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# A first inventory of the labile biochemicals found in Avignon groundwater: can we identify potential bacterial substrates?

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**Abstract.** Groundwater is a major source of water for irrigation of vegetables, especially in the Mediterranean basin. Contamination of aquifer by pathogens has been responsible for numerous disease outbreaks worldwide. Several studies reported that groundwater dissolved organic matter (DOM) can serve as a source of carbon and energy for heterotrophic metabolism of pathogens. In this study, we aimed to investigate the DOM composition of groundwater collected at Avignon. Six liters of groundwater were filtered (0.2 µm) and freeze-dried following appropriate cleaning procedure. The bulk analyses of powder sample were performed using 1D and 2D nuclear magnetic resonance spectroscopy and liquid chromatography coupled with mass spectroscopy. Several components were found at concentrations around 1 µM and comprise: (i) humic and fulvic acids originated from land-derived material or soils and, (ii) various acids, esters and alcohols of different sizes including acetate, lactate and formate, these may result from microbial metabolism. In conclusion, the Avignon groundwater DOM contains a heterogeneous mixture of dissolved organic components with a rather low potential bioreactivity based on the low level of labile biogeochemicals such as carbohydrates.

## Introduction

Water is the main limiting factor for agriculture in Mediterranean basin due to arid and semi-arid climate. Surface water resources are more and more limited in these areas and aquifers are considered as major sources of water for irrigation. Although groundwater is generally considered as safe, microbial contamination of groundwater has been responsible for numerous disease outbreaks worldwide [1]. Nevertheless, irrigation uses mainly untreated and uncontrolled groundwater and this agricultural practice could cause human

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and environmental health risks. The factors that are the most influential in occurrence, survival, dynamic and dispersion of pathogenic bacteria in groundwater are not well understood especially in the agricultural context. Among these factors, there are dissolved oxygen, temperature, pH, pollutants, biological interaction and Dissolved Organic Matter (DOM). DOM is composed of two fractions: the labile or biodegradable fraction, a potential carbon and energy source for heterotrophic pathogens [2] and the refractory fraction considered relatively less important for bacterial growth. The labile fraction consists mainly of sugars, amino acids, peptides and other simple compounds while the refractory fraction is composed mainly of high molecular weight molecules such as humic and fulvic acids.

In this study we report physico-chemical characteristics of Avignon alluvial aquifer and the first analyses of its DOM composition. We provide qualitative information about the organic components found in groundwater, and speculate on their bioavailability and origins [3].

## **1 Material and methods**

### **1.1 Sample collection**

#### *1.1.1 Study area*

Avignon plain is located in south-eastern France under a Mediterranean climate with a wet period in autumn and severe drought and high temperatures in summer. Avignon shallow alluvial aquifer consists of gravels and sands between 4 to 20 m depth and has been extensively described by Nofal [4]. In brief it flows from east to west with a hydraulic gradient between 1.5 and 3.0 ‰, [5], under the Avignon plain that is a triangle of 70 km<sup>2</sup> delimited at north-west by the river Rhône, at south by the river Durance, and at east by the hills of Morières-Les-Avignon. This area is mainly urban but 30 % of the surface remains occupied by various crops that require irrigation. Groundwater samples were collected in this agriculture area from a representative site routinely studied over the last decades by Avignon University (site n° 33, altitude 25.9 m; depth 15 m; lat. 43.9518 and long. 4.8717) and where the groundwater recharge is mainly dependent on irrigation: groundwater level is high in summer during irrigation period and low in winter [4].

#### *1.1.2 Sampling of groundwater*

Six groundwater samples (20 L) were collected from March 2016 to April 2018 for chemical and microbial analyses. Water were hand-sampled in polypropylene unused sterilized flasks using an immersed pump. For DOM analyses, 15 L of groundwater were collected in March 2017 in glass bottles previously cleaned with 2 % HCl and baked at 450 °C for 6 h to minimize organic carbon contamination. The bottles were kept in the dark at 4 °C during 7 days before filtration.

### **1.2 Groundwater analyses**

#### *1.2.1 Microbiological analysis*

The total number of culturable bacteria was determined as previously described on 10 % Tryptic Soy Agar (TSA) [6]. Water subsamples (0.5 to 3.0 L) were filtered (0.22  $\mu\text{m}$ ) under vacuum. Bacteria were desorbed from filters by washing with 1 ml sterile water. Concentrate suspension was diluted and plated on 10 % TSA media.

