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Authors: Oliver Knoop, Marion Woermann, Holger V. Lutze, Bernd Sures, Torsten C. Schmidt

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Ecotoxicological effects prior to and after the ozonation of Tamoxifen

Oliver Knoop [a, b], Marion Woermann [c], Holger V. Lutze [a, b, d], Bernd Sures [b, c]

Torsten C. Schmidt [a, b, d]

[a] Instrumental Analytical Chemistry, University Duisburg-Essen, Universitätsstrasse 5, 45141 Essen, Germany

[b] Centre for Aquatic and Environmental Research (ZWU), University Duisburg-Essen, Universitätsstrasse 2, 45141 Essen, Germany

[c] Aquatic Ecology, University Duisburg-Essen, Universitätsstrasse 5, 45141 Essen, Germany

[d] IWW Water Centre, Moritzstr. 26, Mülheim an der Ruhr, Germany

*Corresponding author (current address):

Torsten C. Schmidt

Instrumental Analytical Chemistry

University of Duisburg-Essen

Universitätsstrasse 5

45141 Essen, Germany

phone: +49-201-1836774

fax: +49-201-1836773

e-mail: torsten.schmidt@uni-due.de

Highlights

- Effects on *D. magna* and *D. subspicatus* were determined for ozonation of tamoxifen
- Increased green algae growth inhibition after ozonation
- Formation of 3 TPs can be correlated with green algae growth inhibition
- No effect induced by secondary TPs

Abstract

The endocrine disrupting micropollutant tamoxifen can induce several effects on aquatic organisms. It is introduced into the environment mainly by wastewater treatment plant effluents. To reduce the discharge of micropollutants into surface waters, ozonation can be used as additional wastewater treatment option. For only few transformation products (TPs) formed by ozonation ecotoxicological data are available. To enable an initial estimation of ecotoxicological potentials of the TPs formed after the ozonation of tamoxifen, acute toxicity (immobilization) to *Daphnia magna* and green algae growth inhibition using *Desmodesmus subspicatus* were determined for several ozone doses spiked at pH 3 and pH 7. The initial immobilization of *D. magna* by tamoxifen was not further observed after ozonation. In contrast, the green algae growth inhibition increased due to ozonation of tamoxifen. Overall, five transformation products were observed. For three TPs, positive correlations of green algae growth inhibition and peak area were determined, whereas two TPs do not induce the residual effects. Based on our observations, TP 270 can be assumed as most potent of the formed TPs concerning green algae growth inhibition. Since the effect is not induced by formed *N*-oxides, green algae growth inhibition could be reduced by sufficient ozone exposure during wastewater treatment.

Keywords:

Ozonation, Transformation Products, Tamoxifen, *Daphnia magna*, *Desmodesmus subspicatus*

1 Introduction

Micropollutants such as pharmaceuticals, personal care products, and other anthropogenic chemicals, can be found in nearly all surface waters and can induce biological effects already in low concentrations [1, 2]. One major source of micropollutants are the effluents of wastewater treatment plants since most of them cannot be removed sufficiently by conventional wastewater treatment [2-4]. Here, advanced treatment processes can be used to reduce the amount of micropollutants emitted into receiving surface waters [5, 6]. Ozonation was shown to be an effective treatment to reduce the discharge of micropollutants and has successfully been tested in large scale [7-10]. Along with a reduction of micropollutant discharge most toxicological effects (e.g. estrogenic activity) can also be reduced by ozonation [11, 12]. However, the reaction of micropollutants with ozone does not result in mineralization but rather in the formation of transformation products (TPs) [13]. Some of these TPs, e.g. *N*-nitrosodimethylamine (NDMA) and bromate (from oxidation of bromide), can also induce toxicological effects [14-17]. but only few TPs are identified yet, compared to the broad range of original micropollutants and for even less of the known TPs toxicological information is available [8, 16, 18]. Accordingly, a combination of analytical and effect monitoring is necessary for the evaluation of a risk reduction by advanced wastewater treatment [19, 20].

Endocrine disruptive compounds (EDCs) affect the hormonal system of organisms and can thus affect aquatic organisms already at very low concentrations [21]. The antineoplastic pharmaceutical tamoxifen (TAM) is used for breast cancer therapy due to its anti-estrogenic activity [22]. TAM can be found in wastewater treatment plant effluents in typical concentrations of 25 to 200 ng L^{-1} [23-25], with 369 ng L^{-1} being the highest reported concentration [26]. Thereby, concentrations in surface waters were found to range between 25 to 50 ng L^{-1} [23, 25, 27] with a maximum of 212 ng L^{-1} in the river Tyne [26]. These TAM concentrations are in the same range as predicted environmental concentrations (PECs) of TAM in Sweden and England (36 to 63 ng L^{-1}) [23, 28].

Effect concentrations with an effect of 50 % (EC_{50}) for acute toxicity of TAM are only available for the immobilization of the invertebrate *Daphnia magna* (EC_{50} = 1530 µg L^{-1} (24 h) [29] and 210 µg L^{-1} (48 h))[30]. Since TAM was found continuously in a river basin in concentrations 3 orders of magnitude below available acute toxicity values

[27], no acute effects are expected. Therefore, chronic toxicity with observable effects at lower concentration levels is of higher interest. For *Daphnia* species, the $EC_{50,\ reproduction}$ has a concentration of 810 ng L^{-1} [29]. For the inhibition of the growth of green algae similar effect levels are reported for the inhibition concentration (IC_{50}) for different green algae species (470 to 980 ng L^{-1}) [30]. However, TAM has an $EC_{50,\ sex\ ratio}$ on zebra fish (*Danio rerio*) populations at concentrations of 150 ng L^{-1} due to its anti-estrogenic activity [31], but no acute toxicity was found during zebrafish embryo tests (≤ 1850 µg L^{-1}) [30]. Based on reported no observable effect concentrations (NOEC) available for *Daphnia magna* [30] the predicted no effect concentration for TAM is 6.7 ng L^{-1}, calculated using a risk assessment factor of 100 [32]. This NOEC is below all reported concentrations [23, 25-27] and therefore underlines the need to further study TAM behavior in advanced wastewater treatment. Here, especially TP formation and their biological effects are of high interest [18].

