



Biotransformation of 2,4,6-trinitrotoluene in a continuous-flow *Anabaena* sp. system

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Abstract

Reductive transformation of 2,4,6-trinitrotoluene (TNT) was observed in a continuous-flow system of *Anabaena* sp. operated for 33 d with a 5.7 d hydraulic retention time and a range of influent TNT concentrations of 1–58 mg/l. The TNT removal efficiency of the continuous-flow system at the highest influent TNT concentration of 58 mg/l was $96.7 \pm 1.7\%$ (mean \pm 95% confidence interval). Culture chlorosis and growth inhibition were not observed during this study. The pseudo-first order TNT transformation rate constant values corresponding to the system performance range (0.14–0.46/h) were lower than the values previously recorded for batch *Anabaena* sp. cultures with less than 10 mg/l initial TNT concentrations, possibly due to an inhibition of the TNT transformation process by either TNT and/or TNT transformation products. Heterotrophic bacterial populations developed in the continuous-flow *Anabaena* sp. cultures also transformed TNT, but at a much lower rate than the *Anabaena* sp. Less than 1% of the overall TNT transformation observed in the continuous-flow system was attributed to the heterotrophic bacterial populations. The only TNT reduction products identified in both the culture media and in biomass extracts were azoxytetranitrotoluene isomers and low levels of aminodinitrotoluene isomers. TNT and TNT transformation products identified in the culture effluent and the biomass extract accounted for only about 24% of the TNT added to the system (on a molar basis). Production of soluble, polar metabolites, uptake, partial mineralization and/or sequestration of TNT and its transformation products by *Anabaena* may be responsible for the relatively low contaminant recovery and mass balance observed in this study. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: *Anabaena* sp.; Cyanobacteria; Inhibition; Phytoremediation; Trinitrotoluene

1. Introduction

As a result of munitions manufacturing, packing and inappropriate waste material disposal, 2,4,6-trinitrotoluene (TNT) has been found in the soil and ground water of numerous sites throughout the United States and worldwide. Because of the toxic and mutagenic effects of TNT and its transformation products, efforts have recently intensified to develop effective and economical remediation technologies. Among these

technologies, biological processes, using either microorganisms (i.e., bioremediation) or plants (i.e., phytoremediation), are being evaluated.

Microbially mediated TNT transformation reactions have recently been reviewed [1–4]. The reductive transformation of TNT typically proceeds with the reduction of one or more aryl nitro groups and the formation of aryl amines (aminodinitrotoluene and diammonitrotoluene isomers and triaminotoluene, the latter requiring very reduced conditions). Nitroso (–NO) and hydroxylamino (–NHOH) intermediates as well as condensation products such as azoxy dimers (–N=NO–) have also been identified. In addition to the reduction of the nitro groups and formation of amino-derivatives, other reactions are possible, some of which

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result in the formation of polar compounds and oxidation products [5–9].

In contrast to the fate and transformation of TNT in systems involving bacteria and fungi, very little is known about the transformation of TNT in algal systems. Most of the previously reported studies were primarily concerned with the toxic effect of TNT and/or its transformation products on microalgae [10–12]. Compared to the biotransformation of xenobiotic organic compounds mediated by heterotrophic microorganisms, reports on the use of microalgae and cyanobacteria for the biotransformation of organic contaminants are relatively scarce and confined to a few classes of xenobiotic compounds such as aromatic hydrocarbons [13–17], phenolic compounds [18], as well as chlorinated and non-chlorinated pesticides and herbicides [19–22].

While assessing the TNT transformation potential of aquatic plants for the development of a process for the phytoremediation of TNT-contaminated surface and ground water, abundant growth of cyanobacteria and algae was observed in these aquatic plant systems [23]. To assess the degradative potential of cyanobacteria, TNT transformation in batch cultures of the cyanobacterium *Anabaena* sp. was investigated [24]. However, TNT transformation by *Anabaena* sp. in continuous-flow cultures simulating full-scale remediation systems has not been reported. The objective of the work presented here was to assess the effectiveness of a continuous-flow *Anabaena* sp. culture in the transformation of TNT at a range of influent TNT concentrations. To our knowledge, this is the first report of TNT transformation by a cyanobacterium in a continuous-flow system.

