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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

Özlem Selçuk Kuşçu [a], Delia Teresa Sponza [b,*]

[a] Department of Environmental Engineering, Faculty of Engineering and Architecture, Süleyman Demirel University, 32360, Isparta, Turkey
[b] Department of Environmental Engineering, Faculty of Engineering, Dokuz Eylül University, Buca Kaynaklar Campus, 35160, İzmir, Turkey

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ABSTRACT

A laboratory scale anaerobic migrating blanket reactor (AMBR) reactor was operated at nitrobenzene (NB) loading rates increasing from 3.33 to 66.67 g NB/m^3 day and at a constant hydraulic retention time (HRT) of 6 days to observe the effects of increasing NB concentrations on chemical oxygen demand (COD), NB removal efficiencies, bicarbonate alkalinity, volatile fatty acid (VFA) accumulation and methane gas percentage. Moreover, the effect of an aerobic completely stirred tank reactor (CSTR) reactor, following the anaerobic reactor, on treatment efficiencies was also investigated. Approximately 91–94% COD removal efficiencies were observed up to a NB loading rate of 30.00 g/m^3 day in the AMBR reactor. The COD removal efficiencies decreased from 91% to 85% at a NB loading rate of 66.67 g/m^3 day. NB removal efficiencies were approximately 100% at all NB loading rates. The maximum total gas, methane gas productions and methane percentage were found to be 4.1, 2.6 l/day and 59%, respectively, at a NB loading rate of 30.00 g/m^3 day. The optimum pH values were found to be between 7.2 and 8.4 for maximum methanogenesis. The total volatile fatty acid (TVFA) concentrations in the effluent were 110 and 70 mg/l in the first and second compartments at NB loading rates as high as 66.67 and 6.67 g/m^3 day, respectively, while they were measured as zero in the effluent of the AMBR reactor. In this study, from 180 mg/l NB 66 mg/l aniline was produced in the anaerobic reactor while aniline was completely removed and transformed to 2 mg/l of cathechol in the aerobic CSTR reactor. Overall COD removal efficiencies were found to be 95% and 99% for NB loading rates of 3.33 and 66.67 g/m^3 day in the sequential anaerobic AMBR/aerobic CSTR reactor system, respectively. The toxicity tests performed with *Photobacterium phosphoreum* (LCK 480, LUMIStox) and *Daphnia magna* showed that the toxicity decreased with anaerobic/aerobic sequential reactor system from the influent, anaerobic and to aerobic effluents.

1. Introduction

Nitrobenzene (NB) has mainly been used in the production of aniline and is also used as a solvent in petroleum refining, as well as being used in the manufacture of other organic compounds such as dinitrobenzenes, dichloroanilines and acetaminophen [1]. More than 95% of nitrobenzene is used in the production of aniline. A small amount is used in the manufacture of rubber chemicals, pesticides, insecticide, wood, petroleum, dyes, soap, shoe, floor polishes, leather dressings, paint solvents and pharmaceuticals [2,3]. Nitrobenzene, as a priority toxic environmental pollutant, mostly occurs in ground water and industrial wastewater due to its moderate water solubility and relatively low vapour pressure. NB is relatively toxic and persistent in the environment and it is listed as a priority pollutant by the U.S. EPA among 129 priority pollutants [4]. If its concentration exceeds 2 mg/l in the water, the wastewater is declared to be hazardous. The US Environmental Protection Agency (EPA) has surveyed nitrobenzene levels reported in effluents from 4000 publicly owned treatment works and industrial sites. The highest value in effluent was >190 mg/l in the organic chemicals and plastics industry [1]. Nitrobenzene was detected in 1 of 33 industrial effluents at a concentration greater than 100 mg/l [5]. Reported nitrobenzene concentrations in raw and treated industrial wastewaters from several industries range from 14 to 591 mg/l [6]. The highest concentrations were associated with wastewaters from the organic chemicals and plastics industries. Nitrobenzene is of moderate to low toxicity to aquatic and terrestrial organisms. Reported lethal dose causes decreasing of 50% of organisms (LC_{50})/(effective concentration affecting 50% of organisms (EC_{50})) data for acute toxicity to freshwater organisms range from 2 to 156 mg/l. The lowest

* Corresponding author.
E-mail address: delya.sponza@deu.edu.tr (D.T. Sponza).

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acute NOEC was 0.46 mg/l for the water flea (*Daphnia magna*) [7].

Combined anaerobic/aerobic processes are a viable alternative for the treatment of xenobiotic compounds which are difficult to treat by traditional processes [8]. The mineralization of some recalcitrant pollutants has been possible by using sequential anaerobic/aerobic treatments [8]. Generally, an aerobic polishing step is needed after the anaerobic system (pretreatment) to meet effluent quality standards [9]. Treatment of NB was carried out using an anaerobic–aerobic reactor system by Aziz et al. [10]. The system was operated at various HRTs (8, 12, 16 and 24 days) and influent NB concentrations (50, 100, 150 and 200 mg/l). The removal efficiency of nitrobenzene was between 75% and 85% when the wastewater was treated by the aerobic process alone. However, the removal efficiency of NB was more than 95% in the acidogenic–aerobic process. Field et al. [11] showed that the NB and COD were removed with removal efficiencies of 96% and 94% in a sequential anaerobic–aerobic bacterial consortia. Majumder and Gupta [4] investigated the treatment performance of wastewater containing NB in a hybrid reactor. Maximum COD and NB removal efficiencies were 96% and 98%, respectively at a HRT of 29.55 h. Tomei and Anesini [12], Kulkarni and Chaudhari [13] mentioned that under anaerobic conditions, nitroaromatics are reduced to aromatic amines to facilitate complete mineralization while aerobic degradation of the intermediates was provided through oxygenation of monooxygenase and dioxygenase catalyzed reactions.

AMBR was developed as a high rate anaerobic treatment system that combines compartmentalization, continuous flow, a short hydraulic retention time and a simple design, but does not feature gas–liquid separation, a feed distribution system or recycling [9]. AMBR staging was beneficial for more favorable conditions for volatile fatty acid degradation, for high level of biomass in the first compartment and for high removal efficiencies of recalcitrant substances since it provided sufficient contact time between microorganisms and toxic substances [9].

Since nitrobenzene is a toxic and persistent nitrogenous organic compound and its concentration ranged from 19 to 591 mg/l in some raw and treated industrial wastewater it was aimed to investigate the sequential anaerobic/aerobic treatability. As limited studies were determined in the literature involving NB removal in AMBR reactor the aim of this study was to investigate the effect of increasing nitrobenzene loading rates on methane gas production, VFA accumulation, COD, and NB removal efficiencies in an AMBR reactor. Furthermore, the effects of the aerobic stage on the removal of the remaining COD, NB, and intermetabolite products were investigated in a sequential anaerobic AMBR/aerobic CSTR tank reactor.

2. Materials and methods

2.1. Anaerobic toxicity assay (ATA)

ATA test was performed at 35 °C using serum bottles with a capacity of 150 ml as described by Owen et al. [14] and Donlon et al. [15]. Serum bottles were filled with 2000 mg VSS/l of biomass, 3000 mg/l of glucose–COD, a suitable volume from the Vanderbilt mineral medium, 667 mg/l of sodiumthioglycollate providing the reductive conditions and 5000 mg/l of $NaHCO_3$ to maintain the neutral pH. Before the ATA test, the serum bottles were batch operated until the variation in daily gas production was less than 15% for at least 7 consecutive days. After observing steady-state conditions, an increasing concentration of nitrobenzene was administered to the serum bottles as slug-doses from concentrated stock solution of this chemical. The effects of nitrobenzene on methane gas production were compared with the control samples. Inhibition was defined as a decrease in cumulative methane compared to the control sample. IC_{50} value indicates 50% inhibition of methane gas production in serum bottles containing toxicant. This value shows the presence of toxicity and that the toxicant concentration caused 50% inhibition in the methane gas production.

