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Groundwater antibiotic pollution and its relationship with dissolved organic matter: Identification and environmental implications[☆]

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ABSTRACT

The occurrence of veterinary antibiotics and hydro-chemical parameters in eleven natural springs in a livestock production area is evaluated, jointly with the characterization of their DOM fingerprint by Orbitrap HRMS. Tetracycline and sulfonamide antibiotics were ubiquitous in all sites, and they were detected at low ng L⁻¹ concentrations, except for doxycycline, that was present at µg L⁻¹ in one location. DOM analysis revealed that most molecular formulas were CHO compounds (49 %–68 %), with a remarkable percentage containing nitrogen and sulphur (16 %–23 % and 11 %–24 %, respectively). Major DOM components were phenolic and highly unsaturated compounds (~90 %), typical for soil-derived organic matter, while approximately 11 % were unsaturated aliphatic, suggesting that springs may be susceptible to anthropogenic contamination sources. Comparing the DOM fingerprint among sites, the spring showing the most different profile was the one with surface water interaction and characterized by having lower CHO and higher CHOS formulas and aliphatic compounds. Correlations between antibiotics and DOM showed that tetracyclines positively correlate with unsaturated oxygen-rich substances, while sulfonamides relate with aliphatic and unsaturated oxygen-poor compounds. This indicates that the fate of different antibiotics will be controlled by the type of DOM present in groundwater.

1. Introduction

Groundwater is an extremely important resource in many European countries, especially in areas where surface water is of limited availability or poor quality. In areas with intensive livestock production, groundwater quality might be seriously compromised by the excessive use of animal manure as organic fertilizer, as it contains high concentrations of veterinary pharmaceuticals and antibiotics (Gros et al., 2019; Marti et al., 2020; Nölvak et al., 2016). Thus, its reuse as soil amendment may be an important source of these pollutants to the aquatic environment (Boy-Roura et al., 2018). Antibiotics can reach groundwater primarily through leaching from fertilized soils (Blackwell et al., 2009; D'Alessio et al., 2019; Hill et al., 2019; Pan and Chu, 2017) or indirectly via surface water recharge (García-Galán et al., 2010).

Besides monitoring antibiotics and other relevant hydro-chemical

parameters (e.g. nitrates), the characterization of dissolved organic matter (DOM) can also be a powerful tool to assess groundwater quality. DOM is a combination of numerous substances, such as humic and fulvic acids, polysaccharides, proteins, lipids, nucleic acids, soluble microbial products, and organic contaminants, such as antibiotics, and it could be considered as a sample fingerprint. The major source of DOM in groundwater is the transport of organic matter from the vadose zone to the subsurface by rainfall recharge (McDonough et al., 2020). However, anthropogenic (e.g. discharges of septic systems) and agricultural activities can have an effect in its composition (Xenopoulos et al., 2021).

High resolution mass spectrometry (HRMS) is a powerful tool for DOM characterization, as it allows the assignment of individual molecular formulae (Hawkes et al., 2020). Among HRMS techniques, high field strength Fourier transform ion cyclotron instruments (FTICR-MS) are widely used. However, in recent years, Orbitrap-HRMS has gained

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popularity, proving its suitability and excellent reproducibility when compared to FTICR-MS (Hawkes et al., 2016). In a comparative study, Orbitrap-MS showed to be a valid alternative to FTICR-MS, as it was capable of detecting most major ionizable organic molecules in typical aquatic mixtures (Hawkes et al., 2020; Hawkes et al., 2016). Groundwater DOM fingerprinting by HRMS has been used to identify the chemical composition and fate of specific DOM components in a petroleum contaminated site (Podgorski et al., 2021), and in groundwaters subject to anthropogenic contamination sources, such as septic tank leakages (Arnold et al., 2014), and to investigate DOM sources and composition (Yu et al., 2020). However, the DOM characterization of groundwater bodies in livestock production areas and the potential impact of manure fertilization on its fingerprint has been seldomly evaluated. Previous studies based on spectrofluorometric techniques, such as excitation emission matrices (EEMs), that characterized the DOM profile in farm and animal wastes, (Baker, 2002), as well as in surface run-off, leachate and farmyard drainage samples following manure applications (Humbert et al., 2019; Naden et al., 2010; Old et al., 2012; Singh et al., 2014) exhibited low aromaticity, high intensities of fulvic-like compounds, and an increase in the tryptophan-like and manure-derived protein-like components, suggesting that natural water bodies impacted by livestock waste and animal manure reuse would have a characteristic DOM signature.

In natural waters, DOM can interact with antibiotics influencing their mobility, transport persistence and bioavailability (Artifon et al., 2019; Carstea et al., 2016; Hernandez-Ruiz et al., 2012; Li et al., 2018; Liu et al., 2019; Sgroi et al., 2017; Wei-Haas et al., 2014; Zhang et al., 2019; Zhou et al., 2016). Previous studies on artificial groundwater recharge with effluent wastewater showed how major DOM components, such as humic and fulvic acids, interact with antibiotics, particularly tetracyclines, and decrease their sorption to soil (Fan et al., 2021), while in another study, a strong affinity between antibiotics, protein-like and humic-like DOM components was also observed, influencing their sorption to soils (Cheng et al., 2020). DOM may then play a critical role in enhancing contaminant transport of the sorptive pollutants that have affinity with it, as co-transport, even when direct interactions between organic matter and the contaminant is weak as defined by the type and properties of both particles, and the environmental factors as groundwater geochemistry and soil mineral composition (Cheng and Saiers, 2015; Zhou and Cheng, 2018). Thus, it is of utmost importance to investigate DOM composition and its potential interactions with antibiotics, to have broader information about the transport, fate, and risks of these contaminants in the environment.

