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Environmental occurrence, fate and transformation of benzodiazepines in water treatment

T. Kosjek [a], S. Perko [a], M. Zupanc [a,b], M. Zanoški Hren [c], T. Landeka Dragičević [c], D. Žigon [a], B. Kompare [d], E. Heath [a,*]

[a] Jožef Stefan Institute, Department of Environmental Sciences, Ljubljana, Slovenia
[b] Ecological Engineering Institute IEI, Maribor, Slovenia
[c] Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb, Croatia
[d] Faculty of Civil and Geodetic Engineering, University of Ljubljana, Ljubljana, Slovenia

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ABSTRACT

Benzodiazepine derivatives are prescribed in large quantities globally and are potentially new emerging environmental contaminants. Unfortunately, a dearth of data exists concerning occurrence, persistence and fate in the environment. This paper redresses this by reviewing existing literature, assessing the occurrence of selected benzodiazepine anxiolytics (diazepam, oxazepam and bromazepam) in wastewater influent and effluent and surface water from Slovenia, evaluating their removal during water treatment and identifying the transformation products formed during water treatment. Their occurrence was monitored in hospital effluent, river water and in wastewater treatment plant influent and effluent. The study reveals the presence of benzodiazepine derivatives in all samples with the highest amounts in hospital effluents: 111 ng L^{-1}, 158 ng L^{-1} and 72 ng L^{-1} for diazepam, bromazepam and oxazepam, respectively. Removal efficiencies with respect to biological treatment of diazepam were 16–18% (oxic), 18–32% (anoxic → oxic), 53–76% (oxic → anoxic) and 83% (oxic → anoxic → oxic → anoxic cascade bioreactors), while the removal oxazepam was 20–24% under anoxic conditions. Coupled biological and photochemical treatment followed by the adsorption to activated carbon resulted in a removal efficiency of 99.99%. Results reveal the recalcitrant nature of benzodiazepine derivatives and suggest that only combinational treatment is sufficient to remove them. In addition, eight novel diazepam and four novel oxazepam transformation products are reported.

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1. Introduction

Benzodiazepines are the drugs of choice in the pharmacotherapy of anxiety and related emotional disorders, sleep disorders, status epileptics, and other convulsive states. They are also used as centrally acting muscle relaxants, for premedication and as inducing agents in anaesthesiology (Neumeyer and Booth, 1995). Their potential shortcomings include tolerance, withdrawal symptoms, and their abuse potential (Riss et al., 2008; Kosjek and Heath, 2011).

Diazepam (DZ, Valium®) is perhaps the most known drug among this group and is long acting because of its active metabolites that have long half-lives (Baldessarini et al., 1996). In humans it is metabolized into either N-desmethyldiazepam or nordazepam (half-life of up to 100 h). Nordazepam is then further 3-hydroxylated into oxazepam (OXA). The presence of

* *Corresponding author*. Tel.: +386 1 4773584.
E-mail address: ester.heath@ijs.si (E. Heath).
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the hydroxyl group enables rapid glucuronidation and excretion with the urine and explains its significantly shorter half-life (4–15 h) when compared to both DZ and nordazepam (Neumeyer and Booth, 1995; Benzodiazepine Equivalents Table, 2011). Nordazepam and OXA are marketed as Calmday® and Serax®, respectively. According to Fürst et al. (2006), besides DZ, the most commonly prescribed anxiolytics in Slovenia are bromazepam (BZ, half-life: 10–20 h) and alprazolam (half-life: 6–12 h). Fürst et al. (2006) also reported significant differences in the use of high doses of DZ and BZ between the individual regions of Slovenia, what is suggestive of their potential abuse.

In environmental research, benzodiazepines are among those pharmaceuticals less commonly addressed (Ternes et al., 2001). Diazepam has been detected in wastewater (WW) originating from hospitals as well as in effluents from municipal wastewater treatment plants. Ternes et al. (2001) and Martínez Bueno et al. (2007) found levels of DZ as high as 53 ng L^{-1} and 87 ng L^{-1}, respectively, in wastewater treatment plant effluent. DZ was further found at 33 ng L^{-1} in German rivers (Ternes et al., 2001), up to 21 ng L^{-1} in rivers in the region of Madrid (Martínez Bueno et al., 2010) and Zuccato et al. (2000) found up to 23.5 ng L^{-1} DZ in drinking water. Further, Heberer (2002) reports the presence of OXA (0.25 μg L^{-1}) in a wastewater treatment plant (WWTP) effluent, while Baker and Kasprzyk-Hordern (2011) found OXA in majority of WWTP effluent, influent and river water samples. In addition, BZ, OXA and DZ were determined in the Llobregat River (north eastern Spain – a source of potable water at mean concentrations of 7 ng L^{-1}, 20 ng L^{-1} and 3 ng L^{-1}, respectively) (Huerta-Fontela et al., 2011).

Benzodiazepines are normally halogenated compounds, and it is suggested that the presence of a halogen in a chemical structure significantly reduces its susceptibility to biodegradation (Johnson et al., 2008). Most data concerning the efficiency of WW treatment is for DZ, and reveals that <10% is removed during classical biological treatment, while anaerobic sludge treatment is only slightly more efficient (10–50%); removal is a result of adsorption to activated sludge, rather than degradation (Ternes et al., 2004; Löffler et al., 2005). Similarly, OXA is persistent to both, aerobic or anaerobic biodegradation and field-based experiments also reveal its recalcitrance (Patterson et al., 2010, 2011). To our knowledge, other than the papers cited no other published degradation data on OXA is available.

DZ is considered to undergo photochemical degradation under environmental conditions which may constitute a feasible mechanism for its removal from surface waters (Boreen et al., 2003). In comparison to classical treatment technologies, advanced oxidation methods are more efficient at eliminating DZ (Belden et al., 2007; Calisto and Esteves, 2009). Degradation of DZ was improved in the presence of ferrioxalate, either under black-light or solar irradiation and the removal efficiency was 80% after 60 min irradiation (Bautitz and Nogueira, 2010). DZ is relatively resistant to ozonation (Verlicchi et al., 2010), but it can be oxidised by •OH radicals during ozone treatment (Ternes et al., 2004). Unfortunately, there are no published data for the remaining benzodiazepines regarding their persistence to abiotic treatment methods.

