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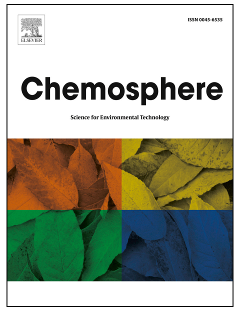

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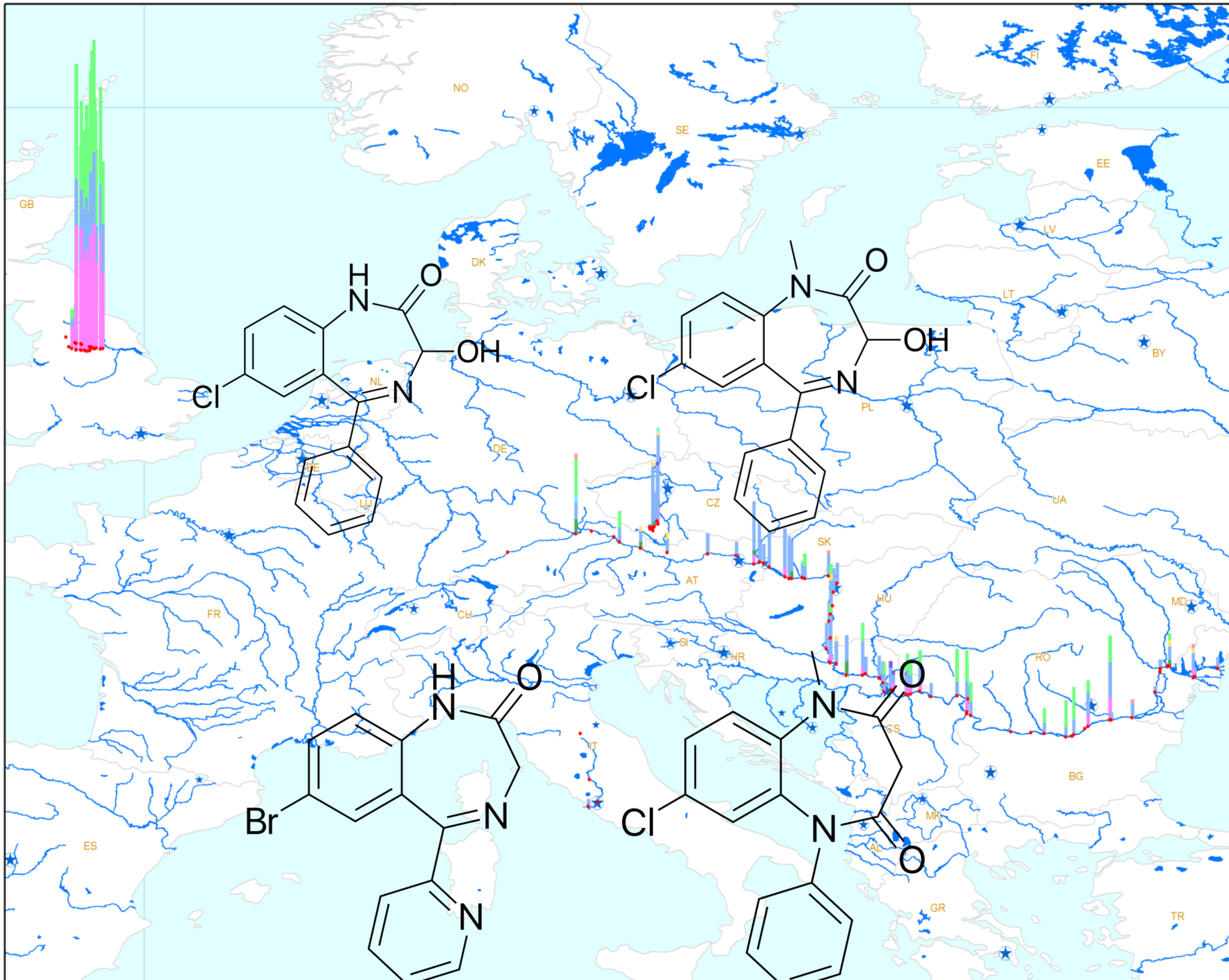

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Screening of benzodiazepines in thirty European rivers

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Keywords

Anxiolytics, oxazepam, temazepam, clobazam, bromazepam

Abstract

Pharmaceuticals as environmental contaminants have received a lot of interest over the past decade but, for several pharmaceuticals, relatively little is known about their occurrence in European surface waters. Benzodiazepines, a class of pharmaceuticals with anxiolytic properties, have received interest due to their behavioral modifying effect on exposed biota. In this study, our results show the presence of one or more benzodiazepine(s) in 86% of the analyzed surface water samples (n=138) from 30 rivers, representing seven larger European catchments. Of the 13 benzodiazepines included in the study, we detected 9, which together showed median and mean concentrations (of the results above limit of quantification) of 5.4 and 9.6 ng L^{-1}, respectively. Four benzodiazepines (oxazepam, temazepam, clobazam, and bromazepam) were the most commonly detected. In particular, oxazepam had the highest frequency of detection (85%) and a maximum concentration of 61 ng L^{-1}. Temazepam and clobazam were found in 26% (maximum concentration of 39 ng L^{-1}) and 14% (maximum concentration of 11 ng L^{-1}) of the samples analyzed, respectively. Finally, bromazepam was found only in Germany and in 16 out of total 138 samples (12%), with a maximum concentration of 320 ng L^{-1}. This study clearly shows that benzodiazepines are common micro-contaminants of the largest European river systems at ng L^{-1} levels. Although these concentrations are more than a magnitude lower than those reported to have effective effects on exposed biota, environmental effects cannot be excluded considering the possibility of additive and sub-lethal effects.

1. Introduction

Pharmaceutical residues in the environment are increasingly recognized as a major threat to aquatic ecosystems worldwide (Boxall et al., 2012). Several biochemically active pharmaceuticals have been found in aquatic systems globally, due to a combination of worldwide use and inadequate removing efficiency in sewage treatment plants (STPs) or a complete lack of STPs (Hughes et al., 2013; Verlicchi et al., 2012; Loos et al., 2013; Asimakopoulos and Kannan, 2016). One of the reasons behind the increased concern for pharmaceuticals in the environment is that even though pharmaceuticals are thoroughly investigated for toxicity in humans, studies on ecotoxicological and ecological effects of these potent and bioactive chemicals are severely underrepresented (Boxall et al., 2012).

