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Retrospective screening of high-resolution mass spectrometry archived digital samples can improve environmental risk assessment of emerging contaminants: A case study on antifungal azoles

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ABSTRACT

Environmental risk assessment associated with aquatic and terrestrial contamination is mostly based on predicted or measured environmental concentrations of a limited list of chemicals in a restricted number of environmental compartments. High resolution mass spectrometry (HRMS) can provide a more comprehensive picture of exposure to harmful chemicals, particularly through the retrospective analysis of digitally stored HRMS data. Using this methodology, our study characterized the contamination of various environmental compartments including 154 surface water, 46 urban effluent, 67 sediment, 15 soil, 34 groundwater, 24 biofilm, 41 gammarid and 49 fish samples at 95 sites widely distributed over the Swiss Plateau. As a proof-of-concept, we focused our investigation on antifungal azoles, a class of chemicals of emerging concern due to their endocrine disrupting effects on aquatic organisms and humans. Our results demonstrated the occurrence of antifungal azoles and some of their (bio)transformation products in all the analyzed compartments (0.1–100 ng/L or ng/g d.w.). Comparison of actual and predicted concentrations showed the partial suitability of level 1 fugacity modelling in predicting the exposure to azoles. Risk quotient calculations additionally revealed risk of exposure especially if some of the investigated rivers and streams are used for drinking water production. The case study clearly shows that the retrospective analysis of HRMS/MS data can improve the current knowledge on exposure and the related risks to chemicals of emerging concern and can be effectively employed in the future for such purposes.

1. Introduction

Aquatic and terrestrial ecosystems are contaminated by thousands of man-made organic contaminants that may impact environmental and human health. Hence, it is important to evaluate the environmental risks associated with these chemicals to propose effective remediation solutions that can protect or restore aquatic ecosystems. Under the current regulatory environmental risk assessment (ERA), the exposure (e.g. Predicted Environmental Concentration, PEC or Measured Environmental Concentration, MEC) and effect (e.g. Predicted No Effect Concentration, PNEC; Environmental Quality Standards, EQS) of individual chemicals are evaluated (Perrodin, 2011). However, aquatic organisms and humans are exposed to broad mixtures of known and unknown compounds that are highly variable along spatial and temporal scales. The current ERA therefore only provides a partial assessment of the potential impact of these mixtures since a true depiction of ERA would require the characterization of a broader range of chemicals (i.e. chemical exposome) (Wild, 2012) and an assessment of relevant environmental mixtures. This holds particularly true for chemical classes including substances with similar structures that can act additively or even synergistically, which are often released simultaneously into the environment (Kretschmann, 2015; Rösch, 2017).

Of particular concern among the emerging class of contaminants is the antifungal azoles family that includes both triazole and imidazole

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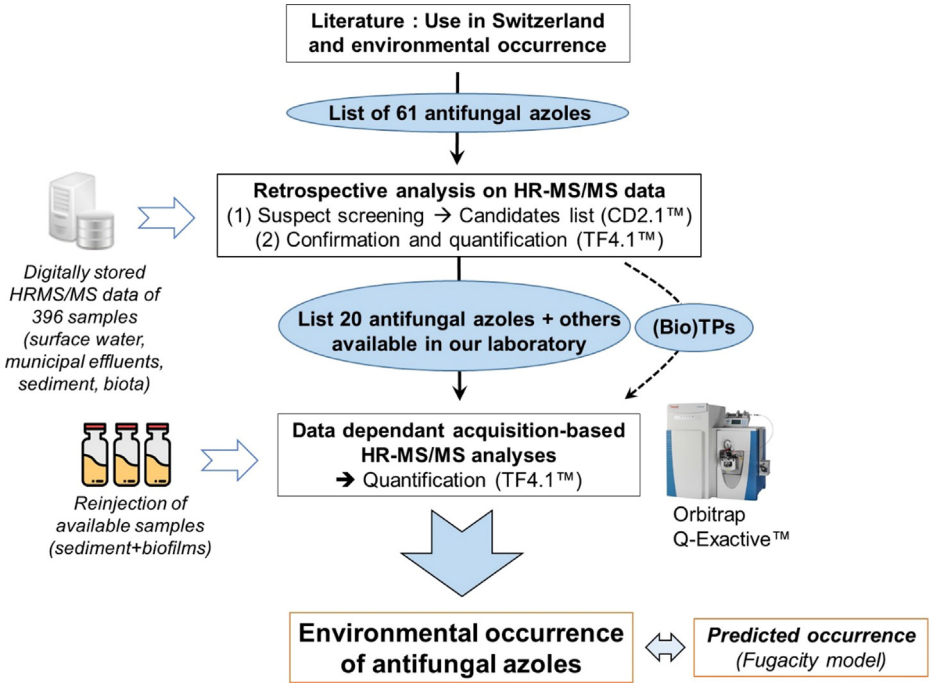

Fig. 1. Workflow for comprehensive azole exposure determination using a retrospective screening strategy.

classes. They are widely used as agricultural fungicides or pharmaceuticals to treat mycoses, parasitic infections and cancer in humans since the 1960s (Sheehan et al., 1999; Miller et al., 2002; Corcoran et al., 2010). Initially designed to inhibit the CYP51 enzyme responsible for the biosynthesis of the ergosterol (an essential building block of the fungal cell membrane), they also disrupt a broad range of other non-targeted CYPs involved in steroidogenesis (e.g. CYP19) and xenobiotic detoxification (e.g. CYP1A) in mammals and aquatic species (e.g. fish) (Chambers, 2014; Matthiessen and Weltje, 2015). Thus, they have the capacity to act as endocrine disruptors and may affect survival, development, growth, reproduction and behaviour of non-target organisms (Matthiessen and Weltje, 2015; Chen and Ying, 2015; Skolness, 2013). For instance, antifungal azoles inhibit aromatase, an endogenous enzyme responsible for the conversion of androgens to estrogens and could lead to masculinization of wild fish population (Matthiessen and Weltje, 2015; Hinfray et al., 2006; Cheshenko, 2008). Azole exposure has been also related to the reduction of fish growth *in vitro* and *in vivo,* although the underlying mechanism is still unknown (Stadnicka-Michalak et al., 2015). Beyond their potential impact on aquatic organisms, there is also an evidence of their involvement in antifungal resistance – a threat to human food resources and human health (Bromley, 2014).

While there is a paucity of information on concentration-additive actions of azoles, synergistic effects have been reported in several species including aquatic organisms (Kretschmann, 2015), suggesting the need for particular caution in the hazard and risk assessment of these chemicals. Because of their broad use, substantial amounts of azoles may reach the environment through urban wastewater, surface runoff and pesticide application (Peng, 2012). Hence, they are widely detected in wastewater, sewage treatment plant sludge and river surface waters (Kahle, 2008; Casado, 2014). Frequently detected azoles have already been proposed or included for regulatory monitoring (Götz, 2010; OSPAR, List, 2013). Nevertheless, data remain scarce on their occurrence in lakes (Moschet, 2014; Peng, 2014), sediments (Huang, 2010), soils (Huang, 2018) and biota (Munz et al., 2018; Richmond, 2018), and they could be widely distributed in different compartments due to their broad range of physico-chemical properties (e.g. Log Kow between 0.25 and 7.41).