Eventhough the K_{ow} of these compounds do not indicate sorption to be an important removal process, recent researches suggest this is not the case. Ternes et al. (2004) classify DZ as easily adsorbable to activated carbon (99% removal, 0.2 mg L^{-1} of activated carbon) while Calisto and Esteves (2009) report that temazepam and OXA may undergo more abiotic losses by sorption to humic substances. Löffler et al. (2005) found DZ to be a highly persistent pharmaceutical with rapid and extensive sorption onto sediments as well as being highly stable in soils, ground waters and during the WWs treatment. In the same study OXA was reported as moderately persistent in water/sediment systems.

It is clear that available data regarding the presence, cycling and fate of benzodiazepine derivatives in the environment are insufficient. This study aims to address this deficiency by (i) identifying and determining the environmental concentrations of benzodiazepines residues, (ii) evaluating their removal during biological and photochemical water treatment and (iii) studying the formation of stable transformation products during wastewater treatment. In addition we also make suggestions about how to improve current wastewater treatment.

2. Experimental

2.1. Chemicals

Authentic compounds DZ and BZ were donated by a cooperating body, while OXA was curtsy of Belupo, d.d. (Croatia). The internal standard [$^2H_5$]-oxazepam (OXA-d5: hydrogen atoms of the C_6H_5 phenyl group were replaced by deuterium atoms, 99.9%) was purchased from Sigma–Aldrich (St. Louis, MO, USA), and [$^2H_5$]-diazepam (DZ-d5: hydrogen atoms of the C_6H_5 phenyl group were replaced by deuterium atoms, 99.9%) was purchased from LGC Standards GmbH (Wesel, Germany). Both deuterated internal standards were used for the trace-level analytical method development and validation, and for the determination of occurrence of the selected pharmaceuticals in the environment. All standards were of highest available purity (>99%). For derivatisation we used acetic anhydride (Sigma Aldrich, USA) and pyridine (Merck, Germany). All applied solvents (ethylacetate, methanol) and chemicals (hydrogen peroxide 30%) were of analytical grade purity.

2.2. Sample preparation

Fourhundred milliliters river water (RW) and 200 mL WW samples were first filtered through glass microfiber prefilters (Machery Nagel, Dueren, Germany) and afterwards through 1.2 μm cellulose nitrate (Whatman, Kent, UK) filters. After filtration the internal standards DZ-d5 and OXA-d5 were added. The concentration of the internal standards was 4.4 nM in the case of treatment experiments; 0.44 nM (DZ-d5) and 1.32 nM (OXA-d5) for determination of actual WW samples; and 0.22 nM (DZ-d5) to 0.44 nM (OXA-d5) for natural water samples. The sample pH was 6.5–7.0 and was not adjusted before the extraction. Samples were extracted by Oasis™ (Waters Corp., Milford, MA, USA) 60 mg/3 mL solid-phase extraction (SPE) cartridges, previously conditioned

with 3 mL ethylacetate, 3 mL methanol and equilibrated by 3 mL of deionised water. After the enrichment phase the cartridges were dried under stream of nitrogen and the analytes were eluted by 1 mL acetone, 1 mL of acetone/ethylacetate (3:7) and 1 mL ethylacetate. The eluate was transferred to a glass vial and dried under a gentle stream of nitrogen. The derivatisation agents (15 μL acetic anhydride and 5 μL pyridine) were added to the dry extract, which was vortexed and left at 80 °C for 15 h. After derivatisation the solvent was removed (N_2). The derivatised extracts were then suspended in ethylacetate (0.1 mL for WW sample, 0.2 mL for RW samples and 0.5 mL in case of treatment experiments).

For the determination of transformation products we applied a similar sample preparation procedure as that described above, excluding the following steps: internal standards were not used, derivatisation was not performed, and the dry extracts were dissolved in an initial composition of LC mobile phase.

2.3. Analysis

2.3.1. Quantitative analysis

HP 6890 series (Hewlett–Packard, Waldbron, Germany) gas chromatograph fitted with a single quadrupole mass selective detector (GC-MSD) was used. The GC oven was programmed as follows: an initial temperature of 80 °C was ramped at 30 °C/min to 250 °C, held for 4 min, ramped at 5 °C/min to 280 °C, at 30 °C/min to 300 °C, and finally held for 1 min and 2 min post run at 300 °C. The total GC run time was 19.34 min. A DB-5 MS 30 m × 0.25 mm × 0.25 μm (Agilent J&W, CA, USA) capillary column was used, with He as the carrier gas (37 cm s^{-1}). One-microlitre samples were injected at 250 °C in splitless mode, and the transfer line was maintained at 280 °C. The MS was operated in EI ionisation mode at 70 eV. In SCAN mode, masses from *m*/*z* 50 to 550 were scanned, while in SIM mode, the following ions were monitored: *m*/*z* 219, 253 and 254 for acetyl-OXA, *m*/*z* 224, 258 and 259 for acetyl-OXA-d5, *m*/*z* 220, 299 and 301 for acetyl-BZ, *m*/*z* 256, 283 and 284 for DZ and *m*/*z* 261, 288 and 289 for DZ-d5. The GC-MSD used Chemstation software for instrumental control and data processing.

