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Ultraviolet Treatment and Biodegradation of Dibenzothiophene: Identification and Toxicity of Products

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Abstract

Biodegradation of pollutants often results in incomplete mineralization and formation of degradation products with unknown chemical and toxicological characteristics. Ultraviolet (UV) irradiation, a common technology used in water and wastewater treatment, may help reduce aqueous concentrations of degradation products produced during biological treatment and their associated hazard. Combined biological and UV transformations may be important in natural systems as well. We investigated the effects of UV irradiation (254 nm) on dibenzothiophene (DBT), a sulfur-containing polyaromatic hydrocarbon, in artificial seawater, and its effects on biodegradation products produced from mixed-community microbial transformations of DBT, including DBT sulfone, DBT sulfoxide, hydroxylated and carboxylated benzothiophenes, thiosalicylic acid, and others. Toxicity of solutions following UV exposure was monitored using bioluminescent bacteria (*Vibrio fischeri*) and by evaluating cardiac deformities in *Fundulus heteroclitus* embryos. The highest UV fluence reduced DBT concentration by 28% when DBT was present as the sole organic solute. In post-biodegradation solution, the same fluence reduced initial concentration of DBT by 81%, and 11 DBT biodegradation products to trace levels. Regardless of UV fluence, DBT by itself produced minimal effects in *Fundulus* embryos but was moderately toxic to *V. fischeri*. Post-biodegradation solutions were highly toxic to both test organisms. The highest UV fluence slightly reduced toxicity of post-biodegradation solution to *V. fischeri*, but exacerbated cardiac deformities in *Fundulus* embryos. Toxicity could not be attributed to specific products, and was likely a result of mixture effects. These results emphasize that toxicity can increase during remediation and that multiple assays may be necessary for evaluation. The novel approach of combined biodegradation/UV treatment is promising, although further research is needed to reduce toxicity in the case of DBT.

Keywords

Dibenzothiophene; Aerobic biodegradation; Toxicity; Ultraviolet treatment; *Fundulus heteroclitus*

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Supplemental Data, Figure S1

UV molar absorption spectrum of dibenzothiophene in artificial seawater medium (DBT solution) and the spectrum of relative intensity of UV light from the low-pressure mercury vapor UV lamp used for treating test solutions.

Introduction

Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by microorganisms can play an important role in natural and engineered environments. Sulfur-containing PAHs, such as the model compound dibenzothiophene (DBT), can be recalcitrant to biodegradation, and are not always completely mineralized, resulting in formation of numerous degradation products whose characteristics and fates are poorly understood. Successful remediation requires a timely reduction in hazard, which is not always achieved with bioremediation alone. Several bench-scale and field studies have reported transient or lasting increases in toxicity, an indicator of hazard, with PAH biodegradation that were often attributed to degradation products [1–3].

Improving bioremediation of recalcitrant compounds may require the development of novel strategies, including combining biodegradation with a second treatment step, ultraviolet (UV) treatment, which is commonly used for disinfection in drinking water and wastewater treatment systems, may be an effective means to break down original contaminants and degradation products remaining after biodegradation. Little is known about UV transformations of contaminants in natural systems, such as contaminated estuaries. Most studies exploring combined UV and biodegradation employ UV as a pretreatment intended to enhance bioavailability and facilitate microbial attack [4–7], however, these studies have yielded mixed results. Research by Guieysse and Viklund [6] showed complete removal of several PAHs including benzo[*a*]pyrene by dissolving the compounds in organic solvent for UV irradiation followed by transfer into silicone oil for biodegradation in a two-phase reactor. Lehto et al. [4] evaluated UV irradiation of individual and mixed PAHs (anthracene, pyrene, benzo[*a*]anthracene, and dibenz[*a,h*]anthracene) as well as creosote in deionized water at saturation followed by degradation by an enrichment culture derived from creosote-contaminated sediment. Ultraviolet pretreatment enhanced biodegradation of individual PAHs, except for dibenz[*a,h*]anthracene, had no effect on biodegradation of the PAH mixture, and reduced biodegradation of PAHs in creosote compared to biodegradation without UV. Few studies have investigated UV treatment following biodegradation as a sequential treatment process. Karetnikova et al. [8] found that biodegradation by *Penicillium tardum* strain H-2 eliminated the test contaminant *p*-cresol while subsequent UV irradiation from a high-pressure mercury lamp greatly reduced its biodegradation products.