In this study, we investigated the occurrence of antibiotics, hydrochemical parameters and DOM composition in groundwater in a region with intensive livestock production, and evaluated, for the first time, the interaction between antibiotics and specific DOM components in the proper hydrogeological framework. Natural springs were selected because they are good surrogates for the assessment of groundwater recharge quality, as spring flow integrates groundwater from local parts of the aquifer system and permits identifying human pressures on water resources quality (Katz et al., 2001). Our main goal was to assess how animal manure reuse as fertilizer in agriculture affects groundwater quality by: (a) evaluating antibiotic occurrence, hydro-chemical parameters and DOM fingerprint at 11 different sites during 3 different seasons, b) assessing differences among sites according to their chemical (e.g. antibiotics and environmental parameters) and DOM profile and hydrological behavior and c) establishing potential relationships between antibiotics and specific DOM components.

2. Materials and methods

2.1. Study area and sampling

Samples were collected from eleven natural springs, some of which are intended for human consumption, from the Osona catchment

(Barcelona province, NE Catalonia; Fig. S1, adapted after Boy-Roura et al. (2013)). This is one of the regions with major intensive agricultural and livestock production within the country. Approximately 90 % of the manure generated by livestock production (mainly pig manure) is spread as fertilizer in crops and only a minor fraction (<10 %) is treated in treatment plants. The most common crops in the area are wheat, followed by barley and sorghum, among other minor crops (Boy-Roura et al., 2013). In 2009, a total of 38 out of 51 municipalities in the region, which represents 70 % of its area and the totality of its agricultural land, were declared as vulnerable to nitrate pollution from agricultural sources by the Catalan Parliament, 2009. Springs were classified into four groups (Fig. S1) according to Hydrological Response Types (HRT) and based on their behavior as indicated by discharge, electrical conductivity (EC) and nitrate content, as described elsewhere (Boy-Roura et al., 2013). Details about spring characteristics in each HRT can be found in the supplementary material (Table S1). HRT categories considered other variables as well, such as the characteristics of the geological setting and the land use, which both play an important role in determining the discharge, hydro-chemical features and nitrate content in the springs (Boy-Roura et al., 2013).

Three different sampling campaigns were performed, during April 2018, September 2018 and January 2019. Temperature, pH, electrical conductivity (EC), dissolved oxygen and redox potential (corrected to standard hydrogen electrode values) were measured in-situ using hand-held probes. Other parameters such as dissolved organic carbon (DOC), total nitrogen (TN), nitrates (N-NO_3), nitrite (N-NO_2), phosphates (P-PO_4), anions and cations were also measured, and more information is found in the supplementary material. For the analysis of antibiotics, samples were taken in 1L amber polyethylene bottles (PET), while for DOM analysis, 1L amber glass bottles were used. Samples were transported refrigerated (4°C) to the laboratory and those for DOM and antibiotic analysis were stored at -20°C until analysis.

2.2. Antibiotics analysis

The methodology used for the determination of antibiotics was adapted from the one described in detail elsewhere (Gros et al., 2013) and explained in the supplementary material. Briefly, after filtration, 500 mL of sample were measured, an appropriate volume of a 0.1M Na_2EDTA solution was added, and sample pH was adjusted. Samples were extracted and pre-concentrated in triplicate by using solid phase extraction (SPE). Instrumental analysis was performed by ultra-high-performance-liquid chromatography (Acquity UHPLC system, Waters Corporation, MA, USA) coupled to a quadrupole-linear ion trap tandem mass spectrometer (5500 QTRAP, AB Sciex, Foster City, USA). Method performance parameters are described in the supplementary material (Tables S2 and S3).

2.3. DOM extraction and HRMS analysis

The concentration of DOM in groundwater is too low for direct injection into the Orbitrap MS. Thus, solid phase extraction (SPE) was used to concentrate the DOM as described elsewhere (Farré et al., 2019; Li et al., 2016), and details are provided in the supplementary material. Prior to Orbitrap analysis, SPE extracts were diluted with ultrapure water to 1:1 methanol/water (v/v) mixture to have a final concentration of approximately 30 mg L^{-1} of DOC. Diluted extracts were filtered through $0.2\ \mu\text{m}$ PTFE syringe filters. In this study, DOM extraction efficiency was not measured. However, we estimate that it is approximately 60 % on a carbon basis, based on previous research using the same cartridges and similar extraction procedure (Dittmar et al., 2008).

Extracts were analyzed using an Orbitrap LTQVelos (Thermo Scientific, Germany), and instrumental conditions are included in the supplementary material. The Orbitrap was externally calibrated for mass accuracy on the day of analysis using the manufacturer's guidelines and negative calibration solution. DOM was analyzed by direct infusion,

adapting the methodology described elsewhere (Hawkes et al., 2016). Electrospray ionization (ESI) in negative mode was selected because it provides a larger range of chemical formulae for DOM analysis than positive ionization (PI). Furthermore, with PI, problematic sodium complexes may occur (Li and Minor, 2015) and the split between NaH and C₂ is only 2.4 mDa, making these peaks coalesce (Farré et al., 2019). Furthermore, with PI there is poor resolution of protonated peaks for sodium adducts (Koch et al., 2005).

2.4. Detection limit, formula assignment and data analysis

Detection limits and formula assignment was done following an adapted procedure from the one described in Farré et al. (2019). Briefly, noise peaks were removed from each individual sample's mass list using an Excel macro, following the detection limit criteria described in Riedel and Dittmar (2014). Data processing and formula assignment was done using an in-house R methodology, with the Rstudio software (RStudio, Inc), as described elsewhere (Hawkes et al., 2016). For formula assignment, formulas up to C₄₋₄₀H₈₀O₄₀NS with and without one ¹³C were allowed. Formulas were assigned according to the following rules: O/C < 1, H/C 0.3–2.5, m/z 200–800, double bond equivalents minus oxygen (DBE-O) > -10 or DBE-O < 10. Masses occurring in only one sample were removed. Peaks detected in the procedural blanks were also removed and signal intensities were normalized to the sum of all signals. The intensities of each molecular mass detected after subtracting the blank were compared between samples.