2.3.2. Identification of transformation products

The chromatographic separation was performed on a Waters Acquity ultra-performance liquid chromatograph (Waters Corp., Milford, MA, USA), equipped with a binary solvent delivery system and an autosampler. The injection volume was 5 μL. Separation was achieved using a 3-cm-long Acquity UPLC™ BEH C18 (Waters Corp., Milford, MA, USA) column with 1.7-μm particle size and 2.1-mm internal diameter. Compounds were analysed under positive [ESI(+)] ion conditions. The mobile phases used were (A) 0.1% formic acid and (B) acetonitrile. The elution gradient was linearly increased from 10 to 80% B in 5 min, decreased back to 10% in 0.3 min and then finally kept isocratic for 1.2 min. The total runtime was 6.5 min. Flow rate was 0.3 mL min^{-1} and the column temperature was maintained at 40 °C. The UPLC system was interfaced to a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (QqToF Premier, Waters Corp.). The instrument was equipped with an electrospray ionisation interface. The capillary voltage was set to 3.0 kV, while the sampling cone voltage was varied between 20 and 30 V. Source and desolvation temperatures were set to 130 and 250 °C, respectively. The nitrogen desolvation gas flow rate was 530 L h^{-1}. For MS experiments, the first quadrupole was operated in rf-only mode, while detection was performed in the ToF mass analyser. MS data were acquired over an *m*/*z* range of 100–1000 at collision energy of 4 V. For MS/MS operation, the acquisition range was between *m*/*z* 50 and 400, and argon was used as the collision gas at a pressure of $4.5\,10^{-3}$ mbar in the T-wave collision cell. The MS/MS experiments were performed with collision energy, varied between 15 and 40 V, to generate product ion spectra providing the most structural information. Data were collected in centroid mode, with a scan accumulation time set to 0.25 s and an interscan delay of 0.02 s. The data station operating software was MassLynx v4.1. Prior to analysis, the instrument was calibrated over a mass range 50–1000, using a sodium formate calibration solution. Reproducible and accurate mass measurements, at a mass resolution of 10 000, were obtained using an electrospray dual sprayer with leucine enkephalin ($[M-H]^-$ 554.2615, $[M\,H]^+$ 556.2271) as the reference compound. The latter was introduced into the mass spectrometer alternating with the sample *via* a Waters Lock Spray device. Elemental composition of TPs was calculated from accurate masses determined by high resolution mass spectrometry (HRMS) at following conditions: C:0–25, H:0–30, N:0–3, O:0–10; Cl:0–1 ± 5.0 ppm tolerance.

For enhanced detection of the transformation products data were acquired in centroid mode and afterwards processed by the MetaboLynx™ application manager embedded into MassLynx v4.1. software (Waters Corp.). The algorithm was programmed to detect products of expected transformation pathways (i.e. hydroxylation and demethylation) and also to detect unexpected components. The latter were examined in *m*/*z* 100–400 scanning range with 10 Da size of a step scan. The presence of transformation products was investigated in treated samples, while untreated samples were used as control samples.

2.4. Sampling

Sampling was performed in three Slovene towns in winter and spring 2011. Each batch comprised from two to six sampling points. Sampling point locations are schematically shown in the Supporting material. Town A has approximately 35 000 inhabitants and on its outskirts is a pharmaceutical industry marketing DZ and BZ. Grab sampling was performed prior and post the inhabitated area. Town B has a population of *cca* 105 000 with a WWTP and a hospital (approx 250 beds and 10 000 patients hospitalised annually). Time proportional 24-hrs sampling included two hospital waste streams (1 and 2), a WWTP influent (3) and effluent (4) and receiving waters upstream (5) and downstream (6) of the WWTP outflow.

In town C (360 000 inhabitants, one of the largest hospitals in Central Europe with psychiatric clinic, in total *cca* 3000 beds and *cca* 105 000 patients hospitalised annually), grab samples were taken before (1) and after (2) the discharge of the town's main WWTP effluent (3) into a river. The WWTP (mechanical and biological treatment) treats WW for 360 000 population equivalents (PE).

2.5. Biodegradation experiments

2.5.1. Setup and operation of a pilot wastewater treatment plant (PWWTP)

Biodegradation experiments were performed in flow-through pilot bioreactors, which were operated either as individual units or were coupled into cascades as described in Table 1. The individual unit is a 4L flow-through oxic bioreactor, which is in details described in Kosjek et al. (2007, 2009). The anoxic conditions were established by removing the air supply, while constantly mixing the bioreactors' content using a magnetic stirrer bar. The bioreactor is shown in Kosjek et al. (2007). All bioreactors/cascades, except for "D" were operated in two parallels. The bioreactors (or in case of a sequence the first bioreactor) were fed with a mineral-nutrient medium (Kosjek et al., 2007) and 100 $\mu g\,L^{-1}$ DZ or OXA was continuously added to the bioreactor influents as described in Table 1.

The bioreactors fed with DZ were operated in parallel, for the first 19 weeks as two individual oxic bioreactors (parallels X1 and X2, encoded "A", Table 1), then three weeks were allowed for adaptation of the anoxic→oxic sequence (parallels A1 → X1 and A2 → X2, "B", Table 1), and thereafter sampling was performed for the following 7 weeks (week 23–29). Further, the bioreactors were swapped into sequence oxic→anoxic (parallels X1 → A1 and X2 → A2, "C", Table 1), and again after a three-week adaptation period, sampling was performed from week 33 to 45. Finally, the two oxic (X1 and X2) and two anoxic (A1 and A2) bioreactors were coupled into a single sequence X2 → A2 → X1 → A1 (code "D").

Separately, OXA was added into two oxic bioreactors O1 and O2, as evident from Table 1 (code "E").

2.5.2. Assessment of the PWWTP operation

The operation of reactors was assessed by monitoring the total chemical oxygen demand (COD_t), NO_3–N, NO_2–N and NH_4–N in the influents and effluents of bioreactors. Along with these parameters the concentration of mixed liquor suspended solids (MLSS), temperature and concentration of oxygen were monitored inside the bioreactors. All measurements were performed on the same day as the sampling for pharmaceuticals.

COD was determined using a DR/2010 spectrophotometer (Hach, Düsseldorf, Germany) and a Hach measuring kit in an appropriate concentration range (0–1500 mg L^{-1} for influent and 0–150 mg L^{-1} for effluent samples). NO_3–N was determined using powder pillows NitraVer 5 MR (0–4.5 mg L^{-1}) with Method 353 on a DR/2010 spectrophotometer, and NO_2–N by NitriVer 3 LR (0–0.3 mg L^{-1}) with Method 371. For NH_4–N the Nessler method (Method 380) was employed (0–2.5 mg L^{-1}). Where necessary, samples were appropriately diluted to fit into a defined concentration range.

To determine MLSS, 15 mL of a sample was filtered through a previously dried and weighed filter using a vacuum crucible and dried at 105 °C to constant weight. Oxygen levels and temperature were measured simultaneously using a HQ30d probe (Hach, Düsseldorf, Germany).