TAM reacts fast with ozone and is readily transformed during ozonation of wastewater effluents [33]. Thereby several TPs are formed, of which two can enhance the anti-estrogenic effect of TAM [34]. Two TPs formed during ozonation were also observed during advanced oxidation processes and photolysis, for which a residual acute toxicity for *Aliivibrio fischeri* is reported [29, 35]. Structures of conceivable TPs formed in ozonation of TAM were proposed previously (see Figure 1) [36]. Contribution of hydroxyl radicals for TAM abatement was calculated using the approach of Lee & von Gunten (2016) [18], arriving at ≤ 1 % contribution of hydroxyl radicals to TAM transformation. Hence, the contribution of radical reactions to TP formation can be neglected.

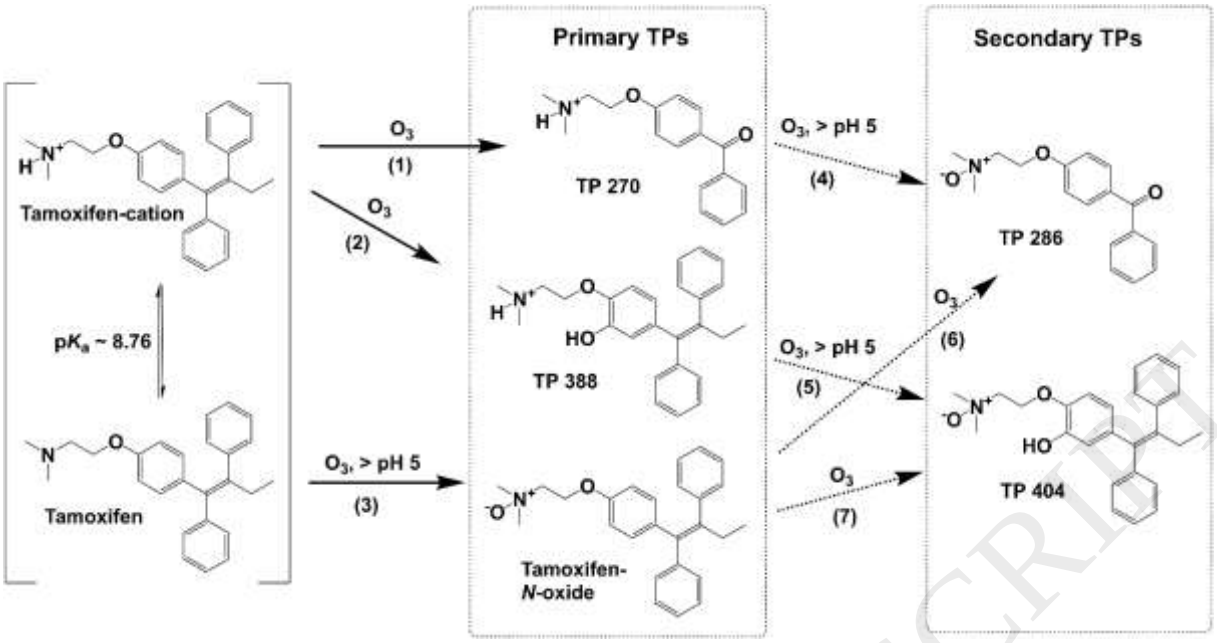

Figure 1: Proposed transformation products and major reaction pathways for the ozonation of TAM at stated pH ranges. TP 270, TP 388, and TAM-*N*-oxide as primary TPs, and TP 286 and TP 404 as secondary TPs[36]. Note that reaction 3 will dominate at pH > 5 [36], due to the high second order rate constants for the reaction of tertiary amines and ozone [37]. Formation of *N*-oxides (reactions 3, 4 & 5) require presence of the corresponding deprotonated species and are most important at a pH > 5, whereas Criegee reaction (1 & 6) and hydroxylation of the benzene ring (2 & 7) are most important at pH < 5. Reaction of the phenolic moieties of TP 388 and TP 404 with ozone have yet not been reported.

In order to gain first information about possible adverse effects of TPs, standardized tests such as the OECD 201 [38] and OECD 202 [39] guidelines can be applied as they are easy to handle. Nevertheless, precursor specific effects, such as anti-estrogenic activity are of high interest and should also be monitored. The anti-estrogenic effect after the ozonation of TAM has already been reported elsewhere [34].

The current study therefore focuses on (I) effect on acute toxicity of tamoxifen on *Daphnia magna* and (II) effect on growth inhibition of the algae *Desmodesmus subspicatus* following ozonation of tamoxifen, both at pH 3 and pH 7.

