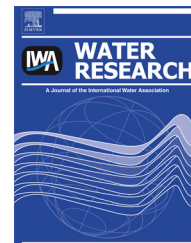


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Comparisons between abiotic nitration and biotransformation reactions of phenolic micropollutants in activated sludge

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ABSTRACT

The transformation of selected phenolic substances was investigated during biological wastewater treatment. A main emphasis was put on the relevance of abiotic processes leading to toxic nitrophenolic transformation products (TPs). Due to their environmental relevance, the antiseptic *ortho*-phenylphenol (OPP), the plastics additive bisphenol A (BPA) and the psychoactive drug dextrorphan have been studied. Batch experiments confirmed that nitro- and nitroso-phenolic TPs can be formed under acidic conditions when nitrite is present. HNO_2 , N_2O_3 and $\cdot\text{NO}$ and $\cdot\text{NO}_2$ radicals are likely involved in the abiotic process. It was found that the process was promoted by the freezing of water samples, since this can lead to an unexpected pH drop. However, under conditions present at wastewater treatment plants (neutral pH, low nitrite concentrations), the formation of appreciable concentrations is rather unlikely through this process, since HNO_2 concentrations are extremely low and $\cdot\text{NO}$ and $\cdot\text{NO}_2$ radicals will also react with other wastewater constituents. Thus, the transformation of phenolic substances such as OPP and BPA is mainly caused by biotic transformation. In addition to hydroxylation as a common reaction under aerobic conditions, the formation of sulfate conjugates was detected with the original compounds as well as with nitrophenolic TPs. Therefore, even when nitro-phenolic substances are formed it is likely that they are further transformed to sulfate conjugates. In raw wastewater and WWTP effluent nitrated BPA and NO_2 -dextrorphan were not detected. Only nitro-OPP was found in the influent of a WWTP with 2.3 ng/L, but it was not identified in the WWTP effluents. The concentrations of dextrorphan increased slightly during WWTP passage, possibly due to the cleavage of the glucuronide-conjugate, its human metabolite form, or demethylation of the prodrug dextromethorphan.

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1. Introduction

An important source of micropollutants in surface waters is municipal or industrial wastewater, which is usually emitted via wastewater treatment plants (WWTPs) into rivers and

streams. During wastewater treatment, biological and chemical processes intended for nutrient removal and the removal of easily biodegradable organic compounds may additionally transform refractory micropollutants. As a consequence, transformation products (TPs) of micropollutants are formed

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and emitted via WWTP effluents into the aquatic environment. Micropollutants containing phenol moieties have received particular attention in this regard, both due to the range of transformation processes which befall many phenols during wastewater treatment (Beel et al., 2013; Chen et al., 2011; Quintana et al., 2005; Skotnicka-Pitak et al., 2008) and their potential for having toxic effects on aquatic organisms, including antibacterial and endocrine disrupting properties (Garg et al., 2001). Understanding of the transformation processes of phenolic micropollutants aids i) their quantification in WWTP effluents and ii) identifying sources of TPs. During biological wastewater treatment, metabolic or co-metabolic reactions can impact the fate of many phenolic compounds. Biotic degradation reactions of phenolic compounds include ring hydroxylation reactions or oxidation of ring substituents, followed by ring cleavage, for example via the *ortho* or *meta* pathway (Reineke and Beek, 2001). Additionally, abiotic reactions, e.g. hydroxylation of an α , β -unsaturated ketone (Wick et al., 2011) or the formation of a nitrobenzene from an aniline moiety in the presence of nitrite (Nödler et al., 2012) are potential transformation routes of micropollutants. Hence, both biotic and abiotic transformation processes could transform these substances in biological wastewater treatment.

An abiotic transformation process of recent interest is the nitration of phenol moieties and the formation of nitrophenolic TPs during biological wastewater treatment (Chiron et al., 2010; Sun et al., 2012). Wick et al. (2011) reported the formation of nitrophenolic TPs in activated sludge batch experiments spiked with morphine. Due to their elevated (eco)toxicity, nitrophenols are of environmental concern (Tomei et al., 2003). For instance, the phenolic compound bisphenol A (BPA) exhibited estrogenic effects to goldfish (Toyoizumi et al., 2008) and other aquatic organisms (Oehlmann et al., 2006), but after transformation to dinitro-BPA the estrogenic activity decreased while genotoxicity increased (Toyoizumi et al., 2008). Recent studies on nitration of phenolic compounds during wastewater treatment have found evidence for different mechanisms but a similar extent of nitration. Acetaminophen for instance, had a reported transformation of 5% to nitroacetaminophen (Chiron et al., 2010) and BPA of 0.2% to dinitro-BPA (Sun et al., 2012) during wastewater treatment in two different WWTPs. In both studies, concentration of substrate phenols was in the 2–6 $\mu\text{g/L}$ range and transformation was reported to occur mostly during biological treatment, in nitrifying reactors or oxidation ditches. Currently, it is unclear which WWTP conditions and agents are favoring the nitration process and which phenolic compounds are more likely to be transformed. Previous reports have attributed two possible agents for the nitration of phenols in WWTPs: nitrite and peroxyxynitrite. Gaulke et al. (2009) proposed that nitrous acid is a reactive species for the nitration of phenols via nitrite. Nitrite is an intermediate for both ammonium oxidation and nitrate reduction and is usually found in low concentrations in nitrifying reactors (0.5–1.0 $\text{mg/L NO}_2^- \text{-N}$ (Randall and Buth, 1984)). The nitration of phenolic compounds by nitrite is known and has been studied under extreme acidic aqueous conditions ($\text{pH} < 1$). The reaction mechanism, initially proposed by Al-

Obaidi and Moodie (1985) and then further underlined by Beake et al. (1994), involves the formation of nitrogen dioxide radicals from nitrous acid. The formation of nitrogen dioxide and nitric oxide radicals from nitrous acid is known to occur in aqueous solution without the influence of an oxidative agent or photolysis (Vione et al., 2004; Khalafi and Rafiee, 2010).

At $\text{pH} < 6$ Chiron et al. (2010) reported that the nitration of acetaminophen by nitrite occurs through a different process similar to a Michael Addition (Matsuno et al., 1989) whereby nitrite adds nucleophilically to the β -carbon of the oxidized benzoquinone imine of acetaminophen. A similar process was suggested for catechols (Khalafi and Rafiee, 2010). In activated sludge at neutral pH, Chiron et al. (2010) suggested a phenolic nitration process involving peroxyxynitrite, while a nucleophilic nitration of acetaminophen did not occur. Peroxyxynitrite is a by-product of cell respiration and is known to be formed through the combination of superoxide and nitric oxide (Ferrer-Sueta and Radi, 2009). The nitration mechanism by peroxyxynitrite also involves the initial formation of nitrogen dioxide radicals. It is reported that high AOB (ammonium oxidizing bacteria) activity promotes the formation of peroxyxynitrite. Studies of the nitration of estrogens, BPA and nonylphenol in activated sludge proposed that nitrite (Sun et al., 2012) or the protonated form, nitrous acid (Gaulke et al., 2009) is the reactive species (Table 1). Nitrite was measured at 0.08–0.34 $\text{mg/L NO}_2^- \text{-N}$ in an oxidation ditch where BPA nitration was detected (Sun et al., 2012). The evidence for a radical mechanism proposed by Moodie was reported by Vione et al. (2004) for phenol at $\text{pH} 2\text{--}5$, while an alternative reaction mechanism in which nitrosation of phenol is followed by oxidation to nitrophenol (Ridd, 1991) was ruled out. However, it is still an open question as to what extent and by which mechanisms, phenolic compounds entering WWTPs are nitrated and to what extent they are discharged into rivers and streams. Furthermore, it is not clear how the discharge of nitrophenolic compounds can be avoided or minimized.