The statistical software R was also used to group compounds based on O/C and H/C ratios and the modified aromaticity index AI_{mod} (Koch and Dittmar, 2006; Koch and Dittmar, 2016; Sanchís et al., 2021) and according to the groups used in Hawkes and co-workers (Hawkes et al., 2020). AI_{mod} was calculated using the following equation:

$$AI_{mod} = (1 + C - 0.5O - S - 0.5(N + P + H)) / (C - 0.5O - S - N - P) \quad (1)$$

In this way, five different groups were selected including: (i) condensed aromatics (AI_{mod} ≥ 0.67) and polycyclic structures in a molecule that are produced during combustion (combustion derived aromatics), but not organisms, (ii) aromatics (0.66 ≥ AI_{mod} > 0.5), that are indicative for plant derived material that has undergone microbial degradation in soil and soil-derived polyphenols, (iii) unsaturated compounds poor in oxygen (H/C < 1.5, AI_{mod} < 0.5, O/C < 0.5), (iv) unsaturated compounds rich in oxygen (H/C < 1.5, AI_{mod} ≥ 0.5, O/C ≥ 0.5), both providing evidence for soil-derived humics (e.g. phenolic and highly unsaturated compounds) and aliphatics (H/C ≥ 1.5), including unsaturated aliphatics, saturated fatty, sulfonic acids, sugars and peptides.

The statistical software R 4.0.2 (R Core Team (2020)) (Koch and Dittmar, 2016) was also used to evaluate the differences in DOM composition across campaigns and between samples, specifically using Bray-Curtis dissimilarity tests (*vegdist* function), which compares signal intensity between each pair of samples for each molecular mass. Principal component analysis (PCA; function *prcomp*) was performed on the hydro-chemical parameters and antibiotics data, to evaluate the potential clustering of the different sites based on these variables. Since multivariate data analysis techniques require complete data matrices as input, and for some antibiotics more than 25 % of the values laid below the limit of detection, censored values were imputed using the non-parametric multiplicative simple imputation procedure (*multRepl*) from package *zCompositions* as described elsewhere (Palarea-Albaladejo and Martín-Fernández, 2015). Principal coordinate analysis (PCoA; *cmdscale* function) was performed using the scores of the PCA for hydro-chemical parameters, the imputed antibiotics and DOM molecular constituents. Finally, the relationship between DOM constituents and antibiotics was investigated by using Spearman rank correlations and represented in van Krevelen diagrams. The *vegan* package for R was used for statistical multivariate analysis (Oksanen et al., 2013).

3. Results and discussion

3.1. Hydro-chemical parameters and antibiotics

Table 1 summarizes the chemical characteristics of the natural springs as well as the antibiotic concentrations detected in the first sampling campaign. Results for the second and third sampling events are presented in the supplementary material (Tables S4 and S5), as hydro-chemical parameters were quite similar among seasons and the number of positive antibiotic detections and corresponding concentrations were much lower. For this reason, the data from these two campaigns was not considered in this study for statistical analysis, and when assessing interactions between antibiotics and DOM components. Concentrations of DOC ranged from 0.59 to 4.28 mg L⁻¹ in the first monitoring, and similar values were observed in the second and third sampling events. Electrical conductivities varied from 631 to 1272 μS cm⁻¹. Lower EC values were observed in sites located in the eastern part of Osona region (e.g. SSO002, FOL006, TAV002 and FOL002, Fig. S1), associated to the occurrence of igneous and carbonate sandstone formations (Boy-Roura et al., 2013). Springs hydrochemistry was of the calcium-bicarbonate water type, consistent with the dominant Osona region geochemistry (Menció et al., 2011). In general, anion and cation concentrations were quite constant across the different sampling events (Table 1, Tables S4 and S5). Sulfate concentrations range from 14 to 176 mg L⁻¹ and, as it has been previously described. Groundwater SO₄²⁻ is assumed to be related to the oxidation of disseminated pyrite (Vitòria et al., 2008). For nitrates, concentrations ranged from 20 to 316 mg L⁻¹ and this range was constant along the different sampling events. Eight out of the eleven sampled springs showed NO₃⁻ concentrations higher than the EU threshold for drinking water (50 mg L⁻¹). Nitrate concentrations stayed uniform despite the hydrological variations and they show a similar trend than those observed in previous monitoring campaigns in the same area, where the highest concentrations were found in springs located in sedimentary formations (geological groups B and C, Table S1 and Fig. S1) with agricultural activity in their recharge areas, while the lowest values were in springs present in crystalline (igneous and metamorphic) rocks (group A, Table S1) and forested areas (Boy-Roura et al., 2013).

For antibiotics, tetracyclines and sulfonamides were detected in several natural springs. Yet tetracyclines were only found in the first monitoring, and concentrations for the first sampling event are summarized in Table 1. Groundwater antibiotic concentrations were surprisingly relevant, ranging from 21 to 365 ng L⁻¹, while doxycycline even reached 2 μg L⁻¹ in one spring. Sulfonamide antibiotics were more ubiquitous than tetracyclines since they were detected throughout the different sampling campaigns (Tables S4 and S5). Nevertheless, their concentrations were considerably lower than those found for tetracyclines, ranging from 5 to 12 ng L⁻¹ in the first sampling, and these values remained uniform in the other monitoring campaigns (Table 1, Tables S4 and S5). The detection of tetracycline antibiotics, especially doxycycline, at such remarkable concentrations, and the widespread occurrence of sulfonamides indicate that manure reuse as fertilizer in crops stands as a potential source of groundwater pollution. Indeed, tetracyclines and sulfonamides are one of the most prescribed antibiotics in livestock production facilities (ECDC, 2017), and high concentrations up to mg L⁻¹ and mg kg⁻¹ have been detected in manure and other livestock wastes (Gros et al., 2019; Marti et al., 2020; Widayarsi-Mehta et al., 2016; Zhou et al., 2013).

The spatial variability in hydro-chemical and antibiotics distribution is shown in a PCA (Figs. S2 and S3, respectively), using imputed values for the antibiotics with reported values below the detection limits. For sulfonamides, no major differences were observed when running the imputation considering the three campaigns or just the first monitoring. However, since tetracyclines had many non-detected values for the last two campaigns, corresponding to more than 80 % of the data, these two campaigns were excluded from the analysis. For this reason, the PCA for

Table 1
Chemical characterization and antibiotic concentrations in natural springs in the first sampling event.

