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Survey of Antifungal in Surface- and Groundwater: A Portuguese Environmental Case Study

Cristina De Mello-Sampayo ^{1,*}, Paula Viana ², Ana Lopes ², Rita Carvalho da Silva ^{3,4}, Rosário de Jesus ², Georgina Sarmiento ⁵, Anabela Almeida ^{6,7}  and Leonor Meisel ^{8,*} 

- ¹ Laboratory of Neuroinflammation, Signaling and Neuroregeneration, Research Institute for Medicines (iMed.Ulisboa), Faculty of Pharmacy, Universidade de Lisboa, 1649-003 Lisbon, Portugal
 - ² Portuguese Environmental Agency (APA), Rua da Murgueira, 9, Zambujal, Alfragide, 2610-124 Amadora, Portugal; paula.viana@apambiente.pt (P.V.); ana.rita@apambiente.pt (A.L.); rosarioj@apambiente.pt (R.d.J.)
 - ³ Research Institute for Medicines (iMed.Ulisboa), Faculty of Pharmacy, Universidade de Lisboa, 1649-003 Lisbon, Portugal; carvalho.da.silva.rp@gmail.com
 - ⁴ Biosafety Unit, Gulbenkian Institute of Science, 2780-156 Oeiras, Portugal
 - ⁵ Analysis Laboratory, Instituto Superior Técnico, University of Lisbon, Av. Rovisco Pais, 1049-001 Lisboa, Portugal; gsarmiento@tecnico.ulisboa.pt
 - ⁶ Vasco da Gama Research Centre (CIVG), Departamento de Ciências Veterinárias, Escola Universitária Vasco da Gama (EUVG), Campus Universitário de Lordemão, Av. José R. Sousa Fernandes 197, 3020-210 Coimbra, Portugal; almeida.anabela@gmail.com
 - ⁷ Coimbra Institute for Biomedical Imaging and Translational Research (CIBIT), University of Coimbra, 3000-548 Coimbra, Portugal
 - ⁸ Laboratory of Systems Integration Pharmacology, Clinical and Regulatory Science, Research Institute for Medicines (iMed.Ulisboa), Faculty of Pharmacy, Universidade de Lisboa, 1600-277 Lisbon, Portugal
- * Correspondence: csampayo@ff.ulisboa.pt (C.D.M.-S.); mmeisel@ff.ulisboa.pt (L.M.)



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Abstract: Antifungals support modern medical and agricultural practices, and extensive screening environmental data are missing. This work aims to survey antifungals for medical and agricultural purposes in surface- and groundwater in Portugal. A passive sampling technique and a high-resolution chromatographic system were used. Antifungals applied in medical practice were not detected as only antifungals for agricultural purposes (fungicides). Thirty-nine different fungicide molecules were found; its detection frequency in surface- and groundwater was dominated by rabenzazole (61%, 92%) and ethoxyquin (54%, 77%); among the five fungicides with similar surface- and groundwater catching rates, ferimzone was the most polluting (54%, 54%); oxadixyl (61%), kresoxim-methyl (61%) and fenamidone (46%) were primarily designated surface water contaminants; for azoles, the occurrence in surface water ranged from a residual (10%) to a moderate detection rate for two compounds (31%—propiconazole and tebuconazole). Surprisingly, only 51% of detected fungicides are authorised. The frequently detected fungicides that are either not authorised (ethoxyquin and its impurity, furmecyclox, oxadixyl), without data in the EU (rabenzazole, ferimzone), or authorised at the national level (fenamidone), should be included in environmental monitoring programmes and followed as emerging (micro)contaminants. Policy makers should gather their efforts to allow the implementation of proper risk management and effective contamination control strategies to achieve Sustainable Development Goals.

Keywords: antifungals; fungicides; surface-groundwater; dodemorph; ethoxyquin; fenamidone; ferimzone; furmecyclox; kresoxim-methyl; oxadixyl; rabenzazole

1. Introduction

Antifungals are antimicrobial substances that selectively eliminate or prevent topical and life-threatening Invasive Fungal Diseases (IFDs). They support modern medical practice and extensive/intensive agriculture, showing a fungicidal or fungistatic mode of

action. Those used in the environment, broadly applied in the agricultural field as well as in timber and paints industries, are designated fungicides [1,2]; this is incorrect terminology since the fungicidal mode of action in phytopathogens may not always happen; azoles are fungistatic rather than fungicidal [2].

Regarding their usage, global trends of a significant increase were found in agriculture, linked to medical practice. Portugal disclosed values in 2019 of about 2000 tones (organic antifungals) for crops and 2.4 tones as pharmaceuticals [3–5]. Likewise, more fungal diseases are now being encountered in a broad variety of human, animal, and plant hosts. The reason for the spread of these infections has been debated, questioning whether climate change could also increase the risk of diseases [1,6,7]. The prevalence of such diseases in humans varies significantly across different countries. Moreover, the number of drug-resistant pathogenic fungal strains in humans has increased due to enhanced usage of prophylactic, empirical and directed therapies [8]. The emergence of drug-resistant fungi in the clinic and agriculture is associated with expanding patient populations at risk of IFDs, including older people, people with compromised immune systems by HIV, cancer chemotherapy or transplant-requiring immune suppression therapy, and those with severe influenza or SARS-CoV-2 virus infections [1]. This drug-resistant fungi emergence has prompted the Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) consortium to update, in April 2021, and the Strategic Research and Innovation Agenda on Antimicrobial Resistance [9] with the inclusion of antifungal resistance. More recently, in 2022, WHO systematically prioritised fungal pathogens and published a fungal priority pathogens list (WHO FPP) [10].

Excessive and routine use of antifungals can cause environmental impacts such as ecological imbalance; their presence in aquatic systems can be toxic to non-target organisms, acting on fundamental biological processes that are not specific to fungi. These drugs reach environmental water bodies through discharge from wastewater treatment plants following domestic use or, when applied to land fields, are transferred to the surface- groundwater via runoff and leaching. Thus, antifungals used in agriculture can also serve as direct environmental drivers for developing drug-resistant fungal strains against all major classes of fungicides, including benzimidazoles, anilinyrimidines, strobilurins, Succinate Dehydrogenase Inhibitors (SDHI) and the sterol biosynthesis inhibitors including the Demethylation Inhibitors (DMIs), azoles [1]. Some environments, so-called ecological ‘hotspots’ [1], such as home and industrial composters, urban environments, and greenhouses, permit the growth of fungus in contact with sub-minimal inhibitory concentrations (MICs) of agricultural DMIs and, thereby, generate conditions that are suitable for fungi to evolve azole resistance in response to drug pressure. Azoles are widely used in both areas (medicine and agriculture) because they have a broad spectrum of antifungal activity. In recent years, azole resistance in human pathogens like *Aspergillus* fungus and several *Candida* yeasts appears to have increased and can severely limit treatment. It has been suggested that this fungi azole resistance has evolved in the environment. Although increasing evidence supports this hypothesis, the connection between azole antifungals’ environmental use and azole-resistance development in *Aspergillus* spp. is not yet proven [2,11–13].