One group of pharmaceuticals that has acquired attention lately is that of benzodiazepines. Benzodiazepines, approximately 50 different active pharmaceutical ingredients on the global market, are anxiolytics, i.e. pharmaceuticals used to treat anxiety disorders. These pharmaceuticals are used frequently and globally, and because they are very persistent to degradation in STPs, they can be found at concentrations ranging from 0.01 to several $\mu g\ L^{-1}$ in treated effluents (Calisto and Esteves, 2009; Kosjek et al., 2012; Verlicchi et al., 2012; Loos et al., 2013; Petrie et al., 2015, Asimakopoulos and Kannan, 2016). In surface waters, benzodiazepines have been found at concentrations ranging from 0.001 to 0.6 $\mu g\ L^{-1}$;(Vulliet and Cren-Olivé, 2011; Hass et al,. 21012, Radovic et al., 2012; Brodin et al., 2013; Hughes et al., 2013; Valcarcel et al., 2013, Mendoza et al., 2014a; Miller et al., 2015; Racamonde et al., 2014a, 2014b Aminot et al., 2015; Arbelaez et al., 2015; Camilleri et al., 2015; Wu et al., 2015; Aminot et al., 2016). In addition, several benzodiazepines are quite resistant to photodegradation, which increases their persistence in aquatic environments (Boreen et al., 2003; Calisto et al., 2011) where they may remain in sediment deposits for decades (Klaminder et al 2015).

Benzodiazepines potentiate the effect of gamma-aminobutyric acid (GABA) on GABA-A receptors, which produce increased inhibitory postsynaptic potentials through the GABA receptors' chloride-linked channels (Sieghart, 1995; Sieghart et al., 2012). Benzodiazepines have a wide clinical use as anxiolytics, hypnotics, anticonvulsants, tranquilizers, and muscle relaxants, and have been in extensive use for half a century (Mohler et al., 2002, Lopez-Munoz et al., 2011). GABA receptors are evolutionary well preserved and can be found in a wide range of vertebrate species (Gunnarsson et al., 2008). It is therefore likely that aquatic organisms exposed to benzodiazepines can show a behavioral pharmacological response, which is not measurable with the current ecotoxicological standard methods (Klaminder et al., 2014). For example, recent studies have shown that $\mu g\ L^{-1}$ of the benzodiazepine oxazepam increased both activity and the feeding rate, while reduced sociality in exposed perch (*Perca fluviatilis*) (Brodin et al., 2013, 2014; Klaminder et al., 2014).

The objective of this study was to investigate the occurrence of the most common benzodiazepines in European rivers, in order to obtain an overview on the presence of these pharmaceuticals in aqueous environments. Due to the large number of different benzodiazepines on the global market and the fact that several benzodiazepines also have active metabolites, a sub-set was selected. Selection was based on European sale statistics and potencies. An efficient and sensitive in-line liquid chromatography mass spectrometry (LC-MS/MS) method was developed for 13 benzodiazepines, and water samples from six European catchments and 30 rivers and their tributaries were included in the study.

2. Methods

2.1. Chemicals

All of the reference standards were classified as analytical grade (>98%) and $^{2}H_5$-oxazepam (Sigma-Aldrich, Steinheim, Germany) was used as internal standard. LC/MS grade quality of methanol and acetonitrile were purchased (Lichrosolv - hypergrade, Merck, Darmstadt, Germany) and the purified water was prepared using a Milli-Q Advantage, including an UV radiation source, ultrapure water system (Millipore, Billerica, USA). Formic acid (Sigma-Aldrich, Steinheim, Germany) was used to prepare the 0.1% mobile phases.

2.2. Sample pretreatment and analytical method

Water samples were filtered using 0.45 µm Filtropur S (Sarstedt, Nümbrecht, Germany) syringe filters and 5 ng of the internal standard $^{2}H_5$-oxazepam were added to each sample (10 mL). Injection was based on an online solid phase extraction (SPE) system using two valves for column switching, which has been described previously (Khan et al., 2012). 1.0 mL was injected using a 1 mL loop, onto an online extraction column (OASIS HLB, 20 mm × 2.1 mm i.d., 15 µm particle size) and then onto an analytical column (Hypersil GOLD aQ, 50 mm × 2.1 mm i.d., 5 µm particles, Thermo Fisher Scientific, San Jose, CA, USA), following a corresponding guard column (20 mm × 2.1 mm i.d, 5 µm particles). The total analysis time of the online extraction and the LC-MS/MS determination was 15 min.

2.3. Quality assurance and quality control

Individual stock solutions of each benzodiazepine were prepared in methanol and stored at -18 °C. Two MS/MS transitions were used for positive identifications of analytes with the criterion that the ratio between the transitions was not allowed to deviate more than +/−30% from the ratio in the corresponding calibration standard. Retention times for all analytes also had to be within +/−2.5% of the retention time in the corresponding calibration standard. Together, this gave four identification points (the highest possible number), as described in the Commission Decision 2002/657/EC concerning the performance of analytical methods and the interpretation of results. Limit of quantification (LOQ) was determined from standard curves based on repeated measurements of low level spiked water (MilliQ and surface water), and the lowest point in the standard curve that had a signal/noise ratio of 10 was considered to be equal to the LOQ. A seven-point calibration curve over the range of 0.5–1000 ng L^{-1} was used for linearity evaluation and quantification. Carry-over effects were evaluated by injecting standards at 1000 ng L^{-1} followed by two mobile phase blanks. Every tenth sample in the analytical runs where either an instrumental or field blank. Precision tests, including the precision of extraction and the instrumental response, were conducted by performing multiple injections (n =10) of a 100 ng L^{-1} calibration standard. Matrix effects were evaluated by constructing standard addition calibration curves using surface water samples fortified to 0; 25; 125; and 250 ng L^{-1}. The slopes of individual benzodiazepines standard addition curves, based on the areas for surface water samples, were compared to equivalent curves prepared based on results for Milli-Q samples. Detailed information about the validation parameters are shown in Table S2, in supporting information.

2.4. Sampling, sample transport and storage

A total of 138 samples were collected in 30 rivers and their tributaries; River Aire (n=13) and River Calder (n=11) in the UK, River Blanice and two tributaries (n=14) in the Czech Republic, Danube River and 15 tributaries (n=68) in Germany, Austria, Slovak Republic, Hungary, Croatia, Serbia, Romania and Bulgaria, one tributary to the River Ems in Germany (n=6), Rhine River and two tributaries (n=18) in Germany, River Tiber and one tributary (n=4) in Italy, and three tributaries (n=4) to the Weser River in Germany. Detailed information about the sampling sites and sampling dates are shown in Table S3, in supporting information.

Aire and Calder River basin

Total number of inhabitants in the Aire and Calder River basin (2,057 km^2) is approximately 2.4 million people. Annual mean flow in the lowest part of the river is 8.6 $m^3 s^{-1}$.