### 1.2.2 Physico-chemical analyses

Water temperature, pH, dissolved oxygen concentration and electrical conductivity were measured at the sampling time. Water sampled for anions and cations analyses were filtered (0.45  $\mu\text{m}$ ) and drops of nitric acid were added in the bottle for cations analysis. Alkalinity was measured immediately after sampling by acid titration. Routine analysis of cations and anions were performed by ionic chromatography. To determine the Total Organic Carbon (TOC) content, water was sampled in brown tinted glass bottle and drops of mercuric chloride were added. TOC content was obtained using the heated persulfate wet oxidation technique.

### 1.2.3 Nuclear Magnetic Resonance (NMR) spectroscopy and Liquid Chromatography coupled with Mass Spectroscopy (LC-MS) analyses.

The sample (6 L) was filtered (0.22  $\mu\text{m}$ ) and freeze-dried, as described by [7], giving 3.4 g of powder (mainly salts). Dissolved Organic Carbon (DOC) was quantified on the filtered sample using a sensitive spectrophotometry method [8].

For NMR analyses, 1 g of powder was dissolved in 3.5 ml of  $\text{D}_2\text{O}$ , freeze dried and then re-dissolved in 0.6 ml of  $\text{D}_2\text{O}$  99,9 % (Sigma-Aldrich) with 1 mM of TSP- $\text{d}_4$ , 98% deuterated, (Sigma-Aldrich) as chemical shift reference. All NMR spectra were acquired with a Bruker Avance III 500 MHz spectrometer at 300K with a cryoprobe Prodigy TCI 5 mm. One-dimensional proton ( $1\text{D } ^1\text{H}$ ) spectra, two dimensional proton-proton spectra (2D  $^1\text{H}$ - $^1\text{H}$  COSY, TOCSY, JRES) and two dimensional proton-13 carbon spectra (HSQC and HMBC) were recorded following usual conditions.

A complementary analysis was performed using liquid chromatography (Agilent 1200 Series Fast LC System) coupled with a MicroTOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Two hundred mg of powder were dissolved in 1 ml of water and 6  $\mu\text{L}$  were injected in a HSST3 150 \* 2.1 mm 1.8  $\mu\text{m}$  column targeting small molecules (< 3000 Da). This column is able to separate polar compounds such as amino-acids, di-peptides, small organic acids, UMP, AMP, sugars, as well as non-polar molecules such as lipids or palmitoleic acid. Analysis was performed by reverse phase chromatography using water in 0.1 % formic acid /ACN in 0.1 % formic acid as mobile phase. A putative identification of main peaks on chromatogram was done based on the Human Metabolome DataBase (HMDB) [9]. These NMR and LC-MS analysis allowed to detect compounds with concentration higher than 1  $\mu\text{M}$ , except those that could be suppress during ionization step.

Concentration of sugars in groundwater was measured using High Pressure Ion Chromatography (HPIC, Dionex ICS-3000). The freeze-dried sample of groundwater was dissolved in water to obtain a saturated solution. Analysis was performed as described by Rusch *et al.* [10].

## 2 Results

### 2.1 Physico-chemical and microbial parameters confirm the oligotrophic status of Avignon groundwater

Analysis of the six samples collected from site n°33 at various dates confirmed that the physico-chemical parameters of Avignon groundwater are relatively stable [4] especially in terms of temperature, pH, dissolved oxygen and electrical conductivity (Table 1). Electrical conductivity of water depends on concentration of ions which come from the dissolved salts. Table 1 shows that  $\text{HCO}_3^-$  is the major anion and  $\text{Ca}^{2+}$  the major cation at this site as reported previously [4].  $\text{NO}_2^-$  and  $\text{PO}_4^-$  were not detected during the experiment.

**Table 1.** Mean values of physico-chemical parameters measured in water from sampling site n°33 of Avignon alluvial aquifer, monitored from March 2016 to April 2018.