Hence, the widespread use of antifungals for agricultural and non-agricultural purposes has resulted in their residues in surface- and groundwater resources being considered emerging water bodies’ (micro)contaminants that raise concerns [14]. The analytical detection of residues in water samples has been possible due to passive sampling methodology but also due to high sensitivity methods with improved resolution, such as ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UHPLC-QqTOF-MS) [15]. In Europe, the Water Framework Directive [16] and the Directive on Environmental Quality Standards [17] provide strategies against water pollution.

To evaluate and monitor the presence of emerging contaminants in surface water, the guidance document [18] recommends using a Polar Organic Chemical Integrative Sampler (POCIS), a methodology based on the passive diffusion of chemicals and their metabolites from the aquatic environment. This integrative passive sampler, by allowing the pre-

concentration of contaminants, increases the ability to detect trace concentrations [15,19]. In Portugal, most occurrence studies have focused on some locally remarkable antifungals; actual data still need to be explored. Antifungals' environmental fate and effects have received far less attention than antibiotics, insecticides, and herbicides. There is evidence of the possible environmental impact of antifungals on surface water ecosystems. However, monitoring data are still lacking, making it difficult to determine their EQS values; therefore, they are not yet included in the EU Priority Lists but are inserted in the third and fourth watch lists of substances [20].

This work aims to qualitatively survey antifungals for agricultural and non-agricultural purposes in surface- and groundwater. It seeks to reflect the current spatial compound profile from North to South of Portugal's mainland over an observational period of three years and four years in surface- and groundwater, respectively. The current approach will support future quantitative occurrence studies of previously detected substances for policy guidance and the implementation of risk minimisation strategies to accomplish the Sustainable Development Goals (SDGs) set by the United Nations.

2. Materials and Methods

2.1. Sampling Stations and Characterisation

The sampling campaign was performed as described in Viana et al., 2021 [21], with slight modification. Briefly, thirteen stations, submitted to diverse environmental pressures, were established for surface water collection, comprising all river districts from North to South of Portugal during 2017–2019. They were placed in the main river basins, tributaries, reservoirs, and transitional and coastal waters, for groundwater monitoring, which started one year earlier (2016 to 2019) and involved ten wells and three piezometers, matching thirteen sampling stations. The period picked for the passive sample deployment on the surface water or groundwater occurred during late spring (2016–2018) and late summer (2019). These seasons were selected because high river flow may compromise POCIS compliance. They were located near animal production or agriculture landscapes, some dominated by rice fields and orchard zones. The sampling stations are shown in Figure 1a,b.

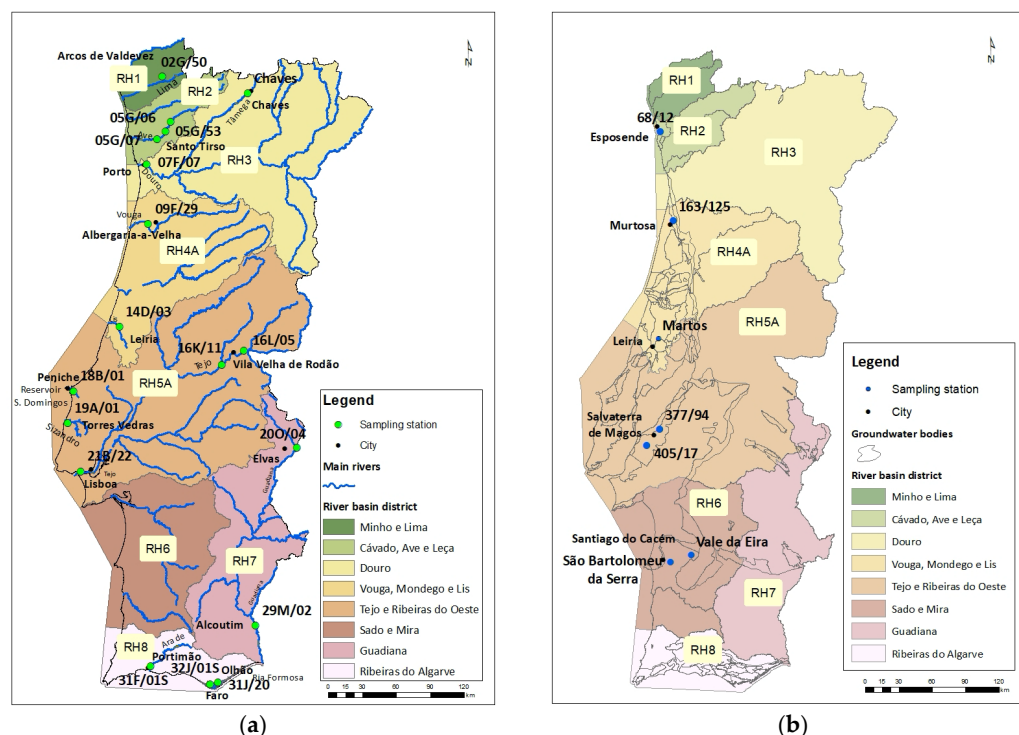


Figure 1. (a) Surface water sampling stations (adapted from Viana et al., 2021 [21]); (b) groundwater sampling stations. Maps provided by the Portuguese Environmental Agency. RH: River basin district.

2.2. Passive Sampler Field Deployment

The sampling of antifungal compounds in surface- and groundwater was done using the passive sampler POCIS. The POCIS used in this allowed the qualitative detection of several organic molecules in the dissolved phase, including antifungals. As stated in Viana et al., 2021 [21], in surface water, two individual POCIS units were held inside a canister placed 50 cm deep into the water column during the seasons of reduced river flow rate. In contrast, one POCIS unit/well and one unit/piezometer were used in groundwater. During the field study, a water level meter was used to measure the depth of 2 m below the groundwater plane. The field deployment lasted for 30 days, which was sufficient to achieve equilibrium between the adsorbed polar organic compounds and active substances in the aqueous medium. The POCIS disks were frozen until extraction in the laboratory.