The objective of the current study was to elucidate the transformation of selected phenolic substances during biological wastewater treatment. The main emphasis was put on the relevance of processes leading to an abiotic nitration in comparison to their enzymatic transformation. Since nitrite is an intermediate in ammonium oxidation, it is possibly responsible for the nitration of phenolic micropollutants. Due to their environmental relevance, the phenolic antiseptic *ortho*-phenylphenol (OPP), the plastics additive and estrogenic compound BPA and the psychoactive drug dextrophan were selected. OPP is an anti-fungal agent used for the preservation of citrus fruit. It is degraded in WWTPs (Rudel et al., 1998), however it is unknown to what extent the degradation is due to an abiotic nitration in activated sludge. Kinetic and mechanistic studies were conducted using OPP as the model phenolic micropollutant, comparisons were then made to the phenolic compounds BPA and dextrophan. BPA is a well-known micropollutant due to its endocrine disrupting activity (Oehlmann et al., 2006). Dextrophan is a human metabolite of the antitussive prodrug dextromethorphan and has been detected in WWTP effluents (Thurman and Ferrer, 2012), however its potential transformation during wastewater treatment has not been studied so far.

2. Experimental

2.1. Chemicals

ortho-Phenylphenol (OPP) was purchased from TCI Europe (Eschborn, Germany) and bisphenol A (BPA) from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Dextrorphan tartrate, acetaminophen, carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazolin-1-oxyl-3-oxid (cPTIO), *N*-acetylcysteine and NaNO₂ were purchased from Sigma Aldrich (Schnellendorf, Germany). LC–MS grade solvents were purchased from LGC Promochem (Wesel, Germany). Purified water was obtained from a Milli-Q water purification system (Millipore, Darmstadt, Germany). The transformation products 4-nitro-6-phenylphenol, 2-nitro-6-phenylphenol and 3,3'-dinitro-bisphenol A were synthesized in the laboratory. Details of the syntheses are given in the Supplementary Data.

2.2. Analytical methods

Quantification of phenols and nitrophenols via LC–MS/MS was carried out on an Agilent HPLC system (1200 Series, Agilent Technologies, Waldbronn, Germany) equipped with a Synergi Polar-RP column (150 × 3.00 mm, 4 μm; Phenomenex, Aschaffenburg, Germany), coupled to a quadrupole-MS/MS (AB Sciex API 4000, Applied Biosystems, Langen, Germany) with ESI operated in positive and negative ionization mode. Mobile phases for gradient elution were A: 0.05% acetic acid in water and B: acetonitrile (gradient for phase A: 0–2 min. 92%, 5–14 min. 60%, 15–18 min. 5%, 19–23 min. 92%). Quantification via UV–VIS was carried out on a Knauer Smartline HPLC

(Knauer GmbH, Berlin, Germany) coupled to a UV–Vis detector. Nitrophenols were detected at 300 nm and phenols at 254 nm. High-resolution mass spectrometry for the identification of TPs was carried out on an Agilent HPLC system (as above) coupled to a QToF-MS (AB Sciex TripleToF 5600, Applied Biosystems) with ESI operated in positive and negative ionization mode and by an Accela HPLC coupled with ESI to an LTQ-Orbitrap-MS (LTQ Orbitrap Velos, Thermo Scientific, Bremen, Germany).

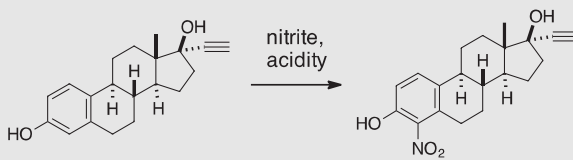
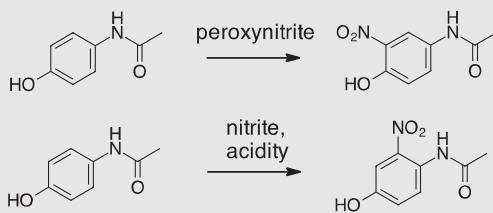
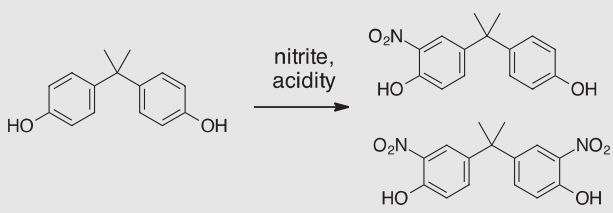
2.3. Experimental setup for kinetic and mechanistic studies of OPP nitration

The phenolic substance OPP was added in varying concentrations (0.5–1.2 mmol/L) to a NaNO₂ solution (5–15 mmol/L) in buffered, purified water (31 mmol/L sodium acetate, pH 2–6). To avoid the photocatalytic formation of radicals, reactions were performed in amber glass flasks. The reaction was monitored by taking 250 μL samples, which were neutralized by diluting to 1 mL with buffered water (pH 12, 50 mmol/L phosphate). Dinitro-BPA was used as an internal standard in the kinetic and mechanistic studies. Analysis of the samples was carried out by LC–MS/MS for the identification of transformation products and both HPLC-UV and LC–MS/MS for their quantification.

2.4. Batch experiments with activated sludge

To study the transformation characteristics of phenols under conditions found in an activated sludge reactor, 400 mL batch experiments were set-up in amber glass flasks. Activated sludge was taken from the nitrifying stage of a municipal

Table 1 – Reported processes for the nitration of phenolic micropollutants in activated sludge.

Precursor compound	Nitration conditions	Reference
17 α -Ethinylestradiol		Gaulke et al., 2009 Khunjar et al., 2011
Acetaminophen		Chiron et al., 2010
Bisphenol A		Sun et al., 2012

WWTP with a capacity of 320,000 population equivalents and a daily flow rate of 61,000 m³. The activated sludge stage is operated with a hydraulic retention time of approximately 7 h, a solids retention time of 12 d and achieves a yearly average N-removal of around 81%, measured as total bound N. The sludge was diluted 20:1 with effluent or used undiluted. Throughout the experiment, the solution was stirred and purged with a mixture of air and CO₂ through a diffuser. CO₂ was added to the gas mixture to stabilize the pH, which would otherwise increase due to purging of dissolved CO₂. For a detailed description of the setup see Wick et al. (2009). The pH was maintained between 6.5 and 7.5 by regulating the gas mixture. In some cases, nitrite concentration and pH were adjusted by addition of acetic acid and NaNO₂. After pH equilibration, OPP, BPA and dextrophan were spiked to the sludge. Samples were filtered (regenerated cellulose, 0.45 μm) and stored at +4 °C. A matrix-matched calibration curve was used for the quantification of OPP and NO₂-OPP. For the calibration and matrix compensation, the sludge was filtered and aliquots were spiked with increasing concentrations of both analytes. This enabled quantification of samples from the batch experiments by LC–MS/MS. Nitrite, nitrate, ammonia and DOC concentrations were measured separately on a DR 5000 photometer (Hach-Lange, Düsseldorf, Germany) using test kits from the same supplier.

