas of the biogas was measured in a GC–MS. The GC–MS is equipped with a flame ionization detector (FID). Sampling loop volume is 2 mL. The sample is injected on the GC column using N_2 as a carrier gas. A CARBORUS carbon molecular sieve is used in the chromatographic column. CARBORUS consists of the seeds made of solid inert support base, covered by a layer of an active carbon in solid stationary phase. The retention time for CH_4 is less than 1 min when using the chromatographic column filled with CARBORUS with grains size of 0.12–0.16 mm and the thickness of the active carbon solid stationary phase of 0.02 mm. One routine measurement needs a minimum of 4 min. Bicarbonate alkalinity (Bic.Alk.) and TVFA concentrations were measured simultaneously using the titrimetric method as suggested by Anderson and Yang [25].

2.4.1. p-Nitrophenol and p-aminophenol measurements

p-NP was measured using Tris–HCl acid at a wavelength of 400 nm in a UV–vis spectrophotometer [5]. p-AP was determined by reaction with p-methyl aminobenzaldehyde following the procedure suggested by Donlon et al. [6].

2.4.2. NB and aniline measurements

NB and aniline measurements were carried out using a high-pressure liquid chromatography (HPLC) (Agilent-1100) following the method developed by the EPA [26]. The chromatographic conditions employed to determine the nitrobenzene and aniline were as follows: C-18 reverse phase HPLC column (Ace 5C18) was used with dimensions of 25.00 cm × 4.60 mm, 5.00 μm, the mobile phase consisted of 50/50 (v/v) methanol/organic-free reagent water. The flow rate and the injection volume were 1.40 mL/min and 10 μl, respectively. Elution was carried out with an isocratic solvent system consisting of 50% methanol and 50% organic-free reagent water. Detections of NB and aniline were performed at wave lengths of 202 and 234 nm, respectively, using an UV detector.

2.4.3. Measurements of intermediate products

Nitrosobenzene and catechol measurements were carried out using a high-pressure liquid chromatography (HPLC) (Agilent-1100) with a method developed by the EPA [26]. Detection was performed at 202 nm for nitrosobenzene and catechol.

The limit of detection (LOD) is estimated from the mean of the blank, the standard deviation of the blank and t value for a 99% confidence interval factor (t). The limit of quantification (LOQ) was measured by determining the probability density function for normally distributed measurements at the blank, at the LOD and at the LOQ by multiyplying the probability constant with standard deviation of the blank. The LODs for NB and aniline were 0.011 ± 0.000119 and 0.0069 ± 0.0009 mg/L, respectively. The LOQs for NB and aniline were 0.0329 and 0.02094 mg/L, respectively.

2.4.4. Toxicity measurements

2.4.4.1. Microtox. Microtox testing was performed according to the standard procedure recommended by the manufacturer [27]. A specific strain of the marine bacterium, *Photobacterium phosphoreum* (LCK 481), was used in this test to determine the toxicity of p-NP and NB. Reduction in the light intensity of the *Photobacterium phosphoreum* at the 5th, 10th and 30th min was chosen to measure the toxicity [27]. In this test, if the inhibitory effect (H) changes between 0% and 5%, the effect is classified as non-toxic. When it is between

5% and 20%, the effect is possibly toxic, and when the inhibitor effect is between 20% and 90%, the effect is said to be toxic [27].

2.4.4.2. Daphnia magna toxicity test. Toxicity was tested using 24-h old *Daphnia magna* as described in Standard Methods [23]. Analysis was carried out using 5 or 10 daphnids (<24 h old) introduced into the test vessel. A 24 h exposure is generally accepted as standard for a *Daphnia* acute toxicity test. Test vessels were diluted (10, 30, 50, 75, 100%) with water containing 10.00 mg/L KCl, 192.00 mg/L $NaHCO_3$, 53.00 mg/L MgSO4 and 183.00 mg/L $CaSO_4{\cdot}2H_2O$. Results were expressed as the mortality percentage of the *Daphnids*. Immobile animals were considered as dead *Daphnids*.

The toxicity measurements were performed at a total HRT of 2.5 days in the AMBR/CSTR reactor system since at this HRT the most favorable operational conditions were reached for maximum p-NP (E = 100%), NB (E = 100%) and COD (E = 98%) removals. The EC_{50} values given in the manuscript were the data obtained on days 4 and 5 from a 15 day sampling period through the continuous operation of the sequential reactor system.

G_L (dilution ratio) is the proportion of wastewater by volume in the test solution diluted by distilled water. Values of % inhibition or dead organism numbers are plotted on a vertical-scale against the G_L values on the horizontal logarithmic axis. In this plot half of the % inhibition corresponded to the $G_L$value. The EC_{50} values were calculated by dividing the initial p-NP and NB concentrations to the G_L value.

2.4.5. Statistical analysis

Differences in sensitivity scores between microorganisms were determined by a parametric *t*-test. The statistical package used for the analysis was SPSSWIN for Windows. Multiple regression analysis between *y* and *x* variables was also performed using SPSSWIN for Windows. Regression analysis was applied to the experimental data in order to determine the regression coefficient R^2.

ANOVA analysis of variance between experimental data was performed to detect *F* and *p* values. In other words, the ANOVA test was used to test for differences between dependent and independent groups. The comparison between the actual variation of the experimental date averages and standard deviation was expressed in terms of *F* ratio. *F* was equal to "found variation of the date averages/expected variation of the date averages". *p* reported the significance level. The linear correlations between the physicochemical properties of p-NP and NB and the acute toxicities of *Daphnia magna* and *Photobacterium phosphoreum* were determined with ANOVA tests. All results are reported at significance levels of p = 0.01 and p = 0.001.

3. Results and discussion

The removal efficiencies and the intermetabolites produced through the sequential anaerobic/aerobic treatment of of p-NP and NB are given in Tables 2a and 2b [10,11].

3.1. Variations in inhibitions in the AMBR/CSTR reactor system treating p-NP and NB during Microtox acute toxicity test

Acute toxicity was examined using the bioluminescent *Photobacterium phosphoreum* bacterium (LCK 480) in the LUMIStox toxicity test. The inhibition percentage (H) values for 5, 15 and 30 min exposure times obtained for the influent, anaerobic and aerobic reactor effluents in the AMBR/CSTR reactor system (at a total HRT of 2.5 days, p-NP and NB loading rates of 26.67 and 40.00 g/m^3 day, respectively) are given in Tables 3a and 3b. Reductions in light intensities produced by *Photobacterium phosphoreum* were measured versus increasing incubation times (at 5th, 15th

Table 2
Intermediate product concentrations and removal efficiencies obtained from both studies through p-NP (a) and NB (b) degradations in anaerobic, aerobic reactors and AMBR/CSTR reactor system at HRT = 1.5 days, 1 day and total HRT = 2.5 days (N = 3, mean ± SD).