2.3. Qualitative Analysis of Antifungals in Surface-Groundwater and Data Analysis

The qualitative analysis of antifungals comprised extracting the adsorbent of POCIS disks followed by qualitative determination using UHPLC-QqTOF-MS, finalising with data analysis. Sample extraction and chromatographic separation were performed as previously described by Viana and colleagues [21], employing the same chemicals and reagents detailed in Supplemental Document S6. The obtained mass spectrometry data were processed via the Data Analysis 4.4 and Target Analysis 1.3 software (Bruker Daltonics, Billerica, MA, USA) as well as using the in-house mass library built for 49 standards (Sigma-Aldrich, St. Louis, MO, USA) plus 620 suspected substances (fungicides, insecticides, herbicides, pharmaceuticals, metabolites). Antifungal compounds were identified using mass libraries for accurate mass screening. The qualitative methodology used in this study allowed the detection of compounds at concentrations as low as 50 ng/L in complex matrices.

2.4. Physicochemical Properties and Key Environmental Fate Attributes of Detected Antifungal

A review targeted the physicochemical properties, mode of action, regulatory status in the EU and some environmental endpoints (e.g., general biodegradability, soil mobility and GUS leaching potential index) of fungicides to interpret and discuss the emergence of antifungals in surface water versus groundwater. The main characteristics were summed up in Tables 1 and S1.

Table 1. Main environmental fate parameters, detection frequency and regulatory status of all detected fungicides.

Active Substance	Water Solub. (20 °C) ¹	Log K _{ow} ¹	Log K _{oc} ¹	DT50 Soil (Days) ¹	DT50 Water/Water-Sediment (Days) ¹	GUS Leaching Index ¹	Detection (Freq. %)	Status during the Survey Period in EU ²
Benalaxyl	Low	3.54	3.70	36.0–66.8	38.0/168.2	2.29	S (23), G (23)	Approved
Metalaxyl	High	1.65 ³	2.20	14.1	106.0/56.0	2.06	S (31), G (31)	Approved
Pyracarbolid	High	2.22	2.18	NA	NA	NA	G (15)	Not Approv.
Methfuroxam	Low	3.16	2.26 ⁴	NA	NA	NA	G (23)	Not Approv.
Mepronil	Low	3.66	2.98	46.0–50.5 ⁵	276.0/NA	1.72	G (23)	Not Approv.
Dimethomorph	Low	2.68	3.80 ⁶	8.6–44.0	10.0/38.0	2.26	S (15)	Approved
Furmecyclox	Moderate	4.38	2.80 ⁴	NA	NA	NA	S (38), G (38)	Not Approv.
Oxycarboxin ^{**}	High	0.77	1.98 ⁴	42.3	9.8/1000.0	NA	G (10)	Not Approv.
Cyflufenamid ^{**}	Low	4.70	3.02 ⁷	25.3	4.3/77.2	1.12	G (10)	Approved
Cycloheximide ^{**}	High	0.55	1.70	NA	NA	NA	G (10)	Not Approv.
Imazalil [*]	Moderate	2.56	NA	6.4	7.8/117.0	0.26	S (10)	Approved
Flusilazole ^{**}	Low	3.90	3.20	94.0	1.0/365.0	1.54	G (10)	Not Approv.
Propiconazole	Moderate	3.72	3.00	35.2	6.0/561.0	1.58	S (31)	Approved ⁸
Tebuconazole ^{**}	Low	3.70	2.85 ⁴	47.1	42.6/365.0	1.86	S (31), G (10)	Approved
Tetraconazole [*]	Moderate	3.56	NA	430.0	2.0/340.0	2.47	S (10)	Approved
Tricyclazole [*]	High	1.40	2.20	130.0	93.0/453.0	3.89	S (10)	Not Approv.
Benomyl [*]	Low	1.40	3.30	67.0	NA	−0.07	S (10)	Not Approv.
Carbendazim ^{**}	Low	1.48	2.35 ⁹	22.0–40.0	7.9/33.7	2.21	S (15), G (10)	Approved ¹⁰

Table 1. Cont.

Active Substance	Water Solub. (20 °C) ¹	Log Kow ¹	Log Koc ¹	DT50 Soil (Days) ¹	DT50 Water/Water-Sediment (Days) ¹	GUS Leaching Index ¹	Detection (Freq. %) ¹	Status during the Survey Period in EU ²
Rabenzazole	Moderate to High	2.62	3.31 ⁴	NA	NA	NA	S (61), G (92)	Not Approv.
Thiabendazole *	Low	2.39	3.60 ⁴	724.0	1.6/4.0	1.94	S (10)	Approved
Probenazole	Moderate	1.40	2.45 ⁴	1.7 ¹¹	NA	NA	G (15)	Not Approv.
Propamocarb	High	0.84	2.86	14.0–30.0	NA	NA	S (15)	Approved
Diethofencarb	Low	2.89	2.42 ⁴	5.4	9.8/24.9	1.09	G (15)	Not Approv.
Isoprothiolane	Moderate	3.30	3.13	NA	NA	NA	S (31)	Not Approv.
Fenamidone	Low	2.80	2.59 ¹²	8.1–15.4	24.0/97.0	1.28	S (46), G (15)	Approved ¹³
Dodemorph	Moderate	4.60	3.22 ⁴	41.0	1.3/23.0	−0.65	S (46), G (46)	Approved
Fenpropimorph *	Low	4.50	3.64 ¹⁴	25.5	2.65/38.0	0.50	S (10)	Not Approv.
Iprobenfos **	High	3.37	3.70	15.0	NA	0.35	S (23), G (10)	Not Approv.
Famoxadone	Low	4.65	3.58	1.6–20.0	0.1/0.7	1.09	G (15)	Approved
Oxadixyl	High	0.65	1.56	75.0	25.0/21.0	4.58	S (61), G (15)	Not Approv.
Fludioxonil *	Low	4.12	5.20	16.0	2.0/575.0	−1.35	S (10)	Approved
Ferimzone	Moderate	2.98	2.57 ⁴	3.0–14.0 ¹⁵	NA	NA	S (54), G (54)	Not Approv.
Pyroquilon *	High	1.57	2.58 ⁴	70.0	NA	NA	S (10)	Not Approv.
Ethoxyquin	Moderate	3.39	3.51	2.2 ¹⁶	NA	6.70 ¹⁷	S (54), G (77)	Not Approv.
Spiroxamine	Moderate	2.89	2.82–3.81 ¹⁸	52.4	0.8/66.2	−0.28	S (15), G (15)	Approved
Azoxystrobin **	Low	2.50	2.77	180.7–248.0	6.1/205.0	3.10	S (31), G (10)	Approved
Picoxystrobin **	Low	3.69 ⁴	2.88–3.08 ¹⁹	19.3	7.5/56.0	1.35	G (10)	Approved ²⁰
Kresoxim-Methyl **	Low	3.40	2.34–2.57 ²¹	1.0–3.0 ²¹	0.85/1.30	0	S (61), G (10)	Approved
Trifloxystrobin **	Low	4.50	3.36 ²²	1.7	1.1/2.40	0.15	G (10)	Approved