2.6. H_2O_2/UV treatment

UV treatment of DZ and OXA was performed using a monochromatic low pressure mercury lamp (LP), with a peak emission at 254 nm. The apparatus was composed of a glass reactor with a volume of 1.5 L. A constant temperature (20–22 °C) was maintained by continuously cooling the treated solution with cold water, and by continuous mixing with a magnetic stirrer at 400 rpm. The pH (6.5–7.0) did not change during the course of the treatment. The UV treatment was performed during different time intervals (10 min, 30 min, 60 min and 120 min) and with and without the addition of H_2O_2 in different concentrations (33 mg L^{-1}, 330 mg L^{-1}, 1.6 g L^{-1} and 3.3 g L^{-1}). The treatment was initially performed in 0.5 L distilled water at 100 $\mu g\,L^{-1}$ concentrations of individual parent compounds, first to determine the transformation products generated during treatment, and secondly, to set the operational parameters for further treatment of actual WW obtained from the bioreactor effluents described in Table 1.

2.7. Adsorption to granulated activated carbon

Activated carbon (AC) with screen size 30 × 60 was provided by the Coast Engineering Laboratory (Redondo Beach, USA). Experiments were performed on effluents of DZ and OXA bioreactors (Table 1) prior or post UV/H_2O_2 treatment, at varying concentrations of AC (from 200 to 4000 mg L^{-1}) and durations of exposure (15 and 60 min). 1200 mg L^{-1} of AC and 60 min of exposure time were found sufficient for elimination of residual DZ and OXA post UV/H_2O_2 treatment.

Table 1 – Bioreactor setup.

Week	1	4	20	23	30	33	46	49	46	49
(Ad)/(Samp)	Ad	Samp	Ad	Samp	Ad	Samp	Ad	Samp	Ad	Samp
Bioreactor setup	X1		A1 → X1		X1 → A1		X1 → A1 → X2 → A2		O1	
	X2		A2 → X2		X2 → A2				O2	
No. of bioreactors per sequence	1		2		2		4		1	
Parent comp.	DZ		DZ		DZ		DZ		OXA	
Bioreactor code	A		B		C		D		E	

A1, A2, anoxic bioreactors fed with DZ; X1, X2, oxic bioreactors fed with DZ; O1, O2, oxic bioreactors fed with OXA; Ad, addition of parent compounds; Samp, sampling.

3. Results and discussion

3.1. Method development and validation

To improve gas-chromatographic response, peak shape and to avoid thermal decomposition of OXA in the injector (Kinani et al., 2007), derivatisation was performed to transform the benzodiazepines into more volatile and thermally stabile derivatives. According to Borrey et al. (2001), acetylation of benzodiazepines with acetic anhydride and pyridine is superior to common derivatisation methods such as silylation or alkylation. The advantages of acetylation are that the reagents and the by-product (acetic acid) are easily evaporated with nitrogen, and any excess reagents will not damage the chromatographic column. In addition, acetylation targets the secondary N-1 atom of OXA and BZ (Fig. 1), whereas DZ which already shows good peak shape and sensitivity has a tertiary N – 1, which will remain underivatised. Derivatisation is considered to be the most variable step in the sample preparation procedure. The optimization of the derivatisation conditions involved testing the acetic anhydride/pyridine ratio, reaction temperature and reaction time. Finally, 15 μL acetic anhydride and 5 μL pyridine at 80 °C for 15 h were sufficient to yield stabile acetyl derivatives of OXA, OXA-d5 and BZ. The derivatives were found to be stabile for at least 14 days.

Other validation parameters involved testing SPE efficiencies, which were 84–101% with standard deviations between 2.9 and 14%. Since the internal standards were added prior to the SPE there was no need for this variation to be taken into account in the overall method performance. The limit of detection of the analytical method (LOD) values were not higher than 3 ng L^{-1} for all three target analytes. The calibration curves were obtained by plotting the target ion response ratio of the analyte to internal standard against the concentration ratio. Both internal standards OXA-d5 and DZ-d5 were taken into account. The method linearity was proven by regression coefficients >0.98 in the range of 10–300 ng L^{-1}. The method repeatability was determined based on three consecutive measurements of the same sample containing the target analytes in the low ng L^{-1} level and ranged from 1.4 to 3.7% within-day and 2.9–11% between-day.

3.2. Occurrence studies

To confirm the identity of target analytes and to enable their secure quantification on the single quadrupole mass detector, we applied acceptability criteria as follows: (1) ion ratios for a given analyte between qualifier and quantification ion were required to be within ±20% of the average ion ratios in respect to the control samples, and (2) retention time was within ±0.20 min of the average for all respective controls (Brooks et al., 2005). The results of the occurrence studies on the presence of OXA, DZ and BZ in Slovene water environment are given in Table 2.

Despite DZ and BZ being marketed as commercial products by the pharmaceutical industry located at River A, none of the selected benzodiazepine derivatives were present at the sampling locations (upstream and downstream of town A, for detailed locations see Supplementary material). There are several possible explanations for this: it is possible that the compounds were not manufactured at the time of sampling or that grab sampling may have missed the contaminants that time or flow proportional composite sampling would have captured. Another solution would be to analyse sediment samples downstream from the suspected point source (pharmaceutical plant) that could provide a historical record of pollution.

River C was sampled in the same manner as River A (grab sampling). The results reveal contamination with two out of three selected benzodiazepine derivatives (OXA and DZ). While OXA was detected only once in a concentration above LOD before the WWTP effluent was discharged into the River C, DZ was detected in all the grab samples in concentrations up to 69 ng L^{-1}. The fact that DZ was detected in all the samples suggests a constant discharge of DZ into River C.

Town B samples were collected in collaboration with a local WWTP. Each was a 24 h composite taken as part of a regular monitoring campaign performed at six locations (see Supplementary material) during two seasons (winter and spring 2011). Results show that OXA, with the exception of both hospital effluents collected during the winter, is present in all the other samples. Compound BZ was present in approximately 60% of samples, while DZ was present in all samples. The presence of BZ in the WW and RW samples may be related to the significantly higher consumption of this pharmaceutical in the area as determined by Fürst et al. (2006).