2.5. Effect of freezing samples during storage

To test the effect of freezing samples as a means of storage, batch experiments were set up with 0.6 mg/L NO₂⁻-N and 1 μg/L OPP and BPA in buffered water (50 mmol/L phosphate). Samples were then stored either by refrigeration at +4 °C, acidification to pH 2 with HCl or frozen at –20 °C. The samples were then analyzed for nitrophenols by LC–MS/MS using the same analytical procedure as described for environmental samples (see below).

2.6. Environmental sampling at WWTPs

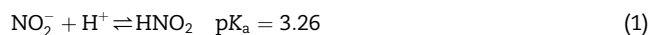
Two German WWTPs equipped for denitrification and nitrification were sampled for the detection of TPs. Technical parameters of the WWTPs are described in the Supplementary Data. NO₂-OPP, dinitro-BPA and the phenolic precursors were quantified by the standard addition method. Special care was taken to avoid freezing samples or exposing them to acidity. 24-h mixed samples of influent (flow proportional) were taken at the start of the treatment process (after grit removal) and after primary clarification. Mixed samples of effluent were taken after secondary settling at WWTP 1 and after sand filtration at WWTP 2. During sample collection the samples were stored at 4 °C. On the day of collection both samples and a blank (Milli-Q) were filtered (GF/6, Whatman). The influent was split into 4 × 150 mL aliquots and the effluent and blank into 4 × 500 mL aliquots. These were stored overnight at 4 °C. Three aliquots of influent, effluent and blank were spiked with increasing amounts of the analytes as standards for quantification via the standard addition method. All aliquots were loaded onto SPE cartridges (Oasis HLB 6 cc, Waters, Eschborn, Germany), which were conditioned with groundwater. The SPE cartridges were eluted with acetone and the organic phase

was reduced to 100 μL by evaporation under a light nitrogen gas flow. The samples were filled to 500 μL with Milli-Q water and analyzed by LC–MS/MS. Details of the analytical method are given in the Supplementary Data.

3. Results and discussion

3.1. Abiotic nitration of ortho-phenylphenol at varying pH values

To study the abiotic nitration and to exclude biological transformation processes, batch experiments without the addition of activated sludge were conducted in buffered solution containing nitrite and ortho-phenylphenol (OPP). The initial rates of reaction decreased rapidly when increasing the pH from 2.0 to 4.5. In Fig. 1a the initial rates of OPP elimination and of nitro-phenylphenol (NO₂-OPP) formation are plotted against the pH. Above pH 5 the formation of NO₂-OPP was not detectable by HPLC-UV. The dotted curves show the results of fitting the experimental data to the equilibrium concentration of nitrous acid (Eqs. (1)–(3)). The quotient in Eq. (3), where [NO₂⁻]₀ is the initial nitrite concentration, is the nitrous acid concentration at equilibrium (for derivation see Supplementary Data Eqs. S1-6). This approach has been reported previously by Vione et al. (2004). The pH-trend for OPP nitration closely mirrors the acid–base equilibrium of nitrous acid, pointing to this as a reactive species.



$$K_a = \frac{[\text{H}^+][\text{NO}_2^-]}{[\text{HNO}_2]} \quad (2)$$

$$\text{initial rate} = k \cdot \frac{[\text{H}^+][\text{NO}_2^-]_0}{K_a + [\text{H}^+]} \quad (3)$$

Three products were identified via LC–HRMS, the ortho- and para-isomer of nitro-2-phenylphenol (NO₂-OPP), and one isomer of nitroso-2-phenylphenol (NO-OPP). For the latter, the location of –NO substitution is unknown, but is assumed to occur at the para-position since in similar experiments with BPA and dextrophan, where the para-position is blocked, nitrosation was not detected. Both isomers of NO₂-OPP had similar rates of formation (see Supplementary Data Fig. S6). Further discussion of NO₂-OPP formation is based on para-NO₂-OPP, however ortho-NO₂-OPP appears to be formed analogously.

The results confirm a strong pH trend and that the rate of abiotic nitration at higher pH (>5) is expected to be extremely low. Furthermore, the results do not support a mechanism in which the nitration occurs via nitrosation by nitrosonium ion followed by an oxidation of the nitrosophenol to the nitrophenol as described by Ridd (1991), since under conditions in which both products are formed (Fig. 1b), the rate of NO₂-OPP formation did not increase with increasing NO-OPP concentration (i.e. an initial rate of zero for NO₂-OPP was not observed). NO-OPP concentrations were also stable for >10 h after reaching equilibrium (data not shown). This implies that NO₂-OPP is, at least to a large degree, a direct product from

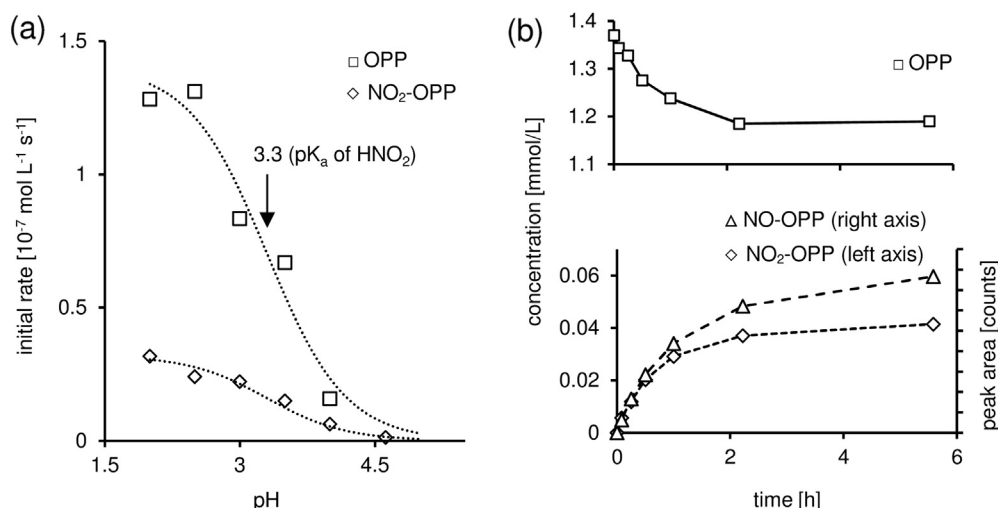
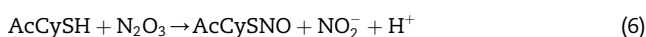


Fig. 1 – (a) pH trend of the initial rate of abiotic OPP elimination and NO₂-OPP formation (absolute values). Conditions: [OPP]₀ = 1 mmol/L, [NaNO₂]₀ = 5 mmol/L. Dotted lines are curves of the acid–base equilibrium of HNO₂, fitted to the experimental data. (b) Formation of NO₂-OPP and NO-OPP, characteristic of two parallel reactions. Conditions: pH 3.5, [NaNO₂]₀ = 5 mmol/L.