Blanice River basin

The Blanice river basin represents a typical midsized river basin in the Czech Republic. The total area of the basin is 860 km^2 with length of the main stream of 93 km and 12 relevant tributaries. Annual mean flow in the lowest part of the river is 4.6 $m^3 s^{-1}$. Total number of inhabitants in the Blanice river basin is approximately 37,000 (mean population density is 43 inhabitants km^{-2}) and the biggest municipality is Prachatice (12,000 inhabitants), situated on Zivny stream (relevant tributary of the Blanice River). The outlet from STP Prachatice creates about 25% of total flow in the Zivny stream.

Danube River basin

The Danube is a river in central and Eastern Europe, which originates in the Black Forest of Germany and flows southeast to the Black Sea; it is the European Union's longest and the continent's second longest river (after the Volga). The Danube River Basin (801,463 km^3) has a population of approximately 83 million people. Annual mean flow in the lowest part of the river is 6500 $m^3 s^{-1}$.

Ems River basin

Ems River is situated in Northwestern Germany and flows through North Rhine-Westphalia and Lower Saxony, before discharging into Wadden Sea at Dollart Bay. Ems River basin (18,000 km^2) has a population of approximately 3 million people. Annual mean flow in the lowest part of the river is 83 $m^3 s^{-1}$.

Rhine River basin

The Rhine River begins in the Swiss canton of Graubünden in the Southeastern Swiss Alps and flows into the North Sea. Rhine River basin (197,177 km^2) has a population of approximately 50 million. The River Rhine is the second longest river in central and western Europe (after the Danube) and the busiest waterway in the world. Population density of the basin is approximately 250 inhabitants km^{-2}, and all major cities in the region are situated on the Rhine or on its larger tributaries. Annual mean flow in the lower part of the river is 2300 $m^3 s^{-1}$.

Tiber River basin

The Tiber River is the third longest river in Italy, rising from the Monte Fumaiolo in Emilia-Romagna and flowing 409 km through Toscana, Umbria and Lazio. In Rome it receives water from one of its main tributary, the river Aniene, then flows through Rome and finally enters the Thyrrenian sea in Fiumicino and in Ostia. The total human population in the Tiber River basin (17375 km^2) is approximately 4.7 million people, giving a population density of approximately 270 inhabitants km^{-2}. Annual mean flow in the lowest part of the river is 260 $m^3 s^{-1}$.

Weser River basin

The Weser River in Northwestern Germany flows through Lower Saxony, then reaching the Hanseatic-town Bremen, before emptying 50 km further North at Bremerhaven into the North Sea. Weser River basin (49,063 km^2) is the fourth largest river basin in Germany and has a population of approximately 9.3 million people. Population density of the basin is approximately 190 inhabitants km^{-2}. Annual mean flow in the lowest part of the river is 200 $m^3 s^{-1}$.

Samplings was performed by taking grab samples (200 mL) 0.5 meter below the surface and please note that the results represent the specific moment when and where the samples were taken. Samples were collected into 500 mL pre-rinsed PET bottles and were kept frozen at −18 °C and shipped to Umeå University by fast courier. Samples were not thawed during transportation and the storage time for the samples did not exceed three months. Fortified surface water samples (100 ng L^{-1}, n=10) were used to investigate the stability during three-month storage and the freeze-thaw cycle.

3. Results and discussion

Benzodiazepines were successfully determined in the water samples, with stable and reproducible results. No carry-over effects were observed, no benzodiazepines were detected in the instrumental or field blanks and R^2 values were above 0.99 for all calibration curves in given concentration range (shown in Table S2 in supporting information). Matrix effects, i.e. the suppression or enhancement of analyte-ion signals due to co-eluting matrix components, are common in LC-MS/MS analysis. This effect is more pronounced in matrix-rich samples such as in influent and effluent samples compared to surface waters and only moderate effects (+/- 10%) were detected for the included benzodiazepines. Signal suppression was the most common matrix effect in this study. Limit of quantifications ranged from 0.5-5 ng L^{-1} (shown in Table S1 in supporting information) and <5% degradation was observed during 3 months storage at -18 °C for all included benzodiazepines.

One or more benzodiazepine(s) were determined in 86% of the analyzed samples (120/138) (Table S3). Measured levels were low; total median and mean concentrations of the results above LOQ were 5.4 and 9.6 ng L^{-1}, respectively and four of the included benzodiazepines (clonazepam, chlordiazepoxide, halazepam, and lorazepam) were not detected in any samples. Four benzodiazepines (oxazepam, temazepam, clobazam, and bromazepam) were most predominantly found; oxazepam had the highest frequency of occurrence, it was measured in 85% of the samples (117/138) and had a maximum concentration of 61 ng L^{-1}; temazepam was found in 26% of the samples (36/138) and had a maximum concentration of 39 ng L^{-1}; clobazam was found in 14% of the samples (19/138) and had a maximum concentration of 11 ng L^{-1}; bromazepam was found in 16 out of 138 samples (12%)

and only in samples from Germany, and had a maximum concentration of 320 ng L^{-1} (Table S4).

3.1. Aire and Calder River basin

Four of the target benzodiazepines were detected in the Aire and Calder Rivers, the highest concentrations were found for temazepam > oxazepam > diazepam > midazolam (Figure 1 and table S4). The total contributions of midazolam and diazepam were negligible, whilst temazepam and oxazepam represented approximately 70% and 30%, respectively. This correlates well with the fact that diazepam, temazepam, and oxazepam are all among the five most prescribed benzodiazepine in England (HSCIC, 2015). It should be noted that lorazepam is the second most prescribed benzodiazepine in England (HSCIC, 2015), but this benzodiazepine was not detect in either River Calder or Aire. None of the studied benzodiazepines were detected in the background samples CA1 or AI1. Midazolam was only detected in one sample from the River Aire and was not detected at all in the River Calder. Diazepam was detected in 8 of the 13 samples from the Aire, ranging from 0.3-1.7 ng L^{-1} and was not detected in the River Calder. The concentration trend in the River Aire shows that the major STPs of Esholt and Leeds are sources of diazepam, although there was also a significant contribution from the small STP at Byram (population served approximately 45,000). Diazepam concentrations decrease rapidly after peaks, which indicate rapid degradation or sorption to sediments (Loeffler et al, 2005), since dilution alone cannot explain the decrease. Both oxazepam and temazepam are degradation products from diazepam, which increases the complexity of the results somewhat. Oxazepam and temazepam were detected in all samples with the exception of background sites and follow very similar concentration trends.