2. Materials and methods

2.1. Chemicals

TNT was purchased from Chem Service (West Chester, PA, USA). Eight compounds commonly observed as the TNT reductive transformation products were purchased from SRI International (Menlo Park, CA, USA). These compounds included several hydroxylamino-, monoamino-, diamino-, and tetranitro-azoxytoluene isomers [24]. At the time this research was conducted, lack of ^{14}C -labeled TNT in our laboratory precluded assessment of the degree of TNT mineralization as well as obtaining better mass recoveries (see below).

2.2. Cultures

Unialgal cultures of *Anabaena* sp. were purchased from the Carolina Biological Supply (Burlington, NC, USA). The stock cultures were maintained in liquid

medium Alga-Gro[®] (Carolina Biological Supply) in 2-l glass Erlenmeyer flasks and were occasionally swirled. This medium contains the following compounds in Tris buffer: biotin, disodium glycerophosphate, thiamine, vitamin B₁₂, disodium EDTA, $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, ZnCl_2 , $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. The initial pH of the culture medium was 7.5. Transfer of the stock cultures in fresh media was practiced every 7 d. All glassware was sterilized by autoclaving at 121°C for 30 min. The cultures were illuminated using two 48 inch long, 40 W cool-white fluorescent bulbs for a 16 h photoperiod and at a light intensity of $100 \mu\text{Einstein}/\text{m}^2 \cdot \text{s}$. The stock cultures were kept at room temperature ($23 \pm 2^\circ\text{C}$).

2.3. Continuous-flow systems

Two identical continuous-flow systems were used. The basic unit of each system consisted of a cylindrical glass reactor (9 in diameter \times 12 in height; 12.5 l total volume), an adjustable, positive displacement pump with a ceramic head (Model RP-C20; Fluid Metering Instruments, Syosset, NY, USA), and Teflon tubing. Compressed air, which was passed through a deionized water trap to prehumidify and purify it, was delivered to the cultures via a stainless steel diffuser at a flow rate of 1.3 standard cubic feet per hour (scfh) after passing through a sterile cotton trap. In order to achieve completely mixed conditions, a magnetic stirrer with a Teflon stir bar was also used. The nominal reactor liquid volume was 9 l. A 9 l glass bottle wrapped with aluminum foil was used to store the influent solution. Continuous addition of the influent solution at an average flow rate of 1.1 ml/min was used resulting in a mean hydraulic retention time of 136 h (ca. 5.7 d).

In order to assess the extent of possible TNT losses by adsorption onto the continuous-flow system components and photodegradation, a preliminary, algal-free continuous-flow test was performed with one of the reactor systems as follows. A solution of $1.94 \pm 0.01 \text{ mg/l}$ TNT (mean \pm standard deviation; $n = 5$) in sterile algal medium was pumped through the system under conditions identical to those anticipated for the continuous-flow TNT transformation experiment. These conditions included aeration of the reactor contents at a flow rate of 1.3 scfh, illumination by fluorescent light as described below, and continuous stirring of reactor contents by a magnetic stirrer. The influent flow rate was set at approximately 1.2 ml/min. The reactor was initially filled to the nominal volume (9 l) with a 1.94 mg/l TNT algal medium solution and was covered with a clear plastic film to avoid evaporative losses. After operating for a period of 280 h (i.e., over two hydraulic retention times), the measured reactor effluent TNT concentration was $1.93 \pm 0.01 \text{ mg/l}$ (mean \pm standard deviation; $n = 15$). Therefore, no discernable decline of the TNT concen-

tration was observed due to adsorption, photochemical degradation, or any other removal mechanism.

