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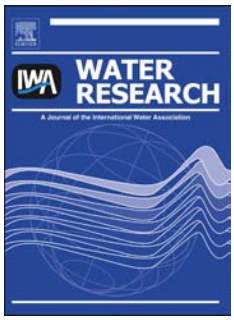

Comparisons between abiotic nitration and biotransformation reactions of phenolic micropollutants in activated sludge

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Highlights

- Postulation of a mechanism for the abiotic nitration of phenolic micropollutants in activated sludge.
- Reaction kinetics allow for an estimation of the extent of nitration for a given set of conditions in activated sludge.
- Identification of sulfate conjugation of phenolic micropollutants as a common microbial process in biological wastewater treatment.

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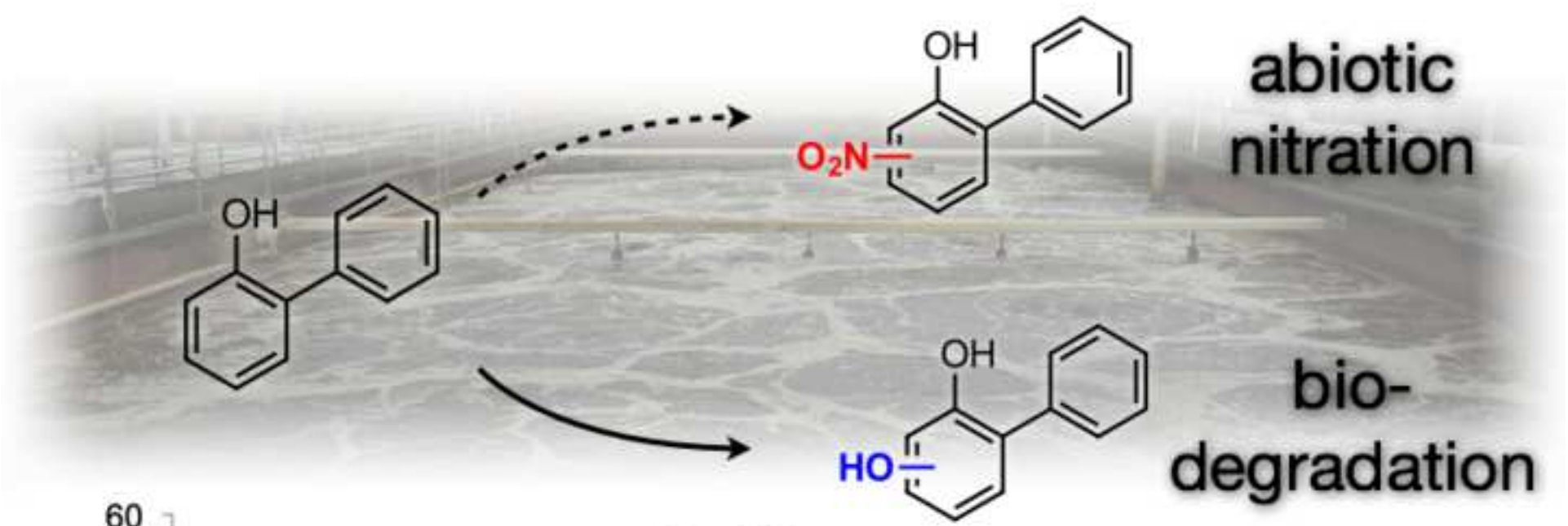

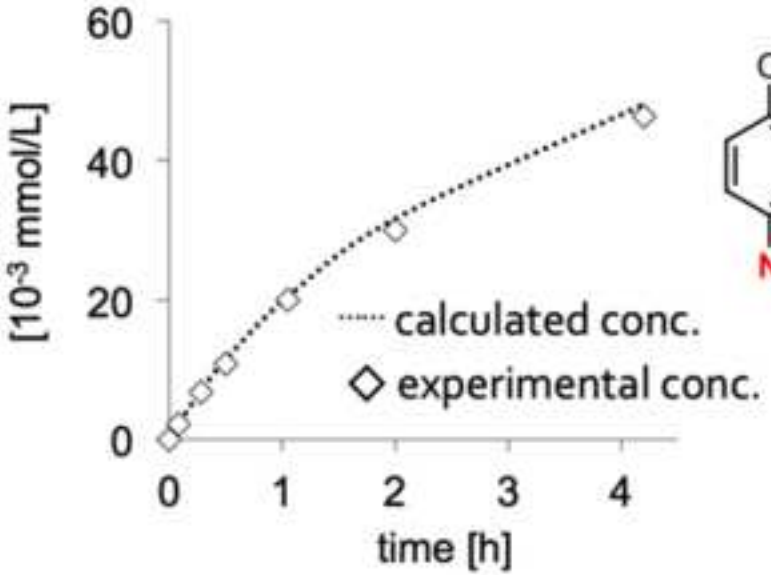

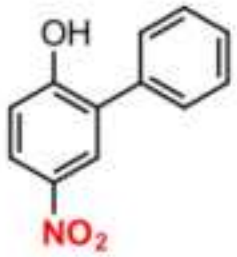

$$\frac{d\,[\text{nitro-OPP}]}{dt} = k_2\,[\text{HNO}_2]\,[\text{OPP}]^{1/2}$$

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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 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 ·NO and ·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 ·NO and ·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, enzymatic 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 into sulfate conjugates. In raw wastewater and WWTP effluent dinitro-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 dextrormethorphan.

Keywords

transformation; phenols; micropollutants; activated sludge; nitration; bisphenol A; 2-phenylphenol; dextrorphan

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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 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 et al. 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 impact 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 2010, Sun 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 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 nitro-acetaminophen (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 μg/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 peroxynitrite. 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 mg/L NO_2^--N (Randall & Buth 1984)). The nitration of phenolic compounds by nitrite is known and has been studied under extreme acidic aqueous conditions (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 from nitrous acid is known to occur in aqueous solution without the influence of an oxidative agent or photolysis (Vione et al. 2004, Khalafi & Raffiee 2010).

At 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 & Raffiee 2010). In activated sludge at neutral pH, Chiron et al. (2010) suggested a phenolic nitration process involving peroxynitrite, while a nucleophilic nitration of acetaminophen did not occur. Peroxynitrite is a by-product of cell respiration and is known to be formed through the combination of superoxide and nitric oxide (Ferrer-Sueta & Radi 2009). The nitration mechanism by peroxynitrite also involves the initial formation of nitrogen dioxide radicals. It is reported that high AOB (ammonium oxidizing bacteria) activity promotes the formation of peroxynitrite. 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 mg/L NO_2^- -N in an oxidation ditch where BPA nitration was detected (Sun et

al. 2012). The evidence for a radical mechanism proposed by Moodie at pH 2-5 was reported by Vione et al. (2004) for phenol, while an alternative reaction mechanism in which nitrosation of the phenol is followed by oxidation to the 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.

Table 1

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 dextrorphan 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 dextrorphan. BPA is a well-known micropollutant due to its endocrine disrupting activity (Oehlmann et al. 2006). Dextrorphan is a human metabolite of the antitussive prodrug dextromethorphan and has been detected in WWTP effluents (Thurman & Ferrer 2012), however its potential transformation during wastewater treatment has not been studied so far.