Overall, addressing accurate exposure assessment of pollutants requires large sampling efforts, including sample storage. However, such strategy is not suitable/manageable in the long term because of high cost of such sampling campaign and the incapacity to store the samples indefinitely.

Retrospective screening of archived digital samples from high resolution tandem mass spectrometry (HRMS/MS) can address these challenges. HRMS/MS not only provides a comprehensive view of the chemical composition based on target (using reference standard), suspect (exact mass as a preliminary information) and non-target analyses (no previous information) (Hollender, 2017; Krauss et al., 2010), it can also be used to create digitally stored archives of samples (i.e. raw data) rather than physical storage. This option offers an opportunity to explore exposure to contaminants of emerging concern retrospectively (Alygizakis and Samanipour, 2018). In particular, suspect-analysis is a very promising approach that allows tentative identification of known and "predicted" chemicals (e.g. (bio)transformation products, (bio)TPs) based on their accurate masses and also on their isotopic patterns and MS^2 spectra included in online databases (e.g. (MassBank, 2020, NIST, 2020, NormanSuspectList, 2020)). Hence, HRMS based analysis allows the identification of environmentally relevant mixtures to be characterized for their hazard.

This work aimed to investigate the potential of using HRMS archives to perform retrospective environmental risk assessment of antifungal azoles. We first characterized their occurrence in various aquatic and terrestrial compartments at sites with different land uses and then assessed the exposure of aquatic organisms from different trophic levels. The measured concentrations were subsequently compared with the predicted data using a partitioning model, and the associated risk was then estimated. For this study, 61 suspected pharmaceutical and pesticidal antifungal azoles and some of their (bio)TPs selected from literature research were investigated in a broad range of samples. These samples included 154 surface water, 46 urban wastewater effluents, 67 sediments, 15 soils, 33 groundwaters, 24 biofilms, and 41 gammarid and 49 fish samples from 95 sites allowing to cover various land uses (urban, agricultural, forest) widely distributed on the Swiss Plateau.

2. Materials & methods

2.1. Retrospective analysis workflow

The occurrence of antifungal azoles in Swiss aquatic and terrestrial ecosystems was investigated through a four-step strategy: (i) literature research (use and occurrence), (ii) retrospective analysis of digitally stored HRMS/MS (i.e. sample archive), (iii) reinjection of available samples (i.e. sediment and biofilms) for confirmation using target analysis and (iv) comparison with partitioning modelling (Fig. 1).

The retrospective screening was applied to a set of digitally stored HRMS/MS data acquired in data independent acquisition mode (DIA, i.e. composite MS^2 spectra of all fragmented ions compiled for each of the 5 or 9 mass windows for the precursor, respectively, independent of an inclusion list) or in data dependent acquisition (DDA, i.e. MS^2 acquisition of both isolated precursor masses from an inclusion list containing targeted azoles and highest precursor masses) using a Q-Exactive-Orbitrap system (Thermo-Scientific) (*section 2 of the supporting information*). These data were from previous studies on urban wastewater effluent, surface water, soil, gammarids and fish samples from 95 sites under various anthropogenic pressures (i.e. urban, agricultural, mixed land use) (Table S1). We further complemented this dataset with sediment samples from selected rivers and lakes as well as biofilms to expand the compartments considered in the partitioning analysis. It was observed that few azoles (i.e. tebuconazole, propiconazole) were already investigated in few of the previous studies. Thus, one aim of the present study was to enlarge the screening of antifungal azoles, providing additional evidence of the relevance of retrospective investigation.

The screening of suspected antifungal azoles and known (bio)TPs (Table S3 and S4) were selected from the literature research (i.e. used in Switzerland, detected in the environment) using Compound Discoverer 2.1 (CD2.1, Thermo-Scientific) (*Section 2 of the supporting information*). This software was used to screen for predicted bioTPs based on known transformation reactions. The resulting list of potentially detected azoles was confirmed with reference standards and were quantified using Trace Finder 4.1 (TF4.1, Thermo-Scientific) through a comparison with an in-house database that checks for MS^2 fragments and retention time. For this purpose, calibration curves with internal standards were used. In total, 23 chemicals (20 azoles + 3 (bio)TPs) were confirmed and quantified (Table S6). Finally, to validate the use of DIA analysis, we reinjected some of the available samples (i.e. sediments and biofilms) and analysed them through DDA.

2.2. Study sites and sampling

In total, 95 sites were investigated and were under various anthropogenic pressures that are widely distributed over Jura and Swiss Plateau. Among these sites, several environmental compartments were examined including 154 surface water, 46 urban effluent, 67 sediment, 15 soils, 31 groundwaters, 24 biofilms, 41 gammarids and 49 fish tissue samples, (Fig. 2, Table S1). Surface water and urban wastewater effluents were sampled as 12–24 h composite samples at different time periods in 2012 and 2014. Groundwater wells were sampled in December 2013 and May 2017. River, stream and lake sediments were sampled in May, June and between September and November in 2014, 2016 and 2017. Biofilms were grown on glass slides at some sampling sites from the 15th of March to the 30th of April 2014 and 2016. Gammarids were collected in September 2014, January 2015 or October 2015. Fish were sampled in May and October 2014, 2015 and 2016. Details on the sampling are provided in (Munz, 2017; Spycher, 2018) (surface water and effluent), (Kiefer, 2019; Hollender, 2018) (groundwater), (sediment and soils), Casado-Martinez et al. *in prep* and (Chiaia-Hernandez and Gunthardt, 2017) (sediment), Tlili et al. (in revision) (biofilms), (Munz et al., 2018) (gammarids) and Fischer et al. in prep (fish).

2.3. Sample preparation and chemical analysis

Surface waters, effluents, groundwaters, gammarids, soils and fish were previously extracted and analyzed using the methodologies in (Spycher, 2018; Munz, 2017; Kiefer, 2019; Hollender, 2018; Munz et al., 2018; Chiaia-Hernandez and Gunthardt, 2017), and by Fischer et al (in prep), respectively. Here, the methods used for the extraction in the respective studies are described whereas extraction and analysis of sediments and biofilms were first performed in this study. Overall, one replicate per sample was analysed whereas the establishment of analytical performance was based on triplicates.