2.2. Experimental setup

The AMBR reactor consisted of a rectangular container (inside dimensions: length = 15 cm, height = 20 cm, width = 15 cm) with an active volume of 13.5 l, which was divided into three compartments. Round openings with a diameter of 2.5 cm from the back of the stainless steel sheets separated the compartments. These openings were placed at the bottom to create sufficient contact between biomass and substrate. Three compartments were mixed equally every 15 min at 60 rpm to ensure gentle mixing. The samples were withdrawn from the AMBR reactor after stopping the mixing process for 15 min. The influent feed was pumped using a peristaltic pump. The outlet of the AMBR was connected to a glass U-tubing to control the level of wastewater. The gas produced was collected via a porthole in the top of the reactor. The operating temperature of the reactor was maintained constantly at 37 ± 1 °C by placing the AMBR reactor on a heater. A digital temperature probe located in the middle part of the second compartment provided the constant operation temperature measurements. This provided a homogenous temperature in all compartments of the AMBR reactor. The aerobic CSTR reactor consisted of an aerobic tank (effective volume = 9 l) and a settler with a volume of 1.32 l. The effluent of the anaerobic AMBR reactor was used as the influent of the aerobic CSTR reactor. The schematic of the lab-scale sequential AMBR and CSTR reactors used in this study is given elsewhere [16]. Partially granulated anaerobic sludge was used as seed in the AMBR reactor and was obtained from an anaerobic up flow anaerobic sludge blanket reactor containing acidogenic and methanogenic partially granulated biomass from the Pakmaya Yeast Beaker Factory in Izmir, Turkey. Activated sludge culture used as seed for the aerobic CSTR reactor was also taken from the activated sludge reactor of Pakmaya Yeast Bakery Factory in Izmir. The suspended solids (SS) in the AMBR reactor were 45 g/l and the mixed liquor solids concentration in the CSTR was 2000–3000 mg/l.

2.3. Composition of synthetic wastewater

The glucose–COD concentration in the synthetic wastewater was constant as 3000 mg/l during continuous operations of the AMBR. Glucose was used as energy and carbon source. In this study the NB concentrations were increased from 20 to 400 mg/l by taking into consideration the NB levels in industrial discharges [6–17]. The COD concentrations in the influent increased from 3008 to 3490 mg/l by the COD originated from the NB concentrations amended to the AMBR reactor. Vanderbilt mineral medium was used together with NB and 3000 mg/l of glucose–COD as feed. To prevent the accumulation of total volatile fatty acid (TVFA) and provide a neutral pH (7.0–7.8); 5000 mg/l of $NaHCO_3$ was added to the feed. Vanderbilt mineral medium was prepared in distilled water by dissolving per liter 0.4 g $MgSO_4$, 0.4 g NH_4Cl, 0.4 g KCl, 0.3 g Na_2S, 0.08 g $(NH_4)_2HPO_4$, 0.05 g $CaCl_2$, 0.04 g $FeCl_2$, 0.01 g $CoCl_2$, 0.01 g KI, 0.01 g $Na(PO_3)$, 0.5 mg $AlCl_3$, 0.5 mg $MnCl_2$, 0.5 mg $CuCl_2$, 0.5 mg $ZnCl_2$, 0.5 mg NH_4VO_3, 0.5 mg $NaMoO_4$, 0.5 mg H_3BO_3, 0.5 mg $NiCl_2$, 0.5 mg NaWO4, 0.5 mg Na_2SeO, and 0.01 g cystein [8].

2.4. Operation conditions

NB concentrations were increased steeply from 20 to 400 mg/l in the feed wastewater since the NB concentrations released from the chemical and petrochemical industries varied between 14 and 591 mg/l [6]. The influent glucose–COD concentrations and flow rate were kept constant at 3000 mg/l and 2.25 l/day, respectively,

Table 1
The operating parameters for the anaerobic AMBR and aerobic CSTR reactors.

Runs	Period (days)	HRT (day)	OLR	NB con.	NBLR	SRT (day)	Runs	Period (days)	HRT (day)	OLR	NB con.	NBLR	SRT (day)
Nitrobenzene removal													
Anaerobic (AMBR)							Anaerobic (AMBR)/Aerobic (CSTR) reactor system						
Run 1	30	6	0.50	20	3.33	450	Run 1	30	9	0.50	20	3.33	470
Run 2	30	6	0.50	40	6.67	460	Run 2	30	9	0.50	40	6.67	480
Run 3	30	6	0.51	60	10.00	500	Run 3	30	9	0.51	60	10.00	520
Run 4	30	6	0.54	100	16.67	550	Run 4	30	9	0.54	100	16.67	570
Run 5	30	6	0.55	180	30.00	600	Run 5	30	9	0.55	180	30.00	620
Run 6	30	6	0.56	250	41.67	650	Run 6	30	9	0.56	250	41.67	670
Run 7	30	6	0.57	300	50.00	690	Run 7	30	9	0.57	300	50.00	710
Run 8	30	6	0.57	400	66.67	700	Run 8	30	9	0.57	400	66.67	730

OLR: Organic loading rate (kg COD/m^3 day), HRT: hydraulic retention time (day), SRT: solid retention time (day), NBLR: NB loading rate (g NB/m^3 day), NB con.: NB concentration (mg/l), (SRT: 20 days in aerobic CSTR reactor).

through operation of 240 days. The operating parameters for the anaerobic AMBR and aerobic CSTR reactors are summarized in Table 1. HRT in the AMBR was constant as 6 days through the operation since the flow rate was kept constant at 2.2 l/day. The organic loading rate was 0.5 kg/m^3 day at an influent glucose–COD concentration of 3000 mg/l. Since increasing NB concentrations (from 20 to 400 mg/l) gives additional COD to the feed the OLR increased from 0.5 to 0.57 kg/m^3 day. No sludge wasting was applied in the AMBR reactor and granulation was provided during the continuous operation. The feed flow was reversed once a week in AMBR reactor over the horizontal plane. A weekly change in flow direction was chosen to prevent a pH drop due to VFA accumulation in the initial compartment of the AMBR reactor. By reversing the flow scheme, the biomass staging was limited and biomass accumulation in the last compartment was prevented. Sufficient contact between substrate and biomass was maintained using intermittent gentle mixing. Sludge retention time (SRT) in the aerobic CSTR reactor was adjusted to 20 days by wasting of mixed biomass from the aeration basin of the reactor. The SRT in the anaerobic reactor varied between 450 and 700 days.

2.5. Analytical methods

Total suspended solid (TSS) and mixed liquor suspended solid (MLSS) in anaerobic granulated and activated sludge were measured by the membrane filtration technique [18]. The soluble COD concentration in samples was measured using the closed reflux colorimetric method following Standard Methods [18]. Biogas production was measured by the liquid displacement method. Total gas was measured by passing the gas through distilled water containing 2% (v/v) H_2SO_4 and 10% (w/v) NaCl [19]. Methane gas was detected by using distilled water containing 3% NaOH (w/v) [20]. Methane percentage in biogas was determined by Dräger (Stuttgart, Germany) Pac-Ex methane gas analyzer. pH was measured with a pH meter (WWT pH 330).