2. Experimental

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 $NaNO_2$

were purchased from Sigma Aldrich (Schnelldorf, 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.

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 x 3.00 mm, 4 µm; Phenomenex, Aschaffenburg, Germany), coupled to a quadrupole-MS/MS (AB Sciex API 4000, Applied Biosystems, Langen, Germany) with ESI operating 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 Acela HPLC coupled with ESI to an LTQ-orbitrap-MS (LTQ Orbitrap Velos, Thermo Scientific, Bremen, Germany).

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 $NaNO_2$ 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.

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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 WWTP with a capacity of 320,000 population equivalents and a daily flow rate of 61,000 m^3. 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 CO_2 through a diffuser. CO_2 was added to the gas mixture to stabilize the pH, which would otherwise increase due to purging of dissolved CO_2. 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 $NaNO_2$. After pH equilibration, OPP, BPA and dextrorphan were spiked to the sludge. Samples were filtered (regenerated cellulose, 0.45 µm) and stored at +4 ºC. A matrix-matched calibration curve was used for the quantification of OPP and NO_2-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.

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 NO_2-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).

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. NO_2-OPP, dinitro-BPA and the phenolic precursors were quantified by the standard addition method. Special care was taken not to freeze samples or expose 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 x 150 mL aliquots and the effluent and blank into 4 x 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 2-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 2-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 (NO_2-OPP) formation are plotted against the pH. Above pH 5 the formation of NO_2-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 (eq. 1-3). The quotient in eq. 3, where $[NO_2^-]_0$ is the initial nitrite concentration, is the nitrous acid concentration at equilibrium (for derivation see Supplementary Data eq. S1-6). This approach has been

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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.

$$\mathrm{NO_2^-} + \mathrm{H^+} \leftrightharpoons \mathrm{HNO_2} \quad \mathrm{pK_a} = 3.26 \tag{1}$$

$$\mathrm{K_a} = \frac{[\mathrm{H^+}][\mathrm{NO_2^-}]}{[\mathrm{HNO_2}]} \tag{2}$$

$$\text{initial rate} = k \cdot \frac{[\mathrm{H^+}][\mathrm{NO_2^-}]_0}{\mathrm{K_a} + [\mathrm{H^+}]} \tag{3}$$

Three products were identified via LC-HRMS, the *ortho*- and *para*-isomer of nitro-2-phenylphenol (NO_2-OPP), and one isomer of nitroso-2-phenylphenol (NO-OPP), for which the location of -NO substitution is unknown, but is assumed to occur at the *para*-position, since in similar experiments with BPA and dextrorphan, where the *para*-position is blocked, nitrosation was not detected. Both isomers of NO_2-OPP had similar rates of formation (see Supplementary Data Fig. S6). Further discussion of NO_2-OPP formation is based on *para*-NO_2-OPP, however *ortho*-NO_2-OPP appears to be formed analogously.

Figure 1

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 NO_2-OPP formation did not increase with increasing NO-OPP concentration (i.e. an initial rate of zero for NO_2-OPP was not observed). NO-OPP concentrations were also stable for >10 h after reaching equilibrium (data not shown). This implies that NO_2-OPP is, at least to a large degree, a direct product from OPP. To confirm this, experiments were carried out using the antioxidant *N*-acetylcysteine, which reacts with HNO_2 and N_2O_3, 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), NO_2-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 where identified in the aqueous nitrite solution, confirming that both N_2O_3 and HNO_2 react with AcCySH (eq. 4-6), analogously to cysteine (CySH), which forms CySNO and the dimer CySSCy (Grossi & Montevecchi 2002). HNO_2 oxidizes AcCySH to AcCyS· radicals, which combine to form the dimer AcCySSCyAc. N_2O_3 is present due to the dissociation of HNO_2 in aqueous solution, (eq. 7 & 8) (Park & Lee 1988) but reacts with thiols. Thus, excess AcCySH effectively eliminates HNO_2 and N_2O_3.

$$\mathrm{AcCySH + HNO_2 \longrightarrow AcCyS\cdot + \cdot NO + H_2O} \quad (4)$$

$$\mathrm{2AcCyS\cdot \longrightarrow AcCySSCyAc} \quad (5)$$

$$\mathrm{AcCySH + N_2O_3 \longrightarrow AcCySNO + NO_2^- + H^+} \quad (6)$$

$$\mathrm{2HNO_2 \rightleftharpoons \cdot NO + \cdot NO_2 + H_2O} \quad (7)$$

$$\mathrm{\cdot NO + \cdot NO_2 \overset{k_+}{\underset{k_-}{\rightleftharpoons}} N_2O_3} \quad (8)$$

Figure 2

Therefore, it can be suggested that HNO_2 and/or N_2O_3 are the predominant agents for the formation of NO_2-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 & Williams 2002). NO-OPP formed via AcCySNO appeared stable with respect to oxidation to NO_2-OPP in the presence of O_2, again suggesting that a consecutive mechanism OPP → NO-OPP → NO_2-OPP does not take place. Furthermore, the product AcCyS-OPP could also be observed by LC-orbitrap-MS using high resolution MS and the MS^2 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.

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245

246 N_2O_3 is known not only to nitrosate thiols (eq. 6) but also to nitrosate phenolic substances (Noble &
247 Williams 2002). N_2O_3 is in equilibrium with the dissociated form ($\cdot NO + \cdot NO_2$, eq. 8), but the equilibrium
248 favors N_2O_3 with $k_+ = 1.1\times10^9\ M^{-1}\ s^{-1}$ versus $k_- = 8.1\times10^4\ s^{-1}$) (Goldstein et al. 2003). Due to the
249 equilibrium, the impact of AcCySH is likely to be similar on both forms. It is reported that the formation
250 of NO-OPP is likely caused by N_2O_3 rather than $\cdot NO$ reacting with OPP (Noble & Williams 2002),
251 however the formation of NO-OPP through the radical coupling of $\cdot NO$ and $\cdot OPP$ radicals cannot be
252 excluded. A radical mechanism including $\cdot NO_2$ might also be responsible for the formation of NO_2-OPP.
253
254 To test the involvement of $\cdot NO_2$ and $\cdot NO$, the nitration reactions were repeated with the addition of
255 carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazolin-1-oxyl-3-oxide (cPTIO), which is a known radical
256 scavenger for both $\cdot NO$ and $\cdot NO_2$ (eq. 9-11; Goldstein et al. 2003). In the presence of cPTIO the
257 equilibrium concentrations of NO-OPP and NO_2-OPP are significantly reduced by 78% and 65%,
258 respectively, and the initial rate of NO-OPP formation is much lower than that for NO_2-OPP formation
259 (Fig. 3). The concentration of cPTIO was not high enough to cause a complete inhibition of the reaction
260 but in a further experiment at lower OPP concentrations, a complete inhibition of NO-OPP was observed
261 (see Supplementary Data Fig. S7). Since cPTIO scavenges specifically $\cdot NO$ and $\cdot NO_2$ radicals, this
262 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.
263 N_2O_3 is known to react as a nitrosating species, however the involvement of the dissociated form of N_2O_3
264 ($\cdot NO + \cdot NO_2$) could not be excluded considering the impact of cPTIO.
265

$$\mathrm{cPTIO} + \cdot\mathrm{NO} \longrightarrow \mathrm{cPTI} + \cdot\mathrm{NO_2} \quad (9)$$

$$\mathrm{cPTIO} + \cdot\mathrm{NO_2} \rightleftharpoons \mathrm{cPTIO^+} + \mathrm{NO_2^-} \quad (10)$$

$$\mathrm{cPTIO^+} + \cdot\mathrm{NO} + \mathrm{H_2O} \longrightarrow \mathrm{cPTIO} + \mathrm{NO_2^-} + 2\mathrm{H^+} \quad (11)$$