This study investigates application of UV treatment following biodegradation of DBT by a mixed microbial culture under the hypothesis that UV treatment may reduce the concentration of DBT and its degradation products remaining after biodegradation, and subsequently reduce residual toxicity. This information is relevant both to the development of new processes for bioremediation, and for a better understanding of transformation in natural systems. One objective of the present study was to compare the effectiveness of UV light in reducing DBT levels in both an aqueous solution of pure DBT, and in media from a mixed microbial culture following DBT biodegradation. A second objective was to determine how UV changes the toxicity of DBT and biodegradation product solutions, using a bioluminescent bacterial assay (a non-specific acute toxicity screening test) and an assay that evaluates cardiac development in *Fundulus heteroclitus* embryos. The final objective of the study was to identify products formed by UV treatment of DBT and compare their trends to products formed via biodegradation alone. By combining chemical analyses with toxicity assays, this research addresses two potentially limiting factors affecting the success of remediation techniques: contaminant reduction and hazard amelioration.

Materials and Methods

Microbial culture care and preparation of test solutions

Biodegradation of dibenzothiophene (DBT, Sigma-Aldrich, USA) was conducted in the dark using a microbial enrichment culture established from creosote-contaminated estuary sediment located near the Atlantic Woods, National Priorities List Site near Portsmouth, Virginia, USA. The culture was maintained in artificial seawater (22.22 g L⁻¹ Instant Ocean™) supplemented with 1 g L⁻¹ NH₄NO₃, 0.2 g L⁻¹ K₂HPO₄, and 0.05 g L⁻¹ FeCl₃·6H₂O (adapted from Chang et al. [9], and Kasai et al. [10]) and adjusted to pH 7.5. All test solutions in this study were based on the artificial seawater media. Effect of UV treatment was evaluated in three test solutions: freshly made culture containing no DBT or DBT degradation products (*Control solution*); freshly made culture containing 1.5 μmol L⁻¹ DBT in the artificial seawater media (*DBT solution*), and culture media from the DBT-degrading microbial community collected at 4, 6, 8, 10, 12, and 16 d after inoculation, combined and stored in amber glass bottles the dark at -40°C until use (up to 3 months) (*Post-biodegradation solution*). All solutions were adjusted to pH 7.55 and filtered through 0.2 μm polycarbonate membranes to ensure solutions contained no particulates or microbial cells.

UV exposures

Monochromatic UV irradiation at 254 nm was produced with a low-pressure (LP) mercury vapor germicidal lamp (G15T8 USHIO ozone free) using the apparatus described by Sharpless and Linden [11]. Ultraviolet spectra of the lamp and DBT are provided in the Supplemental Data, Figure S1. Ultraviolet fluences of 0, 500, 1250, and 2000 mJ cm⁻² were applied, based on exposure time, incident UV irradiance at the surface of the sample, average irradiance over the entire volume of the sample, and initial UV absorbance of the sample measured using a Cary Bio100 spectrophotometer (Varian, USA) [11].

Ultraviolet exposures were performed on 85 ml of test solution stirred gently in an uncovered 70 × 50 mm Pyrex® glass crystallization dish. Samples receiving 0 mJ cm⁻² were prepared in the same manner and placed in the apparatus for five minutes in the dark to serve as UV dose controls. All test solution-UV fluence combinations were repeated in triplicate. After exposure, the sample was immediately removed from the UV apparatus, 1.8 ml was transferred to a glass vial for toxicity assays using bioluminescent bacteria *Vibrio fischeri*, 15 ml was placed in an amber glass bottle for liquid-liquid extraction of DBT and degradation products, and the remaining solution was reserved in a separate amber glass bottle for toxicity assays using *Fundulus heteroclitus* embryos. Aliquots taken for toxicity assays were immediately frozen at -40°C until analysis. Aliquots taken for extraction were acidified to pH < 2 with 6 mol L⁻¹ HCl and extracted three times with dichloromethane (DCM) on and end-over-end shaker in the dark.