The two reactors were initially seeded with inoculum from the same *Anabaena* stock culture at a biomass concentration of 5 mg/l, expressed as particulate organic carbon (POC). The reactors were kept at room temperature ($23 \pm 2^\circ\text{C}$) and placed near a window for access to natural light. In order to supplement the natural light, two 48 inch long, 40 W cool-white fluorescent lamps were also used with a 16 h photoperiod and at a light intensity of $100 \mu\text{Einstein}/\text{m}^2\text{s}$. Sterile algal medium was continuously fed to both reactors, which were operated for 38 d until the biomass concentration reached a steady-state value. During this initial period, neither reactor was amended with TNT. Similar biomass levels (41 mg POC/l) were observed in the duplicate reactors. The contents of both reactors were mixed and then equal volumes were distributed to both reactors to ensure identical initial populations in both reactors. TNT was added to the feed reservoir of one reactor whereas the other reactor was kept TNT-free and served as the biotic control throughout the study. Twice a day, the accumulated, excess culture volume was removed from both reactors by siphoning it into a graduated, glass cylinder. A typical specific growth rate of *Anabaena* sp. observed in batch growth experiments was 0.011/h. This growth rate value corresponds to a hydraulic retention time of 91 h for a completely mixed, continuous-flow system. Therefore, the system hydraulic retention time of 136 h represents a safety factor of 1.5 ($=136/91$). A range of influent TNT concentrations, from 1 to 58 mg/l, was tested. Liquid samples from the end of the influent line and from the TNT-amended reactor were collected by glass syringe, prepared and analyzed for TNT and transformation products as described below.

2.4. Batch assay

Despite efforts to maintain axenic conditions, approximately 17 d after the initial seeding of the continuous-flow reactors, rod-shaped, heterotrophic bacteria populations were observed in both reactors. In order to evaluate the relative contribution of the bacteria populations on the TNT transformation in the subsequent continuous-flow run, a bacterial subculture was obtained by inoculating liquid media with the continuous-flow cultures. The liquid media had the following composition (in mg/l): glucose, 500; K_2HPO_4 , 2000; KH_2PO_4 , 700; NH_4Cl , 150; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 15; NaCl , 10; $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 10; and $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 10. This subculture was incubated at room temperature in the dark and aerated in a way similar to the continuous-flow reactors. A high-density culture of heterotrophic bacteria without observable *Anabaena* cells was obtained after 7 d of incubation. Two batch cultures were then

prepared in algal medium: (a) a culture using as inoculum, the heterotrophic bacteria subculture obtained by enriching the continuous-flow cultures; and (b) a culture seeded with inoculum directly from the continuous-flow cultures, thus containing both *Anabaena* and heterotrophic bacteria. Both cultures were prepared with the same initial biomass concentration (30 mg/l POC) in Alga-Gro[®] medium, amended with 2.5 mg/l initial TNT concentration, and incubated at room temperature with aeration. The bacteria-only culture was maintained in the dark whereas the *Anabaena*/bacteria culture was illuminated. Both cultures were exposed to TNT for the first time. An abiotic (i.e., biomass free), media control was also prepared and tested along with the live cultures.

2.5. Analyses

pH and dissolved oxygen (DO) were measured following standard procedures [25]. Culture biomass was quantified as POC, which was measured using a Shimadzu total organic carbon (TOC) analyzer equipped with a solids sample module (SSM) (Shimadzu Instrument Co., Kyoto, Japan) as previously described [24].