Our results show that all benzodiazepines are being released from the same sources and their concentrations are linked to population density. For each of these benzodiazepines, very clear concentration increases are observed after the major STPs on each river. Maximum concentrations of oxazepam and temazepam, at 28 and 67 ng L^{-1}, respectively, were observed on the River Aire downstream of Leeds STP, which is the largest on the river. The concentration profiles in the River Calder are relatively flat in comparison to the River Aire. Most notable is the significant increase after Halifax STP (21 km), serving a population of 100,000, the third largest STP on the river. Concentrations of oxazepam and temazepam peaked here at 14 and 38 ng L^{-1}, respectively. Halifax STP is in close proximity to Calderdale Royal Hospital, and it is likely that benzodiazepine prescription in this area is relatively high. The river flow at Halifax is approximately one third of the final discharge, meaning a significant concentration increase could be achieved by a relatively small mass input to the river. After Halifax, several smaller concentration peaks were observed after nearly every STP until they peak again just before the confluence with the River Aire. Only oxazepam surpasses the Halifax concentration at the pre-confluence location.

3.2. Blanice River basin

A total of 14 surface water samples were collected and analyzed in the Blanice River basin (Figure 2 and table S4). Six benzodiazepines were determined in these samples, but only oxazepam was found with significantly important frequency, i.e. in 10 of 14 samples collected in the middle and downstream part of the Blanice River and its tributaries. The highest concentrations were found in Zivny Stream downstream

Prachatice (site BL 5; 19 ng L^{-1}) and in the sites situated at lower parts of Blanice River (sites BL 13, BL 14; 20 ng L^{-1}, 17 ng L^{-1}, respectively). The highest number of benzodiazepines (diazepam, midazolam, oxazepam, prazepam, and temazepam) was found at the site BL 13 situated on Blanice River upstream of Vodnany. The main sources of the site contamination are probably smaller municipalities (Bavorov, Svinetice) situated a few kilometers upstream of the BL 13 site where treatment of municipal wastewater is poor. Nevertheless, the presence of prazepam and temazepam in surface water is a bit surprising as neither is registered for human medical purposes in Czech Republic. The main benzodiazepines prescribed in Czech Republic are (in falling order) alprazolam, diazepam, bromazepam, and clonazepam (based on daily defined dose, DDD per 1,000 inhabitants and day) (SUKL, 2010).

3.3. Danube River basin

In the Danube River and its tributaries eight benzodiazepines were found (alprazolam, clobazam, diazepam, flunitrazepam, midazolam, oxazepam, prazepam, and temazepam; Figure 3 and table S4). The two most relevant compounds were oxazepam and clobazam with occurrence frequencies of 85 and 31%, respectively. The maximum concentration found for oxazepam was 15 ng L^{-1}, in sample DA10 (Wildungsmauer, Austria), and for clobazam 11 ng L^{-1} in sample DA2 (Kelheim – gauging station, Germany). The mean and median concentrations for these two most relevant benzodiazepines in the Danube River and its tributaries were 5.9 and 5.4 ng L^{-1} for oxazepam, and 5.8 and 5.0 ng L^{-1} for clobazam (statistics considering only the results > LOQ). Temazepam was present in 19% of the samples, prazepam in 10%, midazolam in 9%, alprazolam in 6%, flunitrazepam in 4%, and diazepam in 3%, with maximum concentrations of 6.7 ng L^{-1} for temazepam, 4.4 ng L^{-1} for prazepam, 2.3 ng

L^{-1} for midazolam, 1.6 ng L^{-1} for alprazolam, 2.0 ng L^{-1} for flunitrazepam, and 2.3 ng L^{-1} for diazepam.

3.4. Ems River basin

Only the tributary Hase River was included in this study and four benzodiazepines were found (bromazepam, diazepam, oxazepam, and temazepam; Figure 4 and table S4). Main benzodiazepines prescribed in Germany are (in falling order) lorazepam, diazepam, bromazepam, oxazepam and alprazolam (based on DDD per 1,000 inhabitants and day) (Arzneiverordnungsreport, 2013). Two compounds had occurrence frequencies of 100 %, oxazepam and bromazepam; the maximum concentration detected for oxazepam was 37 ng L^{-1} in sample EM2 (upstream of Quackenbrück) and for bromazepam 150 ng L^{-1} in sample EM1 and EM3 (both around Quackenbrück). In addition, diazepam was found in 50% of the samples and temazepam in 33%, with maximum concentrations 2.3 ng L^{-1} for diazepam and 14 ng L^{-1} for temazepam. It should be noted that no lorazepam was found, despite that this was the most prescribed benzodiazepine in Germany, which indicates that this compound has a short half-life in surface waters.

3.5. Rhine River basin

In the Rhine River and its tributaries, six benzodiazepines were found (alprazolam, bromazepam, diazepam, flunitrazepam, oxazepam, and temazepam; Figure 4 and table S4), which all are among the most prescribed benzodiazepines in Germany (Arzneiverordnungsreport, 2013). The two most common compounds were oxazepam and bromazepam with occurrence frequencies of 100% and 50%, respectively. The maximum concentration found for oxazepam was 61 ng L^{-1} in sample RH14 (downstream Witten) and for bromazepam 91 ng L^{-1} in sample RH13 (upstream of Witten). Two samples were taken in the River Rhine (RH1, 2) directly downstream Cologne and oxazepam and bromazepam were detected at both sites. In the two tributaries Wupper (RH3-9) and Ruhr River (RH10-18), concentration increases were observed after the major STPs on each river. Highest concentrations of benzodiazepines, 77 ng L^{-1} bromazepam and 26 ng L^{-1} oxazepam, were measured in the Wupper River at site RH9 which is located 4.2 km downstream of the Buchenhofen STP, the largest STP in the Wuppertal region. Highest concentrations in the Ruhr River, 30-91 ng L^{-1} of bromazepam and 36-61 ng L^{-1} of oxazepam, were measured at sites RH11-14, which are located on a stretch of the river that receives effluent from 5 major STPs, including STP Hagen. Other benzodiazepines found were diazepam (in 50% of the samples), temazepam (25%), flunitrazepam (11%), and alprazolam (3.6%), with maximum concentrations of 2.8 ng L^{-1} for diazepam, 17 ng L^{-1} for temazepam, 2.0 ng L^{-1} for flunitrazepam, and 1.6 ng L^{-1} for alprazolam.

3.6. Tiber River basin

The first sampling point was a pristine stretch of River Tiber at the source of the river (TI1); the second sampling site was characterized by agricultural impact (TI2); the third sampling point was at the tributary Aniene (TI3), a stream that suffers from municipal and industrial discharges; the fourth sampling site was an urban impacted area of Tiber River after the city Rome, close to the mouth of the river (TI4) (Figure 5 and table S4). In the Tiber River, only oxazepam was found in the two urban impacted samples TI3 and 4; the concentrations were low: 1.4 ng L^{-1} in TI3 and 2.3 ng L^{-1} in TI4.