OPP. To confirm this, experiments were carried out using the antioxidant *N*-acetylcysteine, which reacts with HNO₂ and N₂O₃, and the nitrogen radical scavenger carboxy-PTIO.

3.2. Impact of *N*-acetylcysteine and *c*-PTIO on OPP abiotic nitration and nitrosation

In the presence of the antioxidant *N*-acetylcysteine (AcCySH), NO₂-OPP was not formed, whereas no change was observed in the formation of NO-OPP (Fig. 2). By LC-Orbitrap-MS, using high-resolution mass spectra, both AcCySNO and AcCySSCyAc dimer were identified in the aqueous nitrite solution, confirming that both N₂O₃ and HNO₂ react with AcCySH (Eqs. (4)–(6)), analogously to cysteine (CySH), which forms CySNO and the dimer CySSCy (Grossi and Montevecchi, 2002). HNO₂ oxidizes AcCySH to AcCyS· radicals, which combine to form the dimer AcCySSCyAc. N₂O₃ is present due to the dissociation of HNO₂ in aqueous solution, (Eqs. (7) and (8)) (Park and Lee, 1988) but reacts with thiols. Thus, excess AcCySH effectively eliminates HNO₂ and N₂O₃.



Therefore, it can be suggested that HNO₂ and/or N₂O₃ are the predominant agents for the formation of NO₂-OPP. Since the NO-OPP formation was not affected by AcCySH addition, different processes must be involved. It can be assumed that AcCySNO leads to the formation of NO-OPP since S-

nitrosothiols are known to act as nitrosating agents of phenolic compounds (Noble and Williams, 2002). NO-OPP formed via AcCySNO appeared stable with respect to oxidation to NO₂-OPP in the presence of O₂, again suggesting that a consecutive mechanism OPP → NO-OPP → NO₂-OPP does not take place. Furthermore, the product AcCyS-OPP could also be observed by LC-Orbitrap-MS using high resolution MS and the MS² fragmentation spectrum (see Supplementary Data), which may be resulting from radical coupling of ·OPP and AcCyS·, suggesting the involvement of ·OPP radicals in the reaction.

N₂O₃ is known not only to nitrosate thiols (Eq. (6)) but also to nitrosate phenolic substances (Noble and Williams, 2002). N₂O₃ is in equilibrium with the dissociated form (·NO + ·NO₂, Eq. (8)), but the equilibrium favors N₂O₃ with $k_+ = 1.1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ versus $k_- = 8.1 \times 10^4 \text{ s}^{-1}$ (Goldstein et al., 2003). Due to the equilibrium, the impact of AcCySH is likely to be similar on both forms. It is reported that the

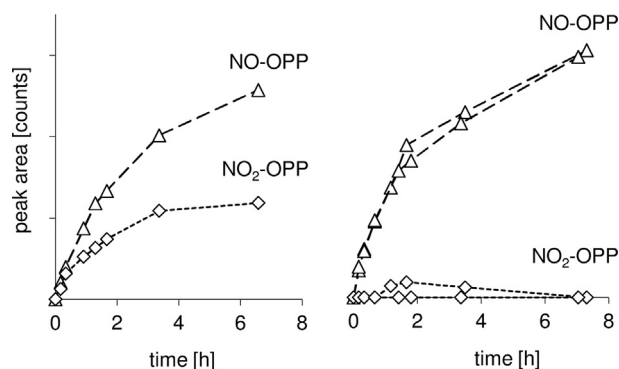
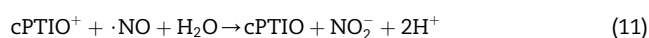
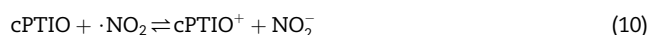
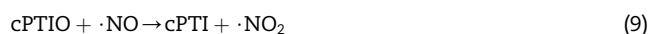


Fig. 2 – The effect of the antioxidant AcCySH on the nitration and nitrosation of OPP; Conditions: pH 4, [OPP]₀ = 1 mmol/L, [NaNO₂]₀ = 5 mmol/L. Left: No addition of AcCySH. Right: Addition of 1 μmol/L AcCySH (duplicate experiment).

formation of nitrosophenol is likely caused by N_2O_3 rather than $\cdot NO$ reacting with phenol (Noble and Williams, 2002), however the formation of NO-OPP through the radical coupling of $\cdot NO$ and $\cdot OPP$ radicals cannot be excluded. A radical mechanism including $\cdot NO_2$ might also be responsible for the formation of NO_2 -OPP.

To test the involvement of $\cdot NO_2$ and $\cdot NO$, the nitration reactions were repeated with the addition of carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazolin-1-oxyl-3-oxide (cPTIO), which is a known radical scavenger for both $\cdot NO$ and $\cdot NO_2$ (Eqs. (9)–(11); Goldstein et al., 2003). In the presence of cPTIO the equilibrium concentrations of NO-OPP and NO_2 -OPP are significantly reduced by 78% and 65%, respectively, and the initial rate of NO-OPP formation is much lower than that for NO_2 -OPP formation (Fig. 3). The concentration of cPTIO was not high enough to cause a complete inhibition of the reaction but in a further experiment at lower OPP concentrations, a complete inhibition of NO-OPP was observed (see Supplementary Data Fig. S7). Since cPTIO scavenges specifically $\cdot NO$ and $\cdot NO_2$ radicals, this confirms that at pH 4 both $\cdot NO$ and $\cdot NO_2$ are involved in the reactions leading to NO-OPP and NO_2 -OPP. N_2O_3 is known to react as a nitrosating species, however the involvement of the dissociated form of N_2O_3 ($\cdot NO + \cdot NO_2$) could not be excluded considering the impact of cPTIO.



In summary, NO_2 -OPP formation was impacted when either HNO_2 , N_2O_3 or possibly $\cdot OPP$ were scavenged by AcCySH, and the involvement of $\cdot NO_2$ or $\cdot NO$ radicals was shown by the cPTIO experiment, therefore NO_2 -OPP should be formed by a radical reaction. Since it is not formed via oxidation of NO-OPP, these experiments support a two-step mechanism in which NO_2 -OPP is being formed by oxidation of OPP by HNO_2 , followed by reaction of $\cdot OPP$ with $\cdot NO_2$ radical to form the nitrophenol, shown by Eqs. (1), (7) and (8) and Scheme 1, as described by Beake et al. (1994) for the nitration of *para*-methoxyphenol by HNO_2 . $\cdot NO_2$, although being

present at a low concentration, would be constantly replenished due to the equilibrium in Eq. (8).

3.3. Kinetics and mechanism of OPP nitration

At conditions typical for a German WWTP, (WWTP 1, see Sec. 3.6) i.e. neutral pH and nitrite concentrations below 1 mg/L NO_2^- -N in the biological wastewater treatment stage, the nitration of phenolic compounds should be extremely low following the abiotic mechanism suggested above. Only when technical problems at WWTPs lead to a drop of pH or an accumulation of nitrite (Randall and Buth, 1984) might an appreciable formation of NO-OPP or NO_2 -OPP occur. In order to predict the potential of NO_2 -OPP formation, a model was developed based on kinetic studies at different pH and nitrite concentrations.