266

267 **Figure 3**

268 In summary, NO_2-OPP formation was impacted when either HNO_2, N_2O_3 or possibly $\cdot OPP$ were
269 scavenged by AcCySH, and the involvement of $\cdot NO_2$ or $\cdot NO$ radicals was shown by the cPTIO
270 experiment, therefore NO_2-OPP should be formed by a radical reaction. Since it is not formed via

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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 ·OPP with ·NO_2 radical to form the nitrophenol, shown by eq. 1, 7, 8 and Scheme 1, as described by Beake et al. (1994) for the nitration of *para*-methoxyphenol by HNO_2. ·NO_2, although being present at a low concentration, would be constantly replenished due to the equilibrium in equation 8.

Scheme 1

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 low 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 & Buth 1984), an appreciable formation of NO-OPP or NO_2-OPP might 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.

Figure 4

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 radical 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 → PhPhO· + ·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 (eq. 12, 13).

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$$\frac{d\,[\mathrm{NO_2\text{-}OPP}]}{dt} = k_1\,[\mathrm{HNO_2}]\,[\mathrm{OPP}]^{1/2} \tag{12}$$

$$-\frac{1}{2}\frac{d\,[\mathrm{HNO_2}]}{dt} = k_2\,[\mathrm{HNO_2}]^2 \tag{13}$$

298

299 The rate constant k_2 is the slope of the reciprocal nitrous acid concentration over time (Fig. 5a).

300 Integration of eq. 13 and solving for $[HNO_2]$ gives:

$$[\mathrm{HNO_2}] = \left(2k_2t + \frac{1}{[\mathrm{HNO_2}]_0}\right)^{-1} \tag{14}$$

301

302 If the OPP concentration is high compared to HNO_2 and/or conversion to NO_2-OPP remains low, then

303 [OPP] can be approximated by $[OPP]_0$. Substituting eq. 14 into eq. 12 and integrating gives:

$$[\mathrm{NO_2\text{-}OPP}] = \frac{k_1\sqrt{[\mathrm{OPP}]_0}}{2k_2}\ln\left(2k_2t\,[\mathrm{HNO_2}]_0 + 1\right) \tag{15}$$

304

305 The pseudo-first order rate constant k_1 ($2.5\cdot10^{-4}$ $L^{1.5}$ $mol^{-1.5}$ s^{-1}) was found by fitting the calculated

306 concentration to the experimental results of Figure 4. Equation 15 was tested by carrying out an

307 experiment at a longer duration and was found to accurately model the experimentally determined

308 concentrations of NO_2-OPP (Fig. 5b).

309

310 **Figure 5**

311

312 The developed model enables the calculation of the NO_2-OPP concentrations formed in the batch systems

313 by the reaction of OPP with HNO_2/·NO_2 radicals. As other wastewater constituents (e.g. further phenolic

314 compounds) are probably also reacting with HNO_2/·NO_2 this model allows prediction of the upper limit

315 of formation (maximum concentration). For instance, at pH 7, a maximum concentration of NO_2-OPP of

316 1 ng/L is predicted after 6 h for 1 µg/L OPP and 1 mg/L NO_2^--N. However, if the nitrite concentration is

317 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

318 be formed under this idealized case where only OPP is reacting with HNO_2. During certain treatment

319 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 $\cdot NO_2$ 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 µg/L BPA and OPP) led to formation of NO_2-OPP, NO_2-BPA and dinitro-BPA (Fig. 6, C & D). The extent for 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. 6, B). Storage at 4 ºC did not cause the artificial formation of nitrophenolic compounds (Fig. 6, A). An explanation could be found in publications reporting a shift to lower pH values when freezing buffered solutions (Sundaramurthi et al. 2010, Goyal & Hafez 1995). Thus, freezing is an inappropriate storage method for samples to be analyzed for phenolic compounds. Sample storage should occur at 4 ºC instead.

Figure 6

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 $\cdot NO_2$. 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.

Figure 7

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 peroxynitrite) are 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 NO_3^--N while the ammonium concentration decreased to 210 mg/L NH_4^+-N. The formation of nitrophenolic compounds was not detected in this experiment.

TPs formed under neutral conditions

Via the LC-MS fragmentation pattern 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.

Scheme 2

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 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

376 experiments of the current study confirms the relevance of these degradation pathways in mixed cultures
377 from municipal WWTPs at substrate concentrations of 200 µg/L. Although the concentration of
378 dextrorphan remained relatively constant (~10% elimination), several hydroxylated dextrorphan-TPs
379 were identified in small concentrations. In total four isomers of hydroxy-dextrorphan TPs were identified
380 with similar MS^2 spectra, possibly due to the formation of diastereomeric pairs from the chiral precursor.
381 Due to the low proportion of dextrorphan conversion, an isolation of TPs for structure confirmation was
382 impossible. In addition, sulfate conjugation products of all three phenols were detected. Sulfo-OPP was
383 quickly eliminated, while the others persisted in the batch experiment. Sulfate conjugation is discussed
384 below in more detail. The characterization of TPs by MS/MS is described in the Supplementary Data.
385
386 Batch experiments were also conducted with the nitrophenolic TPs of OPP and BPA, to test their stability
387 towards (bio)degradation in activated sludge. NO_2-OPP and dinitro-BPA were transformed in the batch
388 experiments during the 6-day period to approximately 50% and 80%, respectively (Fig. 7b). For both
389 nitrophenols the phenolic hydroxyl group was conjugated with sulfate ($-SO_3$; Scheme 2). Further TPs
390 were not observed.
391
392 The sulfate conjugation seems to be a very common microbial process occurring in activated sludge from
393 biological wastewater treatment with a wide substrate spectrum. Sulfate conjugation (sulfurylation) is a
394 widely occurring biological process in cells. It has various functions including detoxification of
395 xenobiotic substances (Malojčić & Glockshuber 2010). Sulfurylation of estrogens has previously been
396 observed by mixed bacterial cultures from activated sludge (Khunjar et al. 2011). Further studies have
397 reported these sulfate conjugates can also be de-conjugated in sludge with resulting release of estrogens
398 (Kumar et al. 2012), an indication of the reversibility of this type of transformation.
399
400 ***Formation of nitrophenols in activated sludge***
401 In an activated sludge medium, a rapid formation of nitrophenols from the three precursor phenols was
402 observed under acidic conditions (pH 3.3-3.5). Figure 7c shows the formation of nitrophenols measured
403 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 maximal formation of NO_2-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 HNO_2 and $\cdot NO_2$ are probably reacting with other sludge constituents (DOC of the sludge ~ 10 mg/L). Therefore, it can be concluded that the nitration process with HNO_2 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.