3.7. Weser River basin

Only tributaries to the Weser River were included in this study and four benzodiazepines were found (bromazepam, diazepam, flunitrazepam and oxazepam; see Figure 4 and table S4), which all are among the most prescribed benzodiazepines in Germany (Arzneiverordnungsreport, 2013). The two compounds with highest occurrence frequencies were oxazepam and diazepam with of 100% and 50%, respectively. The maximum concentration detected for oxazepam was 16 ng L^{-1} and for diazepam 1.0 ng L^{-1}, both in sample WE1 (Wehrder Kanal). In addition, bromazepam and flunitrazepam were found in 25% of the samples, with maximum concentrations 320 ng L^{-1} for bromazepam and 2.0 ng L^{-1} for flunitrazepam.

3.8. General discussion

This screening study on benzodiazepines in European rivers provides important information that can be used in environmental risk assessments of these pharmaceuticals. Detected levels corresponds to earlier studies with oxazepam and temazepam being the benzodiazepines detected in highest concentration in surface waters in Europe, e.g. oxazepam levels were in the range 21-210 ng L^{-1} (Aminot et al., 2015) and 9-310 ng L^{-1} (Camilleri et al., 2015) in France, 2-50 ng L^{-1} (Valcarcel et al., 2013) in Spain and 30-40 ng L^{-1} (Hass et al., 2012) in Germany. Detected levels of diazepam also correspond well with previous reported concentrations of below LOQ or low ng L^{-1} levels in Germany (Hass et al., 2012), France (Aminot et al., 2015) and Spain (Mendoza et al., 2014b; Valcarcel et al., 2013). Bromazepam levels were considerably higher than levels reported in France (Aminot et al., 2015), but this could partially be explained by the distance between sampling sites and STPs, bromazepam was only detected in sampling points directly downstream of STPs, with only a few exceptions. No lorazepam was detected in this study despite the fact it is one of the most prescribed benzodiazepines in both Germany and the UK and previous studies have shown levels of low ng L^{-1} levels (Vulliet and Cren-Olivé, 2011) and in the range of 6-25 ng L^{-1} in France, 1.6-37 ng L^{-1} (Camilleri et al., 2015) and 34-149 ng L^{-1} (Mendoza et al., 2014b) in Spain, and at 34 ng L^{-1} in one sampling point in the Danube (Radovic et al., 2012). No degradation of lorazepam was observed during three-month storage at -18 °C and LOQ of methods used are comparable to these previous studies. Extremely high concentrations of lorazepam have been detected in wastewater in Spain, e.g. a mean of ca 10 µg L^{-1} in influent and 700 ng L^{-1} in effluent, which the authors explained with the use of this pharmaceutical in stockbreeding in the region (Esteban et al., 2012). Our results indicate that smaller- to medium-sized rivers in highly populated regions, e.g. River Aire, Blanice River, and Ruhr River, have concentrations of benzodiazepines that are directly influenced by effluent from STPs. In comparison, the results from the Danube River show that this large river system has lower and more consistent levels of benzodiazepines. Hence, smaller rivers tend to show higher peak concentrations that vary spatially, while larger rivers show less variability and overall lower concentrations. No benzodiazepines were detected at the seven pristine sampling locations included in our study (AI1, CA1, BL1-2, 4, 8 and TI1), which are not influenced by wastewater effluent (Table S4).

This study clearly shows that many benzodiazepines are present in European surface waters at ng L^{-1} levels, and that benzodiazepine concentrations can reach concentrations (200 ng L^{-1}) at which effects on gene expressions in fish has been noted (Oggier et al., 2010). Several studies have also shown effects on exposed fish at µg L^{-1} levels of benzodiazepines (Brodin et al., 2013, 2014; Klaminder et al., 2014, Huerta et al., 2016), i.e. levels an order of magnitude or more higher than the concentrations detected in this study, but concentrations that could still be relevant considering additive effects.

4. Conclusion

In this study we measured 13 benzodiazepines in 138 water samples from six European river basins and 30 rivers and their tributaries.

- One or more benzodiazepine(s) were present in 120 (86%) of the 138 samples with total median and mean concentrations of the results above LOQ of 5.4 and 9.6 ng L^{-1}.
- Four benzodiazepines (oxazepam, temazepam, clobazam, and bromazepam) were most predominantly found with a frequency of occurrence of 85%, 26%, 14%, and 12%, respectively.
- Maximum concentrations measured for oxazepam, temazepam, clobazam, and bromazepam were 61 ng L^{-1}, 39 ng L^{-1}, 11 ng L^{-1}, and 320 ng L^{-1}, respectively.
- This study clearly shows that there are several benzodiazepines present in European surface waters at low ng L^{-1} levels, and although levels are significantly lower than those that have been shown to have effect on exposed biota, environmental effects cannot be dismissed.