	Anaerobic (AMBR)				Aerobic (CSTR)				AMBR/CSTR reactor system				
	Inf. (mg/L)	EC_{50}[a] Inf. (mg/L)	Eff. (mg/L)	% Eff.	EC_{50}[a] Eff. (mg/L)	Inf. (mg/L)	Eff. (mg/L	% Eff.	EC_{00}[a] Eff. (mg/L)	Inf. (mg/L)	Eff. (mg/L)	% Eff.	EC_{00}[a] Eff. (mg/L)
(a)													
COD	3225.00 ± 23	8.47	248.00 ± 23	92 ± 0.1	10.86	248.00 ± 23	15.00 ± 2.1	90 ± 0.8	0.0 Not toxic	3225.00 ± 23	15.00 ± 2.1	98 ± 1	0.0 Not toxic
p-NP	40.00 ± 2		3.18 ± 0.3	92 ± 0.1		3.18 ± 0.3	0	100 ± 0.1		40.00 ± 2	0 ± 0.01	100 ± 1	
p-AP	0		25.00 ± 1.5	-		8.00 ± 1.5	0	100 ± 0.1		25.00 ± 1.5	0	100 ± 0	
phenol	0		5.00 ± 0.2	-		5.00 ± 1.1	0.20 ± 0.01	96 ± 1.2		5.00 ± 0.2	0.20 ± 0.01	96 ± 1.2	
NH_4-N	0		12.00 ± 0.6	-		12.00 ± 1.3	1.40 ± 0.8	88.00 ± 0.5		12.00 ± 0.6	1.40 ± 0.8	88 ± 0.5	
(b)													
COD	3225.00 ± 24	12.00	333.00 ± 49	92 ± 0.4	24.00	333.00 ± 49	50.00 ± 12	84 ± 2	0.0 Not toxic	3225.00 ± 24	50.00 ± 12	98 ± 1	0.0 Not toxic
NB	60.00 ± 2		0.04 ± 0.01	99 ± 1		0.04 ± 0.01	0	100 ± 0.01		60.00 ± 2	0	100 ± 0.1	
Aniline	0		47.00 ± 2	0		47.00 ± 2	0.50 ± 0.01	99.90 ± 0.01		0	0.50 ± 0.01		
Catechol	0		0	0		0	2.40 ± 0.01	0		0	2.40 ± 0.01		

[a] The EC_{50} values were given based on Microtox test results.

Table 3
Inhibition percentages (H) in the AMBR/CSTR reactor system for Microtox test following p-NP (a) and NB removals.

Inhibition H (%)	Time (min.)	Anaerobic influent	Anaerobic effluent	Aerobic effluent
(a)				
H_5	5	74.60	43.50	4.20
H_{15}	15	75.9	45.40	4.10
H_{30}	30	78.10	48.20	4.10
(b)				
H_5	5	63.40	36.10	4.10
H_{15}	15	66.20	42.20	4.10
H_{30}	30	72.10	45.30	4.10

p-NP con.: 40 mg/L, p-NP loading rate: 26.67 g/m^3 day, total HRT = 2.5 days in whole system.
NB con. = 60 mg/L, NB loading rate = 40.00 g/m^3 day, total HRT = 2.5 days in whole system.

and 30th min) due to increasing toxicities in the influent wastewater. In the Microtox test as the exposure time increased from 5 to 15 and 30 min the acute toxicity of p-NP and NB to *Photobacterium phosphoreum* bacteria increased. For example, the inhibition percentages (H) of the influent wastewater containing 40.00 mg/L of p-NP were measured as 74.60%, 75.90% and 78.10%, respectively, after 5, 15 and 30 min of exposure times, respectively. In this paper only the toxicity values at 30 min exposure time is discussed. Thus the wastewater containing 40.00 mg/L of p-NP was toxic after an exposure time of 30 min according to the toxicity evaluation criteria given for luminescent bacteria ($20 < \%H < 90$). After anaerobic treatment, the effluent inhibition decreased from 78.10% to 48.20% at the end of 30 min exposure as shown in Table 3a. This shows that p-NP was transformed to less toxic intermediate products namely p-AP, phenol and NH_4 from its parent organic "p-NP" (See Table 2). The remaining p-NP in the anaerobic effluent caused a reduced acute toxicity when compared to the influent p-NP. The inhibition percentage (H) decreased from 48.20% to 4.00% in the aerobic reactor effluent throughout p-NP removal (Table 3a). The removal efficiency of inhibition was approximately 92% in the aerobic reactor. The aerobic reactor effluent exhibited no toxicity since the p-NP was removed completely in the aerobic reactor taking into consideration the threshold limits given for Microtox test ($0 < H < 5$). The intermediates remaining from the aerobic reactor (0.20 mg/L phenol and 1.40 mg/L NH_4) also showed no acute toxicity (Table 2). Therefore, an EC_{50} value could not be given since the acute toxicity was assessed as zero (EC_{00}). The sequential anaerobic/aerobic reactor system decreased the toxicity significantly, resulting in a removal of acute toxicity of 94%.

The inhibition percentage (H) of the influent wastewater containing 60.00 mg/L NB was 72.00% after 30 min exposure as shown in Table 3b. The H decreased from 72.00% to 45.30% in the effluent of the anaerobic AMBR reactor resulting in an acute toxicity removal of 37.50%. This showed that NB transformed to a less toxic intermediate, namely aniline, in the anaerobic reactor. The inhibition percentage (H) decreased from 45.30% to below 5.00%, indicating the absence of acute toxicity in the aerobic reactor. The removal efficiency of inhibition was approximately 94% in this reactor. The total acute toxicity removal was 93% in sequential anaerobic/aerobic reactor effluent.

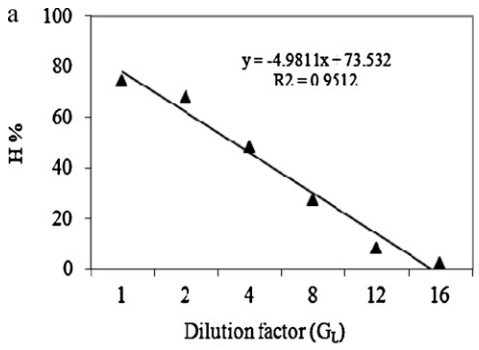
3.2. Variations in EC_{50} values in the AMBR/CSTR reactor system treating p-NP and NB during Microtox acute toxicity test

The influent sample containing 40 mg/L p-NP was diluted at different ratios to determine the EC_{50} value of p-NP. Fig. 1a shows the percentage values of inhibition (H) for the 40 mg/L p-NP versus the G_L values in the influent sample. The G_L value for p-NP, corresponding to half of the percent inhibition in the luminescence of the *Photobacterium phosphoreum*, was calculated as 4.72 from Fig. 1a in the anaerobic reactor influent. The EC_{50} value was calculated to be 8.47 mg/L by dividing the initial p-NP concentration (40.00 mg/L) to the G_L value (Fig. 1a). The linear correlation between inhibition (H%) and G_L was given with an equation $y = -4.9811x + 73.532$ with a R^2 of 0.95 (Fig. 1a). At low G_L values the inhibition of the p-NP increased. The p-NP concentration affecting 50% of the *Photobacterium phosphoreum* (EC_{50} value) was calculated as 10.86 mg/L by dividing the initial p-NP concentration to the G_L value of 3.68 in the anaerobic reactor effluent. The increment in EC_{50} values indicated less toxicity. Under these conditions the inhibition decreased by 62%. The EC_{50} value of influent wastewater causing 50% inhibition in the luminescence of the *Photobacterium phosphoreum* for NB was found as 12.00 mg/L by taking into consideration the influent NB concentration and the dilution ratio G_L of 4.92 (data not shown). Fig. 1b shows the response of *Photobacterium phosphoreum* to the treated wastewater containing NB with a G_L ratio of 2.50 in the anaerobic AMBR reactor effluent. The EC_{50} value increased to 24.00 mg/L in the anaerobic reactor effluent indicating reduced acute toxicity (see Fig. 1b). The linear relationship between G_L and % inhibition was given with an equation of $y = -13.629x + 89.2$ with a R^2 of 0.96.