Table 2

3.6 Analysis of wastewater for the presence of nitrophenolic TPs

The concentrations of three nitrophenolic substances, NO_2-OPP, dinitro-BPA, and NO_2-dextrorphan, and their precursors (OPP, BPA, dextrorphan) were analyzed in wastewater samples from two German WWTPs. Flow-proportional composite samples were taken from the influent and the final effluent over a 24 h period.

Table 3

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-

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433 BPA (Scheme 2) were also identified by LC-MS/MS in raw wastewater and WWTP effluents, suggesting
434 that transformation processes identified in batch experiments may also be occurring during drainage and
435 wastewater treatment. For TP identification, quadrupole-MS/MS in MRM mode was used with
436 characteristic MS^2 fragments for each TP (See Supplementary Data for MS^2 spectra). However, it was
437 impossible to quantify these TPs due to the lack of authentic standards. Nitrophenolic TPs of OPP,
438 dextrorphan and BPA were not detected at the WWTPs as shown in Table 3. NO_2-OPP was detected in
439 raw wastewater with 2.3 ng/L at WWTP 1 and was not found in the WWTP effluent. It can be assumed
440 that NO_2-OPP originated from sources other than biological wastewater treatment. For instance, if
441 favorable conditions in the sewer system were present e.g. a local acidification, this could lead to a
442 nitration of OPP. Alternatively, UV radiation can also promote OPP nitration (Suzuki et al. 1990). This
443 could occur during surface run-off before entering the sewer system. The slight increase of the
444 dextrorphan concentrations, e.g. from 5 ng/L to 15 ng/L in WWTP 2, might be caused by the hydrolysis
445 of O-glucuronide conjugates as suggested by Thurman & Ferrer (2012), who detected dextrorphan in
446 WWTP effluent and a US river. O-Demethylation of dextrormethorphan, the prodrug of dextrorphan,
447 during treatment would also lead to dextrorphan formation.
448
449 These results underline the prediction that NO_2-OPP and dinitro-BPA are not formed in appreciable
450 concentrations during biological wastewater treatment. According to a prediction of NO_2-OPP
451 concentrations using eq. 15 for the conditions found in WWTP 1, taking into consideration the influence
452 of the sludge matrix, not more than 0.1 ng/L NO_2-OPP would be expected. Thus, these concentrations
453 would be far below the quantification limits of the method (see Sec. 2 and Supplementary Data for
454 details). It might be possible that the formation of dinitro-BPA (1.9-3.7 ng/L) reported by Sun et al.
455 (2012) and of nitro-acetaminophen (180-320 ng/L) reported by Chiron et al. (2010) might be caused by
456 different treatment processes, such as the formation of peroxynitrite. However, in our study no indication
457 for the peroxynitrite mechanism was found. Nitro-acetaminophen was included in the analytical method
458 described above and acetaminophen was also spiked into a neutral batch experiment (Table 2, experiment
459 5). Neither in batch experiments, nor in raw wastewater or WWTP effluents was nitro-acetaminophen
460 found despite acetaminophen being permanently present in the raw wastewater.
461

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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 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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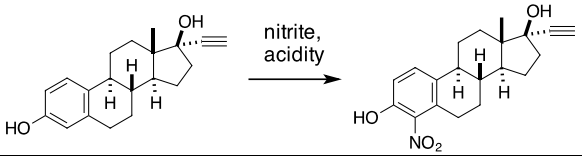
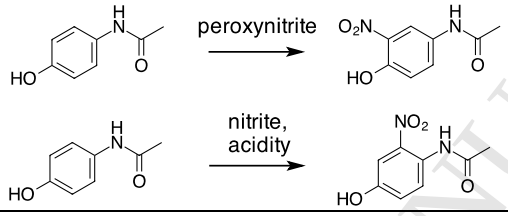
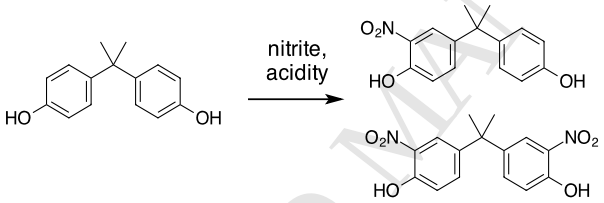
637 ## *Tables*

638

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

Precursor compound	Nitration conditions	Reference
ethinylestradiol		Gaulke et al. 2009 Kunjar et al. 2011
acetaminophen		Chiron et al. 2010
bisphenol A		Sun et al. 2012

640

641 Table 2: Quantification of NO_2-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 modelling[c]
1	3.3	$1.50 \cdot 10^{-4}$	$6.8 \cdot 10^{-7}$	$3.7 \cdot 10^{-8}$	8%
2	3.6	$7.03 \cdot 10^{-5}$	$9.72 \cdot 10^{-7}$	$2.0 \cdot 10^{-8}$	9%
3	3.9	$4.50 \cdot 10^{-5}$	$8.22 \cdot 10^{-7}$	$8.9 \cdot 10^{-9}$	9%
4	4.2	$2.35 \cdot 10^{-5}$	$8.58 \cdot 10^{-7}$	$3.2 \cdot 10^{-9}$	6%
5	7.0	$1.54 \cdot 10^{-9}$	$7.69 \cdot 10^{-7}$	n.d.	–

642 [a] Conditions: activated sludge (batch **1**: 0.2 gSS/L, **2-5**: 4 gSS/L), nitrite addition: batch **1-4**: 3 mg/L NO_2^-

643 -N, batch **5**: no nitrite addition, c_0 = 0.6 mg/L NO_2^--N. [b] $[OPP]_0 < [HNO_2]_0$, however due to low

644 conversion, OPP concentration can be treated as constant. [c] Theoretical formation according to eq. 15. n.d.

645 : not detected.

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648

649 Table 3: Concentrations of parent phenols and nitrophenols detected in two German WWTPs [ng/L]

	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.

650

651 [a] Samples before and after the primary clarifier gave similar concentrations so only the latter is given. [b]
652 LOQs are given in brackets. [c] Due to a lack of an authentic standard no LOQ could be determined. n.d. :
653 not detected. [d] Concentration out of range for standard addition, estimated by matrix-matched calibration
654 curve.

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Figure and Scheme Captions

Figure 1: (a) pH trend of the initial rate of abiotic OPP elimination and NO_2-OPP formation (absolute values). Conditions: $[OPP]_0$ = 1 mmol/L, $[NaNO_2]_0$ = 5 mmol/L. Dotted lines are curves of the acid-base equilibrium of HNO_2, fitted to the experimental data. (b) Formation of NO_2-OPP and NO-OPP, characteristic of two parallel reactions. Conditions: pH 3.5, $[NaNO_2]_0$ = 5 mmol/L.

Figure 2: The effect of the antioxidant AcCySH on the nitration and nitrosation of OPP; Conditions: pH 4, $[OPP]_0$ = 1 mmol/L, $[NaNO_2]_0$ = 5 mmol/L. Left: No addition of AcCySH. Right: Addition of 1 µmol/L AcCySH (duplicate experiment).

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

Figure 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 ºC, $[NaNO_2]_0$ = 9 mmol/L, $[OPP]_0$ = 0.6-1.5 mmol/L. Right: Rate of NO_2-OPP formation with respect to HNO_2 concentration. Conditions: pH 4, 22 ºC, $[NaNO_2]_0$ = 0.8-2.5 mmol/L, $[OPP]_0$ = 1 mmol/L.