¹ Some data retrieved from PPDB: Pesticide Properties Database [22]; water solubility: low <10 mg/L, moderate >10 and <1000 mg/L, and high >1000 mg/L. ² Data retrieved from Substance Information—ECHA—European Union [23]. ³ Data retrieved from [24]. ⁴ Data retrieved from [25]. ⁵ Data retrieved from [26]. ⁶ Data retrieved from [27]. ⁷ Data retrieved from [28]. ⁸ Non-renewal of approval in Reg (EU) 2018/1865 of 28 November 2018 [29]. ⁹ Data retrieved from [30]. ¹⁰ Reg (EU) 2015/408 indicated as appropriate to include in the candidate list for substitution [29]. ¹¹ Data retrieved from [31]. ¹² Data retrieved from [32]. ¹³ Since Reg. (EU) 2018/1043 is only authorised at the national level in Portugal [29]. ¹⁴ Data retrieved from [33]. ¹⁵ Data retrieved from [34]. ¹⁶ Data retrieved from [35]. ¹⁷ Data retrieved from [36]. ¹⁸ Data retrieved from [37]. ¹⁹ Data retrieved from [38]. ²⁰ Non-renewal of approval—Reg. (EU) 2017/1455 of 10 August 2017 [29]. ²¹ Data retrieved from [39]. ²² Data retrieved from [40]. Solub. = solubility; Freq. = frequency; S = surface water; G = groundwater; * fungicide detected once in surface water; ** fungicide detected once in groundwater; and NA = Not available.

3. Results

This qualitative survey revealed thirty-nine systemic active antifungals used in agriculture, of which twenty (51%, 20/39) were “approved for use” concerning placing plant protection products on the market in the EU—status from 2016 to 2019 under Regulation N° 1107/2009 (Table 1). Antifungals used in medical practice were not found. The detected compounds were classified into chemical and sub-groups (Table S1). Thus, according to the fungicides’ chemical group, amide, azole, benzimidazole, morpholine, carbamate, and strobilurin comprised more than one detected substance. The amide group encompasses nine compounds, followed by azole (five substances), the benzimidazole and strobilurin group with four each and morpholine and carbamate with two. The remaining chemical groups were represented only by one antifungal; metabolites were not found. Five identified compounds are on the EU WFD Watch List: azoxystrobin, famoxadone, imazalil, tebuconazole and tetraconazole.

Moreover, among all the detected fungicides, eight showed a 10% detection frequency in surface water, while eleven appeared under the same frequency in groundwater (Table 1). Thus, given the relatively low detection rate, these compounds were removed from the geographic distribution tables (Supplemental Tables S4 and S5); however, they were included in Tables 1 and S1 and discussed whenever they represented an added value for the current study.

3.1. Fungicides Detected in Surface Water

Figure 1a shows the location of the 13 surface water sampling stations evenly distributed throughout Portugal, while Supplemental Tables S2 and S4 describe the characteristics of sampling sites and the list of fungicides detected in surface water.

The number of detections per sampling site ranged from 2 to 13 active substances, as depicted in Supplemental Table S4. Eighty-nine (89) detected fungicides were found in all surface water catchment areas, corresponding to nineteen (19) different compounds. Regarding fungicide contamination, the less contaminated samples were taken in rural regions (Tejo River near the border, stations 7 and 8) or areas where animal production stands out from agricultural production (Caia River—station 10). A high assortment of fungicides was detected in surface water samples from Ave River (station 2), Tejo River estuary (station 9) and Ria Formosa—Olhão (station 11), containing 11, 13 and 10 different fungicides, respectively. Excluding the S. Domingos reservoir (station 6) with potential agricultural pressure, the other sampling sites comprised an urban/agricultural profile. Despite strong urban pressure, the samples collected in the Tinto River (station 4), showed a mix of six distinct agrochemicals.

In addition, the detection rate of each fungicide in surface water was evaluated (Figure 2). The most frequently found fungicides, in decreasing order, were kresoxim-methyl, oxadixyl and rabenzazole, 61% (8/13); ethoxyquin, ferimzone, 54% (7/13); dodemorph, fenamidone, 46% (6/13); furmecyclox, 38% (5/13); azoxystrobin, isoprothiolane, metalaxyl, propiconazole and tebuconazole, 31% (4/13); benalaxyl, iprobenfos, 23% (3/13); and carbendazim, dimethomorph, propamocarb and spiroxamine, 15% (2/13).

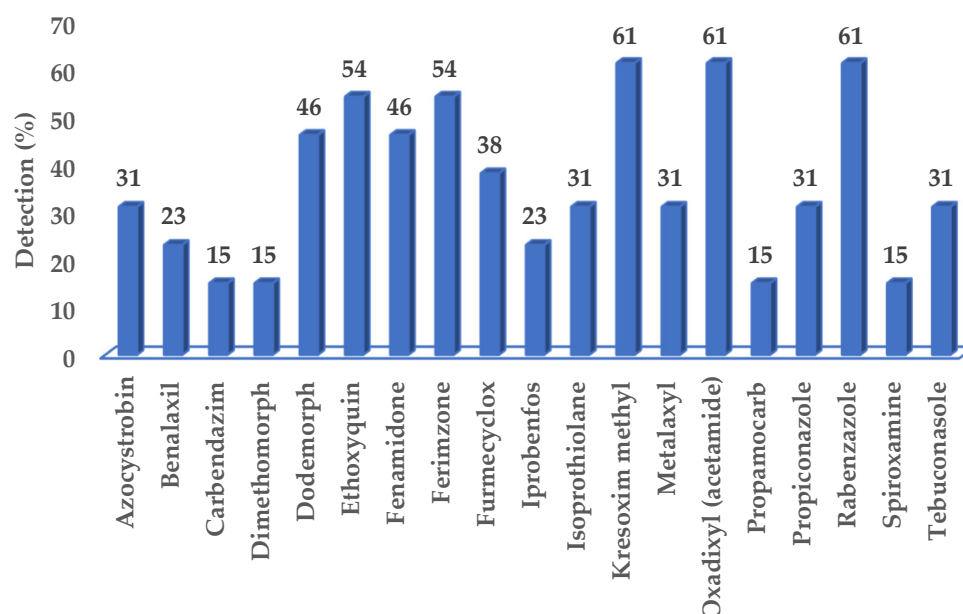


Figure 2. Frequency (%) of fungicides in surface water.