Bicarbonate alkalinity (Bic.Alk.) and total volatile fatty acid (TVFA) concentrations were measured simultaneously using the titrimetric method proposed by Anderson and Yang [21]. The test was carried out as follows: first the pH of the sample was measured, second the sample was titrated with standard sulphuric acid (0.1 N) through two stages (first to pH 5.1, then from 5.1 to 3.5), and finally the VFA and Bic.Alk. concentrations were calculated with a computer program by solving Eqs. (1) and (2).

$$A_1 = \frac{[HCO_3^-]([H]_2 - [H]_1)}{[H]_1 + K_C} + \frac{[VA]([H]_2 - [H]_1)}{[H]_2 + K_{VA}} \quad (1)$$

$$A_2 = \frac{[HCO_3^-]([H]_3 - [H]_1)}{[H]_3 + K_C} + \frac{[VA]([H]_3 - [H]_1)}{[H]_3 + K_{VA}} \quad (2)$$

where A_1 and A_2 are the molar equivalent of the standard acid consumed to the first and second end points; $[HCO_3^-]$ the bicarbonate concentration; [VA] the total volatile fatty acid ion concentration; $[H]_{1,2,3}$ the hydrogen ion concentrations of the original sample and at the first and the second end points; K_C is the conditional dissociation constant of carbonic acid; K_{VA} is the combined dissociation constant of the volatile fatty acids (C_2–C_6), this pair of constants was assumed to be 6.6×10^{-7} for bicarbonate and 2.4×10^{-5} for volatile acids.

2.5.1. Nitrobenzene (NB), aniline, nitrosobenzene, hydroxylamino benzene and 2-amino phenol measurements

Nitrobenzene (NB), aniline, nitrosobenzene, hydroxylamino benzene and 2-amino phenol aniline measurements were carried out using a high-pressure liquid chromatography (HPLC) (Agilent-1100) with a C-18 reverse phase HPLC column, (25-cm × 4.6-mm, 5 μm, (Ace 5C18)) following the method developed by EPA [22]. Initially, all samples were centrifuged in a centrifuge (SED 5X model) to remove any particulate matter and then filtered through a 0.45 μm pore sized Teflon filter using a disposable syringe (Agilent 5185–5835) prior to HPLC analysis. Elution was prepared with isocratic solvent system consisting of 50% methanol and 50% organic-free reagent water. Thereafter it was run at a flow rate of 1.4 ml/min. The autosampler was set for an injection volume of 10 μl. The chromatographic separation of the sample was performed at 25 °C. Detection was performed at 202 nm wave length for NB at 234 nm wave length for 2-amino phenol, at 212–216 nm wave length for nitrosobenzene and hydroxylamino benzene using a UV detector.

2.5.2. Quantification of nitrobenzene (NB), aniline, nitrosobenzene, hydroxylamino benzene and 2-amino phenol

Quantification was carried out by the integration of the peak area. Limits of detection (LOD) for NB and aniline were 0.000119 ± 0.011 and 0.0009 ± 0.0069 mg/l, respectively. Limits of detection (LOD) for nitrosobenzene, hydroxylamino benzene and 2-amino phenol were 0.0007 ± 0.001, 0.0009 ± 0.002 and 0.0009 ± 0.0051 mg/l, respectively. Standard deviation values for eight replicate concentrations were computed and multiplied with Student's t value for 99% confidence limits in minimum concentration over 5 days [23]. $t_{0.99}$ is equal to 2.998 for $n = 8 - 1(7)$. The limits of quantification (LOQ) for NB, aniline, nitrosobenzene, hydroxylamino benzene and 2-amino phenol were 0.0329, 0.02094, 0.0148, 0.0237 and 0.0305 mg/l, respectively.

2.6. Toxicity measurements

2.6.1. LUMIStox toxicity assay

A specific strain of the marine bacterium, *Photobacterium phosphoreum*, was used in this test to determine the toxicity of p-NP and NB. Reductions in light intensity at 5th, 10th and 30th min were chosen to measure the toxicity [24,25]. The standard culture,

P. phosphoreum (LCK480), was obtained from Dr. Lange industrial measurement technique in Germany. Microtox testing was performed according to the standard procedure recommended by the manufacturer [24]. The bioluminescense of the sample was measured in a luminometer (LUMIStox). Before toxicity assay, the pH of sample was adjusted between 5.5 and 8.5 using 0.1N NaOH or HCl. Room temperature was maintained at between 15 and 24 °C. Samples were serially diluted with 2% NaCl (w/v). Sodium chloride (2%) was used as the control. Samples containing bacterial luminescence were measured for 5, 15 and 30 min incubation times in a luminometer. The decrease in bioluminescence indicated the toxic effect of the samples. Toxicity evaluation criteria for luminescent bacteria are explained by the percent inhibition effect (*H*). If the percent inhibitory effect (*H*) changed between 0% and 5%, the effect is 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 toxic [24].

2.6.2. Daphnia magna toxicity test

Toxicity was tested using 24 h born *D. magna* as described in Standard Methods (2005) [26]. Test animals were obtained from the Faculty of Water products in Aegean University in Izmir. After preparing the test solution, experiments were carried out using 5 or 10 daphnids introduced into the test vessel. These vessels were controlled with 100 ml of effective volume at 7–8 pH, providing a minimum dissolved oxygen concentration of 6 mg/l at an ambient temperature of 20–25 °C. Young *D. magna* are used in the test (in first start ≤24 h old). A 24 h exposure is generally accepted for a *Daphnia* acute toxicity test. Results were expressed as mortality percentage of the *Daphnids*. The immobile animals which were not able to move were determined as dead *Daphnids*.

2.7. Statistical analysis

ANOVA analysis of variance between experimental data was performed to detect *F* and *p* values. In other words, ANOVA test was used to test for differences among 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, d.f. indicated the number of degrees of freedom. Regression analysis was applied to the experimental date in order to determine the regression coefficient R^2. The aforementioned test was performed using Microsoft Excel program.

3. Results and discussion

3.1. Anaerobic toxicity assay (ATA) results for NB

The NB concentrations caused 50% decreases in the methanogenic activity (decrease of methane gas production), which were calculated as IC_{50} value. The IC_{50} value for NB was found to be 109 mg/l as shown in Fig. 1. Razo-Flores et al. [20] reported that IC_{50} values of NB were 0.081 mM (9.97 mg/l). In another study, the IC_{50} value for NB was found as 13 mg/l, respectively [8]. In our study, the IC_{50} value of NB was higher than the IC_{50} values reported by Razo-Flores et al. [20] and Speece [8]. This indicated that the NB is less toxic to the anaerobic sludge used in this study. This could be attributed to the resistance of partially granulated sludge to NB. In the previous studies the IC_{50} value for NB was found to be 44 mg/l using an un-granulated suspended anaerobic sludge [27]. The fractal dimension of the granular sludge, 2.79 ± 0.03 mm, was larger than that of some other settling bacteria suggesting that the granular sludge was more compact and denser. Anaerobic granules grown on glucose as carbon source

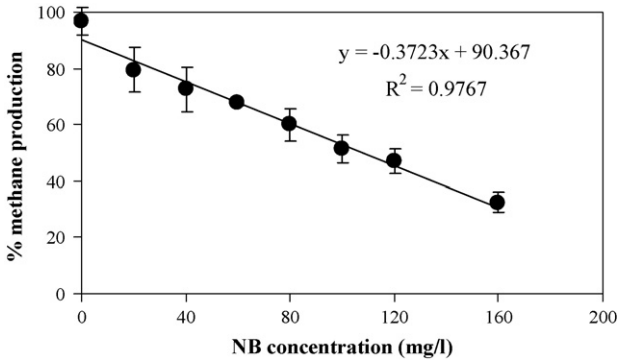

Fig. 1. IC_{50} value for NB (IC_{50} = 109 mg/l).

for the degradation of NB showed good resistance to high NB concentrations in the influent even if unacclimated. The granules exhibited no layered microbial distribution and were packed with different morphotype cells intertwined randomly throughout the cross-section (data not shown). The use of granular sludge as seed proved advantageous over the use of suspended growth anaerobic sludge in terms of resistance to toxicity and rapid acclimation as reported by Del Nery et al. [28] and Mu and Han-Qing [29].