5. Acknowledgment

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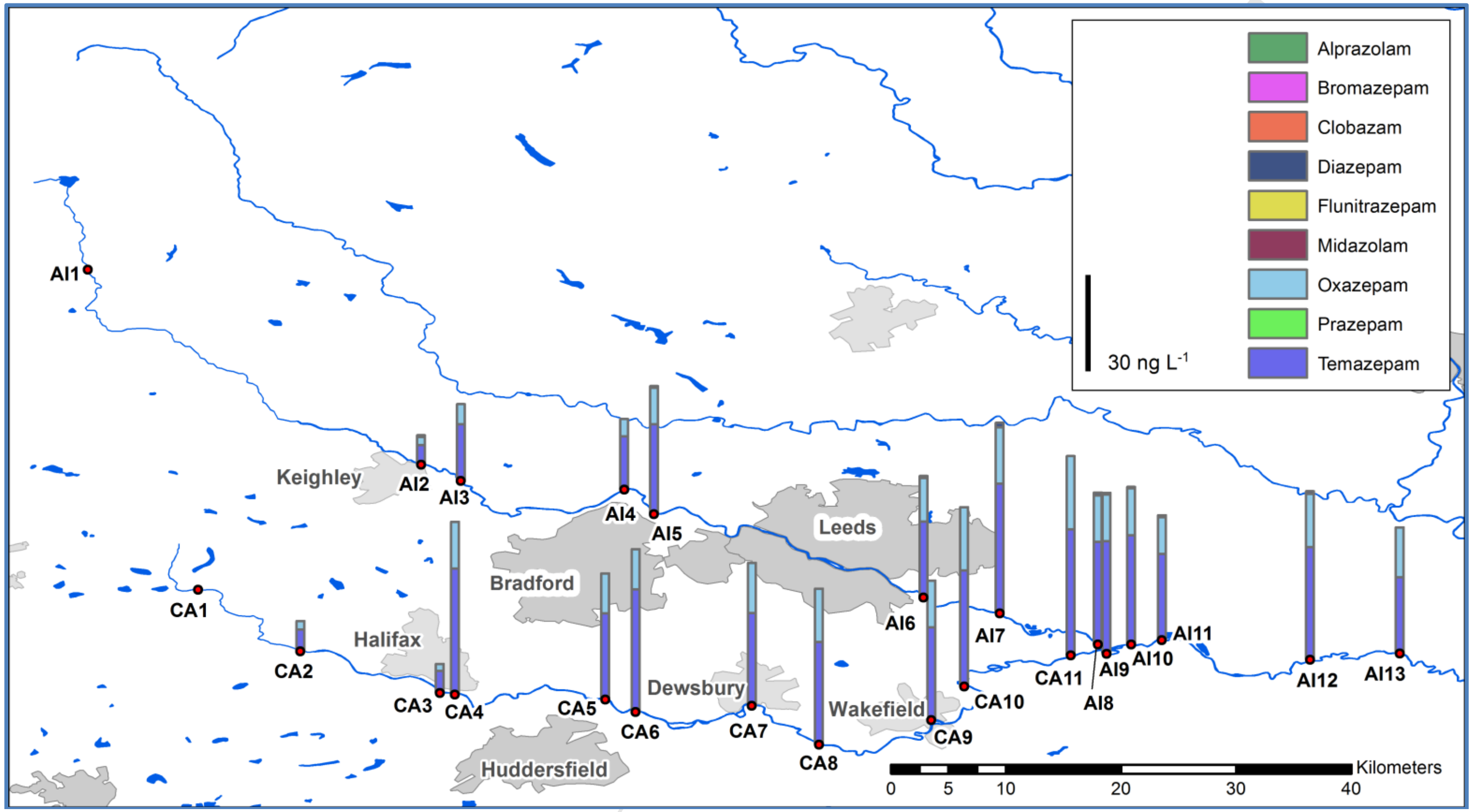
Figure 1. Sampling locations and measured levels of benzodiazepines in UK; A1-13 River Aire, CA1-11 River Calder. Exact positions of the sampling sites and sampling dates are presented in table S1 and all measured values are presented in table S2.

Figure 2. Sampling locations and measured levels of benzodiazepines in the Czech Republic; BL1-3, 7, 11-14 Blanice River, BL4-6 Zivny Stream, BL8-10 Libotynsky Stream. Exact positions of the sampling sites and sampling dates are presented in table S1 and all measured values are presented in table S2.

Figure 3. Sampling locations and measured levels of benzodiazepines in the Danube River basin; DA1-11,13-15, 17, 19-22, 24-28, 30-34, 36, 38-40, 42-47, 49, 50,52, 53, 55, 57, 59-62, 65-68 Danube River, DA12 Morava River, DA16 Moson River, DA18 Vah River, DA23 Rackeve-Soroksar River, DA29 Drava River, DA35 Tisa River, DA37 Sava River, DA41 Velika Morava River, DA48 Timok River, DA51 Iskar River, DA54 Jantra River, DA56 Russenski Lom River, DA58 Arges River, DA63 Siret River, DA64 Prut River. Exact positions of the sampling sites and sampling dates are presented in table S1 and all measured values are presented in table S2.

Figure 4. Sampling locations and measured levels of benzodiazepines in Germany; EM1-6 Hase River, RH 1, 2 River Rhine, RH3-9 Wupper River, RH10-18 Ruhr River, WE1 Hunte River, WE2 Wehrder canal WE3, 4 River Geeste. Exact positions of the sampling sites and sampling dates are presented in table S1 and all measured values are presented in table S2.

Figure 5. Sampling locations and measured levels of benzodiazepines in Italy; TI1,2,4 River Tiber, TI3 Aniene River. Exact positions of the sampling sites and sampling dates are presented in table S1 and all measured values are presented in table S2.



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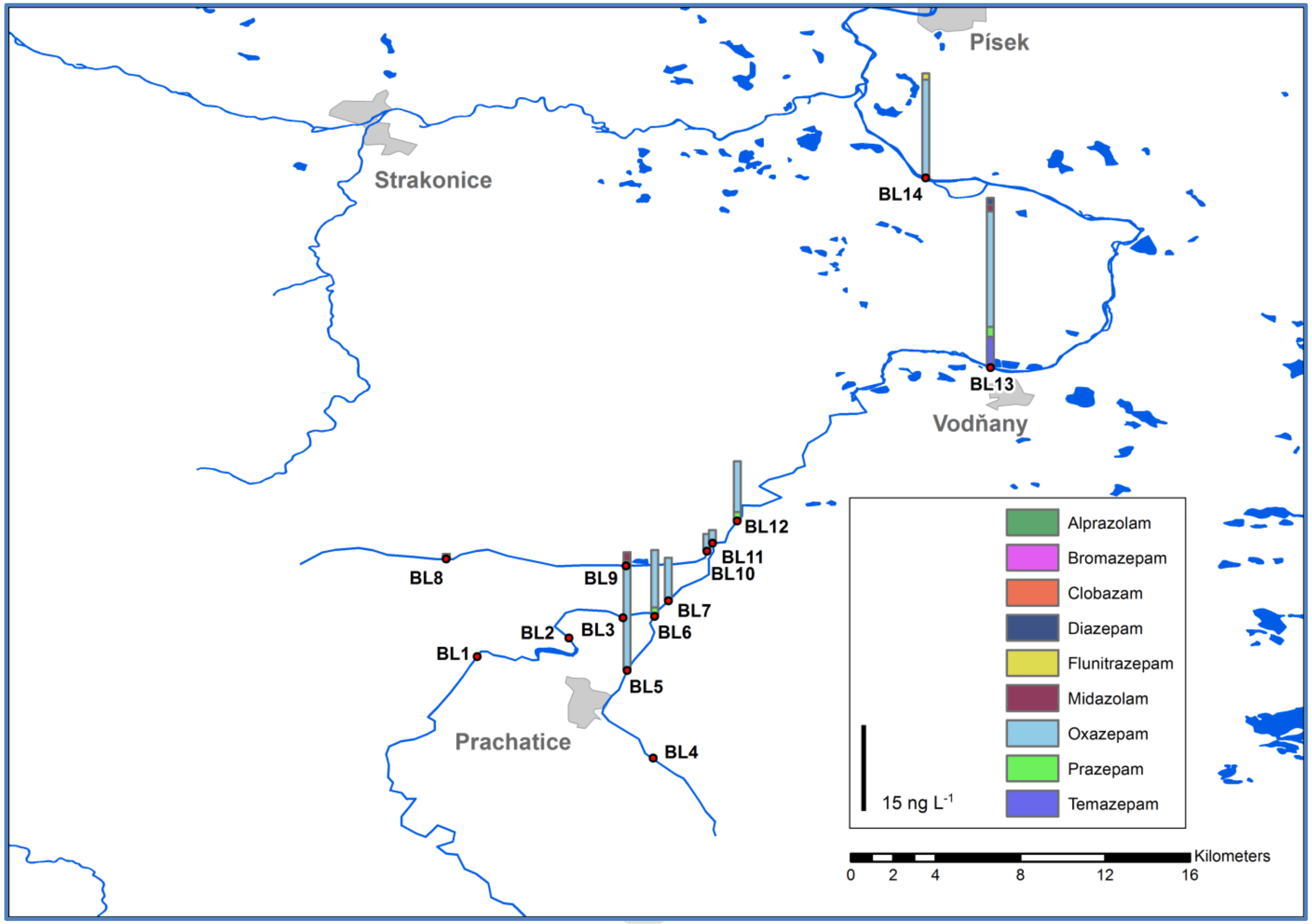