Liquid samples were filtered through $0.2 \mu\text{m}$ nylon filters (Gelman, Ann Arbor, MI, USA) and analyzed for TNT and TNT transformation products by high-performance liquid chromatography (HPLC) as previously described [24]. Calibration curves and UV scans were prepared for the following compounds using stock solutions: TNT, 4-hydroxylamino-2,6-dinitrotoluene (4HA26DNT); 2-amino-4,6-dinitrotoluene (2A46DNT); 4-amino-2,6-dinitrotoluene (4A26DNT); 2,4-diamino-6-nitrotoluene (24DA6NT); 2,6-diamino-4-nitrotoluene (26DA4NT); 4,4',6,6'-tetranitro-2,2'-azoxytoluene (2-2' Azy-TeNT); 2,4',6,6'-tetranitro-2,4'-azoxytoluene (2-4' Azy-TeNT); and 2,2',6,6'-tetranitro-4,4'-azoxytoluene (4-4' Azy-TeNT). Two monoamino-dinitrotoluene isomers, 4A26DNT and 2A46DNT, coeluted. However, for routine HPLC calibrations, only 4A26DNT was used and the HPLC reported data were in terms of 4A26DNT. As a result, the designation ADNT was used throughout this work. An ultrasonication/solvent extraction procedure was used to extract TNT and TNT transformation products from biomass. The extracts were further prepared and analytes quantified as previously described [24].

2.6. Data analysis

The kinetics of TNT disappearance can be described by a mixed, second-order rate expression as follows [23]:

$$-\frac{dC}{dt} = KXC, \quad (1)$$

where C = TNT concentration (mg/l), t = time (h), X = biomass concentration (mg/l), and K = second-order TNT disappearance rate constant (l/mg h). By assuming constant biomass concentration throughout the incubation period of a batch assay, Eq. (1) reduces to

$$-\frac{dC}{dt} = kC, \quad (2)$$

where $k = KX$ is the pseudo-first order TNT disappearance rate constant (1/h) for a given and constant biomass concentration. Integration of Eq. (2) leads to

$$\ln\left(\frac{C}{C_0}\right) = -kt. \quad (3)$$

Eq. (3) was used to determine the value of k based on TNT concentration data over the incubation period of batch TNT biotransformation assays using linear regression. Based on the above analysis, comparison of k values between different systems is only valid if the systems have the same and constant biomass concentration as was practiced in the present study (see below).

For constant biomass concentration and pseudo-first order TNT disappearance kinetics, the materials-balance equation for a completely mixed, continuous-flow reactor can be expressed as follows:

$$V\frac{dC}{dt} = Q(C_0 - C) - kCV, \quad (4)$$

where V is the reactor volume (l), Q the liquid flow rate (l/h), C_0, C the influent and effluent TNT concentration (mg/l), respectively. By integration of Eq. (4) (for $C = 0$ at $t = 0$), the following expression is obtained for the normalized effluent TNT concentration (i.e., C/C_0):

$$\frac{C}{C_0} = \frac{1 - e^{-(1/\theta+k)t}}{1 + k\theta}, \quad (5)$$

where $\theta (= V/Q)$ is the hydraulic retention time (h). Under transient conditions and without a reaction taking place (i.e., for a conservative substance, $k = 0$), Eq. (5) becomes:

$$\frac{C}{C_0} = 1 - e^{-t/\theta}. \quad (6)$$

Under steady-state conditions (i.e., when $t \rightarrow \infty$) Eq. (5) becomes:

$$\frac{C}{C_0} = \frac{1}{1 + k\theta}. \quad (7)$$

Eqs. (5)–(7) were used in the evaluation of the continuous-flow system performance (see below).

3. Results and discussion

3.1. Comparison of TNT transformation by *Anabaena* and heterotrophic bacteria

In order to quantify the relative contribution of *Anabaena* sp. and heterotrophic bacteria—developed in the two continuous-flow systems during the initial operating period during which neither of the reactors was amended with TNT—to the overall TNT transformation observed in the subsequent continuous-flow run, a batch TNT transformation assay was performed with the enriched bacterial culture and the mixed, *Anabaena*/bacteria culture taken directly from the continuous-flow system. Both cultures had the same initial biomass concentration and were not exposed to TNT before. Fig. 1A shows that although TNT depletion was observed in both cultures, it was slower in the bacteria-only culture. In contrast, the TNT concentration in the media control did not change significantly over the incubation period. The biological nature of the observed TNT disappearance in live *Anabaena* sp. cultures has been previously demonstrated [24]. The kinetics of TNT disappearance in both systems were pseudo-first order with respect to TNT concentration (Fig. 2). The pseudo-first order rate constant for the mixed culture was 1.84/h, compared to the bacteria-only culture rate constant of 0.06/h, a thirty-fold difference. However, when the relative biomass concentration of