3.2. The removal of NB in AMBR and sequential AMBR/CSTR reactor system

3.2.1. Effects of increasing NB loading rates on COD and NB removal efficiencies in anaerobic AMBR reactor

The operation of the AMBR was started with an influent NB concentration of 20 mg/l at a NB loading rate of 3.33 g/m^3 day. Then NB concentrations were subsequently increased from 40, 60, 100, 180, 250, 300 to 400 mg/l corresponding to NB loading rates from 6.67, 10.00, 16.67, 30.00, 41.67, 50.00 to 66.67 g/m^3 day. Therefore, the influent COD concentrations increased with increased NB concentrations through operation since additional NB concentration increased the initial COD concentration. Fig. 2 shows the variations of COD and COD removal efficiency in AMBR with increasing NB loading rates. COD removal efficiency remained approximately between 91% and 94% until a NB loading rate of 30.00 g/m^3 day corresponding to a NB concentration of 180 mg/l. After this NB loading rate the COD removal efficiency decreased from 91% to 89% at a NB loading rate of 41.67 g/m^3 day corresponding to a NB concentration of 250 mg/l. This shows that the AMBR reactor exhibits a maximum COD removal efficiency (*E* = 91–94%) until a NB concentration of 180 mg/l corresponding to a NB loading rate of 30.0 g/m^3 day (see Fig. 2). The effluent COD concentration in AMBR was 203 mg/l at a NB loading rate of 10.00 g/m^3 day. A strong linear correla-

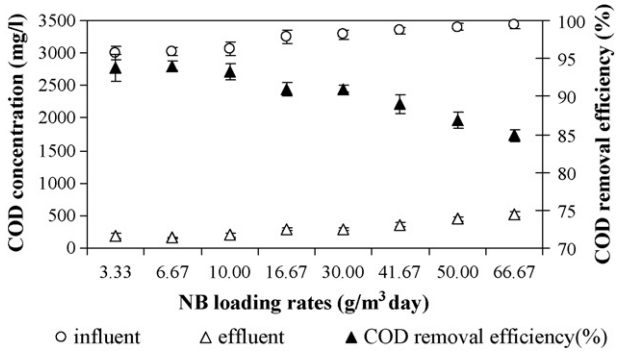

Fig. 2. Effect of increasing NB loading rates on COD removal efficiencies in AMBR at a HRT of 6 days.

tion between COD removal efficiencies and NB loading rates was observed (ANOVA $R = 0.98$; d.f. = 7, $F = 269.9$, $p = 2.43E\text{-}06$). The effluent COD concentrations in AMBR were 295, 359 and 446 mg/l at NB loading rates of 30.00, 41.67 and 50.00 g/m^3 day, respectively, corresponding to COD removal efficiencies of 91%, 89% and 87%. The effluent COD concentration and the removal efficiency were measured as 523 mg/l and 85%, respectively, at a maximum NB loading rate of 66.67 g/m^3 day, corresponding to NB concentration of 400 mg/l.

NB removal efficiency was found as 100% at all NB loading rates (data not shown). The effluent NB concentrations were approximately 0 mg/l at all NB loading rates. 100% of the nitrobenzene was reduced to its corresponding aromatic amines in the anaerobic reactor at an influent NB concentration as high as 400 mg/l (NB loading rate = 66.67 g/m^3 day) and at a HRT of 6 days. The aforementioned NB concentration is higher than the IC_{50} value obtained for NB concentration (IC_{50} = 109 mg/l) causing 50% inhibition on the methane production. This shows that the anaerobic granule microorganisms in AMBR reactor acclimated to high NB concentration. NB at high concentrations is metabolized with the simultaneous utilization of primary substrate (COD) serving as the source of carbon and energy required for growth. Xenobiotic compounds, such as NB, act as secondary substrates that do not contribute to the anabolic process leading to cell growth.

In this study, the optimum NB loading rates and NB concentrations were found to be between 3.33 and 41.67 g/m^3 day and 20 and 250 mg/l, respectively, for maximum COD removal efficiency of 91–94% and a NB removal efficiency of 100% in the AMBR reactor. These results were higher than those in the study performed by Aziz et al. [10]. In this study, NB and COD removal efficiencies were 97% and 16%, respectively, in an acidogen reactor at a HRT of 1 day and an influent NB concentration of 103 mg/l. The NB and COD removals were found to be lower (97% for NB, 89% for COD) in the study performed by Tomei and Annesini [12] under anaerobic conditions, compared to the present study. Similarly, the COD and NB removal efficiencies obtained by Majumder and Gupta [30] (95% and 97% for COD and NB, respectively) at a HRT of 5 days in an hybrid reactor comprising of trickling filter and activated sludge process at an influent NB concentration of 85 mg/l are lower than those of our results. In the study performed by Liu et al. [31], the COD and aniline removal efficiencies were, also, lower than those of our data (between 97% and 98%) in a sequencing batch reactor treating NB and aniline at concentrations varying between 50 and 180 mg/l.

3.2.2. Effects of increasing NB loading rates on the total and the methane gas production in AMBR reactor

The daily total gas, methane gas productions and % methane production were about 3.4 and 2.3 l/day and 54%, respectively, until a NB loading rate of 10.00 g/m^3 day (data not shown). The maximum total gas, methane gas productions and methane percentage were found as 4.1, 2.6 l/day and 59%, respectively, at a NB loading rate of 30.00 g/m^3 day (data not shown). This data exhibited better results than the study performed by Sponza and Kuşçu [32] in an AMBR reactor treating 40 mg/l of NB at a HRT of 10.38 days. In this study, the maximum total gas, methane gas productions and methane percentage were found as approximately 2.3, 1.3 l/day and 56%, respectively, at a NB loading rate of 4.81 g/m^3 day in AMBR reactor. In this present study, 2.7 l/day total gas, 1.98 l/day methane gas and 42% methane gas production were obtained at a maximum NB loading rate of 66.67 g/m^3 day (data not shown). This indicated the inhibitory effect of NB on methane *Archeae* at NB loading rates as high as 66.67 g/m^3 day, corresponding to a NB concentration of 400 mg/l.

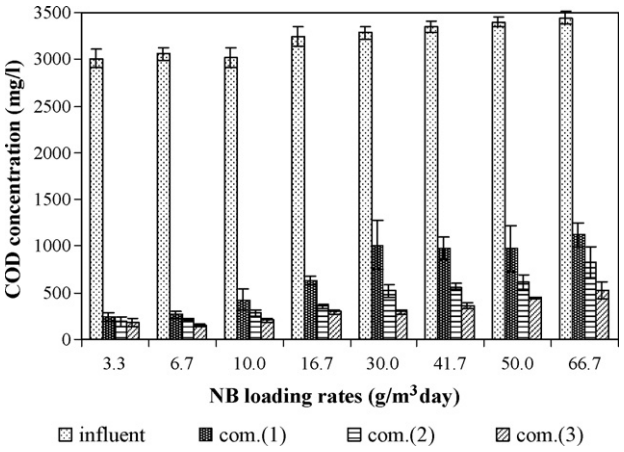

Fig. 3. The COD concentrations in compartments of AMBR at increasing NB loading rates a HRT of 6 days.