2 Materials and Methods

2.1 Chemicals

Tamoxifen (CAS: 10540-29-1) was purchased from Alfa Aesar (Karlsruhe, Germany), tamoxifen-*N*-oxide (analytical standard) from LGC (Ann Arbor, Michigan, USA), dimethylsulfoxide (analytical reagent grade) from VWR (Darmstadt, Germany). Potassium dihydrogen phosphate and dipotassium hydrogen phosphate (Merck) were used for the preparation of a pH 7 buffer (100 mM). Sodium hydroxide (VWR, Darmstadt, Germany) and phosphoric acid (Fisher Scientific, Bremen, Germany) were used for pH adjustment. Purity of all chemicals was ≥ 98 % if not stated otherwise. Ultra-pure water was produced onsite (Purelab Ultra, Elga LabWater, Celle, Germany). For liquid chromatography-mass spectrometry (LC-MS) measurements, LC-MS grade methanol (HiPerSolv CHROMANORM, VWR), triple distilled water, and formic acid (Suprapur, Merck) were used as eluents.

To obtain an aqueous ozone stock solution ozone-containing gas was produced onsite with an ozone generator (BMT 802 X, BMT Messtechnik, Berlin, Germany; feed gas: O_2 6.0, Linde, Düsseldorf, Germany) and bubbled through ice-cooled ultra-pure water. Ozone concentration in the stock solution was determined by UV absorption at 258 nm (ε = 2950 M^{-1} cm^{-1}) [40] using a UV-1650PC UV-visible spectrophotometer (Shimadzu, Kyoto, Japan). Accuracy of spiking ozone was ± 5 % and determined using the indigo method [37].

Reconstituted freshwater, or Aachener Daphnien Medium (ADaM) modified after Klüttgen et al. [41], was prepared and aerated continuously until use. A description of the composition is given in Table S2.

A modified algae growth medium (AAM) was used for algae culture maintenance. For growth inhibition tests, twice the concentration of AAM was prepared as double AAM (DAAM) stock solution to gain same concentrations in the test vessels due to 1:1 (v/v) dilution. Compositions of both media are given in Table S5.

2.2 Experimental set up

2 L stock solutions, containing 10 µM TAM (3.71 mg L^{-1}), were prepared in volumetric flasks using a 5 mM TAM solution in DMSO, yielding a 5 mM DMSO concentration. Further, DMSO was added to gain a final concentration of 10 mM DMSO. The pH was adjusted using phosphoric acid and sodium hydroxide to pH 3 and pH 7 before

adjusting the volume finally with ultrapure water. As samples, aliquots of 150 mL were spiked with the ozone stock solution to gain final concentrations of 0, 5, 10, 15, 20, 30, and 40 µM ozone. Samples were stored overnight and pH was checked the next day. Subsequently, the pH was adjusted to pH 6.5 ± 0.1 by spiking 1.5 mL of a 100 mM phosphate buffer (pH 6.5) to each sample, and sodium hydroxide if required, to gain suitable conditions for the toxicity test. Blanks containing DMSO and phosphate buffer were produced accordingly and spiked with the ozone stock solution to gain final ozone concentrations of 0, 20, and 40 µM. 1 mL of each sample was used for LC/MS analysis. Samples were then stored until toxicity testing in the dark at 4 °C for a maximum of 10 days. The concentration of added radical scavenger DMSO (10 mM) in the samples allowed scavenging of > 95 % of hydroxyl radicals eventually formed. Dilution due to addition of the ozone stock solution was ≤3 %. Ozonation experiments at basic pH were not performed, since previous experiments showed a limited reaction at pH 11 and minor formation of TPs due to a lowered solubility of TAM under basic conditions. Furthermore, the reaction of tamoxifen at pH > 5 is largely controlled by the tertiary amine. Hence, the primary point of attack of ozone can be assumed to be the same at pH 7 and pH > 7 (i.e., tertiary amine) [34].

Experiments in wastewater matrix were not performed to ensure that any changes of the effect were solely based on the TPs formed in the reaction of ozone and TAM.

2.3 LC/MS measurements

Measurements were performed as described previously [34] and are described here only in brief. The LC/MS system used consisted of an Agilent 1100 Series LC and a 6120 quadrupole LC/MS (Agilent, Waldbronn, Germany), using a Kinetex® C8 column (50 x 2.1 mm; 5 µm; 100 Å; Phenomenex, Aschaffenburg) for separation. The applied gradient used methanol (+0.1 %/v formic acid) / water (+0.1 %/v formic acid) started at 45 % methanol at a flow rate of 0.5 mL min^{-1} and was kept constant for 0.5 min. The gradient was then increased to 60 % within 1.5 min and further increased to 70 % within 3 min and kept constant at 70 % for 5 min, before reconditioning at 45 % methanol for 4 min. The injection volume was set to 10 µL and all samples were analyzed in triplicates.

Positive electrospray ionization was set to 3 kV and the nebulizer pressure to 30 psi. Dry gas was heated to 300 °C and the flow rate was set to 10 L min^{-1}. The ions of *m/z* 270.1, 286.1, 372.1, 388.2, and 404.2 were monitored using selected ion mode. 20 %

of the signal time were operated in scan mode. TAM and TAM-N-oxide (*m/z* 388.2) were quantified using an external calibration. For the other TPs no reference standards were available, hence a semi-quantitative evaluation was performed for these, namely TP 270, TP 286, TP 388, and TP 404. Names are based on the corresponding *m/z*.

2.4 Daphnia magna immobilization tests

D. magna acute toxicity test was performed following the OECD guideline 202 [39] to determine the $EC_{50,\ immobility}$ and effect change after ozonation at ozone doses of 0, 20 and 40 µM. For toxicity testing, daphnids younger than 24 h were obtained from cultures with daphnids aged 3-12 weeks. Cultures of each ~20 daphnids are kept in 1 L beakers filled with ADaM at a light cycle of 8/16 h dark/light at (20 ± 1°C), fed three times a week with concentrated algae (*Desmodesmus subspicatus*) and water exchanged three times a week.