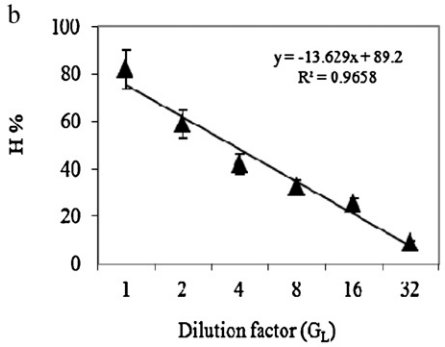
3.3. Daphnia magna acute toxicity test results

The test samples containing 40.00 mg/L p-NP were diluted at 1/1, 1/2, 1/8, 1/16 and 1/24 ratios and ten young *Daphnids* (<24 h old) were added to each test vessel at the beginning of the acute toxicity test ($t = 0$). After 24 h, the EC_{50} value (the p-NP concentration inhibiting the half of the *Daphnia magna* number) of p-NP was found to be 23.52 mg/L in the influent of the AMBR reactor at a G_L value of 1.70 (Fig. 1c). The linear correlation between *Daphnia magna* number and G_L was illustrated with an equation of $y = -1.8x + 8$ and a R^2 of 0.92 (Fig. 1c). At lower G_L values the number of dead *Daphnia magna* increased. No mortalities were detected in *Daphnids* subjected to the effluents of the anaerobic and aerobic reactors, thus confirming the complete elimination of acute toxicity in these samples (data not shown). This shows that the intermediates (p-AP, phenol and NH_4) formed during the treatment of p-NP were not toxic to the *Daphnids* in the anaerobic and aerobic reactor effluents (data not shown). Therefore, an EC_{50} value cannot be given since all *Daphnids* survived in both anaerobic and anaerobic reactor effluents.

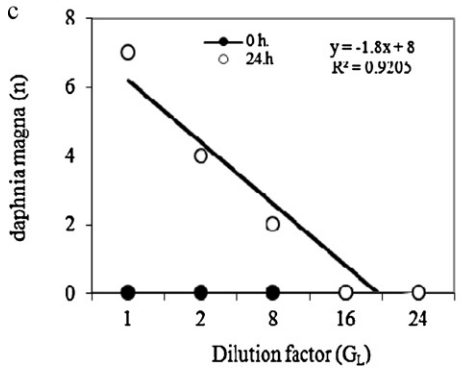
A wastewater sample containing 60.00 mg/L NB was diluted at ratios varying between 1, 1/2, 1/3, 1/4 and 1/8 to calculate the EC_{50} value in the influent. The G_L value was found to be 1.87 in the influent of AMBR reactor for *Daphnia magna*. The EC_{50} value of 60.00 mg/L NB was calculated as 32.08 mg/L for the influent sample (Fig. 1d). Since no mortalities were found in the effluent of anaerobic and aerobic reactors for undiluted samples for *Daphnids* an EC_{50} value could not be ascertained (data not shown). Therefore, an EC_{50} value cannot be given since all Daphnids survived in both anaerobic and anaerobic reactor effluents.



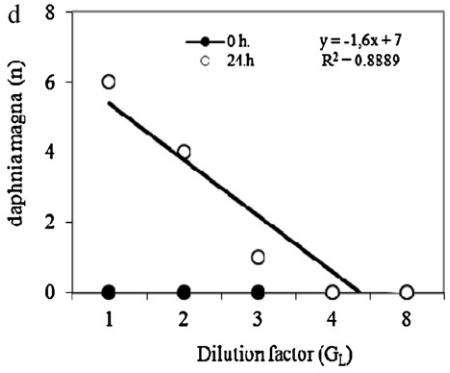
G_L value=4.72; EC_{50} value for influent of AMBR treating p-NP (after 30 min exposure) in the Microtox acute toxicity test (EC_{50} = 8.47 mg/L).



G_L value =2.5; EC_{50} value for the AMBR effluent treating NB (after 30 min exposure) in Microtox acute toxicity test (EC_{50} = 24.00 mg/L).



G_L value =1.70; EC_{50} for the influent of AMBR treating p-NP in *Daphnia magna* acute toxicity test (after 24 h exposure time) (EC_{50} = 23.52 mg/L).



G_L =1.87; EC_{50} for the influent of AMBR treating NB in *Daphnia magna* acute toxicity test (after 24 h exposure time) (EC_{50} = 32.08 mg/L).

Fig. 1. G_L values measured from plots of inhibitions, number of *Daphnia magna* surviving and EC_{50} values in the influent and effluent samples using the Microtox and *Daphnia magna* acute toxicity tests throughout p-NP and NB removals. (a) G_L value = 4.72; EC_{50} value for influent of AMBR treating p-NP (after 30 min exposure) in the Microtox acute toxicity test (EC_{50} = 8.47 mg/L). (b) G_L value = 2.5; EC_{50} value for the AMBR effluent treating NB (after 30 min exposure) in Microtox acute toxicity test (EC_{50} = 24.00 mg/L). (c) G_L value = 1.70; EC_{50} for the influent of AMBR treating p-NP in *Daphnia magna* acute toxicity test (after 24 h exposure time) (EC_{50} = 23.52 mg/L). (d) G_L = 1.87; EC_{50} for the influent of AMBR treating NB in *Daphnia magna* acute toxicity test (after 24 h exposure time) (EC_{50} = 32.08 mg/L).

3.4. Toxicity evaluation

In this study, the EC_{50} values of the intermediates (25.00 mg/L p-aminophenol and 47.00 mg/L aniline) were found as 26.17 and 39.13 mg/L, respectively using Microtox test (Fig. 2a and b), in the anaerobic reactor effluent. This indicated that the EC_{50} values of the parent nitroaromatic compounds were significantly lower (EC_{50} = 8.47 and 12.00 mg/L for p-NP and NB, respectively) than that the EC_{50} values of the intermetabolite products in the anaerobic reactor effluents. This shows that the toxicity decreased in the anaerobic reactor effluent, since the acute toxicities originating from the metabolites in question were significantly decreased. In the present study it was found that the parent nitroorganic compounds were approximately two and three fold more toxic than their corresponding intermetabolite products. The inhibition presumably decreased due to intracellular reduction of the $–NO_2$ group in p-NP to the less toxic $–NH_2$ group in p-AP. Similarly, the benzene bonds present in NB and its metabolites, aniline and cathechol, were cleaved. Therefore the degraded nitroorganics exhibited less toxicity. Although the influent wastewater containing 40.00 mg/L p-NP and 60.00 mg/L NB exhibited toxicities both in Microtox and *Daphnia magna* tests, the effluent of the anaerobic reactor exhibited only slight inhibition to the Microtox test bacteria for both p-NP and NB. No acute toxicity was obtained in the effluent of the anaerobic rector for *Daphnia magna*.

3.5. p-NP and NB toxicities and interspecies correlation

Few investigators reported the acute toxicity of NB and p-NP to Microtox and *Daphnia magna* test organisms (see Table 4). Holdway et al. observed the acute p-NP toxicity to *Daphnia magna* in different polluted water discharges containing nitrogenous organics [28]. Yen et al. reviewed the literature on the Microtox responses to pollution-induced environmental changes by anthropogenic and industrial impacts containing NB [29]. Hung and Masen showed that Microtox organisms exhibited high sensitivity to nitroorganic compounds and nitroorganic discharges [30]. Furthermore, Lei et al. mentioned that microtox organisms were equally sensitive to pesticides, polycyclic aromatic hydrocarbons, p-NP and NB in sediment toxicity assays [31]. Lin et al. showed that Microtox and *D. magna* bioassays were capable of detecting the toxicity of NB and p-NP [32].