3.2. Fungicides Detected in Groundwater

Figure 1b shows the location of the thirteen groundwater sampling stations evenly distributed throughout Portugal, while Supplemental Tables S3 and S5 contain a comprehensive description of the sampling station's characteristics and the list of all fungicides detected in groundwater. Sixty-eight fungicides were detected in all groundwater areas, corresponding to sixteen (16) different compounds.

Concerning the fungicide contamination, the highest contaminated samples were obtained in the wells located in regions with mainly agricultural profiles (Martos-Leiria station 5; Monte Trigo station 10) and in areas in which rice fields surround them, such as Salvaterra de Magos (station 6), Marateca (station 8) and Ermidas de Sado (station 11).

Some of the less contaminated samples were collected by piezometer in three different sites: Formoselha (station 4), Campo Maior (station 9) and Querença (station 13).

The frequency of detected fungicides in groundwater is shown in Figure 3. The most frequently identified fungicides, in decreasing order, were rabenzazole, 92% (12/13); ethoxyquin, 77% (10/13); ferimzone, 54% (7/13), dodemorph, 46% (6/13); furmecyclox, metalaxyl, 38% (5/13); benalaxyl, mepronil and methfuroxam, 23% (3/13); diethofencarb, famoxadone, fenamidone, oxadixyl, pyracarbolid, probenazole and spiroxamine, 15% (2/3).

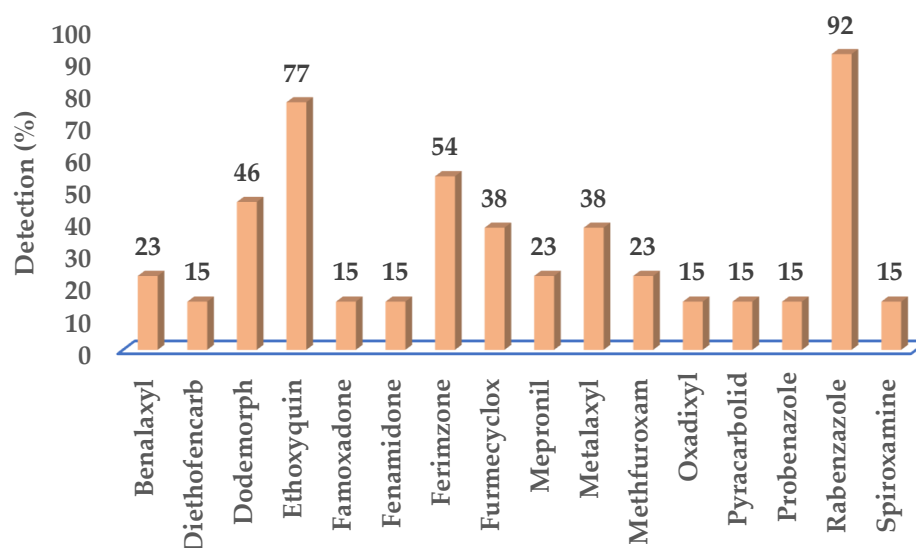


Figure 3. Frequency (%) of fungicides in groundwater.

3.3. Environmental Fate Parameters, Detection Frequency and Regulatory Status of All Detected Fungicides

Table 1 describes the fungicides' main environmental fate parameters, the detection frequency and their regulatory status during the experimental period 2016–2019, while the chemical groups, molecular weight, CAS number and mode of action of detected fungicides are covered by Table S1. The leading fate parameters' values are characterised by the ratio octanol/water partition coefficient (Log Kow < 1 low lipophilicity; <1 and <3 moderate lipophilicity; >3 high lipophilicity; >4.5 very high lipophilicity); the organic carbon partition coefficient in soil (Log Koc < 2.7 high mobility; >2.7 and <3.5 moderate mobility; >3.5 low mobility in the environment); the time dissipation of the initial compound amount in soil or in freshwater (DT50soil, <30 days—non persistent; >30 and <100 days—moderately persistent; >100 and <365 days—persistent; >365 days very persistent; DT50freshwater > 120 days persistent; >180 days very persistent); and the Groundwater Ubiquity Score (GUS < 1.8 improbable to contaminate groundwater; scores transitional >1.8 and <2.8; >2.8 substances leaching to groundwater).

Nevertheless, some endpoints were unavailable for several compounds in the researched literature as depicted in Table 1.

Almost half of all detected substances (49%, 19/39) exhibited moderate to high lipophilicity with a log octanol–water partition coefficient (log Kow) ranging from 3.16 to 4.70, a feature well-reflected by azoles and morpholines. In contrast, the detected benzimidazoles and carbamates showed low lipophilic properties. Moreover, except for fludioxonil and propamocarb, which presented a high adsorption potential to organic carbon (log Koc 5.2–6.0), the outstanding substances displayed log Koc values ranging from 1.6 to 3.8 and exhibited high to moderate mobility in the soil. In general, the stability and persistence of these substances in soil have been demonstrated as non-persistent to a moderate persistent degree, in which DT50 ranged from 1 to 3 days (kresoxim-methyl) to 75 days (oxadixyl). However, five antifungals, for instance, azoxystrobin, flusilazole, tetraconazole, thiabendazole and tricyclazole, presented DT50 values consistent with fate properties of

persistent to very persistent in soil, and detection frequencies in surface- and groundwater compartments of 10%. At the same time, a high number of fungicides indicated low to moderate water solubility, attended by a leaching index potential <1.8 (unlikely to leach) to 2.8 (marginal to leach) (Table 1).

In water, all but three substances displayed a half-live (DT50) below 42.6 days, discarding them of persistent fate; tricyclazole and metalaxyl with DT50 between 93 and 106 days and only mepronil showed a very persistent characteristic (>180 days), displaying a DT50 of 276 days. Taking into account the water/sediment interface (freshwater), excluding oxycarboxin, propiconazole, fludioxonil and tricyclazole with DT50 between 453 and 1000 days, as well as flusilazole, tebuconazole and tetraconazole with DT50 values near one year, the estimated half-lives for the remaining referenced chemicals demonstrated a moderate or easy degradation in the water-sediment system (Table 1).

Considering the primary mode of action classes, the sterol biosynthesis inhibitors, including the amines or morpholines group (Δ^{14} reductase and $\Delta^8 \rightarrow \Delta^7$ isomerase inhibitors) and the well-known DMIs (demethylase inhibitors), were the most representative of the detected fungicides with seven compounds, followed by the quinone outside inhibitors (QoI) and beta-tubulin inhibitors, each one with five substances as well as mitochondrial succinate dehydrogenase inhibitors (SDHI) with three substances (Table S1).