The reaction order determined by the method of initial slopes (Atkins & de Paula, 2002) indicated that at pH 4 the rates of *para*- and *ortho*- NO_2 -OPP formation were first order with respect to HNO_2 and half order with respect to OPP (Fig. 4). The fractional order of 1/2 with respect to OPP is an indication that a dissociation is taking place (Houston, 2006), e.g. formation of OPP radicals by HNO_2 . This would also account for the first order dependence on HNO_2 . In a separate experiment, the rate of HNO_2 elimination was found to be second order in HNO_2 (Fig. 5a). Assuming that the reaction of OPP with nitrous acid ($HNO_2 + PhPhOH \rightarrow PhPhO\cdot + \cdot NO + H_2O$) is the rate-limiting step, the following rate laws can be described based on NO_2 -OPP formation and HNO_2 elimination (Eqs. (12) and (13)).

$$\frac{d[NO_2 - OPP]}{dt} = k_1 [HNO_2] [OPP]^{1/2} \quad (12)$$

$$-\frac{1}{2} \frac{d[HNO_2]}{dt} = k_2 [HNO_2]^2 \quad (13)$$

The rate constant k_2 is the slope of the reciprocal nitrous acid concentration over time (Fig. 5a). Integration of Eq. (13) and solving for $[HNO_2]$ gives:

$$[HNO_2] = \left(2k_2 t + \frac{1}{[HNO_2]_0} \right)^{-1} \quad (14)$$

If the OPP concentration is high compared to HNO_2 and/or conversion to NO_2 -OPP remains low, then $[OPP]$ can be approximated by $[OPP]_0$. Substituting Eq. (14) into Eq. (12) and integrating gives:

$$[NO_2 - OPP] = \frac{k_1 \sqrt{[OPP]_0}}{2k_2} \ln(2k_2 t [HNO_2]_0 + 1) \quad (15)$$

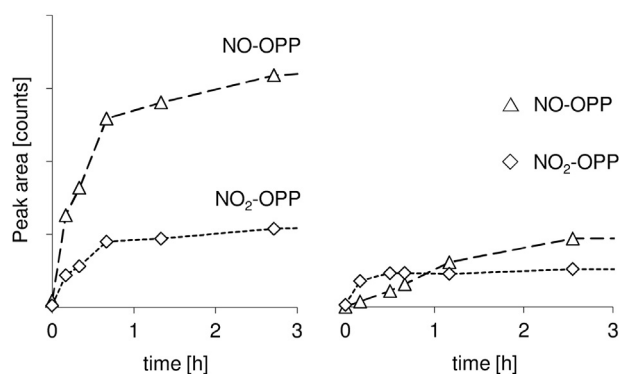
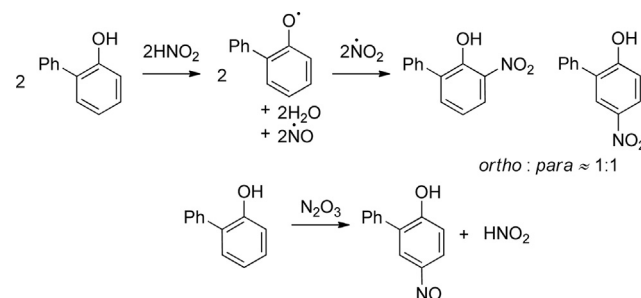


Fig. 3 – The formation of NO_2 -OPP and NO-OPP from two experiments, Left: without cPTIO, Right: with 100 $\mu\text{mol/L}$ cPTIO. Peak areas are relative to an internal standard. Conditions: pH 4, $[OPP]_0 = 1 \text{ mmol/L}$, $[NaNO_2]_0 = 5 \text{ mmol/L}$.



Scheme 1 – Postulated mechanism for the nitration and nitrosation of OPP.

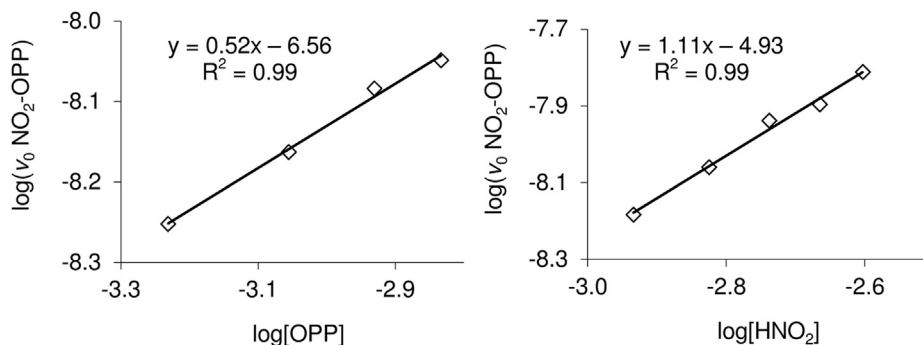


Fig. 4 – Correlation of the initial rate of NO_2 -OPP formation with changing initial concentrations of reactive species (from the method of initial slopes). Left: Rate of NO_2 -OPP formation with respect to OPP concentration. Conditions: pH 4, 22 °C, $[\text{NaNO}_2]_0 = 9 \text{ mmol/L}$, $[\text{OPP}]_0 = 0.6\text{--}1.5 \text{ mmol/L}$. Right: Rate of NO_2 -OPP formation with respect to HNO_2 concentration. Conditions: pH 4, 22 °C, $[\text{NaNO}_2]_0 = 0.8\text{--}2.5 \text{ mmol/L}$, $[\text{OPP}]_0 = 1 \text{ mmol/L}$.

The rate constant k_1 ($2.5 \cdot 10^{-4} \text{ L}^{0.5} \text{ mol}^{-0.5} \text{ s}^{-1}$) was found by fitting the calculated concentration to the experimental results of Fig. 4. Eq. (15) was tested by carrying out an experiment at a longer duration and was found to accurately model the experimentally determined concentrations of NO_2 -OPP (Fig. 5b).

The developed model enables the calculation of the NO_2 -OPP concentrations formed in the batch systems by the reaction of OPP with $\text{HNO}_2/\cdot\text{NO}_2$ radicals. As other wastewater constituents (e.g. further phenolic compounds) are probably also reacting with $\text{HNO}_2/\cdot\text{NO}_2$ this model allows prediction of the upper limit of formation (maximum concentration). For instance, at pH 7, a maximum concentration of NO_2 -OPP of 1 ng/L is predicted after 6 h for 1 $\mu\text{g/L}$ OPP and 1 mg/L NO_2^- -N. However, if the nitrite concentration is increased to 20 mg/L NO_2^- -N and the pH reduced to 6.5, a maximum of 80 ng/L NO_2 -OPP is predicted to be formed under this idealized case where only OPP is reacting with HNO_2 . During certain treatment processes, such as the Sharon–Anammox for nitrification of digester effluents, nitrite concentration reaches 600 mg/L NO_2^- -N (van Dongen et al., 2001). In another example, ammonium oxidation in urine wastewater has been observed at pH 4 and it is reported that at this acidic pH, nitrite

oxidation is a chemical process resulting from the same decomposition reaction of nitrous acid that leads to the formation of $\cdot\text{NO}_2$ radicals (Udert et al., 2005). Under such extreme conditions (low pH, elevated nitrite concentration), higher concentrations of nitrophenolic transformation products are expected (Sec. 3.5).