Surface water and effluent samples were enriched and analyzed using online solid phase extraction (SPE) followed by liquid chromatography (LC) and coupled with electrospray-ionization (ESI) in positive mode to HRMS/MS as described in (Munz, 2017). The groundwater samples collected in 2013 were filtered through a glass microfiber filter (GF/F, 47 mm, 0.7 μm) prior to SPE extraction and injection into the LC-HRMS/MS system as described in (Hollender, 2018). The remainder of the groundwater samples (i.e. from 2017) was enriched with Büchi as described in (Kiefer, 2019). Soil and sediment samples were freeze-dried and then purified and extracted using accelerated solvent extraction (Dionex) at 80 °C with a mixture of ethyl-acetate/acetone (70:30; v:v) as described in (Chiaia-Hernandez and Gunthardt, 2017). Biota samples including biofilms, gammarids and fish were extracted using a QUECHERS-based method as described in (Munz et al., 2018). Separation and detection of the compounds were performed on LC-HRMS/MS. Chromatographic separation was performed using XBridge C18 column (3.5 μm, 2.1 × 50 mm, Waters) with pre-column (2.1 × 10 mm) or an Atlantis T3 C18 column (5 μm, 150 mm, Waters) with methanol and nanopure water, both acidified with 0.1% formic acid, as eluents. Detection was based on QExactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, San Jose, U.S.A.) equipped with an ESI used in positive polarity. Details on sample preparation, chemical analysis, quality control and performance of the methods are provided in *section 2 of the supporting information*. Note, that the analytical performance was established for all the matrix on a new set of samples spiked with the selected antifungal azoles (in triplicates) in parallel to the investigation of newly extracted samples of biofilms and sediments.

2.4. Partitioning and bioaccumulation factor (BAF) calculation.

In the present study, we investigated the partitioning of antifungal azoles in different environmental compartments including aquatic organisms. In particular, we aimed to highlight if the apparent partitioning (i.e. measured environmental concentration) in aquatic compartments fitted with the predicted information from an *in silico* approach (i.e. models). Among others, the fugacity models provide predictions of concentrations/amounts in different aquatic compartments based on the physico-chemical properties of contaminants (log Kow, Henry's law constant, and molecular weight), volume of different compartments and total amount of chemical in the system. Different levels of complexity of the fugacity model can be implemented depending on data availability and the desired outcomes of the study (Mackay, 2009). In this study, the apparent partitioning of the antifungal azoles calculated from the field data was compared to the prediction by the fugacity level I model (i.e. closed system in equilibrium) developed by the University of Trent (Trent, 2020). We used the EQC (equilibrium criterion) – standard environment parameters of the software and adjusted the model with specific lipid fractions (i.e. 0.027 is the average lipid content for gammarids, 0.06 for biofilms and 0.05 for fish) (Table S13). The physico-chemical properties of the azoles were gathered from the literature (Pesticides Properties DataBase (PPDB) (Lewis, 2016); Pubchem (Pubmed, 2020); or Chemspider (Chemspider, 2020) websites) or predicted using EPI suite (EPA, 2012). The chemical usage data required for the modeling was derived for

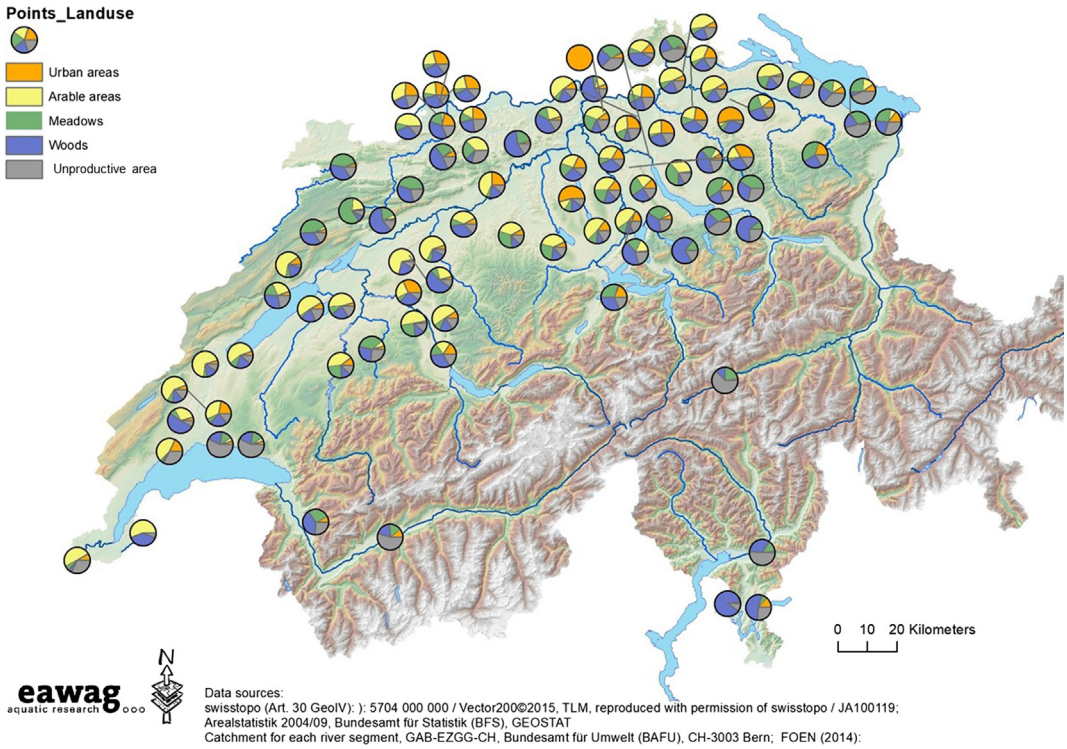

Fig. 2. Sampling sites in Switzerland with their land use.

Switzerland using available information (Federal Office for Agriculture, FOAG, 2020) (Table S15). The overall environmental persistence of the azoles predicted by EPI suite is an indication of the chemical's environmental biodegradation rate under aerobic conditions whereas the environmental DT_{50} (i.e. time leading to the dissipation of half of the compound concentration) values in the different compartments are from PPBD.

We then used the *in situ* measured concentrations or predicted values from the fugacity model to calculate the apparent *in situ* bioaccumulation factor (BAF [L/kg]) of antifungal azoles at different trophic levels as described in Eq. (1),

$$BAFi = \frac{Ci\ biota}{Ci\ water} \tag{1}$$

where Ci biota is the concentration of the compound i in the biota (ng/kg d.w.) and Ci water, its concentration in the surface water (ng/L).

2.5. Risk assessment – quality standard derivation and risk quotient calculations

To calculate the risk associated with antifungal azoles on aquatic organisms and humans, we have used the available (Moschet, 2014; INERIS, 2019) and *ad-hoc* Quality Standard (QS) values as listed in the Tables S17 and S18. In addition to most common quality standard for freshwater pelagic community based on ecotoxicity ($QS_{fw,\ eco}$), we also derived *ad hoc* QS values for sediment for benthic community ($QS_{sed,EqP}$), and surface water used for drinking water regarding human health ($QS_{dw,\ hh}$) to determine the difference in risks to various exposed biota.