Sample preparation and analysis

For each sample, the extracted DCM fractions were combined and concentrated to 200 μl using rapid evaporation (Turbo Vap, Zymark, USA) followed by gentle evaporation under N₂. Half (100 μl) of the extract was derivatized with 0.5 ml ethereal diazomethane solution for 2 h at 4°C in the dark, evaporated to dryness under N₂, and resuspended in 100 μl DCM. To both derivatized and underivatized samples, 10 μl of 200 μmol L⁻¹ 2-naphthol (Sigma-Aldrich, USA) in dichloromethane (DCM) was added as an internal standard, and extracts were analyzed for aqueous DBT, along with DBT degradation products, by gas chromatography with mass spectrometry (Agilent 6890) in electron impact (EI) mode using splitless injection (300°C). Separation of analytes was achieved on a DB-XLB column (30

m, 250 µm nominal diameter, 0.25 µm film thickness; J&W Scientific) using a thermal gradient.

Structures, identification support and references, and identifying ions used in GC/MS analyses of DBT and DBT degradation products are presented in Table 1. References to structure numbers are presented in bold italics; e.g., DBT (**11**). Molecular ions of analytes were confirmed by either negative or positive chemical ionization GC/MS using the conditions listed above for EI mode. Identification and concentration of DBT and several degradation products were determined using authentic standards: DBT sulfone (**14**) (Aldrich), benzothiophene-2,3-dione (**6**) (Ryan Scientific, USA), thiosalicylic acid (**4**) (Aldrich, detected as a methyl ester after derivatization), dithiosalicylic acid (**16**) (Alfa Aesar, Ward Hill, MA, detected as a dimethyl ester after derivatization), and thioindigo (**15**) (TCI America, Portland, OR). With the exceptions of two potential degradation products (**10** and **17**), all DBT degradation products were identified by comparison to an authentic standard, using spectral libraries [12], and/or by comparison to reference spectra from previously published research. Concentration of 2,3-dihydroxybenzothiophene (**7**) was determined semi-quantitatively based on benzothiophene-2,3-dione (**6**). Amounts of all other products were reported as relative response (RR) of peak area for the appropriate quantification ion compared to that of the internal standard 2-naphthol (**5**).

Toxicity to *V. fischeri* and *F. heteroclitus* embryos

Two assays were used to evaluate the effects of UV fluence on toxicity. First, toxicity was assessed as inhibition of luminescence in the bioluminescent bacterium *Vibrio fischeri* (also known as *Photobacterium phosphoreum*), strain NRRL B-11177 (ATCC, Manassas, VA, USA), using a method adapted from McConkey et al. [13], and was similar to other toxicity tests often used to screen soil and water (e.g., Microtox™, Strategic Diagnostics, USA [14, 15]). Briefly, bacterial cells were harvested at 3 d after inoculation, centrifuged for 5 min at 5000 × g, the supernatant was removed, and the cells were resuspended in chilled 2% w/v NaCl to an optical density of 0.82 to 0.86 at 600 nm. Cell suspension was aliquoted to a polystyrene 48-well plate (0.5 ml per well), the plate was incubated in the dark for 10 min at 15° C, and luminescence was measured using a FLUOStar Optima plate reader (BMG LabTech, USA). A 0.5 ml aliquot of each test solution, adjusted to pH 7.5, was added to the wells, incubated for 30 min in the dark at 15°C, and luminescence was measured again. The toxicity of each sample and control was expressed as percent inhibition of luminescence calculated as [13]:

$$\% \text{ Inhibition of luminescence} = \left(1 - \frac{L_f \times C_i}{L_i \times C_f} \right) \times 100$$

where L_i and L_f are initial and final luminescence, respectively, of *V. fischeri* exposed to test solutions, and C_i and C_f are initial and final luminescence, respectively, of *V. fischeri* in assay controls.

A second test, described in Matson et al. [16], was included using *Fundulus heteroclitus* (killifish) embryos to evaluate effects on cardiac development in an aquatic vertebrate. Adult killifish were collected at an uncontaminated reference site on King's Creek in southeastern Virginia (37°17'52.4''N, 76°25'31.4''W) and cared for and spawned as described in Matson et al. [16]. Normal embryos at 24 h post-fertilization were selected for the assay. Each embryo was placed in 5 ml of the test solution in a 20 ml glass scintillation vial and incubated at 28.5°C. For each sample, 10 embryos were dosed, and scored at 7 d after fertilization, or 6 d after dosing. Observed defects were scored 0 (normal), 1 (mild deformities), or 2 (severe deformities). The cardiac defect scoring scale correlates to

hatching success, with a score of 2 indicating no hatching, and a score of 1 indicating approximately 40 to 60% reduction in hatching [16].

Statistical analyses, including linear regression used to evaluate phototransformation rates and analysis of variances used for treatment comparisons, were performed using JMP 7.0 software [17].