Test solutions were prepared by diluting pH 7 TAM or DMSO stock solutions as described in 2.2 for $EC_{50,\ immobility}$ determination with at least 50 mL ADaM in a 100-mL volumetric flask. Highest TAM concentration in the test was hence 5 µM (1.86 mg L^{-1}). Applied DMSO concentrations were tested in advance and did not show any effect on the daphnids or deviation from the ADaM as blank and the highest applied concentration of DMSO (5 mM) was hence used as negative control. For determining the effect after ozonation, aliquots of 34 mL of each sample were diluted accordingly with ADaM to a total volume of 100 mL, resulting in a theoretical TAM concentration of 3.4 µM (1.26 mg L^{-1}) in the test. Dilution was chosen based on results obtained during EC_{50} determination to allow observation of decrease and increase of the effect.

For each tested concentration/sample 4 aliquots of 20 mL were filled into separate 50-mL glass beakers. Subsequently, 20 healthy neonates were preselected and given into the remaining 20 mL of the corresponding concentration/sample before 5 daphnids were distributed into each beaker to avoid dilution in the test vessels (n=20). Physicochemical properties were checked before and after each experiment using a LE621 IP67 dissolved oxygen sensor (Mettler-Toledo, Greifensee, Switzerland) for oxygen saturation and temperature, and pH using test stripes (DOSATEST® pH 6.0 – 10.0, VWR). Daphnia tests were kept in darkness at constant temperature (21 ± 1°C). Mobility was monitored by visual inspection after 24 and 48 hours. pH was in the range of 6.4 – 7.0 and dissolved oxygen 4 – 6 mg L^{-1}. Potassium dichromate was used as positive control to ensure reliability of the test. As defined by OECD

Guideline 202, Annex 1 [39], organisms unable to swim within 15 seconds after gentle agitation of the test vessels were considered immobile. $EC_{50,\ immobility}$ was calculated and dose-response curves were plotted using Graph Pad Prism 5.01.

2.5 Algae growth inhibition tests

Algal growth inhibition was tested using the freshwater green algae *Desmodesmus subspicatus.* following the respective OECD 201 guideline [38]. Algae cultures were maintained and harvested according to ISO 8692 [42], using a modified cultivation medium (AAM). As test vessels, sterile 24-well microplates (Cat. #10062-896, VWR, USA) with a sample volume of 2 mL per well were used. Biomass was determined by measuring the fluorescence of the chlorophyll content with a multimode reader Infinite M200 (Tecan, Switzerland) based on a previously determined factor. For more details see S3.2.

For the determination of the IC_{50} value 5 concentrations were prepared by diluting the pH 7 TAM stock solution as described in 2.2 in the range from 0.5 to 3.7 mg L^{-1} with water in 10-mL volumetric flasks. The highest amount of DMSO (10 mM) gained in the test solutions was used as solvent control. Ultrapure water was used for negative control blanks. Each well was prefilled with 1 mL DAAM containing a predefined biomass of algae and subsequently 1 mL of the according test solutions were added to a well according to the experimental design (Table S8 A). The highest tested TAM concentration in the test solutions was 5 µM (1.86 mg L^{-1}).

Algae growth inhibition of TAM-*N*-oxide was tested in the concentration range of 0.15 – 3 µM (same concentrations were formed during ozonation experiments), using a TAM-*N*-oxide stock solution in DMSO. Thereby the same procedure as described for TAM was used, gaining 3 µM as highest concentration of TAM-*N*-oxide in the test (1.16 mg L^{-1}).

Samples spiked with ozone concentrations of 0, 10, 20, 30, and 40 µM were prepared accordingly, including solvent controls spiked with ozone concentrations of 0, 20, and 40 µM. Here, either samples were used without dilution, gaining a dilution of 1:1 (v/v) in the well, or by reducing the sample amount to 0.5 mL and addition of 0.5 mL ultrapure water, gaining a dilution of 1:3 (v/v), resulting in theoretical TAM concentrations of 1.86 mg L^{-1} and 1.24 mg L^{-1} in the wells, respectively. Overall, 6 replicates per concentration were inserted into microplates using the pre-defined experimental

design (Table S8 B & C). The initial biomass concentration of 0.48 mg L^{-1} in each well was confirmed via fluorescence measurements. Additionally to the standard lid, test plates were sealed with PARAFILM® (Brand, Wertheim, Germany) and incubated using a Celltron shaker (InforsHT, Bottmingen, Switzerland) at 100 rpm, 21 ±1°C and light intensity of 120 µeinstein m^{-2} s^{-1}, measured respectively by a thermometer HI 98128 (Hanna, USA) and a quantum-radiometer-photometer LI-185B (Li-Cor, USA) coupled to a LI-190SB quantum sensor. Prior to measurement of the biomass growth after 24, 48, and 60 h, each well was mixed thoroughly and uncovered microplates were pre-shaken automatically for 30 s again. For further details of the fluorescence measurements see S3.2. Growth rates and percent inhibition of the growth rate were calculated according to OECD 201 [38]. For IC values dose-response curves were plotted as described for acute toxicity and values estimated in accordance with the guideline [38].

3 Results and Discussion

3.1 Effect concentrations

Effect and inhibition concentrations of TAM for an effect of 50 % (EC_{50}/IC_{50}) were determined to allow for comparability with previously reported EC_{50} values. Effect-concentration curves are shown in Figure 2 for *D. magna* (A) and *D. subspicatus* (B) and thereby calculated EC_{50}/IC_{50} values are given in Table 1.