Parameters	Values $\pm$ SD	Units
Temperature	16.4 $\pm$ 0.3	°C
pH	6.98 $\pm$ 0.08	pH unit
Dissolved O <sub>2</sub>	77 $\pm$ 5	%
Electrical conductivity	812 $\pm$ 6	$\mu\text{S}/\text{cm}$
$\text{HCO}_3^-$	338 $\pm$ 18	mg/L
$\text{Ca}^{2+}$	122 $\pm$ 12	mg/L
$\text{SO}_4^{2-}$	109 $\pm$ 5	mg/L
$\text{Cl}^-$	22.2 $\pm$ 0.9	mg/L
$\text{Na}^+$	17.0 $\pm$ 0.5	mg/L
$\text{Mg}^{2+}$	16.4 $\pm$ 0.3	mg/L
$\text{NO}_3^-$	11.2 $\pm$ 0.9	mg/L
$\text{K}^+$	2.4 $\pm$ 0.2	mg/L
$\text{F}^-$	0.16 $\pm$ 0.02	mg/L
$\text{NH}_4^+$	0.021 $\pm$ 0.016	mg/L
$\text{Br}^-$	0.023 $\pm$ 0.015	mg/L
$\text{PO}_4^-$	< 0.01	mg/L
$\text{NO}_2^-$	< 0.01	mg/L

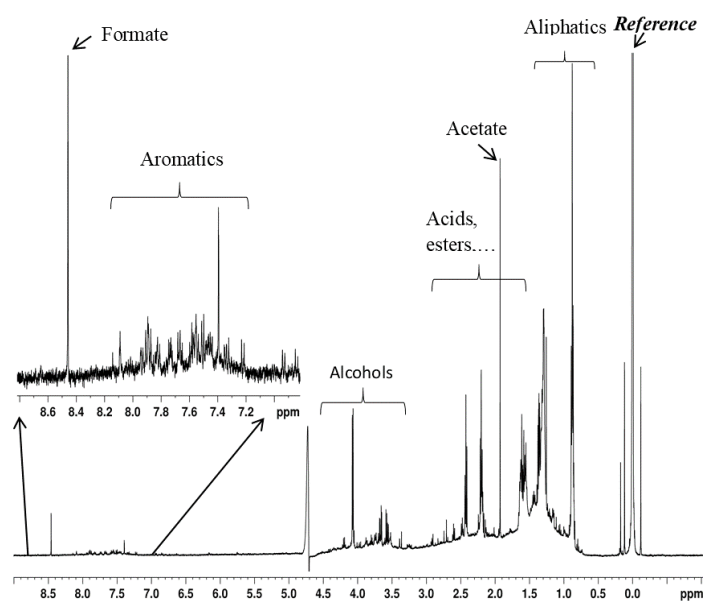
The average concentration of TOC in the six groundwater samples was 1.63  $\pm$  0.69 mg/L. This value was in the range of expected values in oligotrophic environments [11]. We noted a high variability of the values of TOC concentration during the sampling campaign. Such variability was previously reported by Brillard *et al.* [12].

Microbial analysis of the samples indicated that oxic oligotrophic Avignon groundwater contains 2.3  $\pm$  2.3 10<sup>5</sup> culturable bacteria/L. This mean value is 100 times lower than the values found in surface water as shown previously by Hofmann & Griebler on total counts using flow cytometry [11].

## 2.2 DOM from Avignon groundwater differs in composition from river DOM

In order to identify the composition of organic compounds, we analyzed the groundwater sample collected in March 2017. We first quantified DOC of the filtered sample. The DOC concentration was 0.53 mg/L. This value was greater than the TOC value (0.30 mg/L) confirming the better sensitivity of the spectrophotometry method used for DOC measurement [8]. This result also indicates that particulate organic matter was negligible in this sample.