For freshwater pelagic communities, we used the assessment factor (AF) method for chronic exposure based on the most sensitive species since we did not have enough ecotoxicity data for statistical extrapolation methods (e.g. species sensitivity distribution). Eq. (2) (Scientific Committee on Health, 2017) was employed for this approach:

$$QS_{fw,eco}\left[\frac{\mu g}{L}\right] = \frac{NOECmin}{AF} \tag{2}$$

To this end, we applied the NOEC (No Observable Effect Concentration) values found in the literature (INERIS, 2019) and the AF values defined as follows: 10 for chemicals with effect data from at least 3 species representing three trophic levels; 50 for chemicals with effect data from species representing 2 trophic levels; 100 for chemicals with only 1 long-term result. Values of NOEC, AF and calculated QS and those from the literature are provided in Table S17.

For benthic communities, we used the Equilibrium Partitioning (EqP) method to calculate the $QS_{sed,EqP}$ because there are not enough reliable sediment toxicity data available for the azoles. Thus, the $QS_{sed,EqP,ww}$ (i.e. wet weight) was calculated using the following (Eq. (3)) (Scientific Committee on Health, 2017):

$$QS_{sed,EqP,ww}\left[\frac{\mu g}{kg}\right] = \frac{K_{sed-water}}{RHO_{sed}} \times EQS_{fw,eco}\left[\frac{\mu g}{L}\right] \times 1000 \tag{3}$$

where RHO_{sed} is the bulk density of wet sediment (=1300 kg_{ww}/m^3) and $K_{sed\text{-}water}$ is the partition coefficient between sediment and water (m^3/m^3).

$$K_{sed-water} = Fair_{sed} \times K_{air-water} + Fwater_{sed} + Fsolid_{sed} \times \frac{Kp_{sed}}{1000} \times RHOsolid$$

$$\text{and} Kp_{sed} = Foc_{sed} \times K_{oc}$$

where $Fair_{sed}$ is the fraction of air in sediment, $K_{air\text{-}water}$ is the air–water partition coefficient, $Fwater_{sed}$ is the fraction of water in sediment, $Fsolid_{sed}$ is the fraction of solids in sediment, Kp_{sed} is the partition coefficient solid-water in sediment, Foc_{sed} is the weight fraction of organic carbon in sediment, Koc is the partition coefficient between organic carbon and water.

The corresponding value for dry sediment is calculated using Eq. (4):

$$QS_{sed,EqP,dw}[\mu g/kg] = CONV_{sed} \times QS_{sed,EqP,ww}[\mu g/kg] \tag{4}$$

$$\text{with } CONV_{sed} = \frac{RHO_{sed}}{Fsolid_{sed} \times RHO_{solid}}$$

For our calculations, we used the selected $QS_{fw,\ eco}$ and default values of Foc_{sed} (0.05 kg/kg), $Fair_{sed}$ (0 m^3/m^3), $Fwater_{sed}$ (0.8 m^3/m^3), RHO_{solid} (2500 kg_{solid}/m^3_{solid}), RHO_{sed} (1300 kg_{ww}/m^3), $Fsolid_{sed}$ (0.2) and Koc (L/kg) from the literature (INERIS). All the values and resulting calculation are presented in Table S18.

For human health through drinking water consumption, in accordance with the methodology proposed in (Scientific Committee on Health, 2017), we first calculated the $QS_{dw,\ hh}$ with the following (Eq. (5)):

$$QS_{dw,\ hh}[\mu g/L] = \frac{0.1 \times TRV\,[\frac{\mu g}{kg}/j] \times body\ weight\,[kg]}{average\ water\ consumption\left[\frac{L}{j}\right]} \times \frac{1}{Fsafety} \tag{5}$$

where TRV is the toxicological reference value which is the total admissible dose per day; 2 L per day was used for the average water consumption; 70 kg was used for body weight; 0.1 as correcting factor to account for other sources of contamination; F_{safety} is an additional safety factor to account for the endocrine disrupting properties of the chemicals. Because all the $QS_{dw,\ hh}$ values were higher than the value recommended by the directive 98/83EC for pesticides (Commission, 1998), we used the generic value (0.1 μg/L) from the regulation based on precautionary principle (2000/60/EC, 2000)

Finally, since azoles act through a similar mechanism of action (i.e. CYP51 inhibition), we calculated the risk associated to the mixture by summing individual Risk Quotients (RQi) for a sample, as follows (Chèvre, 2006):

$$RQazolesmix = \sum RQi \tag{6}$$

with

$$RQi = \frac{Ci}{QSi} \tag{7}$$

where Ci is the concentration of the compound i in the surface water or the sediment and QSi is the quality standard of the compound i in surface water or sediment.

2.6. Statistical analyses

Statistical analysis of the data was performed using the R software 3.5.1 (R Core Team, 2017). Significant difference between levels of antifungal azoles at urban sites (sampled under low water flow condition) and agricultural sites (sampled under high water flow condition) was estimated by using student *t* test ($p < 0.01$) after checking for normal distribution with the Shapiro-Wilk test. To interpret occurrence data further, we also performed a hierarchical clustering. More details are provided in the *section 3 of the supporting information*.

3. Results and discussion

3.1. HRMS/MS data with DIA is suitable for building a sample archive and implementing retrospective screening

We investigated for the first time the occurrence of antifungal azoles in 430 samples from 95 sites under various chemical pressures in Switzerland using a combination of digitally stored HRMS data (surface waters, groundwaters, urban effluents, gammarids, fish, soils) and the newly collected dataset (sediment and biofilms) all acquired by DIA (Fig. 1). Based on literature and usage of this chemical class in Switzerland, we prioritized a list of 61 chemicals including 22 pharmaceuticals and 39 pesticides (Table S3 and S11). Among these 61 chemicals, 20 could be detected in the samples based on mass accuracy (< 5 ppm) and the isotopic patterns of the molecular ion (Sfit score > 70%; pattern score > 90%) (Table S8). From this screening, the identified candidates were then confirmed and quantified under data evaluation software TF4.1 using the same DIA dataset and our TF database (fragments, retention time, isotope pattern). This group is comprised of 31 azoles and bioTPs. The compilation of calibration curves for reference standards of these 31 compounds and usage of internal standards (ISTD), as the spiking of standards and ISTD into samples of the data archive prior original analysis and re-evaluation allowed this retrospective quantification. Performance of the method for the chemical analysis of azoles was investigated for all the compartments (Table S7). Overall, extraction recoveries (*see supporting information*) ranged between 50% and 97% with few exceptions depending on the compartment and the chemicals. Lower -but still acceptable- absolute recoveries (< 60%) revealed high ion suppression at the source in complex matrices such as biota (Table S7), resulting in higher LOQ_{matrix} (> 1 ng/g d.w.). Note, however, that these recoveries were calculated from newly spiked samples extracted months or years after investigating the digitally archived samples (although they have been prepared and analyzed using the same methodology). This is a major limitation for the retrospective quantification of digitally archived samples.