Group HRT	HYDROCHEMICAL PARAMETERS										
	1	2	3			4					
Spring code	SSO002	FOL006	TAV002	TOR001	FOL002	MDV003	SJV003	CAL004	GUR004	SHV002	SVT002
EC ($\mu\text{s cm}^{-1}$)	630	640	690	730	703	1200	960	1300	1200	880	850
pH	6.7	6.9	7.03	6.8	6.8	6.7	6.7	6.8	6.8	7.07	6.9
Eh ^a (mV)	397	405	412	391	425	370	408	384	398	388	390
Dissolved O ₂ (mg L ⁻¹)	11	8.8	8.9	11	6.9	12	11	7.6	12	12	11.4
DOC (mg L ⁻¹)	1.3	0.59	1.4	0.77	1.07	1.00	1.6	2.2	2.3	4.3	3.5
TN (mg L ⁻¹)	5.1	11	13	29	14	34	11	68	69	16	24
NO ₃ ⁻ (mg L ⁻¹)	20.2	47	55	130	59	150	46	306	320	64	99
NO ₂ ⁻ (mg L ⁻¹)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
NH ₄ ⁺ (mg L ⁻¹)	0.018	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
PO ₄ ³⁻ (mg L ⁻¹)	0.028	0.034	0.032	0.026	0.013	0.018	0.034	0.079	0.017	0.008	0.046
SO ₄ ²⁻ (mg L ⁻¹)	14	35	43	79	83	120	180	110	120	110	120
Cl ⁻ (mg L ⁻¹)	100	14	29	40.01	24	68	107	170	109	62	64
Br ⁻ (mg L ⁻¹)	0.068	<LOQ	<LOQ	0.057	<LOQ	0.14	0.037	0.11	0.10	0.038	<LOQ
F ⁻ (mg L ⁻¹)	0.18	0.056	0.074	0.091	0.104	0.080	0.084	0.071	0.090	0.15	0.16
Na ⁺ (mg L ⁻¹)	23	7.2	13	13	13	38	51	57	38	19	21
K ⁺ (mg L ⁻¹)	1.6	1.6	2.2	5.4	0.31	12	0.96	11	4.4	26	22
Ca ²⁺ (mg L ⁻¹)	79	140	140	140	130	160	204	250	230	140	160
Mg ²⁺ (mg L ⁻¹)	22	12	18	35	10.1	42	15	36	46	29	32
Alcalinity (mg L ⁻¹)	120	310	303	280	220	360	340	320	340	290	320
ANTIBIOTIC CONCENTRATIONS (ng L ⁻¹)											
Tetracycline	47 (± 15)	<LOD	107 (± 28)	40 (± 17)	140 (± 1.9)	<LOD	<LOD	<LOD	<LOD	57 (± 0.28)	130 (± 8.3)
Doxycycline	230 (± 4.2)	21 (± 0.78)	250 (± 13)	2400 (± 2.5)	270 (± 0.01)	<LOD	<LOD	<LOD	<LOD	280 (± 0.13)	280 (± 0.01)
Oxytetracycline	30 (± 2.7)	<LOD	53 (± 6.5)	<LOD	67 (± 18)	<LOD	<LOD	<LOD	<LOD	63 (± 18)	35 (± 19)
Chlortetracycline	64 (± 4.8)	<LOD	97 (± 4.27)	365 (± 7.7)	131 (± 11)	<LOD	<LOD	<LOD	<LOD	110 (± 4.1)	142 (± 11)
Sulfamethoxazole	<LOD	8.4 (± 0.93)	<LOD	<LOD	<LOD	8.3 (± 0.021)	<LOD	12 (± 1.6)	<LOD	<LOD	<LOD
Sulfamethazine	<LOD	4.5 (± 0.1)	<LOD	5.2 (± 0.25)	<LOD	8.1 (± 0.21)	5.7 (± 0.19)	15 (± 0.11)	9.3 (± 0.01)	5.4 (± 0.25)	<LOD

^a Eh values corrected to standard hydrogen electrode values.

antibiotics was performed just considering the data for the first sampling event (C1). For Fig. S2, the first component explained 39 % of the variance while the second explained the 15 %. For antibiotics (Fig. S3), the first component of the PCA explained 56.8 % and the second 28.1 % of the variance between the samples. In both figures, samples were clustered in different groups, clearly differentiating the sites with the highest sulfonamide and tetracycline concentrations (Fig. S3) and springs with more prevalence in sulfonamides were those with the most relevant nitrate, Na⁺, Cl⁻, SO₄²⁻, Ca²⁺ and Mg²⁺ concentrations (Fig. S2). In both cases, samples did not cluster following their classification of their hydrological response type based on geology, land use, hydrochemistry and nitrate content (Table S1 and Fig. S1), as initially expected, indicating that the spatial variability of hydro-chemical parameters and antibiotics among springs might not only be associated to hydrological factors, but probably to other controlling factors, such as husbandry and land use practices as well as to the manure composition and origin applied in the fields (e.g. antibiotic concentrations and the compounds detected may differ from one type of manure to another). Springs in urban and agricultural sites (Table S1) such as CAL004, MDV003, SJV003, GUR004 and FOL006, show higher distribution of sulfonamides, while tetracyclines were more ubiquitous in forested and agricultural locations (TOR001, FOL002, SVT002, SHV002, SSO002).