4. Discussion

Qualitative screening to identify surface- and groundwater antifungals has never been performed on the Portuguese mainland. This survey is crucial for an initial selection of polar organic chemicals of concern, indicating the emergent contaminants that can lead to future quantitative monitoring programmes to implement mitigation strategies as in our previous antibiotics study [21]. The study's sampling stations were located in regions with urban-agricultural-rural environmental pressures.

The current survey showed the occurrence of agricultural antifungals (fungicides) in all sampling sites; unexpectedly, medicinal antifungals (antifungals) were not detected, even in samples from sampling stations located in river basins downstream of urban wastewater treatment plants (UWWTP) (Table S2). This result may represent a differential of specific attributes affecting antifungals against fungicide occurrence: the market size and distinct pathways to enter the environment.

Regarding the correlation between sale figures and environmental detection, it is justifiable to consider the compound use profile on the market [41]. Thus, the consumption of antifungals (2.4 tones, 2019 in Portugal) and the number of primer chemical groups used in human and animal clinics (polyenes, azoles, allylamines, pyrimidine analogues and echinocandins) are explicitly smaller compared with fungicides, showing a value of 2000 tones (2019 in Portugal), and a diversity of chemical groups from which outstand the amides, azoles, benzimidazoles, carbamates, morpholines and strobilurins [1,3,5,7]. Moreover, antifungals enter the environment, mainly following emissions of active ingredients into wastewater. This results in antifungal contamination of sewage sludge or biosolids that can amend the soil and, hence, scarcely appear in surface waters whenever wastewater treatment technologies can remove these substances efficiently. Conversely, fungicides are directly applied to land and transferred mainly to the surface- and groundwater via runoff and leaching [42]. Thus, antifungal occurrence studies revealed the presence of these compounds mainly in raw sewage water of UWWTP, final effluent, or surface water in the low ng/L range [43]. In the present study, the sampling stations were not located near effluents but in river surface water, which might explain the lack of antifungal detection. Contrasting, fungicides are responsible for residues reported worldwide in surface waters and other environmental matrices at higher and year-long varying concentrations [44]. For instance, Jing Zhu et al. detected concentrations of total fungicides in surface water which were significantly increased in September (median = 161.82 ng/L) compared to April (median = 25.47 ng/L) and November (median = 65.49 ng/L) [45].

The highest frequency of detection in surface water for most of the substances were observed in the Ave River, S. Domingos (reservoir), Tejo—Marina de Algés (transitional water) and Ria Formosa (coastal water), which may be due to the diffuse source of fungicide inputs into the water bodies resulting from field agriculture application [46]. Further, the relative amounts of fungicides detected depend highly on the land field use within the monitored catchment area. This fact observed in agricultural regions diverged from rural landscape regions where only two fungicides were identified (Tables S2 and S4). The most elevated frequency of substance detection in groundwater was seen in samples obtained in wells, mainly in agricultural regions, with some surrounded by corn and rice fields. As groundwater is near the surface (well), the fungicides may reach the groundwater through surface runoff and erosion, leaching, spray drift or point source inputs [46]. The observed lower fungicide detection whenever the piezometers were applied corroborates this assumption. Further, only systemic organic fungicides, mainly used to protect fruits, vegetables, vineyards, and cereals (rice and corn) were detected in the groundwater compartment. This broadly shows photochemical persistence, low volatility, stability to hydrolysis, and resistance to microbial degradation (azoles) [22]. Reports of azole occurrence in groundwater were resumed in China, Netherlands and India [47,48]. Nevertheless, these reports of groundwater contamination by pesticides and micro-pollutants like pharmaceuticals have been growing [42], showing that these drugs are potential contaminants of groundwater, and a rising concern as groundwater is one of the most common sources of drinking water and irrigation.

The detection frequency of fungicides in surface- and groundwater was dominated by rabenzazole 61–92% and ethoxyquin 54–77%. Rabenzazole is not approved in the EU and by the ECHA, as it is subject to a pre-registration process [22,23]. Currently, it seems to arrive in Europe through China. Regarding the China Rabenzazole Export Data referenced by the General Administration of Customs, PR China, from 2018.11 to 2019.11, the total export volume of Rabenzazole to Portugal was 193,829 kg, a distinct value from, e.g., Germany (7,723,379 kg), Netherlands (4,664,307 kg), Belgium (3,284,341 kg), France (944,633 kg), Spain (809,951 kg) and UK (721,234 kg) which displayed raised quantities [49]. Occurrence studies for rabenzazole are scarce; only one work by Robinson et al. (2023) identified lower detection frequencies (3%, $n = 38$) of rabenzazole in water samples collected in Southern England rivers [50]. Likewise, the EFSA residue report from 2020 describes a residue quantification rate for rabenzazole of null in 8264 analyses performed in five countries [51]. These facts contradict the positive results obtained in the present study. Rabenzazole is a benzimidazole fungicide used or applied in cereals, vegetables and fruits [52]. Unlike other detected compounds from the same chemical group (benomyl, carbendazim and thiabendazole), rabenzazole is the only one with high solubility in water, which was found in our study with considerable presence in water bodies, mainly in groundwater in the regions RH4A, RH5A and RH6, strongly predicting its use in rice fields. To our knowledge, environmental fate and effects endpoints have not yet been determined; merely a partition coefficient, a bioconcentration factor, a biodegradation half-life and a log K_{oc} were predicted (Table 1), which indicate low bioaccumulation potential and biodegradation capacity, as well as moderately reduced mobile characteristics. Hence, rabenzazole is likelier to leach into groundwater or run off to surface water bodies such as rivers and does not highlight any accumulation concern in soil. In addition, the surrounding catchment locations dominated by rice fields and the sampling timeline coincident with agricultural seasons of pesticide application may have increased the environmental compound concentration, contributing to the high detection observed in the present study. Bioassay results demonstrated the fungicidal activity of rabenzazole against *Magnaporthe oryzae* (Ascomycota) in rice seedlings in paddy fields [53].

Ethoxyquin, like rabenzazole, occurred mainly in groundwater wells with a prominent detection percentage. It is an antioxidant used as a fungicide, a deterrent of scald in pears and apples through pre-harvest and post-harvest dip or spray, and also as a preservative to protect animal feed and fishmeal from oxidation of lipids and fat-soluble vitamins [54,55].