In addition to the parent chemicals, the suspect screening of known and predicted (bio)TPs of antifungal azoles revealed their occurrence in different compartments as reported in Table S9. Among the (Bio)TPs that have available standards in our laboratory, prothioconazole-desthio, prochloraz BTS40308 and BTS44595 could be further confirmed and quantified. Interestingly, although prothioconazole was not detected in the investigated samples, its bioTP was widely detected in different compartments highlighting the need to understand the occurrence of bioTPs. Finally, to investigate if DIA (i.e. composite MS^2) provides similar results to DDA in terms of candidate confirmation prior to their quantification, available samples (i.e. sediment and biofilms) were reinjected and analysed through DDA. As expected, comparison between DIA and DDA did not show notable difference for both qualitative (confirmation of the compounds) and quantitative data (Fig. S3, Table S10). In fact, this step confirmed the occurrence of the 23 compounds previously identified plus 8 additional antifungal azoles that were available as analytical standards in our laboratory and were previously or currently used in Switzerland (Table S6).

Altogether, these results support the use of HRMS/MS data with DIA to build archived digital samples usable for screening. So far, different suspect screening strategies have been reported in the literature (Moschet, 2013; Moschet, 2017; Chiaia-Hernandez, 2014; Gago-Ferrero, 2015). They are typically based on the use of a full HRMS scan from DDA acquisition followed by the re-measurement of the samples to get MS^2 spectra for confirmation. DIA is a more recent development that has been increasingly used since it allows for a quick and comprehensive screening of samples when no prior information about the contaminants in the samples is available (Alygizakis and Samanipour, 2018; Moschet, 2017). Nevertheless, in most cases, the confirmation of the candidate identities also requires an additional DDA to acquire an accurate MS^2 data. In the present study, we showed that the use of MS^2 composite spectra from DIA acquisition allowed the confirmation of all candidate azoles through the comparison with our in-house database. In addition to MS^2 fragments, our database includes isotopic pattern and retention times that are crucial for the confirmation step prior the quantification. It is also important to note that, independent of the original usage of samples, such retrospective screening based on archived digital samples can benefit the addition of a broad range of internals standards into samples prior to analysis and injection in parallel of a range of calibration standards.

3.2. Antifungal azoles widely contaminate the Swiss environment from urban to agricultural activities

This study is the first to investigate the environmental occurrence of numerous antifungal azoles at many sites with different land uses (i.e. urban and agricultural activities as anthropogenic pressures) and their distribution in various compartments including different trophic levels

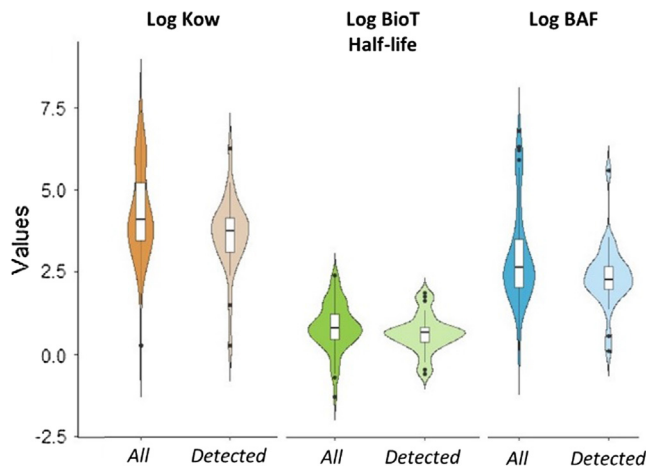

Fig. 3. Physico-chemical ranges of "all" 61 screened and 20 "detected" antifungal azoles (From EPIsuite v1.4). BioT Half-life is the whole body primary biotransformation rate estimate for fish; BAF is the bioaccumulation factor at the highest trophic level.

(primary producers, as biofilms and primary/secondary consumers, as gammarids and fish). Due to the diverse use and the broad range of physico-chemical properties of these compounds, we hypothesized a wide distribution of these contaminants overall (Fig. 3, Table S15). As shown in Fig. 4, azoles can in fact be frequently detected in Swiss ecosystems. Both pharmaceutical and pesticidal azoles showed similar frequency detection pattern, with high frequency in urban effluents (100% and 96% respectively), surface waters (63% and 89%, respectively), sediments (64% and 59%, respectively), but lower frequency in groundwater (25% and 13%, respectively) and in soil (4% and 11%, respectively). Biota samples (i.e. biofilms, gammarids, fish) showed more variability since they were all sampled in sites that were mainly under urban pressure. In these samples, pharmaceutical azoles were detected in 57% of the samples whereas pesticidal azoles were only detected in 8% of them. Regardless of the environmental compartment, the most frequently detected azoles were climbazole, propiconazole and tebuconazole. These substances are among the highly consumed chemicals in medicine or agriculture in Switzerland (up to 100,000 kg/year) (Figs. S5–S9). For instance in our study, the pharmaceutical climbazole was detected in 57% of the sediments, 75% of the surface waters and the pesticide tebuconazole in 60% of the sediments and 89% of the surface waters. Also, the number of detected azoles differed between compartments: those with few detections (i.e. soils, groundwater, biota) *vs* those with high detections (i.e. sediment, surface water, urban effluent) (Figs. S5–S9). The lower number of azoles detected in the biota samples (i.e. 8 in the biofilms, 7 in the gammarids and 3 in the fishes) is likely associated with higher LOQs of the chemicals (> 5 ng/g) due to matrix effects (e.g. ion suppression). This was illustrated by the lower absolute recoveries in the biota samples in comparison to the sediment (LOQ between 0.5 and 1 ng/g d.w sediment) (Table S7). On the other hand, a few detections of azoles in soils are attributed to lower level of contamination as LOQs are in the same range for sediments and soils. Note, that even if there is an overlap between LOQ and concentration ranges in sediments, soils and biota, the values reported in Fig. 4 are only those above the LOQs. In addition, this overlap is associated with very few chemicals with high LOQ (> 5 ng/g d.w., e.g. clotrimazole, ketoconazole, fluconazole, prothioconazole) (Table S7). Nevertheless, such observation highlights that the concentration level in these compartments (biota, sediment and soil) are low and that further method development might be required to reduce the matrix effect.