Among sulfonamides, sulfamethoxazole, which is an antibiotic of human and veterinary use, and that has been detected in sites impacted by urban pollution sources (e.g., wastewater treatment plants, etc.) was more relevant in urban and agricultural sites, being especially relevant in spring CAL004, that is influenced by surface water due to its interaction with groundwater. However, sulfamethazine, which is an antibiotic mostly used in livestock, was mostly detected in springs in agricultural sites. Seasonal variability could be mostly associated to the

time of manure application as fertilizer in the fields but also to both hydrological factors, such as the recharge regime and, also importantly, the reactive transport processes (sorption and degradation) in the sub-surface (Boy-Roura et al., 2018). Higher concentrations in the first survey conducted in April 2018 could be attributed to the application of manure as fertilizer during winter (manure is applied twice per year, normally between November–December and February–March) and their transport to groundwater through subsequent rainfall events. Lower detections in the following campaigns (September 2018 and January 2019) might be explained by the potential dilution due to the strong rainfall events that occurred between April, September 2018, and January 2019 (Fig. S4), together with the potential degradation and sorption of antibiotics to the aquifer sediments.

3.2. Characterization of groundwater DOM

The use of Orbitrap-MS allowed the characterization of the DOM fingerprint of groundwater samples. We were able to resolve between 8992 and 12086 different masses across the different DOM extracts. Bray-Curtis (BC) dissimilarities were obtained first including the samples from all the sampling events, to visualize the differences among sampling seasons (Fig. S5). As shown in Fig. S5, the third sampling event (January 2019) was the one that showed the largest differences in the sample's DOM fingerprint, compared to the first (April 2018) and second monitoring (September 2018), and this could be potentially explained by the lowest number of total molecular formulas detected. Indeed, in a general extent, the DOM composition between the first and second campaigns showed some similarity. Because antibiotics were mostly detected in the first sampling campaign, and that hydro-chemical parameters did not differ among seasons, we decided to further explore the

differences in DOM composition between sites and relate it to antibiotics and hydro-chemical parameters just focusing on the first sampling event. In this way, Fig. 1 shows the heat-map on the BC dissimilarities for the samples collected in the first survey. As it occurred with antibiotics concentrations, the heatmap suggests that there is not a clear relationship between the DOM fingerprint and the spring's hydrological behavior, as described by their HRT classification (Table S1). Sample CAL004 was the one showing the highest differences compared to the other sites, and they were especially remarkable with FOL006 spring, followed by TOR001 and TAV002. This could be explained by the fact that in the spring CAL004 there is an interaction between groundwater and surface water, as indicated by previous hydro-geological field observations (Boy-Roura et al., 2013), having thus an influence in shaping a rather different molecular composition for this spring in comparison with the other sites.

To further explore the differences among sites, the total number of formulas assigned in each spring, the percentage that contain C, H, O, N and S and their classification based on the AI_{mod} was assessed. Table 2 shows the total number of formulas assigned in each spring for the first sampling event, along with the percentage of CHO, CHON, CHOS and CHONS formulas (results for the second and third sampling are presented in Table S6). As indicated in Table 2, molecular formulae were assigned to between 4656 and 5732 peaks. The remaining peaks correspond to noise or isotopologues (Koch et al., 2007). In general, and in all sampling events, most formulas were CHO compounds (49 %–68 %), with CHON and CHOS accounting for smaller fractions, from 16 % to 23 % and from 11 % to 24 % respectively, while CHONS represented a minor proportion (3 %–7.9 %). Some studies showed that intensive farming significantly increased fluvial dissolved organic nitrogen (DON) concentrations (Graeber et al., 2015; Wilson and Xenopoulos, 2009), while a high range of nitrogen containing formulas was also observed in groundwater impacted by septic tank discharges (Arnold et al., 2014).

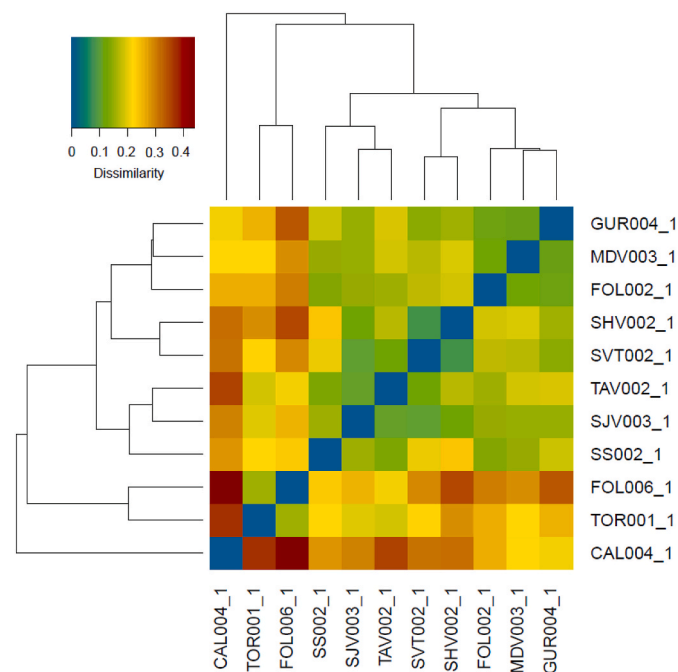


Fig. 1. Heat-map showing the dissimilarities (Bray-Curtis) for the springs collected in the first sampling event. This graph organizes samples according to how different they are, and color codes the magnitude with a number between 0 and 1. The color of the graph represents the dissimilarity among samples (e.g., blue corresponds to samples that show no differences among them (BC = 0), while red is attributed to the samples with the highest differences between them). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2

The total number of formulas and percentages (by number) of the types of formulas assigned to groundwater samples in the first sampling event (April 2018).

SPRING HRT	SPRING CODE	1st SAMPLING				
		Total	% CHO	% CHON	% CHOS	% CHONS
1	SSO002	4681	62	16	18	4.8
2	FOL006	4659	66	18	12	4.8
	TAV002	4675	68	19	11	3.8
3	TOR001	5237	55	22	16	7.1
	FOL002	4656	63	16	16	4.5
	MDV003	5320	55	19	18	7.1
4	SJV003	5320	61	18	15	5.1
	CAL004	5732	51	18	23	7.9
	GUR004	5191	54	20	19	6.9
	SHV002	5250	58	21	15	5.9
	SVT002	5161	58	21	15	5.8

Greater abundances of nitrogen and sulphur-containing molecular formulas were also identified in rivers influenced by anthropogenic inputs, in comparison with primarily forested watersheds, which showed DOM signatures depleted in heteroatomic content (Wagner et al., 2015). Nitrogen and sulphur-containing formulas were quite prevalent in groundwater samples, as described in previous studies (Longnecker and Kujawinski, 2011), where the high proportion of CHON compounds was also attributed to the limited remineralization of dissolved organic nitrogen prior to groundwater reaching the surface (Longnecker and Kujawinski, 2011). As for sulphur-containing formulas, their occurrence could be related with rainwater, since sulphur-containing organic matter can be a dominant component of rainwater (Altieri et al., 2009) which could percolate to the subsurface, but also to livestock waste inputs as well as to pyrite oxidation, especially in springs SVT002, TOR001, TAV002, and from sulfate dissolution in site GUR004.