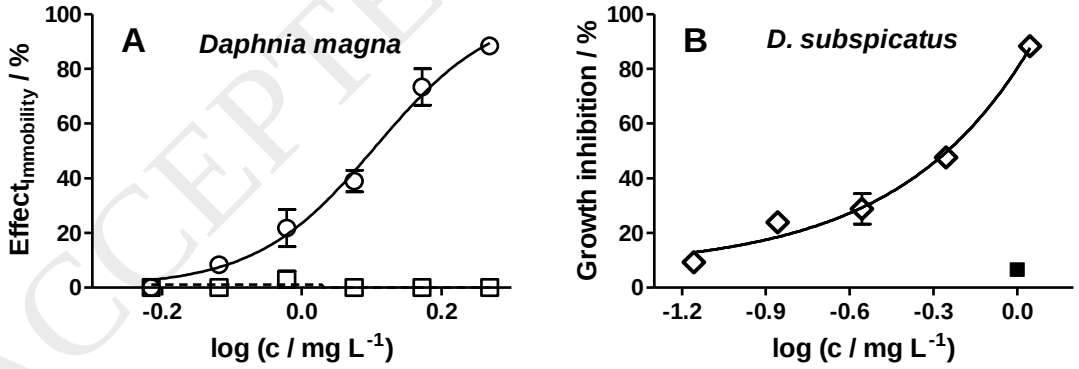

Figure 2: Determination of the $EC_{50,\ immobility}$ of *D. magna* after 24 h (open squares) and ○ 48 h (circles) at pH 7 (A) of TAM and the IC_{50} of the growth inhibition for *D. subspicatus* (diamonds) and DMSO blanks (filled square) after 60 h at pH 7 (B).

The $EC_{50,\ immobilization,\ 48\ h}$ for *D. magna* is in the same range as the values reported previously [29, 30] considering the known variation occurring in these tests [43]. For

D. subspicatus the $IC_{50,\ growth}$ is also in a similar range to the reported value for *Pseudokirchneriella subcapitata* and two further green algae [30], albeit direct comparison is limited since effect concentrations are species specific. Nevertheless, effects at similar concentrations were observed and hence, changes of the effects after ozonation can be evaluated based on tests applied in this study. To the authors' best knowledge, no ecotoxicological data of *D. subspicatus* for TAM are available in the literature. Due to the test design, no higher concentrations were tested and IC values cannot be calculated, but estimated from the dose-response curve in accordance with the guideline [38]. Hence, calculation of the confidence interval was not possible. Higher stock solutions of TAM could not be used due to the limited solubility of TAM.

Table 1: Reported and determined EC_{50}/IC_{50} for TAM. Confidence interval (CI) of 95 % is stated if available. * Calculation of CI for *D. subspicatus* was not possible in this study.

Species	Source	Exposure time / h	Endpoint	EC_{50} / µg L^{-1}	CI (95 %) / µg L^{-1}
Daphnia magna	[30]	48	immobility	210	
Daphnia magna	[29]	24	immobility	1530	
Daphnia magna	**present study**	48	immobility	**1280**	**1080 - 1500**
Species	**Source**	**Exposure time / h**	**Endpoint**	**IC_{50} / µg L^{-1}**	**CI (95 %) / µg L^{-1}**
P. subcapitata	[30]	72	growth	980	830 - 1360
D. subspicatus	**present study**	60	growth	**580**	*****

3.2 Degradation of TAM and formation of TPs

An overview of the concentration of TAM and TAM-*N*-oxide, as well as the semi-quantitative (peak area) formation of the TPs, vs. spiked ozone dose, is given in Figure 3 for pH 3 (A & B) and pH 7 (C & D). Similar results have been reported in a previous study using tertiary butanol as OH-radical scavenger [34]. The formation of propiophenone as possible low molecular weight TP formed by the Criegee reaction was not observed in this study.

3.2.1 pH 3

The concentration of TAM decrements with increasing ozone dose during ozonation at pH 3 (Figure 3 A). Full abatement was observed at an ozone dose of 30 µM, while 20 µM ozone already resulted in 98 % reduction of TAM. Here, only TP 270 and TP 388 were formed (B) and no *N*-oxide formation was observed. At pH 3 TAM is almost

completely dissociated and present as corresponding acid. Due to the protonation at the tertiary amine ozone cannot attack at the amine (p*K*a = 8.76 [44]) and hence absence of *N*-oxide formation can be explained.

3.2.2 pH 7

At pH 7 the initial TAM concentration was reduced to 6 µM instead of 10 µM presumably due to working near the aqueous solubility and potentially subsequent sorpition or precipitation. Since initial concentrations were always measured, this should not affect results of TP formation and effect monitoring. Here, TAM was also completely transformed at an ozone dose of 30 µM. However, the reduction of the TAM concentration at lower ozone doses is somewhat less pronounced than at pH 3 (< 90 % of TAM transformation at an ozone dose of 20 µM) (Figure 3 C). All three primary TPs, TP 270, TP 388, and TAM-*N*-oxide, were formed simultaneously with TAM-*N*-oxide as main product (D). However, the secondary products TP 286 and TP 404 were also observed at the lowest tested ozone dose and are the main products at the highest ozone doses. TPs containing a phenolic moiety such as the suggested structure of TP 404 and TP 388 will be further oxidized at higher ozone doses, though TP 404 persisted at ozone dosages of 60 µM at pH 7 (see Figure S2 D) and no further TPs were detected doses using reversed phase LC/MS, as tested in preliminary experiments. The reason of TP 404 being so persistent is yet unclear. One explanation is that other compounds which escaped detection might have competed for ozone with TP 404. Furthermore, the molecular structure, albeit conceivable, is just proposed. Further transformation of TP 286 due to ozone reactions is highly unlikely due to the deactivation of the aromatic moieties by the ketone [45].