Results

UV effects on DBT and its biodegradation products

Trends of DBT and its biodegradation products in UV irradiated test solutions are shown in Figure 1. Figure 1 panels are organized by compound number, given in bold italics, as given in Table 1. The effects of UV fluence on DBT were evaluated in DBT-saturated fresh artificial seawater media (DBT solution) and in media collected periodically during DBT biodegradation by a mixed microbial community (Post-biodegradation solution) (Fig. 1j). Initial concentrations of DBT in the DBT and Post-biodegradation solutions were $1.49 \pm 0.13 \mu\text{mol L}^{-1}$ and $0.24 \pm 0.03 \mu\text{mol L}^{-1}$, respectively. In both solutions, DBT concentrations decreased with increasing UV fluence, the highest of which (2000 mJ cm^{-2}) reduced initial DBT concentration by 28% ($1.08 \pm 0.23 \mu\text{M}$ final concentration) in DBT solution and 81% ($0.045 \pm 0.016 \mu\text{mol L}^{-1}$ final concentration) in Post-biodegradation solution (Fig. 1j). This suggests that degradation of DBT by UV irradiation alone would require extensive dosing, which may be impractical for contaminant treatment.

Fifteen DBT degradation products were monitored in all test solutions after treatment with UV (Fig. 1). The degradation products monitored included benzoic acid (**1**), 2-hydroxybenzothiophene (**2**), 3-hydroxybenzothiophene (**3**), thiosalicylic acid (**4**), benzothiophene-2,3-dione (**6**), 2,3-dihydroxybenzothiophene (**7**), benzothiophene-3-carboxylic acid (**8**), 3-hydroxybenzothiophene-2-carbaldehyde (**9**), 3-hydroxy-1-benzothiophen-2-one (**10**), benzothiophene-2,3-dicarboxylic acid (**12**), DBT sulfone (**13**), DBT sulfoxide (**14**), thioindigo (**15**), dithiosalicylic acid (**16**), and an unknown product with $M^+ 284 m/z$ (**17**). Note that values for compounds for which no authentic standard was available were reported as relative response (RR), determined as the response of the quantitative ion of the compound of interest to that of the internal standard for GC/MS analyses, 2-naphthol (**5**).

Of the 15 products monitored, only 4 were observed in the DBT solution following UV treatment (Fig. 1, empty squares): 2- and 3-hydroxybenzothiophene (**2**, **3**), 2,3-dihydroxybenzothiophene (**7**), and DBT sulfoxide (**14**). Of these, only DBT sulfoxide (**14**) has been previously reported as a product of DBT desulfurization by UV light [18]. Shiraishi et al. [18] also reported DBT sulfone (**13**) as a DBT photoproduct, but this product was not observed in the current study. Maximum levels of the hydroxylated benzothiophenes (**2**, **3**, **7**) were observed at 500 mJ cm^{-2} UV fluence (Fig. 1b, c, and f), suggesting that ring cleavage of DBT was achievable at the lower fluence tested. The concentrations of these compounds decreased at higher fluences, suggesting they were photodegraded. Photooxidation of sulfur forming DBT sulfoxide (**14**) increased with increasing fluence (Fig. 1m). For DBT sulfoxide and some other DBT degradation products, appropriate standards needed to calculate actual concentrations were not commercially available. Because actual concentrations could not be determined for DBT sulfoxide (**14**) or the hydroxybenzothiophenes (**2**, **3**), a mass balance of phototransformed DBT could not be performed, and it is possible that other photoproducts were formed but not detected in GC/MS analyses.

Trends of DBT degradation products in the UV-treated Post-biodegradation solution, shown as solid lines in Figure 1, fell into three general categories increasing in complexity: consistently increasing or decreasing concentrations with increasing UV fluence, net formation at lower fluence followed by net loss at higher fluence, and alternating increasing and decreasing concentrations with each fluence in more complex trends than in the second category mentioned above. Increasing amounts of a compound indicated net formations, while decreasing concentrations indicated net losses, since simultaneous formation and loss may have been occurring. For most monitored compounds, it was not possible to confirm identities of precursors and photoproducts to establish photolytic pathways, particularly in the Post-biodegradation solution due to its complexity.