Figure 5: (a) Plot of reciprocal nitrous acid concentration as a function of time. The second order rate constant $2 \cdot k_2$ is the slope, 0.31 L/s·mol. (b) Estimated NO_2-OPP concentration using eq. 15 (dotted line) vs. experimental results. Conditions: pH 4, $[OPP]_0$ = 1 mmol/L, $[NaNO_2]_0$ = 5 mmol/L.

Figure 6: Nitrophenol formation resulting from sample storage or preparation: Batch experiments in pH 7 buffered water were spiked with 1 µg/L BPA and OPP, and varying nitrite concentrations: A-C: 0.6, D: 2.4 mg/L NO_2^--N.

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685

686 Figure 7: (a) Concentration of phenolic parent compounds in a batch experiment: c_0 = 200 µg/L.
687 Conditions: activated sludge (0.2 gSS/L), pH 7.2-7.5. (b) Stability of nitrophenols to biodegradation.
688 Conditions: activated sludge (0.2 gSS/L), pH 7.2-7.5, dinitro-BPA and NO_2-OPP, c_0 = 200 µg/L. (c)
689 Formation of nitrophenols in activated sludge. Conditions: activated sludge (0.2 gSS/L), pH 3.3, 4.2 mg/L
690 NO_2^--N.

691

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

693

694 Scheme 2: Biotic transformation products observed from BPA, OPP and dextrorphan. [a] TPs identified in
695 enrichment culture studies (Kohler et al. 1988, Ike et al. 2000).

696

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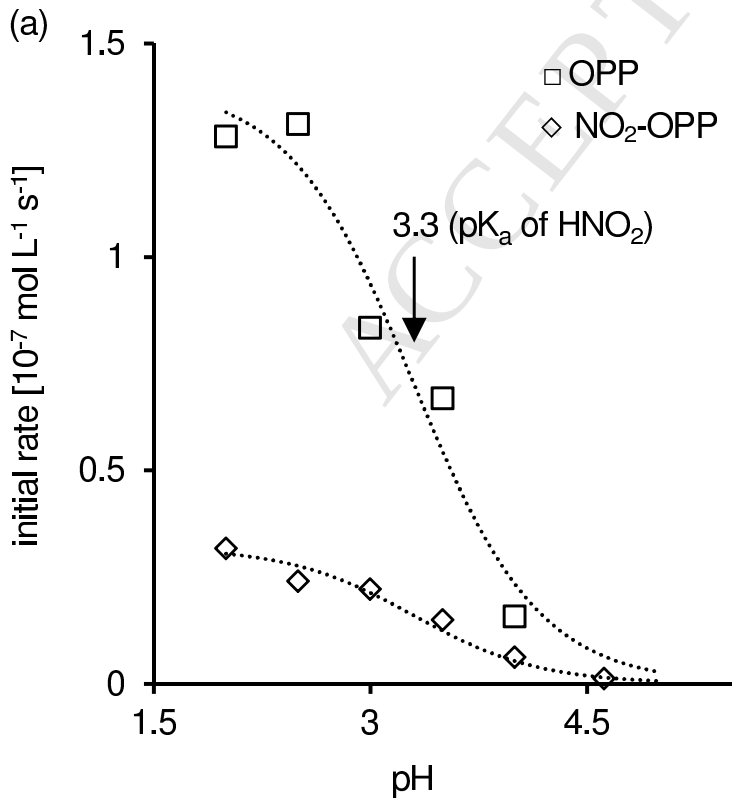