During this study sampling campaign, this compound had already been banned as a fungicide since 2009 [56]; however, it was still approved as a food preservative (antioxidant) for all animal farming feed, including aquaculture and in all food categories. Recently, the European Commission (Regulation (EU) 2022/1375) refused to reauthorise the use of ethoxyquin as an antioxidant, repealing Implementing Regulation (EU) 2017/962 [57]. One of the reasons for its suspension by EFSA is the presence of a suspected mutagenic and carcinogenic impurity: p-phenetidine, which is used as a starting ingredient for ethoxyquin synthesis [55,57]. Monitoring studies of ethoxyquin in human urine samples between 2000 and 2021 showed a significant decline from 2016 to 2021 after the EU decision [54]. When ethoxyquin is used as a fungicide, the applications are made indoors (as it happens for the fruit packing industry); thus, it would probably not leak into surface and groundwater. Nevertheless, the grappling of this substance in the present manuscript makes sense due to the high detection level observed. Few studies address the occurrence of ethoxyquin in the environment. Chau et al. [58] demonstrated in a study performed in Vietnam that ethoxyquin was 1 of the 24 most frequently occurring compounds in surface water. The environmental fate parameters for ethoxyquin may indicate a substance with the potential for bioaccumulation, moderate water solubility, and medium mobility in soil, rapidly degraded ($DT_{50} = 2.2$ days), and a high potential for movement toward groundwater (GUS value 6.70). These ethoxyquin fate properties, together with river aquaculture and farm animal production, could explain the high percentage of ethoxyquin's occurrence in aquatic bodies observed in the present study.

Equivalent surface water and groundwater detection was observed for ferimzone, dodemorph, furmecyclox, metalaxyl, benalaxyl and spiroxamine at 54%/54% to 15%/15%. Among these fungicides, furmecyclox (38%/38%) stands out, a furan derivative with the biologically active group $CH_3-C=C-C=O$, which is currently considered an obsolete compound with use restricted to research. Therefore, its high detection rate in our study does suggest an "off-label" usage to control root or wood rot and even its application in cereals and potatoes [22,59]. Environmental fate and effects data on furmecyclox are sparse. Discussing the possible relationship between the potential environmental pressure and the detection rate is complex.

Regarding the above group, the remaining fungicides, except ferimzone and furmecyclox, were authorised during this study screening period. Despite the different applications (ferimzone—mainly rice farming, dodemorph—ornamental plants, metalaxyl/benalaxyl/spiroxamine—viticulture), they were found as surface- and groundwater contaminants. During application, they can migrate off-site and potentially enter nearby waterways and groundwater resources (e.g., wells). The fate and behaviour are influenced by their chemical properties (e.g., ability to bind to soil, susceptibility to degradation) and environmental factors such as soil type, rainfall, topography, and agricultural management practices [46,60]. Generally, these substances are expected to be adsorbed to organic matter, oxides and clay materials; therefore, the edaphic factors and the amount of fungicide used are crucial. In short, there are various scenarios to consider when assessing the potential for off-site migration of a fungicide. According to our screening, ferimzone is the most polluting (54%/54%) among these five fungicides, having been detected in agricultural areas surrounded by corn and rice fields in the following regions: RH4A, RH5A and RH6, strongly predicting its use in those crops; but it never received authorisation at the EU level. Occurrence studies were not found, and fate parameters in water and water/sediment are lacking. However, a log Kow of 2.98 and 2.57 for log Koc were predicted (Table 1), indicating low bioaccumulation capacity and high surface runoff mobility, explaining its higher leaching potential to groundwater. Dodemorph use has been limited to glasshouse ornamental plants since 2015 [61]. Its presence in surface- and groundwater, with 46% detection frequency, suggests intensive use before the amending Council Directive or illegal usage. As far as we know, studies of environmental occurrence of this active substance are lacking. However, fate predictions suggest that dodemorph is moderately persistent in soil (log Koc of 3.2, soil $DT_{50} = 41$ days) and non-mobile (GUS = 0.65) (Table 1).

The highest detection percentages in surface water and minimal residues in groundwater were dominated by oxadixyl (61%), kresoxim-methyl (61%) and fenamidone (46%). These features made us designate these compounds as primarily surface water contaminants. Oxadixyl, a moderate persistent fungicide, has been employed in potatoes, vegetables and vineyards [62]. Its use is not approved in the EU (Table 1); however, in our study, it appears in surface water with considerable frequency, meaning that it is probably in current use and perhaps applied in excessive amounts. Both substances, kresoxim-methyl and fenamidone, are slightly soluble in water and mobile in soil with low persistency (Table 1). A study about adsorption–desorption and leaching behaviour of kresoxim-methyl in different soils of India stated a moderately low leaching and low groundwater contamination potential for kresoxim-methyl [63]. This fact aligns with our findings, corroborating our primarily surface water contaminant designation.

In the present study, the azole group is depicted by six detected active substances. The occurrence in surface water was a residual (10%) to moderate detection rate (31%). This moderate rate was observed only for two compounds, propiconazole and tebuconazole. Furthermore, the detected azoles showed a pronounced persistence in solid matrices. The DT50 water/sediment system is relatively high, with 117 days for imazalil and up to 561 days for propiconazole (Table 1). The amount used, in Portugal, of each azole-active substance had not been found available. Nevertheless, it has been established that the most used fungicides are expected to be the most detected [64], allowing us to conclude that propiconazole and tebuconazole are the most used within the azole group.

There are many occurrence data sources for pesticides, but comparable data across Europe is limited for fungicides [65]. Even considering this difficulty, we reviewed some available environmental occurrence surveys, which may provide meaningful input regarding relevant compounds. However, some are confined to regions and do not have a country-wide monitoring approach, like in this work. The most frequently detected fungicides in the EU were azoxystrobin, boscalid (UK, Germany and The Netherlands), carbendazim, metalaxyl, tebuconazole (Germany and The Netherlands), propiconazole (Germany) or tricyclazole (Spain) [45,64,66,67]. Except for boscalid, all other fungicides were found in our survey. Additionally, approved and non-approved substances, rarely reported in other EU countries, were identified, such as rabenzazole, oxadixyl, kresoxim-methyl, fenamidone, ferimzone, ethoxyquin, and dodemorph. These findings suggest intensive use of fungicides in Portugal's vineyards, orchards, and corn and rice fields. Summarising, the detected fungicide spectrum indicates a Portuguese use pattern distinct from those described for other European countries.

According to the above-exposed data, some frequently detected fungicides that are either not authorised (ethoxyquin, furmecyclox, oxadixyl), without data in the EU (rabenzazole, ferimzone), or are authorised at the national level (fenamidone), should be included in environmental monitoring programmes and followed as emerging contaminants.