3.4. Uncontrolled nitration of phenolic substances during sample storage

Freezing of neutral (pH 7) water samples containing nitrite (0.6 mg/L) and phenolic compounds (1 $\mu\text{g/L}$ BPA and OPP) led to formation of NO_2 -OPP, NO_2 -BPA and dinitro-BPA (Fig. 6C and D). The extent of nitration was similar to an acidified sample, where a significant formation of NO_2 -OPP (>100 ng/L) can be estimated from Eq. (15) (Fig. 6B). Storage at 4 °C did not cause the artificial formation of nitrophenolic compounds (Fig. 6A). An explanation could be found in publications reporting a shift to lower pH values when freezing buffered solutions (Sundaramurti et al., 2010; Goyal and Hafez, 1995). Thus, freezing is an inappropriate storage method for samples to be analyzed for phenolic compounds. Sample storage should occur at 4 °C instead.

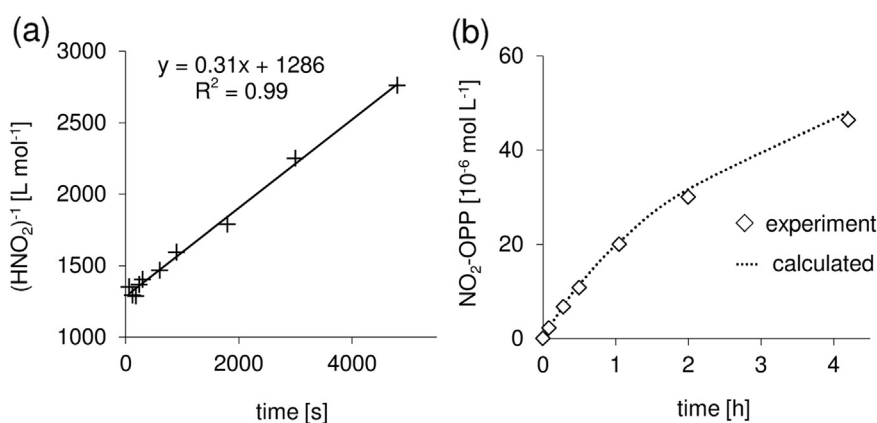


Fig. 5 – (a) Plot of reciprocal nitrous acid concentration as a function of time. The second order rate constant $2k_2$ is the slope, 0.31 $\text{L/s} \cdot \text{mol}$. (b) Estimated NO_2 -OPP concentration using eq. (15) (dotted line) versus experimental results. Conditions: pH 4, $[\text{OPP}]_0 = 1 \text{ mmol/L}$, $[\text{NaNO}_2]_0 = 5 \text{ mmol/L}$.

3.5. Batch experiments with activated sludge

In activated sludge from a municipal WWTP, the formation of nitrophenolic compounds cannot reach the maximum concentration estimated by the kinetic studies, since it i) contains microorganisms enabling an additional biotic transformation of the phenolic compounds and ii) it contains several components that are also able to react with HNO_2 or $\cdot\text{NO}_2$. Dissipation of phenolic compounds and the formation of nitrophenolic substances were monitored in batch experiments with diluted nitrifying activated sludge under varying conditions (pH and nitrite). In addition to OPP, bisphenol A and dextrorphan were spiked to investigate whether the OPP results can be transferred to further phenolic substances.

In batch experiments without alteration of the pH and without artificial addition of nitrite or ammonium, the concentrations of BPA and OPP decreased rapidly, while dextrorphan was found to be more recalcitrant as its concentration remained mainly constant (Fig. 7a). No evidence of nitrophenol formation from any of these three phenolic substances was found. The elimination of BPA and OPP under these conditions is attributed predominantly to biotic transformation processes, as shown below.

To rule out the possibility that other processes associated with ammonium oxidation (e.g. build-up of peroxyxynitrite) were causing a significant nitration, as found by Chiron et al. (2010), the experiment was repeated with an increased ammonium concentration of 240 mg/L NH_4^+-N . During 4 days, in which the system was continually purged with air, it caused nitrate concentrations to increase from 9 to 76 mg/L $\text{NO}_3^- - \text{N}$ while the ammonium concentration decreased to 210 mg/L NH_4^+-N . The formation of nitrophenolic compounds was not detected in this experiment.

3.5.1. TPs formed under neutral pH conditions

Via their LC-MS/MS fragmentation patterns using LC-QToF-MS, several TPs could be identified (Scheme 2), giving insights into the relevant transformation or degradation pathways of these compounds in nitrifying activated sludge.

The TP hydroxy-OPP was formed in the batch experiments containing activated sludge described above and was itself eliminated, suggesting the degradation of OPP proceeds via this catechol intermediate in activated sludge. This OPP-TP

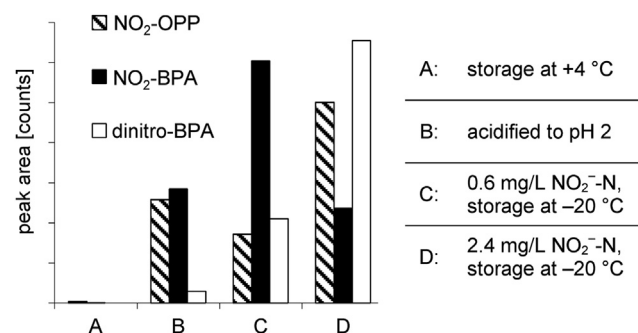


Fig. 6 – Nitrophenol formation resulting from sample storage or preparation: Batch experiments in pH 7 buffered water were spiked with 1 $\mu\text{g/L}$ BPA and OPP, and varying nitrite concentrations: A–C: 0.6, D: 2.4 mg/L $\text{NO}_2^- - \text{N}$.

was previously reported to be formed by a soil bacterium and is the substrate for an oxidative meta cleavage leading to degradation of OPP (Kohler et al., 1988). In the case of BPA, the presence of hydroxy-BPA (1,2-bis(4-hydroxyphenyl)-1-propanol) was identified by LC-QToF-MS as an intermediate species. Fragmentation spectra of this TP suggest a structure that is formed via rearrangement of the quaternary carbon center of BPA (see Supplementary Data). Ike et al. (2000) detected this TP in sludge enrichment cultures degrading BPA (concentrations of 100 mg/L), but it was further degraded to benzoic acid derivatives. Detection of the TPs of OPP and BPA in the batch experiments of the current study confirms the relevance of these degradation pathways in mixed cultures from municipal WWTPs at substrate concentrations of 200 $\mu\text{g/L}$. Although the concentration of dextrorphan remained relatively constant ($\sim 10\%$ elimination), several hydroxylated dextrorphan-TPs were identified in small concentrations. In total four isomers of hydroxy-dextrorphan TPs were identified with similar MS² spectra, possibly due to the formation of diastereomeric pairs from the chiral precursor. Due to the low proportion of dextrorphan conversion, an isolation of TPs for structure confirmation was impossible. In addition, sulfate conjugation products of all three phenols were detected. Sulfo-OPP was quickly eliminated, while the others persisted in the batch experiment. Sulfate conjugation is discussed below in more detail. The characterization of TPs by MS/MS is described in the Supplementary Data.