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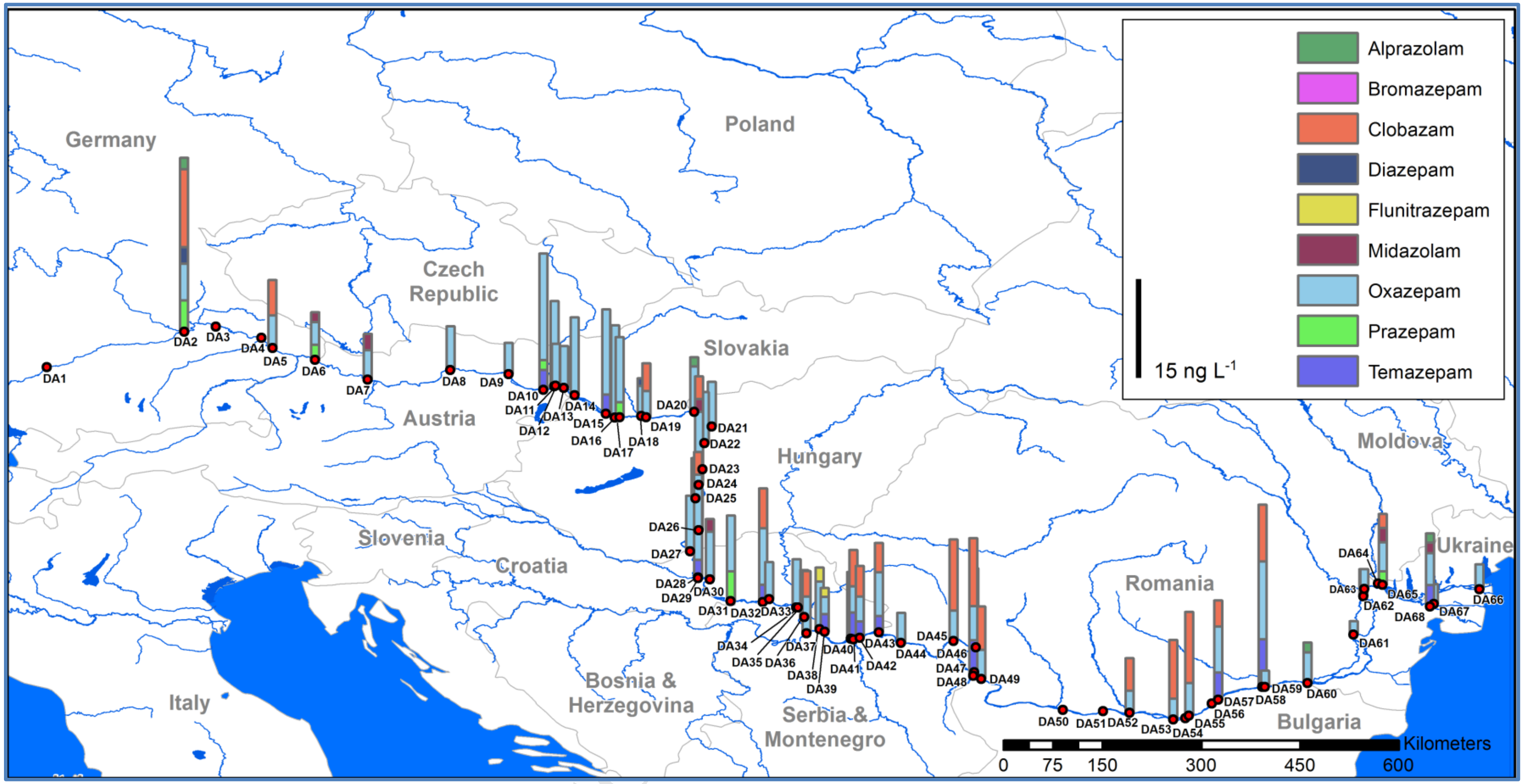