3.2.3. Variation of COD, NB removal efficiencies in compartments of the AMBR reactor at increasing NB loading rates

Figs. 3 and 4 show the initial and the remaining COD and NB concentrations in the compartments of the AMBR, respectively. As shown in these figures, most of the COD and NB were removed in the first compartment. The influent COD concentration was 3008 mg/l (the COD of 20 mg/l NB concentration was 8 mg/l) and then decreased to 246 mg/l (E = 92%) at a NB loading rate of 3.33 g/m^3 day in the effluent of the first compartment. The influent COD concentration was 3446 mg/l (the COD of 400 mg/l NB concentration was 446 mg/l) and then it decreased to 1123 mg/l (E = 67%) in the effluent of the first compartment at a NB loading rate of 66.67 g/m^3 day. The COD removal efficiency decreased with increasing NB loading rates. The COD concentrations in the effluent of the first compartment increased from 266 to 1123 mg/l as the NB loading rates increased from 6.67 to 66.67 g/m^3 day (see Fig. 3). The COD removal efficiencies varied between 20–45% and 15–28% in the second and in the third compartments at a NB loading rates as high as 66.67 g/m^3 day, respectively.

The NB removal efficiencies decreased from 100% to 99% at high NB loading rates such as 41.7, 50.00 and 66.7 g/m^3 day in the first compartment (see Fig. 4). However, the NB concentrations in compartment three were approximately 0 mg/l at all NB loading rates. NB concentrations were 4, 0.1 and 0.01 mg/l in the effluents of the initial, second and the third compartments, respectively, at a NB loading rate of as high as 66.7 g/m^3 day. As aforementioned, the COD concentrations were between 155 and 183, 203 and 295, and

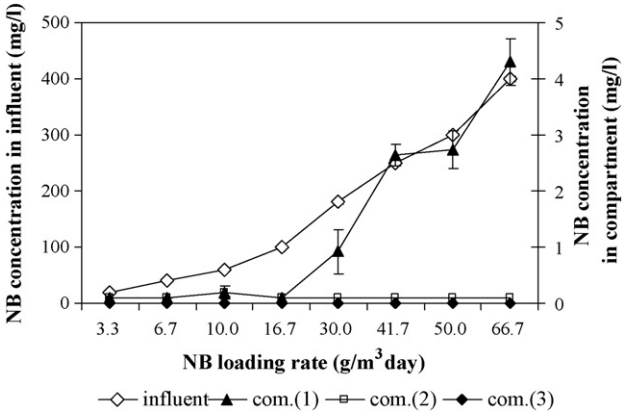

Fig. 4. The NB concentrations in compartments of AMBR at increasing NB loading rates a HRT of 6 days.

359 and 523 mg/l at NB loading rates of 3.33 and 6.67, 10.00 and 30.00, and 41.67 and 66.67 g/m^3 day, respectively, in the effluent of the third compartment of the AMBR reactor. In contrast to COD, the NB was completely removed in the anaerobic reactor. The anaerobic AMBR reactor provides a complete NB removal while the COD was not ultimately removed in the same reactor. For this reason an aerobic CSTR reactor following the anaerobic AMBR reactor was used to remove the rest of the COD remaining from the AMBR reactor.

Similar results were found by Funk et al. [33], Gorontzy et al. [34], Anid et al. [35], Abramowicz [36] and Gerlach et al. [37]. They found that the reduction of nitroaromatics to its corresponding amino groups is in many cases required before ring-fission and mineralization of these contaminants occurred. While the nitro group reduction generally requires anaerobic conditions, the ring-fission and subsequent mineralization is favorable under aerobic conditions. After degrading the NB to aniline the aeration could lead to the fission of aniline resulting in formation of catechol.

3.2.4. The variation of pH, TVFA, bicarbonate alkalinity (Bic.Alk.) and TVFA/Bic.Alk. ratio in compartments of the AMBR reactor at increasing p-NP loading rates

The pH values in the effluent and in the compartments of the AMBR varied between 7.2 and 8.0 (data not shown). These values were between optimum pH values as reported by Speece [8]. The pH values were lower in the first compartment than those of the other compartments since TVFA in the first compartment was higher. The pH values varied between 7.1 and 7.3 in the initial compartment as the NB loading rates increased. The TVFA concentrations were high in the initial compartment at all NB loading rates. As the NB loading rates were increased, the TVFA concentrations increased in the first compartment. A strong linear correlation between TVFA concentrations in the first compartment and NB loading rates was observed ($R = 0.97$; d.f. = 7, $F = 207$, $p = 1.59E\text{-}06$). The TVFA concentration increased from 37 to 179 mg/l in the first compartment as NB loading rates increased from 3.33 to 66.67 g/m^3 day (data not shown). The TVFA concentration was 0 in the second and third compartments of AMBR at NB loading rates of 3.33 and 6.7 g/m^3 day, respectively (data not shown). The Bic.Alk. concentrations were lower in the first compartment than the other compartments due to decline of pH. This indicates the utilization of alkalinity to buffer the TVFA and CO_2 produced from the anaerobic co-metabolism of NB. TVFA/Bic.Alk. ratio gives information necessary to determine the stability of the anaerobic reactor. If the TVFA/Bic.Alk. ratio is lower than 0.4, the reactor is stable. When the TVFA/Bic.Alk. ratio is lower than 0.8, the reactor system is moderately stable or unstable, as reported by Behling et al. [38]. The TVFA/Bic.Alk. ratio varied between 0.00 and 0.04 in the compartments and the effluent of AMBR at increasing NB concentrations, also. These results indicated that the AMBR reactor treating NB was stable also at NB concentrations and at NB loading rates as high as 50.0–66.67 g/m^3 day and 300–400 mg/l, respectively.

3.2.5. Transformation of NB in anaerobic/aerobic sequential reactor system and fate of aniline

A peak of 150 mg/l of aniline was obtained at a retention time of 3.55 min and at a wave length of 234 nm in the anaerobic AMBR reactor effluent (see Fig. 5a). The presence of an aniline peak in the effluent of the anaerobic AMBR indicated that the nitrobenzene converted to aniline under anaerobic conditions [39]. Peres et al. [40] found that the biodegradation of nitrobenzene to aniline occurred via nitrosobenzene. Wu et al. [41] found that nitrosobenzene and hydroxylamino benzene was detected in the anaerobic degradation of NB. Nishino and Spain [42] observed that NB was converted to 2-amino phenol via nitrosobenzene. However no nitrosobenzene, hydroxylamino benzene and 2-amino phenol peaks were observed in the compartments and in the effluent of the

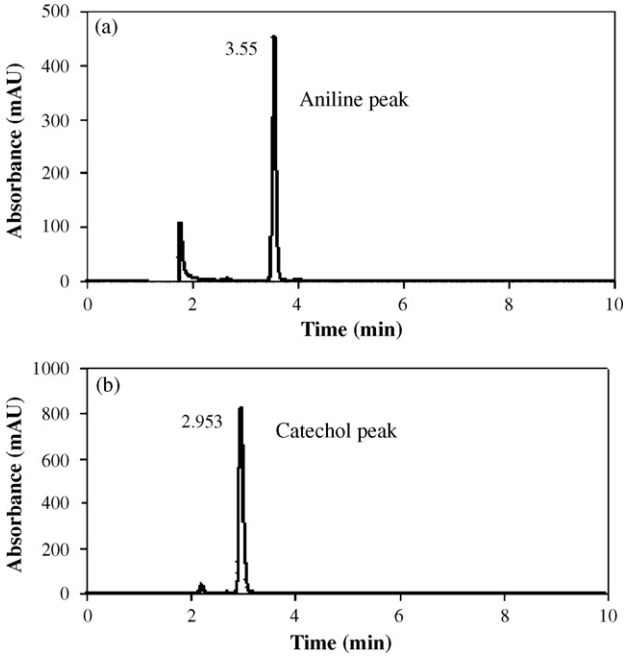

Fig. 5. HPLC chromatograms of anaerobic AMBR effluent (a) (HRT = 6 days; influent NB concentration = 400 mg/l; retention time = 3.55 min; wave length = 234 nm and aerobic CSTR reactor effluent and (b) (HRT = 3 days; influent aniline concentration = 155 mg/l; retention time = 2.95 min; wave length = 234 mAU (mili amper unit)).