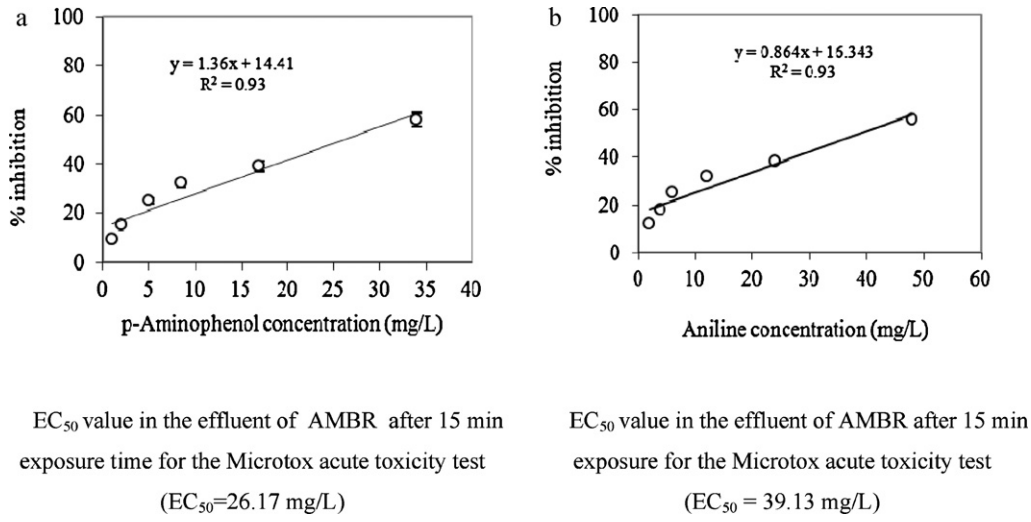


Fig. 2. The EC_{50} values of anaerobic metabolite products through p-NP degradation (p-aminophenol, EC_{50} = 26.17 mg/L) (a) and NB degradation (aniline, EC_{50} = 39.13 mg/L) (b) in Microtox acute toxicity test. (a) EC_{50} value in the effluent of AMBR after 15 min exposure time for the Microtox acute toxicity test (EC_{50} = 26.17 mg/L). (b) EC_{50} value in the effluent of AMBR after 15 min exposure for the Microtox acute toxicity test (EC_{50} = 39.13 mg/L).

In control tests, without p-NP and NB the *Dapnids* and *Photobacterium phosphoreum* bacteria remained active throughout the test period. The EC_{50} and their 95% confidence limits of p-NP and NB were arranged for influent, anaerobic and aerobic reactor effluents for Microtox and *Daphnia magna* tests (Tables 5a and 5b). The results indicated that p-NP was more toxic than NB in the influent and effluent samples of the AMBR reactor system when Microtox was used as test organism. Similarly the p-NP was found to be more toxic than NB in the influent of the AMBR reactor when *Daphnia magna* was used as test organism. In this study, the EC_{50} values suggested that the decreasing rank order toxicity of nitrogenous componds was as follows: p-NP > NB (Tables 5a and 5b).

3.6. Sensitivity ranking in photobacterium phosphoreum and Daphnia magna

To explain the sensitivity of acute toxicity test results based on *Daphnia magna* and *Photobacterium phosphoreum* a table was constructed ranking the samples in order of acute toxicity. Sensitivity ranking indicates the sum of toxicity responses of every organism used in acute toxicity tests. A score of "1" was assigned to the most sensitive test for each sample down to 2 for the least sensitive organism representing two trophic levels which were classified according to the test results. The comparison of toxicity response and sensitivity ranking was assessed in Tables 5a and 5b for p-NP and NB, respectively. The acute toxicity classification of an effluent should always be based on the results of testing all trophic levels at least once.

The sensitivity scores were 10 and 20 in *Photobacterium phosphoreum* and *Daphnia magna*, respectively, through continuous sequential operation of the AMBR/CSTR reactor system during 15 sampling cases. The EC_{50} values measured for *Daphnia magna* differed significantly from *Photobacterium phosphoreum* in the decline of sensitivity scores and exhibited lower mortalities in the influents (*t*-test statistic 8.69, $p \leq 0.05$). The *t*- test statistics showed that *Daphnia magna* has higher EC_{50} values and lower sensitivity scores than *Photobacterium phosphoreum.* The *photobacterium phosphoreum* and *Daphnia magna* had different response to toxicity and mortalities (*t*-test statistic = 8.71, $p = 0.10$) and this difference was significant (*t*-test statistic = 11.76, $p = 0.05$). The *Photobacterium phosphoreum* bacteria had lower EC_{50} values than *Daphnia magna* and these differences were significant (*t*-test statistics = 16.54, $p < 0.10$). The sensitivity score of *Daphnia magna* was higher than *Photobacterium phosphoreum* and the difference was significant (*t*-test statistic = 12.94, $p \leq 0.05$). According to the statistical analysis results, in a sample comparison of the sensitivity between the two trophic levels, the Microtox test seems to be less sensitive than the *Daphnia magna* test. The literature data containing the acute toxicity values of p-NP and NB with *Daphnia magna* and *Photobacterium phosphoreum* showed that p-NP is more toxic than NB and microtox test is more sensitive than water flea (see Table 4). In other words, the *Daphnia magna* was found to be resistant compared to *Photobacterium phosphoreum*. From the acute toxicity tests it can be seen that different organisms were affected differently by the influent and effluent wastewaters. It should be pointed out, however, that the *Photobacterium phosphoreum* bacteria, and water flea tests are reference standards used world-wide for toxicity testing and represent one of the trophic level tests required in toxicity evaluation.

To verify the relationships betwen the acute toxicity data of two organism the EC_{50} values of *Photobacterium phosphoreum* were correlated to those of *Daphnia magna* by statistical analysis using

Table 4
Toxicity of p-NP and NB to *Photobacterium phosphoreum* and *Daphnia magna*.

p-NP		NB		Reference
D.magna	*P.Phosphoreum*	*D. magna*	*P. phosphoreum*	
–	EC_{50} = 0.81	–	EC_{50} = 3.81	Holdway et al. [28]
EC_{50} = 2.25	–	EC_{50} = 13.90	–	Yen et al. [29]
EC_{50} = 4.94	EC_{50} = 0.56	EC_{50} = 2.99	EC_{50} = 2.98	Hung and Masen [30]
EC_{50} = 7.89	EC_{50} = 1.72	EC_{50} = 3.89	EC_{50} = 1.06	Lei [31]
EC_{50} = 9.82	EC_{50} = 2.45	EC_{50} = 2.11	EC_{50} = 1.19	Lin et al. [32]

EC_{50} value was given as mg/L.

Table 5
Acute toxicity test results in the influent and sensitivity ranking in the influent, effluent of the AMBR and effluent of CSTR reactor through 15 day samplings at a HRT of 2.5 days at 40.00 mg/L influent p-NP (a) and 60.00 mg/L NB (b).