There is a lack of data available in the area of persistence or half-life times of benzodiazepine derivatives available. PBT profiler (www.pbtprofiler.net, accessed 21.10.2011) reports half-lives for DZ and OXA in water, soil and sediment to be 38, 75 and 340, respectively; while BZ reported half-lives are 60, 120 and 540. These data indicate DZ and OXA not to be persistent in water (<60 days) while BZ could already be

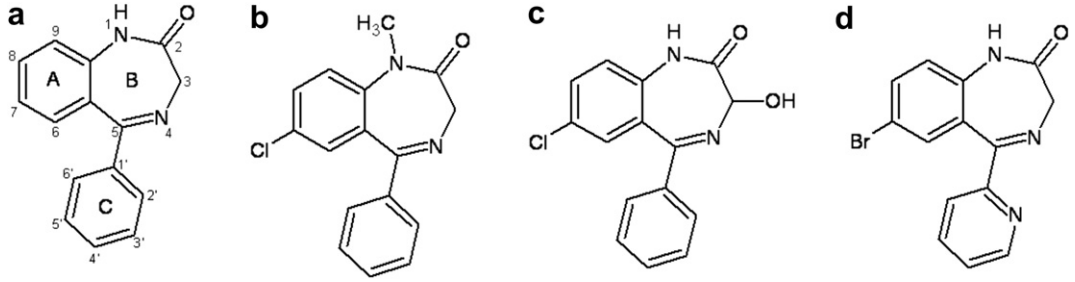


Fig. 1 – Benzodiazepines: (a) 5-phenyl-1,4-benzodiazepin-2-one: ring system, (b) DZ, (c) OXA, (d) BZ.

Table 2 – Concentrations (ng L^{-1}) of OXA, DZ and BZ determined in the wastewater and river water samples.

	Sampling point	Sampling date	Sampling approach	c(OXA) ng/L	c(BZ) ng/L	c(DZ) ng/L
Town A						
River before municipality	1-A	Winter 2011	Grab	<LOD	<LOD	<LOD
River after municipality and pharmaceutical industry	2-A	Winter 2011	Grab	<LOD	<LOD	<LOD
Town B						
Hospital effluent 1	1-B	Winter 2011	Composite 24 h	<LOD	40	27
	1-B	Spring 2011	Composite 24 h	72	<LOD	49
Hospital effluent 2	2-B	Winter 2011	Composite 24 h	<LOD	<LOD	17
	2-B	Spring 2011	Composite 24 h	41	158	111
WWTP influent	3-B	Winter 2011	Composite 24 h	58	<LOD	21
	3-B	Spring 2011	Composite 24 h	54	<LOD	25
WWTP effluent	4-B	Winter 2011	Composite 24 h	28	32	18
	4-B	Spring 2011	Composite 24 h	46	<LOD	22
Stream before effluent	5-B	Winter 2011	Composite 24 h	11	6	17
	5-B	Spring 2011	Composite 24 h	21	9	28
Stream after effluent	6-B	Winter 2011	Composite 24 h	30	17	21
	6-B	Spring 2011	Composite 24 h	31	19	20
Town C						
River before WWTP discharge	1-C	Spring 2011	Grab	<LOD	<LOD	9
River after WWTP discharge	2-C	Spring 2011	Grab	<LOD	<LOD	69
	2-C	Winter 2011	Grab	<LOQ	<LOD	13
WWTP effluent	3-C	Spring 2011	Grab	84	<LOD	21
	3-C	Winter 2011	Grab	133	<LOD	22

classified as moderately persistent (≥60 days). DZ, OXA and BZ are classified as moderately persistent in soil (≥60 days) and very persistent in sediment compartment (>180 days). Basing on these data BZ shows slightly higher persistence when comparing three derivatives. On the other side Löffler et al. (2005) acknowledges the differences between persistence of DZ and OXA where DZ is reported to be highly and OXA moderately persistent pharmaceutical.

3.3. *Biodegradation experiments*

3.3.1. *Assessment of performance of the PWWTP*

Supplementary data gives the recorded values for COD_t, NO_3–N, NO_2–N and NH_4–N. As illustrated in cases B, C and E the COD_t value declined by approximately 95% in the first reactor, disregarding the oxygen concentration. The COD_t was further decreased in the subsequent reactor(s) in a cascade (examples B and C), but this decrease was less significant. High variability of COD_t is observed in the middle container and in the effluent, which may be due to the dead biomass discharged from a reactor.

Based on an increase in NO_3–N concentration (Supplementary data) the nitrification process is confirmed in the oxic reactor "E" and in the oxic bioreactor (IN → M) of the cascade "C", and the predominant nitrification process in the cascade "D".

Slovene guideline (2011) on the quality of WWTP discharge suggests the upper limit for COD at 100 L^{-1} and NH_4–N at 5 L^{-1} (for WWTPs with ≥100 000 PE), and NH_4–N at 10 L^{-1} (for WWTPs from 2000 to 100 000 PE). Considering the COD both, the actual effluents and the middle container effluents, are acceptable for discharge, while the NH_4–N concentrations slightly exceed the limiting values, and therefore an additional removal of NH_4–N is necessary.

Table 3 – Removal of DZ and OXA in individual or cascade bioreactors; expressed as average removal ± standard deviation (number of samples).

Bioreactor/cascade code	Parent compound	Conditions	Removal ± stdev (*n*)		
A	DZ	Oxic		X1: 16 ± 9 (15)	
				X2: 18 ± 8 (14)	
B	DZ	Anoxic → oxic	A1: 13 ± 8 (7)	X1: 14 ± 5 (6)	tot: 18 ± 6 (7)
			A2: 13 ± 10 (7)	X2: 24 ± 10 (6)	tot: 32 ± 12 (7)
C	DZ	Oxic → anoxic	X1: 34 ± 17 (13)	A1: 32 ± 13 (11)	tot: 53 ± 15 (12)
			X2: 67 ± 10 (13)	A2: 29 ± 13 (10)	tot: 76 ± 10 (12)
D	DZ	Oxic → anoxic → oxic → anoxic	X2 → A2 → X1 → A1: tot: 83 ± 8.5 (6)		
E	OXA	Oxic		O1: 20 ± 10 (5)	
				O2: 24 ± 9 (6)	

A1, A2, anoxic bioreactors fed with DZ; X1, X2, oxic bioreactors fed with DZ; tot, total removal; O1, O2, oxic bioreactors fed with OXA.

Table 4 – The results of UV treatment (% removal) of DZ and OXA in DIW and of UV treatment of residual DZ and OXA in biologically treated wastewater (filtered wastewater-FWW and raw wastewater-RWW).