Benzothiophene-2,3-dicarboxylic acid (**12**) and the unidentified product with M^+ 284 m/z (**17**) decreased with increasing fluence, with the highest fluence reducing initial levels of benzothiophene-2,3-dicarboxylic acid (**12**) by 29% and the M^+ 284 m/z product by 98% (Fig. 1k, p). Benzoic acid (**1**), 3-hydroxybenzothiophene (**3**) and thioindigo (**15**) increased up to 1250 $mJ\ cm^{-2}$, but they decreased to zero or near initial concentrations at the highest fluence (Fig. 1a, c, and n). Neither benzoic acid (**1**) nor hydroxybenzothiophenes (**2**, **3**) have been reported as photoproducts of DBT or any of its biodegradation products, and the mechanisms of their formation remain unknown. Thioindigo (**15**) is thought to be formed from two molecules of 3-hydroxybenzothiophene-2-carbaldehyde (**8**) [19], which was present in the Post-biodegradation solution, however, the mechanism of this reaction is not known and the role of UV light in the formation of thioindigo formation from 3-hydroxybenzothiophene-2-carbaldehyde (**8**) or other products observed in the Post-biodegradation solution has not been previously reported.

The majority of the DBT degradation products exhibited alternating net loss or formation with increasing fluence. For example, benzothiophene-2,3-dione (**6**) decreased 71% from an initial concentration of $4.1 \pm 1.04\ \mu mol\ L^{-1}$ to $1.17 \pm 0.07\ \mu mol\ L^{-1}$ at the lowest UV fluence (500 $mJ\ cm^{-2}$, indicating net photolysis (Fig. 1e), then increased to $19 \pm 4.05\ \mu mol\ L^{-1}$ (363% of initial concentration) at a fluence of 1250 $mJ\ cm^{-2}$, and finally decreased to $0.66 \pm 0.07\ \mu mol\ L^{-1}$ at the highest fluence (2000 $mJ\ cm^{-2}$). Other compounds followed similar trends, including DBT sulfone (**14**), DBT sulfoxide (**13**), 2-hydroxybenzothiophene (**2**), 2,3-dihydroxybenzothiophene (**7**), 3-hydroxy-1-benzothiophen-2-one (**9**) and 3-hydroxy-1-benzothiophene-2-carbaldehyde (**8**) (Fig. 1). Thiosalicylic acid (**4**) and dithiosalicylic acid (**16**), showed an equally complex but opposite trend of net formation at both the lowest and highest fluences, and net loss at 1250 $mJ\ cm^{-2}$ (Fig. 14, o). Because the net rate of transformation of a given contaminant was dependent on the rates of formation (which was likely a function of precursor concentrations, as well as fluence) and degradation (which was likely dependent on the contaminant's concentration, as well as fluence), the system dynamics were complicated and so such complex behaviors were not surprising.

Toxicity to *V. fischeri* and *F. embryos*

Effects of UV on the toxicity of the test solutions to bioluminescent bacteria *Vibrio fischeri* and *Fundulus* embryos are shown in Figure 2. The Control solution (i.e., freshly made artificial seawater media) elicited no toxic response in either assay for all fluences tested. Dibenzothiophene solution at all fluences produced mild toxic responses in *Fundulus* embryos, but these responses were not statistically different from those observed with the Control solution. In *V. fischeri*, however, the DBT solution caused 21 to 25% inhibition of luminescence after treatment with 0 to 1250 $mJ\ cm^{-2}$ UV. A statistically significant ($p < 0.05$) increase in toxicity to the bacteria resulted from treatment of the DBT solution with the highest UV fluence. The DBT concentration decreased at this fluence (Fig. 1j), indicating that DBT photoproducts may have caused the increased toxicity to *V. fischeri*. Of the products that were identified in the DBT solution after UV exposure, DBT sulfoxide (**13**)

was the only one exhibiting maximal levels at the highest UV fluence, and therefore was the most likely of these to have raised toxicity [20, 21].

Of the test solutions, the Post-biodegradation solution was most toxic overall to both *V. fischeri* and *Fundulus* embryos. Untreated Post-biodegradation solution produced moderately severe deformities (score of 1.54) in *Fundulus* embryos. Deformity scores increased with increasing UV fluence, reaching 1.97 (out of a maximum of 2) at the highest fluence. Based on this assay, UV treatment exacerbated hazard already attributable to the Post-biodegradation solution, and therefore was not a successful post-biodegradation treatment step. *Vibrio fischeri* responded differently to UV-treated Post-biodegradation solution. Inhibition of luminescence was increased from 37.9% in untreated media to 42.2% at the lowest UV fluence. Toxicity to the bacteria decreased with higher fluences resulting in 32% inhibition of luminescence at 2000 mJ cm⁻², a small but significant ($p < 0.05$) reduction in toxicity compared to the untreated Post-biodegradation solution.