In general, the concentrations of antifungal azoles measured in the present study (Fig. 4) were in the same range as those reported in the effluents (up to 1000 ng/L), surface waters (up to 100 ng/L), sediments (up to 10 ng/g d.w), soil (up to 100 ng/g), groundwater (up to 10 ng/L), and biota (up to 10 ng/g), but data in the latter compartments are scarce (Chen and Ying, 2015; Peng, 2012; Kahle, 2008; Moschet, 2014; Peng, 2014; Huang, 2010). Although both pesticidal and pharmaceutical azoles occurred in the same concentration ranges in each compartment (Fig. 4), there were large differences observed between the compartments: those with very high concentration (urban effluent, soils) and those with very low concentration (biota, sediment, groundwater) levels. This observation agrees with their associated uses in medicine and agriculture as well as their frequency of detection (Fig. 4). For instance, pharmaceutical azoles (i.e. climbazole, fluconazole and ketoconazole) occurred at higher concentrations (up to 100 ng/L) than pesticidal azoles (e.g. cyproconazole, epoxiconazole, tebuconazole) (1–10 ng/L) in almost all urban effluents (Fig. S6). In agricultural soils, 9 pesticides were at higher concentrations (up to 100 ng/g) than the single pharmaceutical (i.e. fluconazole) (< 1 ng/g) (Fig. S4). Similarly, the pharmaceutical climbazole was detected at high concentrations in surface water at urban sites (median at 5 ng/L against 0.5 ng/L in agricultural sites) whereas 12 pesticides occurred at higher concentration in small streams under agricultural pressure (Fig. S8). In addition, some azoles are present at higher level at specific seasons likely due to their timing of use (Spycher, 2018). This is the case of tebuconazole which occurred at high concentrations in March and propiconazole in May in surface water (Fig. S11). Altogether, these

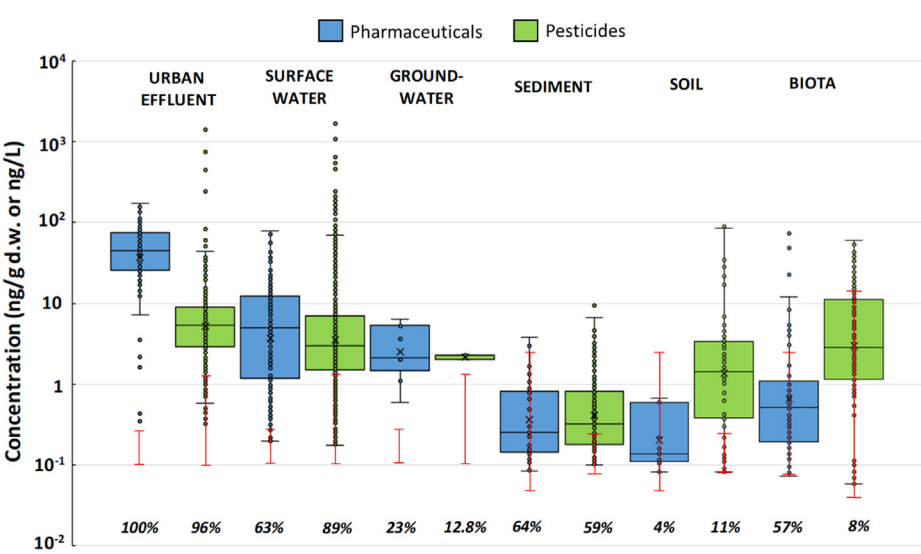

Fig. 4. Level of environmental concentrations and detection frequency of pharmaceutical and pesticidal antifungal azoles in wastewater effluent (n = 46), surface water (n = 154), groundwater (n = 33) sediment (n = 67), soil (n = 15) and biota (biofilms, gammarids, fish) samples (n = 114) in Switzerland. Results are expressed in ng/g d.w. (sediment, soil), ng/g w.w. (biota) or ng/L (urban effluent, surface water, groundwater). The range of LOQs in each compartment is shown in red as detailed in Table S7. The detection frequency in each compartment is expressed as percentage below the graph.

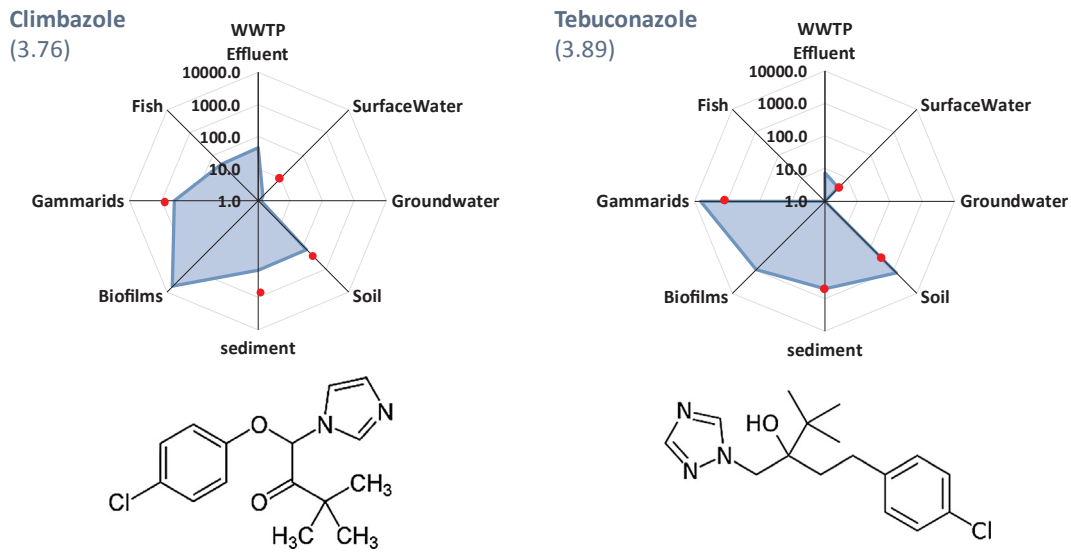

Fig. 5. Distribution of two selected antifungal azoles between environmental compartments. Results are expressed in ng/L (WWTP, surface water or groundwater) or in ng/kg d.w. (sediment, soil) and ng/kg w.w. (biota). Red dots represent the values predicted by the fugacity model. The number in () is the log Kow of each chemical.

results indicated that both pharmaceutical and agricultural practices contribute to the ubiquitous contamination of azoles in Swiss ecosystems, even at reference sites. In fact, some azoles (i.e. climbazole, difenoconazole and propiconazole) have been detected in the surface water at the reference site Goldach (data not shown), however at lower concentrations (ng/L range) than at most of the investigated sites. Overall, our data highlight the need to investigate several environmental compartments since some chemicals can be present in only one compartment and others are spread across several compartments.

3.3. The partitioning of antifungal azoles between surface water, sediment and biota is an interplay of various parameters

Based on the occurrence data, we compared the distribution of antifungal azoles among the compartments with the predicted values by the fugacity model level I. For biota, the distribution of all azoles was predicted in gammarids because relatively large amounts of data exist in the literature for this organism. As for the distribution in biofilms and fish, the prediction was done only for azoles actually detected in these matrices in the present study.