	Assessment of sensitivity							
	Microtox (*Photobacterium phosphoreum*)				*Daphnia magna*			
Days	I SR	ANE SR	AE SR	I-EC_{50} (confidence limits, mg/L)	I SR	ANE SR	AE SR	I-EC_{50} (confidence limits, mg/L)
(a)								
1	1	1	–	8.46 (8.45–8.47)	2	–	–	23.99 (23.99–24.00)
2	1	1	–	8.47 (8.46–8.48)	2	–	–	23.98 (23.98–24.00)
4	1	1	–	8.47 (8.46–8.48)	2	–	–	24.00 (23.99–24.01)
5	1	1	–	8.47 (8.46–8.48)	2	–	–	24.00 (23.98–24.01)
7	1	1	–	8.47 (8.46–8.48)	2	–	–	24.00 (23.99–24.02)
9	1	1	–	8.47 (8.46–8.48)	2	–	–	23.98 (23.96–24.00)
10	1	1	–	8.47 (8.46–8.48)	2	–	–	24.01 (24.00–24.03)
12	1	1	–	8.46 (8.45–8.47)	2	–	–	23.99 (23.97–24.00)
14	1	1	–	8.46 (8.45–8.47)	2	–	–	24.01 (23.99–24.02)
15	1	1	–	8.46 (8.45–8.47)	2	–	–	24.02 (24.00–24.03)
Sum	10	10			20			
(b)								
1	1	1	–	12.00 (11.99–12.01)	2	–	–	48.00 (47.99–48.01)
2	1	1	–	11.99 (11.99–12.00)	2	–	–	47.99 (47.99–48.01)
4	1	1	–	12.00 (12.00–12.01)	2	–	–	48.00 (47.99–48.01)
5	1	1	–	12.00 (11.99–12.01)	2	–	–	48.01 (47.99–48.01)
7	1	1	–	12.00 (11.99–12.01)	2	–	–	47.99 (47.99–48.00)
9	1	1	–	11.99 (11.98–12.01)	2	–	–	47.99 (47.99–48.00)
10	1	1	–	12.01 (11.99–12.00)	2	–	–	48.01 (48.00–48.02)
12	1	1	–	11.99 (11.98–12.00)	2	–	–	48.00 (47.99–48.01)
14	1	1	–	12.00 (12.00–12.01)	2	–	–	48.01 (47.99–48.01)
15	1	1	–	11.98 (11.98–12.02)	2	–	–	47.98 (47.98–48.01)
Sum	10	10			20			

No acute toxicity, I SR: sensitivity ranking in the influent of AMBR reactor; ANE SR: sensitivity ranking in the effluent of AMBR reactor; AE SR: Sensitivity ranking in the aerobic reactor effluent; I-EC_{50}: Acute toxicity in influent.

their EC_{50} values in the influent of AMBR reactor (Fig. 3a). It was found that the EC_{50} values differed in both test organisms for the influent samples. The coefficient of correlation and *t*-test statistics ($r^2 = 0.12$, $p \leq 0.01$, *t*-test statistics = 7.6, $n = 15$) did not show a significant linear relathionship between the acute toxicities of p-NP to *Photobacterium phosphoreum* and to *Daphnia magna* in the influent samples. This result verifies the sensitivity of *Photobacterium phosphoreum* and the resistance of *Daphnia magna* to p-NP by taken into consideration the data obtained above with sensitivity scores. Similarly, the relationships between the acute toxicity datas of NB to *Photobacterium phosphoreum* and to *Daphnia magna* was determined (Fig. 3b) ($r^2 = 0.35$; $p \leq 0.01$, test statistic = 5.89, $n = 15$) in the influent samples of the AMBR. A significant correlation was not found between the acute toxicities of the two organisms to NB. In both cases *Photobacterium phosphoreum* was always more sensitive to acute toxicity than *Daphnia magna* for both p-NP and NB in the influent samples. Furthermore, a relationships between the acute toxicities of the two organisms was not determined in the effluent of the AMBR reactor since the *Daphnia magna* test did not exhibit toxicity to the treated samples.

As aforementioned, the test organisms used to determine the acute toxicity had different EC_{50} values and the two sets of data did not show a significant correlation through the 15 days sampling period (Tables 5a and 5b). The EC_{50} value of 40.00 mg/L p-NP increased from 8.47 to 24.00 mg/L as the trophic level increased from bacteria in Microtox test to water flea in *Daphnia magna* test in the influent samples. This indicated that the water fleas exhibited less acute toxicity to 40.00 mg/L p-NP. The maximum difference in EC_{50} values for *Daphnia magna* and Microtox corresponded to a factor of 2.83 in the influent samples for p-NP. For 15 out of 15 samplings the *Daphnia magna* EC_{50} values were higher than the *Photobacterium* in the influent samples for p-NP (Table 5a). Similarly the EC_{50} values increased from 12.00 to 24.00 mg/L (the acute toxicity decreased) as the trophic level increased from *Photobac-*

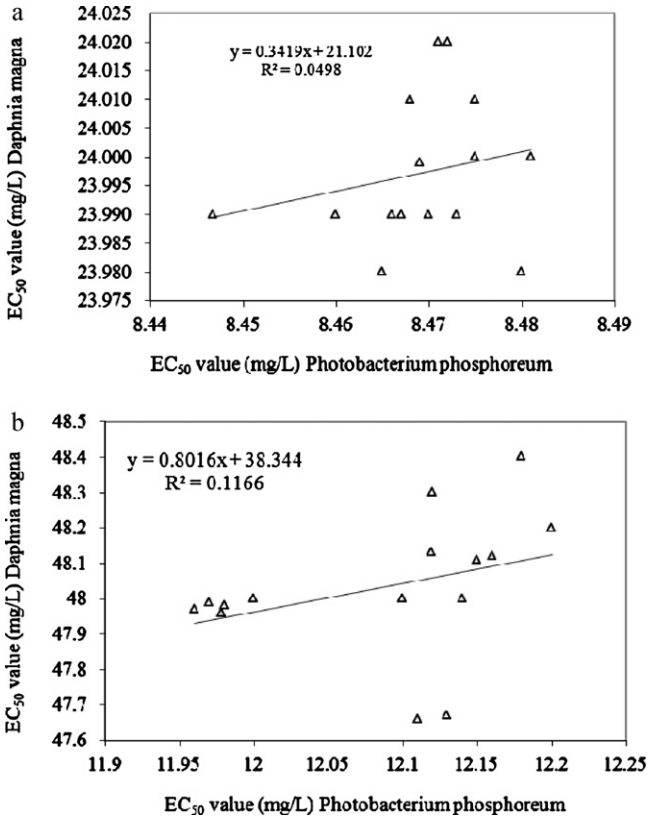


Fig. 3. (a) Correlation between EC_{50} values of *Photobacterium phosphoreum* and *D. magna* at an influent p-NP concentration of 40.00 mg/L in the influent of AMBR reactor ($n = 15$, mean ± SD). (b) Correlation between EC_{50} values of *Photobacterium phosphoreum* and *D. magna* at an influent NB concentration of 60.00 mg/L in the influent of AMBR reactor ($n = 15$, mean ± SD).

terium bacteria in Microtox test to water flea in *Daphnia magna* test in the influent samples for the AMBR reactor treating 60.00 mg/L NB. The acute toxicities decreased in the effluent of anaerobic AMBR reactor by an increase in the EC_{50} values from 8.47 to 10.86 mg/L and from 12.00 to 24.00 mg/L in Microtox test for p-NP and NB, respectively. The difference in sensitivity between the two organisms (*Photobacterium phosphoreum* and *Daphnia magna*) was 4.00 times in the influent samples for NB (Table 2). The differences in test sensitivities can be attributed to the differences in the responses of the two different trophic organisms to different nitroorganic compounds and to their metabolites. As a conclusion, this study showed that the Microtox acute toxicity test is more sensitive than that *Daphnia magna* by factors of 2.83 and 4.00 for p-NP and NB, respectively, in the influent samples.