		Matrix/addition of H_2O_2 (mg L^{-1})									Tested compound
		DIW/ 0 mgL^{-1}	DIW/ 33 mgL^{-1}	DIW/ 330 mgL^{-1}	FWW/ 330 mgL^{-1}	FWW/ 1.6 gL^{-1}	FWW /3.3 gL^{-1}	RWW/ 330 mgL^{-1}	RWW/ 1.6 gL^{-1}	RWW/ 3.3 gL^{-1}	
UV	10	–	47	71	22	22	16	14	17	–	DZ
treatment	30	–	–	96	35	39	–	11	23	–	
(min)	60	–	–	–	–	–	52	43	54	44	
	120	8	–	–	74	86	72	72	82	62	
	10	–	–	53	27	–	–	20	38	–	OXA
	30	–	–	93	72	–	–	69	60	–	
	60	–	–	–	83	–	–	90	83	–	
	120	–	–	–	99	–	–	–	97	–	

3.3.2. *Removal of DZ and OXA*

For DZ different biological treatment methods report poor removal efficiencies, being evident the large amount of diazepam passes unaffected through WWTPs (Ternes et al., 2004; Suarez et al., 2010). Correspondingly, as evident from Table 3 (codes A and B), during the first weeks after the bioreactor setup both the oxic and anoxic bioreactors showed low removal efficiency for DZ. Table 3 summarizes the results of biological treatment of DZ and OXA. Biological treatment was for both compounds performed in two parallels, except for the cascade "D", where both oxic (X1 and X2) and anoxic (A1 and A2) bioreactors fed with DZ were connected into a single cascade. The variability of removal of the two parent compounds was approximately 10%, which is not surprising since bioreactors contain biomass, which is difficult to control.

In general, oxic treatment was more efficient than anoxic treatment, even though we observed an increase in both, the anoxic and oxic removal, over one year of operation. This increase in removal is particularly notorious for the X2 oxic bioreactor, where the elimination efficiency increases from 18% to 67% (Table 3) in one year (see Supplementary data). It is assumed that this phenomenon is the consequence of biomass adaptation, but additional experiments such as enrichment of WW with DZ and the microbiological analysis of the biomass are needed to prove this.

For OXA, elimination under oxic conditions (E, Table 3) was 20% and 24% in parallels O1 and O_2, respectively. The poor biological elimination efficiency of OXA is also reported in two studies by Patterson et al. (2010, 2011), whereas to our knowledge no other reports on the environmental behaviour of OXA exists.

3.4. *Photochemical treatment and sorption to activated carbon*

The potential of UV treatment was investigated for DZ and OXA in distilled water (DIW) and biologically treated WW. The UV treatment of DZ in DIW (direct photolysis) was not efficient removing only 8% of the parent compound in 2 h. By the addition of a H_2O_2 as the source of radicals the removal efficiency was improved significantly, as previously shown in Klavarioti et al. (2009) and Kosjek et al. (2011). Table 4 shows how the addition of 0.01% H_2O_2 increases removal efficiency to 47% under 10 min of UV treatment. By increasing the concentration of H_2O_2 (0.1%) and by prolonging the duration of UV treatment (30 min) we were able to enhance removal efficiency to 96% (Table 4). However, it was observed that further increases in concentration of H_2O_2 up to 1% resulted in a negative effect on DZ removal. This phenomenon has been in the scientific literature explained by excess H_2O_2 playing a role as $^{\bullet}OH$ radical scavenger, thus making the photochemical treatment less effective (Pereira et al., 2007; Matilainen and Sillanpää, 2010).

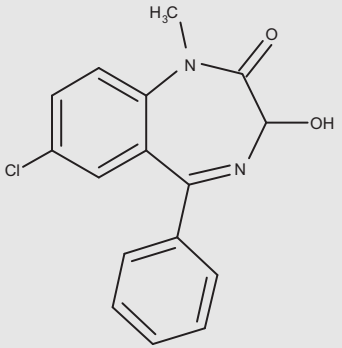
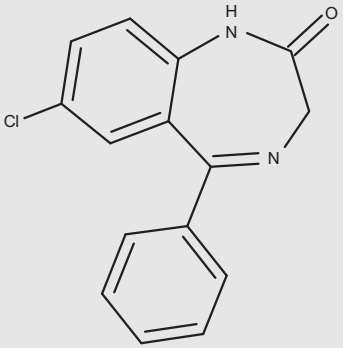
Further, it can be observed in Table 4 that for more complex matrices such as filtered wastewater (FWW) and raw wastewater (RWW) a higher concentration of H_2O_2 and prolonged UV irradiation time are needed to achieve a comparable removal effect. Thus, the addition of 0.5% H_2O_2 at 120 min UV irradiation time was most favorable, achieving 86% and 82% removal of DZ in FWW and RWW, respectively. In comparison, when applying the same treatment conditions (0.1% H_2O_2 and 30 min UV treatment) as those necessary for 96% removal of DZ in DIW, we achieved only 35% and 11% removal of DZ in FWW and RWW, respectively (Table 4).

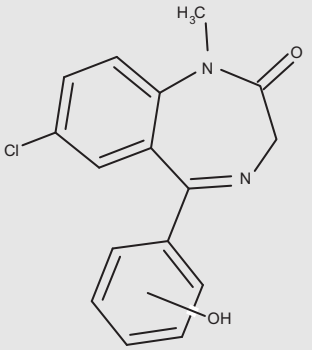
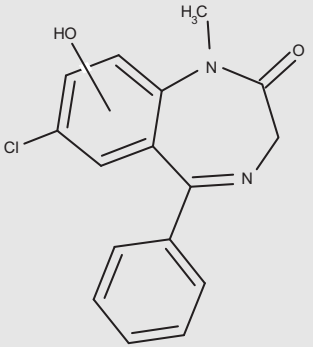
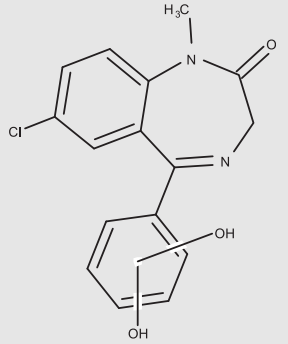
Table 4 further shows the results of the UV/H_2O_2 treatment of OXA in DIW, FWW and RWW under varying conditions, i.e. addition of H_2O_2 and at UV irradiation time. By comparing the results of the photochemical treatment for DZ and OXA (Table 4) it can be concluded that OXA is more prone to oxidation by UV/H_2O_2 treatment than DZ achieving 90% removal at 0.1% addition of H_2O_2 at 60 min UV irradiation time. The likely reason for this is the additional hydroxyl group on the position C-3, which makes OXA more susceptible to $^{\bullet}OH$ radical oxidations.