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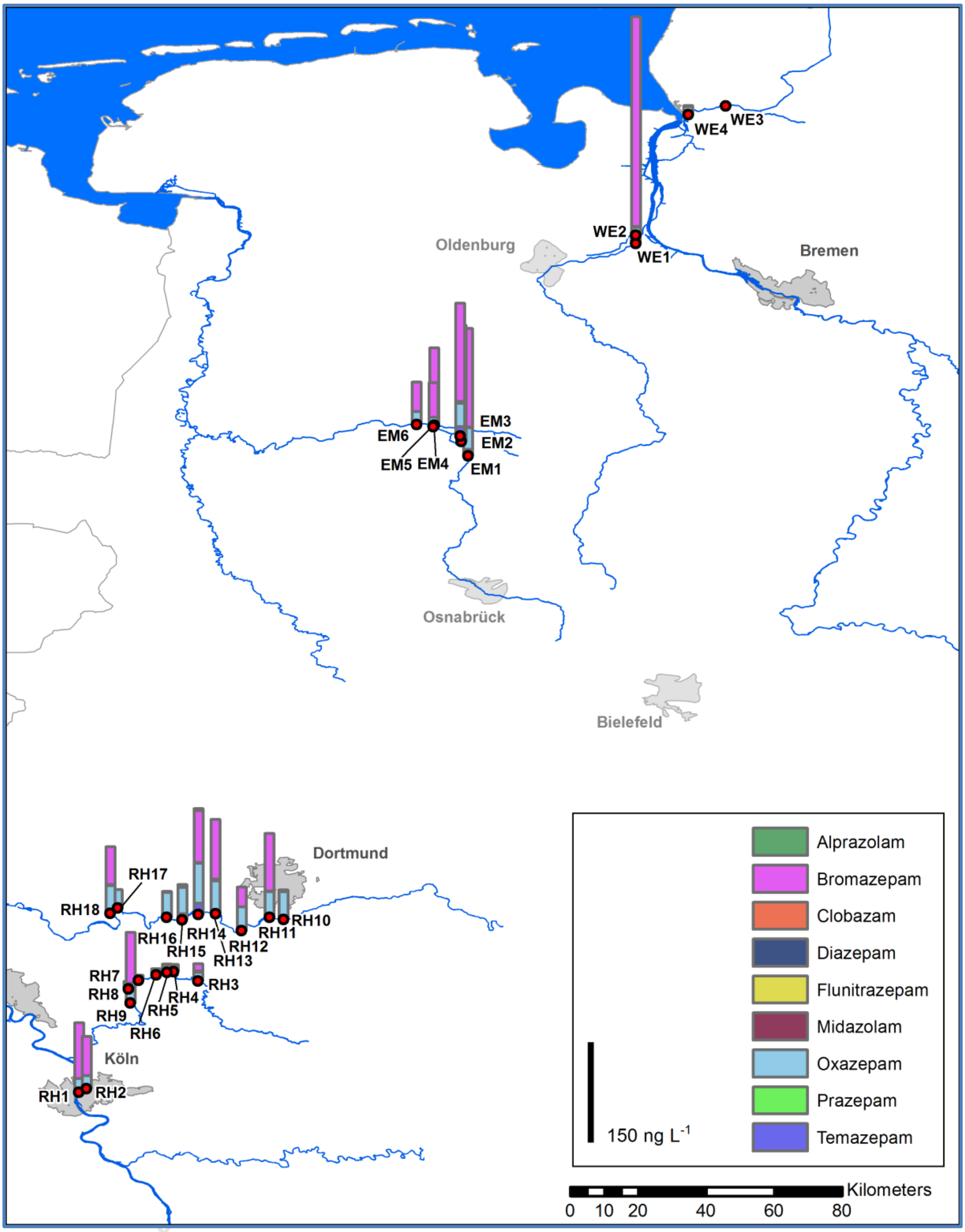

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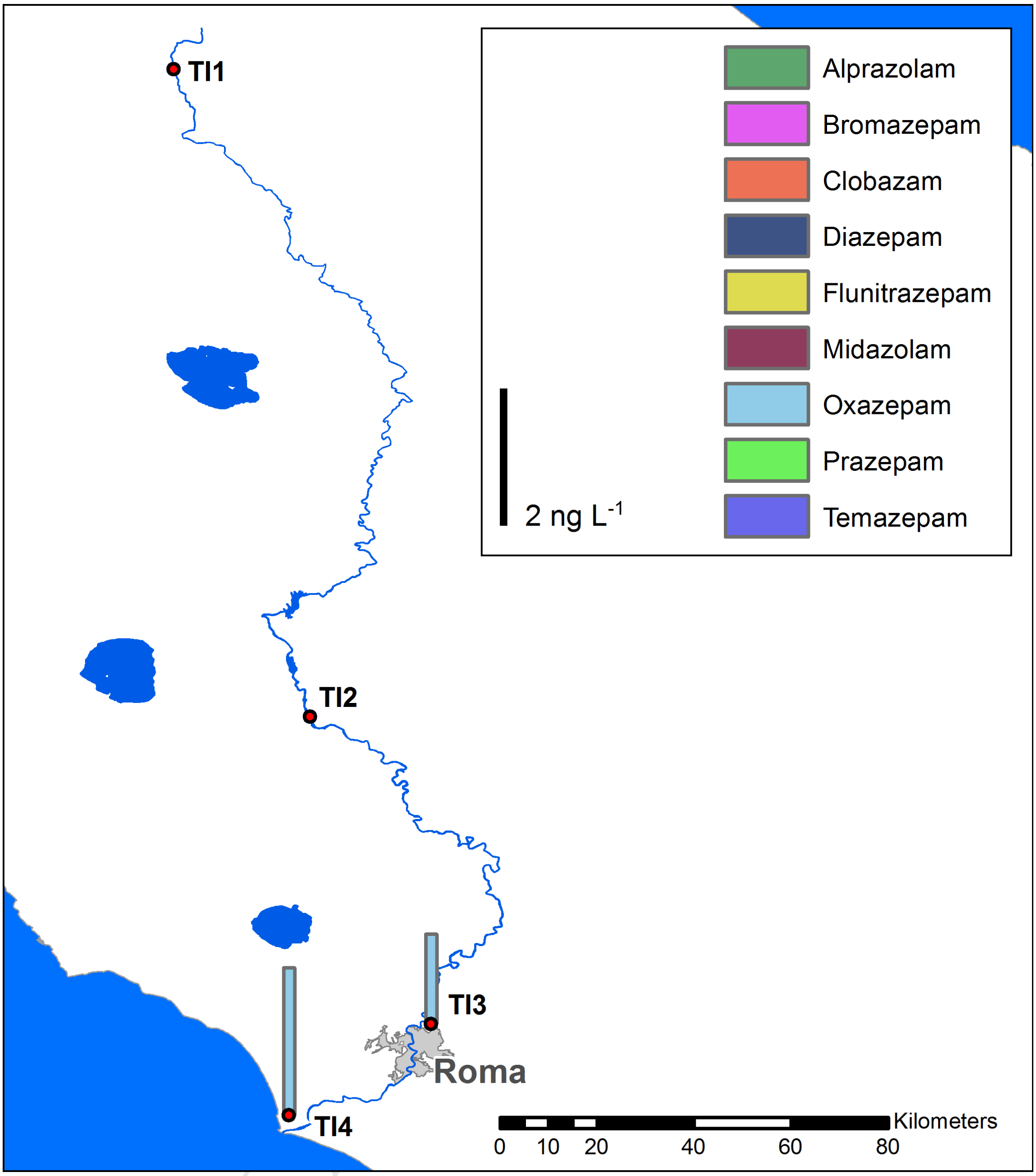

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- We have developed an analytical method to measure 13 benzodiazepines at environmental relevant concentrations
- We have measured 138 water samples from six European river basins and 31 rivers and their tributaries.
- This study clearly shows that there are several benzodiazepines present in European surface waters at ng L^{-1} levels