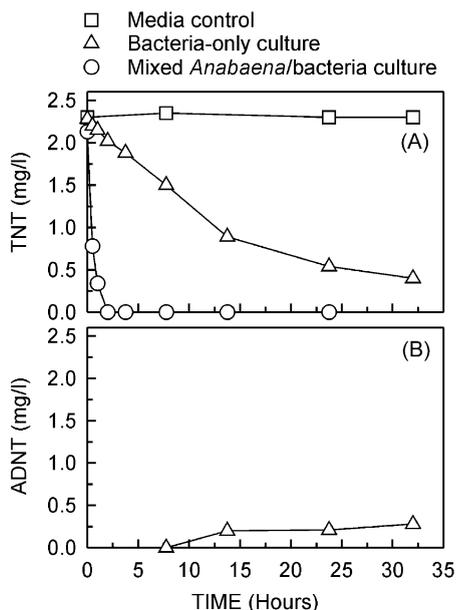


Fig. 1. TNT concentration in the media control, the bacteria-only culture and the mixed *Anabaena*/bacterial culture (A) as well as concentration of ADNT produced in the bacteria-only culture (B) over the incubation period.

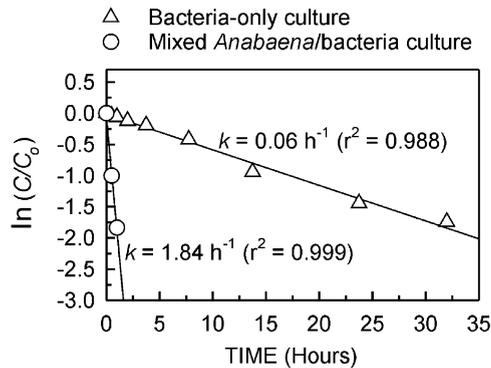


Fig. 2. Regression of normalized TNT concentrations vs. incubation time for the bacteria-only culture and the mixed, *Anabaena*/bacterial culture (initial biomass concentration equal to 30 mg POC/l for both cultures).

heterotrophs in the mixed *Anabaena*/bacteria culture was taken into account, the relative TNT disappearance due to the heterotrophic bacteria populations accounted for less than 1% of the observed overall TNT disappearance rate. These results suggest that although the *Anabaena* sp. culture was not axenic, based on the relative TNT disappearance rates, the observed TNT transformation was attributed mainly to the *Anabaena* sp.

Low levels (i.e., less than 0.1 mg/l) of azoxy-tetra-nitrotoluene isomers were detected in the media of the mixed, *Anabaena*/bacteria culture. In contrast, ADNT was formed in the bacteria-only culture and remained in solution at the conclusion of the experiment (Fig. 1B). In previously performed batch assays with the same *Anabaena* sp. cultures and an initial TNT concentration of 4.5 mg/l, formation and subsequent disappearance of two azoxy-tetra-nitrotoluene isomers (2-2' Azy-TeNT and 2-4' Azy-TeNT) was observed in the culture media when the culture pH ranged from 8.2 to 8.5. However, the only intermediate detected in the culture media of 5% CO₂/air mixed *Anabaena* sp. cultures with pH values ranging from 5.6 to 5.9 was HADNT, which required a significant incubation time for its complete removal [24].