Discussion

Photolysis of DBT and its biodegradation products have been little studied, particularly in seawater. Shemer and Linden [22, 23] observed 23% loss of initial DBT concentration of 2 μ M at 1000 mJ cm⁻¹ (254 nm) in a natural estuary water, results similar to those from the current study in which 72% of initial DBT remained even after the highest UV fluence. These results demonstrate that UV alone would not be an efficient way to reduce contaminant levels. Higher doses may be impractical for treatment due to the energy requirements. Although not a focus of the current study, it may be possible to enhance photooxidation by combining UV with oxidants such as H₂O₂ [22].

Dibenzothiophene photodegradation by UV at 254 nm in the DBT solution yielded DBT sulfoxide (**13**) along with hydroxylated benzothiophenes (**2**, **3**, **7**). Little research has explored products of DBT photolysis at UV 254 in artificial seawater, however, DBT sulfoxide (**13**) has been reported as a photodegradation product in seawater exposed to natural sunlight [24, 25] and in engineered biphasic systems after DBT exposure to high-pressure Hg lamps (>280 nm) [18]. Previous research has also reported DBT sulfone (**14**), benzothiophene-3-carboxylic acid (**9**) and benzothiophene-2,3-dicarboxylic acid (**12**), 2-sulfobenzoic acid, 2-hydroxybiphenyl-2'-sulfinic acid sultine, thiophene carboxylic acids, 5-sulfoisophthalic acid, and acetylbenzothiophenecarboxylic acid as photoproducts of DBT [18, 25–27], yet none of these products was observed in the DBT solution at any fluence, for reasons that are not apparent. Hydroxylated benzothiophenes have not been previously reported as DBT photodegradation products for any light source, yet they were identified in this experiment. Differences in DBT photoproducts observed in the present study compared to those observed in previous research may reflect differences in the chemical composition of the solutions in which DBT was dissolved. For example, the artificial seawater used in this study contained nitrate and Fe, which can contribute to indirect photolysis of aromatic compounds [28, 29], but these were not components of solutions investigated in previous studies [18, 24]. It is unlikely that biodegradation concurrent with UV exposure contributed to the photoproducts observed given that all solutions were filtered through 0.2 μ m, and preliminary trials indicated no significant change in solution composition for solutions incubated in the dark for the lengths of time required for all UV doses.

Even prior to UV treatment, the Post-biodegradation solution was chemically complex, containing a suite of DBT microbial degradation products (Table 1) and inorganic ions from the culture media. Products that showed net formation at any UV dose included DBT sulfone, (**14**), DBT sulfoxide (**13**), benzoic acid (**1**), hydroxybenzothiophenes (**2**, **3**, **7**), benzothiophene-2,3-dione (**6**), thiosalicylic acid (**4**), dithiosalicylic acid (**16**), thioindigo (**15**)

and 3-hydroxybenzothiophene-2-carbaldehyde (**9**) (Fig. 1). As mentioned above, DBT sulfoxide (**13**), DBT sulfone (**14**), benzothiophene-3-carboxylic acid (**9**) and benzothiophene-2,3-dicarboxylic acid (**12**) have been previously reported as photoproducts of DBT [18, 25–27], suggesting that DBT may have been a precursor to these products observed in the Post-biodegradation solution, although they were not observed in the DBT solution that was not biologically treated. Photodegradation pathways may share similarity to established DBT biodegradation pathways [19, 30]. In this case, for example, benzothiophene-2,3-dicarboxylic acid (**12**) may have yielded 2,3-dihydroxybenzothiophene (**7**), which may have subsequently been transformed to 2- and 3-hydroxybenzothiophene (**2,3**), as well as thiosalicylic acid (**4**), which in turn formed dithiosalicylic acid (**16**). However, the chemical complexity of the Post-biodegradation made confirmation of photodegradation pathways problematic. Because preliminary investigations showed no change in solutions incubated in the dark as described above, it is unlikely that changes in UV-exposed DBT degradation products were due to biological activity.