Batch experiments were also conducted with the nitrophenolic TPs of OPP and BPA, to test their stability towards (bio)degradation in activated sludge. NO_2 -OPP and dinitro-BPA were transformed in the batch experiments during the 6-day period to approximately 50% and 80%, respectively (Fig. 7b). For both nitrophenols the phenolic hydroxyl group was conjugated with sulfate ($-\text{SO}_3$; Scheme 2). Further TPs were not observed.

The sulfate conjugation seems to be a very common microbial process occurring in activated sludge from biological wastewater treatment with a wide substrate spectrum. Sulfate conjugation (sulfurylation) is a widely occurring biological process in cells. It has various functions including detoxification of xenobiotic substances (Malojčić and Glockshuber, 2010). Sulfurylation of estrogens has previously been observed by mixed bacterial cultures from activated sludge (Khunjar et al., 2011). Further studies have reported these sulfate conjugates can also be de-conjugated in sludge with resulting release of estrogens (Kumar et al., 2012), an indication of the reversibility of this type of transformation.

3.5.2. Formation of nitrophenols in activated sludge

In an activated sludge medium, a rapid formation of nitrophenols from the three precursor phenols was observed under acidic conditions (pH 3.3–3.5). Fig. 7c shows the formation of nitrophenols measured over time. The formation of NO-OPP was also observed. However it was no longer detected in samples after 6 h, no TPs of NO-OPP could be detected. After 50 h of incubation, nitrite was no longer present and the formation of nitrophenols had slowed down or stopped.

In batch experiments with activated sludge the formation of NO_2 -OPP was quantified at varying pH (3.3–7.0). Using the initial nitrite and OPP concentration and pH, the predicted

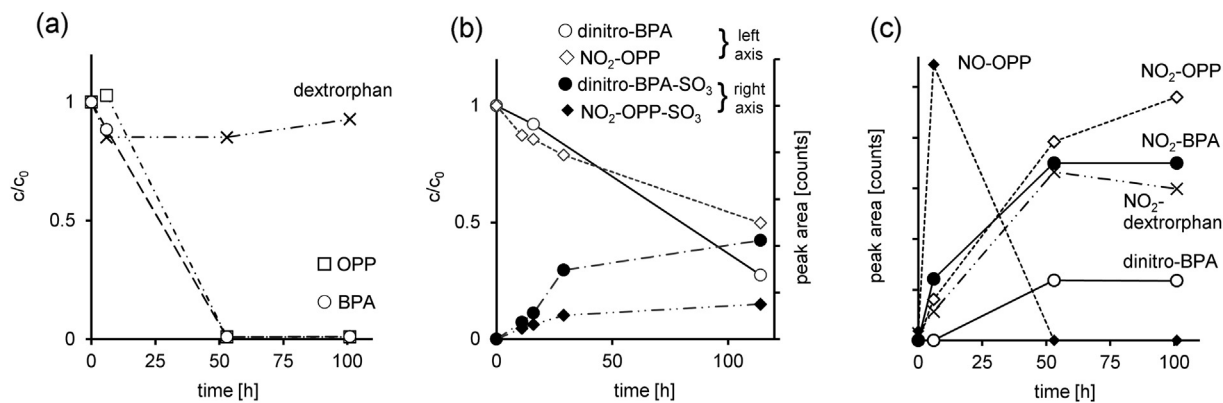


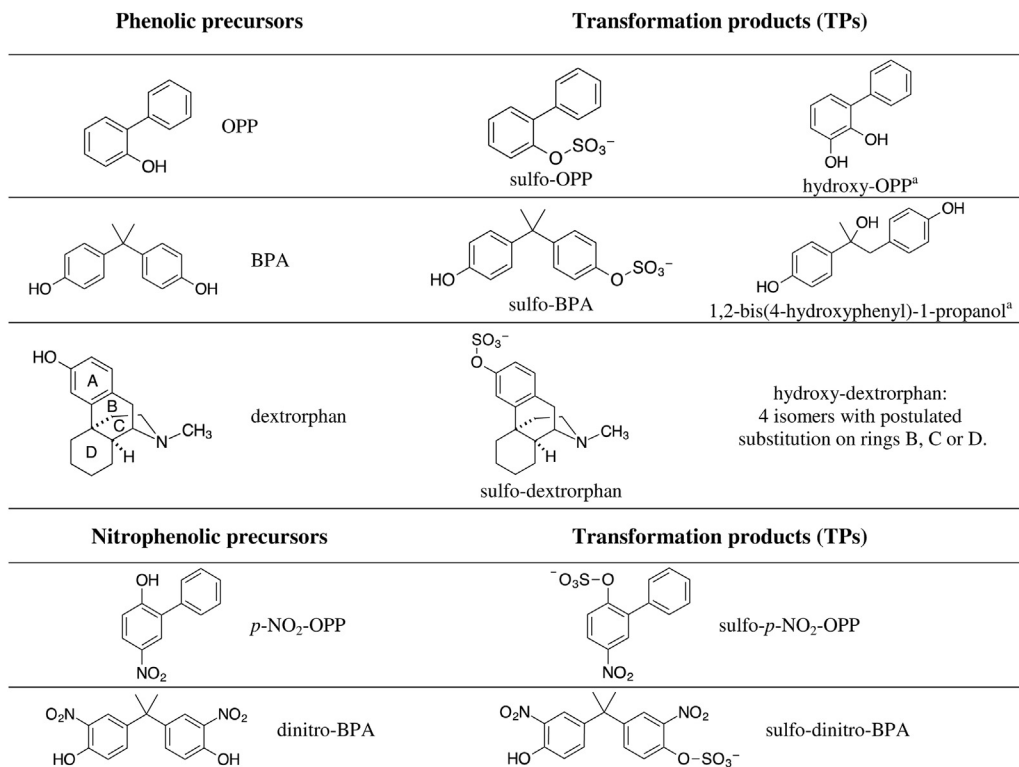
Fig. 7 – (a) Concentration of phenolic parent compounds in a batch experiment: $c_0 = 200 \mu\text{g/L}$. Conditions: activated sludge (0.2 gSS/L), pH 7.2–7.5. (b) Stability of nitrophenols to biodegradation. Conditions: activated sludge (0.2 gSS/L), pH 7.2–7.5, dinitro-BPA and NO₂-OPP, $c_0 = 200 \mu\text{g/L}$. (c) Formation of nitrophenols in activated sludge. Conditions: activated sludge (0.2 gSS/L), pH 3.3, 4.2 mg/L NO₂⁻-N.

maximal formation of NO₂-OPP was calculated with Eq. (15). Only around 10% of the predicted maximum concentrations were detected in the batch experiments with activated sludge (Table 2), since HNO₂ and •NO₂ are probably reacting with other sludge constituents (DOC of the sludge ~10 mg/L). Therefore, it can be concluded that the nitration process with HNO₂ can be neglected in contact with activated sludge. Other processes leading to nitro-phenolic TPs could not be observed, neither with elevated ammonium nor with elevated nitrite concentrations. Thus, the formation of significant levels of nitrophenolic TPs from BPA, OPP and dextrorphan can be

ruled out in batch experiments with activated sludge at the conditions expected at the WWTPs in this study. It seems very unlikely that nitrophenolic substances are formed in biological wastewater treatment.