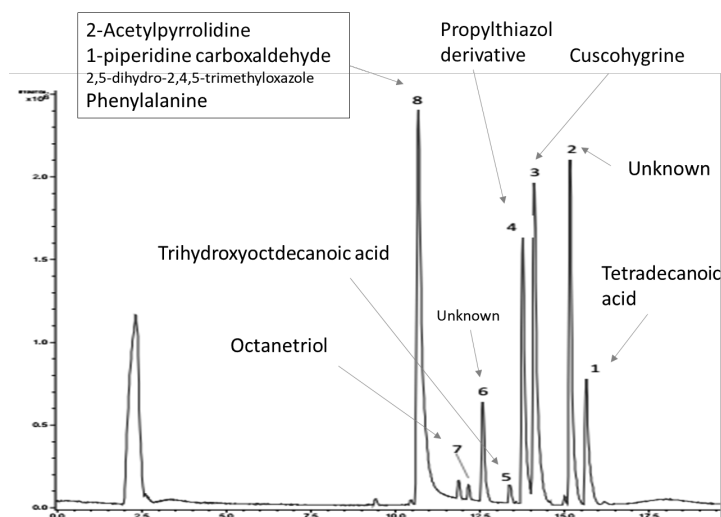
We then performed a NMR analysis to identify DOM functional groups. Figure 1 shows an example of NMR 1D spectrum obtained from the selected groundwater sample. Spectra analysis showed a major contribution of aliphatic functional groups corresponding to hydrophobic molecules like lipids and/or alkanes. These molecules have typically a terrestrial origin and a low potential biodegradability. Contribution of aromatic functional groups to NMR spectra was minor. These functional groups could be attributed to the presence of various fulvic and humic acids derived from soil. These two types of acids are characterized by high condensed, hydrophobic, cyclic molecular structures rich in aliphatic, aromatic, and carboxyl and are generally considered as recalcitrant organic matter.



**Fig. 1.** 1D <sup>1</sup>H spectrum of Avignon groundwater. Organic functions are indicative.

Alcohol resonances corresponding to carbohydrates were minor indicating that carbohydrates could be less abundant in groundwater than in rivers [13]. While photosynthesis could contribute to enrich river water in carbohydrates, microbial activity could play a role in their biodegradation in groundwater. Analysis of 1D NMR spectra also showed the presence of acetate and formate functional groups, showing the presence of potential labile compounds. Two-dimensional NMR analysis of DOM identified the contribution of lactate functional group (data not shown) and two deoxy-sugars, fucose and 2 deoxy-glucose [14]. Acetate, formate and lactate may result from microbial metabolism.

We conducted a LC-MS analysis to reveal the diversity of small molecules. Figure 2 shows an example of chromatogram of the Total Ionic Current (TIC) obtained by LC-MS.



**Fig. 2.** Chromatogram of the Total Ionic Current obtained by LC-MS of Avignon groundwater organic matter, recorded in positive mode. Molecular identifications based on HMDB are indicative.

Figure 2 shows that at least 8 groups of compounds were separated by LC-MS. Their putative molecular identification based on the HMDB database, revealed various components belonging to fatty acids (peaks n°1 and 5), fatty alcohols (peak n°7), alkaloids (peak n°3), aromatic like flavoring agents (peaks n°4 and 8) and cyclic amino acids (peak n°8). Most of them could be originated from plants and are rather recalcitrant. However, some of them such as phenylalanine could be bioavailable for bacteria.

HPIC has been used to identify and quantify sugars in groundwater. Glucose, fructose, sucrose and fucose peaks were observed on chromatogram (data not shown). However, their quantification was not possible due to their very low concentration. These data were in agreement with NMR analyses.

## Conclusion

Avignon groundwater at sampling point n°33, showed rather stable physico-chemical and microbiological features in time. The very low levels of bacterial populations and DOC concentration illustrated the oligotrophic status of Avignon groundwater. The biochemical analysis of DOM shows that groundwater contains a large fraction of recalcitrant condensed compounds derived from terrestrial plants and soil. The recharge of Avignon groundwater at the studied site occurred mainly through irrigation during summer. This suggests that these recalcitrant compounds are originated from both the soil situated above the groundwater and the Durance river-water used to irrigate crops. Condensed compounds of DOM fraction are usually recognized as recalcitrant and unavailable for growth of most heterotrophic bacteria. At the opposite, carbohydrates are largely bioavailable for bacteria. Interestingly, this fraction appeared lesser abundant in groundwater DOM than in river DOM where carbohydrates are among the major components [13]. However, even minor, some labile compounds were potentially identified containing fucose and deoxy-glucose residues, acetate, lactate or formate functional groups.

This study aimed to identify potential substrates for bacterial growth in Avignon groundwater. We showed that high-condensed refractory compounds are dominant. More labile substrates are present but probably at very low concentration. In the case of groundwater contamination with pathogenic bacteria, the ability of a given bacterial strain

to exploit these compounds and survive in such environment remains to be determined. Interestingly powder obtained from Avignon groundwater could be used in direct biodegradation experiments as described by Panagiotopoulos *et al.* [15].

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