anaerobic AMBR reactor through HPLC analysis. This shows that NB is reduced only to aniline under anaerobic conditions as reported by Aziz et al. [10]. Aniline was biodegraded in the aerobic stage. No peak of aniline was observed in the effluent of aerobic CSTR reactor. 12 mg/l Catechol was observed in the oxidative step as reported by Peres et al. [40] (see Fig. 5b) at an initial aniline concentration of 150 mg/l. This showed that the aniline was biodegraded to catechol in the aerobic stage. The mineralization of aniline to catechol was accomplished by subsequent aerobic treatment using activated sludge as reported by Wu et al. [41].

Most microorganisms are only able to reduce the nitro group to an amino group via nitroso and hydroxyl amino functions [40,43–45]. In this study the granular sludge was able to transform the NB via aniline to its corresponding amino aromatic compounds. The effluent aniline concentration increased whenever influent NB loading rates were increased. A strong linear correlation between influent NB loading rates and the effluent aniline concentration was observed ($R^2 = 0.98$; d.f. = 7, $F = 212.65$, $p = 0.0092$). Aniline concentration in the effluent of AMBR reactor increased from 7 to 155 mg/l when the NB concentration in the influent increased from 20 to 400 mg/l (see Fig. 6). In this study the HPLC analysis showed that from the total quantity of 180 mg/l NB introduced to the AMBR reactor approximately 66–68 mg/l aniline was produced. Aziz et al. [10] showed that 1 mol of nitrobenzene reduced to 1 mol of aniline in the anaerobic acidogenic process. According to this result the theoretical assumption of the aniline produced from 180 mg/l of NB should be approximately 165–170 mg/l by taking into consideration the energy losses during the metabolic pathways (data not shown). However, in our study since no other intermediate products were detected (nitrosobenzene, hydroxylamino benzene and 2-amino phenol), with the exception of aniline, it can be concluded that the 180 mg/l NB was converted to 165–170 mg/l aniline and 60% of this aromatic compound (99–102 mg/l) ultimately mineralized to carbondioxide and water under anaerobic conditions in the AMBR reactor. The rest of the aniline (66–68 mg/l) was not mineralized and remained in solutions in the effluent of the anaer-

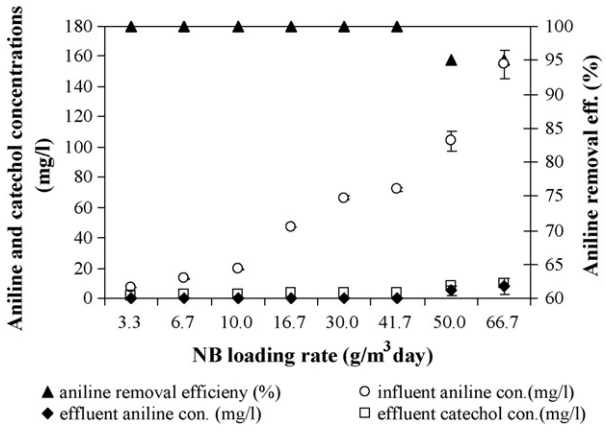

Fig. 6. Aniline removal in aerobic CSTR reactor at increasing NB loading rates (HRT = 3 days).

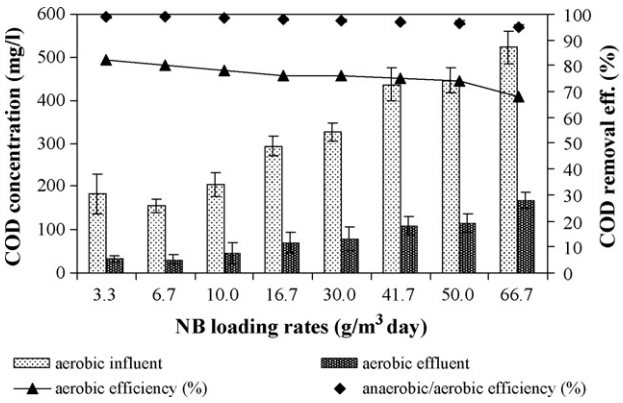

Fig. 7. COD removal in aerobic reactor (HRT = 3 days) and sequential anaerobic (AMBR)/aerobic (CSTR) reactor (HRT = 9 days) at increasing NB loading rates.

obic AMBR reactor. The aniline concentrations measured in the anaerobic reactor effluent indicate the non-metabolized, remaining aniline level (data not shown). Schnell and Schink [46] also reported that aniline can be degraded anaerobically as long as the carbon source was metabolized in granular sludge, thus producing reducing equivalents. The reduction of NB continued to occur and aniline mineralization was observed with the reductive consortium. The NB reduction was not accompanied by a negative influence on aniline mineralization. NB reduction and aniline mineralization were made feasible in the anaerobic AMBR reactor. Peres et al. [40] also indicated that not only does nitrobenzene transformation occur in anaerobic conditions but also aniline mineralization can occur under anaerobic condition. The results obtained in our study confirmed these results.

The first step in the mineralization of aniline was inferred to be conversion to cathechol by dioxygenase, from the combined evidence of growth on cathechol by aniline-induced anaerobic hydrolytic microorganisms to methane. The aromatic ring of the catechol was then cleaved by a second dioxygenase. Both meta [47–49] and ortho [50–52] cleavages have been described for aniline mineralization. Nadeu et al. [53] mentioned that NB converted to hydroxyl amino aromatic, meta ring cleavage to aminomalonic semi-aldehyde and mineralized to cathechol in an aerobic consortia. Friemann et al. [54] found that NB biodegraded to cathechol with release of nitrite under oxic conditions. In our study, although the first and second dioxygenase processes were not studied the catechol concentrations in the effluent of aerobic reactor were very low (between 2 and 10 mg/l) and would not violate the aquatic receiving area standards (see Fig. 6).

3.2.6. *Performance of aerobic reactor and treatment efficiencies of anaerobic/aerobic sequential reactor system*

The aniline removal efficiencies were 100% until a NB loading rate of 50.00 g/m^3 day in the aerobic reactor. For maximum aniline removal efficiencies the optimum NB loading rates varied between 3.33 and 41.67 g/m^3 day in the aerobic CSTR reactor (see Fig. 6). After this loading rate, aniline removal efficiency decreased to 95% for NB loading rates varying between 50.00 and 66.67 g/m^3 day. An aniline concentration of 155 mg/l produced from 400 mg/l of NB concentration was removed with a treatment efficiency of 95% at an NB loading rate of as high as 66.67 g/m^3 day in the aerobic CSTR reactor (data not shown). The aniline concentrations in the effluent of aerobic reactor concentrations were 4 and 8 mg/l at NB loading rates of 50.00 and 66.67 g/m^3 day, respectively (data not shown). The catechol concentrations in the effluent of the aerobic reactor were between 2 and 4 mg/l until a NB loading rate of 41.67 g/m^3 day (see Fig. 6). Then it increased to 8 and 10 mg/l at NB loading rates of 50.00 and 66.67 g/m^3 day, respectively (see Fig. 6). Theoretically, approximately, 370–380 mg/l aniline should be produced from 400 mg/l NB under anaerobic conditions in an AMBR reactor. Since the measured aniline concentration was 150 mg/l in the effluent of AMBR reactor, it can be concluded that 220–230 mg/l aniline was mineralized under anaerobic conditions as aforementioned.