No toxicity response was observed in the effluent of AMBR reactor in *Daphnia magna* acute toxicity tests. This shows that *Daphnia magna* is resistant to the metabolites produced from the anaerobic degradation of p-NP and NB. The sensitivity of the Microtox test is entirely predictible since the benzene and phenol moieties in the NB and p-NP can alter the cell membranes of the *Photobacterium phosphoreum* bacteria resulting in inhibition of these bacteria [33]. Similarly, Shimizu et al. and Naci et al. showed that the Microtox test was more sensitive to some nitro-organics such as 2,4-nitropentanedione and 2-methyl-2,4-nitro pentanediol [34,35].

The p-NP concentration in the effluent of the AMBR reactor was measured as 3.18 mg/L (E = 92%) at an initial p-NP concentration of 40.00 mg/L. If 40.00 mg/L of p-NP were to be released into the receiving bodies, it would cause 78.10% inhibition to the organisms. However, the effluent of the AMBR/CSTR reactor treating 40.00 mg/L p-NP was not toxic. If 60 mg/L NB were to be released into the receiving ecosystem, it would cause 72.00% inhibition to the test organisms. The metabolites produced through anaerobic stage are metabolized in the aerobic reactor and therefore the effluent of the AMBR/CSTR reactor treating 60.00 mg/L NB was not toxic. Therefore, the effluent of the sequential anaerobic/aerobic reactor system can be released into the receiving water without cause for concern since the acute toxicity level is well below the threshold limits given for toxicity evaluation.

3.7. Correlation of acute toxicities with physicochemical properties of NB and p-NP in the influent of the AMBR reactor

In this study, specifically the relative NB and p-NP acute toxicities and their physicochemical characteristics were correlated with linear regression for both *Photobacterium phosphoreum* and *Daphnia magna* (Tables 6a and 6b). The physicochemical properties of p-NP and NB was provided by the literature data [36,37]. The octanol/water partition coefficient ($\log K_{ow}$) has been proposed as a method for evaluating the toxicity of chemicals in aquatic systems. Hydrophobicity expressed as the logarithm of the 1-octanol/water partition coefficient ($\log K_{ow}$) has been frequently used to predict baseline acute toxicity caused by unspecific membrane irritation.The higher the K_{ow} values the stronger the hydrophobicity and the easier the compound is bioconcentrated in an organism [32]. Moreover, the toxic effect of chemicals is probably an accumulative result of the biological interactions associated with different modes and mechanisms. Hence, $\log K_{ow}$ is often insufficient to account for the varying toxicity of the chemicals [32], In this study, it is clear that with *Photobacterium phosphoreum* a high correlation would be found between $\log K_{ow}$ and acute toxicity for the substituted NB and p-NP compounds, including a wide variety of mechanisms of toxic action in both *Photobacterium phosphoreum* and *Daphnia magna* (see Tables 6a and 6b). These chemicals pass through the bio-membrane of the organisms and accumulate before being uptaken due to the hydrophobicity of NB and p-NP themselves. In our previous study, accumulation of NB in *Photobacterium phosphreum* and *Daphnia magna* depended on a complicated kinetic process, including uptake, and metabolism [38]. He et al. [39] also reported that NB with relatively low K_{ow} and high solubility was partially adsorbed on biota. Henry's law constant characterizes the relative amount of a substrate that will enter the organism, hence this parameter has been investigated to identify the uptake of a organic substance by an organism. Henry's law constant was strongly associated with the NB and p-NP transfer rates from the test vessel to the *Photobacterium phosphoreum* and *Daphnia magna* in the influent samples. A significant correlation between Henry's law constants of both nitrogenous compounds and acute toxicities in *Photobacterium phosphoreum* and *Daphnia magna* was observed (see Tables 6a and 6b). Increasing vapor pressure results in more NB diffusing into bacteria from the wastewater samples. It was suggested that the Henry's law constant and vapor pressure had influenced the rate of degradation for some of the aromatic compounds. This result suggests that the acute toxicities of studied compounds to *Daphnia magna* and *Photobacterium phosphoreum* depended on their hydrophobicity. Similarly, significant correlations between water solubility, relative vapour density and relative density of the vapor/air mixture at 20 °C were observed over a wide range of chosen parameters (Tables 6a and 6b).

In this study the acute toxicity is the result of two steps: the penetration of p-NP and NB through the bio-membrane, thus reaching the target site of action, and the interaction of chemicals with the biomacromolecules of the target sites. Generally, the penetration can be considered as a hydrophobicity-controlled process and the penetration ability of chemicals can be modeled by the logarithm of 1-octanol/water partition coefficient.

The toxicity processes involve both toxicant bio-uptake to the sites of interaction and interaction between the chemical and those sites such as Henry's law constant, water solubility, relative vapour density, relative density of the vapor/air mixture, vapor pressure and molecular weigths. Any factor which affects the bio-uptake and the interaction will have an impact on the acute toxicity. Significant relationships have been obtained between the acute toxicity (EC_{50} values in *Daphnia magna* and *Vibrio fisheri*) and the physicochemical properties of NB and p-NP. However, among these relationships it was found that the acute toxicity depends more on solubilities, Henry's law constant, vapor pressure and octanol water coefficient of the substituted nitroorganics with higher coefficient of correlations (R^2). In other words the dominant descriptors for acute toxicity are hydrophobicity, Henry's law constant, vapor pressure and solubility of p-NP and NB.

3.8. Correlations between acute toxicity, reactor performances and intermetabolites

ANOVA test statistics showed that the correlation between y dependent (EC_{50}% values for acute toxicities) and x independent (COD, p-NP and NB concentrations) variables is high, the relationship is strong and significant in the influent of the anerobic reactor for both *Daphnia magna* and *Photobacterium phosphoreum,* respectively (R^2 = 0.91, F = 79.13, p = 0.001; R^2 = 0.95, F = 84.56, p = 0.001). The ANOVA test statistics showed that the regression between y dependent (EC_{50}% values for acute toxicities) and x independent (p-NP removals) variables is low, the relationship is weak and not significant (R^2 = 0.47, F = 3.11, p = 0.001) in the anerobic reactor effluent for *Photobacterium phospherium* through p-NP degradation. Since the p-NP was removed with high yields (p-NP = 3.18 mg/L in the effluent of the AMBR reactor) the acute toxicity was not related to p-NP concentration in the effluent of the AMBR reactor. The toxicity of *Photobacterium phosphoreum* in the effluent of the AMBR reactor originated from the 25.00 mg/L

Table 6
Correlations between EC_{50} values and physicochemical properties of p-NP and NB in Microtox test (a) and *Daphnia magna* (b) for influent wastewater ($n = 15$).