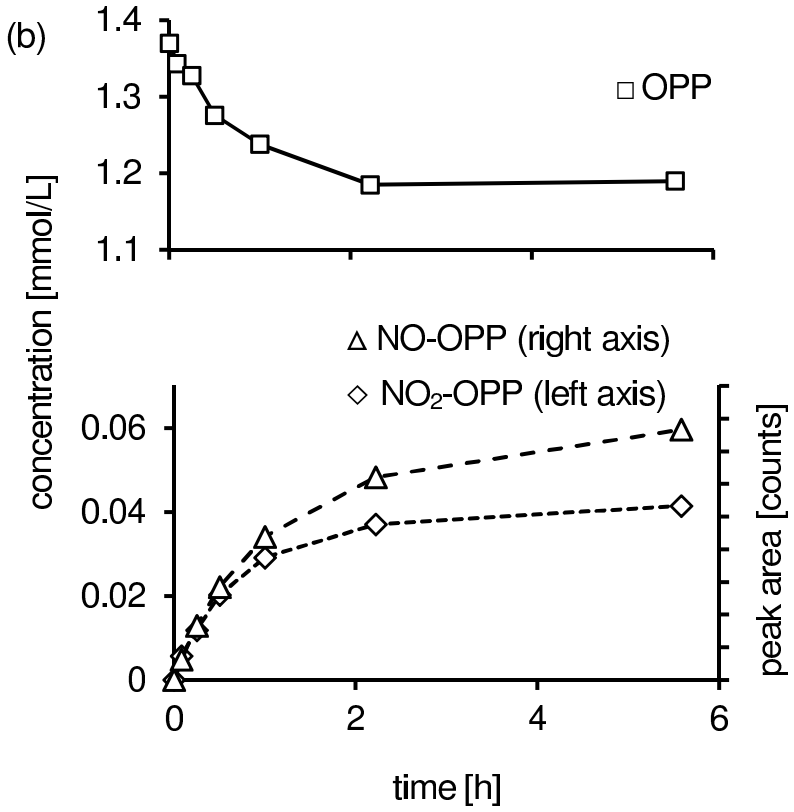

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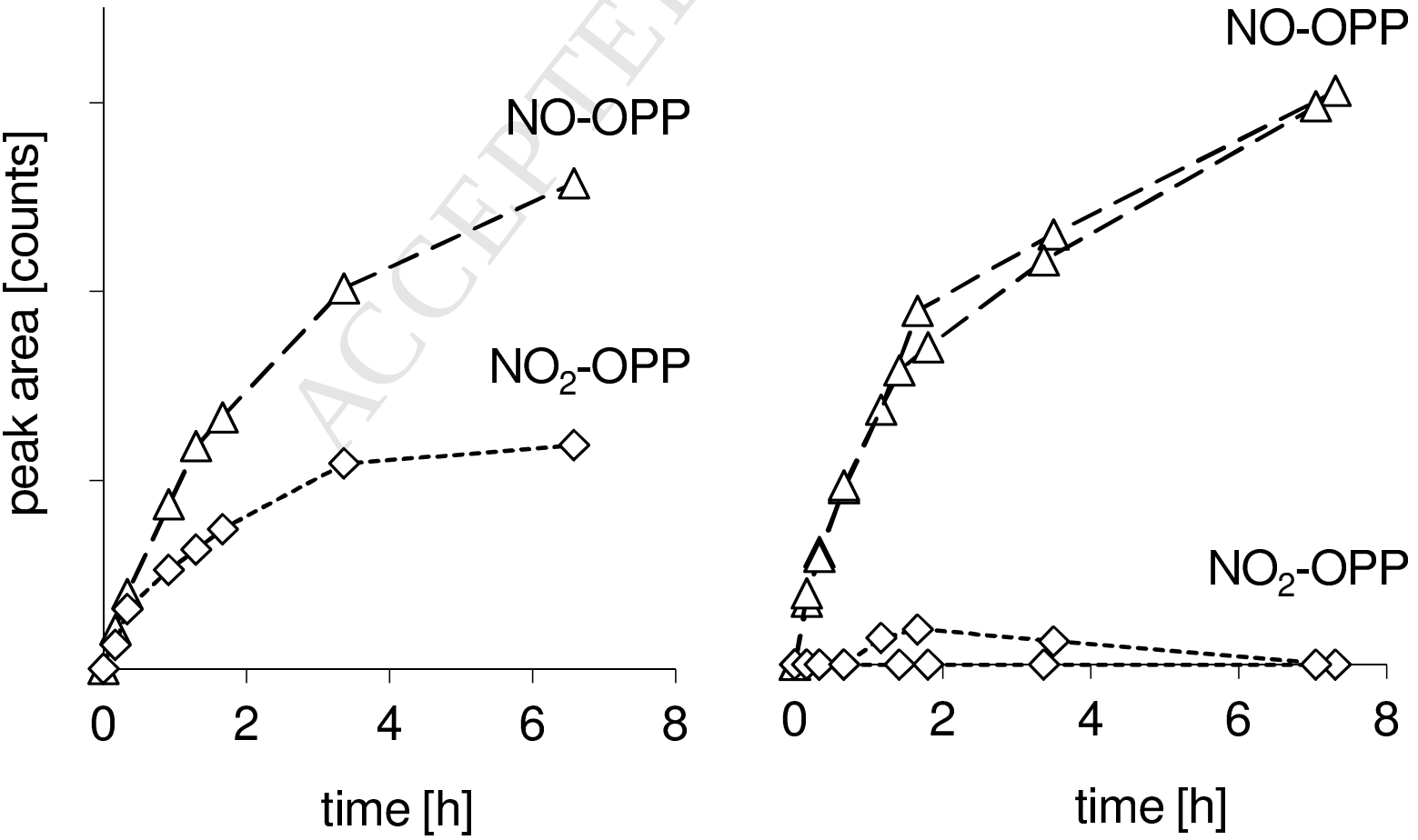

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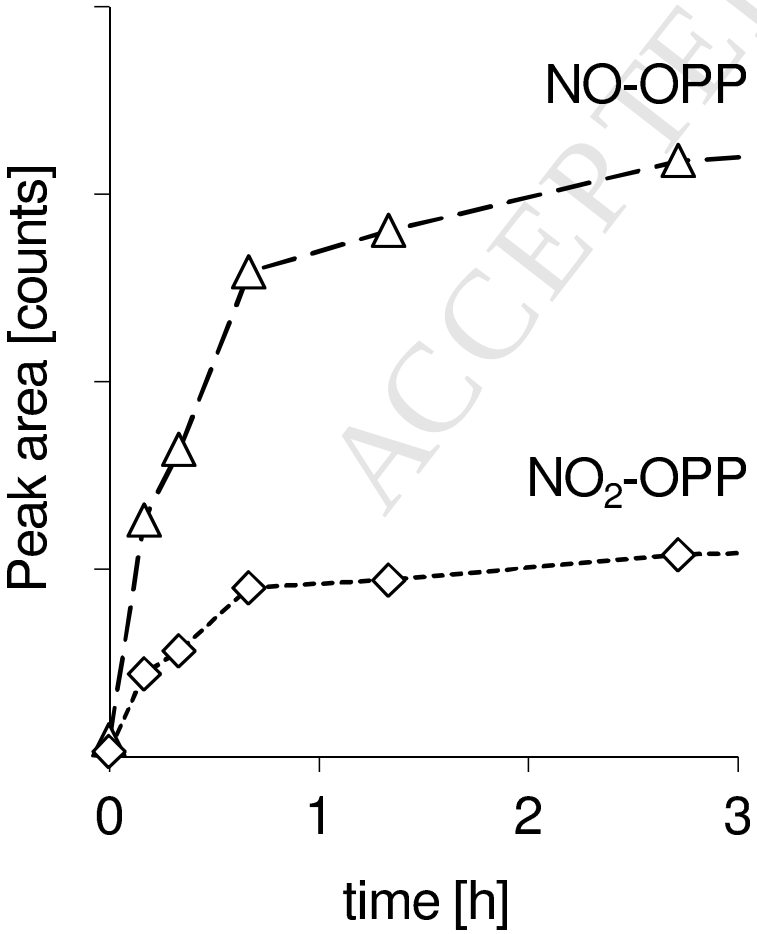

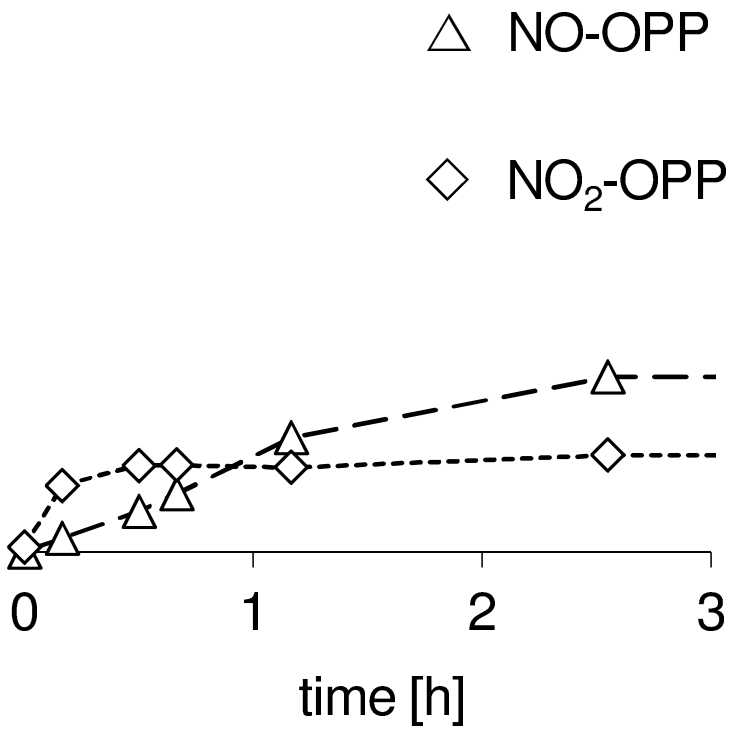

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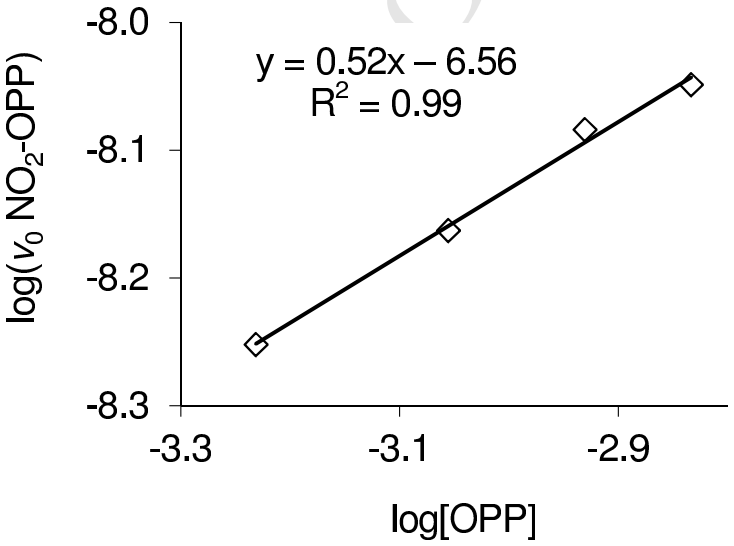