Among the sites under study, CAL004 was the spring with the lowest number of CHO formulas and the highest CHOS containing features. Indeed, some of the differences highlighted in the Bray-Curtis diagram (Fig. 1) between CAL004, FOL006 and TAV002 springs (the ones with major differences) could be explained by the number of CHO and CHOS formulas. TAV002 and FOL006 have the highest CHO and some of the lowest CHOS containing features, in contrast with CAL004. Differences between CAL004 and TOR001 may be mostly attributed to nitrogen containing formulas, being remarkably higher in TOR001 site. The major difference of CAL004 with the other sites might be explained by the surface water inputs to the subsurface that occur upstream from the spring location, acting as a losing stream, having thus an influence on the DOM fingerprint at this site.

Groundwater samples in all springs were dominated by molecules falling in the lignin-like region ($H/C = 0.7$ – 1.5 , $O/C = 0.3$ – 0.7), while minor number of formulas were associated with protein-like substances that cluster in areas with high hydrogen saturation ($H/C \geq 1.5$). This is in contrast to our initial hypothesis, where we expected to detect a larger proportion of formulas associated with protein-like components that are mostly related with farm derived-wastes (Humbert et al., 2019; Naden et al., 2010; Old et al., 2012; Singh et al., 2014). One explanation might be that these compounds remain adsorbed onto the soil, immobilized through bacterial breakdown or degraded by microorganisms, and thus, they may not reach groundwater bodies (Old et al., 2012). The classification of the different DOM compounds in each natural spring is shown in Fig. 2 and revealed a quite similar profile among sites. In all springs, most of the compounds were phenolic and highly unsaturated compounds, that are typical for soil-derived organic matter (Santí-Temkiv et al., 2013; Stenson et al., 2003; Tremblay et al., 2007), with approximately 47 %–61 % being oxygen poor compounds while from 27 % to 40 % were oxygen rich substances. Approximately less than 10 % of the identified compounds were unsaturated aliphatic substances, while 3 %–6 % were aromatics (polyphenolic compounds) and less than 2 %

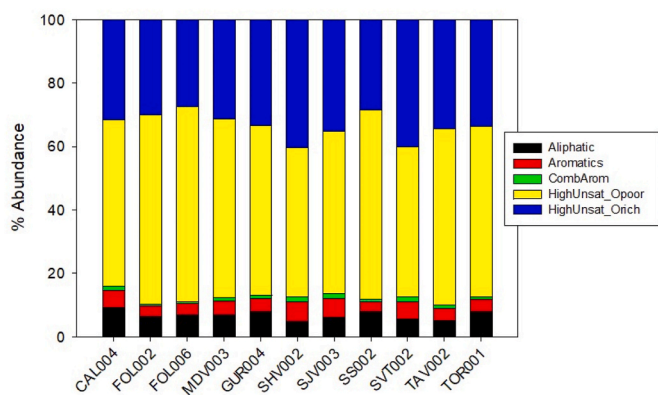


Fig. 2. Relative contribution (i.e. number of formulae) of the different DOM compounds' groups to each sample.

corresponded to combustion derived aromatics.

When analyzing the DOM composition in each individual spring, site CAL004 showed the highest percentage of aliphatic (9 %) and aromatic (6 %) components, in comparison with the other springs. This composition is opposite to the one observed in the springs with greater similarities with CAL004 as highlighted in the Bray-Curtis diagram (Fig. 1), namely FOL006, TAV002 and TOR001. These springs had lower proportion of aliphatic and aromatic compounds with higher unsaturated polyphenols substances. These major differences for site CAL004 are attributed by the influence of surface water to the DOM profile, which might contain higher percentages of these components.

The DOM chemical profile observed for groundwater samples match quite well with previous studies. Humic and fulvic acids, released from organic carbon in the soil/subsurface matrix have been found to be major components in groundwater DOM (Longnecker and Kujawinski, 2011; Ohno and Bro, 2006). Furthermore, other studies also detected high proportions of soil-derived compounds and unsaturated aliphatics, most likely attributed to the desorption of organic matter from soil particles (Lechleitner et al., 2017; Longnecker and Kujawinski, 2011; McDonough et al., 2020; Šantil-Temkiv et al., 2013).

3.3. Relationship of antibiotics and hydro-chemical parameters with DOM

The relationship between antibiotics and DOM constituents was investigated through Spearman rank correlations and represented in van Krevelen diagrams (Fig. 3). In addition, the variability of DOM constituents and their relationship with hydro-chemical parameters and antibiotics concentrations, was further assessed by using principal coordinate analysis (PCoA) (Fig. 4). To avoid autocorrelation effects, a PCA on the hydro-chemical parameters was formerly performed (Fig. S2) and then a data matrix was built just considering the scores of the 11 samples from the first sampling campaign (C1) for the first four axis of the PCA of the hydro-chemical parameters, the imputed antibiotics and DOM molecular constituents for C1.