Parameter	NB			p-NP		
	NB	R^2	p	p-NP	R^2	p
(a)						
Boiling point (°C)	211.00	0.28	NS	279.00	0.05	$p = 0.1$
Melting point (°C)	5.00	0.23	NS	111.00	0.11	$p = 0.1$
Relative density (g/cm^3)	1.20	0.19	NS	1.50	0.09	$p = 0.1$
Henry'slaw constant (atm m^3/mol)	2.40×10^{-5}	0.89	$p = 0.01$	1.99×10^{-4}	0.98	$p = 0.01$
Solubility in water (g/100 mL)	0.20	0.87	$p = 0.01$	1.24	0.91	$p = 0.01$
Vapor pressure (mmHg)	0.25	0.79	$p = 0.01$	0.32	0.75	$p = 0.01$
Relative vapour density (kg/m^3)	4.20	0.76	$p = 0.01$	3.50	0.67	$p = 0.01$
Relative density of the vap./air-mix. at 20 °C	1.00	0.86	$p = 0.01$	1.21	0.73	$p = 0.01$
Flash point:	88.00	0.23	NS	103.00	0.05	NS
Explosive limits vol % in air:	1.80–4.01	0.14	NS	2.00–4.00	0.34	NS
Oct./wat. partition coef. (log P_{ow})	1.86	0.84	$p = 0.01$	1.91	0.89	$p = 0.01$
Molecular mass (g)	123.10 ($C_6H_5NO_2$)	0.75	$p = 0.01$	139.10 ($C_6H_5NO_3$)	0.67	$p = 0.01$
(b)						
Boiling point (°C)	211.00	0.18	NS	279	0.05	$p = 0.1$
Melting point (°C)	5.00	0.19	NS	111-116	0.08	$p = 0.1$
Relative density (g/cm^3)	1.20	0.19	NS	1.50	0.09	$p = 0.1$
Henry's law constant (atm m^3/mol)	2.40×10^{-5}	0.67	$p = 0.01$	1.99×10^{-4}	0.78	$p = 0.01$
Solubility in water (g/100 mL)	0.20	0.77	$p = 0.01$	1.24	0.81	$p = 0.01$
Vapor pressure (mmHg)	0.25	0.70	$p = 0.01$	0.32	0.67	$p = 0.01$
Relative vapour density (kg/m^3)	4.20	0.70	$p = 0.01$	3.5	0.60	$p = 0.01$
Relative density of the vap./air-mix. at 20 °C	1.00	0.83	$p = 0.01$	1.21	0.70	$p = 0.01$
Flash point:	88.00	0.13	NS	103	0.02	$p = 0.1$
Explosive limits vol % in air:	1.80–4.02	0.10	NS	2.00–4.00	0.20	$p = 0.1$
Oct./wat. partition coef. as log P_{ow}	1.86	0.80	$p = 0.01$	1.91	0.82	$p = 0.01$
Molecular mass (g)	123.10 ($C_6H_5NO_3$)	0.75	$p = 0.01$	139.10 ($C_6H_5NO_2$)	0.67	$p = 0.01$

NS: not significant; R^2: correlation coefficient; p. statistical significance.

p-AP, 5.00 mg/L phenol and 12.00 mg/L NH_4-N, which are the anerobic metabolites of p-NP (See Table 2). A significant correlation between acute toxicity and intermetabolites in question was observed ($R^2 = 0.92$, $F = 39.06$, $p = 0.001$). Furthermore, 248.00 mg/L COD concentrations in the effluent of the anaerobic reactor can be contributed to the acute toxicity since this COD might contain some refractory and inert organics. Similarly, the regression between EC_{50}% values for acute toxicities and NB removal efficiencies is low, the relationship is weak and not significant ($R^2 = 0.47$, $F = 9.07$, $p = 0.001$) in the anerobic reactor effluent for *P. phosphoreum* through NB degradation. This showed that the acute toxicity is not dependent on the NB concentration in the effluent of AMBR reactor since this NB concentration is low (0.04 mg/L). The acute toxicity originated from the anaerobic metabolites of NB (47.00 mg/L aniline) and from the COD (333.00 mg/L) in the effluent of AMBR reactor. The inert fraction of COD or some other metabolites measured as COD could have led to acute toxicity in the effluent of the AMBR.

The correlation between acute toxicities and p-AP, phenol and ammonia concentrations is high, the relationship is strong and significant ($R^2 = 0.83$, $F = 12.04$, $p = 0.01$) while the correlation between acute toxicities and COD concentrations in the anerobic reactor effluent is high but not significant for *Photobacterium phosphoreum* ($R^2 = 0.86$, $F = 19.03$, $p = 0.01$). Similarly, the correlation between acute toxicities and aniline concentrations is high, the relationship is strong and significant ($R^2 = 0.86$, $F = 1.03$, $p = 0.01$) in the anerobic reactor effluent while the correlation between acute toxicity and COD concentration is high but not significant for *Photobacterium phosphoreum* ($R^2 = 0.83$, $F = 1.20$, $p = 0.01$).

From these results we conclude that acute toxicity depends strongly on the corresponding intermetabolites of the p-NP and NB in the anaerobic effluent when *Photobacterium phosphoreum* is used as the test organism. Acute toxicity was not observed to be strongly dependent on either the removal of nitroorganic compounds or on their metabolites when *Daphnia magna* was used as the test organism.

4. Conclusions

The results of this study lead us to conclude that the acute toxicities of the p-NP and NB could be reduced significantly with the sequential anaerobic AMBR and aerobic CSTR reactor system. The increment in EC_{50} values indicated the reduction in toxicity from the influent of the AMBR reactor to aerobic reactor effluent. The total sensitivity scores were 10 and 20 for *Photobacterium phosphoreum* and *Daphnia magna* through 15 day sampling, respectively, indicating that *Photobacterium phosphoreum* is more sensitive than *Daphnia magna*. A strong correlation between EC_{50} values and some physicocehemical properties of p-NP and NB in the influent samples showed that the initial toxicity was dependent on the nitroorganics in question and on some of the physicochemical properties in both test organisms. Therefore, it may be concluded that the hydrophobicity, octanol/water partition coefficient and vapour density are the main controlling factors of the toxicity of nitroorganics to the both test organism in the influent. However, the toxicity in the anaerobic effluent which originated from the metabolites of p-NP and NB only appeared in the Microtox tests while no toxicity response was observed in the water flea test.

Acknowledgments

This study was funded by the Turkish National Scientific and Technical Research Foundation (TUBITAK), grant number 106 Y 360. The authors are grateful to TUBITAK for the financial support.

References

[1] J. Ye, A. Singh, W. Owen, Biodegradation of nitroaromatics and other nitrogen-containing xenobiotics, W. J. Microbiol. Biotechnol. 20 (2004) 117–135.
[2] WHO, World Health Organization, Environmental Health Criteria 230, Nitrobenzene. The International Labour Organization and the World Health Organization, and produced within the framework of the Inter-Organization Programme for the Sound Management of Chemicals, 2003, Italy.
[3] EPA, US, Environmental Protection Agency, Nitrobenzene Final Report Final Compilation of Reviewer Comments And Responses to Charge Questions Pre-