Fig. 7 shows the COD removal efficiency in the aerobic reactor and in the sequential anaerobic/aerobic reactor system. COD removal efficiencies were approximately 80% at NB loading rates varying between 3.33 and 50.00 g/m^3 day in the aerobic CSTR reactor while it decreased to 68% at a NB loading rate as high as 66.67 g/m^3 day. The COD concentrations in the effluent of the aerobic reactor were 33, 78 and 167 mg/l at NB loading rates of 3.33, 30.00 and 66.67 g/m^3 day, respectively, at influent COD concentrations of 183, 327 and 523 mg/l, respectively, in the CSTR reactor. Total COD removal efficiencies were found between 95% and 99% for NB loading rates 3.33 and 66.67 g/m^3 day in the sequential anaerobic AMBR/aerobic CSTR reactor system, respectively, at a total HRT of 9 days (6 days in AMBR and 3 days in CSTR).

Since NB is completely transformed to its respective aromatic amines in anaerobic AMBR reactor (in this study only aniline) the aerobic reactor was used only to remove both the COD remain-

Table 2
Toxicity values in AMBR/CSTR reactor system with *Photobacterium phosphoreum* (NB = 180 mg/l, HRT = 1 day).

Inhibition (H) (%)	Time (min)	Anaerobic influent	Anaerobic effluent	Aerobic effluent
H_5	5	92.58	53.5	4[a,b]
H_{15}	15	95.94	55.4	5[a,b]
H_{30}	30	98.1	58.2	6[c]
G_L (dilution ratio)	30	1/8	1	[a]
EC_{50} (mg/l)	30	22.5	66	[b,c]

[a] No dilution, it could be given directly to the receiving area.
[b] No toxicity.
[c] Sligtly toxicity.

Table 3
Toxicity values in AMBR/CSTR reactor system with *Photobacterium phosphoreum* (NB = 180 mg/l, HRT = 6 days).

Inhibition (H) (%)	Time (min)	Anaerobic influent	Anaerobic effluent	Aerobic effluent
H_5	5	92.58	23.5	3[a,b]
H_{15}	15	95.94	25.4	4[a,b]
H_{30}	30	98.1	30.2	4[a,b]
G_L (dilution ratio)	30	1/8	1	[a]
EC_{50} (mg/l)	30	22.5	–	[b]

[a] No dilution, it could be given directly to the receiving area.
[b] No toxicity.

ing from the anaerobic reactor and the aniline which could not be metabolized under anaerobic conditions.

3.2.7. Assessment of toxicity of sequential anaerobic AMBR/aerobic CSTR reactor system

In this study, the toxicity of the effluent of the AMBR and aerobic (CSTR) reactor was determined by bioluminescence test using bacteria *P. phosphoreum* (LCK 480, LUMIStox) and by *D. magna* test. Tables 2 and 3 show the inhibition percentage of 5th, 15th, 30th min in samples taken from the influent synthetic wastewater containing 180 mg/l NB, from the effluents of anaerobic and aerobic reactor through continuous operation at HRTs of 1 day and 6 days. LUMIStox test is an acute toxicity test. Therefore the toxicity results of this test can be obtained in a short time (maximum 30 min). As shown in Table 2 the inhibition percentage (H) of influent was found as 92.58% and 98.1% at incubation times of 5 and 30 min. These results showed that wastewater containing 180 mg/l of NB was toxic due to 92–98% inhibition observed. After anaerobic treatment, the effluent toxicity decreased to 53.5% and 58.2% at incubation times of 5 and 30 min at a HRT of 1 day as shown in Table 2. The toxicity decreased from 92.58% and 98.1% to 23.5% and 30.2% in the effluent of the anaerobic reactor at incubation times of 5 and 30 min at a HRT of 6 days (see Table 3). The results of the study showed that NB transformed to less toxic intermediate aromatic products under anaerobic conditions. Intermediate products such as aniline produced under anaerobic conditions were less toxic than parent organic compounds. It was demonstrated that a long HRT (6 days) compared to a short HRT (1 day) provided sufficient contact time between anaerobic granular sludge and NB resulting in a more acclimated methanogenic bacteria. After aerobic treatment, toxicity decreased from 53.5% and 58.2% to 4% and 6% in the aerobic reactor effluent at incubation times of 5 and 30 min at a HRT of 1 day. The toxicity at 30 min was moderately toxic and exhibited possible toxicity in the aerobic reactor effluent (see Table 2, 6[c], sligtly toxic). At a HRT of 6 days the toxicity in the anaerobic effluent decreased from 23.5% and 30.2% to 3% and 4% resulting in an aerobic effluent without toxicity (see Table 3, 4[a,b], No dilution, it could be given directly to the receiving area; No toxicity). The dilution ratios (G_L) were similar in the anaerobic and aerobic effluents in both HRTs while the effective concentration affected the 50% of the *P. phosphoreum* (EC_{50}) which increased in anaerobic and aerobic effluents as the HRT increased from 1 to 6 days indicating the toxicity decreased in high HRTs since the bacteria had enough contact time with toxicant (see Tables 2 and 3). It is important to note that the toxicity decreased from the anaerobic to aerobic reactor. The results of this study showed that the intermediate organics produced in the anaerobic and aerobic sequentials are less toxic than the parent nitrogenous organic compound "NB".

Table 4 shows the *D. magna* toxicity test results for samples taken from the influent synthetic wastewater containing NB of 180 mg/l and from the effluents of anaerobic AMBR and aerobic CSTR reactors at a HRT of 6 days. *D. magna* test is accepted as an acute toxicity test. The results of this test were expressed as mortality percentage of the *Daphnids*. The test samples containing 180 mg/l NB were diluted, and the experiments were carried out using 10 *daphnids*. The *Daphnids* were added to every test vessel at the beginning of the process ($t = 0$). After 24 h of incubation time, the EC_{50} value of *D. magna* (the concentration affected 50% of *D. magna*) was found as 30 mg/l in feed wastewater diluted 6 times (data not shown). This showed that the feed wastewater was toxic for *Daphnids*. In other words, if the influent wastewater were to be diluted 6 times, 50% of *Daphnids* would be killed. If the influent wastewater were to be diluted 20 times, toxicity would not be observed. The samples taken from the effluent of the anaerobic reactor were diluted at 1, 4/5, 3/5, 2/5 and 1/2 ratios. EC_{50} value (50% inhibition of *Daphnids)* was 52.8 mg/l in the effluent sample diluted at a dilution ratio of 4/5. It was observed that the EC_{50} values increased in the effluent of the anaerobic, indicating the decrease in the toxicity. The other dilution ratios did not show any mortality effect on *Daphnids*. *D. magna* test results showed that no toxicity was observed in the aerobic effluent at all dilutions. The NB and aniline concentrations in the effluent of anaerobic reactor were found to be approximately 0 and 66 mg/l, respectively through a HRT of 6 days and in influent NB concentration of 180 mg/l. If this wastewater were to be diluted at a ratio of 3/5 toxicity would not be caused in the receiving water ecosystems. This shows that the anaerobic reactor decreased or completely removed the toxicity of the influent and produced less toxic intermediates product than that influent. Since the aniline was converted to catechol under aerobic conditions the concentrations of it (2–10 mg/l) measured in the aerobic reactor effluent did not cause toxicity (see Fig. 6).