As shown in Fig. 5, measured *in situ* distributions of climbazole and tebuconazole are in agreement with those predicted by the fugacity model, although the model slightly overpredicted climbazole concentration in soil (1.9 fold), sediment (4 fold) and biota (2.5 fold). This is also the case for flusilazole, cyproconazole and epoxiconazole (1.5–2 fold change in biota), although the fugacity model predicted the occurrence of cyproconazole and epoxiconazole in gammarids whereas they were only detected in the biofilms (Fig. S12). Similarly, only small differences (1.5–3 fold) were noted for difenoconazole, propiconazole and metconazole in abiotic compartments while the predicted concentrations in biota were below the actual LOQ (i.e. non-detects). Additional discrepancies were observed for other azoles (Fig. S12). For instance, the model strongly underestimated the concentration of fluconazole in sediment (120 fold), soils (114 fold) and surface water (12 fold). On the other hand, the model strongly overestimated the concentration of fenamidone and prochloraz in the different compartments (10–20 fold).

Overall, azoles were predicted and observed more in the solid (sediment, soil, biota) than in the aqueous compartments (effluents, surface waters, groundwaters), with concentrations in solid matrices to be ~ 10 to 200 times higher than in water (Fig. S12). This was specifically observed at sites where both solids and waters have been sampled, albeit not at the same time (data not shown). Such partitioning can be partly explained by the log Kow since 24 of the 26 detected azoles have a log Kow value higher than 3 (Table S15). For instance, climbazole (log Kow of 3.76) is 100 fold more concentrated in sediment (1000 ng/kg d.w.) than in the surface water (10 ng/L) while tebuconazole (log Kow of 3.89) is only 10 fold more concentrated in sediment than in the water (Fig. 5). However, fluconazole with a log Kow of 0.25 and triflumizole with the log Kow of 1.5 were also more abundant in sediment and soil than in the surface water suggesting that other parameters than hydrophobicity might be involved in the partitioning (Fig. S12). This was confirmed through the calculation of the concentration in the pore water (in the 1–100 ng/L range) by using the equilibrium partitioning model that demonstrated that most of the detected azoles in the sediment are sorbed to the particulate phase (Table S14).

For all the azoles, differences in persistence (DT_{50}) in sediment and soils compared to water could also contribute to such discrepancies in the mass balance between water and solid phases (Table S15). In fact, the least persistent azoles were also the least detected (i.e., bitertanol, imazalil, fenbuconazole, fuberidazole, prothioconazole) despite the high usage of some of them (prothioconazole). The most persistent and the highly used were the most detected (i.e., climbazole, cyproconazole, difenoconazole, epoxiconazole, flusilazole, metconazole, myclobutanil, penconazole, propiconazole, tebuconazole). Furthermore, compounds with low stability in water and high persistence in solid matrices are more abundant in solid matrices than in water (flusilazole, myclobutanil and penconazole, Table S15). In general, the overall environmental persistence predicted by the EPI suite model is in accordance with the experimental DT_{50} values provided in the literature (PPDB, University of Hertfordshire, 2020) (Table S15). Nevertheless, there are some exceptions such as bromuconazole and paclobutrazole that are described as persistent (i.e. high DT_{50}) but were not detected in the investigated compartments, even though paclobutrazol has a high usage rate in Switzerland (Table S15). Since the use of paclobutrazol includes root treatment, this compound may be slowly released into aquatic ecosystems (i.e. only through leaching from soil to groundwater).

As for the bioaccumulation, all the predicted values from the fugacity model or the EPI suite values were similar or slightly lower (1.4 fold lower) than all the apparent field log BAF values in gammarids,

except for climbazole (1.2 fold higher). These apparent values were also in good agreement (only 1.2–1.5 fold higher) with those reported in the literature from laboratory experiments (Munz et al., 2018) (Table S16). Similar trends were also noted for climbazole, flusilazole and myclobutanil in fish. These slight differences can be explained by accumulation of the chemicals in the exoskeleton of gammarids or skin of fish, which is not considered in both the EPI-suite calculation and the fugacity model. Moreover, some of the antifungal azoles were found at least in 2 trophic levels (i.e. biofilms, gammarids or fish) such as climbazole, flusilazole, epoxiconazole, myclobutanil and tebuconazole. These chemicals have log Kow values of approximately 3.5 (Table S15) and their hydrophobicity could explain their bioaccumulation in biological tissues. The differences found in the field among the trophic levels may be due to the different mechanisms of uptake, elimination and biotransformation in different organisms. This is in agreement with recent findings highlighting that biotransformation of antifungal azoles differs among chemicals (Rösch et al., 2016) and species (Creusot et al. in prep). Such toxicokinetic processes should therefore be investigated further to better understand the measured differences among trophic levels.

Altogether, our results showed that no accurate prediction of the partitioning of azoles can be drawn from the level I fugacity model although this model seems to be a suitable first step in estimating the distribution in aquatic compartments. More complex model (i.e. Level 2 taking into account continuous emissions and transformation; Level 3 taking into account active transport and compartment-specific emission) requiring additional emission and inflow information and/or not freely available model such as RAIDAR (Risk Assessment IDentification and Ranking) may provide better prediction (Arnot, 2006). Overall, the observed discrepancies may reflect an incorrect representation of biotransformation and/or persistence by the models and the interplay of several parameters (e.g. use, persistence, hydrophobicity) or simply that there is no equilibrium between the compartments because of the continuous release of contaminants. As a consequence, digital sample archives as recently proposed by Alygisakis et al. (Alygizakis, 2019) and employed here are useful to providing a more realistic picture of the partitioning of pollutants in the different environmental compartments.

3.4. Antifungal azoles may pose a risk to aquatic ecosystems and humans

In the ERA framework used for substance registration and premarketing authorization, exposure is often estimated using PECs rather than MECs. In this study, MECs of all detected azoles in sediments were below the median PEC values from various modeled FOCUS (FOrum for Co-ordination of pesticide fate models and their Use) scenarios. However, the MECs of difenoconazole, epoxyconazole, metconazole, myclobutanil, propiconazole and tebuconazole in surface water were higher than those predicted by FOCUS (Table S9 from EFSA dossiers, Figs. S5 and S7). Usually, PEC modelling is protective but, in this case, the current risk associated to these contaminants in surface water is underestimated.

Based on the quality standards (QS) available in the literature (Moschet, 2014; INERIS, 2019) and those calculated in this study, we evaluated the risk (ΣRQ) associated with the mixtures of antifungal azoles in all the samples and their respective sites. (Fig. 6, Fig. S13). Based on the $QS_{fw,\ eco}$ (fresh surface water for aquatic organisms), the majority of the samples reflected very good to good water quality, and only very few were in moderate to poor quality (1%). Similarly, based on the comparison with sediment quality standards ($QS_{sed,\ EqP}$), most of the samples were in good to very good quality whereas only one exhibited moderate quality. Finally, when ΣRQ were calculated by using the $QS_{dw,\ hh}$ (fresh surface water for human health via drinking water), surface waters were more at risk with 9% and 5% in moderate and poor quality, respectively ($2 < \Sigma RQ < 10$). It was observed that 16 of the 22 of these poor quality samples represented the worst case scenario since they were sampled in small streams under agricultural pressure (currently not used as a drinking water source, Fig. S13). We have mapped the environmental risk for each site by taking the highest ΣRQ values among all the samples available for sediment or surface water compartments. This showed that 6 sites with poor quality for drinking water were distributed in both urban and arable land use areas and also showed moderate quality for surface water and sediment. Note, that there was a higher risk for humans via drinking water consumption than for aquatic ecosystems since the $QS_{dw,\ hh}$ values employed for the ΣRQ calculation were proposed by the European Commission (98/83/EC) (0.1 μg/L) based on the precautionary principle, but not on effect data.