One measure of the effectiveness of UV as a remediation treatment is its ability to reduce toxicity, however, this measurement can depend on the assay applied. For example, the *V. fischeri* assay reflects a general effect on the bacterium's metabolism, while the *Fundulus* embryo assay specifically addresses cardiac deformities, which might not reflect all toxic effects to the embryo. *Fundulus* embryos appeared to be little affected by DBT alone in the DBT solution, regardless of UV treatment (Fig. 2a), consistent with results by Wassenberg et al. [31]. *Vibrio fischeri*, however, showed considerable toxicity from DBT itself at levels comparable to previously reported values [22], and in this case toxic effects increased at higher UV fluences (Fig. 3b). Based on the *V. fischeri* assay, UV treatment appeared to be counterproductive with respect to the remediation goal of toxicity reduction. The elevated toxicity to *V. fischeri* observed in DBT solution at the highest UV dose may have been related to formation of DBT sulfoxide (**13**), which has been previously shown to be toxic (half maximal inhibitory concentration (IC₅₀) 10 μ M) to *V. fischeri* [20] as well as some plant species [21].

Unlike the DBT solution, the Post-biodegradation solution was very toxic to both *V. fischeri* and *Fundulus* embryos (Fig. 2). However, UV treatment of the Post-biodegradation solution produced different toxicity trends in the two organisms. Whereas UV treatment produced a net decrease in toxicity at the highest fluence in *V. fischeri*, increasing scores of cardiac deformity were observed with increasing UV fluence in the fish embryos (Fig. 2a). Based on a previously-reported correlation between hatching success and deformity scores in the *Fundulus* embryo assay [16], roughly 65% of embryos exposed to untreated (i.e., 0 mJ cm^{-2} UV) Post-biodegradation solution would not be expected to hatch, while no successful hatching would be expected in Post-biodegradation solution treated at the highest UV fluence. From a remediation perspective, both biodegradation and post-biodegradation UV treatment could present a greater problem than the original contaminant DBT if measures to prevent exposure to humans and wildlife were not implemented.

While it is difficult to definitively identify the toxic components in the Post-biodegradation solution because of its chemical complexity, comparison of trends in toxicity to those of DBT and DBT degradation products can provide some candidates. For example, the most extreme toxicity to the embryos exposed to Post-biodegradation solution occurred at the highest UV fluence, which also resulted in nearly complete losses of 12 of the 15 compounds monitored (Fig. 1). The remaining compounds may have contributed to the increased toxicity: benzothiophene-2,3-dicarboxylic acid (**12**), thiosalicylic acid (**4**), and dithiosalicylic acid (**16**). Formation of new photoproducts was not apparent, since chromatograms of UV-treated Post-biodegradation solution did not show new peaks not present in untreated Post-biodegradation solution. The toxicity of benzothiophene-2,3-

dicarboxylic acid (**12**) is unknown. Previous studies have reported low in vivo and in vitro toxicity of thiosalicylic acid (**4**) and dithiosalicylic acid (**16**) in deer mice (*Peromyscus maniculatus*) and house mice (*Mus musculus*) [32], and murine and human kidney cells [33]. None of these studies, however, assayed aquatic vertebrates. It is possible that toxic photoproducts may have formed in the Post-biodegradation solution but were not detected. It is also possible that changes in toxicity were the results of degradation products mixture effects. Overall, results from the current study indicate that post-biodegradation UV treatment may not reduce substantially toxicity even when monitored biodegradation products are reduced.

Conclusions

Post-biodegradation UV irradiation of the medium from a DBT-degrading mixed culture significantly reduced residual DBT and 11 monitored DBT degradation products at the highest UV fluence. However, amelioration of initial toxicity did not accompany loss of degradation products. To the contrary, although high UV fluence did decrease toxicity to *V. fischeri*, high UV fluence exacerbated toxic effects in *Fundulus* embryos. Since reduction of both contaminants and toxicity is a goal of remediation, these findings stress the importance of monitoring toxicity when evaluating potential remediation strategies. The two toxicity assays in this study (*Vibrio fischeri* bioluminescence assay and *Fundulus* embryo deformity assay) yielded assay-dependent trends for both DBT in artificial seawater media and medium from the degradation culture, supporting the use of multiple assays in monitoring toxicity when evaluating remediation approaches. Degradation of DBT in artificial seawater media from UV exposure yielded hydroxybenzothiophenes not previously reported as photodegradation products. This research contributes to fundamental knowledge of phototransformation of condensed thiophenes as well as to the development of novel remediation strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Effect of ultraviolet (UV) fluence on dibenzothiophene (DBT) and DBT biodegradation products in DBT and Post-biodegradation solutions. Numbers in bold italics refer to entries in Table 1. Compounds are presented in order of their listing in Table 1. Relative responses were determined using 2-naphthol (**5**) as an internal standard. Graph letters are starred (*) for products that were only observed in the Post-biodegradation solution. Arrows point to y-axis associated with the data series. Error bars are standard deviations from three replicates.