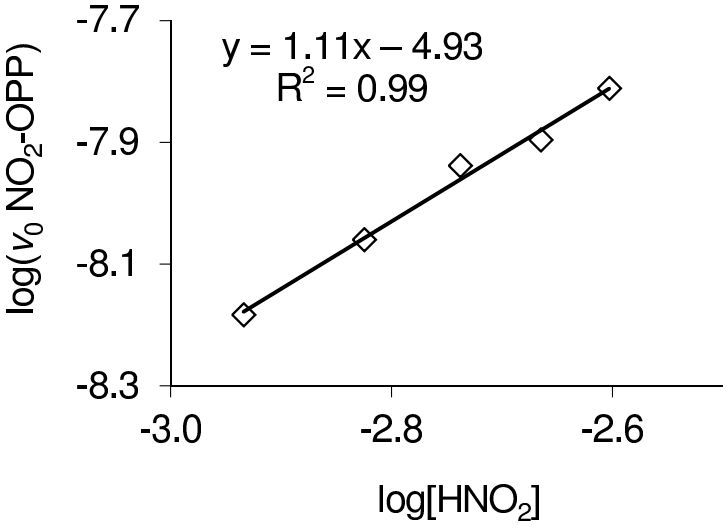

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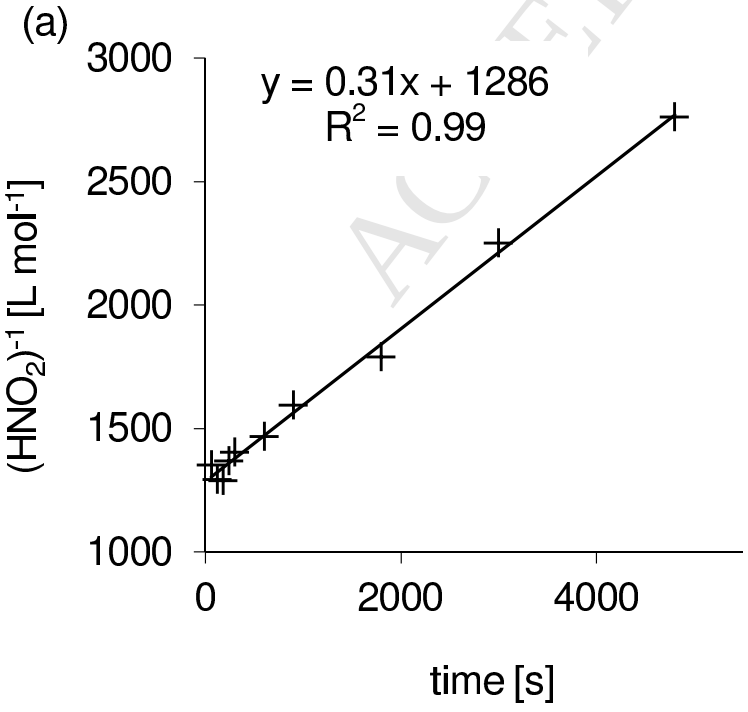

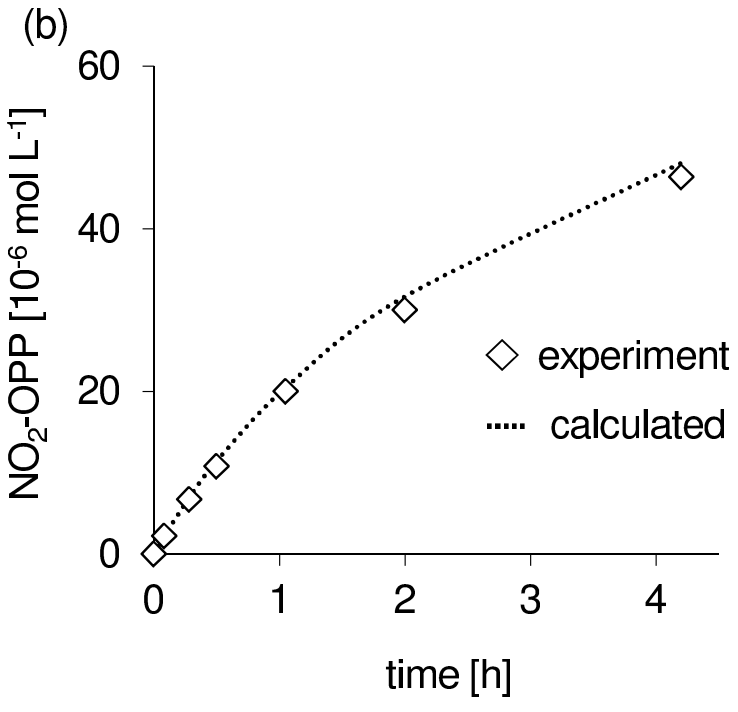

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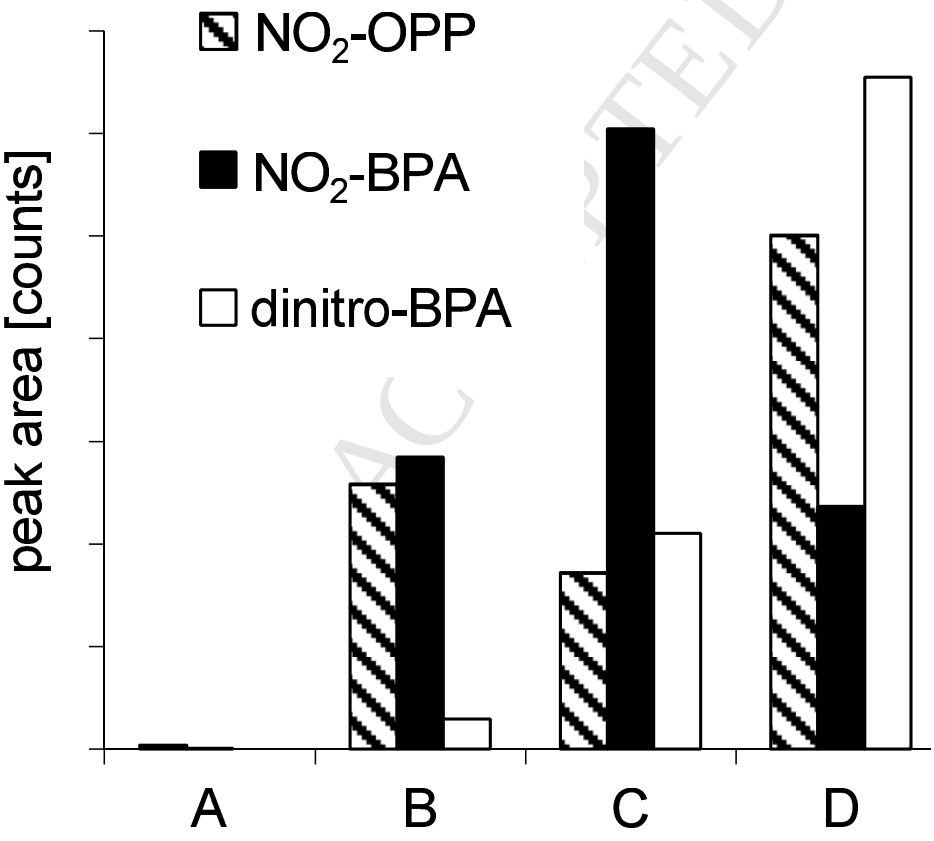