The toxicity studies performed in the present study with NB exhibit better results with granular sludge after sequential anaerobic and aerobic treatment steps. The EC_{50} values increased from 22.5 to 66 mg/l in the effluent of the aerobic reactor, indicating less toxicity while the toxicity was completely removed in the aerobic reactor. The toxicity data given below are comparably higher than those of our study.

The lowest toxic NB concentration reported for microorganisms is for the bacterium *Nitrosomonas*, with an EC_{50} of 0.92 mg/l based upon the inhibition of ammonia consumption [55]. Inhibition of oxygen uptake was used as the criterion for aerobic heterotrophs, with an EC_{50} of 370 mg/l. In anaerobic toxicity tests, the inhibition of gas production was used as the criterion for methanogens, with an EC_{50} of 13 mg/l reported for nitrobenzene [55]. Other reported values are a 72-h no-observed-effect concentration (NOEC) of 1.9 mg/l for the protozoan *Entosiphon sulcatum* and an 8-day lowest-observed-effect concentration (LOEC) of 1.9 mg/l for the blue-green alga *Microcystis aeruginosa* [1]. For freshwater invertebrates, acute toxicity (24- to 48-h LC_{50} values) ranged from 24 mg/l for the water flea (*D. magna*) to 140 mg/l for the snail (*Lymnaea stagnalis*) [1]. For marine invertebrates, the lowest acute toxicity value reported was a 96-h LC_{50} of 6.7 mg/l for the mysid shrimp (*Mysidopsis bahia*) [1]. The lowest chronic test value reported was a 20-day NOEC of 1.9 mg/l for *D. magna*, with an EC_{50}, based on reproduction, of 10 mg/l [1]. Freshwater fish showed similar low sensitivity to nitrobenzene. The 96-h LC_{50} values ranged from 24 mg/l for the medaka (*Oryzias latipes*) to 142 mg/l for the guppy (*Poecilia reticulata*) [1]. There was no effect on mortality or behaviour of medaka at 7.6 mg/l over an 18-day exposure. Yen et al. [56] found that the

Table 4
Toxicity values in the influent, effluent of AMBR and in the effluent of CSTR reactor with *Daphnia magna* (NB = 180 mg/l, HRT = 6 days).

Anaerobic influent			Anaerobic effluent			Aerobic effluent		
Dilution ratio	Alive *Daphnia magna* number		Dilution ratio	Alive *Daphnia magna* number		Dilution ratio	Alive *Daphnia magna* number	
	First start	24 h		First start	24 h		First start	24 h
1	10	0	1	10	0	1	10	10
1/2	10	0	4/5	10	5	4/5	10	10
1/6	10	5	3/5	10	10	3/5	10	10
1/15	10	7	1/2	10	10	1/2	10	10
1/20	10	10	1/4	10	10	1/4	10	10

nitrobenzene concentrations varying between 167 and 340 mg/l were extremely toxic to daphnia and carp in aquatic ecosystems like river and lakes. Deneer et al. [57] investigated the toxicity of various mono and dinitrobenzene derivatives towards *D. magna*, *Chlorella pyrenoidosa* and *P. phosphoreum* establishing quantitative structure–activity relationships (QSARs). It is assumed that the formation of reactive metabolites, probably contributes substantially to the toxicity of most dinitro compounds. Ramos et al. [58] tested the acute toxicity of five NB compounds at concentrations varying between 20 and 300 mg/l to the water flea, the guppy and the pond snail. As expected, the toxicity of these chemicals is higher and the lethal body burdens are lower at lower exposure concentrations. A mixture toxicity investigation was conducted using the bioluminescent marine bacterium *Vibrio harveyi* as the test organism for dual combinations of nitrobenzene and dinitrobenzene in a study performed by Lange and Thomulka [59]. NB concentrations higher than 200 mg/l exhibited high toxicity to the test bacterium *Vibrio harveyi*. Holcombe et al. [7] found that the EC_{50} value of NB varied between 144 and 170 mg/l in a toxicity test performed with Fathead minnow *Pimephales promelas.* Momani [60] which investigated the toxicity of a wastewater pretreated with H_2O_2, Fe^{2+} and photo phenton containing 65 mg/l NB. The toxicity unit of the Microthox bacteria ($TU = 100/EC_{50}$) decreased from 25 to 8 although NB was removed with a treatment efficiency of 100%. The toxicity was attributed to the metabolite products. Wang et al. [61], Donlon et al. [15] and Podeh et al. [62] described the toxicity of NB to anaerobic acetoclastic methanogens bacteria which were extremely sensitive to 50 mg/l NB (EC_{50} values were found between 0.1 and 6 mg/l).

4. Conclusions

The results of this study showed that from the synthetic wastewater containing 180 mg/l NB, 66–68 mg/l aniline was produced in the anaerobic AMBR reactor while aniline was completely removed and it was transformed to 2 mg/l of cathechol in the aerobic CSTR reactor at a NB loading rate of 30.00 g/m^3 day. 180 mg/l NB was treated with a removal efficiency of 100% in the anaerobic AMBR reactor while the COD removal efficiency was 91% at a NB loading rate of 30.00 g/m^3 day resulting in a COD concentration of 180 mg/l in the effluent of the AMBR reactor. The removal efficiency of the COD remaining from the AMBR reactor was removed with a treatment efficiency of 76% in the effluent of aerobic CSTR reactor resulting in an effluent COD concentration of 70 mg/l at a NB loading rate of 30.00 g/m^3 day. The aniline was completely removed in this NB concentration and NB loading rate. The toxicity observed in the anaerobic effluent was attributed to the aniline. The range of the cathechol does not exhibit a toxicity in the effluent of the aerobic reactor. Overall COD removal efficiencies were found to be 95% and 99% for NB loading rates of 3.33 and 66.67 g/m^3 day in the sequential anaerobic AMBR/aerobic CSTR reactor system, respectively. The NB concentrations varying between 20 and 400 mg/l were ultimately removed in the sequential anaerobic AMBR/aerobic CSTR reactor system. The contribution of the aerobic reactor to the sequential AMBR/CSTR reactor system was to remove the COD remaining from the AMBR reactor and the aniline produced in the AMBR reactor at a total HRT of 9 days. Moreover, when NB was amended to a synthetic wastewater at concentrations varying between 20 and 400 mg/l the granular biomass showed good acclimation to NB, with high content of methane composition in the biogas production being observed. The results of the toxicity tests performed with *P. phosphoreum* and *D. magna* showed that toxicity decreased with anaerobic/aerobic sequential reactor system from the influent to anaerobic and to aerobic effluents.

This study has demonstrated that the sequential AMBR/CSTR reactor system can be used effectively as an option for ultimate mineralization of wastewater containing NB at a total HRT of 9 days. The compartmentalized design of the AMBR facilitates the efficient treatment of NB by protecting sensitive methanogens in granular sludge from potentially inhibitory degradation products while CSTR promotes efficient COD reduction remaining from the AMBR. These results provide further evidence that NB can be degraded completely in anaerobic environments while the AMBR reactor is a process limiting the degree of COD degradation in synthetic wastewater. Therefore an aerobic step is necessary for ultimate COD degradation following the anaerobic step and a sequential reactor system consisting of both AMBR and CSTR is recommended for NB removal.

Acknowledgments

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