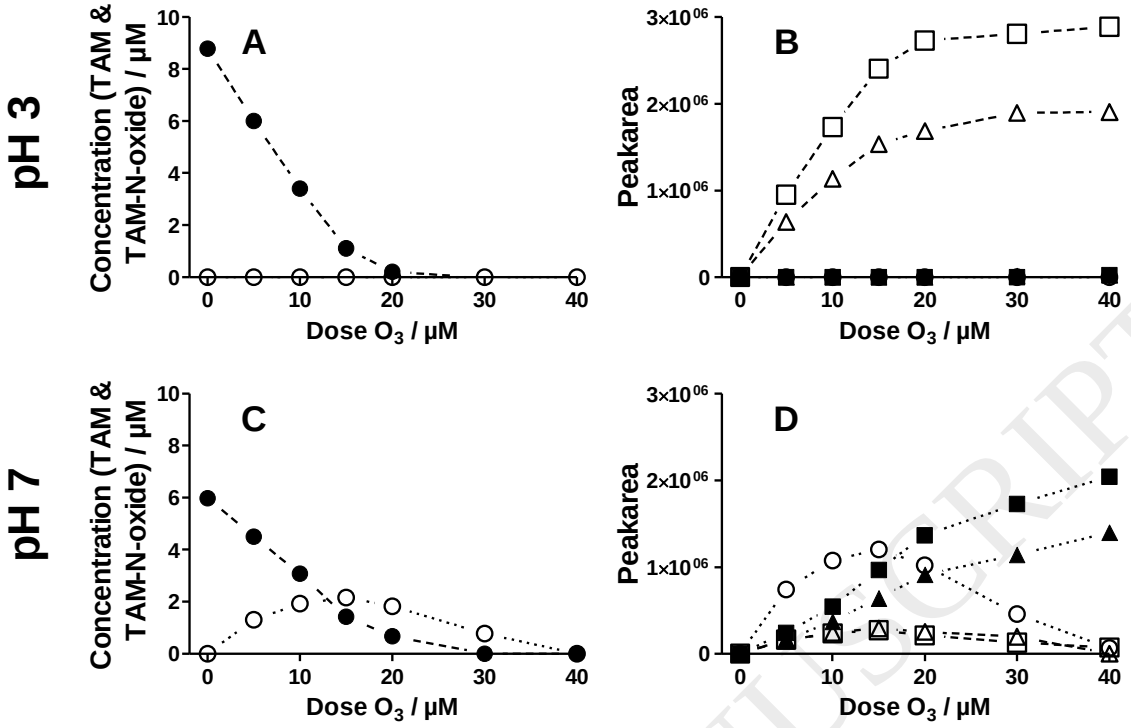

Figure 3: Degradation of ● - TAM at pH 3 (A) and pH 7 (C) during ozonation and formation of ○ - TAM-*N*-oxide. Semiquantititive formation of TPs at pH 3 (B) and 7 (D). □ - TP 270, ■ - TP 286, Δ - TP 388, ▲ - TP 404,. Initial applied concentration of TAM: 10 µM.

3.3 Effects after Ozonation

3.3.1 Daphnia magna immobilization tests

D. magna immobilization of TAM samples ozonated at pH 3 and pH 7 is shown in Figure 4 A & B, respectively. Effect of the initial TAM concentrations was in both cases about 80 %. After ozonation of TAM no further immobilization was observed in the tested samples, spiked with either 20 µM or 40 µM as final ozone concentration at both, pH 3 and pH 7.

3.3.2 Green algae growth inhibition tests

Growth inhibition of the green algae *D. subspicatus* for the ozonation of TAM at pH 3 and pH 7 is shown in Figure 4 C & D, respectively. Two dilutions (1:1 and 1:3 (v/v)) of each sample were tested. At pH 3 (C) the 1:1 (v/v) dilution of the ozonated samples resulted in no significant change in the inhibition of the algae, indicating that the toxicity of the samples was way above the range covered by the test. Therefore, experiments were repeated using the 1:3 (v/v) dilution in the tests. Here, the initial effect increased

by the factor of 2 at an ozone dose of 10 µM and remained constant at about 80 % growth inhibition, which is still at the upper limit of the test.

Samples ozonated at pH 7 showed an increase of the growth inhibition with increasing ozone dose of up to 20 µM, which decreased at higher ozone doses in the 1:1 (v/v) dilution. For the 1:3 (v/v) dilution the initial growth inhibition (30 %) increased by a factor of 2.6 at an ozone concentration of 10 µM (80 %) and subsequently decreased.