pared for Integrated Risk Information System (IRIS) Program, 2007, No: DW-89939822-01-0, USA.
[4] S. Weihua, Z. Zheng, A.S. Rami, Z. Tao, H. Desheng, Degradation and detoxification of aqueous nitrophenol solutions by electron beam irradiation, Rad. Phys. Chem. 65 (2002) 559–563.
[5] E. Razo-Flores, B. Donlon, G. Lettinga, J. Field, Biotransformation and biodegradation of N-substituted aromatics in methanogenic granular sludge, FEMS Microbiol. Rev. 20 (3–4) (1997) 525–538.
[6] B. Donlon, E. Razo-Flores, A.J. Field, G. Lettinga, Toxicity of N-substituted aromatics to acetoclastic methanogenic activity in granular sludge, Appl. Environ. Microbiol. 61 (11) (1995) 3889–3893.
[7] V.L. Gemini, A. Gallego, V.M. Oliveira, C.E. Gomez, G.P. Manfio, S.E. Korol, Biodegradation and detoxification of p-nitrophenol by *Rhodococcus wratislaviensis*, Int. Biodet. Biodegr. 55 (2005) 103–108.
[8] G. Ricco, M.C. Tomei, R. Ramadori, G. Laera, Toxicity assessment of common xenobiotic compounds on municipal activated sludge: comparison between respirometry and Microtoxs, Water Res. 38 (2004) 2103–2110.
[9] A.D. Paola, L. Palmisano, G. Pantaleo, E. Savinov, Heterogeneous photocatalytic degradation of nitrophenols, J. Photochem. Photobiol. A: Chem. 155 (2003) 207–214.
[10] O.S. Kuscu, D.T. Sponza, Performance of *p*-nitrophenol (*p*-NP) fed sequential anaerobic migrating blanket reactor (AMBR)/aerobic completely stirred tank reactor (CSTR) system under increasing organic loading conditions, Enzyme Microbial Technol. 40 (5) (2007) 1026–1034.
[11] O.S. Kuscu, D.T. Sponza, Effects of hydraulic retention time (HRT) and sludge retention time (SRT) on the treatment of nitrobenzene in AMBR/CSTR reactor systems, Environ. Technol. 28 (2007) 285–296.
[12] L. Zhu, B. Ma, L. Zhang, L. Zhang, The study of distribution and fate of nitrobenzene in a water/sediment microcosm, Chemosphere 69 (10) (2007) 1579–1585.
[13] W. Zhang, L. Chen, H. Chen, S.Q. Xia, The effect of $Fe^0/Fe^{2+}/Fe^{3+}$ on nitrobenzene degradation in the anaerobic sludge, J. Hazard. Mater. 143 (1–2) (2007) 57–64.
[14] L. Liu, J. Wang, J. Zhou, F. Yang, C. Jin, Y. Qu, A. Li, L. Zhang, Enhancement of nitroaromatic compounds anaerobic biotransformation using a novel immobilized redox mediator prepared by electropolymerization, Bioresour. Technol. 99 (15) (2008) 6908–6916.
[15] D.T. Sponza, O.S. Kuscu, Effect of increasing nitrobenzene loading rates on the performance of anaerobic migrating blanket reactor and sequential anaerobic migrating blanket reactor/completely stirred tank reactor system, J. Hazard. Mater. 168 (2009) 390–399.
[16] D.T. Sponza, O.S. Kuscu, p-Nitrophenol removal in a sequential anaerobic migrating blanket reactor (AMBR)/aerobic completely stirred tank reactor (CSTR) system, Proc. Biochem. 40 (2005) 1679–1691.
[17] Z. He, J.C. Spain, Comparison of the downstream pathways for degradation of nitrobenzene by Pseudomonas pseudoalcaligenes JS45 (2-aminophenol pathway) and by Comamonassp. JS765 (catechol pathway), Arch. Microbiol. 171 (1999) 309–316.
[18] L.T. Angenent, S. Sung, Development of anaerobic migrating blanket reactor (AMBR), a novel anaerobic treatment system, Water Res. 35 (7) (2001) 1739–1747.
[19] L.T. Angenent, G.C. Banik, S. Sung, Anaerobic migrating blanket reactor treatment of low-strength wastewater at low temperatures, Water Environ. Res. 73 (5) (2001) 567–574.
[20] L.T. Angenent, G.C. Banik, S. Sung, Effect of an organic shock load on the stability of an anaerobic migrating blanket reactor, J. Environ. Eng. 128 (12) (2002) 1020–1109.
[21] K.B.S. Jinadasa, C.H. Mun, M.A. Aziz, W.J. Ng, Acidogenic pretreatment of wastewaters containing 2-nitrophenol, Water Sci. Technol. 50 (2004) 119–124.
[22] R. Speece, Anaerobic Biotechnology, Archae Press, Tennessee, USA, 1996.
[23] APHA-AWWA, Standard methods for the examination of water and wastewater, 8, 21st edition, Water Environment Federation, Washington DC, USA, 1992.
[24] M.I. Beydilli, S.G. Pavlosathis, W.C. Tincher, Decolorization and toxicity screening of selected reactive azo dyes under methanogenic conditions, Water Sci. Technol. 38 (4–5) (1998) 225–232.
[25] G.K. Anderson, G. Yang, Determination of bicarbonate and total volatile acid concentration in anaerobic digesters using a simple titration, Water Environ. Res. 64 (1992) 53–59.
[26] EPA, U.S, Environmental Protection Agency, Environmental Protection Agency (EPA), Nitroorganics and nitroamines by high performance liquid chromatography (HPLC), 8300 method, USA, 1994.
[27] B. Lange, LUMISmini, Operating Manual. Dr. Bruno Lange, Düsseldorf, Germany, 1994.
[28] D. Holdway, K. Lok, M. Semaan, The acute and chronic toxicity of Para nitrophenol to *Daphnia magna* and two hydra species, Environ. Toxicol. 16 (2001) 557–565.
[29] J.H. Yen, K.H. Lin, Y.S. Wang, Acute lethal toxicity of environmental pollutants to aquatic organisms, Environ. Res. B: Ecotoxicol. Environ. Safety 52 (2002) 113–116.
[30] S. Hung Yen, Y. Masen Kung, Toxicity of dinitro- and trinitrotoluenes to *daphnia* and carp SPE International Symposium on Oilfield Chemistry, The Woodlands, Texas, 2009.
[31] B. Lei, S. Huang, M. Qao, T. Li, Z. Wang, Prediction of the environmental fate and aquatic ecological impact of nitrobenzene in the Songhua River using the modified AQUATOX model, J. Environ. Sci. 20 (2008) 769–777.
[32] K.H. Lin, C.G. Jawa, J.H. Yen, Y.S. Wang, Molecular connectivity indices for predicting bioactivities of substituted nitrobenzene and aniline compounds, Ecotoxicol. Environ. Safety 72 (2009) 1942–1949.
[33] M. Ares, L. Grate, M.H. Paulıng (Eds.), A handful of intron-containing genes produces the lion's share of yeast Mrna, Letter to the Editor 3 (1999) 1138–1139.
[34] N. Shimizu, C. Ogino, T. Kawanishi, Y.Y. Hayashi, Fractal analysis of *Daphnia* motion for acute toxicity bioassay, Environ. Toxicol. Water Qual. 17 (5) (2002) 441–448.
[35] D. Naci, E. Jackim, R. Walsh, Comparative evaluation of three rapid marine toxicity tests: sea urchin early embryo growth test, sea urchin sperm cell toxicity test and Microtox, Environ. Toxicol. Chem. 5 (1986) 521–525.
[36] World Health Organization, Nitrobenzene in Environmental Health Criteria 230 Geneva, Switzerland, 2003.
[37] P. Jiang, J. Zhou, A. Zhang, Y. Zhong, Electrochemical degradation of p-nitrophenol with different processes, J. Environ. Sci. (China) 22 (2010) 500–506.
[38] Ö.S. Kuscu, D.T. Sponza, Effect of increasing nitrobenzene loading rates on the performance of anaerobic migrating blanket reactor and sequential anaerobic migrating blanket reactor/completely stirred tank reactor system, J. Hazard. Mater. 168 (2009) 390–399.
[39] M.C. He, Y. Sun, X.R. Li, Z.F. Yang, Distribution patterns of nitrobenzenes and polychlorinated biphenyls in water, suspended particulate matter and sediment from mid- and down-stream of the Yellow River (China), Chemosphere 65 (3) (2006) 365–374.