3.2. Continuous-flow run

The continuous-flow experiment lasted 33 d. Throughout this experimental run, the pH and DO values in both the TNT-free (i.e., control) and the TNT-amended reactors varied from 6.7 to 7.3 and from 7.5 to 8.5 mg/l, respectively. TNT was introduced into the influent of one culture at a concentration of 1 mg/l initially and maintained at this level for over 400 h, or for more than three hydraulic retention times (Fig. 3A). The effluent TNT concentration was below the detection

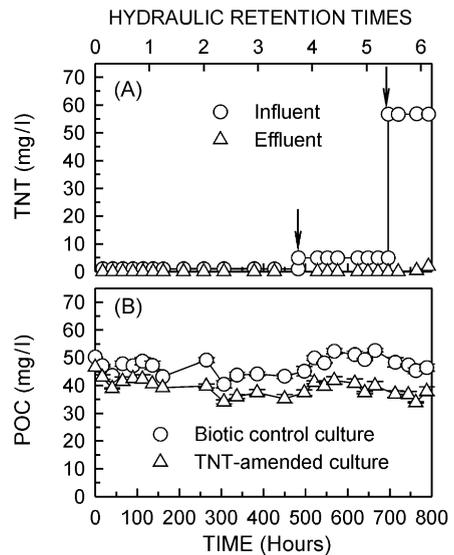


Fig. 3. Influent and effluent TNT concentrations in the TNT-amended system (A) and biomass concentrations (expressed as POC) in both the TNT-free, biotic control and the TNT-amended system (B) over the continuous-flow experimental period (error bars represent one standard deviation of the means) (arrows indicate step increases of the influent TNT concentration; see text).

limit (i.e., below 0.1 mg/l) throughout this period. The influent TNT concentration was then increased to 5 mg/l and maintained for over 200 h, during which period the effluent TNT concentration was again below the detection limit. Finally, the influent TNT concentration was increased to 58 mg/l (approximately half the aqueous solubility of TNT), in order to stress the system and achieve measurable concentrations of TNT and/or TNT transformation products in the effluent. Operation at an influent TNT concentration of 58 mg/l was maintained for approximately 94 h (approximately 0.7 hydraulic retention times) (Fig. 3A). Upon termination of the experiment, the effluent TNT concentration was 1.9 ± 0.4 mg/l (mean \pm standard deviation; $n = 3$).

Based on Eq. (6), for a conservative (non-reactive), completely mixed system operated with an influent concentration of 58 mg/l for 0.7 hydraulic retention times, the reactor TNT concentration should have been equal to 28.9 mg/l. However, the observed reactor (and effluent) TNT concentration was only 1.9 ± 0.4 mg/l which corresponds to a TNT removal of $96.7 \pm 1.7\%$ (mean \pm 95% confidence interval) (Fig. 4). According to Eq. (5), the simulated effluent TNT concentration matching the last measured mean TNT data value (see Fig. 4) corresponds to a pseudo-first order TNT transformation rate (k) of 0.22/h. Taking into account the upper and lower 95% confidence limits for the measured last TNT data value, the corresponding k

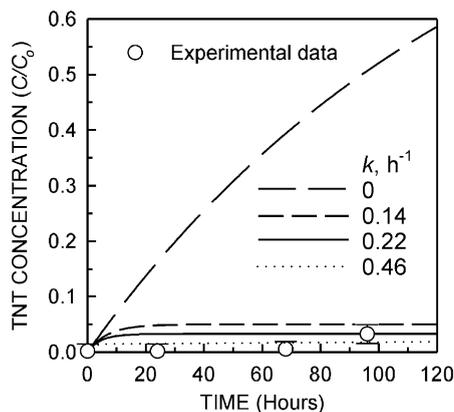


Fig. 4. Experimental data and simulated normalized effluent TNT concentration (C/C_0) over time for a range of pseudo-first order TNT transformation rate constant values (k) (error bars represent 95% confidence intervals) (see text).

values are 0.14 and 0.46/h, respectively (Fig. 4). As evidenced in Fig. 4, even with the lowest k value (0.14/h) the system reaches its steady-state performance in less than 40 h (i.e., in about 0.3 hydraulic retention times).