Overall, the risk associated with azoles was mainly driven by tebuconazole and propiconazole, azoles that were most frequently detected at high concentrations. Nevertheless, it should be noted that our risk assessment suffered from some limitations, leading to an inaccurate depiction of risks of exposure in humans and aquatic ecosystems. In particular, our assessment employed very little ecotoxicological data on azoles since they were missing or restricted to only few species. In addition, no pharmaceutical azoles were included in our RQ calculation because no (eco)toxicity data were available for such chemicals to derive QS values. However, some that were frequently detected at high concentrations (climbazole and fluconazole) might increase the risk. In fact, by using PNEC values from the Norman database (Substance Database, 2020) as QS values (i.e. 0.59 μg/L for climbazole, 1.04 μg/L for fluconazole and 0.0081 μg/L for ketoconazole), the number of samples that have poor quality for drinking water increased from 22 to 30, and 6 samples were considered at poor quality instead of moderate quality for fresh surface water (data not shown). Similarly, transformation products and metabolites, except for prothioconazole-desthio, were also not included in the risk calculation although our data have highlighted their potential occurrence (e.g. prochoraz BTS 44495). Also, $QS_{fw,\ eco}$ values were calculated using the AF methodology while $QS_{sed,\ EqP}$ values were derivated from $QS_{fw,\ eco}$ because of the lack of ecotoxicological data and additional effect data could provide a more accurate picture of the risk. Finally, QS values are generally calculated based on chronic or acute (eco)toxicity tests endpoints at the organism level. It is now well established that effects at lower level of biological organization (i.e. molecular, cellular, e.g. enzymatic activity) could be stronger (Ankley, 2010). This is of particular relevance for antifungal azoles since these chemicals are described as endocrine disruptors and are capable of altering the biosynthesis and the metabolism of endogenous hormones (Matthiessen and Weltje, 2015). Such effects are only partially or not incorporated at all in the QS derivation. In addition, risk calculation is based on the annual average concentration whereas exposure at specific stages during the life cycle can be critical for organism development, in particular for endocrine disrupting agents (WHO, 2012). For instance, some pesticides are applied during specific months and could coincide with the sensitive stage in organisms. This highlights the need to define the exposure at better temporal resolution. To this end, continuous online monitoring of surface water (e.g. Eawag MS2 field project, 2019 appears to be a promising approach.

Altogether, our results showed that conclusions about risk assessment can differ depending on the environmental compartments explored and the corresponding QS values used to calculate the ΣRQ. Nevertheless, even if the risk associated with antifungal azoles seems to be limited in Switzerland for both human and aquatic ecosystems, the fact that some sites appeared at risk raised the question of adverse impacts in countries where the use of these chemicals is higher (Nations, 2020) or the dilution in the surface water is lower.

4. Conclusions

In the present study, the retrospective screening of HRMS/MS data offered the opportunity to investigate for the first time the environmental occurrence of antifungal azoles at numerous sites from different land uses and their distribution in various environmental

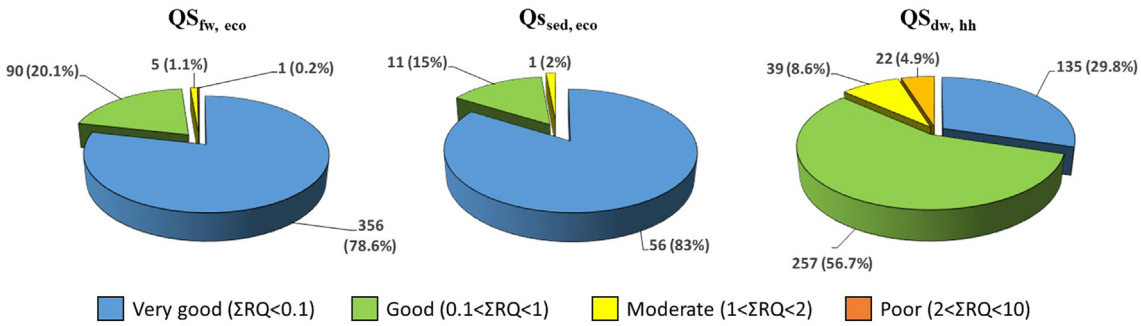

Fig. 6. Chemical quality of water, sediment and drinking water samples based on sum of RQ of azoles calculated from $QS_{fw,\ eco}$ (fresh surface water for aquatic organisms), $QS_{sed,\ EqP}$ (sediment quality for aquatic organisms) and $QS_{dw,\ hh}$ (fresh surface water for human health via drinking water).

compartments. Although the data were limited in terms of partitioning (i.e. no sites with occurrence data in all the compartments at the same sampling time) and seasonality (i.e. only some sites with occurrence data in surface water and sediment at several months along the year), they allowed us to define the exposure more accurately, and hence the current risk associated with antifungal azoles exposures in Switzerland. This was done for aquatic species and also for humans via drinking water risk calculations. However, our approach suffered from some limitations such as the absence of pharmaceuticals in the ΣRQ determination due to the unavailability of effect data. Altogether, this study demonstrated the usefulness of HRMS/MS data-based retrospective analysis to investigate the exposure and the associated risks to emerging or still unknown (e.g. transformation products) contaminants. In particular, because fugacity model might not reflect the actual biotransformation and/or persistence of these chemicals, this study highlighted the need to build HRMS/MS-based digital samples archives for such retrospective investigation. This could in part be addressed by an improvement of freely available software for complex DIA datasets and/or the development of shared MS^2 libraries.

CRediT authorship contribution statement

Nicolas Creusot: Conceptualization, Methodology, Formal analysis, Writing - original draft, Visualization, Project administration, Funding acquisition. **Carmen Casado-Martinez:** Resources, Writing - review & editing, Funding acquisition. **Aurea Chiaia-Hernandez:** Resources, Writing - review & editing, Funding acquisition. **Karin Kiefer:** Resources, Writing - review & editing. **Benoit J.D. Ferrari:** Resources, Writing - review & editing, Funding acquisition. **Qiuguo Fu:** Resources, Writing - review & editing. **Nicole Munz:** Resources, Writing - review & editing. **Christian Stamm:** Resources, Writing - review & editing, Funding acquisition. **Ahmed Tlili:** Resources, Writing - review & editing, Funding acquisition. **Juliane Hollender:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105708.

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