Learning from the One Health Approach towards Sustainability: the detected fungicides present an emerging contaminant source and may pose ecological chronic risks that are partially unknown and even a threat to the SDGs. As discussed and demonstrated above, the frequent application, the moderate to high environmental persistence, and the mobility, raises concerns about the sub-chronic and chronic exposure of these substances to the biota. However, it is currently recognised that their environmental residues might cause adverse effects on the non-target organisms by blocking cellular and physiological homeostasis. Namely, the quinone outside inhibitors that achieved a high detection rate in our study are considered highly toxic and one of the most hazardous fungicide classes. In vivo and in vitro studies demonstrated that they induce metabolic dysfunction in Crustacea, neurotoxicity in the SH-SY5Y neuroblastoma human cell line (azoxystrobin), developmental toxicity in zebrafish larvae (azoxystrobin, fenamidone, famoxadone), transcriptional changes, similar of those found in brain samples from humans with autism or neurodegeneration (fenamidone, famoxadone) or are likely to be carcinogenic to humans (kresoxim-methyl) [68–73]. The beta-tubulin inhibitors, whose mechanism of action is

directed to cell division via disruption of tubulin polymerisation during mitosis of fungi due to inhibition of β -tubulin synthesis [74,75], were represented in this study by the fungicide rabenzazole, for which effect studies on animals/humans were not found. Detected substances with analogous modes of action demonstrated in toxicological studies, (i) reproductive and developmental toxicity, (ii) endocrine-disrupting effects in rats (benomyl, carbendazim) or even (iii) alterations in thyroid hormones, thyroid volume, and thyroid follicular cell adenomas (thiabendazole) [76–80]. Thereby, disrupting hormonal function effects cannot be excluded from rabenzazole exposure. Remarkably, the sterol biosynthesis inhibitors illustrated in this study are dodemorph, spiroxamine and some azoles. Dodemorph demonstrated moderate ecotoxicity to bees, fish and aquatic invertebrates [22]. Although limited data is available on its toxicity to mammals, an external report submitted to EFSA, prepared by Nielsen et al. [81], highlighted that dodemorph delayed pre- and post-natal development and decreased body weight of adult offspring in rats as well as hepatocellular hypertrophy and cell degeneration.

Regarding spiroxamine, it is considered a skin sensitiser; however, the potential of spiroxamine to have endocrine-disrupting properties is currently controversial. While EPA reported no evidence of effects on rat/rabbit development and reproduction, EFSA concluded, in a peer review, that spiroxamine has the potential for endocrine-disrupting effects based on its mode of action. However, the toxicity studies have not been fully completed [82,83]. The azole group is a sterol biosynthesis inhibitor that has an affinity to sterol 14 α -demethylase (CYP51) enzymes [84]. Sterol biosynthesis that depends on the 14 α -demethylase is common in nature [85]; thus, azoles might affect all non-target organisms, including plants, where the enzyme is conserved. In addition to azole's major target (CYP51 inhibition), they also interfere with several other CYP450 enzymes, which has become an enormous concern, such as CYP19 and CYP17 involved in steroidogenesis (suppression of androgens and estrogens) [86] as well as CYP1A [87] intricately involved in xenobiotic detoxification in fish and mammals. Further, Ankley et al. reported decreased egg production and effects on testosterone synthesis in male and female fathead minnows [88]. These observations suggest that this class of fungicides can act as endocrine disruptors.

Another issue is the potential of the environmental pressure of fungicides (environmental contamination) to induce the selection and development of resistance in the fungal community of the contaminated site to major classes of fungicides, such as anilino-pyrimidines, benzimidazoles, succinate dehydrogenase inhibitors, strobilurins and sterol biosynthesis inhibitors [1,42]. The DMIs, including azole fungicides, share the same mechanism of action with medical azoles, which can explain the increased report of azole resistance in naïve patients originating from the environment [89,90]. So, the occurrence of these DMI pesticides and pharmaceutical micro-pollutants in environment niches such as surface- and groundwater could constitute hotspots for antifungal-resistant fungal pathogens. Indeed, it has been reported that these drugs might pose a moderate to high risk for drug resistance [42] based on the quick microbiological risk assessment of risk quotient (RQ) method.

5. Conclusions

A qualitative survey of antifungals for agricultural and non-agricultural purposes in surface- and groundwater was accomplished. Unexpectedly, antifungals applied in medical practice were not found. Overall, our study revealed thirty-nine systemic active antifungals used in agriculture, of which 51% had “approved for use” status; five identified compounds are on the EU WFD Watch List (azoxystrobin, famoxadone, imazalil, tebuconazole and tetraconazole), while the banned/restricted fungicides detected included, for instance, ethoxyquin, furmecyclox, oxadixyl (not authorised), rabenzazole, ferimzone (without data in the EU) and fenamidone (with authorisation at national level). Amazingly, of these—rabenzazole, ethoxyquin, and ferimzone are among the most polluting (frequently detected), as well as oxadixyl and fenamidone (two of the three designated contaminants primarily of surface water), and therefore should be followed as emerging (micro)contaminants.

The detected fungicide spectrum indicates a Portuguese use pattern distinct from those described for other European countries. Furthermore, the detected fungicides present an emerging contaminant source and may pose chronic ecological risks that are partially unknown, illustrating a threat set by the United Nations. In fact, it is currently recognised that their environmental residues might cause adverse effects on non-target organisms. Although limited data is available on its toxicity to mammals, evidence of that has been highlighted for most of the detected fungicides: neurotoxicity, reproductive and developmental toxicity, endocrine-disrupting effects, and alterations in thyroid hormones. Therefore, central nervous system effects and disrupting hormonal function effects cannot be excluded from exposure to these fungicides.

As depicted, this screening approach is essential to identify target fungicides for future quantitative risk assessments and to allow water quality standards to be established. These occurrence studies as well as data on consumption patterns can contribute to country-based fungicide monitoring programs and priority lists, particularly for those frequently detected fungicides that are either not authorised or are authorised at the national level or even without data in the EU. Unfortunately, the usage amounts of the fungicides detected in our study were unknown. Summarising and considering the One Health approach relevance, policy makers should gather their efforts to allow the implementation of proper risk management and effective contamination control strategies to achieve the goal of sustainability targets.

Supplementary Materials: The supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/su16020594/s1>, Table S1: Chemical Groups, Physicochemical Properties and Mode of Action of all Detected Fungicides [91,92]; Table S2: Localisation and Characteristics of Sampling Stations in Surface Water; Table S3: Localisation and Characteristics of Sampling Stations in Groundwater; Table S4: Frequency Detection and Geographic Allocation in Surface Water (2017–2019); Table S5: Frequency Detection and Geographic Allocation in Groundwater (2016–2019); Document S6: Analytical Method Description.

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