Table 5 – Removal of DZ and formation of OXA during coupled biological and abiotic treatment.

	Influent	X2 → A2 → X1 → A1 effluent	Effluent + UV/H_2O_2	Effluent + AC	Effluent + UV/H_2O_2 + AC
c (ng/L) DZ	96.6×10^3	18.4×10^3	170	450	16
c (ng/L) OXA formed during treatment	0	2.2×10^3	166	163	<LOD

Table 6 – Transformation products of DZ: LC retention time (LC – t_R), description of mass spectra (HR measurement and tandem mass fragmentation), treatment conditions and proposed chemical structures.

Compound/ abbreviation	LC – t_R (min)	Accurate mass (calculated) $[M H]^+$	Elemental composition $[M H]^+$	Mass error	MS/MS	MW (nominal)	Treatment conditions	Chemical structure (proposed)
DZ	2.97	285.0795	$C_{16}H_{14}N_2OCl$	−0.5 ppm	285/287, 257/259, 228/230, 222, 193, 154/156	284	Parent compound	Fig. 1(b)
OXA	2.40	287.0587	$C_{15}H_{12}N_2O_2Cl$	−0.3 ppm	287/289, 269/271, 257/259, 241/243, 205, 163/165, 151	286	1. From DZ by biotransformation; 2. Used as a parent compound	Fig. 1(c)
Temazepam	2.72	301.0744	$C_{16}H_{14}N_2O_2Cl$	−1.3 ppm	301/303, 283/285, 271/273, 255/257	300	Photocatalysis, biotransformation	
Nordazepam	2.51	271.0638	$C_{15}H_{12}N_2OCl$	0.0 ppm	271/273, 243/245, 208, 165/167, 140/142	270	Photocatalysis, biotransformation	

TP-C-301: 3 isomers, hydroxylated DZ (ring "C")	1.66 2.29 3.39	301.0744	$C_{16}H_{14}N_2O_2Cl$	0.0 ppm 0.1 ppm −1.0 ppm	301/303, 273/275, 238, 209, 182, 154/156	300	Photocatalysis	
TP-A-301: 2 isomers, hydroxylated DZ (ring "A")	1.75 2.59	301.0744	$C_{16}H_{14}N_2O_2Cl$	2.0 ppm −2.7 ppm	301/303, 273/275, 238, 209, 198, 170/172, 105	300	Photocatalysis	
TP-C-317: 2×hydroxylated DZ	2.28	317.0693	$C_{16}H_{14}N_2O_3Cl$	0.9 ppm	317/319, 289/291, 260/262, 254, 225, 179/181, 182/184, 154/156, 123	316	Photocatalysis	

(*continued on next page*)

Table 6 – (*continued*)

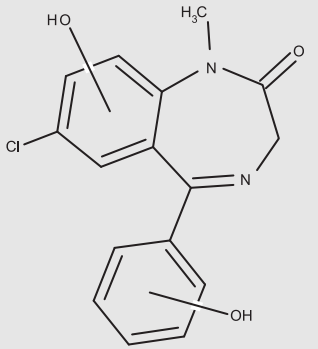
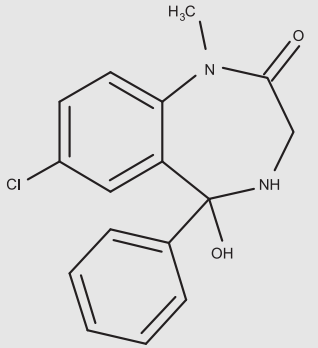
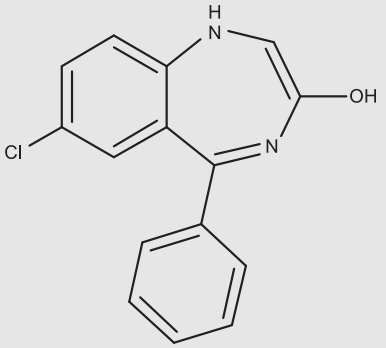
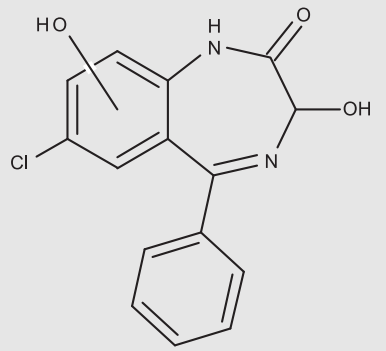
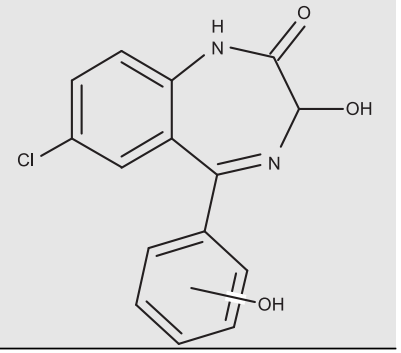
Compound/ abbreviation	LC – t_R (min)	Accurate mass (calculated) $[M\,H]^+$	Elemental composition $[M\,H]^+$	Mass error	MS/MS	MW (nominal)	Treatment conditions	Chemical structure (proposed)
TP-A/C-317: 2×hydroxylated DZ	1.67	317.0693	$C_{16}H_{14}N_2O_3Cl$	−0.9 ppm	317/319, 289/291, 260/262, 199, 182/184, 105	316	Photocatalysis	
TP-303	1.80	303.0900	$C_{16}H_{16}N_2O_2Cl$	−1.0 ppm	303/305, 246/248, 228/230, 193	302	Biotransformation	

Table 7 – Transformation products of OXA: LC retention time (LC – t_R), description of mass spectra (HR measurement and tandem mass fragmentation), treatment conditions and proposed chemical structures.