The k values obtained in previously performed batch assays at an initial TNT concentration range of 1–5 mg/l and with *Anabaena* concentrations similar to those maintained in the continuous-flow system, varied from 1.18 to 1.45/h [24]. It is noteworthy that these k values are higher than the values which correspond to the continuous-flow system performance (0.14–0.46/h) as discussed above. It appears that although toxicity leading to growth inhibition and/or culture chlorosis was not apparent in the continuous-flow culture (as discussed below), TNT and/or its transformation products may have resulted in some inhibition of the TNT transformation process as manifested by the lower k values.

As previously mentioned, the continuous-flow cultures were a mixture of *Anabaena* sp. and heterotrophic bacteria. However, based on the previously discussed TNT transformation kinetics and the population size of heterotrophs relative to that of *Anabaena*, less than 1% of the observed overall TNT transformation rate was attributed to the heterotrophic bacteria populations. It is noteworthy that in any full-scale remediation system, whether natural or engineered, growth of cyanobacteria and algae will undoubtedly occur with heterotrophic bacteria present as a result of a natural symbiosis between these organisms. Cyanobacteria and algae produce oxygen during the light period and exude organic compounds, which support the growth of heterotrophic bacteria. Bacteria, in turn, produce carbon dioxide, which is the carbon source for the autotrophic cyanobacteria and algae. Thus, the continuous-flow system used in the present study better

simulated the conditions of full-scale systems than would have been accomplished by an axenic cyanobacterial culture.

Fig. 3B shows the biomass concentration in both reactors. The mean \pm standard deviation biomass concentration was 47.1 ± 3.1 and 39.2 ± 3.1 mg POC/l in the biotic control and TNT-amended cultures, respectively. The observed fluctuations in biomass concentration in both reactors were attributed to the observed fluctuations in the influent flow rate. In spite of the increase of the influent TNT concentration over the run period, toxicity was not observed as judged by the relatively constant biomass concentration maintained throughout the run as well as the similar color intensity of both cultures (i.e., culture chlorosis was not observed). In contrast to these results, batch assays performed with the same *Anabaena* culture at a range of initial TNT concentrations demonstrated that TNT concentrations above 10 mg/l were toxic and resulted in culture chlorosis and death [24]. Growth inhibition and/or chlorosis have been observed for a number of freshwater algae at TNT levels in the range of 2.5–15 mg/l, different species having a different TNT threshold [10,11]. In a more recent study, 1.4 mg/l TNT resulted in culture chlorosis and 95% growth inhibition of the unicellular green algal species *Selenastrum capricornutum* [12]. Hydroxyl-, amino-, and hydroxylamino-intermediates resulting from the microbial transformation of TNT were less toxic to *S. capricornutum* than TNT [12]. The results of the present study demonstrate the TNT transformation effectiveness of the continuous-flow *Anabaena* culture at relatively high-influent TNT concentrations at which batch culture systems cannot be operated due to TNT toxicity.

Only TNT and traces (i.e., less than 0.1 mg/l) of azoxy-tetranitrotoluene isomers were detected in the effluent during the last test period. Using a solid-phase extraction procedure, triplicate culture samples taken from the TNT-amended continuous-flow reactor were extracted and analyzed by HPLC. The first sample set taken after 420 h, when the influent TNT concentration had been maintained at 1 mg/l for about three hydraulic retention times, did not result in the detection of TNT or TNT transformation products. The second culture sample set was taken at the conclusion of the experiment, when the effluent TNT concentration was 1.9 ± 0.4 mg/l. Taking into account the biomass wastage rate throughout this experiment, and based on the compounds detected in the biomass extracts, the following fractions of TNT and its transformation products were accounted for as biomass associated (values expressed as % of total TNT moles added to the system): ADNT, 0.1; TNT, 0.1; 2,2' Azy-TeNT, 5.9; 2,4' Azy-TeNT, 9.0; and 4,4' Azy-TeNT, 2.6; total, 17.7. Considering both the TNT concentration in the system effluent during the last test period, as well as the

biomass-associated TNT and its transformation products, 24.1% of the total TNT added to the system throughout this experiment was accounted for. The presence of azoxy derivatives in the biomass extracts in combination with the very low levels of the same compounds detected in the culture media indicates that the disappearance of the azoxy compounds from the liquid phase was associated with their binding on the biomass. Formation of relatively low levels of azoxy compounds in the continuous-flow system may be attributed to the circum-neutral pH conditions (pH values between 6.7 and 7.3) of this system.