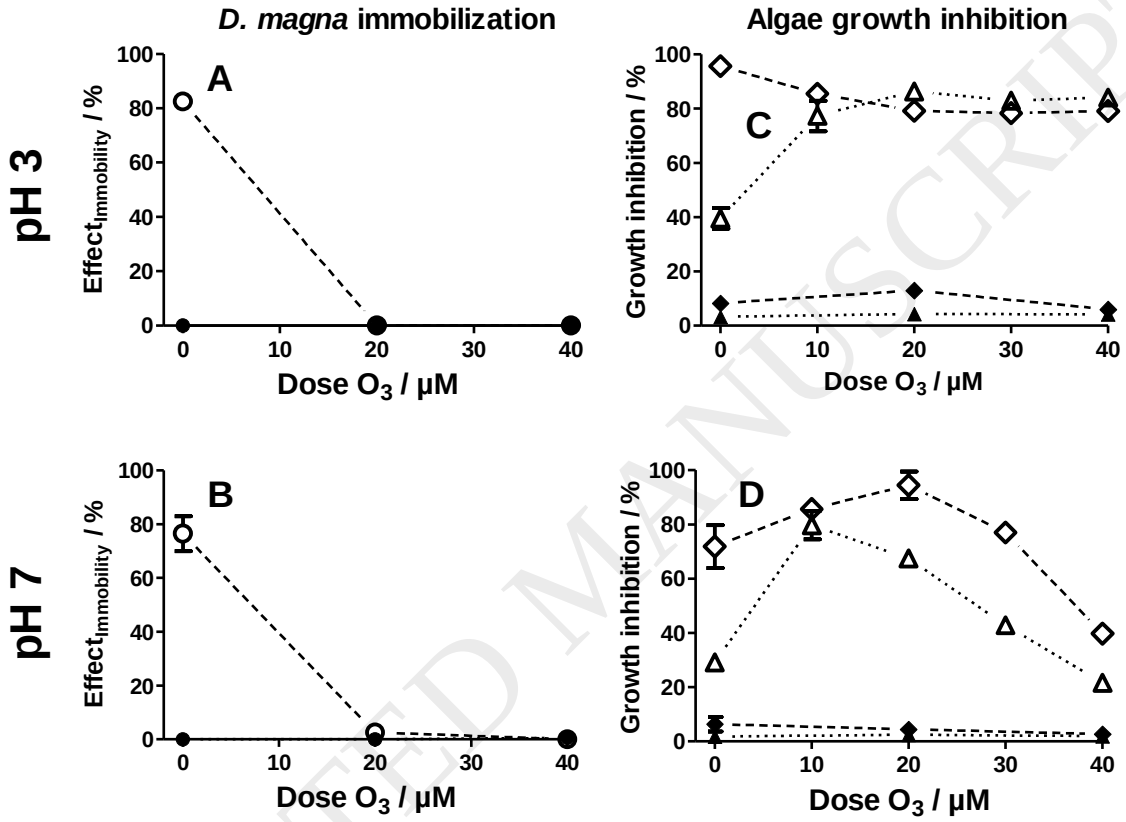

Figure 4: Immobility of *D. magna* after 48 hours at pH 3 (A) and pH 7 (B) of ○ - TAM and ● – DMSO controls over ozone dose in 1:2 (v/v) dilution. Growth inhibition (60 h) of TAM over final ozone dose for *D. subspicatus* at pH 3 (C) and pH 7 (D) with ◊ - in 1:1 and Δ - 1:3 (v/v) dilution of samples in test media. DMSO controls ♦ - 1:1 (v/v) diluted and ▲ - 1:3 (v/v) diluted. Standard deviations of replicates are indicated by error bars that sometimes are smaller than the symbol size.

At pH 3 and 7 growth inhibition was observed although TAM was completely abated. This points to the formation of effect inducing TPs. Indeed, the effect vs. the abatement of TAM shows a positive slope for pH 3. At pH 7 the effect peaks at a TAM degradation

of 2.5 µM (Figure 5 A). To further investigate this observation, the correlation of TP peak area and effect (1:3 (v/v) dilution) were analyzed (Figure 5 B – F).