3.6. Analysis of wastewater for the presence of nitrophenolic TPs

The concentrations of three nitrophenolic substances, NO₂-OPP, dinitro-BPA, and NO₂-dextrorphan, and their precursors (OPP, BPA, dextrorphan) were analyzed in wastewater



Scheme 2 – Biotic transformation products observed from BPA, OPP and dextrorphan. ^aTPs identified in enrichment culture studies (Kohler et al., 1988; Ike et al., 2000).

Table 2 – Quantification of NO₂-OPP formed in batch experiments with activated sludge.

Batch experiment ^a	pH	[HNO ₂] ₀	[OPP] ₀ ^b	[NO ₂ -OPP] after 5 h	NO ₂ -OPP detected compared to modeling ^c
1	3.3	1.50 · 10 ⁻⁴	6.8 · 10 ⁻⁷	3.7 · 10 ⁻⁸	8%
2	3.6	7.03 · 10 ⁻⁵	9.72 · 10 ⁻⁷	2.0 · 10 ⁻⁸	9%
3	3.9	4.50 · 10 ⁻⁵	8.22 · 10 ⁻⁷	8.9 · 10 ⁻⁹	9%
4	4.2	2.35 · 10 ⁻⁵	8.58 · 10 ⁻⁷	3.2 · 10 ⁻⁹	6%
5	7.0	1.54 · 10 ⁻⁹	7.69 · 10 ⁻⁷	n.d.	–

^a Conditions: activated sludge (batch 1: 0.2 gSS/L, 2–5: 4 gSS/L), nitrite addition: batch 1–4: 3 mg/L NO₂⁻-N, batch 5: no nitrite addition, c₀ = 0.6 mg/L NO₂⁻-N.

^b [OPP]₀ < [HNO₂]₀, however due to low conversion, OPP concentration can be treated as constant.

^c Theoretical formation according to Eq. (15). n.d. : not detected.

samples from two German WWTPs. Flow-proportional composite samples were taken from the influent and the final effluent over a 24 h period.

Concentrations of OPP and BPA decreased from the low µg/L range before the activated sludge reactor to the low ng/L range in the WWTP effluent in both sites studied (Table 3). The removal of these compounds is mainly caused by biodegradation as sorption to sludge is negligible (Zhao et al., 2008; Zheng et al., 2011). Several TPs detected in batch experiments, hydroxylated-OPP, sulfo-OPP and sulfo-BPA (Scheme 2) were also identified by LC–MS/MS in raw wastewater and WWTP effluents, suggesting that transformation processes identified in batch experiments may also be occurring during drainage and wastewater treatment. For TP identification, quadrupole-MS/MS in MRM mode was used with characteristic MS² fragments for each TP (See Supplementary Data for MS² spectra). However, it was impossible to quantify these TPs due to the lack of authentic standards. Nitrophenolic TPs of OPP, dextrorphan and BPA were not detected in WWTP effluents as shown in Table 3. Only NO₂-OPP was detected in raw wastewater with 2.3 ng/L at WWTP 1 and was not found in the WWTP effluent. It can be assumed that NO₂-OPP originated from sources other than biological wastewater treatment. For instance, if favorable conditions in the sewer system were present, e.g. a local acidification, this could lead to a nitration of OPP. Alternatively, UV radiation can also promote OPP nitration (Suzuki et al., 1990). This could occur during surface run-off before entering the sewer system. The slight increase of the dextrorphan concentrations, e.g. from 5 ng/L to 15 ng/L in WWTP 2, might be caused by the hydrolysis of O-glucuronide conjugates as suggested by Thurman and Ferrer (2012), who detected dextrorphan in WWTP effluent and a US river. O-Demethylation of dextromethorphan, the prodrug of dextrorphan, during treatment would also lead to dextrorphan formation.

These results underline the prediction that NO₂-OPP and dinitro-BPA are not formed in appreciable concentrations during biological wastewater treatment. According to a prediction of NO₂-OPP concentrations using Eq. (15) for the conditions found in WWTP 1, taking into consideration the influence of the sludge matrix, not more than 0.1 ng/L NO₂-OPP would be expected. Thus, these concentrations would be far below the quantification limits of the method (see Sec. 2 and Supplementary Data for details). It might be possible that the formation of dinitro-BPA (1.9–3.7 ng/L) reported by Sun et al. (2012) and of nitro-acetaminophen (180–320 ng/L) reported by Chiron et al. (2010) might be caused by different treatment processes, such as the formation of peroxyxynitrite. However, in

our study no indication for the peroxyxynitrite mechanism was found. Nitro-acetaminophen was included in the analytical method described above and acetaminophen was also spiked into a neutral batch experiment (Table 2, experiment 5). Neither in batch experiments, nor in raw wastewater or WWTP effluents was nitro-acetaminophen found despite acetaminophen being permanently present in the raw wastewater.

4. Conclusions

The transformation processes of three model phenolic micropollutants, bisphenol A (BPA), *ortho*-phenylphenol (OPP) and dextrorphan, during wastewater treatment has been studied with emphasis on the role of abiotic nitration. It was found that the reaction leading to nitro-phenols is most likely due to the formation of radicals from nitrous acid.

- Kinetic studies under idealized conditions revealed that a significant nitrophenolic TP formation can only be expected in cases of nitrite build-up and/or pH reduction.
- Batch experiments with activated sludge indicated that a significant formation of nitrophenols could be ruled out under typical conditions at the WWTPs included in this study, i.e. neutral pH and low nitrite concentration.
- Since nitrophenols are immediately formed under acidic conditions as well as during freezing or thawing of aqueous

Table 3 – Concentrations of parent phenols and nitrophenols detected in two German WWTPs [ng/L].^{a,b}

	WWTP 1 influent	WWTP 1 effluent	WWTP 2 influent	WWTP 2 effluent
OPP	1660	12	1590	30
NO ₂ -OPP	2.3	<LOQ (2)	<LOQ (2)	<LOQ (2)
BPA	6000 ^d	100	1170	19
Dinitro-BPA	<LOQ (2)	<LOQ (1)	<LOQ (2)	<LOQ (2)
Dextrorphan	4	39	5	15
NO ₂ -dextrorphan ^c	n.d.	n.d.	n.d.	n.d.

^a Samples before and after the primary clarifier gave similar concentrations so only the latter is given.

^b LOQs are given in brackets.

^c Due to a lack of an authentic standard no LOQ could be determined. n.d. : not detected.

^d Concentration out of range for standard addition, estimated by matrix-matched calibration curve.

samples containing nitrite, such conditions have been avoided to prevent an artificial formation of nitrophenolic TPs during sample preparation.

- In batch experiments under neutral conditions, the transformation of OPP, BPA and dextrorphan was observed via biotic pathways including hydroxylation and sulfurylation.
- In accordance with the batch experiments, the formation of nitrophenolic TPs was not observed in WWTPs. Previous findings reporting the contrary may be the result of processes specific to those sites studied.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2013.10.010>.

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