Fig. 3 shows that the antibiotic classes detected show a positive correlation with different DOM components. In this way, tetracycline antibiotics are positively correlated with unsaturated compounds with high oxygen content ($H/C < 1.5$ and O/C between 0.5 and 0.75) while sulfonamides are more related with aliphatic substances and unsaturated compounds with low oxygen content and H/C between 1 and 1.5 and $O/C < 0.5$. For the PCoA (Fig. 4), it resulted in a first principal coordinate, which was the axis of major compositional variation of DOM and that explained 53 % of the variability, and a second axis that explained 25 % of the variability. In the PCoA, springs cluster into several groups. CAL004 is clearly separated from all the other springs, indicating that it is the most different site in terms of DOM composition, as already highlighted (Fig. 1). This site is correlated with phosphates

and nitrates and with sulfonamide antibiotics, especially sulfamethoxazole, which is detected at the highest concentrations (see Table 1). The major differences in DOM composition for site CAL004 could be explained by its interaction with surface water while the occurrence of sulfamethoxazole may reflect the influence of urban contamination sources to surface water that subsequently recharges spring CAL004.

In the PCoA springs GUR004, FOL002, MDV003 and SSO002 form another cluster (Fig. 4). MDV003 and GUR004 also show a correlation with sulfonamide antibiotics, nitrates, sulphates, chloride and calcium, suggesting that these springs are susceptible to anthropogenic contamination sources, such as the use of organic fertilizers and urban pollution sources, as these elements are major components of animal manures, slurries and wastewaters. Furthermore, these sites had some of the highest percentages of aliphatic components as well. Springs TOR001 and FOL006 are also differentiated from the rest of the springs (Fig. 4). Sample point TOR001 is mostly correlated with tetracycline antibiotics, especially doxycycline, which is an antibiotic of major use in livestock production. However, sulfonamide antibiotics were also detected. This spring had high content of aliphatic and highly unsaturated components with high and low oxygen content, which might support the detection of both types of antibiotics (aliphatic components might be related with the occurrence of sulfonamides, Fig. 3). FOL006 is one of the springs with the highest and lowest number of CHO and CHOS formulas, respectively and the highest unsaturated oxygen-poor components. Finally, SHV002, SVT002, TAV002 and SJV003 springs from another cluster in the PCoA (Fig. 4). These sites, with remarkable concentrations for tetracycline antibiotics also correlated with DOC and dissolved oxygen. These four springs have larger abundances of highly unsaturated DOM components with high oxygen content and the lowest aliphatic and unsaturated oxygen-poor compounds, which might explain the detection of tetracycline antibiotics.

The correlation of tetracyclines with highly unsaturated and oxygenated compounds (Fig. 3) might be explained by the fact that at water pH (~ 7), most tetracyclines are zwitterionic, with positive charges that could interact with the negatively charged highly unsaturated phenolic structures of the DOM. Furthermore, tetracyclines might also associate with these DOM components through interactions between hydroxyl groups. This is in good agreement with previous studies where tetracyclines showed strong adsorption to soils with high organic matter and humic acid contents (Albero et al., 2018Eqn 1; Christl et al., 2016; Zhao et al., 2018) and with lignin-based adsorbents (Gao et al., 2021; Gao et al., 2019), which are both rich in unsaturated and phenolic constituents. For sulfonamides, their major affinity for unsaturated compounds with low oxygen content and aliphatic substances (Fig. 3) might be because these compounds are neutral and/or negatively charged at water pH and thus, weaker interactions are expected with phenolic and hydroxyl groups in DOM. These findings also match quite well with previous studies where sulfonamides showed minor sorption to organic matter and humic acids in soils (Albero et al., 2018) while presented high adsorption to manure-based sorbents with high aliphatic content and O-containing components (Tzeng et al., 2016). These results demonstrate that these DOM components play a crucial role in the interaction between DOM as they may enhance co-transport of antibiotics; that is, adsorption on specific DOM molecules may facilitate antibiotic migration in soils and aquifers as it decreases their immobilization by being adsorbed to soil particles. A further in-depth evaluation of the association of antibiotics with specific DOM fractions would allow to identify the factors influencing antibiotic transport and occurrence far away from their sources as well as to consider DOM as a surrogate for potential antibiotic pollution in areas with proved sources of antibiotics. Furthermore, from an environmental perspective, the fact that antibiotics associate with DOM molecules through sorption may enhance their mobility in the subsurface and leads to a new way of considering their mobility and potential risks for humans and ecosystems.