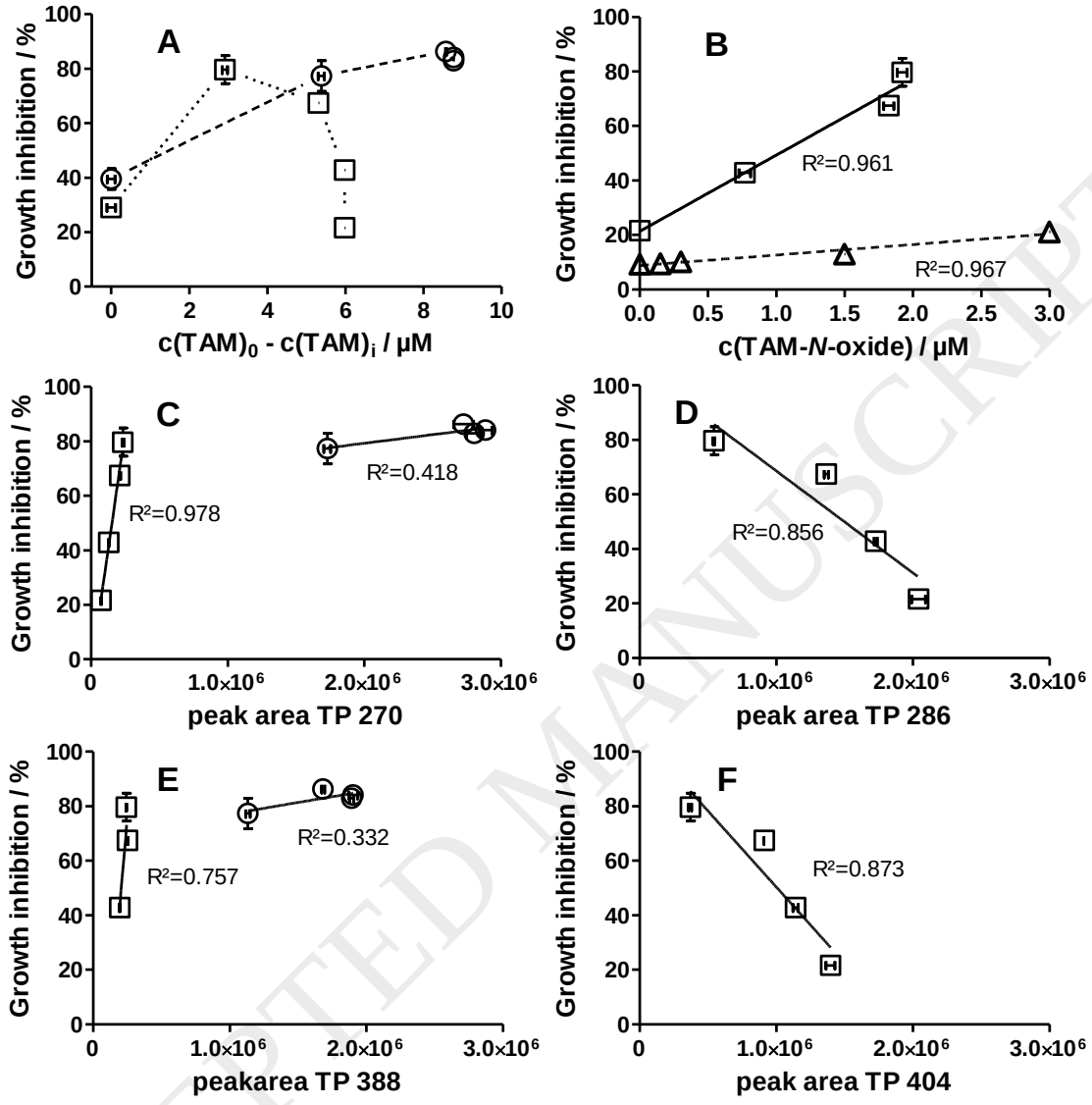

Figure 5: Effect over the abatement of TAM (initial concentration of TAM ($c(TAM)_0$) – concentration of TAM at ozone dose *i* ($c(TAM)_i$)) (A). Correlation of green algae growth inhibition (1:3 (v/v) dilution) and formation of TPs, as concentration or peak area for TAM-*N*-oxide (B), TP 270 (C), TP 286 (D), TP 388 (E), and TP 404 (F) at ○ – pH 3 and □ – pH 7; Δ - growth inhibition of TAM-*N*-oxide, tested individually. Standard deviations are indicated as error bars.

For TP 270 and TP 388, positive correlations were obtained at pH 3 and 7 (Figure 5 C & E). However, for both TPs the obtained slopes vary significantly. At pH 3, solely

effects of > 80 % were observed, resulting in weak correlations for both TPs. Since the observed effects were at the upper limit of the test, the correlation is not valid. Yet, no other TPs were formed at pH 3 which could be responsible for the strong increase of the effect (Figure 4 E). For both TPs also a positive correlation was obtained at pH 7. Here, a high correlation coefficient (R^2=0.978) for TP 270 was observed. TP 388 was present in only 3 of 4 ozonated samples, but also obtained a good correlation (R^2=0.757) at pH 7. Since TP 270 and TP 388 are always present simultaneously one cannot clearly determine the individual contribution of these TPs to the observed effect. Nevertheless, the correlated data for pH 3 and pH 7 are not coherent since effects observed at pH 3 are at the upper limit of the test and hence linearity gained at pH 7 is more meaningful. For TAM-*N*-oxide (Figure 5 B) also a high correlation (R^2 = 0.961) was obtained, indicating the induction of the growth inhibition by TAM-*N*-oxide. As previously mentioned, TAM-*N*-oxide was only observed simultaneously with TP 270 and the individual growth inhibition of 3 µM TAM-*N*-oxide was 21 %. Lower tested concentrations did not differ significantly from the DMSO control (9.1 ± 5.4 %). Therefore, the observed effect is probably not caused by TAM-*N*-oxide, although mixture effects cannot be ruled out [46]. For TP 286 and TP 404 (Figure 5 D & F) negative correlations were obtained and on that account a contribution by these to the effect can be ruled out. Hence, three TPs formed during the ozonation of TAM might proliferate the green algae growth inhibition of TAM. However, at pH 7 TP 388 was not found at the highest ozone dose, thus the remaining growth inhibition of 21 % has to be attributed to TP 270 and/or TAM-*N*-oxide, both remaining present with low intensities. Since TP 270 was always observed with a remaining effect, we assume that this is the TP inducing the strongest green algae growth inhibition, and TP 388 and TAM-*N*-oxide either do only weakly induce the effect and are observed as mixed toxicity effect or are solely incidentally observed. For TAM-*N*-oxide only a minor effect induction was observed. However, individual effects of each TP would be needed to assess if synergistic, additive or inhibitory effects might have influenced the observed effects in the mixture of the TPs as tested here.

4 Conclusion

The TPs formed by ozonation of TAM did not lead to a remaining immobilization effect of *D. magna*. In contrast, an increase of the growth inhibition of the green algae *D. subspicatus* was observed and correlated to the presence of two TPs, namely,

TP 270 and TP 388. Hence, importance of different tests for the ecotoxicological evaluation of TPs and formation of toxicologically relevant TPs could be shown. For the secondary TPs, namely TP 286 and TP 404, no positive correlation to the residual effect was obtained and formation of TAM-*N*-oxide results in a reduced algae growth inhibition. Principally, ozonation my lead to a reduction of the growth inhibition for green algae *D. subspicatus* of TAM at pH values typical for wastewater ozonation (pH 7-8), since mainly *N*-oxides will be formed. However, in practice a reduction of this effect is probably not required since algae growth inhibition of TAM is of minor importance at concentrations typically determined in the environment (ng L^{-1} range). Additionally, wastewater matrix effects are not considered in this study. Individual effects of single TPs could not be determined because beside TAM-*N*-oxide, TPs are not available as authentic standards. Furthermore, the systematic assessment of mixture effects was therefore not possible. For the determination of the ecotoxicological potential of individual TPs, isolation of the TPs will be necessary and will be addressed in future work.

5 Supplementary Material

Supplementary material is available for (S1) validation of LC-MS measurements, (S2) *Daphnia magna* immobilization tests, (S3) Green algae growth inhibition tests.

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