Figure 2.

Toxicity assessed as (a) average deformity in *Fundulus* embryos and (b) inhibition of luminescence in *Vibrio fischeri* for Control solution, dibenzothiophene (DBT) solution ($1.5 \mu\text{mol L}^{-1}$ DBT) and post-biodegradation solution following treatment of the test solutions with low pressure ultraviolet (UV) (254 nm) at fluences of 0, 500, 1250, and 2000 mJ cm⁻². *Fundulus* embryos were dosed with test solutions 24 h post-fertilization and scored 6 d after dosing. Scoring scale: 0 = normal; 1 = mild deformities; 2 = severe deformities. Error bars are standard deviations. For a given assay-solution combination, bars labeled with the same bold italicized letter are not significantly different at $p < 0.05$ in analysis of variance comparisons (Fisher's least significant difference test).

Table 1

Structures and gas chromatography/mass spectrometry identification parameters of compounds identified in extracts of the Post-biodegradation solution collected from a microbial enrichment culture degrading dibenzothiophene, arranged in order of chromatographic retention time. Asterisks (*) indicate the most abundant degradation products based on relative responses of quantifying ions to that of the internal standard, 2-naphthol (5).

Peak	Compound Name	Structure	Identifying Ions ^a	Retention Time ^b (min)	Reference / ID support
1	benzoic acid ^c		136 ^a , 105 ^a , 77	6.666	comparison to authentic standard
2 *	2-hydroxybenzothiophene		150, 121	12.308	[34]
3 *	3-hydroxybenzothiophene		150, 121	12.682	[34]
4	thiosalicylic acid ^c		168, 136, 108	13.272	comparison to authentic standard

Peak	Compound Name	Structure	Identifying Ions ^a	Retention Time ^b (min)	Reference / ID support
5	2-naphthol (internal standard)		144, 115	14.177	comparison to authentic standard
6 *	benzothiophene-2,3-dione		164, 136, 108	14.707	comparison to authentic standard
7 *	2,3-dihydroxybenzothiophene		166, 136, 108	15.64	[35]
8	3-hydroxy-1-benzothiophene-2-carbaldehyde		178, 177, 121	15.65	[36]
9	benzothiophene-3-carboxylic acid		178, 161	17.37	NIST ^d Mass Spectral Library [19]: 91% match

Peak	Compound Name	Structure	Identifying Ions ^a	Retention Time ^b (min)	Reference / ID support
<i>10</i> *	3-hydroxybenzothiophen-2-one		166, 150, 120, 136	17.656	[12]
<i>11</i>	dibenzothiophene (DBT)		184, 139	19.159	comparison to authentic standard

Peak	Compound Name	Structure	Identifying Ions ^a	Retention Time ^b (min)	Reference / ID support
12	benzothiophene-2,3-dicarboxylic acid, dimethyl ester ^c		250, 219, 185	21.39	[37]
13	dibenzothiophene sulfone		216, 187, 168	23.042	comparison to authentic standard

Peak	Compound Name	Structure	Identifying Ions ^a	Retention Time ^b (min)	Reference / ID support
14	dibenzothiophene sulfoxide		<u>200</u> , <u>184</u> , <u>172</u>	23.062	[30]
15	thioindigo		<u>296</u> , <u>240</u> , <u>120</u>	25.479	comparison to authentic standard
16	dithiosalicylic acid ^c		<u>334</u> , <u>167</u>	26.102	comparison to authentic standard
17	Unknown: C ₁₅ H ₈ O ₄ S		<u>284</u> , <u>228</u> , <u>184</u>	26.138	[12]

^aMolecular ions (M⁺) are italicized; quantifying ion is underlined.

^bAnalytes separated on DB-XLB column (30 m, 250 μm nominal diameter, 0.25 μm film thickness; J&W Scientific)

^cCompounds detected as methyl esters after derivatization with diazomethane.

^dNIST: National Institute of Science and Technology.