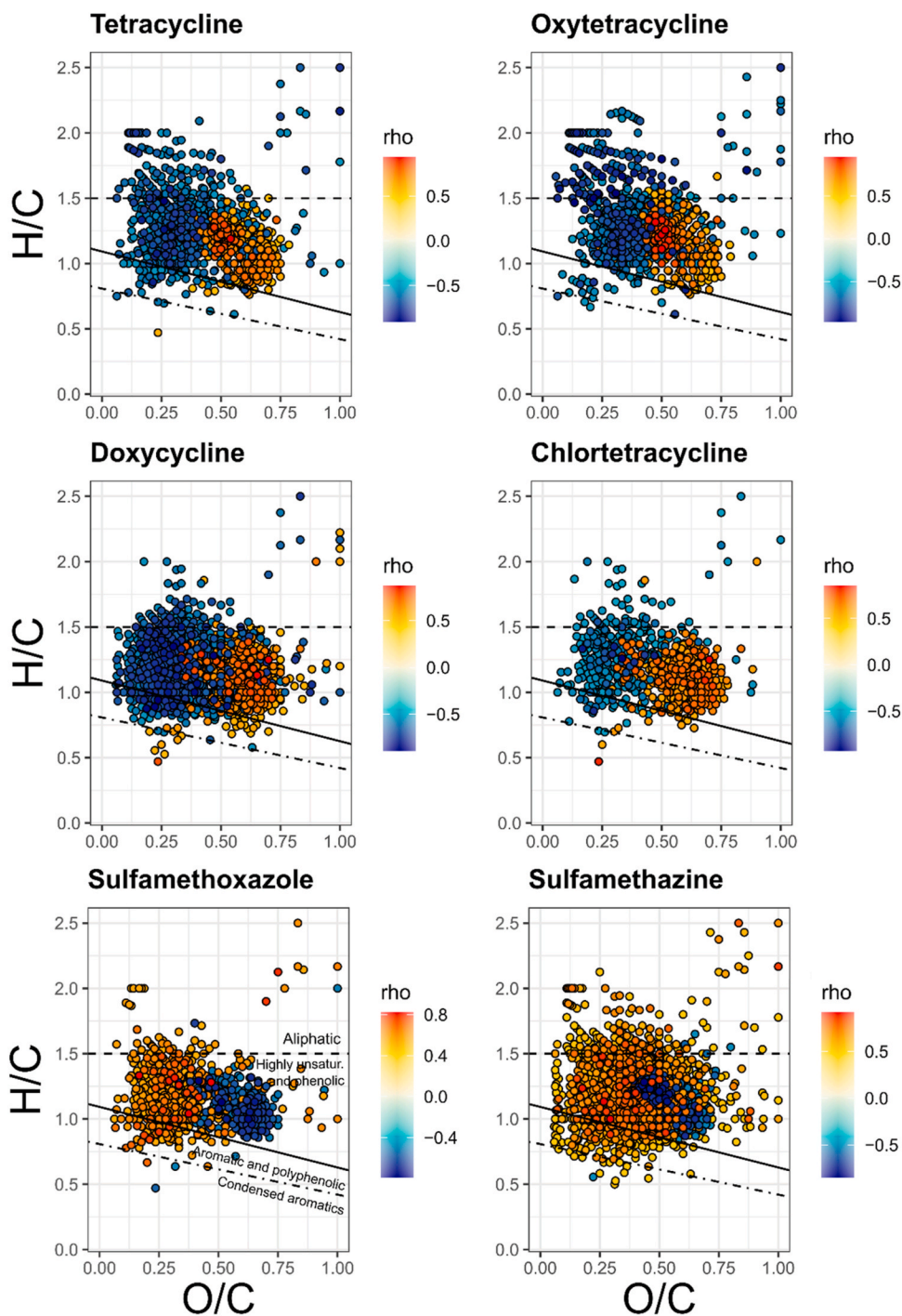


Fig. 3. Van Krevelen diagrams showing the individual compounds with significant Spearman rank correlation coefficients (ρ) with the six studied antibiotics for the samples of the first sampling event (red-colored DOM components show a positive correlation with that specific antibiotic, while blue-colored constituents indicate a negative correlation). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Conclusions

Tetracycline and sulfonamide antibiotics were ubiquitous compounds detected, in general, at low ng L^{-1} levels, in almost all sites. Nitrate concentrations exceeding the 50 mg L^{-1} threshold established by the EU were also detected in several springs, indicating groundwater contamination in the Osona livestock production region. DOM characterization showed that major components were soil-derived substances and, although most formulas were CHO compounds, a remarkable

percentage of nitrogen and sulphur-containing formulas and aliphatic components were found, suggesting that natural springs are susceptible to anthropogenic contamination sources. Neither antibiotic concentrations nor DOM composition could be classified according to their hydrological behavior, as described by their hydrologic response types (HRT). This underlines the fact that their fate in the subsurface is greatly controlled by its complex reactivity (sorption, degradation), more than by hydrological factors as advection and dispersion. In addition, the recharge regime as well as fertilization practices in the recharge

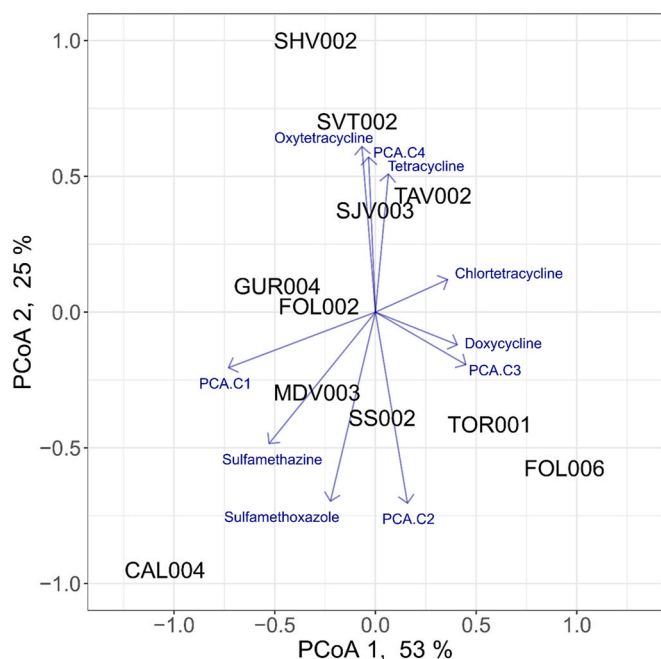


Fig. 4. Principal coordinate analysis for the DOM constituents in the natural springs collected in the first sampling event. The length of the arrows is proportional to the correlation coefficient between the environmental variable and the PCoA scores. Variables are: target antibiotics (tetracycline, oxytetracycline, doxycycline, chlortetracycline, sulfamethoxazole and sulfamethazine); Comp. 1, 2, 3 and 4 (1st to 4th principal components of the PCA on hydrochemical parameters).

areas may also play important roles in the antibiotic and DOM occurrence. Our results also pointed out that tetracyclines positively correlated with highly unsaturated oxygen-rich DOM components while sulfonamides positively related with aliphatic and unsaturated oxygen-poor compounds. The link between antibiotic occurrence and DOM is herein identified as an additional factor influencing antibiotic reactive transport in the subsurface besides sorption and degradation that deserves further in-depth investigation.

Credit author statement

Meritxell Gros: supervision, funding acquisition, conceptualization, sampling, method development, sample analysis, data curation, writing-original draft, review & editing; **Núria Catalán:** conceptualization, data curation, writing-original draft, review & editing; **Josep Mas-Pla:** conceptualization, selection of sampling sites and sampling, writing original-draft, review & editing; **Mira Čelić:** sampling and sample analysis; **Mira Petrović:** funding acquisition, conceptualization, writing-original draft, review & editing; **Maria José Farré:** supervision, conceptualization, method development, data curation, writing original draft, review & editing.

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.envpol.2021.117927>.

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