The observed TNT intermediates in the continuous-flow culture media, as well as those found in culture extracts agree with the results of previously performed batch assays. The only compounds detected in extracts of batch *Anabaena* sp. cultures which transformed TNT were three azoxy-tetranitrotoluene isomers (2-2' Azy-TeNT, 2-4' Azy-TeNT, and 4-4' Azy-TeNT) which, when all combined, accounted for only 4.4% of the initial moles of TNT added to these cultures [24]. In another related study, aminodinitrotoluenes and lower levels of hydroxylaminodinitrotoluene and diamidinotrotoluenes were detected in the media of batch cultures of the aquatic plant *Myriophyllum spicatum*, but these compounds accounted for only 10–20% of the initial TNT mass added to the cultures. A very small fraction (less than 4%) of the initially added TNT moles could be extracted from the plant matrix as TNT and aminodinitrotoluene [23]. Aminodinitrotoluene isomers were detected in culture media and plant extracts of the aquatic plants *M. spicatum* and *M. aquaticum* used to assess the transformation of TNT, but accounted for less than 14% and 4% of the added TNT moles, respectively. About half of the label of the (ring-U-¹⁴C) TNT used in these experiments remained in the plant culture media in a soluble form while the balance, which was in the plant matrix, was made available only by the oxidation of the plant material [26,27].

It has been shown that several aerobic bacterial species possess reductive enzymatic systems which catalyze TNT ring hydrogenation (i.e., addition of a hydride ion to the aromatic ring), and form a hydride–Meisenheimer complex [5,6]. Further hydrogenation of this complex leads to the production of a dihydride–Meisenheimer complex of TNT [6]. These complexes are polar and are generally considered to be dead-end products in the productive breakdown of TNT. However, microbial ring hydrogenation of TNT under aerobic, mixed culture conditions and reductive denitration yielding biodegradable dinitrotoluenes should not be ruled out [28]. TNT transformation by the aquatic plant *M. aquaticum* resulted in the formation of products with significantly higher polarity and aqueous solubility than TNT [9]. In another study, during the transformation of TNT with *M. aquaticum*, significant

accumulation of oxidation products as a result of methyl oxidation and/or aromatic hydroxylation was observed [8]. Such findings, along with the possibility of uptake, partial mineralization and/or sequestration of TNT and its transformation products by *Anabaena* cells may explain the relatively low contaminant recoveries and mass balances observed in the present study.

4. Conclusions

A high TNT removal efficiency (> 96%) was achieved by the continuous-flow *Anabaena* sp. system at the highest influent TNT concentration of 58 mg/l used. In contrast to previously observed chlorosis and growth inhibition of batch *Anabaena* sp. cultures at initial TNT concentrations above 10 mg/l, a stable biomass concentration was maintained in the continuous-flow system operated with a hydraulic retention time equal to 136 h (ca. 5.7 d; corresponding to a specific growth rate of 7.35×10^{-3} /h). These results show that effective TNT removal without toxic culture effects is feasible by using a continuous-flow system. Contaminant recovery and mass balance showed that about 76% of the TNT mass added to the continuous-flow system was not accounted for in either the system effluent or the biomass.

The results of this study expand the relatively short list of xenobiotic compounds which are found to be transformed by cyanobacteria to include nitroaromatic compounds, and further demonstrate the biotransformation potential of this microbial group. However, further investigation aimed at the identification and characterization of TNT transformation intermediates and products, especially their potential toxicity, is necessary before the development of full-scale systems for the remediation of TNT-laden media.

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