Compound/ abbreviation	LC – t_R (min)	Accurate mass (calculated) $[M H]^+$	Elemental composition $[M H]^+$	Mass error	MS/MS	MW (nominal)	Treatment conditions	Chemical structure (proposed)
TP-271	2.89	271.0638	$C_{15}H_{12}N_2OCl$	−1.8 ppm	271/273, 253/255, 218, 190	270	Biotransformation	
TP-A/C-303: 3 isomers, hydroxylated OXA (ring "A" or "C")	1.75, 1.99, 2.63	303.0536	$C_{15}H_{12}N_2O_3Cl$	0.0 ppm, 0.7 ppm, 0.3 ppm	303/305, 285/287, 257/259	302	Photocatalysis at pH2	 or 

To further eliminate the residual DZ and OXA, as well as the transformation products, which are recalcitrant to either biological or photochemical degradation, we investigated their sorption to activated carbon (AC). In our experiment we considered the effect of water matrix, exposure time and concentration of AC. It was observed that the both prolonging exposure time (60 min) and increasing the amount of AC significantly increased the removal efficiency. The results are gathered in the Supplementary data.

3.5. Coupling biological and abiotic water treatment

Removal efficiencies of 99.9% for DZ and OXA may not be satisfactory since detectable concentrations of parent compounds and their transformation products are still present in the effluents. It is important to emphasize that such harsh treatment conditions as presented herein are irrelevant for actual domestic water treatment since the highest determined concentrations of benzodiazepines in Slovene WWTP influents were $<200\ ng\ L^{-1}$, and such treatment would not be cost-effective. However, we believe that concentrations as high as $100\ \mu g\ L^{-1}$ (initial concentration) may occur in treatment of WWs discharged from pharmaceutical production units. In this view, and based on the results of biodegradation and abiotic degradation experiments, we coupled both treatments in series to achieve a factor 10^4 removal in concentration of DZ and OXA.

As illustrated in Table 5, we determined the concentration of DZ prior and post biological treatment (cascade D: X2 → A2 → X1 → A1, Table 1), and coupled to UV or AC only, and to both abiotic treatment methods. It can be noted only the sequenced treatment technology that combines all three treatment methods: biological degradation, photochemical treatment and sorption, results in final concentrations in the low $ng\ L^{-1}$ range.

Table 5 shows that during the biological degradation of DZ substantial amounts of OXA are formed, which are afterwards removed by combining UV/H_2O_2 and AC treatment.

3.6. Transformation during water treatment

By utilizing the capabilities of the QqTOF mass spectrometer, i.e. tandem mass fragmentation and accurate mass measurement, coupled to an ultra-performance liquid chromatography (UPLC), which shows superior performance in terms of separation ability and speed compared to classical high resolution liquid chromatography (HPLC), and by employing mass spectral algorithm (MetaboLynx™) for enhanced detection of transformation products, we determined eleven transformation products (TPs) formed during DZ (bio)degradation (Table 6), whereas four TPs were generated during treatment of OXA (Table 7). As shown in Table 6, the biotransformation of DZ resulted in the formation of OXA, nordazepam, temazepam, which are also known to be human metabolites of DZ and are marketed as individual pharmaceuticals. Nordazepam is formed by N-demethylation on $N-1$ of DZ, and temazepam by hydroxylation of the C-3 atom, whereas OXA comprises both transformation reactions. In contrast to photochemical transformation reactions, biotransformation is less predictable resulting in less common transformations such as hydroxylation on double bond (TP-303, Table 6) and loss of oxygen to form TP-271 (Table 7). Photochemical transformation generally results in the formation of ring-hydroxylated TPs, as previously shown for other pharmaceuticals such as ketoprofen (Kosjek et al., 2011). These ring-hydroxylated TPs often appear as structural isomers (compounds TP-C-301, TP-A-301, TP-C-317, TP-A/C-317 in Table 6, and TP-A/C-303 in Table 7). Resolving the exact position of the –OH group is difficult by mass spectrometry, whereas NMR analyses can provide the missing information in such examples. However, despite its excellent identification capabilities, the NMR is rarely employed in the environmental analysis, since it requires considerably high amounts of analytes and an efficient separation of complex analyte mixtures.

The structures of TPs comprised in Table 6 and Table 7 are proposed basing on comprehensive inspection of their tandem mass spectra, with an aid of HRMS to determine the elemental compositions of protonated analyte molecules and their ion fragments. Yet, to confirm with certainty the chemical structure of the TPs proposed in Table 6 and Table 7, further investigation is needed, which indeed involves the use of authentic standards of the proposed TPs. However, to the best of our knowledge most of the TPs proposed herein (except for the human metabolites: OXA, nordazepam and temazepam) have until now not been recognised, and authentic standards are yet to be synthesized.

4. Conclusions

In conclusion, biodegradation experiments in flow-through pilot bioreactors reveal significant biomass adaptation during incubation during both oxic and anoxic conditions. Operating reactors in oxic–anoxic–oxic–anoxic cascade provides an additional 10% towards total removal of diazepam. Photochemical treatment of benzodiazepine derivatives at concentrations relevant for pharmaceutical industry effluents resulted in their efficient removal. Despite this, chronic exposure to the residual pharmaceuticals could result in adverse effects. For this reason further treatment by adsorption on activated carbon was applied and resulted in sufficient removal of the residual compounds. Such extensive treatment is unrealistic and more feasible combinations will have to be researched but the described treatment combination does highlight the recalcitrant nature of these compounds.

Novel transformation products, eight DZ and four OXA, are reported. But their occurrence and toxicity in the environment are yet to be investigated. Their identification does show that simply reporting the removal efficiencies of just the parent compounds is no longer sufficient and a full assessment of the risks posed by their human metabolites as well as environmental transformation products is required. Finally, as virtually no information on the transformation of benzodiazepine derivatives is currently available, the proposed biotransformation products bring an important contribution to recognising the fate of DZ and OXA during biological WW treatment. Hopefully, because of the increasing attention given to the qualitative determination of pharmaceutical

degradation products in the literature and the development of identification tools in general, knowledge about the fate of this particular group of pharmaceuticals in the environment will become more comprehensive.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.watres.2011.10.056.

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