A:	storage at +4 °C
B:	acidified to pH 2
C:	0.6 mg/L NO_2^--N, storage at –20 °C
D:	2.4 mg/L NO_2^--N, storage at –20 °C

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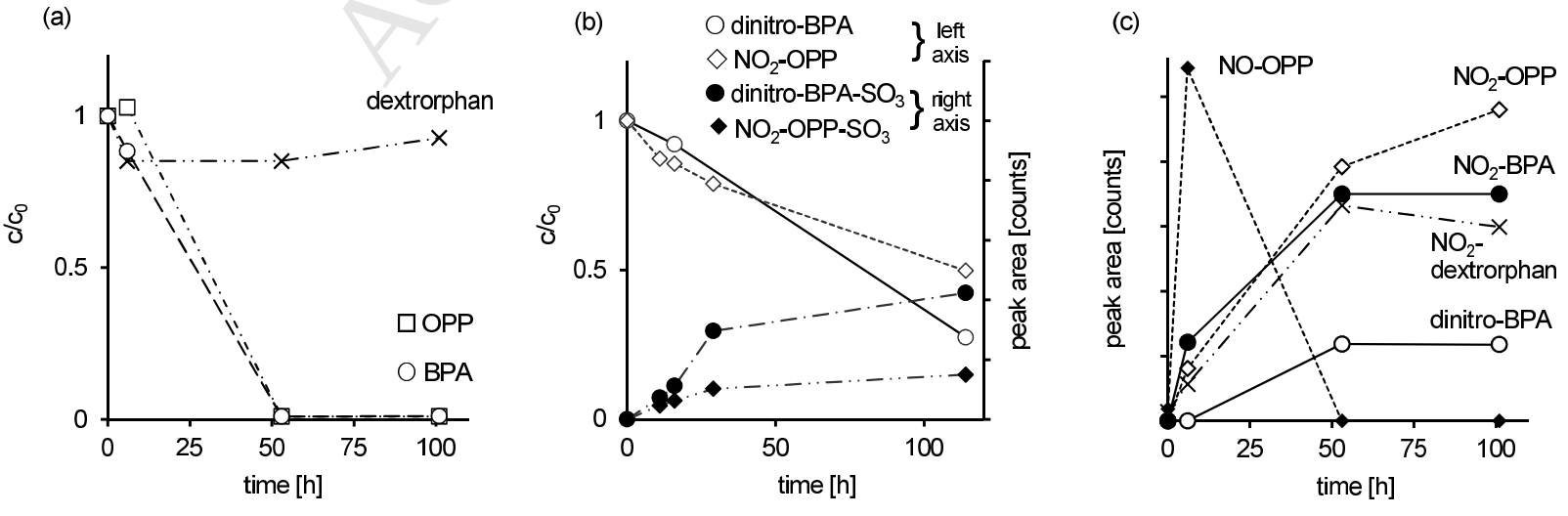

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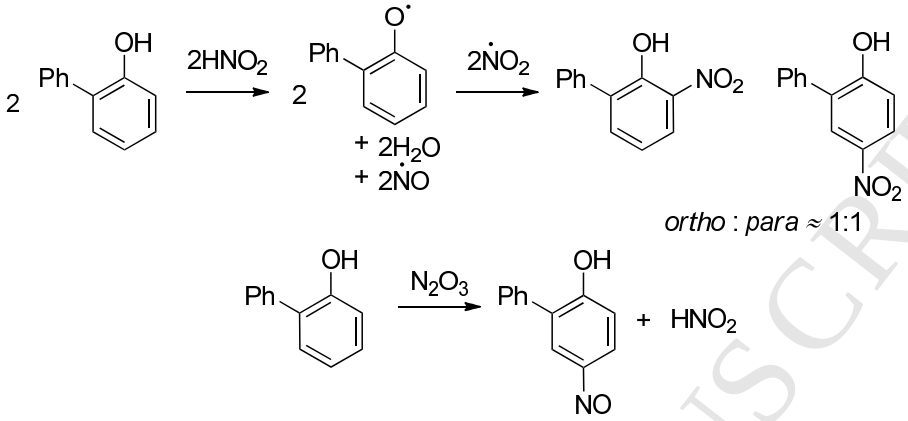

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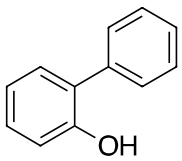
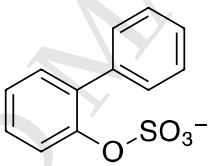
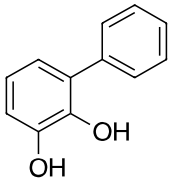
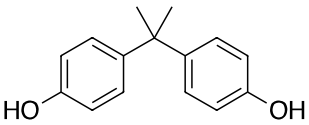
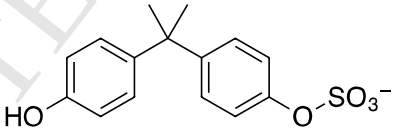
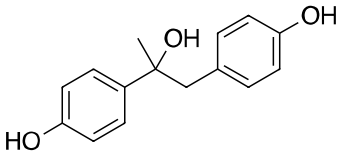
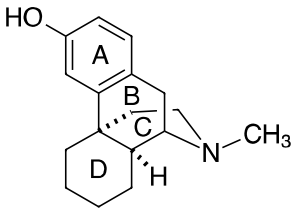
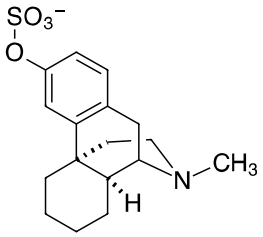
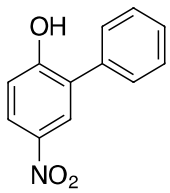
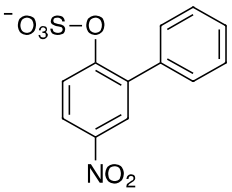
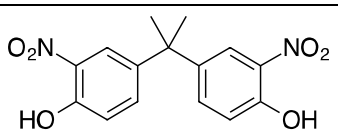
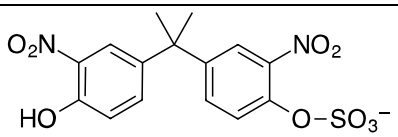
Phenolic precursors	Transformation products (TPs)	
 OPP	 sulfo-OPP	 hydroxy-OPP[a]
 BPA	 sulfo-BPA	 1,2-bis(4-hydroxyphenyl)-1-propanol[a]
 dextrorphan	 sulfo-dextrorphan	hydroxy-dextrorphan: 4 isomers with postulated substitution on rings B, C or D.

Nitrophenolic precursors	Transformation products (TPs)	
 *p*-NO_2-OPP	 sulfo-*p*-NO_2-OPP	
 dinitro-BPA	 sulfo-dinitro-BPA	

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